Uttar Pradesh Rajarshi Tondon
open University

SBSBCH -01
Bio- Analytical Techniques

COURSE INTRODUCTION

The objective of this course to discuss the basic bio-analytical techniques for biomolecule analysis. The bioanalytical techniques also play important role in quantitative measure of the drug and its metabolites. The characterization by modern analytical techniques determines the constituent of sample. The course is organized into following blocks:

Block 1 covers the basics of bio-analytical techniques

Block 2 deals the chromatography and spectroscopy

Block 3 describes in brief about electrophoresis and centrifugation

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SBSBCH -01
Bio- Analytical Techniques

Block-1

Basic of Bio- analytical Techniques

SBSBCH -01

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Introduction

This is the first block on basics of bio-analytical techniques. It consists of the following three units:

Unit-1: This unit covers the general introduction of techniques that is related to the analysis of biological samples. In this unit basic concept of normality, morality and morality has been discussed. Few points related to purification are also mentioned here.

Unit-2: This unit covers the basic knowledge of pH and Buffer. The role of pH and it determination briefly discussed here. The role of buffer solution and their types clearly defined. How buffer is important is discussed here.

Unit-3: This unit covers the study of light in which the properties of Light has been discussed. The different component of light such as light spectra, wave length etc discuss briefly. The nature of light such as plane polarized light, optical rotation, optical rotator is mentioned in this unit. Behavior of light on chromospheres is discussed.

Unit-1: Basic introductions

1.1. Introduction

Bioanalysis is merely running standard analyses for a biological sample. For the bioanalysis the number of sound bioanalytical method has been developed for characterization and identification for unknown biomolecule. These bioanalytical techniques brought a progressive discipline for which the future holds many exciting opportunities to further improvement. In the characterization process the small molecules are typically synthesized or isolated from natural sources, have lower molecular weights, and are usually assayed using chromatographic assays chemical entities (drugs), metabolites, or pharmacodynamic. Large molecules include higher molecular weight peptides, proteins, nucleic acids, lipids, and polysaccharides, and are usually assayed using ligand binding assays. The bioanalytical techniques are very is also useful in isolation and purification of biomolecule such as protein, amino acids, enzymes and antibodies etc. Affinity chromatography is useful step in overall purification strategy of protein. Centrifugation is most important techniques used in separation of biomolecule under the influence of centrifugal forces. Number of gram equivalent weight in a liter of solution is called normality. Molality is an intensive property, and is therefore independent of the amount being measured.

Objectives

- To discuss about bioanalytical techniques
- To discuss about purification of biomolecule
- To discuss about solution units and their concentration measurement.
- To discuss the Filtration and dialysis

1.2. Basic concept of bio analytical techniques

The bio analytical technique is making life easy because it concern with the qualitative and quantitative analysis of biomolecules that are require for living beings. The characterization of these molecules plays vital role in almost all the aspects of living beings and other scientific and research and development. The role of bioanalytical techniques make to easy for separation and isolation of biomolecules are necessary for diagnosis of diseases and its cure. Biochemical studies rely on the availability of appropriate analytical techniques and on the application of these techniques to the advancement of knowledge of the nature of, and relationships between, biological molecules, especially proteins and nucleic acids, and cellular function. Recently introducing to new bioanalytical techniques making lots of opportunity in field of clinical, forensic, environmental and pharmaceutical sciences. In medicine, bio analytical chemistry is the key for clinical laboratory tests which imparts basis of disease diagnosis and chart progress for recovery to the physicians. However, the goal of bioanalytical techniques should be to brought a progressive discipline for future development. In these day a lots of bioanalytical techniques are used in quantitative measurement that are highly concern with drug investigation, its metabolite or a biomarker in biological fluids, such as blood, plasma, serum, urine, and saliva or in tissue extracts. The bioanalytical techniques also play important role in quantitative measure of the drug and its metabolites. The characterization by modern analytical techniques determines the constituent of sample. Recently the classical analytical techniques used in practice are found to be useful in characterization and detection of biomolecule form biological fluids. Some techniques such as titrimetric methods, spectroscopic methods, electro analytical chromatography, centrifugation and radioimmunoassay are very useful in detection of biological sample. A bioanalytical method is a combination of different procedures which are (i) collection, (ii) processing, (iii) storage, and (iv) analysis of a biological sample (blood-cerebrospinal fluid (CSF), serum, plasma, or urine, tissue, and skin).

However, medical and clinical analyses are undergoing the greatest extension of instrumental methods. Interest in identifying biologically active compounds is growing rapidly and providing new challenges for the analytical chemists.

These challenges have been resolved by the introduction of bioanalytical technique as a modern approach to disease diagnosis and therapy. During the past decades, the analyte that have been targeted in bioanalytical studies include amino acid, peptides, proteins, serum enzyme, tumor and cancer genes, carbohydrates, vitamins, catecholamines, cardiac risk factors. In recent years huge advances have been made in our understanding of gene structure and expression and in the application of techniques such as mass spectrometry to the study of protein structure and function.

Characterizations of biological sample are carried out by using following process.

Sample collection and storage

For the analysis for biomolecule the sample collection and storage in main task. The biological sample collected from patients should be reach to analytical section is play important role in accuracy of result. So that there are different types of preservatives should be known to protect the samples from degradation prior to cryopreservation at a reasonable cost. Cryopreservation is a process to store biological samples at very low temperature for prevention of damage. The purpose is to find readily accessible and data-rich biological samples.

Sample preparation

After collection and storage of sample its defects to prepare it sample as per analysis requirement. Biological samples involve plasma, serum, CSF, bile, urine, tissue homogenates, saliva, seminal fluid, and frequently whole blood. Quantitative analysis of drugs and metabolites containing huge amounts of proteins and large numbers of endogenous compounds within these samples is very complicated. There are different types of techniques are used in sample preparation are mentioned in table (1.1)

Table. 1.1: Used of techniques for sample preparation

Sample preparation techniques	Advantages
Liquid phase extraction (LLE)	It is one of the first methods used for extraction. The resulting extract may be directly analyzed.
Solid phase extraction (SPE)	It is a method for the isolation and concentration of selected analyte from a fluid sample by their transfer on a solid phase.
Affinity separation	The affinity sorbent may consist of an immobilized antibody or a molecularly imprinted polymer.
Solid phase micro extraction	It is a solvent free extraction method, consists of

(SPME)	a single extraction step, but the experimental variables must be well controlled. It reduces solvent and sample volume needs and sample preparation time.
Ultrafiltration and microdialysis (MD)	Ultrafiltration consists of filtering the sample through a special size-excluding filter, either by applying pressure (10–100 psi) or by centrifugation. Dialysis and MD can be used to separate an analyte by diffusion through a semi-permeable membrane.

Sample Detection

After sample preparation the biological sample are characterized by as per require biological techniques. The brief discussion of different types of bioanalytical techniques such as spectroscopic techniques (UV-Visible, FTIR spectroscopy), chromatographic, electrophoresis, centrifugation etc are already discuss in this course. The spectrophotometric technique is used to study interactions between electromagnetic radiations and analyte. The concentration of an analyte is determined by using a graph which is called standard analytical curve. An example is determination of iron in blood serum. The considerable advances made in microarray, sequencing technologies and bioinformatics. Analysis is now beginning to provide true insights into the development and maintenance of cells and tissues.

All analytical methods can be characterized by a number of performance indicators that define how the selected method performs under specified conditions. Knowledge of these performance indicators allows the analyst to decide whether or not the method is acceptable for the particular application. The major performance indicators are:

- **Precision (also called imprecision and variability):**

This is a measure of the reproducibility of a particular set of analytical measurements on the same sample of test analyte. If the replicated values agree closely with each other, the measurements are said to be of high precision (or low imprecision).

- **Accuracy (also called trueness, bias and inaccuracy):**

This is the difference between the mean of a set of analytical measurements on the same sample of test analyte and the 'true' value for the test sample.

- **Detection limit (also called sensitivity):**

This is the smallest concentration of the test analyte that can be distinguished from zero with a defined degree of confidence. All methods have their individual detection limits for a given analyte and this may be one of the factors that influence the choice of a specific analytical method for a given study.

- **Analytical range:**

This is the range of concentrations of the test analyte that can be measured reproducibly, the lower end of the range being the detection limit.

- **Analytical specificity (also called selectivity):**

This is a measure of the extent to which other substances that may be present in the sample of test analyte may interfere with the analysis and therefore lead to a falsely high or low value.

- **Analytical sensitivity:**

This is a measure of the change in response of the method to a defined change in the quantity of analyte present

- **Robustness:**

This is a measure of the ability of the method to give a consistent result in spite of small changes in experimental parameters such as pH, temperature and amount of reagents added.

These performance indicators are established by the use of well-characterized test and reference analyte samples. The order in which they are evaluated will depend on the immediate analytical priorities, but initially the three most important may be specificity, detection limit and analytical range.

1.3. Centrifugation

Ultracentrifuge was developed by Edward Greydon Pickels. It most important techniques used in separation of biomolecule under the influence of centrifugal forces. The term centrifuge is Latin world which define the centrum means centre and fugere, means to flee. It is the govern by ostensible forces that draws a gyratory body away from the centre of rotation. The centrifuge is a significant instrument in cellular and molecular biology research laboratory. Centrifugation work on the principle of sedimentation where the acceleration at centripetal force reasons denser constituents to isolate out beside the radiating direction at the bottommost of the tube. With the similar theory lighter substances will be likely to move to the top of the tube. Particle with more density than the solvent residue and the particle with lesser density than, it drift the solution to the top. The greater the difference in density, the faster they move. The instrument used in centrifugation is called centrifuge which is make

density difference between macromolecules and its medium from which they separated. The particle in a centrifugal field will experience a Centrifugal Force defined by:

$$FC = mr\omega^2$$

Where,
m = Mass of the particle
r = Distance from the axis
ω = Angular Velocity = 2 π revolutions / 60 minutes

Types of centrifugation

1. Differential Centrifugation

This is used in successive centrifugation with increasing centrifugal forces. Separation is mainly dependent on particle's mass and size, where heavier particles or cells settle first at lower **g** values.

2. Moving Boundary or Zone Centrifugation

In this, the whole tube is occupied with sample and centrifuged. With centrifugation parting of two units can be achieved, but any unit in the combination may result in the supernatant or in the pellet or it may be distributed in both fractions.

3. Rate Zonal Centrifugation

In this, we keep the sample on the topmost position of the tube in a very thin zone of density gradient in the influence of centrifugal force. The particles will start sedimenting over the gradient in distinct zones as per their size shape and density.

4. Isopycnic or density gradient centrifugation

In isopycnic technique, the density gradient column encompasses the whole range of thicknesses of the sample material. The matter is consistently agitated with the gradient material.

1.4. Normality:

Normality is the strength of solution that is measured in terms of gram equivalent weight in a Litre. Number of gram equivalent weight in a litre of solution is called normality. It is express in N. However, as per the standard definition, normality is described as the number of gram or mole equivalents of solute present in one litre of a solution. When we say equivalent, it is the number of moles of reactive units in a compound.

If any solution is containing 1 gram equivalent of the dissolved solute in 1 litre of its solution is called normal solution. Normality depends on two factors:

a) Dilution

b) Temperature.

Several formula used to calculation of normality that is
When solute present in gram equivalent than use following formula;

Normality = Number of gram equivalents × [volume of solution in litres]$^{-1}$

When solute present in gram than use following formula;

N = Weight of Solute (gram) × [Equivalent weight × Volume (V)]

When normality contains molarity than used following formula;

N = Molarity × Molar mass × [Equivalent mass]$^{-1}$

To calculate the normality for acids use following formula;

N = Molarity × Acidity

The following formula can be used to find the normality of bases;

N = Molarity × Basicity

Calculation of Normality in Titration

Suppose N_1 and V_1 are normality and volume of one solution, and N_2 and V_2 are the normality and volume of another solution, then according to the law of equivalent,

$$N_1 V_1 = N_2 V_2$$

Where,

N_1 = Normality of the Acidic solution

V_1 = Volume of the Acidic solution

N_2 = Normality of the basic solution

V_2 = Volume of the basic solution

This is called normality formula.

Uses of Normality

There are three important use of normality

- It is used for determination of acid-base. The normality is used to indicate hydronium ions (H_3O^+) or hydroxide ions (OH^-) concentrations in a solution.
- Its use to measure the number of ions in precipitation reactions
- It is used in redox reactions to determine the number of electrons that a reducing or an oxidizing agent can donate or accept.

1.5. Morality

One mole of a substance (n) is equal to its molecular mass expressed in grams, where the molecular mass is the sum of the atomic masses of the constituent atoms. Note that the term molecular mass is preferred to the older term molecular weight. The SI unit of concentration is expressed in terms of moles per cubic metre (mol m^{-3}). The chemical in the mixture that is present in the largest amount is called the **solvent**, and the other components are called **solutes**.

Molarity (M) indicates the number of moles of solute per liter of solution (moles/Litre) and is one of the most common units used to measure the concentration of a solution. In other word it is the number of moles of solute dissolved in one litre of solution. **Molarity** or **molar concentration** can be used to convert between the mass or moles of solute and the volume of the solution. Molarity can be used to calculate the volume of solvent or the amount of solute. The molarity is represented by capital M. The molarity (M) calculation is calculated by dividing the moles of solute (n) by the total volume (v) of solution as follows

$$M = \frac{n}{v}$$

M=Molar concentration
n = Mole of solute
v = Litres of solution

The SI unit for molar concentration is mol m^3. However, mol L^{-1} is a more common unit for molarity. A solution that contains 1 mole of solute per 1

liter of solution (1 mol L^{-1}) is called "one Molar" or 1 M. The unit mol L^{-1} can be converted to mol m^3 using the following equation:

1 mol L^{-1} = 1 mol dm^{-3} = 1 mol dm^{-3} = 1 M = 1000 mol m^{-3}

Sample Problem: Calculating Molarity

A solution is prepared by dissolving 42.23 g of NH_4Cl into enough water to make 500.0 mL of solution. Calculate its molarity.

Here known
Mass = 42.23 NH_4 Cl
Molar mass of NH4 Cl = 53.50 g mol^{-1}
Volume of soulution = 500.0 mL = 0.5000 L
Unknown
Molarity = ?

The mass of the ammonium chloride is first converted to moles. Then the Molarity is calculated by dividing by liters. Note the given volume has been converted to liters.

So formula of molarity

$$M = \frac{n}{v}$$

$$M = \frac{0.7893 \text{ mol } NH_4Cl}{V.5000\ L}$$
$$= 1.579 \text{ M}$$

The morality is 1.579 M, meaning that a liter of the solution would contain 1.579 mol NH_4 Cl.

1.6. Molality

Molality (m), or molal concentration, is the amount of a substance dissolved in a certain mass of solvent. It is defined as the moles of a solute per kilograms of a solvent. Molality express as small m. the unit of molality is mol/kg. A solution with a molality of 1 mol/kg is often described as "1 molal" or "1 m. It is calculated by applying following formula such as

$$m = \frac{\text{mole of soult}}{\text{kilograms of solvent}}$$

Molality is an intensive property, and is therefore independent of the amount being measured. This is true for all homogeneous solution

concentrations, regardless of if we examine a 1.0 L or 10.0 L sample of the same solution. The concentration, or molality, remains constant.

Example of molality calculation

If we mass 5.36 g of KCl and dissolve this solid in 56 mL of water, what is the molality of the solution? Remember that molality is *moles* of solute/kg per solvent. KCl is our solute, while water is our solvent. We will first need to calculate the amount of moles present in 5.36 g of KCl:

$$\text{Mole of KCl} = 5.36\text{g x } (\frac{1\ mols}{74.5g}) = 0.0719 \text{ moles of KCl}$$

So equivalent mass of water 56.0 mL in gram calculating by using known density of water (1.0 g/mL)

$$56.0 \text{ mL x} \frac{1.0g\ mols}{mL} = 56.0\ g$$

56.0 g of water is equivalent to 0.056 kg of water. With this information, we can divide the moles of solute by the kg of solvent to find the molality of the solution:

$$\text{Molality} = \frac{mols}{kg\ solvent}$$

$$\text{m} = \frac{0.0719 \text{ moles KCl}}{056 \text{ kg water)}} = 1.3\ m$$

The molality of our KCl and water solution is 1.3 m.

Table: 1.2: Difference between normality, morality and molality

	Normality (N)	**Molarity (M)**	**Molality (m)**
Measure of	Concentration	Concentration	Concentration
Definition	It is equivalent concentration of a solution	The moles of a solute per liters of a solution	The moles of a solute per kilograms of a solvent
Units	N	M	m
Equation	N = Molarity × Basicity	M = moles solute / litres solution	m = moles solute / kg solvent
Ratio of moles to:	Volume (in litres)	Volume (in litres)	Mass (in kilograms)

1.7. About purification

Separation and purification of biomacromolecules either in biopharmaceuticals and fine chemicals manufacturing, or in diagnostics and biological characterization, can substantially benefit from application of microfluidic devices. The purification aim only aim to obtained desire about of pure molecule in shortest period of time. The characteristic of the biomolecule of interest such as solubility, shape, size, hydrophobicity and affinity is highly altering the process of purification.

There are lots of methods are available of the purification of proteins and other biomolecule. The approach to purification of protein that is not previously isolated to be carried out existed precedents and by common sense. The main to obtained purified protein is to known its structural or functional studies. The final degree of purity required depends on the purposes for which the protein will be used, i.e. you may not need a protein sample that is 100% pure for your studies each purification step necessarily involves loss of some of the protein you are trying to purify. The purification of one protein from a cell or tissue homogenate that will typically contain 10,000 – 20,000 different proteins, seems a daunting task, only four different fractionation steps are needed to purify a given protein. Three chromatographic methods used in protein purification.

a. Ion-exchange chromatography:

The column matrix is a synthetic polymer containing bound charged groups; those with bound anionic groups are called cation exchangers, and those with bound cationic groups are called anion exchangers. Ion-exchange chromatography on a cation exchanger is shown here. The affinity of each protein for the charged groups on the column is affected by the pH (which determines the ionization state of the molecule) and the concentration of competing free salt ions in the surrounding solution. Separation can be optimized by gradually changing the pH and/or salt concentration of the mobile phase so as to create a pH or salt gradient.

b. Size-exclusion chromatography:

It is also called gel filtration, separates proteins according to size. The column matrix is a cross-linked polymer with pores of selected size. Larger proteins migrate faster than smaller ones, because they are too large to enter the pores in the beads and hence take a more direct route through the column. The smaller proteins enter the pores and are slowed by their more labyrinthine path through the column.

c. Affinity chromatography:

It separates proteins by their binding specificities. The proteins retained on the column are those that bind specifically to a ligand cross-linked to the beads. After proteins that do not bind to the ligand are washed through the column, the bound protein of particular interest is eluted (washed out of the column) by a solution containing free ligand.

Purification of enzyme:

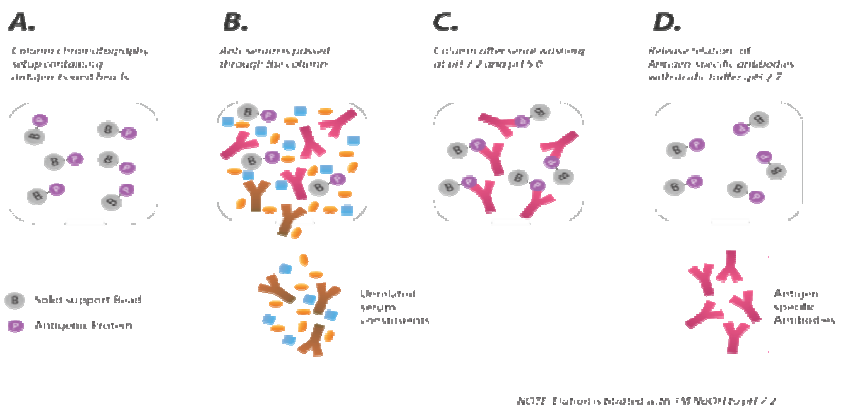
Most enzymes are proteins. The purification and extraction methods employed for enzymes are same as for proteins. To study an enzyme's function, it is essential to obtain its pure preparation from cells which contains multitude of proteins and enzymes.

If the enzyme is a mitochondrial enzyme (or localized to any other sub cellular organelle), differential centrifugation is performed to enrich the enzyme in the crude extract. Crude extract is then subjected to fractionation, a process that separates proteins into different fractions based on size, charge or solubility. First in fractionation procedure used salt as $(NH_4)_2SO_4$ or potassium phosphate which increases the solubility of protein. The salt is added gradually because makes limitation for dissolution of solute and thus dehydration proceeds slowly. The precipitated protein is then resuspended in an appropriate buffer with suitable pH. The resuspended solution is then dialyzed to remove high salt concentration. The solute moves from high to low concentration till its concentration reaches equilibrium. Other agents that can be used for protein precipitation are: (a) inorganic salts, (b) organic solvents, (c) change in pH, (d) polyethylene glycol. The dialyzed protein solution is then subjected to further fractionation procedures. The next method is column chromatography. If it uses the property of size, it is called gel chromatography. In gel chromatography the larger proteins emerge out earlier than the smaller proteins hence, separating the proteins on the basis of size. If it utilizes property of charge, it is ion exchange chromatography. Ion exchange chromatography separates two proteins on the basis of charge. There are two kinds of exchangers: cation exchangers and anion exchangers. Thus, two proteins with opposite charges make two distinct bands and eluted in different fractions. Affinity chromatography can be used if any inhibitor for enzyme of interest is known. The enzyme of interest with affinity for the ligand binds to it while the others appear in the flow through. Then the higher concentration of free ligand is added to the column. Because of competition, the enzyme binds to the free ligand and is eluted out.

Antibody purification

Antibody can be purified from serum by the addition of chaotropic ions in the form of saturated ammonium sulphate. This preferentially precipitates the antibody fraction at around 60% saturation and provides a rapid method for

IgG purification. This method does not work well in tissue culture supernatant as media components such as ferritin are co-precipitated. Ammonium sulphate precipitation may be used as a preparatory method prior to further chromatographic purification. Antibodies from both polyclonal and monoclonal sources can be purified by similar means. In both cases the antibody type is IgG which allows purification by protein A/G affinity chromatography. Purified antibody should be adjusted to 1 mg cm^3 and kept at 4 °C, or 20 °C for long-term storage.



Column chromatography for antigen affinity purification of antibodies

1.8. Filtration

Filtration is a unit operation where separation of insoluble solids from a solid-liquid suspension is done with the application of mechanical or gravity force through a porous membrane. The solids are retained in the porous medium and form a layer, called filter cake. The liquid that passes through the porous medium which is free from any solid particles is called as filtrate. The porous medium is known as filter medium.

Principle of Filtration

The driving force for filtration is most often the pressure difference. In the beginning of the filtration process, filtrate flows easily through the medium with least resistance. The rate of filtration which is the ratio of filtrate volume and time of filtration is high in the beginning. But, as the filtration progresses, the layer of cake deposition upstream gradually increases. So, now the filtrate not only ought to pass through the medium, but also it should cross the layer of cake.

Filtration capacity is the ratio of filtrate volume and the time of filtration cycle. The total filtration time is the addition of filtration time, washing time and transition time for assembling, adjustment of filter etc.

$$\mathbf{FC} = \frac{\mathbf{v}}{\mathbf{t_c}} = \frac{\mathbf{v}}{\mathbf{t} + \mathbf{t_w} + \mathbf{t_r}}$$

Where, t, tw and ttr are filtration time, washing time and transition time respectively.

The resistance offered to the filtrate flow by the medium is called filter medium resistance. The same filtrate which passes through the cake has to pass through the medium. So, an analogy can be built with cake resistance and application of Carmann-Kozeny equation proves to be valid. The filtrate flow through medium would be.

$$\frac{dv}{dt} = \frac{-\Delta p_{mA}}{uR_m}$$

Where, R_m is the filter medium resistance in m^{-1} .

Summary

Affinity chromatography is useful step in overall purification strategy of protein. Centrifugation is most important techniques used in separation of biomolecule under the influence of centrifugal forces. Number of gram equivalent weight in a liter of solution is called normality. Molality is an intensive property, and is therefore independent of the amount being measured. Most enzymes are proteins and hence same procedures employed to extract proteins can be used to extract and purify enzymes. The extraction procedures use differences in charge, solubility and size for separating protein/enzyme of interest from other proteins. The first procedure used relies on the basis of differences in solubility of enzyme in a salt solution. $(NH_4)_2SO_4$ is preferentially used because of high solubility in water. The purification aim only aim to obtained desire about of pure molecule in shortest period of time. The characteristic of the biomolecule of interest such as solubility, shape, size, hydrophobicity and affinity is highly altering the process of purification.

Terminal questions

Q.1: What do you understand for bio analytical techniques? Discuss briefly.

Answer: ----------

Q.2: How the chromatography is useful for the isolation of biomolecule.

Answer: ----------

Q.3: Discuss briefly the purification techniques of biomolecule.

Answer: ----------

Q.4: Discuss the role of centrifuge for protein purification.

Answer: ----------

Q.5: What is solution? Discuss the normality and morality concentration.

Answer: ----------

Q.6: Discuss about enzyme classification.

Answer: ----------

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Unit-2: pH and Buffer

2.1. Introduction

pH is also refers to potential of hydrogen. pH is a symbol and donates the relative concentration of hydrogen ion in solution of extend form of value 0 to 14, the lower the value, the higher the acidity or the more hydrogen ion concentration. In solution water at 25 C has a concentration of hydrogen ion of 10-7, the pH therefore, is

$$pH = -\log[H^+]$$

pH is equal to the negative logarithm of the concentration of H^+ ions in solution: $pH = -\log[H^+]$. The concentration of hydrogen ions dissociating from pure water is 1×10^{-7} moles H^+ ions per liter of water. The pH is calculated as the negative of the base 10 logarithm of this concentration: The negative log of 1×10^{-7} mol m^{-3} is equal to 7.0, which is also known as neutral pH. Human cells and blood each maintain near-neutral pH.

A buffer is an aqueous solution that consists of a mixture of a weak acid and its salt (Acid Buffer) or a weak base with its salt (Basic buffer). Its pH

changes very little when a small amount of strong acid or base is added to it and is thus used to prevent a solution's pH change.

Buffers are solutions that contain a weak acid and it's a conjugate base; as such, they can absorb excess H^+ ions or OH^- ions, thereby maintaining an overall steady pH in the solution. The buffer that maintains the pH of human blood involves carbonic acid (H_2CO_3), bicarbonate ion (HCO^{3-}), and carbon dioxide (CO_2). When bicarbonate ions combine with free hydrogen ions and become carbonic acid, hydrogen ions are removed, moderating pH changes. Similarly, excess carbonic acid can be converted into carbon dioxide gas and exhaled through the lungs; this prevents too many free hydrogen ions from building up in the blood and dangerously reducing its pH; likewise, if too much OH^- is introduced into the system, carbonic acid will combine with it to create bicarbonate, lowering the pH. Without this buffer system, the body's pH would fluctuate enough to jeopardize survival.

Objectives:

- To discuss the hydrogen ion concentration and pH
- To discuss the buffer solution
- To discuss the role of buffer solution in biological system

2.2. Hydrogen ion concentration

Hydrogen ions are spontaneously generated in pure water by the dissociation (ionization) of a small percentage of water molecules into equal numbers of hydrogen (H^+) ions and hydroxide (OH^-) ions. The hydroxide ions remain in solution because of their hydrogen bonds with other water molecules; the hydrogen ions, consisting of naked protons, are immediately attracted to un-ionized water molecules and form hydronium ions (H_3O^+). By convention, scientists refer to hydrogen ions and their concentration as if they were free in this state in liquid water.

H^+ ion of water, k is the basis for the pH scale, it show the actual concentration of hydrogen ion in aqueous solution in range blew 1.0M H^+ and 1.0 M OH^-. All the biological reactions defined this pH scale.

The pH also defines that pH of a solution as the negative logarithm of concentration of hydrogen ion, thus:

$$pH = \log\frac{1}{[H^+]} = -\log[H^+]$$

At mention solution at 25 C the concentration of H^+ ion found 1 x 10^{-7} M. The pH can be calculated as

$$pH = \log\frac{1}{1\ x\ 10^{-7}} = -\log(1\ x\ 10^7)$$

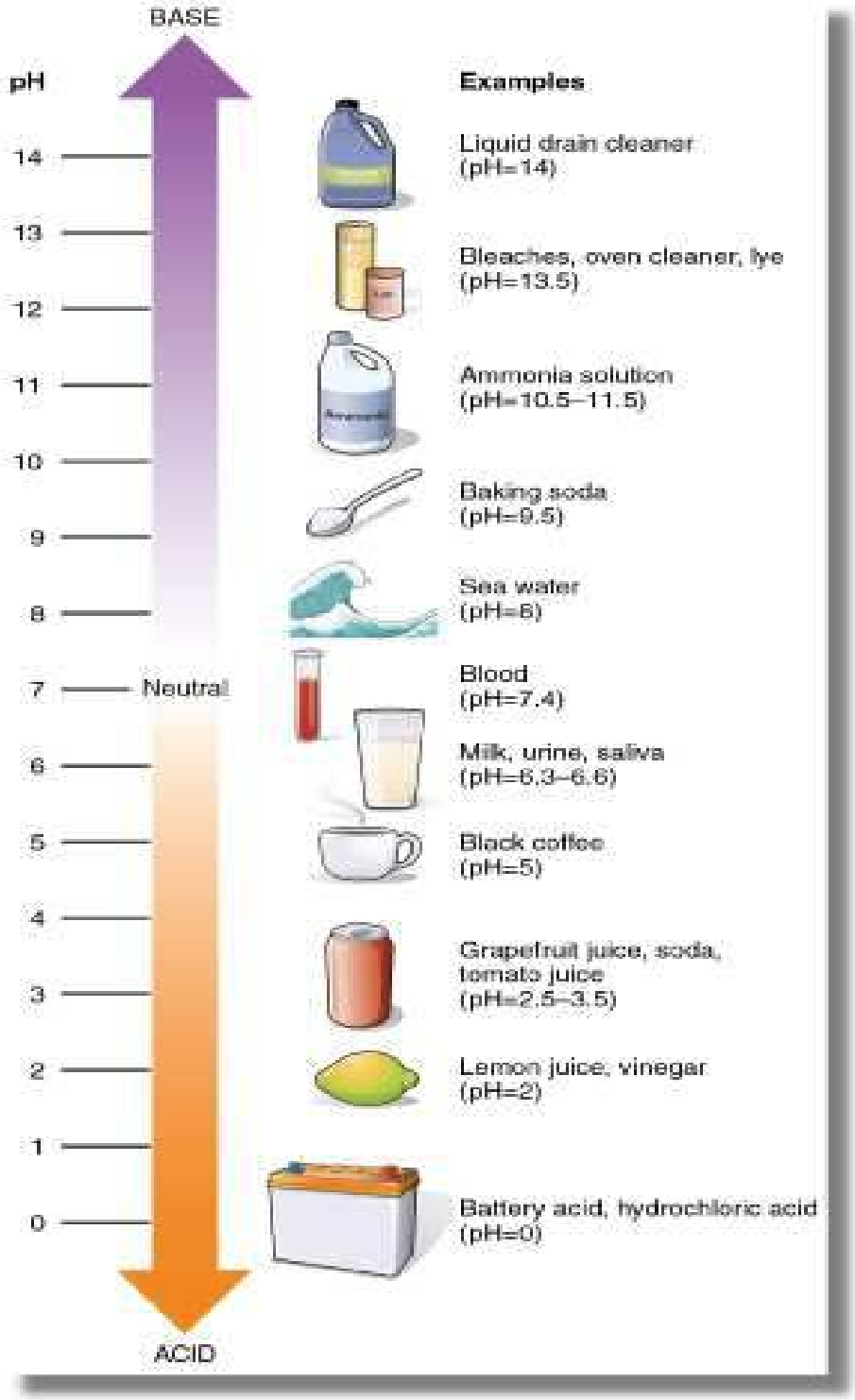
$$= \log 1.0 + \log 10^{7}$$

$$= 0 + 7.0$$

$$= 7.0$$

Solutions in which pH = 7.0 are defined as neutral. Solutions with pH < 7.0 are called "acidic," and solutions in which pH > 7.0 are called "basic." In **Fig. 2.16** are shown the pH Values of some common fluids.

$[H^+]$ M	pH	$[OH^-]$ M	pOH*
$10^{-0(1)}$	0	10^{-14}	14
10^{-1}	1	10^{-13}	13
10^{-2}	2	10^{-12}	12
10^{-3}	3	10^{-11}	11
10^{-4}	4	10^{-10}	10
10^{-5}	5	10^{-9}	9
10^{-6}	6	10^{-8}	8
10^{-7}	7	10^{-7}	7
10^{-8}	8	10^{-6}	6
10^{-9}	9	10^{-5}	5
10^{-10}	10	10^{-4}	4
10^{-11}	11	10^{-3}	3
10^{-12}	12	10^{-2}	2
10^{-13}	13	10^{-2}	1
10^{-14}	14	$10^{-0(1)}$	0



2.3. Buffer solution

A buffer is "a solution is one that resists a change in pH on addition of a small amount of acid [H^+] or base [OH^-] more effectively than an equal volume. Generally buffer solution consists of mixture of acid and conjugated substance. For example mixture of acidic acid and conjugate base and sodium

acetate or of ammonium hydrogen hydroxide and ammonium chloride are buffer solution. If see the titration curve of acetic acid, the pH of a solution undergoes change at near the midpoint (where pH = pKa, and $[CH_3COOH] = [CH_3COO^-]$) of the curve. However the buffering region extends about 1 pH unit on either side of the pKa. In this pH range, buffering power is best because the concentrations of both buffering species, HA and A^-, are the highest. Buffers are selected based on their pKa values and the range of pHs to be buffered. There are some basic factors which determine the effectiveness or capacity of buffer solution. Relative concentration of the conjugate base and weak acid. The hydrogen ion concentration of most body fluids and secretion in on the alkaline side.

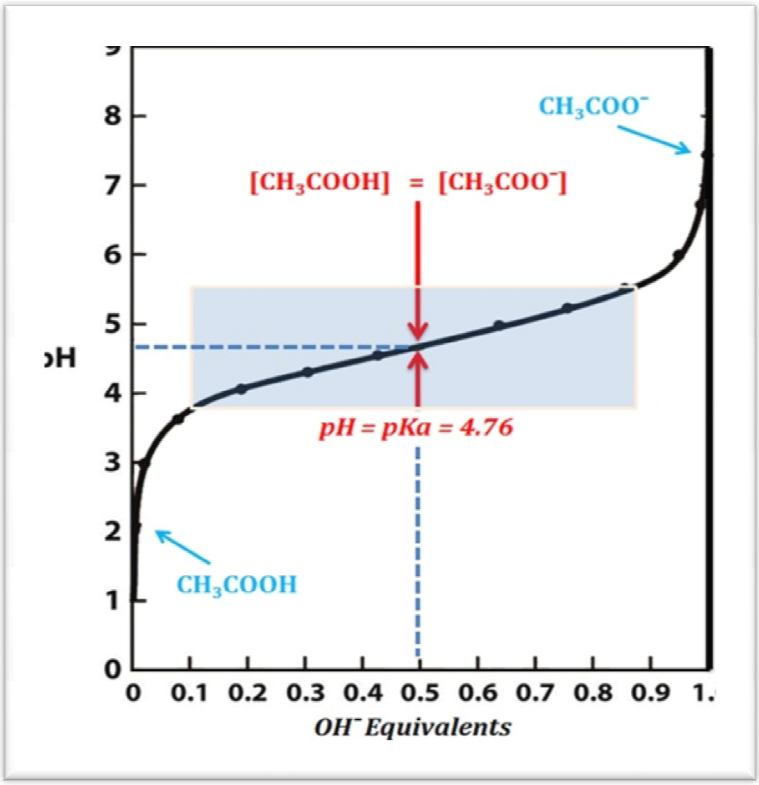


Fig: Buffer region of acidic acid (5.76 50 3.76)

2.4. Types of buffer

Buffers are broadly divided into two types – acidic and alkaline buffer solutions. Acidic buffers are solutions that have a pH below 7 and contain a weak acid and one of its salts. For example, a mixture of acetic acid and sodium acetate acts as a buffer solution with a pH of about 4.75.

Alkaline buffers, on the other hand, have a pH above 7 and contain a weak base and one of its salts. For example, a mixture of ammonium chloride and

ammonium hydroxide acts as a buffer solution with a pH of about 9.25. Buffer solutions help maintain the pH of many different things as shown in the image below.

2.4.1. **Acid Buffer**

A buffer solution that contains large quantities of a weak acid, and its salt with a strong base, is called an acid buffer. On the acidic side such buffer solutions have pH, i.e. pH is below 7 at 298 K. The equation gives the pH of an acid buffer. CH_3COOH, with CH_3COONa.

$$\boldsymbol{pH = pKa + In\frac{[Salt]}{[Acid]}}$$

Where Ka -----acid dissociation constant of the weak acid

2.4.2. **Basic Buffer**

A buffer solution which contains relatively large quantities of a weak base and its salt with a strong acid is called a simple buffer. On the alkaline side these buffers have pH, i.e., pH is higher than 7 at 298 K. For example, NH_4OH and NH_4Cl

The pH of an appropriate buffer is determined by the equation

$$\boldsymbol{pOH = pKb + In\frac{[Salt]}{[Base]}}$$

Where, Kb is base dissociation constant.

There are some example of buffer solution are mentioned

- Blood - contains a bicarbonate buffer system
- Tris buffer
- Phosphate buffer

As mentioned, buffers are beneficial over specific pH ranges. For example, here is the pH range of common buffering agents:

Buffer	pKa	pH Range
Citric Acid	3.13., 4.76, 6.40	2.1 to 7.4
Acetic Acid	4.8	3.8 to 5.8
KH_2PO_4	7.2	6.2 to 8.2
Borate	9.24	8.25 to 10.25

While making a buffer solution, the pH of the solution is changed to get it within the right effective range. A strong acid, such as hydrochloric acid (HCl), is usually added to reduce the pH of acidic buffers. A strong base such as sodium hydroxide (NaOH) solution is added to increase the pH of the alkaline buffers.

2.5. Preparation of a Buffer Solution

If you know the pKa (acid dissociation constant) of the acid and pKb (base dissociation constant) of the base, then you can make a buffer of known pH by controlling the ratio of salt and acid or salt and base. Buffers can either be prepared by mixing a weak acid with its conjugate base or a weak base with its conjugate acid. For example, phosphate buffer, a commonly used buffer in research labs, consists of a weak base (HPO_4^{2-}) and its conjugate acid (H_2PO4^-). Its pH is usually maintained at 7.4.

2.6. Buffer Action

So, how does a buffer work? Let's take the example of a mixture of acetic acid (CH_3COOH) and sodium acetate (CH_3COONa). Here, acetic acid is weakly ionized while sodium acetate is almost completely ionized. The equations are given as follows:

$$CH_3COOH \rightleftharpoons H^+ + CH3COO^-$$

$$CH_3COONa \rightleftharpoons Na^+ + CH3COC^-$$

To this, if you add a drop of a strong acid like HCl, the H^+ ions from HCl combine with CH_3COO^- to give feebly ionized CH_3COOH. Thus, there is

a very slight change in the pH value. Now, if you add a drop of NaOH, the OH^- ions react with the free acid to give undissociated water molecules.

$$CH_3COOH + OH^- \rightleftharpoons CH_3COO^- + H_2O$$

In this way, the OH^- ions of NaOH are removed and the pH is almost unaltered.

2.7. Biological buffer system:

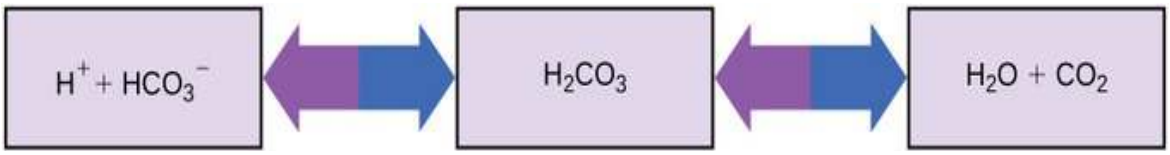
Biological process depends on the pH, however, small change in pH, a large change in the role of process. Biological process affected directly or indirectly by concentration of hydrogen ions. Enzymes and many other molecules on with the act, contain ionizable groups with characteristic pk_a values.

	Bicarbonate buffer
Extracellular fluids	Protein buffer
Extracellular fluids	Phosphate buffer
	Protein
Erythrocytes	Homological buffer

Cell and organism mentaing "specific and constant cytosolic pH. Keeping biomolecule in their optimum ionic sate, usually pH 7. The optimum pH for biological process mentioned by the biological buffers. In the blood, the CO_2^- carbonic acid-bicarbonate system is used for buffering. Inter and intracellular and extracellular fluids of living organism contains conjugated acid base which act as buffer and maintain the normal pH of these fluids.

Maintaining a constant blood pH is critical to a person's well-being. The buffer that maintains the pH of human blood involves carbonic acid (H_2CO_3), bicarbonate ion (HCO_3^-), and carbon dioxide (CO_2). When bicarbonate ions combine with free hydrogen ions and become carbonic acid, hydrogen ions are removed, moderating pH changes. Similarly, excess carbonic acid can be converted into carbon dioxide gas and exhaled through the lungs; this prevents too many free hydrogen ions from building up in the blood and dangerously reducing its pH; likewise, if too much OH^- is introduced into the system, carbonic acid will combine with it to create bicarbonate, lowering

the pH. Without this buffer system, the body's pH would fluctuate enough to jeopardize survival.



Buffers in the body.

This diagram shows the body's buffering of blood pH levels: the blue arrows show the process of raising pH as more CO_2 is made; the purple arrows indicate the reverse process, lowering pH as more bicarbonate is created.

Antacids, which combat excess stomach acid, are another example of buffers. Many over-the-counter medications work similarly to blood buffers, often with at least one ion (usually carbonate) capable of absorbing hydrogen and moderating pH, bringing relief to those that suffer "heartburn" from stomach acid after eating.

2.8. Bicarbonate buffer system

The bicarbonate buffer system is an acid-base homeostatic mechanism involving the balance of carbonic acid (H_2CO_3), bicarbonate ion (HCO_3^-), and carbon dioxide (CO_2) in order to maintain pH in the blood and duodenum, among other tissues, to support proper metabolic function. Catalyzed by carbonic anhydrase, carbon dioxide (CO_2) reacts with water (H_2O) to form carbonic acid (H_2CO_3), which in turn rapidly dissociates to form a bicarbonate ion (HCO^-_3) and a hydrogen ion (H^+) as shown in the following reaction.

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$$

As with H_2CO_3 buffer system, the pH is balanced by the H_2CO_3 of both a weak acid (for example, H_2CO_3) and its conjugate base (for example, HCO_3^-) so that any excess acid or base introduced to the system is neutralized.
In tissue, cellular respiration produces carbon dioxide as a waste product; as one of the primary roles of the cardiovascular system, most of this CO_2 is rapidly removed from the tissues by its hydration to bicarbonate ion. The bicarbonate ion present in the blood plasma is transported to the lungs, where it is dehydrated back into CO_2 and released during exhalation.

2.9. Phosphate-buffer system

A simple phosphate buffer is used ubiquitously in biological experiments, as it can be adapted to a variety of pH levels, including isotonic. This wide range is due to phosphoric acid having 3 dissociation constants, (known in chemistry as a triprotic acid) allowing for formulation of buffers

near each of the pH levels of 2.15, 6.86, or 12.32. Phosphate buffer is highly water soluble and has a high buffering capacity, but will inhibit enzymatic activity and precipitates in ethanol. The buffer is one of the most popular currently used, and is commonly employed in molecular and cell biology, chemistry, and material science, among many others. Phosphate-buffered saline is a buffer solution commonly used in biological research. It is a water-based salt solution containing disodium hydrogen phosphate, sodium chloride and, in some formulations, potassium chloride and potassium dihydrogen phosphate. The buffer helps to maintain a constant pH. The osmolarity and ion concentrations of the solutions match those of the human body (isotonic).

Phosphates inhibit many enzymatic reactions and procedures that are the foundation of molecular cloning, including cleavage of DNA by many restriction enzymes, ligation of DNA, and bacterial transformation.

Phosphate Buffer (Sorenson's buffer) pH 5.8-8

Advantages:

- Most physiological of common buffers.
- Mimics certain components of extracellular fluids.
- Non-toxic to cells.
- pH changes little with temperature.
- Stable for several weeks at 4 °C.

Disadvantages:

- Precipitates more likely to occur during fixation.
- Tends to form precipitates in presence of calcium ions.
- Precipitates uranyl acetate and tends to react with lead salts.
- Becomes slowly contaminated with micro-organisms

1 M sodium phosphate buffer (pH 6.0–7.2)

Mixing 1 M NaH_2PO_4 (monobasic) and 1 M Na_2HPO_4 (dibasic) stock solutions in the volumes designated in the table below results in 1 L of 1 M sodium phosphate buffer of the desired pH. To prepare the stock solutions, dissolve 138 g of $NaH_2PO4.H_2O$ (monobasic; m.w. = 138) in sufficient H_2O to make a final volume of 1 L and dissolve 142 g of Na_2HPO_4 (dibasic; m.w. = 142) in sufficient H_2O to make a final volume of 1 L.

Volume (mL) of 1 M NaH_2PO_4	Volume (mL) of 1 M Na_2HPO_4	Final pH
877	123	6.0
850	150	6.1
815	185	6.2
775	225	6.3
735	265	6.4
685	315	6.5
625	375	6.6
565	435	6.7
510	490	6.8
450	550	6.9
390	610	7.0
330	670	7.1
280	720	7.2

2.10. Acetate buffer

A solution of sodium acetate (a basic salt of acetic acid) and acetic acid can act as a buffer to keep a relatively constant pH level. This is useful especially in biochemical applications where reactions are pH-dependent in a mildly acidic range (pH 4–6).

2.11. Summary

pH is a measure of how acidic/basic water is. The range goes from 0 to 14, with 7 being neutral. pHs of less than 7 indicate acidity, whereas a pH of greater than 7 indicates a base. pH is really a measure of the relative amount of free hydrogen and hydroxyl ions in the water. The letters pH stand for potential of hydrogen, since pH is effectively a measure of the concentration of hydrogen ions (that is, protons) in a substance. The pH scale was devised in

1923 by Danish biochemist Søren Peter Lauritz Sørensen (1868-1969). The pH scale, ranges from 0 (strongly acidic) to 14 (strongly basic or alkaline). A pH of 7.0, in the middle of this scale, is neutral. Blood is normally slightly basic, with a normal pH range of about 7.35 to 7.45. pH ranges from 0 to 14, with 7 being neutral. pHs less than 7 are acidic while pHs greater than 7 are alkaline (basic). Normal rainfall has a pH of about 5.6—slightly acidic due to carbon dioxide gas from the atmosphere. Normal urine pH is slightly acidic, with usual values of 6.0 to 7.5, but the normal range is 4.5 to 8.0. A urine pH of 8.5 or 9.0 is often indicative of a urea-splitting organism, such as Proteus, Klebsiella, or Ureaplasma urealyticum.

A solution containing a mixture of an acid and its conjugate base, or of a base and its conjugate acid, is called a buffer solution. Unlike in the case of an acid, base, or salt solution, the hydronium ion concentration of a buffer solution does not change greatly when a small amount of acid or base is added to the buffer solution. The base (or acid) in the buffer reacts with the added acid (or base).

2.12. Terminal questions:

Q.1: What do you understand about pH?

Answer: ----------

Q.2: Discuss the hydrogen ion concentration.

Answer: ----------

Q.3: Define the Buffer, how it is useful in biological system.

Answer: ----------

Q.4: Write the role of pH in biological system.

Answer: ----------

Q.5: Discuss the different types of buffer solution and its role in biological systems.

Answer: ----------

Q.6: How the 1 M sodium phosphate buffer is prepared? Discuss briefly.

Answer: ----------

2.13. Suggested further readings

1. Principles of Biochemistry: Lehninger, Nelson and Cox. Student Edition, CBS 1439 Publishers and Distributors, Delhi.
2. Fundamentals of Biochemistry: Dr J L Jain, S. Chand and Company
3. Biochemistry: T.A. Brown, Viva book publication. First edition, 2018.
4. Elements of biochemistry: J.L. Jain, S. Chand publication, Seventh Edition.
5. Textbook of Biochemistry and Human Biology: Talwar and Srivastava. Eastern Economy Edition, Prentice Hall, India.

Unit-3: Properties of Light

3.1. Introduction

Light rays have both electrical and magnetic properties and are called electromagnetic radiations (EMR). Many research techniques in biophysics are based on the interaction of EMR with biological objects and are of central importance in understanding bio-molecular structure, function, interaction and mechanisms underlying biochemical and physiological processes in the body. The main source of light on earth is sun which is used by green plants to create sugars that release energy for living systems. Max Planck proposed the quantum nature of light in 1901 to explain the blackbody spectrum emitted by an object at a given temperature. Light is described as electromagnetic wave because its changing electric field creates a changing magnetic field. Electromagnetic radiation consists of radio waves, microwaves, infrared waves, visible light, ultraviolet radiation, X-rays, and gamma rays. A wave is defined as a disturbance which travels and spreads out through some medium. Wave only moves up and down, normal to the direction of propagation of the wave. Such waves are called transverse waves, and light waves are also transverse. Spectral lines are dark and bright lines in otherwise uniform continuous spectrum resulting from absorption or emission of light in a narrow frequency range compared with neighboring frequencies.

Objectives

- To discuss about light and its nature
- To discuss the role of light in biochemistry
- To discuss the nature of plane polarized light
- To discuss optical rotation of light

3.2. What is light?

The light is electromagnetic radiation which can detect by our naked eyes. Everything, which can see by our naked eyes, is reflected by light. Light is not a object which can see but is has power to see object so that we can say that the light is experienced, explored, and exploited by human or living beings. However, as we know that light is electromagnetic radiation, this electromagnetic radiation is actually a electromagnetic spectrum. The electromagnetic spectrum is extremely broad, ranging from low energy radio waves with wavelengths that are measured in meters, to high energy gamma rays with wavelengths that are less than 1×10^{-11} meters. The electromagnetic spectrum which occupy wave length 380 to 700 nanometers (wavelengths visible) is useful to see object by human eye. Electromagnetic radiation, as the name suggests, describes fluctuations of electric and magnetic fields, transporting energy at the Speed of Light (which is ~ 300,000 km/sec through a vacuum). Light can also be described in terms of a stream of photons, massless packets of energy, each travelling with wavelike properties at the speed of light. Visible light is not inherently different from the other parts of the electromagnetic spectrum, with the exception that the human eye can detect visible waves. Radiation lower than 400nm is referred to as Ultra-Violet (UV) and radiation longer than 700nm is referred to as Infra-red (IR), neither of which can be detected by the human eye.

The speed of light in a vacuum is a fundamental physical constant, the currently accepted value of which is exactly 299,792,458 meters per second, or about 186,282 miles per second. In addition, light show dual nature which means sometimes it behaves like a particle (known as photon), which explains how the light travels in straight lines. Sometimes light behaves as the wave, which explains how light bends (or diffract) around an object. If light is consider as stream of photon than is massless packets of energy which travelling with wave like properties at the speed of light. A photon is the smallest quantity (quantum) of energy which can be transported, and it was the realization that light travelled in discrete quanta that was the origins of Quantum Theory. The quantum theory is explained the characteristics of light and to explain the interactions of light with atoms and molecules. However, this theory describes light as consisting of discrete packets of energy,

called photons. Photons are minute energy packet of electromagnetic radiation said by Albert Einstein's (1905) during explanation of the photoelectric effect.

3.3. Electromagnetic radiation

EMR consists of electromagnetic (EM) waves of the electromagnetic (EM) field which are synchronized oscillations of electric and magnetic fields that travel at the speed of light through vacuum and carrying electromagnetic radiant energy. It includes radio waves, microwaves, infrared, (visible) light, ultraviolet, X-rays, and gamma rays. A wave is defined as a disturbance which travels and spreads out through some medium. These waves are also called "electromagnetic radiation" because they radiate from the electrically charged particles. Wave only moves up and down, normal to the direction of propagation of the wave. Such waves are called transverse waves, and light waves are also trans erse. The other kinds of waves are longitudinal, in which the motion is parallel to the direction of motion for example sound wave, which require a medium for their propagation that is known as mechanical waves. Transverse waves can travel in vacuum.

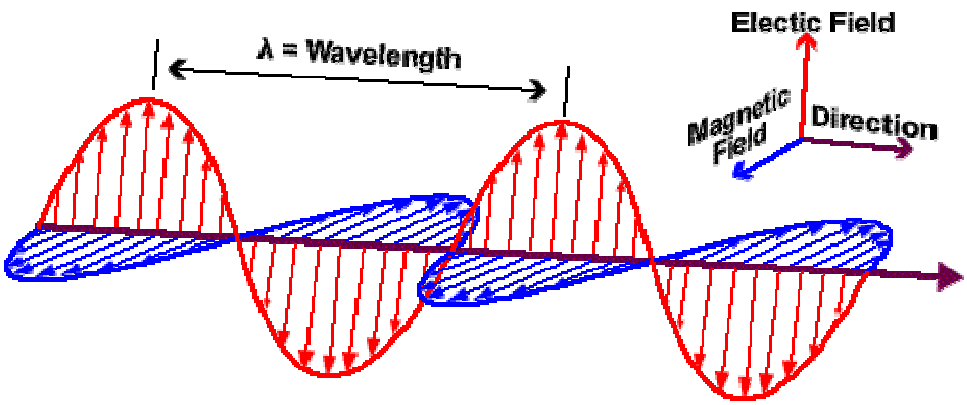


Fig.3.1: Nature of electromagnetic waves

The electromagnetic spectrum encompasses a wide range of energies, of which visible radiation is only a small part. EM radiation spans an enormous range of wavelengths and frequencies. This range is known as the electromagnetic spectrum. The EM spectrum is generally divided into seven regions such as radio waves, microwaves, infrared (IR), visible light, ultraviolet (UV), X-rays and gamma rays. Typically, lower-energy radiation, such as radio waves, is expressed as frequency; microwaves, infrared, visible and UV light are usually expressed as wavelength; and higher-energy radiation, such as X-rays and gamma rays, is expressed in terms of energy per photon.

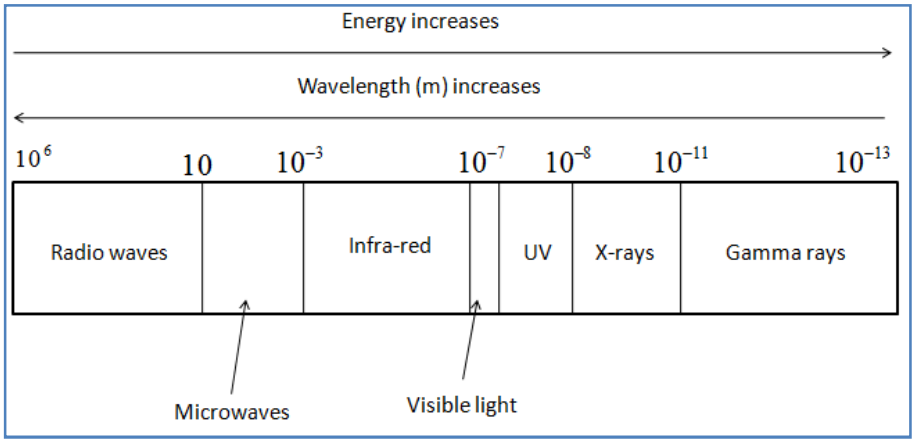


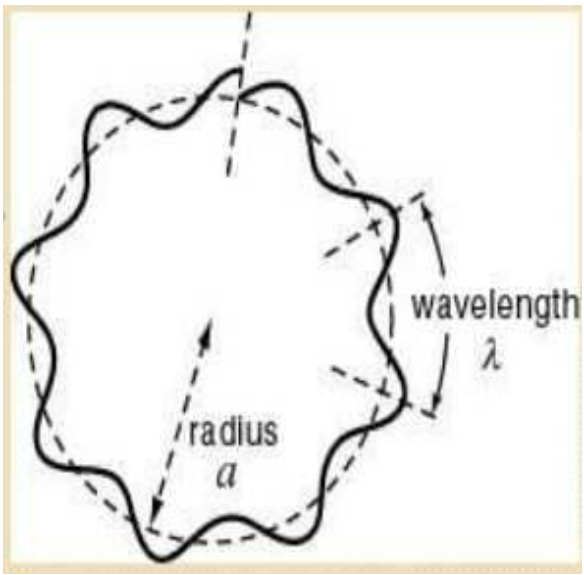
Fig.3.2: Diagram of the electromagnetic spectrum

The energy in electromagnetic radiation exists in the form of photons. The quantum phenomenon of electromagnetic radiation depends upon both; properties of the radiation and the appropriate structural part of the samples involves. The electromagnetic radiation is propagated through free space or through a medium and composed of both electric and magnetic waves. The electric and magnetic waves have oscillations that are perpendicular to each other and also to the direction of travel of the wave.

3.4. Nature of Light

Light is a kind of energy that travels in waves. Light travels very fast and in straight lines. It can travel through a vacuum and many other media. In a vacuum, the speed is a constant, C= 3×10^{8} m/s. the light has some characteristics features such as:

- Light is radiant energy
- Light travels very fast – 300,000 km/sec
- Light can be described either as a wave or as a particle traveling through space
- Light from the Sun arrives with all wavelengths, and we perceive this mixture of colors as white. Phase difference between light waves can produces visible interference effects
- The wave length of a light wave is inversely proportional to its frequency.
- The frequency of a light wave is related to its color.
- The amplitude of a light wave is related to its intensity.



Light has dual nature means light has two different nature, sometimes it behaves like a particle sometimes it behaves like a wave so that

If light behaves as particles

- Particles of light (photons) travel through space.
- These photons have very specific energies. that is, light is quantized.
- Photons strike your eye (or other sensors) like a very small bullet, and are detected.

If light behaves as waves

- A small disturbance in an electric field creates a small magnetic field, which in turn creates a small electric field, and so on, Light propagates itself "by its bootstraps!
- Light waves can interfere with other light waves, canceling or amplifying them! – The color of light is determined by its wavelength.

Frequency and wavelength are related by:

$$\lambda \, x \, \nu = C$$

$$\nu = \frac{C}{\lambda}$$

λ, is wave length in cm^{-1}

ν is the wave speed

c' is the speed of light in m/s

A photon carries energy with it that is related to its wavelength or frequency:

$$E = \frac{hC}{\lambda}$$

Where, E = Energy, h=Planck's constant = 6.626068×10^{-34} m^2 kg / s

3.5. Light spectra

Light spectrum can mean **the visible spectrum**, the range of wavelengths of electromagnetic radiation which our eyes are sensitive to or it can mean a plot (or chart or graph) of the intensity of light vs its wavelength (or, sometimes, its frequency). The realization that visible light is made up of colors is most often attributed to Isaac Newton who used a prism to create a spectrum (rainbow of colors) from a beam of white light, and another to recombine them back into white light. As the full spectrum of visible light travels through a prism, the wavelengths separate into the colors of the rainbow because each color is a different wavelength. Violet has the shortest wavelength, at around 380 nanometers, and red has the longest wavelength, at around 700 nanometers Visible light ranges from about 380 nanometers (nm) to about 750 nm, the window in the Earth's atmosphere which allows us to do astronomy from down here on its surface is a bit wider than the visible spectrum; it goes from about 300 nm to about 1100 nm.

There are seven wavelength ranges in the visible spectrum that coordinate to a different color. Each visible color has a wavelength. As you move from red to violet, the wavelength decreases and energy increases.

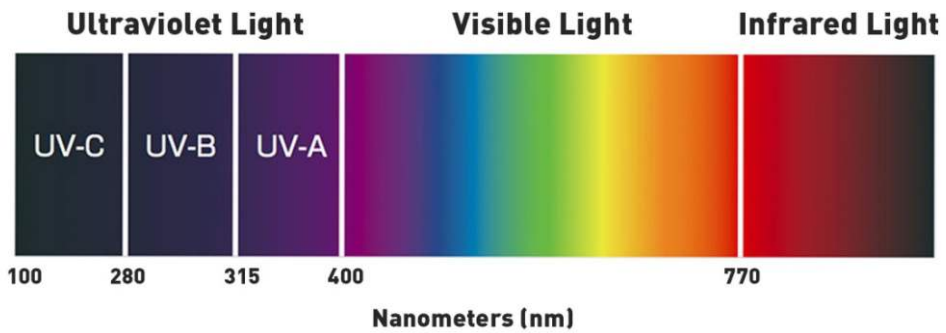


Fig 3.3: Visible color spectrum

Here are the 7 from shortest to longest wavelength.

1. **Violet -** shortest wavelength, around 400-420 nanometers with highest frequency. They carry the most energy.
2. **Indigo -** 420 - 440 nm

3. **Blue** - 440 - 490 nm
4. **Green** - 490 - 570 nm
5. **Yellow** - 570 - 585 nm
6. **Orange** - 585 - 620 nm
7. **Red** - longest wavelength, at around 620 - 780 nanometers with lowest frequency

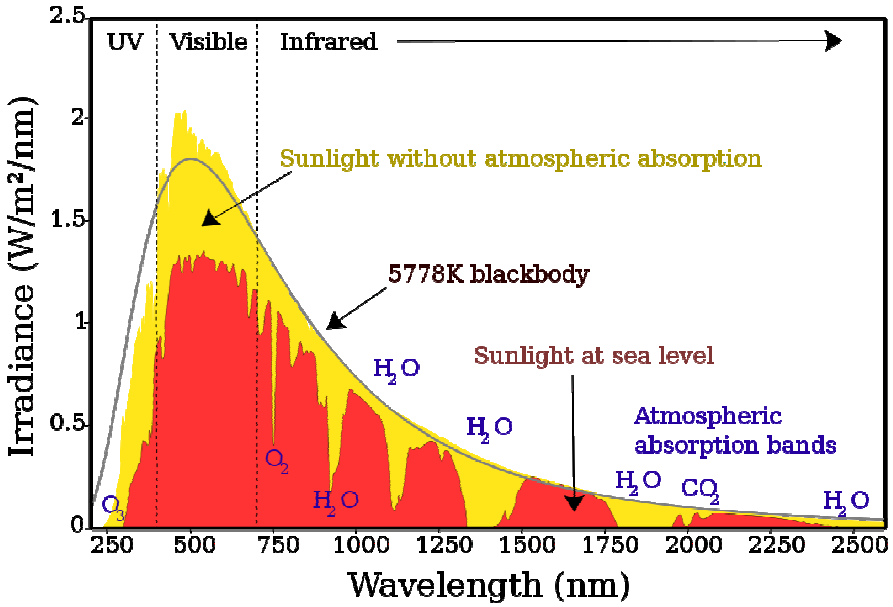


Fig.3.4: Spectrum of solar radiation (earth)

3.6. Wave length

A wavelength is the horizontal distance between the two peaks of the wave. In other word the wave length is distance between corresponding points of two consecutive waves. Light is measured by its wavelength (in nanometers). Wavelength is the distance from one crest to another or from one trough to another, of a wave (which may be an electromagnetic wave, a sound wave, or any other wave). Crest is the highest point of the wave whereas the trough is the lowest. Since wavelength is distance/length, it is measured in units of lengths such as meters, centimeters, millimeters', nanometers, etc. wavelength is usually characterized by the Greek symbol λ. it is equal to the speed (v) of a wave train in a medium divided by its frequency (f):

$$\lambda = \frac{v}{f}$$

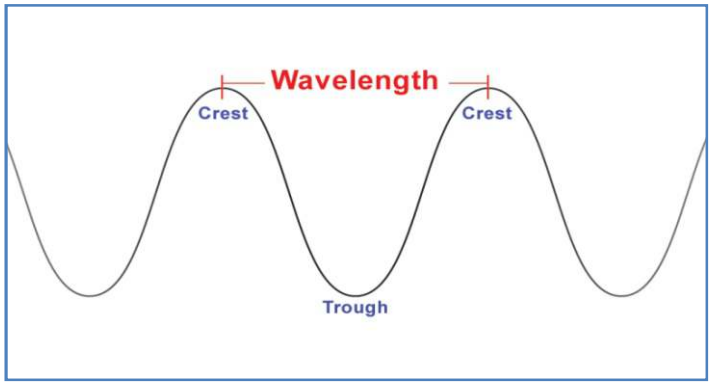


Fig.3.5: Wave length of radiation

Wavelength is inversely proportional to frequency. This means the longer the wavelength, lower the frequency. In the same manner, shorter the wavelength, higher will be the frequency. The wave length of color is vary so that we get different color of spectra. The longer length of radiation gives low energy while shorter wave length gives higher energy. Thus energy (E) is inversely proportional to the wavelength ($\boldsymbol{\lambda}$).

$$\mathbf{E} = \frac{\mathbf{hC}}{\boldsymbol{\lambda}}$$

Where, **c'** is the speed of light in m/s

h=Planck's constant = 6.626068×10^{-34} m^2 kg / s

However, the wave length of UV radiation is shorter than violet light so that uv radiation produce high energy that are harmful for human or living being on earth. Similarly, the wavelength of infra red radiation is longer than the wavelength of red light so that is produce low energy than red light. Thus, radiation of visible range occupies long wave length of radiation with low energy.

3.7. Plane Polarized light

Light is an electromagnetic radiation which accomplishes an isotropic distribution of the electric and magnetic field vectors. If the electric and magnetic field vectors are oscillating at right angle to each other then such light is termed as plane or linearly polarized with respect to the direction of propagation, and all waves vibrating in a single plane are termed plane parallel or plane-polarized.

However, if two linearly polarized light beams are superimposed in such a way that have a phase shift of $\lambda/2$ and their electric and magnetic field vectors are perpendicular to each other, a circularly polarized light is produced. Hence, resultant amplitude vector of the electric field will produce a rotation

on a spiral perpendicular to the direction of the propagating light wave (see Figure 1).

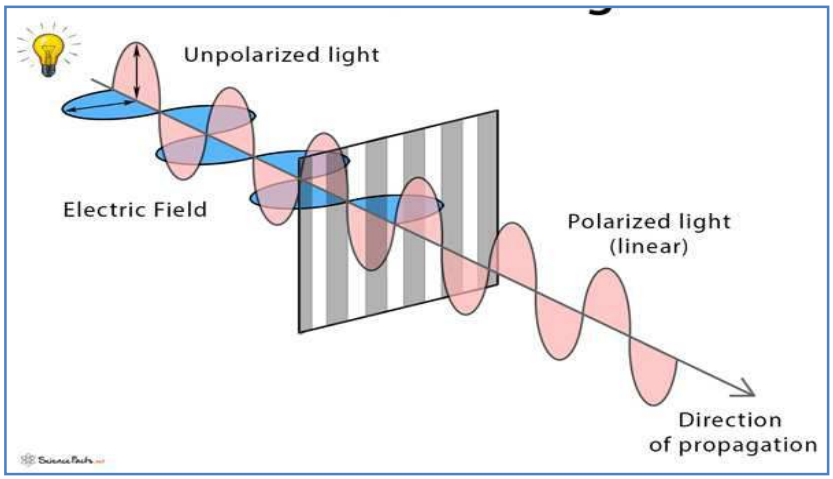


Fig.3.6: Diagram of plane polarized light

However, the circularly polarized light having two different components as left- and righthanded springs which rotates anticlockwise and clockwise, respectively. The frequencies of leftand right-handed springs are related to the frequency of the light.

3.8. Optical rotation

The optical rotation is the angle through which the plane of polarization is rotated when polarized light passes through a layer of liquid. Optical rotation is the effect which is determined by the concentration of chiral molecules and their molecular structure in a substance. Every optical active substance has its own specific rotation. Substances that have the ability to rotate the plane of the polarized light passing through them are called optically active substances.

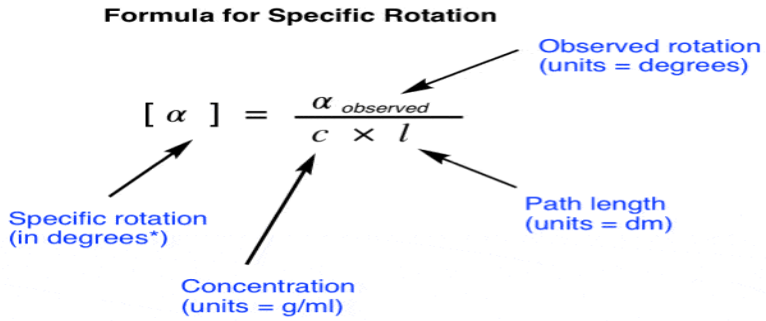
Optically active substances are classified into two types.

Dextrorotatory substances – Substances that rotate the plane of polarization of the light towards the right are known as right-handed or dextrorotatory.

Laevorotatory substances – Substances which rotate the plane of polarization of the light toward the left are known as left-handed or

A measure of the optically activity of a sample is the rotation produced for a 1mm slab for a solid or a 100mm path length for a liquid. This measure is called the specific rotation. The specific rotation depends upon the temperature and upon the wavelength of the light; these quantities also must be specified. The rotation is assigned a positive value if it is clockwise with respect to an observer facing the light source, negative if counterclockwise. Rotation is given in +/- degrees, depending on whether the sample has d- (positive) or l-

(negative) enantiomers. The standard measurement for rotation for a specific chemical compound is called the specific rotation, defined as an angle measured at a path length of 1 decimeter and a concentration of 1g/ml.



3.9. Optial-rotatory dispersion

The rate of change of specific rotation with respect to wavelength is known as optical rotator dispersion (ORD). As we already know that plane polarized light can be regarded as and experimentally broken down into two circularly polarized components, equal in amplitude but opposite in rotation. An optically inactive substance will retard the speeds of two circularly polarized components to the same extent, with no net rotation.

The rotation of plane of polarization by an optically active medium is the result of unequal angles which E_L and E_R make with Z axis. Rotation of electric field component E is depicted in figure 1.

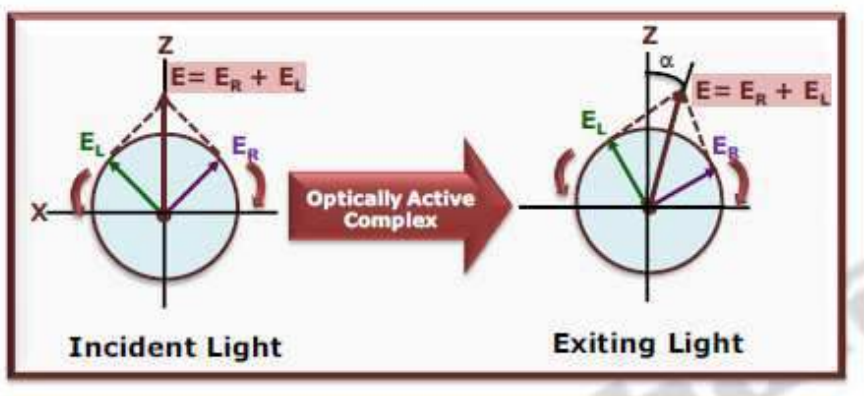


Fig.3.7: representation of optically active substances

It has been shown that since the speed of light in a medium is manifested in the refractive index of the medium, essential property of an optically active substance is that it has different refractive indices for the left and right circularly polarized light, nL and nR, respectively. This is also the reason why an optically active substance is said to be "circularly birefringent.

3.10. Absorbance-chromospheres

The term chromophore was previously used to denote a functional group of some other structural feature of which gives a color to compound. For example- Nitro group is a chromophore because its presence in a compound gives yellow color to the compound. Some of the important chromophores are: ethylene, acetylene, carbonyls, acids, esters and nitrile groups etc. A carbonyl group is an important chromophore, although the absorption of light by an isolated group does not give rise to any colour in the ultra-violet spectroscopy. There is no set rule for the identification of a chromophore. The change in position as well as the intensity of the absorption depends upon a large number of factors.

1. Spectrum having a band near 300 mμ may possess two or three conjugated units.
   Absorption bands near 270-350 mμ with very low intensity ε_{max} 10-100 are because of n-π* transitions of carbonyl group.
2. Simple conjugated chromophores likes dienes or, α β –unsaturated ketones have ε_{max} values, i.e., from 10,000 to 20,000.
3. The absorption with ε_{max} value between 1,000-10,000 reveals the presence of an aromatic system. If aromatic nucleus is substituted with groups which can extends the chromophore, the absorption take place at still higher value of extinction coefficients.

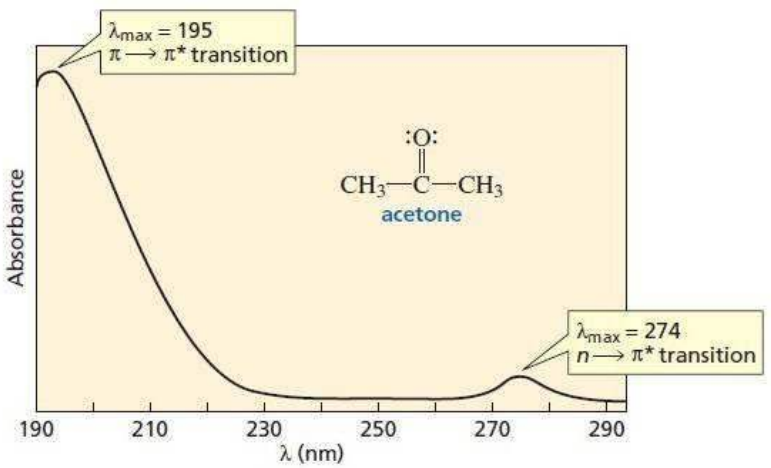


Fig.3.8: UV spectrum of an unsaturated compound.

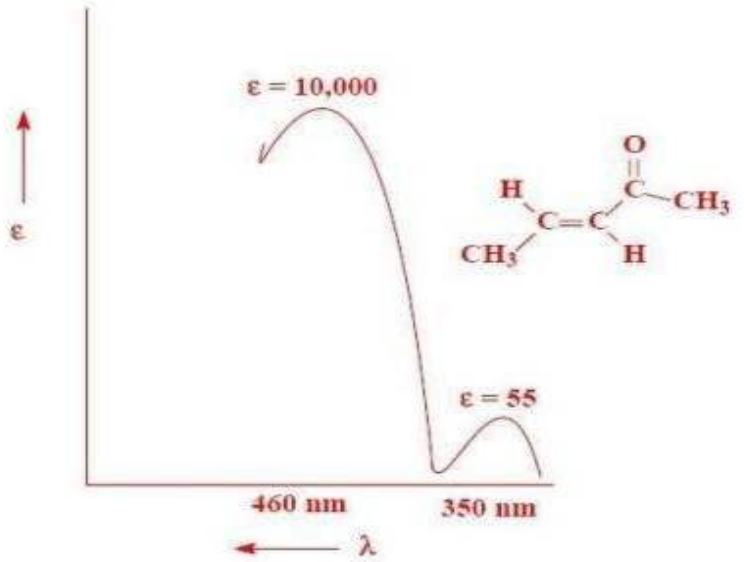


Fig. 3.9: UV spectrum of an α,β-unsaturated ketone.

3.11. Auxochrome

It is a group which itself does not act as a chromophore but when attached to a chromophore, it shifts the adsorption towards longer wavelength along with an increase in the intensity of absorption. Some commonly known auxochromic groups are: -OH, -NH_2, -OR, -NHR, and –NR_2. For example: When the auxochrome –NH_2 group is attached to benzene ring. Its absorption change from λ max 225 (εmax 203) to λ max 280 (εmax1430). All auxochromes have one or more non-bonding pairs of electrons. If an auxochromes is attached to a chromophore, it helps is extending the conjugation by sharing of non-bonding pair of electrons as shown below.

CH_2=CH–NR_2---------->CH_2-CH-NH_2

An auxochrome is a functional group of atoms with one or more lone pairs of electrons when attached to a chromophore, alters both the wavelength and intensity of absorption. If these groups are in direct conjugation with the pi-system of the chromophore, they may increase the wavelength at which the light is absorbed and as a result intensify the absorption. The extended conjugation has been responsible for bathochromic effect of auxochromes.

3.12. Summary

Light is defined as the electromagnetic radiation with wavelengths between 380 and 750 nm which are visible to the human eye. Electromagnetic radiation, such as light, is generated by changes in movement (vibration) of electrically charged particles, such as parts of 'heated' molecules, or electrons in atoms. Light can also be described in terms of a stream of

photons, massless packets of energy, each travelling with wavelike properties at the speed of light. Visible light is not inherently different from the other parts of the electromagnetic spectrum, with the exception that the human eye can detect visible waves. The wave length is distance between corresponding points of two consecutive waves. Light is measured by its wavelength (in nanometers). The realization that visible light is made up of colors is most often attributed to Isaac Newton who used a prism to create a spectrum (rainbow of colors) from a beam of white light, and another to recombine them back into white light. The optical rotation is the angle through which the plane of polarization is rotated when polarized light passes through a layer of liquid. In biological molecules that serve to capture or detect light energy, the chromophore is the moiety that causes a conformational change of the molecule when hit by light. An auxochrome is a functional group of atoms with one or more lone pairs of electrons when attached to a chromophore, alters both the wavelength and intensity of absorption

3.13. Terminal questions

Q.1: What is light? Discus the nature of light.

Answer: ----------

Q.2: What is wave length? Discus it relation with energy and frequency.

Answer: ----------

Q.3: Define the plane polarized light and it nature.

Answer: ----------

Q.4: What are chromophore discuss it absorption of light.

Answer: ----------

Q.5: Discus the role of light in human being.

Answer: ----------

Q.6: What are auxochromophure

Answer: ----------

3.14. Further suggested readings

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SBSBCH -01

Bio- Analytical Techniques

Block -2

Chromatography and Spectroscopy

SBSBCH -01

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Introduction

This is the second block on Chromatography and spectroscopy. It consists of the following three units:

Unit-4: This unit covers the history and types of chromatography. The working principle of exchange, gel filtration and high performance liquid chromatography is discussed in this unit. The chromatography is powerful techniques that used to separate mixture of substance into their individual components. All types of chromatography work on the same principle.

Unit-5: This unit covers the basic concept of spectroscopy. The details discussed here of uv-visible spectroscopy viz. definition and its principle. The spectroscopy, considered as one of the most valuable techniques for the detection of biological sample and useful in qualitative and quantitative detection chemical compounds. This unit covers Beer-Lambert's law, and applications of colorimetery.

Unit-6: This unit also cover spectroscopy where the microbial techniques for isolation and culture of microbes. The antimicrobial activity by using DISC diffusion techniques are discussed in this unit. The use of different solvent system for amino acid, carbohydrate and lipid separation also discussed.

Unit-4.Chromatography

4.1. Introduction

Chromatography is derived from the Greek word *chroma,* which means “color” and *graphein*, which means "to write". So the word chromatography means "color writing". A Russian botanist M.S. Tswett invented chromatography in 1903, while studying the coloring materials in plant life. All chromatography consists of stationary and mobile phase, which may be liquid or gaseous, useful for the separation of biological sample. Mobile phase passed over or through the stationary phase after the mixture of analytes (to be separated) applied to stationary phase. During chromatography, separation of analysts carried out by the phase back and forth occurs between the two phase. So that difference in their distribution coefficient results in their separation. All types of chromatography work on the same principle. There must be exist two phase. Mobile phase should be solvent and stationary phase should be gel/ liquid or solid mixture that is immobilized. Chromatography may be preparative or analytical.

Objective

- To understand the principle of chromatography
- To learn about the process and instrumentation of different chromatography
- To understand the role of different chromatography in biochemistry
- To understand the role of different phase of chromatography

4.2. Principle of Chromatography

Chromatography is a powerful technique used to separate mixture of substance into their individual components, so that the individual components can be thoroughly analyzed. All types of chromatography work on the same principle. There must be exist two phase.

Chromatography is a separation method where the analyte is combined within a liquid or gaseous mobile phase. This is pumped through a stationary phase. Usually one phase is hydrophilic and the other lipophilic. The components of the analyte interact differently with these two phases. Depending on their polarity they spend more or less time interacting with the stationary phase and are thus retarded to a greater or lesser extent. This leads to the separation of the different components present in the sample. Each sample component elutes from the stationary phase at a specific time, its retention time. As the components pass through the detector their signal is recorded and plotted in the form of a chromatogram.

Stationary phase: The stationary means "doesn't move". It can be either a solid or a liquid supported on a thin film on the surface of inert solid, through which the sample contained in the mobile phase percolates.

Mobile phase: This phase flows over the surface of the stationary phase. The mobile phase may be either a liquid or a gas.

The mobile phase flows through the stationary phase and carries the components of the mixture with it. The various components of the mixture travel at different rates, causing them to separate.

In the chromatography substances are separated due to their relative affinities for the stationary and mobile phase. The distribution coefficient 'K' governs the distribution. The fraction with a greater affinity for stationary phase travels slower and at a shorter distance, while that with a lesser affinity travels faster and longer.

$$K = \frac{\text{Concentration of a component in moving phase}}{\text{Concentration of that component in the stationary phase}}$$

4.3. Types of chromatography

There are four main types of chromatography which are based on different kind of stationary and mobile phase.

- Liquid Chromatography,
- Gas Chromatography,
- Thin-Layer Chromatography
- Paper Chromatography

Liquid Chromatography is used in the world to test water samples of lakes and rivers and check pollution. It is used to analyze metal ions and organic compounds in solutions. Liquid chromatography uses liquids which may incorporate hydrophilic, insoluble molecules.

Gas Chromatography is used in airports to detect bombs and forensics in different ways. It is used to analyze fiber on a person's body and also analyze blood found at a crime scene. In gas chromatography, helium is used to move a gaseous mixture through a column of absorbent material.

Thin-layer Chromatography uses an absorbent material on flat glass or plastic plates. This is a simple and rapid method to check the purity of an organic compound. It is used to detect pesticide or insecticide residues in food. Thin-layer chromatography is also used in forensics to analyze the dye composition of fiber.

Paper Chromatography is one of the most common types of chromatography. It uses a strip of paper as the stationary phase. Capillary action is used to pull the solvents up through the paper and separate the solutes.

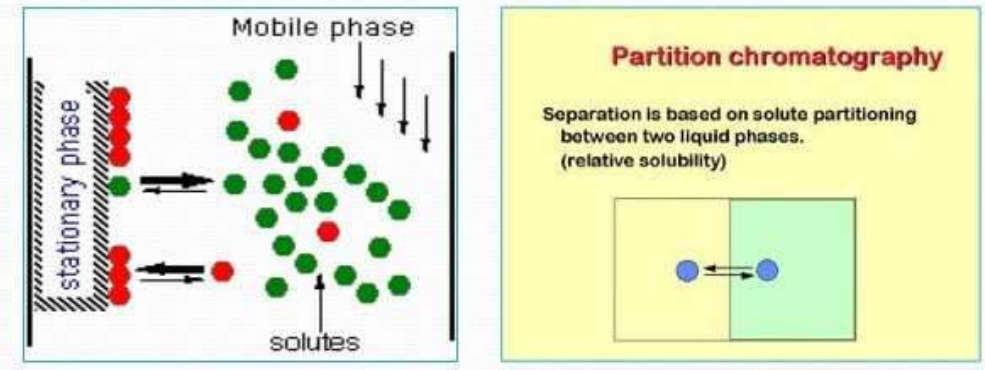
On the other hand on the basis of different application, the chromotagarphy are classified as following types

- Column chromatography.
- Ion-exchange chromatography.
- High Performance liquid chromatography
- Gel-permeation (molecular sieve) chromatography.
- Affinity chromatography.
- Paper chromatography.
- Thin-layer chromatography.
- Gas chromatography.
- Dye-ligand chromatography.
- Bio-affinity chromatography

4.4. Partition chromatography

Partition chromatography is one of the types of various chromatography techniques. It was discovered through the work of Archer Martin and Richard Laurence Millington Synge in 1940s.

The chromatography technique which is based on the partitioning of components of a mixture between stationary and mobile phases is called partition chromatography. It has various types such as paper chromatography, gas – liquid chromatography, liquid -liquid chromatography etc.



Principle

Partition chromatography is based on differential partitioning of components of a sample mixture between two phases – stationary phase and mobile phase. Most commonly it is used in paper chromatography which is a type of partition chromatography. In paper chromatography a paper is used which is called chromatography paper, works as stationary phase. This paper is made up of cellulose which is a polar substance. Mobile phase is liquid which runs on chromatography paper.

The stationary phase immobilizes the liquid surface layer, which becomes stationary phase.

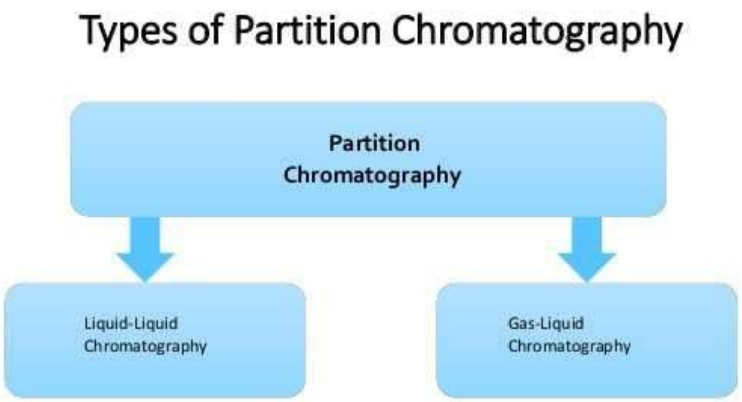
Mobile phases pass over the coated adsorbent and depending upon relative solubility in the coated liquid, separation occurs.

The component of sample mixture appears separated because of differences in their partition coefficient.

Partition of component of a sample between sample and liquid/gas stationary phase retard some components of sample more as compared to others.

This gives basis for separation. Separation of components of a sample mixture occurs because of partition. Stationary phase is coated with a liquid which is immiscible in mobile phase.

4.4.1. Types of Partition Chromatography



- **Liquid -Liquid Chromatography** – In this type of partition chromatography, mobile and stationary both phases are taken in liquid state. Although liquid stationary phase is coated or supported by solid support. It has following sub-types –
    - **Paper Chromatography** – In this type of liquid-liquid chromatography, liquid stationary phase is taken on chromatography paper made up of cellulose which is a polar substance. Water molecules get trapped on it.
    - **Column Chromatography** – In this type of liquid-liquid chromatography, liquid stationary phase is taken on silica gel or alumina cellulose powder.
    - **Thin layer Chromatography** – In this type of liquid -liquid chromatography, a very thin layer of stationary phase is taken that's why it is known as thin layer chromatography. It is used to separate non-volatile mixtures.
- **Gas – Liquid Chromatography** – In this type of partition chromatography, mobile phase is a uncreative gas and stationary phase is non-volatile liquid held on inert solid support. It is also known as vapor-phase chromatography or Gas-liquid partition chromatography (GLPC).

Applications of Liquid -Liquid Chromatography

There are numerous applications of partition chromatography. We are stating its applications according to its various types –

- **Paper Chromatography – Few Applications of Paper Chromatography are Mentioned Below –**
    - It is a qualitative method of identifying components of a sample mixture.
    - It is used in identification of drugs and impurities.
    - It is used in forensic studies.
    - It is used in analytical chemistry to introduce chromatography to students.
    - Used in identification of biomolecules, organic and inorganic compounds.
    - It is a low cost and rapid method of separation.

- **Column Chromatography - Few Applications of Column Chromatography are Mentioned Below –**
    - It is also used in separation of components of a given sample.
    - It is mainly used in purification processes.
    - It is used in separation of diastereomers.
    - It is used in the separation of alkaloids.
    - It is used in isolation and purification of vitamins and hormones.
    - It is used in examination of vegetable oil and pharmaceutical preparations.

- **Thin Layer Chromatography - Few Applications of Thin Layer Chromatography are Mentioned Below –**
    - Reactions of organic compounds are monitored by TLC.
    - TLC is used in separation of inorganic ions.
    - It is used in separation of amino acids.
    - It is used in separation of vitamin E and A.
    - It is used in quantitative analysis.
    - It is used in identification of compounds like acids, alcohols, proteins, alkaloids and amines.

4.5. Ion exchange chromatography

Ion exchange chromatography is the separation technique for charged molecules by their interaction with the oppositely charged stationary phase in the form of ion-exchange resin. Ion exchange chromatography was first used for the analysis of samples from works of art in 1969 when the successful analysis of antique and modern art specimens was reported. Ion-exchange chromatography relies on the charge differences of the solutes for separation. The stationary-phase resins have a charged species associated with them that is displaced (or exchanged with) by the solute species.

As the name suggest, ion exchange chromatography means ion exchanger resin will be there and due to presence of that resin ion exchange will take place i.e. sample is ionized and that sample will exchange with counter ion present in stationary phase and ultimately separation will take place. This technique separates charged or polar molecule in a mixture. Only hydrophilic molecules can be separated out from this technology.

In this type of chromatography separation occurs as a result of formation of ionic or electrostatic bond between the charged group of biomolecules and an ion exchange resin bearing opposite charge. Ions exist in a state of equilibrium between the mobile phase and stationary phases which give rise to two possible formats, anion and cation exchange are referred to as counter ion (Fig. 2.1). These exchangeable matrix counter ions may include protons (H^+), hydroxide groups (OH^-), single charged mono atomic ions (Na^+, K^+, Cl^-), double charged mono atomic ions (Ca^{2+}, Mg^{2+}), and polyatomic inorganic ions (SO_4^{2-}, PO_4^{3-}) as well as organic bases (NR_2H^+) and acids (COO^-). Cations are separated on cation-exchange resin column and anions on an anion exchange resin column.

It is one of the most important adsorption chromatography, performed for separation of peptides, proteins, nucleic acids and related biopolymers which have different molecular sizes and molecular nature with electronic charge. Advantage of using ion chromatography is that only one interaction involved during the separation as compared to other separation techniques; therefore, ion chromatography may have higher matrix tolerance.

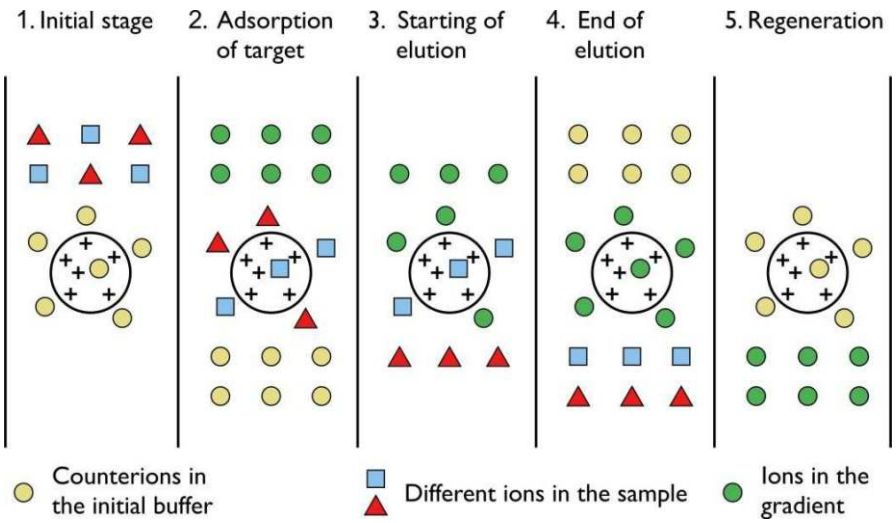


Fig. 4.1: Diagrammatical representation of ion exchange chromatography.

Stationary phase is solid and have resin polymers forming network. Most commonly used cellulose, agarose and polymethacrylate. These stationary phases consist of an immobile matrix which covalently bound to charged molecules. Mobile phase is liquid containing sample that is to be separated.

Principle

This form of chromatography relies on the attraction between oppositely charged stationary phase known as an ion exchange, and analyte.

- The ion exchangers basically contain charged groups covalently linked to the surface of an insoluble matrix.
- The charged groups of the matrix can be positively or negatively charged.
- When suspended in an aqueous solution, the charged groups of the matrix will be surrounded by ions of the opposite charge.
- In this "ion cloud", ions can be reversibly exchanged without changing the nature and the properties of the matrix.

Ion exchange chromatography is based on the reversible electrostatic interaction of charged species with the ion exchange matrix and ultimately separation takes place. On the basis of ions separated, the ion exchange chromatography can be divided into two categories.

a. Anion Exchange Chromatography:

$$\text{Resin-OH}^- + \text{A} \longrightarrow \text{Resin-A} + \text{OH}$$

(in solution) (in eluting solution)

Anion exchanges resin

When molecule of interest is negatively charged then anion exchange chromatography is used. In this process anion in a mobile (liquid) phase exchanges with another anion that is previously bound to a positively charged solid support or matrix. Commonly used anion exchange resins are Q-resin, a Quaternary amine; and DEAE resin, Diethylaminoethane. Anion exchange chromatography is used both for preparative and analytical purposes and may also be used chromatographically, to separate anions and medicinally to remove an anion from gastric contents or bile acids in the intestine.

b. Cation Exchange Chromatography:

$$\text{Resin-H}^+ + \text{M}^+ \longrightarrow \text{Resin-M}^+ + \text{H}^+$$

(in solution) (in eluting solution)

Using cation exchange resin

Cation exchange chromatography is used when the desired molecules to separate are cations in mobile phase. Positively charged molecules are attracted to a negatively charged solid support. S-resin, sulfate derivatives; and CM resins, carboxylate derived ions are commonly used as cation exchange resins. This type of chromatography is used both for preparative and analytical purposes and can separate a large range of molecules from amino acids and nucleotides to large proteins.

Uses of Ion exchange chromatography

- Ion exchange chromatography is used in the purification of water where the positively charged ions are replaced by hydrogen ions, and the negatively charged ions are replaced by hydroxyl ions.
- This method also works as an effective method for the analysis of the products formed after hydrolysis of nucleic acids.

➢ The separation of metals and other inorganic compounds is also facilitated by the ion-exchange chromatography. An important use of ion-exchange chromatography is the routine analysis of amino acid mixtures. The 20 principle amino acids from blood serum or from the hydrolysis of proteins are separated in a few hours, and their concentrations are determined automatically as the amino acid "Autoanalyzer" is based on ion exchange principle.

➢ Separation of many vitamins, other biological amines, carbohydrates, nucleotides, proteins and organic acids and bases etc are performed with the help of ion exchange chromatography.

➢ In biochemistry ion exchange chromatography used for separation of drugs and metabolites from blood, urine, etc which find application in clinical diagnosis. This separation technique effectively used in purification of enzymes after extracting from the tissues.

4.6. Gel filtration chromatography

Gel filtration chromatography is a type of size exclusion chromatography, which separates molecule according to their size and shape. With some exceptions, the separation of the components in the sample mixture correlates with their molecular weight. In these cases, gel filtration chromatography can be worked as analytical tool to find out the molecular weight of an uncharacterized molecule.

Gel filtration is also known as size-exclusion chromatography or molecular-sieve chromatography. In this process, separation is based on the differing ability (due to differing molecular size) of molecules in the sample to enter the pores of the gel-filtration medium. The stationary phase in this technique consists of beads of a hydrated, sponge-like material that has pores of molecular dimensions and with a narrow range of sizes.

Gel filtration chromatography, a type of size exclusion chromatography, can be used to either fractionate molecules and complexes in a sample into fractions with a particular size range, to remove all molecules larger than a particular size from the sample, or a combination of both operations. Gel filtration chromatography can be used to separate compounds such as small molecules, proteins, protein complexes, polysaccharides, and nucleic acids when in aqueous solution. When an organic solvent is used as the

mobile phase, the process is instead referred to as gel permeation chromatography.

It is also an important preparative technique which can be used to separate protein, peptides and oligonucleotides on the basis of size. In gel filtration chromatography separation is achieved by physical means, which make it differ from other types of chromatography. As gel filtration chromatography does not involve any interaction of the sample or the solvent with the matrix in a column. Aqueous solution or buffer solution used as mobile phase in gel filtration chromatography. Gel consisting of porous beads or carbohydrates cross linking agents is used as stationary phase. Most commonly used gels are dextron (sephadex), agarose (sepharose) and polyacrylamide (Bio Gel).

Principle

Separation in gel filtration chromatography is based on the differences in sizes from biomolecules as they pass through a column packed with a chromatographic medium or stationary phase, which is a gel. So, the larger size molecules separate out first from the sample solution, after which smaller size molecules are separated.

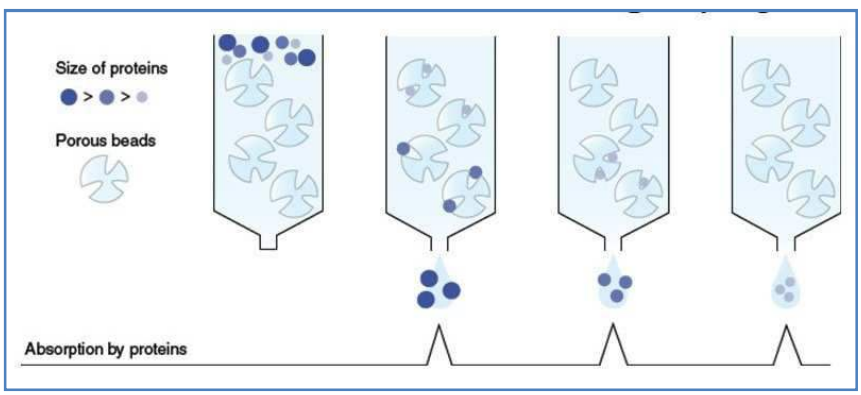


Fig. 2.3: Separation process in gel filtration chromatography

Application

Gel filtration chromatography helps to determine the molecular weight distribution of polymers.

As this technique separate the substance on the basis of different size and mass. So that organic compounds like sugars, polypeptides, polystyrenes, etc can be separated as they have different size.

- Fractionation of molecules and complexes within a predetermined size range
- It can remove small molecules such as nucleotides, primers, dyes, and contaminants from mixture.
- It is effectively used in separation of bound from unbound radioisotopes

- Give some exercise related to gel filtration chromatography
- Gel filtration plays a key role in the purification of enzymes, polysaccharides, nucleic acids, proteins, and other biological macromolecules.
- Gel filtration can also be used to facilitate the refolding of denatured proteins by careful control of changing buffer conditions.
- It is used in protein fractionation experiments.
- Gel filtration technique is also used in molecular weight determination
- Separation of sugar, proteins, peptides, rubbers, and others on the basis of their size.
- Can be used to determine the quaternary structure of purified proteins.

Limitations of Gel Filtration Chromatography

- The limited number of peaks that can be resolved within the short time scale of the run.
- Filtrations must be performed before using the instrument to prevent dust and other particulates from ruining the columns and interfering with the detectors.
- The molecular masses of most of the chains will be too close for the separation to show anything more than broad peaks.

4.7. High Performance Liquid Chromatography

HPLC stands for high-performance liquid chromatography. "Chromatography" is a technique for separation, "chromatogram" is the result of chromatography, and "chromatograph" is the instrument used to conduct chromatography.

As its name suggest, high performance means that this analytical technique used for proper separation, identification and for quantification of components in a mixture**.** HPLC is an instrumental form of liquid chromatography and gives high performance due to the small particle size of the stationary phase. The particle size of stationary phase is 3.5 to 10 micrometer. Due to smaller size, surface area of the particle is high and ultimately HETP (height equivalent to theoretical pressure) increased and thereby helps in achieving more efficient separation of the components of the mixture than those used in conventional liquid chromatography.

In this chromatography, particle size of stationary phase is small and due to small size, packing of stationary phase will be high. Due to tight packing flow rate of mobile phase is reduced. So that to increase the flow rate or to increase efficiency or to increase the separation of the mixture, high pressure is applied. The applied pressure is about 1000-4000 psi. Because of the use of high pressure in this technique, it is sometimes also known as high pressure

liquid chromatography. So we can say HPLC is modern application of liquid chromatography.

HPLC has the ability to separate and identify compounds that are present in any sample that can be dissolved in a liquid, in trace concentrations as low as parts per trillion. Because of this versatility, HPLC is used in a variety of industrial and scientific applications, such as pharmaceutical, environmental, forensics, and chemicals.

Principle

HPLC is highly automated and extremely sensitive technique as compared to column chromatography because the components of a mixture are separated from each other due to their different degrees of interaction with the absorbent particles. This causes different elution rates for the different components and leads to the separation of the components as they flow out the column. Solvents are used as mobile phase. In general organic compounds are analyzed using HPLC and these organic compounds are soluble in polar solvents. Some commonly used solvents as mobile phase are methanol, acetonitrile and water. Acidifiers or basifies or buffer solution are added to the solvent to achieve better separation. As these neutralize the ionized analytes or compounds present in the column. If analyte or compounds present in column will be ionized, so affinity with stationary phase will be lost and proper separation does not take place. Means every analyte immediately come with the mobile phase. Particle size of stationary phase ranges 3.5 to 10 micrometer. There are two types of stationary phase. i) Normal stationary phase for example silica gel and ii) Reversed stationary phase for example octa decyl silane silica gel etc. There are generally two types of columns used. Normal phase column and reversed phase column.

Instrumentation:

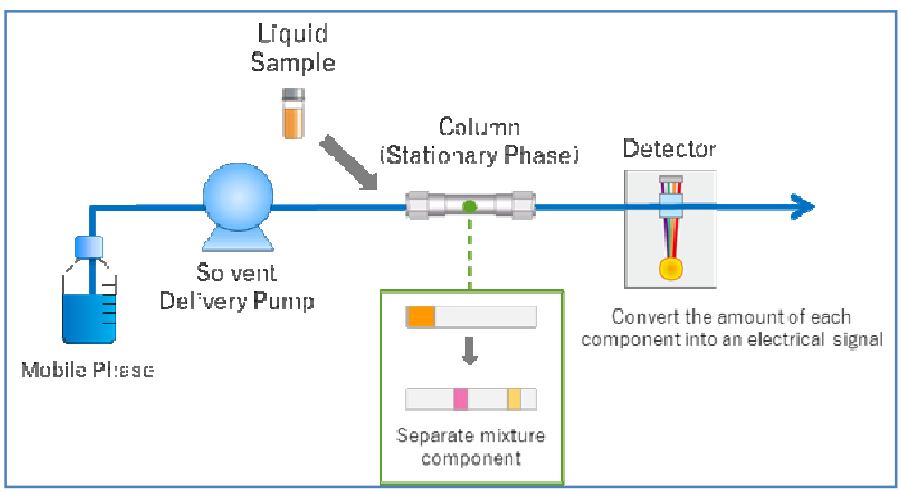


Fig.1 Overview of HPLC

The main components of HPLC are as follows:

1. **Solvent Reservoir:** The solvent reservoir holds the solvent, which is referred to as the mobile phase. Two types of reservoir are used.
    a. **Binary system:** In this type two reservoir are present.
    b. **Quaternary system:** In this type four reservoir are present.

In HPLC we use specific type of solvent that are filtered known as HPLC grade solvent. This solvent is highly pure.

2. **Degasser:** It is used to remove the gases which are dissolved in mobile phase or in solvent. Degassing is done by using vacuum pump. So vacuum pump remove the any type of gas or air present in solvent and makes it suitable for better separation. If these gases interact with mobile phase and go to the column, proper functioning of the column does not take place i.e. proper separation may be hindered.
3. **Solvent Mixing Valve:** This valve is used to mix the solvent. If we use binary system two solvents will get mix together and if we use quaternary system, four solvents mix together.
4. **HPLC Pump:** Two types of pumps are used.
    a. **Constant pressure pump:** Pressure of the pump is constant and flow rate may vary, but this will not do proper separation.
    b. **Constant flow rate pump:** Flow rate is constant and pressure may be changed. So according to our column resistance pressure, will be changed and due to that changed pressure, flow rate of the column does not affected. Generally 1 ml/min flow rate is used in HPLC.
5. **Precolumn or Guard Column:** HPLC has precolumn or guard column to remove impurity of the solvent. As the name suggest precolumn, means it is used before the analytical column. As stationary phase is same in guard column. Solvent first go through the guard column after that it will go to the analytical column. So any type of contamination present in the solvent is removed by this column, before it go to analytical column. Size of the guard column is low as compared to the analytical column. Internal diameter is 4-5 mm and length is 2-10 cm.
6. **Sample Injector: Three types of sample injectors are used.**
    a. **Septum Injector**: In this type one auto selling in present and solvent is injected using micro syringe.
    b. **Stop flow septum less injector:** In this type of injector, firstly we have to stop the mobile phase then we open the upper part of the column and after that we put the sample here and again start the flow of mobile phase.
    c. **Microvolume** sampling injector: This is the modern and sophisticated injector. By using this Injector highest accuracy will be achieved and auto sampling technique is applied by using this method.
7. **Analytical Column:** The length of analytical column is 10-30 cm and internal diameter is 4-5 mm. This column is made up of stainless steel

which can resist the high pressure used in HPLC. During packing of analytical column 14000-15000 psi pressure is applied.

8. **Detector:** It is used to separate compound bands as they elute from the high pressure column. There are several types of detector used in HPLC. Some of them are as follows.
    a. **Bulk property detector:** Like refractive index detector.
    b. **Solute property detector:** Like florescence detector, UV detector.
    c. **Multipurpose detector:** It is combination of 2 or 3 types of detector.
    d. **Electrochemical detector:** Like colometric, amperometric detector.
    e. **Mass detector:** It is also known as LCMS. It is highly sensitive and most commonly used detector in HPLC.

The information is sent from the detector to a computer which generates the chromatogram. The mobile phase exits the detector and is either sent to a waste, or collected, as desired.

Methodology

To run HPLC following process goes on.

- The sampler brings the sample mixture to be evaluated into a stream of mobile phase which is flowing at a defined pressure.
- Now the injected mixture does flow over the stationary phase inside the column under the influence of pressure along with the mobile phase.
- During this flows based on the affinity of individual compounds in the mixture towards stationary and mobile phase, some compounds get eluted out of the column first before others.
- Outside the column they are sent into a detector where individual compounds are detected and recorded in computer installed chromatography software.
- The recordings (preferably in the form of quantitative peaks) are compared with those of standard compound's HPLC values, and the individual compounds are identified. So the overall theory of HPLC is relative separation and detection of compounds.

Application

- Analysis of drugs
- Analysis of synthetic polymers
- Analysis of pollutants in environmental analytics
- Determination of drugs in biological matrices
- Isolation of valuable products
- Product purity and quality control of industrial products and fine chemicals

- Separation and purification of biopolymers such as enzymes or nucleic acids
- Water purification
- Pre-concentration of trace components
- Ligand-exchange chromatography
- Ion-exchange chromatography of proteins
- High-pH anion-exchange chromatography of carbohydrates and oligosaccharides.

4.8. Summary

All types of chromatography work on the same principle and used mobile phase and stationary phase separation of biological samples. The chromatography basically classified into thin layer, ion exchange, affinity, gel filtration, high performance liquid, hydrophobic interaction chromatography. In thin layer chromatography, the stationary phase is used and it is spread over a supporting plate and a mobile phase is also used. This mobile phase migrate over these supporting material or stationary phase against the gravitational force. In ion exchange chromatography, separation occurs as a result of formation of ionic or electrostatic bond between the charged group of biomolecules and an ion exchange resin bearing opposite charge. HPLC has the ability to separate, and identify compounds that are present in any sample that can be dissolved in a liquid in trace concentrations as low as parts per trillion HPLC used for identification and for quantification of components in a biological mixture.

4.9. Terminal Question

Q.1. What is chromatography? Define the role of chromatography in biochemistry.

Answer: ----------

Q.2. Write the principle and types of chromatography.

Answer: ----------

Q.3. Write the principle and application of partition Chromatography.

Answer: ----------

Q.4. Discuss about HPLC in brief.

Answer: ----------

Q.5. Discuss ion exchange chromatography in brief.

Answer: ----------

Q.6. Write the principle of gel filtration chromatography.

Answer: ----------

3.10. Further reading

1. S.M. Khopkar, Basic Concepts of Analytical Chemistry, New Age International Pvt Ltd Publishers,2018
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3. , Organic spectroscopy; first edition.2004
4. David Holme and Hazel Peck, Analytical Biochemistry; Prentice Hall; third edition, 1998).
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Unit-5.Spectroscopy-I

5.1. Introduction

Spectroscopy is the most important tools for the detection of organic and inorganic compounds. In spectroscopy, we study the interaction of radiation with matter to detect the nature of compound. The electromagnetic radiation change their frequency and wavelength when interact with matter and resultant the spectra is obtained. After studying this module, you shall be able to learn about the nature and properties of electromagnetic waves. You will be introduced to the classical picture of light and electromagnetic waves. The different ranges of electromagnetic radiation are found to be responsible for the different spectra of compounds. Spectroscopy generally contains all science such as physics, chemistry, astrophysics, life sciences, geology, or any other science. However, spectroscopy does not directly show us the molecules but its shows different peaks of radiation absorbed by the matter, thus it reveal a lots about it structure. The spectroscopy, which is the main tools in study of matter not only reveal structural properties but also useful in detection of unknown molecules and measurement of concentrations for analytical purposes. Generally, the concept of spectroscopy is clearly appearing in the study of visible light dispersed according to its wavelength, for example by prism. In prism, visible light pass through it reflects at different angle due to nature of prism particle. Spectroscopic data are often represented by an emission spectrum or absorption spectrum. Spectroscopy is a fundamental exploratory tools in the fields of physical, chemical and biological science at atomic and macro scale.

Objectives

- To understand the effects of electromagnetic radiation on the matter
- To understand the role of electromagnetic radiation in spectroscopy

- To discuss different types of spectroscopy and their characteristic feature
- To understand the application of spectroscopy in biochemistry

5.2. Concept of spectroscopy

The word spectroscopy comes from the Latin word *specere*, means "to look at" and the Greek word *skopia*, means "to see". So that spectroscopy refers the techniques that employ light to produce properties of object. However, the light interacts with matter and probe certain features of sample to learn about it consistency or structure. Light is an electromagnetic radiation that comprises several regions of wavelength. The electromagnetic radiations have different energy level radiations which gives phenomenon to probe different molecular features'. The spectroscopy methods are the most useful in biochemistry. The visible spectrum is the portion of the electromagnetic radiation that is visible to the human eye. Unlike X-ray, Gamma ray, the UV ray spectroscopy also play important role in analytical techniques. The absorption wavelength is associated with transition that requires a minimum of energy change

Spectroscopy helps us to know how the incident radiation affects the sample of specimen. However, detailed the study of spectroscopy, the understanding of properties of electromagnetic radiation and it interaction with matter is necessary to every student. The results of spectroscopic data depend on properties of electromagnetic radiation and interacting matter. Spectroscopic data are often represented by an emission spectrum or absorption spectrum. Spectroscopy is a fundamental exploratory tools in the fields of physical, chemical and biological science at atomic and macro scale.

To better understanding of the spectroscopy we should know about the electromagnetic radiation, the interaction of electromagnetic radiations with matter is a quantum phenomenon. Electromagnetic radiation consists of radio waves, microwaves, infrared waves, visible light, ultraviolet radiation, X-rays, and gamma rays. Every radiation has different wavelength, frequency and energy. The wavelength refers for special distance between two consecutive peaks in sinusoidal waveform and is measured in nanometer (nm). The frequency (υ) of electromagnetic radiation defines the number of oscillator made by the wave within the timeframe of 1 second. The energy in electromagnetic radiation exists in the form of photons. The quantum phenomenon of electromagnetic radiation depends upon both; properties of the radiation and the appropriate structural part of the samples involves. The electromagnetic radiation is propagated through free space or through a medium and composed of both electric and magnetic waves. The electric and magnetic waves have oscillations that are perpendicular to each other and also to the direction of travel of the wave.

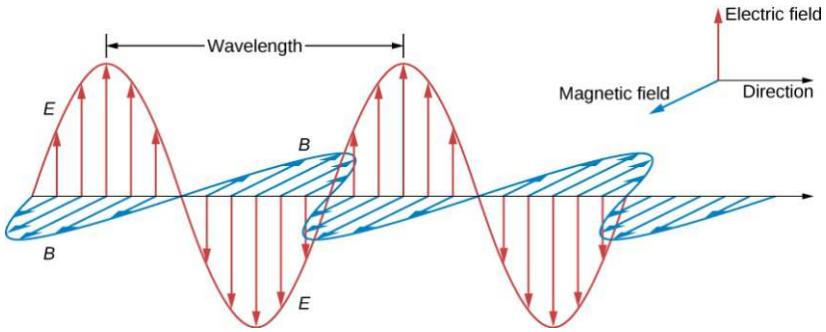


Fig.1.1: Nature of electromagnetic waves

There are many sources of electromagnetic radiation, both natural and man-made. Different electromagnetic radiations are given below in Table (1.1) along with their wavelength and frequency. As the wavelength of waves increases frequency get decreases.

5.3. Ultraviolet and visible light spectroscopy

Spectroscopic techniques employ light to interact with matter and thus probe certain features of a sample to learn about its consistency or structure. Light is an electromagnetic radiation, a phenomenon exhibiting different energies, and dependent on that energy, different molecular features can be probed. Ultraviolet–visible spectroscopy refers to absorption spectroscopy or reflectance spectroscopy concern with ultraviolet and the visible spectral regions of electromagnetic radiation. The instrument that is used in uv-visible spectrometry is called spectrometer.

UV-Visible is a vital tool for the characterization of the functional groups of molecules and complexes. It produces absorbance or transmittance spectra of molecules in the ultraviolet and visible region for quantitative and qualitative analysis of chemical species. The wavelength of absorption is usually reported as λmax which represents the wavelength at the highest point of the curve. Absorption in UV or visible region leads to excitation of bonding electrons. Thus the absorption peak can be correlated with the kind of bonds existing in species. The UV-Visible spectra of ligands and their synthesized complexes in solution were recorded by UV-Visible Instrument (Thermo Scientific) in laboratory. The stock solution of synthesized complexes (0.1 x 10^{-3}M) were prepared by dissolving appropriate and exactly weighted pure solid compound in 50 mL double distilled water. Working solutions were prepared by appropriate dilution of stock solutions. The spectra of working solutions were recorded at room temperature.

The absorbance at a particular wavelength is defined by the Eq. :

$$A = \log_{10} \frac{I_o}{I} = \varepsilon I_o C$$

Or

$$\varepsilon = \frac{A}{CI}$$

where,

A= absorption

I_o= the intensity of the incident radiation

I = the intensity of the transmitted radiation

ε = a constant for each absorbing material, known as the molar absorption coefficient (called the molar extinction coefficient in older texts) which having the units L $mol^{-1}cm^{-1}$, but by convention the units are not quoted. Electronic transaction in energy levels is possible by absorption of radiation due to occurrence following transaction.

The electromagnetic transition into molecules can be classified according to the participating molecular orbital. There are four possible transition exicited with light form the UV/Vis spectrum for some biological molecules such as

σ →σ *, σ →π * {high-energy, λmax <150 nm},

η → σ *,π → σ * {non-bonding electrons (lone pairs) , (λmax=150-250 nm)}

η →π*,π →π* {organic molecular UV-Vis, lone pairs and multiple,λmax = 200-600 nm}

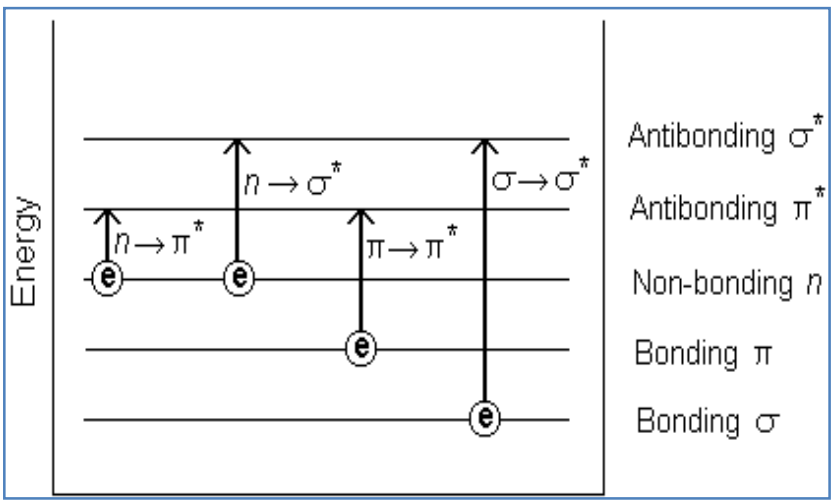


Fig.1.6: Electronic Transition in UV-Vis Spectroscopy

UV-Vis spectroscopy is used for both the quantitative and qualitative determination of different analytes, such as transition metal ions, highly conjugated organic compounds, and biological macromolecules. In qualitative manner, UV-VIS spectroscopy is used to identify the functional group or confirm the identity of compound by matching the absorbance spectrum. The absorption or reflectance in the visible region affects the observed color of the chemical involved. The light source is a tungsten filament bulb for the visible part of the spectrum, and a deuterium bulb for the UV region. Since the emitted light consists of many different wavelengths, A monochromator, consisting of either a prism or a rotating metal grid of high

precision called grating, is placed between the light source and the sample. Wavelength selection can also be achieved by using coloured filters as monochromators that absorb all but a certain limited range of wavelengths

Spectroscopy analysis is commonly carried out in solutions but solids and gases may also be studied. The molecular structures which are responsible for interaction with electromagnetic radiation are called Chromophore. In proteins, there are three types of chromophores relevant for UV/Vis spectroscopy:

- peptide bonds (amide bond);
- certain amino acid side chains (mainly tryptophan and tyrosine); and
- certain prosthetic groups and coenzymes (e.g. porphyrine groups such as in haem)

The UV extends from 100–400 nm and the visible spectrum from 400–800 nm. The 100–200 nm range is called the deep UV. Light source for deep UV range is more difficult so it is not commonly used for UV-Vis measurements.

The presence of several conjugated double bonds in organic molecules results in an extended p-system of electrons which lowers the energy of the π^* orbital through electron delocalisation. In many cases, such systems possess π^* -π^* transitions in the UV/Vis range of the electromagnetic spectrum. Such molecules are very useful tools in colorimetric applications.

The electronic transitions of the peptide bond occur in the far UV. The intense peak at 190 nm, and the weaker one at 210–220 nm is due to the π^* -π^* and n -π^*transitions. A number of amino acids (Asp, Glu, Asn, Gln, Arg and His) have weak electronic transitions at around 210 nm. If protein has prosthetic groups and some metal–protein complexes, may have strong absorption bands in the UV/Vis range.

Principles

The chance for a photon to be absorbed by matter is given by an extinction coefficient which itself is dependent on the wavelength l of the photon. If light with the intensity I_o passes through a sample with appropriate transparency and the path length (thickness) d, the intensity I drops along the pathway in an exponential manner. The characteristic absorption parameter for the sample is the extinction coefficient. α, yielding the correlation

$$I = I_0 e^{-\alpha d}$$

The ratio $T = \frac{I}{I_0}$ is called transmission.

Biochemical samples usually comprise aqueous solutions, where the substance of interest is present at a molar concentration C. Algebraic

transformation of the exponential correlation into an expression based on the decadic logarithm yields the law of Beer–Lambert.

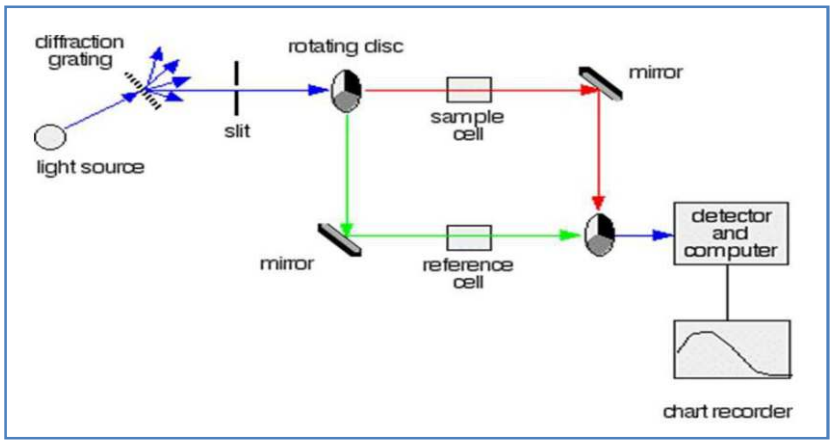


Fig. Diagrammatic representation of Simple double beam spectrometer.

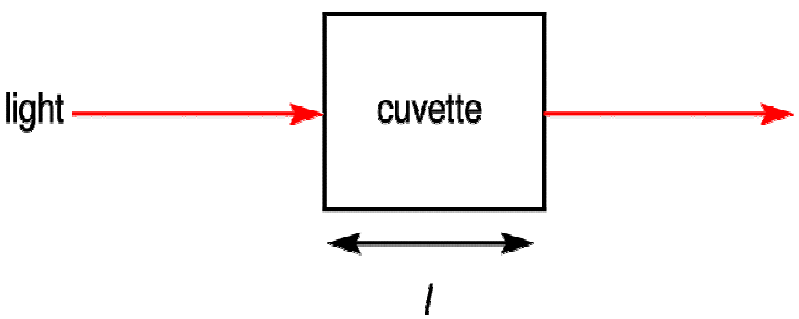
5.4. Beer-Lambert's law

The Beer-Lambert law is a linear relationship between the absorbance and the concentration, molar absorption coefficient and optical coefficient of a solution. For each wavelength of light passing through the spectrometer, the intensity of the light passing through the reference cell is measured. This is usually referred to as Io - that's I for Intensity.

The chance for a photon to be absorbed by matter is given by an extinction coefficient which itself depend on the wavelength (λ) of the photon. If the intensity Io passes through a sample with appropriate transparency and the path length (thickness) l , the intensity I drops along the pathway in an exponential manner.

The absorbance of a transition depends on two external assumptions.

The characteristic absorption parameter for the sample is the extinction coefficient α. Which yield by the equation $I = Io\ e^{-\alpha d}$. The ratio of T = I/Io is called transmission.



$A = \varepsilon cl$

Where, **A** = Absorbance of sample, which display on the spectrophotometer.

E = Molar absorption coefficient M^{-1} cm^{-1}

C = Molar concentration "M"

l = optical length in cm

The Beer-Lambert law is valid for low concentrations only. The absorption and extinction coefficients are additives parameters, which complicates determination of concentrations in samples with more than one absorbing species.

The chance for a photon to be absorbed by matter is given by an extinction coefficient which itself depend on the wavelength (λ) of the photon. If the intensity I_o passes through a sample with appropriate transparency and the path length (thickness) l , the intensity I drops along the pathway in an exponential manner. The characteristic absorption parameter for the sample is the extinction coefficient α. Which yield by the equation $I = I_o e^{-\alpha d}$. The ratio of T = I/Io is called transmission.

The intensity of the light passing through the sample cell is also measured. For that wavelength, given the symbol λ. If λ is less than Io, then the sample has absorbed some of the light (neglecting reflection of light off the cuvette surface). The absorbance of a transition depends on two external assumptions.

1. The absorbance is directly proportional to the concentration (C) of the solution of the sample used in the experiment.
2. The absorbance is directly proportional to the length of the light path, which is equal to the width of the cuvette.

Factors affecting UV/Vis absorption

Biochemical samples are usually buffered aqueous solutions, which has two major advantages. Firstly, proteins and peptides are comfortable in water as a solvent, which is also the 'native' solvent. Secondly, in the wavelength interval of UV/Vis (700–200 nm) the water spectrum does not show any absorption bands and thus acts as a silent component of the sample.

The environment of chromophore can be probed by assessing their absorption that is following:

- Due to bathochromic effect: a wavelength shift to higher values is called red shift or bathochromic effect.
- Due to hypsochromic effect : when a wavelength shift to lower wavelengths is called blue shift or hypsochromic effect
- Due to increase in hyperchromicity (more colour')
- Due to decrease in absorption ('less colour')

Applications

Common applications of differente UV spectroscopy include the determination of the number of aromatic amino acids exposed to solvent, detection of conformational changes occurring in proteins, detection of aromatic amino acids in active sites of enzymes, and monitoring of reactions involving 'catalytic' chromophores (prosthetic groups, coenzymes). This spectroscopy helps to determine the impurities in organic molecules. Additional peak in UV-Vis spectra is due to the presence of impurities in the sample and can be compared with that of standard raw material. UV-Vis is also used as a standard technique to quantify the amount of DNA in a sample, as all the bases absorb strongly at 260 nm. RNA and proteins also absorb at 260 nm, so absorbance at other wavelengths can be measured to check for interferences.

Some of application is common such as Qualitative Analysis of different samples, Solvent perturbation and to know the differente spectra of compounds. To obtain good spectra, the maximum absorbance should be approximately 0.5 which corresponds to concentrations of about 50 uM (assuming e = 10 000 dm^3 mol^{-1} cm^{-1}).

Table 1.4: Common colorimetric and UV absorption assays

Substance	Reagent	Wavelength (nm)
Amino acids	a. Ninhydrin b. Cupric salts	570 (proline : 420) 620
Cysteine residues, thiolates	Ellman reagent (di-sodium-bis-(3-carboxy4-nitrophenyl)-disulphide)	412
Protein	(a) Folin (phosphomolybdate, phosphotungstate, cupric salt)	660
	(b) Biuret (reacts with peptide bonds)	540
	(c) BCA reagent (bicinchoninic acid)	562
	(d) Coomassie Brilliant Blue	595
	(e) Direct	Tyr, Trp: 278, peptide bond : 190
Glucose	Glucose oxidase, peroxidase, o-dianisidine, phosphate buffer	420
Ketohexose	(a) Resorcinol, thiourea, ethanoic acid, HCl	520
DNA	(a) Diphenylamine	595
	(b) Direct	260
RNA	Bial (orcinol, ethanol, FeCl3, HCl)	665

5.5. Applications of colorimetry

Colorimetry is one of the methods of spectral analytical techniques. Colorimeter refers to a device that is used to measure the waves of lights. This kind of measurement helps to find the level of concentration of a particular substance. This is due to the fact that each solution or substance absorbs or transmits a certain amount of light based on its properties and the concentration of particles present in it. The variation of the color of a solution with change in concentration of some solute component in the solution forms the basis of colorimetry. There are several applications of colorimetry and they are mentioned below.

- It is used by hospitals as well as laboratories for analyzing biochemical samples such as urine, cerebrospinal fluids, plasma, biochemical samples, and serum.
- It is widely used to generate a quantitative estimation of the serum components, proteins, glucose, and various biochemical compounds.
- It is also used in food industries and by manufacturing industries to make textiles and paints.

A colorimeter has immense significance in the ever-expanding world of science. Further research is going to enhance its features and functionalities.

5.6. Summary

Spectroscopy is a fundamental tool in the field of physical, chemical and biological sciences which is used in determination of electronic structure, composition of matter from atomic scale to macro scale level. The atomic adsorption and UV-Visible spectroscopy used the visible light for detection of specimen. The atomic microscopy is very useful in detection of heavy metal from the biological samples. There is an interaction between UV visible light and sample to be analyzed. By measuring the absorbance at specific wavelength impurities can be detected by UV visible spectroscopy. Most commonly, this type of spectroscopy is used for quantification of biological samples either directly or via colorimetric assays. In many cases, proteins can be quantified directly using their intrinsic chromophores, tyrosine and tryptophan. Protein spectra are acquired by scanning from 500 to 210 nm. The main advantage of difference spectroscopy is its capacity to detect small absorbance changes in systems with high background absorbance. A difference spectrum is obtained by subtracting one absorption spectrum from another. The qualitative analysis is carried out by the UV-Vis spectroscopy when the atom or molecules absorb UV radiation and identification is done by comparing the absorption spectra with the spectra of known compound. Qualitative analysis is done in UV/Vis regions to identify certain classes of compounds both in the pure state and in biological mixtures (e.g. protein-bound).

5.7. Terminal questions

Q.1. What do you understand for spectroscopy?

Answer:----------

Q.2. Write the principle and application of uv-visible spectroscopy.

Answer:----------

Q.3. Discuss about absorption spectrum of chromophores

Answer:----------

Q.4. UV-Visible work on which law. Discuss the Limbert Beer's Law.

Answer:----------

Q.5. Briefly discuss chromophore and auxochrome in spectroscopy.

Answer:----------

5.8. Further readings

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2. C.N.R. Rao, Ultraviolet and Visible spectroscopy, Butterworth's, Landon, 3rd edition, 1970.
3. G.R. Barrow, Introduction to molecular spectroscopy, McGraw New park 1962.
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Unit-6 Spectroscopy-II

6.1. Introduction

Fourier transform infrared spectroscopy (FTIR) is a technique, used to quickly and definitively identify compounds such as compounded plastics, blends, fillers, paints, rubbers, coatings, resins, and adhesives. FTIR spectrum is recorded between 4000 and 400 cm^{-1}. It is also used to obtain an infrared spectrum of adsorption or emission of a solid, liquid or gas. However, the FTIR technique has been found important in the field of chemistry because of its easy way to indentify the presence of certain functional groups in a molecules and compounds.

In Fourier transform infrared spectroscopy, IR radiation is passed through a sample. Some of the infrared radiation is absorbed by the sample and some of it is passed through (transmitted). The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample. Like a fingerprint no two unique molecular structures produce the same infrared spectrum. This makes infrared spectroscopy useful for several types of analysis. Therefore, infrared spectroscopy can result in a positive identification (qualitative analysis) of every different kind of material. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present. With modern software algorithms, infrared is an excellent tool for quantitative analysis. FTIR technique is non destructive which provides a precise measurement method which requires no external calibration.

Nuclear Magnetic Resonance (NMR) spectroscopy has made a major role in various areas of chemistry, biology and medicine. A wide range of applications of NMR spectroscopy is presented, including the identification and structural studies of complex biomolecule, such as proteins, applications to food analysis, clinical studies, NMR as a microscope etc. High resolution nuclear magnetic resonance spectroscopy (NMR) is the most important technique for structure elucidation of chemical compounds in solution. The NMR spectroscopy is associated with absorption and emission of energy. Various aspects of this technique are still under research, and many functions of the NMR are still pending for a better understanding and acknowledgment.

6.2. Fourier Transform Infrared (FT-IR)

Fourier Transform Infrared (FT-IR) spectrometry is widely used techniques. It is a non destructive and less time consuming technique. FTIR method is used to obtain the infrared spectrum of transmission or absorption of a fuel sample. FTIR identifies the presence of organic and inorganic compounds in the sample. Depending on the infrared absorption frequency range 600–4000 cm−1. FTIR is useful in identifying and characterizing unknown materials, detecting contaminants in a material, finding additives, and identifying decomposition and oxidation. It is a powerful tool for identifying types of chemical bonds in a molecule by producing an infrared absorption spectrum that is like a molecular "fingerprint". The wavelength of light absorbed is characteristic of the chemical bond as can be seen in this annotated spectrum.

Fourier Transform Infrared Spectroscopy (FTIR) identifies chemical bonds in a molecule by producing an infrared absorption spectrum. The spectra produce a profile of the sample, a distinctive molecular fingerprint that can be used to screen and scan samples for many different components. FTIR is an effective analytical instrument for detecting functional groups and characterizing covalent bonding information. FTIR provide following information such as

It provide spectral region is in the infrared

It provide spectra of high resolution

It application involves in weak signals

It provides spectra quickly with in high S/N ratio

It provides high spectral accuracy

FTIR simultaneously collect high spectral resolution data over a wide NIR to FIR range spectra. In FTIR studies of molecules, the absorption information is generally obtained in the form of both wave number and absorption intensity or percent transmittance. The spectrum is generally showing wave number (cm^{-1}) as the x-axis and absorption intensity or percent transmittance as the y-axis. The value of transmittance (T) denotes the radiant

power transmitted by the sample (I) to the radiant power incident on the sample (I_0). Absorbance (A) is the logarithm to the base 10 of the reciprocal of the transmittance (T).

The absorption intensity of molecules vibration can be determined by the Lambert-Beer Law expressed

A = a()*b*c

where A is the measured absorbance,a() is a wavelength dependant adsorptive coefficient, b is the path length,and c is the analyte concentration.

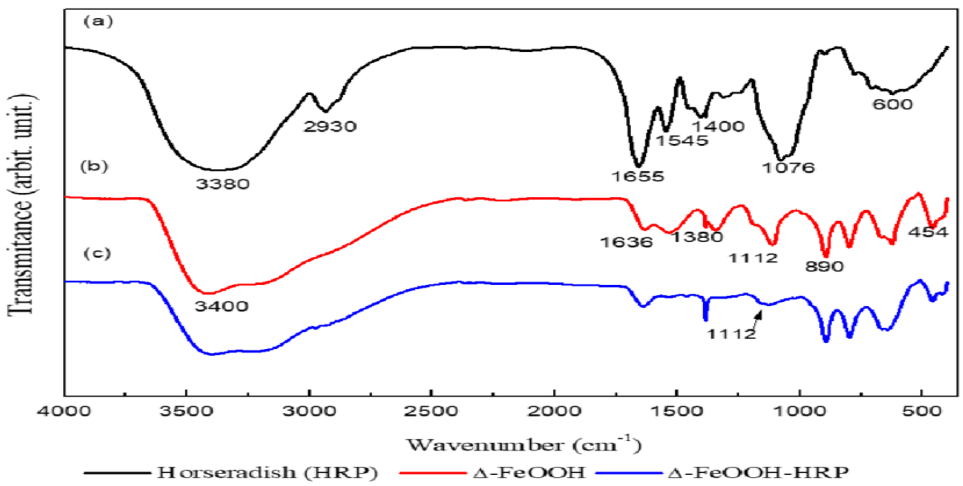


Fig: FTIR spectra of (a) horseradish peroxidase (HRP); (b) Δ-FeOOH particles; and (c) Δ-FeOOH-HRP.

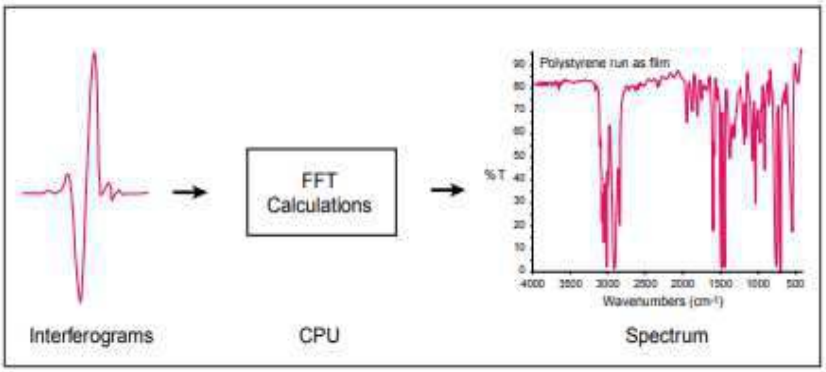
6.3. Principles of FTIR Spectroscopy:

Molecular bonds vibrate at various frequencies depending on the elements and the type of bonds. For any given bond, there are several specific frequencies at which it can vibrate. According to quantum mechanics, these frequencies correspond to the ground state (lowest frequency) and several excited states (higher frequencies). One way to cause the frequency of a molecular vibration to increase is to excite state, the Infrared light from the light source passes through a Michelson interferometer along the optical path.

The Michelson interferometer comprises a beam splitter, moving mirror, and fixed mirror. The light beam split into two by the beam. The two beams reflect off of their respective mirrors and are recombined when they meet back at the beamsplitter. Because the path that one beam travels is a fixed length and the other is constantly changing as its mirror moves, the signal which exits the interferometer is the result of these two beams "interfering" with each other. The resulting signal is called an interferogram which has the unique property that every data point (a function of the moving mirror position) which makes up the signal has information about every infrared frequency which comes from the source. This means that as the interferogram is measured; all frequencies are being measured simultaneously. Thus, the use of the interferometer results in extremely fast measurements.

A means of "decoding" the individual frequencies is required. This can be accomplished via a well-known mathematical technique called the Fourier

transformation. This transformation is performed by the computer which then presents the user with the desired spectral information for analysis



The Sample Analysis Process in FTIR

The FTIR instrument sends infrared radiation of about 10,000 to 100 cm-1 through a sample, with some radiation absorbed and some passed through. The absorbed radiation is converted into rotational and/or vibrational energy by the sample molecules. The resulting signal at the detector presents as a spectrum, typically from 4000 cm-1 to 400cm-1, representing a molecular fingerprint of the sample. The normal instrumental process is as follows:

The Source:

Infrared energy is emitted from a glowing black-body source. This beam passes through an aperture which controls the amount of energy presented to the sample (and, ultimately, to the detector).

The Interferometer:

The beam enters the interferometer where the "spectral encoding" takes place. The resulting interferogram signal then exits the interferometer.

The Sample:

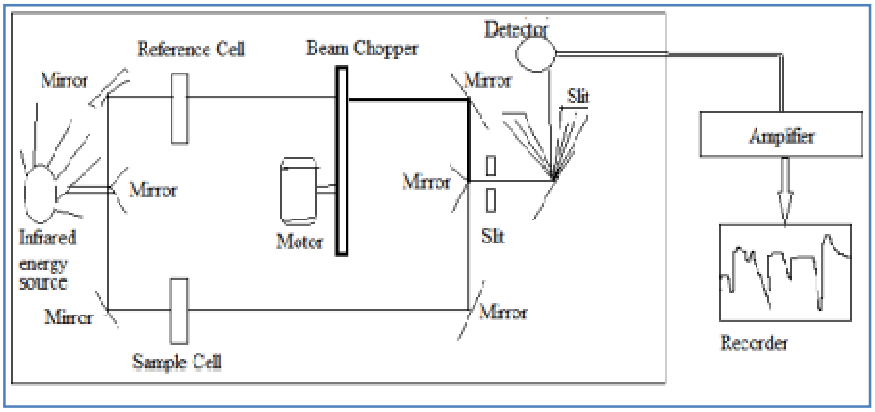
The beam enters the sample compartment where it is transmitted through or reflected off of the surface of the sample, depending on the type of analysis being accomplished. This is where specific frequencies of energy, which are uniquely characteristic of the sample, are absorbed.

The Detector:

The beam finally passes to the detector for final measurement. The detectors used are specially designed to measure the special interferogram signal.

The Computer:

The measured signal is digitized and sent to the computer where the Fourier transformation takes place. The final infrared spectrum is then presented to the user for interpretation and any further manipulation.



6.4. Application of FTIR

FTIR spectra reveal the composition of solids, liquids, and gases. The most common use is in the identification of unknown materials and confirmation of production materials (incoming or outgoing). The information content is very specific in most cases, permitting fine discrimination between like materials. The speed of FTIR analysis makes it particularly useful in screening applications, while the sensitivity empowers many advanced research applications.

Some of the more common applications are:

- Quality verification of incoming/outgoing materials
- Deformulation of polymers, rubbers, and other materials through thermogravimetric infra-red (TGA-IR) or gas chromatography infra-red (GC-IR) analysis
- Microanalysis of small sections of materials to identify contaminants
- Analysis of thin films and coatings
- Monitoring of automotive or smokestack emissions
- Failure analysis

In Environment

Infrared spectroscopy is a valuable technique for monitoring air quality, testing water quality, and analyzing soil to address environmental and health concerns caused by increasing pollution levels. The technique offers a “green” method of testing and fast, accurate results with the added benefit of saving money on the cost of consumables.

In Food Industry

Food manufacturers can use the infrared attenuated total reflectance (ATR) technique for rapid determination of the *trans* fat content of manufactured food products. This analysis is instrumental for compliance with food labeling requirements and to help promote healthy eating habits.

In Forensics

International drug enforcement agencies, police departments, and customs laboratories rely on spectroscopy to quickly identify illegal drugs, crime scene evidence, banned materials, and counterfeit goods. FTIR, FT-Raman, GC-IR, and IR microscopy techniques build a complete understanding of evidence samples and allow forensic scientists to confidently give expert testimony in court. These techniques can provide fast, easy and consistent analysis for:

- Seized drugs: controlled substances and cutting agents
- Clandestine labs: chemical evaluation
- Hit and run: paint and materials
- Textile identification: fibers, coatings, and residues

In Pharmaceuticals

Pharmaceutical laboratories face strong regulatory requirements and market pressures at every step along the product development pipeline. FTIR is an excellent technique for pharmaceutical analysis because it is easy to use, sensitive, fast, and helps ensure regulatory compliance through validation protocols. Applications include:

- Basic drug research and structural elucidation
- Formulation development and validation
- Quality control processes for incoming and outgoing materials
- Packaging testing

In Polymers & plastics

FTIR spectroscopy is used to quickly and definitively identify compounds such as compounded plastics, blends, fillers, paints, rubbers, coatings, resins, and adhesives. It can be applied across all phases of the product lifecycle including design, manufacture, and failure analysis. This makes it a useful tool for scientists and engineers involved in product development, quality control, and problem solving. Key areas where infrared analysis adds value include:

- Material identification and verification
- Copolymer and blend assessment
- Additive identification and quantification
- Contaminant identification—bulk and surface
- Molecular degradation assessment

In Quality Control

Infrared spectroscopy is an ideal analytical tool for both routine quality control (QC) analysis to verify if materials meet specification, and analytical investigations to identify the causes of failures when they occur. The utility of infrared for these purposes arises from the simplicity of sample analysis and data acquisition, coupled with the information-rich spectra that it provides

6.5. Nuclear magnetic resonance (NMR):

Nuclear magnetic resonance (NMR) spectroscopy uses radiofrequency radiation to induce transitions between different nuclear spin states of samples in a magnetic field. NMR spectroscopy can be used for quantitative measurements, but it is most useful in determining the structure of molecules (along with IR spectroscopy and mass spectrometry). The utility of NMR spectroscopy for structural characterization arises because different atoms in a molecule experience slightly different magnetic fields and therefore transitions at slightly different resonance frequencies in an NMR spectrum. Furthermore, splitting of the spectra lines arise due to interactions between different nuclei, which provides information about the proximity of different atoms in a molecule.

Most studies in organic chemistry involve the use of ^{1}H, but NMR spectroscopy with ^{13}C, 15N and ^{31}P isotopes is frequently used in biochemical studies. The resonance condition in NMR is satisfied in an external magnetic field of several hundred mT, with absorptions occurring in the region of radio waves (frequency 40 MHz) for resonance of the ^{1}H nucleus. However, in the NMR the magnet involved is not electron but nuclei of atom of element. Once the basic structure is known, NMR can be used to determine molecular conformation in solution in which the studying physical properties such as conformational exchange, phase changes, solubility, and diffusion are determined. The nuclei of molecules give rise to spectrum which absorbs electromagnetic radiation under magnetic condition. While the proton of nuclei is put under magnetic conditions. The proton present in nuclei shows spin due to absorption of radio wave and act like small magnet. Resonance in this small magnet show spin and process is called NMR, In other word, we can say that, the nuclear magnetic resonance is the phenomenon of nucleolus in which proton and neutron spin about the axis due to electromagnetic radiation.

6.6. NMR Spectrometer:

In NMR the samples in solution are contained in sealed tubes which are rotated rapidly in the cavity to eliminate irregularities and imperfections in sample distribution. In solid samples, the number of spin–spin interactions is greatly enhanced due to intermolecular interactions that are absent in dissolved samples due to translation and rotation movements. As a result, the resonance

signals broaden significantly. However, high-resolution spectra can be obtained by spinning the solid sample at an angle of 54.7°. Advanced computer facilities are needed for operation of NMR instruments, as well as analysis of the acquired spectra.

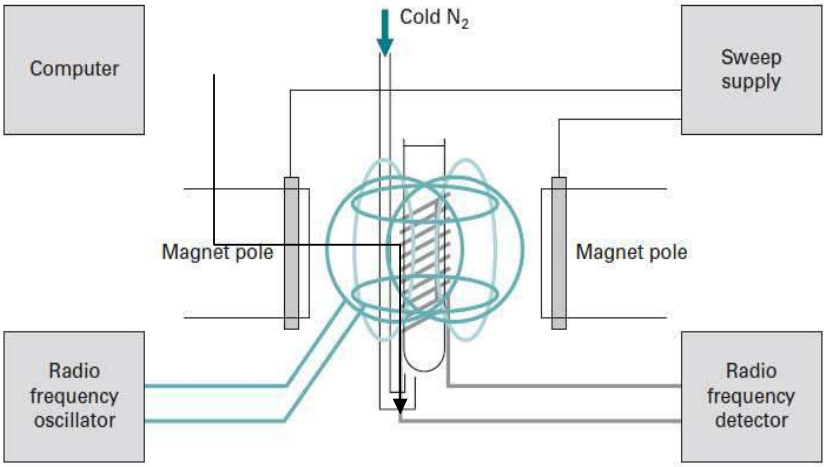


Fig. 6.4: Schematic diagram of an NMR spectrometer with cryoprobe.

6.7. PRINCIPAL OF NMR

The NMR phenomenon is based on the fact that nuclei of atoms have magnetic properties that can be utilized to yield chemical information. Quantum mechanically subatomic particles (protons, neutrons and electrons) have spin. In some atoms (e.g. 12 C, 16O, 32S) these spins are paired and cancel each other out so that the nucleus of the atom has no overall spin. However, in many atoms (1H,13C, 31P, 15N, 19F etc) the nucleus does possess an overall spin. The principle behind NMR is that many nuclei have charge "spin" when they have odd number of proton in their nuclei. When an external magnetic field applied and the nuclear spin occurs, the energy transfer is possible between the base energy to a higher energy level. 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 transfer is measured in several ways and processed in order to yield an NMR spectrum. The molecular environment of proton governs the value to applied external field at which the nucleus resonates. This gives recorded as chemical shift. The chemical field rises from the applied field including secondary field of about 0.15 – 0.2 mT at proton by interacting with adjacent bonding electron.

Nuclear magnetic moment $\mu = g_N u_N [I(I+1)]^{1/2}$

Where I is angular momentum

Also nuclear spin angular momentum $= [I(I+1)]^{1/2} \frac{h}{2\pi}$

$\Delta E = hv\, g u_N B_o$ (bore-Einstein)

g_N = Nuclear g factor, characteristic nucleus

μ_N = Nuclear magnetron = 5.05X 10^{-27} J T^{-1}

B_o = External magnetic field in tesla

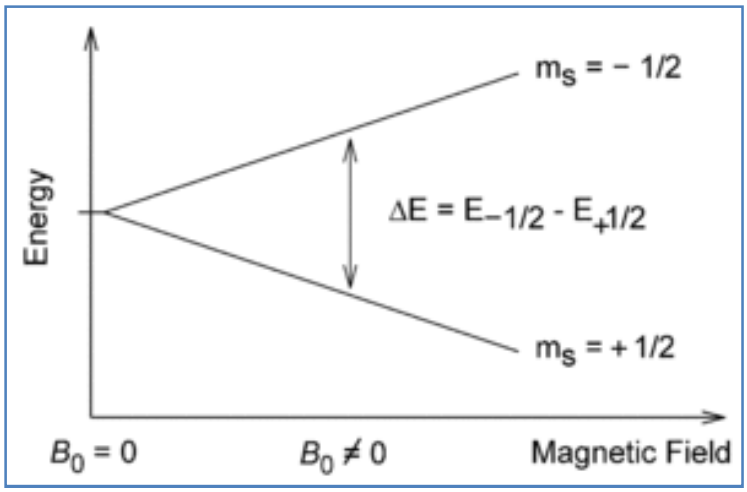


Fig. : Representing the phenomenon of NMR in ^{1}H molecule.

6.8. Types of NMR:

There are different types of NMR spectroscopy but here discussed about two of them.

^{13}C NMR

Carbon forms the backbone of the organic molecules therefore C NMR is also important and gives valuable information about the molecule. The ^{13}C NMR is generated in the same fundamental principle such as proton ^{H}NMR spectrum. Only 1.1 % of naturally occurring carbon is ^{13}C and actually has advantage of less coupling. In ^{13}C NMR spectrum is occurs directly due to present of carbon skeleton in the molecule and the proton present in molecules does not play direct role. The ^{12}C do not absorb radio frequency energy but the other isotope i.e. ^{13}C absorbs the energy but the ^{13}C has a natural abundance of only about 1%. Therefore the sensitivity of ^{13}C is less than the 1H NMR and requires longer time to record.

There is some point regarding ^{13}C NMR as follows:

- The number of signals tell us how many different carbons or set of equivalent carbons
- The splitting of a signal tells us how much hydrogen is attached to each carbon. (N+1 rule)

The chemical shift tells us the hybridization (sp^3, sp^2, sp) of each carbon.

1H NMR

The technique of ^{1}H NMR spectroscopy is central to organic chemistry and other fields involving analysis of organic chemicals, such as forensics and

environmental science. It is based on the same principle as magnetic resonance imaging (MRI). This laboratory exercise reviews the principles of interpreting ^{1}H NMR spectra that you should be learning right now in Chemistry 302. There are four questions you should ask when you are trying to interpret an NMR spectrum.

6.9. NMR phenomenon

Only those nuclei will show NMR phenomenon whose spin number I is greater than 0 (zero). In the nucleolus when the number of neutrons is equal to the number of proton; and neutron are paired the spin will be cancelled due to parallel and anti-parallel spin. Thus the net result of spin will be zero and such nuclei will not show NMR phenomenon. For example molecules of carbon and oxygen (C atomic number = 12, O atomic number = 16) have number of proton and neutron in their nucleus. Whereas as those molecules that have not paired proton and neutron or having unequal number of proton and neutron in their nucleus shows NMR phenomenon because such nuclei will have resultant spin (I) more than zero. Thus, resulting nuclei carry charge, then get spin and generate magnetic field. Thus we can say that nuclei act as a tiny bar magnet.

The magnitude and direction of the magnetic field generate by this nuclei is described by a vector called magnetic moment or magnetic dipole. The nuclei is placed in strong magnetic field and it spin shows two states.

α-spin state: In this state, nuclear spin aligned in the same direction as that of applied magnetic field. α-spin state is of lower energy.

β-spin state: In this state, nuclear spins aligned themselves in the opposite direction of applied magnetic field. β-spin state is of higher energy.

When the electromagnetic radiation of proper frequency is passed, a nucleus with α-spin state absorbs this radiation and converted into higher energy state that is β-spin state. This process is called flipping of nucleus. When the proton is place at magnetic field then it starts processing a certain frequency in the radio wave region and thus it will capable of taking up one of the two orientations with respect to the axis of the external field.

- Alignment with the field
- Alignment against the field

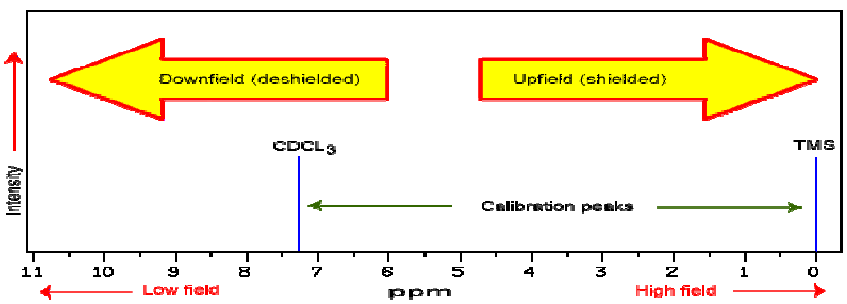
If the proton is processing in the aligned orientation. It can pass into the opposed orientation by absorbing higher energy. It comes back to lower energy aligned orientation by losing energy. The transition between two energy states is called flipping of proton. The transition between two energy states can be brought about by the absorption of electromagnetic radiation in the radio wave region and resulting single peaks is obtained.

6.10. Chemical shift

It indicates the types of protons in the molecule. This results from the fact that the magnetic field at the nucleus varies with the electronic (structural) environment for each type of proton. Different types of proton in the compound have different electronic environment and proton gets absorb at different applied magnetic field. The greater the electron density around a proton, the weaker the effective field it experiences (shielded) resulting in lower frequency transitions. Shielding can occur in cases where there is extra electron density around a carbon or proton, like in a carbanion. Decrease in the electron density will result in an increased effective magnetic field (deshielded) with these protons resonating at a higher frequency. This deshielding effect can be caused by specific functional groups that withdraw electron density from protons, such as the halogens, amines, alcohols, or those containing carbonyls. Chemical shift are measured in reference to a particular standard. Chemical shift of proton are measured with reference to a solvent tetra methyl silane. Chemical shift δ is usually expressed in parts per million (ppm) by frequency, because it is calculated from:

$$\delta = \frac{\nu_{sample} - \nu_{ref}}{\nu_{ref}}$$

Where ν_{sample} is the absolute resonance frequency of the sample and ν_{ref} is the absolute resonance frequency of a standard reference compound, measured in the same applied magnetic field B_0. Since the numerator is usually expressed in hertz, and the denominator in megahertz, δ is expressed in ppm. When molecule is placed in external magnetic field electron will produce secondary magnetic field.



	Down-field	**Up-field**
←	Deshielding	Shielding
←	Down-field	Up-field
←	High frequency	Low frequency
←	High chemical shift values	Low chemical shift values

Secondary magnetic field ⟶ opposed ⟶ shielding ⟶ up-field

Secondary magnetic field ⟶ reinforced ⟶ shielding ⟶ up-field

Up-field/down-field shift is known as chemical shift.

6.11. Applications of NMR

NMR spectroscopy is the use of NMR phenomena to study the physical, chemical, and biological properties of matter. Chemists use it to determine molecular identity and structure. In each carbon the multiplicity of the signal depends upon how many protons are attached to it. The ^{13}C-^{13}C coupling is not possible while ^{1}H-^{13}C coupling is possible.

Molecular structure determination

The NMR is very useful method in structural determination for organic compounds. When the proton or carbon exhibit in the similar chemical environment it gives similar signal. In this case the chemical shift provides a clue about the environment of a particular proton or carbon, and thus allows conclusions as to the nature of functional groups. Spin–spin interactions allow conclusions as to how protons are linked with the carbon skeleton. For structure determination, the fine structure usually is the most useful information because it provides a unique criterion while chemical shifts of some groups can vary over an extended range. The structures of proteins up to a mass of about 50 kDa can be determined with biomolecular NMR spectroscopy. The development of magnets with very high field strengths (currently 900 MHz) continues to push the size limit. Heteronuclear multidimensional NMR spectra need to be recorded for the assignment of all chemical shifts (1H, 13C, 15N). For inter proton NOEs, 13C- and 15N-edited 3D NOESY spectra are required.

Magnetic resonance imaging

The NMR spectroscopy is very useful in the imaging of live samples because the proton is one of the most sensitive nuclides and is present in all biological systems abundantly. The ^{1}H NMR spectroscopy has significance role in the imaging of live samples. The most important compound in biological samples in this context is water. It is distributed differently in different tissues, but constitutes about 55% of body mass in the average human. In NMR, the resonance frequency of a particular nuclide is proportional to the strength of the applied external magnetic field. If an external magnetic field gradient is applied then a range of resonant frequencies are observed, reflecting the spatial distribution of the spinning nuclei.

Magnetic resonance imaging (MRI) can be applied to large volumes in whole living organisms and has a central role in routine clinical imaging of large-volume soft tissues. The number of spins in a particular defined spatial region gives rise to the spin density as an observable parameter. This measure can be combined with analysis of the principal relaxation times (T_1 and T_2). The imaging of flux, as either bulk flow or localized diffusion, adds

considerably to the options available. In terms of whole-body scanners, the entire picture is reconstructed from images generated in contiguous slices. MRI can be applied to

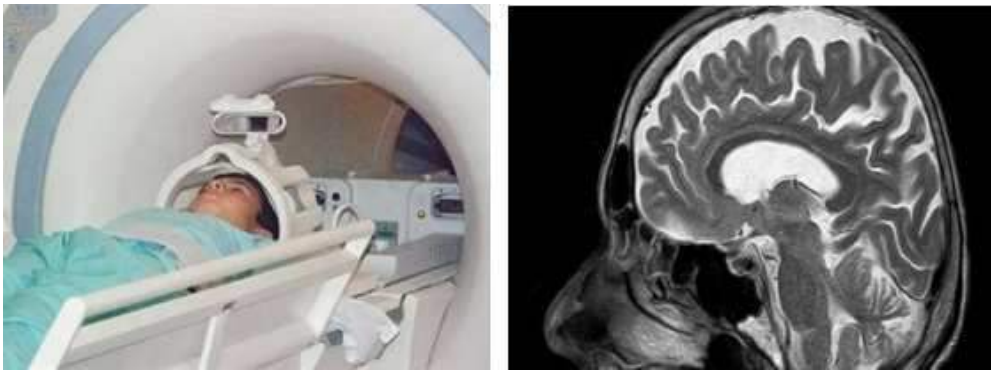


Fig. MRI Image of brain

The whole body or specific organ investigations of head, thorax, abdomen, liver, pancreas, kidney and musculoskeletal regions (**Fig.** 6.8). The use of contrast agents with paramagnetic properties has enabled investigation of organ function, as well as blood flow, tissue perfusion, transport across the blood– brain barrier and vascular anatomy. Resolution and image contrast are major considerations for the technique and subject to continuing development. The resolution depends on the strength of the magnetic field and the availability of labels that yield high signal strengths.

6.12.. Summary

In this unit you have learn that-

The FTIR and NMR would be leading to biochemistry regarding characterization and detection any biological compounds. FTIR spectrum is recorded between 4000 and 400 cm−1. It also used to obtain an infrared spectrum of absorption or emission of a solid, liquid or gas. However, the FTIR technique has been found important in the field of chemistry because of its easy way to indentify the presence of certain functional groups in a molecules and compounds. NMR is an abbreviation for Nuclear Magnetic Resonance. An NMR instrument allows the molecular structure of a material to be analyzed by observing and measuring the interaction of nuclear spins when placed in a powerful magnetic field. The body of organism contains groups of chemicals, and every chemical have different characteristic character and gives their performances accordingly. The chemical phenomenon creates abnormality in organism due to presence of various functional groups or different chemical structure. Thus, the instrumentation techniques are found very useful in understanding of chemical nature and structure of compounds.

6.13. Terminal questions

Q.6. What do you understand about FITR? Write the application of FTIR.

Answer:----------

Q.7. Write the phenomenon of NMR.

Answer:----------

Q.8. Discuss abut chemical shift.

Answer:----------

Q.9. Discuss about ^{1}HNMR .

Answer:----------

Q.10. Writ the application of NMR.

Answer:----------

Q.11. What is signal in NMR spectrum? Describe the principle of ^{13}C NMR.

Answer:----------

6.14. Further readings

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SBSBCH -01

Bio- Analytical Techniques

Block-3

Electrophoresis and Centrifugation and Microbial Techniques

SBSBCH -01

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Introduction

This is the third block on Electrophoresis and Centrifugation. It consists of the following three units:

Unit-7: This unit covers the general introduction of electrophoresis technique. The principle and application of electrophoresis discussed in this unit. Electrophoresis is used full techniques for the separation of biological molecules such as protein, amino acids and some other macromolecules form biological fluids. Separation of protein by PAGE and SD-PAGE is discussed briefly in this unit.

Unit-8: This unit covers the basic knowledge centrifugation technique. The role of centrifugation and its application has been briefly discussed here. The brief discussion of differential and density gradient centrifugation is carried out.

Unit-9: This unit covers the microbial techniques for isolation and culture of microbes. The antimicrobial activity by using DISC diffusion techniques are discussed in this unit. The use of different solvent system for amino acid, carbohydrate and lipid separation also discussed.

Unit-7: Electrophoresis

7.1. Introduction

The term electrophoresis describes the migration of a charged particle under the influence of an electric field. Under the influence of an electric field, these charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge. Electrophoresis is mostly known as electro - kinetic phenomena. The technique of electrophoresis was discovered by Reuss in 1809, when he experimented that soil particles dispersed in water migrate under effect of an applied electric field. There are several factors which determine mobility of particles such as particle size, shape and weight etc. it may be defined as the migration of colloidal particles through a solution under the influence of an electrical field. Electrophoresis is a very broadly used technique which, fundamentally, applies electric current to biological molecules, whether--they're usually DNA, they can be protein or RNA, too and separates these fragments into pieces which are larger or smaller. It's used in a variety of applications Electrophoresis units are available for running either vertical or horizontal gel systems. Vertical slab gel units are commercially available and routinely used to separate proteins in acryl amide gels. The gel is formed between two glass plates that are clamped together but held apart by plastic spacers. The most commonly used units are the so-called minigel apparatus.

Objectives

- To understand basic concept of electrophoresis
- To explain what determines charge or net charge on macromolecule such as protein

- To understand how the SDS-PAGE is useful for macromolecules separation
- To know separation of protein by electrophoresis

7.2. Electrophoresis

Electrophoresis is the separation techniques which work on the principle of migrartion of colloidal particles through a solution under the influence of electric field. However, the colloidal particles moves under the influence of electric field move with a speed dependent on their charge, shape, and size. Electrophoresis has been extensively developed for molecular separations. The electrophoresis is useful in the determination of the number, amount and mobility of components in a given sample, to obtain information about the electrical double layers surrounding the particles. It is also useful into determination of molecular weight of proteins and DNA sequencing. Electrophoresis units are available for running either vertical or horizontal gel systems. Vertical slab gel units are commercially available and routinely used to separate proteins in acrylamide gels. The gel is formed between two glass plates that are clamped together but held apart by plastic spacers. The most commonly used units are the so-called minigel apparatus.

In the electrophoresis techniques, when voltage is applied across the electrodes, it results in the generation of potential gradient (E) which is equal to applied voltage (V) divided by distance between the electrode (d). Thus,

$$E= V/d.$$

It is required to be noted that'd' will vary from equipment to equipment and thus for the same applied voltage, potential gradient will vary.

Electrophoresis mobility 'μ' of an ion is defined as ratio of velocity to potential gradient and is expressed as

$$\mu = v / E = q / f$$

where v is velocity, E is function, f is frictional coefficient and q is charge on molecule.

Molecules with identical or similar charges can be also separated if these molecules experiences different frictional force. Thus, separation of particles differing in charge as well as of similar charge is feasible in electrophoresis. In fact, separation of charged particles based on differences in frictional forces is routinely used in laboratories.

7.3. Principle of electrophoresis

Electrophoresis literally means running in the electric field. The charge molecule moves their counter charge electrode but electric field is removed before it reaches the electrode. Movement of charge species in an electric field

gives differential mobility to the sample based on the charge and consequently resolves them. Fundamentally, the technique is appropriate only to ionic or ionogenic materials, i.e., substances transformable to ionic species. Electrophoresis is recurrently used in forensic science, molecular biology and medicine. All types of electrophoresis are directed by the single set of general principles illustrated by equation

$$\text{Mobility of molecules} = \frac{(\text{Applied voltage})(\text{Net charge on molecule})}{\text{Friction of the molecule}}$$

If migration velocity is υ and E is applied electric field strength then electrophoresis mobility μ. M is positive or negative while neutral species have on mobility.

$$\mu = \frac{\upsilon}{E}$$

$$\upsilon = \frac{EQ}{\eta\pi}$$

where Q= charge of molecule; E = magnitude of applied potential, η = viscosity of medium; π = shape of molecule in terms of radius. M is +ve and, –ve while neutral species have no mobility. Different types of electrophoresis techniques are designated depending upon whether it is carried out in the presence or absence of a supporting media.

The rate at which migration takes place is dependent upon the strength of field, size and shape of molecules, net charge, ionic strength, viscosity and temperature of medium in which molecules are moving. The charged molecules under the stimulus of electric field travel in the direction of oppositely charged electrodes. Those molecules which are positively charged moves towards the cathode and negatively charged molecules moves towards the anode. The charge on the molecules and potential applied through the electrodes is responsible for this movement.

7.4. Classification of Electrophoretic

Electrophoresis is commonly categorised on the basis of the existence or absence of a solid supporting medium or matrix by means of which the charged molecules travel in the electrophoretic system. Generally, Electrophoresis is classified on the basis of two criteria:

1. Initial component distribution
2. Boundary permeability

Electrophoretic techniques can be classified into four main types:

1. **Zone electrophoresis (ZE):** This technique is used for analyzing proteins, nucleic acids, and biopolymers. In zone electrophoresis, different species in a sample are transported in a continuous electrolyte buffer system, subject to a potential gradient. Due to differences in the motilities, the species in

the samples will eventually separate into different, well-resolved peaks. Zone electrophoresis (ZE), is the most widely used mode and simplest form of capillary electrophoresis. The major advantage of presence of supporting media is that it minimizes mixing of the sample and immobilization of the molecule after electrophoresis

2. **Moving-boundary electrophoresis (MBE):** This electrophoresis is carried out in solution, without a supporting media. The sample is dissolved in buffer and molecules move to their respective counter charge electrodes. Moving boundary electrophoresis is carried out in a U shape tube with platinum electrodes attached to the end of both arms. Charged molecule moves to the opposite electrode as they passes through the refracto meter, and change can be measured.
3. **Isotachophoresis (ITP):** Isotachophoresis is a technique based on the principles of moving boundary electrophoresis. Two buffer systems are used: a leading electrolyte and a trailing electrolyte. This method can only be used for either anions or cations, not both at the same time. Any charged substance can be separated by isotachophoresis.
4. **Isoelectric focusing (IEF):** Isoelectric focusing is an electrophoretic method in which proteins are separated on bans of Pls. It makes use of the property of proteins that their net charge is determined by the pH of their local environments. Proteins carry positive, negative or zero net electrical charge, depending on the pH of their surroundings. Proteins are positively charged in solution at pH values below their pl and negatively charge above their isoelectric points thus at pH values below the pl of particular protein it will migrate toward the anode. Protein at its isoelectric point will not move in an electric field.

7.5. Types of Electrophoresis

1. **Affinity electrophoresis:**
   This technique is used for assessment of binding constants, as for example in lectin affinity electrophoresis or categorization of molecules with particular characteristics like glycan content or ligand binding. Affinity electrophoresis may be used as an alternative quantification of the protein.
2. **Capillary electrophoresis**: This techniques is use to isolate species on the basis of their size to charge ratio inside the small capillary filled with an electrolyte
3. **Immunoelectrophoresis**: This technique is used for the isolation and characterization of proteins on the basis of electrophoresis and reaction with antibodies.
4. **Pulsed field gel electrophoresis (PFGE):** This technique is used for the separation of very large DNA pieces using PFGE by application of an electric field to a gel matrix that intermittently changes its course.

5. **Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE):** This techniques is used for protein separation.
6. **Native Gels**: It is also possible to run protein gels without the SDS. These are known as Native Gels in that one does not deliberately denature the protein. Here, the native charge on the protein, divided by its mass, governs the movement of protein in terms of speed and direction.
7. **Electrofocusing Gel:** Electro focusing gel provides rapid analytical fractionation of amphoteric compounds, such as proteins, on the basis of their isoelectric points. The high resolving power of density gradient electro focusing is combined with the convenience and flexibility of gel electrophoresis.
8. **DNA Agarose Gels:** It is a simple method of separating fairly large fragments of DNA from one another by size is to use an Agarose gel. DNA does not need a detergent, since it already has a large number of negative phosphate groups evenly spaced.

7.6. Factor effecting electrophoresis

There are number of factor effecting the electrophoresis such as

- Strength of electric field
- Charge of sample used
- Size of biomolecule
- Binding strength of biomolecule
- Hydrophobicity of molecule taken
- Shape of biomolecule
- Ionic strength of buffer
- pH of solution
- Solution viscosity
- Temperature

7.7. Applications of Electrophoresis

- It is a tool of macromolecular separation
- Many biological complex samples can be separated by using various methods of electrophoresis
- In some cases, identification of molecules is also possible
- Gel method is more commonly used for routine laboratory experiments as well as research oriented separations and identification
- Electrophoresis is a handy tool for biologist and biochemist like the use for chromatographic techniques by organic chemist.
- Many handy instruments are available for conducting such separation experiments

7.8. Separation of protein by electrophoresis

Proteins are separated by the electrophoresis due to their shape, size and charge in nature. During electrophoresis the charged protein molecules are transported through a solvent by an electrical field. Protein separation by the electrophoresisS because it is a simple, rapid, and sensitive analytical tool. Most biological molecules carry a net charge at any pH other than their isoelectric point and will migrate at a rate proportional to their charge density. The mobility of a molecule through an electric field will depend on the following factors: field strength, net charge on the molecule, size and shape of the molecule, ionic strength, and properties of the matrix through which the molecule migrates (e.g. viscosity, pore size etc). Polyacrylamide and agarose are two supporting matrices commonly used in electrophoresis. There are some electrophoresis techniques are the discussed for the protein separation such as

7.8.1. By Polyacrylamide gel electrophoresis (PAGE):

It is an analytical method used to separate components of a protein mixture based on their size. The technique is based upon the principle that a charged molecule will migrate in an electric field towards an electrode with opposite sign. The general electrophoresis techniques cannot be used to determine the molecular weight of biological molecules because the mobility of a substance in the gel depends on both charge and size.

Polyacrylamide gel electrophoreses is carried out by using, Acrylamide solution, Isopropanol/distilled water, Running buffer, Staining, distaining solution, protein samples and molecular weight markers etc. There are following steps involved in the process of Polyacrylamide gel electrophoresis.

Sample preparation

Material may be any protein or nucleic acids. The sample to be analyzed is optically mixed with chemical denaturant in which mainly organic detergent like SDS used for protein. Here the sample is heated about 60 °C for farther promoted for denaturation. After that the tracking dyes is added to the solution for creating higher mobility.

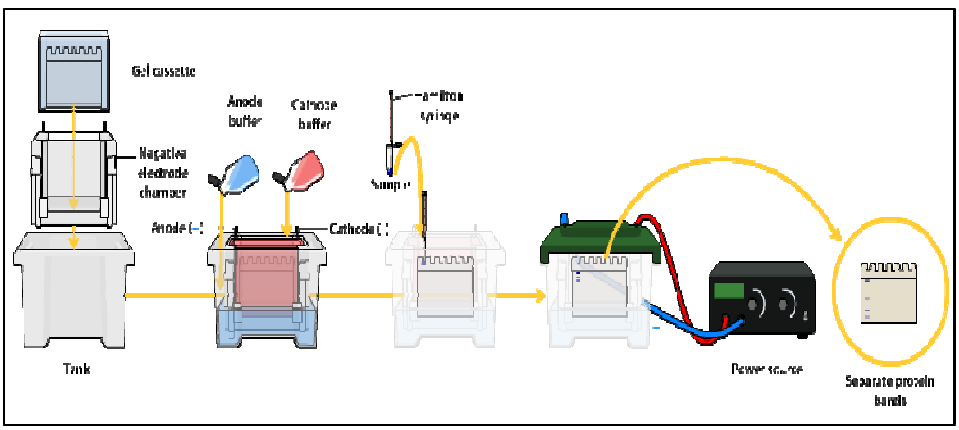
Preparation of Polyacrylamide gel

Cross-linked polyacrylamide gels are formed from the polymerization of acrylamide monomer in the presence of smaller amounts of N, N0 -methylene-bisacrylamide (normally referred to as ‘bis’-acrylamide).Acrylamide monomer is polymerized in a head-to-tail fashion into long chains and occasionally a bis-acrylamide molecule is built into the growing chain, thus introducing a second site for chain extension. The ratio of bisacrylamide to acrylamide can be varied for special purposes, but is generally about 1 part in 35. The acrylamide concentration of the gel can also be varied, generally in the range from 5% to 25%. Gels are usually polymerized between two glass plates in a gel caster,

with a comb inserted at the top to create the sample wells. After the gel is polymerized the comb can be removed and the gel is ready for electrophoresis.

Electrophoresis

An electric field is applied across the gel, causing the negatively charged proteins or nucleic acids to migrate across the gel away from the negative and towards the positive electrode (the anode). Depending on their size, each biomolecule moves differently through the gel matrix: small molecules more easily fit through the pores in the gel, while larger ones have more difficulty. Smaller biomolecule travel farther down the gel, while larger ones remain closer to the point of origin



Detection

The protein is detected after staining where different species biomolecule appear as distinct bands within the gel. It is common to run molecular weight size markers of known molecular weight to be separated. In this process also determine the approximate molecular mass of unknown biomolecule by comparing the distance traveled relative to the marker.

7.8.2. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE):

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used method for analyzing protein mixtures qualitatively. It is particularly useful for monitoring protein purification. The SDS-PAGE method is based on the separation of proteins according to their size; it can also be used to determine the relative molecular mass of proteins. When proteins are separated by electrophoresis through a gel matrix, smaller proteins migrate faster due to less resistance from the gel matrix. Other influences on the rate of migration through the gel matrix include the structure and charge of the proteins. In SDS-PAGE, the use of sodium dodecyl sulfate (SDS, also known as sodium lauryl sulfate) and polyacrylamide gel largely eliminates the influence of the structure and charge. SDS is an anionic detergent. Samples to be run on SDS–PAGE are firstly boiled for 5 min in sample buffer containing

b-mercaptoethanol and SDS. Each protein in the mixture is therefore fully denatured by this treatment and opens up into a rod-shaped structure with a series of negatively charged SDS molecules along the polypeptide chain. On average, one SDS molecule binds for every two amino acid residues. The original native charge on the molecule is therefore completely swamped by the negatively charged SDS molecules.

By heating the protein sample between 70-100°C in the presence of excess SDS and thiol reagent, disulfide bonds are cleaved, and the protein is fully dissociated into its subunits. In the presence of SDS and a reducing agent that cleaves disulfide bonds critical for proper folding, proteins unfold into linear chains with negative charge proportional to the polypeptide chain length. Polymerized acrylamide (polyacrylamide) forms a mesh-like matrix suitable for the separation of proteins of typical size. Protein is separated based on their polypeptide chain length by electrophoresis in a polyacrylamide gel with an appropriate mesh size.

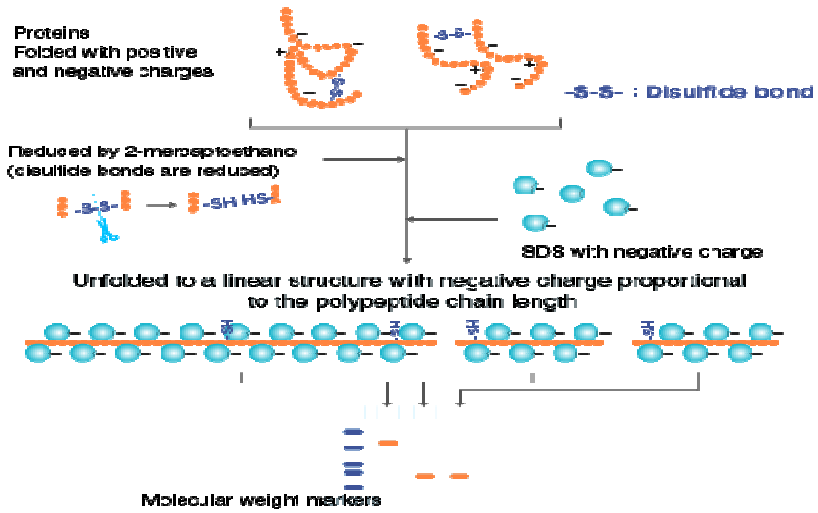


Fig.4.3: Process of SDS-PAGE

Source: https://ruo.mbl.co.jp/bio/e/support/method/sds-page.html

The negatively charged protein–SDS complexes move towards the anode because they have the same charge per unit length, they travel into the separating gel under the applied electric field with the same mobility. However, as they pass through the separating gel the proteins separate, owing to the molecular sieving properties of the gel.

7.9. Summary:

The transport of particles through a solvent by application of an electric field is called as electrophoresis. Electrophoresis is useful in identification and structure determination of big molecules. It is a physical method of analysis which involves separation of the compounds that are capable of acquiring electric change in conducting electrodes. Electrophoresis may be defined as the

migration of the charged particle through a solution under the influence of an external electrical field. The electrophoresis equipment has basically two units such as power pack and Electrophoretic unit. There are several macromolecules such as amino acids, peptides, proteins, and nucleotides etc have ionizable group therefore, at given pH they can be migrated toward different charge under the influence of electric field. Moving boundary electrophoretic is carried out in a U shape tube with platinum electrode attached to the end of both arms. SDS-PAGE is a technique widely used in biochemistry, forensics, genetics, molecular biology and biotechnology to separate biological macromolecules. Rate of movement of macromolecules in an electric field is useful parameter to know any change in amino acid regarding its charge. Electrophoresis is similar to chromatography. As movement of ions or their mobility depends upon the frictional coefficient, which in turn depends on the function of some of the physical properties of the molecules such as weight, molecular shape, size etc.

7.10. Terminal questions

Q.1. What is electrophoresis? Discuss the principle of electrophoresis.

Answer: ----------

Q.2. Write the about moving boundary electrophoresis.

Answer: ----------

Q.3. What is PAGE electrophoresis?

Answer: ----------

Q.4. Define the SDS-Polyacrylamide gel electrophoresis (SDS-PAGE).

Answer: ----------

Q.5. Define the role of electrophoresis in protein separation.

Answer: ----------

Q.6. Write the application of electrophoresis.

Answer: ----------

7.11. Further readings

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Unit-8: Centrifugation

8.1. Introduction

Centrifugation is the techniques in which separate or concentrate materials suspended in a liquid medium. Centrifugation is a separation technique based on the properties of the particle in an applied centrifugal field. Particles which differ in size, shape and density sediment at different rates in a medium held in a tube. The rate of sedimentation depends upon the applied centrifugal field, density and radius of the particle as well as the density and viscosity of the suspending medium. Centrifugation is a process which involves the use of the centrifugal force for the sedimentation of heterogeneous mixtures with a centrifuge, used in industry and in laboratory settings. More-dense components of the mixture migrate away from the axis of the centrifuge, while less- dense components of the mixture migrate towards the axis. In a solution, particles whose density is higher than that of the solvent sink (sediment), and particles that are lighter than it float to the top. A centrifuge is a device for separating particles from a solution according to their size, shape, density, viscosity of the medium and rotor speed.

Objectives:

- To define the centrifugation process
- To understand types and principle of centrifugation
- To discuss the use of centrifugation in process of separation of biomolecules
- To discuss the differential and density grade centrifugation

8.2. What is Centrifugation

Centrifugation is the techniques in which the biological sample is separated from their liquid media by centrifugal forces. The centrifugation is key technique for isolating and analysing cells, subcellular fractions, supramolecular complexes and isolated macromolecules such as proteins or nucleic acids. Today, centrifugation technique represents a critical tool for modern biochemistry and are employed in almost all invasive subcellular studies. While analytical centrifugation is mainly concerned with the study of purified macromolecules or isolated supramolecular assemblies, preparative centrifugation methodology is devoted to the actual separation of tissues, cells, subcellular structures, membrane vesicles and other particles of biochemical interest. However, the centrifugation is based on the density gradient of molecules. The sedimentation rate of particles can be increasing by using centrifugal forces. The rapid spinning imposes high centrifugal forces on suspended particles or even molecules in solution through ultracentrifugation that cause separation of particles on the basis of difference in weight. The centrifugation technique is the not only extremely powerful tools for isolation of biomolecules but also it can be utilised to separate intact organelles and membrane vesicles by centrifugation.

8.3. Basic principle of Centrifugation

The centrifugation process depends on the sedimentation of biological samples, in which the sedimentation occurs due to the influence of earth gravitational forces. Apparently we can say that the rate of sedimentation is increases when centrifugal field is increased. However, the centrifuge separates the particles from solution according to their size, shape, density, velocity of medium and rotor speed. Thus, the biological structures exhibit a drastic increase in sedimentation when they undergo acceleration in a centrifugal field. The relative centrifugal field is usually expressed as a multiple of the acceleration due to gravity.

The centrifugation involves principle of sedimentation, where the acceleration at centrifugal force causes denser substance to separate out along the radial direction at the bottom of tube. However in centrifugation protocol there is some other relevant points should keep in mind such as

- the more dense a biological structure is, the faster it sediments in a centrifugal field;
- the more massive a biological particle is, the faster it moves in a centrifugal field;
- the greater the frictional coefficient is, the slower a particle will move;
- the greater the centrifugal force is, the faster the particle sediments;
- the sedimentation rate of a given particle will be zero when the density of the particle and the surrounding medium are equal.

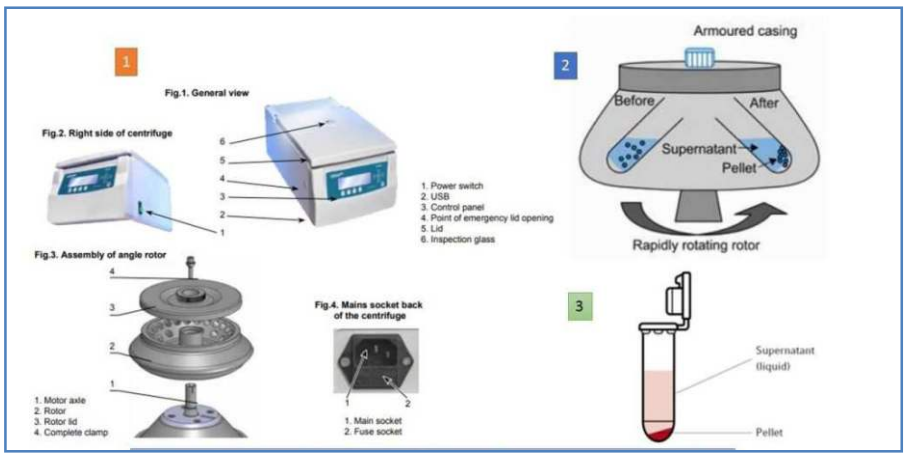


Fig.8.1: Centrifugation procedure and components of separation

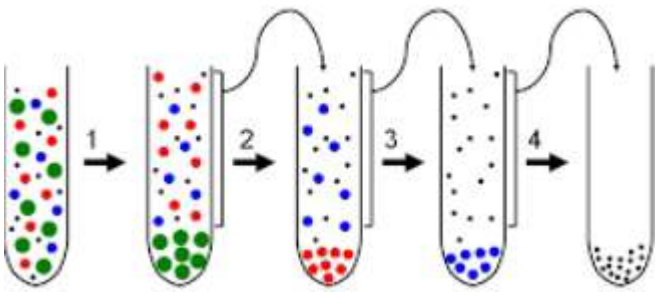
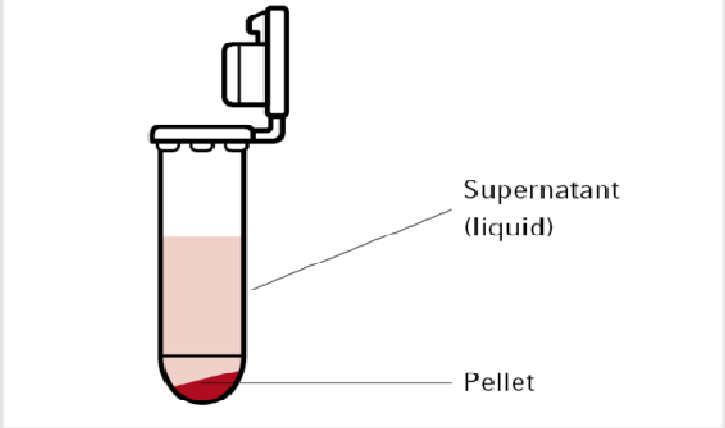


Fig.8.2: Deferent steps of centrifugation



Precipitate (pellet) stands for the concentrated particles in a tube after successful centrifugation.

Supernatant is the remaining solution above the pellet.

If we consider a body of mass m rotating in a circular path of radius r at a velocity υ. The force acting on the body in a radial direction is given by

$$F = \frac{m\upsilon^2}{r}$$

where

F = centrifugal force

m = mass of body

υ = velocity of the body

r = radius of circle of rotation

The gravitational force acting upon the body; G=mg, where G= gravitational force, g = acceleration due to gravity.

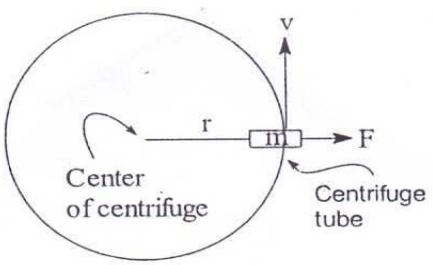


Fig. 3.2 : Illustration of the principle of centrifugation

However, the rate of sedimentation is dependent upon the applied centrifugal field (cm s^{-2}), G, that is determined by the radial distance, r, of the

particle from the axis of rotation (in cm) and the square of the angular velocity, ω of the rotor.

$$G = \omega^2 r$$

RCF (relative centrifugal force), which is the ratio of the centrifugal acceleration at a specified radius and the speed to the standard acceleration of gravity. RCF is measured in force x gravity or g-force. RCF is the dependent on the speed of rotion in rpm and the distance of the particles from the centre of rotation. The formula to calculate the relative centrifugal force (RCF) can be written as:

$$\textbf{RCF (g Force)} = 1.118 \times 10^{-5} \times r \times (RPM)^2$$

where **r** is the radius of the rotor (in centimeters), and **RPM** is the speed of the rotor in rotation per minute. RCF units are therefore dimensionless (denoting multiples of g) and revolutions per minute are usually abbreviated as r.p.m.: RCF $=1.12X10^{-5}$ r.p.m.

- Relative centrifugal force is the measure of the strength of rotors of different types and sizes.
- This is the force exerted on the contents of the rotor as a result of the rotation.
- RCF is the perpendicular force acting on the sample that is always relative to the gravity of the earth.
- The RCF of the different centrifuge can be used for the comparison of rotors, allowing the selection of the best centrifuge for a particular function.

The sedimentation rate or velocity of a biological particle can also be expressed as its sedimentation coefficient (s)

$$S = \frac{v}{\omega^2 r}$$

Where, s is sedimentation coefficient, v is velocity, ω is the angular velocity of rotor and r is radial distance.

Since the sedimentation rate per unit centrifugal field can be determined at different temperatures and with various media. The sedimentation coefficients of biological macromolecules are relatively small, and are usually expressed as Svedberg units, S. One Svedberg unit equals 10–13 s.

8.4. Types of Centrifuge

Centrifugation technique take a central position in modern biochemical, cellular and molecular biological studies. Depending on the particular application, centrifuges differ in their overall design and size. However, a common feature in all centrifuges is the central motor that spins a rotor containing the samples to be separated. Particles of biochemical interest are usually suspended in a liquid buffer system contained in specific tubes or separation chambers that are located in specialised rotors. The biological medium is chosen for the specific centrifugal application and may differ considerably between preparative and analytical approaches.

Many different types of centrifuges are commercially available including:

- large-capacity low-speed preparative centrifuges
- refrigerated high-speed preparative centrifuges
- analytical ultracentrifuges
- preparative ultracentrifuges
- large-scale clinical centrifuges
- small-scale laboratory microfuges

The low speed centrifuge has a maximum speed of 4000-5000 rpm. This intrument usually operate at the room tempreature with no means of temperature control. It is manily used in the sedimentation of red blood cells unit. In this techniques the particles are tightly packed into a pettet and the suspention is separated by decantation. The low speed centrifuge have following types of rotor such as fixed angle and swinging bucket.

High-speed refrigerated centrifuges are absolutely essential for the sedimentation of protein precipitates, large intact organelles, cellular debris derived from tissue homogenisation and microorganisms. They operate at maximum speed of 15,000- 20,000 rpm. Such centrifugal force is not sufficient to sediment smaller microsomal vesicles or ribosomes, but can be employed to differentially separate nuclei, mitochondria or chloroplasts. The high speed centrifugation may also be used in a continuous flow mode with zonal rotors for harvesting of yeast and bacterial cell from large volume of cultural media. In adition, the ultracentrifugation that is advanced centrifuagtion used would useful in biochemistry for the subcellular structures and isolated biomolecules. Another centrifuge such as Preparative ultracentrifugation can be operated at relative centrifugal fields of up to 900 000 g. is also used full in analysis of biomolecules.

8.5. Differential centrifugation

Differential centrifugation owes its origin to the variation in the effect of gravity on different particles or organelles that vary in size, shape and density. In this technique, controlling the gravity of a solution artificially allows the separation of an organelle from the homogeneous solution of particles. Differential centrifugation is simplest form of separation deferential palliating. It separates components of a cell on the basis of their densities and mass. The cell membrane is first ruptured to release the cell's components by using a homogenizer. The resulting mixture is referred to as the homogenate. Size of the particle is the main factor for separation by differential centrifugation. This technique is commonly used to fractionate sub cellular organelles and macromolecules. The homogenate is centrifuged to obtain a pellet containing the densest organelles. Particles larger in size experience more centrifugal force unlike smaller ones thus they sediment faster and thus settled accordingly. Compounds that are the densest will form a pellet at lower centrifuge speeds while the less dense compounds will likely remain in the

liquid supernatant above the pellet. Different pelleting is commonly used for harvesting or producing crude sub cellar fractions for tissue homogenizing, for example a rat liver hematogenate containing nuclei, mitochondria, lysosomes and membrane vesicles centrifuge at low speed for a short time will pellet mainly the larger and more dense nuclei. The final pellet is taken out for further study and supernatant may be discarded or utilized depending on the kind of work.

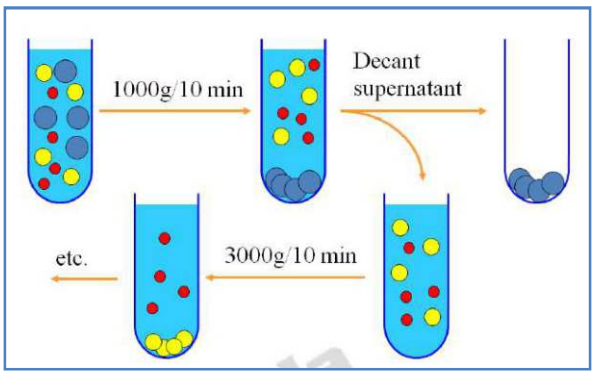


Fig.8.3: Steps of differential centrifugation

Differential centrifugation is based on the difference in the sedimentation rate of the particles of different size and density. It is used for the isolation of sub-cellular organelles. The centrifugal force is proportional to the radius of the centrifugal head and to the square of angular velocity. Therefore, it is possible to use small rotor heads at very high speeds. The tube containing the homogenate is held at an angle to the axis. This technique is commonly used for collecting cells or producing subcellular fractions from a tissue homogenate.

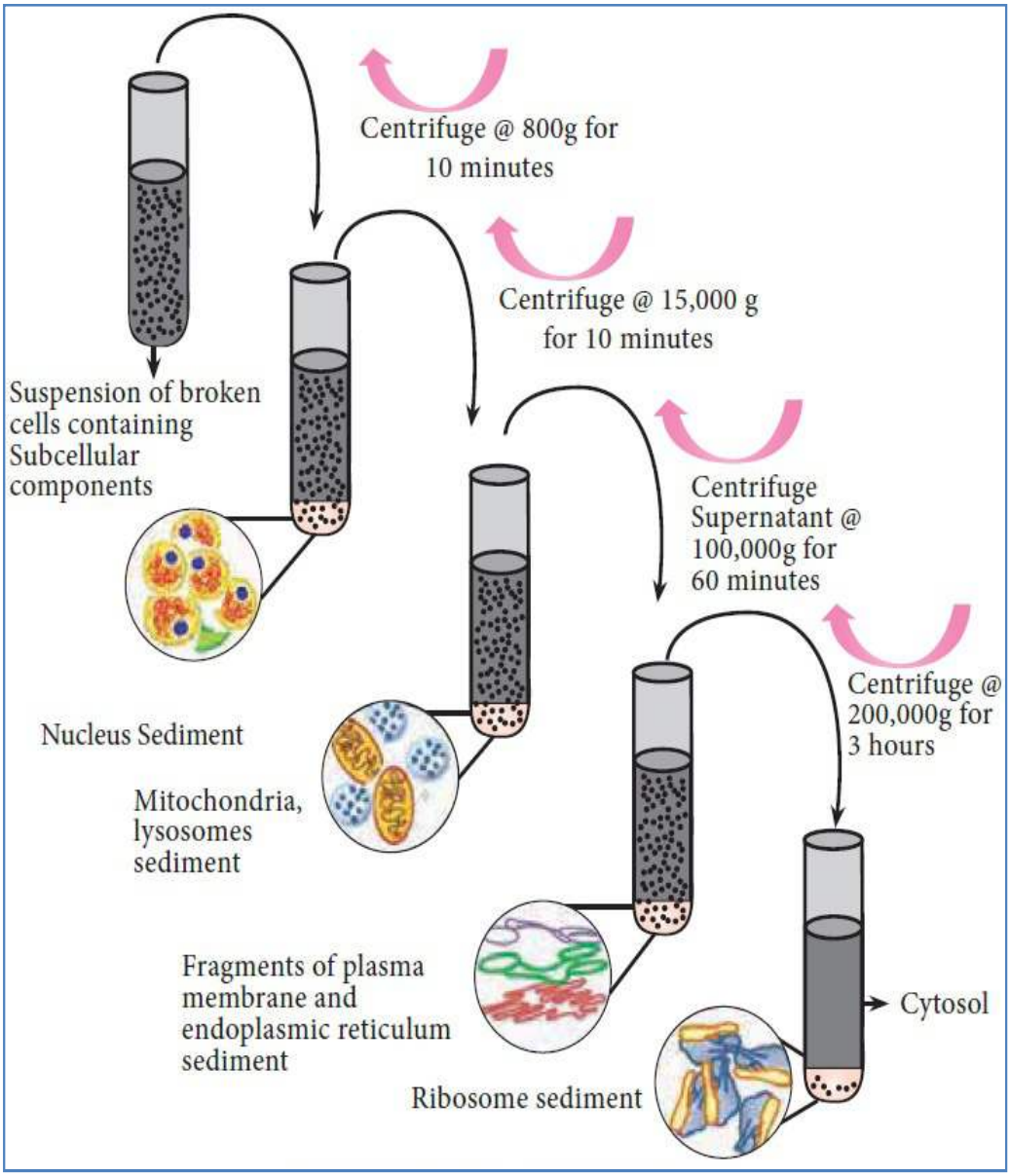


Fig.8.4: Differential centrifugation

The differential sedimentation of a particulate suspension in a centrifugal field is diagrammatically shown in Fig. 8.4. In this process the homogenate mixtures are evenly distributed throughout the centrifuge tube and then move down the tube at their respective sedimentation rate during centrifugation. The largest class of particles forms a pellet on the bottom of the centrifuge tube, leaving smaller-sized structures within the supernatant. However, during the initial centrifugation step smaller particles also become entrapped in the pellet causing a certain degree of contamination. At the end of each differential centrifugation step, the pellet and supernatant fraction are carefully separated from each other. Differential centrifugation is commonly used for the separation of cell organelles and membranes found in the cell. It

can also be used for low-resolution separation of the nucleus. As this technique separates particles based on their sizes, this can be used for the purification of extracts containing larger-sized impurities.

Principle of Differential centrifugation

- Differential centrifugation is based upon the differences in the sedimentation rate of biological particles of different size and density.
- As the increasing centrifugal force is applied, initial sedimentation of the larger molecules takes place.
- Further particles settle down depending upon the speed and time of individual centrifugation steps and the density and relative size of the particles.
- The largest class of particles forms a pellet on the bottom of the centrifuge tube, leaving smaller-sized structures within the supernatant.
- Thus, larger molecules sediment quickly and at lower centrifugal forces whereas the smaller molecules take longer time and higher forces.
- In the case of particles that are less dense than the medium, the particles will float instead of settling.

8.6. Density gradient centrifugation

It is the preferred method for purification of sub cellular organelles and macromolecules. To further separate biological particles of similar size but differing density, ultracentrifugation with preformed or self-establishing density gradients is the method of choice. The density gradient centrifugation is used to separate molecules depending on their size, shape and density. Macromolecules, cells and cell organelles are separated by the density gradient centrifugation. Density gradients is achieved by placing layer after layer of gradient media such as sucrose in a tube in a way that the heaviest layer at the bottom and the lightest at the top in either a continuous or discontinuous mode.

In Fig. 8.4b, the preparative ultracentrifugation of low- to high-density particles is shown. A mixture of particles, such as is present in a heterogeneous microsomal membrane preparation, is layered on top of a preformed liquid density gradient. Depending on the particular biological application, a great variety of gradient materials are available. Caesium chloride is widely used for the bonding of DNA and the isolation of plasmids, nucleoproteins and viruses. Sodium bromide and sodium iodide are employed for the fractionation of lipoproteins and the bonding of DNA or RNA molecules, respectively.

If one wants to separate all membrane species spanning the whole range of particle densities, the maximum density of the gradient must exceed the density of the most dense vesicle species. Both step gradient and continuous gradient systems are employed to achieve this. For isopycnic separation, centrifugation is continued until the desired particles have reached their

isopycnic position in the liquid density gradient. In contrast, during rate separation, the required fraction does not reach its isopycnic position during the centrifugation run.

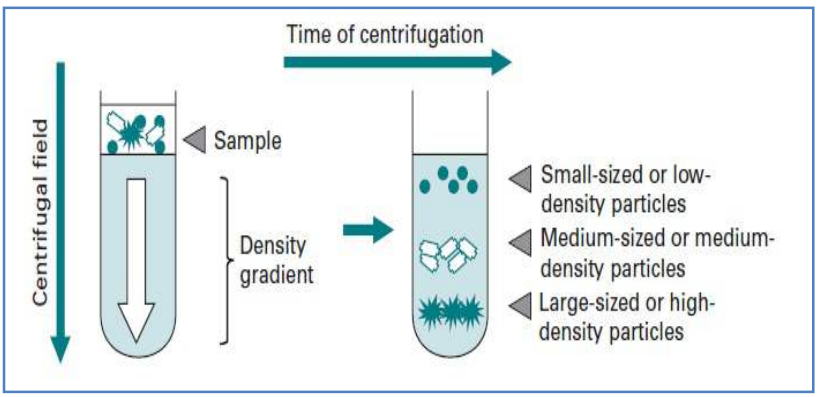


Fig 8.5: Density gradient centrifugation

Principle of Density gradient centrifugation

- Density gradient centrifugation is based on the principle that the molecules settle down under a centrifugal force until they reach to be density which is same as of that medium used.
- In this case, a medium with a density gradient is employed, which either has to decrease density or increasing density.
- Molecules in a sample move through the medium as the sample are rotated creating a centrifugal force.
- The more dense molecules begin to move towards the bottom as they move through the density gradient.
- The molecules then become suspended at a point in which the density of the particles equals the surrounding medium.
- In this way, molecules with different densities are separated at different layers which can then be recovered by various processes.

There are some examples of density gradient centrifugation such as;

- This method was used in the famous experiment, which proved that DNA is semi-conservative by using different isotopes of nitrogen.
- Another example is the use of this technique for the isolation of the microsomal fraction from muscle homogenates and subsequent separation of membrane vesicles with a differing density.

8.7. Application of centrifugation

- A centrifuge is used to separate two miscible substances:
  - ❖ Cells
  - ❖ Sub-cellular components
  - ❖ Proteins -Nucleic acids
- Centrifugation is basis of the size, shape and density of particles

- It utilizes density difference between the particles/macromolecules and the medium in which these are dispersed
- To analyze the hydrodynamic properties of macromolecules and purification of mammalian cells
- In separation of urine components and blood components in forensic and research laboratories.
- It is used to separate fraction of sub-cellular organelles and fractionation of membrane vesicles
- A tube of anti-coagulated whole blood left standing on a bench top will eventually separate into plasma, red blood cell and white blood cell fractions.
- It utilizes density difference between the particles/macromolecules and the medium in which these are dispersed. Dispersed systems are subjected to artificially induced gravitational fields
- Density gradient ultracentrifugation and affinity is used for the isolation of the microsomal fraction from muscle homogenates and subsequent separation of membrane vesicles with a differing density.
- Analytical ultracentrifugation for the determination of the relative molecular mass of a macromolecule can be performed by a sedimentation velocity approach or sedimentation equilibrium methodology.

8.9. Summary

Biological centrifugation is a process that uses centrifugal force to separate and purify mixtures of biological particles in a liquid medium. It is a key technique for isolating and analysing cells, subcellular fractions, supramolecular complexes and isolated macromolecules such as proteins or nucleic acids. The Centrifugation techniques are critical tools for modern biochemistry. Centrifugal force is necessary to separate the most of particles. In addition, the potential degradation of biological compounds during prolonged storage means faster separation techniques are needed. The rate of separation is a suspension of particles by means of gravitational force; it mainly depends on the particles size and density. Differential centrifugation also known as differential sedimentation, is essentially a process of successive centrifugation (single or repeated steps) with increasing centrifugal force (g). Separation of particles through this technique primarily depends on their mass and size, where heavier particles or cells settle first at lower g values (e.g. intact cells can sediment at around 800 g).

Within these extremely high gravitational fields, the ultracentrifuge cell has to allow light passage through the biological particles for proper measurement of the concentration distribution. Analytical ultracentrifugation is most often employed in

• the determination of the purity of macromolecules;

• the determination of the relative molecular mass of solutes in their native state;
• the examination of changes in the molecular mass of supramolecular complexes;
• the detection of conformational changes

8.10. Terminal questions

Q.1: Define the centrifugation techniques and its role in separation of biological samples.

Answer: ----------

Q.2: Write down the basic principle of centrifugation.

Answer: ----------

Q.3: Discuss the types of centrifugation and its applications.

Answer: ----------

Q.4: What is deferential centrifugation? Discuss about it.

Answer: ----------

Q.5: What is analytical ultracentrifugation?

Answer: ----------

Q.6: What is Density gradient centrifugation? Write down its application in biochemistry?

Answer: ----------

8.11. Further readings

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Unit-9: Microbial techniques

9.1. Introduction

Microbes are minute organisms that are invisible to the naked eye. However, they could only be seen under a microscope. Thus they are called microorganisms or microscopic organisms. In nature, the microscopic world makes up almost 60% of the earth's living matter. They make major fraction of biomass on the planet. The term "microbes" is used to describe several different life forms with different size and characteristics. Few of these microbes are as follows like Bacteria, Fungi, Protists, Viruses and Archaea. However, the microbes may be us useful as well as harmful. Certain microbes cause severe infections and diseases, and can also spoil food and other materials. The antimicrobial activity is the process to killing or inhibits the harmful microbes that cause diseases. It is very complex process in which involves living organisms who's every step of life like sustenance, metabolism, respiration, and capacity of reproduction could be affected by the presence of toxic substances that are used as antibacterial agent. Many of the techniques use as separation techniques, in which the constituents of a mixture are separated according to differences in chemical or physical properties such as size, shape, mass, density, or chemical affinity between the constituents.

Objectives:

- To the isolation of bacterial and their microbial activity
- To the DISC diffusion techniques and their types and role
- To discuss about separation of molecules

9.2. Isolation of bacteria

Microbial techniques are those techniques which used for the study of microorganism. Mostly, the microbial techniques are used for conducting survey, culture; identify strain, engineering and manipulation. The development of microbial techniques is very important, because microbes play important role in our life. However, microorganisms are also play important role in agriculture, medicine and industry. The techniques makes easy to understand about the identification, culture, habitat and behavior of microbes. The microbiology and its basic techniques are also related to some other science like plant physiology. Thus basic techniques involve in microbes are very helpful to all in many ways. The basic techniques involve in microbial study are following.

9.2.1. Microbiological media

Generally microbes grow on the media which contain carbohydrate and nitrogen compound, because, in this type of media, carbon is responsible for respiration and nitrogen is for microbial growth. However, the most common media used for microbial growth are nutrient broth and nutrient agar. Nutrient broth is soup like liquid medium that is poured in tube.While nutrient agar is the nutrient broth that is set into jelly form by adding of sweated extracted called agar and pure into plate. Some mineral and vitamins are also requiring for microbial growth which depends on the requirement of bacteria. Some time, selective media are used which play an important role in suppression of bacterial growth. Lots of bacterial media used for culture of microbes are discussed in this chapter.

9.2.2. Sterilization

Sterilization is the process in which media are used for the growth of bacteria or other microbes should be sterilized by heating in an autoclave at 121 °C for about 15 min. However, the sterilization refers to the killing or elimination of all microorganisms, including highly resistant bacteria spores. Sterilization may also refers to removal of all form of life of microbes (bacteria, fungi, viruses, spore forms, etc.) present on a surface, in a fluid, drugs or biological culture media by applying heat, chemicals, irradiation, high pressure, and filtration or combinations. There are three methods of sterilization:

I. Physical Sterilization

For example:

a) by moist and dry heat (boiling at 100°C, steaming, steam sterilizer, red heat, flamming).

b) by filtration (Earthenware filters, Asbestos filters, Sintered glass filters, Membrane filters)

c) by radiation (ionizing and non-ionizing radiation)

II. Chemical Sterilization: Sterilization can also be done by the use of chemicals. Different types of chemical used are such as alcohol, aldehyde, Oxidizing agents, Phenolic compound, and Quaternary ammonium compounds etc.

III. Physiochemical Sterilization: A physiochemical method includes both physical and chemical method. Use of steam-formaldehyde is an example of physiochemical method of sterilization.

9.2.3. Inoculation

It the techniques by which microbes or bacteria introduce into the media. The most common techniques are by spreading on the surface of the agar or in the broth with the help of the heat sterilized loop.

9.2.4. Incubation

After inoculation, the agar or broth congaing media is kept into incubator at required temperature and period of time. Usually, the plate is kept inverted position for preventing the condensation droplet of water from falling on the surface of agar. Some time plate are sealed for prevention of contamination.

9.3. Antimicrobial activity

The antimicrobial activity is the process to killing or inhibits the harmful microbes that cause diseases. It is very complex process in which involves living organisms who's every step of life like sustenance, metabolism, respiration, and capacity of reproduction could be affected by the presence of toxic substances that are used as antibacterial agent. The anti microbial agents are those substance or compounds that used to kill or inhibit the microbial growth and development. Lots of agents are used for this purpose. Antimicrobial may be anti-bacterial, anti-fungal or antiviral. They all have different modes of action by which they act to suppress the infection. The antimicrobial activity is used in practice since log time for food safety and disease control activity. For examples determination of the antimicrobial effect of vapours of crushed garlic against *Mycobacterium* species, *Escherichia coli,* Serratia *marcescens* and *B. subtilus* was studied as early as 1936. Due to benefits of antimicrobial activity there are lots of interest has been developed

for the searching of new antimicrobial agent from various source to combat microbial resistance. The antimicrobial susceptibility also as used an antimicrobial activity for drugs discovery, epidemiology and prediction of therapeutic outcome. Hera lots of antimicrobial activity are used in practice such as *in vitro* investigation of extracts and pure drugs as potential antimicrobial agents. Apart from some other screening activity also involve in antimicrobial activity for a yielding of desire microbes for antibiotic compound extraction. Some most popular methods for antimicrobial activity such as disk-diffusion testing, agar dilution methods are commonly in practice. Other methods are used especially for antifungal testing, such as poisoned food technique.

Some nanoparticles are showing their antimicrobial activity reported by researchers. Metal oxides nanoparticles antibacterial activity has been reported on enormously. Magnetic nanoparticles found to be enhanced antibacterial activity of treated with *Argemone mexicana* L. leaf extract. These particles have been shown to exhibit enhanced antibacterial activity against *E. coli*, *B. subtilis*, and *P. mirabilis*. Al-Adham et al. Different types of metallic and metal oxide nanoparticles such as silver (Ag), silver oxide (AgO), titanium dioxide (TiO), zinc oxide (ZnO), gold (Au), calcium oxide (CaO), silica (Si), copper oxide (CuO), and magnesium oxide (MgO) have been found to show both antibacterial and antiviral activity. Similarly, fungal biofilm development was successfully prevented using biohybrid nanostructured iron oxide nanoparticles and *Satureja hortensis.* Antimicrobial activity of CNTs also depends on their dispersion ability. When CNTs are highly dispersed in the solution, interaction between CNTs and bacteria cells is also more likely to occur.

9.3.1. **Antimicrobial activity by using DISC diffusion techniques**

We know the antimicrobial activity is to be inhibiting the growth of bacteria and prevent the formation of all microbial colonies on that specific area. Some times it's consider the destroy the colonies of microorganism. Recently the antimicrobial activity is considered to be for the screening of desire microbial colonies for drugs and necessary production of antibacterial agents. There are several bioassays such as disk-diffusion, well diffusion and broth or agar dilution are well known and commonly used for the antimicrobial activity. However, the disk diffusion test that is also known as agar diffusion test/ disc-diffusion antibiotic susceptibility test/ disc-diffusion antibiotic sensitivity test etc is a culture-based microbiology assay used in diagnostic and drug discovery laboratories. Agar diffusion was first used by Martinus Beijerinck in 1889 to study the effect of auxins on bacterial growth. In drug discovery laboratories, the disk diffusion test is used to screen natural product extracts for antibacterial activity.

Disc diffusion is most common laboratory methods to determine susceptibility of bacteria isolates to antibiotics. In this method, discs impregnated with known concentrations of antibiotics are placed on agar plate that has been inoculated with a culture of the bacterium to be tested. The plate is incubated at 37°C for 18–24 hours. After diffusion, the concentration of antibiotic usually remains higher near the site of antibiotic disc, but decreases with distance. Susceptibility to the particular antibiotic is determined by measuring the zone of inhibition of bacterial growth around the disc. The bacteria are first isolated in pure culture on a solid medium The medium that supports both test and control strains is selected for carrying out antibiotic susceptibility testing of the bacteria. The pH of the medium is maintained at 7.2–7.4. The broth is inoculated on the medium by streaking with sterile swabs. Only the clinically relevant antibiotics are tested in antibiotic susceptibility tests. The reading is reported after incubating the plate for 18–24 hours at 37°C aerobically.

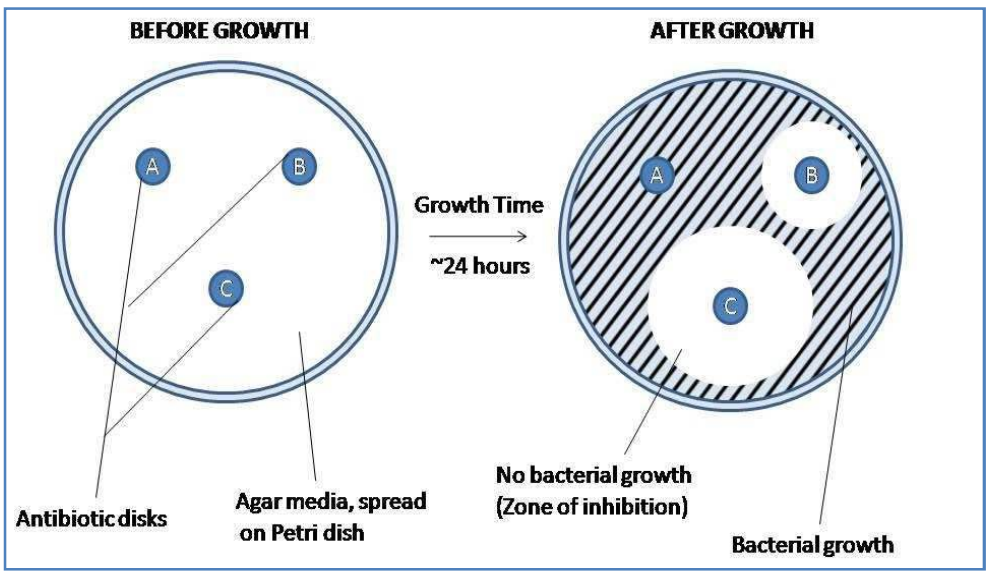

Fig. 9.1: DISC diffusion techniques

Disc diffusion method was used for antibacterial activity. A stock solution of extract was prepared by dissolving 0.1 g of extract with 100 mL of their respective solvents (distilled water and absolute ethanol) to produce a final concentration of 100 mg/mL. The stock solution was then diluted to concentrations of 2.5, 5, 10, 20, 50, and 100 mg/mL of extract. 20 µL of each dilution was impregnated into sterile, blank discs 6 mm in diameter. 5 µL of extract was spotted alternately on both sides of the discs and allowed to dry before the next 5 µL was spotted to ensure precise impregnation. Distilled water and ethanol-loaded discs were used as negative controls for aqueous and ethanol extracts, respectively. All discs were fully dried before the application on bacterial lawn. The positive controls used were vancomycin antibiotic discs for all S. aureus strains. Antibacterial activity was evaluated by measuring the

diameter of the inhibition zone (IZ) around the discs. The assay was repeated trice. Antibacterial activity was expressed as the mean zone of inhibition diameters (mm) produced by the leaf extract.

Types of disc diffusion tests

Disc diffusion tests are of the following types:

1. Kirby–Bauer disc diffusion method:

This test is found most common in antimicrobial activity that is used for determination of antibiotic sensitivity of bacteria isolated from clinical specimens. In this method, both the test strains and the control strains are tested in separate plates. This test is performed in suitable broth solution where it incubated at 37°C for 2–4 hours. The test is carried out in both test strain and the control strain in separated plates. However, the this test the susceptibility of drug is determined from the zones of inhibition of bacterial growth surrounding the antibiotic discs.

2. Stokes disc diffusion method:

In this method, the Petri dish containing the Mueller–Hinton agar is divided horizontally into three parts. The test strain is inoculated in the central area and the control strains on the upper and lower third of the plate. The plates are incubated at 37°C and observed for zones of bacterial inhibition around the discs.

Primary disc diffusion test:

Disc diffusion test for Antibiotic Sensitivity testing is carried out to determine the appropriate antibiotic agent to be used for a particular bacterial strain isolated from clinical specimens.

9.4. Separation of biomolecule

The diversity in biomolecule makes them difficult to separate in their pure form. In order to augment the biological activity of many important biomolecule it is very necessary to separate and purify them in their purest form. For the separation of biomolecule the number of techniques used in practice. Many of the techniques use as separation techniques, in which the constituents of a mixture are separated according to differences in chemical or physical properties such as size, shape, mass, density, or chemical affinity between the constituents. Three key analytical and purification methods are chromatography, electrophoresis, and ultracentrifugation. Each one relies on certain physicochemical properties of biomolecules. These techniques are

useful in the separation of four major types of biomolecules which are carbohydrates, lipids, nucleic acids.

Chromatography is one of the techniques used in the separation of biomolecule. The components are separated due to differential affinity for a mobile versus a stationary phase. When a mixture of proteins is introduced into the mobile phase and allowed to migrate through the column, separation occurs because proteins that have a greater attraction for the solid phase migrate more slowly than do proteins that are more attracted to the mobile phase.

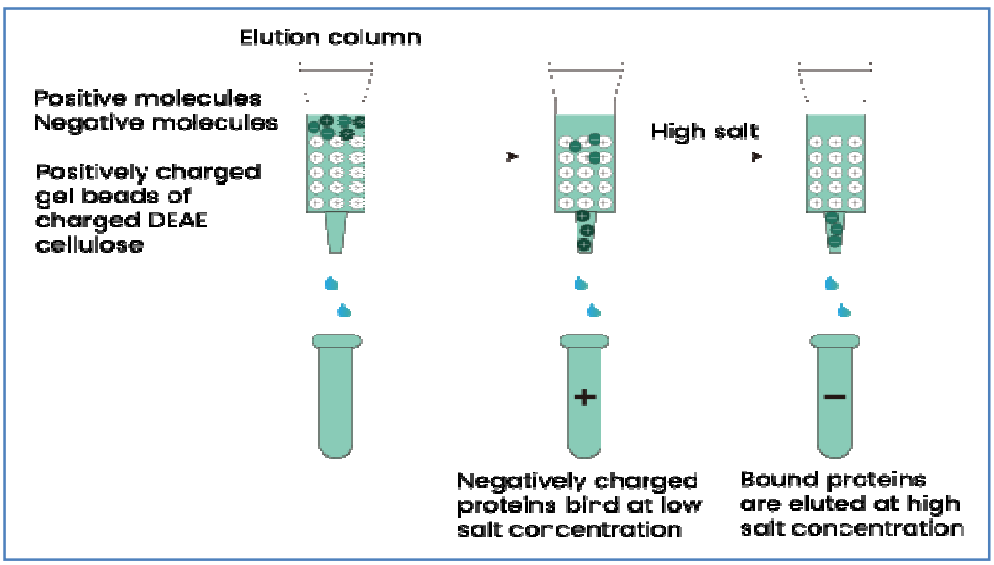

Fig.9.2: Protein purification by ion exchange chromatography

Source: Protein Purification by Ion Exchange Chromatography | Sino Biological

Ion Exchange Chromatography (IEX) Steps in Protein Purification

Equilibration: The first step is the equilibration of the stationary phase. When equilibrium is reached, all stationary phase charged groups are bound with exchangeable counterions, such as chloride or sodium. The pH and ionic strength of the start buffer are selected to ensure proteins of interest bind to the medium.

Sample Application and Wash: The goal in the second step is to bind the target molecule(s) and wash out all unbound material.

Elution: The proteins with the lowest net charge at the selected pH will be the first ones eluted from the column as ionic strength increases. Similarly, the proteins with the highest charge at a certain pH will be most strongly retained and will be eluted last.

Regeneration: A final wash with high ionic strength buffer regenerates the column and removes any molecules still bound. The column is then re-equilibrated in start buffer before starting the next run.

Electrophoresis in biology uses porous gels as the media. The sample mixture is loaded into a gel, the electric field is applied, and the molecules migrate through the gel matrix . Thus, separation is based on both the molecular sieve effect and on the electrophoretic mobility of the molecules. Electrophoresis in agarose or polyacrylamide gels is the most usual way to separate DNA molecules according to size. The technique can be used analytically or preparatively, and can be qualitative or quantitative. Large fragments of DNA such as chromosomes may also be separated by a modification of electrophoresis termed pulsed field gel electrophoresis (PFGE). Agarose gels can be used to separate molecules larger than about 100 bp. For higher resolution or for the effective separation of shorter DNA molecules polyacrylamide gels are the preferred method.

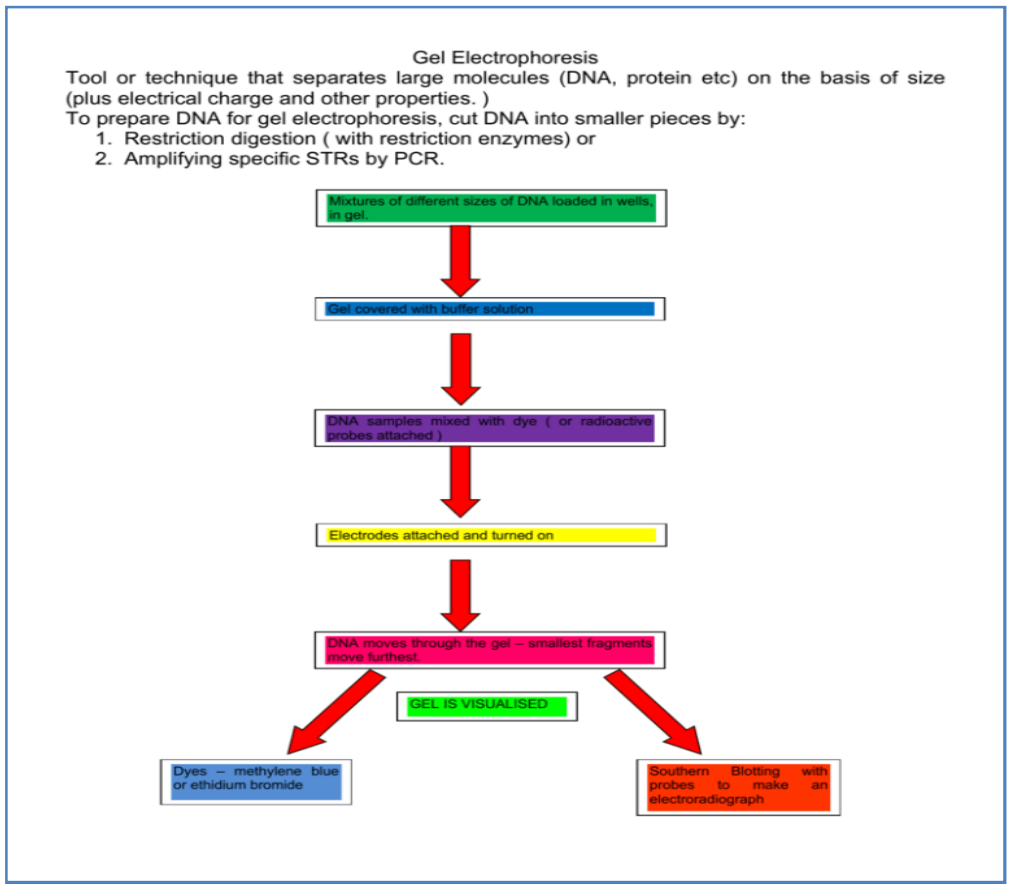

Fig.9.3: Separation of DNA by electrophoresis

Sources: Gel Electrophoresis flow chart (studylib.net)

Cells, organelles, or macromolecules in solution exposed to a centrifugal force will separate because they differ in mass, shape, or a combination of those factors. The instrument used for this process is a centrifuge. An

ultracentrifuge generates centrifugal forces of 600,000 g and more. It is an indispensable tool for the isolation of proteins, DNA, and subcellular particles.

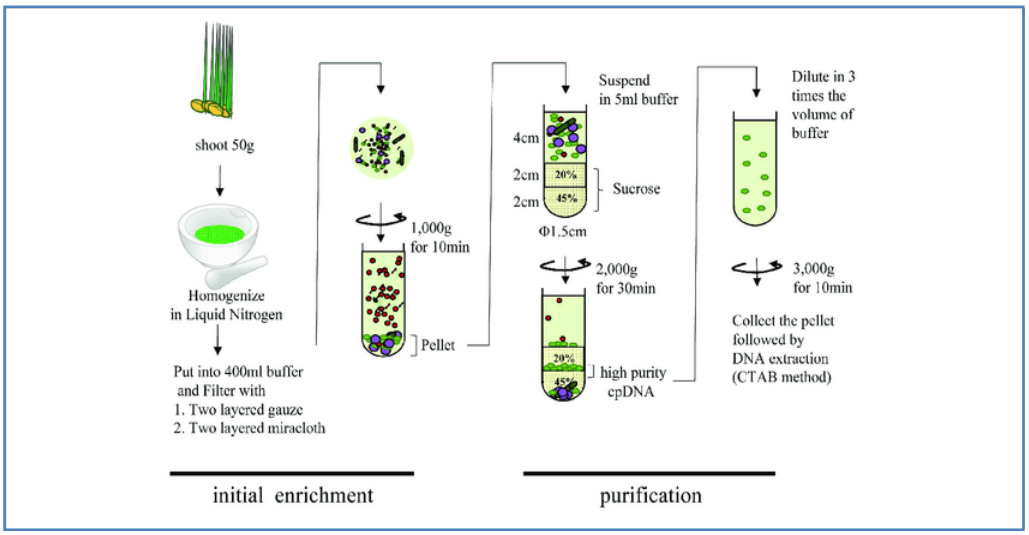

Fig.9.4: DNA isolation using liquid nitrogen coupled with sucrose gradient centrifugation.

Sources: | Flowchart of chloroplast DNA isolation using liquid nitrogen coupled... | Download Scientific Diagram (researchgate.net)

9.5. Solvent system for amino acid

The effect of varying solvent systems on the solubilities of glycine, L-alanine, L-valine, L-phenylalanine, and DL-aminooctanoic acid at 50 °C was studied. The entire concentration spectrum from pure water to pure semipolar solvent was used for each of the solvent systems of methanol-water, ethanol-water, n-propanol water, isopropanol-water, and tertiary butanol-water. Further, the effect of pH variation on the solubilities of the amino acids in each of the solvent systems was studied.

Maximum solubility was found in pure water with a reduction to low solubility in the semipolar solvents in the order of a second degree polynomial equation. To each percent strength of the hydroalcoholic solvent systems, the ratio of water to alcoholic molecules per amino acid molecule remains constant. This would indicate that the lengthening of the nonpolar portion of the chain from the hydrogen of glycine to the methyl phenyl of L-phenylalanine 'does not affect the orientation of the water to alcohol molecules in the solvent system. Each of the amino acids studied demonstrated an ability to differentiate between the hydroalcoholic solvents used.

In an aqueous system, the total solubility is equal to the sum of the original zwitterions solubility plus the solubility of the salt that was found. For those amino acids studied, only single salts were formed. In the hydroalcoholic solvent systems, variation of pH produced minimum solubility at the isoelectric

point with no distinct isoelectric band seen. As the percent alcohol increased in those solvent systems studied, similar increments of acid or base added to the system produced a proportionally greater increase in the magnitude of total solubility of the amino acid.

The importance of amino-acids in viable biological systems is due to the unusual properties of this class of chemical compounds. These substances, possessing both acidic and basic properties within the same molecule, afford the basic linkages leading to complex peptides and proteins, the basic materials of life. Further, ten such amino-acids have been shown to be essential for life processes. Again, due to the basic and acidic character in a given molecule, these substances are dipolar compounds, with either zwitterions species formation or anionic or cationic species formation depending upon the acidity of the fluid environment. The relative concentrations of these species, which coexist with each other, depend completely upon the value of pH.

An important property of the amino acids is that they exhibit an isoelecfric point. This is the point in an electric field at which the zwitterion form will not migrate to either the anode or cathode. Also, at the isoelectric point, the sum of the net charge is zero. If we represent the isoelectric point as pl and the dissociation constants as pK_1 and pK_2 , then .. $pl = 1/2\ pK_1 + 1/2\ pK_2$. Literature values of solubility for amino acids are usually expressed as the solubility in pure water or in aqueous systems adjusted to a pH equal to the isoelectric point. Since the charged form of the amino acid would be dominant, it was assumed that this type of compound would exhibit a high degree of solubility in water.

9.6. Solvent system for Carbohydrate

A rapid method for separating carbohydrates by means of thin-layer chromatography on silica gel G mixed with a small amount of sodium bisulfite has been developed. The solvent system propanol—water (85:15) gave the best resolution of the carbohydrates but did not separate some pentoses. Five spray reagents, *viz.o*-amino-diphenyl—orthophosphoric acid, carbazole—sulfuric acid, dimedone—orthophosphoric acid, phenol—sulfuric acid and thymol—sulfuric acid, were used for the detection of carbohydrates. Carbohydrate compounds are purified in a similar manner as other small molecules. Carbohydrates are often reacted with protecting groups that greatly modify their polar character and allow purification with silica gel. The first examples demonstrate the purification of protected carbohydrates. These compounds are relatively easy to purify on RediSep silica columns because the protecting groups mask the hydroxyl groups which greatly reduce the polarity of the compound and allow solubility in organic solvents. Normal phase solvents are easily removed from the purified compounds due to their low boiling points.

9.7. Solvent system for lipid

Chromatography of lipids on a column of suitable material is a versatile and useful procedure for fractionation of lipid mixtures on a preparative scale. Three types of column chromatography are in general use: adsorption (solid-liquid) chromatography; ion-exchange chromatography; and partition (liquid-liquid) chromatography. Chromatography on silicic-acid-impregnated paper is a very useful and versatile analytical method. Thin layer chromatography is considered as one of the most effective and versatile techniques for separation of intact complex lipids and their lipid moieties, and for neutral lipids. Gas-liquid partition chromatography (GLC) is the method of choice for rapid, quantitative, analysis of volatile lipid components such as hydrocarbons, fatty alcohols, fatty acid esters, sterols, and so on. Generally, lipids are dissolved into a number of solvents, such as methanol, ethanol, butanol, isopropanol, chloroform, n-hexane, acetone, benzene, and cyclohexane; however, hexane, chloroform, and methanol are regarded as the most potential solvents to extract microalgal lipid. For the purposes of lipid separation, particularly for neutral lipids such as triglycerides, we will be using a silica coated plastic plate (stationary phase) and an organic, largely nonpolar solvent mobile phase consisting of petroleum ether: diethyl ether: acetic acid at a ratio of 84:15:1. The lipids are then visualized using resublimed iodine, which will bind to double bonds found in lipid hydrocarbon chains and aromatic compounds. The polarity index for petroleum ether (0.1) positions this solvent component as extremely nonpolar, and will allow the most nonpolar lipid in the mixture to be "dissolved." Whereas acetic acid, which has a high polarity index (6.2), is much more polar since it has the capacity to ionize, and serves as a solvent for the more polar species in this mixure. However, do note that the components in this solvent are not provided in equal quantities; it is both the polarity index as well as the relative amount of each solvent component that dictates how it will carry specific lipid species up the TLC plate.

Solvent Component	**Polarity Index**	**Ratio**
Petroleum Ether	0.1	84
Diethyl Ether	2.8	15
Acetic Acid	6.2	1

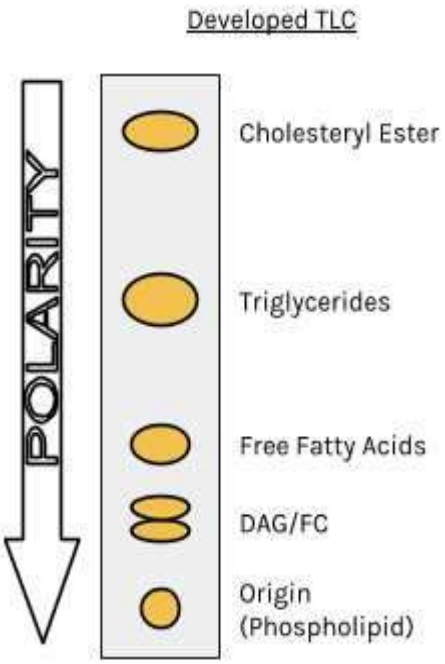

Fig.9.5: Chromatography of lipids on a column

9.8. Summary

Microbial techniques are those techniques which used for the study of microorganism. Mostly, the microbial techniques are used for conducting survey, culture, identify strain, engineering and manipulation. The microbiology and its basic techniques are also related to some other science like plant physiology. Thus basic techniques involve in microbes are very helpful to all in many ways. Sterilization is the process in which media are used for the growth of bacteria or other microbes should be sterilized by heating in an autoclave at 121 °C for about 15 min. However, the sterilization refers to the killing or elimination of all microorganisms, including highly resistant bacteria spores. Many of the techniques use as separation techniques, in which the constituents of a mixture are separated according to differences in chemical or physical properties such as size, shape, mass, density, or chemical affinity between the constituents. Chromatography is one of the techniques used in the separation of biomolecule. The components are separated due to differential affinity for a mobile versus a stationary phase. Maximum solubility was found in pure water with a reduction to low solubility in the semipolar solvents in the order of a second degree polynomial equation. Carbohydrate compounds are purified in a similar manner as other small molecules.

Carbohydrates are often reacted with protecting groups that greatly modify their polar character and allow purification with silica gel.

9.9. Terminal questions

Q.1: Define the isolation techniques.

Answer: ----------

Q.2: Write the basic principle of centrifugation.

Answer: ----------

Q.3: Discus the antibacterial activity.

Answer: ----------

What do understand by separation of biomolecule?

Answer: ----------

Q.4: Discuss the amino acid separation?

Answer: ----------

Q.5: What is Density gradient centrifugation?

Answer: ----------

9.10. Further readings

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