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SCHOOL OF BIO AND CHEMICAL ENGINEERING
DEPARTMENT OF BIOTECHNOLOGY

UNIT – I – ANALYTICAL TECHNIQUES – SBB2104

1.1 Bright field microscopy

Bright field Microscopy is the simplest of all the optical micrscopy 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 absorance of some 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.

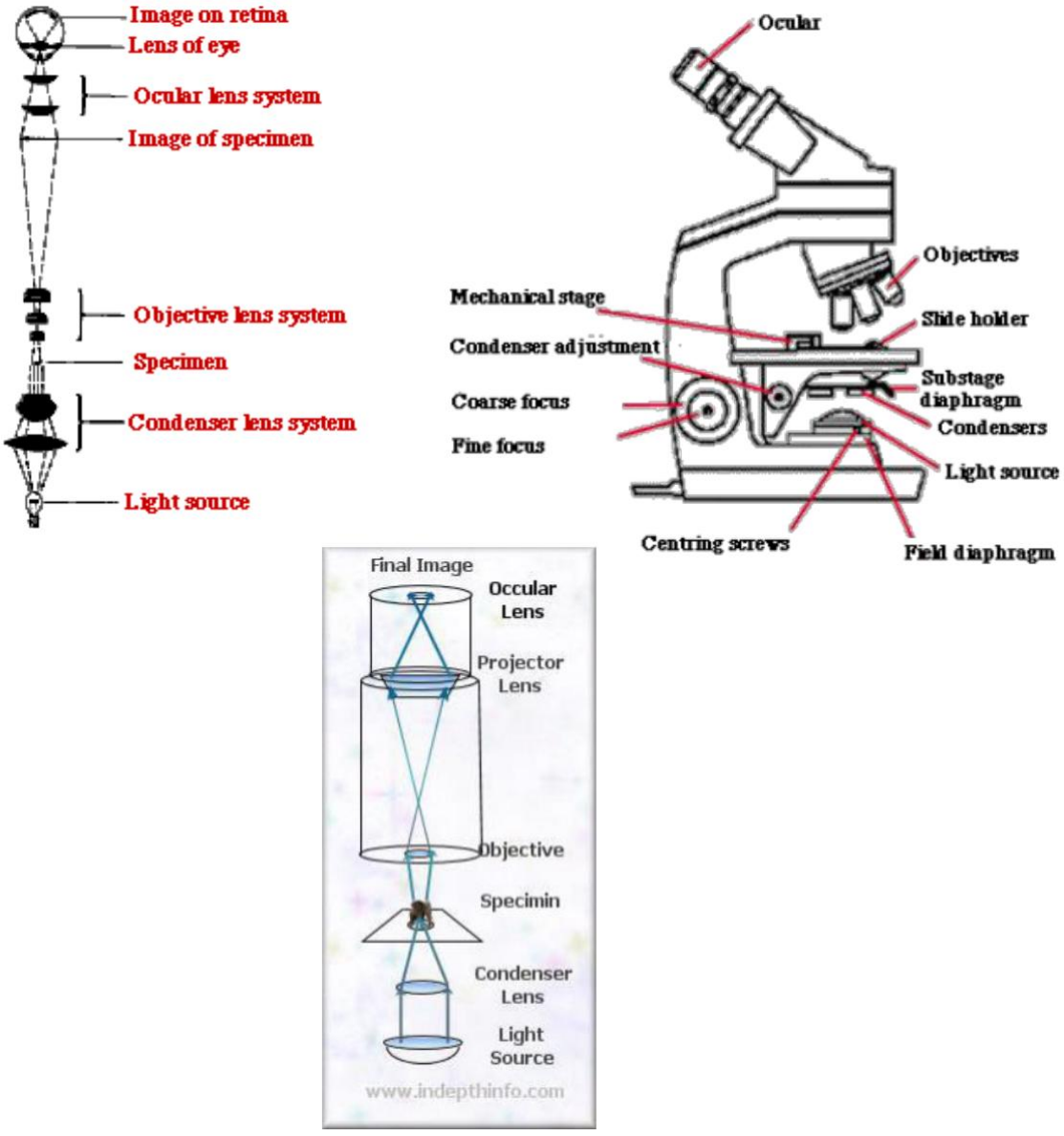

Figure 1 Light path of bright field microscope

Light path - The light path of a bright field microscope is extremely simple, no additional components are required beyond the normal light microscope setup (Fig 1). The light path therefore consists of:

Transillumination light source, commonly a halogen lamp in the microscope stand; A halogen lamp, also known as a tungsten halogen lamp or quartz iodine lamp, is an incandescent lamp that has a small amount of a halogen such as iodine or bromine added. The combination of the halogen gas and the tungsten filament produces a halogen cycle chemical reaction which redeposits evaporated tungsten back onto the filament, increasing its life and maintaining the clarity of the envelope

C**ondenser lens** which focuses light from the light source onto the sample. A condenser is one of the main components of the optical system of many transmitted light compound microscopes. A condenser is a lens that serves to concentrate light from the illumination source that is in turn focused through the object and magnified by the objective lens.

Objective lens : In an optical instrument, the objective is the optical element that gathers light from the object being observed and focuses the light rays to produce a real image. Objectives can be single lenses or mirrors, or combinations of several optical elements. Microscope objectives are characterized by two parameters: magnification and numerical aperture. The typically ranges are 4× , 10x , 40x and 100×. 4. oculars to view the sample image.

An **eyepiece**, or **ocular lens**, is a type of lens that is attached to a variety of optical devices such as microscopes. It is so named because it is usually the lens that is closest to the eye when someone looks through the device. The objective lens or mirror collects light and brings it to focus creating an image. The eyepiece is placed near the focal point of the objective to magnify this image. The amount of magnification depends on the focal length of the eyepiece.

Magnification is the process of enlarging something only in appearance, not in physical size. Typically magnification is related to scaling up visuals or images to be able to see more detail, increasing resolution.

Resolving power is the ability of an imaging device to separate (i.e., to see as distinct) points of an object that are located at a small angular distance..

In optics, the **numerical aperture** (NA) of an optical system is a dimensionless number that characterizes the range of angles over which the system can accept or emit light. In most areas

of optics, and especially in microscopy, the numerical aperture of an optical system such as an objective lens is defined by

NA = n Sin

where n is the index of refraction of the medium in which the lens is working (1.0 for air, 1.33 for pure water, and up to 1.56 for oils; see also list of refractive indices), and θ is the half-angle of the maximum cone of light that can enter or exit the lens. In general, this is the angle of the real marginal ray in the system

1.1.1 Working Performance

Bright field microscopy typically has low contrast with most biological samples as few absorb light to a great extent. Staining is often required to increase contrast, which prevents use on live cells in many situations. Bright field illumination is useful for samples which have an intrinsic colour, for example chloroplasts in plant cells.

Light is first emitted by the light **source** and is directed by the **condenser lens** on to the specimen, which might be a loose object, a prepared plate or almost anything. A microscope can even be applied to small parts of larger objects, though with a bit more difficulty. (The light does not absolutely need to originate below the specimen.)

The light from the specimen then passes through the objective lens. This lens is often selected from among three or four and is the main determinant for the level of magnification. It bends the light rays and in the case of this example sends them to a **projector lens**, which reverses their direction so that when the image reaches the eye it will not appear "upside-down". Not all microscopes have a projector lens, so the viewer may be seeing a reverse image. In these cases, when the slide is moved, it will appear to be moving in the opposite direction to the viewer.

The light rays then travel to the oracular lens or "eye piece". This is often a 10X magnification lens, meaning it magnifies the magnified image an additional ten times. The image is then projected into the eye. It is very seldom that a specimen is in focus the moment it is placed beneath a microscope. This means that some adjustment will have to be made. Unlike in telescopes, the focal length between lenses remains constant when adjusting the focus. The lens apparatus is brought closer to or further from the object. The focus adjustment is often along the neck of the tube containing the lenses, but it might just as well move the slide up and down. The best way to make this adjustment is to make a course adjustment so that it is too close to the

object and then back off with the fine adjustment2. This helps to ensure that the specimen is not inadvertently smashed by the lens.

1.1.2 Advantages

- The name "brightfield" is derived from the fact that the specimen is dark and contrasted by the surrounding bright viewing field. Simple light microscopes are sometimes referred to as 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.

1.1.3 Disadvantages

Certain disadvantages are inherent in any optical imaging technique.

- By using an aperture diaphragm for contrast, past a certain point, greater contrast adds distortion. However, employing an iris diaphragm will help compensate for this problem.
- Brightfield microscopy can't be used to observe living specimens of bacteria, although when using fixed specimens, bacteria have an optimum viewing magnification of 1000x.
- Brightfield microscopy has very low contrast and most cells absolutely have to be stained to be seen; staining may introduce extraneous details into the specimen that should not be present.
- Also, the user will need to be knowledgeable in proper staining techniques.
- Lastly, this method requires a strong light source for high magnification applications and intense lighting can produce heat that will damage specimens or kill living microorganisms.

1.2Phase contrast microscopy

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. Phase shifts themselves are invisible, but become visible when shown as brightness variations.

When light waves travels through a medium other than vacuum, interaction with the medium causes the wave amplitude and phase to change in a manner dependent on properties of the

medium. Changes in amplitude (brightness) arise from the scattering and absorption of light, which is often wavelength dependent and may give rise to colors. Photographic equipment and the human eye are only sensitive to amplitude variations. Without special arrangements, phase changes are therefore invisible. Yet, often these changes in phase carry important information.

1.2.1 History and Background Information

Frits Zernike, a Dutch physicist and mathematician, built the first phase contrast microscope in 1938.

It took some time before the scientific community recognized the potential of Zernike's

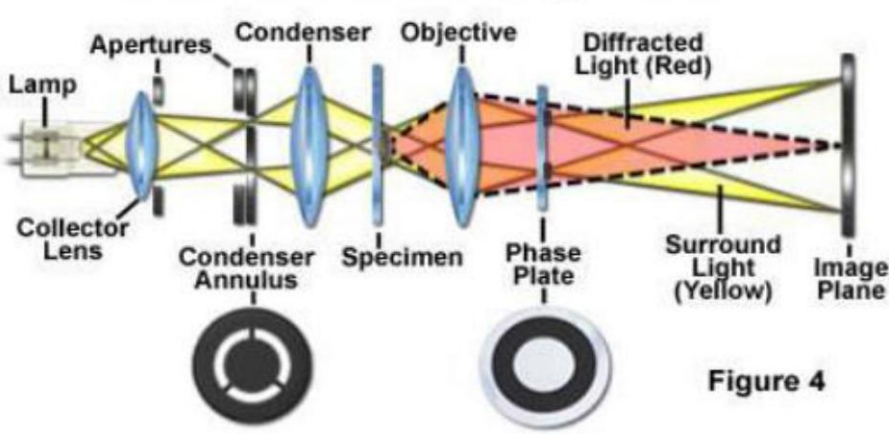

Figure 4

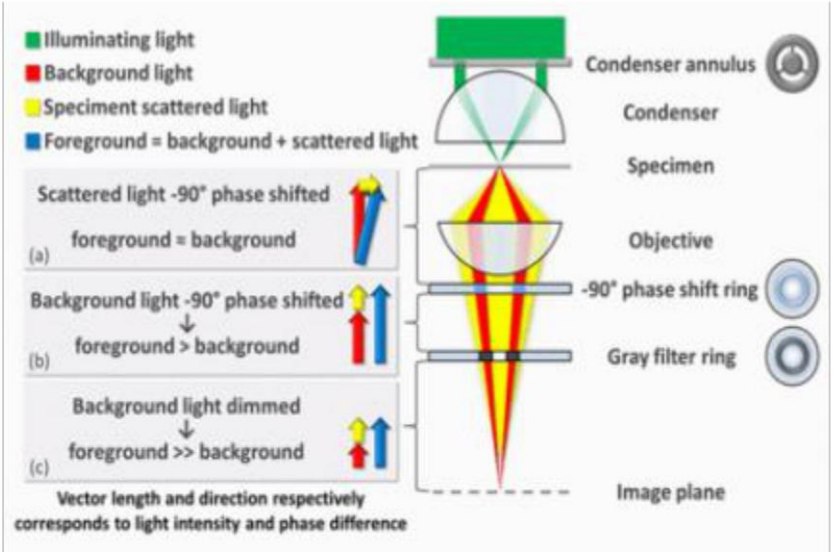

Figure 2 Phase contrast microscope

discovery; he won the Nobel Prize in 1953 and the German-based company Zeiss began manufacturing his phase contrast microscope during World War II. .

1.2.2 Working Principle

The basic principle to make phase changes visible in phase contrast microscopy is to separate the illuminating background light from the specimen scattered light, which make up the foreground details, and to manipulate these differently.

The ring shaped illuminating light (green) that passes the condenser annulus is focused on the specimen by the condenser. Some of the illuminating light is scattered by the specimen (yellow). The remaining light is unaffected by the specimen and form the background light (red). When observing unstained biological specimen, the scattered light is weak and typically phase shifted by -90° — relative to the background light. This leads to that the foreground (blue vector) and the background (red vector) nearly have the same intensity, resulting in a low image contrast (a). In a phase contrast microscope, the image contrast is improved in two steps. The background light is phase shifted -90° by passing it through a phase shift ring. This eliminates the phase difference between the background and the scattered light, leading to an increased intensity difference between foreground and background (b). To further increase contrast, the background is dimmed by a gray filter ring (c). Some of the scattered light will be phase shifted and dimmed by the rings. However, the background light is affected to a much greater extent, which creates the phase contrast effect (Fig 2).

The above describes negative phase contrast. In its positive form, the background light is instead phase shifted by +90°. The background light will thus be 180° out of phase relative to the scattered light. This results in that the scattered light will be subtracted from the background light in (b) to form an image where the foreground is darker than the background.

12.3 Applications in Microscopy

The possible applications of Zernike's phase contrast microscope in microscopy are evident in the fields of molecular and cellular biology, microbiology and medical research.

Specimens that can be observed and studied include live microorganisms such as protozoa, erythrocytes, bacteria, molds and sperm, thin tissue slices, lithographic patterns, fibers, glass fragments and sub-cellular particles such as nuclei and organelles.

1.2.4 Advantages

The advantages of the phase contrast microscope include:

- The capacity to observe living cells and, as such, the ability to examine cells in a natural state
- Observing a living organism in its natural state and/or environment can provide far more information than specimens that need to be killed, fixed or stain to view under a microscope
- High-contrast, high-resolution images
- Ideal for studying and interpreting thin specimens
- Ability to combine with other means of observation, such as fluorescence
- Modern phase contrast microscopes, with CCD or CMOS computer devices, can capture photo and/or video images
- In addition, advances to the phase contrast microscope, especially those that incorporate technology, enable a scientist to hone in on minute internal structures of a particle and can even detect a mere small number of protein molecules.

1.2.5 Disadvantages

- Disadvantages and limitations of phase contrast:
- Annuli or rings limit the aperture to some extent, which decreases resolution
- This method of observation is not ideal for thick organisms or particles
- Thick specimens can appear distorted
- Images may appear grey or green, if white or green lights are used, respectively, resulting in poor photomicrography
- Shade-off and halo effect, referred to a phase artifacts
- Shade-off occurs with larger particles, results in a steady reduction of contrast moving from the center of the object toward its edges
- Halo effect, where images are often surrounded by bright areas, which obscure details along the perimeter of the specimen
- Modern advances and techniques provide solutions to some of these confines, such as the halo effect.
- Anodized phase contrast utilizes amplitude filters that contain neutral density films to minimize the halo effect. Essentially, this is attempting to reverse the definition achieved through phase contrast annuli, but the halo effect can never be eliminated completely.

- The pros that phase contrast has brought to the field of microscopy far exceed its limitations. This is easily seen with the myriad of advances in the fields of cellular and microbiology as well as in medical and veterinary sciences.

1.2.6 Conclusion

The **phase contrast microscope** opened up an entire world of microscopy, providing incredible definition and clarity of particles never seen before.

1.3 Fluorescence microscope

Figure 3 An upright fluorescence microscope (Olympus BX61) with the fluorescent filter cube turret above the objective lenses, coupled with a digital camera.

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. 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 (Fig 3).

1.3.1 Principle

The specimen is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by the fluorophores, causing them to emit light of longer wavelengths (i.e., of a different color than the absorbed light). The illumination light is separated from the much weaker

emitted fluorescence through the use of a spectral emission filter. Typical components of a fluorescence microscope are a light source (xenon arc lamp or mercury-vapor lamp are common; more advanced forms are high-power LEDs and lasers), the excitation filter, the dichroic mirror (or dichroic beamsplitter), and the emission filter (see figure below). The filters and the dichroic are chosen to match the spectral excitation and emission characteristics of the fluorophore used to label the specimen.In this manner, the distribution of a single fluorophore (color) is imaged at a time. Multi-color images of several types of fluorophores must be composed by combining several single-color images.

Most fluorescence microscopes in use are epifluorescence microscopes, where excitation of the fluorophore and detection of the fluorescence are done through the same light path (i.e. through the objective). These microscopes are widely used in biology and are the basis for more advanced microscope designs, such as the confocal microscope and the total internal reflectio fluorescence microscope (TIRF).

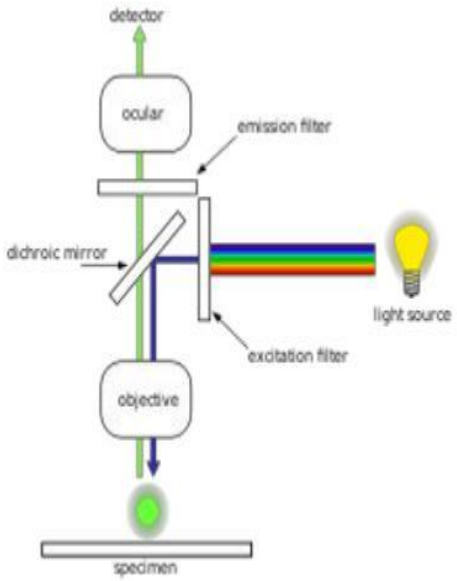

Figure 4 Schematic of a fluorescence microscope.

The majority of fluorescence microscopes, especially those used in the life sciences, are of the epifluorescence design shown in the diagram. Light of the excitation wavelength is focused onthe specimen through the objective lens. The fluorescence emitted by the specimen is focused to the detector by the same objective that is used for the excitation which for greatest sensitivitywill have a very high numerical aperture. Since most of the excitation light is

transmitted through the specimen, only reflected excitatory light reaches the objective together with the emitted light and the epifluorescence method therefore gives a high signal to noise ratio. An additional barrier filter between the objective and the detector can filter out the remaining excitation light from fluorescent light (Fig 3).

1.3.2 Light sources

Fluorescence microscopy requires intense, near-monochromatic, illumination which some widespread light sources, like halogen lamps cannot provide. Four main types of light source are used, including xenon arc lamps or mercury-vapor lamps with an excitation filter, lasers, supercontinuum sources, and high-power LEDs. Lasers are most widely used for more complex fluorescence microscopy techniques like confocal microscopy and total internal reflection fluorescence microscopy while xenon lamps, and mercury lamps, and LEDs with a dichroic excitation filter are commonly used for widefield epifluorescence microscopes (Fig 4).

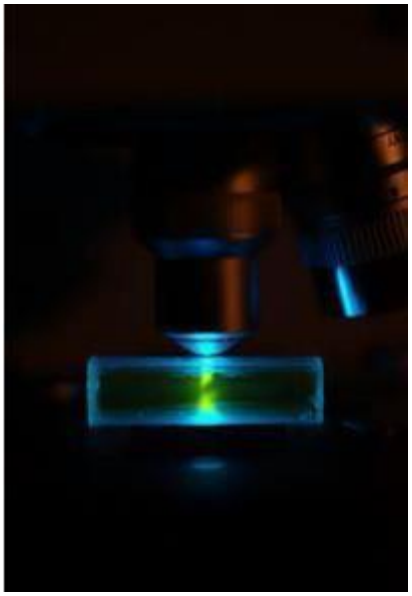

Figure 5 Light source

1.3.3 Sample preparation

A sample of herring sperm stained with SYBR green in a cuvette illuminated by blue light in an epifluorescence microscope. The SYBR green in the sample binds to the herring sperm DNA and, once bound, fluoresces giving off green light when illuminated by blue light.

In order for a sample to be suitable for fluorescence microscopy it must be fluorescent. There are several methods of creating a fluorescent sample; the main techniques are labelling with

fluorescent stains or, in the case of biological samples, expression of a fluorescent protein. Alternatively the intrinsic fluorescence of a sample (i.e., autofluorescence) can be used.[1] In the life sciences fluorescence microscopy is a powerful tool which allows the specific and sensitive staining of a specimen in order to detect the distribution of proteins or other molecules of interest. As a result there is a diverse range of techniques for fluorescent staining of biological samples

1.3.4 Biological fluorescent stains

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 (excited by UV wavelength light) and DRAQ5 and DRAQ7 (optimally excited by red light) which all bind the minor groove of DNA, thus labelling the nuclei of cells. Others are drugs or toxins which bind specific cellular structures and have been derivatised with a fluorescent reporter. A major example of this class of fluorescent stain is phalloidin which is used to stain actin fibres in mammalian cells.

There are many fluorescent molecules called fluorophores or fluorochromes such as fluorescein, Alexa Fluors or DyLight 488, which can be chemically linked to a different molecule which binds the target of interest within the sample.

1.4 Electron microscope

An electron microscope is a microscope that uses a beam of accelerated electrons as a source of illumination. As the wavelength of an electron can be up to 100,000 times shorter than that of visible light photons, electron microscopes have a higher resolving power than light microscopes and can reveal the structure of smaller objects. A transmission electron microscope can achieve better than 50 pm resolution[1] and magnifications of up to about 10,000,000x whereas most light microscopes are limited by diffraction to about 200 nm resolution and useful magnifications below 2000x. Transmission electron microscopes use electrostatic and electromagnetic lenses to control the electron beam and focus it to form an image. These electron optical lenses are analogous to the glass lenses of an optical light microscope. Electron microscopes are used to investigate the ultrastructure of a wide range of biological and inorganic specimens including microorganisms, cells, large molecules, biopsy samples, metals, and crystals. Industrially, electron microscopes are often used for quality control and failure analysis. Modern electron

microscopes produce electron micrographs using specialized digital cameras and frame grabbers to capture the image.

1.4.1Types

14.1.1 Transmission electron microscope (TEM) The original form of electron microscope, the transmission electron microscope (TEM) uses a high voltage electron beam to illuminate the specimen and create an image. The electron beam is produced by an electron gun, commonly fitted with a tungsten filament cathode as the electron source. The electron beam is accelerated by an anode typically at +100 keV (40 to 40018keV) with respect to the cathode, focused by electrostatic and electromagnetic lenses, and transmitted through the specimen that is in part transparent to electrons and in part scatters them out of the beam. When it emerges from the specimen, the electron beam carries information about the structure of the specimen that is magnified by the objective lens system of the microscope. The spatial variation in this information (the "image") may be viewed by projecting the magnified electron image onto a fluorescent viewing screen coated with a phosphor or scintillator material such as zinc sulfide.

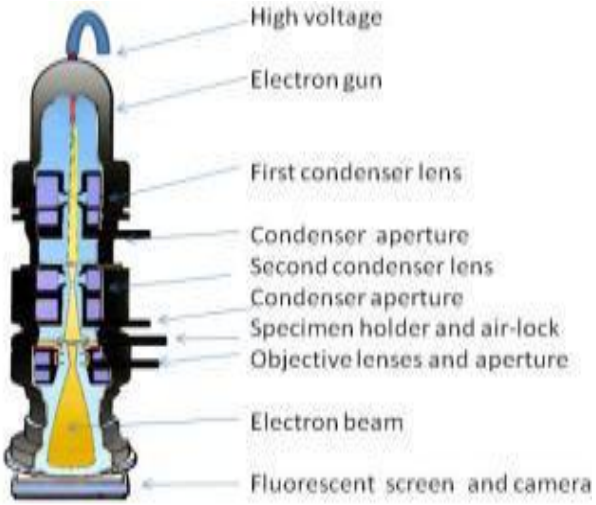

Figure 6 Transmission electron microscope

Alternatively, the image can be photographically recorded by exposing a photographic film or plate directly to the electron beam, or a high-resolution phosphor may be coupled by means of a lens optical system or a fibre optic light-guide to the sensor of a digital camera. The image detected by the digital camera may be displayed on a monitor or computer.

The resolution of TEMs is limited primarily by spherical aberration, but a new generation of aberration correctors have been able to partially overcome spherical aberration to increase

resolution. Hardware correction of spherical aberration for the high-resolution transmission electron microscopy (HRTEM) has allowed the production of images with resolution below 0.5 angstrom (50 picometres) and magnifications above 50 million times. The ability to determine the positions of atoms within materials has made the HRTEM an important tool for nano-technologies research and development.

Transmission electron microscopes are often used in electron diffraction mode. The advantages of electron diffraction over X-ray crystallography are that the specimen need not be a single crystal or even a polycrystalline powder, and also that the Fourier transform reconstruction of the object's magnified structure occurs physically and thus avoids the need for solving the phase problem faced by the X-ray crystallographers after obtaining their X-ray diffraction patterns of a single crystal or polycrystalline powder.

The major disadvantage of the transmission electron microscope is the need for extremely thin sections of the specimens, typically about 100 nanometers. Biological specimens are typically required to be chemically fixed, dehydrated and embedded in a polymer resin to stabilize them sufficiently to allow ultrathin sectioning. Sections of biological specimens, organic polymers and similar materials may require special treatment with heavy atom labels in order to achieve the required image contrast.

1.4.1.2 Scanning electron microscope (SEM)

The SEM produces images by probing the specimen with a focused electron beam that is scanned across a rectangular area of the specimen (raster scanning). When the electron beam interacts with the specimen, it loses energy by a variety of mechanisms. The lost energy is converted into alternative forms such as heat, emission of low-energy secondary electrons and high-energy backscattered electrons, light emission (cathodoluminescence) or X-ray emission, all of which provide signals carrying information about the properties of the specimen surface, such as its topography and composition. The image displayed by an SEM maps the varying intensity of any of these signals into the image in a position corresponding to the position of the beam on the specimen when the signal was generated. In the SEM image of an ant shown below and to the right, the image was constructed from signals produced by a secondary electron detector, the normal or conventional imaging mode in most SEMs.

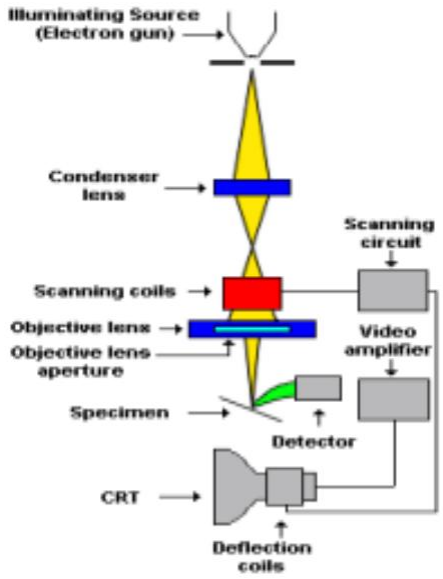

Figure 7 Scanning electron microscope

Generally, the image resolution of an SEM is at least an order of magnitude poorer than that of a TEM. However, because the SEM image relies on surface processes rather than transmission, it is able to image bulk samples up to many centimeters in size and (depending on instrument design and settings) has a great depth of field, and so can produce images that are good representations of the three-dimensional shape of the sample. Another advantage of SEM is its variety called environmental scanning electron microscope (ESEM) can produce images of sufficient quality and resolution with the samples being wet or contained in low vacuum or gas. This greatly facilitates imaging biological samples that are unstable in the high vacuum of conventional electron microscopes.

1.4.2 Color

In their most common configurations, electron microscopes produce images with a single brightness value per pixel, with the results usually rendered in grayscale. However, often these images are then colorized through the use of feature-detection software, or simply by hand editing using a graphics editor. This may be done to clarify structure or for aesthetic effect and generally does not add new information about the specimen.

1.4.3 Sample preparation

Materials to be viewed under an electron microscope may require processing to produce a suitable sample.

The technique required varies depending on the specimen and the analysis required:

- Chemical fixation – for biological specimens aims to stabilize the specimen's mobile macromolecular structure by chemical crosslinking of proteins with aldehydes such as formaldehyde and glutaraldehyde, and lipids with osmium tetroxide.

- Negative stain – suspensions containing nanoparticles or fine biological material (such as viruses and bacteria) are briefly mixed with a dilute solution of an electron-opaque solution such as ammonium molybdate, uranyl acetate (or formate), or phosphotungstic acid. This mixture is applied to a suitably coated EM grid, blotted, then allowed to dry. Viewing of this preparation in the TEM should be carried out withoutdelay for best results. The method is important in microbiology for fast but crude morphological identification, but can also be used as the basis for high resolution 3D reconstruction using EM tomography methodology when carbon films are used for support. Negative staining is also used for observation of nanoparticles.

- Cryofixation – freezing a specimen so rapidly, in liquid ethane, and maintained at liquid nitrogen or even liquid helium temperatures, so that the water forms vitreous (noncrystalline) ice. This preserves the specimen in a snapshot of its solution state. An entire field called cryo-electron microscopy has branched from this technique. With the development of cryo-electron microscopy of vitreous sections (CEMOVIS), it is now possible to observe samples from virtually any biological specimen close to its native state.[citation needed]

- Dehydration – or replacement of water with organic solvents such as ethanol or acetone, followed by critical point drying or infiltration with embedding resins. Also freeze drying.

- Embedding, biological specimens – after dehydration, tissue for observation in the transmission electron microscope is embedded so it can be sectioned ready for viewing. To do this the tissue is passed through a 'transition solvent' such as propylene oxide (epoxypropane) or acetone and then infiltrated with an epoxy resin such as Araldite, Epon, or Durcupan; tissues may also be embedded directly in water-miscible acrylic resin. After the resin has been polymerized (hardened) the sample is thin sectioned (ultrathin sections) and stained – it is then ready for viewing.

- Embedding, materials – after embedding in resin, the specimen is usually ground and polished to a mirror-like finish using ultra-fine abrasives. The polishing process must be performed carefully to minimize scratches and other polishing artifacts that reduce image quality.

- Metal shadowing – Metal (e.g. platinum) is evaporated from an overhead electrode and applied to the surface of a biological sample at an angle. The surface topography results in variations in the thickness of the metal that are seen as variations in brightness and contrast in the electron microscope image.

- Replication – A surface shadowed with metal (e.g. platinum, or a mixture of carbon and platinum) at an angle is coated with pure carbon evaporated from carbon electrodes at right angles to the surface. This is followed by removal of the specimen material (e.g. in an acid bath, using enzymes or by mechanical separation) to produce a surface replica that records the surface ultrastructure and can be examined using transmission electron microscopy.

- Sectioning – produces thin slices of specimen, semitransparent to electrons. These can be cut on an ultramicrotome with a diamond knife to produce ultra-thin sections about 60–90 nm thick. Disposable glass knives are also used because they can be made in the lab and are much cheaper.

- Staining – uses heavy metals such as lead, uranium or tungsten to scatter imaging electrons and thus give contrast between different structures, since many (especially biological) materials are nearly "transparent" to electrons (weak phase objects). In biology, specimens can be stained "en bloc" before embedding and also later after sectioning. Typically thin sections are stained for several minutes with an aqueous or alcoholic solution of uranyl acetate followed by aqueous lead citrate.

- Freeze-fracture or freeze-etch – a preparation method particularly useful for examining lipid membranes and their incorporated proteins in "face on" view. The fresh tissue or cell suspension is frozen rapidly (cryofixation), then fractured by breaking or by using a microtome while maintained at liquid nitrogen temperature. The cold fractured surface (sometimes "etched" by increasing the temperature to about −100 °C for several minutes to let some ice sublime) is then shadowed with evaporated platinum or gold at an average angle of 45° in a high vacuum evaporator. A second coat of carbon, evaporated perpendicular to the average surface plane is often performed to improve stability of the replica coating. The specimen is returned to room temperature and pressure, then the extremely fragile "pre-shadowed" metal replica of the fracture surface is released from the underlying biological material by careful chemical digestion with acids, hypochlorite solution or SDS detergent. The still-floating replica is thoroughly washed free from residual chemicals, carefully fished up on fine grids, dried then viewed in the TEM.

- Ion beam milling – thins samples until they are transparent to electrons by firing ions (typically argon) at the surface from an angle and sputtering material from the surface. A subclass of this is focused ion beam milling, where gallium ions are used to produce an electron transparent membrane in a specific region of the sample, for example through a device within a microprocessor. Ion beam milling may also be used for cross-section polishing prior to SEM analysis of materials that are difficult to prepare using mechanical polishing.

- Conductive coating – an ultrathin coating of electrically conducting material, deposited either by high vacuum evaporation or by low vacuum sputter coating of the sample. This is done toprevent the accumulation of static electric fields at the specimen due to the electron irradiation required during imaging. The coating materials include gold, gold/palladium, platinum, tungsten, graphite, etc.

- Earthing – to avoid electrical charge accumulation on a conductive coated sample, it is usually electrically connected to the metal sample holder. Often an electrically conductive adhesive is used for this purpose.

1.4.4 Disadvantages

Electron microscopes are expensive to build and maintain, but the capital and running costs of confocal light microscope systems now overlaps with those of basic electron microscopes. Microscopes designed to achieve high resolutions must be housed in stable buildings (sometimes underground) with special services such as magnetic field cancelling systems.

The samples largely have to be viewed in vacuum, as the molecules that make up air would scatter the electrons. An exception is the environmental scanning electron microscope, which allows hydrated samples to be viewed in a low-pressure (up to 20 Torr or 2.7 kPa) and/or wet environment.

Scanning electron microscopes operating in conventional high-vacuum mode usually image conductive specimens; therefore non-conductive materials require conductive coating (gold/palladium alloy, carbon, osmium, etc.). The low-voltage mode of modern microscopes makes possible the observation of non-conductive specimens without coating. Non-conductive materials can be imaged also by a variable pressure (or environmental) scanning electron microscope.

Small, stable specimens such as carbon nanotubes, diatom frustules and small mineral crystals (asbestos fibres, for example) require no special treatment before being examined in the electron microscope. Samples of hydrated materials, including almost all biological specimens have to be prepared in various ways to stabilize them, reduce their thickness (ultrathin sectioning) and increase their electron optical contrast (staining).

2. pH meter

German chemist Fritz Haber (1868–1934) and his student Zygmunt Klemensiewicz (1886–1963) developed the glass electrode idea in 1909. The modern, electronic pH meter was invented about a quarter century later, around 1934/5, when American chemist Arnold Beckman (1900–2004) figured out how to hook up a glass electrode to an amplifier and voltmeter to make a much more sensitive instrument.

A typical pH meter has two basic components: the meter itself, which can be a moving-coil meter (one with a pointer that moves against a scale) or a digital meter (one with a numeric display), and either one or two probes that you insert into the solution you're testing. To make electricity flow through something, you have to create a complete electrical circuit; so, to make electricity flow through the test solution, you have to put two electrodes (electrical terminals) into it. If your pH meter has two probes (like the one in the photo at the top of this article), each one is a separate electrode; if you have only one probe, both of the two electrodes are built inside it for simplicity and convenience.

The electrodes aren't like normal electrodes (simple pieces of metal wire); each one is a mini chemical set in its own right. The electrode that does the most important job, which is called the glass electrode, has a silver-based electrical wire suspended in a solution of potassium chloride, contained inside a thin bulb (or membrane) made from a special glass containing metal salts (typically compounds of sodium and calcium). The other electrode is called the reference electrode and has a potassium chloride wire suspended in a solution of potassium chloride.

The potassium chloride inside the glass electrode (shown here colored orange) is a neutral solution with a pH of 7, so it contains a certain amount of hydrogen ions (H^+). Suppose the unknown solution you're testing (blue) is much more acidic, so it contains a lot more hydrogen ions. What the glass electrode does is to measure the difference in pH between the orange solution and the blue solution by measuring the difference in the voltages their hydrogen ions produce. Since we know the pH of the orange solution, we can figure out the pH of the blue solution.

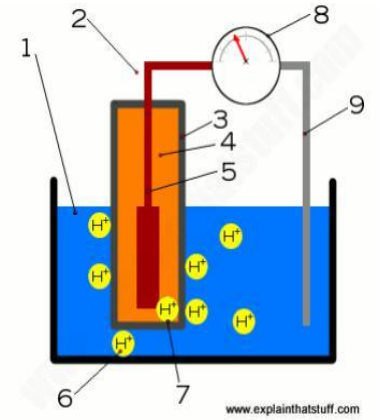

Figure 8 Key parts of a pH meter: (1) Solution being tested; (2) Glass electrode, consisting of

(3) a thin layer of silica glass containing metal salts, inside which there is a potassium chloride solution (4) and an internal electrode (5) made from silver/silver chloride. (6) Hydrogen ions formed in the test solution interact with the outer surface of the glass. (7) Hydrogen ions formed in the potassium chloride solution interact with the inside surface of the glass. (8) The meter measures the difference in voltage between the two sides of the glass and converts this "potential difference" into a pH reading. (9) Reference electrode acts as a baseline or reference for the measurement—or you can think of it as simply completing the circuit.

2.1 Working Procedure

When you dip the two electrodes into the blue test solution, some of the hydrogen ions move toward the outer surface of the glass electrode and replace some of the metal ions inside it, while some of the metal ions move from the glass electrode into the blue solution. This ion-swapping process is called ion exchange, and it's the key to how a glass electrode works. Ion-swapping also takes place on the inside surface of the glass electrode from the orange solution. The two solutions on either side of the glass have different acidity, so a different amount of ion-swapping takes place on the two sides of the glass. This creates a different degree of hydrogen-ion activity on the two surfaces of the glass, which means a different amount of electrical charge builds up on them. This charge difference means a tiny voltage (sometimes called a potential difference, typically a few tens or hundreds of millivolts) appears between the two sides of the glass, which produces a difference in voltage between the silver electrode (5) and the reference electrode (8) that shows up as a measurement on the meter.

Although the meter is measuring voltage, what the pointer on the scale (or digital display) actually shows us is a pH measurement. The bigger the difference in voltage between the orange

(inside) and blue (outside) solutions, the bigger the difference in hydrogen ion activity between. If there is more hydrogen ion activity in the blue solution, it's more acidic than the orange solution and the meter shows this as a lower pH; in the same way, if there's less hydrogen ion activity in the blue solution, the meter shows this as a higher pH (more alkaline).

2.2 Making accurate pH measurements

For pH meters to be accurate, they have to be properly calibrated (the meter is accurately translating voltage measurements into pH measurements), so they usually need testing and adjusting before you start to use them. You calibrate a pH meter by dipping it into buffers (test solutions of known pH) and adjust the meter accordingly. Another important consideration is that pH measurements made this way depend on temperature. Some meters have built-in thermometers and automatically correct their own pH measurements as the temperature changes; those are best if fluctuations in temperature are likely to occur while you're making a number of different measurements. Alternatively, you can correct the pH measurement yourself, or allow for it by calibrating your instrument and making pH measurements at broadly the same temperature.

3. Centrifugation

Centrifugation is a process which involves the application of the centripetal force for the sedimentation of heterogeneous mixtures with a centrifuge, and is used in industrial and laboratory settings. This process is used to separate two immiscible substances, but also to analyze the hydrodynamic properties of macromolecules. More-dense components of the mixture migrate away from the axis of the centrifuge, while less-dense components of the mixture migrate towards the axis. Chemists and biologists may increase the effective gravitational force on a test tube so as to more rapidly and completely cause the precipitate (pellet) to gather on the bottom of the tube. The remaining solution(supernatant) may be discarded with a pipette

There is a correlation between the size and density of a particle and the rate that the particle separates from a heterogeneous mixture, when the only force applied is that of gravity. The larger the size and the larger the density of the particles, the faster they separate from the mixture. By applying a larger effective gravitational force to the mixture, like a centrifuge does, the separation of the particles is accelerated. This is ideal in industrial and lab settings because

particles that would naturally separate over a long period of time can be separated in much less time. The rate of centrifugation is specified by the angular velocity usually expressed as revolutions per minute (RPM), or acceleration expressed as g. The conversion factor between RPM and g depends on the radius of the centrifuge rotor. The particles' settling velocity in centrifugation is a function of their size and shape, centrifugal acceleration, the volume fraction of solids present, the density difference between the particle and the liquid, and the viscosity.

The most common application is the separation of solid from highly concentrated suspensions, which is used in the treatment of sewage sludges for dewatering where less consistent sediment is produced. In the chemical and food industries, special centrifuges can process a continuous stream of particle-laden liquid. Centrifugation is the most common method used for uranium enrichment, relying on the slight mass difference between atoms of U238 and U235 in uranium hexafluoride gas.

3.1 Centrifugation in biological research

3.1.1 Microcentrifuges

Microcentrifuges are used to process small volumes of biological molecules, cells, or nuclei. Microcentrifuge tubes generally hold 0.5 - 2.0 mL of liquid, and are spun at maximum angular speeds of 12,000–13,000 rpm. Microcentrifuges are small enough to fit on a table-top and have rotors that can quickly change speeds. They may or may not have a refrigeration function.

3.1.2 High-speed centrifuges

High-speed or superspeed centrifuges can handle larger sample volumes, from a few tens of millilitres to several litres. Additionally, larger centrifuges can also reach higher angular velocities (around 30,000 rpm). The rotors may come with different adapters to hold various sizes of test tubes, bottles, or microtiter plates.

3.1.3 Fractionation process

General method of fractionation: Cell sample is stored in a suspension which is:

- Buffered - neutral pH, preventing damage to the structure of proteins including enzymes (which could affect ionic bonds)
- Isotonic (of equal water potential) - this prevents water gain or loss by the organelles
- Cool - reducing the overall activity of enzyme released later in the procedure
- Cells are homogenised in a blender and filtered to remove debris
- The homogenised sample is placed in an ultracentrifuge and spun in low speed - nuclei settle out, forming a pellet
- The supernatant (suspension containing remaining organelles) is spun at a higher speed - chloroplasts settle out
- The supernatant is spun at a higher speed still - mitochondria and lysosomes settle out
- The supernatant is spun at an even higher speed - ribosomes, membranes settle out. The ribosomes, membranes and Golgi complexes can be separated by another technique called density gradient centrifugation.

3.1.4 Ultracentrifugation

Ultracentrifugation makes use of high centrifugal force for studying properties of biological particles. Compared to microcentrifuges or high-speed centrifuges, ultracentrifuges can isolate much smaller particles, including ribosomes, proteins, and viruses. Ultracentrifuges can also be used in the study of membrane fractionation. This occurs because ultracentrifuges can reach maximum angular velocities in excess of 70,000 rpm. Additionally, while microcentrifuges and supercentrifuges separate particles in batches (limited volumes of samples must be handled manually in test tubes or bottles), ultracentrifuges can separate molecules in batch or continuous flow systems. In addition to purification, analytical ultracentrifugation (AUC) can be used for determination of the properties of macromolecules such as shape, mass, composition, and conformation. Samples are centrifuged with a high-density solution such as sucrose, caesium chloride, or iodixanol. The high-density solution may be at a uniform concentration throughout the test tube ("cushion") or a varying concentration ("gradient"). Molecular properties can be modeled through sedimentation velocity analysis or sedimentation equilibrium analysis. During the run, the particle or molecules will migrate through the test tube at different speeds depending on their physical properties and the properties of the solution, and eventually form a pellet at the bottom of the tube, or bands at various heights.

3.1.5 Differential Centrifugation

Differential Centrifugation is a type of centrifugation in which one selectively spins down components of a mixture by a series of increasing centrifugation forces. This method is commonly used to separate organelles and membranes found in cells. Organelles generally differ from each other in density in size, making the use of differential centrifugation, and centrifugation in general, possible. The organelles can then be identified by testing for indicators that are unique to the specific organelles.

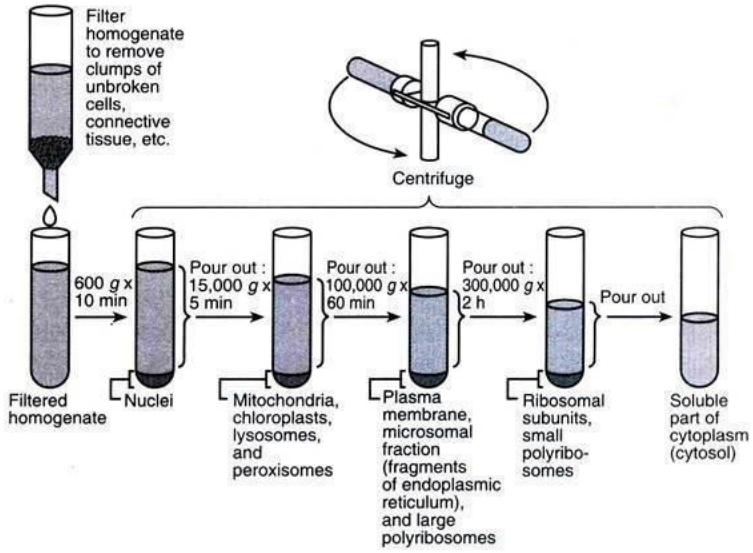

Figure 9 Isolation of different cell organelles by differential centrifugation

3.1.6 Density gradient centrifugation

Density gradient centrifugation Is considered one of the more efficient methods of separating suspended particles. Density gradient centrifugation can be used both as a separation technique and as a method of measuring the densities of particles or molecules in a mixture. A tube, after being centrifuged by this method, has particles in order of density based on height. The object or particle of interest will reside in the position within the tube corresponding to its density.Linderstorm-Lang, in 1937, discovered that density gradient tubes could be used for density measurements. He discovered this when working with potato yellow-dwarf virus. This method was also used in Meselson and Stahl's famous experiment in which they proved that DNA replication is semi-conservative by using different isotopes of nitrogen. They used density

gradient centrifugation to determine which isotope or isotopes of nitrogen were present in the DNA after cycles of replication.Nevertheless, some non-ideal sedimentations are still possible when using this method. The first potential issue is the unwanted aggregation of particles, but this can occur in any centrifugation. The second possibility occurs when droplets of solution that contain particles sediment. This is more likely to occur when working with a solution that has a layer of suspension floating on a dense liquid, which in fact have little to no density gradient.

(a) Rate Zonal Technique: Particle separation by the rate zonal technique is based upon differences in the size, shape and density of particles, the density and viscosity of the medium and the applied centrifugal field. Subcellular organelles, which have different densities but are similar in size, do not separate efficiently using this method, but sepa¬ration of proteins of similar densities and differing only 3 folds in relative molecular mass can be achieved easily. The technique involves carefully layering a sample solution on top of preformed liquid density gradient, the highest density of which does not exceed that of densest particle to be separated. The function of gradient is primarily to stabilize the liquid column in the tube against the movements resulting from conventional currents and secondarily to produce a gradient that helps to improve the resolution of gradient. The sample is then centrifuged until the desired degree of separation is achieved. Since the technique is time dependent, centrifugation must be terminated before any of the separated zone pellets at he bottom of tube. The technique is employed for the separation of enzymes, RNA-DNA hybrids, ribosomal subunit, subcellular organelle, etc.

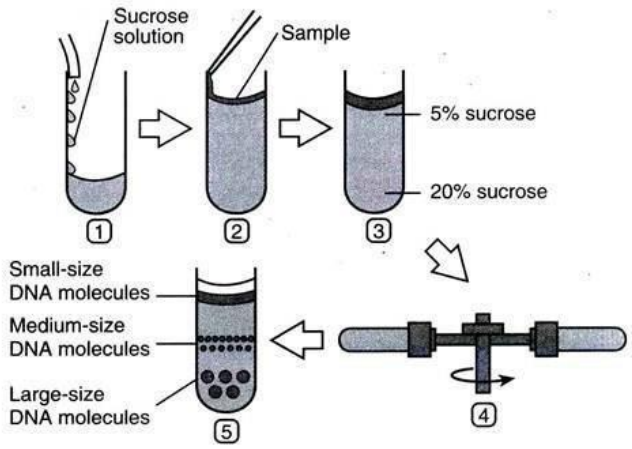

Figure 10 Principle of rate zonal centrifugation technique

(b) Isopycnic Centrifugation Technique: Isopycnic centrifugation depends solely upon the buyout density and not on its shape, size and time, the size of the particle affecting only the rate at which it reaches its isopycnic position in the gradient. The technique is used to separate particles of similar size but of different density. Hence soluble proteins which have very similar densities cannot be usually separated by this method, where as sub cellular organelles can be effectively separated.

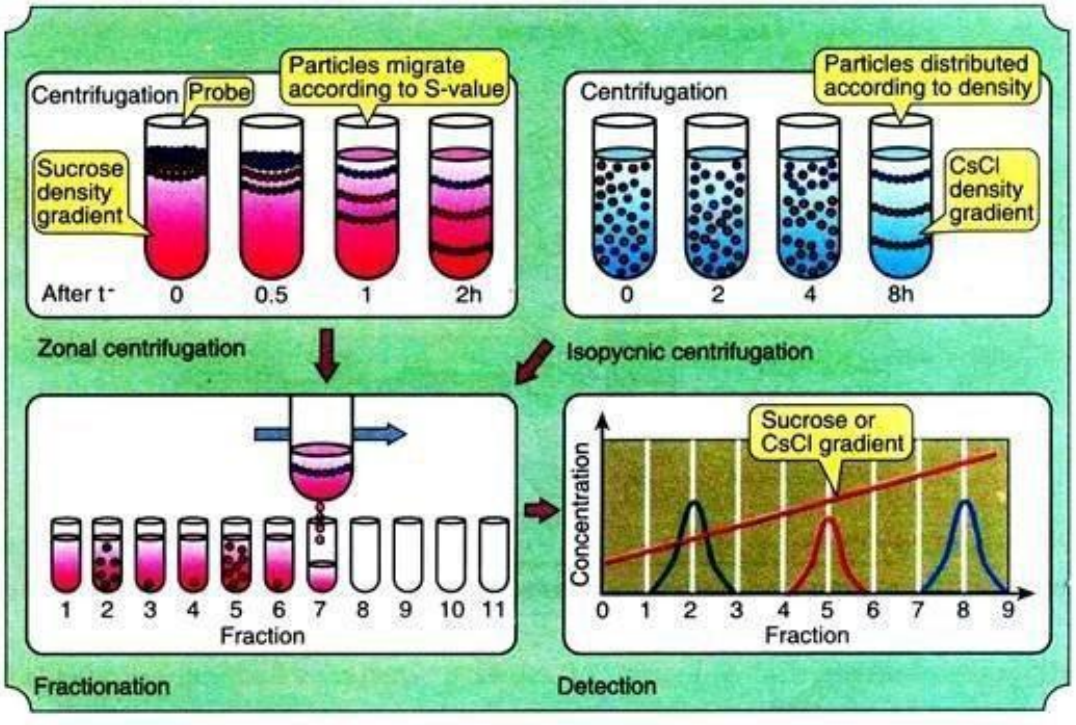

Figure 11 Density gradient centrifugation

The methods are a combination of sedimentation and flotation and involve layering the sample on top of a density gradient that spans the whole range of the particle densities that are to be separated. The maximum density of the gradient, therefore, must always exceed the density of the densest particle. During centrifugation, sedimentation of the particle occurs until the buoyant density of the particle and density of the gradient are equal. At this point of isodensity no further sedimentation occurs, irrespective of how long centrifugation continues, because the particles are floating on the cushion of material that has density greater than their own.

Isopycnic centrifugation, in contrast to the rate zonal technique, is an equilibrium method, the particle banding to form zones each at their own characteristic buoyant density. In case when not all components in a mixture of particle are required, a gradient range can be selected in which unwanted materials will be sediment at the bottom of the tube and whole of the particles of interest will float at their respective isopynic positions. Such a technique involves a combination of both the rate zonal and isopynic approaches.

Table 1 Densities of various important biological molecules

Material	Density (g/cm^3)
Microbial cells	1.05–1.15
Mammalian cells	1.04–1.10
Organelles	1.10–1.60
Proteins	1.30
DNA	1.70
RNA	2.00

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SCHOOL OF BIO AND CHEMICAL ENGINEERING
DEPARTMENT OF BIOTECHNOLOGY

UNIT – II – ANALYTICAL TECHNIQUES – SBB2104

1. Colorimeter

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 most 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 (Fig 1):

- 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

1.1 Filters

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

1.2 Cuvette

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.

1.3 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.

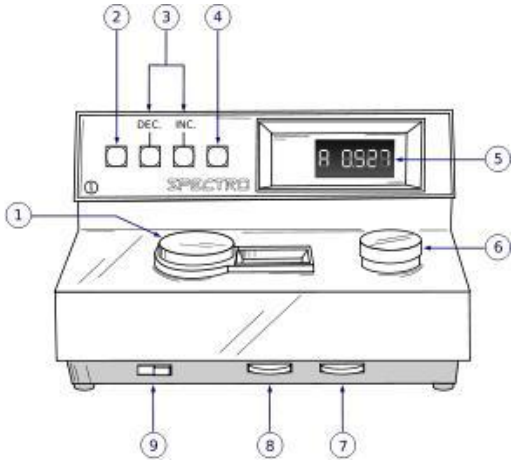


Figure 1 Colorimeter

(1) Wavelength selection, (2) Printer button, (3) Concentration factor adjustment, (4) UV mode selector (Deuterium lamp), (5) Readout, (6) Sample compartment, (7) Zero control (100% T), (8) Sensitivity switch, (9)ON/OFF switch

2. Visible and Ultraviolet Spectroscopy

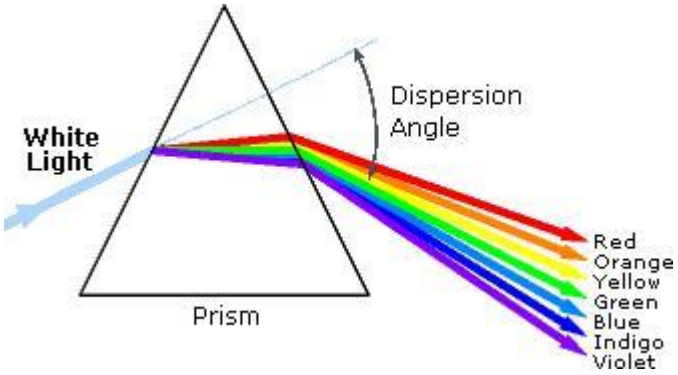


Figure 2 Visible light

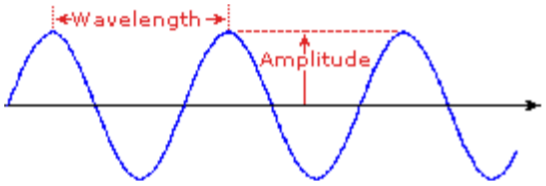
An obvious difference between certain compounds is their color. Thus, quinone is yellow; chlorophyll is green; the 2,4- dinitrophenylhydrazone derivatives of aldehydes and ketones range in color from bright yellow to deep red, depending on double bond conjugation; and aspirin is colorless. In this respect the human eye is functioning as a spectrometer analyzing the light reflected from the surface of a solid or passing through a liquid. Although we see sunlight (or white light) as uniform or homogeneous in color, it is actually composed of a broad range of radiation wavelengths in the ultraviolet (UV), visible and infrared (IR) portions of the spectrum.

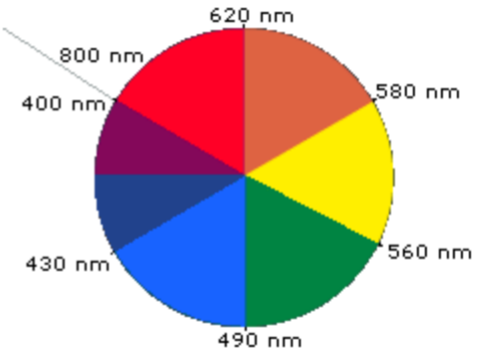
As shown on the right, the component colors of the visible portion can be separated by passing sunlight through a prism, which acts to bend the light in differing degrees according to wavelength. Electromagnetic radiation such as visible light is commonly treated as a wave phenomenon, characterized by a wavelength or frequency (Fig 2).

2.1 Wavelength

It is defined as the distance between adjacent peaks (or troughs), and may be designated in meters, centimeters or nanometers (10^{-9} meters). **Frequency** is the number of wave cycles that travel past a fixed point per unit of time, and is usually given in cycles per second, or hertz (Hz). Visible wavelengths cover a range from approximately 400 to 800 nm. The longest visible wavelength is red and the shortest is violet.

The wavelengths of what we perceive as particular colors in the visible portion of the spectrum are displayed and listed below. In horizontal diagrams, such as the one on the bottom left, wavelength will increase on moving from left to right.





Violet: 400 - 420 nm
Indigo: 420 - 440 nm
Blue: 440 - 490 nm
Green: 490 - 570 nm
Yellow: 570 - 585 nm
Orange: 585 - 620 nm
Red: 620 - 780 nm

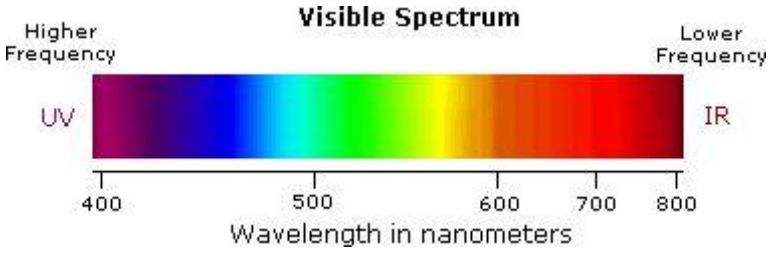


Figure 3 Visible spectrum

2.2 The Electromagnetic Spectrum

The visible spectrum constitutes but a small part of the total radiation spectrum. Most of the radiation that surrounds us cannot be seen, but can be detected by dedicated sensing instruments. This **electromagnetic spectrum** ranges from very short wavelengths (including gamma and x-rays) to very long wavelengths (including microwaves and broadcast radio waves). The following chart displays many of the important regions of this spectrum, and demonstrates the inverse relationship between wavelength and frequency (shown in the top equation below the chart).

2.3 UV-Visible Absorption Spectra

To understand why some compounds are colored and others are not, and to determine the relationship of conjugation to color, we must make accurate measurements of light absorption at different wavelengths in and near the visible part of the spectrum. Commercial optical spectrometers enable such experiments to be conducted with ease, and usually survey both the near ultraviolet and visible portions of the spectrum (fig 3).

The visible region of the spectrum comprises photon energies of 36 to 72 kcal/mole, and the near ultraviolet region, out to 200 nm, extends this energy range to 143 kcal/mole. Ultraviolet radiation having wavelengths less than 200 nm is difficult to handle, and is seldom used as a routine tool for structural analysis.

The energies noted above are sufficient to promote or excite a molecular electron to a higher energy orbital. Consequently, absorption spectroscopy carried out in this region is sometimes called "electronic spectroscopy". As a rule, energetically favored electron promotion will be from the **highest occupied molecular orbital (HOMO)** to the **lowest unoccupied molecular orbital (LUMO)**, and the resulting species is called an **excited state**. When sample molecules are exposed to light having an energy that matches a possible electronic transition within the molecule, some of the light energy will be absorbed as the electron is promoted to a higher energy orbital. An optical spectrometer records the wavelengths at which absorption occurs, together with the degree of absorption at each wavelength. The resulting spectrum is presented as a graph of absorbance (A) versus wavelength, as in the isoprene spectrum shown below. Since

isoprene is colorless, it does not absorb in the visible part of the spectrum and this region is not displayed on the graph.

2.4 Absorbance usually ranges from 0 (no absorption) to 2 (99% absorption), and is precisely defined in context with spectrometer operation. Because the absorbance of a sample will be proportional to the number of absorbing molecules in the spectrometer light beam (e.g. their molar concentration in the sample tube), it is necessary to correct the absorbance value for this and other operational factors if the spectra of different compounds are to be compared in a meaningful way. The corrected absorption value is called "molar absorptivity", and is particularly useful when comparing the spectra of different compounds and determining the relative strength of light absorbing functions (chromophores).

Molar absorptivity (ε) is defined as:

$$\text{Molar Absorptivity, } \varepsilon = A / c\, l$$

(where *A*= absorbance, **c** = sample concentration in moles/liter & **l** = length of light path through the sample in cm.)

The only molecular moieties likely to absorb light in the 200 to 800 nm region are pi-electron functions and hetero atoms having non-bonding valence-shell electron pairs. Such light absorbing groups are referred to as **chromophores**. A list of some simple chromophores and their light absorption characteristics is provided on the left above. The oxygen non- bonding electrons in alcohols and ethers do not give rise to absorption above 160 nm. Consequently, pure alcohol and ether solvents may be used for spectroscopic studies. The presence of chromophores in a molecule is best documented by UV-Visible spectroscopy, but the failure of most instruments to provide absorption data for wavelengths below 200 nm makes the detection of isolated chromophores problematic. Fortunately, conjugation generally moves the absorption maxima to longer wavelengths, as in the case of isoprene, so conjugation becomes the major structural feature identified by this technique.

Molar absorptivities may be very large for strongly absorbing chromophores (>10,000) and very small if absorption is weak (10 to 100). The magnitude ofε reflects both the size of the chromophore and the probability that light of a given wavelength will be absorbed when it strikes the chromophore.

3. Fluorescence spectroscopy

Fluorescence spectroscopy (also known as fluorometry or spectrofluorometry) is a type of electromagnetic spectroscopy which analyzes fluorescence from a sample. It involves using a beam of light, usually ultraviolet light, that excites the electrons in molecules of certain compounds and causes them to emit light; typically, but not necessarily,visible light. A complementary technique is absorption spectroscopy. In the special case of single molecule fluorescence spectroscopy, intensity fluctuations from the emitted light are measured from either single fluorophores, or pairs of fluorophores. Devices that measure fluorescence are called fluorometers

Molecules have various states referred to as energy levels. Fluorescence spectroscopy is primarily concerned with electronic and vibrational states. Generally, the species being examined has a ground electronic state (a low energy state) of interest, and an excited electronic state of higher energy. Within each of these electronic states are various vibrational states.

In fluorescence , the species is first excited, by absorbing a photon, from its ground electronic state to one of the various vibrational states in the excited electronic state. Collisions with other molecules cause the excited molecule to lose vibrational energy until it reaches the lowest vibrational state of the excited electronic state.

The molecule then drops down to one of the various vibrational levels of the ground electronic state again, emitting a photon in the process. As molecules may drop down into any of several vibrational levels in the ground state, the emitted photons will have different energies, and thus frequencies. Therefore, by analysing the different frequencies of light emitted in fluorescent spectroscopy, along with their relative intensities, the structure of the different vibrational levels can be determined.

For atomic species, the process is similar; however, since atomic species do not have vibrational energy levels, the emitted photons are often at the same wavelength as the incident radiation. This process of re-emitting the absorbed photon is "resonance fluorescence" and while it is characteristic of atomic fluorescence, is seen in molecular fluorescence as well.

In a typical fluorescence (emission) measurement, the excitation wavelength is fixed and the detection wavelength varies, while in a fluorescence excitation measurement the detection wavelength is fixed and the excitation wavelength is varied across a region of interest. An **emission map** is measured by recording the emission spectra resulting from a range of excitation wavelengths and combining them all together. This is a three dimensional surface data set: emission intensity as a function of excitation and emission wavelengths, and is typically depicted as a contour map.

3.1 Instrumentation

Two general types of instruments exist: filter fluorometers that use filters to isolate the incident light and fluorescent light and spectrofluorometers that use a diffraction grating monochromators to isolate the incident light and fluorescent light. Both types use the following scheme: the light from an excitation source passes through a filter or monochromator, and strikes the sample. A proportion of the incident light is absorbed by the sample, and some of the molecules in the sample fluoresce. The fluorescent light is emitted in all directions. Some of this fluorescent light passes through a second filter or monochromator and reaches a detector, which is usually placed at 90° to the incident light beam to minimize the risk of transmitted or reflected incident light reaching the detector.

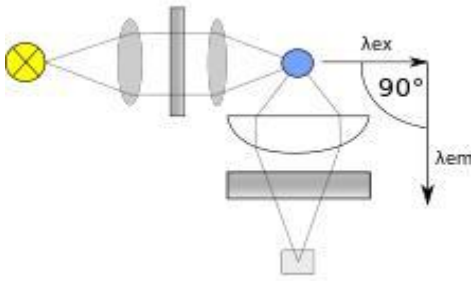


Figure 4 A simplistic design of the components of a fluorimeter

Various light sources may be used as excitation sources, including lasers, LED, and lamps; xenon arcs and mercury-vapor lamps in particular. A laser only emits light of high irradiance at a very narrow wavelength interval, typically under 0.01 nm, which makes an excitation

monochromator or filter unnecessary. The disadvantage of this method is that the wavelength of a laser cannot be changed by much. A mercury vapor lamp is a line lamp, meaning it emits light near peak wavelengths. By contrast, a xenon arc has a continuous emission spectrum with nearly constant intensity in the range from 300- 800 nm and a sufficient irradiance for measurements down to just above 200 nm (fig 4).

Filters and/or monochromators may be used in fluorimeters. A monochromator transmits light of an adjustable wavelength with an adjustable tolerance. The most common type of monochromator utilizes a diffraction grating, that is, collimated light illuminates a grating and exits with a different angle depending on the wavelength. The monochromator can then be adjusted to select which wavelengths to transmit. For allowing anisotropy measurements the addition of two polarization filters are necessary: One after the excitation monochromator or filter, and one before the emission monochromator or filter.

As mentioned before, the fluorescence is most often measured at a 90° angle relative to the excitation light. This geometry is used instead of placing the sensor at the line of the excitation light at a 180° angle in order to avoid interference of the transmitted excitation light. No monochromator is perfect and it will transmit some stray light, that is, light with other

wavelengths than the targeted. An ideal monochromator would only transmit light in the specified range and have a high wavelength- independent transmission. When measuring at a 90° angle, only the light scattered by the sample causes stray light. This results in a better signal-to-noise ratio, and lowers the detection limit by approximately a factor 10000, when compared to the 180° geometry. Furthermore, the fluorescence can also be measured from the front, which is often done for turbid or opaque samples .

The detector can either be single-channeled or multichanneled. The single-channeled detector can only detect the intensity of one wavelength at a time, while the multichanneled detects the intensity of all wavelengths simultaneously, making the emission monochromator or filter unnecessary. The different types of detectors have both advantages and disadvantages.

The most versatile fluorimeters with dual monochromators and a continuous excitation light source can record both an excitation spectrum and a fluorescence spectrum. When measuring

fluorescence spectra, the wavelength of the excitation light is kept constant, preferably at a wavelength of high absorption, and the emission monochromator scans the spectrum. For measuring excitation spectra, the wavelength passing though the emission filter or monochromator is kept constant and the excitation monochromator is scanning. The excitation spectrum generally is identical to the absorption spectrum as the fluorescence intensity is proportional to the absorption.

3.2 Analysis of data

At low concentrations the fluorescence intensity will generally be proportional to the concentration of the fluorophore.

Unlike in UV/visible spectroscopy, 'standard', device independent spectra are not easily attained. Several factors influence and distort the spectra, and corrections are necessary to attain 'true', i.e. machine-independent, spectra. The different types of distortions will here be classified as being either instrument- or sample-related. Firstly, the distortion arising from the instrument is discussed. As a start, the light source intensity and wavelength characteristics varies over time during each experiment and between each experiment. Furthermore, no lamp has a constant intensity at all wavelengths. To correct this, a beam splitter can be applied after the excitation monochromator or filter to direct a portion of the light to a reference detector.

Additionally, the transmission efficiency of monochromators and filters must be taken into account. These may also change over time. The transmission efficiency of the monochromator also varies depending on wavelength. This is the reason that an optional reference detector should be placed after the excitation monochromator or filter. The percentage of the fluorescence picked up by the detector is also dependent upon the system. Furthermore, the detector quantum efficiency, that is, the percentage of photons detected, varies between different detectors, with wavelength and with time, as the detector inevitably deteriorates.

Two other topics that must be considered include the optics used to direct the radiation and the means of holding or containing the sample material (called a cuvette or cell). For most UV, visible, and NIR measurements the use of precision quartz cuvettes is necessary. In both cases, it is important to select materials that have relatively little absorption in the wavelength range of interest. Quartz is ideal because it transmits from 200 nm-2500 nm; higher grade quartz can even

transmit up to 3500 nm, whereas the absorption properties of other materials can mask the fluorescence from the sample.

Correction of all these instrumental factors for getting a ‘standard’ spectrum is a tedious process, which is only applied in practice when it is strictly necessary. This is the case when measuring the quantum yield or when finding the wavelength with the highest emission intensity for instance.

As mentioned earlier, distortions arise from the sample as well. Therefore some aspects of the sample must be taken into account too. Firstly, photodecomposition may decrease the intensity of fluorescence over time. Scattering of light must also be taken into account. The most significant types of scattering in this context are Rayleigh and Raman scattering. Light scattered by Rayleigh scattering has the same wavelength as the incident light, whereas in Raman scattering the scattered light changes wavelength usually to longer wavelengths. Raman scattering is the result of a virtual electronic state induced by the excitation light. From this virtual state, the molecules may relax back to a vibrational level other than the vibrational ground state. In fluorescence spectra, it is always seen at a constant wavenumber difference relative to the excitation wavenumber e.g. the peak appears at a wavenumber 3600 cm^{-1} lower than the excitation light in water.

Other aspects to consider are the inner filter effects. These include reabsorption. Reabsorption happens because another molecule or part of a macromolecule absorbs at the wavelengths at which the fluorophore emits radiation. If this is the case, some or all of the photons emitted by the fluorophore may be absorbed again. Another inner filter effect occurs because of high concentrations of absorbing molecules, including the fluorophore. The result is that the intensity of the excitation light is not constant throughout the solution. Resultingly, only a small percentage of the excitation light reaches the fluorophores that are visible for the detection system. The inner filter effects change the spectrum and intensity of the emitted light and they must therefore be considered when analysing the emission spectrum of fluorescent light

4. Atomic Absorption Spectroscopy (AAS)

Atomic absorption spectroscopy (**AAS**) is a spectroanalytical procedure for the quantitative determination of chemical elements using the absorption of optical radiation (light) by free atoms in the gaseous state.

In analytical chemistry the technique is used for determining the concentration of a particular element (the analyte) in a sample to be analyzed. AAS can be used to determine over 70 different elements in solution or directly in solid samples used in pharmacology, biophysics and toxicology research.

4.1 Principle

The technique makes use of absorption spectrometry to assess the concentration of an analyte in a sample. It requires standards with known analyte content to establish the relation between the measured absorbance and the analyte concentration and relies therefore on the Beer-Lambert Law.

In short, the electrons of the atoms in the atomizer can be promoted to higher orbitals (excited state) for a short period of time (nanoseconds) by absorbing a defined quantity of energy (radiation of a given wavelength). This amount of energy, i.e., wavelength, is specific to a particular electron transition in a particular element. In general, each wavelength corresponds to only one element, and the width of an absorption line is only of the order of a few picometers (pm), which gives the technique its elemental selectivity. The radiation flux without a sample and with a sample in the atomizer is measured using a detector, and the ratio between the two values (the absorbance) is converted to analyte concentration or mass using the Beer-Lambert Law.

4.2 Instrumentation

In order to analyze a sample for its atomic constituents, it has to be atomized. The atomizers most commonly used nowadays are flames and electrothermal (graphite tube) atomizers. The atoms should then be irradiated by optical radiation, and the radiation source could be an element-specific line radiation source or a continuum radiation source. The radiation then passes through amonochromator in order to separate the element-specific radiation from any other radiation emitted by the radiation source, which is finally measured by a detector (fig 5).

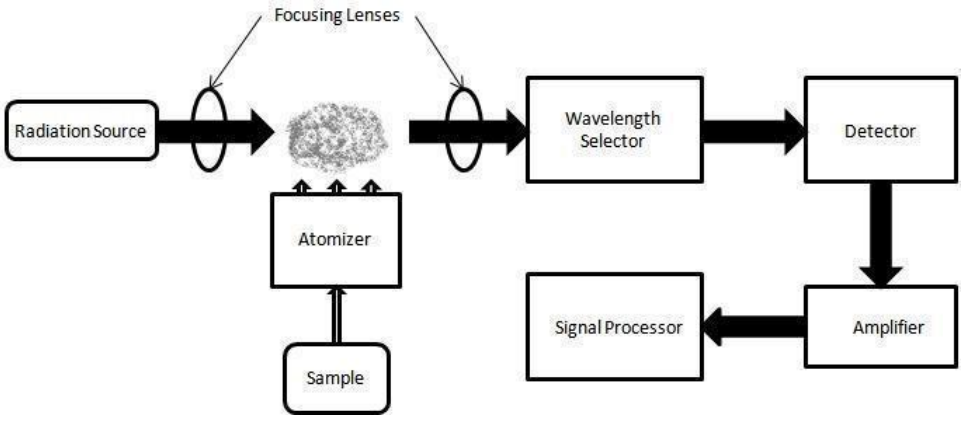


Figure 5 Atomic Adsorption Spectroscopy

4.1 Atomizers

The atomizers most commonly used nowadays are (spectroscopic) flames and electrothermal (graphite tube) atomizers. Other atomizers, such as glow-discharge atomization, hydride atomization, or cold- vapor atomization might be used for special purposes.

4.2 Flame atomizers

The oldest and most commonly used atomizers in AAS are flames, principally the air-acetylene (compressed high-purity oxygen + C_2H_2) flame with a temperature of about 2300 °C and the nitrous dioxide system (N2O)-acetylene flame with a temperature of about 2700 °C. The latter flame, in addition, offers a more reducing environment, being ideally suited for analytes with high affinity to oxygen.

Liquid or dissolved samples are typically used with flame atomizers. The sample solution is aspirated by a pneumatic analytical nebulizer, transformed into an aerosol, which is introduced into a spray chamber, where it is mixed with the flame gases and conditioned in a way that only the finest aerosol droplets (< 10 μm) enter the flame. This conditioning process is responsible that only about 5% of the aspirated sample solution reaches the flame, but it also guarantees a relatively high freedom from interference.

On top of the spray chamber is a burner head that produces a flame that is laterally long (usually 5– 10 cm) and only a few mm deep. The radiation beam passes through this flame at its longest axis, and the flame gas flow-rates may be adjusted to produce the highest concentration of free

atoms. The burner height may also be adjusted, so that the radiation beam passes through the zone of highest atom cloud density in the flame, resulting in the highest sensitivity.

The processes in a flame include the stages of desolvation (drying) in which the solvent is evaporated and the dry sample nano-particles remain, vaporization (transfer to the gaseous phase) in which the solid particles are converted into gaseous molecule, atomization in which the molecules are dissociated into free atoms, and ionization where (depending on the ionization potential of the analyte atoms and the energy available in a particular flame) atoms may be in part converted to gaseous ions.

Each of these stages includes the risk of interference in case the degree of phase transfer is different for the analyte in the calibration standard and in the sample. Ionization is generally undesirable, as it reduces the number of atoms that are available for measurement, i.e., the sensitivity.

In flame AAS a steady-state signal is generated during the time period when the sample is aspirated. This technique is typically used for determinations in the mg L^{-1} range, and may be extended down to a few μg L^{-1} for some elements.

4.3 Electrothermal atomizers

Although a wide variety of graphite tube designs have been used over the years, the dimensions nowadays are typically 20–25 mm in length and 5–6 mm inner diameter. With this technique liquid/dissolved, solid and gaseous samples may be analyzed directly. A measured volume (typically 10–50 μL) or a weighed mass (typically around 1 mg) of a solid sample are introduced into the graphite tube and subject to a temperature program. This typically consists of stages,
such as drying – the solvent is evaporated; pyrolysis – the majority of the matrix constituents are removed; atomization – the analyte element is released to the gaseous phase; and cleaning – eventual residues in the graphite tube are removed at high temperature.
The graphite tubes are heated via their ohmic resistance using a low-voltage high-current power supply; the temperature in the individual stages can be controlled very closely, and temperature ramps between the individual stages facilitate separation of sample components.

4.4 Radiation sources

We have to distinguish between line source AAS (LS AAS) and continuum source AAS (CS AAS). In classical LS AAS, as it has been proposed by Alan Walsh, the high spectral resolution required for AAS measurements is provided by the radiation source itself that emits the spectrum of the analyte in the form of lines that are narrower than the absorption lines. Continuum sources, such as deuterium lamps, are only used for background correction purposes. The advantage of this technique is that only a medium-resolution monochromator is necessary for measuring AAS; however, it has the disadvantage that usually a separate lamp is required for each element that has to be determined. In CS AAS, in contrast, a single lamp, emitting a continuum spectrum over the entire spectral range of interest is used for all elements.

5. Mass Spectrometry

The Mass Spectrometer In order to measure the characteristics of individual molecules, a mass spectrometer converts them to ions so that they can be moved about and manipulated by external electric and magnetic fields. The three essential functions of a mass spectrometer, and the associated components, are:

i. **The Ion Source** A small sample is ionized, usually to cations by loss of an electron.
ii. **The Mass Analyze** The ions are sorted and separated according to their mass and charge.
iii. **The Detector** The separated ions are then measured, and the results displayed on a chart.

Because ions are very reactive and short-lived, their formation and manipulation must be conducted in a vacuum. Atmospheric pressure is around 760 torr (mm of mercury). The pressure under which ions may be handled is roughly 10-5 to 10-8 torr (less than a billionth of an atmosphere). Each of the three tasks listed above may be accomplished in different ways. In one common procedure, ionization is effected by a high energy beam of electrons, and ion separation is achieved by accelerating and focusing the ions in a beam, which is then bent by an external magnetic field. The ions are then detected electronically and the resulting information is stored and analyzed in a computer. The heart of the spectrometer is the ion source. Here molecules of the sample (black dots) are bombarded by electrons (light blue lines) issuing from a heated filament. This is called an EI (electron-impact) source. Gases and volatile liquid samples are allowed to leak into the ion source from a reservoir (as shown). Non- volatile solids and liquids

may be introduced directly. Cations formed by the electron bombardment (red dots) are pushed away by a charged repeller plate (anions are attracted to it), and accelerated toward other electrodes, having slits through which the ions pass as a beam. Some of these ions fragment into smaller cations and neutral fragments. A perpendicular magnetic field deflects the ion beam in an arc whose radius is inversely proportional to the mass of each ion. Lighter ions are deflected more than heavier ions. By varying the strength of the magnetic field, ions of different mass can be focused progressively on a detector fixed at the end of a curved tube (also under a high vacuum).

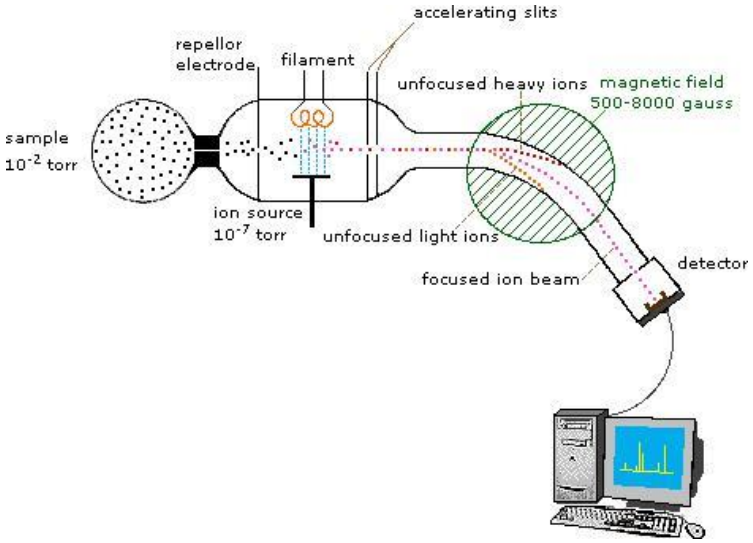


Figure 6 Mass Spectroscopy

When a high energy electron collides with a molecule it often ionizes it by knocking away one of the molecular electrons (either bonding or non-bonding). This leaves behind a **molecular ion**. Residual energy from the collision may cause the molecular ion to fragment into neutral pieces (colored green) and smaller **fragment ions** (colored pink and orange). The molecular ion is a radical cation, but the fragment ions may either be radical cations (pink) or carbocations (orange), depending on the nature of the neutral fragment (Fig 6).

5.1 An outline of what happens in a mass spectrometer

Atoms and molecules can be deflected by magnetic fields - provided the atom or molecule is first turned into an ion. Electrically charged particles are affected by a magnetic field although electrically neutral ones aren't. The sequence is :

5.2 Ionisation

The atom or molecule is ionised by knocking one or more electrons off to give a positive ion. This is true even for things which you would normally expect to form negative ions (chlorine, for example) or never form ions at all (argon, for example). Most mass spectrometers work with positive ions.

5.3 Acceleration

The ions are accelerated so that they all have the same kinetic energy.

5.4 Deflection

The ions are then deflected by a magnetic field according to their masses. The lighter they are, the more they are deflected.

The amount of deflection also depends on the number of positive charges on the ion - in other words, on how many electrons were knocked off in the first stage. The more the ion is charged, the more it gets deflected.

5.5 Detection

The beam of ions passing through the machine is detected electrically (fig 7).

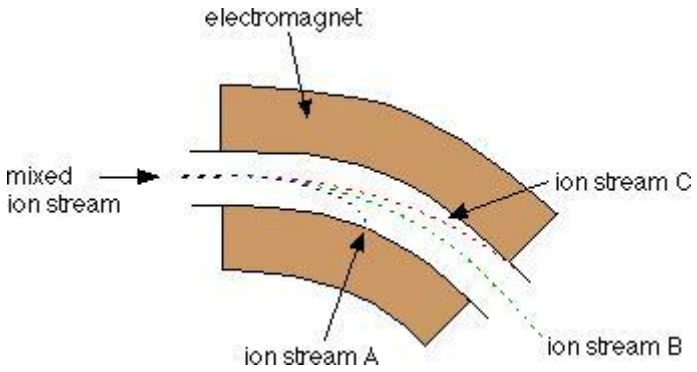


Figure 7 Detection in mass spectroscopy

Different ions are deflected by the magnetic field by different amounts. The amount of deflection depends on:

- the mass of the ion. Lighter ions are deflected more than heavier ones.
- the charge on the ion. Ions with 2 (or more) positive charges are deflected more than ones with only 1 positive charge.

These two factors are combined into the ***mass/charge ratio.*** Mass/charge ratio is given the symbol m/z (or sometimes m/e).

For example, if an ion had a mass of 28 and a charge of 1+, its mass/charge ratio would be 28. An ion with a mass of 56 and a charge of 2+ would also have a mass/charge ratio of 28.

Ion stream A is most deflected - it will contain ions with the smallest mass/charge ratio. Ion stream C is the least deflected - it contains ions with the greatest mass/charge ratio.

It makes it simpler to talk about this if we assume that the charge on all the ions is 1+. Most of the ions passing through the mass spectrometer will have a charge of 1+, so that the mass/charge ratio will be the same as the mass of the ion.

Assuming 1+ ions, stream A has the lightest ions, stream B the next lightest and stream C the heaviest. Lighter ions are going to be more deflected than heavy ones.

Only ion stream B makes it right through the machine to the ion detector. The other ions collide with the walls where they will pick up electrons and be neutralised. Eventually, they get removed from the mass spectrometer by the vacuum pump.

5.6 What the mass spectrometer output looks like

The output from the chart recorder is usually simplified into a "stick diagram". This shows the relative current produced by ions of varying mass/charge ratio. The stick diagram for molybdenum looks lilke this (fig 8).

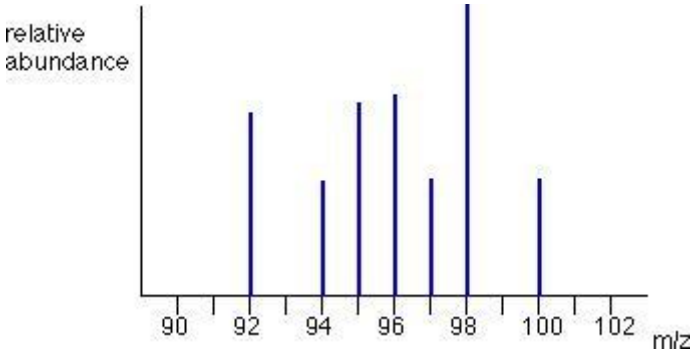


Figure 8 Mass Spectrometer Outcome

You may find diagrams in which the vertical axis is labelled as either "relative abundance" or "relative intensity". Whichever is used, it means the same thing. The vertical scale is related to the current received by the chart recorder - and so to the number of ions arriving at the detector:

the greater the current, the more abundant the ion. The commonest ion has a mass/charge ratio of 98. Other ions have mass/charge ratios of 92, 94, 95, 96, 97 and 100.That means that molybdenum consists of 7 different isotopes. Assuming that the ions all have a charge of 1+, that means that the masses of the 7 isotopes on the carbon-12 scale are 92, 94, 95, 96, 97, 98 and 100.

6. Nuclear Magnetic Resonance Spectroscopy

6.1 Background

Over the past fifty years nuclear magnetic resonance spectroscopy, commonly referred to as nmr, has become the preeminent technique for determining the structure of organic compounds. Of all the spectroscopic methods, it is the only one for which a complete analysis and interpretation of the entire spectrum is normally expected. Although larger amounts of sample are needed than for mass spectroscopy, nmr is non-destructive, and with modern instruments good data may be obtained from samples weighing less than a milligram.

The nuclei of many elemental isotopes have a characteristic spin (**I**). Some nuclei have integral spins (e.g. I = 1, 2, 3 ....), some have fractional spins (e.g. I = 1/2, 3/2, 5/2 .....), and a few have no spin, I = 0

(e.g.12C, 16O, 32S, ...). Isotopes of particular interest and use to organic chemists are 1H, 13C, 19F and ^{31}P, all of which have I = 1/2. Since the analysis of this spin state is fairly straightforward, our discussion of nmr will be limited to these and other I = 1/2 nuclei.

6.2 The following features lead to the nmr phenomenon

1. A spinning charge generates a magnetic field, as shown by the animation on right. The resulting spin-magnet has a magnetic moment (**μ**) proportional to the spin (Fig 9)

2. In the presence of an external magnetic field (**B0**), two spin states exist, **+1/2** and**-1/2**.

The magnetic moment of the lower energy +1/2 state is aligned with the external field, but that of the higher energy -1/2 spin state is opposed to the external field. Note that the arrow representing the external field points North.

3. The difference in energy between the two spin states is dependent on the external magnetic field strength, and is always very small. The two spin states have the same energy when the

external field is zero, but diverge as the field increases. At a field equal to Bx a formula for the energy difference is given (remember I = 1/2 and μ is the magnetic moment of the nucleus in the field).

Strong magnetic fields are necessary for nmr spectroscopy. The international unit for magnetic flux is the tesla (**T**). The earth's magnetic field is not constant, but is approximately 10^{-4} T at ground level. Modern nmr spectrometers use powerful magnets having fields of 1 to 20 T. Even with these high fields, the energy difference between the two spin states is less than 0.1 cal/mole. To put this in perspective, recall that infrared transitions involve 1 to 10 kcal/mole and electronic transitions are nearly 100 time greater. For nmr purposes, this small energy difference (ΔE) is usually given as a frequency in units of MHz (10^6 Hz), ranging from 20 to 900 Mz, depending on the magnetic field strength and the specific nucleus being studied. Irradiation of a sample with radio frequency (rf) energy corresponding exactly to the spin state separation of a specific set of nuclei will cause excitation of those nuclei in the +1/2 state to the higher -1/2 spin state. Note that this electromagnetic radiation falls in the radio and television broadcast spectrum. Nmr spectroscopy is therefore the energetically mildest probe used to examine the structure of molecules. The nucleus of a hydrogen atom (the proton) has a magnetic moment μ = 2.7927, and has been studied more than any other nucleus.

4. For spin 1/2 nuclei the energy difference between the two spin states at a given magnetic field strength will be proportional to their magnetic moments.

7. Electron Spin Resonance Spectroscopy

7.1 Theory

When a molecule or compound with an unpaired electron is placed in a strong magnetic field, the spin of the unpaired electron can align in two different ways creating two spin states, ms = ±1/2. The alignment can either be along the direction (parelle) to the magnetic filed which corresponds to the lower energy state ms = - ½ or opposite (antiparallel) to the direction of the applied magnetic field ms = + ½ . The two allignments have different energies and this difference in energy lifts the degeneracy of the electron spin states.

The energy difference is given by:

$\Delta E = E+ - E- = hv = gmßB$

During the experiment the values of h, v, and ß does not change and g value decrease as B increases. The g-factor is a unitless measurement of the intrinsic magnetic moment of the electron, and its value for a free electron is 2.0023. The concept of g can be roughly equated to

that of chemical shift in NMR. EPR spectrum is the absorption of microwave frequency radiation ploted against the magnetic field intensity

7.2 Working principles of EPR:

In an EPR experiment the field of the spectrometer magnet is swept linearly to excite some of the electrons in the lower energy level to the upper energy level while the sample is exposed to fixed microwave irradiation. The free or the upaired electrons have a small magnetic field and orient themselves parallel to the larger field produced by the spectrometer's magnet. At a particular magnetic field strength the microwave irradiation will cause some of the free electrons to "flip" and orient against the spectrometer's magnetic field. This separation between the lower and the higher energy level is exactly matched by our microwave frequency. The condition where the magnetic field and the microwave frequency are "just right" to produce an EPR resonance (or absorption) is known as the resonance condition is detected by the spectrometer., EPR spectroscopy can be carried out by either

1. varying the magnetic field and holding the frequency constant or
2. varying the frequency and holding the magnetic field constant (as is the case for NMR spectroscopy). Typically in a comericial spectrometer works by varying the magnetic field and holding the frequency constant. EPR spectrometers working at frequencies ranging from several hundred MHz to several hundred Ghz are in use. 1-2 GHz (L-band) and 2-4 GHz (S-band), 8-10 GHZ (X-Band), 35 Ghz (Q- band) and 95 GHz (W-band). The most commonly used EPR spectrometer is in the range of 9-10 GHz (X-band).

7.3 Methods to record EPR spectra

Continous wave method: the sample is irradiated continuously with microwave radiation of fixed frequency while the magnetic field is slowly swept and the microwave absorption is measured for each field position Pulse EPR: short pulse of high microwave radiation are sent to the sample and the response in the absence radiation are recorded.

Measuring a CW-EPR spectroscopy can be influenced by various parameters, both instrumental and experimental. The significant aspect of the measurement is to get a high resolved EPR spectrum from a low concentration sample of interest which is dependend on the the sensivity and the resolution of the spectrometer. Microwave bridge and Resonator governs the sensitivity

of the signal. Magnet, Magnet field controller, Magnet power supply and signal channel control the resolution of the spectrum obtained.

7.4 Application of EPR:

Only direct method to detect the presence of free radicals and to identify the paramagnetic species

Provides information on :

1. molecular structure near the unpaired electron.
2. EPR spectra lineshape gives insight to dynamic processes molecular motions or fluidity.
3. probes the structure of "active sites" in metalloproteins.
4. dose measurements for sterilization of medical goods and foods,
5. detection of irradiated foods, and the dating of early human artifacts.

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SCHOOL OF BIO AND CHEMICAL ENGINEERING
DEPARTMENT OF BIOTECHNOLOGY

UNIT – III – ANALYTICAL TECHNIQUES – SBB2104

1. Chromatography

Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis.

- A wide range of chromatographic procedures makes use of differences in size, binding affinities, charge, and other properties to separate materials.
- It is a powerful separation tool that is used in all branches of science and is often the only means of separating components from complex mixtures.
- Chromatography is a very useful technique as it allows the separation of components of a mixture on the basis of their nature, structure, size, and other properties.
- Chromatography, in general, is based on the principle that components of a mixture are separated when the mixture added to a mobile phase is moved through a stationary phase (which mostly is a solid surface), resulting in some components of the mixture being attached to the stationary phase. At the same time, the rest is passed along with the mobile phase.
- Thus, there are two essential components of all chromatography techniques.

1.1 Stationary phase

The stationary phase in chromatography is the phase that is either a solid or liquid particle attached to a glass or a metal surface on which the components of the mixture to be separated is absorbed selectively.

- The term stationary refers to the fact that this phase remains stationary while the other phase moves.
- Most substances used as stationary phases are porous, thus allowing the attachment of components during chromatography.
- The stationary phase to be selected for a chromatographic process depends on the nature of the components to be separated and the type of chromatography.

- Depending on the type of chromatography gel beads, thin uniform paper, silica, glass, some gases, or even liquid components are used as a stationary phase.

1.2 Mobile phase

The mobile phase in chromatography is the phase that is either liquid or gas that is passed through a chromatographic system where the components of the mixture are separated at different raters by adsorbing them to the stationary phase.

- The mobile phase is the solvent that carries the mixture as it moves down the stationary phase.
- The term mobile indicates that the phase is moving down the chromatographic system, whereas the other phase remains stationary.
- Substances used as mobile phases are selected for a chromatographic process depending on the nature of the components to be separated and the type of chromatography.
- Alcohol, water, acetic acid, acetone, or some gases are the commonly used mobile phase in different chromatographic techniques.

1.3 Types of chromatography

The type of chromatography where the stationary phase is hydrophilic and the mobile phase is hydrophobic is called a **normal-phase chromatography.** The other type is the **reverse- phase chromatography**, in which the stationary phase is non-polar while the mobile phase is polar.

Based on the purpose, chromatography can be either Analytical chromatography or Preparative chromatography.

1.4 Analytical chromatography

In this type, the molecules are separated for analysis of the component, i.e. for identification or quantification purposes.

1.5 Preparative chromatography

Preparative chromatography involves the separation and isolation of one or more components of the sample. The components isolated can be used for some other analytical or experimental studies. Hence this is used as preparatory step for the next experiment.

2. Paper chromatography

Paper chromatography is a separation technique where the separation is performed on a specialized paper (Fig 1).

2.1 Principle of Paper chromatography

- Paper chromatography is of two types based on two different principles.
- The first is the paper adsorption chromatography that is based on the varying degree of interaction between the molecules and the stationary phase.
- The molecules having higher affinity remain adsorbed for a longer time decreasing their speed of movement through the column.
- However, the molecules with lower affinity move with a faster movement, thus allowing the molecules to be separated in different fractions.
- The second type of paper chromatography is the paper partition chromatography. It is based on the principle that the moisture on the cellulose paper acts as a stationary phase for the molecules moving with the mobile phase.
- The separation of the molecules is thus based on how strongly they adsorb onto the stationary phase.
- An additional concept of ‘retention factor’ is applied during the separation of molecules in the paper chromatography.
- The retention value for a molecule is determined as a ratio of distance traveled by the molecule to the distance traveled by the mobile phase.
- The retention value of different molecules can be used to differentiate those molecules.

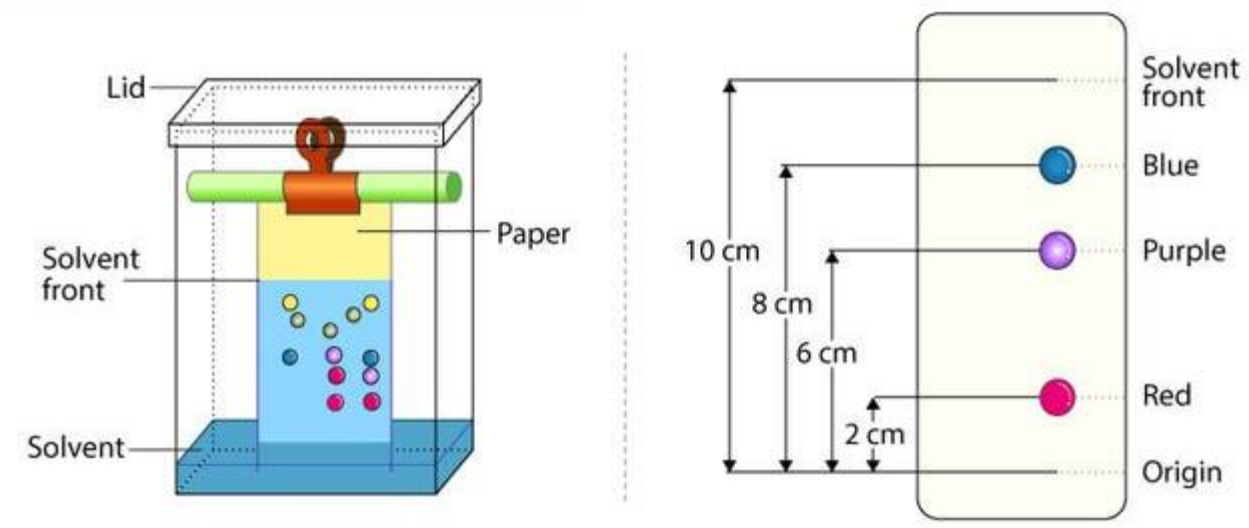

Figure 1 Paper chromatography

2.2 Steps of Paper chromatography

- The stationary phase is selected as a fine quality cellulosic paper.
- Different combinations of organic and inorganic solvents are taken as the mobile phase.
- About 2-200 µl of the sample solution is injected at the baseline of the paper, and it is allowed to air dry.
- The sample loaded paper is then carefully dipped into the mobile phase not more than the height of 1 cm.
- After the mobile phase reaches near the edge of the paper, the paper is taken out.
- The retention factor is calculated, and the separated components are detected by different techniques.

2.3 Retention Factor, Rf

The retention factor, Rf, is a quantitative indication of how far a particular compound travels in a particular solvent. The Rf value is a good indicator of whether an unknown compound and a known compound are similar, if not identical. If the Rf value for the unknown compound is close or the same as the Rf value for the known compound then the two compounds are most likely similar or identical. The retention factor, Rf, is defined as Rf = distance the solute (D1) moves divided by the distance traveled by the solvent front (D2) (Fig 2).

Rf = D1 / D2

where

D1 = distance that color traveled, measured from center of the band of color to the point where the food color was applied

D2 = total distance that solvent traveled

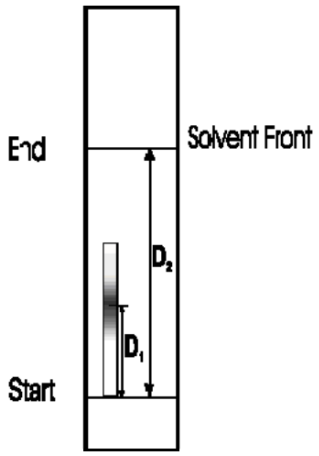

Figure 2 Retention Factor

2.4 Uses of Paper chromatography

- Paper chromatography is performed to detect the purity of various pharmaceutical products.
- It can also be employed to detect contamination in various samples, like food and beverages.
- This method can also be used for the separation of impurities from various industrial products.
- The analysis of the reaction mixtures in chemical labs is also conducted via paper chromatography.

3. Thin-layer chromatography

Thin-layer chromatography is a separation technique where the stationary phase is applied as a thin layer on a solid support plate with a liquid mobile phase (Fig 3).

3.1 Principle of Thin-layer chromatography (TLC)

- This chromatography technique is based on the principle that components of a mixture are separated when the component having an affinity towards the stationary phase binds to the stationary phase. In contrast, other components are eluted with the mobile phase.
- The substrate/ ligand is bound to the stationary phase so that the reactive sites for the binding of components are exposed.
- Now, the mixture is passed through the mobile phase where the components with binding sites for the substrate bind to the substrate on the stationary phase while the rest of the components are eluted out with the mobile phase.
- After separation, the molecules are seen as spots at a different location throughout the stationary phase.
- The detection of molecules is performed by various techniques.

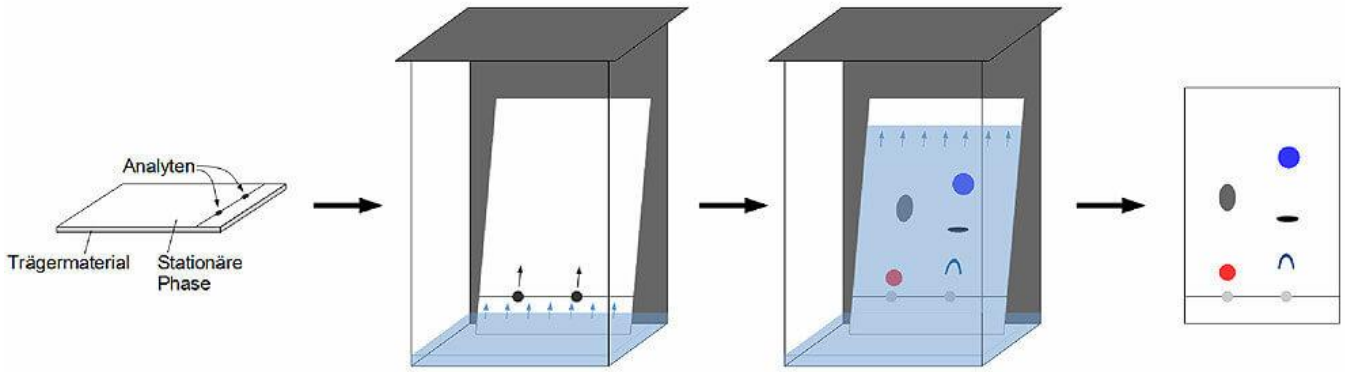

Figure 3 Thin-layer chromatography (TLC)

3.2 Steps of Thin-layer chromatography (TLC)

- The stationary phase is uniformly applied on the solid support (glass, thin plate or aluminum foil) and dried.
- The sample is injected as spots on the stationary phase about 1 cm above the edge of the plate.
- The sample loaded plate is then carefully dipped into the mobile phase not more than the height of 1 cm.
- After the mobile phase reaches near the edge of the plate, the plate is taken out.

- The retention factor is calculated as in paper chromatography, and the separated components are detected by different techniques.

3.3 Uses of Thin-layer chromatography (TLC)

- Thin-layer chromatography is routinely performed in laboratories to identify different substances present in a mixture.
- This technique helps in the analysis of fibers in forensics.
- TLC also allows the assay of various pharmaceutical products.
- It aids in the identification of medicinal plants and their composition.

4. Column chromatography

Column chromatography is the separation technique where the components in a mixture are separated on the basis of their differential adsorption with the stationary phase, resulting in them moving at different speeds when passed through a column (Fig 4).

It is a solid-liquid chromatography technique in which the stationary phase is a solid & mobile phase is a liquid or gas.

4.1 Principle of Column chromatography

- This technique is based on the principle of differential adsorption where different molecules in a mixture have different affinities with the absorbent present on the stationary phase.
- The molecules having higher affinity remain adsorbed for a longer time decreasing their speed of movement through the column.
- However, the molecules with lower affinity move with a faster movement, thus allowing the molecules to be separated in different fractions.
- Here, the stationary phase in the column chromatography also termed the absorbent, is a solid (mostly silica) and the mobile phase is a liquid that allows the molecules to move through the column smoothly.

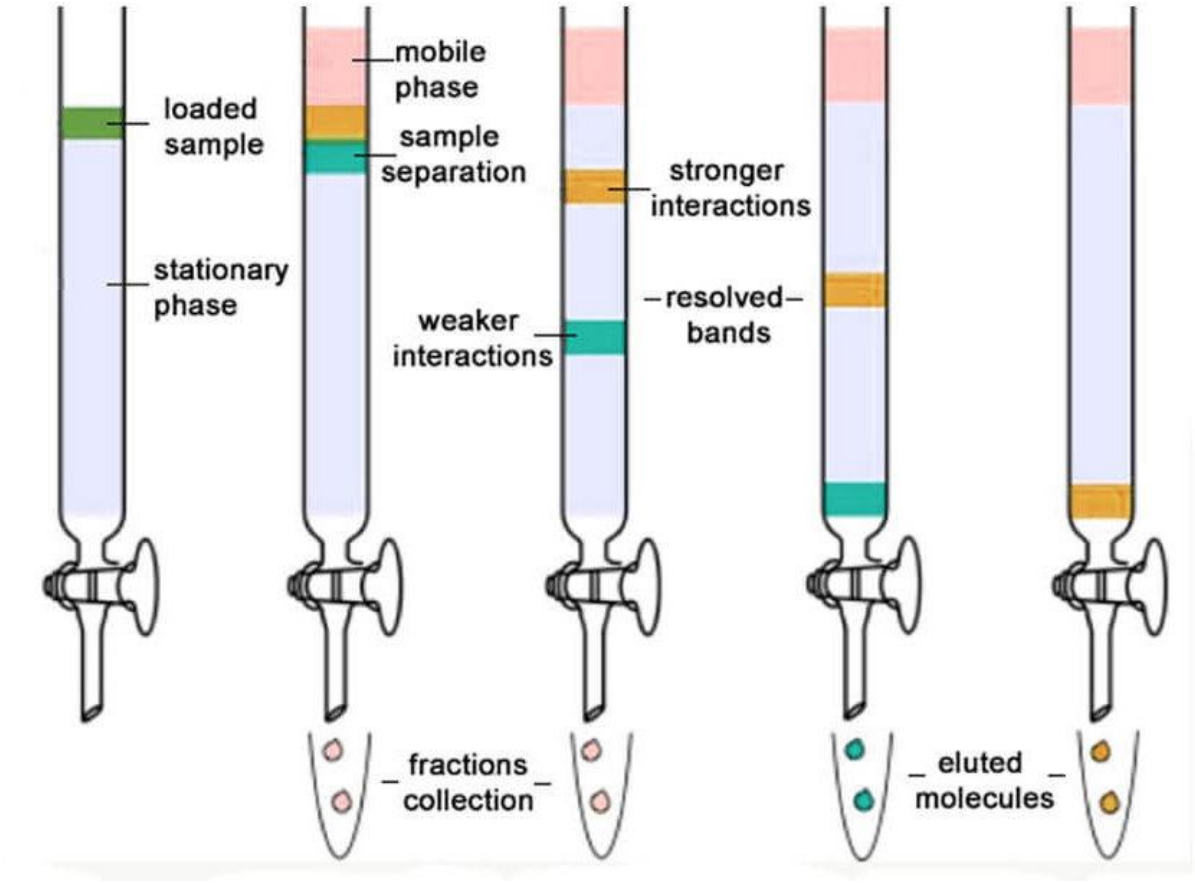

Figure 4 Column chromatography

4.2 Steps of Column chromatography

- The column is prepared by taking a glass tube that is dried and coated with a thin, uniform layer of stationary phase (cellulose, silica).
- Then the sample is prepared by adding the mixture to the mobile phase. The sample is introduced into the column from the top and is allowed to pass the sample under the influence of gravity.
- The molecules bound to the column are separated by elution technique where either solution of the same polarity is used (isocratic technique), or different samples with different polarities are used (gradient technique).
- The separated molecules can further be analyzed for various purposes.

4.3 Uses of Column chromatography

- Column chromatography is routinely used for the separation of impurities and purification of various biological mixtures.
- This technique can also be used for the isolation of active molecules and metabolites from various samples.
- Column chromatography is increasingly used for the detection of drugs in crude extracts.

5. Ion exchange chromatography

Ion exchange chromatography is the separation technique for charged molecules by their interaction with the oppositely charged stationary phase in the form of ion-exchange resin. (Fig 5).

5.1 Principle of Ion exchange chromatography

- This technique is based on the principle of attraction of charged resin and the oppositely charged analyte. Here the exchange of negatively/ positively charged ions takes place to remove the charged molecules.
- The stationary phase is first coated with particular charges where the components of the mixture with opposite charges will bind.
- A cation or anion exchange resin with a higher affinity to the charged components then binds the components, displacing the oppositely charged resin.
- The cation or anion exchange resin-component complex then is removed by using different buffers.

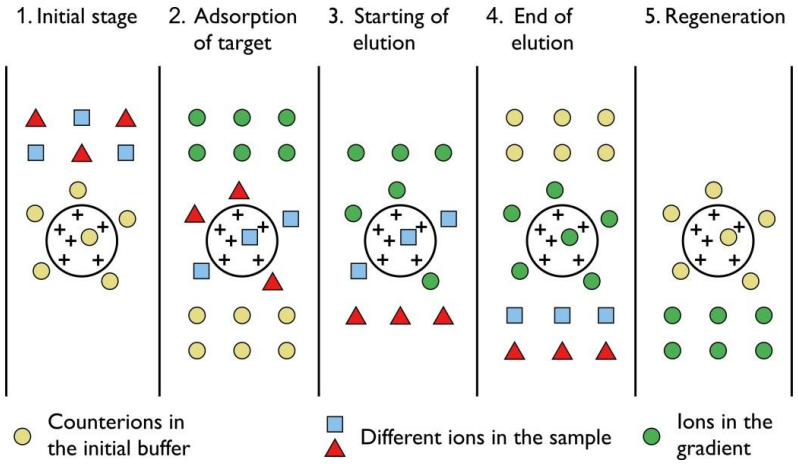

Figure 5 Ion exchange chromatography.

5.2 Steps of Ion exchange chromatography

- A column packed with charged resin that can either be positively charged or negatively charged is taken as the stationary phase.
- The mixture with the charged particles is then passed down the column where the charged molecules bind to the oppositely charged resins.
- If a cation exchange resin is used, the positively charged molecules now bind to the cation exchange resin displacing the negatively charged resin.
- Similarly, if an anion exchange resin is used, the negatively charged molecules bind to the anion exchange resin displacing the positively charged resin.
- Now an appropriate buffer is applied to the column to separate the complex of charged exchange resins and the charged molecules.

5.3 Uses of Ion exchange chromatography

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

5.4 Anion exchange chromatography

Anion exchange chromatography is the separation technique for negatively charged molecules by their interaction with the positively charged stationary phase in the form of ion-exchange resin (Fig 6).

5.5 Principle of Anion exchange chromatography

- This technique is based on the principle of attraction of positively charged resin and the negatively charged analyte. Here the exchange of positively charged ions takes place to remove the negatively charged molecules.
- The stationary phase is first coated with positive charges where the components of the mixture with negative charges will bind.

- An anion exchange resin with a higher affinity to the negatively charged components then binds the components, displacing the positively charged resin.
- The anion exchange resin-component complex then is removed by using different buffers.

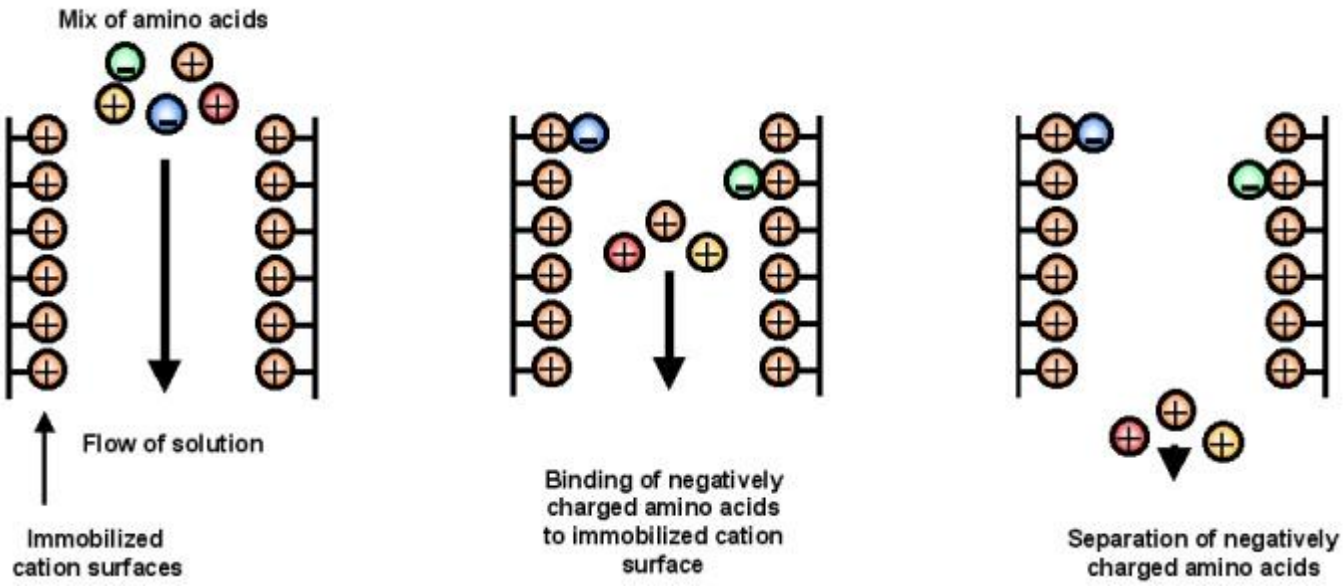

Figure 6 Anion exchange chromatography

5.6 Steps of Anion exchange chromatography

- A column packed with positively charged resin is taken as the stationary phase.
- The mixture with the charged particles is then passed down the column where the negatively charged molecules bind to the positively charged resins.
- The anion exchange resin is then passed through the column where the negatively charged molecules now bind to the anion exchange resin displacing the positively charged resin.
- Now an appropriate buffer is applied to the column to separate the complex of anion exchange resins and the charged molecules.

5.7 Uses of Anion exchange chromatography

- Anion exchange chromatography is used to separate proteins and amino acids from their mixtures.
- Negatively charged nucleic acids can be separated, which helps in further analysis of the nucleic acids.
- This method can also be used for water purification where the anions are exchanged for hydroxyl ions.

- Anion exchange resins can be used for the separation of metals as they usually have negatively charged complexes that are bound to the anion exchangers.

5.8 Cation exchange chromatography

Cation exchange chromatography is the separation technique for positively charged molecules by their interaction with negatively charged stationary phase in the form of ion-exchange resin.

5.9 Principle of Cation exchange chromatography

- This technique is based on the principle of attraction of negatively charged resin and the positively charged analyte. Here the exchange of negatively charged ions takes place to remove the positively charged molecules.
- The stationary phase is first coated with negative charges where the components of the mixture with positive charges will bind.
- A cation exchange resin with a higher affinity to the positively charged components then binds the components, displacing the negatively charged resin.
- The cation exchange resin-component complex then is removed by using different buffers.

5.10 Steps of Cation exchange chromatography

- A column packed with negatively charged resin is taken as the stationary phase.
- The mixture with the charged particles is then passed down the column where the positively charged molecules bind to the negatively charged resins.
- The cation exchange resin is then passed through the column where the positively charged molecules now bind to the cation exchange resin displacing the negatively charged resin.
- Now an appropriate buffer is applied to the column to separate the complex of cation exchange resins and the charged molecules.

5.11 Uses of Cation exchange chromatography

- Cation exchange chromatography is used for the analysis of the products obtained after the hydrolysis of nucleic acids.
- This can also be used for the separation of metals where the metal ions themselves bind to the negatively charged resins to remove the negatively charged complexes.

- Cation exchange chromatography helps in purification of water by exchanging the positively charged ion by the hydrogen ions.
- It is also used to analyze the rocks and other inorganic molecules.

6. Affinity chromatography

Affinity chromatography is a separation technique where the components of a mixture are separated based on their affinity towards the stationary phase of the system (Fig 7).

6.1 Principle of Affinity chromatography

- This chromatography technique is based on the principle that components of a mixture are separated when the element having an affinity towards the stationary phase binds to the stationary phase. In contrast, other components are eluted with the mobile phase.
- The substrate/ ligand is bound to the stationary phase so that the reactive sites for the binding of components are exposed.
- Now, the mixture is passed through the mobile phase where the components with binding sites for the substrate bind to the substrate on the stationary phase while the rest of the components are eluted out with the mobile phase.
- The components attached to the stationary phase are then eluted by changing the pH, ionic strength, or other conditions.

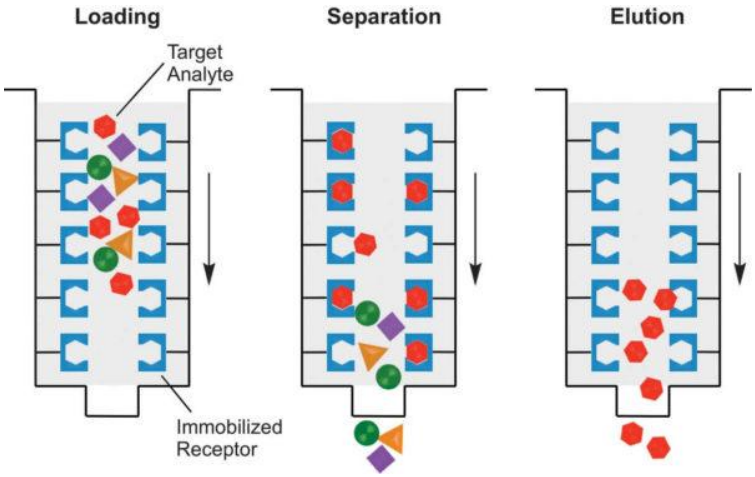

Figure 7 Affinity chromatography.

6.2 Steps of Affinity chromatography

- The column is prepared by loading it with solid support like agarose or cellulose, onto which the substrate/ ligand with the spacer arm, is attached.
- The mobile phase containing the mixture is poured into the column at a constant rate.
- Once the process is complete, the ligand-molecule complex is eluted from the stationary phase by changing the conditions that favor the separation of ligand and components of the mixture.

6.3 Uses of Affinity chromatography

- Affinity chromatography is used as a staple separation technique from enzymes and other proteins.
- This principle is also applied in the in vitro antigen-antibody reactions.
- This technique is used for the separation of components as well as the removal of impurities from a mixture.
- Affinity chromatography can be used in the detection of mutation and nucleotide polymorphisms in nucleic acids.

7. Gel-filtration chromatography

Gel-filtration chromatography is a form of partition chromatography used to separate molecules of different molecular sizes (Fig 8).

This technique has also frequently been referred to by various other names, including gel-permeation, gel-exclusion, size- exclusion, and molecular- sieve chromatography.

7.1 Principle

- Molecules are partitioned between a mobile phase and a stationary phase as a function of their relative sizes.
- The stationary phase is a matrix of porous polymer which have pores of specific sizes.
- When the sample is injected with the mobile phase, the mobile phase occupies the pores of the stationary phase.
- If the size of the molecules is appropriate enough to enter the pores, they remain in the pores partly or wholly.

- However, molecules with a larger size are retained from entering the pores, causing them to be moved with the mobile phase, out of the column.
- If the mobile phase used in an aqueous solution, the process is termed gel filtration chromatography.
- If the mobile phase used is an organic solvent, it is termed as gel permeation chromatography.

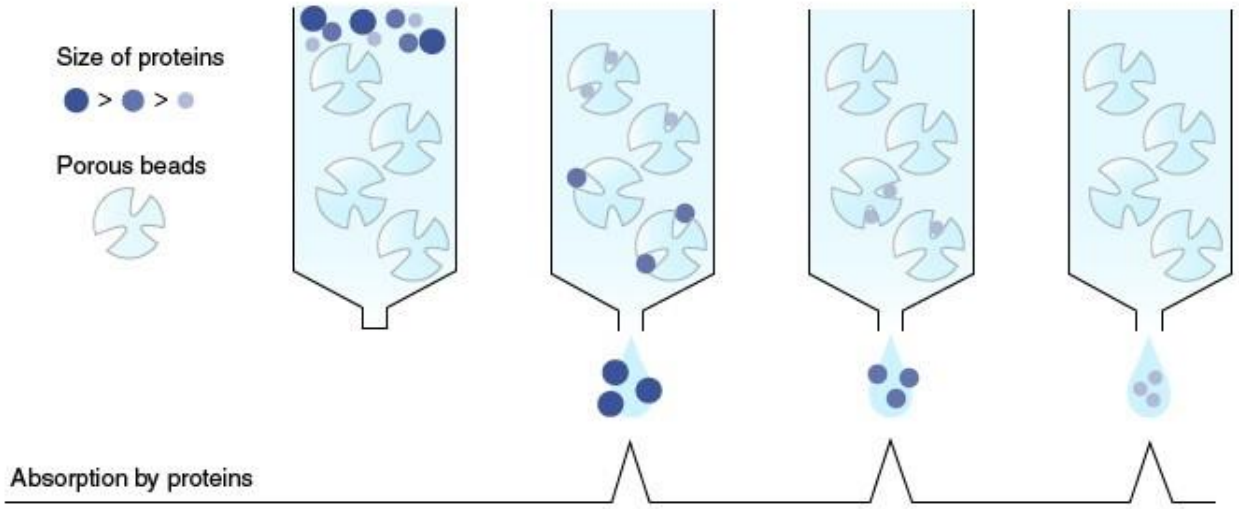

Figure 8 Gel-filtration chromatography.

7.2 Steps

- The column is filled with semi-permeable, porous polymer gel beads with a well-defined range of pore sizes.
- The sample, mixed with the mobile phase, is then injected into the column from the top of the column.
- The molecules bound to the column are separated by elution solution where either solution of the same polarity is used (isocratic technique), or different samples with different polarities are used (gradient technique).
- Elution conditions (pH, essential ions, cofactors, protease inhibitors, etc.) can be selected, which will complement the requirements of the molecule of interest.

7.3 Uses

- One of the principal advantages of gel-filtration chromatography is that separation can be performed under conditions specifically designed to maintain the stability and activity of the molecule of interest without compromising resolution.
- The absence of a molecule-matrix binding step also prevents unnecessary damage to fragile molecules, ensuring that gel-filtration separations generally give high recoveries of activity.
- Because of its unique mode of separation, gel-filtration chromatography has been used successfully in the purification of proteins and peptides from various sources.
- Gel-filtration chromatography has been used to separate various nucleic acid species such as DNA, RNA, and tRNA as well as their constituent bases, adenine, guanine, thymine, cytosine, and uracil.

8. High-performance liquid chromatography

High-performance liquid chromatography is a modified form of column chromatography where the components of a mixture are separated on the basis of their affinity with the stationary phase (Fig 9).

8.1 Principle of HPLC

- This technique is based on the principle of differential adsorption where different molecules in a mixture have a varying degree of interactions with the absorbent present on the stationary phase.
- The molecules having higher affinity remain adsorbed for a longer time decreasing their speed of movement through the column.
- However, the molecules with lower affinity move with a faster movement, thus allowing the molecules to be separated in different fractions.
- This process is slightly different from the column chromatography as in this case; the solvent is forced under high pressures of up to 400 atmospheres instead of allowing it to drip down under gravity.

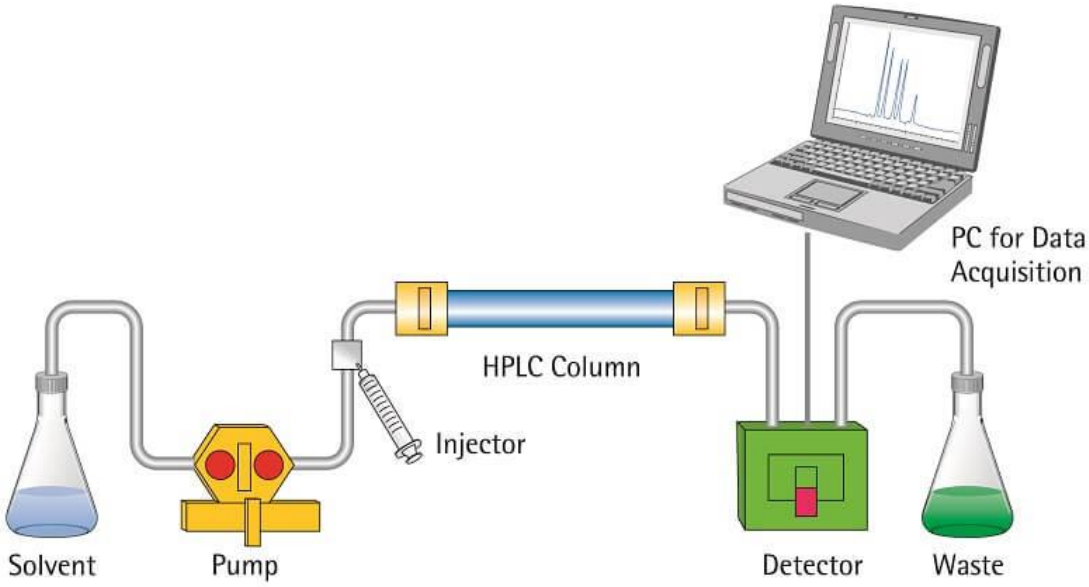

Figure 9 High-performance liquid chromatography (HPLC)

8.2 Steps of HPLC

- The column is prepared by taking a glass tube that is dried and coated with a thin, uniform layer of stationary phase (cellulose, silica).
- Then the sample is prepared by adding the mixture to the mobile phase. The sample is introduced into the column from the top, and a high-pressure pump is used to pass the sample at a constant rate.
- The mobile phase then moves down to a detector that detects molecules at a certain absorbance wavelength.
- The separated molecules can further be analyzed for various purposes.

8.3 Uses of HPLC

- High-performance liquid chromatography is used in the analysis of pollutants present in environmental samples.
- It is performed to maintain product purity and quality control of various industrial productions.
- This technique can also be used to separate different biological molecules like proteins and nucleic acids.

- The increased speed of this technique makes the process faster and more effective.

9. Gas chromatography

Gas chromatography is a separation technique in which the molecules are separated on the basis of their retention time depending on the affinity of the molecules to the stationary phase (Fig 10).

The sample is either liquid or gas that is vaporized in the injection point.

9.1 Principle of Gas chromatography

- Gas chromatography is based on the principle that components having a higher affinity to the stationary phase have a higher retention time as they take a longer time to come out of the column.
- However, the components having a higher affinity to the stationary phase have less retention time as they move along with the mobile phase.
- The mobile phase is a gas, mostly helium, that carries the sample through the column.
- The sample once injected in converted into the vapor stage is then passed through a detector to determine the retention time.
- The components are collected separately as they come out of the stationary phase at different times.

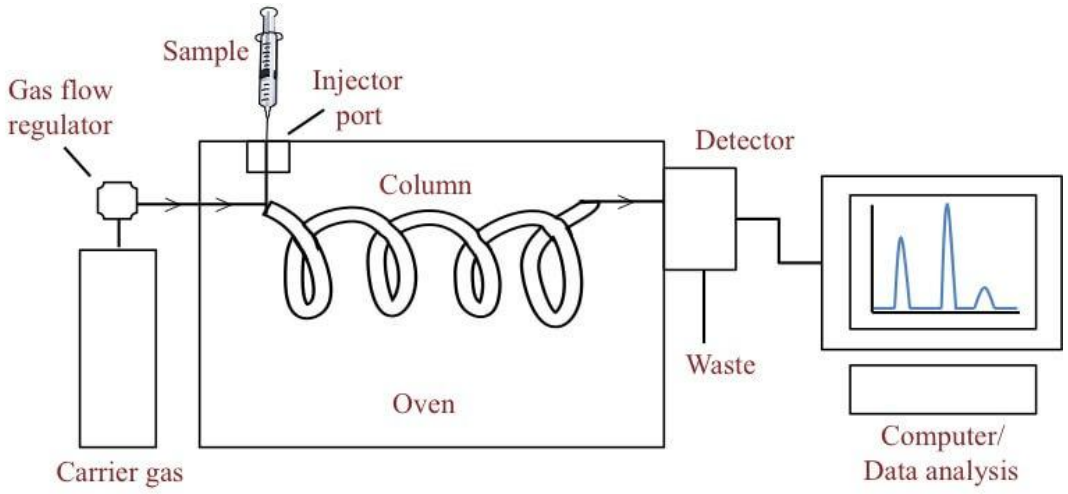

Figure 10 Gas chromatography. Image Source: Bitesize Bio.

9.2 Steps of Gas chromatography

- The sample is injected into the column where it is vaporized into a gaseous state. The vapourised component than mixes with the mobile phase to be carried through the rest of the column.
- The column is set with the stationary phase where the molecules are separated on the basis of their affinity to the stationary phase.
- The components of the mixture reach the detector at different times due to differences in the time they are retained in the column.

9.3 Uses of Gas chromatography

- This technique is used to calculate the concentration of different chemicals in various samples.
- This is used in the analysis of air pollutants, oil spills, and other samples.
- Gas chromatography can also be used in forensic science to identify and quantify various biological samples found in the crime scene.

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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – IV – ANALYTICAL TECHNIQUES – SBB2104

1. Electrophoresis

- The term electrophoresis describes the migration of a charged particles under the influence of an electric field.
- Various essential biological molecules, such as amino acids, peptides, proteins, nucleic acids, nucleotides, have ionizable group, which at given pH exist in a solution as electrically charged species either as cation (+ve) and anion (-ve) are separated by electrophoresis
- Under the influence of electric field these charged particles will migrates either to cathode or anode depending on the nature of their net charge

1.1 Principle of electrophoresis:

- When a potential difference is applied, the molecules with different overall charge will begin to separate owing to their different electrophoretic mobility. Even the molecules with similar charge will begins to separate if they have different molecular sizes, since they will experience different frictional forces. Therefore, some form of electrophoresis rely almost totally on the different charges on the molecules for separation while some other form exploits difference in size (molecular size) of molecules.
- Electrophoresis is regarded as incomplete form of electrolysis because the electric field is removed before the molecules in the samples reaches the electrode but the molecules will have been already separated according to their electrophoretic mobilities.
- The separated samples are then located by staining with an appropriate dye or by autoradiography, if the sample is radiolabeled.

Electrophoresis uses an electric field applied across a gel matrix to separate large molecules such as DNA, RNA, and proteins by charge and size. Samples are loaded into the wells of a gel matrix that can separate molecules by size and an electrical field is applied across the gel. This field causes negatively charged molecules to move towards the positive electrode. The gel matrix, itself, acts as a sieve, through which the smallest molecules pass rapidly, while longer molecules are slower-moving.

For DNA and RNA, sorting molecules by size in this way is trivial, because of the uniform negative charge on the phosphate backbone. For proteins, which vary in their charges, a clever trick must be employed to make them mimic nucleic acids - see polyacrylamide gel electrophoresis (PAGE) below. Different kinds of gels have different pore sizes. Like sieves with finer or coarser meshes, some gels do a better job of separating smaller molecules while others work better for larger ones. Gel electrophoresis may be used as a preparative technique (that is, when purifying proteins or nucleic acids), but most often it is used as an analytical tool.

1.2 Factor affecting electrophoresis:

i. Nature of charge:

- Under the influence of an electric field these charged particles will migrate either to cathode or anode depending on the nature of their net charge.

ii. Voltage:

- When a potential difference (voltage) is applied across the electrodes, it generates a potential gradient (E), which is the applied voltage (v) divided by the distance "d" between the two electrodes i.e. p.d. (E) = V/d.
- When this potential gradient 'E' is applied, the force as the molecule bearing a charge of 'q' coulombs is 'E_q' Newtons.
- It is this force that drives the molecule towards the electrodes.

iii. Frictional force:

- There is also a frictional force that retards the movement of this charged molecule.
- This frictional force is the measure of the hydrodynamic size of the molecule, the shape of the molecule, the pore size of the medium in which the electrophoresis is taking place and the viscosity of the buffer.
- The velocity 'v' of the charged molecule is an electric field is therefore given by the equation. $U=E_q/f$, where 'f'= frictional coefficient

iv. Electrophoretic mobility:

- More commonly a term electrophoretic mobility ( ) of an ion is used, which is the ratio of the velocity of the ion and the field strength. i.e. =U/E.
- When a p.d. is applied, the molecule with different overall charges will begin to separate owing to their different electrophoretic mobility.
- Even the molecule with similar charges will begin to separate if they have different molecular sizes, since they will experience different frictional forces.

V. current:

- Ohm's law: V/I=R
- It therefore appears that it is possible to accelerate an electrophoretic separation by increasing the applied voltage, which ultimately results in corresponding increase in the current flowing.
- The distance migrated by the ions will be proportional to both current and time.

vi. Heat:

- One of the major problems for most forms of electrophoresis, that is the generation of heat.
- During electrophoresis, the power (W) generated in one supporting medium is given by $W= I^2R$
- Most of the power generated is dissipated as heat.
- The following effects are seen on heating of the electrophoretic medium has:
    - An increased rate of diffusion of sample and buffer ions which leads to the broadening of the separated samples.
    - Formation of convention currents, which leads to mixing of separated samples.
    - Thermal instability of samples that are sensitive to heat.
    - A decrease of buffer viscosity and hence reduction in the resistance of the medium.

- If a constant voltage is applied, the current increases during electrophoresis owing to the decrease in resistance and this rise in current increases the heat output still further.
- For these reasons, often a stabilized power supply is used, which provides constant power and thus eliminates fluctuations in heating.
- Constant heat generation is however a problem. For which the electrophoresis is run at very low power (low current) to overcome any heating problems, but this can lead to poor separation as a result of the increased amount of diffusion due to long separation time.
- Compromise condition have to be found out with reasonable power settings, to give acceptable separation time and an appropriate cooling system, to remove liberated heat. While such system works fairly well, the effect of heating are not always totally eliminated.

Vii. Elecrtroendosmosis:

- The phenomenon of electroendosmosis (aka- electro-osmotic flow) is a final factor that can affect electrophoretic separation.
- This phenomenon is due to the presence of charged groups on the surface of the support medium.
- For instance, paper has some carboxyl group present, agarose contains sulfate groups depending on the purity grade and the surface of glass walls used in capillary electrophoresis contains silanol (Si-OH) groups.
- These groups, at appropriate pH, will ionize, generating charged sites.
- It is these charges that generate electroendosmosis.
- In case of capillary electrophoresis, the ionized sianol groups creates an electrical double layer, or a region of charge separation, at the capillary wall/electrolytic interface.
- When voltage is applied cations in the electrolyte near the capillary walls migrate towards the cathode, pulling electrolyte solution with them.
- This creates a net electroosmotic flow towards cathode.

1.3 Types of support media used in electrophoresis:

- The earliest supports used were filter paper or cellulose acetate strips, wetted in electrophoresis buffer. Nowadays these media are not is use.
- Nowadays either an agarose gel or polyacrylamide gels are used.
- **Agarose gel:**
  - Agarose- a linear polysaccharide (M.W. 12000 Da) made up of the basic repeat unit of agarobiose (which comprises alternating units of galactose and 3,6-anhydrogalactose.
  - It is one of the components of agar, that is a mixture of polysaccharides from seaweeds.
  - It is used at a concentration between 1% and 3%.
  - Agarose gel is formed by suspending dry agarose in aqueous buffer and then boiling the mixture till it becomes clear solution, which is then poured and allowed to cool at room temperature to form rigid gel.
  - The gelling properties is attributed to inter and intramolecular H-bonding within and between long agarose chains.
  - The pore size of the gel is controlled by the initial concentration of agarose, large pore size corresponds to low concentration and vice versa.
  - Although free from charges, substitution of the alternating sugar residues with carboxyl, methoxyl, pyruvate, and sulfate groups occur to varying degrees which can result in electroendosmosis during electrophoresis.
  - Agarose is therefore sold in different purity grades, based on the sulfate concentration- the lower the sulfate concentration, the higher the purity.
  - These gels are used for the electrophoresis of both proteins and nucleic acids.
  - For proteins, the pore size of a 1% agarose gel is large relative to the sizes of proteins.
  - Therefore, used in techniques such as immune-electrophoresis or flat-bed isoelectric focusing, where proteins are required to move unhindered in the gel matrix according to their native charge.

- Such large pure gels are also used to separate much larger molecules such as RNA and DNA, because the pore sizes are still large enough for RNA and DNA molecule to pass through the gel.
- An advantage of using agarose in the availability of low melting point agarose (62-65^{o}C).
- This gel can be reliquefied by heating to 65^{o}C and thus, for example DNA samples separated can be cut out of the gel, returned to solution and recovered.

- **Polyacrylamide gel:**
  - Cross-linked polysaccharide gel are formed from the polymerization of acrylamide monomer in the presence of small amount of N,N'-methylene bis acrylamide (aka- bis-acrylamide).
  - Bis-acryl amide is basically two acrylamide molecules linked by a methylene group, and is used as a cross-linking agent.
  - Acrylamide monomers is polymerized in head to tail fashion into long chain, thus introducing a second site for chain extension.
  - Proceeding in this way, a cross-linked matrix of fairly well-defined structure is formed.
  - The polymerization of acrylamide is an example of free radical catalysis and is initiated by the addition of ammonium persulfate and the base N, N, N', N'- tetra-methylene diamine (TEMED).
  - TEMED catalyses decomposition of the persulphate ion to give free radical.
    - $S_2O_8^{2-} + e^{-}$ à $SO_4^{2-} + SO^{\bullet}_4$
    - $R^{\bullet} + M$ à $RM^{\bullet}$
    - $RM^{\bullet} + M$ à $RMM^{\bullet}$
    - $RMM^{\bullet} + M$ à $RMMM^{\bullet}$ and so on…
  - Photopolymerisation is an alternative method that can be used to polymerize acrylamide gels.
  - The ammonium persulphate and TEMED are replaced by riboflavin and when the gel is poured, it is placed in front of a bright light for 2-3hrs.

- Photodecomposition of riboflavin generates a free radical that initiates polymerization.
- Acrylamide gels are defined in terms of the total percentage of acrylamide present, and the pore size in the gel can be varied by changing the concentration of both acrylamide and bis-acrylamide.
- The acrylamide gel can be made with a content between 3% and 30% acrylamide.
- Thus, the low percentage gels (e.g., 4%) have large pore size and are used for electrophoresis of protein- example flat bed isoelectric focusing, or stacking gel system of an SDS-PAGE.
- Low percentage acrylamide gels are also used to separate DNA.
- Gels between 10% and 20% acrylamide are used in techniques such as SDS-gel electrophoresis, where smaller pore size now introduces a sieving effect that contributes to the separation of proteins according to their size.

2. Agarose Gel Electrophoresis

Agarose gel electrophoresis is a technique used to separate nucleic acids primarily by size. Agarose (Fig 1) is a polysaccharide obtained from seaweeds (Figure 8.11). It can be dissolved in boiling buffer and poured into a tray, where it sets up as it cools (Figure 8.12) to form a slab. Agarose gels are poured with a comb in place to make wells into which DNA or RNA samples are placed after the gel has solidified. The gel is immersed in a buffer and a current is applied across the slab. Double-stranded DNA has a uniform negative charge that is independent of the sequence composition of the molecule. Therefore, if DNA fragments are placed in an electric field they will migrate from the cathode (-) towards the anode (+). The rate of migration is directly dependent on the ability of each DNA molecule to worm or wiggle its way through the sieving gel. The agarose matrix provides openings for macromolecules to move through. The largest macromolecules have the most difficult time navigating through the gel, whereas the smallest macromolecules slip through it the fastest (Fig 2).

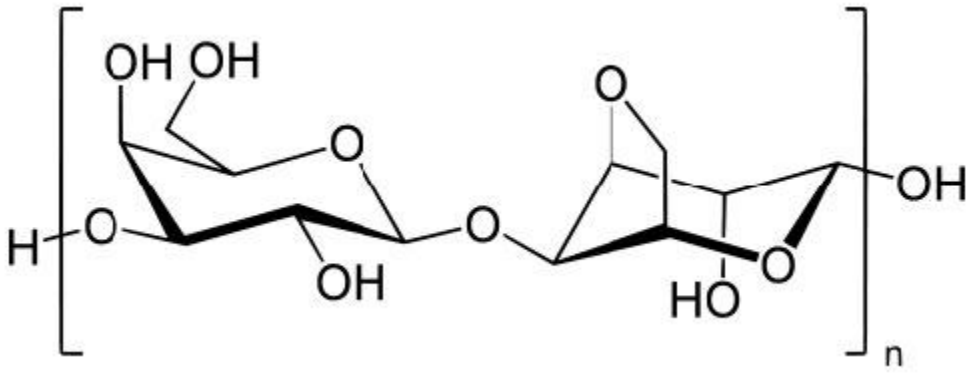

Figure 1 Structure of the agarose polysaccharide.

Because electrophoresis uses an electric current as a force to drive the molecules through the matrix, the molecules being separated must be charged. Since the size to charge ratio for DNA and RNA is constant for all sizes of these nucleic acids, the molecules simply sort on the basis of their size - the smallest move fastest and the largest move slowest.

All fragments of a given size will migrate the same distance on the gel, forming the so-called "bands" on the gel. Visualization of the DNA fragments in the gel is made possible by addition of a dye, such as ethidium bromide, which intercalates between the bases and fluoresces when viewed under ultraviolet light (Figure 8.13) By running reference DNAs of known sizes alongside the samples, it is possible to determine the sizes of the DNA fragments in the sample. It is useful to note that, by convention, DNA fragments are not described by their molecular weights (unlike proteins), but by their length in base-pairs(bp) or kilobases (kb) (Fig 3).

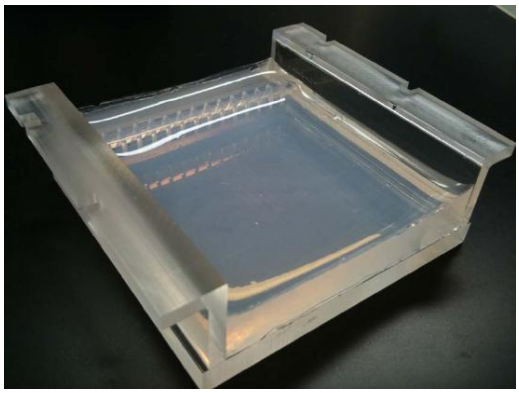

Figure 2 Agarose gel electrophoresis separation of DNA - orange bands are DNA fragments.

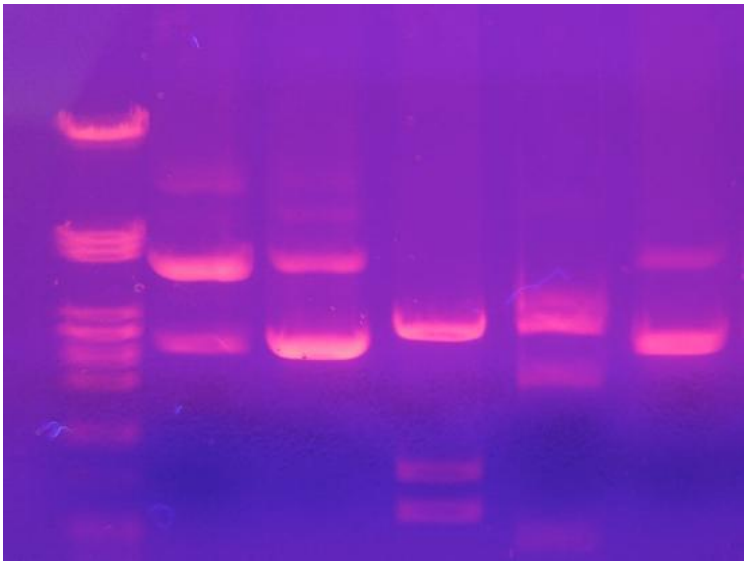

Figure 3 DNA bands visualized with ethidium bromide staining.

3. Polyacrylamide gel electrophoresis (PAGE)

Like DNA and RNA, proteins are large macromolecules, but unlike nucleic acids, proteins are not necessarily negatively charged. The charge on each protein depends on its unique amino acid sequence. Thus, the proteins in a mixture will not necessarily all move towards the anode.

Additionally, whereas double-stranded DNA is rod-shaped, most proteins are globular (folded). Further, proteins are considerably smaller than nucleic acids, so the openings of the matrix of the agarose gel are simply too large to effectively provide separation. Consequently, unaltered (native) proteins are not very good prospects for electrophoresis on agarose gels. To separate proteins by mass using electrophoresis, one must make several modifications.

3.1 Gel matrix

First, a matrix made by polymerizing and cross-linking acrylamide units is employed. A monomeric acrylamide (Fig 4) is polymerized and the polymers are cross-linked using N,N'-Methylene-bisacrylamide (Fig 5) to create a mesh-like structure. One can adjust the size of the openings of the matrix/mesh readily by changing the percentage of acrylamide in the reaction. Higher percentages of acrylamide give smaller openings and are more effective for separating smaller molecules, whereas lower percentages of acrylamide are used when resolving mixtures

of larger molecules. (Note: polyacrylamide gels are also used to separate small nucleic acid fragments, with some acrylamide gels capable of separating pieces of DNA that differ in length by just one nucleotide.)

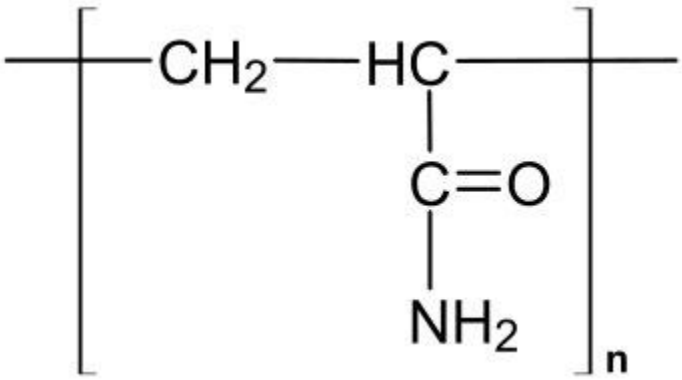

Figure 4 Acrylamide monomer.

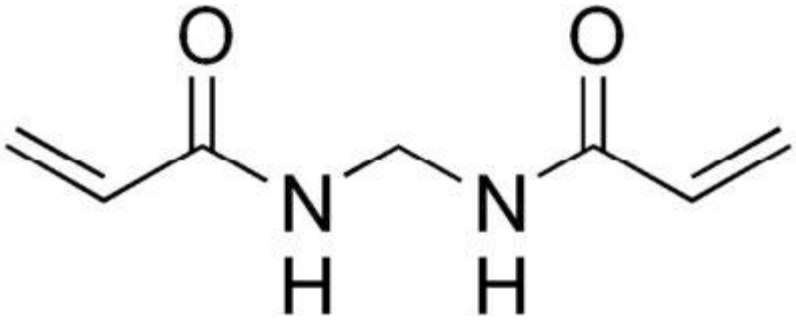

Figure 5 N,N'-Methylenebisacrylamide - acrylamide crosslinking reagent.

3.2 Charge alteration by SDS

A second consideration is that proteins must be physically altered to "present" themselves to the matrix like the negatively charged rods of DNA. This is accomplished by treating the proteins with the anionic detergent, SDS (sodium dodecyl sulfate). SDS denatures the proteins so they assume a rod-like shape and the SDS molecules coat the proteins such that the exterior surface is loaded with negative charges, masking the original charges on the proteins and making the charge on the proteins more proportional to their mass, like the backbone of DNA.

Since proteins typically have disulfide bonds that prevent them from completely unfolding in detergent, samples are boiled with mercaptoethanol to break the disulfide bonds and ensure the

proteins are as rod-like as possible in the SDS. Reagents like mercaptoethanol (and also dithiothreitol) are sulfhydryl-containing reagents that become oxidized as they reduce disulfide bonds in other molecules (Fig 6)

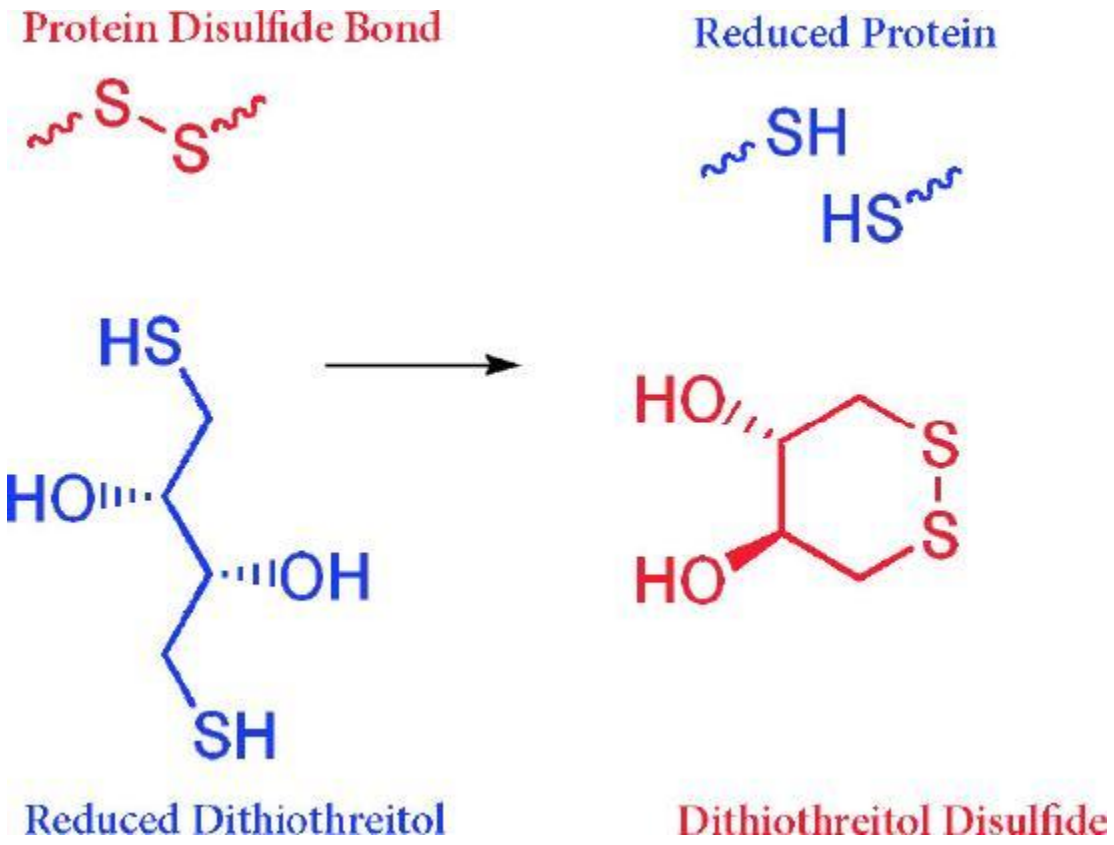

Figure 6 Reduction of disulfide bonds by dithiothreitol.

3.3 Stacking Gel

A third consideration is that a "stacking gel" may be employed at the top of a polyacrylamide gel to provide a way of compressing the samples into a tight band before they enter the main polyacrylamide gel (called the resolving gel). Just like DNA fragments in agarose gel electrophoresis get sorted on the basis of size (largest move slowest and smallest move fastest), the proteins migrate through the gel matrix at velocities inversely related to their size. Upon completion of the electrophoresis, proteins may be visualized by staining with compounds that bind to proteins, like Coomassie Brilliant Blue (Fig 7) or silver nitrate.

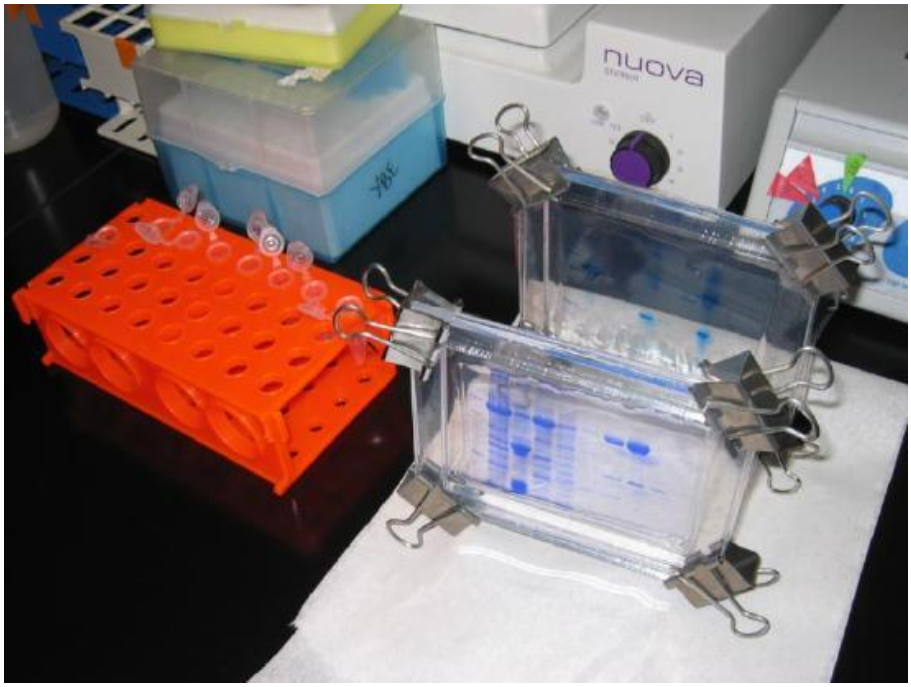

Figure 7 Two SDS-PAGE gels - Proteins are the blue bands (stained with Coomassie Blue).

3.4 **Non-denaturing gel electrophoresis**

The SDS_PAGE technique described above is the commonest method used for electrophoretic separation of proteins. In some situations, however, proteins may be resolved on so-called "native" gels, in the absence of SDS. Under these conditions, the movement of proteins through the gel will be affected not simply by their mass, but by their charge at the pH of the gel, as well. Proteins complexed with other molecules may move as single entity, allowing the isolation of the binding partners of proteins of interest.

4. Isoelectric focusing

Proteins vary considerably in their charges and, consequently, in their pI values (pH at which their charge is zero). This can be exploited to separate proteins in a mixture. Separating proteins by isoelectric focusing requires establishment of a pH gradient in a tube containing an acrylamide gel matrix. The pore size of the gel is adjusted to be large, to reduce the effect of sieving based on size. Molecules to be separated are applied to the gel containing the pH gradient

and an electric field is applied. Under these conditions, proteins will move according to their charge.

Positively charged molecules, for example, move towards the negative electrode, but since they are traveling through a pH gradient, as they pass through it, they reach a region where their charge is zero and, at that point, they stop moving. They are at that point attracted to neither the positive nor the negative electrode and are thus "focused" at their pI (Fig 8). Using isoelectric focusing, it is possible to separate proteins whose pI values differ by as little as 0.01 units.

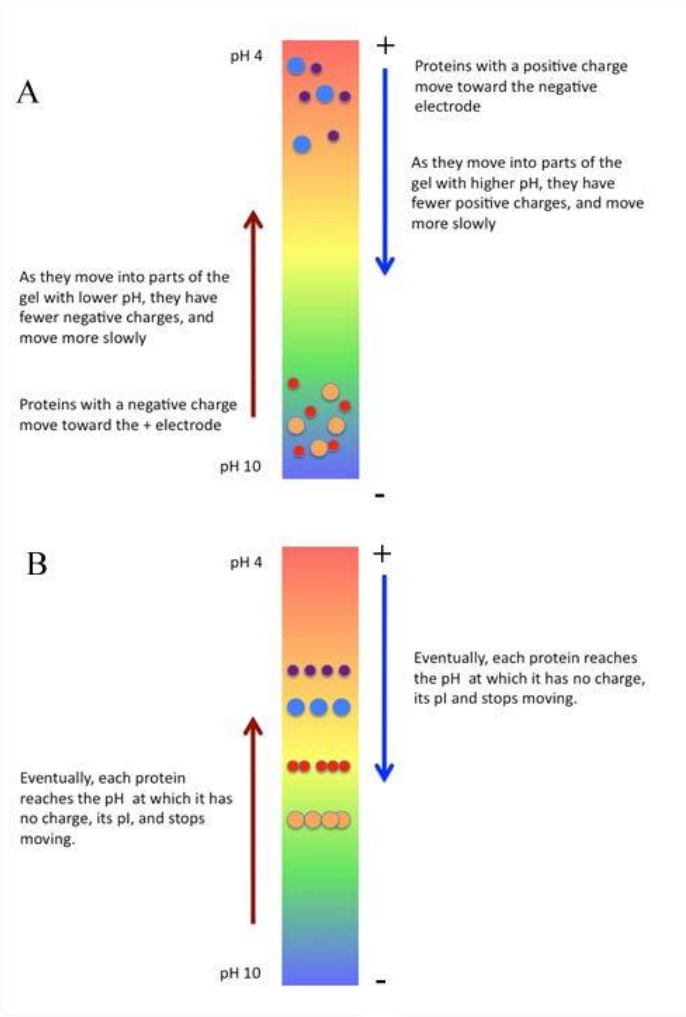

Figure 8 Isoelectric focusing: A. At the start of the run; B. at the end of the run

5. 2D gel electrophoresis

Both SDS-PAGE and isoelectric focusing are powerful techniques, but a clever combination of the two is a powerful tool of proteomics - the science of studying all of the proteins of a cell/tissue simultaneously. In 2-D gel electrophoresis, a lysate is first prepared from the cells of interest. The proteins in the lysate are separated first by their pI, through isoelectric focusing and then by size by SDS-PAGE.

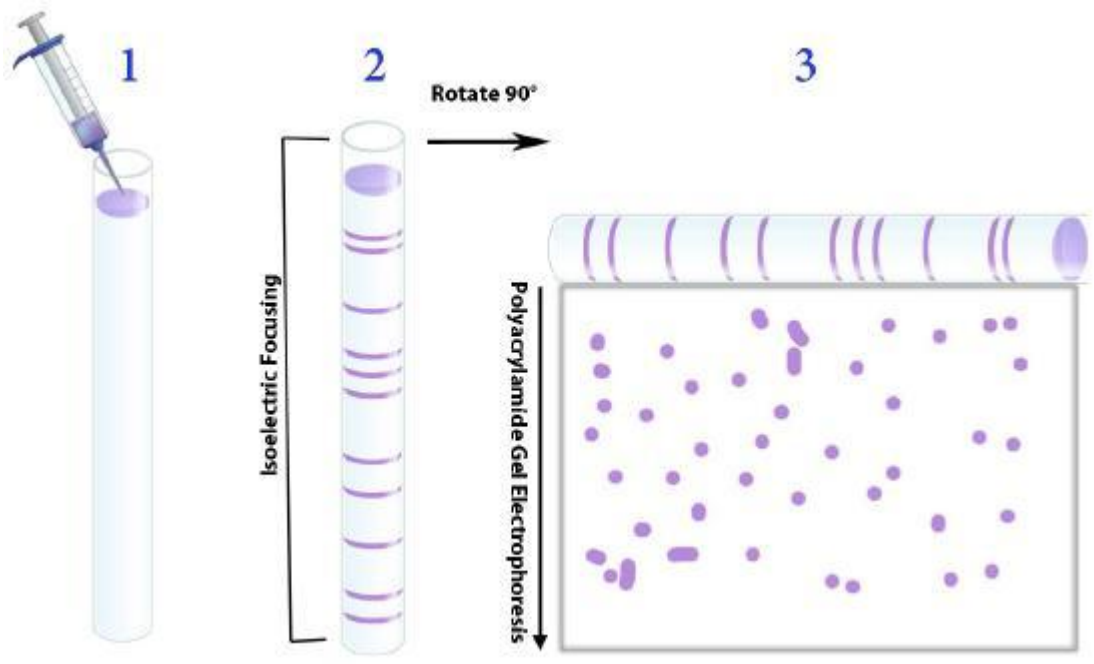

Figure 9 Scheme for performing 2-D gel analysis.

The mixture of proteins is first applied to a tube or strip (Figure 8.19, Step 1) where isoelectric focusing is performed to separate the proteins by their pI values (Step 2). Next, as shown in the figure, the gel containing the proteins separated by their pIs is turned on its side and applied along the top of a polyacrylamide slab for SDS-PAGE to separate on the basis of size (Step 3). The proteins in the isoelectric focusing matrix are electrophoresed into the polyacrylamide gel and separated on the basis of size (Fig 9). The product of this analysis is a 2-D gel as shown in Figure 10. The power of 2-D gel electrophoresis is that virtually every protein in a cell can be separated and appear on the gel as a spot defined by its unique size and pI. In the figure, spots in the upper left correspond to large positively charged proteins, whereas those in the lower right

are small negatively charged ones. Every spot on a 2-D gel can be eluted and identified by using high throughput mass spectrometry. This is particularly powerful when one compares protein profiles between different tissues or between control and treated samples of the same tissue.

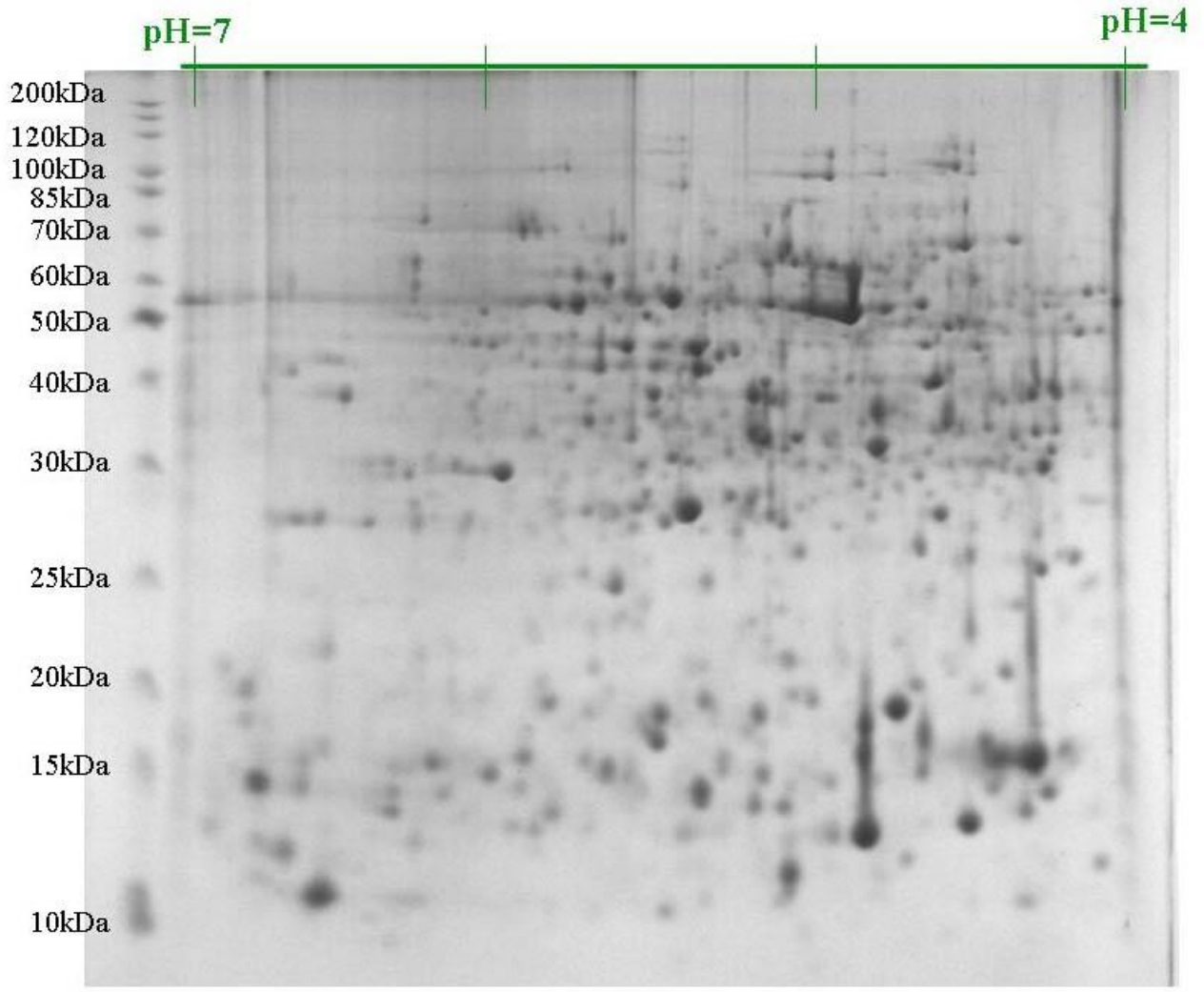

Figure 10 Result of 2-D gel electrophoresis separation.

5.1 Protein profiles comparison

Comparison of 2-D gels of proteins from non-cancerous tissue and proteins from a cancerous tissue of the same type provides a quick identification of proteins whose level of expression differs between the two. Information such as this can be useful in designing treatments or in understanding the mechanism(s) by which the cancer develops.

6. Southern Blotting

Southern blotting is a technique for detecting specific **DNA** fragments in a complex mixture. The technique was invented in mid-1970s by Edward Southern. It has been applied to detect Restriction Fragment Length Polymorphism (**RFLP**) and Variable Number of Tandem Repeat (**VNTR**) Polymorphism. The latter is the basis of DNA fingerprinting.

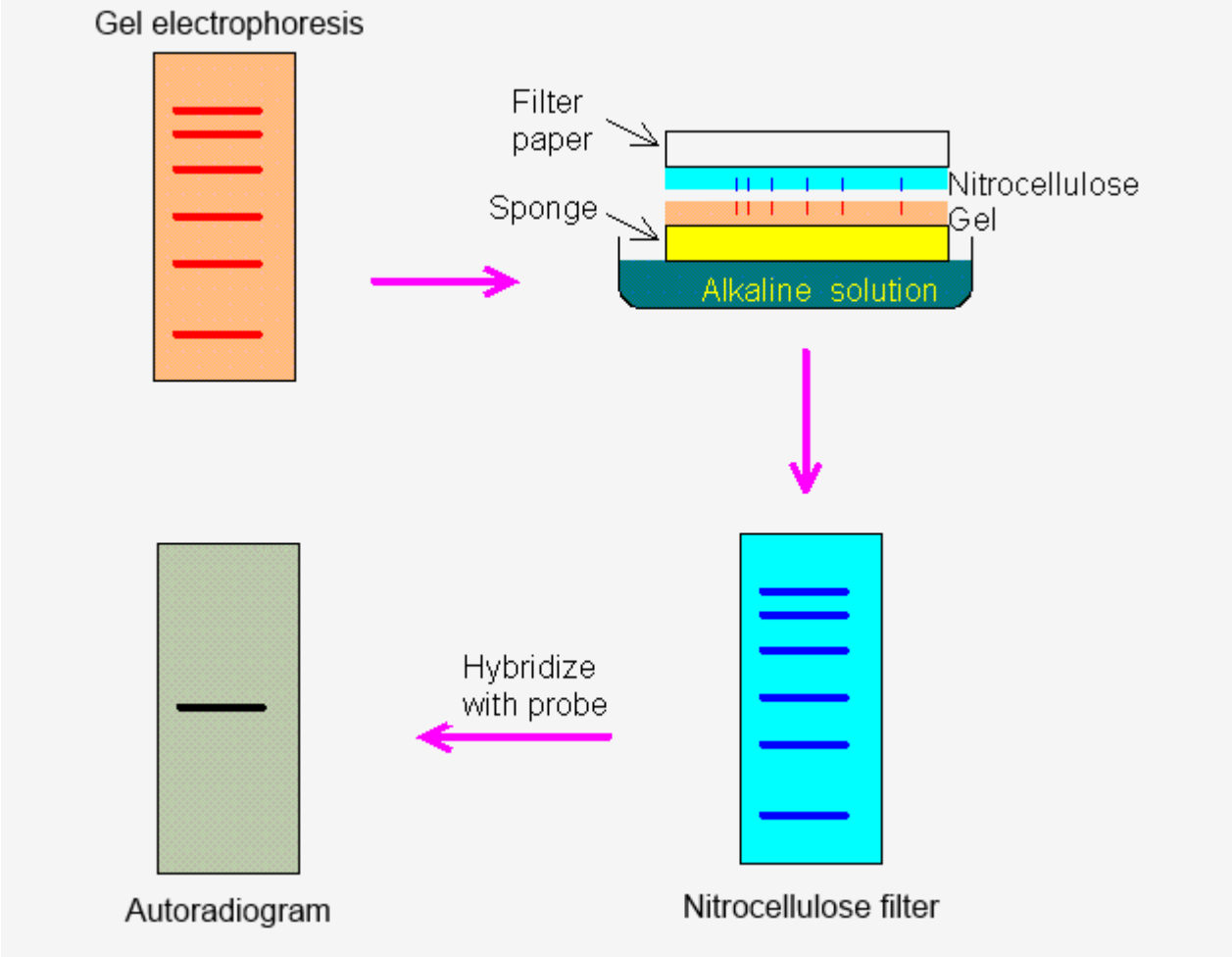

Figure 11**.** Southern blotting. **(a)** The DNA to be analyzed is digested with restriction enzymes and then separated by agarose gel electrophoresis. **(b)** The DNA fragments in the gel are denatured with alkaline solution and transferred onto a nitrocellulose filter or nylon membrane by blotting, preserving the distribution of the DNA fragments in the gel. **(c)** The nitrocellulose filter is incubated with a specific probe.

The location of the DNA fragment that hybridizes with the probe can be displayed by autoradiography.

6.1 Principle of Southern Blotting

The process involves the transfer of electrophoresis-separated DNA fragments to a carrier membrane which is usually nitrocellulose and the subsequent detection of the target DNA fragment by probe hybridization. Hybridization refers to the process of forming a double-stranded DNA molecule between a single-stranded DNA probe and a single-stranded target DNA. Since the probe and target DNA are complementary to each other, the reaction is specific which aids in the detection of the specific DNA fragment (Fig 11).

6.2 Steps involved in southern blotting

a. DNA extraction and purification

DNA is first separated from target cells following standard methods of genomic DNA extraction and then purified.

b. Restriction Digestion

Restriction endonucleases are used to cut high-molecular-weight DNA strands into smaller fragments. One or more restriction enzymes can be used to achieve such fragments.

c. Electrophoresis

The separation may be done by agarose gel electrophoresis in which the negatively charged DNA fragments move towards the positively charged anode, the distance moved depending upon its size.

d. Depurination

Partial depurination is done by the use of dilute HCl which promotes higher efficiency transfer of DNA fragments by it breaking down into smaller pieces.

e. Denaturation

DNA is then denatured with a mild alkali such as an alkaline solution of NaOH. This causes the double stranded DNA to become single-stranded, making them suitable for hybridization. DNA is then neutralized with NaCl to prevent re-hybridization before addition of the probe.

f. Blotting

The denatured fragments are then transferred onto a nylon or nitrocellulose filter membrane which is done by placing the gel on top of a buffer saturated filter paper, then laying nitrocellulose filter membrane on the top of gel. Finally some dry filter papers are placed on top of the membrane. Fragments are pulled towards the nitrocellulose filter membrane by capillary action and result in the contact print of the gel.

g. Baking

The nitrocellulose membrane is removed from the blotting stack, and the membrane with single stranded DNA bands attached on to it is baked in a vacuum or regular oven at 80 °C for 2-3 hours or exposed to ultraviolet radiation to permanently attach the transferred DNA onto the membrane.

h. Hybridization

The membrane is then exposed to a hybridization probe which is a single DNA fragment with a specific sequence whose presence in the target DNA is to be determined. The probe DNA is labeled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye.

i.Washing

After hybridization, the membrane is thoroughly washed with a buffer to remove the probe that is bound nonspecifically or any unbound probes present.

j. Autoradiograph

The hybridized regions are detected autoradiographically by placing the nitrocellulose membrane in contact with a photographic film which shows the hybridized DNA molecules. The pattern of hybridization is visualized on X-ray film by autoradiography in case of a radioactive or fluorescent probe is used or by the development of color on the membrane if a chromogenic detection method is used.

6.3 Application

- Identifying specific DNA in a DNA sample.
- Preparation of RFLP (Restriction Fragment Length Polymorphism) maps
- Detection of mutations, deletions or gene rearrangements in DNA
- For criminal identification and DNA fingerprinting (VNTR)
- Detection and identification of trans gene in transgenic individual
- Mapping of restriction sites
- For diagnosis of infectious diseases
- Prognosis of cancer and prenatal diagnosis of genetic diseases
- Determination of the molecular weight of a restriction fragment and to measure relative amounts in different samples.

7. Restriction Fragment Length Polymorphism (RFLP)

Polymorphism refers to the DNA sequence variation between individuals of a species. If the sequence variation occurs at the restriction sites, it could result in RFLP. The most well known example is the RFLP due to β globin gene mutation (Fig 12).

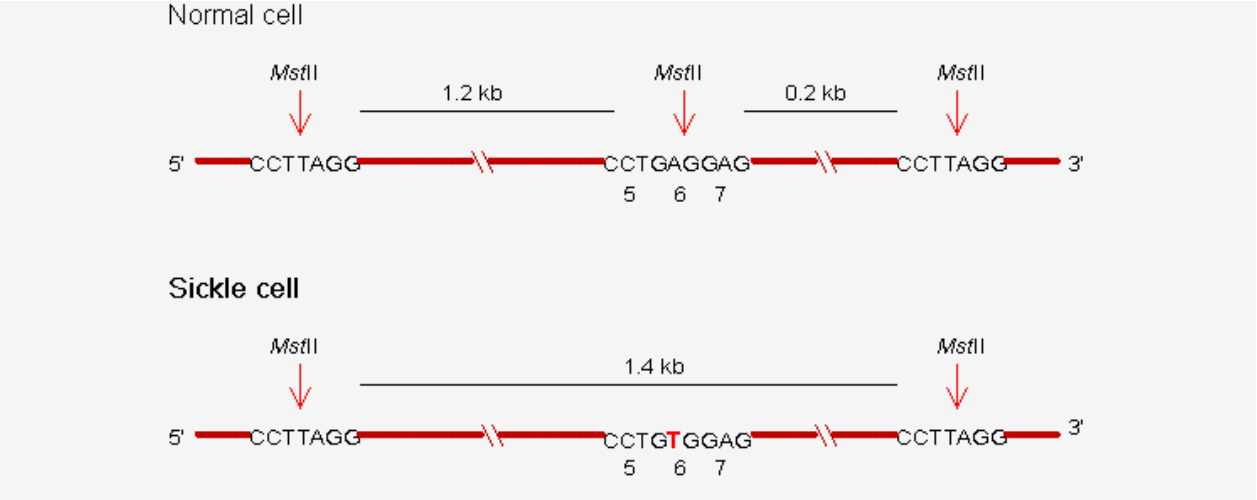

Figure 12 RFLP resulting from β-globin gene mutation. In the normal cell, the sequence corresponding to 5th to 7th amino acids of the β-globin peptide is CCTGAGGAG, which can be recognized by the restriction enzyme MstII. In the sickle cell, one base is mutated from A to T, making the site unrecognizable by MstII. Thus, MstII will generate 0.2 kb and 1.2 kb fragments in the normal cell, but generate 1.4 kb fragment in the sickle cell. These different fragments can be detected by the southern blotting.

8. Northern Blotting

Northern Blotting is a technique used for the study of gene expression. It is done by detection of particular RNA (or isolated mRNA). mRNA is generally represented as 5% of the overall RNA sequence. This method reveals the identity, number, activity, and size of the particular gene. This blotting technique can also be used for the growth of a tissue or organism. In different stages of differentiation and morphogenesis the abundance of an RNA changes and this can be identified using this technique. It also aids in the identification of abnormal, diseased or infected condition at the molecular level. The northern blot technique was developed in 1977 by James Alwine, David Kemp and George Stank at Stanford University. The technique got its name due to the similarity of the process with Southern blotting. The primary difference between these two techniques is that northern blotting concerns only about RNA.

8.1 Principle

As all normal blotting technique, northern blotting starts with the electrophoresis to separate RNA samples by size. Electrophoresis separates the RNA molecules based on the charge of the nucleic acids. The charge in the nucleic acids is proportional to the size of the nucleic acid sequence. Thus the electrophoresis membrane separates the Nucleic acid sequence according to the size of the RNA sequence. In cases where our target sequence is an mRNA, the sample can be isolated through oligo cellulose chromatographic techniques, as mRNA are characterized by the poly(A)-tail. Since gel molecules are fragile in nature, the separated sequences are transferred to the nylon membranes. The selection of nylon membrane is contributed to the factor that nucleic acids are negatively charged in nature. Once the RNA molecules are transferred it is immobilized by covalent linkage. The probe is then added, the probe can be complementary an ss DNA sequence. Formamide is generally used as a blotting buffer as it reduces the annealing temperature (Fig 13).

8.2 Procedure

1. The tissue or culture sample collected is first homogenized. The samples may be representative of different types of culture for comparison or it can be for the study of different stages of growth inside the culture.
2. The RNA sequence is separated in the electrophoresis unit an agarose gel is used for the purpose of the nucleic acid separation.
3. Now the separated RNA sequence is transferred to the nylon membrane. This is done by two mechanisms capillary action and the ionic interaction.

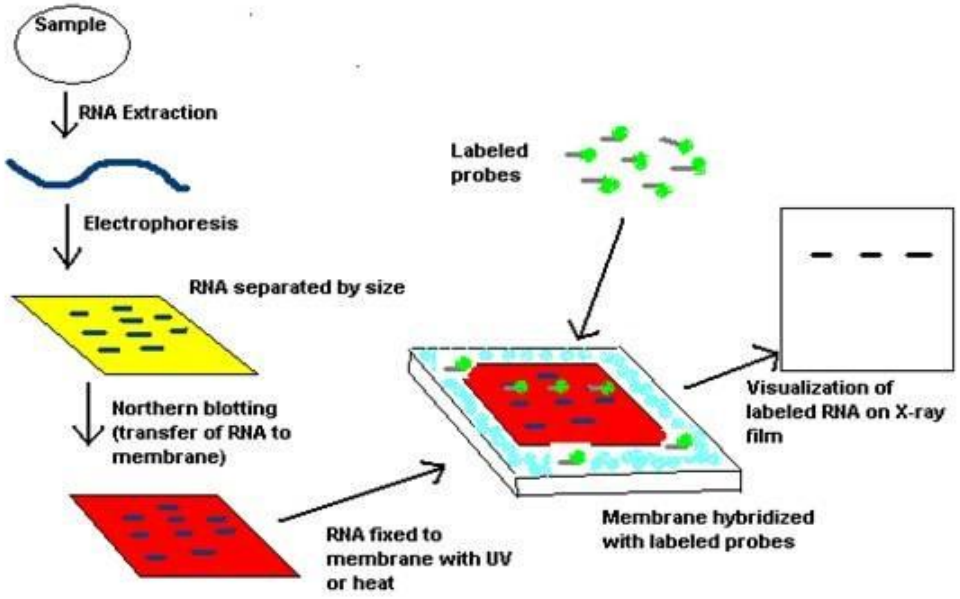

Figure 13 Northern Blotting

4. The transfer operation is done by keeping the gel in the following order. First, the agarose gel is placed on the bottom of the stack, followed by the blotting membrane. On top of these paper towels a mild weight (glass plate) is placed. The entire setup is kept in a beaker containing transfer buffer.
5. RNA transferred to the nylon membrane is then fixed using UV radiation.
6. The fixed nylon membrane is then mixed with probes. The probes are specifically designed for the gene of interest, so that they will hybridize with RNA sequences on the blot corresponding to the sequence of interest.
7. The blot membrane is washed to remove unwanted probe
8. Labeled probe is detected by chemiluminescence or autoradiography. The result will be dark bands in x ray film.

9. Western blotting

Western blotting (or immunoblotting) is a widely used method to detect proteins as well as posttranslational modifications on proteins, using antibody based probes to obtain specific information about target proteins from complex samples. It is a routine method in a molecular

biology, biochemistry and cell biology field with the multitude of applications. It can provide semi-quantitative or quantitative data about the target protein in simple or complex biological samples.

Since western blotting is a multistep protocol, variations and errors can occur at any step reducing the reliability and reproducibility of this technique. Recent reports suggest that a few key steps, such as the sample preparation method, the amount and source of primary antibody used, as well as the normalization method utilized, are critical for reproducible western blot results. This method relies on the fact that most epitopes (sites recognized by antibodies, generally comprising several amino acids) inspite of denaturation of proteins can still be recognized. Due to high affinities of antibody toward their epitopes, it is a very sensitive method and even picogram quantities of a target protein can be detected. The two primary advantages of western blotting are sensitivity and specificity. Western blotting has advantages over other protein detection techniques. Silver staining, another technique of protein detection detects 10 ng of protein and all proteins in a given sample. Whereas, western blotting can detect as little as 0.1ng of protein, and it selectively detects only the protein of interest. Thus a complex mixture containing only traces of the desired protein may be analyzed accurately with this technique. Western blotting was first described by Harry Towbin in 1979. It was in 1981 when W. Neal Burnette developed an improved version of the method and gave the name “western blotting” simply because of the location of laboratory. Towbin’s landmark paper has been cited more than 54,000 times (Fig 14).

Below is a general procedure for blotting, and every step is critical for obtaining high-quality, reliable and analyzable data.

1. Homogenize the sample.
2. Separation of the molecule of interest by an electrophoresis membrane.
3. Transferring the molecules to a nitro cellulosic membrane/ nylon membrane.
4. Hybridization or identification of the molecule

Western blotting is the technique used for separation or identification of protein molecules. This technique can be used for both the active 3D protein and denatured long peptide chains. The 3-D protein in its active structure has sulfur-hydroxyl bonds in the structure. This methodology classifies protein based on the molecular weight and charge. These techniques have application in the identification of a wide variety of infectious diseases like HIV, Hepatitis B, Herpes type 2,

and feline immunodeficiency disease. The identification of these diseases is done by using the antibody of the particular disease as the probe and these probes are produced in vitro condition. Western blotting is also used for research purpose. This technique can be used in the study of the properties and activity of a protein molecule of interest.

9.1 PROCEDURE

9.1.1 Sample Preparation

1. Wash cells in the tissue culture flask or dish by adding cold Phosphate Buffered Saline (PBS) and rocking gently. Flask or dish should be kept on ice throughput the process. Discard PBS.
2. Add PBS and use a cell scraper to dislodge the cells. Pipette the mixture into microcentrifuge tubes.
3. Centrifuge at 1500 RPM for 5 minutes. Discard the supernatant.
4. Add 180 ul of ice cold lysis buffer solution to 20 ul of fresh protease inhibitor. This prevents the protease enzyme. Incubate for 30 minutes.
5. Incubate for 30 min on ice, and centrifuge this solution for 10 minutes at 12000 RPM at 4^0c and the sample solution is ready.
6. Transfer supernatant (or protein mix) to a fresh tube and store on ice or frozen at -20°C or -80°C.
7. Measure the concentration of protein using a spectrophotometer and determine the volume of protein extract to ensure 50 μg in each well.
8. Add 5 μL sample buffer to the sample, and make the volume in each lane equalized using double distilled H_2O (dd H_2O). Mix well.
9. Heat the samples with a dry plate for 5 minutes at 100°C.

9.1.2 Gel Preparation

The gel has two parts stacking gel and separation gel. 10% stacking gel and 6% separating gel are generally used. Add the stacking gel solution into the assembly carefully and then add H_2O to the top. Wait for 15–30 minutes until the gel solidifies. Overlay the stacking gel with the separating gel, after removing the water. Insert the comb, ensuring that there are no air bubbles. Wait until the gel is solidified.

9.1.3 Electrophoresis

Pour the running buffer into the electrophorator. Place gel inside the electrophorator and connect to a power supply. (Tip: When connecting to the power source always connect red to red, and black to black). Make sure buffer covers the gel completely, and remove the comb carefully. Load marker (6 μL) followed by samples (15 μL) in to each well. Run the gel at 40 volts until the sample reaches the stacking gel and changed into 80 volts from the separation gel. Run the gel for approximately an hour, or until the dye front runs off the bottom of the gel

9.1.4 Fixing and blotting

Fixing is done 5% of bovine serum albumin solution. Cut 6 filter sheets to fit the measurement of the gel and one polyvinylidene fluoride (PDVF) membrane with the same dimensions. Wet the sponge and filter paper in transfer buffer, and wet the PDVF membrane in methanol. Separate glass plates and retrieve the gel. Create a transfer sandwich as follows – Sponge, 3 Filter Papers, Gel PVDF, 3 Filter Papers (Ensure there are no air bubbles between the gel and PVDF membrane, and squeeze out extra liquid). Relocate the sandwich to the transfer apparatus, which should be placed on ice to maintain 4°C. Add transfer buffer to the apparatus, and ensure that the sandwich is covered with the buffer. Place electrodes on top of the sandwich, ensuring that the PVDF membrane is between the gel and a positive electrode. Blotting is carried in two ways, capillary blotting or through electroblotting. Usually, electroblotting is carried out at 40 volts. For capillary blotting, the gel is stacked in the following order: electrophoretic gel followed by blotting membrane followed by wet tissue and lid glass plate.

9.1.5 Blocking and Incubation

Block the membrane with 5% skims milk in TBST for 1 hour. Add primary antibody in 5% bovine serum albumin (BSA) and incubate overnight in 4°C on a shaker. Wash the membrane with TBST for 5 minutes. Do this 3 times. (Tip: All washing and antibody incubation steps should be done on a shaker at room temperature to ensure even agitation). Add secondary antibody in 5% skim milk in TBST, and incubate for 1 hour. Wash the membrane with TBST for 5 minutes. Do this 3 times.

9.1.6 Detection

Detection and identification can be carried out by a number of methods like radiography, chemiluminescence, colorimetric and x-ray methods.

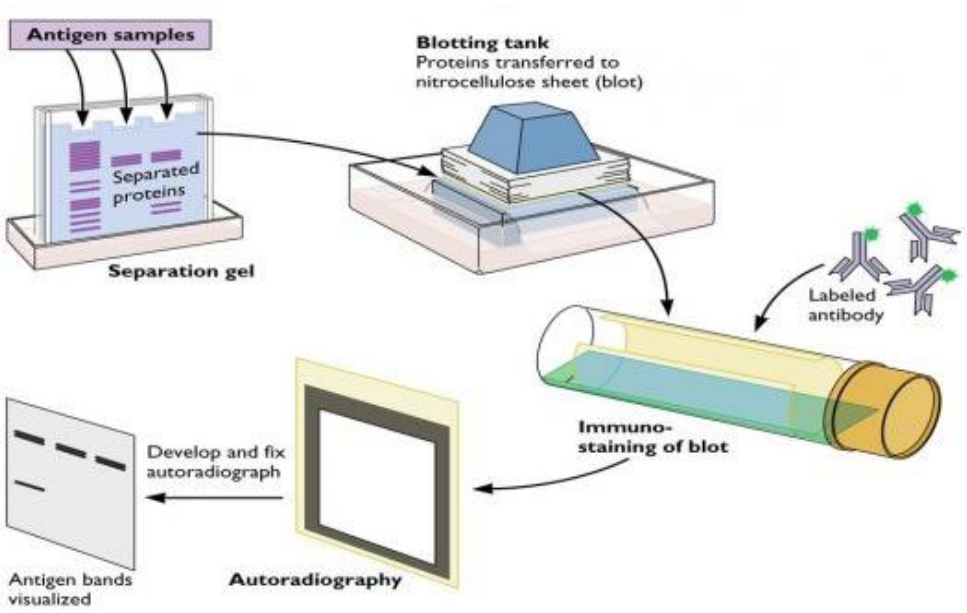

Figure 14 Western Blotting

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DEPARTMENT OF BIOTECHNOLOGY

UNIT – V – ANALYTICAL TECHNIQUES – SBB2104

1. Autoclave

An autoclave is a machine that provides a physical method of sterilization by killing bacteria, viruses, and even spores present in the material put inside of the vessel using steam under pressure.

- Autoclave sterilizes the materials by heating them up to a particular temperature for a specific period of time.
- The autoclave is also called a steam sterilizer that is commonly used in healthcare facilities and industries for various purposes.
- The autoclave is considered a more effective method of sterilization as it is based on moist heat sterilization.

1.1 Autoclave Parts

The simplest form of the autoclave is the pressure cooker types or laboratory bench autoclaves. The following is the detailed description of different components/ parts of an autoclave (Fig 1)

Figure 1 Autoclave Parts

a. Pressure Chamber

- The pressure chamber is the main component of a steam autoclave consisting of an inner chamber and an outer jacket.
- The inner chamber is made up of stainless steel or gunmetal, which is present inside the out chamber made up of an iron case.
- The autoclaves used in healthcare laboratories have an outer jacket that is filled with steam to reduce the time taken to reach the sterilization temperature.
- The inner chamber is the case where the materials to be sterilized are put.
- The size of the pressure chamber ranges from 100 L to 3000 L.

b. Lid/ Door

- The next important component of an autoclave is the lid or door of the autoclave.
- The purpose of the lid is to seal off the outside the atmosphere and create a sterilized condition on ht inside of the autoclave.
- The lid is made airtight via the screw clamps and asbestos washer.
- The lid consists of various other components like:

Pressure gauge

- A pressure gauge is present on the lid of the autoclave to indicate the pressure created in the autoclave during sterilization.
- The pressure gauge is essential as it assures the safety of the autoclave and the working condition of the operation.

Pressure releasing unit/ Whistle

- A whistle is present on the lid of the autoclave is the same as that of the pressure cooker.
- The whistle controls the pressure inside the chamber by releasing a certain amount of vapor by lifting itself.

Safety valve

- A safety valve is present on the lid of autoclave, which is crucial in cases where the autoclave fails to perform its action or the pressure inside increases uncontrollably.
- The valve has a thin layer of rubber that bursts itself to release the pressure and to avoid the danger of explosion.

c. Steam generator/ Electrical heater

- An electrical steam generator or boiler is present underneath the chamber that uses an electric heating system to heat the water and generate steam in the inner and the outer chamber.
- The level of water present in the inner chamber is vital as if the water is not sufficient; there are chances of the burning of the heating system.
- Similarly, if the water is more than necessary, it might interfere with the trays and other components present inside the chamber.

d. Vacuum generator (if applicable)

- In some types of autoclaves, a separate vacuum generator is present which pulls out the air from the inside of the chamber to create a vacuum inside the chamber.
- The presence of some air pockets inside the chamber might support the growth of different microorganisms. This is why the vacuum chamber is an important component of an autoclave.

e. Wastewater cooler

- Many autoclaves are provided with a system to cool the effluent before it enters the draining pipes.
- This system prevents any damage to the drainage pipe due to the boiling water being sent out of the autoclave.
- The autoclave works on the principle of moist heat sterilization where steam under pressure is used to sterilize the material present inside the chamber.
- The high pressure increases the boiling point of water and thus helps achieve a higher temperature for sterilization.
- Water usually boils at 100°C under normal atmospheric pressure (760 mm of Hg); however, the boiling point of water increases if the pressure is to be increased.
- Similarly, the high pressure also facilitates the rapid penetration of heat into deeper parts of the material, and moisture present in the steam causes the coagulation of proteins causing an irreversible loss of function and activity of microbes.
- This principle is employed in an autoclave where the water boils at 121°C at the pressure of 15 psi or 775 mm of Hg.

- When this steam comes in contact on the surface, it kills the microbes by giving off latent heat.
- The condensed liquid ensures the moist killing of the microbes.
- Once the sterilization phase is completed (which depends on the level of contamination of material inside), the pressure is released from the inside of the chamber through the whistle.
- The pressure inside the chamber is then restored back tot eh ambient pressure while the components inside remain hot for some time (Fig 2).

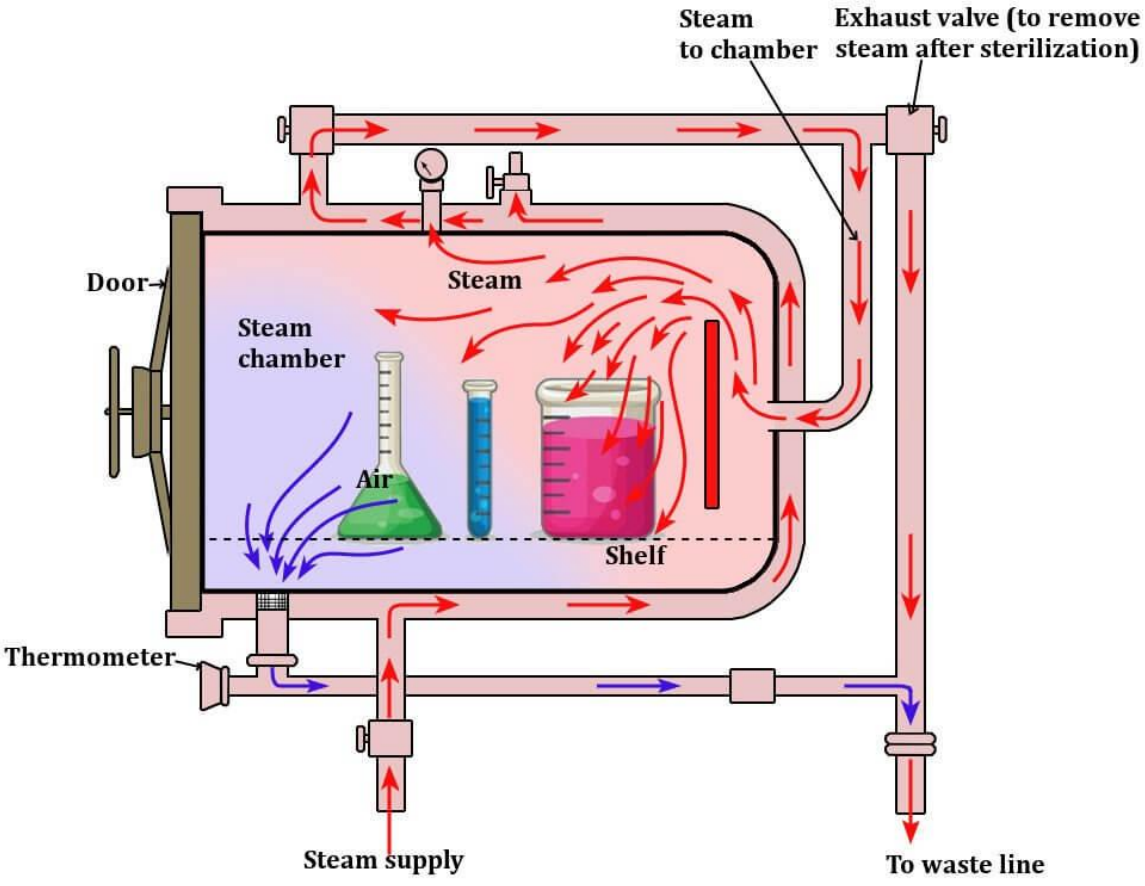

Figure 2 Autoclave Principle or Working.

1.2 Procedure

In general, an autoclave is run at a temperature of 121° C for at least 30 minutes by using saturated steam under at least 15 psi of pressure. The following are the steps to be followed while running an autoclave:

- Before beginning to use the autoclave, it should be checked for any items left from the previous cycle.
- A sufficient amount of water is then put inside the chamber.
- Now, the materials to be sterilized are placed inside the chamber.
- The lid is then closed, and the screws are tightened to ensure an airtight condition, and the electric heater is switched on.
- The safety valves are adjusted to maintain the required pressure in the chamber.
- Once the water inside the chamber boils, the air-water mixture is allowed to escape through the discharge tube to let all the air inside to be displaced. The complete displacement can be ensured once the water bubbles cease to come out from the pipe.
- The drainage pipe is then closed, and the steam inside is allowed to reach the desired levels (15 lbs in most cases).
- Once the pressure is reached, the whistle blows to remove excess pressure from the chamber.
- After the whistle, the autoclave is run for a holding period, which is 15 minutes in most cases.
- Now, the electric heater is switched off, and the autoclave is allowed to cool until the pressure gauge indicates the pressure inside has lowered down to that of the atmospheric pressure.
- The discharge pipe is then opened to allow the entry of air from the outside into the autoclave.
- Finally, the lid is opened, and the sterilized materials are taken out of the chamber.

1.3 Types of Autoclave

There are different types of autoclaves present in the market, some of which are (Fig 3)

1.3.1 Pressure cooker type/ Laboratory bench autoclaves (N-type)

- These, as domestic pressure cookers, are still in use in many parts of the world.
- The more modern type has a metal chamber with a secure metal lid that can be fastened and sealed with a rubber gasket.
- It has an air and steam discharge tap, pressure gauge, and safety valve. There is an electric immersion heater in the bottom of the chamber.

1.3.2 Gravity displacement type autoclave

- This is the common type of autoclave used in laboratories.
- In this type of autoclave, the steam is created inside the chamber via the heating unit, which then moves around the chamber for sterilization.
- This type of autoclave is comparatively cheaper than other types.

1.3.3 Positive pressure displacement type (B-type)

- In this type of autoclave, the steam is generated in a separate steam generator which is then passed into the autoclave.
- This autoclave is faster as the steam can be generated within seconds.
- This type of autoclave is an improvement over the gravity displacement type.

1.3.4 Negative pressure displacement type (S-type)

- This is another type of autoclave which contains both the steam generator as well as a vacuum generator.
- Here, the vacuum generator pulls out all the air from inside the autoclave while the steam generator creates steam.
- The steam is then passed into the autoclave.
- This is the most recommended type of autoclave as it is very accurate and achieves a high sterility assurance level.
- This is also the most expensive type of autoclave.

Figure 3 Types of Autoclave

1.4 Uses

Autoclaves are important devices to ensure the sterilization of materials containing water as they cannot be sterilized by dry heat sterilization. Besides, autoclaves are used for various other purposes.

- They are used to decontaminate specific biological waste and sterilize media, instruments, and labware.
- Regulated medical waste that might contain bacteria, viruses, and other biological materials are recommended to be inactivated by autoclaving before disposal.

- In medical labs, autoclaves are used to sterilize medical equipment, glassware, surgical equipment, and medical wastes.
- Similarly, autoclaves are used for the sterilization of culture media, autoclavable containers, plastic tubes, and pipette tips.

1.5 Precautions

Although autoclaves are pretty simple to use, there are certain rules of precautions to be followed while operating an autoclave. Some of the important precautions to be followed while running an autoclave are:

- Autoclaves should not be used to sterilize water-proof or water-resistant substances like oil or powders.
- The autoclave should not be overcrowded, and the materials should be loaded in a way that ensures sufficient penetration of articles by the steam.
- TheThe items to be autoclaved should always be placed in a secondary container.
- Only autoclavable bags are to be used to autoclave packaged waste.
- To ensure sufficient penetration, articles should be wrapped in something that allows penetration by steam, and materials like aluminum foils should not be used.
- The items placed inside the chamber should not touch the sides or top of the chamber.
- The wastes and clean items should be autoclaved separately.
- Attempts to open the lid when the autoclave is working should never be made.
- Liquid components should never be autoclaved in sealed containers.
- The liquid inside the containers should only be filled $2/3^{rd}$ of the total volume to prevent the spilling of the liquid.
- Plastic or polyethylene trays or containers should not be used as they might melt and damage the autoclave.
- Besides, never autoclave flammable, reactive, corrosive, toxic or radioactive materials, household bleach, or paraffin-embedded tissue.
- The paper should not be placed directly inside an autoclave as it is a combustible substance. It should be autoclaved in a waste bag on a bio bag setting to prevent fire.

2. Hot air oven

2.1Introduction of Hot air oven

Hot air oven is the most common method of sterilization in the laboratory working on dry heat. Sterilization is the process of removing or destroying all microorganisms including viruses, bacteria and their spores from the article or surface without destroying its quality and quantity. It is a physical method of sterilization due to dry heat. Factors influencing sterilization by heat are nature of heat i.e dry or moist, temperature and time, number of microorganism, nature of microorganisms, type of microorganism and presence of organic material. Mode of action: protein denaturation, oxidative destruction of essential cell constituents and toxic effects of elevated level of electrolytes. It works on the principle of conduction where heat is absorbed by the exterior surface of an item and then passed inward to the next layer. This method was introduced by Louis Pasture.

2.2 Principle of hot air oven

Electrical device that work on the principle of dry and hot air convection (that is circulation of heated air), conduction and radiation. Hot air convection process is of two types. a. Gravity convection process: Heated air expands and possess less density than cooled air which rises up and displaces the cooler air (the cooler air descends). It produces inconsistent temperature within the chamber thus has a slow turn over. b. Mechanical convection: Use of fitted blower or fan that actively forces heated air throughout all areas of the chamber. This dry heat destroys bacterial endotoxins (or pyrogens) which are difficult to eliminate by other means. This property makes it applicable for sterilizing glass bottles which are to be filled aseptically. Dry heat kills by oxidation, protein denaturation and toxic effects of elevated levels of electrolytes and it is more efficient.

2.3 Structure of Hot air oven and Functions

It consists of the following parts (Fig 4)

- An insulated chamber surrounded by an outer case containing electric heaters
- A fan

- Shelves
- Thermostat
- Door locking controls

Metallic cabinet with heating filament and fan fixed in the walls. Thermostat, temperature control, double walled :(inner being poor conductor and outer being metallic and air filled space in between the layers) insulation keeps the heat in and conserve energy. Electrically heated, and provided with a fan or a blower to ensure rapid and uniform. Heating Mechanism:- Killing effect of dry heat on microorganism is due to i) destructive oxidation of essential cell constituents, ii) protein denaturation and iii) toxic effect of elevated level of electrolytes.

2.4 Uses of Hot air oven

Sterilization of articles that withstand high temperature and not get burned e.g. Glass-wares, powders, forceps, scissors, scalpels, glass syringes, pharmaceutical products like liquid paraffin, fats, grease and dusting powder etc.

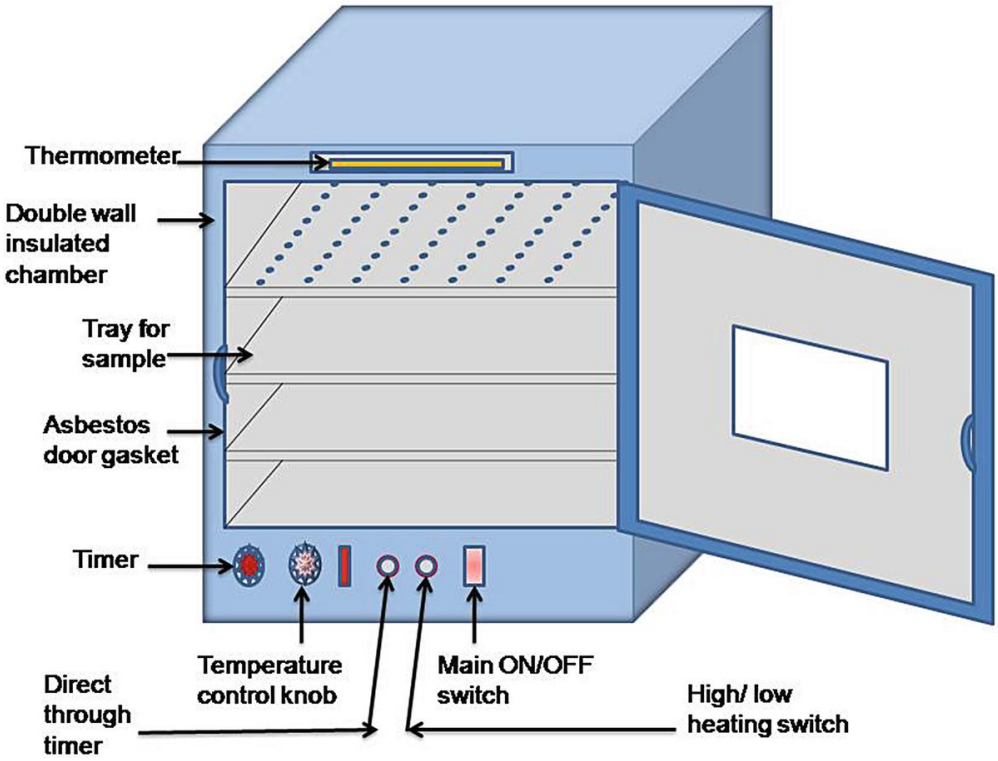

Figure 4 Hot Air Oven

2.5 Handling procedure of Hot air oven

Wrap the articles or enclose in container of cardboard, aluminium or paper. Mouths of flasks, test tube sand both ends of pipettes must be plugged with cotton wool. Articles to be sterilized such as Petri plates and pipettes may be arranged inside metal canisters and then placed. Place the articles at sufficient distances so as to allow free circulation of air in between them and to ensure uninterrupted air flow. Shut the door and switch on the hot air oven. When the thermometer shows that the oven air has reached sterilizing temperature, heating is continued for the required period of time (e.g. 160°C for an hour). Allow the temperature to fall up to 40°C (approximately 2 hours), prior to removal of sterilized materials; which prevents breakage of glassware.

2.6 Advantages

1. Do not require water and there is not much pressure build up within the oven making safer to work.
2. Smaller than autoclave but can still be as effective.
3. Higher temperature can be reached compared to other means.
4. This treatment kills the bacterial endotoxin, not all treatments can do this.
5. Effective method of sterilization of heat stable articles only method of sterilizing oils and powders.
6. Protective of sharps or instruments with a cutting edge (fewer problems with dulling of cutting edges).
7. It does not leave any chemical residue.
8. It is nontoxic and does not harm the environment.

2.7 Disadvantage

1. Some organisms like prion may not be killed or inactivated.
2. Plastic wares or heat sensitive materials can’t be sterilized.
3. Glasses may become smoky due to high sterilization temperatures: Temperature holding period is at 160°C for 1 hour, 170°C 30 minutes where as at 180°C 20 minutes.
4. Dry heat penetrates materials slowly and unevenly and thus time consuming method because of slow rate of heat penetration and microbial killing.

5. It requires a continuous source of electricity.

2.8 Precautions

1. Sterilize dry substances.
2. It shouldn't be overloaded.
3. Rubber goods, fabrics, any inflammable or volatile substances should not be put inside the oven.
4. Oven is allowed to cool gradually for about 2 hours or up to 40°C before the door is opened.

3. Laminar flow hood

A Laminar flow hood/cabinet is an enclosed workstation that is used to create a contamination-free work environment through filters to capture all the particles entering the cabinet.

- These cabinets are designed to protect the work from the environment and are most useful for the aseptic distribution of specific media and plate pouring.
- Laminar flow cabinets are similar to biosafety cabinets with the only difference being that in laminar flow cabinets the effluent air is drawn into the face of the user.
- In a biosafety cabinet, both the sample and user are protected while in the laminar flow cabinet, only the sample is protected and not the user.

3.1 Parts

A laminar flow cabinet consists of the following parts (Fig 5)

a. Cabinet
    - The cabinet is made up of stainless steel with less or no gaps or joints preventing the collection of spores.
    - The cabinet provides insulation to the inner environment created inside the laminar flow and protects it from the outside environment.
    - The front of the cabinet is provided with a glass shield which in some laminar cabinets opens entirely or in some has two openings for the user's hands to enter the cabinet.

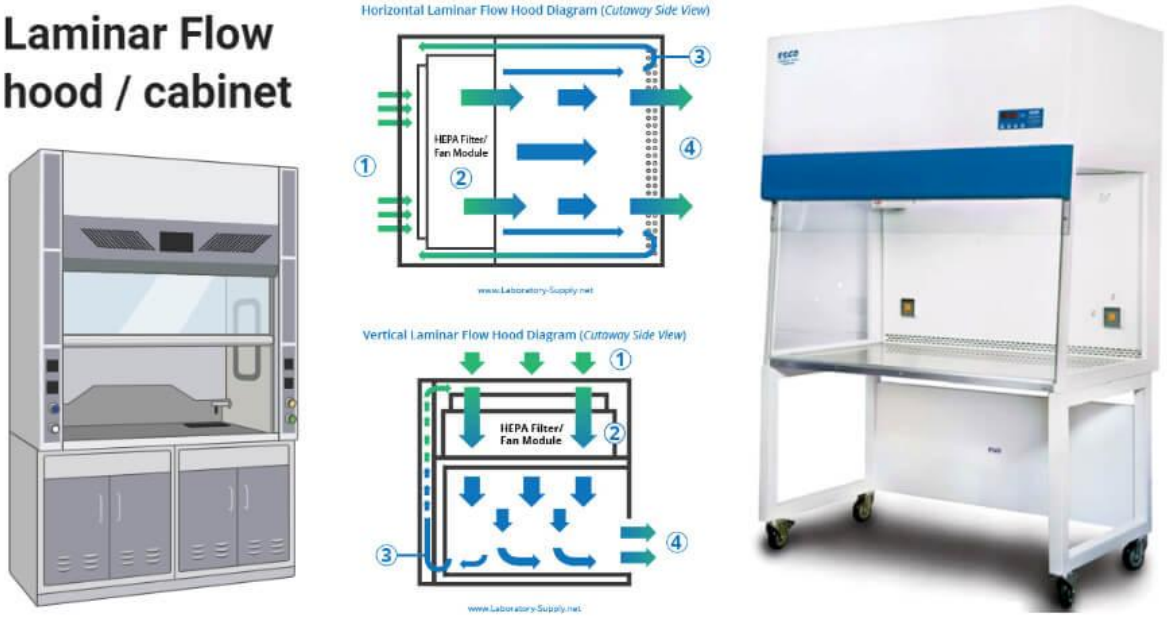

Figure 5 Laminar Flow Hood

b. Working station
    - A flat working station is present inside the cabinet for all the processes to be taken place.
    - Culture plates, burner and loops are all placed on the working station where the operation takes place.
    - The worktop is also made up of stainless steel to prevent rusting.

c. Filter pad/ Pre-filter
    - A filter pad is present on the top of the cabinet through which the air passes into the cabinet.
    - The filter pad traps dust particles and some microbes from entering the working environment within the cabinet.

d. Fan/ Blower
    - A fan is present below the filter pad that sucks in the air and moves it around in the cabinet.
    - The fan also allows the movement of air towards the HEPA filter sp that the remaining microbes become trapped while passing through the filter.

e. UV lamp
    - Some laminar flow hoods might have a UV germicidal lamp that sterilizes the interior of the cabinet and contents before the operation.
    - The UV lamp is to be turned on 15 minutes before the operation to prevent the exposure of UV to the body surface of the user.

f. Fluorescent lamp
    - Florescent light is placed inside the cabinet to provide proper light during the operation.

g. HEPA filter
    - The High-efficiency particulate air filter is present within the cabinet that makes the environment more sterile for the operation.
    - The pre-filtered air passes through the filter which traps fungi, bacteria and other dust particles.
    - The filter ensures a sterile condition inside the cabinet, thus reducing the chances of contamination.

3.2 Working

- The principle of laminar flow cabinet is based on the laminar flow of air through the cabinet.
- The device works by the use of inwards flow of air through one or more HEPA filters to create a particulate-free environment.
- The air is taken through a filtration system and then exhausted across the work surface as a part of the laminar flow of the air.
- The air first passes through the filter pad or pre-filter that allows a streamline flow of air into the cabinet.
- Next, the blower or fan directs the air towards the HEPA filters.
- The HEPA filters then trap the bacteria, fungi and other particulate materials so that the air moving out of it is particulate-free air.
- Some of the effluent air then passes through perforation present at the bottom rear end of the cabinet, but most of it passes over the working bench while coming out of the cabinet towards the face of the operator.

- The laminar flow hood is enclosed on the sides, and constant positive air pressure is maintained to prevent the intrusion of contaminated external air into the cabinet.

3.3 Procedure

The procedure to be followed while operating a laminar flow cabinet is given below:

- Before running the laminar flow cabinet, the cabinet should be checked to ensure that nothing susceptible to UV rays is present inside the cabinet.
- The glass shield of the hood is then closed, and the UV light is switched on. The UV light should be kept on for about 15 minutes to ensure the surface sterilization of the working bench.
- The UV light is then switched off, and a time period of around 10 minutes is spared before the airflow is switched on.
- About 5 minutes before the operation begins, the airflow is switched on.
- The glass shield is then opened, and the fluorescent light is also switched on during the operation.
- To ensure more protection, the working bench of the cabinet can be sterilized with other disinfectants like 70% alcohol.
- Once the work is completed, the airflow and florescent lamp both are closed and the glass shield is also closed.

3.4 Types

Depending on the direction of movement of air, laminar flow cabinets are divided into two types (Fig 6)

3.4.1 Vertical laminar flow cabinet

- In the vertical flow cabinets, the air moves from the top of the cabinet directly towards the bottom of the cabinet.
- A vertical airflow working bench does not require as much depth and floor space as a horizontal airflow hood which makes it more manageable and decreases the chances of airflow obstruction or movement of contaminated air downstream.

- The vertical laminar flow cabinet is also considered safer as it doesn't blow the air directly towards the person carrying out the experiments.

3.4.2 Horizontal laminar flow cabinet

- In the horizontal laminar flow cabinets, the surrounding air comes from behind the working bench, which is then projected by the blower towards the HEPA filters.
- The filtered air is then exhausted in a horizontal direction to the workplace environment.
- One advantage of this cabinet is that airflow parallel to the workplace cleanses the environment with a constant velocity.
- The elluent air directly hits the operator, which might reduce the security level of this type of laminar flow cabinets.

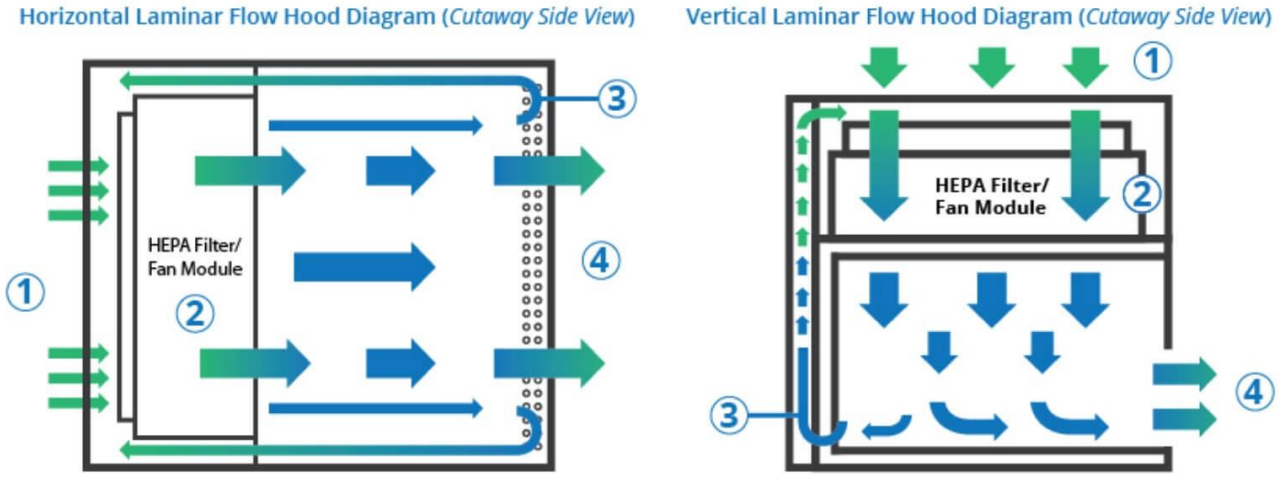

Figure 6 Types of Laminar flow hood

3.5 Uses

The following are some common uses of a laminar flow cabinet in the laboratory:

- Laminar flow cabinets are used in laboratories for contamination sensitive processes like plant tissue culture.
- Other laboratories processes like media plate preparation and culture of organisms can be performed inside the cabinet.

- Operations of particle sensitive electronic devices are performed inside the cabinet.
- In the pharmaceutical industries, drug preparation techniques are also performed inside the cabinet to ensure a particulate-free environment during the operations.
- Laminar flow cabinets can be made tailor-made for some specialized works and can also be used for general lab techniques in the microbiological as well as the industrial sectors.

3.6 Precautions

While operating the laminar airflow, the following things should be considered:

- The laminar flow cabinet should be sterilized with the UV light before and after the operation.
- The UV light and airflow should not be used at the same time.
- No operations should be carried out when the UV light is switched on.
- The operator should be dressed in lab coats and long gloves.
- The working bench, glass shield, and other components present inside the cabinet should be sterilized before and after the completion of work.

4. Incubator

Incubator, in microbiology, is an insulated and enclosed device that provides an optimal condition of temperature, humidity, and other environmental conditions required for the growth of organisms.

An incubator is a piece of vital laboratory equipment necessary for the cultivation of microorganisms under artificial conditions.

An incubator can be used for the cultivation of both unicellular and multicellular organisms.

4.1 Parts of Incubator

A microbial incubator is made up of various units, some of which are (Fig 7)

a. Cabinet

- The cabinet is the main body of the incubator consisting of the double-walled cuboidal enclosure with a capacity ranging from 20 to 800L.

- The outer wall is made up of stainless steel sheets while the inner wall is made up of aluminum.
- The space between the two walls is filled with glass wool to provide insulation to the incubator.

.Figure 7 Incubator

- The insulation prevents heat loss and in turn, reduces the electric consumption, thereby ensuring the smooth working of the device.
- The inner wall of the incubator is provided with inward projections that support the shelves present inside the incubator.

b. Door

- A door is present in all incubators to close the insulated cabinet.
- The door also has insulation of its own. It is also provided with a glass that enables the visualization of the interior of the incubator during incubation without disturbing the interior environment.
- A handle is present on the outside of the door to help with the maneuvering of the door.

c. Control Panel

- On the outer wall of the incubator is a control panel with all the switches and indicators that allows the parameters of the incubator to be controlled.
- The control panel also has a witch to control the thermostat of the device.

d. Thermostat

- A thermostat is used to set the desired temperature of the incubator.
- After the desired temperature is reached, the thermostat automatically maintains the incubator at that temperature until the temperature is changed again.

e. Perforated shelves

- Bound to the inner wall are some perforated shelves onto which the plates with the culture media are placed.
- The perforations on the shelves allow the movement of hot air throughout the inside of the incubator.
- In some incubators, the shelves are removable, which allows the shelves to be cleaned properly.

f. Asbestos door gasket

- The asbestos door gasket provides an almost airtight seal between the door and the cabinet.
- This seal prevents the outside air from entering the cabinet and thus, creating an isolated hot environment inside the cabinet without being interrupted by the external environment.

g. L-shaped thermometer

- A thermometer is placed on the top part of the outer wall of the incubator.

- One end of the thermometer provided with gradations remains outside of the incubator so that temperature can be read easily.
- The next end with the mercury bulb is protruded slightly into the chamber of the incubator.

h. HEPA filters

- Some advanced incubators are also provided with HEPA filters to lower the possible contamination created due to airflow.
- AN air-pump with filters creates a closed-loop system so that the air flowing inside the incubator generates less contamination.

i. Humidity and gas control

- The CO_2 incubators are provided with a reservoir underneath the chamber that contains water.
- The water is vapourised to maintain the relative humidity inside the chamber.
- Similarly, these incubators are also provided with gas chambers to give the desired concentration of CO_2 inside the incubator.

4.2 Principle

- An incubator is based on the principle that microorganisms require a particular set of parameters for their growth and development.
- All incubators are based on the concept that when organisms are provided with the optimal condition of temperature, humidity, oxygen, and carbon dioxide levels, they grow and divide to form more organisms.
- In an incubator, the thermostat maintains a constant temperature that can be read from the outside via the thermometer.
- The temperature is maintained by utilizing the heating and no-heating cycles.
- During the heating cycle, the thermostat heats the incubator, and during the no-heating period, the heating is stopped, and the incubator is cooled by radiating heat to the surrounding.
- Insulation from the outside creates an isolated condition inside the cabinet, which allows the microbes to grow effectively.

- Similarly, other parameters like humidity and airflow are also maintained through different mechanisms that create an environment similar to the natural environment of the organisms.
- Similarly, they are provided with adjustments for maintaining the concentration of CO2 to balance the pH and humidity required for the growth of the organisms.
- Variation of the incubator like a shaking incubator is also available, which allows for the continuous movement of the culture required for cell aeration and solubility studies.

4.3 Procedure

Once the cultures of organisms are created, the culture plates are to be placed inside an incubator at the desired temperature and required period of time. In most clinical laboratories, the usual temperature to be maintained is 35–37°C for bacteria.

The following are the steps to be followed while running an incubator:

- Before using the incubator, it should be made sure that no remaining items are present in the incubator from the previous cycles. However, in some cases, if the same incubator is being used for multiple organisms, and they require the same set of parameters, they can be placed together in the same incubator.
- The door of the incubator is then kept closed, and the incubator is switched on. The incubator has to be heated up to the desired temperature of the growth of the particular organism. The thermometer can be used to see if the temperature has reached.
- In the meantime, if the organism requires a particular concentration of CO_2 or a specific humidity, those parameters should also be set in the incubator.
- Once all the parameters are met, the petri dish cultures are placed on the perforated shelves upside down, i.e., media uppermost. This is necessary because if the plates are incubated normally, condensation collects on the surface of the medium and prevents the formation of isolated colonies.
- If it is necessary to incubate Petri dish cultures for several days, the plates are sealed with adhesive tapes or are placed in plastic bags or plastic food containers.

- Now, the door is locked, and the plates are kept inside for the required time before taking them out.

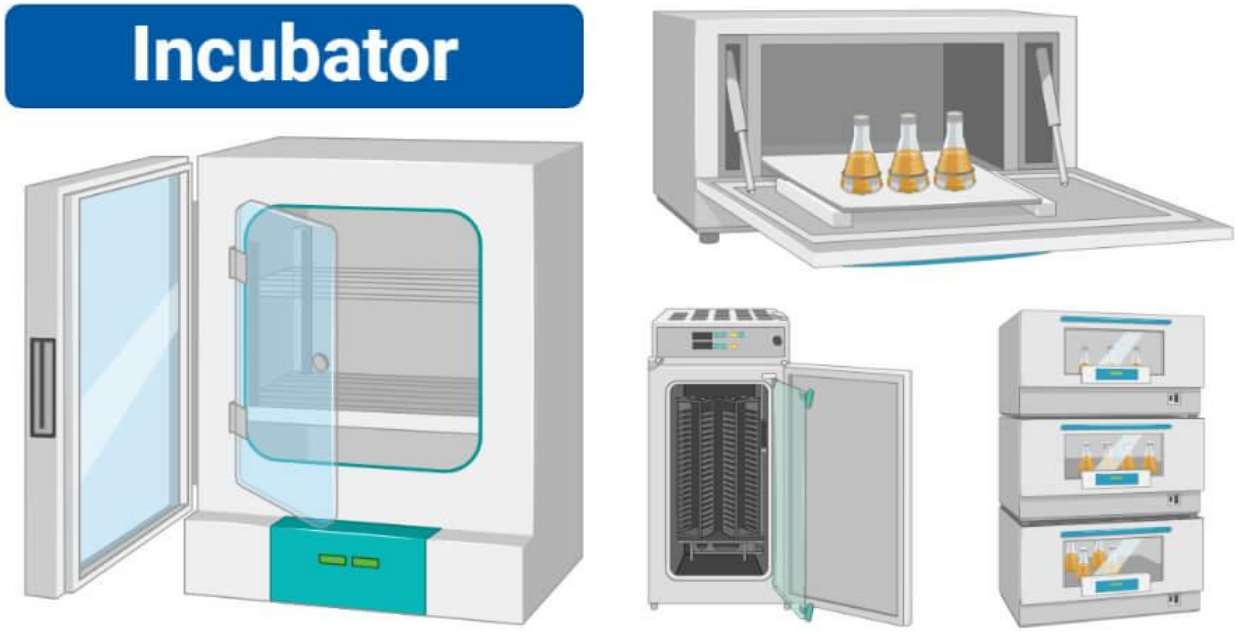

Figure 8 Types of incubator

4.4 Types

On the basis of the presence of a particular parameter or the purpose of the incubator, incubators are divided into the following types (Fig 8)

4.4.1 Benchtop incubators

- This is the most common type of incubator used in most of the laboratories.
- These incubators are the basic types of incubators with temperature control and insulation.

4.4.2 CO2 incubators

- CO2 incubators are the special kinds of incubators that are provided with automatic control of CO2 and humidity.
- This type of incubator is used for the growth of the cultivation of different bacteria requiring 5-10% of CO2 concentration.
- For humidity control, water is kept underneath the cabinet of the incubator.

4.4.3 Cooled incubators

- For incubation at temperatures below the ambient, incubators are fitted with modified refrigeration systems with heating and cooling controls.
- This type of incubator is called the cooling incubator.
- In the cooling incubator, the heating and cooling controls should be appropriately balanced.

4.4.4 Shaker incubator

- A thermostatically controlled shaker incubator is another piece of apparatus used to cultivate microorganisms.
- Its advantage is that it provides a rapid and uniform transfer of heat to the culture vessel, and its agitation provides increased aeration, resulting in acceleration of growth.
- This incubator, however, can only be used for broth or liquid culture media.

4.4.5 Portable incubator

- Portable incubators are smaller in size and are used in fieldwork, e.g. environmental microbiology and water examination.

4.5 Uses

Incubators have a wide range of applications in various areas including cell culture, pharmaceutical studies, hematological studies, and biochemical studies.

Some of the uses of incubators are given below:

- Incubators are used to grow microbial culture or cell cultures.
- Incubators can also be used to maintain the culture of organisms to be used later.
- Some incubators are used to increase the growth rate of organisms, having a prolonged growth rate in the natural environment.
- Specific incubators are used for the reproduction of microbial colonies and subsequent determination of biochemical oxygen demand.
- These are also used for breeding of insects and hatching of eggs in zoology.
- Incubators also provide a controlled condition for sample storage before they can be processed in the laboratories.

4.6 Precautions

The following precautions are to be followed while running an incubator:

- As microorganisms are susceptible to temperature change, the fluctuations in temperature of the cabinet by repeatedly opening the door should be avoided.
- The required parameters growth of the organism should be met before the culture plates are placed inside the cabinet.
- The plates should be placed upside down with the lid at the bottom to prevent the condensation of water on to the media.
- The inside of the incubators should be cleaned regularly to prevent the organisms from settling on the shelves or the corners of the incubator.
- While running the incubator for an extended period of time, sterile water should be placed underneath the shelves to prevent the culture media from drying out.

5.Biosensor

A biosensor is a biological detection system consists of a biological component combined with a transducer to perform measurement of a biochemical quantity. The development of biosensors started with the invention of enzyme electrodes by Leland C. Clark in 1962.

A typical biosensor includes a bioelement such as an enzyme, antibody, or a cell receptor, and a sensing element or a transducer. These two elements are combined together through a number of methods such as covalent bonding, matrix entrapment, physical adsorption and membrane entrapment.

5.1 Working Principle of Biosensors

Biosensors are operated based on the principle of signal transduction. These components include a bio-recognition element, a biotransducer and an electronic system composed of a display, processor and amplifier.

The bio-recognition element, essentially a bioreceptor, is allowed to interact with a specific analyte. The transducer measures this interaction and outputs a signal. The intensity of the signal output is proportional to the concentration of the analyte. The signal is then amplified and processed by the electronic system (Fig 9).

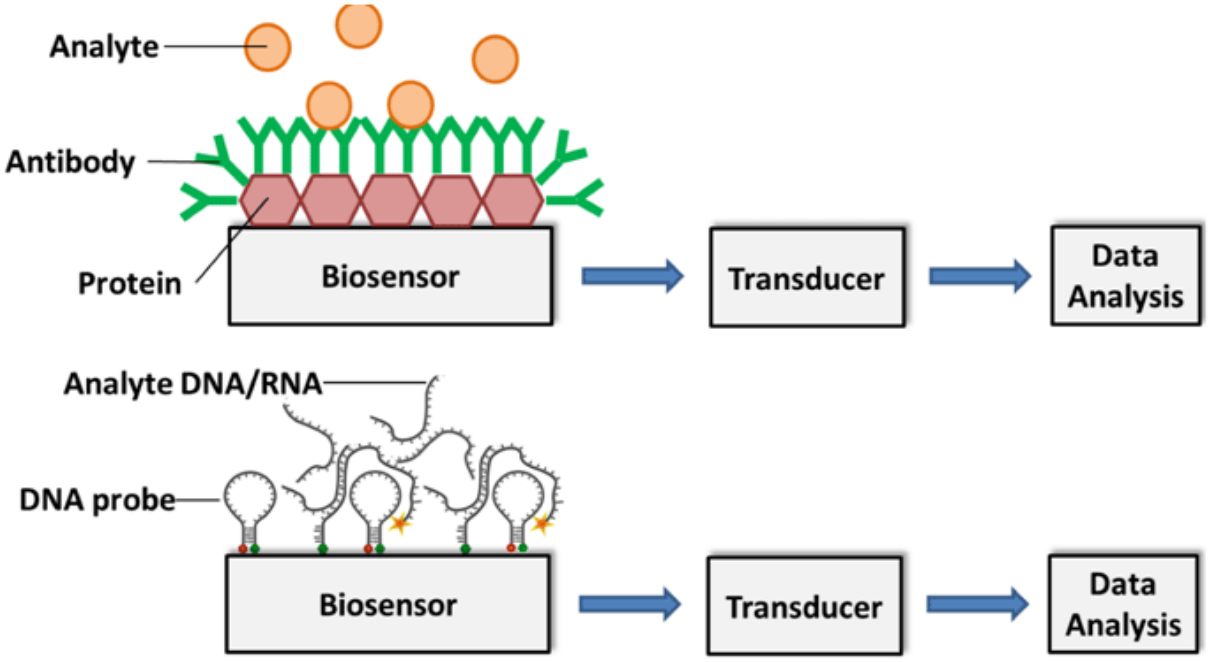

Figure 9 Biosensor

5.2 Types of Biosensors

Depending on the mechanism of transduction, biosensors are classified as follows:

- Resonant Biosensors – These sensors employ an acoustic wave transducer combined with the bioreceptor. Interaction between the analyte and bioreceptor forces a change in the mass of the bioreceptor. As a result, the resonant frequency of the acoustic transducer also changes, which is then evaluated and this value can then be interpreted by the end-user.
- Optical Detection Biosensors – These type of sensors include a silicon wafer to which protein molecules are attached through covalent bonds. The wafer is subjected to UV light which makes the antibodies inactive. The wafer is then diced and placed in the

analyte, which encourages formation of diffusion grating that generates a signal which can be measured and amplified.

- Thermal Detection Biosensors – In these sensors, the immobilized enzymes are coupled with temperature detectors. A heat reaction is initiated when the analyte is made to interact with the enzyme. The concentration of the analyte can thus be evaluated with respect to the measurement of a heat reaction.
- Ion Sensitive Biosensors – These sensors work on the principle that the interaction of ions with a semiconductor changes the electric potential of the semiconductor surface. The potential changes can then be measured to evaluate the desired parameter.
- Electrochemical Biosensors – The principle behind the operation of these sensors involves the generation of ions by various chemical events that change the electrical properties of the analyte solution. The concentration of the analyte is then measured with respect to this change.

5.3 Advantages of Biosensors

- Rapid and continuous measurement
- High specificity
- Very less usage of reagents required for calibration
- Fast response time
- Ability to measure non-polar molecules that cannot be estimated by other conventional devices.

5.4 Applications of Biosensors

Some of the major applications of biosensors are listed below:

- Monitoring glucose level in diabetes patients
- Food analysis
- Environmental applications
- Protein engineering and drug discovery applications
- Wastewater treatment.