KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed University Established Under Section 3 of UGC Act 1956)
Coimbatore - 641021.
(For the candidates admitted from 2015 onwards)

DEPARTMENT OF BIOCHEMISTRY

SUBJECT : **BIOINSTRUMENATION AND GOOD LABORATORY PRACTICE**
SEMESTER : I
SUBJECT CODE **:** 17BCP103 **CLASS :** I M.Sc.(BC)

Program Learning Outcome

This course provides impact knowledge about the technical aspects of various experiment on centrifugation, colorimetry, chromatographic, electrophoretic and radioisotope methods. In addition, this course teaches the good laboratory practices that are mandatory to execute these experiments.

UNIT I

Centrifugation: Types of centrifuges, Principles and applications of analytical and preparative centrifuges, density gradient and ultra centrifugation. Relative molecular mass determination and sedimentation coefficient. Sub cellular fractionation of cellular components. Applications. Separation of cells on the basis of density.

Colorimetry: Beer's law and Lambert's law. Principle of photoelectric colorimeter, Spectroscopy – Properties of electromagnetic radiations, Instrumentation and applications of UV Visible and mass spectroscopy, FTIR, NIR, reverse spectroscopy. Spectrofluorimetery, atomic spectroscopy, NMR spectroscopy. Advantatages and disadvantages and advancements of spectroscopic methods.

UNIT II

Chromatography: Principles, Types – paper chromatography, thin layer chromatography and HPTLC, Column chromatography - Ion exchange chromatography, affinity chromatography, gel filtration chromatography, Low pressure liquid chromatography (LPLC) and High Performance Liquid Chromatography (HPLC)- Normal and Reverse Phase Gas -liquid chromatography Mass spectroscopy (GC – MS), LC-MS, MALDI-TOF, ICPMS, Application of Chromatography. Separation of phytoconstituents using TLC.

UNIT III

Electrophoresis: Principle, instrumentation and applications of agarose gel electrophoresis, sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE), native PAGE, isoelectric focusing, immunoelectrophoresis, 2D gel electrophoresis. Pulse field gel electrophoresis, capillary electrophoresis, gel documentation – Applications. Blotting techniques.

UNIT IV

Radioisotopic techniques : Introduction, nature of radio activity, types and rate of radioactive decay, units of radio activity, detection and measurement of radioactivity-Geiger-Muller counter, solid and liquid scintillation counter. Autoradiography, X-ray diffraction and circular dichorism. Non radioactive, fluorescent methods. Applications of radioisotopes in biological sample analysis.

Flowcytometry: Principles and applications**.**

UNIT V

Good Laboratory Practices: Quality concepts, personal protective equipment. General safety-biological safety, chemical safety and fire safety. data generation and storage, quality control documents, retention samples, records, audits of quality control facilities. List of Regulations to be followed**.** Laboratory safety procedure- glass ware, equipment safety, hands protection, precaution to be undertaken to prevent accident and contamination. GLP – an overview and basic information, Scope. Principles of GLP: Test Facility Organization and Personnel, Test Systems, Test and Reference Items, Standard Operating Procedures, Performance of the Study, Reporting of Study Result, Storage and Retention of Records and Materials. Responsibilities in GLP. Implementing of GLP in non GLP analytical laboratory.

TEXT BOOKS

Weinberg, S., (1995). Good Laboratory Practice Regulations, 3rd edition, CRC Press, U.S.A.

Harburn, K., (1990). Quality Control of Packing Materials in Pharmaceutical Industry, CRC Press, U.S.A.

Chatwal, G.R., and Anand, S.K., (2003). Instrumental Methods of Chemical Analysis. 5th Edition, Himalaya Publishing House, Mumbai.

Sharma, B.K., (2004). Instrumental Methods of Chemical Analysis, 24th Edition, Goel Publishing House, Meerut.

REFERENCES

Richard, A.G., Richard, G., (2009). New Drug Approval Process Drugs and the Pharmaceutical Sciences), 5th edition CRC Press, U.S.A.

Wenclawiak, B.W., Koch, M., Hadjicostas, E. (2004). Quality Assurance in Analytical Chemistry: Training and Teaching. 1st edition, springer. U.S.A.

Wilson, K., and Walker, J., (2010). Principles and Techniques of Biochemistry and Molecular Biology, 7th Low Price Edition, Cambridge University Press, India.

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SUBJECT : BIOINSTRUMENTATION AND GLP
SEMESTER : I
SUBJECT CODE : 17BCP103 CLASS : I M.Sc.BC

CENTRIFUGATION TECHNIQUES:

Introduction:

- The centrifuge is an instrument, which is used to spin substances at high speed.
- It is often used to separate particles present in a liquid.
- The mixture is placed in a tube that pivots so that when the machine starts to move, the tube can swing out horizontally.
- The centrifugal force tries to push the mixture away from the centre of centrifuge.

BASIC PRINCIPLES OF SEDIMENTATION:

- This method is based on the principle of sedimentation.
- From everyday experience, the effect of sedimentation due to the influence of the Earth's gravitational field (g = 981 cms-2) versus the increased rate of sedimentation in centrifugal field (g>981 cms-2) is apparent.
- To give a simple but illustrative example, crude sand particles added to a bucket of water travel slowly to the bottom of the bucket by gravitation, but sediment much faster when the bucket is swung around in a circle.
- Similarly, biological structures exhibit a drastic increase in sedimentation when they undergo acceleration in a centrifugal field.
- The relative centrifugal field is usually expressed as a multiple of the acceleration due to gravity.

- Below is a short description of equations used in practical centrifugation classes.
- The more dense a biological structure is the faster it sediments in a centrifugal field.
- The more massive a biological particle is, the faster it moves in a centrifugal field.
- The denser the biological buffer system is, the slower the particle will move in a centrifugal field.
- The greater the frictional coefficient is, the slower a particle will move.
- The greater the centrifugal force is the faster the particle sediments.
- The sedimentation rate of a given particle will be zero when the density of the particle and the surrounding medium are equal.
- When particle sediments, it must displace some of the solution in which it is suspended, resulting in an up thrust on the particle equal to the weight of the liquid displaced.
- If a particle is assumed to be a sphere of known volume and density, then the net force(f) is experienced when the centrifugal force at an angular velocity of ω radians/sec is given by:

$$S = \text{Volume} \times \text{Density} \times \omega 2r$$

Or,

$$F = 4/3\ \Pi\ r \quad p3\ (\rho p - \rho m)\ \omega 2\ r$$

Where,

$4/3\ \Pi\ r\ p3$ = Volume of sphere of radius 'r'.

ρp = Density of the particle.

ρm = Density of the suspended medium.

ν = Distance of the particle from the center of rotation.

ω = Angular velocity of rotor.

DESKTOP CENTRIFUGES:

- Centrifuges are essential devices, each of the clinical laboratories.
- This may be a desktop or floor-type, refrigerated or cooling.

- Centrifuge is mainly used to create the fractionation due to fluid samples with high g forces of spin.
- Desktop centrifuge designed to meet the laboratory setting to limited space requirements.
- These are used to separate liquid solid;, also dealing with blood samples used.
- Desktop centrifuge is in a variety of different specifications, sizes and abilities. Independent Electoral Commission Centra, Beckman and Adams is a leading manufacturer of desktop centrifuge.

Desktop centrifuge with innovative features

- Desktop centrifuge important feature is to break the power system strong guarantee automatic acceleration and deceleration.
- Another important feature is their silence and vibration free operation.
- The device is designed with a full timer, brushless motor and has a clear lid safety switch.
- It also has a maintenance-free brushless motor; with power interrupt the user's security design.
- The device and the cooling or not cooling at different speeds to choose from.
- Refrigeration unit is an independent unit, but the time dependence of their work, because it's centrifuge for power.
- The device has a multi-functional, four rotor, test tubes and bottles can accommodate all possible sizes.
- Desktop centrifuge have such characteristics:
- Removable rotor bowl for easy cleaning.
- Reagents, and consumables can be controlled.
- Speed control knob.
- Keep the programs running longer.
- Temperature, velocity and time display settings
- Find the real purchasing the best product distributor
- To analyze the working conditions before purchasing, quality and warranty desktop centrifuge specifications.

- A leading supplier of laboratory bench centrifuge at low cost high quality products, and to ensure service for years.

Desktop Centrifuges

LARGE CAPACITY REFRIGERATED CENTRIFUGES

- Controlled by microcomputer, touching panel and LED display.
- The compressor imported from Europe operating without Freon for environment protection, pre-cooling when power on.
- The brushless direct drive motor with high start torque and frequency inversion.
- Imbalance, over-speed, over-temperature and door interlocking protection function for the safety of people and instrument.
- The parameter can be changed at operating state.
- Automatic computing and setting RCF.adjustable rise-and-fail speed from grade 0 to 9
- Fitting to the adapters of 5ml.7ml .10ml.15ml.30ml.50ml, etc.
- Widely used in the filed of radicalization immunity, biochemistry, pharmacy, blood separation and purification
- Hold-on, transient centrifuge.

ANALYTICAL AND PREPARATIVE ULTRACENTRIFUGATION:

Ultracentrifuge:

- The ultracentrifuge is a centrifuge optimized for spinning a rotor at very high speeds, capable of generating acceleration as high as 2,000,000 g (approx. 19,600 km/s²).

- There are two kinds of ultracentrifuges, the preparative and the analytical ultracentrifuge. Both classes of instruments find important uses in molecular biology, biochemistry and polymer science.

Analytical ultracentrifuge

- In an analytical ultracentrifuge, a sample being spun can be monitored in real time through an optical detection system, using ultraviolet light absorption and/or interference optical refractive index sensitive system.

- This allows the operator to observe the evolution of the sample concentration versus the axis of rotation profile as a result of the applied centrifugal field.

- With modern instrumentation, these observations are electronically digitized and stored for further mathematical analysis.

- Two kinds of experiments are commonly performed on these instruments: sedimentation velocity experiments and sedimentation equilibrium experiments.

- Sedimentation velocity experiments aim to interpret the entire time-course of sedimentation, and report on the shape and molar mass of the dissolved macromolecules, as well as their size-distribution.

- The size resolution of this method scales approximately with the square of the particle radii, and by adjusting the rotor speed of the experiment size-ranges from 100 Da to 10 GDa can be covered.

- Sedimentation velocity experiments can also be used to study reversible chemical equilibria between macromolecular species, by either monitoring the number and molar mass of macromolecular complexes, by gaining information about the complex composition from multi-signal analysis exploiting differences in each components spectroscopic signal, or by following the composition dependence of the sedimentation rates of the macromolecular system, as described in Gilbert-Jenkins theory.

- Sedimentation equilibrium experiments are concerned only with the final steady-state of the experiment, where sedimentation is balanced by diffusion opposing the concentration

gradients, resulting in a time-independent concentration profile. Sedimentation equilibrium distributions in the centrifugal field are characterized by Boltzmann distributions.

- This experiment is insensitive to the shape of the macromolecule, and directly reports on the molar mass of the macromolecules and, for chemically reacting mixtures, on chemical equilibrium constants.

- The kinds of information that can be obtained from an analytical ultracentrifuge include the gross shape of macromolecules, the conformational changes in macromolecules, and size distributions of macromolecular samples.

- For macromolecules, such as proteins, which exist in chemical equilibrium with different non-covalent complexes, the number and subunit stoichiometry of the complexes and equilibrium constant constants can be studied.

Preparative ultracentrifuge

- Preparative ultracentrifuges are available with a wide variety of rotors suitable for a great range of experiments.

- Most rotors are designed to hold tubes that contain the samples.

- Swinging bucket rotors allow the tubes to hang on hinges so the tubes reorient to the horizontal as the rotor initially accelerates.

- Fixed angle rotors are made of a single block of metal and hold the tubes in cavities bored at a predetermined angle.

- Zonal rotors are designed to contain a large volume of sample in a single central cavity rather than in tubes.

- Some zonal rotors are capable of dynamic loading and unloading of samples while the rotor is spinning at high speed.

- Preparative rotors are used in biology for pelleting of fine particulate fractions, such as cellular organelles (mitochondria, microsomes, and ribosomes) and viruses.

- They can also be used for gradient separations, in which the tubes are filled from top to bottom with an increasing concentration of a dense substance in solution.

- Sucrose gradients are typically used for separation of cellular organelles.

- Gradients of caesium salts are used for separation of nucleic acids.

- After the sample has spun at high speed for sufficient time to produce the separation, the rotor is allowed to come to a smooth stop and the gradient is gently pumped out of each tube to isolate the separated components.

APPLICATIONS OF ULTRACENTRIFUGATION:

- The analytical ultracentrifuge has found the following wide applications in Biology.
- To determine relative molecular mass of macromolecules such as, proteins and DNA.
- To investigate the purity of DNA preparations, viruses and proteins.
- To detect conformational changes in macromolecules such as DNA and protein.
- Characterize assembly and disassembly mechanisms of bio molecular complexes
- Determine subunit stoichiometries
- Thermodynamic and hydrodynamic information
- Measure equilibrium constants and thermodynamic parameters for self- and hetero-associating systems.

Sedimentation velocity

- Sedimentation velocity is an analytical ultracentrifugation (AUC) method that measures the rate at which molecules move in response to centrifugal force generated in a centrifuge.
- This sedimentation rate provides information about both the molecular mass and the shape of molecules. In some cases this technique can also measure diffusion coefficients and molecular mass.
- In the biotechnology industry sedimentation velocity is used much more frequently than sedimentation equilibrium and thus when biotech scientists say "AUC" or "analytical ultracentrifugation" they typically really mean "sedimentation velocity".
- Sedimentation velocity is particularly valuable for:verifying whether a sample is entirely homogeneous in mass and conformation detecting aggregates in protein samples and quantifying the amount of aggregate comparing the conformations for samples from different lots, manufacturing processes, or expression systems (comparability studies), or comparing different engineered variants of the same protein/peptide,establishing whether the native state of a protein or peptide is a monomer, dimer, trimer, etc.,determining the overall shape of non-glycosylated protein and peptide molecules in solution, measuring the distribution of sizes in samples which

contain a very broad range of sizes, detecting changes in protein conformation, for example partial unfolding or transitions to "molten globule" states.studying the formation and stoichiometry of tight complexes between proteins (for example receptor-ligand or antigen-antibody complexes)

- In the sedimentation velocity method a sample is spun at very high speed (usually 40-60 K rpm) in an analytical ultracentrifuge. The high centrifugal force rapidly depletes all the protein from the region nearest the center of the rotor (the meniscus region at the air/solution interface), forming a boundary which moves toward the outside of the rotor with time (see example below), until finally all the protein forms a pellet at the outside of the cell.

- The concentration distribution across the cell at various times during the experiment is measured while the sample is spinning, using either absorbance or refractive index detection in our Beckman ProteomeLab XL-I.

- A major advantage of this method over sedimentation equilibrium is that experiments usually require only 3-5 hours, as opposed to the several days typical of sedimentation equilibrium. Thus sedimentation velocity can be used with samples that are too labile for sedimentation equilibrium.

- The major drawback relative to sedimentation equilibrium applies to interacting systems (proteins that reversibly self-associate or protein-protein complexes), where the non-equilibrium nature of the measurement can lead to significant changes in species distributions over the course of an experiment. Further, for interacting systems it is generally more difficult and less accurate to derive binding constants (Kd's) from sedimentation velocity data.

- An important strength of sedimentation velocity is its ability to study samples over a fairly wide range of pH and ionic strength conditions (and often directly in formulation buffers), and at temperatures from 4 to 40 °C. The amount of protein required depends on the application, but each sample is usually ~0.45 ml at typical protein concentrations of 0.1-1 mg/ml (45-450 micrograms total).

- Protein concentration can range as low as ~10 micrograms/ml or as high as ~40 mg/ml in some cases (but generally the concentration should be 2 mg/mL or below). Up to 3 samples can be run at one time.A sedimentation velocity case study: a monoclonal antibody.

- The graph below shows scans across the centrifuge cell, recording the absorbance at 280 nm versus position within the cell. These scans were taken starting at 13 minutes after initiating a run at 45,000 rpm (the black data set in the graph), and then every ~12 minutes thereafter (blue, green, cyan, etc.).

- The sharp vertical spike at 6.02 cm indicates the position of the air-solution meniscus. In the first data set the sedimentation of the antibody has already depleted its concentration in the

region near the meniscus and formed a sedimentation boundary.

- At later times in the run the depleted region expands and the boundary moves away from the center of the rotor, until by the time of the last data set the concentration of antibody has dropped to essentially zero throughout the upper half of the cell.

- The rate at which the sedimentation boundary moves is a measure of the sedimentation coefficient of the protein. The sedimentation coefficient depends on the molecular weight (larger proteins sediment faster) and also on molecular shape. Unfolded proteins or one with highly elongated shapes will experience more hydrodynamic friction, and thus will have smaller sedimentation coefficients than a folded, globular protein of the same molecular weight.

- The minimum width of the sedimentation boundary is related to the diffusion coefficient of the molecule; the presence of multiple species with similar sedimentation coefficients will cause the boundary to be broader than expected on the basis of diffusion alone. In this case the majority of the boundary is reasonably narrow, but the slow rise of the data on the right side of the boundary suggests the presence of some faster moving species.

- When viewed as in the graph above the data is difficult to interpret. What we often want to know is how much material is sedimenting at various sedimentation coefficients. By taking many scans close together in time (the graph above shows only a small number of the scans), subtracting them in pairs, and doing some mathematical manipulation these data can be transformed into the sedimentation coefficient distribution, g(s*), which is shown below.

- This distribution resembles a chromatogram, and in many ways is similar to a size-exclusion chromatogram except the peaks come in the opposite order. Like a chromatogram, the area under each peak gives the total amount of that species.

- For this antibody sample we see only one distinct peak, centered at a sedimentation coefficient of ~6.5 S, which corresponds to the native antibody 'monomer' (really a covalent heterotetramer of 2 light and 2 heavy chains). A sedimentation coefficient of 6.5 S is actually rather low for a 150 kDa species, which is consistent with high hydrodynamic friction from its highly asymmetric, non-globular 'Y' shape.

- Although we see only a single peak in the g(s*) distribution, a more detailed analysis quickly reveals that this sample is not homogeneous. The red curve is a fit of these data as a single species. This fit clearly fails to account for the data over the region from 8-12 S, indicating the presence of some dimer and possibly also some trimer.

- The fact that the distribution has not returned fully to zero at 12 S also suggests that there may also be small amounts of even faster sedimenting species present.

- From the width of the main peak we can also calculate the apparent diffusion coefficient of the monomer. In turn, from the ratio of sedimentation coefficient to diffusion coefficient we can calculate a mass of 151 kDa for this species, which matches the expected value well within 3-5% error expected for masses determined in this fashion.

Lastly, we can apply a new analysis algorithm to further enhance the resolution of the species distribution.

- In this figure the full distribution is shown in the main graph, while the inset magnifies the vertical scale by 10X in order to better show the minor components. With the enhanced resolution we now see a fully baseline-resolved dimer peak at ~9.4 S (4.7% of the total protein), and small peaks at ~13.7 S and 17.5 S (1.8% and 1.1%, probably trimer and tetramer). In addition there is 0.7% of a low mass contaminant at 2 S (possibly free light chain).

- Thus from this one velocity experiment we have been able to quantify the amount and mass of the main component, the content and sedimentation coefficients of 3 aggregates and a low mass contaminant, and to obtain information about the conformation of the main component.

Other applications

A sedimentation coefficient distribution for a sample of adenovirus (~150 MDa, used to deliver vectors for gene therapy). This illustrates the broad range of sizes and molecule types that can be studied using this technique. The inset shows a 20-fold expanded scale to allow the many minor peaks to be seen. This material turns out to be quite heterogeneous, having only 57% of the material as the main peak (virus monomer), plus many rapidly-sedimenting viral aggregates and some slowly-sedimenting species that may be fragments or empty capsids.

SEPARATION OF CELL ORGANELLES:

- To study the functions of any organelle in depth, it is first necessary to isolate it in a relatively pure form, free of concentration by other organelles.

- The process of separation of cell organelles is known as subcellular fractionation.

- As a first step toward isolating a specific organelle, organs (liver, brain, and kidney) are homogenized in a suitable homogenizing medium at 4º C.

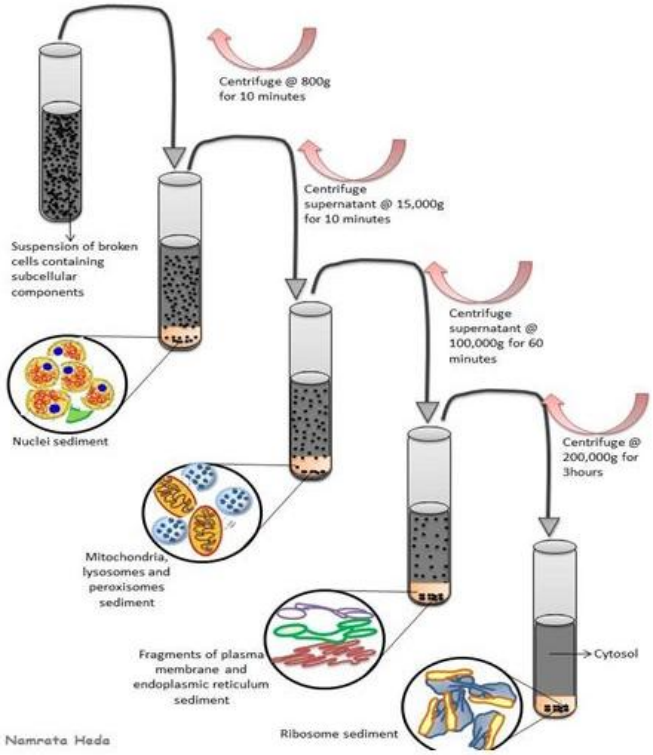
- The resulting suspension, containing many contact organelles, is known as a homogenate.

- Fractionation of the contents of a homogenate is done by a classical biochemical technique called differential centrifugation.

- This methodis based upon the differences in the sedimentation rate of particles of different size and density.
- This method uses a series of four different centrifugation steps at successively greater speeds.
- Each step yields a pellet and a supernatant.
- The supernatant from each step is subjected to centrifugation in the next step.
- This procedure provides four pellets, namely, nuclear, mitochondrial, lysosomal and microsomal fractions.
- At the end of each step, the pellet is washed several times by resuspending in the homogenization medium followed by recentrifugation under the same conditions.
- This procedure minimizes contamination of other subcellular organelles and gives a fairly pure preparation of pellet fraction.
- The purity of organelles obtained by differential centrifugation is measured by estimating some marker activity.
- A marker is one that is almost exclusively present in one particular organelle.
- A marker may be an enzyme molecule or a biochemical compound.
- Various fractions, their functions and markers

S. No	Organelle	Function	Marker
1.	Plasma membrane	Regulates entry and exit of compounds	5' Nucleotidase
2.	Nucleus	Site of DNA-directed RNA synthesis	DNA
3.	Mitochondrion for urea formation.	Citric acid cycle, ammonia release	DHases
4.	Lysosome	Site of many hydrolases.	Acid phosphatase
5.	ER	Oxidation of many xenobiotics	Glucose6-pase.
6.	Cytosol	Enzymes of glycolysis.	LDH

- The microsomal fraction contains mostly a mixture of smooth endoplasmic reticulum and free ribosomes.
- The contents of the final supernatant correspond approximately to those of Cytosol.



Fractionation of Cells

Although biochemical analysis requires disruption of the anatomy of the cell, gentle fractionation techniques have been devised to separate the various cell components while preserving their individual functions. Just as a tissue can be separated into its living constituent cell types, so the cell can be separated into its functioning organelles and macromolecules. In this section we consider the methods that allow organelles and proteins to be purified and analyzed biochemically.

Organelles and Macromolecules Can Be Separated by Ultracentrifugation:

- Cells can be broken up in various ways: It can be subjected to osmotic shock or ultrasonic vibration, forced through a small orifice, or ground up in a blender. These procedures break many of the membranes of the cell (including the plasma membrane and membranes of the endoplasmic reticulum) into fragments that immediately reseal to form small closed vesicles. If carefully applied, however, the disruption procedures leave organelles such as nuclei, mitochondria, the Golgi apparatus, lysosomes, and peroxisomes largely intact.

- The suspension of cells is thereby reduced to thick slurry (called a homogenate or extract) that contains a variety of membrane-enclosed organelles, each with a distinctive size, charge, and density. Provided that the homogenization medium has been carefully chosen (by trial and error for each organelle), the various components—including the vesicles derived from the endoplasmic reticulum, called microsomes—retain most of their original biochemical properties.

- The different components of the homogenate must then be separated. Such cell fractionations became possible only after the commercial development in the early 1940s of an instrument known as the preparative ultracentrifuge, in which extracts of broken cells are rotated at high speeds. This treatment separates cell components by size and density: in general, the largest units experience the largest centrifugal force and move the most rapidly.

- At relatively low speed, large components such as nuclei sediment to form a pellet at the bottom of the centrifuge tube; at slightly higher speed, a pellet of mitochondria is deposited; and at even higher speeds and with longer periods of centrifugation, first the small closed vesicles and then the ribosomes can be collected.

- All of these fractions are impure, but re-suspending the pellet and repeating the centrifugation procedure several times can remove many of the contaminants.

The preparative ultracentrifuge:

- The sample is contained in tubes that are inserted into a ring of cylindrical holes in a metal rotor. Rapid rotation of the rotor generates enormous centrifugal forces, which cause particles in the sample to sediment. The vacuum reduces friction, preventing heating of the rotor and allowing the refrigeration system to maintain the sample at 4°C.

- Centrifugation is the first step in most fractionations, but it separates only components that differ greatly in size. A finer degree of separation can be achieved by layering the

homogenate in a thin band on top of a dilute salt solution that fills a centrifuge tube. When centrifuged, the various components in the mixture move as a series of distinct bands through the salt solution, each at a different rate, in a process called velocity sedimentation.

- For the procedure to work effectively, the bands must be protected from convective mixing, which would normally occur whenever a denser solution (for example, one containing organelles) finds itself on top of a lighter one (the salt solution). This is achieved by filling the centrifuge tube with a shallow gradient of sucrose prepared by a special mixing device. The resulting density gradient—with the dense end at the bottom of the tube—keeps each region of the salt solution denser than any solution above it, and it thereby prevents convective mixing from distorting the separation.

- When sediment through such dilute sucrose gradients, different cell components separate into distinct bands that can be collected individually. The relative rate at which each component sediments depends primarily on its size and shape—being normally described in terms of its sedimentation coefficient, or s value. Present-day ultracentrifuges rotate at speeds of up to 80,000 rpm and produce forces as high as 500,000 times gravity. With these enormous forces, even small macromolecules, such as tRNA molecules and simple enzymes can be driven to sediment at an appreciable rate and so can be separated from one another by size.

- Measurements of sedimentation coefficients are routinely used to help in determining the size and subunit composition of the organized assemblies of macromolecules found in cells.The ultracentrifuge is also used to separate cellular components on the basis of their buoyant density, independently of their size and shape. In this case the sample is usually sedimented through a steep density gradient that contains a very high concentration of sucrose or cesium chloride.

- Each cellular component begins to move down the gradient but it eventually reaches a position where the density of the solution is equal to its own density. At this point the component floats and can move no farther. A series of distinct bands is thereby produced in the centrifuge tube, with the bands closest to the bottom of the tube containing the components of highest buoyant density. This method, called equilibrium sedimentation, is so sensitive that it is capable of separating macromolecules that have incorporated heavy isotopes, such as 13C or 15N, from the same macromolecules that contain the lighter, common isotopes (12C or 14N).

- In fact, the cesium-chloride method was developed in 1957 to separate the labeled from the unlabeled DNA produced after exposure of a growing population of bacteria to nucleotide precursors containing 15N; this classic experiment provided direct evidence for the semiconservative replication of DNA.

- Studies of organelles and other large subcellular components isolated in the ultracentrifuge have contributed enormously to our understanding of the functions of different cellular components. Experiments on mitochondria and chloroplasts purified by centrifugation,

for example, demonstrated the central function of these organelles in converting energy into forms that the cell can use.

- Similarly, resealed vesicles formed from fragments of rough and smooth endoplasmic reticulum (microsomes) have been separated from each other and analyzed as functional models of these compartments of the intact cell.

- An extension of this approach makes it possible to study many other biological processes free from all of the complex side reactions that occur in a living cell, by using purified cell-free systems. In this case, cell homogenates are fractionated with the aim of purifying each of the individual macromolecules that are needed to catalyze a biological process of interest.

- For example, the mechanisms of protein synthesis were deciphered in experiments that began with a cell homogenate that could translate RNA molecules to produce proteins. Fractionation of this homogenate, step by step, produced in turn the ribosomes, tRNAs, and various enzymes that together constitute the protein-synthetic machinery.

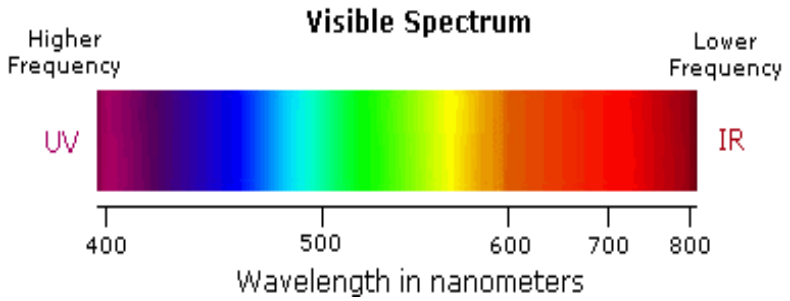
- Once individual pure components were available, each could be added or withheld separately to define its exact role in the overall process. A major goal today is the reconstitution of every biological process in a purified cell-free system, so as to be able to define all of its components and their mechanism of action.

Colorimetry:

Colors and absorption Spectra

The electromagnetic spectrum is comprised of all known types of electromagnetic radiation, including X-rays and radio waves, and is organized by wavelength, which is determined by the energy of the radiation. Shorter wavelengths correspond to higher energy radiation. Visible light is the very small portion of the spectrum (380 nm to 800 nm) that the human eye is sensitive to. The colors of most substances arise from the interaction between visible light and the electrons within molecular bonds.

Specifically, the light at specific wavelengths is absorbed, causing these electrons to occupy different energy levels.

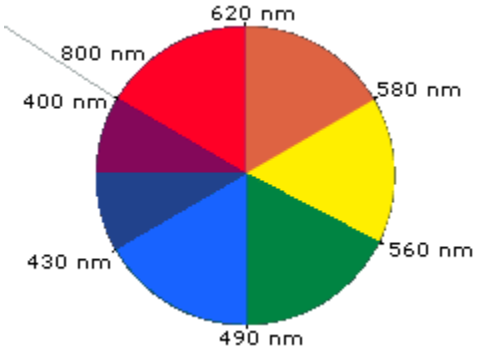


Wavelengths that the molecule doesn't absorb are transmitted, or reflected, to the observer's eye as the perceived color of the molecule, especially when it is dissolved in a transparent solvent such as water. For example, nickel sulfate, NiSO4, dissolved in water appears greenish-blue because it transmits those wavelengths associated with blue-green-yellow portion of the visible spectrum. All other visible wavelengths are absorbed.

- **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

When white light passes through or is reflected by a colored substance, a characteristic portion of the mixed wavelengths is absorbed. The remaining light will then assume the complementary color to the wavelength(s) absorbed. The color wheel shown on the right demonstrates this relationship. Here, complementary colors are diametrically opposite each other. Thus, absorption of 420-430 nm lights renders a substance yellow, and absorption of 500-520 nm lights makes it red. Green is unique in that it can be created by absoption close to 400 nm as well as absorption near 800 nm.

Early humans valued colored pigments, and used them for decorative purposes. Many of these were inorganic minerals, but several important organic dyes were also known. These included the crimson pigment, kermesic acid, the blue dye, indigo, and the yellow saffron pigment, crocetin. A rare dibromo-indigo derivative, punicin, was used to color the robes of the royal and wealthy. The deep orange hydrocarbon carotene is widely distributed in plants, but is not sufficiently stable to be used as permanent pigment, other than for food coloring. A common feature of all these colored compounds, displayed below, is a system of extensively conjugated pi-electrons.



The operation of colorimeters is based on the property of colored solutions of absorbing light passing through them. The absorption increases with increasing concentration *c* of the coloring substance. All colorimeter measurements are taken in monochromatic light in the region of the spectrum that is most strongly absorbed by the particular substance in the solution and most weakly absorbed by the solution's other components. Therefore, colorimeters are equipped with light filters; the use of various light filters with narrow spectral ranges for transmitted light makes possible separate determination of the concentration of the various components of the same solution.

Colorimeters are divided into visual and objective (photoelectric) types. In visual colorimeters the light passing through the solution being measured illuminates one part of the field of vision, and light passing through a solution with a known concentration of the same substance is incident on the other part. By changing the thickness *I* of the layer of one of the solutions being compared or the intensity *I* of the light beam, the viewer attempts to make the color tones of the two halves of the field of vision indistinguishable to the eye. The concentration of the solution under study may be determined from the known relationships for *I*, *l*, and *c* (the Bouguer-Lambert-Beer 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.

$$I_t = I_o^{e\text{-}KC}$$

Lambert's Law

According to Lambert's law the amount of light transmitted decreases exponentially with increase in thickness of the colored solution.

$$I_t = I_o^{e\text{-}kt}$$

Therefore, together Beer-Lambert's law is:

$$I_E/I_o = e^{-KCT}$$

where,

I_E = intensity of emerging light

I_o = intensity of incident light

e = base of neutral logarithm

K = a constant

C = concentration

T = thickness of the solution

Photoelectric Colorimeter

Photoelectric colorimeters give higher accuracy of measurement than the visual type. Selenium and vacuum photocells, photomultipliers, photoresistors, and photodiodes are used as light detectors in photoelectric colorimeters. The strength of the photocurrent of the detectors is determined by the intensity of the incident light and thus by the extent of absorption of the light beam in the solution (absorption increases with increasing concentration). In addition to photoelectric colorimeters with reading of the photocurrent strength, compensating colorimeters are also common; in this type the difference in the signals corresponding to the standard and test solutions is set to zero (compensated) by an electric or optical compensator (for example, a photometer wedge). The reading in this case is taken from the compensator scale. Compensation makes possible minimization of the effect on accuracy of measurement conditions, such as temperature and instability of properties of the colorimeter's parts. The readings of colorimeters do not immediately give concentration values for the substances studied. Conversion to concentration values requires the use of calibration curves obtained by measuring solutions of known concentrations.

Colorimeter measurements are relatively simple and rapid. The accuracy of such measurements is often not less than that of more complicated methods of chemical analysis. The lower limits for determinable concentration are 10^{-3} to 10^{-18} moles per liter, depending on the type of substance.

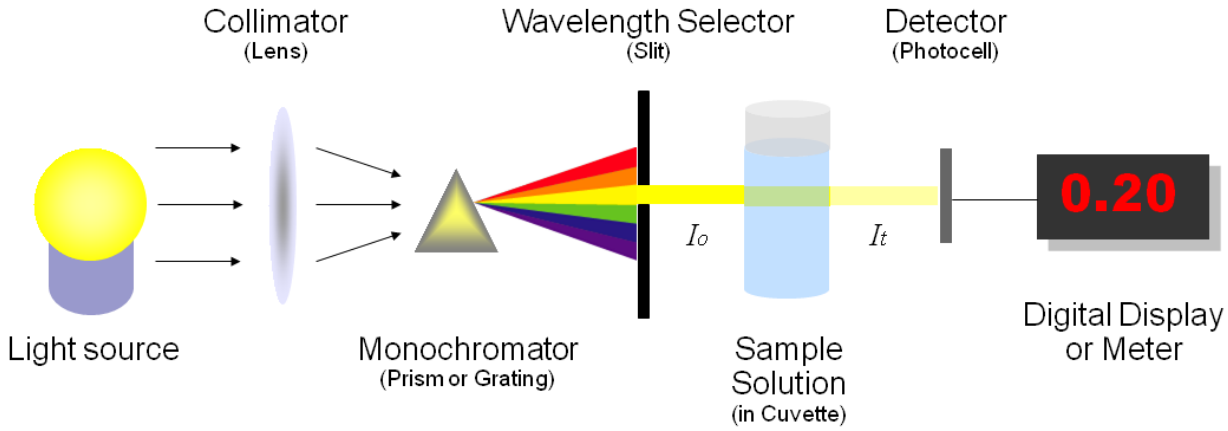
Principles:

Colorimetry is the techniques that is frequently used in biochemical investigations. This involves the quantitative estimation of colors. This measure the quantity of a substance in a mixture, you could use the technique of colorimetry, by allowing the substance to bind with color forming chromogens. The difference in color results in the difference in the absorption of light, which is made use of here in this technique called colorimetry.

- Light from a suitable source is passed through a light filter to select the most appropriate wavelength of light, some of which is then absorbed by the solution held in a special glass cuvette (a sort of 'test tube').
- The amount of light absorbed is called, and measured as, the absorbance which is a function of the coloured solute concentration.
- Most expensive instruments use a double beam system of two cuvets, one is a 'blank' of water and one the actual coloured solution under test, two photocells and sophisticated optics of lenses and mirrors which need not concern as at all.
- Cheaper colorimeters (i.e. in school and illustrated above) allow you to put in a cuvet of 'colourless' water, zero the instrument i.e. set it to read zero absorbance, replace with a cuvet of the coloured solution and simply read of the 'absorbance'.
- The 'zeroing' is necessary because even the apparently 'colourless blank' of glass cuvet and water can absorbed a tiny amount of light. This procedure eliminates this error.

- The filter is chosen to select the band of wavelengths which are most strongly absorbed by the coloured solution e.g. this is illustrated on the diagram above, and in the table below, by using a yellow filter to use in measuring the concentration of a blue coloured solution like copper (II) sulphate or its ammine/amine complex.
- Although the table illustrates the 'complementary' colour relationship between the solution and the filter, in practice it is better to try several filters on a typical concentration of the solution under test to see which filter gives the highest absorption value i.e. gives you maximum sensitivity and hence maximum accuracy in your measurements.

The difference in color results in the difference in the absorption of light, which is made use of here in this technique called colorimetry.



Instrumentation

The instrument use for colorimetry is colorimeter. This appartus will comprise of the following parts:

1. light source
2. filter (the device that selects the desired wavelength)
3. cuvette chamber (the transmitted light passes through compartment wherein the solution containing the colored solution are kept in cuvette, made of glass or disposable plastic)
4. detector (this is a photosensitive element that converts light into electrical signals)
5. Galvanometer (measures electrical signal quantitatively)

Steps for operating the photoelectric colorimeter:

- Choose the glass filter recommended (see table below) in the procedure and insert in the filter.

- Fill two of the cuvette with blank solution to about three-fourth and place it in the cuvette slot.
- Switch on the instrument and allow it to warm up for 4 – 5 minutes.
- Adjust to zero optical density.
- Take the test solution i another cuvette and read the optical density.
- Take the standard solution in varying concentration and note down the optical density as S1, S2, S3, S4, S5 and so on.
- A graph is plotted taking concentration of standard solution versus the optical density.
- From the graph the concentration of the test solution or the unknown solution can be calculated.

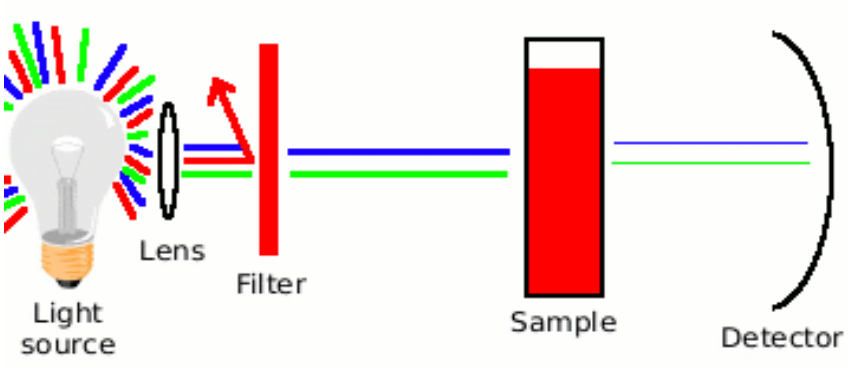


Table for choosing the wavelength of absorption:

Si. No.	Color of the Solution	Colour Absorbed	Wavelength of Absorption
1.	Yellow to Green	Violet	400 nm – 435 nm
2.	Yellow to Orange	Blue	435 nm – 490 nm
3.	Red	Blue to Green	490 nm – 500 nm
4.	Purple	Green	500 nm – 560 nm
5.	Violet	Yellow to Green	560 nm – 580 nm
6.	Blue to Green	Yellow to Orange	580 nm – 650 nm
7.	Bluish Green	Red	650 nm – 700 nm

SPECTROSCOPY

Properties of Electromagnetic Radiation

Electromagnetic radiation is characterized by a broad range of wavelengths and frequencies, each associated with a specific intensity (or amplitude) and quantity of energy. This interactive tutorial

explores the relationship between frequency, wavelength, and energy, and enables the visitor to adjust the intensity of the radiation and to set the wave into motion.

The tutorial initializes with a visible light wave appearing in the window having a wavelength of 650 nanometers (red light) and amplitude of 61 candelas. Energies associated with waves in the tutorial appear beneath the window and are given in units of kJ/mole.

To adjust the wavelength (and simultaneously, the frequency) of the wave, translate either the Wavelength or Frequency sliders to the left or right. As the sliders are relocated, the new values for wavelength and frequency appear above the sliders, the wave color changes to match the value for visible light associated with the wavelength, and the energy associated with the wave appears beneath the tutorial window. The amplitude of the wave can be adjusted with the Amplitude slider, and the resulting intensity values will appear above the slider, measured in units of candelas. In order to stop propagation of the wave, click on the Propagation Stop button located in the lower right-hand side of the tutorial window. The wave can be restarted by again clicking on the button, which changes into a Start button when the wave is halted.

An electromagnetic wave moves or propagates in a direction that is at right angles to the vibrations of both the electric and magnetic oscillating field vectors, carrying energy from its radiation source to undetermined final destination. The two fields are mutually perpendicular. By convention, and to simplify illustrations, the vectors representing the electric and magnetic oscillating fields of electromagnetic waves are often omitted, although they are understood to still exist.

Whether transmitted to a radio from the broadcast station, heat radiating from the oven, furnace or fireplace, X-rays of teeth, or the visible and ultra-violet light emanating from the sun, the various forms of electromagnetic radiation all share fundamental wave-like properties. Every form of electromagnetic radiation, including visible light, oscillates in a periodic fashion with peaks and valleys, and displaying a characteristic amplitude, wavelength, and frequency that defines the direction, energy, and intensity of the radiation.

The standard unit for all electromagnetic radiation is the magnitude of the wavelength (in a vacuum), which is usually reported in terms of nanometers for the visible light portion of the spectrum. Each nanometer represents one-thousandth of a micrometer, and is measured by the distance between two successive peaks (see Figure 1). The corresponding frequency of the radiation wave, the number of sinusoidal cycles (oscillations or complete wavelengths) that pass a given point per second, is proportional to the reciprocal of the wavelength. Frequency is usually measured in Hertz (Hz) or cycles per second (cps). Thus, longer wavelengths correspond to lower frequency radiation and shorter wavelengths correspond to higher frequency radiation.

The different wavelengths and frequencies of various forms of electromagnetic radiation are fundamentally similar in that they all travel at the same speed--about 186,000 miles per second (approximately 300,000 kilometers per second), commonly known as the speed of light (and identified with the variable **c**). Electromagnetic radiation (including visible light) travels 149 million kilometers (93 million miles) from the sun to Earth in about 8 minutes.

The wavelength of light, and all other forms of electromagnetic radiation, is related to the frequency by a relatively simple equation:

$$n = c/l$$

wherec is the speed of light (measured in meters per second), n is the frequency of the light in hertz (Hz), and l is the wavelength of the light measured in meters. From this relationship one can conclude that the wavelength of light is inversely proportional to frequency. An increase in frequency produces a proportional decrease in the wavelength of light with a corresponding increase in the energy of the photons that compose the light. Upon entering a new medium (such as glass or water from air), the speed and wavelength of light is reduced, although the frequency remains unaltered.

Electromagnetic radiation (EMR) is a form of energy that is produced by oscillating electric and magnetic disturbance, or by the movement of electrically charged particles traveling through a vacuum or matter. The electric and magnetic fields come at right angles to each other and combined wave moves perpendicular to both magnetic and electric oscillating fields thus the disturbance. Electron radiation is released as photons, which are bundles of light energy that travel at the speed of light as quantized harmonic waves. This energy is then grouped into categories based on its wavelength into the electromagnetic spectrum. These electric and magnetic waves travel perpendicular to each other and have certain characteristics, including amplitude, wavelength, and frequency.

General Properties of all electromagnetic radiation:

1. Electromagnetic radiation can travel through empty space. Most other types of waves must travel through some sort of substance. For example, sound waves need either a gas, solid, or liquid to pass through in order to be heard.
2. The speed of light is always a constant. (Speed of light : 2.99792458×10^{8} m s^{-1})
3. Wavelengths are measured between the distances of either crests or troughs. It is usually characterized by the Greek symbol \(\lambda\).

Waves and their Characteristics

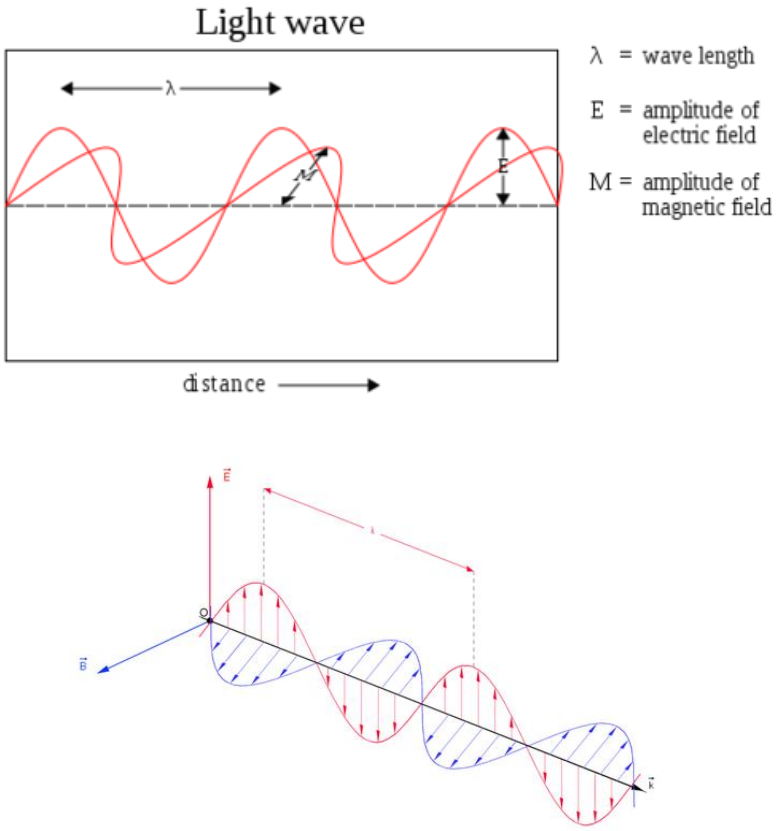


Fig. 1 & 2: Electromagnetic Waves

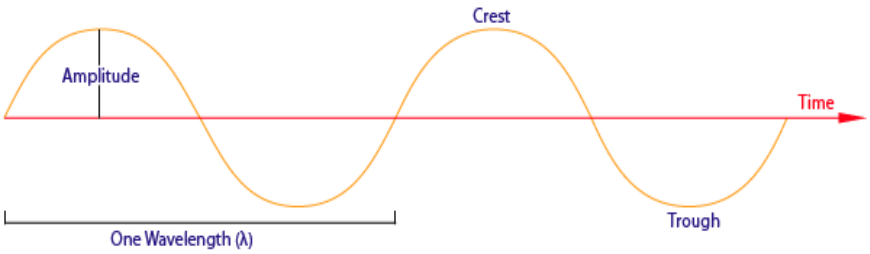
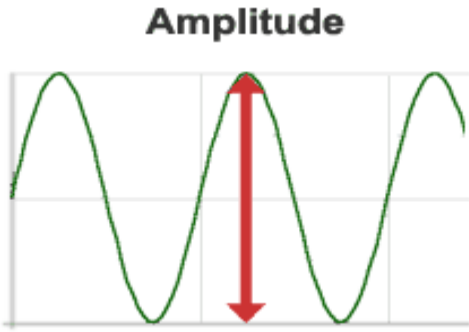


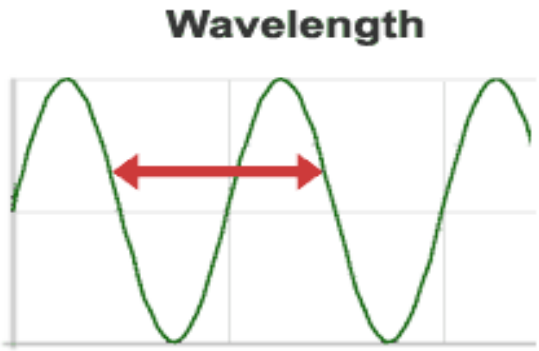
Fig. 3: An EM Wave

Amplitude



Amplitude is the distance from the maximum vertical displacement of the wave to the middle of the wave. This measures the magnitude of oscillation of a particular wave. In short, the amplitude is basically the height of the wave. Larger amplitude means higher energy and lower amplitude means lower energy. Amplitude is important because it tells you the intensity or brightness of a wave in comparison with other waves.

Wavelength



Wavelength (\(\lambda\)) is the distance of one full cycle of the oscillation. Longer wavelength waves such as radio waves carry low energy; this is why we can listen to the radio without any harmful consequences. Shorter wavelength waves such as x-rays carry higher energy that can be hazardous to our health. Consequently lead aprons are worn to protect our bodies from harmful radiation when we undergo x-rays. This wavelength frequently relationship is characterized by:

\[c = \lambda\nu \]

where

- c is the speed of light,
- \(\lambda \) is wavelength, and
- \(\nu \) is frequency.

Shorter wavelength means greater frequency, and greater frequency means higher energy. Wavelengths are important in that they tell one what type of wave one is dealing with.

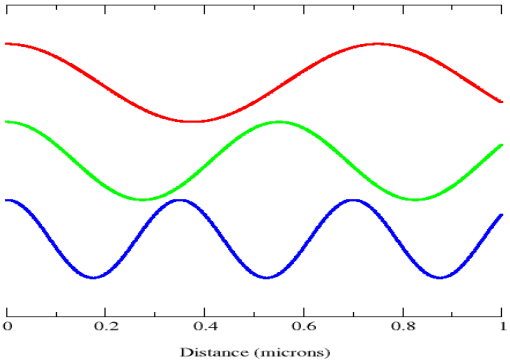
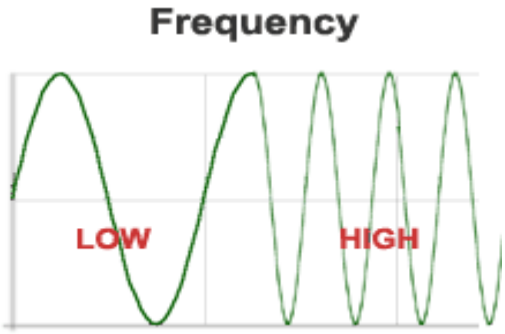


Fig. 4: Different Wavelengths and Frequencies

Frequency



Frequency is defined as the number of cycles per second, and is expressed as sec^{-1} or Hertz (Hz). Frequency is directly proportional to energy and can be express as:

\[E = h\nu \]

where

- E is energy,
- h is Planck's constant, (h= 6.62607 x 10^{-34} J), and
- \(\nu\) is frequency.

Period

Period (T) is the amount of time a wave takes to travel one wavelength. It is measured in seconds (s).

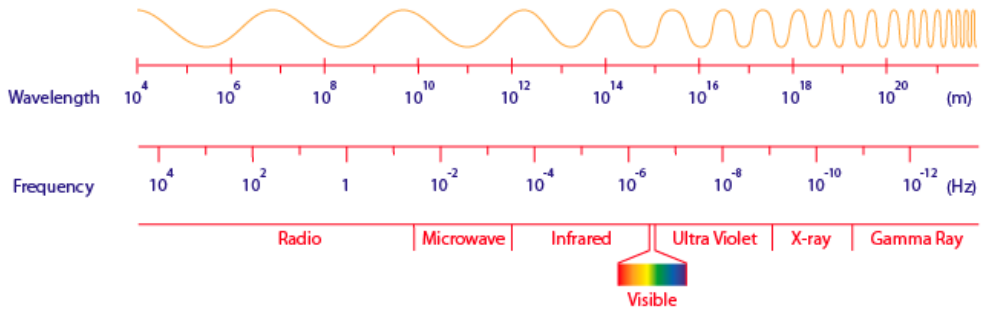
Velocity

The velocity of wave in general is expressed as:

\[velocity = \lambda\nu \]

For Electromagnetic wave, the velocity is 2.99x108m/s or 186,000 miles per second.

Electromagnetic spectrum



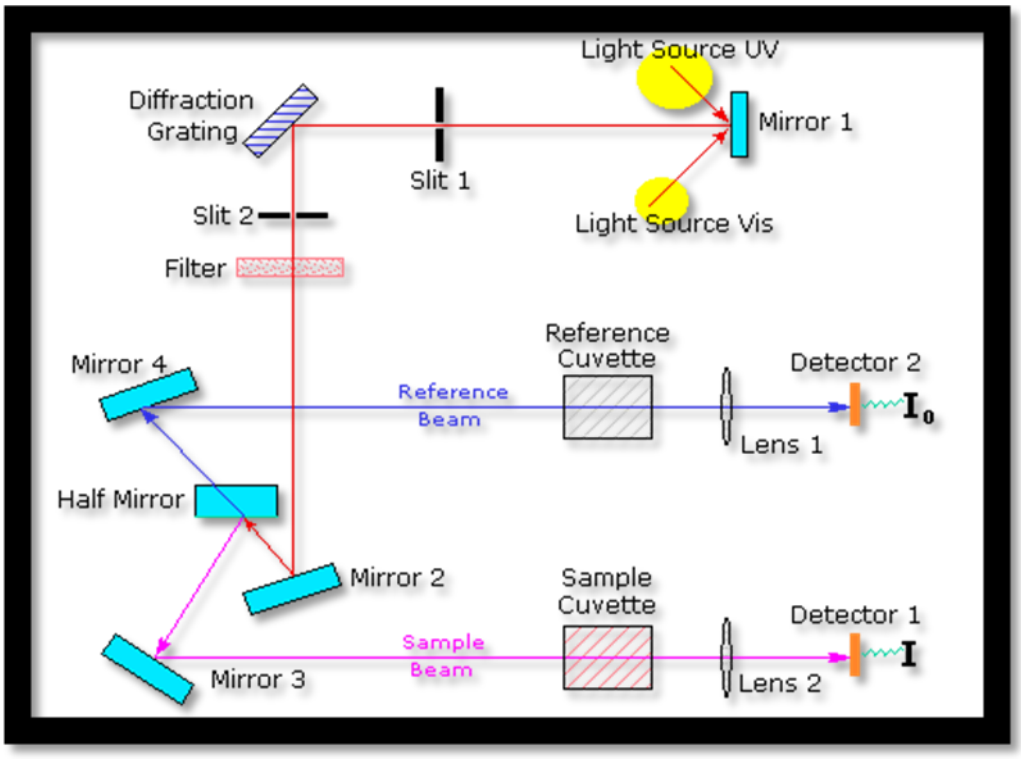
In general, as a wave's wavelength increases, the frequency decreases, and as wave's wavelength decreases, the frequency increases. When electromagnetic energy is released as the energy level increases, the wavelength decreases and frequency decreases. Thus, electromagnetic radiation is then grouped into categories based on its wavelength or frequency into the electromagnetic spectrum. The different types of electromagnetic radiation shown in the electromagnetic spectrum consists of radio waves, microwaves, infrared waves, visible light, ultraviolet radiation, X-rays, and gamma rays. The part of the electromagnetic spectrum that we are able to see is the visible light spectrum.

UV/Visible Spectroscopy

The absorption spectrum (plural, spectra), or more correctly the absolute absorption spectrum, of a compound may be shown as a plot of the light absorbed by that compound against wavelength. Such a plot for a colored compound will have one or more absorption maxima (λmax's) in the visible region of the spectrum (400 to 700 nm). Absorption spectra in the ultraviolet (200 to 400 nm) and visible regions are due to energy transitions of both bonding and nonbonding outer electrons of the molecule. Usually delocalized electrons are involved such as the B bonding electrons of C=C and the lone pairs of nitrogen and oxygen. Since most of the electrons in a molecule are in the ground state at room temperature, spectra in this region give information about this state and the next higher one. As the wavelengths of light

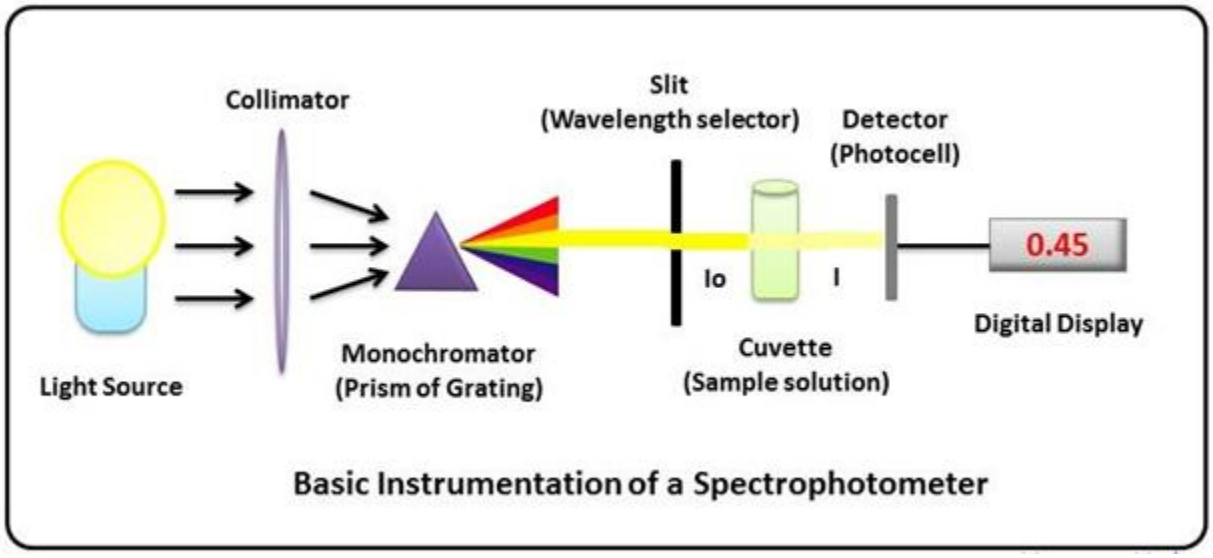
absorbed are determined by the actual transitions occurring, specific absorption peaks may be recorded and related to known molecular substructures.

The term chromophore is given to that part of a molecule that gives rise independently to distinct parts of an absorption spectrum, for example the carbonyl group. Conjugated double bonds lower the energy required for electronic transitions and results in an increase in the wavelength at which a chromophore absorbs. This is referred to as a bathochromic shift, whereas a decrease in conjugation, caused for example by protonating a ring nitrogen atom, causes a hypochromic shift which leads to a decrease in wavelength. Hyperchromic and hypochromic effects refer to an increase and a decrease in absorbance respectively.



Instrumentation.

To obtain an absorption spectrum, the absorbance of a substance must be measured at a series of wavelengths. Absorption in the visible and ultraviolet regions can be measured by a UV/visible spectrophotometer. UV/Vis spectrometers consist of three basic components, (i) a light source and a mechanism to select a specific wavelength of light in the UV/visible region of the spectrum, (ii) a chamber where a cuvette containing a test solution can be introduced into the light path, and (iii) a photocell that can determine the amount of light absorbed by the sample (or the intensity of light transmitted through the sample).



The light source is usually a tungsten lamp for the visible region of the spectrum, and either ahydrogen or deuterium lamp for ultraviolet wavelengths. Cuvettes are optically transparent cells that hold the material(s) under study and are used to introduce samples into the light path. A reference cuvette optically identical to, and containing the same solvent (and impurities) as the test cuvette is always required for setting the spectrophotometer to read zero absorbance at each wavelength used. For accurate work, the optical matching of the two cuvettes should always be checked. Glass and plastic absorb strongly below 310 nm and are not useful for measuring absorbance below that wavelength. Quartz or silica cells are used when measuring absorption of ultraviolet wavelengths by a solution since they are transparent to wavelengths greater than 180 nm.

Application

- UV/Vis spectroscopy is routinely used in analytical chemistry for the quantitative determination of different analyses, such as transition metal ions, highly conjugated organic compounds, and biological macromolecules. Spectroscopic analysis is commonly carried out in solutions but solids and gases may also be studied.
- Solutions of transition metal ions can be colored (i.e., absorb visible light) because d electrons within the metal atoms can be excited from one electronic state to another.
- The colour of metal ion solutions is strongly affected by the presence of other species, such as certain anions or ligands. For instance, the colour of a dilute solution of copper sulfate is a very light blue; adding ammonia intensifies the colour and changes the wavelength of maximum absorption (λmax).

- Organic compounds, especially those with a high degree of conjugation, also absorb light in the UV or visible regions of the electromagnetic spectrum. The solvents for these determinations are often water for water-soluble compounds, or ethanol for organic-soluble compounds. (Organic solvents may have significant UV absorption; not all solvents are suitable for use in UV spectroscopy. Ethanol absorbs very weakly at most wavelengths.)
- Solvent polarity and pH can affect the absorption spectrum of an organic compound. Tyrosine, for example, increases in absorption maxima and molar extinction coefficient when pH increases from 6 to 13 or when solvent polarity decreases.
- While charge transfer complexes also give rise to colours, the colours are often too intense to be used for quantitative measurement.
- The wavelengths of absorption peaks can be correlated with the types of bonds in a given molecule and are valuable in determining the functional groups within a molecule.
- The spectrum alone is not, however, a specific test for any given sample. The nature of the solvent, the pH of the solution, temperature, high electrolyte concentrations, and the presence of interfering substances can influence the absorption spectrum. Experimental variations such as the slit width (effective bandwidth) of the spectrophotometer will also alter the spectrum.
- To apply UV/Vis spectroscopy to analysis, these variables must be controlled or accounted for in order to identify the substances present.

Mass Spectroscopy

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:

The Ion Source: A small sample is ionized, usually to cations by loss of an electron.

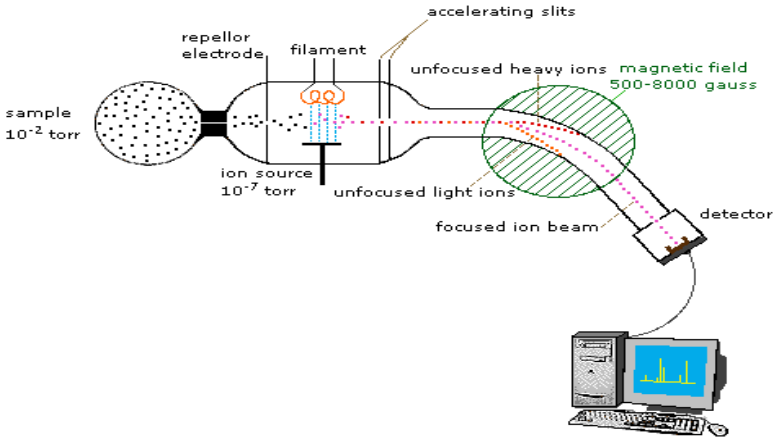
The Mass Analyzer: The ions are sorted and separated according to their mass and charge.

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

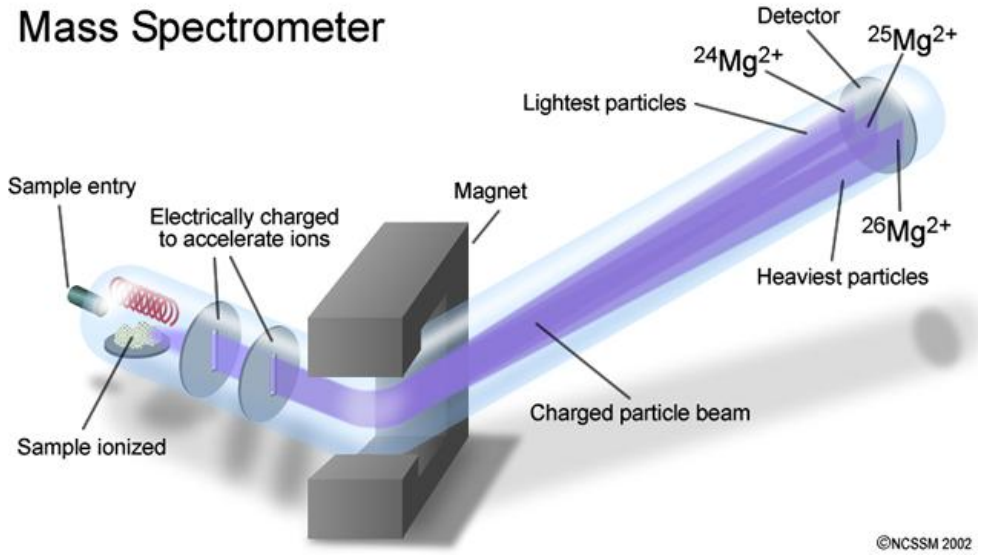
is achieved by accelerating and focusing the ions in a beam, which is then bent by an external magnetic field. The ions are then detected electronically and the resulting information is stored and analyzed in a computer. A mass spectrometer operating in this fashion is outlined in the following diagram. 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).



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 (colored red in the following diagram). 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. An animated display of this ionization process will appear if you click on the ion source of the mass spectrometer.



Nature of Mass Spectra

A mass spectrum will usually be presented as a vertical bar graph, in which each bar represents an ion having a specific mass-to-charge ratio (m/z) and the length of the bar indicates the relative abundance of the ion. The most intense ion is assigned an abundance of 100, and it is referred to as the base peak. Most of the ions formed in a mass spectrometer have a single charge, so the m/z value is equivalent to mass itself. Modern mass spectrometers easily distinguish (resolve) ions differing by only a single atomic mass unit (amu), and thus provide completely accurate values for the molecular mass of a compound. The highest-mass ion in a spectrum is normally considered to be the molecular ion, and lower-mass ions are fragments from the molecular ion, assuming the sample is a single pure compound.

Applications:

- Mass spectrometry has both qualitative and quantitative uses. These include identifying unknown compounds, determining the isotopic composition of elements in a molecule, and determining the structure of a compound by observing its fragmentation.
- Other uses include quantifying the amount of a compound in a sample or studying the fundamentals of gas phase ion chemistry (the chemistry of ions and neutrals in a vacuum).
- MS is now in very common use in analytical laboratories that study physical, chemical, or biological properties of a great variety of compounds.

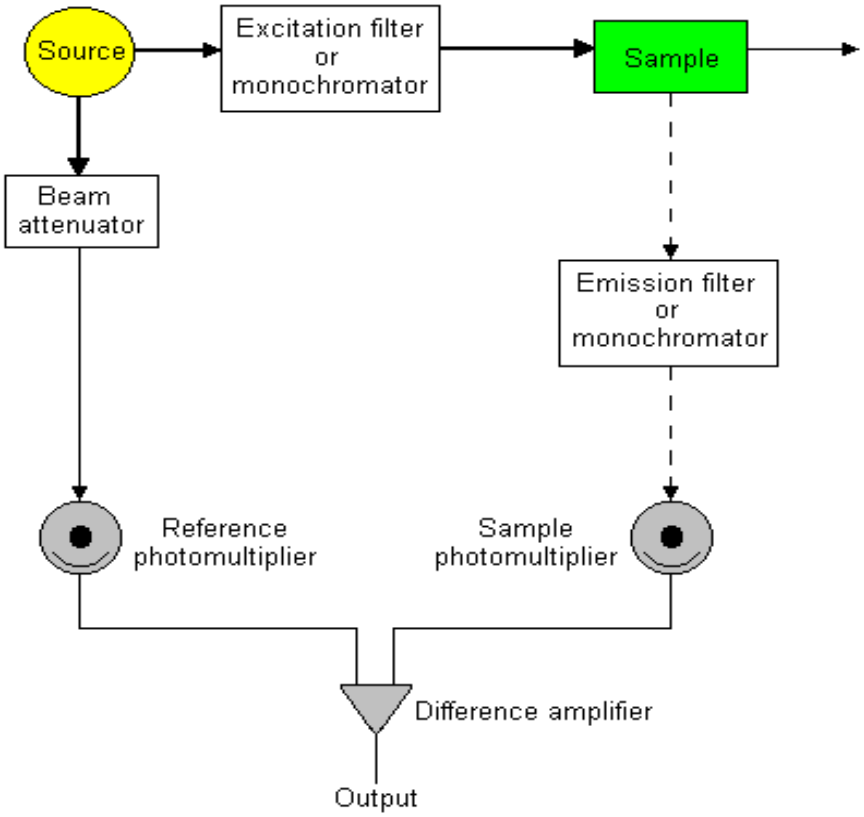
- As an analytical technique it possesses distinct advantages such as:

  - Increased sensitivity over most other analytical techniques because the analyzer, as a mass-charge filter, reduces background interference
  - Excellent specificity from characteristic fragmentation patterns to identify unknowns or confirm the presence of suspected compounds.
  - Information about molecular weight.
  - Information about the isotopic abundance of elements.
- Temporally resolved chemical data.

- A few of the disadvantages of the method is that often fails to distinguish between optical and geometrical isomers and the positions of substituent in o-, m- and p- positions in an aromatic ring.
- Also, its scope is limited in identifying hydrocarbons that produce similar fragmented ions.
- Mass spectrometry is also used to determine the isotopic composition of elements within a sample.
- Differences in mass among isotopes of an element are very small, and the less abundant isotopes of an element are typically very rare, so a very sensitive instrument is required.

Spectrofluorimeter

A spectrofluorimeter is an instrument which takes advantage of fluorescent properties of some compounds in order to provide information regarding their concentration and chemical environment in a sample. A certain excitation wavelength is selected, and the emission is observed either at a single wavelength, or a scan is performed to record the intensity versus wavelength, also called an emission spectra.



Instrumental components

Sources

Generally, the source must be more intense than that required for UV-Vis. absorption spectroscopy; magnitude of the emitted radiation is directly proportional to the power of the source.

Filter fluorometers often employ a low-pressure mercury vapour lamp. This source produces intense lines at certain wavelengths. One of these lines will usually be suitable for excitation of a fluorescent sample.

Spectrofluorometers, which need a continuous radiation source, are often equipped with a 75-450 W high-pressure xenon arc lamp.

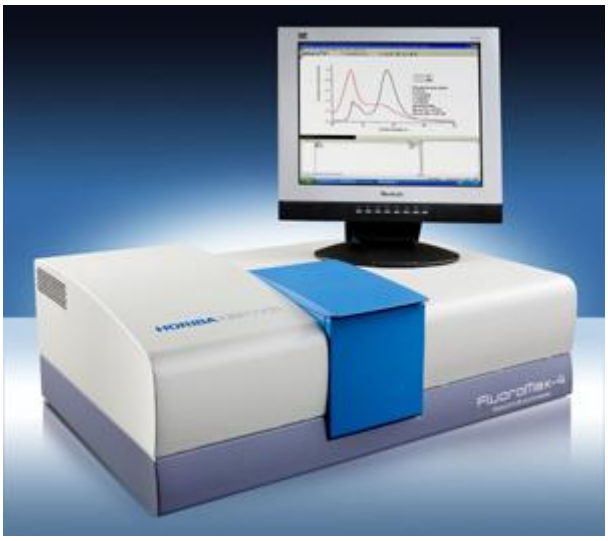
Lasers are sometimes used as excitation sources. A tunable dye laser, using a pulsed nitrogen laser as the primary source can produce monochromatic radiation between 360 and 650 nm. Since the radiation produced *is* monochromatic, there is no need for an excitation monochromator.

Filters and monochromators

Fluorometers use either interference or absorption filters. Spectrofluorometers are usually fitted with grating monochromators.

Detectors

Fluorescence signals are usually of low intensity. Photomultiplier tubes are in common use as detectors. Diode-array detectors are sometimes used.

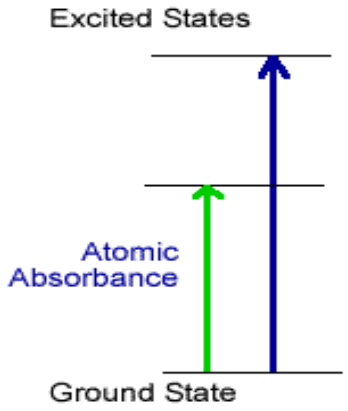


Applications:

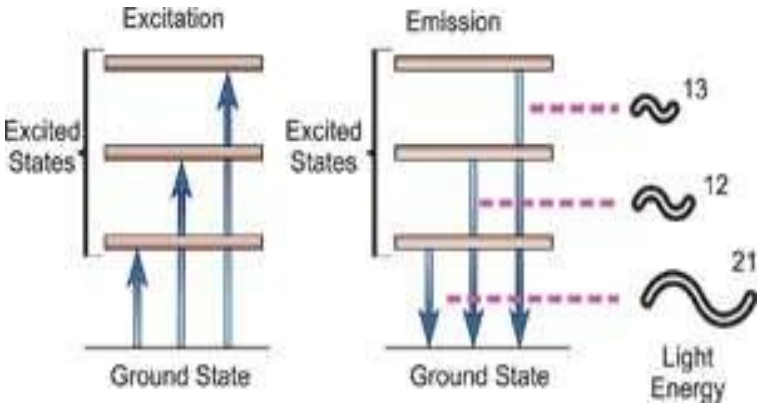
- Generally, spectrofluorometers use high intensity light sources to bombard a sample with as many photons as possible.
- This allows for the maximum number of molecules to be in an excited state at any one point in time.
- The light is either passed through a filter, selecting a fixed wavelength, or a monochromator, which allows a wavelength of interest to be selected for use as the exciting light.

Atomic Spectroscopy

Atomic spectroscopy exploits different energetic transitions experienced by atoms that are associated with either the absorption or emission of photons. When these transitions involve the excitation and relaxation of the valence (outer or bonding) shell electrons of metal atoms and ions, the corresponding photons have energies within the ultraviolet and visible regions of the spectrum. A good example of this is the dark absorption lines in the solar spectrum, which are caused by heavier elements present in the outer layers of the sun.



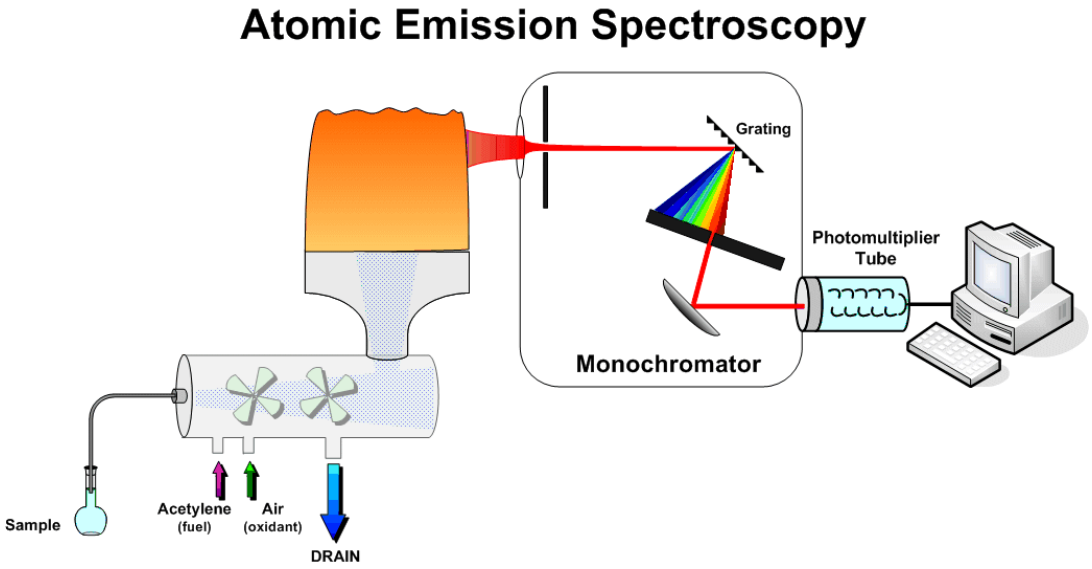
The figure shows a high energy photon with Ephoton = hν being absorbed, resulting in a 2s→3s electron excitation; similarly, a 3d→3p electron relaxation results in the emission of a lower energy photon. By convention, the change in electron energy ΔE = Ef − Ei, where f and i refer to the final and initial states, respectively; so ΔE = Ephoton, and the sign of Ephoton tells you whether the photon is being absorbed or emitted. Since Ef and Ei depend on the number electrons and protons within an atom (or monatomic ion), the wavelengths associated with atomic absorption and emission are considered characteristic for a particular element.



Absorption and Emission:

In atomic absorption (AA) spectroscopy, absorption of a photon results in excitation of an electron from a lower to higher energy atomic orbital (AO). An instrument measures the absorbance, A, which is defined as the logarithm of the ratio of incident to transmitted radiant power of the photon beam, A = log(P0 ÷ P), at a wavelength specific to the element of interest. Samples are typically analysed using a flame atomic absorption spectrophotometer.

In atomic emission (AE) spectroscopy, thermal or electrical energy from an arc, flame, spark, or plasma is used to excite and electron from a lower to higher energy AO; when the excited electron returns to its original AO (i.e. the ground state), it may do so by emitting a photon. The instrument measures the intensity, I, of these emitted photons as a function of wavelength.



Because AO energies are well-defined, atomic absorption and emission spectra consist of discrete, narrow lines. This allows the concentration of metallic elements in different samples to be determined selectively, with lower limits at or below 1 mg/L (1 ppm). Techniques such as graphite furnace atomic absorption spectrophotometry (GFAAS) allow concentration to be measured down to µg/L (ppb) levels. Actual limits-of-detection vary withelement, technique, and sample matrix.

Applications:

- Atomic spectroscopy has many useful applications. Since the emission spectrum is different for every element, it acts as an atomic fingerprint by which elements can be identified.
- Some elements were discovered by the analysis of their atomic spectrum.
- Helium, for example, was discovered while scientists were analyzing the absorption spectrum of the sun.
- Emission spectrum is especially useful to astronomers who use emission and absorption spectra to determine the makeup of far away stars and other celestial bodies.

By which optical phenomenon does the splitting of white light into seven constituent colours occur?	Refraction	Reflection	Dispersion	Interference	Dispersion
Which colour of light deviates maximum in the dispersion of white light by prism?	Violet	Blue	Green	Red	Violet
Which of the following are the primary colours of light?	Red, Blue, Yellow	Red, Green, Violet	Yellow, Green, Blu	Red, Green, Blue	Red, Green, Blue
A colorimeter is used to _____.	determine the heat of a	determine the heat s	store the heat from	determine the heat of	determine the heat given off/
The amount of light is absorbed is directly proportional to the length of the medium through which the l	Beer's law	Lambert's law	Beer and Lambert's	Planck's law	Lambert's law
The intensity of the color is directly proportional to the concentration of the colored particles in the solution.	Beer's law	Lambert's law	Beer and Lambert's	Planck's law	Beer's law
The correct order for the basic features of a mass spectrometer is_____	acceleration, deflection	ionisation, accelerat	acceleration, ionisa	acceleration, deflecti	ionisation, acceleration, defle
Which one of the following statements about ionisation in a mass spectrometer is incorrect?	gaseous atoms are ioni	atoms are ionised s	atoms are ionised s	it doesn't matter how	it doesn't matter how much e
Beer's Law states that	absorbance is proportic	absorbance is propo	absorbance is equa	absorbance is equal to P/P0	absorbance is proportional to
A UV-VIS spctrophotometer has ------light sources.	Two	Three	One	Four	Two
In colorimetry, Beer-Lamberts law is used to evaluate_____.	quantitative measurem	qualitative measure	absorbance measur	adsorption spectrum	quantitative measurements
According to the Beer-Lambert law, on which of the following does absorbance NOT depend?	Colour of the solution	Solution concentrat	Distance that the li	Extinction coefficien	Colour of the solution
What is the name of an instrument used to measure the absorbance of a coloured compound in solution	Coulometer	Colourmeter	Colorimeter	Calorimeter	Colorimeter
The optically transparent cells are made up of glass/ plastic/quartz for spectrophotometry are --------.	Cuvettes	test tubes	Vials	Microtip	Cuvettes
The source of visible radiation in spectrophotometer is_____ lamp.	hydrogen	deuterium	tungsten filament	mercury	hydrogen
Flourimeter employs a ----- vapour lamp.	Tungsten	Hydrogen	Mercury	Deuterium	Mercury
Cuvettes used in spectrophotometer is having an optical path length of_____ cm.	2	3	0.5	1	1
Which of the following is used as light source in colorimeter?	Hydrogen lamp	Deuterium lamp	Tungsten lamp	Sodium lamp	Tungsten lamp
In colorimeter, the bandwidth is selected by_____.	filter	monochromotor	prism	gratings	filter
Lambert's law is applicable to_____ solution.	concentrated	dilute	very dilute	both concentrated ar	concentrated
Colorimetry is a form of _____.	Photometry	Electrophoresis	Chromatography	Spectrofluorimetry	Photometry
_____ is a spectroanalytical procedure for the quantitative determination of chemical elemen	Atomic absorption spe	Spectrofluorimetry	Chromatography	Electrophoresis	
Atomic absorption spectrometry analyze _____ in biological fluids	metals	pigments	metabolites	compound	metals
The absorbed wavelengths in atomic absorption spectrum appear as	dark background	dark lines	light background	light lines	dark lines
The lines which appear in absorption and emission spectrum are	same	different	very different	far apart	same
The background in atomic absorption spectrum is	bright	dark	brown	purple	bright
Ionization of analyte atoms in flame/plasma can be suppressed by:	EDTA or other comple	Addition of KCl to	Addition of oxyanic	Internal standards me	Addition of KCl to the matri
The visible portion of the electromagnetic spectrum occurs between _____ and _____ nm	1 and 10	10 and 100	400 and 740	800 and 1200	400 and 740
Which part of the spectrophotometer is adjusted to select the desired wavelength?	light source	filter	sample	photodetector	filter
Which part of the spectrophotometer is adjusted to select the desired wavelength?	the lowest wavelength	the highest wavelen	the wavelength at w	the wavelength at wh	the wavelength at which the
Using a standard curve, if you know the absorbance of an unknown sample, what else can be determined about the unknown?	the wavelength of maxi	the molecular weig	the concentration o	the identify of the sar	the concentration of the samp
Blank contains the ___ but not the dissolved chemical.	Solvent	solute	filter	absorption spectrum	Solvent
Basically, what is the function of an absorption spectrum?	It converts light energy	It is a graph of a ch	It is a graph of a ch	It is the amount of radiation retained by a sample	It is a graph of a chemical rel
Basically, what is the path of light through a spectrophotometer?	meter, photodetector, f	meter, filter sample	light source, filter, s	light source, sample,	light source, filter, sample, pl
The ratio of transmitted light (I) to that of incident light (I_o) is referred to as	transmittance	absorbance	incidence	radiance	transmittance
The working of colorimeter is based on the principle of	Beer's law	Lambert's law	Beer - Lambert's L	Planck's law	Beer - Lambert's Law
The spectrophotometer primarily differs from colorimeter by covering the ultraviolet region of the electromagnetic spectrum	200-400 nm	400-800 nm	300-700 nm	400-500 nm	200-400 nm
Which of the following techniques would be used to detect a metabolite labelled with ^2H?	Infra red spectroscopy	Nuclear magnetic r	Scintillation counti	Mass spectrometry	Mass spectrometry
_____ is used to determine the structural formula of an unknown chemical compound	Infra red spectroscopy	Nuclear magnetic r	Scintillation counti	Mass spectrometry	Mass spectrometry
Who was credited for the invention of the mass spectrometer?	J.J. Thompson	Ernest Rutherford	James Chadwick	Neils Bohr	J.J. Thompson
Visible wavelengths cover a range from approximately	400 to 800 nm	300 to 700 nm	200 to 700 nm	300 to 600 nm	400 to 800 nm
_____ has a shorter wavelength than the visible violet light	Ultraviolet radiation	Infrared radiation	visible spectrum	electromagnetic spec	Ultraviolet radiation
_____ has a longer wavelength than visible red light	Ultraviolet radiation	Infrared radiation	visible spectrum	electromagnetic spec	Infrared radiation
Sunlight consists of the entire	Ultraviolet radiation	Infrared radiation	visible spectrum	electromagnetic spec	electromagnetic spectrum
The visible _____ light has a wavelength of about 590 nm	Orange	Red	violet	indigo	Orange
The visible _____ light has a wavelength of about 570 nm	yellow	red	violet	indigo	yellow
The visible _____ light has a wavelength of about 510 nm	green	red	violet	indigo	green
The visible _____ light has a wavelength of about 650 nm	green	red	violet	indigo	red
The visible _____ light has a wavelength of about 475 nm	green	Blue	violet	indigo	blue
The visible _____ light has a wavelength of about 445 nm	green	blue	violet	indigo	indige
The visible _____ light has a wavelength of about 400 nm	green	Blue	violet	indigo	Violet
The _____ light is a mixture of the colors of the visible spectrum	green	white	violet	indigo	white
The visible colors from shortest to longest wavelength are:	violet, blue, green, yell	blue, violet, green,	green, violet, blue,	violet	violet, blue, green, yellow, o
Light with such short wavelengths is called	Ultraviolet light	Infrared light	visible spectrum	electromagnetic spec	Ultraviolet light
Energy with wavelengths too short to see is	bluer than blue	redder than red	greener than green	brighter than bright	
The visible indigo light has a wavelength of about _____ nm	200	800	700	445	445
The visible blue light has a wavelength of about _____ nm.	800	475	445	425	475
The visible red light has a wavelength of about _____ nm	650	475	445	425	650
The visible orange light has a wavelength of about _____ nm	590	475	445	425	590
The visible violet light has a wavelength of about _____ nm	400	475	445	425	400

absorbed during some process

ection, detection
nergy you use to ionise the atoms

both the path length and concentration of the absorbing species

x

chemical's absorption value is the lowest

oles

lating the absorbance to wavelength
hotodetector, meter

range, and red

KARPAGAM ACADEMY OF HIGHER EDUCATION
(Deemed University Established Under Section 3 of UGC Act 1956)
Coimbatore - 641021.
(For the candidates admitted from 2015 onwards)
DEPARTMENT OF BIOCHEMISTRY

SUBJECT : BIOINSTRUMENTATION AND GLP
SEMESTER : I
SUBJECT CODE : 17BCP103 CLASS : I M.Sc.BC

UNIT II Chromatography: Principles, Types – paper chromatography, thin layer chromatography and HPTLC, Column chromatography - Ion exchange chromatography, affinity chromatography, gel filtration chromatography, Low pressure liquid chromatography (LPLC) and High Performance Liquid Chromatography (HPLC)- Normal and Reverse Phase Gas -liquid chromatography Mass spectroscopy (GC – MS), LC-MS, MALDI-TOF, ICPMS, Application of Chromatography. Separation of phytoconstituents using TLC.

Definition:

- Chromatography (from Greek χρῶμα chroma "color" and γράφειν graphein "to write") is the collective term for a set of laboratory techniques for the separation of mixtures.
- The mixture is dissolved in a fluid called the "mobile phase", which carries it through a structure holding another material called the "stationary phase".
- The various constituents of the mixture travel at different speeds, causing them to separate.
- The separation is based on differential partitioning between the mobile and stationary phases.
- Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus changing the separation.
- Chromatography may be preparative or analytical.
- The purpose of preparative chromatography is to separate the components of a mixture for more advanced use (and is thus a form of purification).
- Analytical chromatography is done normally with smaller amounts of material and is for measuring the relative proportions of analytes in a mixture. The two are not mutually exclusive.

THE PRINCIPLE OF CHROMATOGRAPHY:

- In chromatography a liquid is pumped through a bed of particles.
- The liquid is called the mobile phase and the particles the stationary phase.
- A mixture of the molecules that shall be separated is introduced into the mobile phase.
- In the animation below the blue molecules shall be separated from the red molecules and a mixture containing these two types of molecules is introduced into the mobile phase in front of the stationary phase.
- The mixture of the red and blue molecules is then transported by the mobile phase through the stationary phase.
- The molecules in the mixture that adsorbs the most to the stationary phase, in this particular case the red molecules, is moving slowest through the particle bed. The red molecules become separated from the blue.

Partition co-efficient:In the physical sciences, a partition-coefficientis the ratio of concentrations of a compound in a mixture of two immisciblephases at equilibrium. These coefficients are a measure of the difference in solubility of the compound in these two phases. The two phases are often restricted to mean two immiscible solvents. In this context, a partition coefficient is the ratio of concentrations of a compound in the two phases of a mixture of two immiscible liquids at equilibrium.Normally one of the solvents chosen is aqueous while the second is hydrophobic such as 1-octanol. Hence both the partition and distribution coefficient are measures of how hydrophilic ("water-loving") or hydrophobic ("water-fearing") a chemical substance is. Partition coefficients are useful in estimating the distribution of drugs within the body. Hydrophobic drugs with high octanol/water partition coefficients are preferentially distributed to hydrophobic compartments such as the lipid bilayers of cells while hydrophilic drugs (low octanol/water partition coefficients) preferentially are found in aqueous compartments such as blood serum.

TYPES OF CHROMATOGRAPHY:

Adsorption Chromatography

- Adsorption chromatography is probably one of the oldest types of chromatography around.

- It utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase.
- The equilibration between the mobile and stationary phase accounts for the separation of different solutes.

Partition Chromatography

- This form of chromatography is based on a thin film formed on the surface of a solid support by a liquid stationary phase.
- Solute equilibriates between the mobile phase and the stationary liquid.

Ion Exchange Chromatography

- In this type of chromatography, the use of a resin (the stationary solid phase) is used to covalently attach anions or cations onto it.
- Solute ions of the opposite charge in the mobile liquid phase are attracted to the resin by electrostatic forces.

Molecular Exclusion Chromatography

- Also known as gel permeation or gel filtration, this type of chromatography lacks an attractive interaction between the stationary phase and solute.
- The liquid or gaseous phase passes through a porous gel, which separates the molecules according to its size.
- The pores are normally small and exclude the larger solute molecules, but allow smaller molecules to enter the gel, causing them to flow through a larger volume.
- This causes the smaller molecules to pass through the column at a faster rate than the larger molecules.

Affinity Chromatography

- This is the most selective type of chromatography employed.
- It utilizes the specific interaction between one kind of solute molecule and a second molecule that is immobilized on a stationary phase.
- For example, the immobilized molecule may be an antibody to some specific protein.
- When this molecule passes solutes containing a mixture of proteins, only the specific protein is reacted to this antibody, binding it to the stationary phase.
- This protein is later extracted by changing the ionic strength or pH.

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.
- Removing liquid flows between the fibers of the cellulose but these are not the stationary phase.
- The true stationary phase is the very thin film of liquid usually water adhering o the surface of the fibers. (Water is adsorbed on the fibers/ cellulose by strong hydrogen bonds with –OH of the cellulose).
- The substrate to be separated is distributed between the two liquids, stationary liquid that is held on the fibers of the paper and moving liquid in developing solvent.
- It uses a strip of paper and capillary action is used to pull the solvents up through the paper to separate the solutes.
- A small concentrated spot of solution that contains the sample is applied to a strip of chromatography paper about 2 cm away from the base of the plate, usually using a capillary tube for maximum precision.
- This sample is absorbed onto the paper and may form interactions with it.
- Any substance that reacts or bonds with the paper cannot be measured using this technique.

- The paper is then dipped in to a suitable solvent, such as ethanol or water, taking care that the spot is above the surface of the solvent, and placed in a sealed container.

- The solvent moves up the paper by capillary action, which occurs as a result of the attraction of the solvent molecules to the paper, also this can be explained as differential absorption of the solute components into the solvent.

- As the solvent rises through the paper it meets and dissolves the sample mixture, which will then travel up the paper with the solvent solute sample.

- Different compounds in the sample mixture travel at different rates due to differences in solubility in the solvent, and due to differences in their attraction to the fibers in the paper.

- This method has been largely replaced by thin layer chromatography

SEPARATION OF AMINO ACIDS BY ASCENDING CHROMATOGRAPHY:

- Cut the chromatography sheet carefully to a convenient size (40 x 24cm). Draw a line with pencil across the sheet about 5cm away from one end. Mark a number of points at intervals of 3cm.

- Apply a small volume (say, 25mL) of each amino acid as a separate small spot using a microsyringe. A stream of hot air from a hair-dryer facilitates fast drying of spot. The spot should be as small as possible for better resolution.

- Similarly spot different known aliquots of sample extract.

- After spotting, place the sheet in a stainless steel trough in the chromatography chamber; firmly hold it by placing a long steel rod over the sheet. The spot-end of the sheet should be in the trough (descending chromatography). Otherwise, the sheet may be rolled as a cylinder, tied together with fine thread and placed upright with the spots as the bottom in a large Petridish for upward movement of solvent (ascending chromatography).

- Add the organic (phase) solvent to the trough/petri dish and close the chamber airtight. Develop the chromatogram, preferably overnight or longer, until the solvent moves almost to the other end.

- Note the solvent front and dry the chromatogram free of solvent in a fume chamber.

- Spray the chromatogram with the ninhydrin reagent using an atomizer. Dry the paper for about 5 min at room temp followed by at 100°C in an oven for 2-3 min.

Amino acids appear as purple spots; hydroxyproline and proline give yellow colored spots.Mark all the spots and calculate their Rfvalues by the formula.

Rf = Distance (cm) moved by the solute from the origin

Distance (cm) moved by the solvent from the origin

- The amino acids present in the sample are then identified by comparing the Rf values with that of the authentic amino acids, co-chromatographed.
- For quantitative estimation, cut each spot into several small bits and transfer to the bottom of the test tube. Add 3mL of elution mixture. Shake the tubes vigorously for 15 min. Decant the liquid and elute the pieces with another 2mL of elution mixture. Repeat the elution with small aliquots until the bits are colorless. Combine and clear the eluate by centrifuging at 10,000rpm for 10 min. Read the intensity of purple color at 570nm in a colorimeter. Use the spot of leucine (50mg) run as standard for comparison.

THIN LAYER CHROMATOGRAPHY:

- The surface of the silica gel is polar and, because of the -OH groups, can form hydrogen bonds with suitable compounds around it as well as Van der Waals dispersion forces and dipole-dipole attractions.
- The other commonly used stationary phase is alumina - aluminium oxide. The aluminium atoms on the surface of this also have -OH groups attached.
- Spot the material at the origin (bottom) of the TLC plate.
- Place the plate into a glass jar with a small amount of a solvent in the glass jar.
- This solvent acts as the moving phase.
- Remove the plate from the bottle when the solvent is close to the top of the plate.

Visualization of the spots

- Non-polar compounds are less strongly attracted to the plate and spend more time in the moving phase.
- This compound will move faster and will appear closer to the top of the plate.

- Polar compounds will be more strongly attracted to the plate and will spend less time in the moving phase and appear lower on the plate.
- It is used to detect pesticide or insecticide residues in food.
- Thin-layer chromatography is also used in forensics to analyze the dye composition of fibers.

PRINCIPLE AND SEPARATION OF PHOSPHOLIPIDS:

Principle:

- Similar to other chromatographic methods TLC is also based on the principle of separation.
- The separation depends on the relative affinity of compounds towards stationary and mobile phase.
- The compounds under the influence of mobile phase (driven by capillary action) travel over the surface of stationary phase.
- During this movement the compounds with higher affinity to stationary phase travel slowly while the others travel faster.
- Thus separation of components in the mixture is achieved.
- Once separation occurs individual components are visualized as spots at respective level of travel on the plate.
- Their nature or character are identified by means of suitable detection techniques

Separation of phospholipids:

- A particularly frequent approach is to obtain information on the various phospholipids components of the lipid extract under investigation.
- This can be achieved easily and efficiently by subjecting a sample to thin-layer chromatography (TLC).
- There is a huge number of TLC techniques described for that purpose, some of them using one-dimensional migration others using two successive migrations in two orthogonal directions (two-dimensional).

- We describe below some techniques that have proven easy and reliable in our hands but others could be found in specialized books and scientific journals.
- The proposed simple one-dimensional TLC procedure is routinely used in our laboratory and may be considered as efficient as common two-dimensional techniques.
- It has the peculiarity to allow a good separation of phosphatidylinositol and phosphatidylserine owing to the impregnation of TLC plates with boric acid.
- Other one-dimensional techniques are used for the separation of less frequent phospholipid forms (polyphosphoinositides, mono-and dimethyl phosphatidylethanolamine) or for quantitative estimation by densitometry.
- For some specialized purposes, a two-dimensional procedure is also described.

ADSORPTION CHROMATOGRAPHY:

- Chromatography in which separation is based mainly on differences between the adsorption affinities of the sample components for the surface of an active solid.

Chromatography Adsorbents Description

- Chromatography adsorbents are regularly used in pharmaceutical & chemical manufacturing units, where prime concern of chromatography is to make final pure compounds or make an impurity profile studies for pharmaceutical & Herbal products.
- Column chromatography techniques help in extractions, synthesis & purifications of natural products & Active Pharmaceutical Ingredients.
- Sorbead India with technical tie up with Swambe chemicals manufactures various types of Silica & alumina for chromatographic separations, with an aim to offer various particle sizes & various pore diameters products, which is required for complex chromatographic separations.
- The Silica Gel & Alumina Brockmann I-II grades, which are manufactured, are as Normal phase, Flash grade & gravity grade with different pore diameters.
- Alumina is being offered as basic, acidic and neutral grades.
- Sorbead India measures the pore diameter using the BET method, which guarantees the best and most accurate results during manufacturing process.
- Sorbead manufactures the following chromatography Adsorbents for industrial and laboratory use

1. Aluminum Oxide- Aluminum oxide is a whitish colored powder which is used for chromatography and is highly porous and water adsorbing. With a bulk density of 800-920 gms per liter and a surface area of 180-240 square meters per gram, these adsorbents are suited for various applications like column chromatographic separations, Food Colours, Dyes & Spectroscopic solvents, Herbal extractions of Natural products, isolation and antibiotics purification.

2. Silica Gels – Silica gels have a different mesh size, which are between 35-800. The Silica gel particle size distribution offers an excellent flow rates and a high level of stability. The typical surface area of these gels is 400-800 square meters per gm. Sorbead India adopts a highest quality standard for such a sensitive & accurate product, which ensures a batch-to-batch reproducible result for any chromatographic separations.

Separation:

- Classical column chromatography and TLC were originally used for determination of carotenoids.
- However, these methods are time-consuming, need large sample amounts, their separation efficiency is not particularly high, and they suffer from a poor reproducibility of results and low recoveries of the analytes.
- Among the high-performance separation methods, gas chromatography (GC) is unsuitable, primarily because of low volatility and thermo ability of Carotenoids (however, the volatility of carotenoids can be increased by reducing the double bonds).
- Capillary zone electrophoresis (CZE) is inapplicable because of the absence of charge on the carotenoid molecules.

Adsorption Chromatography

- Therefore, the most common method used in the analysis of carotenoids is HPLC employing various detection techniques.
- Bothnormal- and reversed-phase systems are used, either in isocratic or gradient elution modes.
- Reversed-phase systems several disadvantages, namely, lower column stability.
- In reversed-phase systems, non-aqueous mobile phases are recommended, in view of the pronounced hydrophobicity of carotenoids that makes their separations in mobile phases

containing water difficult or impossible. Various mixtures of Solvents, mostly of methanol, acetonitrile and tetrahydrofuran, have been successfully applied to the purpose.

- Antioxidants, such as BHT, are added to the mobile phase, and the temperature of the HPLC column should be maintained low and constant (around 20 0C), to prevent decomposition of carotenoid samples during the HPLC analysis and improve the reproducibility of quantitative analysis.

- An example is the analysis of carotenoids in orange juice (a- and b-carotenes, lutein, zeaxanthin, b-cryptoxanthin) on a C-18 reversed phase, using a ternary mobile phase consisting of a mixture of acetonitrile - methanol – dichloromethane (60:35:5), with additions of the BHT antioxidant (0.1 %), triethylamine (0.1 %), and ammonium acetate (0.05 mol.L-1 solution in methanol).

- Triethylamine and ammonium acetate minimize the effects of acidity generated by the free silanol groups present on the silica support.

- One of the great problems of carotenoid analyses lies in the unavailability of standard compounds caused by natural instability of carotenoids.

- Some carotenoids, e.g., b-carotene, can be obtained commercially but their purity is insufficient for their use as chromatographic standards and thus they must be purified under spectrophotometric control.

- However, the purity of standard solutions must also be verified chromatographically (HPLC).

- The importance of certified materials in analyses for carotenoids in vegetables has been emphasized in an inter laboratory study.

- Carotenoids can be purified using classical column chromatography on alumina, silica gel, magnesium oxide or carbonate, calcium hydroxide or carbonate, Cellite and further adsorbents, with various solvent systems.

- Preparative TLC can be used but preparative HPLC is most common Degradation and isomerization of carotenoids must be prevented during the preparation of standards, by preparing and storing stock solutions in darkness, at -20oC, under a protective atmosphere (nitrogen, argon) and using solvents containing antioxidants.

- An interesting procedure, based on recrystallization and fractional dissolution, has been used for the obtaining of pure all-trans b-carotene.

- This paper also describes the spectral characteristics for the pure and a partially degraded product and points out a high reactivity with atmospheric and dissolved oxygen. The procedure is based on recrystallization and fractional dissolution.

- So far the best separations of various carotenoids have been attained on a C-30 chemically bonded phase.

ION EXCHANGE CHROMATOGRAPHY:

- Imagine if we had a tube whose surfaces were coated with an immobilized cation.

- These would have electrostatic attraction for anions.

- If a solution containing a mixture of positively and negatively charged groups flows through this tube, the anions would preferentially bind, and the cations in the solution would flow through this is the basis of ion exchange chromatography.

- The example above is termed "anion exchange" because the inert surface is interacting with anions

- If the immobile surface was coated with anions, then the chromatography would be termed "cation exchange" chromatography (and cations would selectively bind and be removed from the solution flowing through strength of binding can be affected by pH, and salt concentration of the buffer.

- The ionic species "stuck" to the column can be removed (i.e. "eluted") and collected by changing one of these conditions.

- For example, we could lower the pH of the buffer and protonate anions.

- This would eliminate their electrostatic attraction to the immobilized cation surface. Or, we could increase the salt concentration of the buffer, the anions in the salt would "compete off" bound anions on the cation surface.

PRINCIPLE:

- To optimize binding of all charged molecules, the mobile phase is generally a low to medium conductivity (i.e., low to medium salt concentration) solution.

- The adsorption of the molecules to the solid support is driven by the ionic interaction between the oppositely charged ionic groups in the sample molecule and in the functional ligand on the support.
- The number and location of the charges on the molecule and on the functional group determine the strength of the interaction.
- By increasing the salt concentration (generally by using a linear salt gradient) the molecules with the weakest ionic interactions start to elute from the column first.
- Molecules that have a stronger ionic interaction require a higher salt concentration and elute later in the gradient.
- The binding capacities of ion exchange resins are generally quite high.
- This is of major importance in process scale chromatography, but is not critical for analytical scale separations.

Buffer pH

- As a rule, the pH of the mobile phase buffer must be between the pI (isoelectric point) or pKa (acid dissociation constant) of the charged molecule and the pKa of the charged group on the solid support.
- For example, in cation exchange chromatography, using a functional group on the solid support with a pKa of 1.2, a sample molecule with a pI of 8.2 may be run in a mobile phase buffer of pH 6.0.
- In anion exchange chromatography a molecule with a pI of 6.8 may be run in a mobile phase buffer at pH 8.0 when the pKa of the solid support is 10.3.

Salt Gradients

- As in most other modes of chromatography (SEC being the exception) a protein sample is injected onto the column under conditions where it will be strongly retained.
- A gradient of linearly increasing salt concentration is then applied to elute the sample components from the column.
- An alternative to using a linear gradient is to use a step gradient.
- This requires less complicated equipment and can be very effective to elute different fractions if the appropriate concentrations of salt are known, usually from linear gradient experiments.

Varying pH

- Many chromatographers also use changes in pH to affect a separation.
- In cation exchange chromatography, raising the pH of the mobile phase buffer will cause the molecule to become less protonated and hence less positively charged.
- The result is that the protein no longer can form an ionic interaction with the negatively charged solid support, which ultimately results in the molecule to elute from the column.
- In anion exchange chromatography, lowering the pH of the mobile phase buffer will cause the molecule to become more protonated and hence more positively (and less negatively) charged.
- The result is that the protein no longer can form an ionic interaction with the positively charged solid support, which causes the molecule to elute from the column.

DIFFERENT TYPES OF RESINS:

- In ion-exchange chromatography, four basic types of resins are commonly used.

Strongly acidic cation exchange resins:

- Sulphonated polystyrene resins belong to this class.
- They are useful in the pH range from 1 to 14.
- They are used mainly in the fractionation of cations, inorganic separations, vitamins, peptides and amino acids.

Strongly basic anion exchange resins:

- Quaternary ammonium polystyrene resins belong to this class and are effective between pH 0 and 12.
- They are useful in the fraction of anions, halogens, alkaloids, B-complex vitamins, fatty acids, etc.

Weakly acidic cation exchange resins:

- Carboxylic polymethacrylate is an example of this group.
- The resins are effective between pH 5 and 14.
- They are useful in the fractionation of cations, transition elements, amino acids, antibiotics and organic bases.

Weakly basic anion exchange resins:

- Phenol, formaldehyde and polyamine polystyrene resins belong to this class.
- They are effective in the pH range 0 to 9.
- They are useful in the fractionation of anionic complexes of metals, anions of different valencies, vitamins and amino acid.

SEPARATION OF AMINO ACIDS:

- The strength of the acidity or basicity of these groups and their number per unit volume of resin determine the type and strength of binding of an exchanger.
- Fully ionized acidic groups such as sulfonic acids result in an exchanger with a negative charge, which binds cations very strongly.

- Weakly acidic or basic groups yield resins whose charge (and binding capacity) depends on the pH of the eluting solvent.
- The choice of the appropriate resin depends on the strength of binding desired.
- The bare charges on such solid phases must be counterbalanced by oppositely charged ions in solution ("counterions").
- Washing a cation exchange resin, such as Dowex-50, which has strongly acidic phenyl-SO3- groups, with a NaCl solution results in the formation of the so-called sodium form of the resin. Operation of a cation exchange column for the separation of the amino acids Asp, Ser and Lys.

The separation of amino acids on a cation exchange column.

- When the mixture whose separation is desired is added to the column, the positively charged solute molecules displace the Na+ ions and bind to the resin.
- A gradient of an appropriate salt is then applied to the column, and the solute molecules are competitively (and sequentially) displaced (eluted) from the column by the rising concentration of cations in the gradient, in an order that is inversely related to their affinities for the column.
- The separation of a mixture of amino acids on such a column
- Operation of a cation exchange column, separating a mixture of Asp, Ser, and Lys.
- (a) The cation exchange resin in the beginning, Na+ form.
- (b) A mixture of Asp, Ser, and Lys is added to the column containing the resin.
- (c) A gradient of the eluting salt (e.g., NaCl) is added to the column. Asp, the least positively charged amino acid, is eluted first.
- (d) As the salt concentration increases, Ser is eluted.
- (e) As the salt concentration is increased further, Lys, the most positively charged of the three amino acids, is eluted last

GEL FILTERATION:

- Gel filtration does not rely on any chemical interaction with the protein; rather it is based on a physical property of the protein - that being the effective molecular radius (which relates to mass for most typical globular proteins).
- Gel filtration resin can be thought of as beads, which contain pores of a defined size range.
- Large proteins, which cannot enter these pores, pass around the outside of the beads.
- Smaller proteins, which can enter the pores of the beads, have a longer, tortuous path before they exit the bead.
- Thus, a sample of proteins passing through a gel filtration column will separate based on molecular size: the big ones will elute first and the smallest ones will elute last (and "middle" sized proteins will elute in the middle).
- If your protein is unusually "small" or "large"in comparison to contaminating proteins then gel filtration may work quite well.

Principle:

- Gel media A gel is a heterogeneous phase system in which a continuous liquid phase, usually aqueous, is contained within the pores of a continuous solid phase,

the gel matrix.

- In gels made for gel filtration, the pores have a carefully

controlled range of sizes, and the matrix is chosen for its chemical and

physical stability, and inertness (lack of adsorptive properties).

- Gels may be formed from polymers by cross-linking to form a

three-dimensional network; for example Sephadex that is formed by cross-linking dextran. Some polymers, like agarose, form gels spontaneou-

sly under the appropriate conditions.

- Composite gels may be prepared by, for example, grafting a second polymer onto a pre-formed matrix.

- Superdex is such a gel. Dextran chains arecovalently bonded to a highly cross-linked agarose gel matrix.

- Compositegels are of interest since they can combine valuable properties from morethan one gel-forming system

ESTIMATION OF MOLECULAR SIZE AND MOLECULAR WEIGHT OF A BIOMACROMOLECULE:

Molecular size:

- Most molecules are far too small to be seen with the naked eye, but there are exceptions. DNA, a macromolecule, can reach macroscopic sizes, as can molecules of many polymers.

- The smallest molecule is the diatomic hydrogen (H2), with a bond length of 0.74 Å.

- Molecules commonly used as building blocks for organic synthesis have a dimension of a few Å to several dozen Å.

- Single molecules cannot usually be observed by light (as noted above), but small molecules and even the outlines of individual atoms may be traced in some circumstances by use of an atomic force microscope.
- Some of the largest molecules are macromolecules or supermolecules

Molecular Weight:

- The log of a molecule's molecular weight is proportional to the distance that molecule has migrated.
- Therefore, the first step is to generate a standard curve using molecules of know size (the molecular weight markers).
- When using semilog paper (see the next page), the molecular weights (in bp for DNA and kiloDaltons, kDa, for proteins) is plotted on the Y-axis and the distance the molecule migrated is plotted on the X-axis.
- When generating a standard curve, you will obtain a straight line (use a best-fit line).
- Once your standard curve is ready, measure the distance traveled by your molecule of interest.
- Find that distance on the X-axis, and go up until you intersect with your standard curve.
- Move over to the Y-axis and that will indicate the molecular weight of the molecule you are studying.
- Use the graph paper below and the DNA gel shown to the right to determine the molecular weight of the unknown band indicated with an arrow.

AFFINITY CHROMATOGRAPHY:

- Affinity chromatography separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatography matrix.
- The technique offers high selectivity, hence high resolution, and usually high capacity for the protein(s) of interest.

- Purification can be in the order of several thousand fold and recoveries of active material are generally very high.
- Affinity chromatography is unique in purification technology since it is the only technique that enables the purification of a biomolecule on the basis of its biological function or individual chemical structure.
- Purification that would otherwise be time-consuming, difficult or even impossible using other techniques can often be easily achieved with affinity chromatography.
- The technique can be used to separate active biomolecules from denatured or functionally different forms, to isolate pure substances present at low concentration in large volumes of crude sample and also to remove specific contaminants.

PRINCIPLE AND SEPARATION OF AN ENZYME:

Principle

- The immobile phase is typically a gel matrix, often of agarose; a linear sugar molecule derived from algae.
- Usually the starting point is an undefined heterogeneous group of molecules in solution, such as a cell lysate, growth medium or blood serum.
- The molecule of interest will have a well-known and defined property which can be exploited during the affinity purification process.
- The process itself can be thought of as an entrapment, with the target molecule becoming trapped on a solid or stationary phase or medium.
- The other molecules in solution will not become trapped, as they do not possess this property.
- The solid medium can then be removed from the mixture, washed and the target molecule released from the entrapment in a process known as elution.
- Possibly the most common use of affinity chromatography is for the purification of recombinant proteins.

Separation

- The goal of affinity chromatography is to separate all the molecules of a particular specificity from the whole gamut of molecules in a mixture such as a blood serum.

- For example, the antibodies in a serum sample specific for a particular antigenic determinant can be isolated by the use of affinity chromatography.

Step 1:

- An immunoadsorbent is prepared. This consists of a solid matrix to which the antigen (shown in blue) has been coupled (usually covalently).
- Agarose, sephadex, derivatives of cellulose, or other polymers can be used as the matrix.

Step 2:

- The serum is passed over the immunoadsorbent.
- As long as the capacity of the column is not exceeded, those antibodies in the mixture specific for the antigen (shown in red) will bind (noncovalently) and be retained. Antibodies of other specificities (green) and other serum proteins (yellow) will pass through unimpeded.

Step 3: Elution

- A reagent is passed into the column to release the antibodies from the immunoadsorbent.
- Buffers containing a high concentration of salts and/or low pH are often used to disrupt the noncovalent interactions between antibodies and antigen.
- A denaturing agent, such as 8 M urea, will also break the interaction by altering the configuration of the antigen-binding site of the antibody molecule.
- Another, gentler, approach is to elute with a soluble form of the antigen.
- These compete with the immunoadsorbent for the antigen-binding sites of the antibodies and release the antibodies to the fluid phase.

Step 4:Dialysis

- The eluate is then dialyzed against, for example, buffered saline in order to remove the reagent used for elution.

Thin layer chromatography is	partition chromatography	electrical mobility of ionic species	**adsorption chromatography**	migration of charged ions in elect
Relative flow (Rf) value ranges from	**0 to 1**	0 to 2.0	+2 to -2	+1 to -1
Sucrose can be determined after silylation using which chromatographic technique	HPLC	Gel chromatography	**Gas liquid chromatography**	Paper chromatography
The relationship between concentration, temperature & potential of a solution is given by	Ilkovic equation	Henderson equation	**Nernst equation**	Hassalbach equation
Ion exchange chromatography is based on the	**electrostatic attraction**	electrical mobility of ionic species	adsorption chromatography	partition chromatography
The locating agent of amino acids is	Diazo reagent	**ninhydrin spray**	Amphoteric oxides	neutral oxides
Chromatography can be used to	form mixtures	change mixture compositions	**separate mixtures into pure su**	is not a separation technique
In gas chromatography, the basis for separation of the components of the volatile material is the difference in	**partition coefficients**	conductivity	molecular weight	molarity
Proteins can be visualized directly in gels by	**staining them with the dye**	using electron microscope only	measuring their molecular weigh	Spectrophotometer
Electrophoresis of histones and myoglobin under non-denaturing conditions (pH = 7.0) results in	both proteins migrate to the anode	histones migrate to anode and my	**histones migrate to the cathode**	both proteins migrate to the catho
In isoelectric focusing, proteins are separated on the basis of their	relative content of positively charged residu	relative content of negatively char	size	**relative content of positively an**
In SDS-PAGE, the protein sample is first	**treated with a reducing agent and then w**	fractionated by electrophoresis th	treated with a oxidizing agent an	treated with acetic acid
SDS is a(n)	**anionic detergent**	cationic detergent	not an detergent	Chargeless
Proteins are separated in an SDS-PAGE experiment on the basis of their	positively charged side chains	**molecular weight**	negatively charged side chains	different isoelectric points
DNA possesses	No charge	a positive charge	**a negative charge**	a supercharge
The rate at which DNA migrates through the gel is mainly determined by	**molecular size of the DNA**	salt levels	Protein	lipid
What is ethidium bromide?	buffer	**dye**	DNA solution	restriction enzyme
Why do scientists load DNA of known sizes into the agarose gel?	**It makes it easier to determine sizes of ur**	To fill in all the slots on the gel sc	To practice loading the DNA bef	So you know how long the gel ne
The colour of cathode is	Red	**Black**	Blue	Green
The colour of anode is	**Red**	Black	Blue	Green
Which of the following is not required for SDS-PAGE	TEMED	APS	SDS	**Tryptophan**
Which amino acid gives yellow colour with ninhydrin	**Proline**	Alanine	glutamine	serine
Galactose is a constitutent of	Lactose	Agarose	**lactose and agarose**	not a constituent of lactose and ag
pH of stacking gel is	10	**6.8**	8.8	9
pH of separating gel is	10	6.8	**8.8**	9
Protein with less molecular weight moves faster during electrophoresis.This statement is	**TRUE**	FALSE	hypothetical	will not comment
Electrophoresis technique was first developed in	1947	1950	**1937**	2000
Electrophoresis technique was first developed by	**Tiselius**	James	Watson	Crick
Ampholytes contain	Positive charge	Negative charge	**both positive and negative charge**	no charge
Why glycerol is added in loading buffer	**to give weight to sample**	to give charge to sample	to give weight and charge	it is not added in loading buffer
Mercaptoethanol reduces	peptide bond	**disulfide bond**	Hydrogen bond	vander waals force
Which causes decomposition of persulphate ion to give free radical during SDS PAGE	APS	**TEMED**	SDS	acrylamide
Urea at 3 to 12 M concentration disrupts	peptide bond	**disulfide bond**	Hydrogen bond	vander waals force
EtBr intercalates DNA and is visible under	dark condition	**UV**	visible light	IR
Glycinate ion in stacking gel is	**poorly ionized**	completely ionized	not ionized	ionized
Which of the following is referred as "leading ion" in SDS PAGE	**Chloride**	Glycinate	APS	Protein
Which of the following is referred as "trailing ion" in SDS PAGE	Chloride	**Glycinate**	APS	Protein
Amount of time an analyte stays in column is referred as	retention volume	retention ratio	**retention time**	Void
Amount of solvent required for an analyte to come out of column is referred as	**retention volume**	retention ratio	retention time	Void
During gel chromatography the molecules are separated according to	Charge	Charge/mass ratio	**Mass**	applied electric field
Dextran is a polysaccharide composed of	**Glucose**	Fructose	Galactose	Fucose
In chromatography, Rf is referred as	retention force	retardation factor	**relative flow**	random factor
Ion exchange chromatography uses	cationic exchanger	anion exchanger	no exchanger	**cation and anion exchanger**
In TLC the plates are dried after applying stationary phase at	4 Celsius	**40 Celsius**	100 Celsius	55 Celsius
Antimony trichloride is used for the detection of	Steroids	Terpenoids	Carbohydrates	**Steroids and terpenoids**
Acridine orange is used for the detection of	**Nucleic acids**	Terpenoids	Carbohydrates	Steroids
Anisaldehyde is used for the identification of	**Carbohydrates**	Proteins	Fats	Vitamins
Weakly acidic cation is effective at pH	3	**5**	Zero	2
DEAE-sephadex exchanger is an example for	strong cationic	weak cationic	strong anionic	**weak anionic**
A good adsorbent used in chromatography should ideally be	Inert	Stable	Cheap	**inert, stable and cheap**
Agarose gel electrophoresis is referred as	**Horizontal**	Vertical	Slanting	Running
Electrophoresis can be done in paper. This statement is	**TRUE**	FALSE	Imaginary	Hypothetical
Identification of DNA is referred as	Western blotting	Northern blotting	Eastern blotting	**Southern blotting**
Identification of RNA is referred as	Western blotting	**Northern blotting**	Eastern blotting	Southern blotting
Identification of proteins is referred as	**Western blotting**	Northern blotting	Eastern blotting	Southern blotting
Amount of SDS bound to protein per gram protein is	1.4 gram	2.5 gram	**1.4 gram**	8 gram
Generally, the loading dye used for agarose gel electrophoresis is available at	**6X**	10X	1X	8X
500 base pair DNA fragments are generally identified using	**agarose gel electrophoresis**	acrylamide electrophoresis	PAGE	Chromatography
Bromophenol blue migration in agarose gel electrophoresis coincides with	**300 bp**	10 bp	500 bp	1000 bp
EtBr used in agarose gel electrophoresis	**Mutagen**	Neurotoxin	Safe	Nephrotoxin

adsorption chromatography
0 to 1
Gas liquid chromatography
Nernst equation
electrostatic attraction
ninhydrin spray
separate mixtures into pure substa
partition coefficients
staining them with the dye
histones migrate to the cathode an
relative content of positively and n
treated with a reducing agent and
anionic detergent
molecular weight
a negative charge
molecular size of the DNA
dye
It makes it easier to determine size
Black
Red
Tryptophan
Proline
lactose and agarose
6.8
8.8
TRUE
1937
Tiselius
both positive and negative charge
to give weight to sample

disulfide bond
TEMED
disulfide bond
UV
poorly ionized
Chloride
Glycinate
retention time
retention volume
Mass
Glucose
relative flow
cation and anion exchanger
40 Celsius
Steroids and terpenoids
Nucleic acids
Carbohydrates
5
weak anionic
inert, stable and cheap
Horizontal
TRUE
Southern blotting
Northern blotting
Western blotting
1.4 gram
6X
agarose gel electrophoresis
300 bp
Mutagen

ınces

d myoglobin migrates to the anode
egatively charged residue
then with anionic detergent followed by fractionation by electrophoresis

s of unknowns using comparison techniques.

KARPAGAM ACADEMY OF HIGHER EDUCATION
(Deemed University Established Under Section 3 of UGC Act 1956)
Coimbatore - 641021.
(For the candidates admitted from 2015 onwards)
DEPARTMENT OF BIOCHEMISTRY

SUBJECT : BIOINSTRUMENTATION AND GLP
SEMESTER : I
SUBJECT CODE : 17BCP103 CLASS : I M.Sc.BC

UNIT III Electrophoresis: Principle, instrumentation and applications of agarose gel electrophoresis, sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE), native PAGE, isoelectric focusing, immunoelectrophoresis, 2D gel electrophoresis. Pulse field gel electrophoresis, capillary electrophoresis, gel documentation – Applications. Blotting techniques.

ELECTROPHORESIS:

- Electrophoresis is the motion of dispersed particles relative to a fluid under the influence of a spatially uniform electric field
- A technique for separating the components of a mixture of charged molecules (proteins, DNAs, or RNAs) in an electric field within a gel or other support.
- The movement of electrically charged molecules in an electric field often resulting in their separation.

PRINCIPLES:

- Migration of charged particles in an electric field.
- The rate of travel of the particle depends upon the following major factors.

a) The charge of the particle

b) Applied electrical field

c) Temperature and

d) Nature of the suspended medium.

- Many biologically important molecules such as amino acids, peptides, proteins, nucleotides and nucleic acids possess ionizable groups.
- Under the influence of an electric field, these charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge.
- There are two main types of electrophoretic methods.
- When the separation is carried out in absence of a supporting or stabilizing medium, the method is called free solution method.
- When the separation is carried out in presence of a stabilizing medium, such as paper gel, the technique is known as zone electrophoresis.

TYPES OF ELECTROPHORESIS:

Agarose Gel Electrophoresis

- Agarose gels are commonly used to sort DNA and RNA molecules based on size.
- The agarose gel concentration can be varied, based on the size of the molecules that need to be isolated.

SDS-PAGE Electrophoresis

- Sodium dodecyl sulfate - polyacrylamide gel electrophoresis is used to separate proteins based on size.
- The proteins are unfolded, or denatured, using SDS detergent, and run on a polyacrylamide gel.

PAPER ELECTROPHORESIS:

- Paper electrophoresis technique is a simple and less expensive one.
- It requires micro quantities of plasma for separation.
- The serum under investigation is mixed with bromophenol blue, a blue coloured stain, and spotted at the centre of a strip of a special filter paper, saturated with barbitone of pH 8.6.

Graphical representation of the concentration of serum protein fractions

- When an electric current of proper amperage and voltage is passes through the paper, charged protein fractions bearing different charges migrate at different rates.
- If the pH of the serum is adjusted by the addition of a proper buffer to a value alkaline to the isoelectric points of all the fractions of plasma protein, they all will carry negative charges, but of different magnitudes.
- The different fraction\s of plasma will migrate toward the anode at characteristically different rates.
- After a run of about 5 to 6 hours, the paper is dried and stained with a solution containing bromophenol blue.
- In human serum, five different bands can be identified on paper electrophoresis.
- They are designated in the order of decreasing mobility as albumin, alpha1 – globulin, alpha2 – globulin, beta globulin, fibrinogen and gamma – globulin.
- Albumin being the fastest moving fraction of the proteins of plasma forms the last band of the paper.
- Gamma globulin, which is the slowest moving protein, forms a band at the other end.
- The rest of the fractions take their positions in between theses two bands.

AGAROSE GEL ELECTROPHORESIS:

Agarose gels provide a simple method for analyzing preparations of DNA. Although the base compositions of individual DNA molecules vary, the basic chemical structure of DNA is the same for all DNA molecules. DNA molecules share the same charge/mass ratio, which imparts similar electrophoretic properties to DNAs of widely varying lengths.

Procedure

- An electrophoresis chamber and power supply
- Gel casting trays, which are available in a variety of sizes and composed of UV-transparent plastic. The open ends of the trays are closed with tape while the gel is being cast, then removed prior to electrophoresis.
- Sample combs, around which molten agarose is poured to form sample wells in the gel.

- Electrophoresis buffer, usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE).

- Loading buffer, which contains something dense (e.g. glycerol) to allow the sample to "fall" into the sample wells, and one or two tracking dyes, which migrate in the gel and allow visual monitoring or how far the electrophoresis has proceeded.

- Ethidium bromide, a fluorescent dye used for staining nucleic acids. NOTE: Ethidium bromide is a known mutagen and should be handled as a hazardous chemical - wear gloves while handling.

- Transilluminator (an ultraviolet lightbox), which is used to visualize ethidium bromide-stained DNA in gels. NOTE: always wear protective eyewear when observing DNA on a transilluminator to prevent damage to the eyes from UV light.

- To pour a gel, agarose powder is mixed with electrophoresis buffer to the desired concentration, then heated in a microwave oven until completely melted. Most commonly, ethidium bromide is added to the gel (final concentration 0.5 ug/ml) at this point to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60C, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature or, if you are in a big hurry, in a refrigerator.

- After the gel has solidified, the comb is removed, using care not to rip the bottom of the wells. The gel, still in its plastic tray, is inserted horizontally into the electrophoresis chamber and just covered with buffer. Samples containing DNA mixed with loading buffer are then pipeted into the sample wells, the lid and power leads are placed on the apparatus, and a current is applied. You can confirm that current is flowing by observing bubbles coming off the electrodes.DNA will migrate towards the positive electrode, which is usually colored red.

- When adequate migration has occured, DNA fragments are visualized by staining with ethidium bromide. This fluorescent dye intercalates between bases of DNA and RNA. It is often incorporated into the gel so that staining occurs during electrophoresis, but the gel can also be stained after electrophoresis by soaking in a dilute solution of ethidium bromide. To visualize DNA or RNA, the gel is placed on a ultraviolet transilluminator. Be aware that DNA will diffuse within the gel over time, and examination or photography should take place shortly after cessation of electrophoresis.

Migration of DNA Fragments in Agarose

Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the log10 of their molecular weight. In other words, if you plot the distance from the well that DNA fragments have migrated against the log10 of either their molecular weights or number of base pairs, a roughly straight line will appear.

Agarose Concentration: By using gels with different concentrations of agarose, one can resolve different sizes of DNA fragments. Higher concentrations of agarose facilite separation of small DNAs, while low agarose concentrations allow resolution of larger DNAs.

The image to the right shows migration of a set of DNA fragments in three concentrations of agarose, all of which were in the same gel tray and electrophoresed at the same voltage and for identical times. Notice how the larger fragments are much better resolved in the 0.7% gel, while the small fragments separated best in 1.5% agarose. The 1000 bp fragment is indicated in each lane.

Voltage: As the voltage applied to a gel is increased, larger fragments migrate proportionally faster that small fragment. For that reason, the best resolution of fragments larger than about 2 kb is attained by applying no more than 5 volts per cm to the gel (the cm value is the distance between the two electrodes, not the length of the gel).

Electrophoresis Buffer: Several different buffers have been recommended for electrophoresis of DNA. The most commonly used for duplex DNA are TAE (Tris-acetate-EDTA) and TBE (Tris-borate-EDTA). DNA fragments will migrate at somewhat different rates in these two buffers due to differences in ionic strength. Buffers not only establish a pH, but provide ions to support conductivity. If you mistakenly use water instead of buffer, there will be essentially no migration of DNA in the gel! Conversely, if you use concentrated buffer (e.g. a 10X stock solution), enough heat may be generated in the gel to melt it.

Effects of Ethidium Bromide: Ethidium bromide is a fluorescent dye that intercalates between bases of nucleic acids and allows very convenient detection of DNA fragments in gels, as shown by all the images on this page. As described above, it can be incorporated into agarose gels, or added to samples of DNA before loading to enable visualization of the fragments within the gel. As might be expected, binding of ethidium bromide to DNA alters its mass and rigidity, and therefore its mobility.

Applications

- Estimation of the size of DNA molecules following restriction enzyme digestion, e.g. in restriction mapping of cloned DNA.
- Analysis of PCR products, e.g. in molecular genetic diagnosis or genetic fingerprinting
- Separation of DNA fragments for extraction and purification.
- Separation of restricted genomic DNA prior to Southern transfer, or of RNA prior to Northern transfer.

Agarose gels are easily cast and handled compared to other matrices and nucleic acids are not chemically altered during electrophoresis. Samples are also easily recovered. After the experiment is finished, the resulting gel can be stored in a plastic bag in a refrigerator.

Electrophoresis is performed in buffer solutions to reduce pH changes due to the electric field, which is important because the charge of DNA and RNA depends on pH, but running for too long can exhaust the buffering capacity of the solution. Further, different preparations of genetic material may not migrate consistently with each other, for morphological or other reasons.

POLYACRYLAMIDE GEL ELECTROPHORESIS:

- To separate proteins on the basis of their size and charge

Theory

- PAGE (Polyacrylamide Gel Electrophoresis), is an analytical method used to separate components of a protein mixture based on their size.
- The technique is based upon the principle that a charged molecule will migrate in an electric field towards an electrode with opposite sign.
- The general electrophoresis techniques cannot be used to determine the molecular weight of biological molecules because the mobility of a substance in the gel depends on both charge and size.
- To overcome this, the biological samples needs to be treated so that they acquire uniform charge, then the electrophoretic mobility depends primarily on size.
- For this different protein molecules with different shapes and sizes, needs to be denatured(done with the aid of SDS) so that the proteins lost their secondary, tertiary or quaternary structure.
- The proteins being covered by SDS are negatively charged and when loaded onto a gel and placed in an electric field, it will migrate towards the anode (positively charged electrode) are separated by a molecular sieving effect based on size.
- After the visualization by a staining (protein-specific) technique, the size of a protein can be calculated by comparing its migration distance with that of a known molecular weight ladder (marker).

Principle behind separation:

- Separation of charged molecules in an electric field is based on the relative mobility of charged species which is related to frictional resistance

Charge of the species:

PAGE is working upon the principle in which, the charged molecule will migrate towards the appositively charged electrode through highly cross linked matrix.

- Separation occurs due to different rates of migration occurs by the magnitude of charge and frictional resistance related to the size.

Relative Mobility:

where,

Z = charge on the molecule

E = Voltage applied and ,

f = frictional resistance

Rf is measured by:

Direction of movement is determined from Z : -

if $Z < 0$, then $\rightarrow +$

if $Z > 0$, then $\rightarrow -$

if $Z = 0$, then no movement

- The gel is used is divided into an upper "stacking" gel of low percentage (with large pore size) and low pH (6.8) , where the protein bands get squeezed down as a thin layer migrating toward the anode and a resolving gel (pH 8.8) with smaller pores.

- Cl - is the only mobile anion present in both gels. When electrophoresis begins, glycine present in the electrophoresis buffer, enters the stacking gel, where the equilibrium favors zwitter ionic form with zero net charge.

- The glycine front moves through the stacking gel slowly, lagging behind the strongly charged, Cl- ions.

- Since these two current carrying species separate, a region of low conductivity, with high voltage drop, is formed between them.
- This zone sweeps the proteins through the large pores of the stacking gel, and depositing it at the top of the resolving gel as a narrow band.

Stacking gel interactions:

- Stacking occurs by the differential migration of ionic species, which carry the electric current through the gel.
- When an electrical current is applied to the gel,the negatively charged molecules start migrating to the positively charged electrode .
- Cl- ions, having the highest charge/mass ratio move faster, being depleted and concentrated at anode end.
- SDS coated proteins has a higher charge/mass ratio than glycine so it moves fast, but slower than Cl-.
- When protein encounters resolving gel it slows the migration because of increased frictional resistance, allowing the protein to stack in the gel.

Resolving Gel Interactions:

- When glycine reaches resolving gel it becomes positively charged and migrates much faster than protein due to higher charge/mass ratio.
- Now proteins are the main carrier of current and separate according to their molecular mass by the sieving effect of pores in gel.

Materials Required For PAGE

- Acrylamide solutions (for resolving & stacking gels).
- Isopropanol /distilled water.
- Gel loading buffer.
- Running buffer.
- Staining, destaining solutions.
- Protein samples.
- Molecular weight markers.

The equipment and supplies necessary for conducting SDS-PAGE includes:

- An electrophoresis chamber and power supply.
- Glass plates(a short and a top plate).
- Casting frame .
- Casting stand.
- Combs.

Objectives:

Separation of proteins based on their molecular weight.

1. Gloves should be worn, while performing SDS-PAGE.
2. To ensure proper alignment,all the requirements should be clean.
3. Special attention should be paid while using acrylamide (since it is a neurotoxin).

Stock solutions

- 30% Polyacrylamide solution(29g acrylamide+1g bisacrylamide in 50 mL of water, dissolve completely using a magnetic stirrer, make the volume upto 100mL).Keep the solution away from sunlight.
- 5 M Tris, pH 8.8 (Add 121.14g of Tris in 100 ml water. Adjust the pH to 8.8 and make up the final volume to 200ml).
- 5 M Tris, pH 6.8 (Add 60.57g of Tris in 40 ml water. Adjust the pH to 6.8 and themake up the final volume to 100 ml).
- 10% SDS (10 g SDS in 100mL distilled water).
- 10% ammonium persulfate (0.1 g in 1 ml water). It should be freshly prepared.
- 10x SDS running buffer (pH ~8.3) - Take 60.6 g Tris base,288g Glycine and 20g SDS in separate beakers and dissolve them using distilled water. When properly dissolved, mix three of them and make upto 2L.(working standard is 1X buffer)

Gel loading buffer:

To make 10 ml of 4X stock:

- 2.0 ml 1M Tris-HCl pH 6.8.

- 0.8 g SDS.
- 4.0 ml 100% glycerol.
- 0.4 ml 14.7 M
- β-mercaptoethanol.
- 0.5 M EDTA.
- 8 mg bromophenol Blue.

Staining solution:

- Weigh 0.25g of Coomassie Brilliant Blue R250 in a beaker. Add 90 ml methanol: water (1:1 v/v) and 10ml of Glacial acetic acid ,mix properly using a magnetic stirrer. (When properly mixed, filter the solution through a Whatman No. 1 filter to remove any particulate matter and store in appropriate bottles)

Destaining solution:

- Mix 90 ml methanol: water (1:1 v/v) and 10ml of Glacial acetic acid using a magnetic stirrer and store in appropriate bottles.

Resolving gel (10%)		Stacking gel (5%)
dH20	4.0 ml	
30% acrylamide mix	3.3 ml	
1.5M Tris pH8.8	2.5 ml	
10% SDS	0.1 ml	
10% ammonium persulfate	0.1 ml	
TEMED	0.004ml	
dH20	5.65 ml	
30% acrylamide mix	1.65 ml	
1.0M Tris pH 6.8	2.5 ml	
10% SDS	0.1 ml	
10% ammonium persulfate	0.1 ml	

TEMED 0.004ml

Thin layer chromatography is	partition chromatography	electrical mobility of ionic species	**adsorption chromatography**	migration of charged ions in elect	**adsorption chromatography**
Relative flow (Rf) value ranges from	**0 to 1**	0 to 2.0	+2 to -2	+1 to -1	**0 to 1**
Sucrose can be determined after silylation using which chromatographic technique	HPLC	Gel chromatography	**Gas liquid chromatography**	Paper chromatography	**Gas liquid chromatography**
The relationship between concentration, temperature & potential of a solution is given by	Ilkovic equation	Henderson equation	**Nernst equation**	Hassalbach equation	**Nernst equation**
Ion exchange chromatography is based on the	**electrostatic attraction**	electrical mobility of ionic species	adsorption chromatography	partition chromatography	**electrostatic attraction**
The locating agent of amino acids is	Diazo reagent	**ninhydrin spray**	Amphoteric oxides	neutral oxides	**ninhydrin spray**
Chromatography can be used to	form mixtures	change mixture compositions	**separate mixtures into pure su**	is not a separation technique	**separate mixtures into pure substa**
In gas chromatography, the basis for separation of the components of the volatile material is the difference in	**partition coefficients**	conductivity	molecular weight	molarity	**partition coefficients**
Proteins can be visualized directly in gels by	**staining them with the dye**	using electron microscope only	measuring their molecular weigh	Spectrophotometer	**staining them with the dye**
Electrophoresis of histones and myoglobin under non-denaturing conditions (pH = 7.0) results in	both proteins migrate to the anode	histones migrate to anode and my	**histones migrate to the cathod**	both proteins migrate to the catho	**histones migrate to the cathode an**
In isoelectric focusing, proteins are separated on the basis of their	relative content of positively charged residu	relative content of negatively char	size	**relative content of positively ar**	**relative content of positively and n**
In SDS-PAGE, the protein sample is first	**treated with a reducing agent and then v**	fractionated by electrophoresis th	treated with a oxidizing agent an	treated with acetic acid	**treated with a reducing agent and**
SDS is a(n)	**anionic detergent**	cationic detergent	not an detergent	Chargeless	**anionic detergent**
Proteins are separated in an SDS-PAGE experiment on the basis of their	positively charged side chains	**molecular weight**	negatively charged side chains	different isoelectric points	**molecular weight**
DNA possesses	No charge	a positive charge	**a negative charge**	a supercharge	a negative charge
The rate at which DNA migrates through the gel is mainly determined by	**molecular size of the DNA**	salt levels	Protein	lipid	**molecular size of the DNA**
What is ethidium bromide?	buffer	**dye**	DNA solution	restriction enzyme	dye
Why do scientists load DNA of known sizes into the agarose gel?	**It makes it easier to determine sizes of u**	To fill in all the slots on the gel so	To practice loading the DNA bef	So you know how long the gel ne	**It makes it easier to determine size**
The colour of cathode is	Red	**Black**	Blue	Green	**Black**
The colour of anode is	**Red**	Black	Blue	Green	**Red**
Which of the following is not required for SDS-PAGE	TEMED	APS	SDS	**Tryptophan**	**Tryptophan**
Which amino acid gives yellow colour with ninhydrin	**Proline**	Alanine	glutamine	serine	**Proline**
Galactose is a constitutent of	Lactose	Agarose	**lactose and agarose**	not a constituent of lactose and ag	**lactose and agarose**
pH of stacking gel is	10	**6.8**	8.8	9	**6.8**
pH of separating gel is	10	6.8	**8.8**	9	**8.8**
Protein with less molecular weight moves faster during electrophoresis.This statement is	**TRUE**	FALSE	hypothetical	will not comment	**TRUE**
Electrophoresis technique was first developed in	1947	1950	**1937**	2000	**1937**
Electrophoresis technique was first developed by	**Tiselius**	James	Watson	Crick	**Tiselius**
Ampholytes contain	Positive charge	Negative charge	**both positive and negative charge**	no charge	**both positive and negative charge**
Why glycerol is added in loading buffer	**to give weight to sample**	to give charge to sample	to give weight and charge	it is not added in loading buffer	**to give weight to sample**
Mercaptoethanol reduces	peptide bond	**disulfide bond**	Hydrogen bond	vander waals force	**disulfide bond**
Which causes decomposition of persulphate ion to give free radical during SDS PAGE	APS	**TEMED**	SDS	acrylamide	**TEMED**
Urea at 3 to 12 M concentration disrupts	peptide bond	**disulfide bond**	Hydrogen bond	vander waals force	**disulfide bond**
EtBr intercalates DNA and is visible under	dark condition	**UV**	visible light	IR	**UV**
Glycinate ion in stacking gel is	**poorly ionized**	completely ionized	not ionized	ionized	**poorly ionized**
Which of the following is referred as "leading ion" in SDS PAGE	**Chloride**	Glycinate	APS	Protein	**Chloride**
Which of the following is referred as "trailing ion" in SDS PAGE	Chloride	**Glycinate**	APS	Protein	**Glycinate**
Amount of time an analyte stays in column is referred as	retention volume	retention ratio	**retention time**	Void	**retention time**
Amount of solvent required for an analyte to come out of column is referred as	**retention volume**	retention ratio	retention time	Void	**retention volume**
During gel chromatography the molecules are separated according to	Charge	Charge/mass ratio	**Mass**	applied electric field	**Mass**
Dextran is a polysaccharide composed of	**Glucose**	Fructose	Galactose	Fucose	**Glucose**
In chromatography, Rf is referred as	retention force	retardation factor	**relative flow**	random factor	**relative flow**
Ion exchange chromatography uses	cationic exchanger	anion exchanger	no exchanger	**cation and anion exchanger**	**cation and anion exchanger**
In TLC the plates are dried after applying stationary phase at	4 Celsius	**40 Celsius**	100 Celsius	55 Celsius	**40 Celsius**
Antimony trichloride is used for the detection of	Steroids	Terpenoids	Carbohydrates	**Steroids and terpenoids**	**Steroids and terpenoids**
Acridine orange is used for the detection of	**Nucleic acids**	Terpenoids	Carbohydrates	Steroids	**Nucleic acids**
Anisaldehyde is used for the identification of	**Carbohydrates**	Proteins	Fats	Vitamins	**Carbohydrates**
Weakly acidic cation is effective at pH	3	**5**	Zero	2	5
DEAE-sephadex exchanger is an example for	strong cationic	weak cationic	strong anionic	**weak anionic**	**weak anionic**
A good adsorbent used in chromatography should ideally be	Inert	Stable	Cheap	**inert, stable and cheap**	**inert, stable and cheap**
Agarose gel electrophoresis is referred as	**Horizontal**	Vertical	Slanting	Running	**Horizontal**
Electrophoresis can be done in paper. This statement is	**TRUE**	FALSE	Imaginary	Hypothetical	**TRUE**
Identification of DNA is referred as	Western blotting	Northern blotting	Eastern blotting	**Southern blotting**	**Southern blotting**
Identification of RNA is referred as	Western blotting	**Northern blotting**	Eastern blotting	Southern blotting	**Northern blotting**
Identification of proteins is referred as	**Western blotting**	Northern blotting	Eastern blotting	Southern blotting	**Western blotting**
Amount of SDS bound to protein per gram protein is	14 gram	2.5 gram	**1.4 gram**	8 gram	**1.4 gram**
Generally, the loading dye used for agarose gel electrophoresis is available at	**6X**	10X	1X	8X	**6X**
500 base pair DNA fragments are generally identified using	**agarose gel electrophoresis**	acrylamide electrophoresis	PAGE	Chromatography	**agarose gel electrophoresis**
Bromophenol blue migration in agarose gel electrophoresis coincides with	**300 bp**	10 bp	500 bp	1000 bp	**300 bp**
EtBr used in agarose gel electrophoresis	**Mutagen**	Neurotoxin	Safe	Nephrotoxin	**Mutagen**

ances

ıd myoglobin migrates to the anode
negatively charged residue
then with anionic detergent followed by fractionation by electrophoresis

es of unknowns using comparison techniques.

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(For the candidates admitted from 2015 onwards)
DEPARTMENT OF BIOCHEMISTRY

SUBJECT : BIOINSTRUMENTATION AND GLP
SEMESTER : I
SUBJECT CODE : 17BCP103 CLASS : I M.Sc.BC

UNIT IV Radioisotopic techniques : Introduction, nature of radio activity, types and rate of radioactive decay, units of radio activity, detection and measurement of radioactivity-Geiger-Muller counter, solid and liquid scintillation counter. Autoradiography, X-ray diffraction and circular dichorism. Non radioactive, fluorescent methods. Applications of radioisotopes in biological sample analysis. Flowcytometry: Principles and applications.

ISOTOPES:

Definition:

- Most atoms have several naturally occurring isotopes (click here for a list of elements that have no isotopes).
- An isotope is an atom, which contains a different number of neutrons in its nucleus than some other atom of the same element.
- This means that different isotopes of an element will have different masses, since both the protons and the neutrons contribute about equally to the mass of an atom.

IMPORTANT STABLE AND RADIOACTIVE ISOTOPES USED IN BIOCHEMICAL RESEARCH:

- In general, isotopes are the elements having the same atomic number but

differing in mass number.

- Thus, in isotopes the number of neutrons is different.
- Isotopes are of two types, namely, stable and radioactive.

- The stable isotopes are those whose nuclei do not undergo spontaneous decomposition. e.g. 14N, 15N, 180, 13C, 33S, etc. Radioisotopes are those whose nuclei decompose spontaneously with the emission of radiations. 14C, 35S, 32P, 3H, etc.
- The stable isotopes are measured quantitatively with a mass spectrometer.
- This technique is less sensitive and more tedious than that for measuring radioactive isotopes.
- However, since no useful radioactive isotopes of oxygen and nitrogen exist, the mass spectrometer remains an essential tool for studying these elements.
- The radioactive isotopes are usually more useful as tracers than the stable isotopes

since the analytical methods for their measurement are extremely sensitive.

- The most widely used apparatus for the determination of radioactive isotopes is Geiger-Muller counter and Scintillation counter.

USE OF RADIOACTIVE ISOTOPES IN CLINICAL DIAGNOSIS AND THERAPY:

- 131I has a half-life of about 8 days.
- This radioisotope is mainly used for in vivo experiments.
- a) The turnover rate of a substance in a biological system (i.e., the rate of synthesis and breakdown) can be studied by tracer techniques.
- For example, 131I – labeled immunoglobulin is injected, the quantity of the labeled molecules in the circulation will be proportional to the catabolism of the immunoglobulin.
- By such studies, it is shown that the half-life of IgH is 15 days, of IgM and of IgA is about 5 days.
- b) The thyroid uptake studies of 131I are used extensively to detect functional derangements of thyroid gland.
- About 15 mCi of 131I is given intravenously.
- After a few hours, the patient is monitored at the neck region by a movable gamma-ray counter, which will pick up the radiation emitted by the thyroid gland.
- The normal values are about 25 per cent uptake by thyroid within 2 hours and about 50 per cent uptake within 4 hours.

- In hyperthyroidism, there will be increased uptake and hypothyroidism shows the reverse effect.

- c) Kidney scanning is done by injecting 131I – labeled hippuran or 131I-labelled diodrast.

- Both are excreted by kidney within a few minutes after injection.

- Two radioactivity detectors are placed in the renal region, behind the patient and scanning is done.

- Anatomical and physiological defects in the renal excretion could be easily identified.

- Living organisms cannot distinguish the normal atom from the radioactive atom.

- Therefore, when a radiolabelled compound is administered, these molecules are absorbed, metabolized and excreted by the body similar to normal molecules.

- Based on this, radioisotopes provide the following applications in the field of biological science:

- Tracing metabolic pathways:Radioisotopes are frequently used for identifying metabolic pathways. This involves application of trace amount of a radioactive substrate to experimental animals and then tracing the radioactivity in different products.

- Isotope dilution analysis: Many compounds present in living organisms cannot be accurately measured because they are present in such low amounts.

- Radiodating: The determination of the age of the rocks, fossils or sediments using radioisotopes is known as radiodating.

- Autoradiography: Autoradiography is a very sensitive method used in a wide variety of biological experiments.

- Radioimmuno assay (RIA): Radioimmuno assay is one of the most important techniques used in the clinical and biochemical fields for the quantitative analysis of hormones, steroids and drugs.

- Pharmacological studies: Isotopes are widely used in the development of new drugs. This is a much complicated process because before a drug is used in the treatment of pathological conditions, a number of factors must be ascertained.

- Clinical diagnosis: Radioisotopes are widely used for identifying various diseases. Thyroid function tests using131I are employed in the diagnosis of hypo- and hyperthyroidism.

- Mechanism of absorption, accumulation and transportation: Radioisotopes are widely used in various research laboratories to study the mechanism and rate of absorption, accumulation and transportation of inorganic and organic compounds by both plants and animals.

- Molecular biology techniques:Many radioisotopes are used in recent advances made in molecular biology such as DNA and RNA sequencing. DNA replication, transcription, synthesis of cDNA and recombinant DNA technology.

- Industrial microbiology: In industrial microbiology, mutants are desirable in various microbiological studies.

- Binding studies: Radioisotopes are used in studies such as enzyme-substrate binding, ligand or receptor binding and elucidation of mechanism of enzyme action.

- Sterilization of food and equipment: For sterilization of prepackaged foods, such as milk and meat, very strong 'Y-emitters are widely used in the food industry.

- Ecological studies: The migratory patterns and behavior patterns of many animals (e.g., birds and fishes) are monitored using radiotracers.

- Membrane transport studies: Transport of ions into the tissues of the body is extensively studied with the help of isotopes.

RADIATION HAZARDS AND PRECAUTIONS TO BE TAKEN IN HANDLING RADIOACTIVE ISOTOPES:

- Atoms of a given element with different atomic weights (mass numbers) are called

isotopes.

- The ratio of protons to neutrons is an important factor in determining whether

an element is radioactive or not. Radio isotopes emit particles or electromagnetic

radiation as a result of changes in the composition of the atomic nucleus.

- This phenomenon, which is termed as radioactivity or radioactive decay, leads to the production of a stable isotope.

- A radioactive compound may emit radiations in several ways as mentioned below to achieve stability.

- Negatron emission: A negatron is an electron. In negatron emission a neutron is converted to a proton.
- It results in the ejection of a negatively charged particle which is known as negatron (β-ve).
- Since the origin of electron is from the nuclear site, the term negatron is commonly used.

Neutron → Proton + Negatron

- The negatron emission results in loss of a neutron and gain of proton.
- Since the atomic number is increased by one, the N:Z ratio decreases.
- The mass number is not changed.
- Therefore, a new isotope is produced. 32p is an example of negatron emission.

$^{32}P_{15} \rightarrow {}^{32}P_{16} + \beta - ve$

- Many of the commonly used radioisotopes are obtained by this mechanism.
- Examples are 3H, 14C, 35S and 32P.
- To label any organic compound, 3H and 14C are extensively used.
- 35S labelled methionine is used to study protein synthesis.
- 32P-labelled nucleic acid is a powerful tool in molecular biology
- Positron emission: positrons are positively charged p-particles. Some radioisotopes decay by emitting positrons. During positron emission, a proton is converted to a neutron.

Proton → Neutron + Positron (β+ve)

- A proton loss and a gain of neutron are the result of positron emission.
- Therefore, the N: Z ratio increases. While the mass number remains constant, the atomic number decreases by one. An example of an isotope decaying by positron emission is 11C.

$^{11}C_{6} \rightarrow {}^{11}B_{5} + \beta + ve$

- Alpha emission: The alpha particles are heavier than other radiations because an alpha particle is a helium nucleus. It consists of two protons and two neutrons. Decay of 238U is an example.

238U92 234Th90 +4He2

- Alpha emitting isotopes are highly toxic if ingested.
- This is due to the large mass and the ionizing power of the atomic particle
- Electron capture (k-capture): In this an electron orbiting in the innermost k shell is captured by the nucleus resulting in a proton to neutron conversion.
- Electron capture does not affect the nuclear mass but the value of Z (atomic number) is reduced by 1.
- The vacancy left by the captured electron is filled by rearrangement of the remaining orbital electrons.
- This transition is characterized by the emission of electromagnetic radiation (x-rays).

54Mn25 54Cr24 +X-ray

- Gamma ray emission: Gamma rays are produced from a transformation in the nucleus of an atom.
- Excitation of orbital electrons leads to emission of gamma rays and it is always associated with alpha or beta particle emission.

131I63 131Xe64 +β + Y

- Gamma emission alone does not lead to a change in atomic or mass numbers.
- Gamma radiation has low ionizing power but high penetration.
- Properties of alpha particles:Alpha particles have considerable energy (3 to 8 MeV). When they react with matter, they cause excitation.
- Properties of beta particles: Beta particles are very small and rapidly moving particles. They carry a negative charge. They are less ionizing and more penetrating than alpha particles.
- Properties of gamma rays: Since gamma rays are electromagnetic radiations, they do not possess any charge or mass. They rarely collide with neighbouring atoms and travelgreat distances before dissipating their energy.
- Safety measures: One of the greatest disadvantages of using radioisotopes is their toxicity. After absorption, radiation produces ionization and forms free radicals.
- Rad: It is a measure of energy librated in a specific material by exposure to radiation. One rad equals to 100 ergs per gram of material.

- Rem: It is defined as the dose in rads multiplied by the quality factor.
- The beta – and gamma – radiations are assigned a quality factor of 1 and the alpha – radiation is assigned a quality factor of 20.
- The whole body tolerable dose of radiation is set at a maximum of 5 rems per year.

Precautions:

When handling radioisotopes, the following cautions are to be kept:

- Keep maximum distance from radiation source.
- Wear disposable gloves when doing tracer experiments.
- α and β – emitters should be separated by a Perspex shield.
- Gamma emitters should be separated by a lead shield.
- Radioactive work should be done in a Govt. authorized laboratory only.
- Try to minimize the time of exposure.
- Do not eat, drink or smoke in a radioactive laboratory.
- Do not use mouth to pipette out radioactive solutions.
- Radioactive wastes should be disposed as per the suggested rules.

Radioactivity Units

- The size or weight of a quantity of material does not indicate how much radioactivity is present. A large quantity of material can contain a very small amount of radioactivity, or a very small amount of material can have a lot of radioactivity.
- For example, uranium-238, with a 4.5 billion year half-life, has only 0.00015 curies of activity per pound, while cobalt-60, with a 5.3 year half-life, has nearly 513,000 curies of activity per pound. This "specific activity," or curies per unit mass, of a radioisotope depends on the unique radioactive half-life and dictates the time it takes for half the radioactive atoms to decay.
- In the United States, the amount of radioactivity present is traditionally determined by estimating the number of curies (Ci) present. The more curies present, the greater amount of radioactivity and emitted radiation.

- Common fractions of the curie are the millicurie (1 mCi = 1/1,000 Ci) and the microcurie (1 μCi = 1/1,000,000 Ci). In terms of transformations per unit time, 1 μCi = 2,220,000 dpm.

- The System International of units (SI system) uses the unit of becquerel (Bq) as its unit of radioactivity. One curie is 37 billion Bq. Since the Bq represents such a small amount, one is likely to see a prefix noting a large multiplier used with the Bq as follows:

- 37 GBq = 37 billion Bq = 1 curie

- 1 MBq = 1 million Bq = ~27 microcuries

- 1 GBq = 1 billion Bq = ~27 millicuries

- 1TBq = 1 trillion Bq = ~27 curies

- The becquerel (symbol Bq) (pronounced: 'be-kə-rel) is the SI derived unit of radioactivity. One Bq is defined as the activity of a quantity of radioactive material in which one nucleus decays per second. The Bq unit is therefore equivalent to an inverse second, s−1. The becquerel is named after Henri Becquerel, who shared a Nobel Prize with Pierre and Marie Curie in 1903 for their work in discovering radioactivity.

- For a given mass m (in grams) of an isotope with atomic mass m_a (in g/mol) and a half-life of t_{1/2} (in s), the amount of radioactivity can be calculated using:

- A_{Bq} = \frac{m}{m_a}N_A\frac{\ln(2)}{t_{1/2}}

- With N_A=6.022 141 79(30)×1023 mol−1, the Avogadro constant.

- Since m/ma is the number of mols (n), the amount of radioactivity A can be calculated by:

- A_{Bq} = nN_A\frac{\ln(2)}{t_{1/2}}

- For instance, one gram of potassium contains 0.000117 gram of 40K (all other naturally occurring isotopes are stable) that has a t_{1/2} of 1.277×109 years = 4.030×1016 s, and has an atomic mass of 39.964 g/mol, so the radioactivity is 30 Bq.

- 1 Bq = 1 s−1

- A special name was introduced for the reciprocal second (s−1) to represent radioactivity to avoid potentially dangerous mistakes with prefixes. For example, 1 μs−1 could be taken to mean 106 disintegrations per second: 1•(10−6 s)−1 = 106 s−1. Other names considered were hertz (Hz), a special name already in use for the reciprocal second, and fourier (Fr). The hertz is now only used for periodic phenomena.

- This SI unit is named after Henri Becquerel. As with every International System of Units (SI) unit whose name is derived from the proper name of a person, the first letter of its symbol is upper case (Bq).

G-M counter, counting statistics and absorption cross-section

Introduction:

- Geiger-Müller (GM) counters were invented by H. Geiger and E.W. Müller in 1928, and are used to detect radioactive particles. A typical GM Counter consists of a GM tube having a thin end window (e.g. made of mica), a high voltage supply for the tube, a scalar to record the number of particles detected by the tube, and a timer which will stop the action of the scalar at the end of a preset interval.

- The sensitivity of the GM tube is such that any particle capable of ionizing a single atom of the filling gas of the tube will initiate an avalanche of electrons and ions in the tube.

- The collection of the charge thus produced results in the formation of a pulse of voltage at the output of the tube.

- The amplitude of this pulse, on the order of a volt or so, is sufficient to operate the scalar circuit with little or no further amplification. The pulse amplitude is largely independent of the properties of the particle detected, and gives therefore little information as to the nature of the particle.

- Even so, the GM Counter is a versatile device, which may be used for counting alpha particles, beta particles, and gamma rays, albeit with varying degrees of efficiency.

- Apparatus:

- □ Set-up for ST-350 Counter

- □ GM Tube and stand

- □ shelf stand, serial cable, and a source holder

- □ Radioactive Source (e.g., Cs-137, Sr-90, or Co-60)

Principle of the Method:

- All nuclear radiations, whether they are charged particles or gamma rays, it will ionize

atoms/molecules while passing through a gaseous medium. This ionizing property of a

nuclear radiation is utilized for its detection. Geiger-Muller counter, commonly called as GM counter or simply as Geiger tube is one of the oldest and widely used radiation detectors.

- It consists of a metallic tube with a thin wire mounted along its axis. The wire is insulated from the tube using a ceramic feed-through, The central wire (anode) is kept at a positive

potential of a few hundreds of volt or more with respect to the metallic tube, which is

grounded. The tube is filled with argon gas mixed with 5-10% of ethyl alcohol or halogens (chlorine or bromine).

- When an ionizing radiation enters the Geiger tube some of the energy of the radiation

may get transferred to a gas molecule within the tube. This absorption of energy results

in ionization, producing an electron-ion pair (primary ions). The liberated electrons move

towards the central wire and positive ions towards the negatively charged cylinder. The

- electrons now cause further ionization by virtue of the acceleration due to the intense electric field. These secondary ions may produce other ions and these in turn still other ions before reaching the electrodes. This cascading effect produces an avalanche of ions. In an avalanche created by a single original electron many excited gas molecules are formed by electron collisions in addition to secondary ions. In a very short time of few nanoseconds these excited molecules return to ground state through emission of photons in the visible or ultraviolet region.

- These photons are the key element in the propagation of the chain reaction that makes up the Giger discharge. If one of these photons interacts by photoelectric absorption in some other region of the tube a new electron is liberated creating an avalanche at a different location in the tube.

- The arrival of these avalanches at the anode causes a drop in the potential between the central wire and the cylinder. This process gives rise to a very large pulse with an amplitude independent of the type and energy of the incident radiation. The pulse is communicated to the amplifier through an appropriate RC circuit, and then to a counter which is called as scaler. Suitable arrangements are made to measure the counts for a preset time interval.

MEASUREMENT OF RADIOACTIVITY BY SCINTILLATION COUNTERS:

Scintillation and principle:

- There are three commonly used methods of detecting and quantifying radioactivity. They are based on the
- Ionization of gases,
- The excitation of solids or liquids and
- The ability of radioactivity to expose photographic emulsions (autoradiography).
- In the second method mentioned above, the effect leads the excited compounds (known as the fluor) to emit photons of light.
- This fluorescence can be detected and quantified.
- This process is known as scintillation and when the light is detected by a photomultiplier, it forms the basis of scintillation counting.
- The electric pulse that results from the conversion of light energy to electrical energy in the photomultiplier is directly proportional to the energy of the original radioactive event.
- Instruments which measure the magnitude of scintillation with the help of photomultipliers are known as scintillation counters.
- Scintillation counting provides two kinds of information:
- a) Quantitative: The number of scintillations is proportional to the amount of radioactivity.
- b) Qualitative: The intensity of light given out is proportional to the energy of radiation.

Scintillation counter and its uses:

- The formation of fluorescence due to excitation by radioactivity is known as

scintillation.

- The light emitted in scintillation can be detected by coupling it to a

photomultiplier which converts the photon energy into an electrical pulse whose magnitude remains proportional to the energy of the original radioactive count.

- Instruments which measure scintillation are known as scintillation counters.
- There are two types of scintillation counters, namely, solid or external scintillation counters and liquid or internal scintillation counters.

- In solid scintillation counting, the sample is placed close to a fluor crystal (ZnS for a-emitters, anthracene for f3-emitters and Nal for y-emitters) which, in turn, is placed adjacent to a photomultiplier.
- This photomultiplier is connected to a high voltage supply and a scaler.
- Solid scintillation counting is useful for measurement of y-emitting isotopes.
- But they are not useful for measuring weak f3-emitting radioisotopes

such as 3H, 14C and 35S.

- In liquid or internal scintillation counting, the radioactive sample is dissolved or suspended in a scintillation system composed of solvent and primary and secondary Scintillators.
- The radiation from the suspended sample molecules collides with a solvent

molecule imparting a discrete amount of its energy to the solvent molecule.

- The solvent molecule, which has become excited, emits light as it comes back to ground state.
- This process is known as phosphorescence.
- The phosphorescence is due to the peculiar chemical nature of the solvent.
- The solvent emits light of a very short wavelength which falls in the range of 260 to 340 nm.
- Since this range is too short to be detected by most existing instruments,

a second molecule known as primary fluor (e.g., PPO) is added to the system.

- This compound absorbs light in the range of 260 to 340 nm and emits light of a longer wave length (340 to 430 nm).
- This longer wave length light can be measured by most of the modern instruments.
- For instruments which are not capable of measuring this longer

wavelength light, another substance called secondary fluor (e.g., POPOP) is added.

- The secondary fluor absorbs light emitted by the primary fluor and emits light 'with a maximum in the visible region.
- The photomultiplier converts the light energy into an identical signal (electrical) that can be easily manipulated and measured.

Measurement of radioactivity by liquid scintillation counters:

- The phenomenon of fluorescence due to excitation by radiation is known as scintillation.
- The light emitted in scintillation can be detected by coupling it to a photomultiplier which converts the photon energy into an electrical pulse.
- The magnitude of the generated electrical impulse is proportional to the energy of the original radioactive count.
- Instruments which measure the magnitude scintillation with the help of photomultipliers are known as scintillation counters.
- Liquid scintillation counting is the best method for counting of soft β-emitting isotopes such as 3H, 14C, 32P and 35S.
- It can also be used to detect α – particles, positrons and weak X-rays.
- The sample for counting consists of three components, namely, the radioisotope, an organic solvent or solvent mixture and one or more organic fluors.
- The radiation is absorbed by the solvent {xylene, toluene or 1, 4-dioxane) which, in turn, transfers the energy to the fluor.
- In response to this, the fluor fluoresces or scintillates.
- The emitted light photons are collected by a photomultiplier and converted into an amplified pulse, which is recorded as a count corresponding to the particle or radiation emitted.

Liquid scintillation counting

- The above figure depicts the sequence of events occurring in a liquid

scintillation counter.

- The p-particles from the radioactive source have a short range

in the fluid and interact with solvent molecules which transfer their excitation

energy to the fluor which, in turn, is excited to a higher electronic energy level.

- When the excited fluor returns to its ground state, it emits the energy as photons.
- The number of photons produced per p-particle depends on the energy of the

p-particle released.

- The two major requirements of a good fluor are:
- It should display a high efficiency of p-energyabsorption.
- The absorbed energy should be re-emitted at a wavelength best suited for detection.
- But, in practice, such a fluor is not available.
- To achieve the above two points, a combination of two fluors is used.
- The primary fluor absorbs the p-particles efficiently and the secondary fluor emits the transferred energy from the primary fluor to the visible region of the spectrum as a photon.
- A typical primary fluor is 2,5-diphenyl oxazole (PPO). It is used with 1,4-bis•

(5-phenyl oxazol-2-yl) benzene (POPOP) as the secondary fluor.

- The organic solvents usually will not accept aqueous samples.
- Addition of ethanol solves this problem to some extent but a loss of efficiency occurs.
- Emulsifier-based cocktails are frequently used for counting aqueous solutions.
- They contain an emulsifier such as Triton X-100 and can accept up to 50 per cent water (v/v).
- Thus, a final pulse consisting of a large number of electrons is

produced.

- The number of electrons emitted at an anode for each incident electron is a

function of the applied accelerating voltage.

- The size of the pulse can be controlled by altering the high voltage applied.
- The pulses generated by photomultiplier then strike an amplifier which permits

additional amplification of the pulse.

- The pulse height analyzer comprises circuits which accept or reject pulses

according to their voltage.

- The energy of the 3-particle.
- The high voltage setting in the photomultiplier and
- The amplifier gain setting.
- High energy pulses from cosmic radiation,
- Low energy pulses from chemiluminescence of the sample bottle glass.
- Low energy pulses generated by the random thermal emission of

photocathode of the photomultiplier tube.

- Background counts will be usually in the range of 20 to 40 cpm and this value is then subtracted from the observed sample count.

AUTORADIOGRAPHY

 An autoradiograph is an image on an x-ray film or nuclear emulsion produced by the pattern of decay emissions (e.g., beta particles orgamma rays) from a distribution of a radioactive substance.

 Alternatively, the autoradiograph is also available as a digital image (digital autoradiography), due to the recent development of scintillation gas detectors or rare earth phosphorimaging systems.

 The film or emulsion is opposed to the labeled tissue section to obtain the autoradiograph (also called an autoradiogram). The auto- prefix indicates that the radioactive substance is within the sample, as distinguished from the case of historadiography or microradiography, in which the sample is X-rayed using an external source.

 Some autoradiographs can be examined microscopically for localization of silver grains (such as on the interiors or exteriors of cells or organelles) in which the process is termed micro-autoradiography. For example, micro-autoradiography was used to examine whether atrazine was being metabolized by the hornwort plant or by epiphytic microorganisms in the biofilm layer surrounding the plant

Autoradiography

Application

- Autoradiography has been used in biology on the macroscopic level to study the uptake of radioactive tracers by both plant leaves and animal organs. Since the 1960's the technique has been applied to successively smaller structures, such as individual cells, chromosomes and organelles within a cell, strands of DNA, and protein molecules. It is easier to understand the microscopic applications after first looking at a large-scale example.

- In one experiment, bean plants were grown in a nutrient solution containing radioactive phosphorus. The phosphorus moved from the roots to the leaves as expected, shown by an autoradiograph of a leaf pressed against photographic film.

- When the bean plant is allowed to continue growing in a nonradioactive solution, autoradiography shows that radioactive phosphorus is withdrawn from older leaves and translocated to new leaves and buds. Evidently, nutrients not only travel up from the roots but also move around the plant.

- In another experiment, a solution containing phosphorus was sprayed directly onto the leaf surface and was shown to migrate away from it. Redistribution of nutrients on an even larger scale takes place in deciduous trees, where as much as 90 percent of some minerals are withdrawn from leaves before they fall.

Differential centrifugation is based on the differences in _____ of biological particles of different _____	Size,density	Sedimentation rate, sizes ar	Size,structure	mass,size	Sedimentation rate, sizes and
Which of the following statements about the basic principle of sedimentation is False?					
After centrifugation of milk, the supernatant is	Fat	Whey	casein	water	Whey
Which of the following is not an example of a use of alpha, beta or gamma radioactivity?				To measure thickness in paper n	
Solute used in density gradient centrifugation is /are _____.	ammonium sulphate	caesium chloride	arabinose	dextrin	caesium chloride
Operating force of analytical ultra centrifuge is -------		60000g	600000g	6000g	600000g
Microfuge comes under the category of_____ speed centrifuge		high	ultra	very high	medium
Optical systems are equipped in analytical ultracentrifuges to monitor_____ of the sample.	the sedimentation	the purification	the separation	both a and c	the sedimentation
Separation of subcellular components is done by_____ centrifugation	differential	velocity sediment	ultra	medium speed	differential
Sedimentation coefficient of a particle is measured by_____ centrifugation	differential	density gradient	isopycnic	differential and isopycnic	differential
In isopycnic centrifugation the gradient is established using_____	cesium salts	sodium salts	sucrose	potassium salts	cesium salts
In solid scintillation counter which fluor crystal is placed closed to sample?	ZnS	NCS	Ammonia	Silica	ZnS
Solid scintillation counting is useful for measurement of _____ isotopes.		beta emitter	gamma emitter	proton	gamma emitter
The solvent molecule, which has become excited, emits light as it comes back to ground state is know as____			fl alpha emitter	beta emitter	ph
The secondary fluor in liquid scintillation counter is_____	PPO	POPOP	ZnS	NaI	POPOP
What is used to measure radioactive decay?	An MG tube	A GM tube	A PM tube	An EM tube	A GM tube
What is the meaning of the term half-life?	Half the time it takes for the entire	The length of time it takes for the rac			Th
What is alpha radioactivity?		A helium nucleus	An electromagnetic waves	An electron	A helium nucleus
Type of radioactivity is the most penetrating?	Alpha	Beta	Gamma	Delta	Gamma
Type of radioactivity is the least ionizing?	Alpha	Beta	Gamma	All equally ionising	Gamma
Emission of which one of the following leave both atomic number and mass number unchanged?	Positron	Neutron	Alpha particle	Gamma radiation	Gamma radiation
Which type of radiation is the least penetrating?	Alpha	Beta	Gamma	X-ray	Alpha
A Geiger- Muller tube is a _____	gas ionization detector	cloud chamber	fluorescence	spectrophotom	gas ionization detector
A radioactive decay series is_____.	an equation that relates the half-lif	a relationship between the half-life o	a process by which a non-radioac		a s
A nuclide with a long half-life_____.	has fewer atoms left after one hou		c		has
A Geiger-Muller counter can measure which of the following types of radiation?	Alpha particles	Electron capture events	Beta particles	All ionizing radiation	All ionizing radiation
Why is alpha -particle emission less hazardous than gamma -ray or X-ray emission as long as it is ou			a		alp
Which of the following emissions is common in carbon-14?	Beta -emission	lambda -ray emission.Electron captu		alpha-emission	Beta -emission
Self-absorption would least likely effect the efficiency of counting_____.	alpha radiation		b X-rays	gamma-rays	gar
A GM detector is used for counting___	beta particles	gamma rays	alpha particles	neutron	gar
The spontaneous disintegration of the nuclei of some of the isotopes of certain elements are called---	Radioactivity		R Radioactive decay	Radiation	Radioactive decay
Geiger Muller counter is related to ----------	Chromatography	Electrophoresis	Radioactivity	Centrifugation	Ra
Ultra centrifuges are run under vacuum to avoid_____.	heat		c air circulation	speed	hea
The isotope used to diagnose kidney function is_____	131Xe	131I	42K	131Ihippuric acid	131Ihippuric acid
Which isotope is used for the determination of exchangeable potassium in coronary blood flow?	Potassium-42	Palladium-103			Potassium-42
GM counter measures radioactivity depends on_____	ionization	excitation	both a and b	radiation	ionization
The following radioisotope is used in cancer therapy_____	51Cr	131I	Yttrium-90	131Xe	Yttrium-90
Gamma rays can be stopped by_____	paper	Aluminium foil	Lead block	silver block	Lead block
The common quenching agent used in Geiger Muller counter is_____		chloroform	benzene	acetic acid	eth
Cell organelles can be separated from the cell homogenate through	Chromatography	Differential centrifugation	X-ray diffraction	Auto-radiography	Differential centrifugation
Which of the following define homogenisation?			E		Bre
Which one is a structure which is not present in intact cell but obtained during cell fractionation?			E Ribosomes	Microsomes	Microsomes
Which of the following represents the various fractions obtained during differential centrifugation?	Plastidal, microsomal and cytosol		N		Nu
Radioactive isotopes like C^{14}, H^{3}, I^{131}, P^{32}, S^{35} are used in one of the following techniques:			M	chromatography	Au
Pure fractions of cellular components can be obtained by		Differential centrifugation	Autoradiography		Differential centrifugation
Autoradiography will produce:			Ir		Me
The order of sedimentation of subcellular structures during differential centrifugation is:			I		Nu
Radioactivity was discovered by			W		He
The International System of Units (SI) unit of radioactive activity is the			c		be
An older unit of radioactivity is the			c		Cu
_____ refers to the particles which are emitted from nuclei as a result of nuclear instability.			R		Ra
_____ occurs when an atomic nucleus breaks down into smaller particles			R		Ra
One _____ is defined as the activity of a quantity of radioactive material in which one nucleus d			C		bec
The _____ is a non-SI unit of radioactivity			C		Cu
An _____ is an image on an x-ray film or nuclear emulsion produced by the pattern of decay er			M		Au
_____ is any technique used to produce an image of the 2D distribution of a radioactive substance			M		Au
Which one of the following can be used to confirm whether drinking water contains a gamma emittin	Spectrophotometer		M		Sci
Which of the following techniques would be used to detect a metabolite labelled with ^{3}H?			S Scintillation counting	Scintillation counting	
Which of the following techniques would be used to detect a metabolite labelled with ^{14}C?			S Scintillation counting	Scintillation counting	
_____ is a common procedure used to separate certain organelles from whole cells for further an			I		Dif

density.
e more massive a biological particle is, the slower it moves in a centrifugal field.

e use of X-rays for medical examinations.

osphorescence.

e length of time it takes for the radioactivity of a sample to decrease by half.

eries of steps that shows how a radioactive nuclide decays into a non-radioactive nuclide.
s a smaller decay constant than a nuclide with a short half-life.

ha rays and X-rays are hazardous because the rays are highly penetrating, causing damage inside the body quite easily.

mma-rays
mma-rays

dioactivity
t

yl formate.

aking of cells and suspending them in 0.25M sucrose solution

clear, mitochondrial and microsomal
toradiography

tallic silver grains
cleus-mitochondria-lysosome-ribosome
nri Becquerel
cquerel
rie
dioactivity
dioactivity
xquerel
rie
toradiography
toradiography
ntillation counter

ferential centrifugation

KARPAGAM ACADEMY OF HIGHER EDUCATION
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(For the candidates admitted from 2015 onwards)
DEPARTMENT OF BIOCHEMISTRY

SUBJECT : **BIOINSTRUMENTATION AND GLP**
SEMESTER : **V**
SUBJECT CODE : **17BCP103** **CLASS** : **I M.Sc.BC**

UNIT V

Good Laboratory Practices: Quality concepts, personal protective equipment. General safety- biological safety, chemical safety and fire safety. data generation and storage, quality control documents, retention samples, records, audits of quality control facilities. List of Regulations to be followed. Laboratory safety procedure- glass ware, equipment safety, hands protection, precaution to be undertaken to prevent accident and contamination. GLP – an overview and basic information, Scope. Principles of GLP: Test Facility Organization and Personnel, Test Systems, Test and Reference Items, Standard Operating Procedures, Performance of the Study, Reporting of Study Result, Storage and Retention of Records and Materials. Responsibilities in GLP. Implementing of GLP in non GLP analytical laboratory.

The word quality is often used indiscriminately for many different meanings. Quality can be defined as "fitness for use," "customer satisfaction," "doing things right the first time," or "zero defects." These definitions are acceptable because quality can refer to degrees of excellence. Webster's dictionary defines quality as "an inherent characteristic, property or attribute.". Quality control is the science of keeping these characteristics or qualities within certain bounds.

Basic Elements in a Quality System

There are three basic elements in a quality system: Quality Management, Quality Control, and Quality Assurance.

- ***Quality Management:*** Quality management is the means of implementing and carrying out quality policy. They perform goal planning and manage quality control and quality assurance activities. Quality management is responsible for seeing that all quality goals and objectives are implemented and that corrective actions have been achieved. They periodically review the quality system to ensure effectiveness and to identify and review any deficiencies.

- ***Quality Control:*** The term quality control describes a variety of activities. It encompasses all techniques and activities of an organization that continuously monitor

and improve the conformance of products, processes or services to specifications. Quality control may also include the review of processes and specifications and make recommendations for their improvement. Quality control aims to eliminate causes of unsatisfactory performance by identifying and helping to eliminate or at least narrow the sources of variation. Quality control has the same meaning as variation control of productcharacteristics. The objective of a quality control program is to define a system in which products meet design requirements and checks and feedback for corrective actions and process improvements. Quality control activities should also include the selecting and rating of suppliers to ensure that purchased products meet quality requirements.

- ***Quality Assurance:*** The term quality assurance describes all the planned and systematic actions necessary to assure that a product or service will satisfy the specified requirements. Usually this takes the form of an independent final inspection. The distinction between quality control and quality assurance is stated in an ANSI/ASQ standard: "Quality control has to do with making quality what it should be, and quality assurance has to do with making sure quality is what it should be." The quality assurance function should represent the customer and be independent of the quality control function, which is an integral part of the manufacturing operation.

personal protective equipment

Always wear proper eye protection in the lab.

– safety glasses or goggles

• Do not wear contact lenses in the lab.

• Wear proper gloves.

• Wear aprons or lab coats.

• Wear closed-toe shoes.

• Wear hearing protection if the noise level isgreater than 85 dBA.

• Wash arms and hands immediately afterworking with allergens, carcinogens,pathogenic organisms, or toxic chemicals.

General safety-biological safety, chemical safety and fire safety

• Avoid working alone.

• Clean up spills.

• Do not store or consume food or beverages in the lab.

• Do not smoke in the lab.

• Do not deliberately smell or taste chemicals.

• Do not use damaged glassware.

• Always shield Dewar (Vacuum) flasks.

• Wash up before leaving.No horseplay, practical jokes, or other acts ofcarelessness.

• Do not pipette by mouth.

• Wear proper PPE.

• Keep your work area clean.

• Properly label all chemical containers.

• Observe the SDS– Safety Data Sheet

• Post appropriate warning signs.

• Inspect all equipment for defects

• Be alert to unsafe conditions and correct them when detected.

All electrical equipment and services must be

grounded.

• Replace frayed or deteriorated electrical cords.

• Extension cords are for temporary use only.

• Do not place electrical cords where they will be subjected to wear by friction or heat or where they

may present a shock or fire hazard.

• Do not place electrical cords above ceiling tiles, through doorways or walls, or where they will present

a trip hazard.

• Store flammable chemicals in “Flammable Storage” or “Explosion Proof ” refrigerators or freezers only.

• Use explosion proof equipment when explosive vapors are present.

• Use proper guards – rotating parts– sharp edges– hot surfaces– machine belts, pto's

• Do not use defective equipment.

Data Generation And Storage, Quality Control Documents, Retention Samples, Records, Audits Of Quality Control Facilities.

Good data and record management are critical elements of the pharmaceutical quality system

and a systematic approach should be implemented to provide a high level of assurance that

across the product life cycle all GxP records and data are accurate, consistent, trustworthy

and reliable.The data governance programme should include policies and governance procedures that

address the general principles listed below for a good data management program. These

principles are clarified with additional detail in sections below.

Applicability to both paper and electronic data.

The requirements for good data and recordmanagement that assure robust control of data validity apply equally to paper and electronic data. Organizations subject to GxP should be fully aware that reverting from automated/ computerized to manual/paper-based systems does not in itself remove the need for robustmanagement controls.

Applicability to contract givers and contract acceptors. The principles of these guidelines apply to contract givers and contract acceptors. Contract givers are ultimately responsible for the robustness of all decisions made on the basis of GxP data, including those that are made on the basis of data provided to them by contract acceptors. Contract givers therefore should perform due diligence to assure themselves that contract acceptors have in place appropriate programmes to ensure the veracity, completeness and reliability of provided data. Good documentation practices: To achieve robust decisions and data sets based need to be reliable and complete. Good documentation practices (GDP) should be followed in order toensure all records, both paper and electronic, allow the full reconstruction of the relatedactivities.

Management governance.

To establish a robust and sustainable good data management system it is important that senior management ensure that appropriate data management governance programmes are in place.

Elements of effective management governance should include:

• application of modern quality risk management principles and good data management

principles to the current quality management system to integrate those elements that

assure the validity, completeness and reliability of data. For example, monitoring of risks and application of appropriate quality metrics can help management gain the awareness necessary for good decision-making to reduce data integrity risks;

• management should ensure personnel are not subject to commercial, political, financial and other organizational pressures or incentives that may adversely affect the quality and integrity of their work;

• management should allocate adequate human and technical resources such that the workload, work hours and pressures on those responsible for data generation and record keeping do not increase errors;

• management should also make staff aware of the importance of their role in ensuring data integrity and the relationship of these activities to assuring product quality and protecting patient safety.

Quality culture. Management, together with the quality unit, should establish and maintain a working environment often referred to as a quality culture that minimizes the risk noncompliantrecords and erroneous records and data. An essential element is the transparent and open reporting of deviations, errors, omissions and aberrant results at all levels of the organization. Steps should be taken to prevent and detect and correct weaknesses in systems and procedures that may lead to data errors so as to continually the improve scientific robustness of decision making of the organization.

Quality risk management and sound scientific principles. Assuring robust decision making requires valid and complete data, appropriate quality and risk management systems,adherence to sound scientific and statistical principles. For example, the scientific principle of being an objective, unbiased observer regarding the outcome of a sample analysis requires that suspect results be investigated and rejected from the reported results only if they are clearly due to an identified cause. Adhering to good data and record-keeping principles requires that any rejected results be recorded, together with a documented justification for their rejection, and that this documentation is subject to review and retention. Data life cycle. Continual improvement of products to ensure and enhance their safety, efficacy and quality requires a data governance approach to ensure management of data integrity risks throughout all phases of the process by which data are recorded, processed, reviewed, reported, retained, retrieved and subject to ongoing review. In order to ensure that the organization, assimilation and analysis of data into information facilitates evidence based and reliable decision-making, data governance should address data ownership and accountability for data process(es) and risk management of the data lifecycle.

Design of record-keeping methodologies and systems. Record-keeping methodologies and systems, whether paper or electronic, should be designed in a way that encourages compliance with the principles of data integrity. Examples include but are not restricted to: restricting access to changing clocks for recording timed events; ensuring batch records are accessible at locations where activities take place so that adhoc data recording and later transcription to official records is not necessary;• controlling the issuance of blank paper templates for data recording so that all printed forms can be reconciled and accounted for;Working document restricting user access rights to automated systems in order to prevent (or audit trail) dataamendments;• ensuring automated data capture or printers are attached to equipment such as balances;• ensuring proximity of printers to relevant activities;• ensuring ease of access to locations for sampling points (e.g. sampling points for water systems) such that the temptation to take shortcuts or falsify samples is minimized;• ensuring access to original electronic data for staff performing data checking activities.Maintenance of record-keeping systems. The systems implemented and maintained for both paper and electronic record-keeping should take account of scientific and technical progress.Systems, procedures and methodology used to record and store data should be periodicallyreviewed and updated as necessary.

QUALITY RISK MANAGEMENT TO ENSURE GOOD DATA MANAGEMENT

All organizations performing work subject to GxP are required by applicable existing WHOguidance to establish, implement and maintain an appropriate quality management system,the elements of which should be documented in their prescribed format such as a quality manual or other appropriate documentation. The quality manual, or equivalent documentation, should include a quality policy statement of management's commitment to an effective quality management system and good professional practice. These policies should include expected ethics and proper code of conduct to assure the reliability and completeness of data, including mechanisms for staff to report any questions or concerns to management. Within the quality management system, the organization should establish the appropriate infrastructure, organizational structure, written policies and procedures, processes and

systems to both prevent and detect situations that may impact data integrity and in turn the risk-based and scientific robustness of decisions based upon that data. Quality risk management is an essential component of an effective data and record validity program. The effort and resource assigned to data and record governance should be commensurate with the risk to product quality. The risk-based approach to record and data management should ensure that adequate resources are allocated and that control strategies for the assurance of the integrity of GxP data are commensurate their potential impact on product quality and patient safety and related decision-making. Control strategies that promote good practices and prevent record and data integrity issues from occurring are preferred and are likely to be the most effective and cost-effective. For example, security controls that prevent persons from altering a master processing formula will reduce the probability of invalid and aberrant data occurring. Such preventive measures, when effectively implemented, also reduce the degree of monitoring required to detect uncontrolled change. Record and data integrity risks should be assessed, mitigated, communicated and

reviewed throughout the data life cycle in accordance with the principles of quality risk management.

Example approaches that may enhance data reliability are given in these guidelines but should be viewed as recommendations. Other approaches may be justified and shown to be equally effective in achieving satisfactory control of risk. Organizations should therefore Working document design appropriate tools and strategies for management of data integrity risks based upon their specific GxP activities, technologies and processes. A data management program developed and implemented, based upon sound quality risk management principles, is expected to leverage existing technologies to their full potential, streamline data processes in a manner that not only improves good data management but also the business process efficiency and effectiveness, thereby reducing costs and facilitating continual improvement.

MANAGEMENT GOVERNANCE AND QUALITY AUDITS

Assuring robust data integrity begins with management which has the overall responsibility for the technical operations and provision of resources to ensure the required quality of GxP operations. Senior management has the ultimate responsibility to ensure an effective quality system is in place to achieve the quality objectives, and that staff roles, responsibilities and authorities, including those required for effective data governance programs, are defined, communicated and implemented throughout the organization. Leadership is essential to establish and maintain a company-wide commitment to data reliability as an essential element of the quality system. The building blocks of behaviours, procedural/policy considerations and basic technical controls together form the basis of a good data governance foundation upon which future revisions can be built. For example, a good data governance program requires the necessary management arrangements to ensure personnel are not subject to commercial, political, financial and other pressures or conflicts of interest that may adversely affect the quality of their work and integrity of their data. Management should also make staff aware of the relevance of data integrity and importance of their role in protecting the safety of the patient and the reputation of the organization for quality products and services. Management should create a work environment in which staff are encouraged to communicate failures and mistakes, including data reliability issues, so that corrective and preventative actions can be taken and the quality of an organization's products and services enhanced. This includes ensuring adequate information flow between staff at all levels. Senior management should actively discourage any management practices that might reasonably be expected to inhibit the active and complete reporting of such issues. Management reviews and regular reporting of quality metrics facilitate these objectives. This requires designation of a quality manager who has direct access to the highest level of management in order to directly communicate risks so that senior management is aware and can allocate resources to address any issues. To fulfil this role the quality unit should conduct and report to management formal, documented risk reviews of the key performance indicators of the quality management system. These should include metrics related to data

integrity to help identify opportunities for improvement. For example: • tracking and trending the occurrence of invalid and aberrant data may reveal unforeseen variability in processes and procedures previously believed to be robust, opportunities to enhance analytical procedures and their validation, validation of processes, training of personnel or sourcing of raw materials and components

Good Laboratory Practice (GLP)

Introduction In the early 70's FDA (United States Food and Drug administration) have realized cases of poor laboratory practice throughout the United States. FDA decided to check over 40 toxicology labs in-depth. They revealed lot dishonest activities and a lot of poor lab practices. Examples of some of these poor lab practices found were equipment not been calibrated to standard form, therefore giving wrong measurements, incorrect or inaccurate accounts of the actual lab study and incompetent test systems. Although the term "good laboratory practice" might have been used informal already for some time in many laboratories around the world GLP originated in the United States and it had a powerfull effect world wide.

Quality Management in the present context can be considered a modem version of the hitherto much used concept "Good Laboratory Practice" (GLP) with a somewhat wider interpretation. The OECD Document defines GLP as follows: *"Good Laboratory Practice (GLP) is concerned with the organizational process and the conditions under which laboratory studies are planned, performed, monitored, recorded, and reported."*

Thus, GLP prescribes a laboratory to work according to a system of procedures and protocols. This implies the organization of the activities and the conditions under which these take place are controlled, reported and filed. GLP is a policy for all aspects of the laboratory which influence the quality of the analytical work. When properly applied, GLP should then:

- allow better laboratory management (including quality management)
- improve efficiency (thus reducing costs)
- minimize errors
- allow quality control (including tracking of errors and their cause)
- stimulate and motivate all personnel
- improve safety
- improve communication possibilities, both internally and externally.

The result of GLP is that the performance of a laboratory is improved and its working effectively controlled. An important aspect is also that the standards of quality are documented and can be demonstrated to authorities and clients. This results in an improved reputation for the laboratory (and for the institute as a whole). In short, the message is:

- say what you do
- do what you say

- do it better
- be able to show what you have done

The basic rule is that all relevant plans, activities, conditions and situations are recorded and that these records are safely filed and can be produced or retrieved when necessary. These aspects differ strongly in character and need to be attended to individually.

Standard Operating Procedures (SOP)

According to EPA(Environmental Protection Agency) GLP regulations, "Raw data" means any laboratory worksheets, records, memoranda, notes, or exact copies thereof, that are the result of original observations and activities of a study and are necessary for the reconstruction and evaluation of the report of that study. Logbooks for recording temperatures or equipment use, repair, and maintenance, field or laboratory notebooks, forms for field or laboratory observations, training reports, computer printouts, recorded data from automated instrument are examples of raw data. It's so hard and not necessary for anyone remember all these details and that's one of the functions of the Standard Operating Procedures (SOPs). 8.1 In FDA it is said that :"If it is not documented..., it did not happen!" or, it's a rumor!" GLPs SOPs Can't do Guarantee "good science", guarantee good documentation, replace common sense, prevent all mistakes (Cobb, 2007). SOPs are written procedures for a laboratories program. They are approved protocols indicating test objectives and methods. Standard Operating Procedures are intended to ensure the quality and integrity of the data generated by the test facility. Revisions to Standard Operating Procedures should be approved by test facility management (OECD, 1998). 8.1.1 They define how to carry out protocol-specified activities. SOPs are most often written in a chronological listing of action steps. They are written to explain how the procedures are supposed to work SOP of routine inspection, cleaning, maintenance, testing and calibration, actions to be taken in response to equipment failure, analytical methods, definition of raw data, keeping records, reporting, storage, mixing, and recovery of data. (Standard Operating Procedures should have been written and approved by test facility management that are intended to ensure the quality and integrity of the data generated by that test facility. Revisions to Standard Operating Procedures should be approved by test facility management. Each separate test facility unit or area should have at once available current Standard Operating Procedures relevant to the activities being performed therein.

Performance of the study

Performance of the Study should be monitorized carefully. All the standards supplied bythe GLP should be followed from the beginning of the study to the end by the final report. For each study, a written plan should exist prior to the initiation of the study (Seiler, 2005). The study plan should contain the following information: Identification of the study, the test item and reference item, information concerning the sponsor and the test facility, dates, test methods, issues (where applicable)and records. (OECD, 1998) The study plan should be approved by dated signature of the Study Director and verified for GLP compliance. Deviations from the study plan should be described, explained, recognized and dated in a timely fashion by the Study Director and/or Principal Investigator(s) and maintained with the study raw data. 9.1.1 In the study plan the identification of the study, the test item and reference item information should exist: A descriptive title; a statement which reveals the nature

and purpose of the study; Identification of the test item by code or name; The reference item to be used. Information Concerning the Sponsor and the Test Facility should be declared. It should comprise:Name and address of the sponsor, any test facilities and test sites involved, Study Director, Principal Investigator(s), and the phase(s) of the study delegated by the Study Director and under the responsibility of the Principal Investigator(s) with the date of approval of the study plan by signature of the Study Director, of the study plan by signature of the test facility management and sponsor if required by national regulation or legislation in the country where the study is being performed, the proposed experimental starting and completion dates, reference to the OECD Test Guideline or other test guideline or method to be used, the justification for selection of the test system characterisation of the test system, such as the species, strain, substrain, source of supply, number, body weight range, sex, age and other pertinent information. It should also contain the method of administration and the reason for its choice; The dose levels and/or concentration(s), frequency, and duration of administration/application; detailed information on the experimental design, including a description of the chronological procedure of the study, all methods, materials and conditions, type and frequency of analysis, measurements, observations and examinations to be performed, and statistical methods to be used. Specimens from the study should be identified to confirm their origin. Such identification should enable traceability, as appropriate for the specimen and study. The study should be conducted in accordance with the study plan. All data generated during the conduct of the study should be recorded directly, punctually, correctly, and legibly by the individual entering the data. These entries should be signed or initialled and dated. Any change in the raw data should be made in order to understand the previous entry easily, should indicate the reason for change and should be dated and signed or initialled by the individual making the change. 9.1.2 Computerised system design should always supply for the retention of full audit trails to show all changes to the data without obscuring the original data. It should be possible to associate all changes to data with the persons having made those changes. Reason for changes should be given.

Reporting of study results

All studies generate raw data that are the original data gathered during the conduct of a procedure. They are essential for the reconstruction of studies and contribute to the traceability of the events of a study. Raw data are the results of the experiment upon which the conclusions of the study will be based. Some of the raw data may be used directly, and some of them will be treated statistically. The results and their interpretations provided by the scientist in the study report must be a true and accurate reflection of the raw data. A final report should be prepared for each study. The study report, like all the other scientific aspects of the study, is the responsibility of the Study Director. He/she must ensure that it describes the study accurately. Reports of Principal Investigators or scientists involved in the study should be signed and dated by them. The final report should be signed and dated by the Study Director to indicate acceptance of responsibility for the validity of the data.If necessary, corrections and additions to a final report should be in the form of amendments. Amendments should clearly specify the reason for the corrections or additions and should be signed and dated by the Study Director. The Study Director is responsible for the scientific interpretation included in the study report and is also responsible for declaring to what extent the study was conducted in compliance with the GLP Principles. The GLP

Principles list the essential elements to be included in a final study report. The final report should include, the following information: A descriptive title; identification of the test item by code or name, characterisation of the test item including purity, stability and homogeneity. Information concerning the sponsor and the test facility should imply;name and address of the sponsor, any test facilities and test sites involved, the study Director, the Principal Investigator(s) and the phase(s) of the study, delegated and scientists having contributed reports to the final report, experimental starting and completion dates. A Quality Assurance Programme statement listing the types of inspections made and their dates, including the phase(s) inspected, and the dates any inspection results should be reported to management and to the Study Director and Principal Investigator(s). This statement should also serve to confirm that the final report reflects the raw data. It should contain the Description of Materials and Test Methods. A summary of results should be given. All information and data required by the study plan;A presentation of the results, including calculations and determinations of statistical significance;An evaluation and discussion of the results and, where appropriate, conclusions. It should imply the location(s) where the study plan, samples of test and reference items, specimens, raw data and the final report are to be stored. 10.1.3 A computerised system to be used in a GLP area should include both the dating and timing of the original entry and the retention of a full audit trail. Such identification could be possible either by the use of personal passwords recognised by the computer or by digital signatures. Furthermore, the system should not accept any changes to data without concomitant entry of a reason or justification. In manual recording the entries made on a sheet of paper can be dated and signed to bear witness to the validity of data and to accept responsibility. Therefore GLP wants to ensure that data safety and integrity remains the same in electronically as in manually recorded data, irrespective of how they were recorded, and that reconstruction of the way in which the final results and conclusions were obtained remains fully possible The Study Director must sign and date the final report to indicate acceptance of responsibility for the validity of all the data.

Storage and retention of records and materials

Storage and retention of records and materials should be prepared appropriately. The following should be retained in the archives for the period specified by the appropriate authorities : the study plan, raw data, samples of test and reference items, specimens, and the final report of each study records of all inspections performed by the Quality Assurance Programme, as well as master schedules, records of qualifications, training, experience and job descriptions of personnel; records and reports of the maintenance and calibration of apparatus; validation documentation for computerised systems. In the absence of a necessitated retention period, the final arrangement of any study materials should be documented.

Responsibilities in GLP. Implementing of GLP in non GLP analytical laboratory.

When implementing GLP in a test facility, and particularly during training, it is important to clearly differentiate between the formal, regulatory use of the term Good Laboratory Practice and the general application of “good practices” in scientific investigations. Since the term “Good Laboratory Practice” is not a trade-mark protected term, any laboratory may consider that it is following good practices in its daily work. This does not comprise GLP compliance. It must be clearly understood that only adherence to, and compliance with, all the requirements of the

OECD GLP Principles constitutes real compliance with GLP. Therefore, the use of similar terminology to describe quality practices outside the scope of GLP proper should be strongly discouraged.

The device to maintain a safe laboratory environment for all begins with
When a chemical splashes in the eye rinse for
Which of the following types of personal protective equipment (PPE) is frequently used
Chemical, reagents, broth cultures should be pipperted by
Good work practices include
What is the name of procedure performed under sterile conditions to eliminate contamination in hopes to obtain a pure culture of one type of microorganism
After a biohazard spill is covered with paper towels and disinfectant solution, it must sit for
What is needed for the source of nutrient for the growth and reproduction of microbes
To prevent the contamination of microscopes and surrounding areas disinfect /clean used slides prepared by students with
Which of the following extinguishers is suitable for a fire involving flammable liquids
Ethanol is
Which of the following is the principles of SSLP ?
How many types of inspection
SOP is otherwise known as
What good laboratory must contain?
The prevention of large-scale loss of biological integrity is
Which of the following is not a laboratory safety rule?
Which piece of laboratory equipment is best-suited for accurately measuring the volume of a liquid?
Which piece of laboratory equipment can be used to store chemicals for long periods of time?
The independent variable in an experiment is:
Qualitative results refers to
When drawing a graph that measures family average income over a period of 30years , the independent variable is
Accuracy is defined as
How many significant figures are present in the number 30,450?
The key component of GLP system of quality is
Microscope is wiped dry using
Which one of the following is correct?
Before operating inoculation chamber the palm should be wiped with
Which one of the following are GLP regulations on requirements
A "class –A" fire extinguisher can be used to treat fires involving which as fuel sources
Which of the following id not a type of firefighting equipment
Why shouldn't carbon dioxide extinguishers be used in confined spaces
What is the correct definition of fire
What is the extraction as practiced in the organic chemistry laboratory
Lathe gloves
What is spectroscopy?
What piece of laboratory equipment is best suited for accurately measuring the volume of a liquid
What piece of laboratory equipment can be used to store chemical for log periods of time
Qualitative results refers to
Accuracy is defined as

A. prevention
A. 10 seconds
A. safety glasses
A. mouth
A. smelling and tasting chemicals
A. sterilization technique
A. 5 minutes
A. pathogens
A. 75% ethanol and lens paper
A. carbon dioxide extinguisher (black)
A. Glass ware
A. GLP systems
A. 7
A. Standard operating procedures
A. Area should be free from smoke , smell, dust
A. Fire safety
A. You should never mix acids with bases
A. Graduated cylinder
A. Burette
A. The variable you hope to observe in an experiment
A. Results that can be observed during an experiment
A. Accurate
A. A measure of how often an experimental value can be repeated
A. Three
A. Quality unit
A. 70% isopropyl alcohol +30% water
A. acid can be added to water
A. Ethanol
A. 21CFR58
A. Ordinary combustibles (wood and plastics)
A. Fire blanket
A. they might explode
A. a chemical reaction from which heat and light are emitted
A. the removal of one solid material from other
A. may be reused only if they have not be been germinated
A. distillation is when a liquid is evaporated and then recondensed in another container
A. graduated cylinder
A. burette
A. results that can be observed during an experiment
A. a measure of how often an experimental value can be repeated

B. ubiquity
B. 30 seconds
B. lab coats
B. ear
B. not washing hand before and after lab
B. aseptic technique
B. 60 minutes
B. bacteria
B. aerosne and ieeo paper
B. powder extinguisher (blue)
B. FDA regulations
B. Reporting of study results
B. 8
B. System operating procedures
B. Maintenance and calibration data
B. Bio safety
B. You should tie back your long hair
B. Beaker
B. Evaporating dish
B. The variable you change in an experiment
B. Results those are difficult to observe during an experiment
B. Average
B. The closeness of a measure value to the real value
B. Four
B. Quantity unit
B. distilled water
B. water can be added to acid
B. distilled water
B. 48CFR160
B. electrical equipment
B. fuize vest
B. harmful fumes may be inhaled
B. hot orange stuff
B. the separation of one substance from the another based on solubility
B. may be reused as long as they are clean
B. distillation is when material heated to melting and then separated
B. beaker
B. evaporating dish
B. results that is difficult to observe during an experiment
B. the closeness of a measured value to the real value

C. microbiology
C. 5 minutes
C. face shields
C. pipette
C. confining long hair and loose clothing
C. disinfectal technique
C. 90 minutes
C. reagents
C. 5% methylene blue and lens paper
C. foam extinguisher (cream)
C. Analytical laboratory
C. Test and reference substances
C. 5
C. Safety operating procedures
C. Air conditional the lab with humidity control
C. Chemical safety
C. You should never add water to acid
C. Erlenmeyer flask
C. Beaker
C. The variable that isn't changed in an experiment
C. Results that require numerical data
C. Years
C. The number of significant figures used in a measurement
C. Five
C. Quality reading unit
C. 75% ethanol
C. both a and b
C. sanitizer
C. 21CFR211
C. combustible metals
C. sprinkler
C. they could cause claustrophobia
C. mixture of carbon dioxide and nitrogen
C. the removal of painful or impacted teeth
C. should never be reused
C. distillation is when a substance is dissolved, heated and then precipitated
C. Erlenmeyer flask
C. beaker
C. results that require numerical data
C. the number of significant figures used in a measurement

D. accidents
D. 15 minutes
D. all of the above
D. none
D. using damaged equipment and glassware
D. pathogen technique
D. 25 minutes
D. media
D. water and lens paper
D. polka dot extinguisher (dotty)
D. MFRs rules
D. All the above
D. 1
D. Stationary operating procedures
D. Both A and C
D. Test systems
D. All the above
D. More than one of the above
D. More than one of the above
D. None of these is correct
D. None of these is correct
D. It is impossible to say
D. None of these
D. None of these
D. Quality assurance unit
D. only with water
D. none of these
D. all of the above
D. a and b only
D. flammable to combustible liquids
D. ice cubes
D. they might not show up if its dark
D. a yellow coloured solution
D. none of these
D. both a and b only
D. none of these
D. more than one of the above
D. more than one of the above
D. none of these is correct
D. none of these

A. prevention
D. 15 minutes
D. all of the above
C. pipette
C. confining long hair and loose clothing
B. aseptic technique
B. 60 minutes
D. media
A. 70% ethanol and lens paper
A. carbon dioxide extinguisher (black)
B. FDA regulations
B. All the above
C. 2
A. Standard operating procedures
D. Both A and C
B. Bio safety
A. You should never mix acid with bases
A. Graduated cylinder
C. Beaker
B. The variable you change in an experiment
D. None of these correct
C. Years
B. The closeness of a measure value to the real value
B. Four
D. Quality assurance unit
A. 70% isopropyl alcohol +30% water
A. acid can be added to water
A. Ethanol
D. a and b only
C. combustible metals
D. ice cubes
B. harmful fumes may be inhaled
A. a chemical reaction from which heat and light are emitted
B. the removal of one substance from the another based on solubility
C. should never be reused
A. distillation is when a liquid is evaporated and then recondensed in another container
A. graduated cylinder
C. beaker
D. none of these is correct
B. the closeness of a measured value to the real value

Reg. No. _____

[17BCP103]

KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed University *established Under Section 3 of UGC Act 1956*)

DEPARTMENT OF BIOCHEMISTRY

I M.Sc., Biochemistry - First Semester

17BCP103 – BIOINSTRUMENTATION AND GLP

FIRST INTERNAL EXAMINATIONS - AUGUST 2017

Time: 2 hrs
Date: 30.08.2017 **Maximum: 50 marks**

PART A (20 x 1 = 20 marks)

1. Angular velocity is denoted by the term

   a) alpha b) beta
   c) omega d) delta

2. Subcellular fractionation is done using which centrifugation?

   a) Differential b) Density gradient
   c) Percoll centrifugation d) Isoelectric

3. Violet light absorbs

   a) Blue b) Green
   c) Yellow d) Orange

4. Maximum absorbance for yellow colour is read at.

   a) 400 b) 500
   c) 600 d) 700

5. Absorbance is directly proportional to

   a) Intensity of colour b) Path length
   c) Instrument d) Intensity and pathlength

6. Change in fluorescence is referred as

   a) Stoke shift b) Beer law
   c) Lambert law d) Emerson effect

7. Example of an extracellular enzyme is

   a) Lactate dehydrogenase b) Cytochrome oxidase
   c) Pancreatic lipase d) Hexokinase

8. Red light absorbs _____ colour

a) Blue
b) Green
c) Yellow
d) Orange

9. DNA has maximum absorbance at

a) 300
b) 500
c) 260
d) 280

10. Protein has maximum absorbance at

a) 300
b) 500
c) 260
d) 280

11. Thin layer chromatography is

a) partition chromatography
b) electrical mobility of ionic species
c) adsorption chromatography
d) migration of charged ions in electric field

12. Relative flow (Rf) value ranges from

a) 0 to 1
b) 0 to 2.0
c) +2 to -2
d) +1 to -1

13. After centrifugation of milk, the supernatant is

a) fat
b) whey
c) casein
d) water

14. Chromatography can be used to

a) form mixtures
b) change mixture compositions
c) separate mixtures into pure substances
d) separation according to charge

15. Electrophoresis of histones and myoglobin under non-denaturing conditions (pH = 7.0) results in

a) both proteins migrate to the anode
b) histones migrate to anode and myoglobin migrates to the cathode
c) histones migrate to the cathode and myoglobin migrates to the anode
d) both proteins migrate to the cathode

16. In isoelectric focusing, proteins are separated on the basis of their

a) relative content of positively charged residue only
b) relative content of negatively charged residue only
c) size
d) relative content of positively and negatively charged residue

17. In SDS-PAGE, the protein sample is first

a) treated with a reducing agent and then with anionic detergent followed by fractionation by electrophoresis
b) fractionated by electrophoresis then treated with an oxidizing agent followed by anionic detergent

c) treated with a oxidizing agent and then with anionic detergent followed by fractionation by electrophoresis
d) treated with acetic acid

18. Chromatography generally involves

a) Two phases b) Single phase
c) Three phase d) No phase

19. SDS is a(n)

a) anionic detergent b) Cationic detergent
c) not an detergent d) Chargeless

20. Proteins are separated in an SDS-PAGE experiment on the basis of their

a) positively charged side chains b) molecular weight
c) negatively charged side chains d) different isoelectric points

Part - B

Answer ALL questions (3x2=6marks)

21. Define sedimentation coefficient.
22. List out the types of centrifugation.
23. State Beer-Lambert law.

Part - C

Answer ALL questions (3x8=24marks)

24. a) How do you separate subcellular organelles using centrifugation?

or

b) Explain in detail about density gradient centrifugation.

25. a) Discuss the principles, instrumentation and applications of colorimeter.

or

b) List out the principles, instrumentation and applications of spectrophotometer.

26. a) Elaborate the principles, instrumentation and applications of spectrofluorimeter.

or

b) Explain immunofluorescence and FRAP.

Reg. No. _____

[17BCP103]

Enable | Enlighten | Enrich
(Deemed to be University)
(Under Section 3 of UGC Act 1956)

KARPAGAM ACADEMY OF HIGHER EDUCATION

DEPARTMENT OF BIOCHEMISTRY

I M.Sc., Biochemistry - First Semester

17BCP103 – BIOINSTRUMENTATION

SECOND INTERNAL EXAMINATIONS - SEPTEMBER 2017

Time: 2 hrs
Date: **Maximum: 50 marks**

PART A (20 x 1 = 20 marks)

1. SDS detergent is
   a) Cationic b) Anionic c) uncharged d) Positronic

2. DNA is loaded in which electrode?
   a) Cathode b) Anode c) Middle electrode d) Rhode

3. pH of stacking gel is
   a) 6.8 b) 8.8 c) 10.8 d) 3.8

4. pH of separating gel is
   a) 6.8 b) 8.8 c) 10.8 d) 3.8

5. Which of the following is primary fluor
   a) PPO b) POPOP c) PPPP d) PAP

6. Which of the following is secondary fluor
   a) PPO b) POPOP c) PPPP d) PAP

7. Ampholytes are
   a) Postitively charged b) Negatively charged
   c) Uncharged d) not used in IEF

8. What does pI indicate?

a) acidity b) basicity c) isoelectric point d) it does not indicate anything

9. The colour of cathode is

a) Red b) Black c) Blue d) Green

10. The colour of anode is

a) Red b) Black c) Blue d) Green

11. What is alpha radioactivity?

a) A proton b) A helium nucleus c) An electromagnetic wave d) An electron.

12. Type of radioactivity is the most penetrating?

a) Alpha b) Beta c) Gamma d) Delta.

13. Emission of which one of the following leave both atomic number and mass number unchanged?

a) Positron b) Neutron c) Alpha particle d) Gamma radiation.

14. Cuvettes used in spectrophotometer is having an optical path length of_____ cm.

a) 2 b) 3 c) 0.5 d) 1.

15. Which of the following is used as light source in colorimeter?

a) Hydrogen lamp b) Deutrium lamp c) Tungsten lamp d) Sodium lamp

16. The rate at which DNA migrates through the gel is mainly determined by

a) molecular size of the DNA b) salt levels c) Protein d) Lipid

17. Solid scintillation counting is useful for the measurement of _____ isotopes.

a) alpha emitter b) beta emitter c) gamma emitter d) proton

18. The solvent molecule, which has become excited, emits light as it comes back to ground state is know as_____.

a) phosphorescence b) fluorescence c) alpha emitter d) beta emitter

19. Solute used in density gradient centrifugation is /are _____.

a) ammonium sulphate b) caesium chloride c) arabinose d) dextrin.

20. The correct order for the basic features of a mass spectrometer is_____

a) acceleration, deflection, detection, ionisation

b) ionisation, acceleration, deflection, detection

c) acceleration, ionisation, deflection, detection

d) acceleration, deflection, ionisation, detection.

Part - B

Answer ALL questions **(3x2=6marks)**

21. What is Cerenkov radiation
22. Define isoelectric focusing
23. Give any two features of GLP

Part - C

Answer ALL questions **(3x8=24marks)**

24. a) Explain the principle, instrumentation and working of SDS PAGE.

or

b) Explain the principle, instrumentation and working of agarose gel electrophoresis.

24. a) Explain the principle, instrumentation and working of 2D electrophoresis

or

b) Explain the working principle of GM Counter

25. a) Discuss the salient features of good laboratory practices

or

b) What is scintillation counting? Explain in detail.

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