UNIT: I BASIC INSTRUMENTATION

Weighing Balance:

A Beam balance (or Beam scale) is a device to measure weight or mass. These are also known as mass scales, weight scales, mass balance, weight balance, or simply scale, balance, or balance scale. The traditional scale consists of two plates

Principle:

The basis of the rapid and exact working method of our Weigh Cells is the Principle of Electro Magnetic Force Restoration (EMFR).

The weighing pan is attached to an electromagnetic coil, through which electric current is flowing. The coil floats in a magnetic field created by an amplifier. The amplifier maintains the right current to keep the lever (remember, balance operate on the lever principle) balanced with the mass on the pan. As more weight is applied to the pan, the current is increased to maintain the level's position. The counteracting force that is created is measured and "translated" by various electronics to obtain a readable result. The resulting electrical current is then "translated" into a displayed number that is shown to the user.

Protocol & Working

Methods for Weighing Two common methods used to weigh a chemical are "weighing by difference" or "taring the balance".

Weighing by Difference

The mass of the chemical is calculated by subtracting the weight of an empty container from the total weight of the container and chemical. Place an empty container on the pan, close the draft shield doors and wait a few seconds for the display to read a constant mass. Record the mass of the empty container to three decimal places; do not round off. Remove the container from the pan, spoon the chemical into the container, and record the mass of both container and chemical. The mass of the chemical is the difference of the two recorded masses. Remember to handle the container with tongs or tissue; moisture from fingers can cause an error in the apparent mass.

Taring the Balance

The balance is set to ignore the mass of the container so the mass of the added chemical is measured directly. Place the empty container on the pan and close the draft shield. Wait a few seconds for the display to register a constant mass. Press the bar so the display reads "0.000 g". The balance is now set to "ignore" the mass of the container (a process called "taring" the balance). Now if a chemical is added to the container, the balance displays only the mass of that chemical. When the container and chemical is removed from the pan a negative weight will be displayed. (This negative weight is the mass of the original empty container which the balance was instructed to ignore.) To erase this weight from memory, press the bar again. The display should read "0.000g".

Applications:

Used for weighing Chemicals and other Biological specimen precisely

pH Meter

A **pH meter** is a scientific instrument that measures the hydrogen-ion activity in water-based solutions, indicating its acidity or alkalinity expressed as pH.

Principle

pH meter basically works on the fact that interface of two liquids produces a electric potential which can be measured. In other words when a liquid inside an enclosure made of glass is placed inside a solution other than that liquid, there exists an electrochemical potential between the two liquids.

Components

It is basically an electrode consisting of 4 components:

A measuring electrode:

It is a tube made up of glass and consists of a thin glass bulb welded to it, filled up with Potassium Chloride solution of known pH of 7. It also contains a block of silver chloride attached to a silver element. It generates the voltage used to measure pH of the unknown solution.

A Reference Electrode:

It is a glass tube consisting of potassium chloride solution in intimate contact with a mercury chloride block at the end of the potassium chloride. It is used to provide a stable zero voltage connection to the complete the whole circuit.

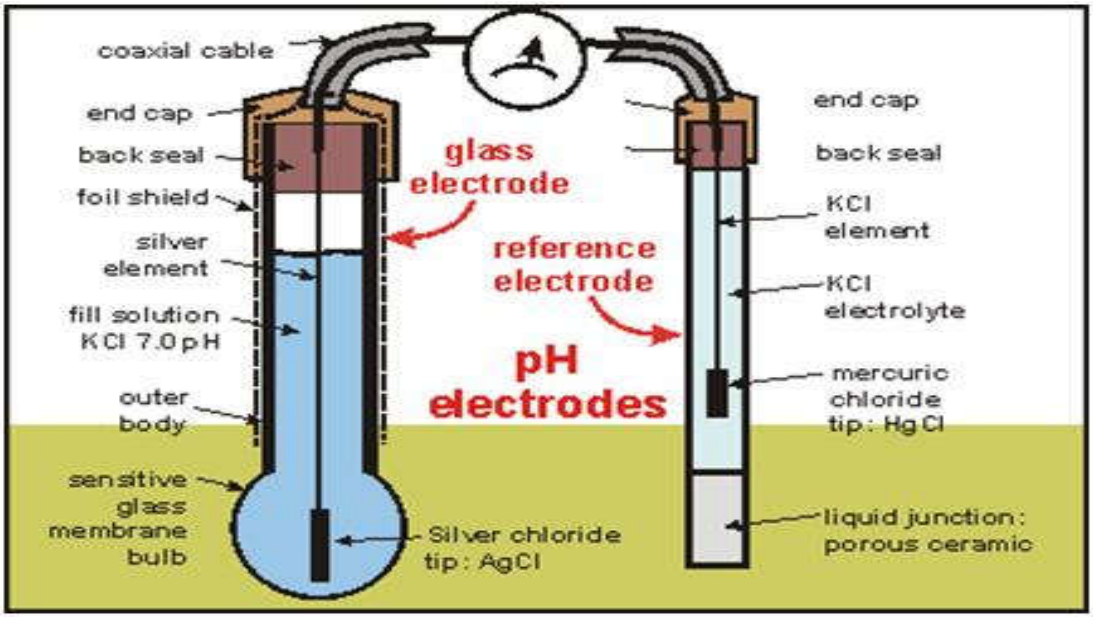
Preamplifier:

It is a signal conditioning device and converts the high impedance pH electrode signal to a low impedance signal. It strengthens and stabilizes the signal, making it less susceptible to electrical noise.

Transmitter or Analyzer:

It is used to display the sensor's electrical signal and consists of a temperature sensor to compensate for the change in temperature.

Protocol & Working:



The electrode is placed inside the beaker filled with a solution whose pH is to be measure. The glass bulb welded at the end of the measurement electrode consists of lithium ions doped to it which makes it act as an ion selective barrier and allows the hydrogen ions from the unknown solution to migrate through the barrier and interacts with the glass, developing an electrochemical potential related to the hydrogen ion concentration. The measurement electrode potential thus changes with the hydrogen ion concentration. On the other hand, the reference electrode potential doesn't changes with the hydrogen ion concentration and provides a stable potential against which the

measuring electrode is compared. It consists of a neutral solution which is allowed to exchange ions with the unknown solution through a porous separator, thus forming low resistance connection to complete the whole circuit. The potential difference between the two electrodes gives a direct measurement of the hydrogen ion concentration or pH of the system and is first preamplified to strengthen it and then given to the voltmeter.

$$U = EpH - Eref$$

EpH – Voltage potential of measurement electrode

Eref – Voltage potential of reference electrode

Applications

it is used to measure acidity levels in water & wastewater treatment, medicines, chemicals substances & petrochemicals. However, it is mostly used in food & drinks, drugs, mining, water treatment plants, and other manufacturing. Major end-users are food science, pharmaceuticals & biotechnology, environmental research & pollution centre, and others.

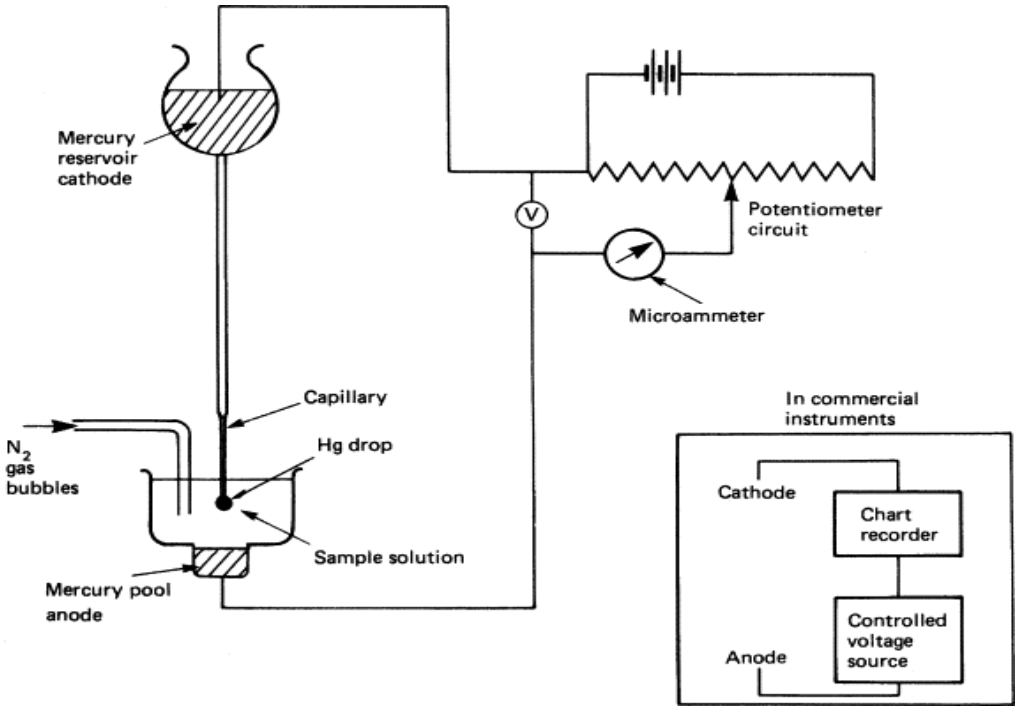
Polarography

It is a type of voltammetry where the working electrode is a dropping mercury electrode (DME) or a static mercury drop electrode (SMDE), which are useful for their wide cathodic ranges and renewable surfaces.

Principle

The simple principle of polarography is the study of solutions or of electrode processes by means of electrolysis with two electrodes, one polarizable and one unpolarizable, the former formed by mercury regularly dropping from a capillary tube. Polarography is a specific type of measurement that falls into the general category of linear-sweep voltammetry where the electrode potential is altered in a linear fashion from the initial potential to the final potential. As a linear sweep method controlled by convection/diffusion mass transport, the current vs. potential response of a polarographic experiment has the typical sigmoidal shape. What makes polarography different from other linear sweep voltammetry measurements is that polarography makes use of the dropping mercury electrode (DME) or the static mercury drop electrode.

Protocol & Working:



The solution to be analyzed is placed in a glass cell containing two electrodes. One electrode consists of a glass capillary tube from which mercury slowly flows in drops, and the other is commonly a pool of mercury.

The cell is connected in series with a galvanometer (for measuring the flow of current) in an electrical circuit that contains a battery or other source of direct current and a device for varying the voltage applied to the electrodes from zero up to about two volts. With the dropping mercury electrode connected (usually) to the negative side of the polarizing voltage, the voltage is increased by small increments, and the corresponding current is observed on the galvanometer.

The current is very small until the applied voltage is increased to a value large enough to cause the substance being determined to be reduced at the dropping mercury electrode. The current increases rapidly at first as the applied voltage is increased above this critical value but gradually attains a limiting value and remains more or less constant as the voltage is increased further.

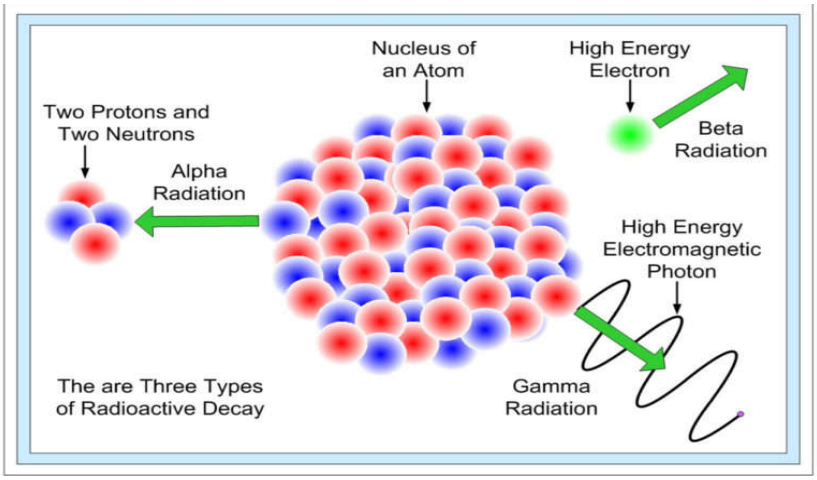
Applications:

- ✓ Polarography is used widely employed for the analysis of trace metals in the alloys including ultra-pure metals, minerals/metallurgy, environmental analysis, foodstuffs, beverages and body-fluids, toxicology and clinical analysis.
- ✓ In the biological systems it is used to determine vitamins, alkaloids, hormones, terpenoid substances and so on.
- ✓ In medical field polarography is used to analyze natural colouring substance of drugs and pharmaceutical preparations, determining pesticide or herbicide residues in food stuffs, and in the structure determination of many organic compounds.
- ✓ Since a fresh, smooth, reproducible drop is produced at regular intervals of time contamination or surface poisoning will be limited.

Radioactivity

Radioactivity refers to the particles which are emitted (radiations) from nuclei as a result of nuclear instability. The most common types of radiation are called alpha, beta, and gamma radiation

Types of Radioactive Decay



α Decay

In alpha decay, an energetic helium ion (alpha particle) is ejected, leaving a daughter nucleus of atomic number two less than the parent and of atomic mass number four less than the parent.

An example is the decay (symbolized by an arrow) of the abundant isotope of uranium, $_{235}U$, to a thorium daughter plus an alpha particle

It is Composed of two protons and two neutrons, the alpha particle is a nucleus of the element helium

β Decay

In beta decay, an energetic negative electron is emitted, producing a daughter nucleus of one higher atomic number and the same mass number.

An example is the decay uranium, $_{235}U$, to a Neptunium daughter plus an beta particle

γ Decay

Gamma decay, usually accompanies alpha or beta decay. Gamma rays are photons and are without rest mass or charge. Alpha or beta decay may either simply proceed directly to the ground (lowest energy) state of the daughter nucleus or precede wholly or partly to higher energy states (excited states) of the daughter. In the latter case, gamma emission may occur as the excited states transform to lower energy states of the same nucleus.

An example of gamma ray production due to radionuclide decay is the decay scheme for cobalt-60

Alpha decay

$$^{235}_{92}U \longrightarrow {}^{231}_{90}Th + {}^{4}_{2}He$$

Beta decay

$$^{235}_{92}U \longrightarrow {}^{235}_{93}Np + {}^{0}_{-1}e$$

Gamma decay

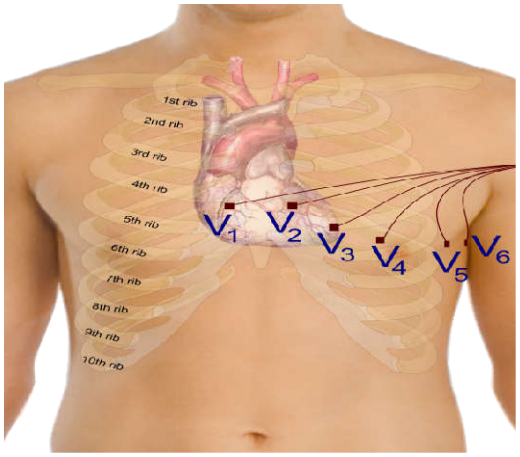
$$^{235}_{92}U \longrightarrow {}^{231}_{92}Th + {}^{0}_{0}\gamma$$

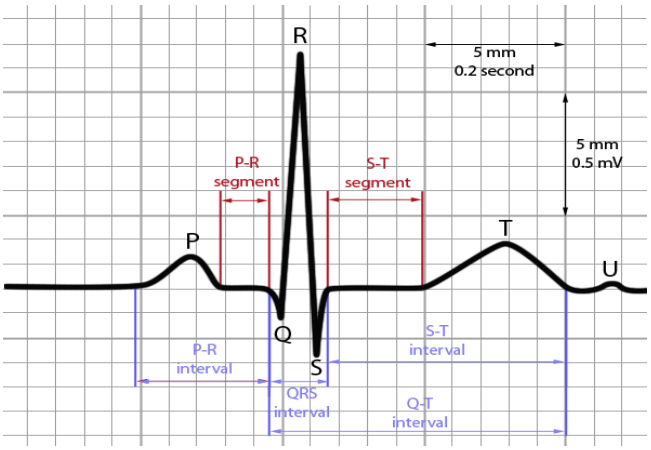
Electrocardiography

It is the process of producing an **electrocardiogram** (**ECG**), a recording or a graph of voltage versus time of the electrical activity of the heart using electrodes placed on the skin. These electrodes detect the small electrical changes that are a consequence of cardiac muscle depolarization followed by repolarization during each cardiac cycle

Electrodes & Leads

Electrodes are the actual conductive pads attached to the body surface. Any pair of electrodes can measure the electrical potential difference between the two corresponding locations of attachment and lead is the physical connection between the electrodes





RA	On the right arm, avoiding thick muscle.
LA	In the same location where RA was placed, but on the left arm.
RL	On the right leg, lower end of inner aspect of calf muscle. (Avoid bony prominences)
LL	In the same location where RL was placed, but on the left leg.
V_1	In the fourth intercostal space (between ribs 4 and 5) just to the right of the sternum (breastbone).
V_2	In the fourth intercostal space (between ribs 4 and 5) just to the left of the sternum.
V_3	Between leads V_2 and V_4.
V_4	In the fifth intercostal space (between ribs 5 and 6) in the mid-clavicular line.
V_5	Horizontally even with V_4, in the left anterior axillary line.
V_6	Horizontally even with V_4 and V_5 in the mid-axillary line.

Commonly, 10 electrodes attached to the body are used to form 12 ECG leads, with each lead measuring a specific electrical potential difference

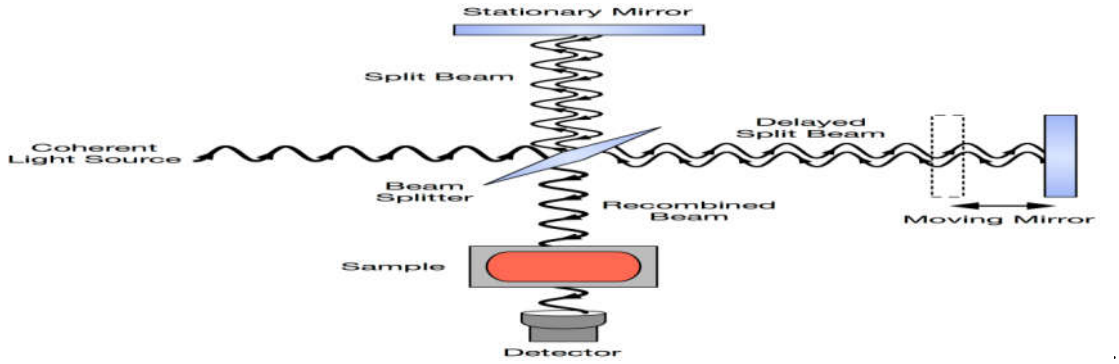
FTIR

Fourier-transform infrared spectroscopy (FTIR) is a technique used to obtain an infrared spectrum of absorption or emission of a solid, liquid or gas.

Principle:

FTIR spectrometer operates on a different principle called Fourier transform.

Instrumentation & Working



Like other Spectroscopy, we still have a source, a sample and a detector, but everything else is different. Now, we send all the source energy through an interferometer and onto the sample. In every scan, all source radiation gets to the sample! The interferometer is a fundamentally different piece of equipment than a monochromator. The light passes through a beamsplitter, which sends the light in two directions at right angles. One beam goes to a stationary mirror then back to the beamsplitter. The other goes to a moving mirror. The motion of the mirror makes the total path length variable versus that taken by the stationary-mirror beam. When the two meet up again at the beamsplitter, they recombine, but the difference in path lengths creates constructive and destructive interference:

An interferogram:

The recombined beam passes through the sample. The sample absorbs all the different wavelengths characteristic of its spectrum, and this subtracts specific wavelengths from the interferogram. The detector now reports variation in energy versus time for all wavelengths simultaneously. A laser beam is superimposed to provide a reference for the instrument operation.

Energy versus time is an odd way to record a spectrum, until you recognize the relationship between *time* and *frequency*: they are reciprocals! A mathematical function called a Fourier transform allows us to convert an intensity-vs.-time spectrum into an intensity-vs.-frequency spectrum.

Applications:

- ✓ Identification of functional group and structure elucidation.
- ✓ Identification of substances.
- ✓ Studying the progress of the reaction.
- ✓ Detection of impurities.
- ✓ Quantitative analysis.

UNIT: II MICROSCOPY

Light Microscope

The optical microscope, often referred to as the light microscope, is a type of microscope that uses visible light and a system of lenses to magnify images of small subjects.

A simple microscope uses a lens to enlarge an object through angular magnification alone, giving the viewer an erect enlarged virtual image.

The use of a single convex lens or groups of lenses is found in simple magnification devices such as the magnifying glass, loupes, and eyepieces for telescopes and microscopes. A simple microscope is actually a convex lens of small focal length, which is used for seeing the magnified images of small objects.

Principle

A simple microscope works on the principle that when a tiny object is placed within its focus, a virtual, erect and magnified image of the object is formed at the least distance of distinct vision from the eye held close to the lens.

Magnification of Simple Microscope

The magnifying power of a simple microscope is given by:

$$M = 1 + D/F$$

Where, D = least distance of distinct vision

F = focal length of the convex lens

- The focal length of the convex lens should be small because smaller the focal length of the lens, greater will be its magnifying power.
- The maximum magnification of a simple microscope is about 10, which means that the object will appear 10 times larger by using the simple microscope of maximum magnification.

Instrumentation

The parts of a simple microscope may be:

(i) Mechanical parts

(ii) Optical parts

Mechanical Parts

These parts support the optical parts and help in their adjustment for focusing the object.

Metal Stand

- It has a heavy base plate and a vertical rod fitted to it, which provide support and stability to other parts of the microscope.

Stage

- It is a rectangular metal plate fitted to the vertical rod.
- It has a central hole for light to pass from below.
- Slide with specimen to be observed is kept on the stage, in such a way that, the specimen remains just on the central hole.
- Some microscopes have a pair of slanting wings projecting from the both the sides of the stage. They provide support to hand for manipulating the object.

Optical Parts

The components of the optical parts are as follows:

Mirror

- A plano-convex mirror is fitted below the stage to the vertical rod by means of a frame. It focuses the surrounding light on the object to be observed.

Lens

- A biconvex lens is fitted above the stage, to the vertical rod, by means of a frame.
- It magnifies the size of the object and the enlarged virtual image formed is observed by keeping the eye above it. For proper focusing, the lens can be moved up and down by the frame.

Applications

- It is usually used for study of microscopic algae, fungi and biological specimen.
- The simple microscope is commonly used by watch makers to see the magnified view of small parts of a watch.
- It is also used by the jewelers to see the magnified view of the fine parts of jewellery.
- Simple microscope is used to see the enlarged image of letters of a book, textures of fibers or threads of a cloth.

- Simple microscope is used to see the magnified view of different particles of different types of soils.
- It is used by palmists to see enlarged view of the lines of our hand.
- Simple microscope is used by skin specialists to find out various diseases of skin.
- It is also used to see the details of stamp and engravings.

Light microscopes use visible light or ultraviolet rays to illuminate specimens. They include brightfield, darkfield, phase-contrast, and fluorescent instruments.

This is similar to the ordinary light microscope; however, the condenser system is modified so that the specimen is not illuminated directly.

Bright-field Microscopy

It is the simplest of all the optical microscopy illumination techniques. Sample illumination is transmitted (i.e., illuminated from below and observed from above) white light, and contrast in the sample is caused by attenuation of the transmitted light in dense areas of the sample.

Bright-field microscopy is the simplest of a range of techniques used for illumination of samples in light microscopes, and its simplicity makes it a popular technique. The typical appearance of a bright-field microscopy image is a dark sample on a bright background, hence the name.

Principle

In brightfield microscopy a specimen is placed on the stage of the microscope and incandescent light from the microscope's light source is aimed at a lens beneath the specimen. This lens is called a condenser.

The condenser usually contains an aperture diaphragm to control and focus light on the specimen; light passes through the specimen and then is collected by an objective lens situated in a turret above the stage.

The objective magnifies the light and transmits it to an oracular lens or eyepiece and into the user's eyes. Some of the light is absorbed by stains, pigmentation, or dense areas of the sample and this contrast allows you to see the specimen.

Advantages

- Simplicity of setup with only basic equipment required.
- Living cells can be seen with bright-field microscopes
- Brightfield microscopy is very simple to use with fewer adjustments needed to be made to view specimens.
- Some specimens can be viewed without staining and the optics used in the brightfield technique don't alter the color of the specimen.
- It is adaptable with new technology and optional pieces of equipment can be implemented with brightfield illumination to give versatility in the tasks it can perform

Dark field Microscope

The condenser directs the light obliquely so that the light is deflected or scattered from the specimen, which then appears bright against a dark background.

Living specimens may be observed more readily with darkfield than with brightfield microscopy.

Principle

- A dark field microscope is arranged so that the light source is blocked off, causing light to scatter as it hits the specimen.
- This is ideal for making objects with refractive values similar to the background appear bright against a dark background.

- When light hits an object, rays are scattered in all azimuths or directions. The design of the dark field microscope is such that it removes the dispersed light, or zeroth order, so that only the scattered beams hit the sample.
- The introduction of a condenser and/or stop below the stage ensures that these light rays will hit the specimen at different angles, rather than as a direct light source above/below the object.
- The result is a "cone of light" where rays are diffracted, reflected and/or refracted off the object, ultimately, allowing the individual to view a specimen in dark field.

Applications

- Dark-field microscopy is a very simple yet an effective technique.
- It is well suited for uses involving live and unstained biological samples, such as a smear from a tissue culture or individual, water-borne, single-celled organisms.
- Considering the simplicity of the setup, the quality of images obtained from this technique is impressive.
- Dark-field microscopy techniques are almost entirely free of artifacts, due to the nature of the process.
- A researcher can achieve dark field by making modifications to his/her microscope.

Phase Contrast Microscope

- Unstained living cells absorb practically no light. Poor light absorption results in extremely small differences in the intensity distribution in the image.
- This makes the cells barely, or not at all, visible in a brightfield microscope.
- Phase-contrast microscopy is an optical microscopy technique that converts phase shifts in light passing through a transparent specimen to brightness changes in the image. It was first described in 1934 by Dutch physicist Frits Zernike

Principle

When light passes through cells, small phase shifts occur, which are invisible to the human eye. In a phase contrast microscope, these phase shifts are converted into changes in amplitude, which can be observed as differences in image contrast.

Instrumentation

- Partially coherent illumination produced by the tungsten-halogen lamp is directed through a collector lens and focused on a specialized annulus (labeled condenser annulus) positioned in the substage condenser front focal plane.
- Wavefronts passing through the annulus illuminate the specimen and either pass through undeviated or are diffracted and retarded in phase by structures and phase gradients present in the specimen.
- Undeviated and diffracted light collected by the objective is segregated at the rear focal plane by a phase plate and focused at the intermediate image plane to form the final phase contrast image observed in the eyepieces.

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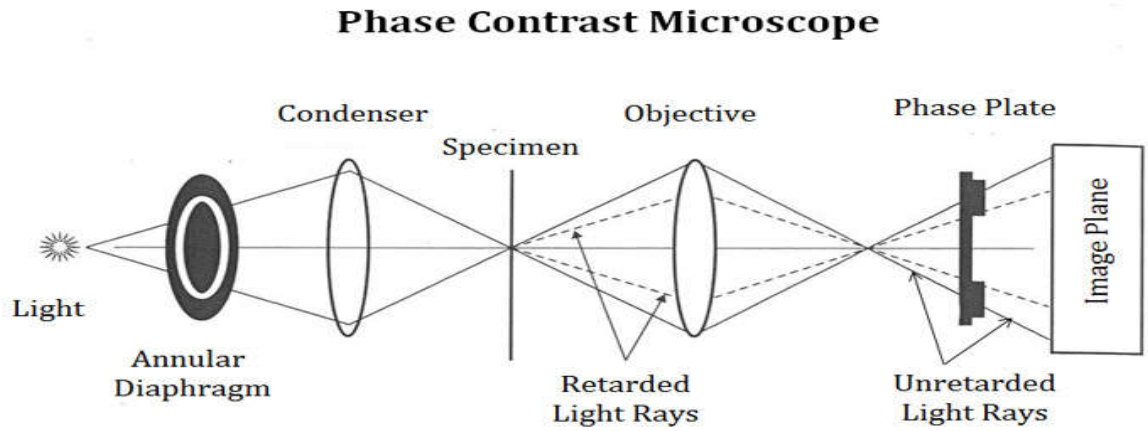


Working

Phase-contrast microscopy is basically a specially designed light microscope with all the basic parts in addition to which an annular phase plate and n annular diaphragm are fitted.

The Annular Diaphragm

- It is situated below the condenser.
- It is made up of a circular disc having a circular annular groove.
- The light rays are allowed to pass through the annular groove.
- Through the annular groove of the annular diaphragm, the light rays fall on the specimen or object to be studied.
- At the back focal plane of the objective develops an image.
- The annular phase plate is placed at this back focal plane.

The Phase Plate

- It is either a negative phase plate having a thick circular area or a positive phase plate having a thin circular groove.
- This thick or thin area in the phase plate is called the conjugate area.
- The phase plate is a transparent disc.
- With the help of the annular diaphragm and the phase plate, the phase contrast is obtained in this microscope.
- This is obtained by separating the direct rays from the diffracted rays.
- The direct light rays pass through the annular groove whereas the diffracted light rays pass through the region outside the groove.
- Depending upon the different refractive indices of different cell components, the object to be studied shows different degree of contrast in this microscope

Applications

To produce high-contrast images of transparent specimens, such as

- living cells (usually in culture),
- microorganisms,
- thin tissue slices,
- lithographic patterns,
- fibers,
- latex dispersions,
- glass fragments, and
- subcellular particles (including nuclei and other organelles)

Fluorescence Microscope

A fluorescence microscope is an optical microscope that uses fluorescence and phosphorescence instead of, or in addition to, reflection and absorption to study properties of organic or inorganic substances.

- Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation while phosphorescence is a specific type of photoluminescence related to fluorescence.

- Unlike fluorescence, a phosphorescent material does not immediately re-emit the radiation it absorbs.
- The fluorescence microscope was devised in the early part of the twentieth century by August Köhler, Carl Reichert, and Heinrich Lehmann, among others

Principle

1. Most cellular components are colorless and cannot be clearly distinguished under a microscope. The basic premise of fluorescence microscopy is to stain the components with dyes.
2. Fluorescent dyes, also known as fluorophores of fluorochromes, are molecules that absorb excitation light at a given wavelength (generally UV), and after a short delay emit light at a longer wavelength. The delay between absorption and emission is negligible, generally on the order of nanoseconds.
3. The emitted light can then be filtered from the excitation light to reveal the location of the fluorophores.
    - Fluorescence microscopy uses a much higher intensity light to illuminate the sample. This light excites fluorescence species in the sample, which then emit light of a longer wavelength.
    - The image produced is based on the second light source or the emission wavelength of the fluorescent species — rathe rthan from the light originally used to illuminate, and excite, the sample.

Working

Light of the excitation wavelength is focused on the specimen through the objective lens. The fluorescence emitted by the specimen is focused to the detector by the objective. Since most of the excitation light is transmitted through the specimen, only reflected excitatory light reaches the objective together with the emitted light.

Forms

The “fluorescence microscope” refers to any microscope that uses fluorescence to generate an image, whether it is a more 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 fluorescent image.

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)

Instrmentation

Typical components of a fluorescence microscope are:

Fluorescent dyes (Fluorophore)

- A fluorophore is a fluorescent chemical compound that can re-emit light upon light excitation.
- Fluorophores typically contain several combined aromatic groups, or plane or cyclic molecules with several π bonds.
- Many fluorescent stains have been designed for a range of biological molecules.
- Some of these are small molecules which are intrinsically fluorescent and bind a biological molecule of interest. Major examples of these are nucleic acid stains like DAPI and Hoechst, phalloidin which is used to stain actin fibres in mammalian cells.

A light source

- Four main types of light source are used, including xenon arc lamps or mercury-vapor lamps with an excitation filter, lasers, and high- power LEDs.
- Lasers are mostly used for complex fluorescence microscopy techniques, while xenon lamps, and mercury lamps, and LEDs with a dichroic excitation filter are commonly used for wide field epifluorescence microscopes.

The excitation filter

- The exciter is typically a bandpass filter that passes only the wavelengths absorbed by the fluorophore, thus minimizing excitation of other sources of fluorescence and blocking excitation light in the fluorescence emission band.

The dichroic mirror

- A dichroic filter or thin- film filter, is a very accurate color filter used to selectively pass light of a small range of colors while reflecting other colors.

The emission filter

- The emitter is typically a bandpass filter that passes only the wavelengths emitted by the fluorophore and blocks all undesired light outside this band – especially the excitation light.

- By blocking unwanted excitation energy (including UV and IR) or sample and system autofluorescence, optical filters ensure the darkest background.

Applications

- To identify structures in fixed and live biological samples.
- Fluorescence microscopy is a common tool for today's life science research because it allows the use of multicolour staining, labelling of structures within cells, and the measurement of the physiological state of a cell.

Electron Microscope

An electron microscope is a microscope that uses a beam of accelerated electrons as a source of illumination.

- It is a special type of microscope having a high resolution of images, able to magnify objects in nanometres, which are formed by controlled use of electrons in vacuum captured on a phosphorescent screen.
- Ernst Ruska (1906-1988), a German engineer and academic professor, built the first Electron Microscope in 1931, and the same principles behind his prototype still govern modern EMs.

Principle

Electron microscopes use signals arising from the interaction of an electron beam with the sample to obtain information about structure, morphology and composition.

- The electron gun generates electrons.
- Two sets of condenser lens focuses the electron beam on the specimen and then into a thin tight beam.
- To move electrons down the column, an accelerating voltage (mostly between 100 kV-1000 kV) is applied between tungsten filament and anode.
- The specimen to be examined is made extremely thin, at least 200 times thinner than those used in optical microscope. Ultra thin sections of 20-100 nm are cut which is already placed on the specimen holder.
- The electronic beam passes through the specimen and electrons are scattered depending upon the thickness or refractive index of different parts of the specimen.

- The denser regions in the specimen scatter more electrons and therefore appear darker in the image since fewer electrons strike that area of the screen. In contrast, transparent regions are brighter.
- The electron beam coming out of the specimen passes to objective lens, which has high power and forms the intermediate magnified image.
- The ocular lenses then produce the final further magnified image.

Instrumentation

EM is in the form of a tall vacuum column which is vertically mounted. It has the following components:

Electron gun

- Electron gun is a heated tungsten filament, which generates electrons.

Electromagnetic lenses

- Condenser lens focuses the electron beam on the specimen. A second condenser lens forms the electrons into a thin tight beam.
- The electron beam coming out of the specimen passes down the second of magnetic coils called objective lens, which has high power and forms the intermediate magnified image.
- A third set of magnetic lenses called projector (ocular) lenses produce the final further magnified image.
- Each of these lenses acts as image magnifier all the while maintaining an incredible level of details and resolution.

Specimen Holder

The specimen holder is an extremely thin film of carbon or collodion held by a metal grid.

Image viewing and Recording System

- The final image is projected on a fluorescent screen.
- Below the fluorescent screen is a camera for recording the image.

TEM (Transistion Electron Microscope)

The transmission electron microscope is used to view thin specimens through which electrons can pass generating a projection image.

- The TEM is analogous in many ways to the conventional (compound) light microscope.
- TEM is used, among other things, to image the interior of cells (in thin sections), the structure of protein molecules (contrasted by metal shadowing), the organization of molecules in viruses and cytoskeletal filaments (prepared by the negative staining technique), and the arrangement of protein molecules in cell membranes (by freeze-fracture).

SEM (Scanning Electron Microscope)

- Conventional scanning electron microscopy depends on the emission of secondary electrons from the surface of a specimen.
- Because of its great depth of focus, a scanning electron microscope is the EM analog of a stereo light microscope.
- It provides detailed images of the surfaces of cells and whole organisms that are not possible by TEM. It can also be used for particle counting and size determination, and for process control.
- It is termed a scanning electron microscope because the image is formed by scanning a focused electron beam onto the surface of the specimen in a raster pattern

Applications

- Electron microscopes are used to investigate the ultra structure 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.
- Science of microbiology owes its development to electron microscope.

Confocal Microscopy

It is the most frequently confocal laser scanning microscopy (CLSM) or laser confocal scanning microscopy (LCSM), is an optical imaging technique for increasing optical resolution and contrast of a micrograph by means of using a spatial pinhole to block out-of-focus light in image formation. Capturing multiple two-dimensional images at different depths in a sample enables the reconstruction of three-dimensional structures (a process known as optical sectioning) within an object. This technique is used extensively in the scientific and industrial communities and typical applications are in life sciences, semiconductor inspection and materials science.

Principle

Light travels through the sample under a conventional microscope as far into the specimen as it can penetrate, while a confocal microscope only focuses a smaller beam of light at one narrow depth level at a time. The CLSM achieves a controlled and highly limited depth of focus.

Methodology

Four types of confocal microscopes are commercially available:

Confocal laser scanning microscopes use multiple mirrors (typically 2 or 3 scanning linearly along the x- and the y- axes) to scan the laser across the sample and "descan" the image across a fixed pinhole and detector.

Spinning-disk (Nipkow disk) confocal microscopes use a series of moving pinholes on a disc to scan spots of light. Since a series of pinholes scans an area in parallel, each pinhole is allowed to hover over a specific area for a longer amount of time thereby reducing the excitation energy needed to illuminate a sample when compared to laser scanning microscopes. Decreased excitation energy reduces phototoxicity and photobleaching of a sample often making it the preferred system for imaging live cells or organisms.

Microlens enhanced or **dual spinning-disk** confocal microscopes work under the same principles as spinning-disk confocal microscopes except a second spinning-disk containing micro-lenses is placed before the spinning-disk containing the pinholes. Every pinhole has an associated microlens. The micro-lenses act to capture a broad band of light

and focus it into each pinhole significantly increasing the amount of light directed into each pinhole and reducing the amount of light blocked by the spinning-disk. Microlens enhanced confocal microscopes are therefore significantly more sensitive than standard spinning-disk systems. Yokogawa Electric invented this technology in 1992.

Programmable array microscopes (PAM) use an electronically controlled spatial light modulator (SLM) that produces a set of moving pinholes. The SLM is a device containing an array of pixels with some property (opacity, reflectivity or optical rotation) of the individual pixels that can be adjusted electronically. The SLM contains microelectromechanical mirrors or liquid crystal components. The image is usually acquired by a charge coupled device (CCD) camera.

Applications

Biology and medicine

Clinically, CLSM is used in the evaluation of various eye diseases, and is particularly useful for imaging, qualitative analysis, and quantification of endothelial cells of the cornea. It is used for localizing and identifying the presence of filamentary fungal elements in the corneal stroma in cases of keratomycosis, enabling rapid diagnosis and thereby early institution of definitive therapy. Research into CLSM techniques for endoscopic procedures (endomicroscopy) is also showing promise. the pharmaceutical industry, it was recommended to follow the manufacturing process of thin film pharmaceutical forms, to control the quality and uniformity of the drug distribution.

Optics and crystallography

CLSM is used as the data retrieval mechanism in some 3D optical data storage systems and has helped determine the age of the Magdalen papyrus.

UNIT: III SPECTROSCOPY

Calorimeter

A colorimeter is a device used in colorimetry.

In scientific fields the word generally refers to the device that measures the absorbance of particular wavelengths of light by a specific solution. This device is commonly used to determine the concentration of a known solute in a given solution by the application of the Beer-Lambert law, which states that the concentration of a solute is proportional to the absorbance.

The essential parts of a colorimeter are:

A light source (often an ordinary low-voltage filament lamp); an adjustable aperture; a set of colored filters; a cuvette to hold the working solution; a detector (usually a photoresistor) to measure the transmitted light; a meter to display the output from the detector.

In addition, there may be:

A voltage regulator, to protect the instrument from fluctuations in mains voltage; a second light path, cuvette and detector. This enables comparison between the working solution and a "blank", consisting of pure solvent, to improve accuracy.

Filters

Changeable Filters are used in the colorimeter to select the wavelength which the solute absorbs the most, in order to maximize accuracy. The usual wavelength range is from 400 to 700 [nanometer] (nm). If it is necessary to operate in the [ultraviolet]range then some modifications to the colorimeter are needed. In modern colorimeters the filament lamp and filters may be replaced by several (light-emitting diode)of different colors..

Cuvettes

In a manual colorimeter the cuvettes are inserted and removed by hand. An automated colorimeter (as used in an AutoAnalyzer) is fitted with a flowcell through which solution flows continuously.

Output

The output from a colorimeter may be displayed by an analogue or digital meter and may be shown as transmittance (a linear scale from 0-100%) or as absorbance (a logarithmic scale from zero to infinity). The useful range of the absorbance scale is from 0-2 but it is desirable to keep within the range 0-1 because, above 1, the results become unreliable due to scattering of light.

In addition, the output may be sent to a chart recorder, data logger, or computer.

Spectrophotometer

A spectrophotometer is a special instrument that measures how much light a substance absorbs. Every substance will transmit (reflect back) and absorb light slightly differently. Like how a fingerprint identifies each individual human, knowing exactly how much red (or green, or blue, etc.) gets absorbed allows us to identify and quantify different materials.

Parts of Spectrophotometer

There are 7 essential parts of a spectrophotometer

Light source – In spectrophotometer three different sources of light are commonly used to produce light of different wavelength. The most common source of light used in the spectrophotometer for the visible spectrum is a tungsten lamp. For Ultraviolet radiation, commonly used sources of are the hydrogen lamp and the deuterium lamp. Nernst filament or globar is the most satisfactory sources of IR (Infrared) radiation.

Monochromator – To select the particular wavelength, prism or diffraction grating is used to split the light from the light source.

Sample holder – Test tube or Cuvettes are used to hold the colored solutions. They are made up of glass at a visible wavelength.

Beam splitter – It is present only in double beam spectrophotometer. It is used to split the single beam of light coming from the light source into two beams.

Mirror – It is also present only and double beam spectrophotometer. It is used to the right direction to the splitted light from the beam splitter.

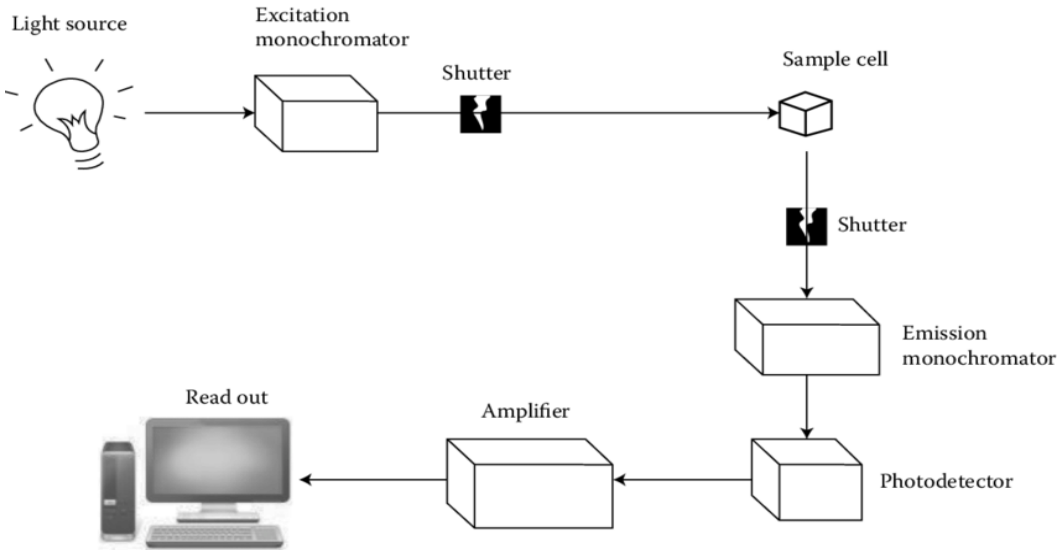
Photodetector system – When light falls on the detector system, an electric current is generated that reflects the galvanometer reading.

Measuring device – The current from the detector is fed to the measuring device – the galvanometer. The meter reading is directly proportional to the intensity of light.

Working of the Spectrophotometer

When using a Spectrophotometer, it requires being calibrated first which is done by using the standard solutions of the known concentration of the solute that has to be determined in the test solution. For this, the standard solutions are filled in the Cuvettes and placed in the Cuvette holder in the spectrophotometer that is similar to the colorimeter.

There is a ray of light with a certain wavelength that is specific for the assay is directed towards the solution. Before reaching the solution the ray of light passes through a series of the diffraction grating, prism, and mirrors. These mirrors are used for navigation of the light in the spectrophotometer and the prism splits the beam of light into different wavelength and the diffraction grating allows the required wavelength to pass through it and reaches the Cuvette containing the standard or Test solutions. It analyzes the reflected light and compares with a predetermined standard solution.



When the monochromatic light (light of one wavelength) reaches the Cuvette some of the light is reflected, some part of the light is absorbed by the solution and the remaining part is transmitted through the solution which falls on the photodetector system. The

photodetector system measures the intensity of transmitted light and converts it into the electrical signals that are sent to the galvanometer.

The galvanometer measures the electrical signals and displays it in the digital form. That digital representation of the electrical signals is the absorbance or optical density of the solution analyzed.

If the absorption of the solution is higher than there will be more light absorbed by the solution and if the absorption of the solution is low then more lights will be transmitted through the solution which affects the galvanometer reading and corresponds to the concentration of the solute in the solution.

By putting all the values in the formula given in the below section one can easily determine the concentration of the solution.

In double beam spectrophotometers, the beam splitters are present which splits the monochromatic light into two beams one for the standard solution and the other for test solution. In this, the absorbance of Standard and the Test solution can be measured at the same time and any no. of test solutions can be analyzed against one standard. It gives more accurate and precise results, eliminates the errors which occur due to the fluctuations in the light output and the sensitivity of the detector.

Applications of the Spectrophotometer

The spectrophotometer is commonly used for the determination of the concentration of colored as well as colorless compounds by measuring the optical density or its absorbance.

It can also be used for the determination of the course of the reaction by measuring the rate of formation and disappearance of the light absorbing compound in the range of the visible & UV region of electromagnetic spectrum.

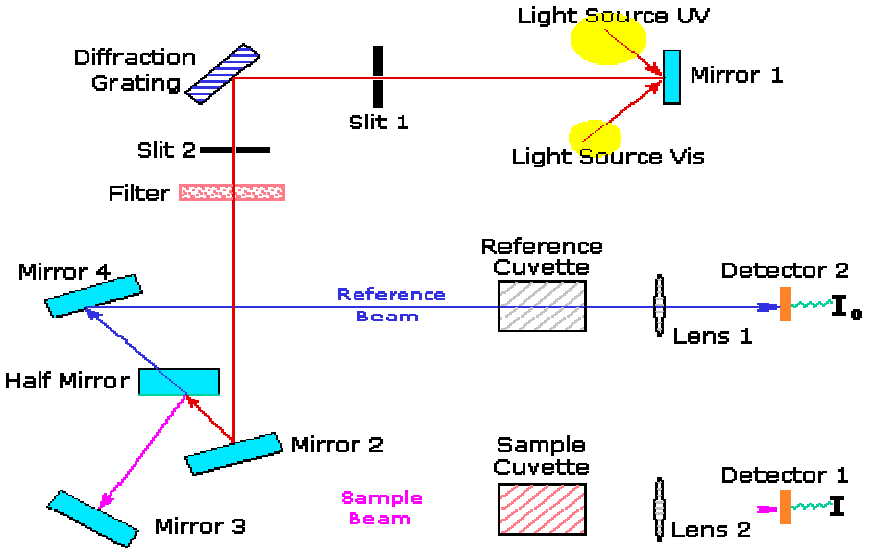
By spectrophotometer, a compound can be identified by determining the absorption spectrum in the visible region of the light spectrum as well as the UV region of the electromagnetic spectrum

Ultraviolet–Visible Spectroscopy or Ultraviolet–Visible Spectrophotometry

(UV–Vis or UV/Vis) refers to absorption spectroscopy or reflectance spectroscopy in part of the ultraviolet and the full, adjacent visible spectral regions. This means it uses light in the visible and adjacent ranges

Principle

Molecules containing bonding and non-bonding electrons (n-electrons) can absorb energy in the form of ultraviolet or visible light to excite these electrons to higher anti-bonding molecular orbitals. The more easily excited the electrons



Instrumentation and working of UV spectroscopy

Instrumentation and working of the UV spectrometers can be studied simultaneously. Most of the modern UV spectrometers consist of the following parts-

Light Source- Tungsten filament lamps and Hydrogen-Deuterium lamps are most widely used and suitable light source as they cover the whole UV region. Tungsten filament lamps are rich in red radiations; more specifically they emit the radiations of 375 nm, while the intensity of Hydrogen-Deuterium lamps falls below 375 nm.

Monochromator- Monochromators generally composed of prisms and slits. The most of the spectrophotometers are double beam spectrophotometers. The radiation emitted from the primary source is dispersed with the help of rotating prisms. The various wavelengths of the light source which are separated by the prism are then selected by the slits such the rotation of the prism results in a series of continuously increasing wavelength

to pass through the slits for recording purpose. The beam selected by the slit is monochromatic and further divided into two beams with the help of another prism.

Sample and reference cells- One of the two divided beams is passed through the sample solution and second beam is passé through the reference solution. Both sample and reference solution are contained in the cells. These cells are made of either silica or quartz. Glass can't be used for the cells as it also absorbs light in the UV region.

Detector- Generally two photocells serve the purpose of detector in UV spectroscopy. One of the photocell receives the beam from sample cell and second detector receives the beam from the reference. The intensity of the radiation from the reference cell is stronger than the beam of sample cell. This results in the generation of pulsating or alternating currents in the photocells.

Amplifier- The alternating current generated in the photocells is transferred to the amplifier. The amplifier is coupled to a small servometer. Generally current generated in the photocells is of very low intensity, the main purpose of amplifier is to amplify the signals many times so we can get clear and recordable signals.

Recording devices- Most of the time amplifier is coupled to a pen recorder which is connected to the computer. Computer stores all the data generated and produces the spectrum of the desired compound

Applications of UV Spectroscopy

1. **Detection of functional groups**- UV spectroscopy is used to detect the presence or absence of chromophore (isolated covalently bonded group that shows a characteristic absorption in the **ultraviolet or visible region 200-800 nm**) in the compound, where this may be benzyl, nitryl, Bromo or iodo atoms.

2. **Detection of extent of conjugation**- The extent of conjugation in the polyenes can be detected with the help of UV spectroscopy. With the increase in double bonds the absorption shifts towards the longer wavelength. If the double bond is increased by 8 in the polyenes then that polyene appears visible to the human eye as the absorption comes in the visible region.

3. **Identification of an unknown compound**- An unknown compound can be identified with the help of UV spectroscopy. The spectrum of unknown compound is

compared with the spectrum of a reference compound and if both the spectrums coincide then it confirms the identification of the unknown substance.

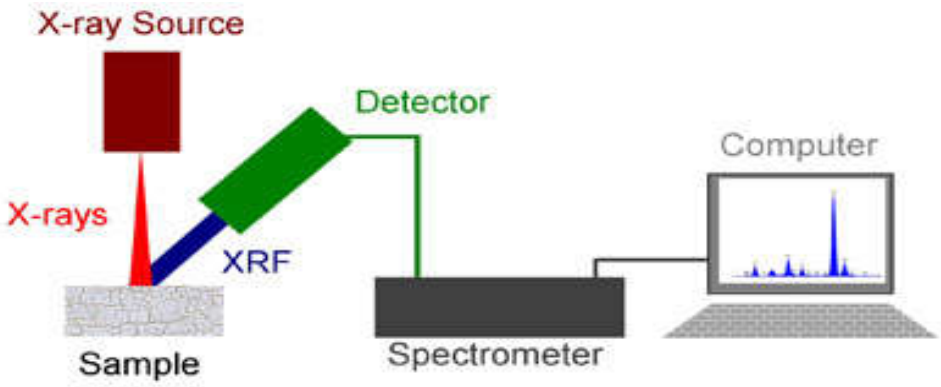
4. **Determination of the purity of a substance**- Purity of a substance can also be determined with the help of UV spectroscopy. The absorption of the sample solution is compared with the absorption of the reference solution. The intensity of the absorption can be used for the relative calculation of the purity of the sample substance

X- Ray Spectrometer

X-ray **spectroscopy** is a general term for characterization of materials by using x-ray excitation.

Principle

XRF works on methods involving interactions between electron beams and x-rays with samples.It is made possible by the behavior of atoms when they interact with radiation.When materials are excited with high-energy, short wavelength radiation (e.g., X-rays), they can become ionized.When an electron from the inner shell of an atom is excited by the energy of a photon, it moves to a higher energy level. When it returns to the low energy level, the energy which it previously gained by the excitation is emitted as a photon which has a wavelength that is characteristic for the element (there could be several characteristic wavelengths per element). Thus atomic X-rays emitted during electronic transitions to the inner shell states in atoms of modest atomic number.These X-rays since have characteristic energies related to the atomic number, and each element therefore has a characteristic X-ray spectrum which can be used to identify the element.



Components for X-ray spectroscopy are:

1. X-ray generating equipment (X-ray tube)
2. Collimator
3. Monochromators
4. Detectors

X-ray generating equipment (X-ray tube)

- X-rays can be generated by an X-ray tube.
- The high velocity electrons collide with a metal target, the anode, creating the X-rays.

Collimators

- A collimator is a device that narrows a beam of particles or waves.
- Collimation is achieved by using a series of closely spaced ,parallel metal plates or by a bundle of tubes ,0.5 or less in diameter.

Monochromator

- Monochromator separates and transmit a narrow portion of the optical signal chosen from a wider range of wavelengths available at the input.

X-ray Detectors

- Responsible for detecting the fetch X rays passing through the sample

Working

- An XRF spectrometer works because if a sample is illuminated by an intense X-ray beam, known as the incident beam, some of the energy is scattered, but some is also absorbed within the sample in a manner that depends on its chemistry.
- The incident X-ray beam is typically produced from a Rh target, although W, Mo, Cr and others can also be used, depending on the application.
- When x-ray hits sample, the sample emits x-rays along a spectrum of wavelengths characteristic of the type of atoms present.
- If a sample has many elements present, the use of a Wavelength Dispersive Spectrometer allows the separation of a complex emitted X-ray spectrum into characteristic wavelengths for each element present.
- Various types of detectors used to measure intensity of emitted radiation.

- ❖ The intensity of the energy measured by these detectors is proportional to the abundance of the element in the sample.
- ❖ The exact value for each element is derived from standards from prior analyses from other techniques.

Applications

X-Ray spectrometry is used in a wide range of applications, including

- ➢ Research in igneous, sedimentary, and metamorphic petrology
- ➢ Soil surveys & Mining (e.g., measuring the grade of ore)
- ➢ Cement production
- ➢ Ceramic and glass manufacturing
- ➢ Metallurgy (e.g., quality control)
- ➢ Environmental studies (e.g., analyses of particulate matter on air filters)
- ➢ Petroleum industry (e.g., sulfur content of crude oils and petroleum products)

ELISA Reader

It is a highly sophisticated instrument whose job it is to detect the presence of a protein in a liquid sample. ELISA stands for enzyme-linked immunosorbent assay. The ELISA reader is one of the **most popular versions of a device called a plate reader** which is designed to determine the presence of any sort of biological, chemical or physical presences in a small microtiter sample,

Principle

The tests done by the ELISA reader are typically done as a diagnostic procedure in medicine. The ELISA reads data from the liquid sample by attaching antigens to the antibodies in the small microtiter sample. The resulting reaction, **usually a color change of some kind,** indicates to whoever is using the ELISA reader, whether a protein is present in the sample. This can help doctors make diagnoses about the health of their patients

Applications

As mentioned above, since they are part of the health care and pharmaceutical industries, the most likely person to be using an ELISA reader is a scientist. Academic research laboratories and diagnostic centers who are staffed by trained scientists and

physicians are the people best suited to understand what an ELISA does and how to use it. ELISA readers have several detection modes that are **used by scientists and diagnosticians** to fully understand what is in the sample they are examining.

In addition to organic matter taken from patients, quality control technicians also often use the ELISA reader to make sure that the chemicals that need to be present in certain products used professionally are indeed there. They can also be used to test the safety of certain substances to ensure that they have not been corrupted. As the biotech industry begins to grow and genetic testing becomes more and more common, ELISA readers are becoming increasingly savvy and increasingly sophisticated.

Atomic Absorption Spectroscopy

Atomic absorption spectrometry (AAS) is an analytical technique that measures the concentrations of elements. Atomic absorption is so sensitive that it can measure down to parts per billion of a gram (μg dm–3) in a sample. The technique makes use of the wavelengths of light specifically absorbed by an element. They correspond to the energies needed to promote electrons from one energy level to another, higher, energy level.

Principle

A liquid sample is allowed to convert into free atoms (desolvated and atomized). These free atoms absorb the light of a specific wavelength. The remaining unabsorbed light is detected and recorded. The intensity of absorption is directly proportional to the concentration of the sample.

Instrumentation

1. The atomizer (burner) to dry the sample and produce atoms.
2. Sample container.
3. Fuel and oxidant to burn the sample by heat.
4. Hollow cathode lamp to produce light of the desired wavelength.
5. Detector to detect the absorption intensity.
6. Amplifier and data recorder.

.

Light source: The light source should produce a narrow spectrum with little background noise. Besides the light should be stable and have sufficient intensity.
Two types of light sources can be used based on the requirement.

1. Hollow cathode lamp: This is most widely used as a light source. Inside the lamp, the cathode is coated with a metal of analyte to be analyzed

2. Electrode-less discharge lamps: These lamps are less conventional in regular use but are essential of determination of Arsenic and selenium. A bulb containing an element of interest (with argon gas) is present in the lamp. This element is excited using microwave energy or radio frequency energy

Sample container: This is a beaker-like a container of the sample which is placed below the burner preferably. A capillary tube drains the sample to the tip of the burner.

The burner (atomizer): Here the sample from the capillary rises to the tip of the burner. Here it is burned with the flame. This flame is produced by a fuel and oxidant combination. The sample after evaporation leaves a fine residue of neutral atoms.

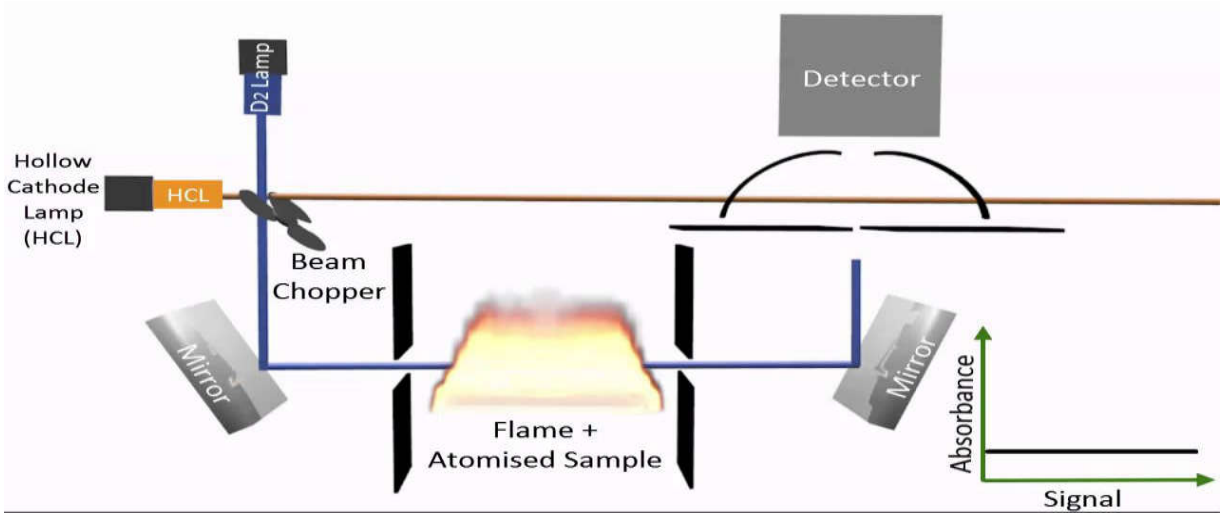
Fuel and oxidant: This is a very important part of the entire process to be remembered. If the heat produced is not sufficient then the sample doesn't form neutral atoms. If the heat of the burner is more, the sample molecules may ionize instead of forming atoms. So, both are undesirable for experimentation. Hence a proper combination of fuels and oxidant are to be used to produce recommended temperatures. Commonly used flues include propane, Hydrogen, and acetylene and oxidants are mostly air or oxygen.

Monochromator: As discussed before, elements have specific absorption line. But some elements also have secondary absorption lines. Further, there is also emission from the lamp and the flame. Hence, we need to isolate the desired spectral line for the measurement of absorption. To achieve this a monochromator which can filter and provide a resolution of <1nm is employed.

Detector: This part of the instrument detects the intensity of radiation absorbed by the elements. The detector consists of a photomultiplier tube or simple photocell. The current or potential recorded for the sample absorption is recorded in computer software and then analyzed.

Read device: This can be a display computer. It displays the absorbance at a specific wavelength.

Working



Atoms of different elements absorb characteristic wavelengths of light. Analysing a sample to see if it contains a particular element means using light from that element. For example with lead, a lamp containing lead emits light from excited lead atoms that produce the right mix of wavelengths to be absorbed by any lead atoms from the sample. In AAS, the sample is atomised – ie converted into ground state free atoms in the vapour state – and a beam of electromagnetic radiation emitted from excited lead atoms is passed through the vaporised sample. Some of the radiation is absorbed by the lead atoms in the sample. The greater the number of atoms there is in the vapour, the more radiation is absorbed. The amount of light absorbed is proportional to the number of lead atoms. A calibration curve is constructed by running several samples of known lead concentration under the same conditions as the unknown. The amount the standard absorbs is compared with the calibration curve and this enables the calculation of the lead concentration in the unknown sample

Applications

Clinical analysis: Analysing metals in biological fluids such as blood and urine.

Environmental analysis: Monitoring our environment

Pharmaceuticals: In some pharmaceutical manufacturing processes, minute quantities of a catalyst used in the process (usually a metal) are sometimes present in the final product.

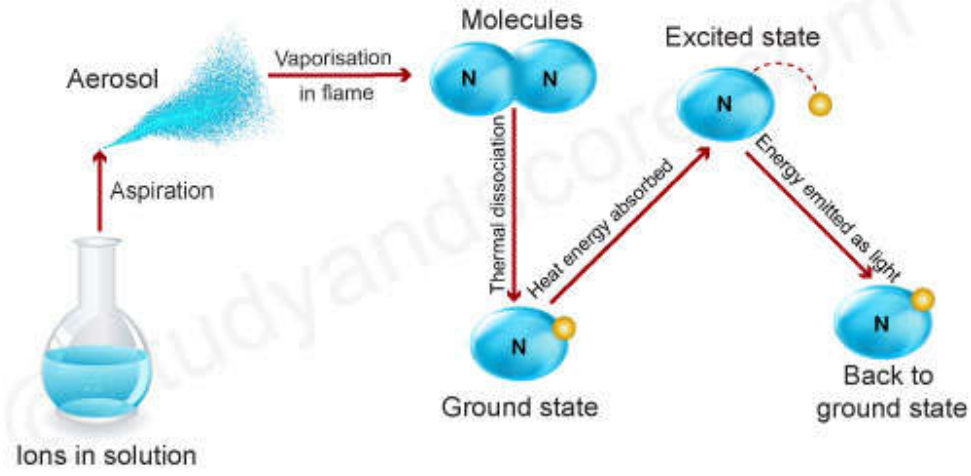
Industry: Many raw materials are examined and AAS is widely used to check that the major elements are present and that toxic impurities are lower than specified.
Mining: By using AAS the amount of metals such as gold in rocks can be determined to see whether it is worth mining the rocks to extract the gold.

Flame Photometry: Its Principle, Instrumentation & Applications

Flame photometry is a process wherein the emission of radiation by neutral atoms is measured.The neutral atoms are obtained by introduction of the sample into flame and hence it is named as flame photometry. Since radiation is also emitted, it is also called as flame emission spectroscopy.

Principle

When a solution of metallic salt is sprayed as fine droplets into a flame. Due to the heat of the flame, the droplets dry leaving a fine residue of salt. This fine residue converts into neutral atoms.



Due to the thermal energy of the flame, the atoms get excited and after that return to ground state. In this process of return to ground state, excited atoms emit radiation of specific wavelength. This wavelength of radiation emitted is specific for every element.

This specificity of the wavelength of light emitted makes it a qualitative aspect, while the intensity of radiation depends on the concentration of element. This makes it a quantitative aspect.

Process seems to be simple and applicable to all elements. But in practice, only a few elements of Group IA and group II A (like Li, Na, k & Ca, Mg) are only analyzed

Instrumentation

1. Burner
2. Monochromators
3. Detectors
4. Recorder and display.

Burner: This is a part which produces excited atoms. Here the sample solution is sprayed into fuel and oxidant combination. A homogenous flame of stable intensity is produced.

There are different types of burners like Total consumption burner, Laminar flow and Mecker burner.

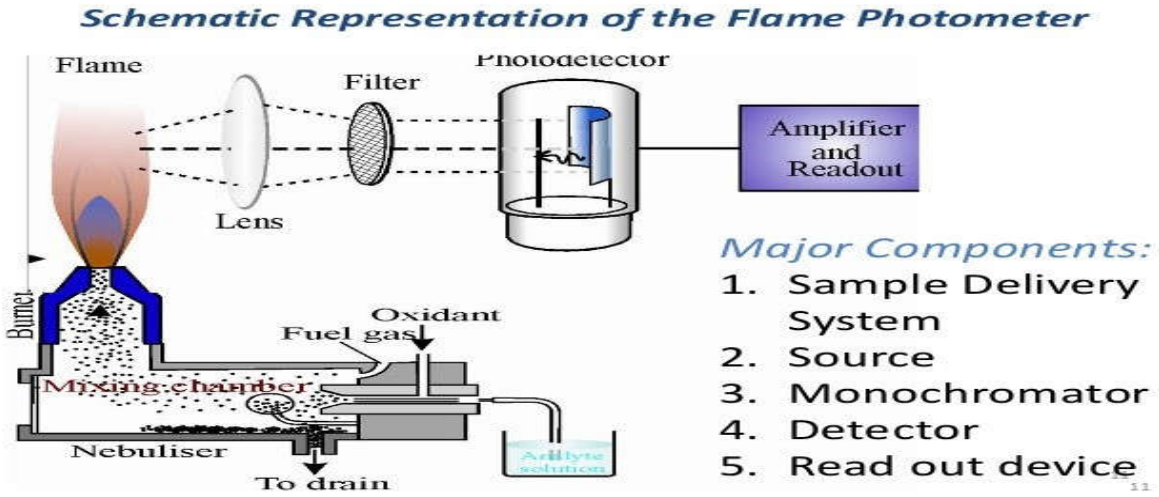
Fuel and oxidants: Fuel and oxidant are required to produce the flame such that the sample converts to neutral atoms and get excited by heat energy.

The temperature of flame should be stable and also ideal. If the temperature is high, the elements in sample convert into ions instead of neutral atoms. If it is too low, atoms may not go to excited state. So a combination of fuel and oxidants is used such that there is desired temperature.

Monochromators: Filters and monochromators are needed to isolate the light of specific wavelength from remaining light of the flame. For this simple filters are sufficient as we study only few elements like Ca, Na, K and Li. So a filter wheel with filter for each element is taken. When a particular element is analyzed, the particular filter is used so that it filters all other wavelengths.

Detector: Flame photometric detector is similar to that used in spectrophotometry. The emitted radiation is in the visible region, i.e., 400nm to 700nm. Further, the radiation is specific for each element, so simple detectors are sufficient for the purpose of photovoltaic cells, phototubes, etc.

Recorders and display: These are the devises to read out the recording from detectors.



Working

- The solvent is first aspirated to obtain fine solid particles.
- These molecules in the solid particles are moved towards the flame to produce gaseous atoms and ions.
- These ions absorb the energy from the flame get excited to high energy levels from the ground state.
- But as these ions are unstable, they return back to ground state. While returning they emit characteristic radiation.
- The intensity of emitted light is proportional to the concentration of the element.

Applications

1. For qualitative analysis of samples by comparison of spectral emission wavelengths with that of standards.

2. For quantitative analysis to determine the concentration of group IA and IIA elements.

For example,

a) Concentration of calcium in hard water.

b) Concentration of Sodium, potassium in Urine

c) Concentration of calcium and other elements in bio-glass and ceramic materials

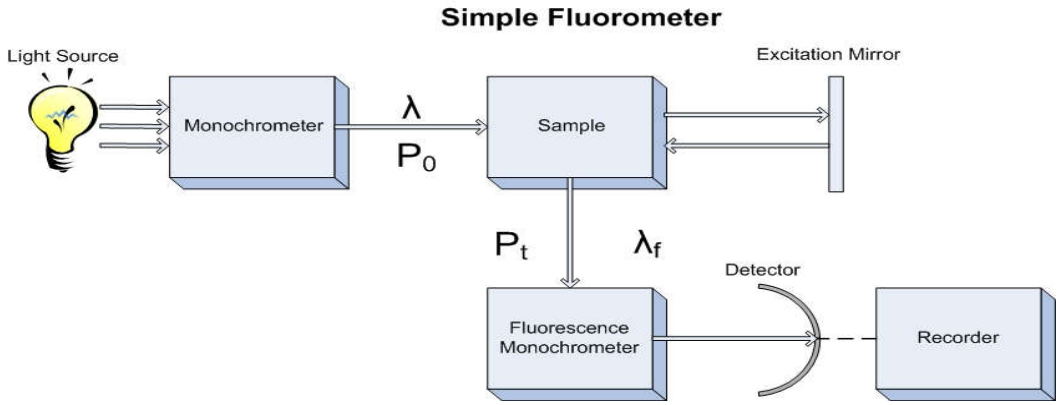
Flourimeter

A fluorometer or fluorimeter is a device used to measure parameters of visible spectrum fluorescence

Principle & Working

Fluorometers quantify biological analytes as a function of fluorescence. This requires the sample to be bound to a specific fluorescent agent and loaded into the instrument in a polypropylene tube. Fluorophores absorb light of a distinct excitation wavelength and emit, or fluoresce, light of reduced energy thus a longer wavelength. This behavior can be modified so that the fluorescent reagents are restricted from emitting light unless bound to a specific molecule, such as dsDNA. It is, therefore, possible to directly correlate the fluorescent intensity (RFU) of the assay to the number of desirable biomolecules within the sample.

This methodology is extremely sensitive, providing biomolecular detection at sensitivities of up to 0.0005 nanograms per microliter (ng/µl). Fluorometry also delivers exclusive detection for desirable analytes, eliminating measurement inaccuracies caused by a contaminant or unknown elements in the sample. Concentrations of unknowns can then be mathematically quantified against the fluorescent intensity of known biomolecules within the assay



Applications

Dairy industry

Fluorimetry is widely used by the dairy industry to verify whether pasteurization has been successful

Spectroflurometer

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.

Principle

Fluorometers quantify biological analytes as a function of fluorescence. This requires the sample to be bound to a specific fluorescent agent and loaded into the instrument in a polypropylene tube. Fluorophores absorb light of a distinct excitation wavelength and emit, or fluoresce, light of reduced energy thus a longer wavelength. This behavior can be modified so that the fluorescent reagents are restricted from emitting light unless bound to a specific molecule, such as dsDNA. It is, therefore, possible to directly correlate the fluorescent intensity (RFU) of the assay to the number of desirable biomolecules within the sample.

Instrumentation

A spectrofluorometer is an analytical instrument used to measure and record the fluorescence of a sample. While recording the fluorescence, the excitation, emission or both wavelength may be scanned. With additional accessories, variation of signal with time, temperature, concentration, polarization, or other variables may be monitored. Fluorescence spectrometers use laser sources, which contains wavelength selectors, sample illumination, detector and corrected spectra.

Illuminator source: The continuous light source is 150 W ozone free xenon arc lamp. Light from the lamp is collected by a diamond turned elliptical mirror, and then focused on the entrance slit of the excitation monochromator. The lamp housing is separated from the excitation monochromator by a quartz window. This vents heat out of

the instrument, and protects against the unlikely occurrence of lamp failure. Resolution over the entire spectral range and minimize spherical aberrations and re diffraction.

Monochromators: It contains two monochromators : Excitation monochromator and Emission monochromator. They use all reflective optics to maintain high resolution over the entire spectral range, and minimize spherical aberrations and re diffraction.

Gratings: The essential part of a monochromator is a reflection grating. A grating disperses the incident light by means of its vertical grooves. A spectrum is obtained by rotating the gratings contain 1200 grooves mm 1, and are blazed at 330 nm (excitation) at 500 nm (emission). Each grating is coated with MgF2 for protection against oxidation.

Slits: The entrance and exit ports of each monochromator have continuously adjustable slits. The width of the slits on the excitation monochromator determines the band pass of light incident on the sample. The emission monochromator's slits control the intensity of the fluorescence signal recorder by the signal detector. When setting slit width, the trade off is intensity of signal versus spectral resolution. The wider the slits are, the more light falls on the sample and detector, but the resolution decreases. The narrower slits are, the higher the resolution gets but at the expense of signal.

Shutters: An excitation shutter is located just after the excitation monochromator's exit slit. The shutter protects sample from photo bleaching or photo degradation from prolonged exposure to the light source. An emission shutter is placed just before the emission monochromator's entrance and protects the detector from bright light.

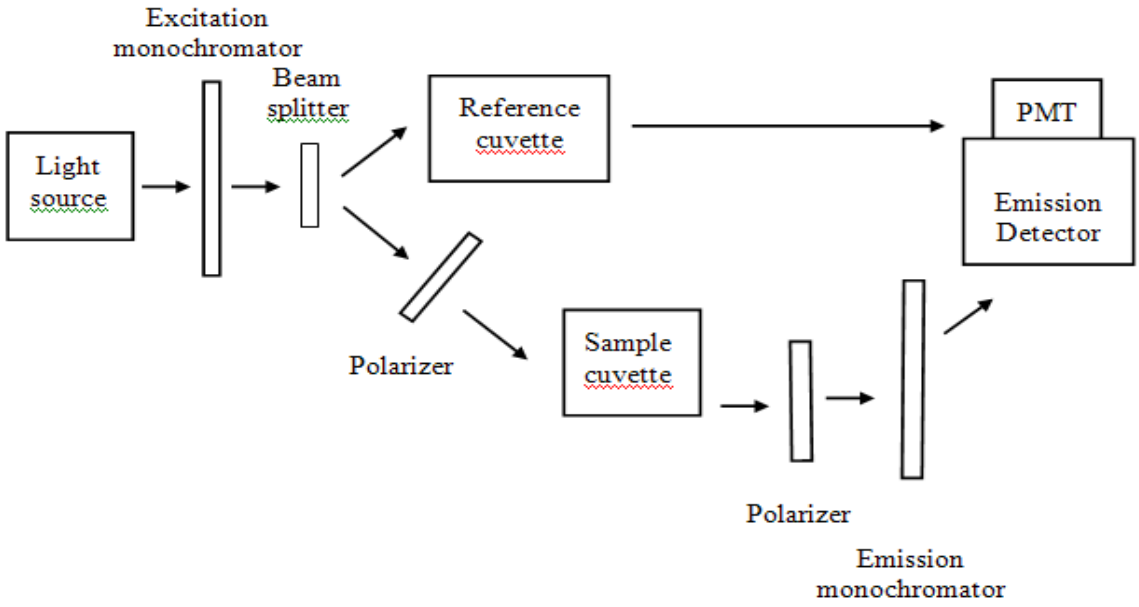
Sample compartment: The sample compartment accommodates various optional accessories, as well as fiber optic bundles to take the excitation beam to a remote sample and return the emission beam to the emission monochromator.

Detectors: It contains two detectors: Signal detector and reference detector. The signal detector is a photon counting detector. This detector is an R928P photomultiplier tube, which sends the signal to a photon counting module. The reference detector monitors the xenon lamp, in order to correct for wavelength and time dependent output of the lamp. This detector is a UV enhance silicon photodiode, which is just before the sample compartment.

Computer Control: The entire control of the FluoroMax 4 originates in your PC with our revolutionary new Fluor Essenc software and is transmitted through a serial link. On start up, the system automatically calibrates and presents itself for new experiments or stored routines instantly called from memory.

Working

Generally, spectrofluorometers use high intensity light sources to bombard a sample with as many photons as possible. This allows for the maximum number of molecules to be in an excited state at any one point in time. The light is either passed through a filter, selecting a fixed wavelength, or a monochromator, which allows a wavelength of interest to be selected for use as the exciting light. The emission is collected at the perpendicular to the emitted light. The emission is also either passed through a filter or a monochromator before being detected by a photomultiplier tube, photodiode, or charge-coupled device detector. The signal can either be processed as digital or analog output.



Applications

Environmental Significance: To detect environmental pollutants such as polycyclic aromatic hydrocarbons, organothiophosphorous, pesticides & carbamate insecticides

Geology: Many types of calcite and amber will fluoresce under shortwave UV. Rubies, emeralds, and the Hope Diamond exhibit red fluorescence under short-wave UV light; diamonds also emit light under X ray radiation.

Analytical chemistry: To detect compounds from HPLC flow

Biochemistry: Used generally as a non-destructive way of tracking or analysis of biological molecules (proteins)

Fingerprints can be visualized with fluorescent compounds such as ninhydrin.

Medicine: Blood and other substances are sometimes detected by fluorescent reagents, particularly where their location was not previously known.

UNIT IV SEPERATION TECHNIQUES

Centrifugation

Centrifugation is a technique of separating substances which involves the application of centrifugal force. The particles are separated from a solution according to their size, shape, density, viscosity of the medium and rotor speed.

Principle:

- ✓ 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 piece of equipment that puts an object in rotation around a fixed axis (spins it in a circle), applying a potentially strong force perpendicular to the axis of spin (outward).
- ✓ The centrifuge works using the sedimentation principle, where the centripetal acceleration causes denser substances and particles to move outward in the radial direction.
- ✓ At the same time, objects that are less dense are displaced and move to the center.
- ✓ In a laboratory centrifuge that uses sample tubes, the radial acceleration causes denser particles to settle to the bottom of the tube, while low- density substances rise to the top

Protocol for working:

Place the centrifuge on a firm, level surface. Because of the high speeds at which the centrifuge spins, it needs to be housed on a solid, flat surface. Find a level table or counter that is secure and can properly support the weight of the centrifuge.

Choose the proper rotor to use at the speed you need. The rotor is the piece that spins your sample. Some rotors have arms that you can attach smaller buckets to, while other rotors are one piece that you place the tubes directly into. Rotors are designated for specific tubes and speeds. Use a rotor that fits the tubes of your sample and is within the limit of speed needed.

If your samples are in the wrong type of tube, you may need to transfer them to the proper type before spinning.

Load the tubes opposite each other in the centrifuge. If you are only spinning 1 sample, you will need to make a balance tube to load directly opposite the sample tube. If you are spinning more than 2 tubes, only the ones directly opposite each other have to be equal in mass.

Enter the centrifugation speed. Centrifugation speed is often given in rotations per minute (rpm), or how many times the rotor completes a full rotation in one minute. Speed can also be given in relative centrifugal force (RCF) or the G-force on the rotor. Modern day centrifuges are digital and have settings for both.

The speed at which you spin your samples is dependent upon what you are spinning. Do some online research if you aren't sure what speed to use.

Keep a safe distance while the centrifuge is running. Bumping or moving the centrifuge during use can cause an imbalance and lead to injury. Larger centrifuges can spin at very high speeds and are extremely dangerous if they become unbalanced.

Turn off the centrifuge if it is wobbling. After starting the centrifuge, stay close until it gets up to full speed in case it needs to be turned off. When a centrifuge is off balance it may shake or wobble. This can damage the equipment and can also lead to injury in severe cases. If you cannot get to the power button, simply unplug the centrifuge.

Open the lid only after the rotor has completely stopped. Many modern centrifuges have a lock on them that will not unlock until the rotor has come to a stop. Older centrifuges may not have this feature so make sure the rotor is no longer moving before opening the lid. Do not touch the rotors while they are moving.

Keep power cords away from the ground to avoid trip hazards that could move the centrifuge.

Remove the tubes carefully after the centrifuge has completely stopped spinning. You want to gently remove the tubes so that the separated suspensions do not mix again. You also want to check to see if any of the samples have leaked or tubes have broken. If a spill occurs, clean the rotor and buckets immediately.

Rf Value (Retardation Factor)

Thus, in order to obtain a measure of the extent of movement of a component in a paper chromatography experiment, “Rf value” is calculated

$$\textbf{Rf Value} = \frac{\text{Distance from Baseline travelled by Solute}}{\text{Distance from Baseline travelled by Solvent (Solvent Front)}}$$

Chromatography

It is a laboratory technique for the separation of a mixture. The mixture is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase.

Principle

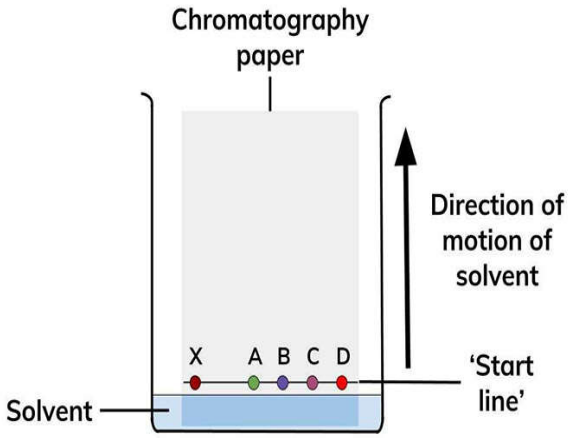
It works on the principle of the movement of Mobile Phase over the Stationary Phase

Procedure

- A 15cm by 1cm chromatography strip was cut.
- A horizontal line 1cm from one end is drawn with a pencil. This is the application base (origin) where the test material is applied.
- A small drop of test material is placed on the centre of the application line.
- About 2.0 -2.5 ml of solvent is transferred at the base of the container bottle or glass test tube using a long neck glass pipette.
- The chromatography strip is now inserted from the tap slot and hold from falling inside by inserting a straw in the slot. The straw's outward pressure will hold the strip in place against the edge of the slot edge
- The strip is lowered through the needles and further down till its end is about 2mm below the solvent superficial layer.
- This is left till the solvent rises at least 70% of the length. If the solvent seems that it has stopped flowing (no change in 5 mins) the strip should be removed.
- This is left to dry in air, preferably by suspending vertically in air
- The paper is placed in 5-10ml solvent (eg ethanol).
- When the paper is clear from the test material (hence dissolved in the solution) it is removed using a metal tweezers
- The solution can be concentrated by letting to evaporate in room temperature. A fastest way is to apply heat some distance above an electric flame

Paper Chromatography (Ascending , Descending , Circular)

- Paper chromatography (PC) is a planar chromatography whereby chromatography procedures are run on a specialized paper.
- PC is considered to be the simplest and most widely used of the chromatographic techniques because of its applicability to isolation, identification and quantitative determination of organic and inorganic compounds.
- It was first introduced by German scientist Christian Friedrich Schonbein (1865).



Principle

The principle of separation is mainly partition rather than adsorption. Substances are distributed between a stationary phase and mobile phase. It emphasizes the movement of mobile phase over the stationary phase

Instrumentation

1. Stationary phase & papers used
2. Mobile phase
3. Developing Chamber
4. Detecting or Visualizing agents

1. STATIONARY PHASE AND PAPERS

- Whatman filter papers of different grades like No.1, No.2, No.3, No.4, No.20, No.40, No.42 etc
- In general the paper contains 98-99% of α-cellulose, 0.3 – 1% β -cellulose.

2. PAPER CHROMATOGRAPHY MOBILE PHASE

- Pure solvents, buffer solutions or mixture of solvents can be used.

Examples-

Hydrophilic mobile phase

- Isopropanol: ammonia:water 9:1:2
- Methanol : water 4:1
- N-butanol : glacial acetic acid : water 4:1:5

Hydrophobic mobile phases

- dimethyl ether: cyclohexane kerosene : 70% isopropanol
- The commonly employed solvents are the polar solvents, but the choice depends on the nature of the substance to be separated.
- If pure solvents do not give satisfactory separation, a mixture of solvents of suitable polarity may be applied.

3. CHROMATOGRAPHIC CHAMBER

- The chromatographic chambers are made up of many materials like glass, plastic or stainless steel. Glass tanks are preferred most.
- They are available invarious dimensional size depending upon paper length and development type.
- The chamber atmosphere should be saturated with solvent vapor.

Procedure

In paper chromatography, the sample mixture is applied to a piece of filter paper, the edge of the paper is immersed in a solvent, and the solvent moves up the paper by capillary action.

The basic steps include:

Selection of Solid Support

Fine quality cellulose paper with defined porosity, high resolution, negligible diffusion of sample and favoring good rate of movement of solvent.

Selection of Mobile Phase

Different combinations of organic and inorganic solvents may be used depending on the analyte.

Example. Butanol: Acetic acid: Water (12:3:5) is suitable solvent for separating amino-acids.

Saturation of Tank

The inner wall of the tank is wrapped with the filter paper before solvent is placed in the tank to achieve better resolution.

Sample Preparation and Loading

If solid sample is used, it is dissolved in a suitable solvent. Sample (2-20ul) is added on the base line as a spot using a micropipette and air dried to prevent the diffusion.

Development of the Chromatogram

Sample loaded filter paper is dipped carefully into the solvent not more than a height of 1 cm and waited until the solvent front reaches near the edge of the paper.

Different types of development techniques can be used:

Ascending Paper Chromatography

- **Like conventional type, the solvent flows against gravity.**
- **The spots are kept at the bottom portion of paper and kept in a chamber with mobile phase solvent at the bottom.**

Descending Paper Chromatography

- **This is carried out in a special chamber where the solvent holder is at the top.**
- **The spot is kept at the top and the solvent flows down the paper.**
- **In this method solvent moves from top to bottom so it is called descending chromatography.**

Circular / Radial Paper Chromatography

- **Spot is kept at the centre of a circular paper.**
- **The solvent flows through a wick at the centre & spreads in all directions uniformly.**

Drying of Chromatogram

After the development, the solvent front is marked and the left to dry in a dry cabinet or oven.

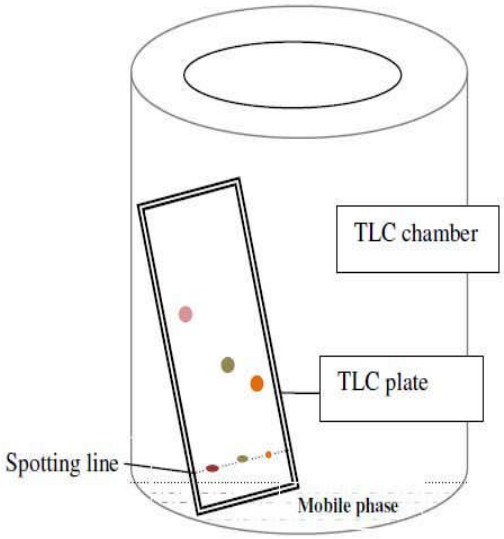
Detection

Colourless analytes detected by staining with reagents such as iodine vapour, ninhydrin etc.

Radiolabeled and fluorescently labeled analytes detected by measuring radioactivity and florescence respectively

Thin Layer Chromatography

It can be defined as a method of separation or identification of a mixture of components into individual components by using finely divided adsorbent solid / (liquid) spread over a plate and liquid as a mobile phase.



Principle

- Thin-layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide (alumina), or cellulose. This layer of adsorbent is known as the stationary phase.
- After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved.
- It is thus based on the principle of adsorption chromatography or partition chromatography or combination of both, depending on adsorbent, its treatment and nature of solvents employed. The components with more affinity towards stationary phase travels slower.
- Components with less affinity towards stationary phase travels faster.

Instrumentation

TLC system consists of:

TLC plates, preferably readymade with a stationary phase: These are stable and chemically inert plates, where a thin layer of stationary phase is applied on its whole

surface layer. The stationary phase on the plates is of uniform thickness and is in a fine particle size.

TLC chamber- This is used for the development of TLC plate. The chamber maintains a uniform environment inside for proper development of spots. It also prevents the evaporation of solvents, and keeps the process dust free.

Mobile phase- This comprises of a solvent or solvent mixture The mobile phase used should be particulate-free and of the highest purity for proper development of TLC spots. The solvents recommended are chemically inert with the sample, a stationary phase.

A filter paper- This is moistened in the mobile phase, to be placed inside the chamber. This helps develop a uniform rise in a mobile phase over the length of the stationary phase.

Procedure

The stationary phase is applied onto the plate uniformly and then allowed to dry and stabilize. These days, however, ready-made plates are more commonly used.

1. With a pencil, a thin mark is made at the bottom of the plate to apply the sample spots.
2. Then, samples solutions are applied on the spots marked on the line in equal distances.
3. The mobile phase is poured into the TLC chamber to a leveled few centimeters above the chamber bottom.
4. A moistened filter paper in mobile phase is placed on the inner wall of the chamber to maintain equal humidity (and also thereby avoids edge effect).
5. Now, the plate prepared with sample spotting is placed in TLC chamber so that the side of the plate with the sample line is facing the mobile phase. Then the chamber is closed with a lid.
6. The plate is then immersed, such that the sample spots are well above the level of mobile phase (but not immersed in the solvent) for development.
7. Sufficient time is given for the development of spots.
8. The plates are then removed and allowed to dry.
9. The sample spots are then seen in a suitable UV light chamber, or any other methods as recommended for the given sample.

Visualization

Some common techniques for visualizing the results of a TLC plate include

- UV light
- Iodine Staining: is very useful in detecting carbohydrates since it turns black on contact with Iodine
- KMnO4 stain (organic molecules)
- Ninhydrin Reagent: often used to detect amino acids and proteins

Applications

- In monitoring the progress of reactions
- Determining the purity of a substance.
- Analyzing ceramides and fatty acids
- Detection of pesticides or insecticides in food and water
- Analyzing the dye composition of fibers in forensics
- Assaying the radiochemical purity of radiopharmaceuticals
- Identification of medicinal plants and their constituents

HPTLC-High Performance Thin Layer Chromatography

- ✓ **HPTLC is a** sophisticated form of Thin-Layer chromatography (TLC). In full form, it is High-Performance Thin Layer Chromatography. This is so called because of the features like the greater efficiency and higher resolution.
- ✓ It has added advantages like auto-sampling, faster development of spots and also easy detection and quantification of separated compounds.

The advancement concerning better efficiency and resolution is due to the

Use of precoated HPTLC plates with an optimized stationary phase. The stationary phase has smaller particles with size less than 10µ. It is also uniformly spread and dried.

In TLC chromatography a thick solution of stationary phase is poured on to the plates. The solution is spread and allowed to dry. This is a time-consuming process. Further, there are chances of non-uniform distribution of stationary phase on the plate which can lead to errors in the chromatogram.

A choice to use a wide variety of stationary phases is possible in HPTLC. Silica gel is used for normal phase chromatography. While materials like C8, C18 are used for reverse phase chromatography.

Auto-sampling is present which help minimize handling errors. The techniques like manual spotting and streaking in TLC are time-consuming with possible human error.

Greater efficiency in the separation due to the presence of smaller and uniform sized particles in the stationary phase. Smaller particle size enhances the surface area of adsorption. While the uniform size enabled better flow and movement of sample particles.

Better and efficient solvent usage: There is a minimal requirement of solvents due to the presence of advanced development chambers which requires fewer amounts of solvents for developing the chromatograms.

Detectors and recorders: Auto-detection *of compounds* from chromatogram is possible by using built-in UV and fluorescent detectors system.

Recording and storage *of data* from chromatograms are done by utilizing HPTCL data software in a computer.

Advantages of HPTLC over TLC:

- Samples in minute quantities like in nano-gram range can be detected using HPTLC.
- Handling and human errors are minimum due to automation.
- Better accuracy and sensitivity than TLC.

Application of HPTLC:

- For detection and analysis of components of phytochemicals obtained from plants. For analysis of compounds of medicinal chemistry & organic chemistry.
- In TLC one can identify the elements of an extract. Whereas using this method, one can even estimate its concentration.
- Compounds having a complicated structure or those available in very scarce quantities can be analyzed.

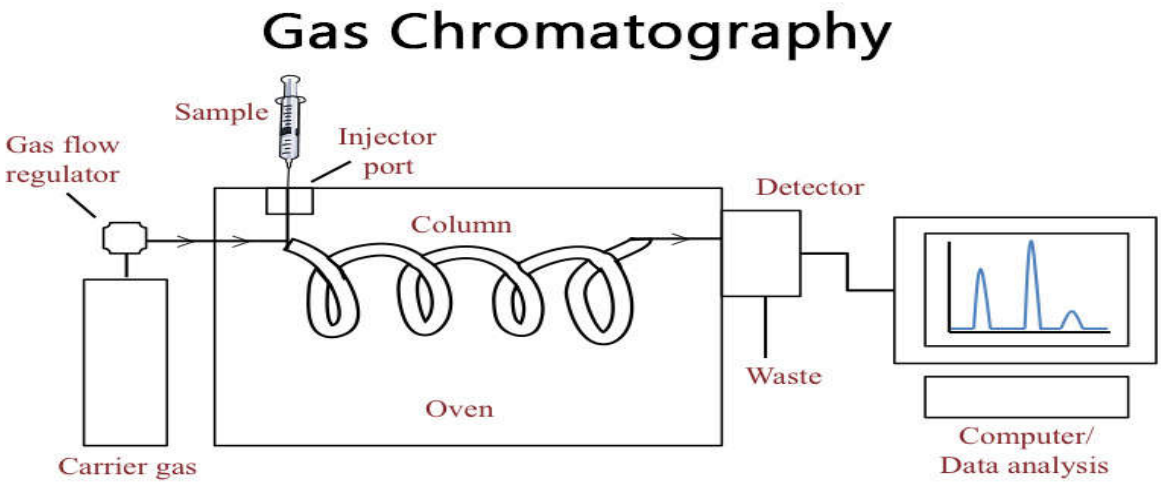
Gas Chromatography

- Gas chromatography differs from other forms of chromatography in that the mobile phase is a gas and the components are separated as vapours.
- It is thus used to separate and detect small molecular weight compounds in the gas phase.
- The sample is either a gas or a liquid that is vaporized in the injection port. The mobile phase for gas chromatography is a carrier gas, typically helium because of its low molecular weight and being chemically inert.
- Pressure is applied and the mobile phase moves the analyte through the column. The separation is accomplished using a column coated with a stationary phase.

Principle

- The equilibrium for gas chromatography is partitioning, and the components of the sample will partition (i.e. distribute) between the two phases: the stationary phase and the mobile phase.
- Compounds that have greater affinity for the stationary phase spend more time in the column and thus elute later and have a longer retention time (tR) than samples that have higher affinity for the mobile phase.
- Affinity for the stationary phase is driven mainly by intermolecular interactions and the polarity of the stationary phase can be chosen to maximize interactions and thus the separation

Instrumentation



Gas chromatography is mainly composed of the following parts:

Carrier gas in a high pressure cylinder with attendant pressure regulators and flow meters

- Helium, N_2, H, Argon are used as carrier gases.
- Helium is preferred for thermal conductivity detectors because of its high thermal conductivity relative to that of most organic vapours.
- N_2 is preferable when large consumption of carrier gas is employed.
- Carrier gas from the tank passes through a toggle valve, a flow meter, (1-1000 ml / min), capillary restrictors, and a pressure gauge (1-4 atm).
- Flow rate is adjusted by means of a needle valve mounted on the base of the flow meter and controlled by capillary restrictors.
- The operating efficiency of the gas chromatograph is directly dependant on the maintenance of a constant gas flow.

Sample injection system

- Liquid samples are injected by a micro syringe with needle inserted through a self scaling, silicon-rubber septum into a heated metal block by a resistance heater.
- Gaseous samples are injected by a gas tight syringe or through a by-pass loop and valves.
- Typical sample volumes range from 0.1 to 0.2 ml.

The separation column

- The heart of the gas chromatography is the column which is made of metals bent in U shape or coiled into an open spiral or a flat pancake shape.
- Copper is useful up to 250^0
- Swege lock fittings make column insertion easy.
- Several sizes of columns are used depending upon the requirements.

Liquid phases

- An infinite variety of liquid phases are available limited only by their volatility, thermal stability and ability to wet the support.
- No single phase will serve for all separation problems at all temperatures.

Supports

- The structure and surface characteristics of the support materials are important parameters, which determine the efficiency of the support and the degree of separation respectively.
- The support should be inert but capable of immobilizing large volume of liquid phase as a thin film over its surface.
- Surface area should be large to ensure rapid attainment of equilibrium between stationary and mobile phases.
- Support should be strong enough to resist breakdown in handling and be capable of packed into a uniform bed.

Detector

- Detectors sense the arrival of the separated components and provide a signal.
- These are either concentration dependant or mass dependant.
- The detector should be close to the column exit and correct temperature to prevent decomposition.

Recorder

- The recorder should be generally 10 mv (full scale) fitted with a fast response pen (1 sec or less). The recorder should be connected with a series of good quality resistances connected across the input to attenuate the large signals.
- An integrator may be a good addition.

Procedure

Step 1: Sample Injection and Vapourization

1. A small amount of liquid sample to be analysed is drawn up into a syringe.
2. The syringe needle is positioned in the hot injection port of the gas chromatograph and the sample is injected quickly.
3. The injection of the sample is considered to be a “point” in time, that is, it is assumed that the entire sample enters the gas chromatograph at the same time, so the sample must be injected quickly.
4. The temperature is set to be higher than the boiling points of the components of the mixture so that the components will vaporise.

5. The vaporised components then mix with the inert gas mobile phase to be carried to the gas chromatography column to be separated.

Step 2: Separation in the Column

- Components in the mixture are separated based on their abilities to adsorb on, or bind to, the stationary phase.
- A component that adsorbs most strongly to the stationary phase will spend the most time in the column (will be retained in the column for the longest time) and will therefore have the longest retention time (Rt). It will emerge from the gas chromatograph last.
- A component that adsorbs the least strongly to the stationary phase will spend the least time in the column (will be retained in the column for the shortest time) and will therefore have the shortest retention time (Rt). It will emerge from the gas chromatograph first.
- If we consider a 2 component mixture in which component A is more polar than component B then:

1. component A will have a longer retention time in a polar column than component B
2. component A will have a shorter retention time in a non-polar column than component B

Step 3: Detecting and Recording Results

1. The components of the mixture reach the detector at different times due to differences in the time they are retained in the column.
2. The component that is retained the shortest time in the column is detected first. The component that is retained the longest time in the column is detected last.
3. The detector sends a signal to the chart recorder which results in a peak on the chart paper. The component that is detected first is recorded first. The component that is detected last is recorded last

Applications

- GC analysis is used to calculate content of a chemical product, for example in assuring the quality of products in the chemical industry; or measuring toxic substances in soil, air or water.

- Gas chromatography is used in the analysis of air-borne pollutants, performance enhancing drugs in athlete's urine samples, oil spills and essential oils in perfume preparation
- GC is very accurate if used properly and can measure picomoles of a substance in a 1 ml liquid sample, or parts-per-billion concentrations in gaseous samples.
- Gas Chromatography is used extensively in forensic science. Disciplines as diverse as solid drug dose (pre-consumption form) identification and quantification, arson investigation, paint chip analysis, and toxicology cases, employ GC to identify and quantify various biological specimens and crime-scene evidence

High Performance Liquid Chromatography

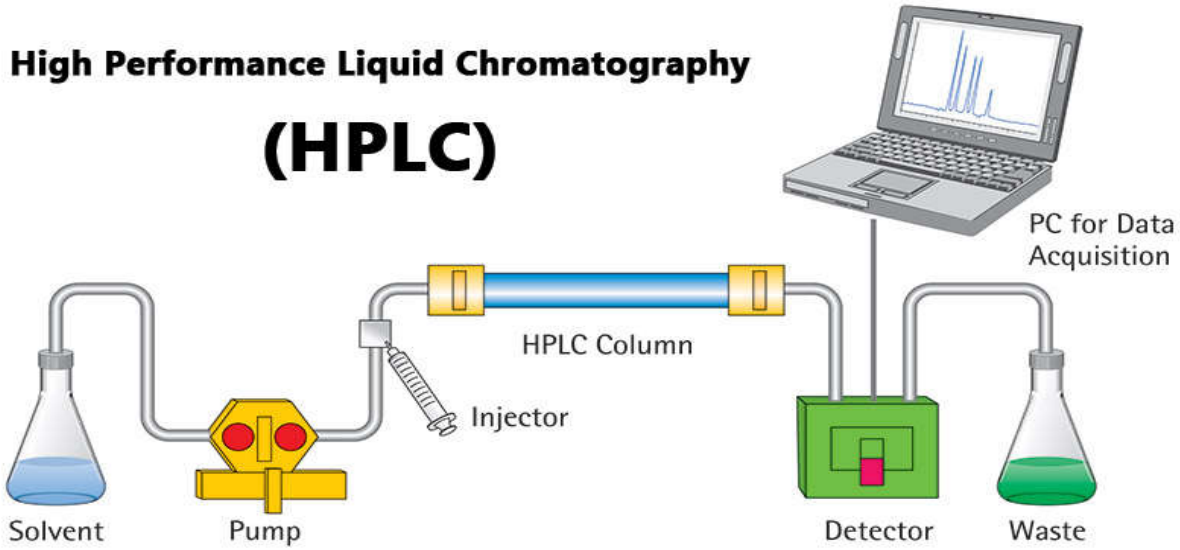
High performance liquid chromatography or commonly known as HPLC is an analytical technique used to separate, identify or quantify each component in a mixture.

- The mixture is separated using the basic principle of column **chromatography** and then identified and quantified by spectroscopy.
- In the 1960s the column chromatography LC with its low-pressure suitable glass columns was further developed to the HPLC with its high-pressure adapted metal columns.
- HPLC is thus basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres.

Principle

- Chromatography is a technique to separate mixtures of substances into their components on the basis of their molecular structure and molecular composition
- This involves a stationary phase (a solid, or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mobile phase flows through the stationary phase and carries the components of the mixture with it. Sample components that display stronger interactions with the stationary phase will move more slowly through the column than components with weaker interactions.

Instrumentation



1. Solvent Resorvoir

Mobile phase contents are contained in a glass resorvoir. The mobile phase, or solvent, in HPLC is usually a mixture of polar and non-polar liquid components whose respective concentrations are varied depending on the composition of the sample.

2. Pump

A pump aspirates the mobile phase from the solvent resorvoir and forces it through the system's column and detecter. Depending on a number of factors including column dimensions, particle size of the stationary phase, the flow rate and composition of the mobile phase, operating pressures of up to 42000 kPa (about 6000 psi) can be generated.

3. Sample Injector

The injector can be a single injection or an automated injection system. An injector for an HPLC system should provide injection of the liquid sample within the range of 0.1-100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).

4. Columns

Columns are usually made of polished stainless steel, are between 50 and 300 mm long and have an internal diameter of between 2 and 5 mm. They are commonly filled with a stationary phase with a particle size of 3–10 µm.

Columns with internal diameters of less than 2 mm are often referred to as microbore columns. Ideally the temperature of the mobile phase and the column should be kept constant during an analysis.

5. Detector

The HPLC detector, located at the end of the column detect the analytes as they elute from the chromatographic column. Commonly used detectors are UV-spectroscopy, fluorescence, mass-spectrometric and electrochemical detectors.

6. Data Collection Devices

Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret.

Applications

The HPLC has developed into a universally applicable method, so that it finds its use in almost all areas of chemistry, biochemistry and pharmacy.

- Analysis of drugs & Analysis of synthetic polymers
- Analysis of pollutants in environmental analytics
- Determination of drugs in biological matrices
- Isolation of valuable products
- Water purification
- Pre-concentration of trace components
- Ligand-exchange chromatography & Ion-exchange chromatography of proteins

Column chromatography

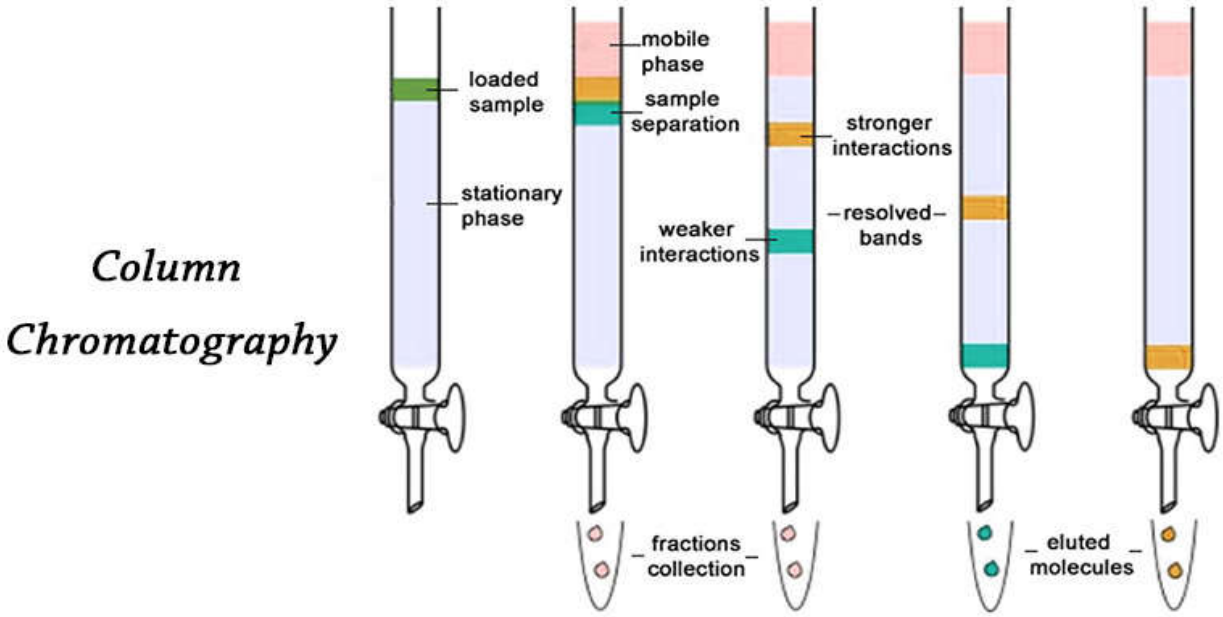
- It is a technique in which the substa
- nces to be separated are introduced onto the top of a column packed with an adsorbent, passed through the column at different rates that depend on the affinity of each substance for the adsorbent and for the solvent or solvent mixture, and are usually collected in solution as they pass from the column at different times.

- It is a solid – liquid technique in which the stationary phase is a solid & mobile phase is a liquid or gas.
- It was developed by the American chemist D.T Day in 1900 while M.S. Tswett, the Polish botanist, in 1906 used adsorption columns in his investigations of plant pigments.

Principle

- In column chromatography the stationary phase is packed into a glass or metal column.
- The mixture of analytes is then applied and the mobile phase, commonly referred to as the eluent, is passed through the column either by use of a pumping system or applied gas pressure.
- The stationary phase is either coated onto discrete small particles (the matrix) and packed into the column or applied as a thin film to the inside wall of the column.
- As the eluent flows through the column the analytes separate on the basis of their distribution coefficients and emerge individually in the eluate as it leaves the column

Instrumentation



A typical column chromatographic system using a gas or liquid mobile phase consists of the following components:

A stationary phase:

- Chosen to be appropriate for the analytes to be separated.

A column:

- In liquid chromatography these are generally 25- 50 cm long and 4mm internal diameter and made of stainless steel whereas in gas chromatography they are 1-3m long and 2- 4mm internal diameter and made of either glass or stainless steel.
- They may be either of the conventional type filled with the stationary phase, or of the microbore type in which the stationary phase is coated directly on the inside wall of the column.

A mobile phase and delivery system:

- Chosen to complement the stationary phase and hence to discriminate between the sample analytes and to deliver a constant rate of flow into the column.

An injector system:

- To deliver test samples to the top of the column in a reproducible manner.

A detector and chart recorder:

- To give a continuous record of the presence of the analytes in the eluate as it emerges from the colum
- Detection is usually based on the measurement of a physical parameter such as visible or ultraviolet absorption or fluorescence.
- A peak on the chart recorder represents each separated analyte.

A fraction collector: For collecting the separated analytes for further biochemical studies

Procedure

A. Preparation of the Column

- The column mostly consists of a glass tube packed with a suitable stationary phase.
- A glass wool/cotton wool or an asbestos pad is placed at the botton of the column before packing the stationary phase.
- After packing, a paper disc kept on the top, so that the stationary layer is not disturbed during the introduction of sample or mobile phase.

B. Introduction of the Sample

- The sample which is usually a mixture of components is dissolved in minimum quantity of the mobile phase.

- The entire sample is introduced into the column at once and get adsorbed on the top portion of the column.
- From this zone, individual sample can be separated by a process of elution.

C. Elution

- By elution technique, the individual components are separated out from the column.
- It can be achieved by two techniques:
- **Isocratic elution technique:** Same solvent composition or solvent of same polarity is used throughout the process of separation.

Eg. Use of chloroform alone.

- **Gradient elution technique:** Solvents of gradually ↑ polarity or ↑ elution strength are used during the process of separation.

E.g. initially benzene, then chloroform, then ethyl acetate then chloroform

D. Detection of Components

- If the compounds separated in a column chromatography procedure are colored, the progress of the separation can simply be monitored visually.
- If the compounds to be isolated from column chromatography are colorless.
- In this case, small fractions of the eluent are collected sequentially in labelled tubes and the composition of each fraction is analyzed by TLC

Applications

Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids. Its major application includes:

- Separation of mixture of compounds.
- Removal of impurities or purification process.
- Isolation of active constituents.
- Isolation of metabolites from biological fluids.
- Estimation of drugs in formulation or crude extracts.

Ion Exchange Chromatography

- 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 in 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

Principle

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.

Instrumentation

Typical IC instrumentation includes: pump, injector, column, suppressor, detector and recorder or data system.

Pump

- The IC pump is considered to be one of the most important components in the system which has to provide a continuous constant flow of the eluent through the IC injector, column, and detector.

Injector

- Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. Liquid samples may be injected directly and solid samples need only to be dissolved in an appropriate solvent. Injectors should provide the possibility of injecting the liquid sample within the range of 0.1 to 100 ml of volume with high reproducibility and under high pressure (up to the 4000 psi).

Columns

- Depending on its ultimate use and area of application, the column material may be stainless steel, titanium, glass or an inert plastic such as PEEK. The column can vary in diameter from about 2mm to 5 cm and in length from 3 cm to 50 cm depending on whether it is to be used for normal analytical purposes, microanalysis, high speed analyses or preparative work.
- Guard column is placed anterior to the separating column. This serves as a protective factor that prolongs the life and usefulness of the separation column. They are dependable columns designed to filter or remove particles that clog the separation column

Suppressor

- The suppressor reduces the background conductivity of the chemicals used to elute samples from the ion-exchange column which improves the conductivity measurement of the ions being tested. IC suppressors are membrane-based devices which are designed to convert the ionic eluent to water as a means of enhancing the sensitivity.

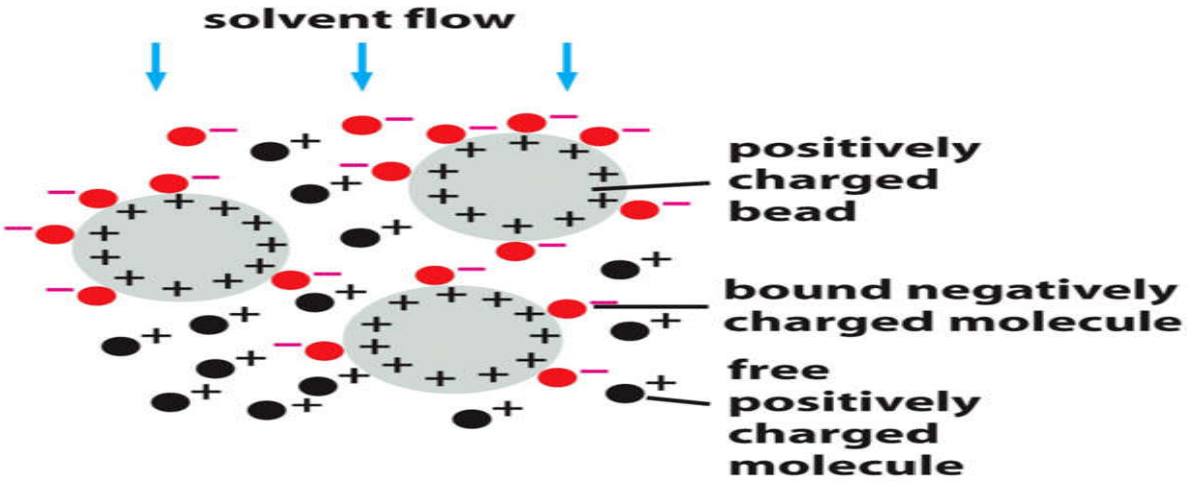
Detectors

- Electrical conductivity detector is commonly use.

Data system

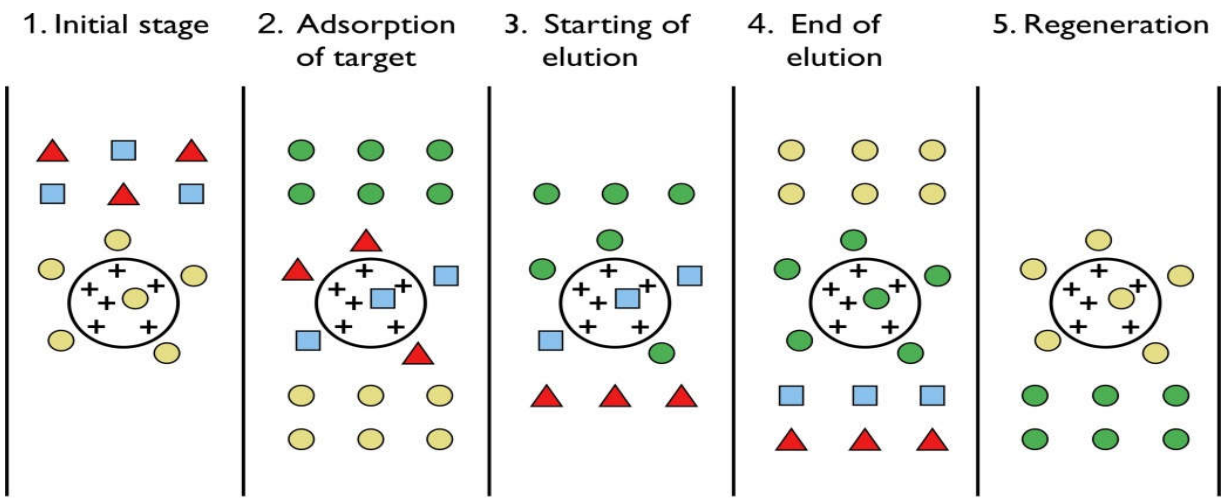
- In routine analysis, where no automation is needed, a pre-programmed computing integrator may be sufficient. For higher control levels, a more intelligent device is necessary, such as a data station or minicomputer.

Procedure



ION-EXCHANGE CHROMATOGRAPHY

- Ion exchange separations are carried out mainly in columns packed with an ion-exchanger.
- These ionic exchangers are commercially available. They are made up of styrene and divinyl benzene. Example. DEAE-cellulose is an anionic exchanger, CM-cellulose is a cationic exchanger.
- The choice of the exchanger depends upon the charge of particle to be separated. To separate anions "Anionic exchanger" is used, to separate cations "Cationic exchanger" is used.
- First the column is filled with ion exchanger then the sample is applied followed by the buffer. The tris-buffer, pyridine buffer, acetate buffer, citrate and phosphate buffers are widely used.
- The particles which have high affinity for ion exchanger will come down the column along with buffers.
- In next step using corresponding buffer separates the tightly bound particles.
- Then these particles are analyzed spectroscopically.



➢

Applications

- This is most effective method for water purification. Complete deionization of water (or) a non-electrolyte solution is performed by exchanging solute cations for hydrogen ions and solute anions for hydroxyl ions. This is usually achieved by method is used for softening of drinking water.
- In the analysis of products of hydrolysis of nucleic acids. In this way, information is gained about the structure of these molecules and how it relates to their biological function as carriers of hereditary information.
- Chelating resins are used to collect trace metals from seawater.
- To analyze lunar rocks and rare trace elements on Earth.

Affinity Chromatography

- 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.

Principle

Separation of a desired protein using affinity chromatography relies on the reversible interactions between the protein to be purified and the affinity ligand coupled

to chromatographic matrix. As stated earlier, most of the proteins have an inherent recognition site that can be used to select the appropriate affinity ligand. The binding between the protein of interest and the chosen ligand must be both specific and reversible.

Instrumentation

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.

Spacer arm

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

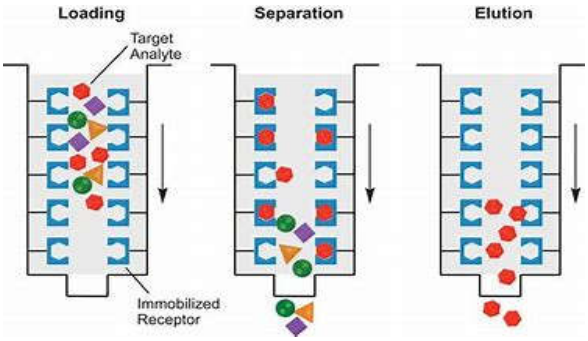
Ligand

- 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.

Procedure

- Affinity medium is equilibrated in binding buffer.
- Sample is applied under conditions that favor specific binding of the target molecule(s) to a complementary binding substance (the ligand). Target substances bind specifically, but reversibly, to the ligand and unbound material washes through the column.

- Elution is performed specifically, using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Target protein is collected in a purified, concentrated form.
- Affinity medium is re-equilibrated with binding buffer



These events can be summarized into the following three major steps:

Preparation of Column

- The column is loaded with solid support such as sepharose, agarose, cellulose etc.
- Ligand is selected according to the desired isolate.
- Spacer arm is attached between the ligand and solid support.

Loading of Sample

- Solution containing a mixture of substances is poured into the elution column and allowed to run at a controlled rate.

Elution of Ligand-Molecule Complex

- Target substance is recovered by changing conditions to favor elution of the bound molecules.

Applications

Widely Used In

- 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 invitro antigen-antibody reactions
- Detection of Single Nucleotide polymorphisms and mutations in nucleic acids

LC-MS (Liquid Chromatography – Mass Spectroscopy)

Liquid chromatography–mass spectrometry (**LC-MS**) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS).

Principle

Liquid chromatography / Mass Spectroscopy (LC / MS) is a technique which combines high performance liquid chromatography HPLC, a powerful analytical separation technique with mass spectroscopy,a powerful analysis & detection technique

What is (LC) Liquid Chromatography?

Liquid chromatography (LC) is a separation process used to isolate the individual components of a mixture. This process involves mass transfer of a sample through a polar mobile phase and non-polar stationary phase.

How (LC) Liquid Chromatography Works?

The device is a column packed with the porous medium made of a granular solid material (i.e., stationary phase), such as polymers and silica, where the sample is injected and the solvent (i.e., mobile phase) passes to transport the sample.

When a sample is injected, it is adsorbed on the stationary phase, and the solvent passes through the column to separate the compounds one by one, based on their relative affinity to the packing materials and the solvent. The component with the most affinity to the stationary phase is the last to separate. This is because high affinity corresponds to more time to travel to the end of the column.

What is (MS) Mass Spectrometry?

Mass spectrometry (MS) ionizes atoms or molecules to facilitate their separation and detection in accordance with their molecular masses and charges (mass to charge ratio). MS is used in various applications, e.g., biochemicals and atomic physics.

How (MS) Mass Spectrometry Works?

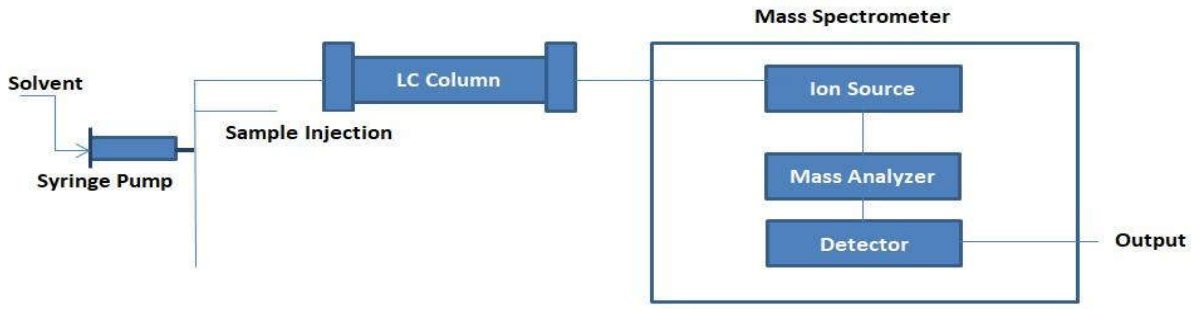
The main processes that take place in a mass spectrometer are:

1. Sample Introduction: sample is converted into a gaseous phase (except with gaseous samples or samples that are thermally unstable) and is introduced through the inlet to the ionization chamber

2. Ionization: gaseous sample is ionized to generate cations (in most cases but a few types of MS work with anions)
3. Separation: ions separate according to their mass/charge ratio by a mass analyzer
4. Detection: a detector is used to determine the species and quantity of each ion.

What is LC-MS?

The combined technique between MS and HPLC is commonly known as LC-MS. Combining the two analytical methods reduces experimental error and improves accuracy. This technique is very useful in applications that involve a huge number of compounds, such as environmental effluents.



Simple Schematic of LC-MS System

How LC-MS Works

LC-MS involves separating mixtures in accordance with their physical and chemical properties, then identifying the components within each peak and detecting based on their mass spectrum. The flow rates used in LC-MS should be less than those used for HPLC. This is to ensure complete ionization and to maintain the detection sensitivity of the MS, which starts to decrease beyond 200 µL/min. Therefore, the column in LC-MS is much smaller to accommodate the smaller solvent flow rates and sample volumes.

This makes syringe pumps very convenient for LC-MS because they are very accurate and can deliver very low flow rates. In addition, it is possible to use syringe pumps for sample injection into the system as they can deliver very precise sample dosing

UNIT: V ELECTROPHORESIS

Electrophoresis

Electrophoresis is the motion of dispersed particles relative to a fluid under the influence of a spatially uniform electric field. Electrophoresis of positively charged particles (cations) is sometimes called **cataphoresis**, while electrophoresis of negatively charged particles (anions) is sometimes called **anaphoresis**.

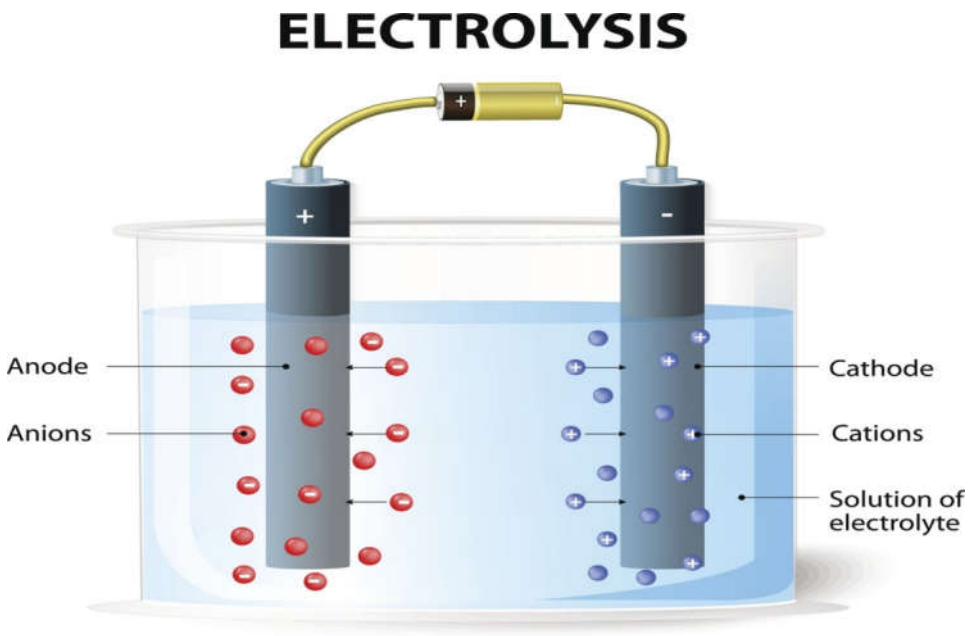
Principle

Electrophoresis is general term that involves the migration and separation of charged ions under the influence of electric current.

It consists of two electrode anode & cathode and electrolyte which serve as conducting medium

Applications

- Electrophoresis is used in laboratories to separate macromolecules based on size, and they are also used extensively in DNA, RNA and protein analysis



Native & Denatured Electrophoresis

Native gel electrophoresis varies from denaturing gel electrophoresis in that the proteins analyzed remain folded and retain their charges.

Both types of gel electrophoresis operate using the same basic principle. Protein samples are loaded into the top of a polyacrylamide gel. An electric current is passed through the gel, and the proteins migrate through the gel. In denaturing gels, the proteins are coated with SDS, which gives them a strong negative charge. As a result, denatured proteins migrate through the gel based primarily on their molecular weight.

Native Gel Electrophoresis

Native gel electrophoresis is a method by which proteins are separated on a gel without having been denatured In native gel electrophoresis, the proteins are still folded, native PAGE also has the potential for separating proteins of identical molecular weight which cannot be resolved with SDS - PAGE. In addition, proteins on native PAGE usually retain their activity. This allows enzymes to be detected by sensitive and specific activity stains and delicate proteins to be resolved and recovered in a biologically active form.

Native gel electrophoresis is used to study binding to other compounds, aggregation and conformation. Native gels are necessary for many of these techniques, as denaturing protein causes it to lose its structure and any binding affinities it may have. The last benefit of native gel electrophoresis is that it is possible to extract the proteins after running the gel.

Denatured gel electrophoresis is a method by which proteins are separated on a gel as denatured

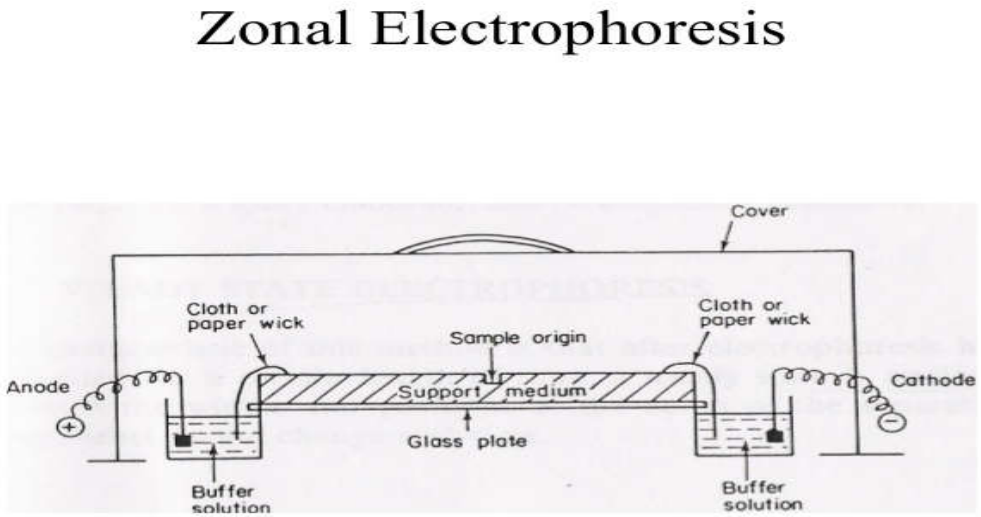
The most commonly used denaturant is sodium dodecyl sulfate (SDS). SDS is an amphipathic surfactant. It denatures proteins by binding to the protein chain with its hydrocarbon tail, exposing normally buried regions and coating the protein chain with surfactant molecules. The polar head group of SDS adds an additional benefit to the use of this denaturant. Proteins solubilized in SDS bind the detergent uniformly along their length to a level of 1.4g SDS/g protein. This creates a charge/mass ratio which is consistent between proteins, make it denatured

Zone Electrophoresis

Zone Electrophoresis or Capillary electrophoresis is a technique that is used to separate out molecules by their size and electrical charge. It is also often called capillary zone electrophoresis or capillary gel electrophoresis. Capillary electrophoresis analysis relies on the principle that molecules possess differing electrical charges and differing weights. Thus, when molecules in a substrate are exposed to an electrical field, different molecules will move in different directions and at differing speeds within the substrate.

Principle & Procedure

When a substrate has an electrical field applied to it by means of one electrode at either end of the field, positively charged molecules will move towards the negative electrode, and negatively charged molecules will move towards the positive electrode. The relative speed at which the molecule will move through the substrate is determined by a characteristic known as the hydrodynamic size of the molecule. The hydrodynamic size of a molecule depends on two factors, its mass, and the strength of its positive or negative charge. A large molecule with a strong positive charge will tend to move relatively rapidly towards the negative electrode in an electrical field. A small molecule with a weak negative charge will tend to move much more slowly towards the positive electrode in an electrical field.



Once a charge has been applied to a sample for a set length of time, and the molecules being analyzed have been separated, it is necessary to show the scientist where the different molecules are located in the substrate. In many modern capillary electrophoresis systems this is achieved with ultraviolet (UV) light. The UV illumination can show a user the physical path that has been traced by a molecule, or set of molecules, through the substrate. In other systems, the molecules to be analyzed are first treated with chemicals to give them a fluorescent property. Fluorescent detection methods can be very sensitive, but are not suitable for every type of molecule.

Applications

The applications of capillary electrophoresis in science are many and varied. Capillary electrophoresis sequencing is a process, used particularly by geneticists, in which capillary electrophoresis is used to analyze molecules of the genetic material Deoxyribo Nucleic Acid (DNA). Food scientists may use capillary electrophoresis protein analysis to determine the different protein content characteristics of food. In medical research, this process has many applications, such as the analysis of antibodies, and the study of how they bind to other molecules.

Isoelectro Focussing

Isoelectric focusing (**IEF**), also known as **electrofocusing**, is a technique for separating different molecules by differences in their isoelectric point (pI). 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

Principle & Procedure

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.

A protein that is in a pH region below its isoelectric point (pI) will be positively charged and so will migrate toward the cathode (negatively charged electrode). As it migrates through a gradient of increasing pH, however, the protein's overall charge will decrease until the protein reaches the pH region that corresponds to its pI. At this point it has no net charge and so migration ceases (as there is no electrical attraction toward either electrode). As a result, the proteins become focused into sharp stationary bands with each protein positioned at a point in the pH gradient corresponding to its pI. The technique is capable of extremely high resolution with proteins differing by a single charge being fractionated into separate bands.

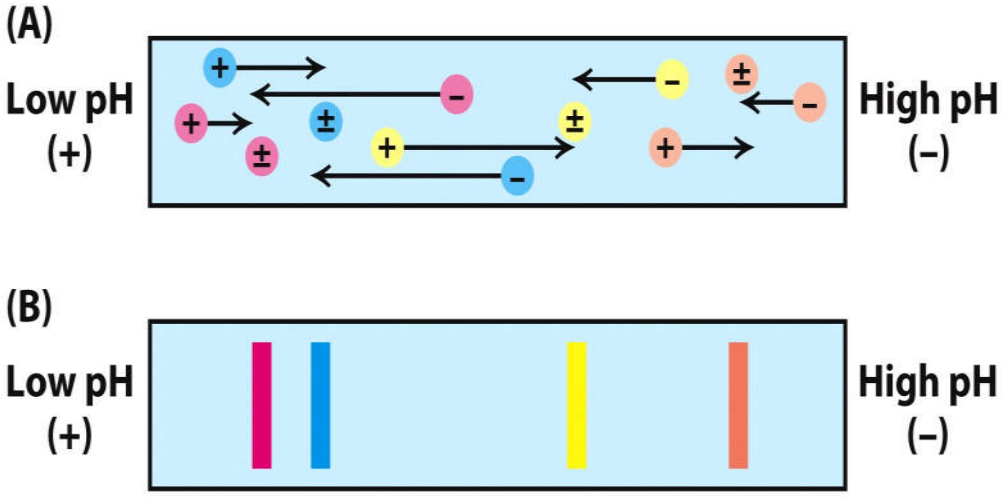


Figure 3.11
Biochemistry, Seventh Edition

Molecules to be focused are distributed over a medium that has a pH gradient (usually created by aliphatic ampholytes). An electric current is passed through the medium, creating a "positive" anode and "negative" cathode end. Negatively charged molecules migrate through the pH gradient in the medium toward the "positive" end while positively charged molecules move toward the "negative" end. As a particle moves toward the pole opposite of its charge it moves through the changing pH gradient until it reaches a point in which the pH of that molecules isoelectric point is reached. At this point the molecule no longer has a net electric charge (due to the protonation or deprotonation of the associated functional groups) and as such will not proceed any further within the gel. The gradient is established before adding the particles of interest by first subjecting a

solution of small molecules such as polyampholytes with varying pI values to electrophoresis.

Applications

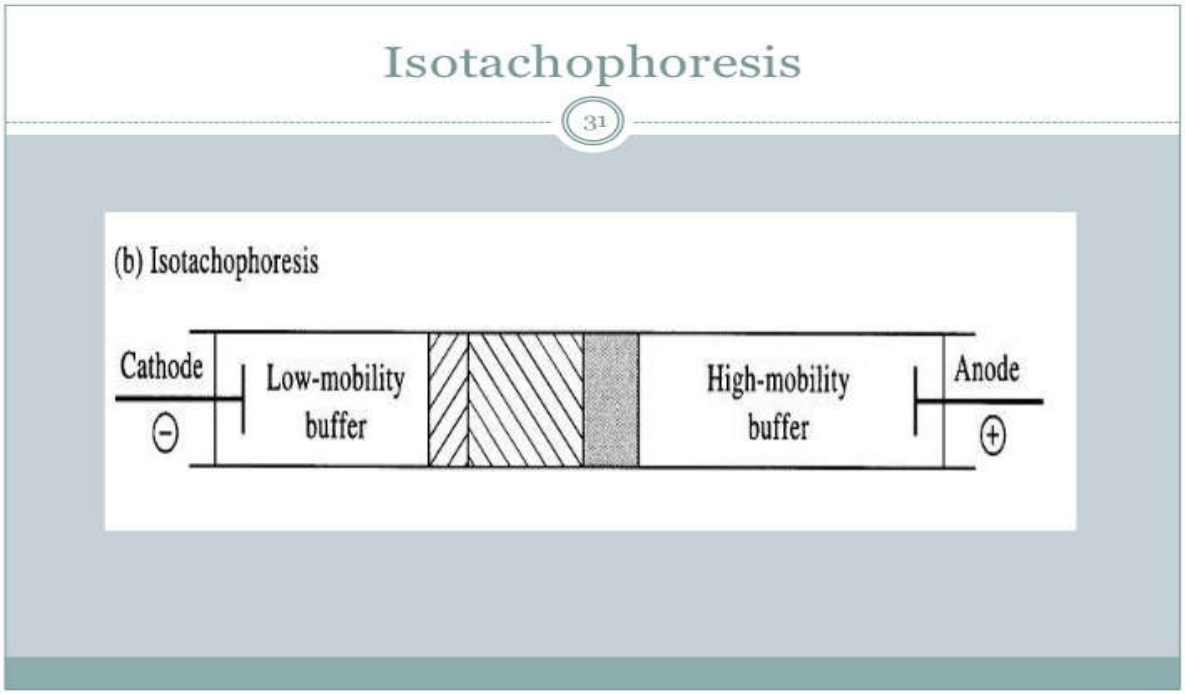
The method is applied particularly often in the study of proteins, which separate based on their relative content of acidic and basic residues, whose value is represented by the pI.

Isoelectric focusing can resolve proteins that differ in pI value by as little as 0.01.

Isoelectric focusing is the first step in two-dimensional gel electrophoresis, in which proteins are first separated by their pI value and then further separated by molecular weight through SDS-PAGE

Isotachophoresis

Isotachophoresis (**ITP**) is a technique in analytical chemistry used for selective separation and concentration of ionic analytes. It is a form of electrophoresis, in which charged analytes are separated based on ionic mobility, a factor which tells how fast an ion migrates through an electric field



Overview

In conventional ITP separations, a discontinuous buffer system is used. The sample is introduced between a zone of fast leading electrolyte (LE) and a zone of slow terminating (or: trailing) electrolyte (TE). Usually, the LE and the TE have a common counterion, but the coions (having charges with the same sign as the analytes of interest) are different: the LE is defined by coions with high ionic mobility, while the TE is defined by coions with low ionic mobility. The analytes of interest have intermediate ionic mobility. Application of an electric potential results in a low electrical field in the leading electrolyte and a high electrical field in the terminating electrolyte. Analyte ions situated in the TE zone will migrate faster than the surrounding TE coions, while analyte ions situated in the LE will migrate slower; the result is that analytes are focused at the LE/TE interface.

ITP is a displacement method in which focusing ions of a certain kind displace other ions. If present in sufficient amounts, focusing analyte ions can displace all electrolyte coions, reaching a plateau concentration. Multiple analytes with sufficiently different ionic mobilities will form multiple plateau zones. Indeed, plateau mode ITP separations are readily recognized by stairlike profiles, each plateau of the stair representing an electrolyte or analyte zone having (from LE to TE) increasing electric fields and decreasing conductivities. In peak mode ITP, analytes amounts are insufficient to reach plateau concentrations, such analytes will focus in sharp Gaussian-like peaks. In peak mode ITP, analyte peaks will strongly overlap, unless so-called spacer compounds are added with intermediate ionic mobilities between those of the analytes; such spacer compounds are able to segregate adjacent analyte zones.

A completed ITP separation is characterized by a dynamic equilibrium in which all co-ionic zones migrate with equal velocities

Polymerase Chain Reaction

- PCR is an enzymatic process in which a specific region of DNA is replicated over and over again to yield many copies of a particular sequence.
- The most widely used target nucleic acid amplification method is the polymerase chain reaction (PCR).
- This method combines the principles of complementary nucleic acid hybridization with those of nucleic acid replication applied repeatedly through numerous cycles.
- This method is able to amplify a single copy of a **nucleic acid** target, often undetectable by standard hybridization methods, and multiply to 10^7 or more copies in a relatively short period.
- This thus provides ample target that can be readily detected by numerous methods.

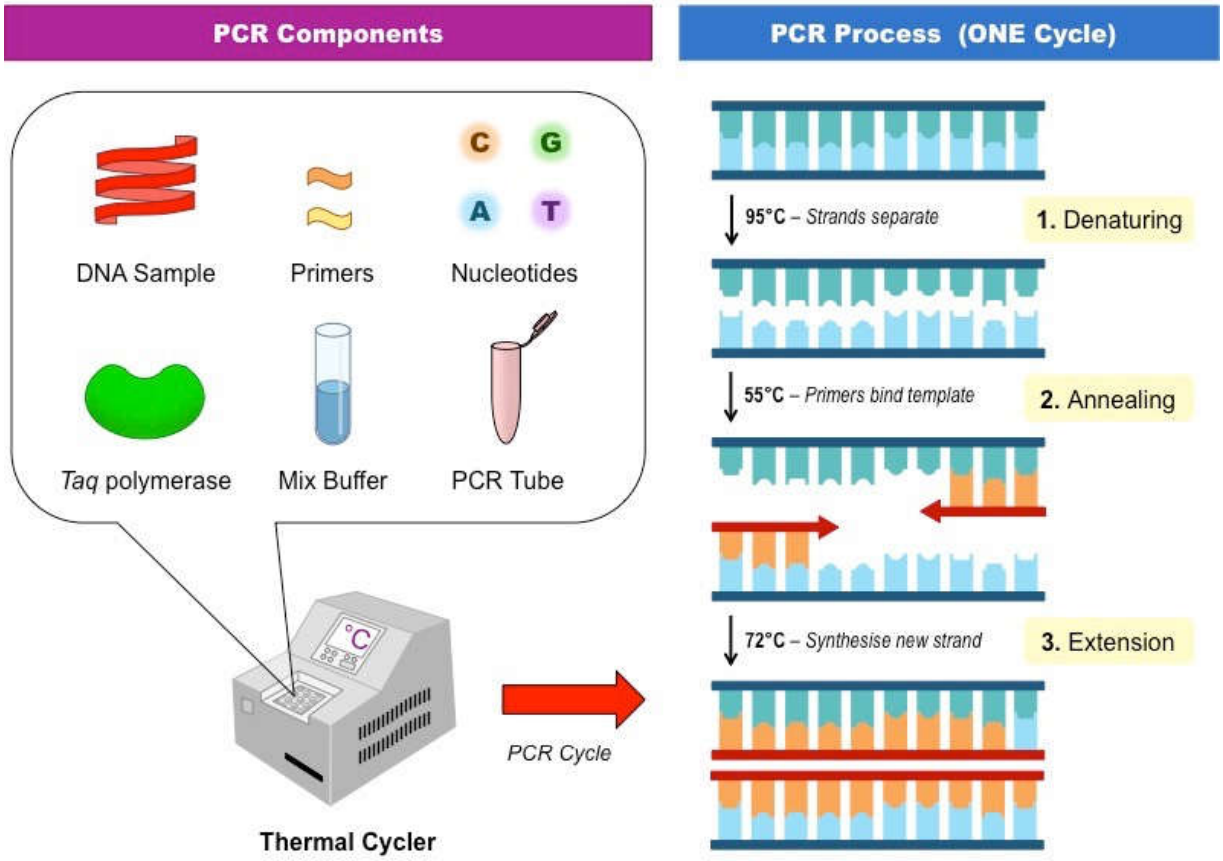
Principle

The target sequence of nucleic acid is denatured to single strands, primers specific for each target strand sequence are added, and **DNA** polymerase catalyzes the addition of deoxynucleotides to extend and produce new strands complementary to each of the target sequence strands (cycle 1). In cycle 2, both double-stranded products of cycle 1 are denatured and subsequently serve as targets for more primer annealing and extension by DNA polymerase. After 25 to 30 cycles, at least 10^7 copies of target DNA may be produced by means of this thermal cycling.

Requirements

- A PCR reaction contains the target double-stranded DNA, two primers that hybridize to flanking sequences on opposing strands of the target, all four deoxyribonucleoside triphosphates and a DNA polymerase along with buffer, co-factors of **enzyme** and water.
- Since the reaction periodically becomes heated to high temperature, PCR depends upon using a heat-stable DNA polymerase.
- Many such heat-stable enzymes from thermophilic bacteria (bacteria that live in high temperature surroundings) are now available commercially.
- The first one and the most commonly used is the Taq polymerase from the thermophilic bacterium *Thermus aquaticus*.

Steps involved



Conventional PCR involves 25 to 50 repetitive cycles, with each cycle comprising three sequential reactions:

1. **Denaturation** of target nucleic acid
2. **Primer annealing** to single-strand target nucleic acid extension of primer target duplex.
3. **Extension** of the primer-target duplex.

Denaturation

- The reaction mixture is heated to 95°C for a short time period (about 15–30 sec) to denature the target DNA into single strands that can act as templates for DNA synthesis.

Primer annealing

- The mixture is rapidly cooled to a defined temperature which allows the two primers to bind to the sequences on each of the two strands flanking the target DNA.
- Primers are short, single-stranded sequences of nucleic acid (i.e., oligonucleotides usually 20 to 30 nucleotides long) selected to specifically hybridize (anneal) to a particular nucleic acid target, essentially functioning like probes.
- This annealing temperature is calculated carefully to ensure that the primers bind only to the desired DNA sequences (usually around 55^{o}C).
- One primer binds to each strand. The two parental strands do not re-anneal with each other because the primers are in large excess over parental DNA.

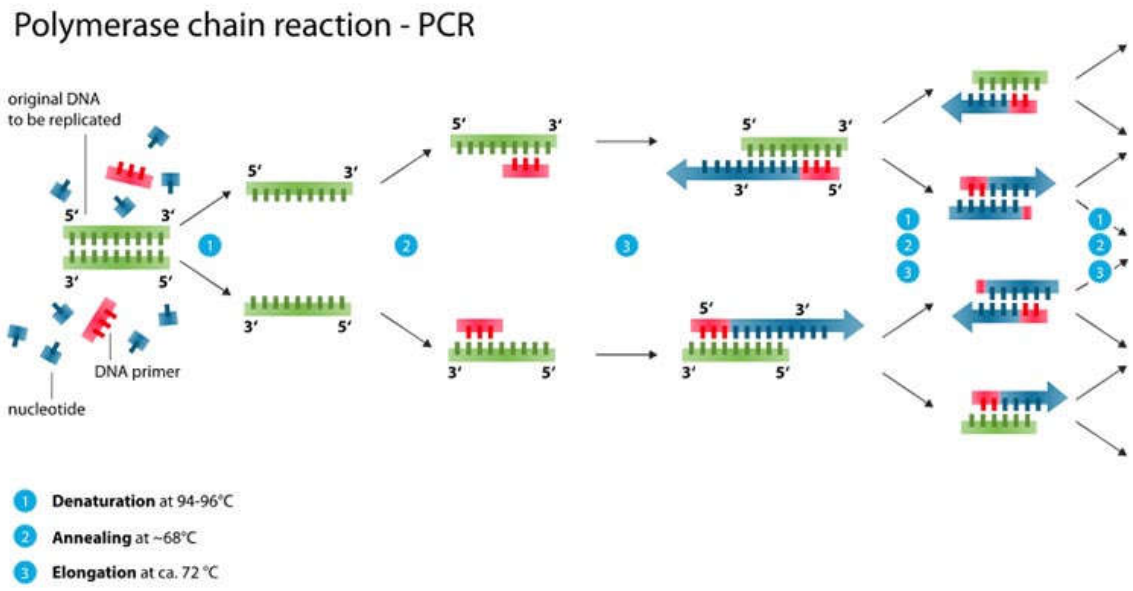
Extension

- The temperature of the mixture is raised to 72°C (usually) and kept at this temperature for a pre-set period of time to allow DNA polymerase to elongate each primer by copying the single-stranded templates.
- Annealing of primers to target sequences provides the necessary template format that allows the DNA polymerase to add nucleotides to the 3' terminus (end) of each primer and extend sequence complementary to the target template
- Taq polymerase is the enzyme commonly used for primer extension, which occurs at 72°C. This enzyme is used because of its ability to function efficiently at elevated temperatures and to withstand the denaturing temperature of 94°C through several cycles.
- The ability to allow primer annealing and extension to occur at elevated temperatures without detriment to the polymerase increases the stringency of the reaction, thus decreasing the chance for amplification of non-target nucleic acid (i.e., nonspecific amplification).

The three steps of the PCR cycle are repeated.

- Thus in the second cycle, the four strands denature, bind primers and are extended. No other reactants need to be added. The three steps are repeated for a third cycle and so on for a set of additional cycles.
- By the third cycle, some of the PCR products represent DNA sequence only between the two primer sites and the sequence does not extend beyond these sites.

- As more and more reaction cycles are carried out, the double-stranded DNA are synthesized more in number. After 20 cycles, the original DNA has been amplified a million-fold and this rises to a billion fold (1000) million after 30 cycles



Analysis

- Gel electrophoresis of the amplified product is commonly employed after amplification.
- The amplified DNA is electrophoretically migrated according to their molecular size by performing agarose gel electrophoresis.
- The amplified DNA forms clear bands which can be visualized under ultra-raviolet (UV) light.

Applications

- PCR can amplify a single DNA molecule from a complex mixture, largely avoiding the need to use DNA cloning to prepare that molecule. Variants of the technique can similarly amplify a specific single RNA molecule from a complex mixture.
- DNA sequencing has been greatly simplified using PCR, and this application is now common.
- By using suitable primers, it is possible to use PCR to create point mutations, deletions and insertions of target DNA which greatly facilitates the analysis of gene expression and function.

- PCR is exquisitely sensitive and can amplify vanishingly small amounts of DNA. Thus, using appropriate primers, very small amounts of specified bacteria and viruses can be detected in tissues, making PCR invaluable for medical diagnosis.

Maldi Tof

MALDI stands for **M**atrix-**A**ssisted **L**aser **D**esorption **I**onization. In this ionization method samples are fixed in a crystalline matrix and are bombarded by a laser. The sample molecules vaporize into the vacuum while being ionized at the same time without fragmenting or decomposing.

TOF stands for **T**ime **o**f **F**light, a mass spectrometry method that separates ions by their mass to charge ratio and determines that mass to charge ratio by the time it takes for the ions to reach a detector.

This technology generates characteristic mass spectral fingerprints which is compared with large library of mass spectra. As the spectral fingerprints are unique signatures for each microorganism accurate microbial identification at the genus and species levels is done using bioinformatics pattern profiling

Working Principle of MALDI-TOF Mass Spectrometry

The MALDI TOF process is a two-phase procedure;

1. Ionization Phase
2. Time of Flight Phase

Ionization Phase:

Initially, the samples are fixed in a crystalline matrix in a target plate and are bombarded by a laser. The sample molecules vaporize into the vacuum while being ionized at the same time. High voltage is then applied to accelerate the charged particles.

The second step is the time-of-flight mass spectrometry phase.

1. In the linear mode, particles will impinge upon the linear detector within a few nanoseconds after ionization. Higher mass molecules will arrive later than lighter ones. Flight time measurement make it possible to determine molecule masses directly. Each peak in the spectrum corresponds to the specific mass of the particle along the time axis, starting with the ionization moment.

2. In the reflector mode, the particles are diverted so that they fly towards a second detector. In addition to extending the flight distance, the reflector also focuses the masses. The combination of these two effects makes for higher resolution than in the linear mode.

Procedure

- Pick a bacterial colony and smear onto a target plate.
- Add 1-2 ml of matrix consisting α-Cyano-4-hydroxycinnamic acid (CHCA) dissolved in acetonitrile (50%) and 2.5% trifluoroacetic acid on to it and dry it on the target plate (at room air)
- Place the target plate in to the plate chamber of the mass spectrometer, close it and perform the analysis.

Applications

MALDI-TOF MS is being used for routine diagnostic or diagnostic-like purposes in clinic, veterinary, pharma and food microbiology (food quality control) laboratories as well as for environmental monitoring, biodefense and various biological research.