DNR college(A); Bhimavaram.
Dept. of Biochemistry
Paper: 2 ; Semester-II ; Analytical Techniques

Unit-1.Biological concept and cell distribution method

Water as biological solvent, Buffer, measurements of pH, Electrodes, Biological relevance to pH, pKa values, Electrical conductivity, Analysis of Water and Pond water, Total dissolved salts(TDS), BOD, COD, Soil analysis (texture, organic matter and elements), Methods of Tissues homogenation(Potter-Elevejham, mechanical blender, Sonicator and enzymatic

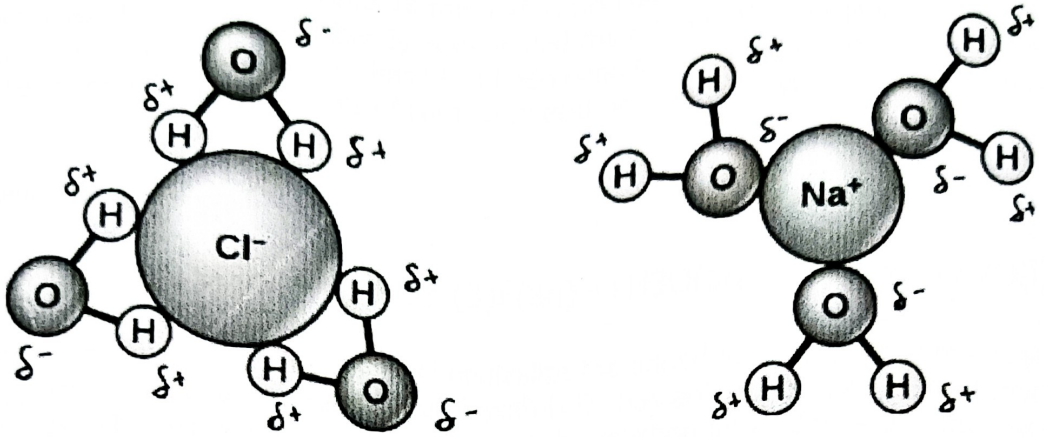
Water as biological solvent

Thanks to its ability to dissolve a wide range of solutes, water is sometimes called the "universal solvent." However, this name isn't entirely accurate, since there are some substances (such as oils) that don't dissolve well in water. Generally speaking, water is good at dissolving ions and polar molecules, but poor at dissolving nonpolar molecules. (A **polar** molecule is one that's neutral, or uncharged, but has an asymmetric internal distribution of charge, leading to partially positive and partially negative regions.)

Water interacts differently with charged and polar substances than with nonpolar substances because of the polarity of its own molecules. Water molecules are polar, with partial positive charges on the hydrogens, a partial negative charge on the oxygen, and a bent overall structure. The unequal charge distribution in a water molecule reflects the greater electronegativity, or electron-greediness, of oxygen relative to hydrogen: the shared electrons of the O-H bonds spend more time with the O atom than with the Hs. In the image below, the partial positive and partial negative charges on a water molecule are represented by the symbols δ^++start superscript, plus, end superscript and δ^--start superscript, minus, end superscript, respectively.

- Because of its polarity, water can form electrostatic interactions (charge-based attractions) with other polar molecules and ions. The polar molecules and ions interact with the partially positive and partially negative ends of water, with positive charges attracting negative charges

(just like the + and - ends of magnets). When there are many water molecules relative to solute molecules, as in an aqueous solution, these interactions lead to the formation of a three-dimensional sphere of water molecules, or **hydration shell**, around the solute. Hydration shells allow particles to be dispersed (spread out) evenly water



Buffer

A buffer is able to resist pH change because the two components (conjugate acid and conjugate base) are both present in appreciable amounts at equilibrium and are able to neutralize small amounts of other acids and bases (in the form of H_3O^+ and OH^-) when the are added to the solution. To clarify this effect, we can consider the simple example of a Hydrofluoric Acid (HF) and Sodium Fluoride (NaF) buffer. Hydrofluoric acid is a weak acid due to the strong attraction between the relatively small F^- ion and solvated protons (H_3O^+), which does not allow it to dissociate completely in water. Therefore, if we obtain HF in an aqueous solution, we establish the following equilibrium with only slight dissociation ($K_a(HF) = 6.6x10^{-4}$, strongly favors reactants):

$$HF_{(aq)}+H_2O_{(l)}\rightleftharpoons F^-_{(aq)}+H_3O^+_{(aq)}(1)(1)HF(aq)+H2O(l)\rightleftharpoons F(aq)-+H3O(aq)+$$

We can then add and dissolve sodium fluoride into the solution and mix the two until we reach the desired volume and pH at which we want to buffer. When Sodium Fluoride dissolves in water, the reaction goes to completion, thus we obtain:

$$NaF_{(aq)}+H_2O_{(l)}\rightarrow Na^+_{(aq)}+F^-_{(aq)}(2)(2)NaF(aq)+H2O(l)\rightarrow Na(aq)++F(aq)-$$

Since Na^+ is the conjugate of a strong base, it will have no effect on the pH or reactivity of the buffer. The addition of NaFNaF to the solution will, however, increase the concentration of F^- in the buffer solution, and, consequently, by Le Chatelier's Principle, lead to slightly less dissociation of the HF in the previous equilibrium, as well. The presence of significant amounts

of both the conjugate acid, HFHF, and the conjugate base, F^-, allows the solution to function as a buffer. This buffering action can be seen in the titration curve of a buffer solution.

As we can see, over the working range of the buffer. pH changes very little with the addition of acid or base. Once the buffering capacity is exceeded the rate of pH change quickly jumps. This occurs because the conjugate acid or base has been depleted through neutralization. This principle implies that a larger amount of conjugate acid or base will have a greater buffering capacity.

If acid were added:

$$F_{-(aq)}+H_3O_{+(aq)} \rightleftharpoons HF_{(aq)}+H_2O_{(l)}(3)(3)F(aq)-+H3O(aq)+ \rightleftharpoons HF(aq)+H2O(l)$$

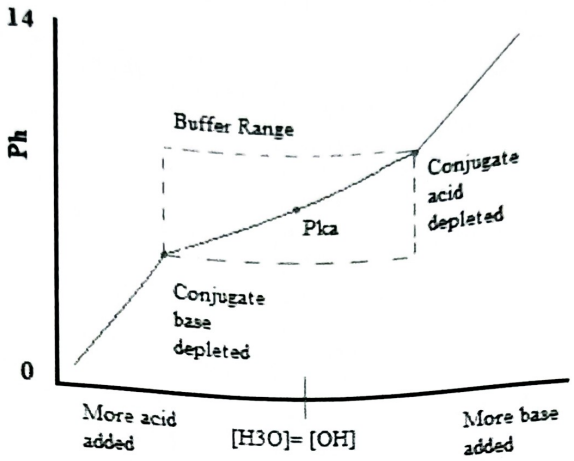
In this reaction, the conjugate base, F^-, will neutralize the added acid, H_3O^+, and this reaction goes to completion, because the reaction of F^- with H_3O^+ has an equilibrium constant much greater than one. (In fact, the equilibrium constant the reaction as written is just the inverse of the K_a for HF: $1/K_a(HF) = 1/(6.6x10^{-4}) = 1.5x10^{+3}$.) So long as there is more F^- than H_3O^+, almost all of the H_3O^+ will be consumed and the equilibrium will shift to the right, slightly increasing the concentration of HF and slightly decreasing the concentration of F^-, but resulting in hardly any change in the amount of H_3O^+ present once equilibrium is re-established.

If base were added:

$$HF_{(aq)}+OH_{-(aq)} \rightleftharpoons F_{-(aq)}+H_2O_{(l)}(4)(4)HF(aq)+OH(aq)- \rightleftharpoons F(aq)-+H2O(l)$$

In this reaction, the conjugate acid, HF, will neutralize added amounts of base, OH^-, and the equilibrium will again shift to the right, slightly increasing the concentration of F^- in the solution and decreasing the amount of HF slightly. Again, since most of the OH^- is neutralized, little pH change will occur.

These two reactions can continue to alternate back and forth with little pH change.



Selecting proper components for desired pH

Buffers function best when the pK_a of the conjugate weak acid used is close to the desired working range of the buffer. This turns out to be the case when the concentrations of the conjugate acid and conjugate base are approximately equal (within about a factor of 10). For example, we know the K_a for hydroflouric acid is 6.6×10^{-4} so its $pK_a = -\log(6.6 \times 10^{-4}) = 3.18$. So, a hydrofluoric acid buffer would work best in a buffer range of around pH = 3.18.

For the weak base ammonia (NH_3), the value of K_b is 1.8×10^{-5}, implying that the K_a for the dissociation of its conjugate acid, NH_4^+, is $K_w/K_b = 10^{-14}/1.8 \times 10^{-5} = 5.6 \times 10^{-10}$. Thus, the pK_a for $NH_4^+ = 9.25$, so buffers using NH_4^+/NH_3 will work best around a pH of 9.25. (It's always the pK_a of the conjugate acid that determines the approximate pH for a buffer system, though this is dependent on the pK_b of the conjugate base, obviously.)

When the desired pH of a buffer solution is near the pK_a of the conjugate acid being used (i.e., when the amounts of conjugate acid and conjugate base in solution are within about a factor of 10 of each other), the Henderson-Hasselbalch equation can be applied as a simple approximation of the solution pH, as we will see in the next section.

Why is the pH Scale Logarithmic?

The pH scale is commonly used to represent hydrogen ion activity. On the pH scale, pH values below 7 represent acidic solutions (hydrogen ion activity greater than hydroxide ion

activity) while values above 7 represent basic solutions. At pH = 7, hydrogen ion and hydroxide ion activity are equal.

As can be seen in table 1, the possible range of hydrogen (H^+) and hydroxide (OH^-) ion activity can span many orders of magnitude. In order to easily manage and represent the wide range of ion activities, a logarithmic pH scale is used.

	pH	H^+ Activity	OH^- Activity
	0	1.E+00	1
	1	1.E-01	0.1
	2	1.E-02	0.01
acid	3	1.E-03	0.001
	4	1.E-04	0.0001
	5	1.E-05	0.00001
	6	1.E-06	0.000001
neutral	7	**1.E-07**	**0.0000001**
	8	1.E-08	0.00000001
	9	1.E-09	0.000000001
	10	1.E-10	0.0000000001
base	11	1.E-11	0.00000000001
	12	1.E-12	0.000000000001
	13	1.E-13	0.0000000000001
	14	1.E-14	0.00000000000001

Hydrogen ion and hydroxide ion activities on the pH scale.

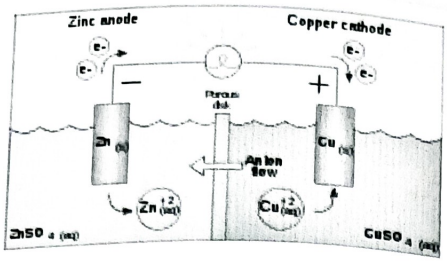
A change on the pH scale of 1.0 pH unit indicates that hydrogen ion activity differs by an order of magnitude (i.e. factor of 10). For example, hydrogen ion activity at pH 4 is 10 times greater than at pH 5.

Due to the logarithmic nature of the pH scale, it is incorrect to simply average pH values and report them. Instead, it is more appropriate to report the median pH value or provide the range of pH values observed.

Electrode

An **electrode** is an **electrical conductor** used to make contact with a nonmetallic part of a **circuit** (e.g. a **semiconductor**, an **electrolyte**, a **vacuum** or air). Electrodes are essential parts of **batteries** that can consist of a variety of materials depending on the type of battery.

Anode and cathode in electrochemical cell



Schematic of a voltaic (galvanic) cell

Electrodes are an essential part of any battery. The first electrochemical battery made was devised by Alessandro Volta and was aptly named the Voltaic cell[2] This battery consisted of a stack of copper and zinc electrodes separated by brine-soaked paper disks. Due to fluctuation in the voltage provided by the voltaic cell it wasn't very practical. The first practical battery was invented in 1839 and named the Daniell cell after John Frederic Daniell. Still making use of the zinc–copper electrode combination. Since then many more batteries have been developed using various materials (see List of batteries). The basis of all these is still making use of two electrodes which can be divided in two categories: Anodes and Cathodes.

Anode

A term coined by William Whewell at Faraday's request, derived from the Greek words . Anode is the electrode through which the conventional current enters from the electrical circuit of an electrochemical cell (battery) into the non-metallic cell. The electrons then flow to the other side of the battery. Note the difference in the flow of current and the flow of electrons, this is due to the discovery of the flow of current prior to the discovery of the electron. Benjamin Franklin surmised that the electrical flow moved from positive to negative.[4] The electrons flow away from the anode and the conventional current towards it. From both can be concluded that the charge of the anode is negative. The electron entering the anode comes from the oxidation reaction that takes place next to it.

Cathode

The cathode is in many ways the opposite of the anode. The name (also coined by William Whewell) comes from the Greek words κάθά (kata), 'downwards' and ὀδός (hodós), 'a way'. It is the positive electrode, meaning the electrons from the electrical circuit through the cathode into the non-metallic part of the electrochemical cell. At the cathode, the reduction reaction takes place with the electrons arriving from the wire connected to the cathode and are absorbed by the oxidizing agent.

pKa Values

It is helpful to have a way of comparing Bronsted-Lowry acidities of different compounds. If the chemistry of protons involves being passed from a more acidic site to a less acidic site, then the site that binds the proton more tightly will retain the proton, and the site that binds protons less tightly will lose the proton. If we know which sites bind protons more tightly, we can predict in which direction a proton will be transferred.

Figure AB9.1. In which direction will the equilibrium lie? Which base gets the proton?

There is an experimentally-determined parameter that tells us how tightly protons are bound to different compounds. "Experimental" often implies to students "untested" or "unreliable", but here it means that someone has done the work to measure how tightly the proton is bound. Experimental in this sense means "based on physical evidence".

This experimental parameter is called "the pKa". The pKa measures how tightly a proton is held by a Bronsted acid. A pKa may be a small, negative number, such as -3 or -5. It may be a larger, positive number, such as 30 or 50. The lower the pKa of a Bronsted acid, the more easily it gives up its proton. The higher the pKa of a Bronsted acid, the more tightly the proton is held, and the less easily the proton is given up.

The pKa scale as an index of proton availability.

- Low pKa means a proton is not held tightly.
- pKa can sometimes be so low that it is a negative number!
- High pKa means a proton is held tightly.

nitric acid hydrobromic acid acetic acid formic acid

Electrical Conductivity Definition

Electrical conductivity is the measure of the amount of electrical current a material can carry or it's ability to carry a current. Electrical conductivity is also known as specific conductance. Conductivity is an intrinsic property of a material.

Units of Electrical Conductivity

Electrical conductivity is denoted by the symbol σ and has SI units of siemens per meter (S/m). In electrical engineering, the Greek letter κ is used. Sometimes the

Greek letter γ represents conductivity. In water, conductivity is often reported as specific conductance, which is a measure compared to that of pure water at 25°C.

Relationship Between Conductivity and Resistivity

Electrical conductivity (σ) is the reciprocal of the electrical resistivity (ρ):

$\sigma = 1/\rho$

where resistivity for a material with a uniform cross section is:

$\rho = RA/l$

where R is the electrical resistance, A is the cross-sectional area, and l is the length of the material

Electrical conductivity gradually increases in a metallic conductor as the temperature is lowered. Below a critical temperature, resistance in superconductors drops to zero, such that an electrical current could flow through a loop of superconducting wire with no applied power.
In many materials, conduction occurs by band electrons or holes. In electrolytes, entire ions move, carrying their net electrical charge. In electrolyte solutions, the concentration of the ionic species is a key factor in the conductivity of the material.

Analysis of fresh water and pond water

pH Testing

What is pH? If middle school science class feels like an age and a day behind you, here's a quick reminder. pH is a measure of the concentration of hydrogen ions in a solution. The more of these hydrogen ions there are in a solution, the more acidic that water is. Acidity effects taste of water, but it can also affect how health water is to consume. Drinking water that's not neutral enough in acidity can make people sick!

Other Types of Testing

There are some basic water tests that don't have anything to do with chemical testing: conductivity, odor, sediment, and turbidity. Not

relevant in all situations, these tests create a measure of the more physical traits of a water sample.

Biochemical oxygen demand (BOD)

It is the amount of **dissolved oxygen** (DO) needed (i.e. demanded) by aerobic biological organisms to break down organic material present in a given water sample at certain temperature over a specific time period. The BOD value is most commonly expressed in milligrams of oxygen consumed per litre of sample during 5 days of incubation at 20 °C and is often used as a surrogate of the degree of **organic pollution of water**.

BOD reduction is used as a gauge of the effectiveness of wastewater treatment plants. BOD of wastewater effluents is used to indicate the short-term impact on the oxygen levels of the receiving water.
BOD analysis is similar in function to chemical oxygen demand (COD) analysis, in that both measure the amount of organic compounds in water. However, COD analysis is less specific, since it measures everything that can be chemically oxidized, rather than just levels of biologically oxidized organic matter.

chemical oxygen demand (COD)

It is an indicative measure of the amount of **oxygen** that can be consumed by **reactions** in a measured **solution**. It is commonly expressed in **mass** of oxygen consumed over **volume** of solution which in SI units is milligrams per litre (**mg/L**). A COD test can be used to easily quantify the amount of **organics** in **water**. The most common application of COD is in quantifying the amount of oxidizable **pollutants** found in **surface water** (e.g. **lakes** and **rivers**) or **wastewater**. COD is useful in terms of **water quality** by providing a metric to determine the effect an **effluent** will have on the receiving body, much like **biochemical oxygen demand (BOD)**.

Soil Testing

A soil test is important for several reasons: to optimize crop production, to protect the environment from contamination by runoff and leaching of excess fertilizers, to aid in the diagnosis of plant culture problems, to improve the nutritional balance of the growing media and to save money and conserve energy by applying only the amount of fertilizer needed. Pre- plant media analyses provide an indication of potential nutrient deficiencies, pH imbalance or excess soluble salts. This is particularly important for growers who mix their own media. Media testing during the growing season is an important tool for managing crop nutrition and soluble salts levels. To use this tool effectively, you must know

how to take a media sample to send for analysis or for in-house testing, and be able to interpret media test results.

Determining the pH and fertility level through a soil test is the first step in planning a sound nutrient management program. Soil samples from soilless mixes are tested differently than samples from field soil. There are three commonly used methods of testing soilless media using water as an extracting solution: 1:2 dilution method, saturated media extract (SME), and leachate Pour Thru. The values that represent each method of testing are different from each other. For example, 2.6 would be "extreme" (too high) for the 1:2 method, "normal" for SME, and "low" for leachate Pour Thru. Likewise, values for specific nutrients are likely to differ with testing methods. Always use the interpretative data for the specific soil testing method used to avoid incorrect interpretation of the results.

Homogenization

.Potter elvehjem homogeniser

A device used to disrupt tissues. A cylindrical glass or hard polymer pestle rotates in a close-fitting tube and a suspension of the tissue particles is subjected to shearing forces as the pestle moves up and down and presses the suspension through the space between the rotating pestle and the tube

- **Mechanical blender**

A **blender** (sometimes called a **mixer** or **liquidiser** in British English) is a kitchen and laboratory appliance used to mix, crush, purée or emulsify food and other substances. A stationary blender consists of a blender container with a rotating metal blade at the bottom, powered by an electric motor that is in the base. Some powerful models can also crush ice and other frozen foods. The newer immersion blender configuration has a motor on top connected by a shaft to a rotating blade at the bottom, which can be used with any container.

Sonication Process

During sonication, cycles of pressure form thousands of microscopic vacuum bubbles in the solution. The bubbles collapse into the solution in a process known as cavitation. This causes powerful waves of vibration that release an enormous energy force in the cavitation field, which disrupts molecular interactions such as interactions between molecules of water, separates clumps of particles, and facilitates mixing. For example, in dissolved gas vibrations, the gas bubbles come together and more easily leave the solution.

The energy from sound waves creates friction in the solution, which creates heat. To stop a sample from heating up and degrading, keep it on ice before, during and after sonication.

If cells and proteins are too fragile to withstand sonication, a gentler alternative is enzyme digestion or grinding with sand.

Unit-2.Microscopy and Centrifugation

Microscopy: Basic principals of light microscopy, phase contrast, electron microscopy, florescent microscopy, and their applications. Centrifugation techniques, principle, application- differential, density gradient, Ultra centrifugation- preparative and analytical.

Microscopy?

Microscopy is the technical field of using microscopes to view samples & objects that cannot be seen with the unaided eye (objects that are not within the resolution range of the normal eye).

| Light Microscopy

Principles

The light microscope is an instrument for visualizing fine detail of an object. It does this by creating a magnified image through the use of a series of glass lenses, which first focus a beam of light onto or through an object, and convex objective lenses to enlarge the image formed

Phase-contrast microscopy

Phase-contrast microscopy (PCM) is an optical microscopy technique that converts phase shifts in light passing through a transparent specimen to brightness changes in the image. Phase shifts themselves are invisible, but become visible when shown as brightness variations.

Phase-contrast microscopy is particularly important in biology. It reveals many **cellular** structures that are invisible with a **bright-field microscope**, as exemplified in the figure. These structures were made visible to earlier microscopists by **staining**, but this required

additional preparation and death of the cells. The phase-contrast microscope made it possible for biologists to study living cells and how they proliferate through **cell division**. It is one of the few methods available to quantify cellular structure and components that does not use **fluorescence**.[1] After its invention in the early 1930s,[2] phase-contrast microscopy proved to be such an advancement in microscopy that its inventor **Frits Zernike** was awarded the **Nobel Prize in Physics** in 1953.[3]

Electron microscope

An **electron microscope** is a microscope that uses a beam of accelerated electrons as a source of illumination. As the wavelength of an electron can be up to 100,000 times shorter than that of visible light photons, electron microscopes have a higher resolving power than light microscopes and can reveal the structure of smaller objects. A scanning transmission electron microscope has achieved better than 50 pm resolution in annular dark-field imaging mode[1] and magnifications of up to about 10,000,000× whereas most light microscopes are limited by diffraction to about 200 nm resolution and useful magnifications below 2000×.

Electron microscopes are used to investigate the **ultrastructure** of a wide range of biological and inorganic specimens including **microorganisms**, **cells**, large **molecules**, **biopsy** samples, **metals**, and **crystals**. Industrially, electron microscopes are often used for quality control and **failure analysis**. Modern electron microscopes produce electron **micrographs** using specialized digital cameras and **frame grabbers** to capture the images.

Fluorescence microscope

A **fluorescence microscope** is an optical microscope that uses fluorescence instead of, or in addition to, scattering, reflection, and attenuation or absorption, to study the properties of organic or inorganic substances.[1][2] "Fluorescence microscope" refers to any microscope that uses fluorescence to generate an image, whether it is a simple set up like an epifluorescence microscope or a more complicated design such as a confocal microscope, which uses optical sectioning to get better resolution of the fluorescence image.

Principle

The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit light of longer wavelengths (i.e., of a different color than the absorbed light). The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. Typical components of a fluorescence microscope are a light source (xenon arc lamp or mercury-vapor lamp are common; more advanced forms are high-power LEDs and lasers), the excitation filter, the dichroic mirror (or dichroic beamsplitter), and the emission filter (see figure below). The filters and the dichroic beamsplitter are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen.[1] In this manner, the distribution of a single fluorophore (color) is imaged at a time. Multi-color images of several types of fluorophores must be composed by combining several single-color images.[1]
Most fluorescence microscopes in use are epifluorescence microscopes, where excitation of the fluorophore and detection of the fluorescence are done through the same light path (i.e. through the objective). These microscopes are widely used in biology and are the basis for more advanced microscope designs, such as the confocal microscope and the total internal reflection fluorescence microscope (TIRF).

Definition of Centrifugation

It is a unit operation working for separation separating the consequent present in a dispersion with the help of centrifugal force for example centrifugal force includes the earth revolves around the sun. It is a technique which involves the application of centrifugal force to separate particles from a solution according to their size, shape, density, the viscosity of the medium and rotor speed.

Types of Centrifugation Techniques

1) Density gradient centrifugation

It allows separation of many or all components in a mixture and allows for measurement also. There are 2 forms of density gradient centrifugation one is rate zonal centrifugation and the second is I saw pyknic or sedimentation equilibrium centrifugation.

A)Rate zonal centrifugation – In rate zonal centrifugation the solution has a density gradient. The sample has a density therefore greater than all the layers the solution. The sample is applied in a thin zone at the top of the centrifuge tube on a density gradient. Under centrifugal force, the particles will begin segmenting through the

gradient. The particles will begin segmenting in separate zones according to their size, shape and density.

B) Isopycnic or sedimentation equilibrium centrifugation – In this type of centrifugation, the solution contains a greater range of densities. The density gradient contains the whole range of densities of the particle in the sample pool stop each particle with sediment only to the position in the centrifuge tube at which the gradient density is equal to the phone density. In sedimentation centrifugation separation of particles occur into the zone based on their density difference, independent of time.

2) Differential Centrifugation

Differential centrifugation is a common procedure in microbiology and cytology useful to separate certain organelles for further analysis of specific parts of cells. In the process, a tissue temple is first homogenised generalized to break the cell membranes and mix up the cell contents. The homogenate is then subjected to repeated centrifugation, each time remove in the palate and increasing the centrifugal force.

3) Ultracentrifugation

Svedberg coined the term " ultracentrifugation".He was a chemist. He used the ultra fuse to determine the MW and subunit structure of emo globin. The first commercial ultra fuse was produced in 1940. An important tool in biochemical research is the centrifuge, which through rapid spinning imposes hi centrifugal forces on suspended particles, or even molecules in solution, and causes separation of such matter on the basis ultracentrifugation of differences in weight. For example, red cells may be separated from the plasma of blood, nuclei from mitochondria and cell homogenate, and one protein from another in complex mixtures.

Principle of Centrifugation

1) The centrifuge involve the s principle of sedimentation.

2) The principle of the centrifugation technique is to separate the particles suspended in liquid media under the influence of a centrifugal field. These are placed either in tubes or bottles in a rotor in the centrifuge.

3) Sedimentation is a phenomenon where suspended material settles out of the fluids by gravity. The suspended material can be particles such as clay or powder. Example, tea leaves falling to the bottom in a teacup.

4) The particles having size more than 5 micrometres are separated by simple filtration process while the particles having size 5 micrometre or less do not sediment under gravity. The central force is useful to separate those particles.

Application of Centrifugation

1) Production of bulk drugs.

2) Production of biological products.

3) Evaluation of suspensions and emulsion.

4) Determination of molecular weight of collides.

5) Separating chalk powder from water.

6) Removing fat from milk to produce skimmed milk.

7) The clarification and stabilization of the wine.

8) Biopharmaceutical analysis of drugs.

9) Use in water treatment.

10) Removing water from lettuce after washing it in a salad spinner.

11) Separating particles from an airflow using cyclonic separation.

Unit-3.Chromatographic Techniques

Chromatography: Principles and applications, types of Chromatography techniques- paper Chromatography- solvents, rf values, application,; Thin layer chromatography- Principle, choice of absorbent and solvent, rf values, application; Gel filtration, Ion exchange- Principles, resins, actions of resins, applications, seperation of metal ions; Affinity Chromatography.

Chromatography

The chromatography technique is one of the most powerful methods for separating a sample, such as a synthesized mixture or a biological crude extract, into its single components. The chromatography separation technique is based on substances partitioning between two phases: a stationary phase with a large surface and a mobile phase which moves through the stationary phase.

Principles of Chromatography

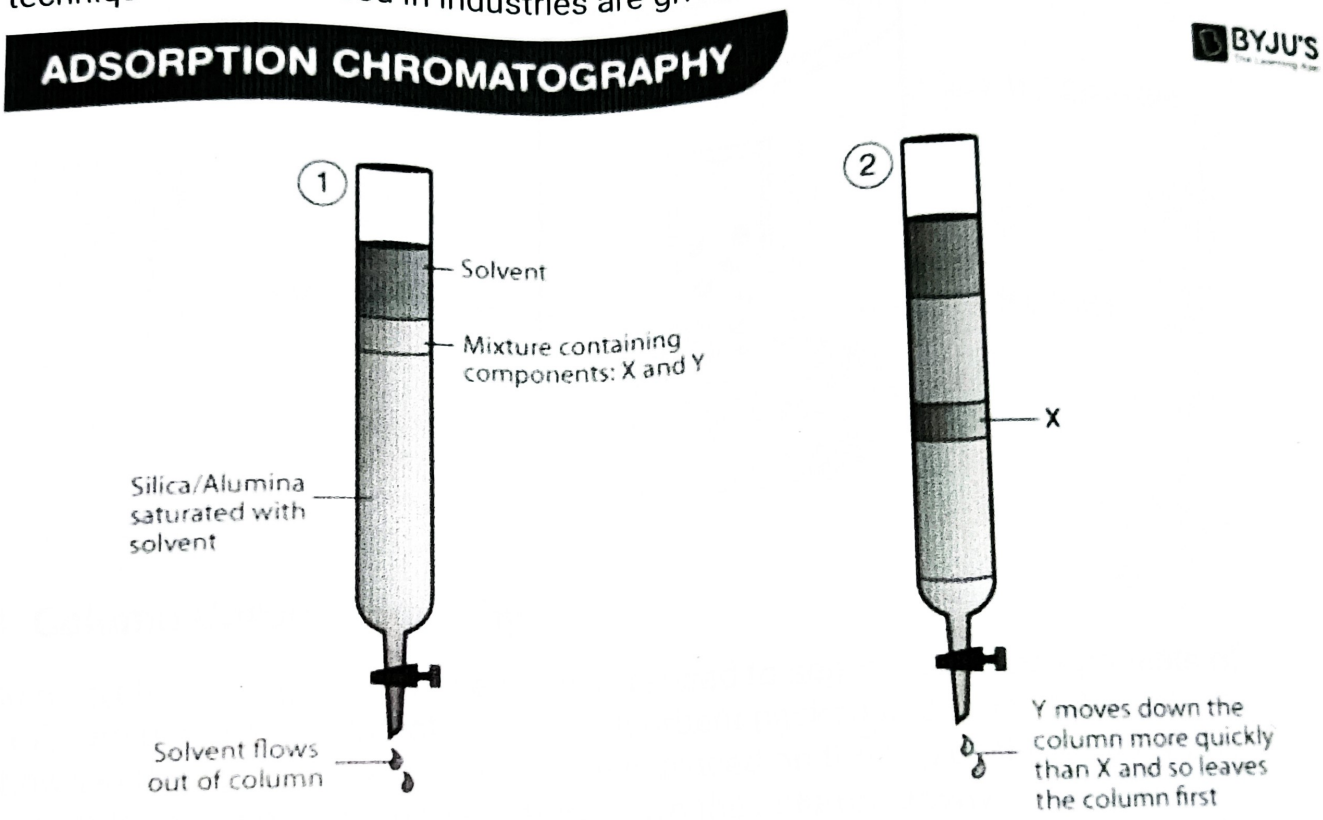
Chromatography is a separation method where the analyte is combined within a liquid or gaseous mobile phase., which is pumped through a stationary phase. Usually one phase is hydrophilic and the other lipophilic. The components of the analyte interact differently with these y=two phases. Depending on of 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.

Types of Chromatography

The four main types of chromatography are

1. Adsorption Chromatography

In the process of adsorption chromatography, different compounds are adsorbed on the adsorbent to different degrees based on the absorptivity of the component. Here also, a mobile phase is made to move over a stationary phase, thus carrying the components with higher absorptivity to a lower distance than that with lower absorptivity. The main types of chromatographic techniques that are used in industries are given as under.

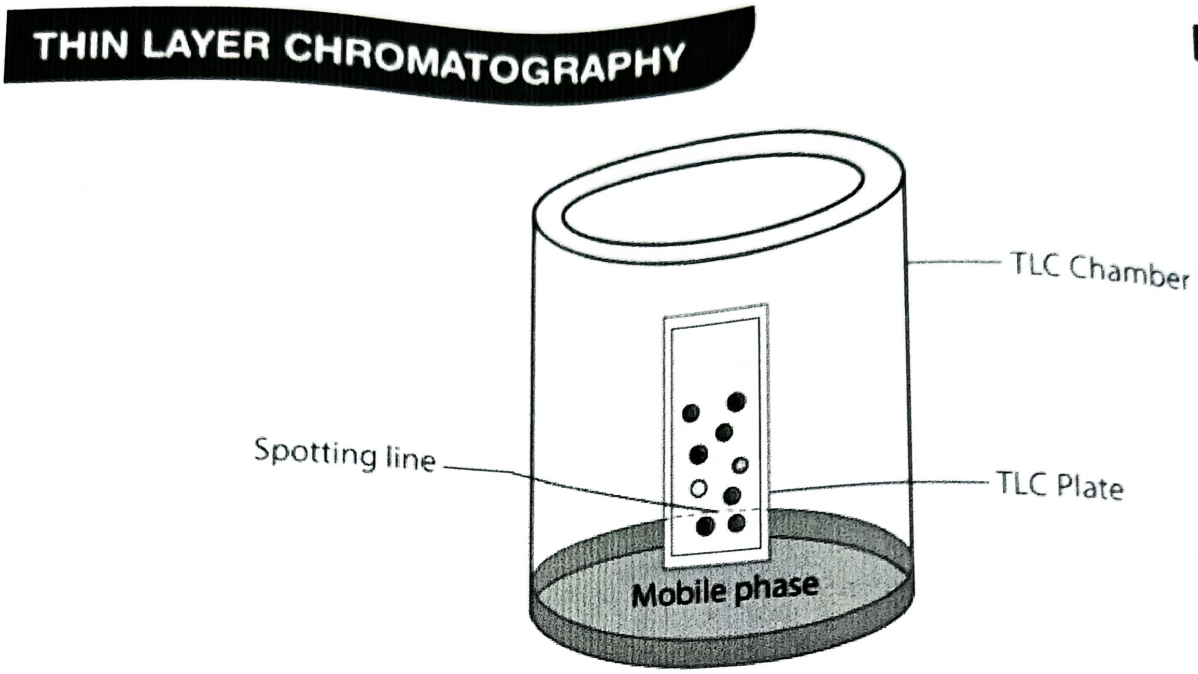


2. Thin Layer Chromatography

In the process of thin-layer chromatography (TLC), the mixture of substances is separated into its components with the help of a glass plate coated with a very thin layer of adsorbent, such as silica gel and alumina, as shown in the figure below.

The plate used for this process is known as chrome plate. The solution of the mixture to be separated is applied as a small spot at a distance of 2 cm above one end of the plate. The plate is then placed in a closed jar containing a fluid

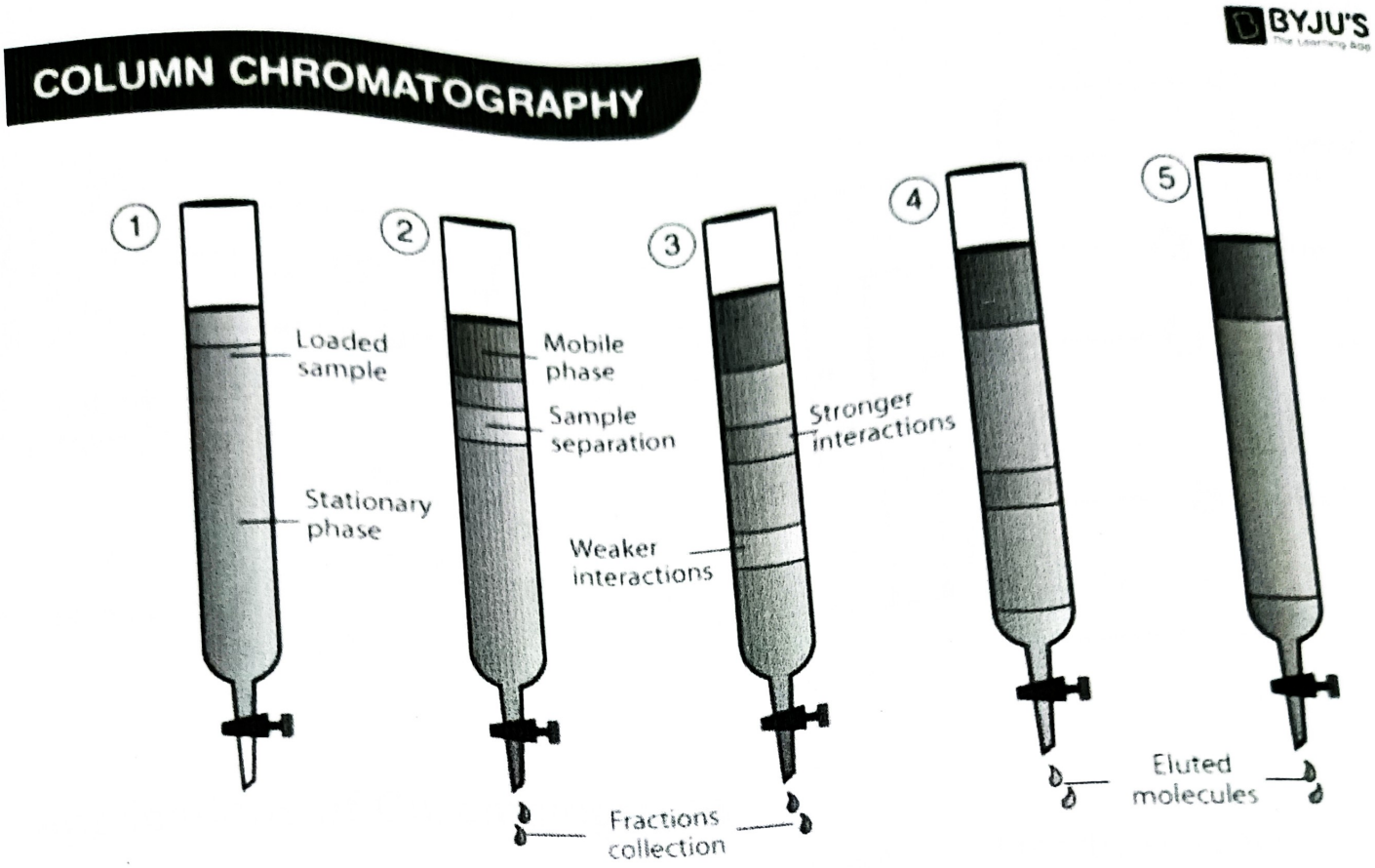
termed as an eluant, which then rises up the plate carrying different components of the mixture to different heights.



3. Column Chromatography

Column chromatography is the technique used to separate the components of a mixture using a column of suitable adsorbent packed in a glass tube, as shown in the figure below. The mixture is placed on the top of the column, and an appropriate eluant is made to flow down the column slowly.

Depending upon the degree of adsorption of the components on the wall adsorbent column, the separation of the components takes place. The component with the highest absorptivity is retained at the top, while the other flow down to different heights accordingly.

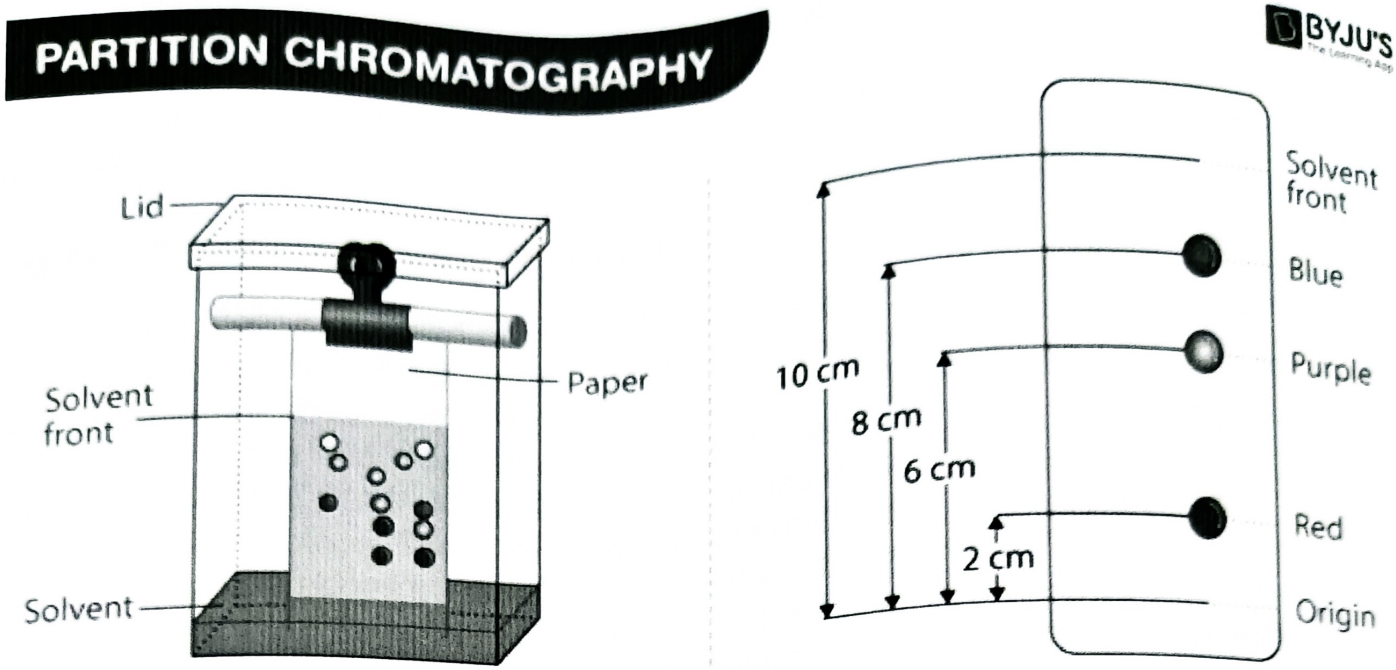


Column Chromatography

4. Partition chromatography

In this process, a continuous differential partitioning of components of a mixture into a stationary phase and mobile phase takes place. The example of partition chromatography can be seen in paper chromatography. In this process, chromatography paper is used as a stationary phase which is suspended in a mixture of solvents that act as a mobile phase.

Here, we put a spot at the base of the chromatographic paper with the mixture to be separated and as the solvent rises up this paper, the components are carried to different degrees depending upon their retention on the paper. The components are thus separated at different heights.



Applications of Chromatography

In bio analytical chemistry, chromatography is mainly used for the separation, isolation and purification of proteins from complex sample matrices. In cells for example, proteins occur alongside numerous other compounds such as lipids and nucleic acids. In order to be analysed, these proteins must be separated from all the other cell components. Then the proteins of interest might have to be isolated from other proteins and purified further.

Chromatography is an essential part of almost any protein purification strategy. A number of different chromatographic techniques are used for the purification and analysis of proteins. They can be classified according to the physical principle involved in the separation process. Typical examples include reversed phase chromatography, ion exchange chromatography, affinity chromatography and size exclusion chromatography.

Paper Chromatography

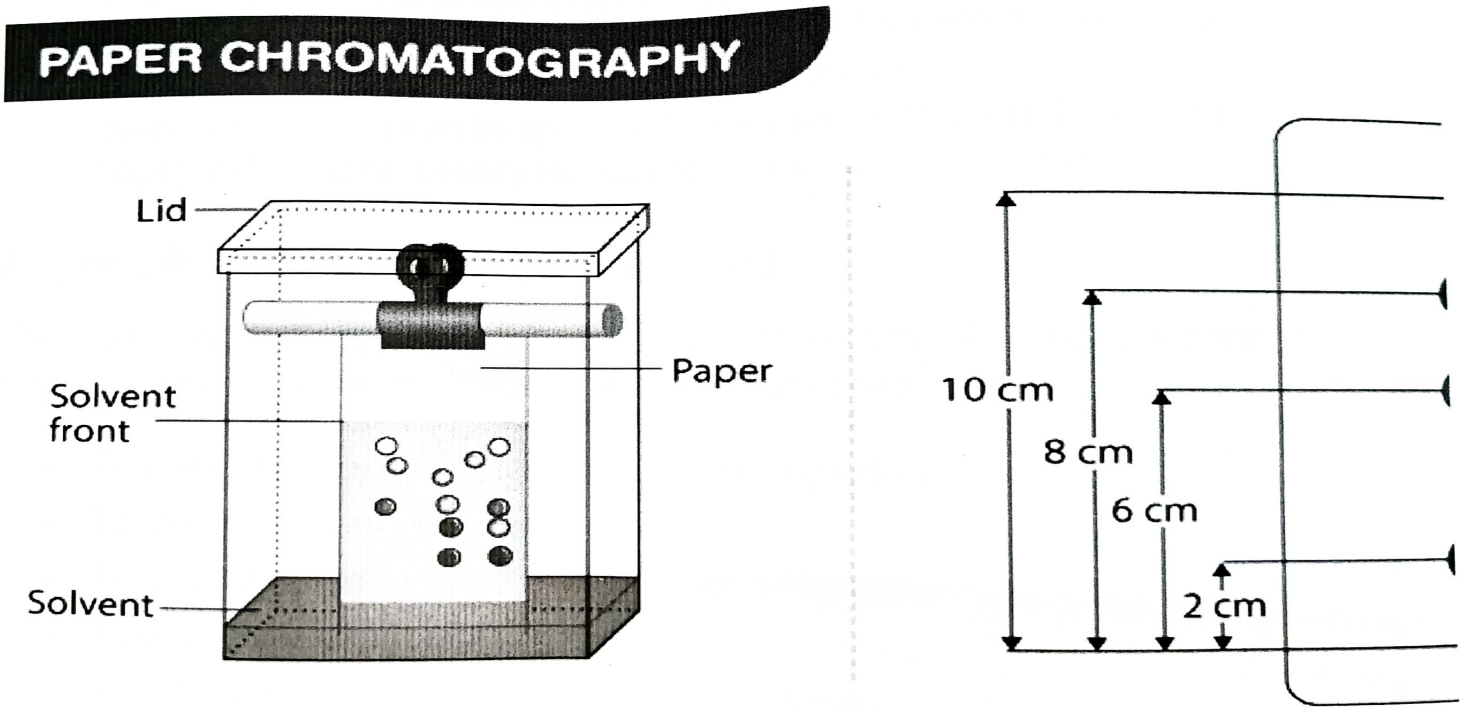
Chromatography technique that uses paper sheets or strips as the adsorbent being the stationary phase through which a solution is made to pass is called paper chromatography. It is an inexpensive method of separating dissolved

chemical substances by their different migration rates across the sheets of paper. It is a powerful analytical tool that uses very small quantities of material. Paper chromatography was discovered by Synge and Martin in the year 1943.

Paper Chromatography Principle

The principle involved can be partition chromatography or adsorption chromatography. Partition chromatography because the substances are partitioned or distributed between liquid phases. The two phases are water held in pores of the filter paper and the other phase is a mobile phase which passes through the paper. When the mobile phase moves, the separation of the mixture takes place. The compounds in the mixture separate themselves based on the differences in their affinity towards stationary and mobile phase solvents under the capillary action of pores in the paper. Adsorption chromatography between solid and liquid phases, wherein the solid surface of the paper is the stationary phase and the liquid phase is the mobile phase.

Paper Chromatography Diagram



Paper Chromatography Procedure

Below we have explained the procedure to conduct Paper Chromatography Experiment for easy understanding of students.

1. **Selecting a suitable type of development:** It is decided based on the complexity of the solvent, paper, mixture, etc. Usually ascending type or radial paper chromatography is used as they are easy to perform. Also, it is easy to handle, the chromatogram obtained is faster and the process is less time-consuming.
2. **Selecting a suitable filter paper**: Selection of filter paper is done based on the size of the pores and the sample quality.
3. **Prepare the sample:** Sample preparation includes the dissolution of the sample in a suitable solvent (inert with the sample under analysis) used in making the mobile phase.
4. **Spot the sample on the paper:** Samples should be spotted at a proper position on the paper by using a capillary tube.
5. **Chromatogram development:** Chromatogram development is spotted by immersing the paper in the mobile phase. Due to the capillary action of paper, the mobile phase moves over the sample on the paper.
6. **Paper drying and compound detection**: Once the chromatogram is developed, the paper is dried using an air drier. Also, detecting solution can be sprayed on the chromatogram developed paper and dried to identify the sample chromatogram spots.

Paper Chromatography Applications

There are various applications of paper chromatography. Some of the uses of Paper Chromatography in different fields are discussed below:

- To study the process of fermentation and ripening.
- To check the purity of pharmaceuticals.
- To inspect cosmetics.
- To detect the adulterants.
- To detect the contaminants in drinks and foods.
- To examine the reaction mixtures in biochemical laboratories.
- To determine dopes and drugs in humans and animals

Thin Layer Chromatography

Thin Layer Chromatography is a technique used to isolate non-volatile mixtures. The experiment is conducted on a sheet of aluminium foil, plastic, or glass which is coated with a thin layer of adsorbent material. The material usually used is aluminium oxide, cellulose, or silica gel.

On completion of the separation, each component appears as spots separated vertically. Each spot has a retention factor (R_f) expressed as:

R_f = dist. travelled by sample / dist. travelled by solvent

The factors affecting retardation factor are the solvent system, amount of material spotted, absorbent and temperature. TLC is one of the fastest, least expensive, simplest and easiest chromatography technique.

Thin Layer Chromatography Principle

Like other chromatographic techniques, thin-layer chromatography (TLC) depends on the separation principle. The separation relies on the relative affinity of compounds towards both the phases. The compounds in the mobile phase move over the surface of the stationary phase. The movement occurs in such a way that the compounds which have a higher affinity to the stationary phase move slowly while the other compounds travel fast. Therefore, the separation of the mixture is attained. On completion of the separation process, the individual components from the mixture appear as spots at respective levels on the plates. Their character and nature are identified by suitable detection techniques.

Thin Layer Chromatography Diagram

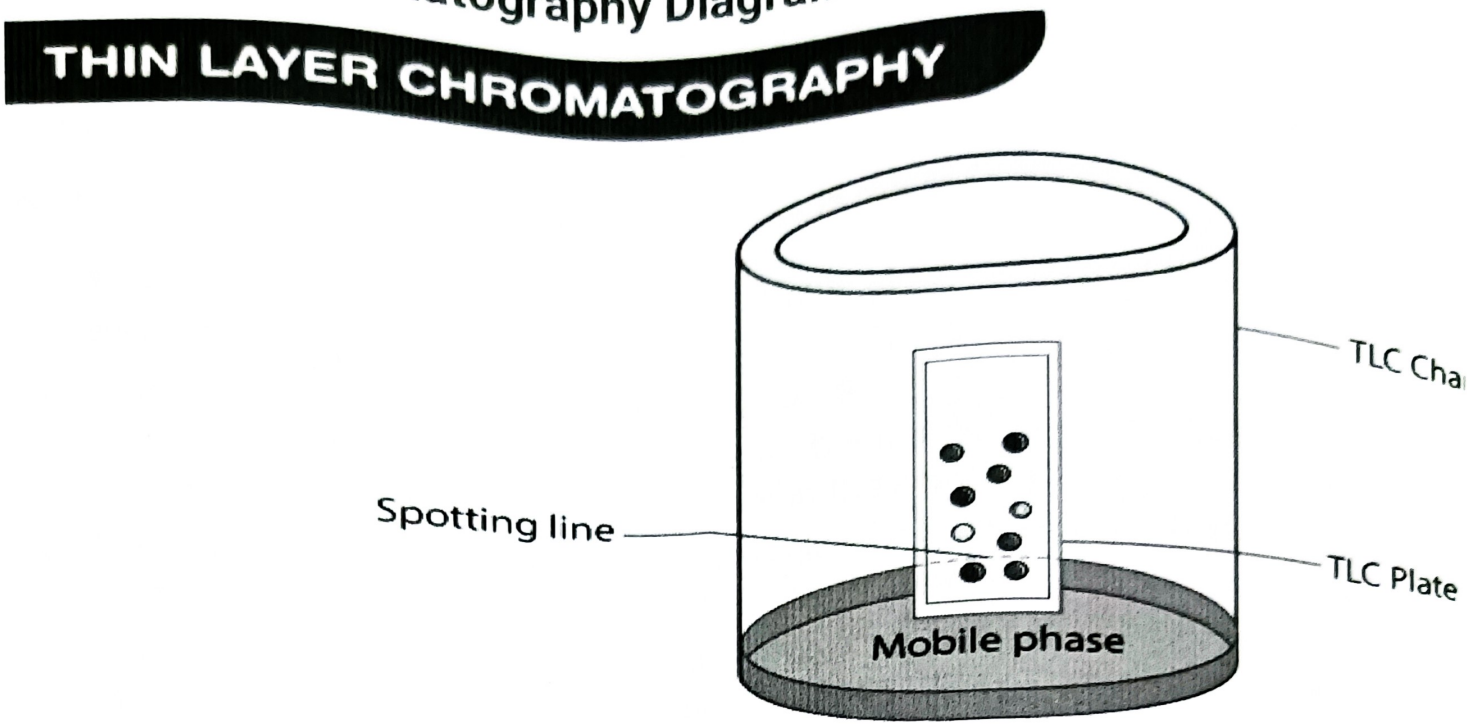


Diagram of Thin Layer Chromatography

Thin Layer Chromatography Procedure

Before starting with the Thin Layer Chromatography Experiment, let us understand the different components required to conduct the procedure along with the phases involved.

1. Thin Layer Chromatography Plates – ready-made plates are used which are chemically inert and stable. The stationary phase is applied on its surface in the form of a thin layer. The stationary phase on the plate has a fine particle size and also has a uniform thickness.
2. Thin Layer Chromatography Chamber – Chamber is used to develop plates. It is responsible to keep a steady environment inside which will help in developing spots. Also, it prevents the solvent evaporation and keeps the entire process dust-free.
3. Thin Layer Chromatography Mobile phase – Mobile phase is the one that moves and consists of a solvent mixture or a solvent. This phase should be particulate-free. The higher the quality of purity the development of spots is better.

4. Thin Layer Chromatography Filter Paper – It has to be placed inside the chamber. It is moistened in the mobile phase.

Thin Layer Chromatography Experiment

The stationary phase that is applied to the plate is made to dry and stabilize.

- To apply sample spots, thin marks are made at the bottom of the plate with the help of a pencil.
- Apply sample solutions to the marked spots.
- Pour the mobile phase into the TLC chamber and to maintain equal humidity, place a moistened filter paper in the mobile phase.
- Place the plate in the TLC chamber and close it with a lid. It is kept in such a way that the sample faces the mobile phase.
- Immerse the plate for development. Remember to keep the sample spots well above the level of the mobile phase. Do not immerse it in the solvent.
- Wait till the development of spots. Once the spots are developed, take out the plates and dry them. The sample spots can be observed under a UV light chamber.

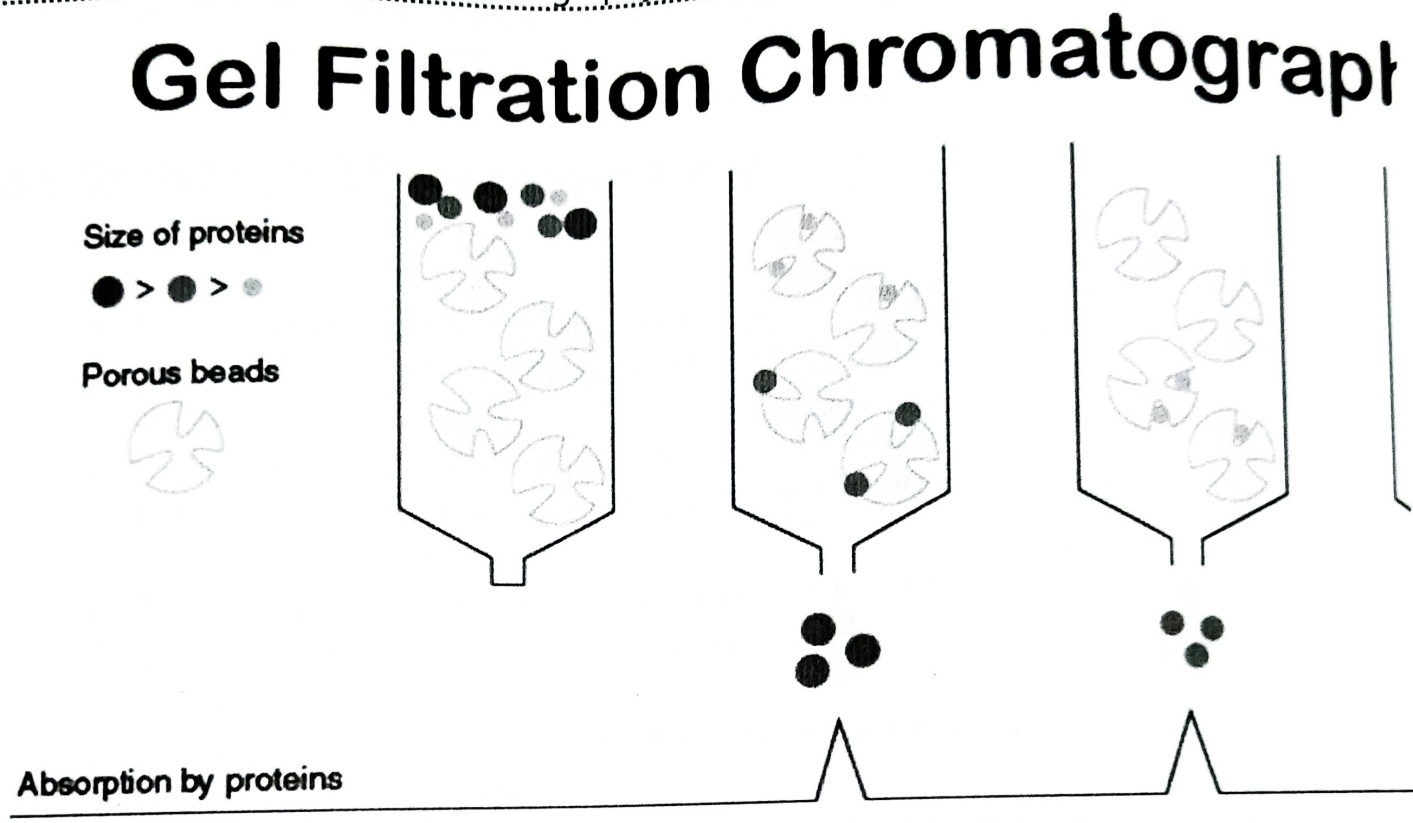
Thin Layer Chromatography Applications

- The qualitative testing of Various medicines such as sedatives, local anaesthetics, anticonvulsant tranquilisers, analgesics, antihistamines, steroids, hypnotics is done by TLC.
- TLC is extremely useful in Biochemical analysis such as separation or isolation of biochemical metabolites from its blood plasma, urine, body fluids, serum, etc.
- Thin layer chromatography can be used to identify natural products like essential oils or volatile oil, fixed oil, glycosides, waxes, alkaloids, etc.
- It is widely used in separating multicomponent pharmaceutical formulations.
- It is used to purify of any sample and direct comparison is done between the sample and the authentic sample.
- It is used in the food industry, to separate and identify colours, sweetening agent, and preservatives

- It is used in the cosmetic industry.
- It is used to study if a reaction is complete

Gel Filtration Chromatography

Principle of Gel Filtration Chromatography



To perform a separation, the gel filtration medium is packed into a column to form a packed bed. The medium is a porous matrix in the form of spherical particles that have been chosen for their chemical and physical stability, and inertness (lack of reactivity and adsorptive properties). The packed bed is equilibrated with a buffer which fills the pores of the matrix and the space in between the particles. The liquid inside the pores is sometimes referred to as the stationary phase and this liquid is in equilibrium with the liquid outside the particles, referred to as the mobile phase.

- The stationary phase used is a porous polymer matrix whose pores are completely filled with the solvent to be used as the mobile phase.

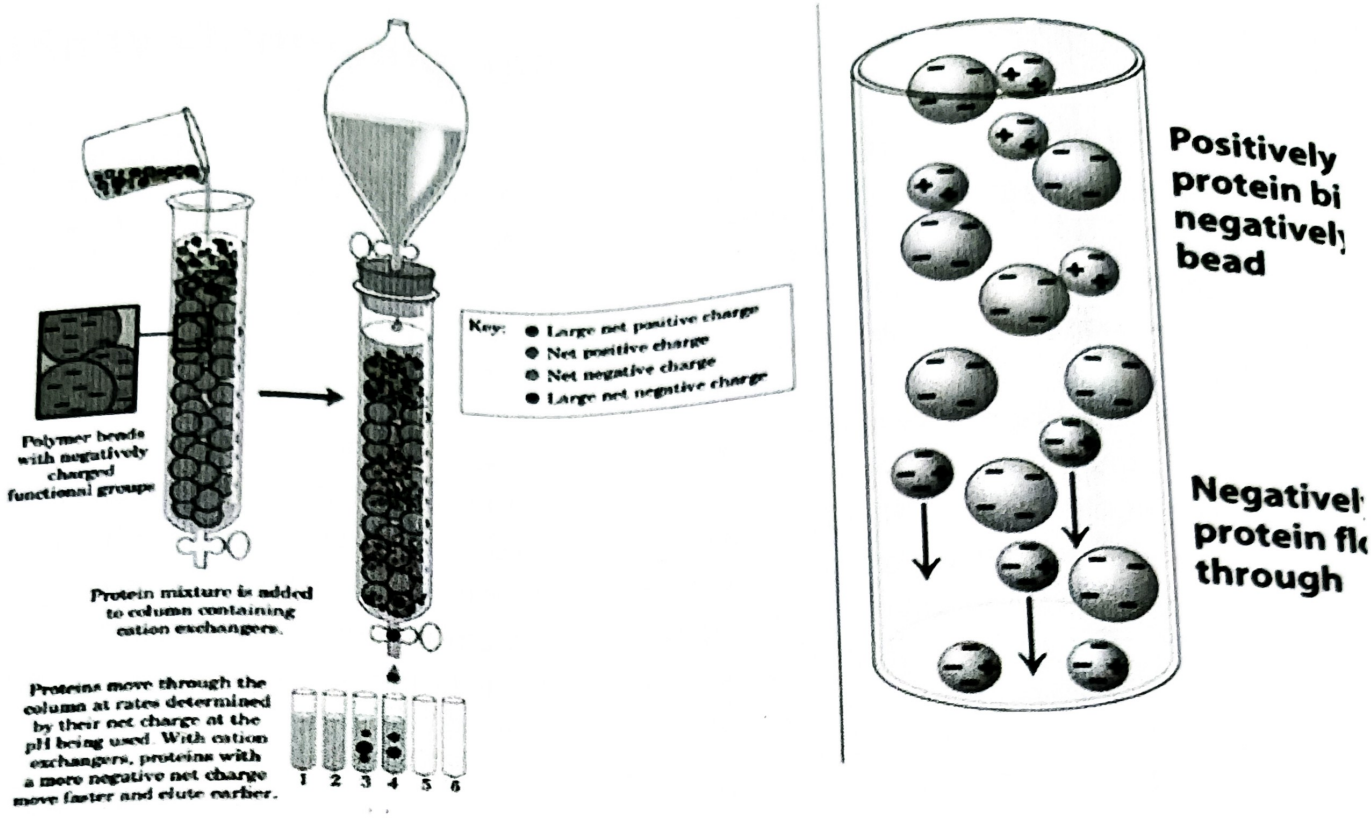
- The molecules in the sample are pumped through specialized columns containing such microporous packing material (gel).
- The basis of the separation is that molecules above a certain size are totally excluded from the pores, while smaller molecules access the interior of the pores partly or wholly.
- The flow of the mobile phase hence will cause larger molecules to pass through the column unhindered, without penetrating the gel matrix, whereas smaller molecules will be retarded according to their penetration of the gel.

Ion Exchange Chromatography

- **Chromatography** is the separation of a mixture of compounds into its individual components based on their relative interactions with an inert matrix.
- Ion exchange chromatography (or ion chromatography) is a process that allows the separation of ions and polar molecules based on their affinity to ion exchangers.
- The principle of separation is thus by reversible exchange of ions between the target ions present in the sample solution to the ions present on ion exchangers.
- In this process two types of exchangers i.e., cationic and anionic exchangers can be used.

1. **Cationic exchangers** possess negatively charged group, and these will attract positively charged cations. These exchangers are also called "Acidic ion exchange" materials, because their negative charges result from the ionization of acidic group.
2. **Anionic exchangers** have positively charged groups that will attract negatively charged anions. These are also called "Basic ion exchange" materials.

- Ion exchange chromatography is most often performed in the form of column chromatography. However, there are also thin-layer chromatographic methods that work basically based on the principle of ion exchange.



Working Principle of ion exchange chromatography

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

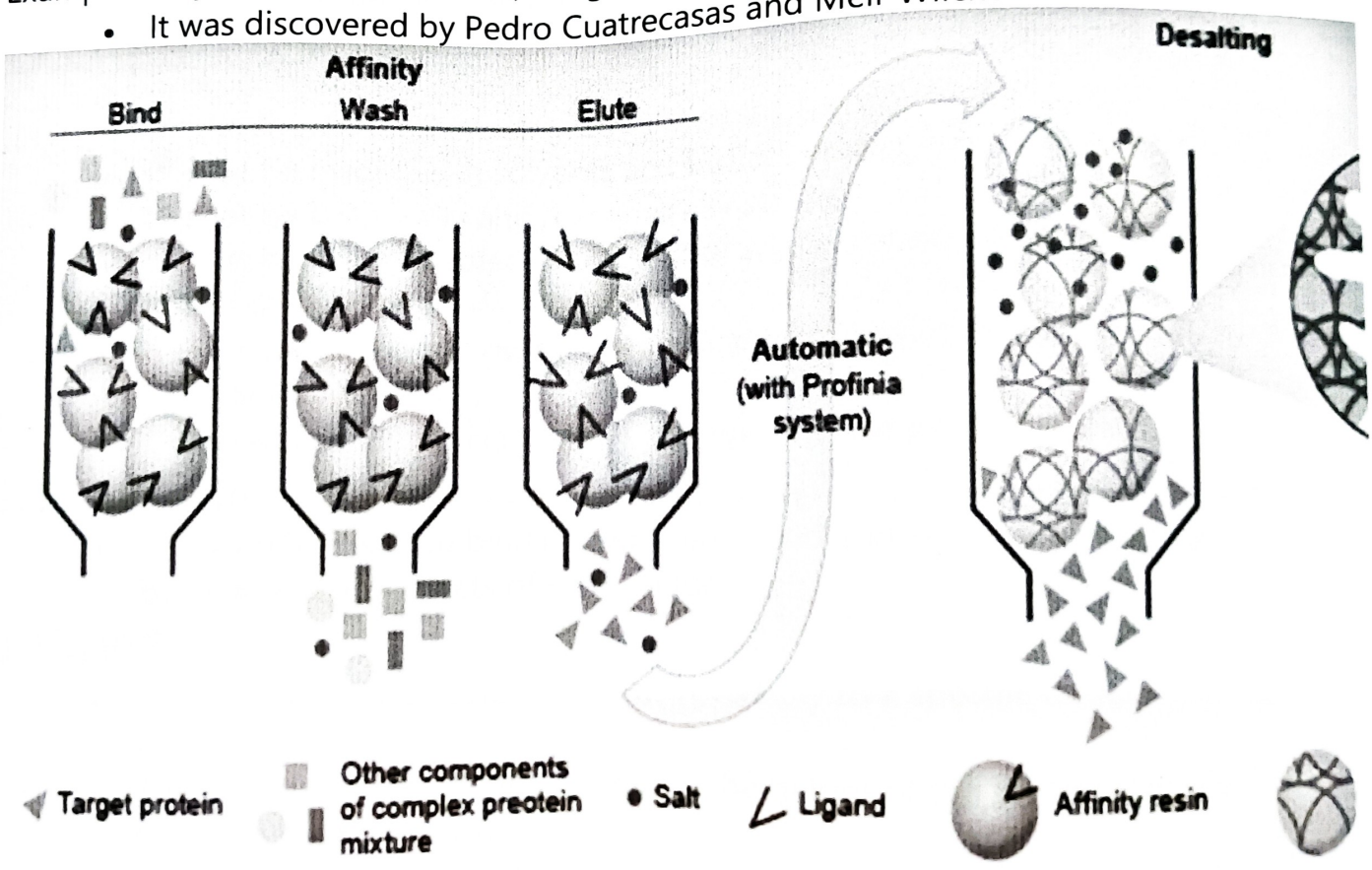
- The ion exchangers basically contain charged groups covalently linked to the surface of an insoluble matrix.
- The charged groups of the matrix can be positively or negatively charged.
- When suspended in an aqueous solution, the charged groups of the matrix will be surrounded by ions of the opposite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

Affinity Chromatography

- Chromatography is an important biophysical technique that enables
- the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis.
- It is a separation technique in which a mobile phase carrying a mixture is caused to move in contact with a selectively absorbent stationary phase.
- Affinity chromatography is a type of liquid **chromatography** for the separation, purification or specific analysis of sample components.
- It utilizes the reversible biological interaction or molecular recognition called affinity which refers to the attracting forced exerted in different degrees between atoms which cause them to remain in combination.

Example: Enzyme with an inhibitor, antigen with an antibody, etc.

- It was discovered by Pedro Cuatrecasas and Meir Wilcheck.



Principle of Affinity Chromatography

- The stationary phase consists of a support medium, on which the substrate (ligand) is bound covalently, in such a way that the reactive groups that are essential for binding of the target molecule are exposed.
- As the crude mixture of the substances is passed through the chromatography column, substances with binding site for the immobilized substrate bind to the stationary phase, while all other substances is eluted in the void volume of the column.
- Once the other substances are eluted, the bound target molecules can be eluted by methods such as including a competing ligand in the mobile phase or changing the pH, ionic strength or polarity conditions

Components of Affinity Chromatography

1. Matrix

- The matrix is an inert support to which a ligand can be directly or indirectly coupled.
- In order to for the matrix to be effective it must have certain characters:
- Matrix should be chemically and physically inert.
- It must be insoluble in solvents and buffers employed in the process
- It must be chemically and mechanically stable.
- It must be easily coupled to a ligand or spacer arm onto which the ligand can be attached.
- It must exhibit good flow properties and have a relatively large surface area for attachment.
- The most useful matrix materials are agarose and polyacrylamide.

2. Spacer arm

- It is used to improve binding between ligand and target molecule by overcoming any effects of steric hindrance.

3. Ligand

- It refers to the molecule that binds reversibly to a specific target molecule.
- The ligand can be selected only after the nature of the macromolecule to be isolated is known.
- When a hormone receptor protein is to be purified by affinity chromatography, the hormone itself is an ideal candidate for the ligand.

- For antibody isolation, an antigen or hapten may be used as ligand.
- If an enzyme is to be purified,a substrate analog, inhibitor, cofactor, or effector may be used as a the immobilized ligand.

Applications of Affinity Chromatography

- Affinity chromatography is one of the most useful methods for the separation and purification of specific products.
- It is essentially a sample pu**Colorimetr**technique, used primarily for biological molecules such as proteins.

Its major application includes:

- Separation of mixture of compounds.
- Removal of impurities or in purification process.
- In enzyme assays
- Detection of substrates
- Investigation of binding sites of enzymes
- In in vitro antigen-antibody reactions
- Detection of Single Nuceotide polymorphisms and mutations in nucleic acids

Unit-4. Spectroscopy and Tracer techniques

Electromagnetic radiation, Beer Lambert's law, Colorimetry and spectrophotometry, flame photometry, Tracer techniques: radio active isotopes, units of radiation, half life, beta and gamma emitters, use of radioisotopes in biology and ELISA

It is an magnetic disturbance traveling through space at the speed of light (2.998 × 108 m/s). It contains neither mass nor charge but travels in packets of radiant energy called photons, or quanta. Examples of EM radiation include radio waves and microwaves, as well as infrared, ultraviolet, gamma, and x-rays. Some sources of EM radiation include sources in the cosmos (e.g., the sun and stars), radioactive elements, and manufactured devices. EM exhibits a dual wave and particle nature

Electromagnetic radiation travels in a waveform at a constant speed. The wave characteristics of EM radiation are found in the relationship of velocity to wavelength (the straight line distance of a single cycle) and frequency (cycles per second, or hertz, Hz), expressed in the formula

$$c=\lambda v$$

where c = velocity, λ = wavelength, and v = frequency.

Because the velocity is constant, any increase in frequency results in a subsequent decrease in wavelength. Therefore, wavelength and frequency are inversely proportional. All forms of EM radiation are grouped according to their wavelengths into an electromagnetic spectrum, seen in Figure 1-3.

The particle-like nature of EM radiation manifests in the interaction of ionizing photons with matter. The amount of energy (E) found in a photon is equal to its frequency (v) times Planck's constant (h):

$$E=vh$$

Photon energy is directly proportional to photon frequency. Photon energy is measured in eV or keV (kilo-electron volts). The energy range for diagnostic x-rays is 40 to 150 keV. Gamma rays, x-rays, and some ultraviolet rays possess sufficient energy (>10 keV) to cause ionization.

The energy of EM radiation determines its usefulness for diagnostic imaging. Because of their extremely short wavelengths, gamma rays and x-rays are capable of penetrating large body parts. Gamma rays are used in radionuclide imaging. X-rays are used for plain film and computed tomography (CT) imaging. Visible light is applied to observe

and interpret images. Magnetic resonance imaging (MRI) uses radiofrequency EM radiation as a transmission medium

Beer-Lambert Law

The Beer-Lambert law, known by various names such as the Lambert-Beer law, Beer-Lambert–Bouguer law or the Beer's law states the following:

For a given material, the sample path length and concentration of the sample are directly proportional to the absorbance of the light.

Various Names for Beer-Lambert Law

The Beer-Lambert law is known by so many names because more than one law is involved.

- In 1729, Pierre Bouguer discovered the law.
- Later, in 1760, Johann Heinrich Lambert quoted Bouger's discovery saying that the absorbance of a sample is directly proportional to the path length of light. Although Lambert dint claim the discovery, he was often credited with it.
- In 1852, August Beer discovered a related law which stated that the absorbance is proportional to the concentration of the sample.

Beer-Lambert Law Equation

The Beer-Lambert law equation is as follows:

$$I=I_0e^{-\mu(x)}I=I0e-\mu(x)$$

Where,

I is the intensity

I_0 is the initial intensity

μ is the coefficient of absorption

x is the depth in meter

Beer-Lambert Law Applications

This law finds applications in various fields such as:

Analytical chemistry

This analysis mainly concentrates on the separation, quantification, and identification of matter by spectrophotometry. There is no involvement of extensive pre-processing of the sample to get the results. For example, bilirubin count in a blood sample can be determined by using a spectrophotometer.

In atmosphere

Solar or stellar radiation in the atmosphere can be described using this law. The law in atmospheric applications has a modified equation:

$$T=e^{-m(T_a+T_g+T_{RS}+T_{NO2}+T_w+T_{O3}+T_r+..)}T=e-m(Ta+Tg+TRS+TNO2+Tw+TO3+Tr+..)$$

Where,

a is the aerosols

g is the mixed gases

RS is the Raman scattering effect.

NO_2 is Nitrogen dioxide

w is the water vapour absorption

O_3 is Ozone

r is Rayleigh scattering

Colorimeter

A colorimeter is a device that is used in Colorimetry. It refers to a device which helps specific solutions to absorb a particular wavelength of light. The colorimeter is usually used to measure the concentration of a known solute in a given solution with the help of the Beer-Lambert law. The colorimeter was invented in the year 1870 by Louis J Duboscq.

Check out the derivation of Beer-Lambert law here.

Principle of Colorimeter

It is a photometric technique which states that when a beam of incident light of intensity I_o passes through a solution, the following occur:

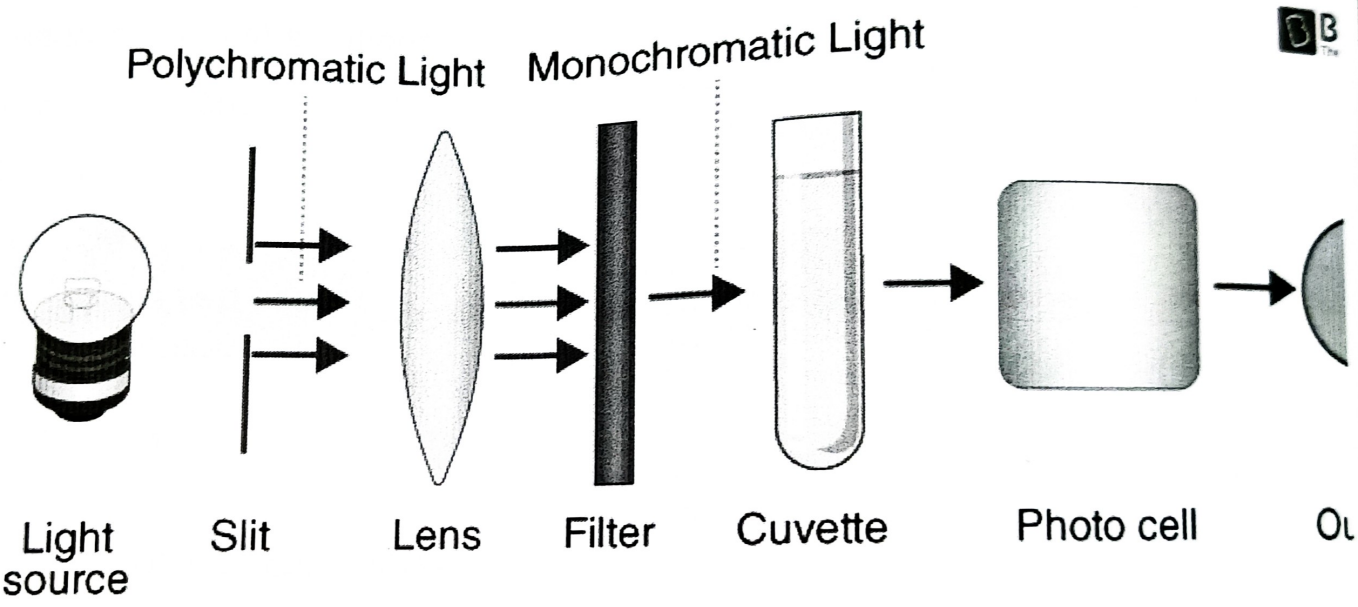
- A part of it is reflected which is denoted as I_r
- A part of it is absorbed which is denoted as I_a
- Rest of the light is transmitted and is denoted as I_t

Therefore, $I_o = I_r + I_a + I_t$

To determine I_a the measurement of I_o and I_t is sufficient therefore, I_r is eliminated. The amount of light reflected is kept constant to measure I_o and I_t.

Colorimeter is based on two fundamental laws of photometry

Diagram of Colorimeter



Working of Colorimeter

Step 1: Before starting the experiment it is important to calibrate the colorimeter. It is done by using the standard solutions of the known solute concentration that has to be determined. Fill the standard solutions in the cuvettes and place it in the cuvette holder of colorimeter.

Step 2: A light ray of a certain wavelength, which is specific for the assay is in the direction of the solution. The light passes through a series of different lenses and filters. The coloured light navigates with the help of lenses, and the filter helps to split a beam of light into different wavelengths allowing only the required wavelength to pass through it and reach the cuvette of the standard test solution.

Step 3: When the beam of light reaches' cuvette, it is transmitted, reflected, and absorbed by the solution. The transmitted ray falls on the photodetector system where it measures the intensity of transmitted light. It converts it into the electrical signals and sends it to the galvanometer.

Step 4: The electrical signals measured by the galvanometer are displayed in the digital form.

Step 5: Formula to determine substance concentration in test solution.

$A = \epsilon cl$

For standard and test solutions

ϵ and l are constant

$A_T = C_T$ ..... (i)

$A_S = C_S$ ..... (ii)

From the above two equations,

$A_T \times C_S = A_S \times C_T$

$C_T = (A_T/A_S) \times C_S$

Where,

C_T is the test solution concentration

A_T is the absorbance/optical density of test solution

C_S is the standard concentration

A_S is the absorbance / optical density of standard solution

Uses of Colorimeter

- It is used in laboratories and hospitals to estimate biochemical samples such as urine, cerebrospinal fluid, plasma, serum, etc.

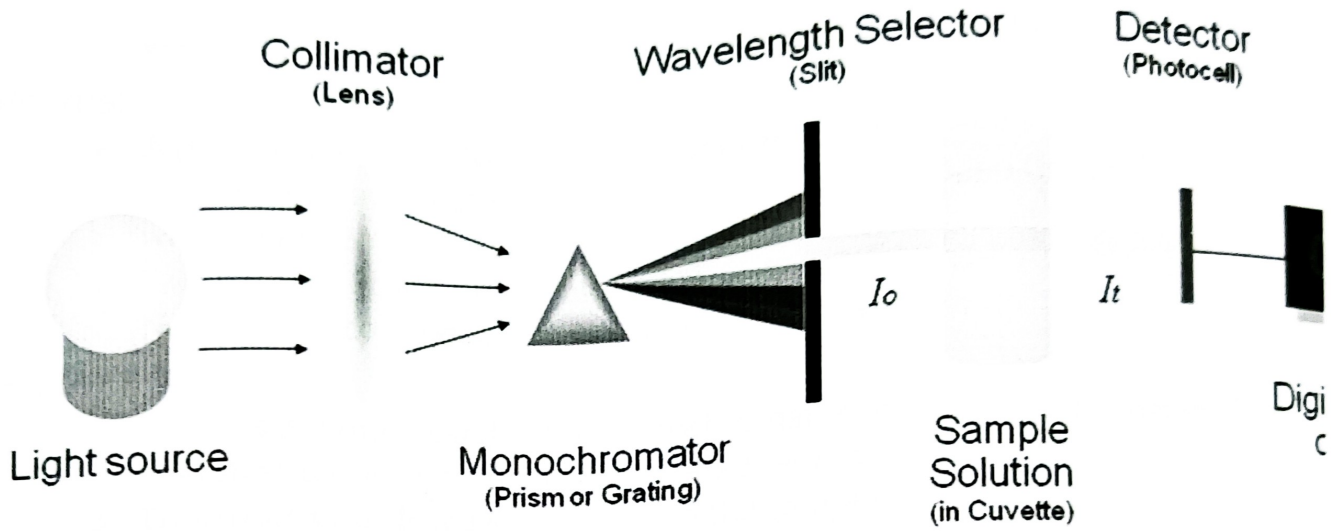
- It is used in the manufacturing of paints.
- It is used in textile and food industry.
- It is used in the quantitative analysis of proteins, glucose, and other biochemical compounds.
- It is used to test water quality.
- It is used to determine the concentration of haemoglobin in the blood.

Spectrophotometer-

- A spectrophotometer is an instrument that measures the amount of light absorbed by a sample.
- Spectrophotometer techniques are mostly used to measure the concentration of solutes in solution by measuring the amount of the light that is absorbed by the solution in a cuvette placed in the spectrophotometer.
- Scientist Arnold J. Beckman and his colleagues at the National Technologies Laboratory (NTL) invented the Beckman DU spectrophotometer in 1940.

Spectrophotometer

Principle, Instrumentation, Applications



Principle of Spectrophotometer

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

1. In the spectrophotometer, a prism (or) grating is used to split the incident beam into different wavelengths.
2. By suitable mechanisms, waves of specific wavelengths can be manipulated to fall on the test solution. The range of the wavelengths of the incident light can be as low as 1 to 2nm.
3. The spectrophotometer is useful for measuring the absorption spectrum of a compound, that is, the absorption of light by a solution at each wavelength.

Instrumentation of Spectrophotometer

The essential components of spectrophotometer instrumentation include:

1. A table and cheap radiant **energy source**

- Materials that can be excited to high energy states by a high voltage electric discharge (or) by electrical heating serve as excellent radiant energy sources.

2. A **monochromator**, to break the polychromatic radiation into component wavelength (or) bands of wavelengths.
- A monochromator resolves polychromatic radiation into its individual wavelengths and isolates these wavelengths into very narrow bands.

Prisms:

- A prism disperses polychromatic light from the source into its constituent wavelengths by virtue of its ability to reflect different wavelengths to a different extent
- Two types of Prisms are usually employed in commercial instruments. Namely, 600 cornu quartz prism and 300 Littrow Prism.

Grating:

- Gratings are often used in the monochromators of spectrophotometers operating ultraviolet, visible and infrared regions.

3. **Transport vessels** (cuvettes), to hold the sample
- Samples to be studied in the ultraviolet (or) visible region are usually glasses (or) solutions and are put in cells known as "CUVETTES".
- Cuvettes meant for the visible region are made up of either ordinary glass (or) sometimes Quartz.

4. A Photosensitive **detector** and an associated **readout system**
- Most detectors depend on the photoelectric effect. The current is then proportional to the light intensity and therefore a measure of it.
- Radiation detectors generate electronic signals which are proportional to the transmitter light.
- These signals need to be translated into a form that is easy to interpret.
- This is accomplished by using amplifiers, Ammeters, Potentiometers and Potentiometric recorders.

Applications

Some of the major applications of spectrophotometers include the following:

- Detection of concentration of substances
- Detection of impurities
- Structure elucidation of organic compounds
- Monitoring dissolved oxygen content in freshwater and marine ecosystems

- Characterization of proteins
- Detection of functional groups
- Respiratory gas analysis in hospitals
- Molecular weight determination of compounds

The visible and UV spectrophotometer may be used to identify classes of compounds in both the pure state and in biological preparations.

Spectrofluorometer

A **Spectrofluorometer** is an instrument which takes advantage of fluorescent properties of some compounds in order to provide information regarding their concentration and chemical environment in a sample. A certain excitation wavelength is selected, and the emission is observed either at a single wavelength, or a scan is performed to record the intensity versus wavelength, also called an emission spectrum.[1] The instrument is used in fluorescence spectroscopy.

Photoelectric flame photometer

photoelectric flame photometer is a device used in inorganic chemical analysis to determine the concentration of certain metal ions, among them sodium, potassium, lithium, and calcium. Group 1 and Group 2 metals are quite sensitive to Flame *Photometry* due to their low excitation energies.

In principle, it is a controlled flame test with the intensity of the flame color quantified by photoelectric circuitry. The intensity of the colour will depend on the energy that had been absorbed by the atoms that was sufficient to vaporise them. The sample is introduced to the flame at a constant rate. Filters select which colours the photometer detects and exclude the influence of other ions. Before use, the device requires calibration with a series of standard solutions of the ion to be tested.
Flame photometry is crude but cheap compared to flame emission spectroscopy or ICP-AES, where the emitted light is analysed with a monochromator. Its status is similar to that of the colorimeter (which uses filters) compared to the spectrophotometer (which uses a monochromator). It also has the range of metals that could be analysed and the limit of detection are also considered

Radioactive tracer

radioactive tracer, **radiotracer**, or **radioactive label**, is a chemical compound in which one or more atoms have been replaced by a radionuclide so by virtue of its radioactive decay it can be used to explore the mechanism of chemical reactions by tracing the path that the radioisotope follows from reactants to products. **Radiolabeling** or **radiotracing** is thus the radioactive form of isotopic labeling. In biological contexts, use of radioisotope tracers are sometimes called **radioisotope feeding** experiments.

Radioisotopes of hydrogen, carbon, phosphorus, sulfur, and iodine have been used extensively to trace the path of biochemical reactions. A radioactive tracer can also be used to track the distribution of a substance within a natural system such as a cell or tissue,[1] or as a flow tracer to track fluid flow. Radioactive tracers are also used to determine the location of fractures created by hydraulic fracturing in natural gas production.[2] Radioactive tracers form the basis of a variety of imaging systems, such as, PET scans, SPECT scans and technetium scans. Radiocarbon dating uses the naturally occurring carbon-14 isotope as an isotopic label.

SI Unit of Radioactivity

The SI unit of radioactivity is **becquerel (Bq)** and this term is named after Henri Becquerel. Unit of radioactivity is defined as:

The activity of a quantity of radioactive material where one decay takes place per second.

1 becquerel = 1 radioactive decay per second = 2.703×10^{-11}

Other Radioactivity Units:

There are usually three radioactivity units. An older radioactivity unit is the curie (Ci) and the name has been taken from **Pierre** and **Marie Curie**.

- It is defined as that quantity of any radioactive substance which gives $3.7 \times 10^{10} s^{-1}$ disintegration (dps).
- Sometimes **millicurie (mc)** and **microcurie (mc)** are also used.

Another unit is **Rutherford (rd)** and it is defined as the amount of radioactive substance which gives 10^6 disintegrations s^{-1} (dps).

1 curie = 3.7×10^{10} radioactive decays per second

1 becquerel = 1 radioactive decay per second = 2.703×10^{-11} Ci.

1 rutherford = 1.106 radionuclide decays per second

half-life

half-life, in radioactivity, the interval of time required for one-half of the atomic nuclei of a radioactive sample to decay (change spontaneously into other nuclear species by emitting particles and energy), or, equivalently, the time interval required for the number of disintegrations per second of a radioactive material to decrease by one-half.

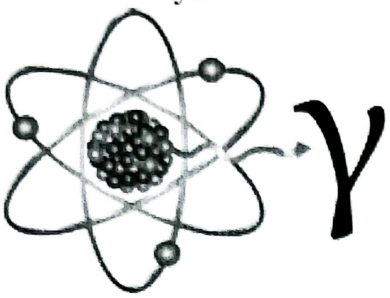
The radioactive isotope cobalt-60, which is used for radiotherapy, has, for example, a half-life of 5.26 years. Thus after that interval, a sample originally containing 8 g of cobalt-60 would contain only 4 g of cobalt-60 and would emit only half as much radiation. After another interval of 5.26 years, the sample would contain only 2 g of cobalt-60. Neither the volume nor the mass of the original sample visibly decreases, however, because the unstable cobalt-60 nuclei decay into stable nickel-60 nuclei, which remain with the still-undecayed cobalt.

Beta Particles

Beta particles (β) are small, fast-moving particles with a negative electrical charge that are emitted from an atom's nucleus during radioactive decay. These particles are emitted by certain unstable atoms such as hydrogen-3 (tritium), carbon-14 and strontium-90.

Beta particles are more penetrating than alpha particles, but are less damaging to living tissue and DNA because the ionizations they produce are more widely spaced. They travel farther in air than alpha particles, but can be stopped by a layer of clothing or by a thin layer of a substance such as aluminum. Some beta particles are capable of penetrating the skin and causing damage such as skin burns. However, as with alpha-emitters, beta-emitters are most hazardous when they are inhaled or swallowed.

Gamma Rays



Gamma rays (γ) are weightless packets of energy called photons. Unlike alpha and beta particles, which have both energy and mass, gamma rays are pure energy. Gamma rays are similar to visible light, but have much higher energy. Gamma rays are often emitted along with alpha or beta particles during radioactive decay.

Gamma rays are a radiation hazard for the entire body. They can easily penetrate barriers that can stop alpha and beta particles, such as skin and clothing. Gamma rays have so much penetrating power that several inches of a dense material like lead, or even a few feet of concrete may be required to stop them. Gamma rays can pass completely through the human body; as they pass through, they can cause ionizations that damage tissue and DNA

Radioactivity is generally used in life sciences for highly sensitive and direct measurements of biological phenomena, and for visualizing the location of biomolecules radiolabelled with a radioisotope.
All atoms exist as stable or unstable isotopes and the latter decay at a given half-life ranging from attoseconds to billions of years; radioisotopes useful to biological and experimental systems have half-lives ranging from minutes to months. In the case of the hydrogen isotope tritium (half-life = 12.3 years) and carbon-14 (half-life = 5,730 years), these isotopes derive their importance from all organic life containing hydrogen and carbon and therefore can be used to study countless living processes, reactions, and phenomena. Most short lived isotopes are produced in cyclotrons, linear particle accelerators, or nuclear reactors and their relatively short half-lives give them high maximum theoretical specific activities which is useful for detection in biological systems

ELISA

ELISA stands for enzyme-linked immunoassay. It is a commonly used laboratory test to detect antibodies in the blood. An antibody is a protein produced by the body's immune system when it detects harmful substances, called antigens.

In the most simple form of an ELISA, antigens from the sample to be tested are attached to a surface. Then, a matching antibody is applied over the surface so it can bind the antigen. This antibody is linked to an enzyme and then any unbound antibodies are removed. In the final step, a substance containing the enzyme's substrate is added. If there was binding, the subsequent reaction produces a detectable signal, most commonly a color change.

Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support (usually a polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA). After the antigen is immobilized, the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme or can itself be detected by a secondary antibody that is linked to an enzyme through bioconjugation. Between each step, the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are non-specifically bound. After the final wash step, the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample.

Of note, ELISA can perform other forms of ligand binding assays instead of strictly "immuno" assays, though the name carried the original "immuno" because of the common use and history of development of this method. The technique essentially requires any ligating reagent that can be immobilized on the solid phase along with a detection reagent that will bind specifically and use an enzyme to generate a signal that can be properly quantified. In between the washes, only the ligand and its specific binding counterparts remain specifically bound or "immunosorbed" by antigen-antibody interactions to the solid phase, while the nonspecific or unbound components are washed away. Unlike other spectrophotometric wet lab assay formats where the same reaction well (e.g., a cuvette) can be reused after washing, the ELISA plates have the reaction products immunosorbed on the solid phase, which is part of the plate, and so are not easily reusable.

Unit-5.Electrophorosis

Unit-5 - Electrophoresis.

Electrophoresis: principles and applications of paper, polyacrylamide(native and SDS), and agarose gel electrophoresis, isoelectric focusing, immune-electrophoresis – types and applications

Electrophoresis

Electrophoresis is a laboratory technique used to separate DNA, RNA, or protein molecules based on their size and electrical charge. An electric current is used to move molecules to be separated through a gel. Pores in the gel work like a sieve, allowing smaller molecules to move faster than larger molecules. The conditions used during electrophoresis can be adjusted to separate molecules in a desired size range.

In electrophoresis, there are two primary factors that control how quickly a particle can move and in what direction. First, the charge on the sample matters. Negatively charged species are attracted to the positive pole of an electric field, while positively charged species are attracted to the negative end. A neutral species may be ionized if the field is strong enough. Otherwise, it doesn't tend to be affected.

The other factor is particle size. Small ions and molecules can move through a gel or liquid much more quickly than larger ones.

While a charged particle is attracted to an opposite charge in an electric field, there are other forces that affect how a molecule moves. Friction and the electrostatic retardation force slow the progress of particles through the fluid or gel. In the case of gel electrophoresis, the concentration of the gel can be controlled to determine the pore size of the gel matrix, which influences mobility. A liquid buffer is also present, which controls the pH of the environment.

As molecules are pulled through a liquid or gel, the medium heats up. This can denature the molecules as well as affect the rate of movement. The voltage is controlled to try to minimize the time required to separate molecules, while maintaining a good separation and keeping the chemical species intact. Sometimes electrophoresis is performed in a refrigerator to help compensate for the heat.

Types of Electrophoresis

Electrophoresis encompasses several related analytical techniques. Examples include:

- **affinity electrophoresis** - Affinity electrophoresis is a type of electrophoresis in which particles are separated based on complex formation or bio specific interaction
- **capillary electrophoresis** - Capillary electrophoresis is a type of electrophoresis used to separate ions depending mainly on the atomic radius, charge, and viscosity. As the name suggests, this technique is commonly performed in a glass tube. It yields quick results and a high resolution separation.
- **gel electrophoresis** - Gel electrophoresis is a widely used type of electrophoresis in which molecules are separated by movement through a porous gel under the influence of an electrical field. The two main gel materials are agarose and polyacrylamide. Gel electrophoresis is used to separate nucleic acids (DNA and RNA), nucleic acid fragments, and protien

Polyacrylamide gel electrophoresis (**PAGE**) is a technique widely used in **biochemistry**, **forensic chemistry**, **genetics**, **molecular biology** and **biotechnology** to separate biological **macromolecules**, usually **proteins** or **nucleic acids**, according to their **electrophoretic mobility**. Electrophoretic mobility is a function of the length, conformation and charge of the molecule. **Polyacrylamide** gel electrophoresis is a powerful tool used to analyze RNA samples. When polyacrylamide gel is denatured after electrophoresis, it provides information on the sample composition of the RNA species

Hydration of acrylonitrile results in formation of acrylamide molecules (C_3H_5NO) by nitrile hydratase.[2] Acrylamide monomer is in a powder state before addition of water. Acrylamide is toxic to the human nervous system, therefore all safety measures must be followed when working with it. Acrylamide is soluble in water and upon addition of free-radical initiators it polymerizes resulting in formation of polyacrylamide.[2] It is useful to make polyacrylamide gel via acrylamide hydration because pore size can be regulated. Increased concentrations of acrylamide result in decreased pore size after polymerization. Polyacrylamide gel with small pores helps to examine smaller molecules better since the small molecules can enter the pores and travel through the gel while large molecules get trapped at the pore openings.
As with all forms of gel electrophoresis, molecules may be run in their native state, preserving the molecules' higher-order structure. This method is called **native-PAGE.** Alternatively, a chemical denaturant may be added to remove this structure and turn the molecule into an unstructured molecule whose mobility depends only on its length (because the protein-SDS complexes all have a similar mass-to-charge ratio). This procedure is called SDS-PAGE. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a method of separating molecules based on the difference of their molecular weight. At the pH at which gel electrophoresis is carried out the SDS molecules are negatively charged and bind to proteins in a set ratio, approximately one molecule of SDS for every 2 amino acids.[3]:164–79 In this way, the detergent provides all proteins with a uniform charge-to-mass ratio. By binding to the proteins the detergent destroys their secondary, tertiary and/or quaternary structure denaturing them and turning them into

negatively charged linear polypeptide chains. When subjected to an electric field in PAGE, the negatively charged polypeptide chains travel toward the anode with different mobility. Their mobility, or the distance traveled by molecules, is inversely proportional to the logarithm of their molecular weight.[4] By comparing the relative ratio of the distance traveled by each protein to the length of the gel (Rf) one can make conclusions about the relative molecular weight of the proteins, where the length of the gel is determined by the distance traveled by a small molecule like a tracking dye.[5]

For nucleic acids, urea is the most commonly used denaturant. For proteins, sodium dodecyl sulfate (SDS) is an anionic detergent applied to protein samples to coat proteins in order to impart two negative charges (from every SDS molecule) to every two amino acids of the denatured protein.[3]:161–3 2-Mercaptoethanol may also be used to disrupt the disulfide bonds found between the protein complexes, which helps further denature the protein. In most proteins, the binding of SDS to the polypeptide chains impart an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis. Proteins that have a greater hydrophobic content – for instance, many membrane proteins, and those that interact with surfactants in their native environment – are intrinsically harder to treat accurately using this method, due to the greater variability in the ratio of bound SDS.[6] Procedurally, using both Native and SDS-PAGE together can be used to purify and to separate the various subunits of the protein. Native-PAGE keeps the oligomeric form intact and will show a band on the gel that is representative of the level of activity. SDS-PAGE will denature and separate the oligomeric form into its monomers, showing bands that are representative of their molecular weights. These bands can be used to identify and assess the purity of the protein.

Agarose gel electrophoresis is a method of **gel electrophoresis** used in **biochemistry**, **molecular biology**, **genetics**, and **clinical chemistry** to separate a mixed population of macromolecules such as DNA or proteins in a matrix of **agarose**, one of the two main components of **agar**. The proteins may be separated by charge and/or size (**isoelectric focusing** agarose electrophoresis is essentially size independent), and the **DNA** and **RNA** fragments by length.[1] Biomolecules are separated by applying an **electric field** to move the charged molecules through an agarose matrix, and the biomolecules are separated by size in the agarose gel matrix

Agarose gel is easy to cast, has relatively fewer charged groups, and is particularly suitable for separating DNA of size range most often encountered in laboratories, which accounts for the popularity of its use. The separated DNA may be viewed with stain, most commonly under UV light, and the DNA fragments can be extracted from the gel with relative ease. Most agarose gels used are between 0.7–2% dissolved in a suitable electrophoresis buffer.

Isoelectric focusing

Isoelectric focusing (**IEF**), also known as **electrofocusing**, is a technique for separating different **molecules** by differences in their **isoelectric point** (pI).[1][2] It is a type of zone **electrophoresis** usually performed on **proteins** in a **gel** that takes advantage of the fact that overall charge on the molecule of interest is a function of the **pH** of its surroundings.

IEF involves adding an **ampholyte** solution into **immobilized pH gradient** (IPG) gels. IPGs are the acrylamide gel matrix co-polymerized with the pH gradient, which result in completely stable gradients except the most alkaline (>12) pH values. The immobilized pH gradient is obtained by the continuous change in the ratio of *immobilines*. An immobiline is a weak acid or base defined by its pK value

Immunoelectrophoresis-

- Immunoelectrophoresis refers to **precipitation** in agar under an electric field.
- It is a process of a combination of immuno-diffusion and electrophoresis.
- An antigen mixture is first separated into its component parts by electrophoresis and then tested by double immuno-diffusion.
- Antigens are placed into wells cut in a gel (without antibody) and electrophoresed. A trough is then cut in the gel into which antibodies are placed.
- The antibodies diffuse laterally to meet diffusing antigens, and lattice formation and precipitation occur permitting determination of the nature of the antigens.
- The term "Immunoelectrophoresis" was first coined by Grabar and Williams in 1953.

Principle of Immunoelectrophoresis

When an electric current is applied to a slide layered with gel, the antigen mixture placed in wells is separated into individual antigen components according to their charge and size. Following electrophoresis, the separated antigens are reacted with specific antisera placed in troughs parallel to the electrophoretic migration and diffusion is allowed to occur. Antiserum present in the trough moves toward the antigen components resulting in the formation of separate precipitin lines in 18-24 hrs, each indicating reaction between individual proteins with its antibody.

Applications of Immunoelectrophoresis

- The test helps in the identification and approximate quantization of various proteins present in the serum. Immunoelectrophoresis created a breakthrough in protein identification and in immunology.
- Immunoelectrophoresis is used in patients with suspected monoclonal and polyclonal gammopathies.
- The method is used to detect normal as well as abnormal proteins, such as myeloma proteins in human serum.
- Used to analyze complex protein mixtures containing different antigens.
- The medical diagnostic use is of value where certain proteins are suspected of being absent (e.g., hypogammaglobulinemia) or overproduced (e.g., multiple myeloma).
- This method is useful to monitor antigen and antigen-antibody purity and to identify a single antigen in a mixture of antigens.
- Immunoelectrophoresis is an older method for qualitative analysis of M-proteins in serum and urine.
- Immunoelectrophoresis aids in the diagnosis and evaluation of the therapeutic response in many disease states affecting the immune system.