SPECTROPHOTOMETER

A spectrophotometer measures either the amount of light reflected from a sample object or the amount of light that is absorbed by the sample object. The instrument operates by passing a beam of light through a sample and measuring the intensity of light reaching a detector.

A molecule or substance that absorbs light is called a chromophore. Chromophores exhibit unique absorption spectra and can be defined by a wavelength of maximum absorption, or λmax, of a broad absorbtion band due to the vibrational levels. The absorption spectra can consist of several absorption maxima of various amplitudes. A large number of biological molecules absorb light in the visible and ultraviolet (UV) range.

The net affect of absorption is that the intensity of the light decreases as it passes through a solution containing a chromophore. The amount of light absorbed depends on the nature of the chromophore, the concentration of the chromophore, the thickness of the sample, and the conditions (eg., pH, solvent,etc.) under which absorption is measured.

Absorption is governed by the Beer-Lambert Law:
Beer's Law: According to Beer's law when monochromatic light passes through the colored solution, the amount of light transmitted decreases exponentially with increase in concentration of the colored substance.

Spectrophotometers produce monochromatic light and then accurately measure the light intensity. The major components of a spectrophotometer are the light source, a monochromator, sample holder, a light detector (phototube), and a meter. In most instruments a tungsten lamp is used for the visible range and either high pressure H_2or D_2 lamps are used for UV range. Monochromatic light is generated by either1) a movable prism, 2) a diffraction gradient, or 3) filters. Monochromatic light is projected through the sample and then measured by a photomultiplier tube. A photomultiplier tube converts the energy of the light photons into electrons. The voltage resulting from

these electrons is measured by a meter and converted to an absorbance value. The I_o (initial intensity) is determined by calibrating the instrument with a 'buffer blank'. The relative difference in the light intensity between the blank and the sample is then expressed as the absorbance (A). Spectrophotometers often include accessories such as chart recorders or microprocessors for data analysis.

The signal changes as the amount of light absorbed by the liquid changes. If development of color is linked to the concentration of a substance in solution then that concentration can be measured by determining the extent of absorption of light at the appropriate wavelength. For example hemoglobin appears red because the hemoglobin absorbs blue and green light rays much more effectively than red. The degree of absorbance of blue or green light is proportional to the concentration of hemoglobin.

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There are two kinds of spectrophotometers: single beam and double beam. A double beam spectrophotometer compares the light intensity between two light paths. One path containing a reference sample. The other the test sample. A single beam spectrophotometer measures the relative light intensity of the beam before and after a test sample is inserted. A double beam machine makes comparison readings easier and more stable. But a single beam machine can have measure a wider range of light frequencies. Single beam machines have simple optical systems and are more compact.

<u>Applications of spectrophotometry are:</u>

Determining the concentration of substances in solution is the most common use of the spectrophotometer. Exact concentrations can be determined in cases where ε (ε is the molar extinction coefficient) are known and the measurement is carried out under the prescribed conditions. The substance being measured does not necessarily need to absorb radiation if it can scatter radiation. For example, measuring the A_{600} (A is absorbance) is a quick and easy way to monitor bacterial growth and determine the number of bacteria in cultures. In addition, since compounds exhibit unique absorption profiles, spectrophotometry can also be used to identify unknown compounds.

Spectrophotometry is also a convenient method to measure enzyme activity in cases where the substrate and the product exhibit different λmax. Either the disappearance of substrate or the appearance of product over time is measured.

Advantages of spectrophotometry are:

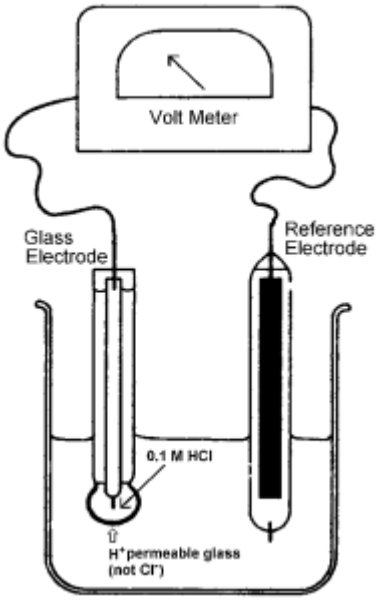
1) It is often non-destructive (i.e., can measure and recover sample),
2) It is selective (often a particular compound in a mixture can be measured without separation techniques),
3) It has a short time interval of measurement (10-14 seconds).

PH METER

A pH meter is an electronic device used for measuring the pH of a liquid. It is composed of 1) a reference electrode, 2) a glass electrode, and 3) a voltmeter. (The two electrodes are usually combined into a single electrode.) It measures the electro-chemical potential between a known liquid inside the glass electrode (membrane) and an unknown liquid outside. Because the thin glass bulb allows mainly the agile and small hydrogen ions to interact with the glass, the glass electrode measures the electro-chemical potential of hydrogen ions or the *potential of hydrogen*.

The calomel reference electrode consists of a glass tube with a potassium chloride (KCl) electrolyte which is in intimate contact with a mercuric chloride element at the end of a KCL element. It is a fragile construction, joined by a liquid junction tip made of porous ceramic or similar material.

The glass electrode is made of a special glass that is permeable to H+, but not other cations or anions. The glass electrode consists of a sturdy glass tube with a thin glass bulb welded to it. Inside is a known solution of potassium chloride (KCl) buffered at a pH of 7.0. A silver electrode with a silver chloride tip makes contact with the inside solution.


Volt Meter
Glass Electrode
Reference Electrode
0.1 M HCl
H+ permeable glass (not Cl-)

CHROMATOGRAPHY

Chromatography, firstly introduced by the Russian botanist Micharl Iswett is a method for separating the components of a mixture by differential distribution of the components between a stationary phase and mobile (moving) phase. It is now the most extensive technique of separation and purification of coloured/colourless organic compounds.

Separation of two sample components in chromatography is based on their different distribution between two non-miscible phases. The one, the stationary phase, a liquid or solid, is fixed in the system. The other, the mobile phase, a fluid, is flowing through the chromatographic system. In gas chromatography the mobile phase is a gas, in liquid chromatography it is a liquid.

Chromatography is used to:

Analyze – examine a mixture, its components, and their relations to one another

Identify – determine the identity of a mixture or components based on known components

Purify – separate components in order to isolate one of interest for further study

Quantify – determine the amount of the a mixture and/or the components present in the sample

Real-life examples of uses for chromatography:

Pharmaceutical Company – determine amount of each chemical found in new product

Hospital – detect blood or alcohol levels in a patient's blood stream

Law Enforcement – to compare a sample found at a crime scene to samples from suspects

Environmental Agency – determine the level of pollutants in the water supply

Manufacturing Plant – to purify a chemical needed to make a product.

Terminology:

Differential – showing a difference, distinctive

Affinity – natural attraction or force between things

Mobile Medium – gas or liquid that carries the components (mobile phase)

Stationary Medium – the part of the apparatus that does not move with the sample (stationary phase)

Rf value-It is measure of the rate of flow of a particular substance in the mixture during chromatographic separation. It is the ratio of the distance travelled by the solute to the distance travelled by the solvent.

Types of Chromatography

Based on the nature of stationary phase:

A)Adsorption chromatography-stationary phase is a finely divided adsorbent such as alumina or silica gel and the mobile phase can be a gas or more commonly a liquid.

B)Partition chromatography- stationary phase is a liquid. The liquid may be immobilized on a solid support.

A) Adsorption chromatography

Liquid solid chromatography-(mobile phase is a liquid-Column Chromatography, Thin-layer Chromatography, Iron exchange chromatography), and Gas Solid Chromatography

B) Partition Chromatography

Liquid-liquid chromatography (paper chromatography) and Gas liquid or gas chromatography.

Column Chromatography

Stationary phase is confined to a glass or plastic tube and the mobile phase is allowed to flow through the solid adsorbent. With the proper solvents, packing conditions, some components in the sample will travel the column more slowly than others resulting in the desired separation. The most common stationary phase for column

chromatography is silica gel, followed by alumina. A pump may be needed to control the flow rate of buffers through the column. However, gravity can also be used.

Thin-Layer Chromatography

Stationary phase-a thin layer of adsorbent like silica gel coated over a glass or plastic sheet to form a thin layer. Compared to paper-faster runs, better separations, the choice between different adsorbents.

Gas Chromatography / Gas liquid Chromatography

For the separation of volatile compounds of fatty acids, hydrocarbons, sterols and other lipids. The stationary phase is adhered to the inside of a small diameter glass tube (a capillary column) or a solid matrix inside a larger metal tube (a packed column) it is widely used in analytical chemistry. Solid stationary phase (often a liquid silicone-based material) and a mobile gas (most often helium).

Paper Chromatography

Paper Chromatography is one of the most common types of chromatography in which filter paper serves as a support for immobile liquid phase. The true stationary phase is the very thin film of liquid usually water adhering to the surface of the fibers. It uses a strip of paper and capillary action is used pull the solvents up through the paper to separate the solutes. A drop of the mixture is put on one end of a long strip of the Whatman filter paper. The filter paper is hung in a manner that the end with the drop of the mixture dips into the solvent mixture kept in the tray/jar. As the liquid is drawn up on the paper, different substances in the mixture begin to separate according to their molecular weight, size and solubility in the solvent and rise up to different heights on the paper. It is then analysed by using certain chemicals for further investigation. The components of the mixture move up the paper with the solvent at different rates, Rf, due to their differing interactions with the stationary and mobile phases.

$$Rf = \frac{\text{Distance the solute moves}}{\text{Distance the solvent front moves}}$$

High Performance (Pressure) Liquid Chromatography

HPLC is not a distinct chromatographic technique, but an advancement of technology. The flow rates in conventional chromatography is limited because of the compression

of the support matrices used in the columns. These low flow rates result in diffusion and loss of resolution. New resins that can withstand packing and high flow rates allow for higher resolution have been developed. This allows for separations to be carried out under higher pressures (i.e., high flow rates) resulting in increased resolution. All of the same types of chromatographic media are available for HPLC as in conventional chromatography. HPLC is more widely used for the separation of small molecules but can be applied to the separation of proteins in some applications. Fast protein liquid chromatography (FPLC) is a similar concept as HPLC, but specificially designed for protein separations. FPLC also uses special columns and pumps to achieve high flow rates and therefore faster separations. In general the flow rates obtained with FPLC are not as great as those achieved with HPLC.

Ion exchange chromatography (IEC)

Ion exchange chromatography (IEC) is a specific type of adsorption chromatography based upon charge-charge interactions. The stationary phase consists of fixed charges on a solid support. The fixed charges on the stationary phase can be either negative or positive and are respectively referred to as cation exchange or anion exchange chromatography. Counter ions will interact with the fixed charged groups and can 'exchange' with solute molecules. In other words, substances to be separated will replace the counter ions associated with the chromatography medium and stably bind to the exchanger via electrostactic interactions. Conditions in which some solute molecules are electrostatically bound to the exchanger and other solute molecules are not bound can be used to separate solutes.

ELECTROPHORESIS

Electrophoresis is a technique used to separate and sometimes purify macromolecules - especially proteins and nucleic acids - that differ in size, charge or conformation. When charged molecules are placed in an electric field, they migrate toward either the positive or negative pole according to their charge.

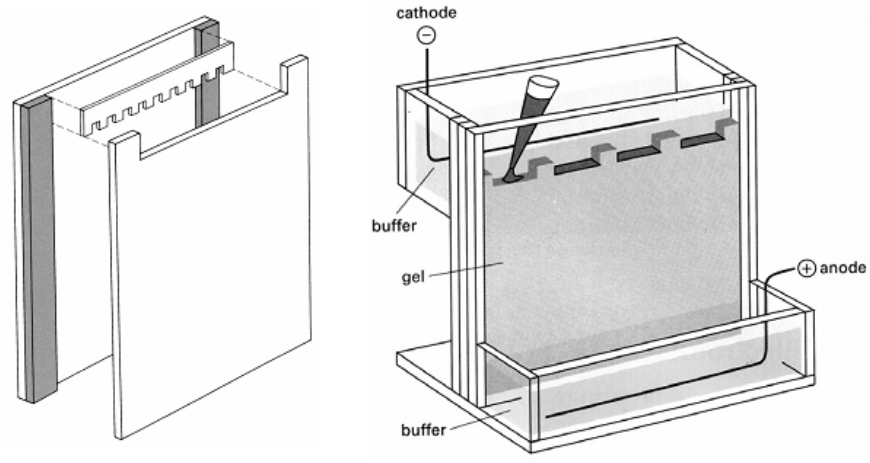
Proteins and nucleic acids are electrophoresed within a matrix or "gel". Most commonly, the gel is cast in the shape of a thin slab, with wells for loading the sample.

The gel is immersed within an electrophoresis buffer that provides ions to carry a current and some type of buffer to maintain the pH at a relatively constant value. The gel itself is composed of either agarose or polyacrylamide.

Agarose gels have a large range of separation, but relatively low resolving power. Polyacrylamide gels have a rather small range of separation, but very high resolving power.

Equipment to conduct gel electrophoresis is relatively simple. They consist of a mold to form the gels, an apparatus to hold the gel and contain buffers, and a power supply capable of delivering the required voltage or current. There are many types of apparatus for carrying out electrophoresis depending on the application. Gels can be either in a vertical or horizontal configuration. Polyacrylamide gels are run in a vertical fashion and agarose gels tend to be run in a horizontal position.

Gels can either be formed as cylinders by using glass tubing as a mold (often called tube gels) or formed as rectangular slabs. These slab gels are formed by polymerizing the acrylamide solution between glass plates separated by spacers. Typically the gel is 0.75-1.5 mm thick. At the top a 'comb' is used to form sample wells. Slab gels allow multiple samples to be compared on the same gel, thus eliminating gel-to-gel variations.



The formed gel is placed into the apparatus to that the top and bottom of the gel are in

contact with chambers containing buffer. These chambers contain electrodes which are connected to a power supply. Thus an electric field is generated across the gel when a voltage is applied. The buffer in the chambers is generally different that the buffer making up the gel for protein electrophoresis and in some applications the buffers in the lower and upper chambers may be different. In most applications the buffers are such that the protein has a negative charge and therefore the anode (positve pole) will be in the lower chamber and the cathode (negative pole) will be in the upper chamber. However, there are applications in which the proteins of interest may be positively charged and therefore the electrodes will be reversed.

Polyacrylamide gel electrophoresis in the presence of SDS (sodium dodecyl sulfate) is the most common form of protein gel electrophoresis. SDS completely disrupts protein-protein interactions and denatures almost all proteins resulting in a complete unfolding of proteins.

CENTRIFUGATION

The centrifuge is an essential instrument in cell and molecular biology research. It is primarily used to separate biological components based upon differential sedimentation properties. By spinning samples at very high speeds, the components of a given mixture are subjected to centrifugal force, which causes more dense particles to migrate away from the axis of rotation and lighter ones to move toward it. These particles can sediment at the bottom of the tube into what's known as a pellet, and this isolated specimen, or the remaining solution, the supernatant, can be further processed or analyzed. Many types of centrifuges are available for various applications. All centrifuges basically consist of a motor which spins a rotor containing the experimental sample. The differences between centrifuges are in the speeds at which the samples are centrifuged and the volumes of samples.

The centrifugal force generated is proportional to the rotation rate of the rotor (in rpm) and the distance between the rotor center and the centrifuge tube. Therefore, a given centrifuge may use multiple rotor sizes to give flexibilty in choosing centrifugation conditions. Typically, the material to be "spun" is placed in a

centrifuge tube which is then placed in a rotor. The rotor is a generally a dense metal which dissipates heat quickly, and is of sufficient mass that it generates momentum, i.e., once its spinning it requires little energy to keep it going. Centrifuges generally work under vacuum and are refrigerated to reduce heating caused by frictional forces as the rotor spins. Rotors are usually stored in refrigeration units to keep them at or near the operating temperature.

Types of Centrifuge depends on:

- Maximum speed of sedimentation
- Presence /absence of vacuum
- Temperature control refrigeration.
- Volume of sample andcapacity of centrifugation tubes.

Small Benchtop -With or without refrigeration, slow speed (eg up to 4000 RPM) common in clinical lab (blood/plasma/serum separation) can take approx (up to) 100 tubes, depending on diameter.

Microcentrifuges- ("microfuge", "Eppendorf"), take tubes of small volumes (up to 2 mL), very common in biochemistry/molecular biology/ biological labs, can generate forces up to ~15,000 x g, with or without refrigeration.

High Speed centrifuges-15,000 – 20,000 RPM, centrifugal field of 100,000 g, large sample capacity depending on rotor, normally refrigerated, research applications, Differentiation separation of nucleus, mitochondrial, protein precipitate, large intact organelle, cellular debris, bulky protein aggregates.

Ultracentrifuges- 65,000 RPM (100,000's x g), limited lifetime, Expensive, require special rotors, care in use – balance critical, research applications, The high speeds used in such devices generate considerable amounts of heat ,Therefore cooling arrangements and vaccuum are required in ultracentrifuges.

Large-capacity preparative centrifuge- Centrifugal fields of 3000 to 7000g, Efficient separation of coarse precipitates or whole cells.