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وزارة التعليم العالي و البحث العلمي

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Ministry of Higher Education and Scientific Research

University of RELIZANE
Faculty of Science and Technology
Department of Biological Sciences

COURSE HANDOUT

For Microbiology Bachelor's Degree Students

Title:

The Analysis Techniques

Prepared by:

Dr. MELLALI Sarah

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Preface

Since October 20, 2019, I have been a Lecturer at the Faculty of Science and Technology, Department of Biological Sciences at the University of Relizane. In this role, I have had the opportunity to teach a wide range of subjects, including Analytical Techniques, Molecular Biology, Genetic Engineering, Cell Culture, Biological Analysis Techniques, Bioinformatics, Cell Biology, Zoology, Microbial Biochemistry, Scientific English, Computer Data Processing, Mycology Algology Virology and Food Microbiology.

In preparation for my promotion to the rank of Professor, I have chosen to present a booklet that covers the course material for Analytical Techniques. This module is a methodological unit in the curriculum of the third year of the Bachelor's degree in Microbiology at the Faculty of Science and Technology, Department of Biological Sciences at the University of Relizane.

This module is of crucial importance as a necessary and essential element for the subsequent modules of the third year of the Bachelor's degree program in Microbiology. It plays a fundamental role in preparing students to carry out their research work successfully. Indeed, mastering the analytical techniques acquired through this module will allow students to make an informed choice of the most appropriate technique for analyzing their samples. This acquired skill will also be of great value in pursuing master's studies, where students will have the opportunity to deepen their knowledge and engage in more advanced research projects. Thus, this module constitutes an essential foundation for the development of the skills necessary to carry out advanced research work in the field of microbiology and offers a solid basis for higher studies.

This course details three main axes in Biological Analysis Techniques, namely: Spectral Methods; Fractionation Methods and Electron Microscopy.

The course outline for this subject is as follows:

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Chapter I:
Spectral Methods

1. Spectrophotometry:

1.1. Definition:

Spectrophotometry is a quantitative measurement technique that evaluates the absorbance or optical density of a chemical substance in solution, allowing its concentration to be deduced. Indeed, the relationship between the concentration of a substance and its light absorption is described by Beer-Lambert's law, according to which the more concentrated a solution is, the more light it absorbs.

Absorbance, also known as optical density, opacity, or extinction coefficient depending on the context, reflects a substance's ability to absorb light passing through it.

Among the types of radiation commonly used in spectrophotometry are ultraviolet (UV), visible, and infrared (IR). The visible and UV spectrum, although widely explored and crucial for understanding chemical bonds at an experimental level, offers limited richness in terms of structural information. However, these domains remain valuable for quantitative analysis, particularly through the application of Beer-Lambert's law.

To measure the optical density of a solution, a spectrophotometer is used, which must be specifically calibrated for the wavelength at which the target substance absorbs light (Figure 1) .

Figure 1: Spectrophotometer device

1.2. The electromagnetic spectrum:

The electromagnetic spectrum is a vast and captivating realm that encompasses all forms of electromagnetic radiation, categorized by their frequency or wavelength. This spectrum stretches from the infinitesimal realm of high-energy gamma rays to the expansive waves of radio waves, encompassing a universe of phenomena that shape our world and beyond **(Figure 2)** .

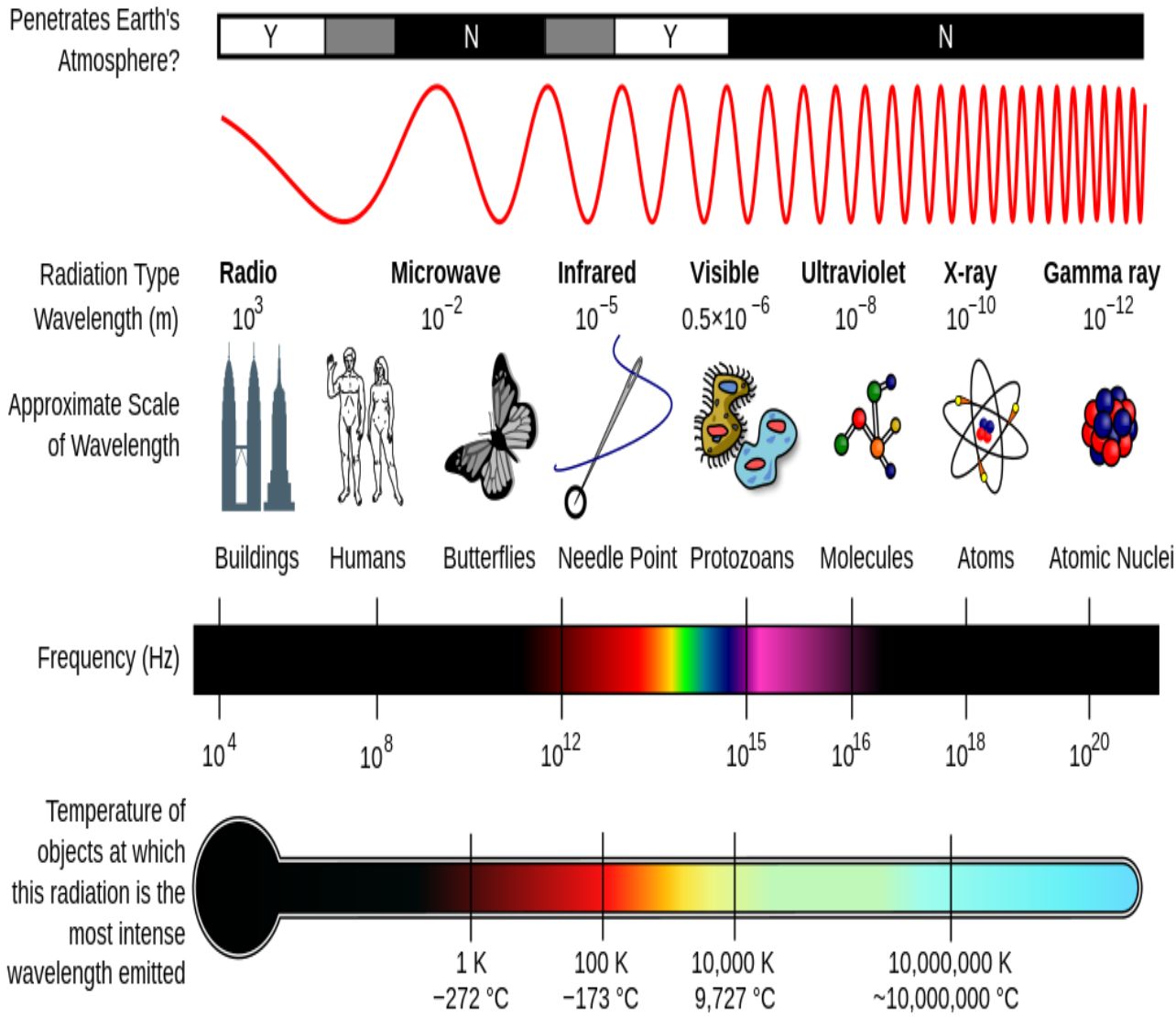

Figure 2: Electromagnetic spectrum

1.2.1. The decomposition of white light:

The familiar glow of white light, as we witness in the splendor of a rainbow, is not a single entity but rather a harmonious blend of countless hues. This seemingly pure light is, in fact, a composite of various monochromatic radiations, each with its unique wavelength, typically ranging from 400 to 800 nanometers when propagating in a vacuum. Unlike the precise, single-wavelength nature of laser light, white light is polychromatic, embracing a diverse spectrum of colors.

1.2.2. The Realm of ultraviolet radiation:

Beyond the visible spectrum lies the realm of ultraviolet (UV) radiation, often referred to as "black light" due to its invisibility to the human eye. UV radiation, with its shorter wavelengths than visible light but longer than X-rays, spans a range of 190 to 400 nanometers. Its presence can only be detected indirectly, either through the fluorescence it induces in certain materials or by employing specialized detection equipment.

1.3. UV-visible spectroscopy:

Spectrophotometry, a powerful analytical technique, delves into the intricate interplay between light and matter. As light encounters a substance, a portion of it is transmitted, while the remainder is absorbed. Colored substances, or chromophores, possess the ability to capture visible light, while certain solutions can absorb ultraviolet radiation. Infrared radiation, in contrast, is generally not exploited in spectrophotometry as its absorption is more dependent on the solution's temperature than its concentration. For the study of infrared radiation, infrared spectroscopy is the preferred technique (Figure 3).

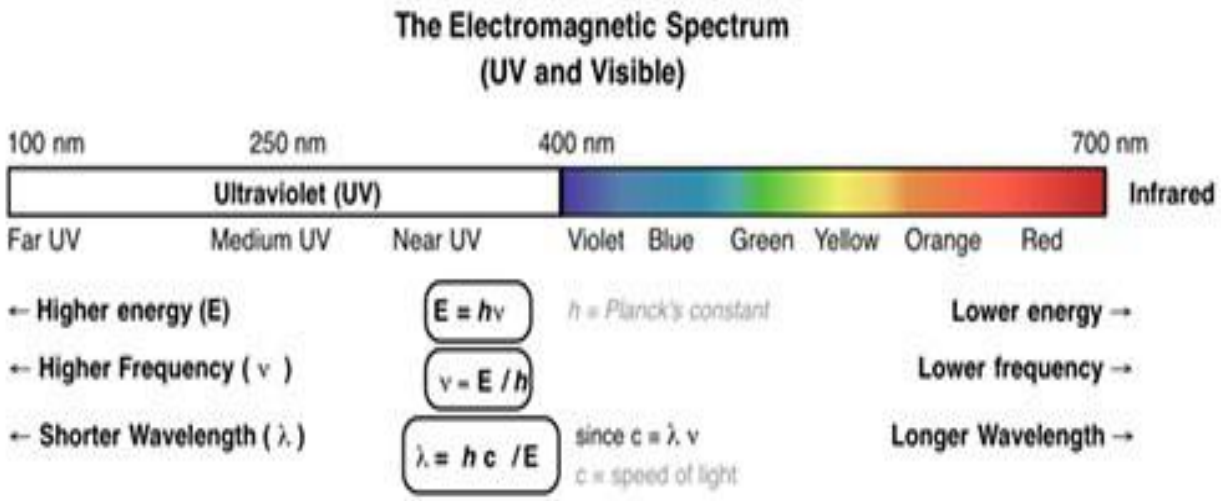

Figure 3: Range of wave lengths of spectrophotometers

1.4. Principle of spectrophotometer:

The spectrophotometer technique is to measure light intensity as a function of wavelength. It does this by diffracting the light beam into a spectrum of wavelengths, detecting the intensities with a charge-coupled device, and displaying the results as a graph on the detector and then on the display device.

In the spectrophotometer, a prism (or) grating is used to split the incident beam into different wavelengths.

By suitable mechanisms, waves of specific wavelengths can be manipulated to fall on the test solution. The range of the wavelengths of the incident light can be as low as 1 to 2nm.

The spectrophotometer is useful for measuring the absorption spectrum of a compound, that is, the absorption of light by a solution at each wavelength (Figure 4).

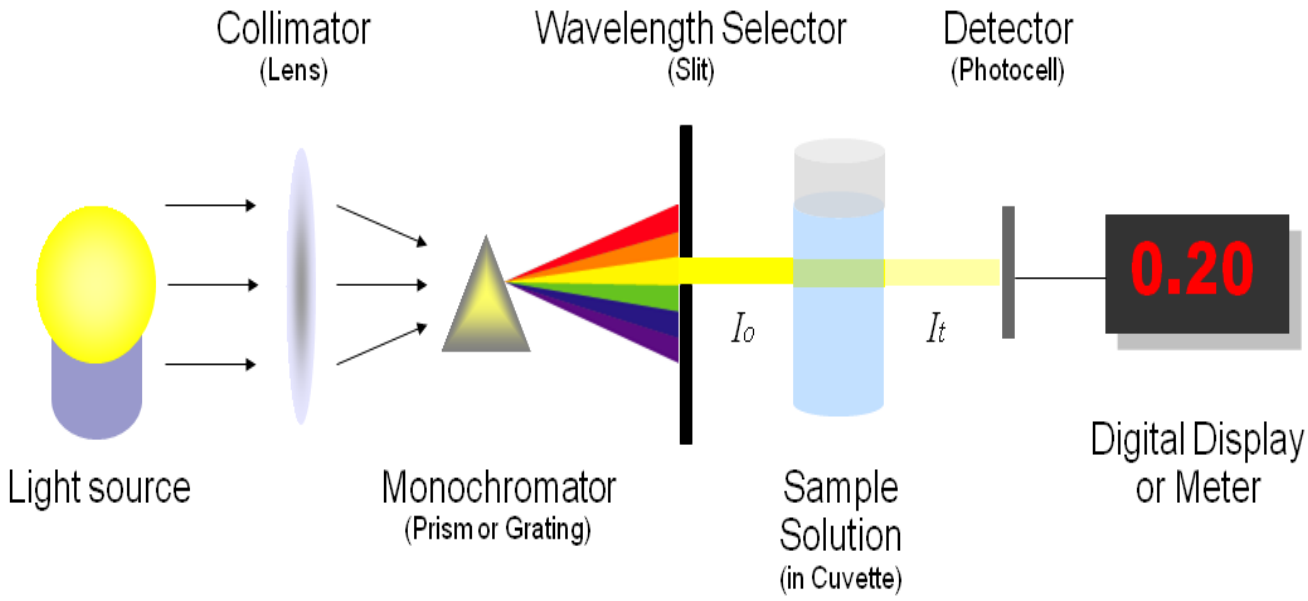

Figure 4: Principle of the spectrophotometer

1.4.1. **Beer-Lambert law:**

The relationship between absorbance and solute concentration is governed by the Beer-Lambert law, a fundamental principle in spectrophotometry. This law states that the absorbance of a solution is directly proportional to the path length (l) of the light through the solution and the concentration (c) of the absorbing solute. Mathematically, it is expressed as:

$A = \varepsilon \times c \times l$

where ε is the molar extinction coefficient, a characteristic property of the absorbing solute at a specific wavelength.

The intensity of incident light is symbolized as (I0) which is the original light or "total light". When the light passes through a blank solution, it is only minimally absorbed and is symbolized as transmitted light (It). There are important values which are Transmittance (**T)** Absorbance (**A).**

$$\mathbf{T} = \frac{\mathbf{I_t}}{\mathbf{I_0}}$$

$$\mathbf{A} = -\log_{10} \mathbf{T}$$

It = the intensity of light after passing through the cuvette.

I0= the intensity of light before passing through the cuvette.

In spectrophotometry, we need to measure the intensity of light that crossed a blank solution, and then measure the intensity of light that crossed or passed through a sample.

- ✓ **Calculation of Transmittance and Absorbance**

The number of photons transferred or absorbed totally is dependent on the concentration of the sample and the length of the cuvette .

The transmittance and absorption relation is:

Absorbance (A) = −log (T)

–log (T)= - log (lt/l0)

1.4.2. Conditions for the validity of the Beer-Lambert law:

The Beer-Lambert law, a fundamental principle in spectrophotometry, establishes a direct relationship between the absorbance of a solution and the concentration of the absorbing solute. However, for this relationship to hold true, certain conditions must be met.

A. **Monochromatic light:**

The light source used in spectrophotometry must emit monochromatic light, meaning light of a single, defined wavelength. This ensures that the absorbance is measured at a specific wavelength, allowing for accurate determination of solute concentration.

B. **Moderate concentration:**

The concentration of the absorbing solute should not be too high. Typically, a concentration range of around 10^{-2} mol/L is considered appropriate. At higher concentrations, interactions between solute molecules can lead to deviations from the Beer-Lambert law.

C. **Non-fluorescent solutions:**

The solution being analyzed should not exhibit fluorescence, a phenomenon where some absorbed light is re-emitted in all directions by the solute molecules. Fluorescence can

interfere with the measurement of absorbance, affecting the accuracy of concentration determination.

D. **Preserved chemical equilibrium:**

If the solute is involved in a chemical equilibrium, dilution for analysis should not alter the equilibrium position. For instance, in a solution containing $Cr_2O_7^{2-}$ (orange) and H_2O, dilution should not cause a significant shift towards the formation of $HCrO_4^-$ (incolore en milieu acide), as this would affect the absorbance.

E. **Clarity of the solution:**

The solution being analyzed should be clear and free of any precipitates or turbidity. Suspended particles can scatter light, causing deviations from the Beer-Lambert law and affecting the accuracy of absorbance measurements.

By adhering to these conditions, spectrophotometry can provide reliable and accurate measurements of solute concentration, making it a valuable tool in various scientific fields.

I.5. Spectrophotometer:

Spectrophotometer is an instrument that measures the intensity of light after it passes through a sample solution. Depending on the range of wave length of the light source, spectrophotometers can be classified into two different types :

UV range spectrophotometer: Uses light over the ultraviolet range, and wave length ranges between 185 - 400 nm.

Visible range spectrophotometer**:** Uses a tungsten light range and wave length ranges between 400 - 700 nm.

Note: Notation of wavelength is Lambda (λ)

Nanometer: It is the unit of measuring wavelength in nano-meter (nm = 1*10-9 m).

1.5.1. The essential components of spectrophotometer:

Spectrophotometer consists of (Figure 5):

A. Light source: It can be two kinds:

• Tungsten lamp ; produces light at visible region.

• Hydrogen lamp; produces light at ultraviolet region.

B. Collimator: It is a focusing device that transmits an intense straight beam of light.

C. Monochromator: It is a device that divides the light beam into it's component wavelengths.

D. Selector: It selects the required wavelength.

E. Cuvette: It is a compartment in which the sample is placed. There is two kinds:

• Glass cuvettes; used in the visible region.

• Quartz cuvettes; used in the ultraviolet region.

• The glass cuvettes absorbs light in the ultraviolet region ..

Thus the amount of light measured by spectrophotometer will be the absorbance of sample + the glass cuvette.

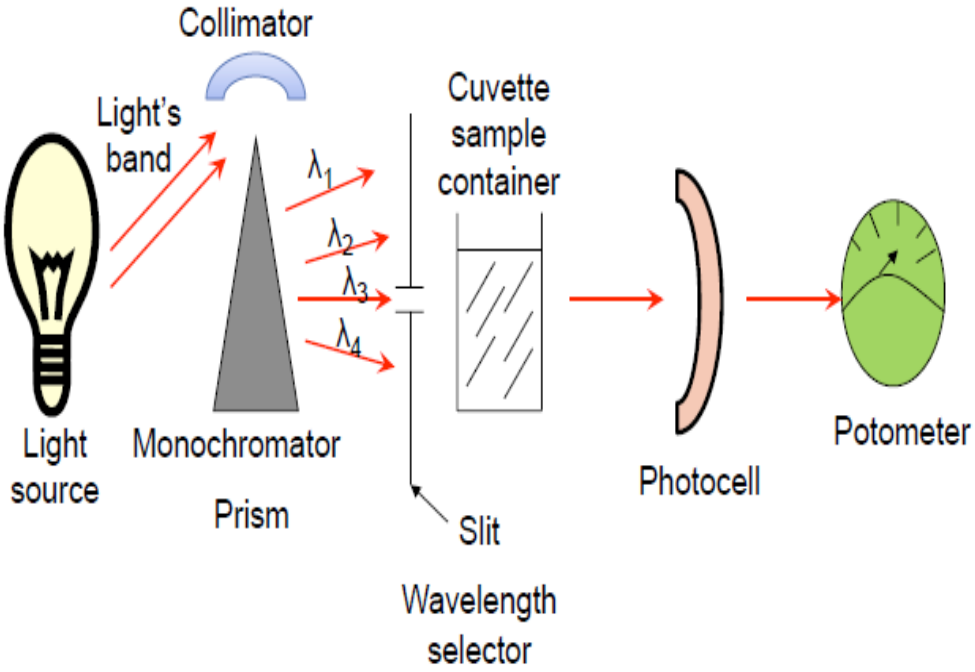

Figure 5: The components of the spectrophotometer.

1.6. Applications:

Spectrophotometry's power lies in its ability to analyze light-matter interactions. Here's a glimpse into its diverse applications:

- ✓ **Chemical Analysis:** Identify unknown substances and quantify components in mixtures.
- ✓ **Biomolecular Science:** Study structure and function of biomolecules, and determine their concentrations.
- ✓ **Environmental Monitoring:** Detect and quantify pollutants, and assess water quality.
- ✓ **Clinical Chemistry:** Analyze blood components for diagnosis and monitor drug effectiveness.
- ✓ **Food & Pharmaceutical Industries:** Ensure quality and safety of products and monitor drug development.

2. Atomic absorption spectrophotometry:

2.1. Definition:

Atomic absorption spectrometry studies the absorption of light by the free atom. It is one of the main techniques involving atomic spectroscopy in the UV-visible range used in chemical analysis. It allows the determination of about sixty chemical elements (metals and non-metals). Flame atomic absorption spectrometry allows the mono-elemental determination of major cations in the order of mg/L in liquid samples.

Each element has a specific number of electrons associated with its nucleus. The normal and most stable orbital configuration of the electrons is called the ground state. When energy is supplied to an atom, the latter absorbs it and adopts an electronic configuration called an excited state. This state is unstable and the atom immediately returns to its ground state, thus releasing light energy.

2.2. Principle:

Flame atomic absorption is a method that allows the determination of mainly metals in solution. This elemental analysis method requires that the measurement be made from an analyte (element to be determined) transformed into a state of free atoms. The sample is heated to a temperature of 2000 to 3000 degrees to destroy the chemical combinations in which the elements are engaged. Atomic absorption spectrometry is based on the theory of quantization of the energy of the atom. Its energy varies as one of its electrons passes from one electronic orbit to another. Generally, only the external electrons of the atom are concerned (Figure 6 and 7).

The absorbed photons being characteristic of the absorbing elements, and their quantity being proportional to the number of atoms of the absorbing element, the absorption allows the measurement of the concentrations of the elements to be determined. Atomic absorption analysis uses the Beer-Lambert law. If there are several elements to be determined, this manipulation is carried out for each element of the sample by setting a fixed wavelength. It is therefore necessary to choose a suitable source for each manipulation to illuminate the element that one wishes to excite.

- The sample is reduced to atomic vapor.

- The atoms in the ground state absorb the specific radiation.

- The absorbance is proportional to the quantity of atoms of the element to be determined.

In AAS, the atomic vapors are obtained by:

- Atomization by nebulization in a flame

- Electrothermal atomization

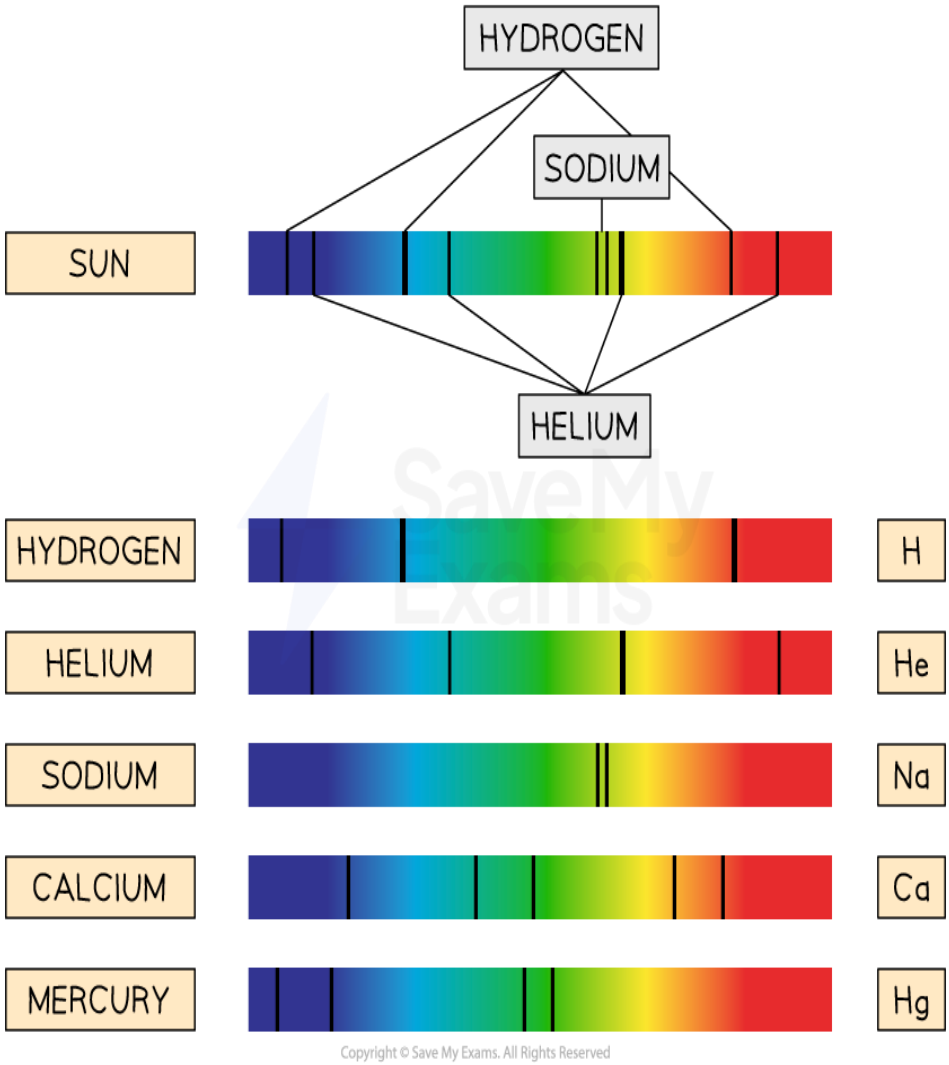

Figure 6: Atomic Absorption Spectra of the chemical composition of the Sun

Note: The dark lines on the absorption spectrum of the Sun tell us about its chemical composition, which is that it contains hydrogen, helium and sodium, but not calcium or mercury. By comparing the absorption spectra of a star to the spectra of known elements here on Earth, astronomers can determine the chemical composition of the star. For example, the absorption spectrum of the Sun tells us that it is predominantly made of hydrogen and helium gas

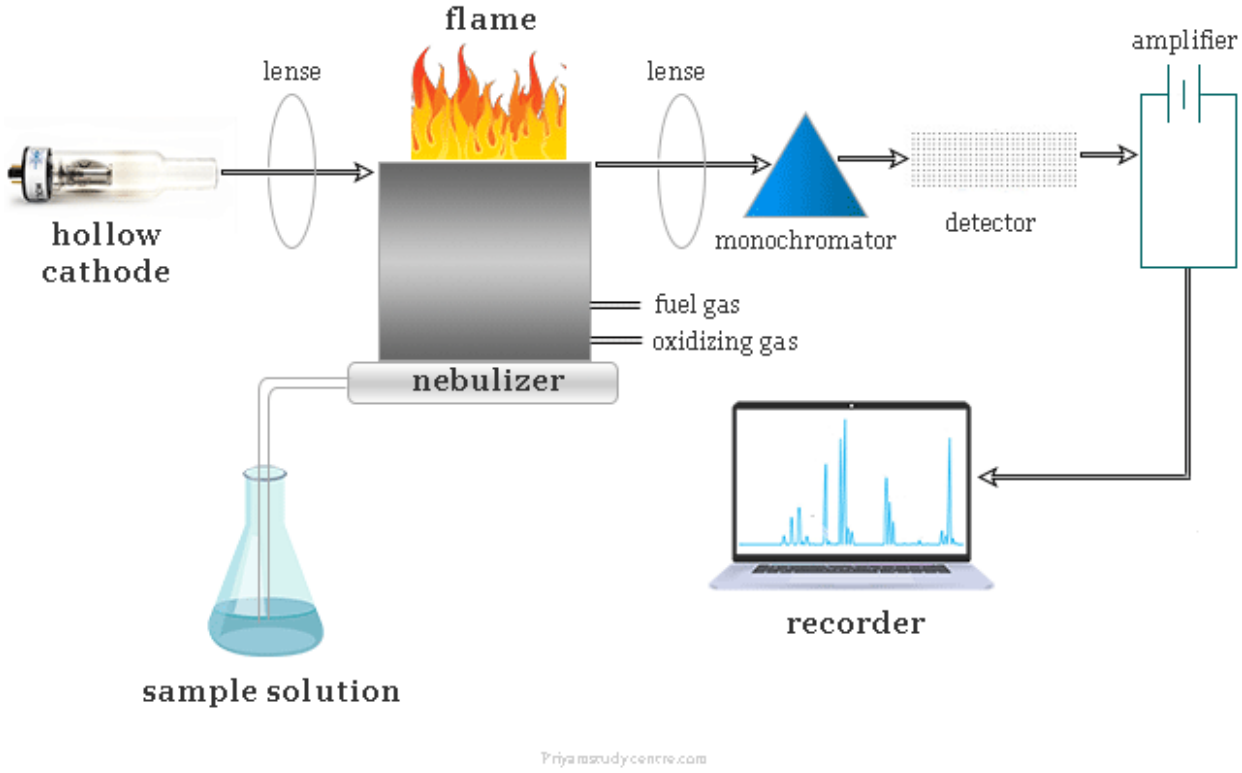

Figure 7: The atomic absorption spectroscopy equipment

2.3. Atomic absorption spectroscopy flame:

Fuel and an oxidant are used to create an atomic absorption spectroscopy flame. Generally, natural gas, propane, butane, hydrogen, and acetylene are employed as fuels to generate a flame. Air, oxygen, nitrous oxide, and a mixture of nitrous oxide and acetylene are used as oxidants for flame creation in atomic absorption spectroscopy.

A table listing various flames with their maximum temperatures is provided below.

Table 1: Atomic absorption spectroscopy flame

Atomic absorption spectroscopy flame			
Fuel	Air oxidant	Oxygen oxidant	Nitrous oxide oxidant
Hydrogen	2100 °C	2780 °C	–
Acetylene	2200 °C	3050 °C	2955 °C
Propane	1950 °C	1950 °C	–

Low temperatures are used for metals like copper (Cu), lead (Pb), zinc (Zn), and

cadmium (Cd). These metals are easily vaporized at low temperatures. Some metals are not easily atomized or vaporized. Therefore, a high temperature is required for the vaporization of such metals. Such high temperatures can be attained by using an oxidant in the flame along with fuel gas in atomic absorption spectroscopy.

For example, an oxyacetylene flame was used for the analysis of aluminum, titanium, and rare earth elements in an AAS instrument.

2.4. Hollow cathode lamp:

A continuous source of radiation is required for atomic absorption spectroscopy (AAS) instruments. However, the extreme narrowness of the absorption lines in these sources causes issues. To address this, a hollow cathode glow discharge lamp is used to provide sharp emission lines for the specific element being analyzed.

The hollow cathode lamp has two electrodes, with the cup-shaped cathode made of the element being determined. To avoid spurious radiation, the lamp output is either mechanically chopped or electrically pulsed.

The metal used in the cathode matches the element being analyzed. The lamp is filled with a noble gas at low pressure, which creates a glow discharge emission from the hollow cathode.

Multiple types of hollow cathode lamps are available, including multi-element hollow cathode lamps. These versatile lamps allow analysis of different samples without having to change lamps, and are widely used in atomic absorption spectroscopy instruments.

2.5. Application of atomic absorption spectroscopy:

Today, the atomic absorption spectroscopy technique is the most powerful tool in analytical chemistry, forensic science, environmental analysis, and food industries. It is popular for analysts due to several advantages. The most important advantage is the speed of analysis. It can analyze various samples within a day.

Secondly, it is possible to determine all elements at trace concentration.

Thirdly, it is not always essential to separate the element before analysis because AAS can be used to determine one element in presence of another.

The atomic absorption spectroscopy principle or instrumentation can be used to analyze sixty-seven metals and several nonmetals such as phosphorus and boron.

3. Atomic emission photometry:

3.1. Definition and principle:

Atomic emission spectroscopy (AES) is a chemical analysis technique that examines samples through the electronic transitions of atoms, utilizing flame or argon plasma sources. The excitation source affects the intensity of the emission observed. It provides enough energy to vaporize the sample and induce electronic excitation in the gaseous atomic particles. As a result, the spectra obtained are either band spectra or line spectra.

In atomic emission spectroscopy, measurements are possible because each spectral line corresponds to a specific wavelength. Recently, we have employed inductively coupled plasma atomic emission spectroscopy for sample analysis.

Electrons within an atom can exist at a stable energy level known as the ground state or lowest energy state. When energy is added, one or more electrons are excited to higher energy levels. As these excited electrons return to their ground state, they release energy in the form of radiation (Figure 8) .

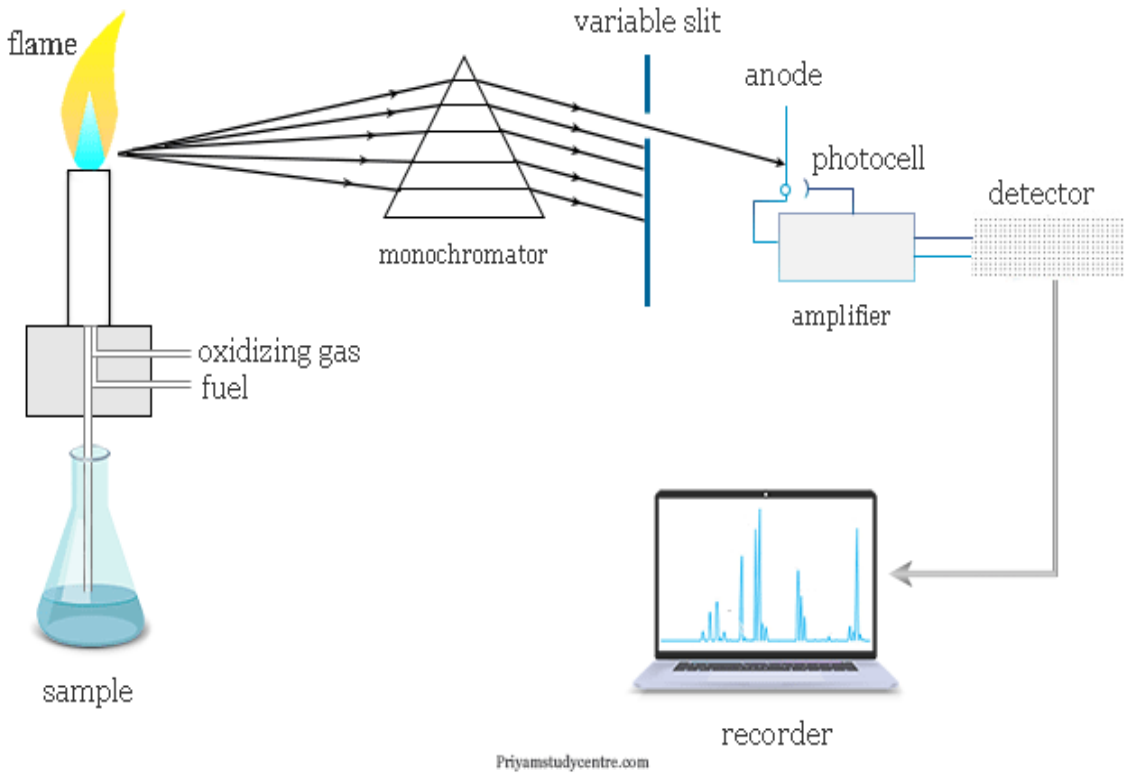

Figure 8: Atomic emission spectroscopy equipment

3.2. Atomic emission spectra:

In atomic emission spectroscopy, the flame in the spectrometer converts solid or liquid samples into a vaporized state and breaks them down into simpler molecules or atoms.

The flame then excites the electrons in these atoms to higher energy levels. As the excited electrons return to their ground state, they emit radiation. The process involves evaporating any water or solvent, leaving behind dry salts in the flame.

With additional heating, the dry salts are vaporized, and the molecules are dissociated into neutral atoms, which are responsible for the emission phenomena.

The vaporized neutral metal atoms are excited by the thermal energy of the flame, leading to the emission of characteristic spectra with specific wavelengths. Typically, line spectra are observed from atoms or ions, while molecules produce band spectra.

3.3. Flame emission spectroscopy:

Flame emission spectroscopy or flame photometry involves excitation by bringing the gaseous sample into flame or spraying the sample into flame or directly inserting by use of a small loop of platinum wire. Radiation from flame enters a dispersing device to isolate the desired region of the spectrum.

Each element emits light with a characteristic wavelength. It can be dispersed by a grating or prism and detected by a spectrometer. When sodium atomic ions emit light in a flame, they display a brilliantly bright yellow emission. Similarly, potassium characteristically colours the flame lilac and strontium colours it red (Figure 9).

Figure 9 : Characteristic flame colorations of atomic ions

3.4. Inductively coupled plasma emission spectroscopy (ICP):

Atomic emission spectrometry (ICP-AES), sometimes termed OES – optical emission spectrometry, is a process whereby the light energy emitted by atoms or ions is measured and used to calculate the concentration of the element of interest. The emission occurs when thermal or electrical energy is available to excite a free atom or ion to an unstable energy state – this is

what happens in the plasma – and the light energy is then released when the atom or ion returns to its stable ground state as it passes through the plasma.

The plasma is generated by a radio frequency field and a highly ionised gas, usually argon, and the temperature in the plasma is in the region of 10,000°C . This temperature allows complete atomisation of elements, thus minimising chemical interference effects. In addition, no primary light source is required, and up to 30 elements can commonly be determined in one run of about three to four minutes per sample (Figure 10) .

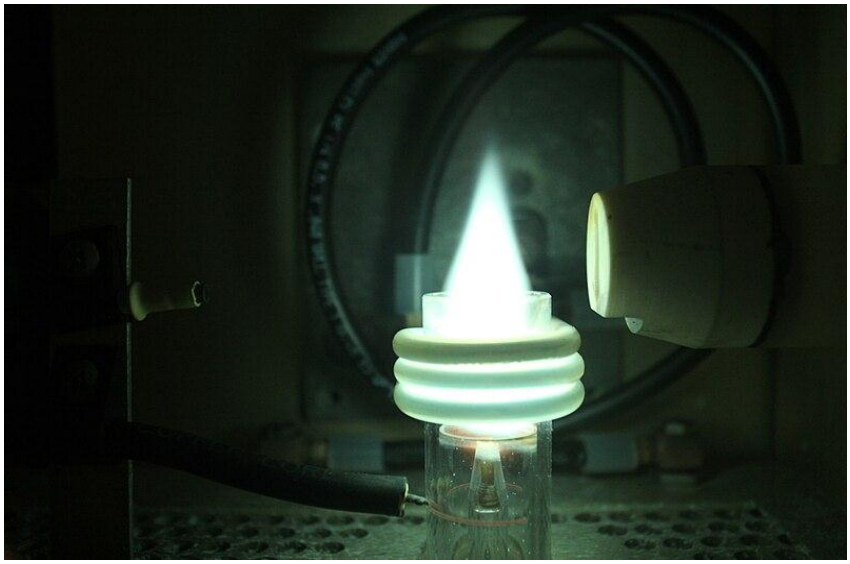

Figure 10: Inductively coupled plasma atomic emission source

3.5. The difference between atomic absorption and emission phenomena:

Atomic absorption spectrometry (AAS) and atomic emission spectrometry (AES) are two widely used techniques for the analysis of more than 70 elements, sometimes at trace levels .

- **Atomic absorption** is the phenomenon observed when an atom in the ground state absorbs an electromagnetic radiation at a specific wavelength and is promoted to an excited state (Figure 11). This results in a spectrum of dark lines on a bright background (Absorption spectrum).

❖ **Atomic emission** is the phenomenon observed when an electromagnetic radiation is emitted by excited atoms or ions returning to the ground state. This results in a spectrum of bright lines on a dark background (Emission spectrum) (Figure 12) (Table 2).

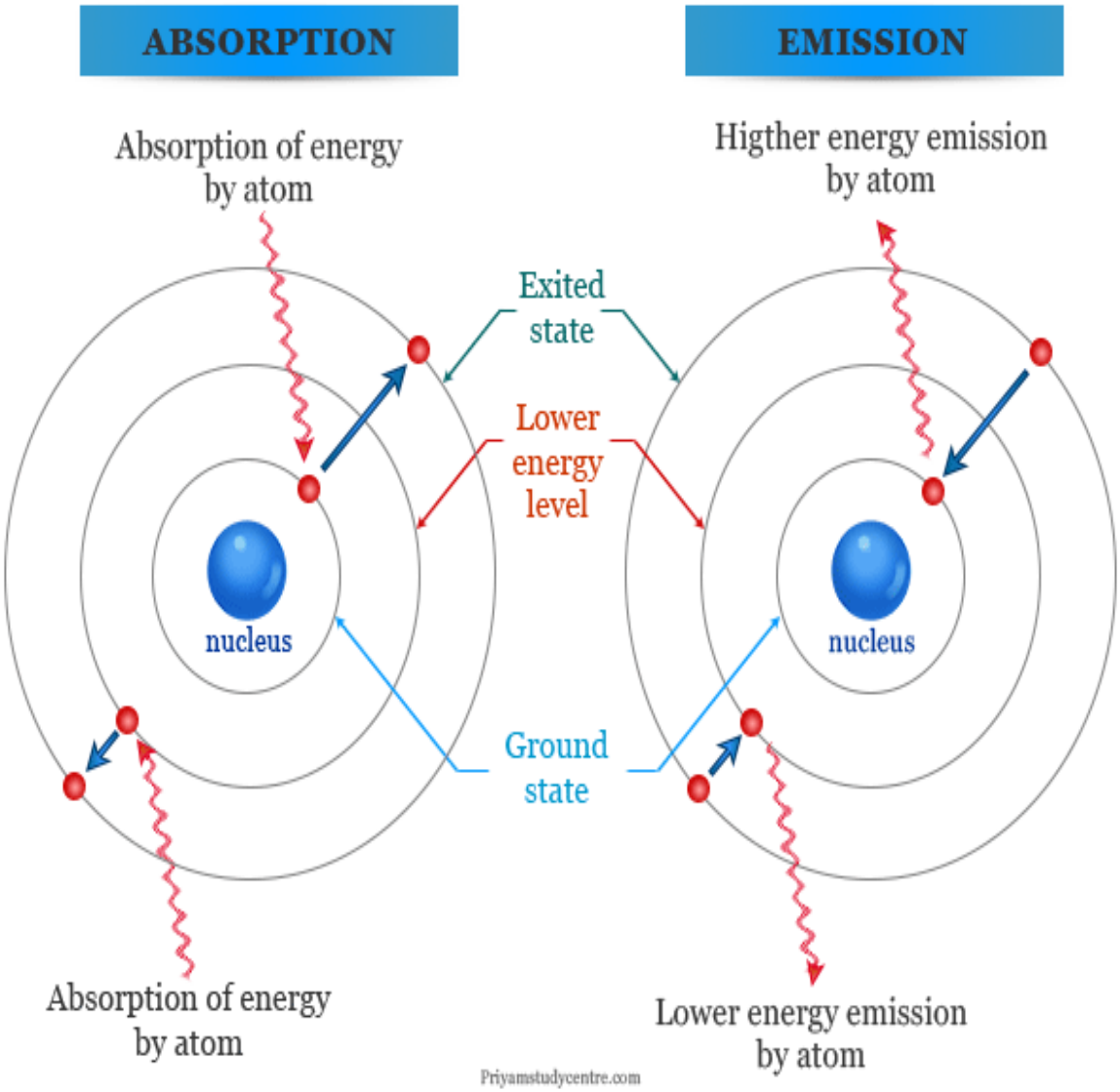

Figure 11 : Differences between absorption and emission spectroscopy

Important: If excitation energy is large then the emitted energy is also large. It forms several lines for atomic emission spectroscopy measurement.

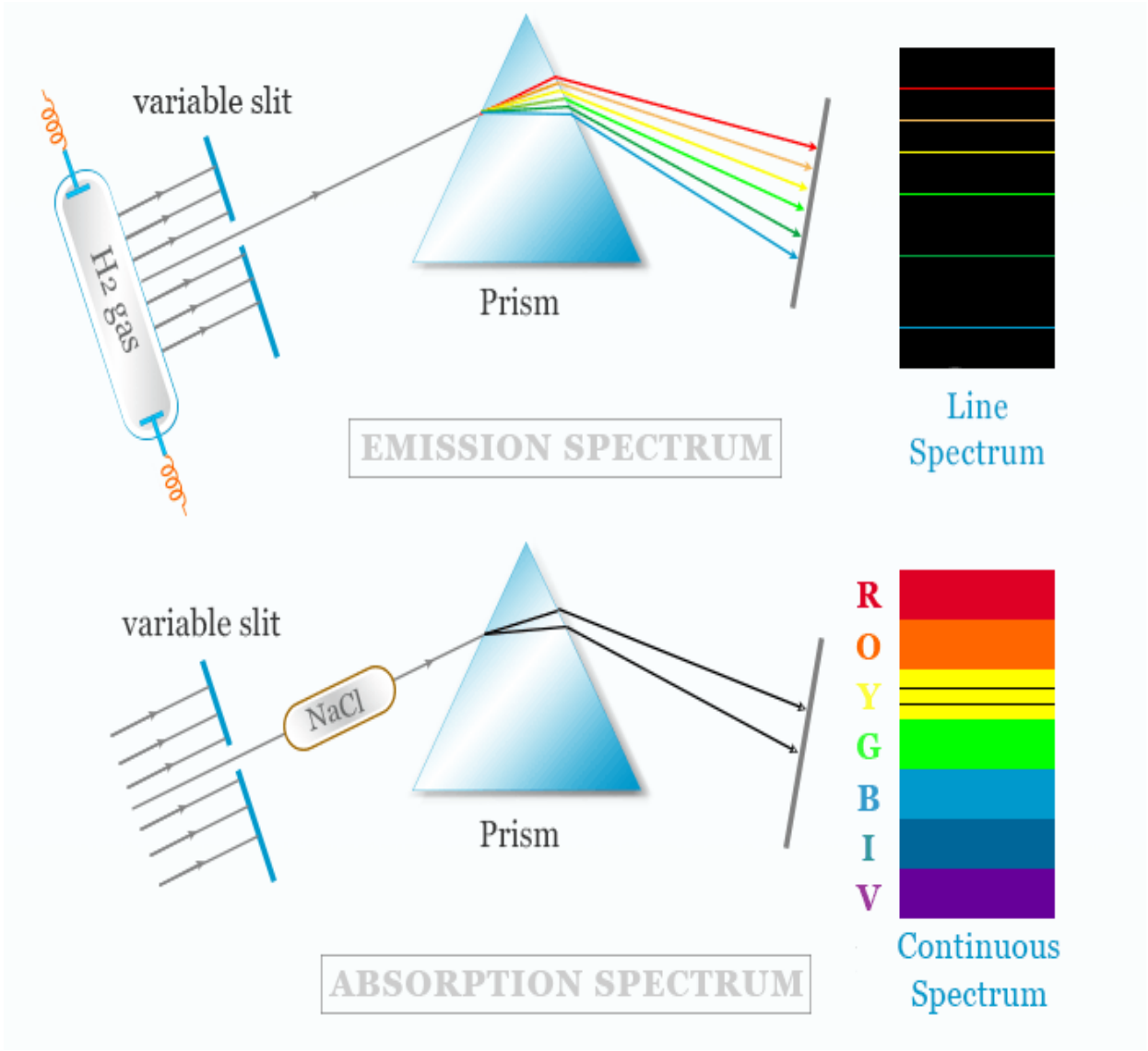

Figure 12: Differences between absorption and emission spectrum

Both techniques involve free atoms in the vapor state. The instrumentation will therefore produce an atomic vapor from the sample, which induces the destruction of the molecule to be analyzed, thus making it possible to simultaneously determine all forms of the same element.

The two techniques both involve free atoms in the vapor state, but they differ in the way the atoms interact with electromagnetic radiation:

- In atomic absorption, the atoms in the ground state absorb specific wavelengths of light, which is observed as dark lines in the spectrum.

- In atomic emission, the excited atoms emit specific wavelengths of light, which is observed as bright lines in the spectrum.

The key difference is that absorption measures the reduction in light intensity as atoms absorb light, while emission measures the light intensity emitted by excited atoms.

Table 2 : Atomic absorption vs atomic emission spectroscopy

Atomic Absorption Spectroscopy (AAS)	Atomic Emission Spectroscopy (AES)
Atomic absorption spectroscopy is used to find the concentration of metals atom in a solution	Atomic emission spectroscopy is used to find out the concentration of the analyte by emission of light
A fixed amount of energy is absorbed by the electrons of an atom	The discrete energy emitted during de excitation
From ground state to an excited state	From excited state to ground state
Electromagnetic radiation is absorbed	Electromagnetic radiation is emitted
It can not be used for direct analysis of solid samples	It can be used for the direct analysis of solid samples
The spectrum obtained is dark lines are gaps	Colored spectrum is obtained
It requires a light source	It does not require a light source
It depends upon a number of ground-state atoms	It depends upon the number of excited-state atoms

4. Molecular absorption spectrophotometry:

4.1. Definition and principle:

Molecular absorption spectrometry (MAS), originally developed in the 1970s, is a technique to determine non-metals in flames and graphite furnaces by monitoring the absorbance of diatomic molecules.

Molecular spectroscopy deals with the interaction of molecules with electromagnetic radiations. Due to such interaction, a spectrum (absorption pattern) is generated. When molecules interact with various electromagnetic radiations, these particles move from one energy

level to another. This type of motion produces the molecular spectrum. Unlike atoms, molecules show interactions between different electrons and nuclei. Although the mechanisms are quite the same, the process is more complex in the case of molecules (Figure 13) .

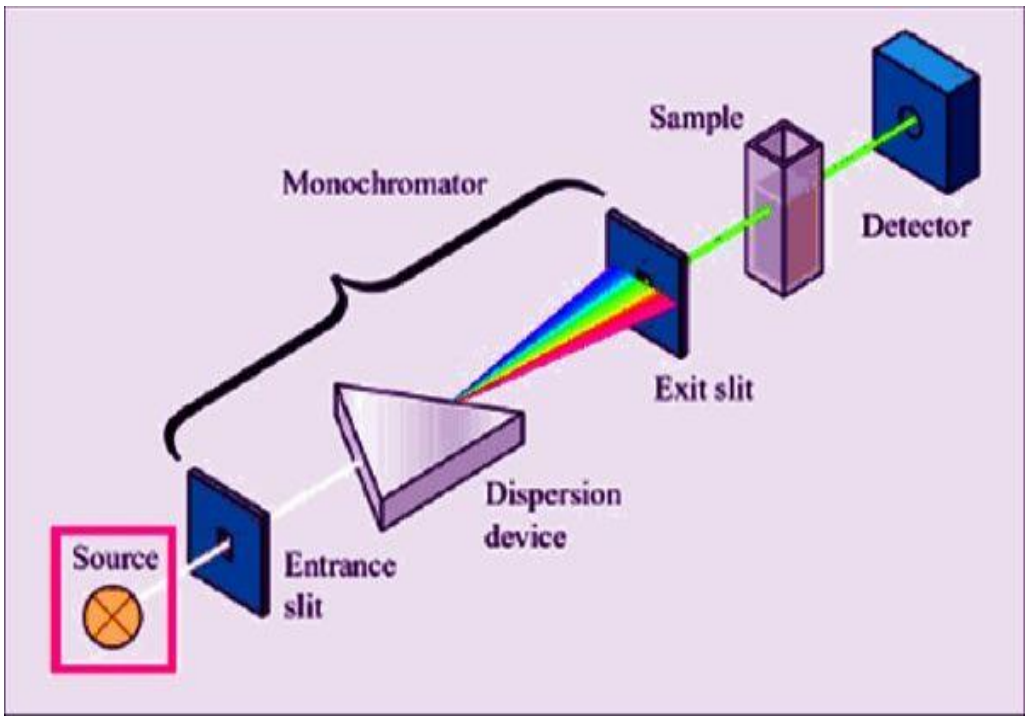

Figure 13: Molecular absorption spectrophotometry

4.2. Comparison of atomic and molecular absorption spectra:

Atomic absorption spectra consist of discrete absorption lines with dark spaces, in contrast to the continuous spectrum seen in white light passing through a prism. These spectra result from the absorption of photons during transitions from lower-energy to higher-energy states.

The narrow width of the absorption lines is determined by the fixed energy difference between ground and excited states, along with other contributing factors. The uncertainty principle governs the natural line widths, approximately 5-10 nm, while additional broadening mechanisms increase the line width to around 3-10 nm.

According to the Born-Oppenheimer approximation rule, a molecule's total energy is the sum of its constituent particles' energies, encompassing translational, vibrational, rotational, and electronic motions.

These motions give rise to molecular absorption spectra, representing transitions to higher energy levels associated with each motion, resulting in distinct spectra for each type of transition. However, translational energy is often disregarded due to its minimal contribution.

Rotational spectra (observed in microwave region) rely on angular momentum and electron spins, vibration spectra (observed in IR and Raman regions) stem from molecular vibrations due to absorbed energy, and electronic spectra (observed in UV to near-IR regions) involve electron transitions between orbitals, with certain constraints.

4.3. Comparison of applications of AAS and MAS :

Atomic and molecular absorption spectroscopy have diverse and overlapping fields of Atomic absorption spectroscopy aids in detecting toxic heavy metals, performing quality control in drug manufacturing, quantifying precious metals in mining operations, and analyzing drinking water for contaminants. It also finds use in analyzing trace metals in various samples, including soils, geological samples, petroleum products, and biological fluids.

On the other hand, molecular absorption spectroscopy provides valuable compositional and structural information about substances, aiding in environmental monitoring, food analysis, forensic investigations, and material characterization. It is particularly valuable in studying biomolecules, detecting biomarkers, and analyzing complex mixtures.

4.4. Comparing the advantages and disadvantages of AAS and MAS :

Atomic absorption spectroscopy offers high accuracy, typically within a range of 0.5% to 5% and sensitivity for elemental analysis, particularly for metals. It has been instrumental in revolutionizing practices in fields like medicine and pharmaceuticals by detecting trace toxins

and previously unknown elements in materials.

Atomic absorption spectroscopy is well-suited for accessing inaccessible places, such as mines, for testing rocks. Modern AAS systems are comparatively inexpensive and can accurately detect specific elements.

However, atomic absorption spectroscopy is limited to metals due to their easily readable atoms and clear absorption lines. In addition, it is a destructive analysis technique as it involves converting the sample into an atomic gas through evaporation and atomization.

On the other hand, molecular absorption spectroscopy provides structural information about molecules and has a wide range of applications. It is non-destructive, efficient, highly sensitive for trace analysis, can analyze organic and inorganic molecules, and has a larger scope of substances.

However, molecular absorption spectroscopy may face challenges due to overlapping absorption bands and lack of specificity, which can hinder accurate quantification and identification of compounds.

The choice between atomic absorption spectroscopy and molecular absorption spectroscopy depends on the specific analytical requirements. AAS is advantageous for elemental analysis and high selectivity for metals, while MAS offers insights into molecular composition and versatility across different substances.

5. Spectrofluorimetry:

5.1 Photoluminescence, fluorescence and phosphorescence:

Photoluminescence is light emission from matter after the absorption of photons. There are two types: fluorescence and phosphorescence. Fluorescence is relatively intense and fast, with timescales on the order of picoseconds (ps) to nanoseconds (ns). In contrast, phosphorescence is weaker and slower, with timescales on the order of microseconds (μs) or longer. Both fluorescence and phosphorescence are redshifted compared to the excitation wavelength.

The Jablonski diagram, named after Aleksander Jabłoński, is a helpful tool for understand the different types of photophysical processes in molecules and is shown in Figure 5.4 This diagram describes the ground and excited electronic states of molecules, vibrational and rotational levels within both the ground and excited electronic states, the electron spin properties of these states, and the types of possible transitions between all of these states **(Figure 14).**

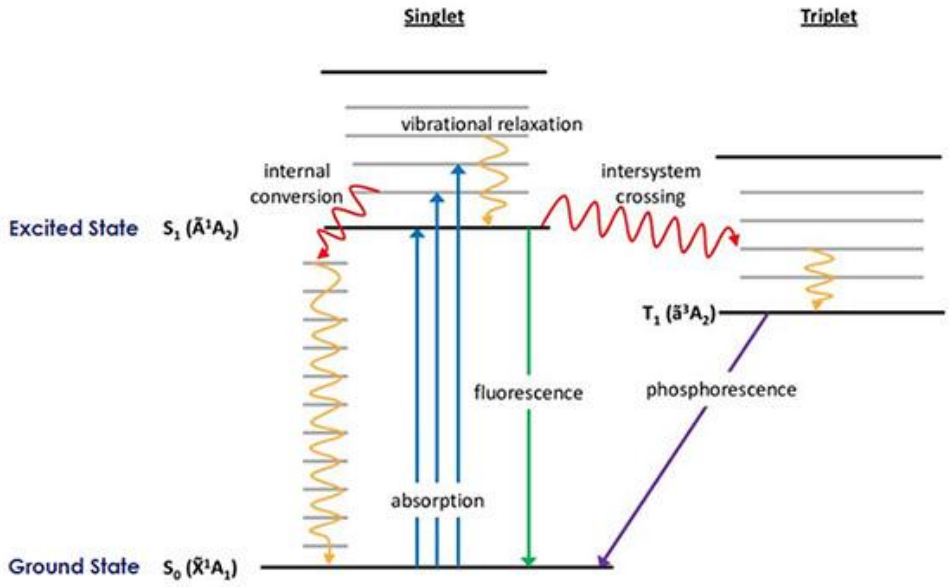

Figure 14: The Jablonski diagram

Fluorescence occurs when an electron in an excited electronic state relaxes to the ground electronic state by emitting a photon. This happens when both the ground and excited states are singlet spin states.

What can also happen is that an electronically excited electron in a singlet state can undergo an intersystem crossing (IC) into an electronically excited triplet spin state. The relaxation of an electron in an excited triplet state to a ground state by direct photon emission is quantum mechanically forbidden. However, this type of transition can and does happen due to spin-orbit coupling, and this process is the origin of phosphorescence**.**

5.2. Definition and principle of spectrofluorimetry:

Fluorescence spectroscopy or fluorimetry or spectrofluorimetry is a technique to detect and analyze the fluorescence in the sample. Fluorescence is the emission of light by a substance (fluor) that has absorbed light or other electromagnetic radiation. In this emission phenomenon, a beam of light (usually UV light) excites the electron in a molecule which moves from ground state to higher energy excited state. When the electron falls back to the ground state, it emits fluorescence. Fluorescence spectroscopy is mainly concerned with electronic (ground state and excited state) and vibrational states.

In molecular species, energy transition may occur in different vibrational levels of a particular excited state because the energy of the vibrational level of excited state matches with the energy of vibrational level of ground state and therefore in such energy transition, some energy is lost as heat (also known a non-radiative transition) until it reaches the lowest vibrational level of the excited state. After losing some energy as non-radiative transition and reaching the lowest vibrational level of the excited state, the electrons follow radiative transition. Radiative transition occurs when electrons falls back from higher energy excited state to lower energy ground state within the molecule, then energy emitted is measured as

light. Therefore, in most cases, the emitted light has a longer wavelength (lower energy) than the absorbed radiation (higher energy). During radiative transition, the electrons or molecules may descend into any of several vibrational levels in the ground state, as a result the emitted photons will have different energies, and thus different frequencies. It is a form of luminescence when the emitted light is in the visible range. A fascinating example of fluorescence is when the absorbed radiation is in the ultraviolet region (invisible to the human eye) of the electromagnetic spectrum and the emitted light is in the visible region.

Similar phenomenun occurs in some atomic or molecular species. There are some chromophores which are inflexible and rigid molecules and therefore, may have limited range of vibrational energy levels. In such molecules, the vibrational energy level of the excited state often does not overlap with those of the ground state. When chromophores of this type absorb light, it is not possible for them to return to the ground by simply losing their excess energy as heat. Instead, they undergo a radiative transition in which the absorbed energy is reemitted as light with the same frequency. This process of re-emitting the absorbed photon is “resonance fluorescence” and this is seen in molecular fluorescence.

A schematic diagram of a fluorescence spectrometer is shown in Figure 15. A light source, which can be either a broadband light source or a narrow-band laser, is focused by a lens onto a sample where electrons in the sample are excited from the ground state to the excited state. When the electron relaxes from the excited state to the ground state by emitting a photon, the emitted fluorescence is collected by placing a lens, usually at a 90o angle from the excitation path to minimize the background from the excitation source, and focused into a monochromator where it is detected. The data is then sent to a recorder, where the measured fluorescence is reported as the intensity of the light emitted as a function of the wavelength of the emission.

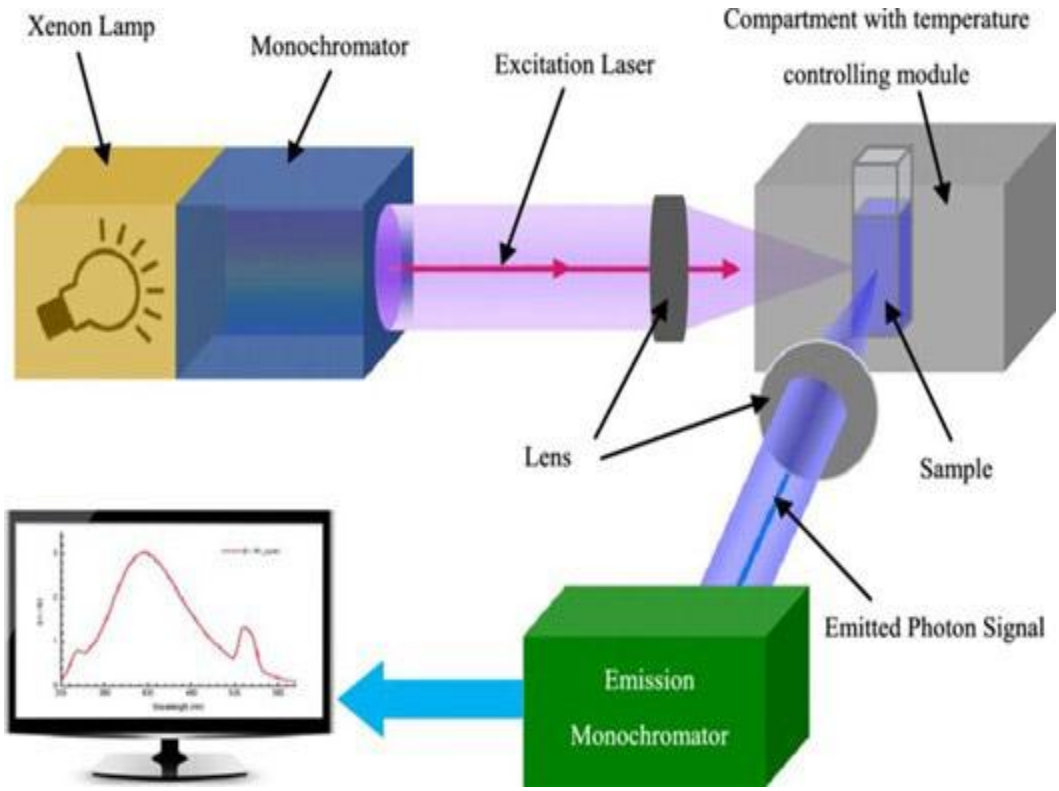

Figure 15: Schematic diagram of a fluorescence spectrometer.

5.3. Stokes shift:

Stokes shift is named after Irish physicist George G. It is the difference between the avelength of absorption maxima and the emission maxima.

Wavelength of absorbed radiation (having low wavelength units and higher energy) is enoted by aWavelength of emitted (fluorescence) radiation (having higher wavelength units values and lower energy is denoted by b

Stokes shift = b-a.

Good results are achieved with the compounds having the greater Stokes shift. Greater the Stokes shift, lesser will be the interference as the excitation and the emission spectra do not overlap.

Chromophores which exhibit the phenomenon of fluorescence are called fluors or

fluorophores. Fluorophores are organic molecules of 20-100 Daltons. Fluorescent molecules absorb the electromagnetic radiation in visible region and emit the radiation at a higher wavelength in the visible. Example: ethidium bromide (493 nm/620 nm). Most commonly fluorescent molecules absorb the electromagnetic radiation in the UV range and emits in visible range. Example: green fluorescent protein (360 nm/508 nm).

- ✓ **Intrinsic fluors:** The native compound exhibits the property due to the presence of aromatic groups in amino acid side-chains in the case of proteins for example tyrosine, tryptophan and phenylalanine. Cofactors such as FMN, FAD and NAD also exhibit fluorescence.
- ✓ **Extrinsic fluors:** Non-fluorescent compounds can be detected by coupling a fluorescent probe (or fluor). Examples are 1- Anilino-8-naphthalene suphonate, fluorescein (for protein), ethidium bromide and acridine orange (for DNA).

Fluors have characteristics emission spectrum (fluorescence) or as well as characteristic absorbance spectrum which depends upon its structure and chemical environment.

•Most electrons will occupy the ground state and lowest vibrational level (S0V0) at room temperature.

•Electrons are elevated to the high energy excitation state S1, S2, etc by the absorption of photons provided by the electromagnetic radiation. The excitation occurs in less than 10-15 s

•The life time of excited state is very short, ranging from 0.5 to 8 ns (0.5 to 8 x 10-9 s) or in some cases it may range up to 2s (this situation can arise as a consequence of a phenomenon associated with electrons called magnetic spin)

•Non radiative transition of electrons leads to a rapid loss of energy in the form of heat. This occurs by the collision degradation resulting in the lowest vibrational energy in the lowest

excited state (S1V0).

•Electrons after reaching the lowest vibrational level of the excited state return to the ground state in less than 10-8 s and the emitted energy is stated as fluorescence.

•In fluorescence emission measurement, the excitation wavelength is fixed but the detection wavelength varies. In fluorescence excitation measurement, the detection is fixed but the emission wavelength varies (Figure 16).

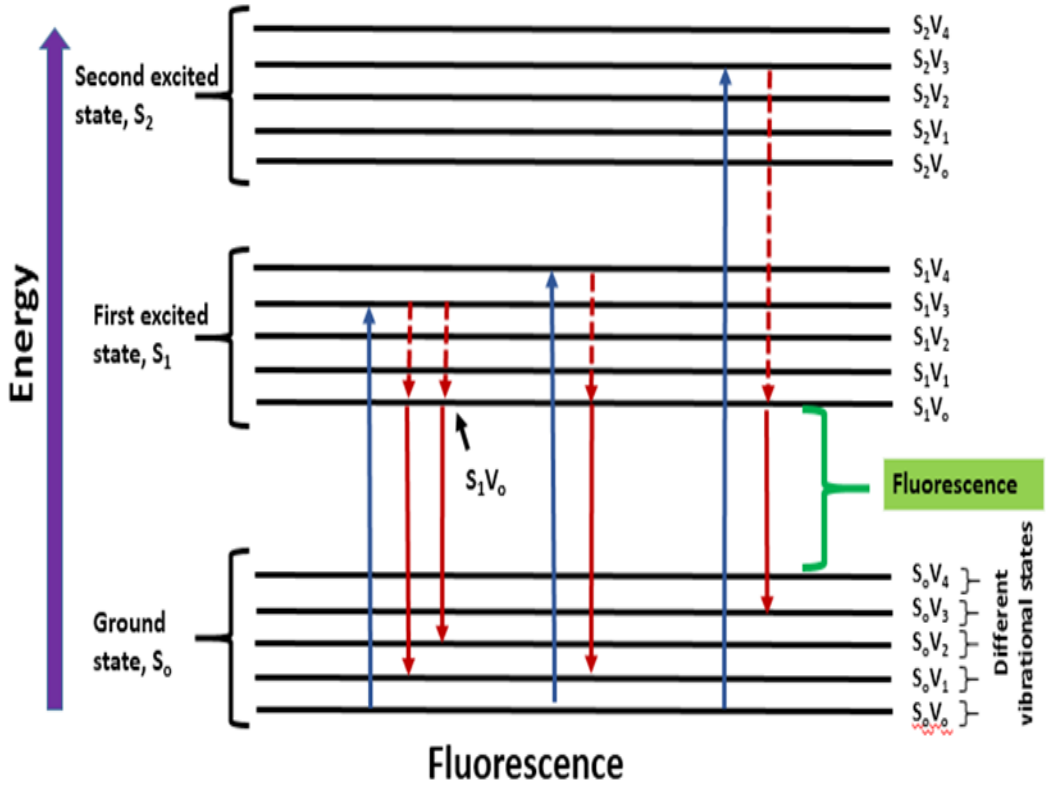

Figure 16: The principle of fluorescence

5.4. Instrumentation:

A. **Light source:** Mercury lamp emits light near peak wavelengths. Xenon arc exhibits continuous emission spectrum with constant intensity in 300-800 nm range, but can also used for justabove 200 nm.

B. **Monochromators:** Most common type of monochromators utilizes a diffraction grating. Two monochromators are used. One monochromator (excitation monochromator) is used for the selection of the excitation wavelength from incident beam. Fluorescent sample will emit the fluorescence in all the directions. Angle of 90° is chosen for emitted florescence and second monochromator (emission monochromator) is used for determination of fluorescence spectrum. The excitation wavelengths which are frequently being selected are in the ultraviolet region and the emission wavelength is in the visible region.

C. **Detector:** Detector can be a single-channeled (detects the one wavelength at a time) or multi- channeled (detects all emitted wavelength) both having advantages and disadvantages. Detector is a sensitive photocell, (eg: red sensitive photomultiplier for wavelengths greater than500nm) (Figure 17) .

There are two setup for the illumination of the sample :

(i) 90° illumination (as discussed above)

Pre-filter effects arises due to the absorption of radiation before reaching to the fluorescent molecule and **post-filter effects** arises due to the decrease in fluorescence emitted by the fluorescent molecule before escaping the cuvette. These effects increase with the increase in sample concentration. The use of microcuvettes alleviates this effect to some extent.

(ii) Front face illumination: This type of illumination setup removes pre and post-filter effects. In front face illumination, cuvette with one optical face is used and the excitation and the emission occur at the same face. This set up is less sensitive than 90° illumination.

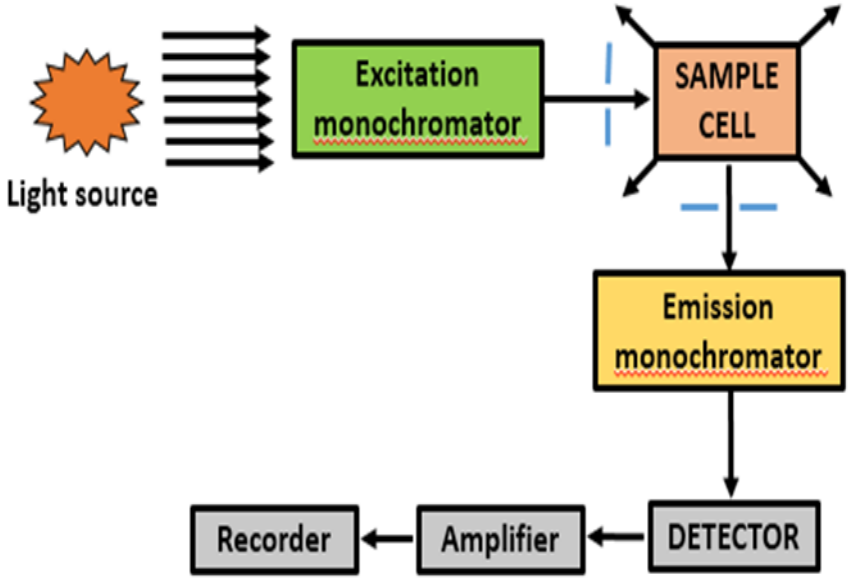

Figure 17: The components of the spectrofluorometry

5.5. Applications of spectrofluorometry:

Spectrofluorometry finds widespread applications in various biological fields:

- **Identifying Biomolecules:** Unique fluorescence spectra allow for the identification of specific biomolecules like proteins, nucleic acids (DNA and RNA), and vitamins.
- **Studying Protein Structure and Interactions:** Fluorescence can be used to label specific amino acids within proteins, providing insights into protein folding, interactions with other molecules, and conformational changes.
- **Quantifying Biomolecules:** The intensity of fluorescence emission can be correlated with the concentration of fluorescent molecules in a sample, enabling researchers to quantify biomolecules for various assays.
- **Cell and Tissue Imaging:** Fluorescence microscopy utilizes fluorescent probes to visualize specific molecules within living cells and tissues, aiding in cell biology research and disease diagnosis.

- **Environmental Monitoring:** Certain pollutants possess fluorescence properties, allowing spectrofluorometry to be used for their detection and monitoring in environmental samples.

6. Nuclear magnetic resonance:

6.1. Definition:

Nuclear Magnetic Resonance (NMR) spectroscopy, also referred to as Magnetic Resonance Spectroscopy (MRS), is a spectroscopic technique used to observe the local magnetic fields around atomic nuclei.It is a spectroscopy technique that is based on the absorption of electromagnetic radiation in the radiofrequency range of 4 to 900 MHz by the nuclei of atoms.

Over the past fifty years, NMR has become the preeminent technique for determining the structure of organic compounds. Of all the spectroscopic methods, NMR is the only one for which a complete analysis and interpretation of the entire spectrum is typically expected.

6.2. Principle of nuclear magnetic resonance:

The principle behind NMR is that many nuclei have spin and all nuclei are electrically charged. When an external magnetic field is applied, an energy transfer becomes possible between the base energy level and a higher energy level (generally a single energy gap).

The energy transfer takes place at a wavelength that corresponds to radio frequencies, and when the spin returns to its base level, energy is emitted at the same frequency.

The signal that matches this energy transfer is measured in various ways and processed in order to yield an NMR spectrum for the nucleus being analyzed.

6.3. Instrumentation of nuclear magnetic resonance spectroscopy:

- ✓ **Sample holder**

Glass tube with 8.5 cm long, 0.3 cm in diameter.

- ✓ **Permanent magnet**

It provides a homogeneous magnetic field at 60-100 MHZ

✓ **Magnetic coils**

These coils induce a magnetic field when current flows through them

✓ **Sweep generator**

To produce an equal amount of magnetic field pass through the sample

✓ **Radio frequency transmitter**

A radio transmitter coil transmitter that produces a short powerful pulse of radio waves

✓ **Radio frequency receiver**

A radio receiver coil that detects radio frequencies emitted as nuclei relax to a lower energy level

✓ **Read out systems**

A computer that analyses and records the data (Figure 18) .

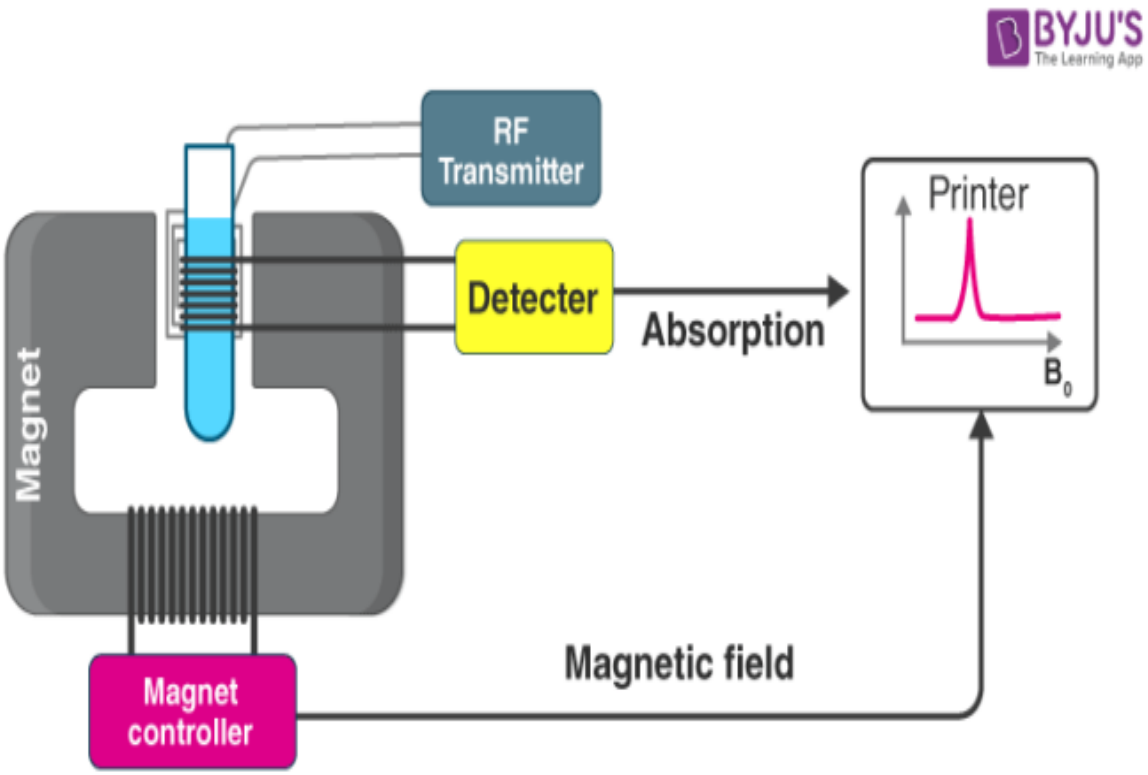

Figure 18 : Nuclear magnetic resonance spectroscopy instrumentation

6.4. Applications of NMR spectroscopy:

NMR spectroscopy is a non-destructive and non-invasive technique that allows for the determination of molecular structure and dynamics. The applications of NMR are diverse and include the following research and industrial domains:

In biology, NMR is applied to the study of macromolecules, such as proteins, lipids, and nucleic acids. The most biologically relevant NMR-active nuclei, including 13C, 1H, 15N, 31P, 23Na, and 19F, are used to understand the biochemical pathways involved in the metabolism of amino acids, lipids, and carbohydrates.

In chemistry, NMR is widely used for both qualitative and quantitative analyses to monitor reactions, identify structures, and evaluate the purity of samples.

In polymer science, it allows for the analysis of monomer ratios, molecular weight, tacticity, sequencing, chain length, and degree of branching, as well as the identification of end groups.

In the pharmaceutical industry, it is used to determine the purity and quantity of active ingredients, excipients, and impurities in pharmaceutical products.

In the oil industry, it is employed to evaluate hydrocarbons in crude oil and its products.

In medicine, Magnetic Resonance Imaging (MRI) is an application of NMR that allows for the analysis of soft tissues to identify damaged or diseased tissues.

Chapter II:
Fractionation Methods

1. Introduction:

The exploration of fractionation methods plays a central role in the fields of biology and chemistry, offering crucial tools for isolating and analyzing various compounds. Each method, whether it be filtration, sedimentation, dialysis, chromatography, or electrophoretic techniques, contributes distinctively to the resolution of molecular puzzles.

This diverse panorama of fractionation techniques forms an essential foundation for researchers aspiring to understand the complexity of biological and chemical samples. In this quest, the methods presented in this chapter emerge as indispensable allies, shaping the landscape of modern scientific research.

2. Filtration:

2.1. Definition:

Filtration is a physical separation technique used to separate particles in a heterogeneous mixture based on their size. It is widely used in various fields such as agri-food, pharmacy, and by many aquatic species.

It involves passing the mixture through a porous medium called a filter. Particles larger than the pore size of the filter are retained, while smaller particles and the liquid pass through the filter, forming the filtrate.

To facilitate the operation and increase the liquid flow rate, which depends on the pressure drop in the channels of the filtering medium, suction can be applied to the filter or the pressure on the liquid to be filtered can be increased.

Continuous or discontinuous filtration is used when treating liquids or gases with very low solid content, especially when solid particles have a low sedimentation

rate.

- ✓ Microfiltration separates particles on the micrometer scale.
- ✓ Sterile filtration is a specific case where the particles are microorganisms.

Common applications of filtration involve the separation of solid particles dispersed in a liquid to obtain:

- ✓ Clarified liquid, free from solid particles.
- ✓ Solids drained of excess liquid.

2.2. Principle:

Filtration is a separation based on the diameter of solid particles of different sizes dispersed in a liquid. The pressure difference forces the liquid to pass through the filter while the solid particles remain on the surface.

Two phenomena often accompany filtration:

- ❖ The first phenomenon is clogging: The penetration of particles into the interstices (small empty spaces between the parts of the filter) of the filtering material leads to clogging. This alters the porosity and slows down filtration.
- ❖ The second phenomenon is adsorption: It results from the electric charge of the filtering material, which leads to the retention of certain products by the filter, even though their dimensions allow them to pass through the filter pores.

2.3. Equipment:

Filtration equipment includes filters and funnels.

2.3.1. Filters:

The choice of filter type depends on the characteristics of the fluid and the particles to be separated. There are two types of filters: depth filters and membrane filters. The use of either type depends on the purpose of the experiment, the quality and quantity of the material to be filtered. They can be used separately or together, as needed. The filters should be chemically and physically inert towards the liquid to be filtered, insoluble, and undergo no physical changes (swelling, shrinking, distortion).

A. Depth filters:

These filters retain particles within a network of fibers (paper, asbestos, cellulose, cotton, fiberglass, etc.) or canaliculi (sintered glass, sand, charcoal, etc.). The efficiency of a depth filter increases with its thickness, but decreases when pressure applied to the filter increases (Figure 19).

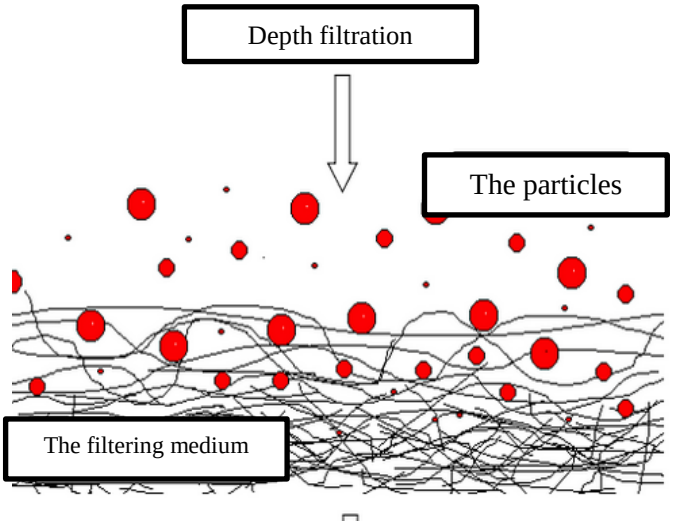

Figure 19 : Depth filtration.

The materials used in depth filters are:

- ✓ **Traditional filter papers:** which differ in their shapes (rectangular sheets, circular, pleated, etc.), texture (loose, fine), porosity, and purity (raw, purified, ashless, etc.). There

are ashless filter papers, demineralized through acid washing, with high purity that, after combustion, add no foreign elements to the precipitate. There is a color or numbering code defining the paper's porosity.

- ✓ **Textiles:** gauze, cotton, wool.
- ✓ **Fibers:** fiberglass, asbestos.
- ✓ **Diatomaceous earth, clays, and porcelain.**
- ✓ **Sintered materials:** Sintered glass is obtained by controlled temperature compression of glass microbeads. Different porosities are achieved based on the diameter of these grains and the sintering temperature. The porosities are coded from 0 (large pores) to 4 (narrow pores).

B. Membrane filters (screens or surface filters):

The most commonly used are Millipore membranes (Figure 20). They consist of a thin plastic sheet with calibrated pores. The filtering membranes are made of cellulose, cellulose acetate, cellulose nitrate, or Teflon. The pore diameter is small, ranging from 5 to 35 nm for ultrafiltration and from 0.1 to 8 µm for microfiltration.

Microfiltration membranes allow for rapid filtration despite the small pore diameter, thanks to their thinness and high pore density (10^{10} pores/cm2). These membranes are composed of only 15 to 35% of their volume as material, with the rest occupied by pores.

Figure 20: Millipore membrane.

2.3.2. Funnels

These are cone-shaped instruments, ending with a tube and designed to hold a filtering material. Two types of funnels are distinguished:

A. Ordinary funnels: These can be made of glass, porcelain, or polycarbonate (Figure 21).

Figure 21: Ordinary funnel.

B. Special funnels: These are funnels with a perforated flat part on which filter paper is placed. They can be made of glass or porcelain. Two types of funnels are distinguished in this category:

- Buchner funnels: Used for the filtration of significant quantities of solids (Figure 22).

Figure 22: Buchner funnels.

- Hirsch funnels: Used for the recovery of small quantities of solids (Figure 23).

Figure 23: Hirsch funnels.

2.4. Classification of filtration:

The classification of filtration can be done based on two main criteria: the fluid flow mode and the particle dimensions.

2.4.1. According to the fluid flow mode:

There are two main filtration techniques based on the fluid flow mode **(Table 3):**

A. Frontal filtration (conventional):

The most well-known technique, involves passing the fluid to be filtered perpendicularly to the surface of the filter. This is the technique used, for example, in coffee filters. As the particles are retained by the filter, this technique is limited by the accumulation of particles on its surface, gradually clogging it .

B. Tangential filtration:

Also known as cross-flow filtration, involves the fluid flowing parallel to the filter membrane, which it passes through under the effect of pressure. As a result, relatively small particles pass through the filter, while larger particles continue their path via the flow (which is a filtration process aimed at separating particles in a liquid based on their size). Clogging occurs much less rapidly in this case.

However, this technique is reserved for filtering very small particles, ranging in size from nanometers to micrometers (Figure 24).

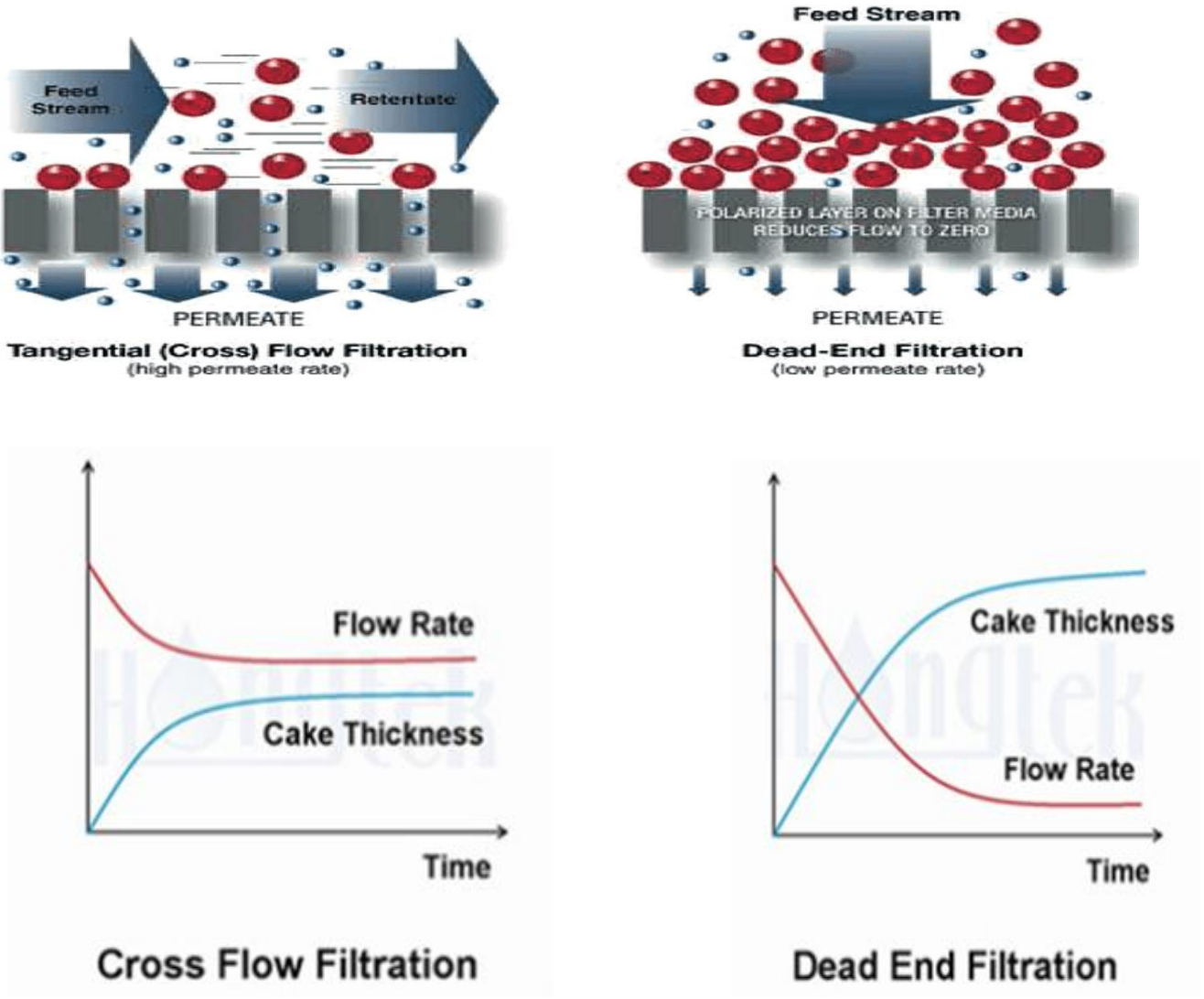

Figure 24: Principle of frontal (dead-end filtration) and tangential filtration.

Note: In the case of tangential filtration, a portion of the liquid, called permeate (filtrate), passes through the membrane due to a pressure gradient. The larger particles concentrate in the liquid that has not passed through the membrane, referred to as retentate.

Tableau 3 : Comparaison entre la filtration frontale & tangentielle

Frontal filtration:	Tangential filtration:
-The most conventional- filtration occurs perpendicular to the surface of the membrane. The particles to be removed are retained by the filtering medium (forming the filter cake) and gradually clog it, resulting in a decrease in the filtrate flow rate. This type of process never reaches a steady state since it requires a series of filtrations and cleaning or replacement of the filtering medium. Frontal filtration is primarily used to filter suspensions with low particle loadings.	The fluid flows parallel to the filtering membrane and passes through it under the effect of pressure. Clogging occurs much slower in this process. However, this technique is reserved for filtering very small particles, ranging in size from nanometers to micrometers.

2.4.2. According to the particle dimensions:

The filtration operation can also be classified differently based on the pore size of the filter:

A. Clarifying Filtration: When the pore diameter ranges from 10 to 450 micrometers.

B. Microfiltration: Used to retain micrometer-sized particles, with pore diameters ranging from 10 nm to 10 micrometers. It is commonly used in the food and pharmaceutical industries.

C. Ultrafiltration: This technique retains smaller particles, with pore diameters ranging from 1 to 10 nm. It is often used for separating proteins, viruses, and colloids.

D. Reverse Osmosis: When the pore diameter ranges from 0.1 to 1 nm, primarily used for water purification by removing salts and other contaminants.

E. Sterile Filtration: When the pore diameter is larger than 0.22 micrometers (allowing for the retention of microorganisms).

F. Nanofiltration: It is used to retain nanometer-sized particles, typically ranging from 0.001 to 0.01 micrometers. Nanofiltration is often employed to remove salts from water.

2.5. Types of Filtration:

Filtration involves separating the components of a liquid-solid mixture by passing it through a filtering medium. It is much faster than sedimentation. Several filtration processes exist:

- **Gravimetric Filtration.**
- **Vacuum Filtration.**
- **Pressure Filtration.**
- **Ultrafiltration.**

2.5.1. Gravimetric filtration (gravity filtration):

In this method, filters, typically made of paper, conical or pleated, are used, and the liquid flows through them under the force of its own weight. In laboratory settings, a funnel equipped with a filter paper is commonly used. The pressure difference is created by the height of the liquid above the filter (Figure 25).

A. Principle:

It is based on using a filter with mesh openings that allow water to pass through while retaining the particles it contains. Filtration thus produces a homogeneous liquid. The small openings in the filter paper do not allow any particles larger than bacteria to pass through. The large particles retained on the filter paper constitute the residue, while the liquid that passes through the filter is called the filtrate.

B. Setup:

To perform filtration, a filter and a supporting device, such as a filter holder, are required. Most often, a funnel is used.

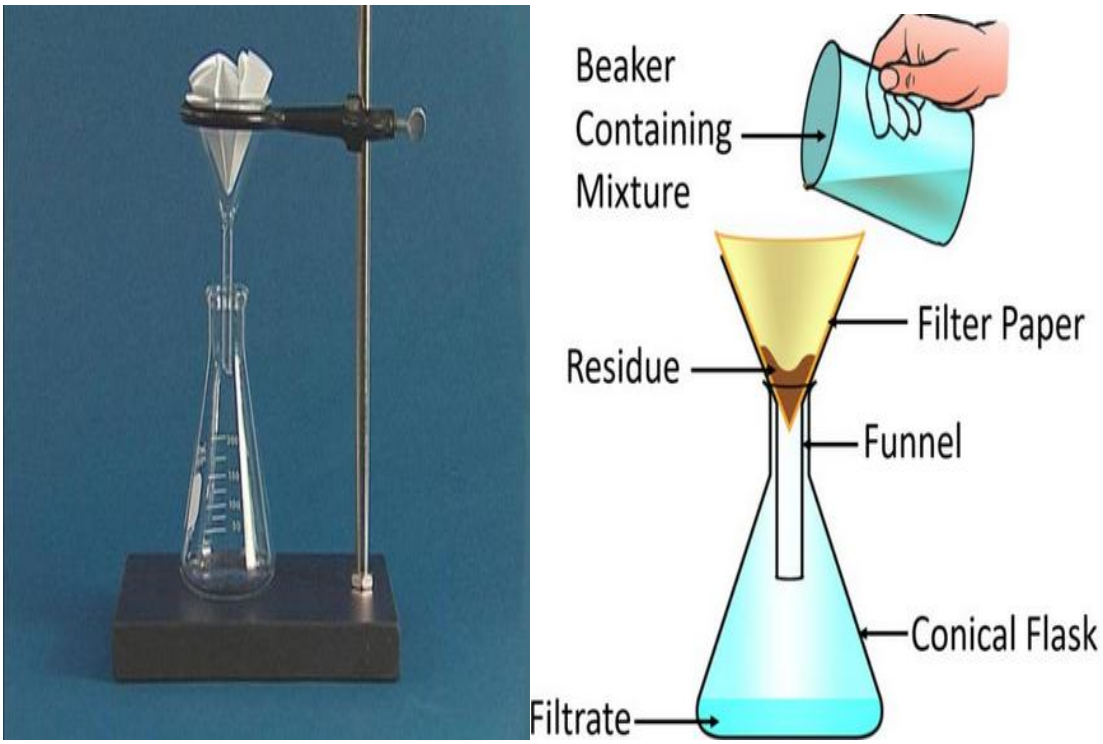

Figure 25: Gravity filtration setup.

C. Disadvantages:

Gravimetric filtration has the following disadvantages:

- Filtration is slow.

- Recovery of the solid phase is challenging, especially when it is in low abundance.

- The separation is incomplete, as the solid retains a significant amount of liquid.

- This method is generally slow and does not allow for optimal separation of the solid and liquid. To overcome these drawbacks, vacuum filtration is often employed.

2.5.2. Vacuum filtration:

The filtration rate is increased by creating a vacuum downstream of the filtering material (Figure 26). This filtration mode is commonly used for fritted glassware and filter membranes. Special Büchner funnels, adapted to a suction flask in which a vacuum is created,

are utilized. The funnel is fitted onto the flask through a rubber cone, which ensures a tight seal when the vacuum is established. In cases where the solid consists of particles that are too fine and may pass through the filter, a fritted glass funnel, onto which the mixture is directly poured, can be used. Different porosities of fritted glass are available, and the appropriate one should be chosen based on the size of the solid particles to be filtered.

A. Büchner filter: It is a cylindrical porcelain filter with large holes on which a circular filter paper, large enough to cover the entire surface, is placed.

B. Fritted glass filter: This is a glass funnel containing a fritted glass disc with a fixed porosity. This type of filter is used in extreme pH conditions where paper filters would not withstand the conditions. However, it cannot be used with hydrofluoric acid solutions as it reacts with the silica in the glass.

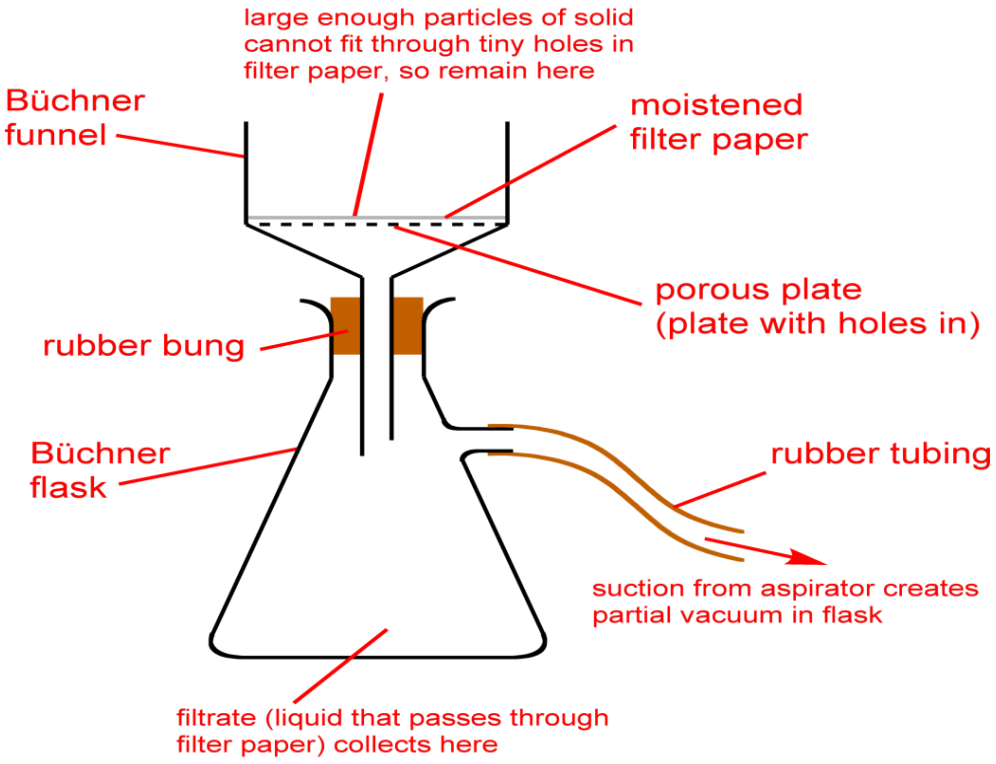

Figure 26: Vacuum filtration diagram.

2.5.3. Pressure filtration:

The filtration rate is increased by applying pressure to the liquid being filtered through a filtering material represented by a filter membrane (Figure 27). Pressure filtration prevents foaming and solvent evaporation and is commonly used in industry. This pressure filtration system with filter membranes is also available in the form of filter cartridges (Millipore) that can be attached to a syringe for filtering small volumes of solution. In the laboratory, sterile microfiltration using the Swinnex Millipore device is a form of pressure filtration. This device consists of two plastic pieces that are screwed together, enclosing a filter membrane.

Figure 27: Pressure filtration.

2.5.4. Ultrafiltration:

A. Definition:

It is the separation of macromolecules in a dispersing phase solution. It involves a membrane with a very low porosity (25 nm) that can retain proteins and nucleic acids. It allows for the concentration of macromolecule solutions and the removal of most low-molecular-weight contaminants (such as salts and carbohydrates).

B. Applications of ultrafiltration:

The applications of ultrafiltration and microfiltration are primarily analytical. In addition to clarification and sterile filtration, the following applications are also mentioned:

- ❖ Microbiological analysis and sterility testing.
- ❖ Gravimetric analysis.
- ❖ Isolation of cells from cerebrospinal fluid.
- ❖ Dust analysis.
- ❖ Virus isolation.

2.6. Applications:

Filtration is a technique used in various fields, including:

- Water treatment: Filtration is used to purify water by removing suspended particles, bacteria, and viruses.

- Chemical industry: Filtration is used to separate chemicals and catalysts.

- Food industry: Filtration is used to clarify fruit juices, syrups, and oils.

- Pharmaceutical industry: Filtration is used to purify medications and vaccines.

- Laboratory: Filtration plays a crucial role in sample preparation by removing solid impurities and separating cells, bacteria, and viruses. It is also widely used for liquid clarification, making solutions transparent by removing suspended particles. These diverse applications highlight the importance of filtration in scientific research, laboratory analysis, and various industrial sectors.

2.7. Terminology:

- ❖ **Permeate:** Corresponds to the filtrate; it is the fraction of the processed liquid that passes through the filter membrane.

- **Retentate:** Also referred to as concentrate; it is the fraction of the processed liquid that does not pass through the filter membrane.
- **Permeate flux (denoted as J):** The flow rate of filtrate per square meter of membrane surface area ($L/h/m^2$).
- **Cut-off threshold:** The molecular weight of molecules retained 90% by the membrane under specific operating conditions. It is expressed in Daltons (Da) or g/mol.
- **Transmembrane pressure:** The pressure difference across the membrane, denoted as ΔPm, existing between the feed (retentate side) and permeate.
- **Retention rate:** The proportion of molecules retained by the membrane compared to their concentration in the feed.

3. Sedimentation

3.1. Definition of sedimentation:

Sedimentation is a physical process that involves allowing a liquid containing particles to settle at the bottom of a container under the influence of gravity. Sedimentation is a technique used to separate a dispersion of a solid in a liquid or a dispersion of a liquid in another immiscible liquid of different density. This separation can occur naturally under the action of gravity when the dispersion consists of a substance denser than the liquid (decantation). Centrifugation can replace the acceleration due to gravity (g) with a centrifugal acceleration generated by a high-speed rotating rotor (6 to 10,000 revolutions per minute).

3.2. Centrifugation:

3.2.1. Definition of centrifugation:

Centrifugation is a technique that allows the separation of components in a mixture based on their density under the action of centrifugal force. It enables the recovery of a

precipitate (pellet) and a supernatant. The mixture to be separated can consist of two liquid phases or solid particles suspended in a liquid.

3.2.2. Principle of centrifugation:

Centrifugation allows the separation of components with significantly different sizes and masses in a liquid. The components in a sample are subjected to two forces:

- **Gravity:** This is the force exerted from top to bottom.

- **Archimedes' buoyant force:** This is the force exerted from bottom to top.

For a given rotational speed, each rotor has a relative centrifugal force in x.g (relative gravitational force or acceleration) that can be expressed in revolutions per minute (rpm) using the conversion formula:

$$\mathbf{g = 1.119 \,.\, 10^{-5} \,.\, r \,.\, N^2}$$

Where g is the relative centrifugal force, r is the radius of rotation of the rotor (in cm), and N (rpm) represents the rotational speed.

Example:

A rotor has a maximum rotation radius of 10 cm. At what speed should it be rotated to achieve an acceleration of 100,000 x gmax?

According to the formula:

RPM = √(FGR / (r * 1.119 * 10-5),

RPM = √ (100 000 / (10 * 1.119 * 10-5)= 29 894 , approximately 30 000 RPM

3.2.3. Centrifugation equipment:

The centrifuge is the apparatus used for centrifugation. It consists of a rotating axis enclosed in a centrifugation chamber. Except for benchtop centrifuges, which have relatively

limited rotational speed and usage time, it is necessary to prevent sample heating. Therefore, the centrifuge chamber must be refrigerated (Figure 28).

The samples to be centrifuged must be balanced in pairs. Each pair should be placed symmetrically with respect to the axis of rotation.

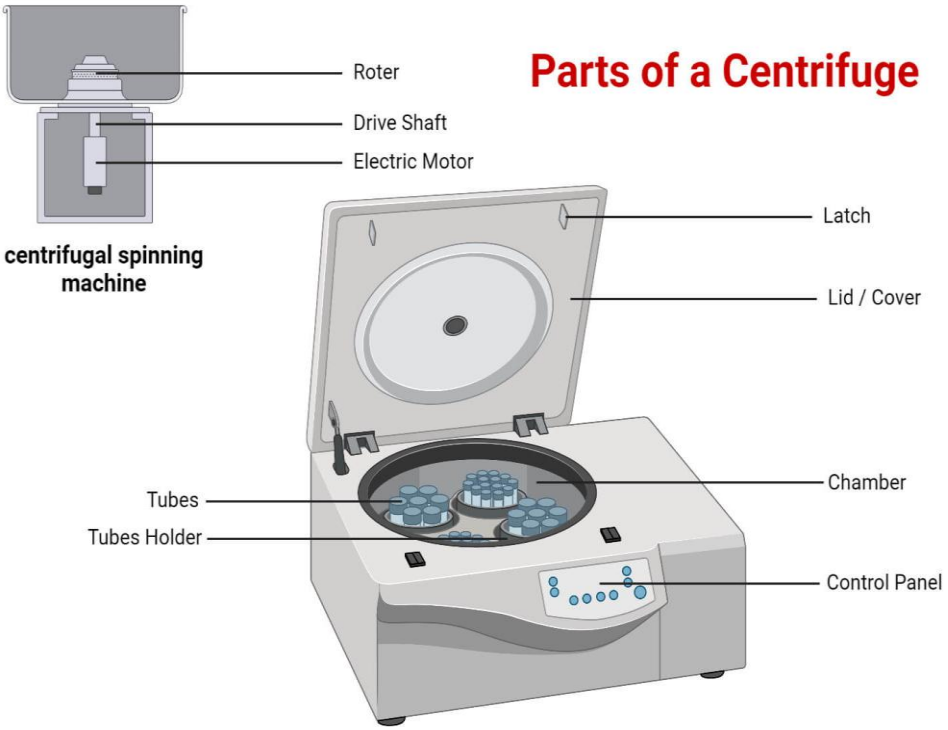

Figure 28: Parts of centrifuge.

3.2.3.1. Types of centrifuges:

The motor's power, which drives the rotation, is the main limiting factor determining the rotational speed of the rotor. The heavier and larger the rotor, the greater the effort required by the motor.

A. Classification based on experimental needs:

Depending on the experimental requirements (accelerations, volume of material to be centrifuged, working temperature), several types of centrifuges have been developed.

- Tabletop centrifuges (or clinical centrifuges):

These are the simplest models characterized by low accelerations (1000 to 3000 g). They can be refrigerated.

- Floor-standing centrifuges:

These devices are slightly more complex and characterized by accelerations of around 20,000 g. These centrifuges can handle relatively large volumes. Some rotors can even accommodate four or six 250 ml bottles. All models are refrigerated.

- Ultracentrifuges:

As the name suggests, these devices can achieve very high accelerations (up to 300,000 g). All models are refrigerated. The rotors can only hold about ten 40 ml tubes.

- Microcentrifuges:

These are centrifuges specifically designed for micro-volumes. They can be refrigerated and reach accelerations of around 12,000 to 15,000 g.

- **Analytical ultracentrifuges:**

They are mainly used to analyze the size and mass of particles and proteins. They are less commonly used.

B. Classification based on the axis of rotation:

There are three categories of centrifuge machines based on the axis of rotation:

- Horizontal centrifuges:

They are named so because the pots rotate horizontally. Conical-bottom centrifuge tubes are used to clarify a liquid (to recover the supernatant and discard the pellet), while round-bottom tubes are used to recover the pellet. This type of centrifuge has some disadvantages, such as poor aerodynamics of the rotor, and the sedimenting particles have to pass through a large thickness of liquid.

- Vertical centrifuges:

These are centrifuges with a discrotor that rotates vertically. They are commonly used for high-speed centrifugation and can handle larger volumes. The sedimentation process occurs in a thin layer of liquid, allowing for more efficient separation.

- Angle-head centrifuges:

These centrifuges have a rotor that is angled between the horizontal and vertical positions. They offer a compromise between the advantages of horizontal and vertical centrifuges.

3.2.4. Types of centrifugation

There are three main types of centrifugation (Figure 29).

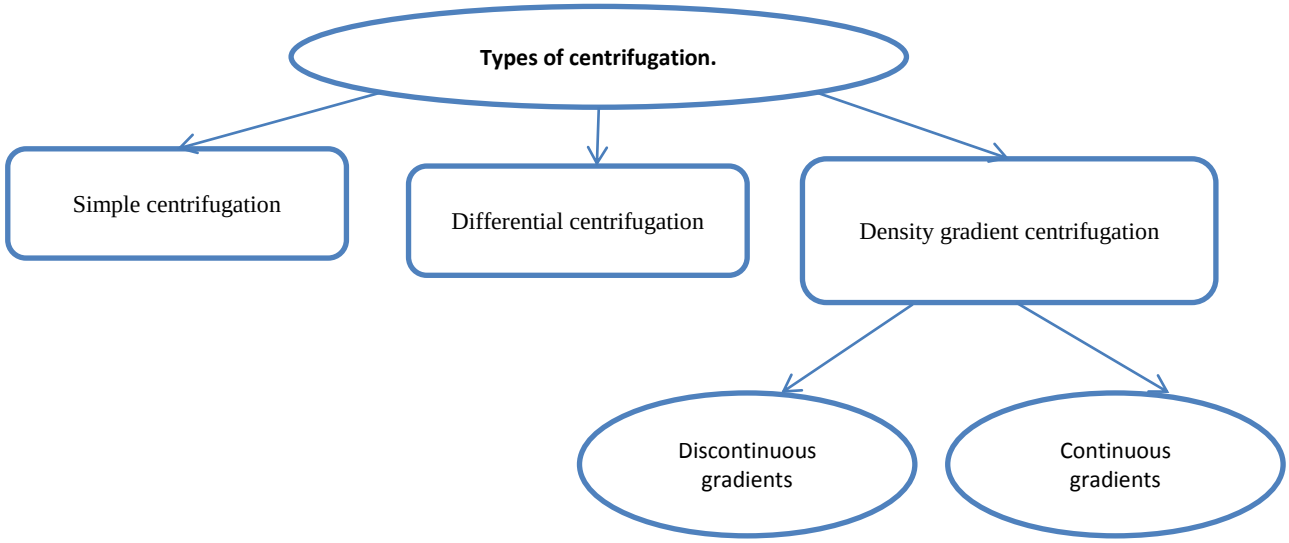

Figure 29: Types of centrifugation.

A. **Simple centrifugation**:

Simple centrifugation refers to a basic form of centrifugation technique where a sample is subjected to centrifugal force to separate its components based on their density and sedimentation rates. It involves spinning the sample at a specific speed and duration to induce the separation. The heavier components or particles settle at the bottom, forming a pellet, while the lighter components remain in the supernatant. Simple centrifugation is commonly

used for basic separation purposes and does not involve complex gradient systems or multiple centrifugation steps.

An example of simple centrifugation is the separation of blood components. When a blood sample is subjected to centrifugation, the heavier components, such as red blood cells, will sediment at the bottom of the centrifuge tube, forming a pellet. The lighter components, such as plasma or serum, will remain in the supernatant. This allows for the separation of different blood components for various diagnostic or research purposes.

B. Differential centrifugation:

Differential centrifugation is a technique used to separate different components of a sample based on their size and density differences. It involves a series of sequential centrifugation steps at increasing speeds or durations. During each step, the centrifugal force causes particles of different sizes and densities to sediment at different rates, allowing for their separation. The supernatant is collected after each centrifugation step, leaving behind the pellet containing the heavier particles. By adjusting the centrifugation parameters, such as speed and time, it is possible to isolate specific components from a mixture.

C. Density gradient centrifugation:

Density gradient centrifugation is a technique used to separate particles based on their buoyant density. In this method, a density gradient is created within the centrifuge tube by layering solutions of different densities. When the sample is centrifuged, the particles migrate through the density gradient until they reach a region where their density matches that of the surrounding medium. This results in the formation of distinct bands or zones containing particles of similar density. Density gradient centrifugation is particularly useful for separating particles with small density differences, such as subcellular organelles, viruses, and nucleic acids.

3.3. Sedimentation vs. centrifugation:

Sedimentation and centrifugation are related processes used for particle separation, but there are some differences between them.

Sedimentation occurs naturally under the influence of gravity, allowing particles to settle at the bottom of a container. It is a slower process that relies on the density difference between the particles and the surrounding liquid. Sedimentation is often used for larger particles or when the density difference is significant.

On the other hand, centrifugation is a faster and more controlled process that uses centrifugal force to separate particles based on their density. It involves spinning the sample at high speeds, creating a centrifugal force that drives the particles towards the bottom of the tube. Centrifugation is commonly used for smaller particles or when a more rapid separation is required.

Centrifugation offers several advantages over sedimentation:

- It is faster and more efficient in separating particles due to the higher gravitational forces generated.
- It allows for better control and optimization of separation conditions, such as rotational speed and centrifuge tube design.
- It can handle larger volumes and higher particle concentrations.
- It is applicable to a wider range of particle sizes and densities.

However, sedimentation also has its advantages:

- It is a simpler and more cost-effective process since it relies on gravity alone.
- It can be used in situations where centrifugation equipment is not available or practical.
- It is suitable for larger particles or when the density difference is significant.

3.4. Ultracentrifugation:

3.4.1. Definition:

Ultracentrifugation is an advanced centrifugation technique that utilizes extremely high centrifugal forces to separate and analyze the components of a biological sample, typically macromolecules such as proteins, nucleic acids, or viral particles. This method is based on the same fundamental principle as centrifugation but allows for much higher rotational speeds, typically in the range of 50,000 to 100,000 revolutions per minute or even higher.

3.4.2. Principle:

Ultracentrifugation relies on the principle of accelerated sedimentation. By spinning a rotor at extremely high speeds, particles suspended in a liquid experience significant centrifugal forces that accelerate their sedimentation.

3.4.3. Types of ultracentrifugation:

A. Analytical ultracentrifugation: Used to separate and analyze molecules based on their molecular weight and shape.

B. Preparative ultracentrifugation: Used to isolate specific particles, such as cellular organelles or cellular fractions.

3.5. Applications:

3.5.1. Centrifugation:

A. Food industry: Clarification of juices, separation of solid particles, and concentration of certain food products.

B. Chemistry: Purification and separation of chemicals, including the separation of complex mixtures.

C. Biotechnology: Preparation of biological samples, clarification of cell cultures, and separation of biomolecules.

D. Biological research: Separation of cellular components, sample preparation for analysis, and isolation of cellular organelles.

E. Clinical medicine: Separation of blood components for diagnostic analyses, such as separating plasma and blood cells.

3.5.2. Ultracentrifugation:

A. Molecular biology research: In-depth analysis of biological macromolecules such as proteins, nucleic acids, and protein complexes.

B. Biochemistry: Study of molecular interactions, determination of molecular masses, and characterization of proteins.

C. Virology: Study of viruses, purification of viral particles, and analysis of the physical and chemical properties of virions.

D. Cell studies: Fractionation of cellular organelles to study cellular structure and function.

E. Structural biology: Analysis of complex molecular structures, including understanding the formation of protein complexes.

F. Medicine and pharmacy: Study of drugs, analysis of lipoproteins, and characterization of blood components.

G. Environmental science research: Analysis of environmental samples, particularly for the detection and quantification of contaminants.

These applications reflect the versatility of centrifugation and ultracentrifugation techniques in various scientific and industrial fields, contributing to significant advances in research and understanding of biological and chemical systems.

3.6. Terminology:

A. Sedimentation: The term "sedimentation" is used to describe the process by which denser particles settle at the bottom of a liquid under the influence of gravity.

B. Gravity: Gravity is a mutual attraction force exerted by the mass of one object on another due to their respective masses. This force is responsible for the phenomenon commonly referred to as "weight." Gravity is an omnipresent force that pulls all massive objects toward the center of the Earth.

4. Dialysis and electrodialysis

4.1. Definition of dialysis:

Dialysis is a membrane separation process for molecules or ions in a solution. Small molecules that pass through the dialysis membrane are referred to as diffusible molecules, while larger molecules are considered non-diffusible.

4.2. Basic principles:

Dialysis is based on the principles of diffusion through a permeable or semi-permeable membrane. Diffusible molecules move across the membrane according to the concentration gradient and enter the dialysate. This results in a net movement of molecules from the more concentrated side to the less concentrated side. At equilibrium, the concentrations of diffusible species are equal on both sides. If the volume of the liquid outside the dialysis sac is much larger than the volume of the solution being dialyzed, this equality of concentrations means that the majority of diffusible molecules have actually been removed from the solution. This removal of diffusible molecules can be enhanced by repeating the process. Molecules larger than the pore size of the membrane remain on the retentate side .

4.3. Equipment:

The solution to be dialyzed is placed in a tube made of a semi-permeable membrane, often cellulose or a derivative. Both ends of the tube are sealed, forming a "dialysis sac." This sac is placed in the solution against which dialysis is desired (the counter-dialysis liquid) (Figure 30).

There are two types of setups for dialysis. The first and most common type is closed-system dialysis. In this setup, the dialysis sac containing the solution to be dialyzed is placed in a large container. Diffusible molecules are given time to equilibrate. This process can be accelerated by agitating the counter-dialysis liquid (using a magnetic stir bar). The smaller the ratio between the volume of retentate and dialysate, the more diluted the diffusible molecules

will become during this process. If a greater dilution is desired, the volume of the dialysate can be increased or closed-system dialysis can be repeated two or three times.

For further reduction of diffusible molecules, the second type of setup, continuous dialysis, can be used. In this technique, the dialysis sac is exposed to a continuous flow of the desired solvent, usually water. As the flow is continuous, the small molecules cannot reach equilibrium concentration and continuously diffuse out of the dialysis sac.

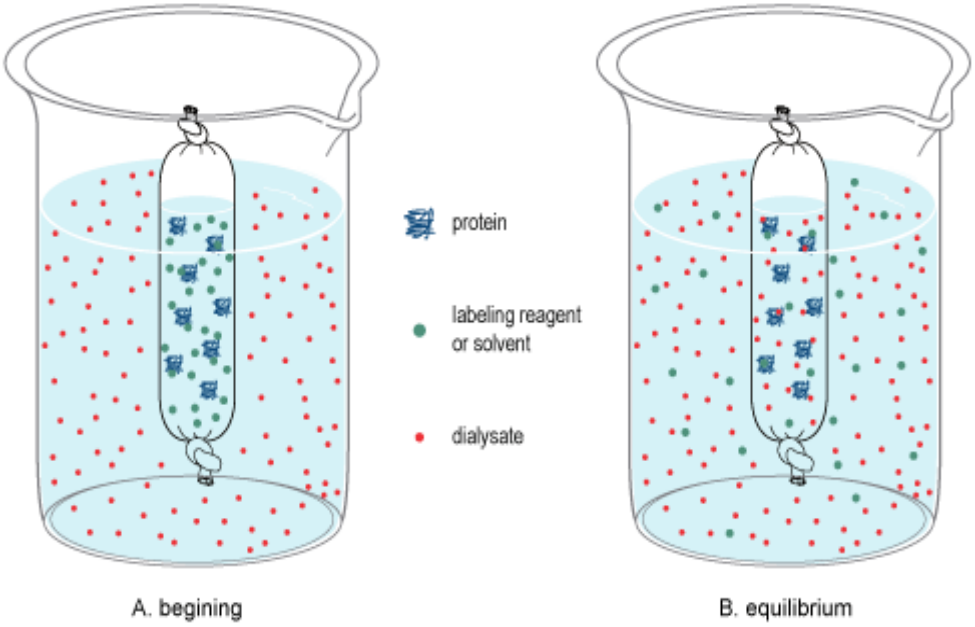

Figure 30: Dialysis setup.

4.4. Applications:

Dialysis is used in the fields of biochemistry, analysis, medicine, industrial applications, and hemodialysis. In food control, dialysis is used for purification, such as purifying before chromatography, detecting food colorants, or measuring aflatoxin levels in milk. In biological analyses, it is used for separating the element to be measured from complex environments (proteins, hemoglobin, triglycerides).

Industrial applications cover three distinct areas: preparation, purification, and recovery. For example:

- Removing mineral salts from molasses to facilitate sucrose crystallization.

- Recovering sodium hydroxide in the cellulose industry.

4.5. Definition and principle of electrodialysis:

Electrodialysis is a technique used for extracting ions from a solution. It involves the transfer of ions through membranes under the influence of an electric field. The device used for this process, an electrodialyzer, consists of multiple compartments and ion-exchange membranes (anionic and cationic).

Under the electric field, the ion-exchange membranes selectively allow the transfer of charged species based on their charge sign, with cations transferring through cation-exchange membranes (CEMs) and anions transferring through anion-exchange membranes (AEMs). The selective transfer of charged species occurs through an ion-exchange mechanism between the ions in the solution and the counter-ions in the membrane. For example, CEMs carry functional groups with a negative charge (Figure 31).

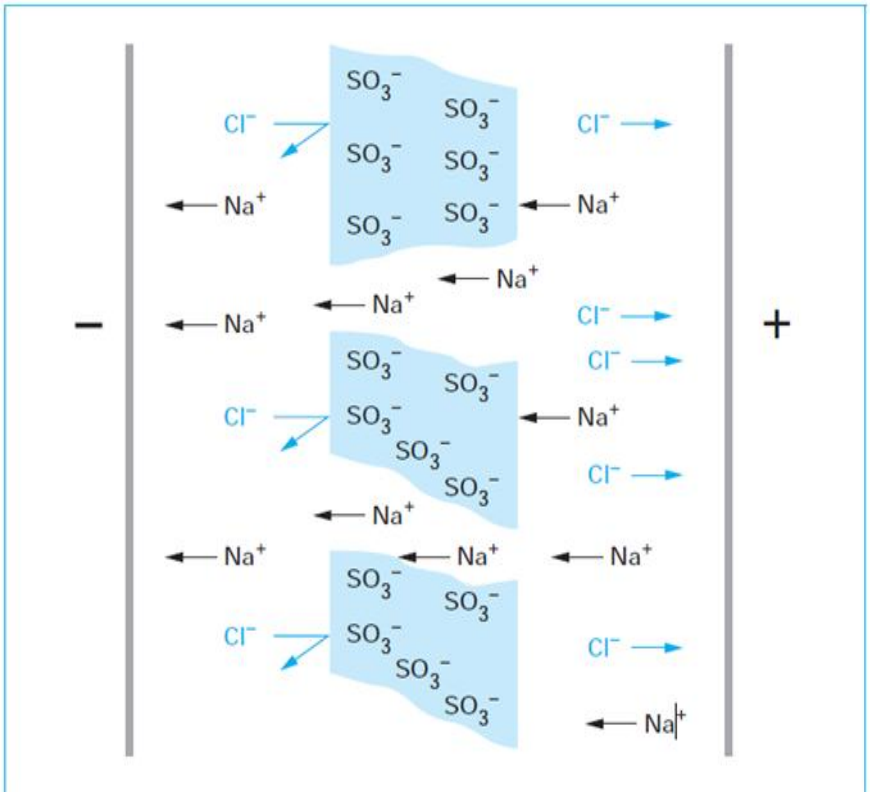

Figure 31: Schematic representation of the operation of a cation-exchange membrane.

4.6. Applications:

Numerous applications have been developed in recent years, including integrating these technologies into processes for purification or organic molecule production. In the agri-food industry, electrodialysis is used for demineralization of whey to recover proteins, production of organic acids (lactic, gluconic, acetic), demineralization of sweet juices, and deacidification of fruit juices.

5. Chromatographic methods

5.1. Definition and principle:

Chromatography is a physical technique for the separation of chemical species that allows the identification and quantification of different compounds in a mixture. The sample containing one or more species is carried by a mobile phase (liquid, gas, or supercritical fluid) along a stationary phase (paper, gelatin, silica, polymer, grafted silica, etc.). Each species moves at its own velocity, depending on its characteristics, the characteristics of the two phases, and the differences in affinity between them. This physicochemical analysis technique can be coupled with a detector for qualitative or quantitative analysis of the sample.

Depending on the chromatographic technique employed, the separation of the components carried by the mobile phase results either from their successive adsorption and desorption on the stationary phase or from their different solubility in each phase.

5.2. Principle:

Chromatography is based on the transport of a dissolved sample by a mobile phase through a stationary phase. The stationary phase retains the substances in the sample to varying degrees based on the intensity of weak interaction forces (such as van der Waals forces, hydrogen bonds, etc.) between the different molecular species and the stationary phase. The different components of the sample generally have a characteristic velocity that

allows them to be separated and identified. This separation velocity is strongly dependent on the nature of the mobile phase and the stationary phase.

Often, the sample is analyzed by comparison with known substances in the sample or by comparison with the results of the analysis of a standard solution (a commercial solution containing known substances at well-known concentrations). These substances serve as references and allow the identification or quantification of each species by comparing the separation velocities (and potentially other information provided by the detection). This is called analytical chromatography.

In other cases, the goal is to purify and separate fractions for further identification using other techniques. This is called preparative chromatography. This analytical method allows the identification and quantification of compounds in a mixture and can be coupled with a mass spectrometer for the identification of unknown compounds. To fully exploit it, it is important to know the different retention factors and to use columns with good efficiency.

Chromatography also allows for precise quantification. The main quantification methods are internal standardization, standard addition method, and internal calibration. External calibration can also be performed under certain conditions.

A. Stationary phase in chromatography:

The stationary phase may be a column, plate, or paper in chromatographic technique. It is a solid phase or a liquid phase coated on the surface of a solid phase.

Depending upon the nature of the stationary phase, three chromatographic techniques have been used widely in the laboratory. These techniques are,

- Liquid-solid absorption or column chromatography (LSC)
- Paper chromatography (PC)
- Thin-layer chromatography (TLC) (Figure 32)

B. Mobile phase in chromatography:

The mobile phase (liquid or gas) moves through the stationary phase when different components are separated due to different rates of migration (Figure 33).

1. If the mobile phase is liquid, it is called liquid chromatography (LC).
2. If the mobile phase is gas then it is called gas chromatography (GC).

The migration of molecules depends on their affinity to the stationary and mobile phases. Therefore, the components which are attached less by the stationary phase will move faster in mobile phases.

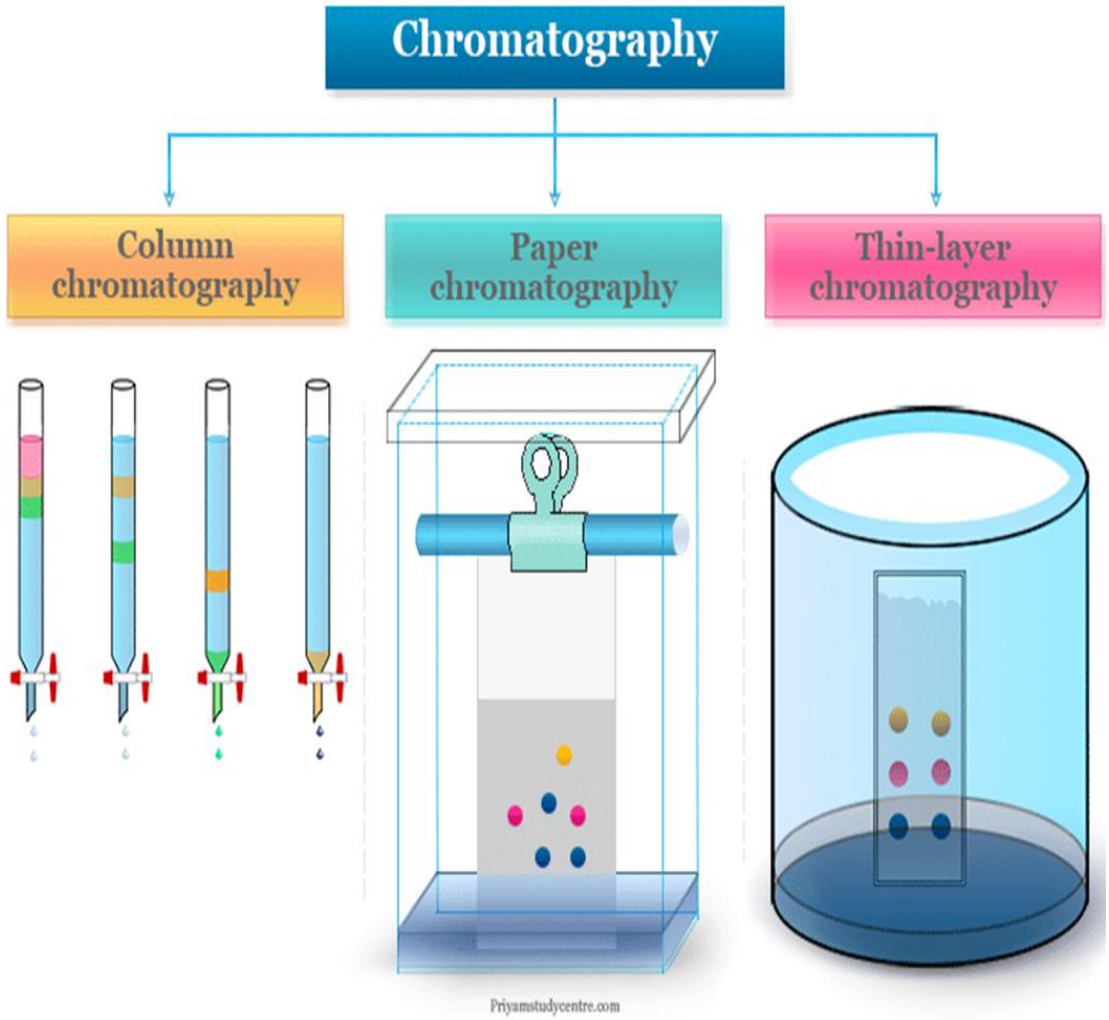

Figure 32: Classification of chromatography based on the processes used

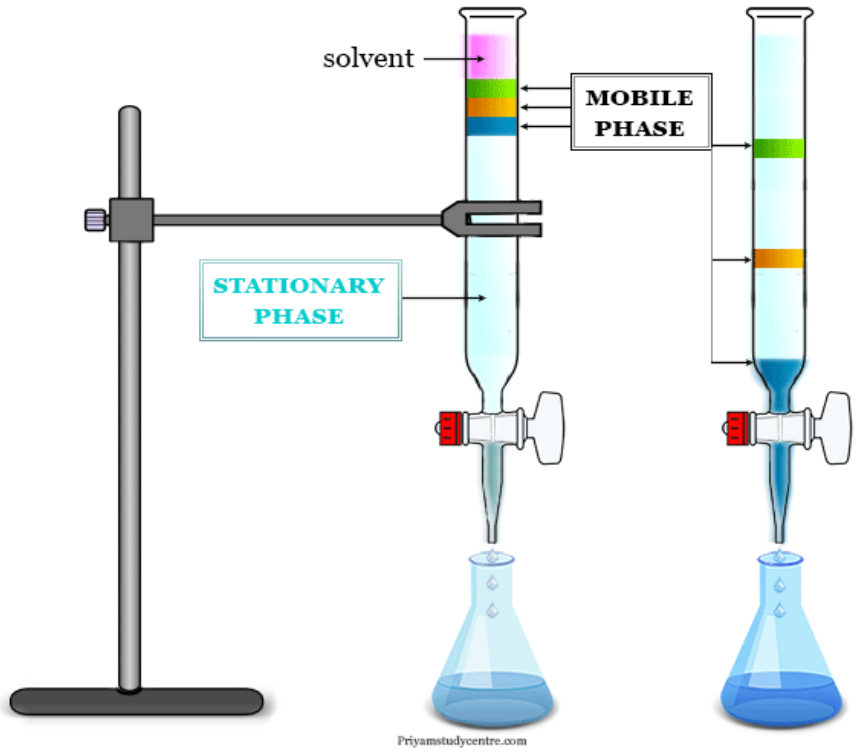

Figure 33: Stationary phase and mobile phase in chromatography

5.3. Classification:

There are numerous types of chromatography, which can be classified according to three different modalities:

- ❖ Classification based on the nature of the phases
- ❖ Classification based on the chromatographic phenomenon involved
- ❖ Classification according to the support of the stationary phase

5.3.1. Classification based on the nature of the phases:

Chromatographic methods can be classified into three categories based on the nature of the mobile phase: liquid-phase chromatography (LPC), gas-phase chromatography (GPC), and supercritical fluid chromatography (SFC or CPS). These three types of chromatography mainly differ in the nature of the mobile phase used to carry the samples through the chromatographic column.

A. Liquid-Phase Chromatography (LPC):

In LPC, the mobile phase is a liquid, usually called a solvent or eluent, which carries the sample through the stationary phase.

Different techniques of LPC include high-performance liquid chromatography (HPLC), ultra-high-performance liquid chromatography (UHPLC), size exclusion chromatography (SEC), low-pressure liquid chromatography (LPLC), etc.

LPC is often used to analyze samples of various natures, such as organic compounds, biomolecules, drugs, natural products, etc.

B. Gas-Phase Chromatography (GPC):

In GPC, the mobile phase is an inert gas, such as helium or nitrogen, which carries the sample through the stationary phase.

GPC techniques are used to analyze volatile or semi-volatile compounds, such as hydrocarbons, organic compounds, pesticides, aromatic compounds, solvents, etc.

GPC is often preferred for its excellent resolution, fast analysis, and high sensitivity, especially in fields such as atmospheric gas analysis, environmental analysis, petroleum product analysis, etc.

C. Supercritical Fluid Chromatography (SFC or CPS):

Supercritical fluid chromatography uses a supercritical fluid, typically carbon dioxide (CO2) above its critical point, as the mobile phase to carry the samples through the stationary phase.

In SFC, the supercritical fluid acts as both a gas and a liquid, offering unique characteristics such as densities close to liquids and diffusivities close to gases.

Supercritical fluid chromatography columns typically use porous supports for the stationary phase, allowing selective interactions with the components of the sample.

SFC is commonly used to analyze thermosensitive and less volatile compounds, such as triglycerides, steroids, pesticides, polar compounds, etc.

This technique offers advantages such as good selectivity, low solvent consumption, a wide range of applications, and the ability to separate difficult-to-separate isomeric compounds using other chromatographic techniques.

5.3.2. Classification based on the chromatographic phenomenon employed:

Chromatographic methods can also be classified based on the chromatographic phenomenon employed for the separation of components. Some common types include (Table 4) :

Table 4: Separation criteria of chromatographic techniques

Separation Factors	Chromatographic Technique
Solubility in a liquid solvent	**Partition Chromatography**
Size and shape	**Exclusion Chromatography**
Polarity	**Adsorption Chromatography and Reverse Phase Chromatography**
Electric charge	**Ion Exchange Chromatography**
Specific atom groupings forming specific sites	**Affinity Chromatography**

A. Adsorption Chromatography:

Adsorption chromatography is based on the differential adsorption of components from the sample onto the surface of the stationary phase. The stationary phase is usually a solid material with a large surface area, such as silica gel or activated alumina.

The separation occurs because different components have different affinities for the stationary phase, leading to different retention times and elution order.

This technique is widely used for the separation and purification of organic compounds, especially in thin-layer chromatography (TLC) and column chromatography (Figure 34) .

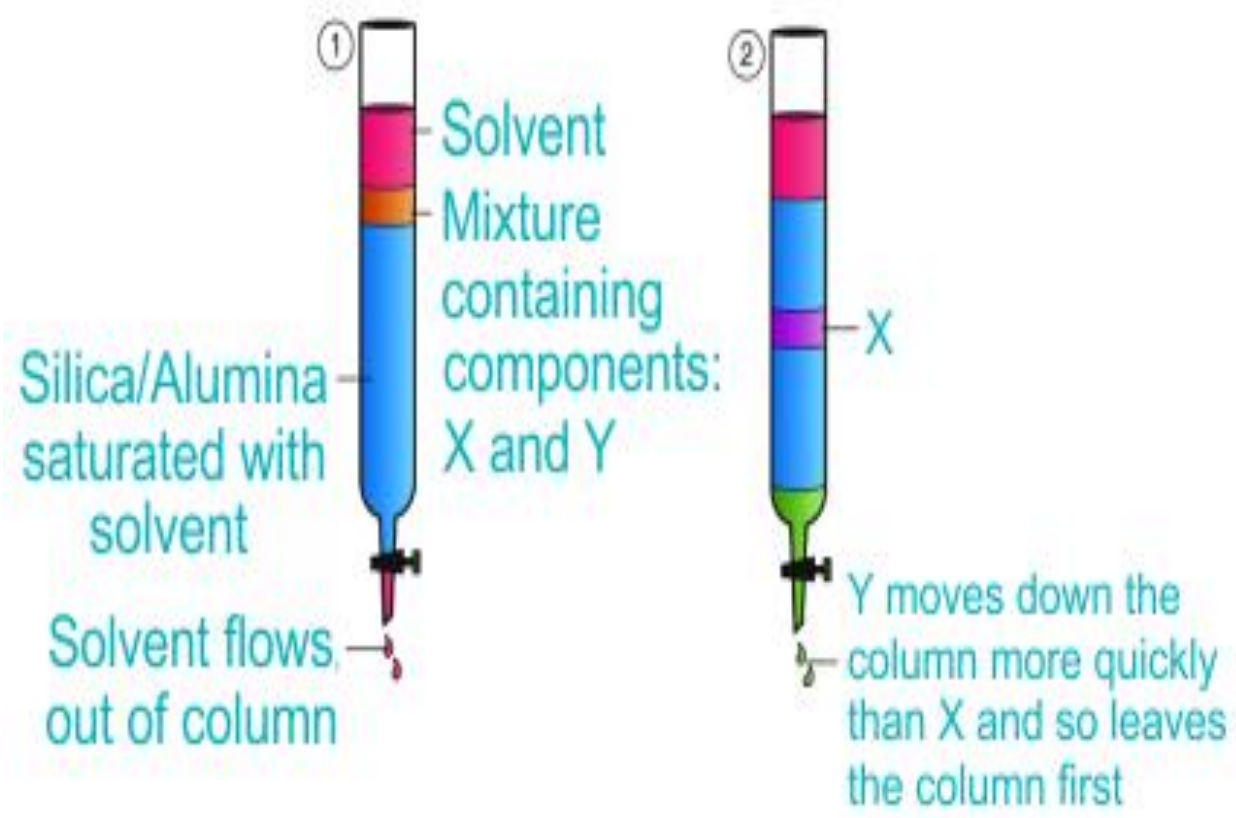

Figure 34: Adsorption chromatography

B. Partition Chromatography:

Partition chromatography relies on the differential partitioning of components between two immiscible phases: the stationary phase and the mobile phase.

The stationary phase is often a liquid supported on a solid material, such as a liquid adsorbed on a solid support or a liquid immobilized within a porous material.

The separation is achieved by repeated partitioning of the components between the two phases, leading to differential retention and elution.

Common examples of partition chromatography include gas-liquid chromatography (GLC) and reversed-phase liquid chromatography (RPLC).

❖ **Difference between adsorption and partition chromatography**

These are differences between adsorption and partition chromatography (Table 5):

Table 5: Difference between adsorption and partition chromatography:

Adsorption chromatography	Partition chromatography
The chromatography in which the stationary phase is a solid adsorbent material, and the separation is based on the adsorption of the sample components onto the solid surface	A type of liquid-liquid chromatography (LLC) in which the sample components are separated based on their partition coefficient between two immiscible liquid phases.
In this type, a substance leaves the noble phase to become adsorbed on the surface of solid stationary phase.	In this, types of substance being separated are distributed throughout both the stationary and mobile phases.
Examples of adsorption chromatography: Column chromatography Thin layer chromatography Gas solid chromatography	Examples of partition chromatography are: Paper chromatography Gas liquid chromatography

C. Ion-exchange chromatography:

Ion-exchange chromatography separates charged species based on their interactions with charged sites on the stationary phase. The stationary phase contains charged functional groups, such as resins or gels, which attract or repel ions present in the sample.

The separation is achieved by controlling the mobile phase's pH and ionic strength, allowing for selective retention and elution of ions. Ion-exchange chromatography is commonly used for the analysis of inorganic ions, amino acids, proteins, and other biomolecules (Figure 35).

Ion-exchange chromatography can be further classified into two main types:

❖ **Anion-exchange chromatography:**

In anion-exchange chromatography, the stationary phase contains positively charged functional groups, which attract and retain negatively charged ions (anions) from the sample. The mobile phase typically consists of a buffer solution with an appropriate pH and ionic strength. Anion-exchange chromatography is commonly used for the separation and analysis of inorganic and organic anions, such as chloride, sulfate, nitrate, organic acids, etc.

❖ **Cation-exchange chromatography:**

In cation-exchange chromatography, the stationary phase contains negatively charged functional groups, which attract and retain positively charged ions (cations) from the sample. The mobile phase composition is similar to anion-exchange chromatography, with the appropriate pH and ionic strength. Cation-exchange chromatography is often employed for the separation and analysis of inorganic and organic cations, such as sodium, potassium, calcium, ammonium, etc.

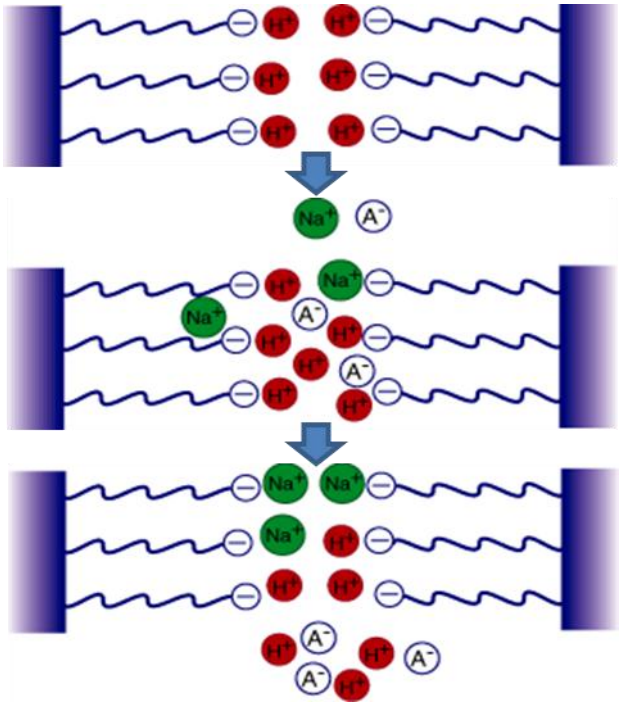

Figure 35 : Ion exchange phenomenon

D. Affinity chromatography:

Affinity chromatography utilizes the specific interactions between a target analyte and a ligand immobilized on the stationary phase.

The stationary phase is designed to selectively bind the target analyte, while other components pass through unretained.

This technique is particularly useful for the purification and isolation of specific biomolecules, such as proteins, enzymes, antibodies, and nucleic acids.

Affinity chromatography often involves the use of affinity tags, antibodies, or specific binding agents for target molecule capture (Figure 36).

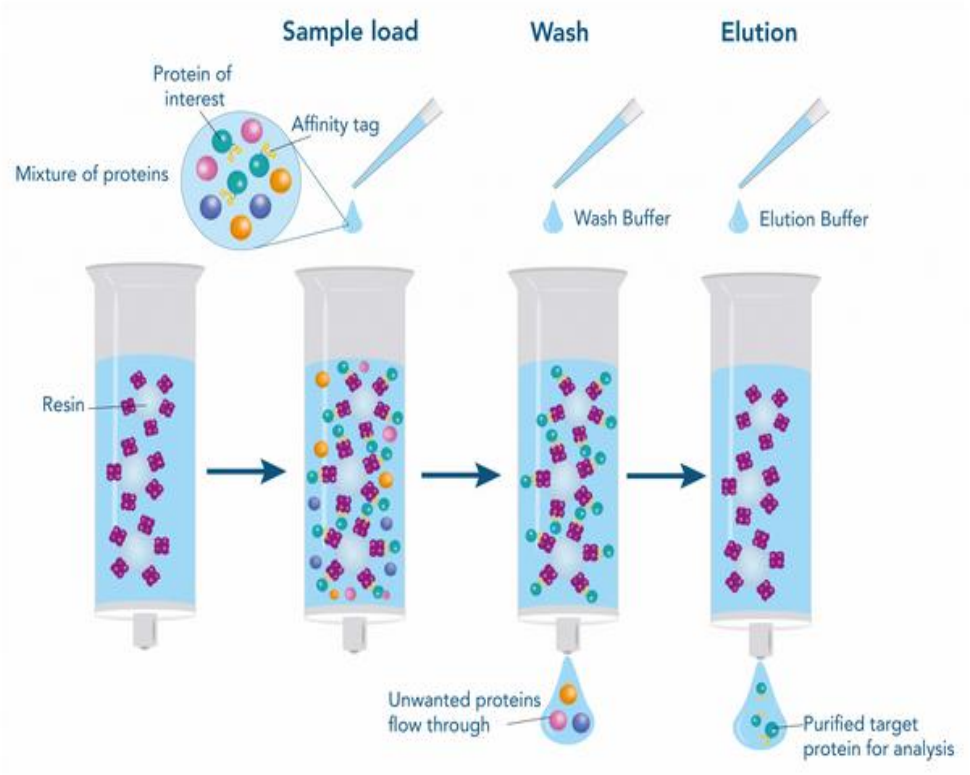

Figure 36: Principle of affinity-based protein purification

E. Size Exclusion Chromatography (SEC):

Size exclusion chromatography separates components based on their size or molecular weight (Figure 37).

The stationary phase consists of porous particles that allow smaller molecules to enter the pores, resulting in longer retention times.

Larger molecules are excluded from the pores and elute earlier.

SEC is commonly used for the analysis of polymers, proteins, peptides, oligonucleotides, and other macromolecules.

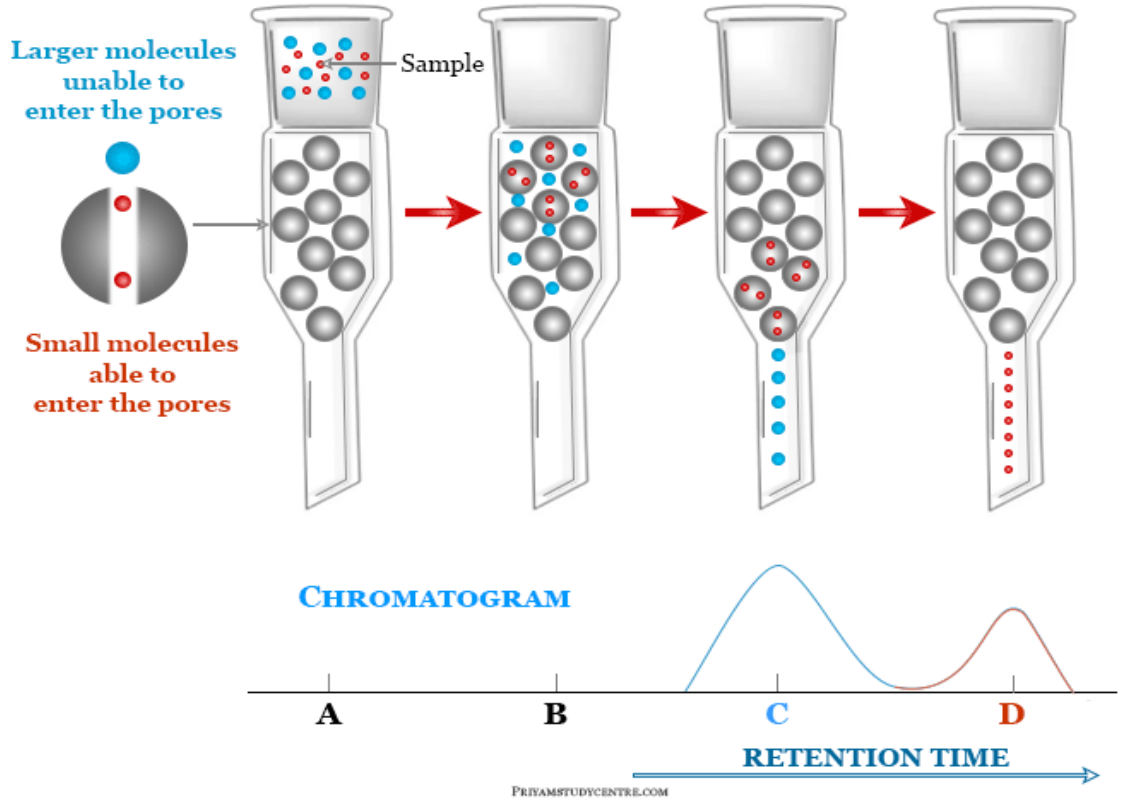

Figure 37: Size exclusion chromatography principle

5.3.3. Classification according to the support of the stationary phase:

Based on the packaging of the stationary phase, we can distinguish:

- **Column chromatography (including HPLC and GC):** the stationary phase is in a narrow tube, and the mobile phase progresses by gravity or pressure difference.
- **Planar chromatography (covering TLC and paper chromatography):** the stationary phase is on the surface of a flat support (TLC) or in a porous cellulose sheet (paper chromatography), and the mobile phase moves by capillarity or gravity.

5.4. Condition for chromatographic separation

The condition for chromatographic separation is crucial to obtain accurate and reproducible results. In this section, we will discuss the necessary conditions for effective separation in two main types of chromatography: low-pressure chromatography and high-pressure chromatography.

5.4.1. Low-Pressure Chromatography:

Low-pressure chromatography is a commonly used technique for separating compounds that are not thermally stable or sensitive to high pressure. It includes various methods such as column chromatography, paper chromatography, thin-layer chromatography, etc. Here are the important conditions for effective low-pressure separation:

A. Solvent selection: The solvent should be chosen based on the nature of the samples to be separated and their interactions with the stationary phase. It should be miscible with the sample and sufficiently fluid to allow proper migration of the compounds.

B. Stationary phase: The stationary phase can be made of various materials such as resins, gels, polymers, etc. It should be chosen based on the chemical properties of the compounds to be separated to ensure selective interaction with them.

C. Flow rate and sample volume: The sample flow rate should be controlled to avoid overloading the chromatographic column, which could compromise resolution. Additionally, the injected sample volume should be carefully adjusted to ensure adequate separation of the compounds.

5.4.2. High-Pressure Chromatography:

High-pressure chromatography, also known as high-performance liquid chromatography (HPLC), is a more advanced and faster separation method compared to low-pressure chromatography. It is widely used for analyzing complex samples such as

pharmaceutical mixtures, biological samples, etc. Here are the important conditions for effective high-pressure separation (Figure 38) :

A. Pressure and flow rate: HPLC utilizes high-pressure pumps to maintain a constant solvent flow rate through the chromatographic column. The pressure should be maintained within the limits recommended by the column manufacturer to avoid any damage and ensure effective separation.

B. Choice of chromatographic column: The chromatographic column should be chosen based on the properties of the samples to be separated, such as their polarity, molecular size, etc. Stationary phases with different particle sizes and stationary phases are available to meet various analytical requirements.

C. Temperature conditions: Temperature can influence chromatographic separation by affecting the interactions between the compounds and the stationary phase. Precise control of the column and detector temperature can improve the resolution and reproducibility of the analyses.

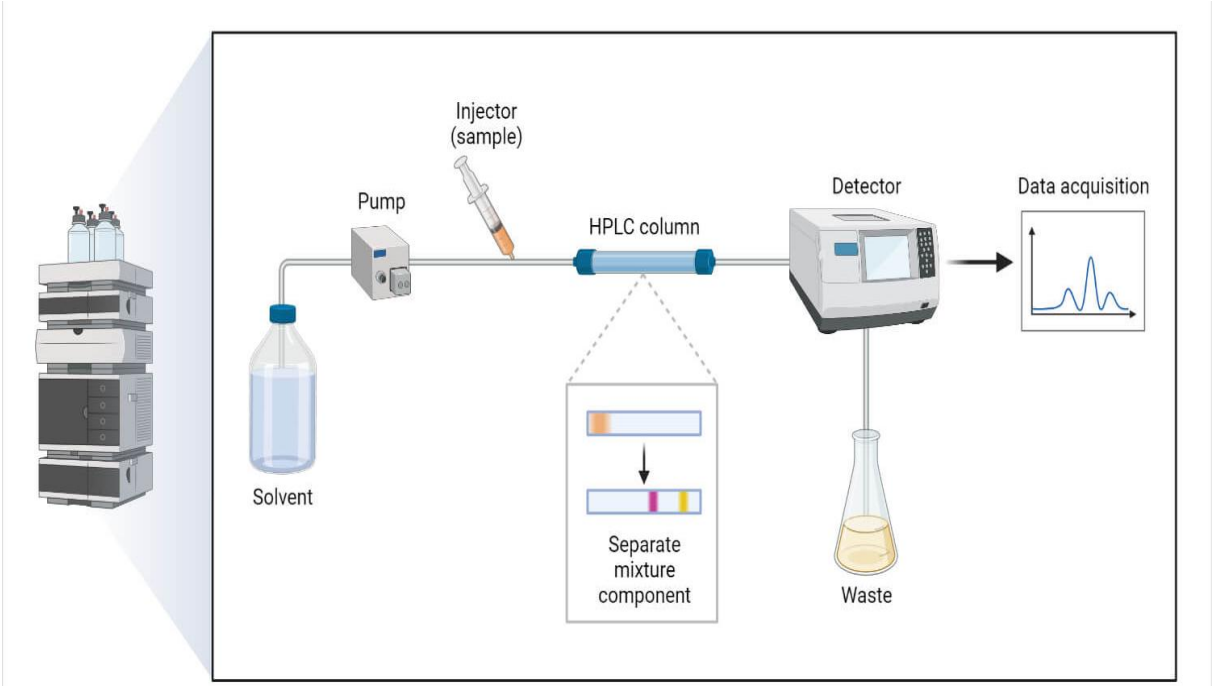

Figure 38: high-performance liquid chromatography (HPLC)

6. Electrophoretic methods

6.1. Definition:

Electrophoresis is a method of separating molecules on the basis of their ability to move in an electric field. Electrophoresis has become the most extensively used method for analyzing biomolecules in biochemistry or molecular biology, including genetic components such as DNA or RNA, proteins, and polysaccharides (Figure 39).

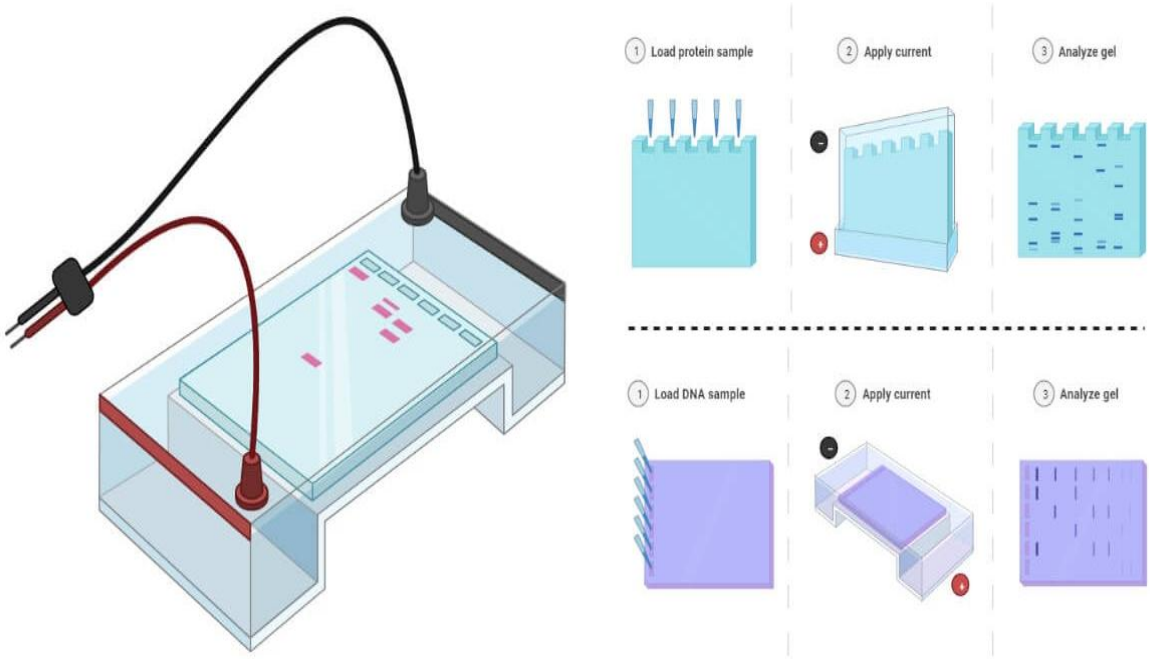

Figure 39 : Gel electrophoresis system

6.2. Principle of electrophoresis:

When a potential difference is introduced, molecules with different overall charges start to segregate due to differences in electrophoretic mobility. Even molecules with equal charges will begin to split if their molecular sizes differ because they would encounter distinct frictional forces. As a result, certain forms of electrophoresis rely almost entirely on the various charges on molecules for separation, whilst others rely on differences in the size (molecular size) of molecules (Figure 40).

The migration and separation of charged particles (ions) under the influence of an

electric field are referred to as electrophoresis. An electrophoretic system consists of two electrodes of opposite charge (anode, cathode) linked by a conducting substance known as an electrolyte. The separation effect on ionic particles is caused by changes in velocity (v), which is the product of particle mobility (m) and field strength (E):

V = ME

An ionic particle's mobility (m), which is constant under specific electrophoretic circumstances, is dictated by the particle's size, shape, charge, and temperature during separation.

The rate of movement of charged molecules is affected by the following factors:

(a) The electric field's strength, size, and shape.

(b) The sample's relative hydrophobicity.

(c) The buffer's ionic strength and temperature.

(d) The biomolecule's molecular size.

(e) The biomolecule's net charge density.

(f) The biomolecule's shape.

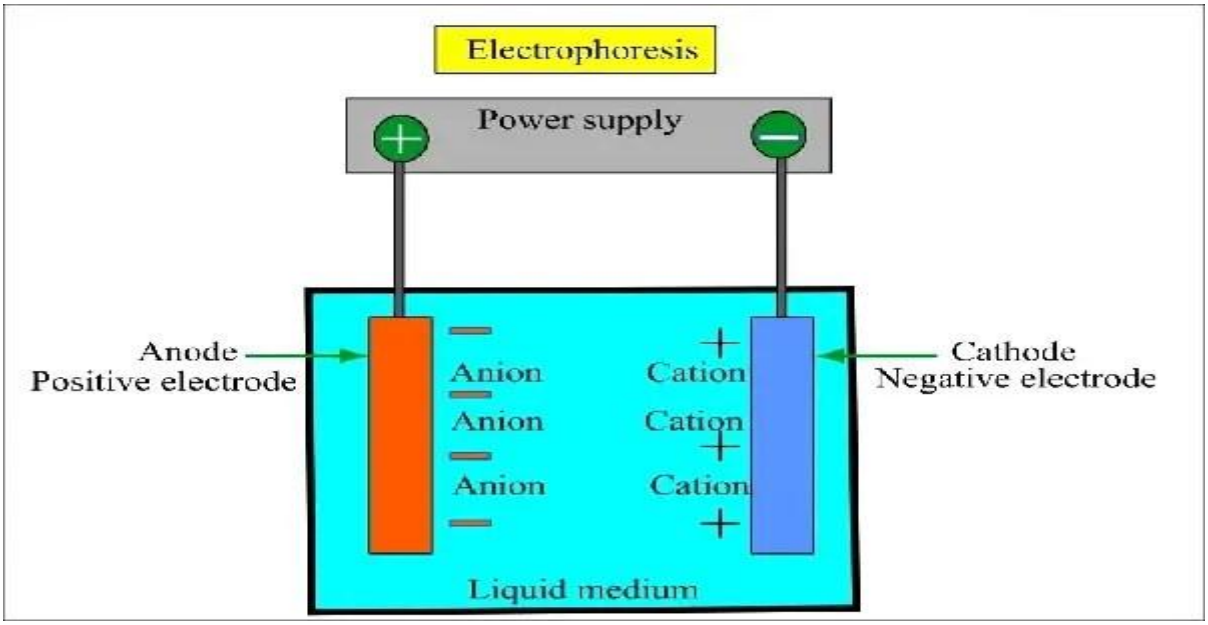

Figure 40: General principle of gel electrophoresis

6.3. Electrophoresis instrument

Modern electrophoresis equipment and systems vary based on its types and forms. However, all the electrophoretic system possesses two essential components (Figure 41):

A. Power pack:

Power supply drives the movement of ionic species in the medium and allows adjustment and control of either the current or the voltage.

B. An electrophoresis unit

An electrophoretic system depends on its type but essentially consists of two electrodes of opposite charge (anode and cathode), connected by a conducting medium called an electrolyte. In addition, a supportive medium is present in electrophoretic systems like gel and paper electrophoresis.

- Buffer (Electrolyte)

Buffers carry applied electric current and provide appropriate pH for the process. Conducting (running) buffers like Tris borate EDTA (TBE) and Tris-acetate acid EDTA (TAE) are commonly used.

- Supportive Medium

The supportive medium is the matrix (gel), in which biomolecules are separated. It can be in the slab or capillary form. The supportive mediums used are sugar polymers like agarose gel, polyacrylamide gel, starch gel, and cellulose acetate gel.

The medium runs either vertical or horizontal gel systems in gel electrophoresis. Horizontal: agarose gel electrophoresis, and vertical: SDS-PAGE. The higher the pore size, the higher the speed traveled by charged particles.

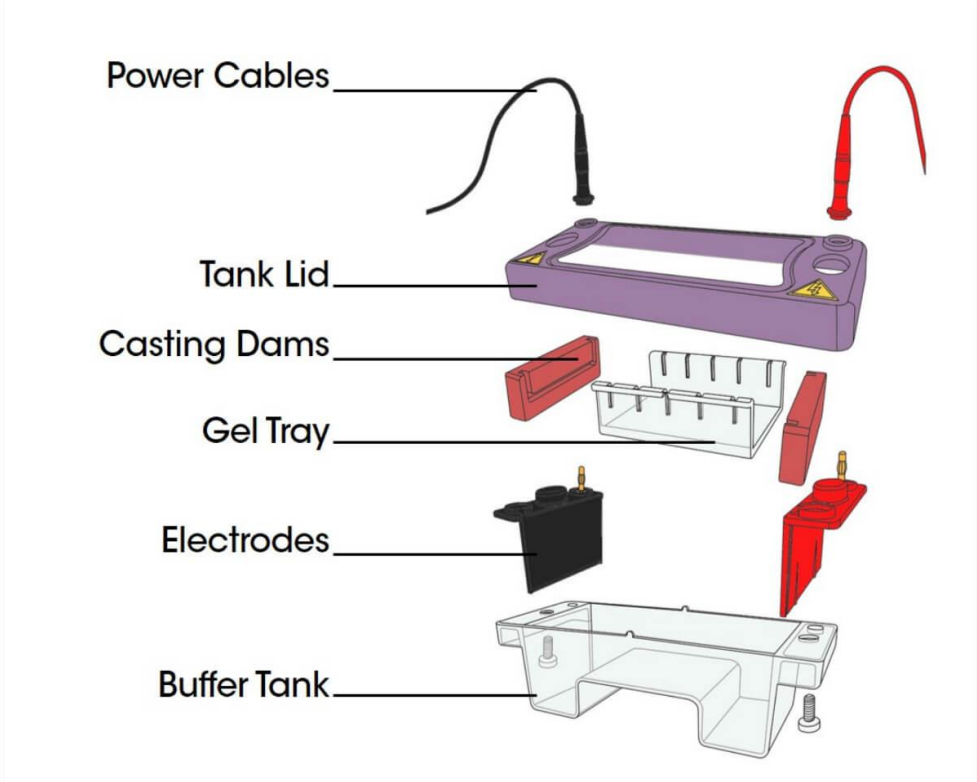

Figure 41: Parts of gel electrophoresis apparatus

6.4. Parameters and experimental conditions:

When performing electrophoresis, several parameters and experimental conditions need to be considered:

- In native electrophoresis, the analysis is carried out in a buffer solution that maintains the native structure and charge of the molecules. The pH, buffer composition, and voltage are carefully chosen to preserve the natural state of the molecules during separation.
- On the other hand, electrophoresis in a dissociating or denaturing medium involves using agents like SDS or urea to disrupt the native structure of the molecules. This allows for size-based separation and is commonly used in protein analysis.

6.4. Different types of electrophoresis:

6.4.1. Support electrophoresis:

Support electrophoresis is a technique that utilizes supporting matrices or supports to facilitate the separation of molecules based on their size or charge. Various types of support

materials can be used, such as agarose, cellulose acetate, or polyacrylamide gels.

A. Agarose gel electrophoresis:

Agarose gel electrophoresis is commonly employed for the separation of nucleic acids, particularly DNA fragments. Agarose gels are easy to prepare and handle, and their porosity can be adjusted by altering the agarose concentration. DNA samples are loaded into wells created in the gel, and an electric field is applied. The negatively charged DNA molecules migrate through the gel matrix towards the positive electrode, with smaller fragments moving faster and traveling farther from the loading wells than larger fragments. This technique allows for the visualization and analysis of DNA fragments, such as in DNA fingerprinting or genetic testing.

B. Cellulose acetate electrophoresis:

Cellulose acetate electrophoresis is another support electrophoresis technique used for the separation of charged biomolecules. It is commonly utilized for the analysis of serum proteins, such as in the diagnosis of certain diseases or monitoring protein abnormalities. In this method, a cellulose acetate sheet is immersed in a buffer solution, and the sample is applied as a line or spot on the sheet. An electric field is then applied, causing the proteins to migrate through the cellulose acetate matrix based on their charge and size. After electrophoresis, the proteins are visualized through staining or immunodetection methods.

C. Polyacrylamide gel electrophoresis (PAGE):

Polyacrylamide gel electrophoresis (PAGE) is a widely used technique for the separation of proteins based on their size and charge. Polyacrylamide gels can be prepared with different concentrations to achieve different pore sizes, allowing for fine-tuned separation. SDS-PAGE, a variant of polyacrylamide gel electrophoresis, involves the denaturation of proteins with sodium dodecyl sulfate (SDS) and a reducing agent, which unfolds the proteins and coats them with a negative charge. This equalizes the charge-to-mass

ratio of the proteins, allowing for separation based solely on size during electrophoresis. SDS-PAGE is commonly used for protein purification, characterization, and analysis in various research fields, including biochemistry and molecular biology (Figure 42).

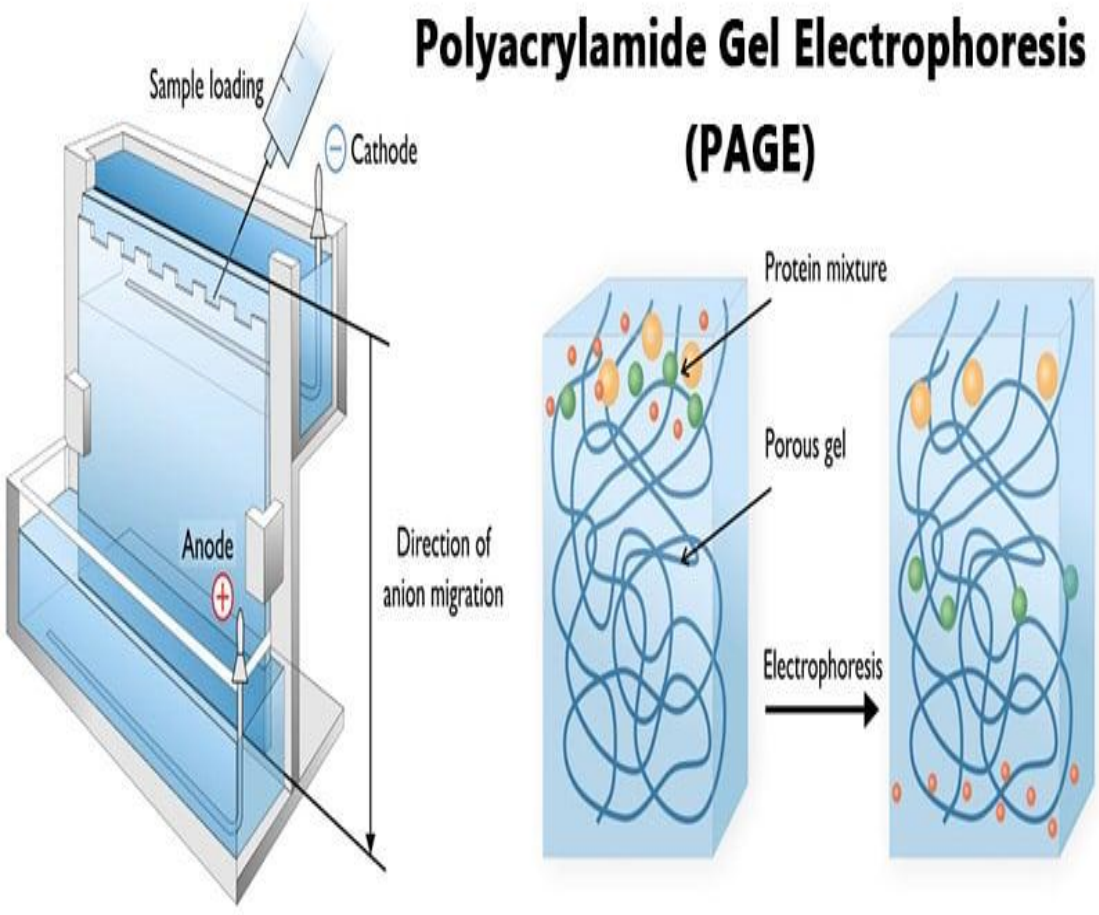

Figure 42: Principle of Polyacrylamide Gel Electrophoresis (PAGE)

6.4.2. Isoelectric focusing:

Isoelectric focusing (IEF) is a high-resolution technique used to separate proteins or peptides based on their isoelectric points (pI). The pI of a molecule is the pH at which it carries no net charge. In IEF, a pH gradient is established within a gel matrix, and an electric field is applied perpendicular to the gradient (Figure 43).

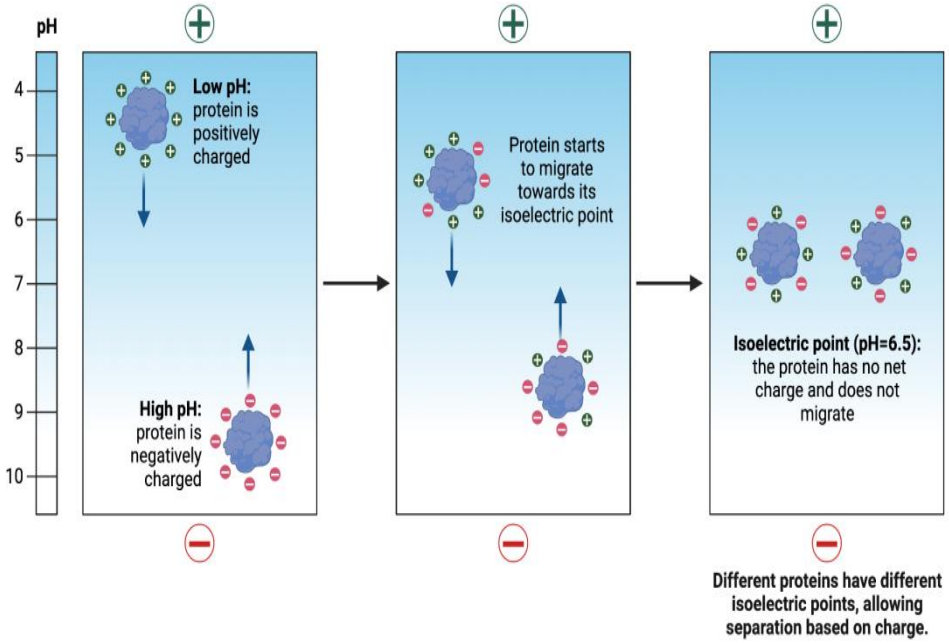

Figure 43: Principle of isoelectric focusing

The process begins by preparing a gel with a pH gradient using either chemical or immobilized pH gradient (IPG) techniques. IPG strips are commonly used, consisting of a gel strip with immobilized buffering molecules that establish a pH gradient when rehydrated and placed in the gel apparatus. The sample, containing a mixture of proteins or peptides, is then applied to the gel strip.

When the electric field is applied, the charged proteins or peptides migrate through the gel strip towards their respective isoelectric points. Once a molecule reaches its pI, it becomes electrically neutral and stops migrating. This results in the separation of proteins or peptides along the length of the gel strip, with each molecule accumulating at its specific pI position.

IEF provides exceptional resolution because it separates molecules based on their charge rather than size. It is particularly useful for analyzing complex protein mixtures, identifying protein isoforms, and investigating post-translational modifications. After separation, the proteins can be further analyzed or visualized using staining techniques, such as Coomassie Brilliant Blue or silver staining, or transferred to other techniques, such as SDS-

PAGE, for additional analysis.

IEF is a widely employed technique in proteomics research, allowing for the detailed characterization of protein samples based on their charge properties. It has applications in diverse areas, including biomarker discovery, protein profiling, and the study of protein variants and modifications in disease research.

6.4.3. Two-dimensional electrophoresis:

Two-dimensional electrophoresis (2-DE) is a widely used analytical technique in biochemistry and proteomics for separating complex mixtures of proteins based on their molecular weight and charge.

In a two-dimensional electrophoresis experiment, proteins are first separated based on their charge using a technique called isoelectric focusing (IEF). In IEF, proteins migrate through a gel matrix that contains a pH gradient. The proteins move until they reach a position in the gel where the pH matches their isoelectric point (pI), which is the pH at which they have no net charge. This separation in the first dimension results in a series of protein spots distributed along the gel based on their pI values.

After the first dimension separation, the proteins are then subjected to a second separation based on their molecular weight using a technique called SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). In SDS-PAGE, proteins are denatured and treated with sodium dodecyl sulfate, a detergent that gives the proteins a uniform negative charge. The proteins are then separated based on their size by electrophoresis through a polyacrylamide gel (Figure 44).

By combining the first dimension separation (based on charge) with the second dimension separation (based on molecular weight), two-dimensional electrophoresis allows for the separation of complex protein mixtures into individual spots on the gel. These spots can then be further analyzed, for example, by staining with specific dyes or by transferring

them onto membranes for protein identification using mass spectrometry.

Two-dimensional electrophoresis has been instrumental in protein research and has found applications in various fields, including biomarker discovery, protein expression profiling, and comparative proteomics. It provides a powerful tool for studying the complexity and dynamics of protein mixtures and has contributed to our understanding of cellular processes, disease mechanisms, and the identification of potential therapeutic targets.

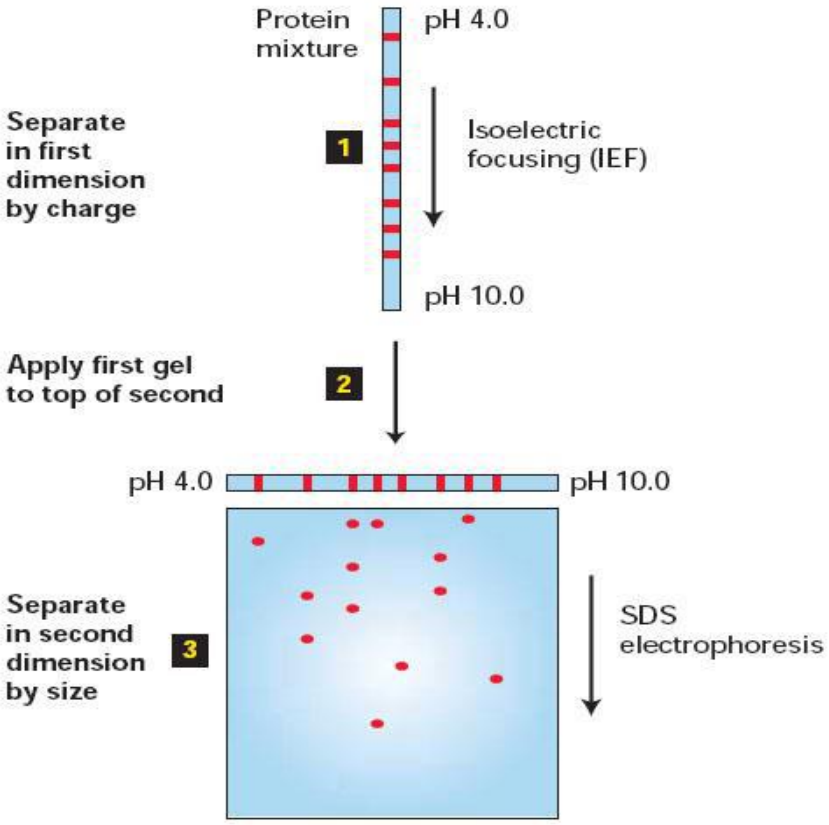

Figure 44: 2D Electrophoresis principle

6.4.4. Immunoelectrophoresis:

Immunoelectrophoresis is a laboratory technique that combines electrophoresis and immunodiffusion to separate and identify specific proteins based on their antigen-antibody interactions (Figure 45).

In immunoelectrophoresis, a mixture of proteins is first separated by electrophoresis based on their charge in a gel matrix. This separation is typically performed using agarose or

polyacrylamide gels. The proteins migrate in an electric field, with negatively charged proteins moving towards the anode and positively charged proteins moving towards the cathode.

After the initial electrophoresis step, an antibody against a specific protein of interest is applied to the gel. The antibody recognizes and binds to the target protein, forming antigen-antibody complexes. The protein-antibody complexes then migrate towards their respective charges, resulting in the formation of precipitation arcs or lines in the gel. These arcs or lines represent the presence and relative concentration of the target protein.

Immunoelectrophoresis allows for the separation and identification of specific proteins based on their antigenic properties. It is particularly useful in the analysis of complex protein mixtures, such as serum or plasma samples, where multiple proteins may be present. By using specific antibodies, it is possible to detect and quantify individual proteins or groups of proteins in the sample.

Immunoelectrophoresis has been widely used in clinical diagnostics and research. It has applications in the detection of disease markers, analysis of immune responses, and characterization of specific proteins or protein patterns. The technique provides valuable information about the presence, quantity, and interactions of proteins, contributing to the understanding of various diseases and biological processes.

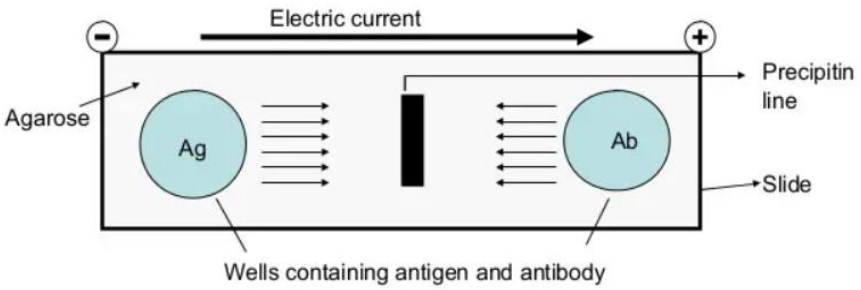

Figure 45: Immunoelectrophoresis principle

6.5. Applications of electrophoresis

- ✓ DNA fingerprinting to separate DNA fragments to investigate crime scenes and paternity testing.
- ✓ Detection of genetic variations and proteins implicated in health and illness.
- ✓ It is employed in the detection and purification of nucleic acids and proteins for scientific purposes.
- ✓ It helps to find pathogens in the blood, other tissues, or sources like food.
- ✓ It facilitates the identification and purification of proteins or nucleic acids frequently examined in greater detail using mass spectrometry or DNA sequencing.
- ✓ It is used in blotting methods to analyze macromolecules and evolutionary studies.
- ✓ It facilitates the evaluation of results of Polymerase Chain Reaction (PCR).
- ✓ Vaccine development and manufacturing both benefit from electrophoresis.
- ✓ To differentiate species and evolutionary relationships, taxonomy-DNA profiling is performed.

Chapter III:
Electron Microscopy

1. Electron microscopy:

An electron microscope uses a beam of accelerated electrons for imaging. Since the wavelength of electrons is much shorter than that of visible light, it results in a much higher imaging resolution in electron microscope compared to the light microscope.

Two types of electron microscopy are used in analysis: Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) both equipped with Energy Dispersive X-Ray Spectrometer (EDS/EDX) (Figure 46).

The SEM uses an electron beam with lower acceleration voltage that scans the surface of the sample to create a 3D image of its microstructure. Thus, in the SEM, the sample thickness is limited only by the chamber size. In contrast, in the TEM the beam penetrated the sample limiting its thickness (ultrathin sections). As a result, the ultrastructure becomes visible. Detecting transmitted electrons also means that the beam acceleration voltage, therefore also the resolution, of the TEM is usually higher compared to the SEM.

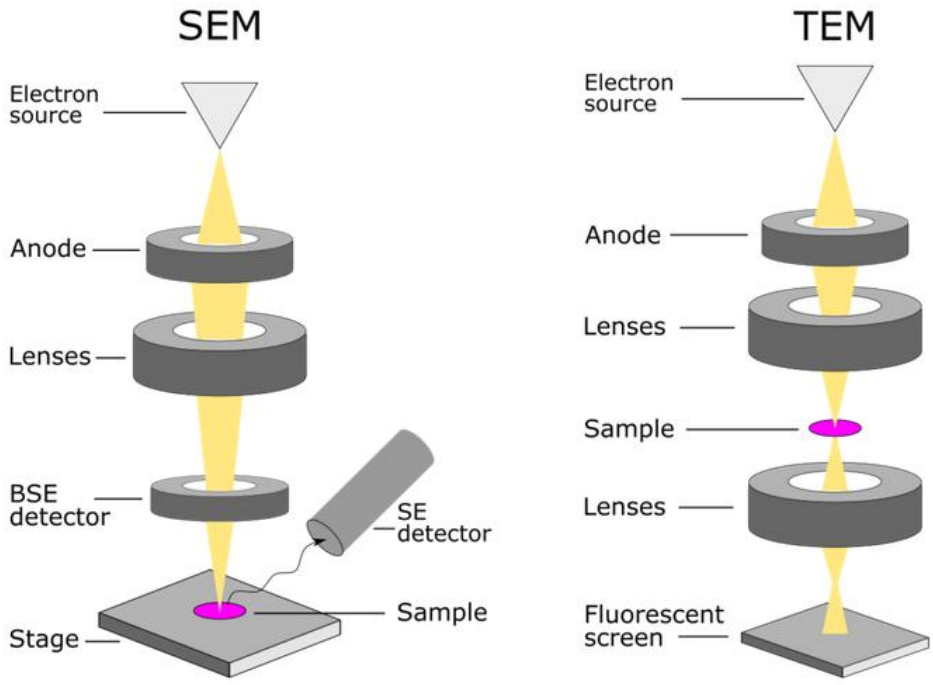

Figure 46: Schematic diagram of scanning electron microscope and transmission electron microscope

2. Transmission electron microscopy:

2.1. Definition:

Transmission electron microscopy (TEM) is a form of microscopy which in which a beam of electrons transmits through an extremely thin specimen, and then interacts with the specimen when passing through it. The formation of images in a TEM can be explained by an optical electron beam diagram (Figure 47)

TEMs provide images with significantly higher resolution than visible-light microscopes (VLMs) do because of the smaller de Broglie wavelength of electrons. These electrons allow for the examination of finer details, which are several thousand times higher than the highest resolution in a VLM. Nevertheless, the magnification provide in a TEM image is in contrast to the absorption of the electrons in the material, which is primarily due to the thickness or composition of the material.

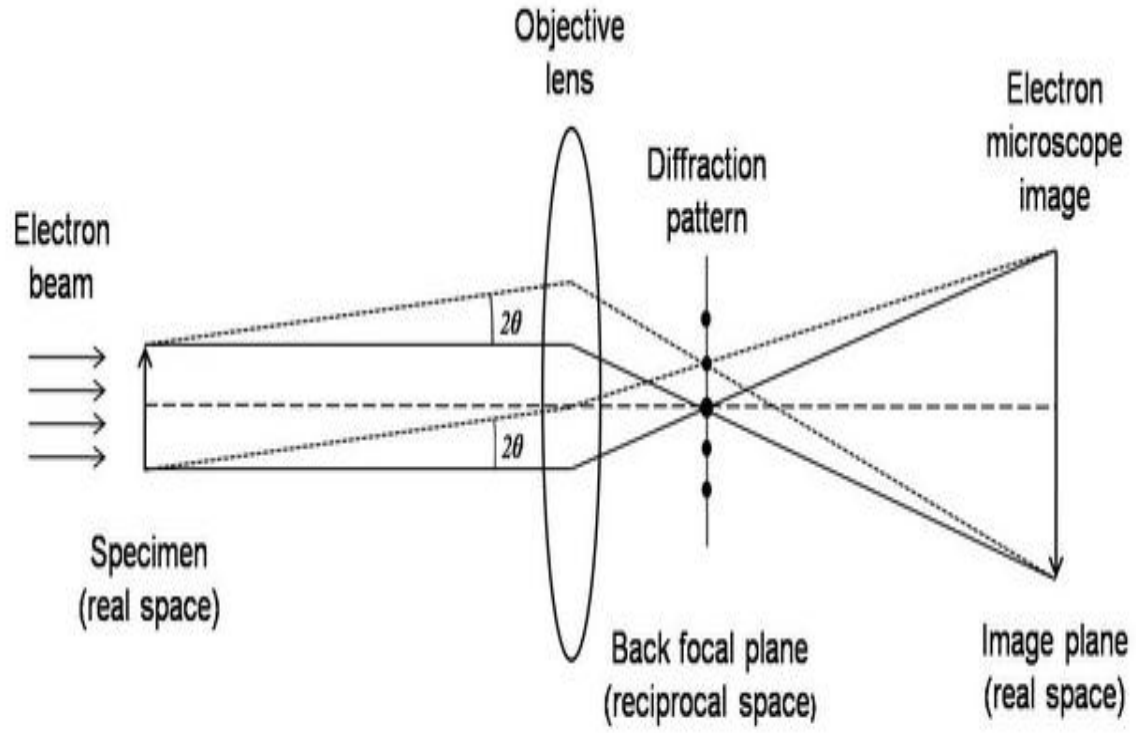

Figure 47: The optical electron beam diagram of TEM.

2.2. Principle of transmission electron microscope:

The working principle of the Transmission Electron Microscope (TEM) is similar to the light microscope. The major difference is that light microscopes use light rays to focus and produce an image while the TEM uses a beam of electrons to focus on the specimen, to produce an image.

Electrons have a shorter wavelength in comparison to light which has a long wavelength. The mechanism of a light microscope is that an increase in resolution power decreases the wavelength of the light, but in the TEM, when the electron illuminates the specimen, the resolution power increases increasing the wavelength of the electron transmission. The wavelength of the electrons is about 0.005nm which is 100,000X shorter than that of light, hence TEM has better resolution than that of the light microscope, of about 1000times.

This can accurately be stated that the TEM can be used to detail the internal structures of the smallest particles like a virion particle.

2.3. Components of TEM:

A Transmission Electron Microscope (TEM) consists of three essential systems:

- **Electron gun and condenser system (illumination system):**

The electron gun produces the electron beam, while the condenser system focuses the beam onto the object (sample).

- **Image-producing system:**

This system consists of the objective lens, movable specimen stage, and a series of lenses including the objective, intermediate, and projector lenses. These lenses focus the electrons passing through the specimen to form a real and highly magnified image.

- **Image-recording system:**

The image-recording system converts the electron image into a form perceptible to the human eye. It typically includes a fluorescent screen for viewing and focusing the image, as well as a

digital CCD camera for permanent records. Additionally, a vacuum system with pumps, gauges, valves, and power supplies is required.

2.3.1. Illumination system (electron gun and condenser system):

A. Electron Gun:

The electron gun consists of a heated, sharply pointed rod-shaped lanthanum hexaboride cathode. The cathode is surrounded by a control grid called the Wehnelt cylinder, with a central aperture aligned on the column axis. The cathode and control grid are negatively charged at the desired accelerating voltage and insulated from the rest of the instrument. The final electrode of the electron gun is the anode, which is a disk with an axial hole. Electrons emitted from the cathode accelerate toward the anode. Proper control and alignment of the electron gun are critical for satisfactory operation.

B. Condenser system:

The condenser system, located between the electron gun and the specimen, controls the intensity and angular aperture of the electron beam. It typically uses a double condenser system, where the first lens converges the beam onto the object, and the second lens images the reduced source onto the object. This configuration helps minimize disturbances in the specimen caused by heating and irradiation.

2.3.2. Image-producing system:

A. Objective lenses and projector lenses:

The specimen grid is held in a small holder on a movable specimen stage. The objective lens, usually with a short focal length (1-5 mm), produces a real intermediate image, which is further magnified by the projector lens or lenses. A single projector lens can provide a range of magnification of 5:1, and interchangeable pole pieces in the projector allow for a wider range of magnifications. Modern instruments often employ two projector lenses, including an intermediate lens, to enable a greater range of magnification without a significant increase in the

physical length of the microscope column.

For image stability and brightness, the microscope is typically operated at a final magnification of 1,000-250,000x on the screen. Higher magnifications can be achieved through photographic or digital enlargement. The quality of the final image in the electron microscope depends largely on the accuracy of mechanical and electrical adjustments that align the lenses with each other and the illuminating system. The lenses require highly stable power supplies, and electronic stabilization to better than one part in a million is necessary for the highest resolution. Modern electron microscopes are controlled by computers, with dedicated software readily available.

2.3.3. Image-recording system (fluorescent screen and digital photographic unit):

TEM provides information in the form of variations in electron intensity within the image. The electron image is monochromatic and can be made visible to the eye in two ways. First, the electrons can be directed onto a fluorescent screen located at the base of the microscope column. Alternatively, the image can be captured digitally for display on a computer monitor. Computerized images are typically stored in formats such as TIFF or JPEG.

The components of a TEM work together to generate and manipulate the electron beam, focus it onto the specimen, produce magnified images, and record the resulting data for observation and analysis (Figure 48).

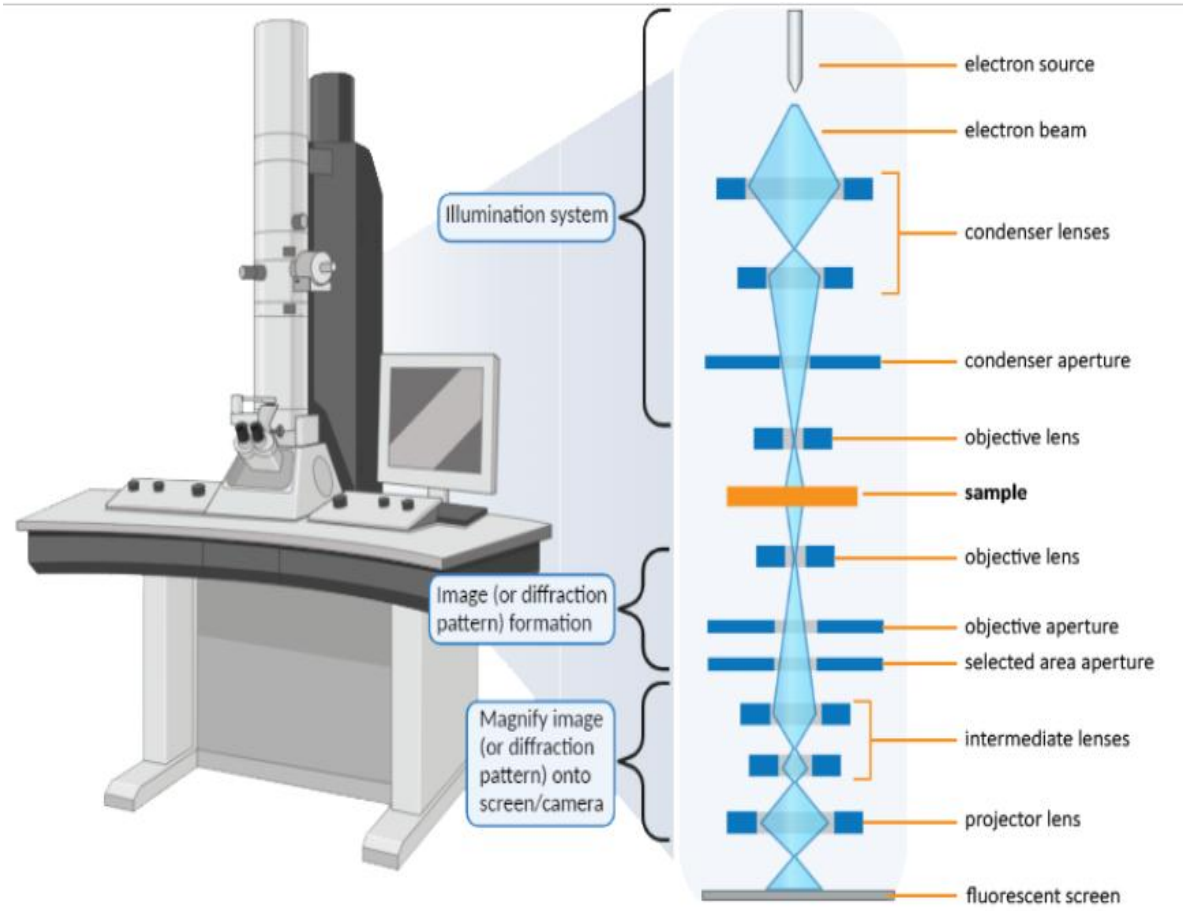

Figure 48: Simplified TEM diagram highlighting the major components throughout the column.

2.4. Biological sample preparation techniques for TEM:

Biological sample preparation techniques for Transmission Electron Microscopy (TEM) involve several steps to ensure the preservation, visualization, and analysis of the ultrastructure of biological specimens:

A. Tissue isolation: This step involves the isolation of the specific tissue or organ of interest from the organism. The tissue is carefully dissected and handled to maintain its structural integrity.

B. Fixation: The isolated tissue is then subjected to fixation, which involves the use of chemical fixatives to preserve the cellular structure and prevent degradation. Common fixatives used in TEM include glutaraldehyde, OsO4 (osmium tetroxide), and

occasionally KMnO4 (potassium permanganate). Glutaraldehyde helps to cross-link proteins and stabilize the cellular components, while OsO4 and KMnO4 provide contrast by staining lipids and other cellular structures.

C. Embedding: After fixation, the tissue is dehydrated using a series of alcohol washes and then embedded in a plastic resin. The resin infiltrates the tissue and hardens to form a solid block, providing support and stability for subsequent processing.

D. Ultramicrotomy: The embedded tissue block is cut into ultra-thin sections (typically around 50-100 nanometers thick) using an ultramicrotome. This instrument uses a diamond or glass knife to slice the block, and the sections are collected on grids or support films.

E. Post-staining: Thin sections are often stained with heavy metals, such as uranyl acetate and lead citrate, to enhance contrast and improve visualization of cellular structures under the electron beam. These stains selectively bind to different cellular components, highlighting specific features.

F. Photography: Finally, the prepared samples are loaded onto a TEM grid and placed in the electron microscope for imaging. Electron beams are used to illuminate the sample, and the resulting electron scattering patterns are recorded by detectors. Images are captured and can be further processed for analysis.

These steps in biological sample preparation for TEM ensure that the delicate cellular structures are preserved, contrast is enhanced, and high-resolution imaging is achieved for detailed analysis and understanding of biological samples at the ultrastructural level.

2.5. Difference between TEM and SEM:

The main differences between TEM and SEM are summarized in table 4:

Table 6: Difference between TEM and SEM:

Feature	TEM	SEM
Electron Beam	Broad, static beams	**Beam focused to finepoint;sample is scanned line by line**
Voltages Needed	Accelerating voltage much lower; not nec-essary to penetrate the specimen	**SEM voltage ranges from60-300,000 volts**
Interaction of the beam electrons	Specimen must be very thin	**Wide range of specimensallowed; simplifies sample preparation**
Imaging	Electrons must pass through and be trans-mitted by the specimen	**Information needed is collected near the sur-face of the specimen**
Image Rendering	**Transmitted electrons are collectively focused by the objective lens and magnified to createa real image**	**Beam is scanned along the surface of the sampleto build up the image**

2.6. Applications of TEM:

- ✓ Transmission Electron Microscope is ideal for a number of different fields such as life sciences, nanotechnology, medical, biological and material research, forensic analysis, gemology and metallurgy as well as industry and education.
- ✓ TEMs provide topographical, morphological, compositional and crystalline information.

- ✓ The images allow researchers to view samples on a molecular level, making it possible to analyze structure and texture.
- ✓ This information is useful in the study of crystals and metals, but also has industrial applications.
- ✓ TEMs can be used in semiconductor analysis and production and the manufacturing of computer and silicon chips.
- ✓ Technology companies use TEMs to identify flaws, fractures and damages to micro-sized objects; this data can help fix problems and/or help to make a more durable, efficient product.
- ✓ Colleges and universities can utilize TEMs for research and studies.
- ✓ Although electron microscopes require specialized training, students can assist professors and learn TEM techniques.
- ✓ Students will have the opportunity to observe a nano-sized world in incredible depth and detail.

2.7. Advantages of TEMs

- ✓ TEMs offer the most powerful magnification, potentially over one million times or more
- ✓ TEMs have a wide-range of applications and can be utilized in a variety of different scientific, educational and industrial fields
- ✓ TEMs provide information on element and compound structure
- ✓ Images are high-quality and detailed
- ✓ TEMs are able to yield information of surface features, shape, size and structure
- ✓ They are easy to operate with proper training

2.8. Disadvantages of TEMs

- ✓ Some cons of electron microscopes include:
- ✓ TEMs are large and very expensive

- ✓ Laborious sample preparation
- ✓ Potential artifacts from sample preparation
- ✓ Operation and analysis requires special training
- ✓ Samples are limited to those that are electron transparent, able to tolerate the vacuum chamber and small enough to fit in the chamber
- ✓ TEMs require special housing and maintenance
- ✓ Images are black and white
- ✓ Electron microscopes are sensitive to vibration and electromagnetic fields and must be housed in an area that isolates them from possible exposure.
- ✓ A Transmission Electron Microscope requires constant upkeep including maintaining voltage, currents to the electromagnetic coils and cooling

3. Scanning electron microscopy:

3.1. Definition:

Scanning Electron Microscope (SEM) is a type of electron microscope that scans surfaces of microorganisms that uses a beam of electrons moving at low energy to focus and scan specimens. The development of electron microscopes was due to the inefficiency of the wavelength of light microscopes. Electron microscopes have very short wavelengths in comparison to the light microscope which enables better resolution power (Figure 49).

Figure 49: Scanning electron microscope

3.2. Principle of scanning electron microscope

Unlike the Transmission Electron Microscope which uses transmitted electrons, the scanning electron Microscope uses emitted electrons. The Scanning electron microscope works on the principle of applying kinetic energy to produce signals on the interaction of the electrons. These electrons are secondary electrons, backscattered electrons, and diffracted backscattered electrons which are used to view crystallized elements and photons. Secondary and backscattered electrons are used to produce an image. The secondary electrons are emitted from the specimen play the primary role of detecting the morphology and topography of the specimen while the backscattered electrons show contrast in the composition of the elements of the specimen.

The source of the electrons and the electromagnetic lenses are from tungsten filament lamps that are placed at the top of the column and it is similar to those of the transmission electron Microscope.

The electrons are emitted after thermal energy is applied to the electron source and allowed to move in a fast motion to the anode, which has a positive charge. The beam of

electrons activates the emission of primary scattered (Primary) electrons at high energy levels and secondary electrons at low-energy levels from the specimen surface. The beam of electrons interacts with the specimen to produce signals that give information about the surface topography and composition of the specimen.

The specimen does not need special treatment for visualization under the SEM, even air-dried samples can be examined directly. However, microbial specimens need fixation, dehydration, and drying in order to maintain the structural features of the cells and to prevent collapsing of the cells when exposed to the high vacuum of the microscope. The samples are mounted and coated with thin layer of heavy metal elements to allow spatial scattering of electric charges on the surface of the specimen allowing better image production, with high clarity.

Scanning by this microscope is attained by tapering a beam of electrons back and forth over a thin section of the microscope. When the electrons reach the specimen, the surface releases a tiny staw of electrons known as secondary electrons which are then trapped by a special detector apparatus.

When the secondary electrons reach and enter the detector, they strike a scintillator (a luminescence material that fluoresces when struck by a charged particle or high-energy photon). This emits flashes of light which get converted into an electric current by a photomultiplier, sending a signal to the cathode ray tube. This produces an image that looks like a television picture that can be viewed and photographed.

The quantity of secondary electrons that enter the detector is highly defined by the nature of the specimen i.e raised surfaces to receive high quantities of electrons, entering the detector while depressed surfaces have fewer electrons reaching the surface and hence fewer electrons enter the detector. Therefore raised surfaces will appear brighter on the screen while depressed surfaces appear darker.

3.3. Apparatus description:

The major components of the Scanning Electron Microscope include (Figure 38):

- Electron Source : This is where electrons are produced under thermal heat at a voltage of 1-40kV. the electrons condense into a beam that is used for the creation of an image and analysis. There are three types of electron sources that can be used i. e Tungsten filament, Lanthanum hexaboride, and Field emission gun (FEG)
- Lenses: it has several condenser lenses that focus the beam of electrons from the source through the column forming a narrow beam of electrons that form a spot called a spot size.
- Scanning Coil : they are used to deflect the beam over the specimen surface.
- Detector : It's made up of several detectors that are able to differentiate the secondary electrons, backscattered electrons, and diffracted backscattered electrons. The functioning of the detectors highly depends on the voltage speed, the density of the specimen.
- The display device (data output devices)
- Power supply
- Vacuum system

Like the transmission electron Microscope, the Scanning electron microscope should be free from vibrations and any electromagnetic elements (Figure 50).

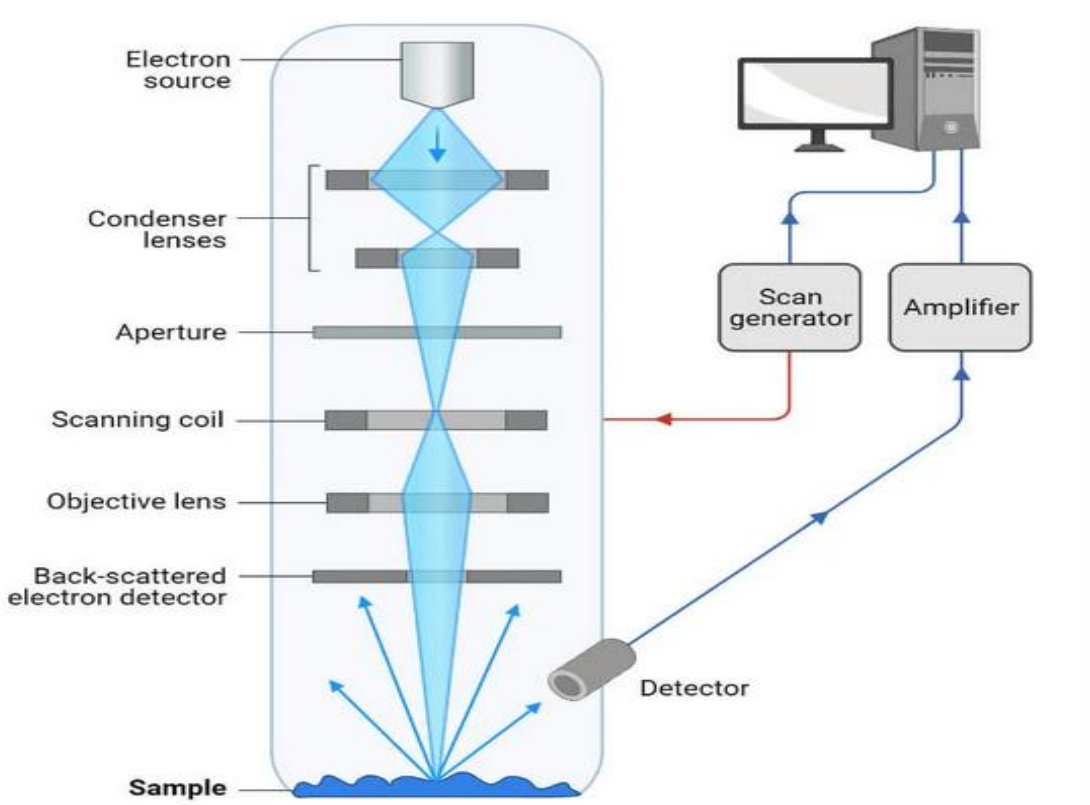

Figure 50: Parts of a scanning electron microscope

3.4. Applications of the scanning electron microscope

It is used in a variety of fields including Industrial uses, nanoscience studies, Biomedical studies, Microbiology

- Used for spot chemical analysis in energy-Dispersive X-ray Spectroscopy.
- Used in the analysis of cosmetic components which are very tiny in size.
- Used to study the filament structures of microorganisms.
- Used to study the topography of elements used in industries.

3.5. Advantages of the scanning electron microscope

- They are easy to operate and have user-friendly interfaces.
- They are used in a variety of industrial applications to analyze surfaces of solid objects.
- Some modern SEMs are able to generate digital data that can be portable.
- It is easy to acquire data from the SEM, within a short period of time of about 5 minutes.

3.6. Limitations of the scanning electron microscope

- They are very expensive to purchase
- They are bulky to carry
- They must be used in rooms that are free of vibrations and free of electromagnetic elements
- They must be maintained with a consistent voltage
- They should be maintained with access to cooling systems

The combination of the working principles of the Scanning Electron Microscope (SEM) and the Transmission Electron Microscope (TEM) formed the Scanning-Transmission Electron Microscope (STEM). The Scanning- Transmission Electron Microscope (STEM), uses a convergent beam of electrons to focus on a probe on the specimen, and the probe is then scanned on its surface collecting signals which are then collected as point-to-point to form an image.

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