KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed to be University)
(Established Under Section 3 of UGC Act 1956)
Coimbatore - 641021.
(For the candidates admitted from 2019 onwards)

DEPARTMENT OF BIOCHEMISTRY

SUBJECT : BIOINSTRUMENTATION AND GLP

SEMESTER : I

SUBJECT CODE: 19BCP103 **CLASS : I MSc. BC**

SCOPE

OBJECTIVES

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.

Suggested Readings

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.

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.

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SUBJECT : **BIOINSTRUMENTATION AND GLP**

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LECTURE PLAN
DEPARTMENT OF BIOCHEMISTRTY

S.No	Lecture Duration Hour	Topics to be Covered	Support Material/Page Nos
		UNIT-I	
1	1	Types of centrifuges, Principles and applications of analytical and preparative centrifuges	S7: 109
2	1	Density gradient and ultra centrifugation	S3:34
3	1	Relative molecular mass determination and sedimentation coefficient.	S7: 118;S3:36
4	1	Sub cellular fractionation of cellular components. Applications. Separation of cells on the basis of density	S7: 121
5	1	Beer's law and Lambert's law. Principle of photoelectric colorimeter	S7:558
6	1	Spectroscopy – Properties of electromagnetic radiations,	S7:551
7	1	Instrumentation and applications of UV Visible and mass spectroscopy	S7:557
8	1	FTIR, NIR, reverse spectroscopy	
9	1	Spectrofluorimetery, atomic spectroscopy, NMR spectroscopy.	S3:139
10	1	Advantatages and disadvantages and advancements of spectroscopic methods.	S4:269,294
	Total No Of Hours Planned For Unit 1=10		
		UNIT-II	
1	1	Principles, Types – paper chromatography, thin layer chromatography	S7:485

2	1	Column chromatography - Ion exchange chromatography, affinity chromatography	S3:44,46
3	1	Gel filtration chromatography	S4:201
4	1	Low pressure liquid chromatography (LPLC) and High Performance Liquid Chromatography (HPLC)	S7:500
5	1	Gas -liquid chromatography Mass spectroscopy (GC – MS), LC-MS	S3:66
6	1	MALDI-TOF, ICPMS, Application of Chromatography	S4:215
7	1	Separation of phytoconstituents using TLC.	S4:415
	Total No Of Hours Planned For Unit II=07		
		UNIT-III	
1	1	Principle, instrumentation and applications of agarose gel electrophoresis	S7:473; S4:245
2	1	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE), native PAGE	S7:457,473 S4:238
3	1	Isoelectric focusing	S7:462
4	1	Immunoelectrophoresis, 2D gel electrophoresis	S3:99
5	1	Pulse field gel electrophoresis	S7:476
6	1	Capillary electrophoresis, gel documentation	S7:478
7	1	Applications. Blotting techniques.	S7:469
	Total No Of Hours Planned For Unit III=7		
		UNIT-IV	
1	1	Introduction, nature of radio activity, types and rate of radioactive decay	S7:621
2	1	Units of radio activity	S4:308
3	1	Detection and measurement of radioactivity	S7:628
4	1	Geiger-Muller counter, solid and liquid scintillation counter	S3:79
5	1	Autoradiography	S3:189
6	1	X-ray diffraction and circular dichorism	S4:286
7	1	Non radioactive, fluorescent methods	S4:269
8	1	Applications of radioisotopes in biological sample analysis	S7:188
9	1	Flowcytometry: Principles and applications**.**	W1
	Total No Of Hours Planned For Unit IV=09		

		UNIT-V	
1	1	Quality concepts, personal protective equipment.	S1:14-18
2	1	General safety-biological safety, chemical safety and fire safety. data generation and storage	S1:96
3	1	Quality control documents, retention samples, records, audits of quality control facilities. List of Regulations to be followed**.**	S1:1-18
4	1	Laboratory safety procedure- glass ware, equipment safety, hands protection, precaution to be undertaken to prevent accident and contamination. GLP – an overview and basic information	S1:87
5	1	Principles of GLP: Test Facility Organization and Personnel, Test Systems, Test and Reference Items	S1:10
6	1	Standard Operating Procedures, Performance of the Study, Reporting of Study Result,	S1:40,102
7	1	Storage and Retention of Records and Materials	S1:43-48
8	1	Responsibilities in GLP	S1:97
9	1	Quality concepts, personal protective equipment.	S1:14-18
10	1	General safety-biological safety, chemical safety and fire safety. data generation and storage	S1:96
11	1	Quality control documents, retention samples, records, audits of quality control facilities. List of Regulations to be followed**.**	S1:1-18
12	1	Laboratory safety procedure- glass ware, equipment safety, hands protection, precaution to be undertaken to prevent accident and contamination. GLP – an overview and basic information	S1:87
	Total no of Hours Planned for unit V=12		
Total Planned Hours		**45**	

Suggested Readings

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.

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

UNIT-I

SYLLABUS

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.

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, thetube 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:

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

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/s^2).
- 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.

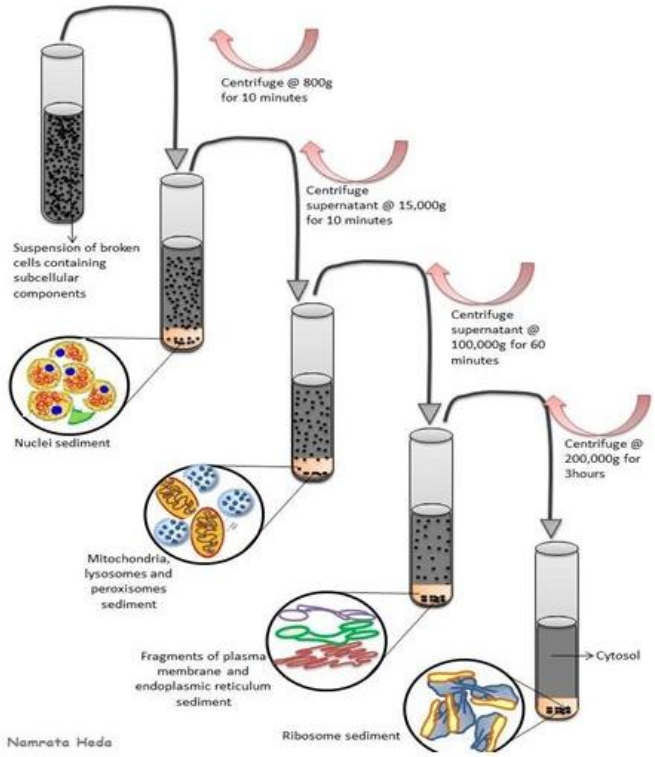
SEPARATION OF CELL ORGANELLES:

- 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	Citric acid cycle, ammonia release	DHases
	for urea formation.		
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.

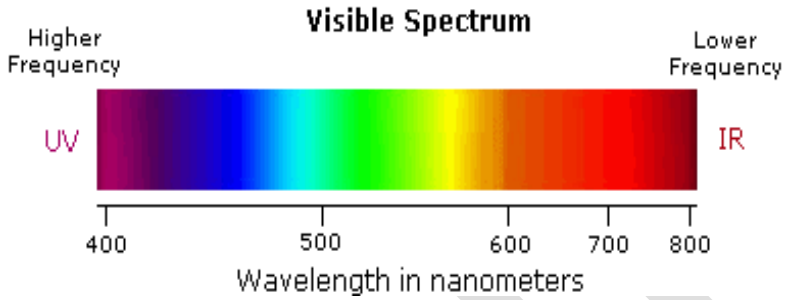
- Each cellular component begins to move down the gradient but it eventually reaches a position where the density of the solution is equal to its own density. At this point the component floats and can move no farther. A series of distinct bands is thereby produced in the centrifuge tube, with the bands 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.



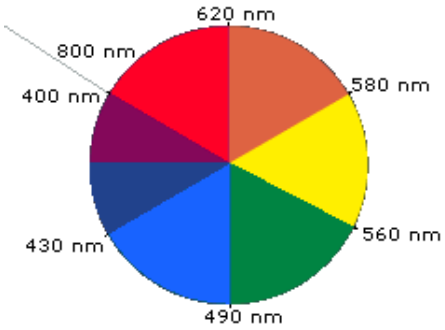
Wavelengths that the molecule doesn't absorb are transmitted, or reflected, to the observer's eye as the perceived color of the molecule, especially when it is dissolved in a transparent solvent such as water. For example, nickel sulfate, NiSO4, dissolved in water appears greenish-blue because it transmits 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.

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

Lambert's Law

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

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

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.

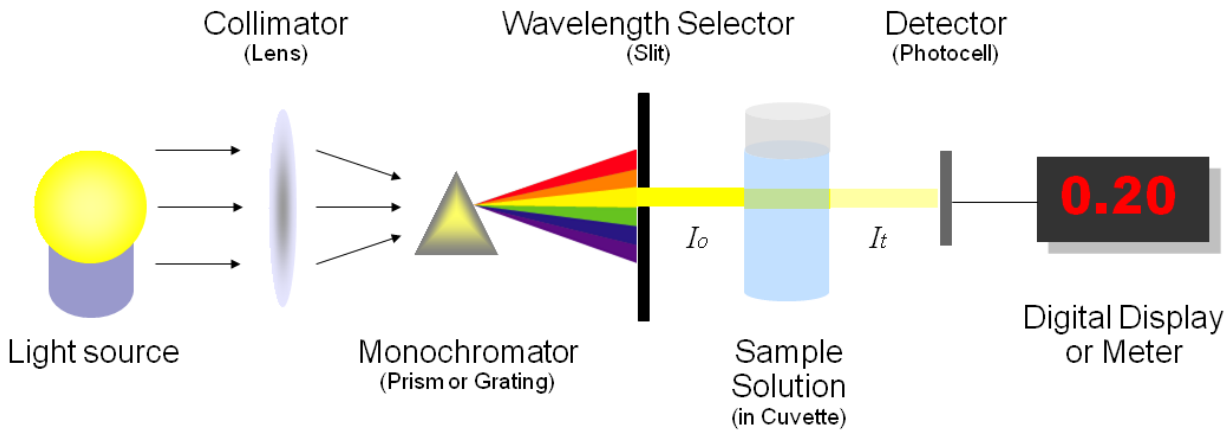
Principles:

Colorimetry is the techniques that are 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 apparatus 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.

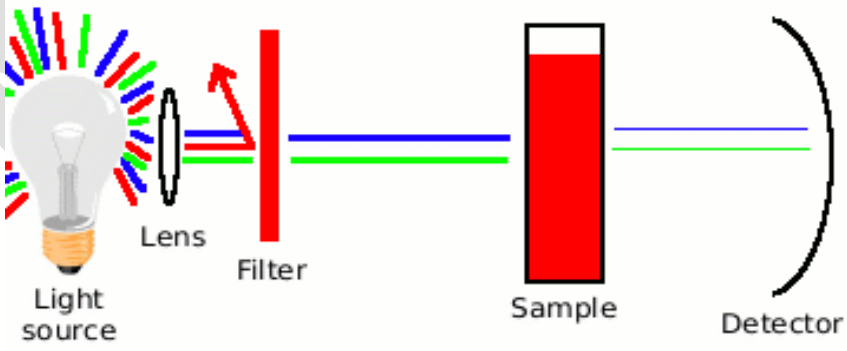


Table for choosing the wavelength of absorption:

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

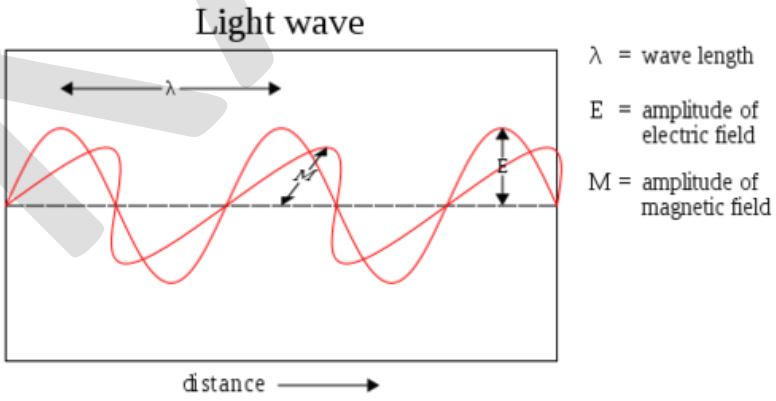
as glass or water from air), the speed and wavelength of light is reduced, although the frequency remains unaltered.

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. Theseelectric 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



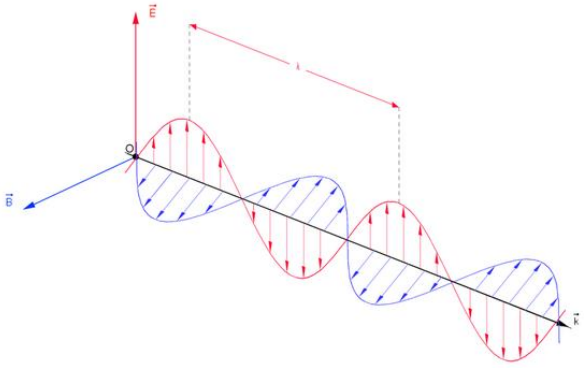


Fig. 1 & 2: Electromagnetic Waves

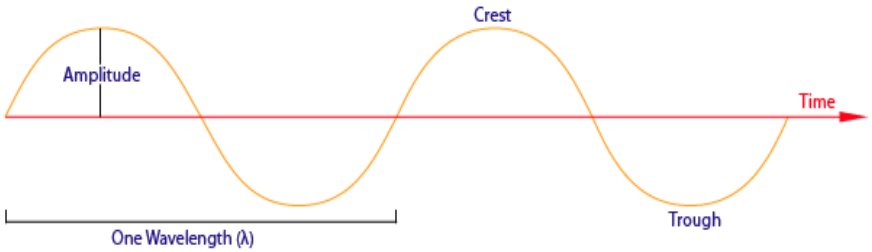
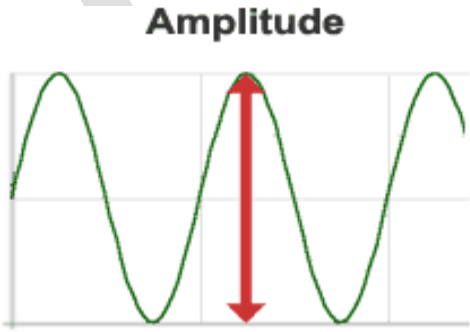


Fig. 3: An EM Wave

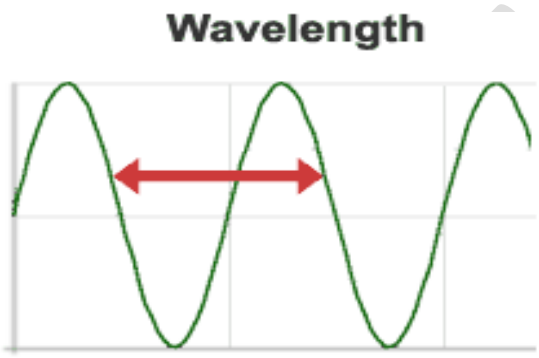
Amplitude



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.

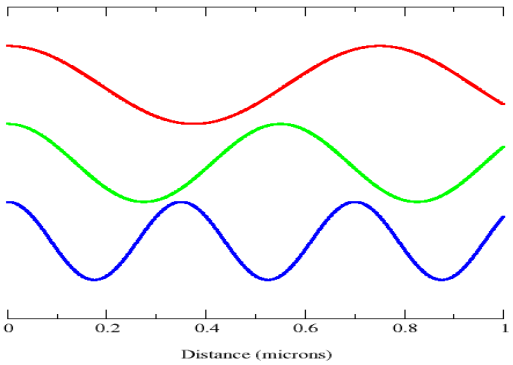
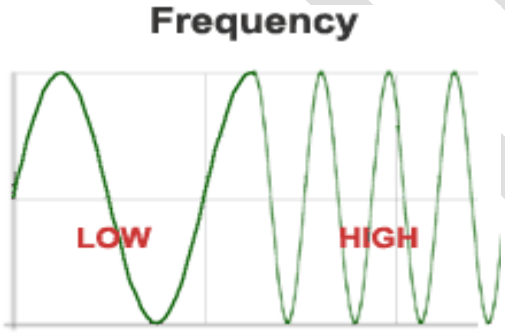


Fig. 4: Different Wavelengths and Frequencies

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, ($h= 6.62607 \times 10^{-34}$ J), and
- ν is frequency.

Period

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

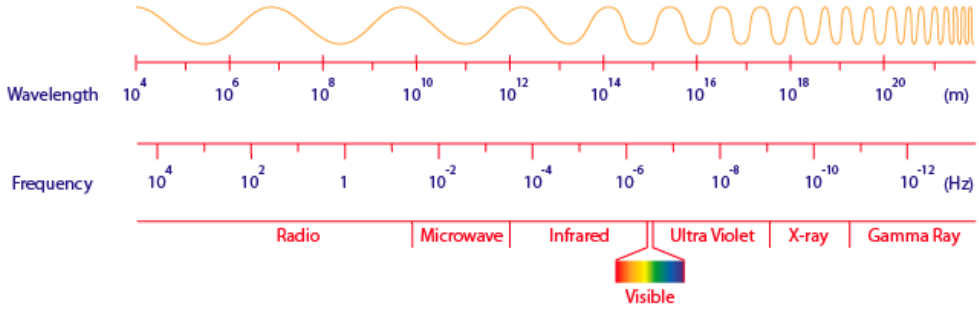
Velocity

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



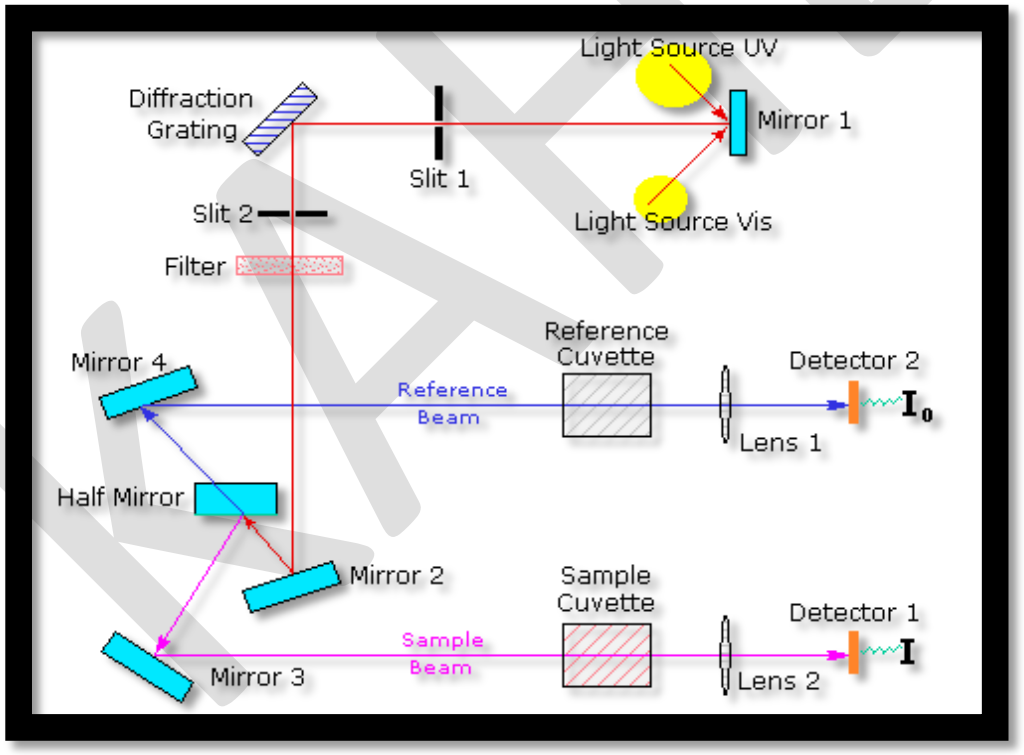
In general, as a wave's wavelength increases, the frequency decreases, and as wave's wavelength decreases, the frequency increases. When electromagnetic energy is released as the energy level increases, the wavelength decreases and frequency decreases. Thus, electromagnetic radiation is then grouped 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.

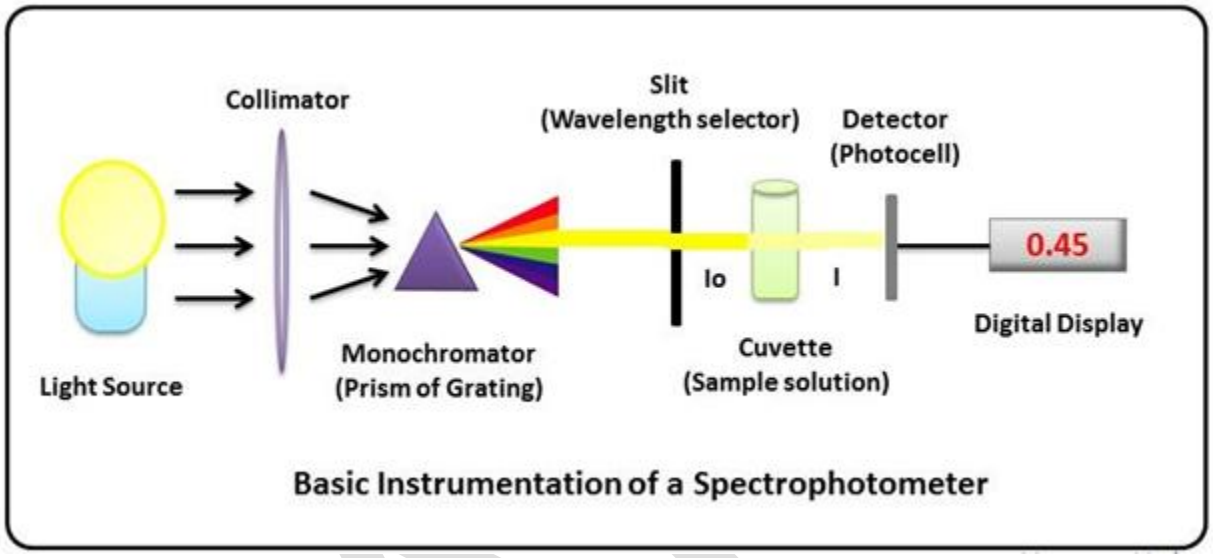
The term chromophore is given to that part of a molecule that gives rise independently to distinct parts of an absorption spectrum, for example the carbonyl group. Conjugated double bonds lower the energy required for electronic transitions and results in an increase in the wavelength at which a chromophore 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).



Basic Instrumentation of a Spectrophotometer

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.

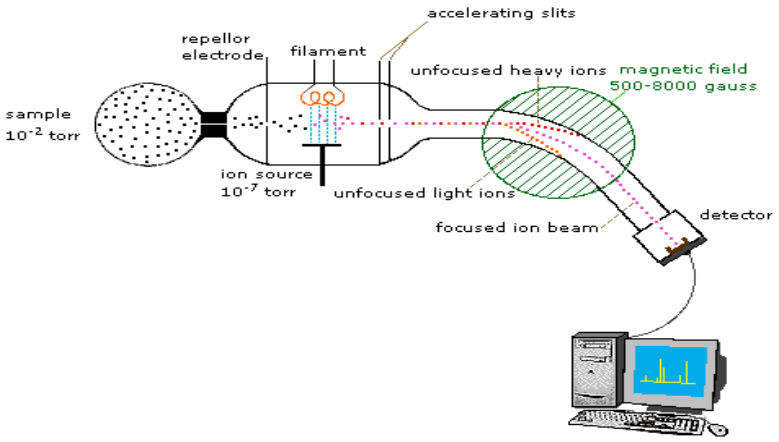
The Mass Analyzer: The ions are sorted and separated according to their mass and charge.

The Detector: The separated ions are then measured, and the results displayed on a chart.

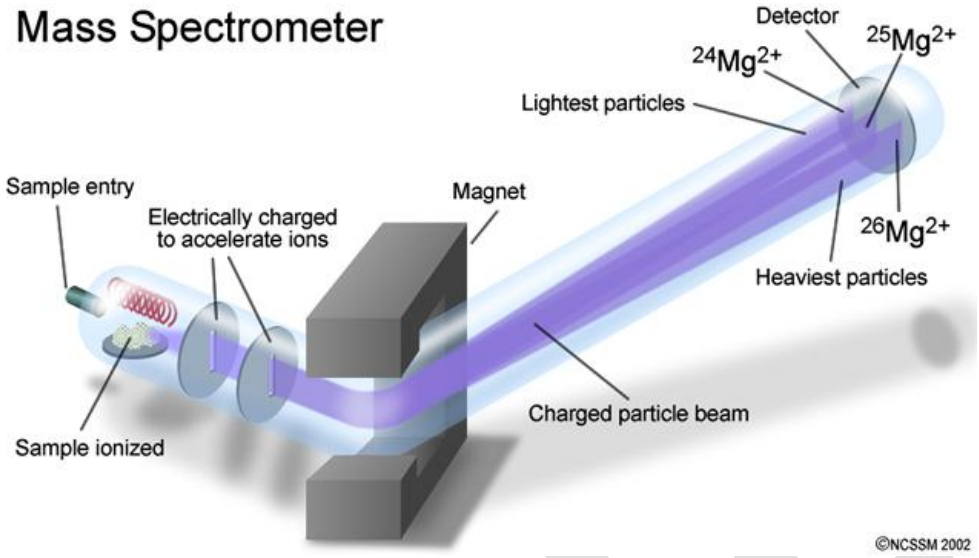
Because ions are very reactive and short-lived, their formation and manipulation must be conducted in a vacuum. Atmospheric pressure is around 760 torr (mm of mercury). The pressure under which ions may be handled is roughly 10^{-5} to 10^{-8} torr (less than a billionth of an atmosphere). Each of the 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:

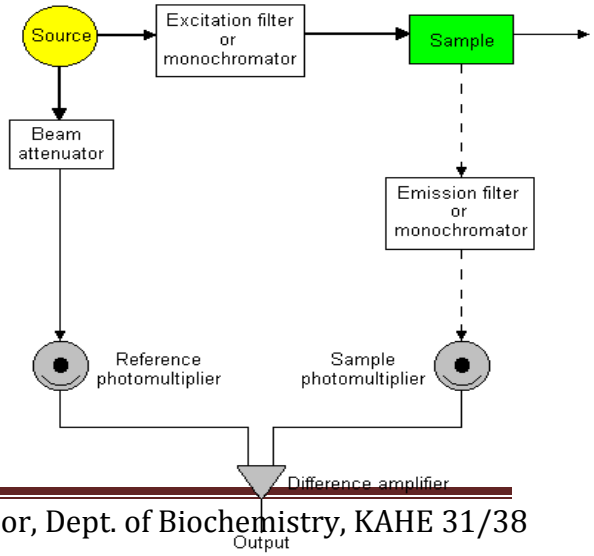
- Mass spectrometry has both qualitative and quantitative uses. These include identifying unknown compounds, determining the isotopic composition of elements in a molecule, and determining the structure of a compound by observing its fragmentation.
- Other uses include quantifying the amount of a compound 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.

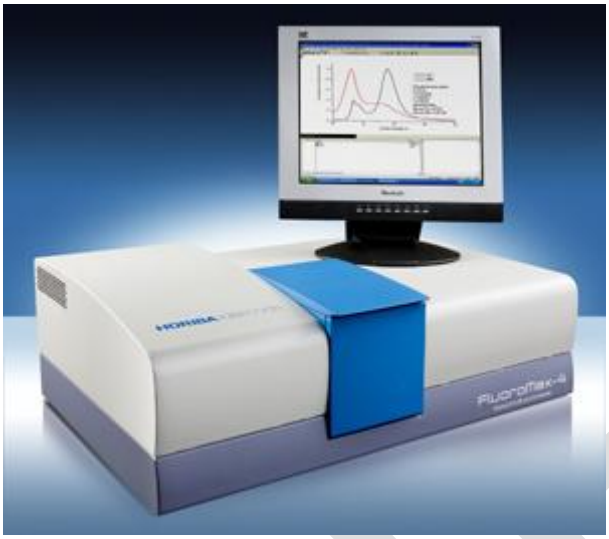
Lasers are sometimes used as excitation sources. A tunable dye laser, using a pulsed nitrogen laser as the primary source can produce monochromatic radiation between 360 and 650 nm. Since the radiation produced *is* monochromatic, there is no need for an excitation monochromator.

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.

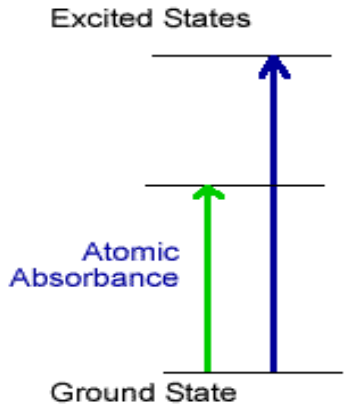


Applications:

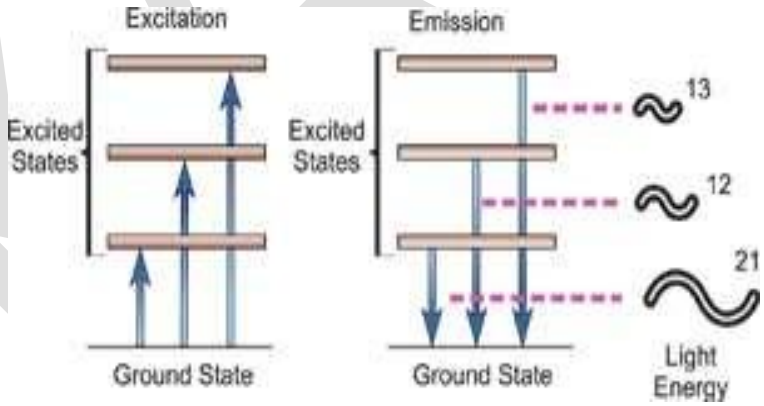
- Generally, spectrofluorometers use high intensity light sources to bombard a sample with as many photons as possible.
- This allows for the maximum number of molecules to be in an excited state at any one point in time.
- The light is either passed through a filter, selecting a fixed wavelength, or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

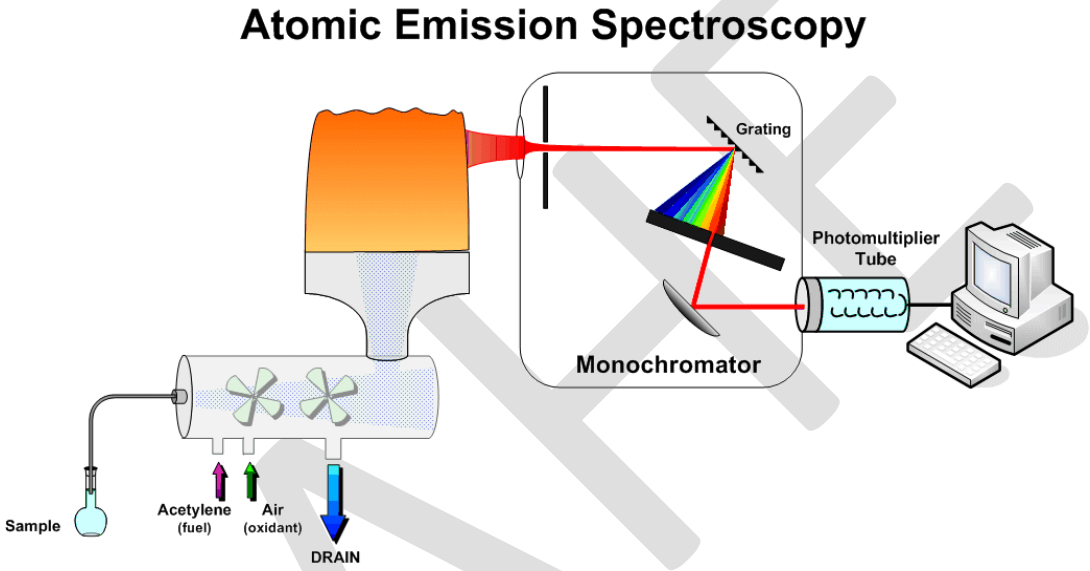


Absorption and Emission:

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.

Spectrofluorimeter Instrumentation

Fluorescence spectroscopy works most accurately at very low concentrations of emitting fluorophores. UV/Vis spectroscopy, in contrast, is least accurate at such low concentrations. One major factor adding to the high sensitivity of fluorescence applications is the spectral selectivity. Due to the Stokes shift, the wavelength of the emitted light is different from that of the exciting light. Another feature makes use of the fact that fluorescence is emitted in all directions. By placing the detector perpendicular to the excitation pathway, the background of the incident beam isreduced.

The schematic of a typical spectrofluorimeter has two monochromators, one for tuning the wavelength of the exciting beam and a second one for analysis of the fluorescence emission. Due to the emitted light always having a lower energy than the exciting light, the wavelength of the excitation monochromator is set at a lower wavelength than the emission monochromator. The better fluorescence spectrometers in laboratories have a photon-counting detector yielding very high sensitivity. Temperature control is required for accurate work as the emission intensity of a fluorophore is dependent on the temperature of the solution. Two geometries are possible for the measurement, with the ° arrangement most commonly used. Pre- and post-filter effects can arise owing to absorption of light prior to reaching the fluorophore and the reduction of emitted radiation.Thesephenomenaarealsocalledinnerfiltereffectsandaremoreevidentinsolutions with high concentrations. As a rough guide, the absorption of a solution to be used for fluorescence experiments should be less than 0.05. The use of microcuvettes containing less material can also be useful. Alternatively, the front-face illumination geometry can be used which obviates the inner filter effect. Also, while the ° geometry requires cuvettes with two neighbouring faces being clear (usually, fluorescence cuvettes have four clear faces), the front- face illumination technique requires only one clear face, as excitation and emission occur at the same face. However, front-face illumination is less sensitive than the° illumination.

Applications

There are many and highly varied applications for fluorescence despite the fact that relatively few compounds exhibit the phenomenon. The effects of pH, solvent composition and the polarisation of fluorescence may all contribute to structural elucidation. Measurement of fluorescence lifetimes can be used to assess rotation correlation coefficients and thus particle

sizes. Non-fluorescent compounds are often labeled with fluorescent probes to enable monitoring of molecular events. This is termed extrinsic fluorescence as distinct from intrinsic fluorescence where the native compound exhibits the property. Some fluorescent dyes are sensitive to the presence of metal ions and can thus be used to track changes of these ions in invitro samples, as well as whole cells. Since fluorescence spectrometers have two monochromators, one for tuning the excitation wavelength and one for analyzing the emission wavelength of the fluorophore, one can measure two types of spectra: excitation and emission spectra. For fluorescence excitation spectrum measurement, one sets the emission monochromator at a fixed wavelength ($_{em}$) and scans a range of excitation wavelengths which are then recorded as ordinate (x-coordinate) of the excitation spectrum; the fluorescence emission at $_{em}$ is plotted as abscissa. Measurement of emission spectra is achieved by setting a fixed excitation wavelength ($_{exc}$) and scanning a wavelength range with the emission monochromator. To yield a spectrum, the emission wavelength lem is recorded as ordinate and the emission intensity at $_{em}$ is plotted asabscissa.

Fourier transform infrared spectroscopy (FTIR)

Is a technique which is used to obtain an infrared spectrum of absorption or emission of a solid, liquid or gas. An FTIR spectrometer simultaneously collects high spectral resolution data over a wide spectral range. This confers a significant advantage over a dispersive spectrometer which measures intensity over a narrow range of wavelengths at a time.

The term Fourier transform infrared spectroscopy originates from the fact that a Fouriertransform (a mathematical process) is required to convert the raw data into the actual spectrum. Fourier transform spectroscopy is a less intuitive way to obtain the same information. Rather than shining a monochromatic beam of light at the sample, this technique shines a beam containing many frequencies of light at once, and measures how much of that beam is absorbed by the sample. Next, the beam is modified to contain a different combination of frequencies, giving a second data point. This process is repeated many times. Afterwards, a computer takes all these data and works backwards to infer what the absorption is at eachwavelength.

The beam described above is generated by starting with a broadband light source one containing the full spectrum of wavelengths to be measured. The light shines into a Michelsoninterferometer a certain configuration of mirrors, one of which is moved by a motor. As this mirror moves, each wavelength of light in the beam is periodically blocked, transmitted, blocked, transmitted, by the interferometer, due to wave interference. Different wavelengths are modulated at different rates, so that at each moment, the beam coming out of

the interferometer has a differentspectrum.

As mentioned, computer processing is required to turn the raw data (light absorption for each mirror position) into the desired result (light absorption for each wavelength). The processing required turns out to be a common algorithm called the Fourier transform (hence the name, "Fourier transform spectroscopy"). The raw data is sometimes called an "interferogram".

There are three principal advantages for an FT spectrometer compared to a scanning (dispersive) spectrometer.

- The multiplex or Fellgett's advantage. This arises from the fact that information from all wavelengths is collected simultaneously. It results in a higher Signal-to-noise ratio for a given scan-time. For a spectrum with m resolution elements, this increase is equal to the square root of m. alternatively; it allows a shorter scan-time for a given resolution. In practice multiple scans are often averaged, increasing the signal-to-noise ratio by the square root of the number ofscans.
- The throughput or Jacquinot's advantage. This results from the fact that in a dispersive instrument, the monochromator has entrance and exit slits which restrict the amount of light that passes through it. The interferometer throughput is determined only by the diameter of the collimated beam coming from the source. Although no slits are needed, FTIR spectrometers do require an aperture to restrict the convergence of the collimated beam in the interferometer. This is because convergent rays are modulated at different frequencies as the path difference is varied. Such an aperture is called a Jacquinot stop.[1]For a given resolution and wavelength this circular aperture allows more light through than a slit, resulting in a higher signal-to-noiseratio.
- The wavelength accuracy or Connes advantage. The wavelength scale is calibrated by a laser beam of known wavelength that passes through the interferometer. This is much more stable and accurate than in dispersive instruments where the scale depends on the mechanical movement of diffraction gratings. In practice, the accuracy is limited by the divergence of the beam in the interferometer which depends on theresolution.

Another minor advantage is less sensitivity to stray light that is radiation of one wavelength appearing at another wavelength in the spectrum. In dispersive instruments, this is the result of imperfections in the diffraction gratings and accidental reflections. In FT instruments there is no direct equivalent as the apparent wavelength is determined by the modulation frequency in the interferometer

Nuclear magnetic resonance(NMR)

NMR is a physical phenomenon in which nuclei in a magnetic field absorb and re-emit electromagnetic radiation. This energy is at a specific resonance frequency which depends on the strength of the magnetic field and the magnetic properties of the isotope of the atoms; in practical applications, the frequency is similar to VHF and UHF television broadcasts (60– 1000 MHz). NMR allows the observation of specific quantum mechanical magnetic properties of the atomic nucleus. Many scientific techniques exploit NMR phenomena to study molecularphysics,crystals, and non-crystalline materials through nuclear magnetic resonancespectroscopy. NMR is also routinely used in advanced medical imaging techniques, such as in magnetic resonance imaging(MRI).

All isotopes that contain an odd number of protons and/or neutrons (see Isotope) have an intrinsic magnetic moment and angular momentum, in other words a nonzero spin, while all nuclides with even numbers of both have a total spin of zero. The most commonly studied nuclei are 1H and 13C, although nuclei from isotopes of many other elements (e.g. 2H,6Li,10B,11B, 14N,15N,17O,19F,23Na,29Si, 31P, 35Cl, 113Cd, 129Xe,195Pt)

have been studied by high-field NMR spectroscopy as well.

Instrumentation

Schematically, an analytical NMR instrument is very similar to an EPR instrument, except that instead of a klystron generating microwaves two sets of coils are used to generate and detect radio frequencies. Samples in solution are contained in sealed tubes which are rotated rapidly in the cavity to eliminate irregularities and imperfections in sample distribution. In this way, an averageanduniformsignalisreflectedtothereceivertobeprocessedandrecorded.Insolid samples, the number of spin–spin interactions is greatly enhanced due to intermolecular interactions that are absent in dissolved samples due to translation and rotation movements. As a result, the resonance signals broaden significantly. However, high-resolution spectra can be obtained by spinning the solid sample at an angle of ° (magic anglespinning).

The sophisticated pulse sequences necessary for multidimensional NMR require a certain geometric layout of the radio frequency coils and sophisticated electronics. Advanced computer facilities are needed for operation of NMR instruments, as well as analysis of the acquired spectra.

Applications

Medicine

The application of nuclear magnetic resonance best known to the general public is magnetic resonance imaging for medical diagnosis and magnetic resonance microscopy in research settings, however, it is also widely used in chemical studies, notably in NMR spectroscopy such as proton NMR,carbon-13 NMR, deuterium NMR and phosphorus-31 NMR. Biochemical information can also be obtained from living tissue (e.g. human brain tumors) with the technique known as in vivo magnetic resonance spectroscopy or chemical shift NMR Microscopy.

Chemistry

By studying the peaks of nuclear magnetic resonance spectra, chemists can determine the structure of many compounds. It can be a very selective technique, distinguishing among many atoms within a molecule or collection of molecules of the same type but which differ only in terms of their local chemical environment. NMR spectroscopy is used to unambiguously identify known and novel compounds, and as such, is usually required by scientific journals for identity confirmation of synthesized new compounds.

Purity determination

NMR is primarily used for structural determination, however it can also be used for purity determination, providing that the structure and molecular weight of the compound is known. This technique requires the use of an internal standard of a known purity. Typically this standard will have a high molecular weight to facilitate accurate weighing, but relatively few protons so as to give a clear peak for later integration e.g. 1,2,3,4-tetrachloro-5-nitrobenzene. Accurately weighed portions of both the standard and sample are combined and analyzed byNMR.

Flow cytometer

Modern flow cytometers are able to analyze several thousand particles every second, in "real time," and can actively separate and isolate particles having specified properties. A flow cytometer is similar to a microscope, except that, instead of producing an image of the cell, flow cytometry offers "high-throughput" (for a large number of cells) automated quantification of set parameters. To analyze solid tissues, a single-cell suspension must first be prepared.

A flow cytometer has five main components:

- a flow cell - liquid stream (sheath fluid), which carries and aligns the cells so that they pass single file through the light beam forsensing
- a measuring system - commonly used are measurement of impedance (or conductivity) and optical systems - lamps (mercury,xenon); high-power water-cooled lasers (argon, krypton, dyelaser);low-powerair-cooledlasers(argon(488nm),red-HeNe(633nm),green-HeNe, HeCd (UV)); diode lasers (blue, green, red, violet) resulting in light signals
- a detector and Analogue-to-Digital Conversion (ADC) system - which converts analogue measurements of forward-scattered light (FSC) and side-scattered light (SSC) as well as dye- specific fluorescence signals into digital signals that can be processed by a binarycomputer
- an amplification system - linearorlogarithmic
- a computer for analysis of thesignals.

The process of collecting data from samples using the flow cytometer is termed 'acquisition'. Acquisition is mediated by a computer physically connected to the flow cytometer, and the software which handles the digital interface with the cytometer. The software is capable of adjusting parameters (e.g., voltage, compensation) for the sample being tested, and also assists in displaying initial sample information while acquiring sample data to ensure that parameters are set correctly. Early flow cytometers were, in general, experimental devices, but technological advances have enabled widespread applications for use in a variety of both clinical and research purposes. Due to these developments, a considerable market for instrumentation, analysis software, as well as the reagents used in acquisition such as fluorescently labeled antibodies has developed.

Modern instruments usually have multiple lasers and fluorescence detectors. The current record for a commercial instrument is ten lasers and 18 fluorescence detectors. Increasing the number of lasers and detectors allows for multiple antibody labeling, and can more precisely identify a target population by their phenotypic markers. Certain instruments can even take digital images of individual cells, allowing for the analysis of fluorescent signal location within or on the surface ofcells.

Applications

The technology has applications in a number of fields, including molecularbiology,pathology, immunology,plant biology and marine biology. It has broad application inmedicine (especially in transplantation, hematology, tumor immunology and chemotherapy, prenatal diagnosis, genetics and sperm sorting for sex preselection). Also, it is

extensively used in research for the detection of DNA damage, caspase cleavage and apoptosis. In marine biology, the autofluorescent properties of photosynthetic planktoncan be exploited by flow cytometry in order to characterise abundance and community structure. In protein engineering, flow cytometry is used in conjunction with yeast displayand bacterial display to identify cell surface-displayed protein variants with desiredproperties.

POSSIBLE QUESTIONS

UNIT-I

PART-A (20 MARKS)

(Q.NO 1 TO 20 Online Examination)

PART-B (2 MARKS)

1. Write about the Basic Terminology of Sedimentation rate?
2. Define Absorbance with example.
3. What is the difference between settling and sedimentation?
4. What are the two types of rotors found in high-powered centrifuges?
5. What is the meaning of the word "derated"?
6. What is the beer lambert law? What are the correlations derived from it.]
7. What is chemiluminescence? What are its uses in diagnostic?
8. What is fluorescence? How is it used in diagnostics?

PART-C (6 MARKS)

1. Define Svedberg equation. Explain in detail about the principles behind centrifugation.
2. Explain in detail on Density gradient centrifugation.
3. Explain in detail on isopycnic centrifugation.
4. Explain in detail on rate zonal centrifugation.
5. What is the principle of colorimetry? Draw a simple labelled diagram of a colorimeter
6. What is the principal of a flame photometerr?
7. Write a short note on spectrophotometry?
8. Write in detail on FTIR.
9. Explain about NMR.
10. What is the process involved in flow cytometer?

UNIT I

Questions	Option A	Option B	Option C	Option D	Answer
. What is the normal blood pH	7.8	7.4	6.8	6.4	7.4
Equivalent weight X Normality=	Amount of substance dissolved in one litre	Amount of substance dissolved in 100 ml	Amount of substance dissolved in 1 ml	Mole Fraction	Amount of substance dissolved in one litre
$[H^+]_i$ is always fixed in glass electrode and is generally	0.1 M	1 M	5 M	Zero	0.1 M
pKa is	Negative logarithm of Ka	Positive logarithm of Ka	Negative anti-logarithm of Ka	Positive anti-logarithm of Ka	Negative logarithm of Ka
Amount of substance in grams dissolved in 1 kg of solvent is referred as	1 Molal	1 Molar	1 Normal	1 Percentage	1 Molal
pH is	Negative logarithm of hydrogen ion concentration	Positive logarithm of hydrogen ion concentration	Negative anti-logarithm of hydrogen ion concentration	Positive anti-logarithm of hydrogen ion concentration	Negative logarithm of hydrogen ion concentration

a solution of NaOH is found to be completely neutralized by 8 ml of a given solution of HCI . if we take 20 ml of the same solution of NaOH, the amount of HCI solution (the same solution as before) required to neutralize	4 ml	8 ml	12 ml	16 ml	16 ml
pH of 1M HCl and 1M CH_3COOH are	same	different	zero	2	different
Serial dilution results in	Gradual decrease in the concentration of solutes	Gradual increase in the concentration of solutes	doesnot decrease the concentration of solutes	doesnot increase the concentration of solutes	Gradual decrease in the concentration of solutes
Buffer solution contains	Weak acid and its salt	Strong acid and its salt	only weak acid	only strong acid	Weak acid and its salt
pKa of carbonic acid is	6.1	7.4	1.2	9.2	6.1

Buffering action is maximum when the pH	Equals pKa	Higher than pKa	Lower than pKa	Zero	Equals pKa
Chromatography generally involves	Two phases	single phase	Three phase	No phase	Two phases
Chromatography can be used to	form mixtures	change mixture compositions	separate mixtures into pure substances	all of these	separate mixtures into pure substances
One milligram of substance dissolved in one litre is referred as	1 ppm	1 M	1 N	1%	1 ppm
Amino acids can be determined using	Ninhydrin	Phenolpthalein	Phenol Red	Diazo reagent	Ninhydrin
Cations have	Positive charge	Negative charge	No charge	It is impossible to predict the charge on a cation	Positive charge

If I dilute 5 mL of 0.15 M NaCl to a final volume of 5 L, what is the final concentration of NaCl?	0.00015 M	0.0015 M	15000 M	none of these	0.00015 M
Buffers keep the pH of a solution from changing by:	converting strong acids to weak ones	converting weak acids to strong ones	converting weak bases to strong ones	more than one of the above answers	converting strong acids to weak ones
Which of the following is not an acid?	HNO3	CH3COOH	H2SO4	All of these are acids	All of these are acids
Which of the following dye changes from indicate red and yellow in acidic and alkaline environment?	Methyl red	Bromothymol blue	Phenolpthalein	Alizarin yellow	Methyl red

Which of the following dye changes from indicate yellow and red in acidic and alkaline environment?	Methyl red	Bromothymol blue	Phenolphthalein	Alizarin yellow	Alizarin yellow
Which of the following dye changes from indicate yellow and blue in acidic and alkaline environment?	Methyl red	Bromothymol blue	Phenolphthalein	Alizarin yellow	Bromothymol blue
Which of the following dye changes from indicate colourless and red in acidic and alkaline environment?	Methyl red	Bromothymol blue	Phenolphthalein	Alizarin yellow	Phenolphthalein
Isotonic solutions have	same osmotic pressure	same temperature	exhibit similar chemistry	same charge	same osmotic pressure

Flow of solvent from dilute to a concentrated solution across a semipermeable membrane is referred as	electrolysis	endosmosis	osmosis	reverse osmosis	osmosis
For the preparation of 100 ml of 10% SDS, how much of SDS should be used	100 grams	10 grams	100 milligrams	10 milligrams	10 grams
For the preparation of 10 ml of 10% SDS, how much of SDS should be used	100 grams	1 gram	100 milligrams	10 milligrams	1 gram
For the preparation of 100 ml of 5% APS, how much of APS should be used	5 grams	50 grams	500 milligrams	60 milligrams	5 grams

For optimal buffer action, which of the following is essential	pH	Molarity	pH and Molariy	acid	
Laboratory concentration of commercially available hydrochloric acid is	5 M	12 M	20 M	24 M	12 M
Laboratory concentration of commercially available sulphuric acid is	5 M	12 M	18 M	24 M	18 M
Acidosis is defined as	a blood pH below 7.4	a blood pH greater than 7.4	a blood pH of 7.4	a condition of low H+ concentration	a blood pH below 7.4
In Universal indicators, red color shows	strong acids	strong alkalis	weak acids	weak bases	strong acids
Soap is	acidic	alkaline	neutral	basic	alkaline
Orange juice is	acidic	alkaline	neutral	basic	acidic
Lemon juice has a pH of	2	6	8	12	2

An aqueous solution with pH = 0 is	Strongly acidic	Strongly basic	Neutral	weakly basic	Strongly acidic
Which one of the following types of medicines is used for treating indigestion?	Antibiotic	Analgesic	Antacid	antiseptic	Antacid
The pH of the gastric juices released during digestion is	less than 7	equal to 7	more than 7	equal to 0	less than 7
Which of the following is acidic in nature?	lime water	lime juice	Human blood	antacid	lime juice
Which of the following is used for the dissolution of gold?	Hydrochloric acid	Sulphuric acid	Nitric acid	Aqua regia	Aqua regia
Which of the following is not an mineral acid	Hydrochloric acid	Sulphuric acid	Citric acid	Nitric acid	Citric acid
The acid present in lemon is	Tartaric acid	Ascorbic acid	Citric acid	Oxalic acid	Citric acid

The acid present in grape is	Tartaric acid	Ascorbic acid	Citric acid	Oxalic acid	Tartaric acid
The acid present in tomato is	Tartaric acid	Ascorbic acid	Citric acid	Oxalic acid	Oxalic acid
The acid present in apple is	Tartaric acid	Malic acid	Citric acid	Oxalic acid	Malic acid
The most important buffering system for maintaining proper blood pH is	the charges on the amino acids	the bicarbonate buffer system	phosphate groups of serum phosphoproteins	albumin	the bicarbonate buffer system
The acid having a highest H+ ion concentration is one with	pH = 7.0	pH = 1.2	pH = 2.3	pH = 8.2	pH = 1.2
Aqueous solution of which of the following turns blue litmus red?	NaNO3	CuSO4	NH4OH	CH3COONa	CH3COONa
An element common to all acids is?	Chlorine	Nitrogen	Oxygen	Hydrogen	Hydrogen
Bases on ionization release	Hydrogen ions	Sodium ions	Chlorine ions	Hydroxide ions	Hydroxide ions

A solution turns red litmus to blue its pH is likely to be	2	5	7	10	10
Which of the following represents a base?	KOH	KCI	CH3OH	CH3COOH	KOH
Fruit juices, such as orange juice contain	Boric acid	Citric acid	Sulphuric acid	Nitric acid	Citric acid
Which of the following solution will turn phenolphthalein pink?	HCI (aq)	CO2(aq)	KOH(aq)	CH3OH(aq)	KOH(aq)
A common substance that contains acetic acid is	Vinegar	Ammonia water	Salad oil	Soap	Vinegar

When HCI (aq) is exactly neutralized by NaOH (aq), the hydrogen ion concentration in the resulting mixture is.	Always less than the concentration of the hydroxide ions	Always greater then the concentration of hydroxide ions	Always equal to the concentration of the hydroxide ions	Sometimes greater and sometimes less than the concentration of the hydroxide ions.	Always equal to the concentration of the hydroxide ions
Which of the following statement is correct about an aqueous solution of an acid and of a base?	Higher the pH, stronger the acid	Higher the pH, weaker the acid	Lower the pH, stronger the base	Acid and base have equal pH	Higher the pH, weaker the acid
Which of the following is not a base	NaOH	KOH	CH3OH	NH4OH	CH3OH

UNIT-II

SYLLABUS

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.

Chromatography

Chromatography is a versatile method of separating many different kinds of chemical mixtures. The process or technique of separating molecules or components in mixture solutions (gas or liquid) according to the differential absorption and elution.Invented in 1906 by Russian botanist Mikhail Tsvet. Chromatography is the physical separation of a mixture into its individual components. Used in qualitative and quantitative analysis of biological and chemical substances.

Basics of Chromatography

In any chromatographic technique, a **stationary phase** usually a solid, thick liquid or bonded coating that stays fixed in one place, and a **mobile phase** or **eluent** (usually a liquid or gas) moves through it or across it. A sample to be separated, when placed on the stationary phase, will gradually move along in the same direction as the mobile phase. If a sample compound (or **analyte**) has no interaction with the stationary phase, it will run right through and come out of the system (**elute**) at the same rate as the mobile phase. On the other hand, if an analyte has no interaction with the mobile phase, it will stick directly to the stationary phase and never elute. Neither of these are goodoutcomes.

Types of Chromatography

- ✓ AdsorptionChromatography
- ✓ PartitionChromatography
- ✓ Ion ExchangeChromatography
- ✓ Molecular ExclusionChromatography
- ✓ AffinityChromatography

Definition:

- Chromatography (from Greek χρῶμα chroma "color" and γράφεινgraphein "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.

$$Rf = \frac{\text{Distance (cm) moved by the solute from the origin}}{\text{Distance (cm) moved by the solvent from the origin}}$$

- The am g the Rf values with that of the

- 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) Thecation 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

morethanone 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.

Ion exchange chromatography:

Ion exchange chromatography is a process that allows separation of ions and polar molecules change. Ion exchange chromatography is coined by Smail, Stevens and Raumann. It can be used for almost any kind of charged molecules including large proteins, small nucleotides and amino acids. Ion exchange chromatography retains analyte molecules on the column based on coulombic (ionic) interactions. The stationary phase surface displays ionic functional groups that interact with analyte ions of opposite charge.

This type of chromatography is further subdivided into two. They are cation exchange chromatography and anion exchange chromatography.

The ionic compound consisting of the cationic species M^+ and the anionic species B can be retained by the stationary phase. Cationic exchange chromatography retains positively charged cations because the stationary phase displays a negatively charged functional group. Anion exchange chromatography retains anion using positively charged functional group. Ion exchange is classified into resins, gels and inorganic exchanger.

Resins:

Resins are amorphous particles of organic materials. Ion exchange resins are used for the separation of small molecules.

Classification of ion exchange resins:

Strongly acidic cation exchanger – Sulphonic acid groups attached to styrene and divinyl benzenecopolymer.

Weakly acidic cation exchanger – Carboxylic groups attached to acrylic and divinyl benzene copolymer.

Strongly basic anion exchanger – Quaternary ammonium groups attached to styrene and divinyl benzene copolymer.

Weakly basic anion exchanger – poly alkyl amine groups attached to styrene and divinyl benzene copolymer.

Ion exchange gels:

Cellulose and dextran ion exchangers, which are polymers of the sugar glucose, posses larger pore size and lower charge densities. Because they are much softer than polystyrene resins, dextran and its relatives are called gels. Ion exchange gels consist of,

Column: Glass, stainless steel or polymers, Length: Diameter, Ratio: 20:100, Packing column: Wet packing method, Application of the sample: After packing, sample is added to the top of the column, use syringe or pipette.

Mobile phase: Acids, alkalis, buffers.

Elution: Components of mixture separate and move down the column at different rates depending upon the affinity of the ion for ion exchanger.

The elutes are collected at different stages, **Analysis of elute:** Spectrophotometric, flame **Method:** A sample is introduced either manually or with an auto sampler, into a sample loop of known volume. The mobile phase carries the sample from the loop onto a column that contains some form of stationary phase material. Stationary phase material is used as a resin or gel matrix consisting of agarose or cellulose beads with covalently bonded charged functional groups. The target analytes are retained on the stationary phase but can be eluted by increasing the concentration of a similarly charged species that will displace the analyte ions from the stationary phase. The analyte of interest must then be detected by some means, typically by conductivity or UV/Visible light absorbance. A chromatography data system (CDS) is usually needed to control anIC.

Advantages:

- It is a non-denaturing technique. It can be used at all stages and scales of purification
- An IEX separation can be controlled by changing pH, salt concentration and/or the ion exchangemedia
- It can serve as a concentrating step. A large volume of dilute sample can be applied to a media, and the adsorbed protein subsequently eluted in a smallervolume
- It offers high selectivity; it can resolve molecules with small differences incharge.

Disadvantages:

Costly equipment and more expensive chemicals Turbidity should be below 10 ppm.

Affinity chromatography:

Affinity chromatography is essential sample purification technique used primarily for biological molecules such as proteins. It is a method of separating a mixture of proteins or nucleic acids (molecules) by specific interaction of those molecules with a component known as a ligand, which is immobilized on a support. If a solution of a mixture of proteins is passed over the column, one of

the proteins binds to the ligand on the basis of specificity and high affinity. The other proteins in the solution wash through the column because they were not able to bind to the ligand.

Principle:

Affinity chromatography is one of the most diverse and powerful methods for purification of a specific molecule or a group of molecules from complex mixtures. It is based on highly specific biological interactions between two molecules such as interactions between enzyme and substrate, receptor and ligand or antibody and antigen. These interactions which are typically reversible are used for purification by placing one if the interacting molecules referred to as affinity lignand onto a solid matrix to create a stationary pase while a target molecule are in the mobile phase. Many of the commonly used ligands coupled to affinity matrices are now commercially available and are ready to use.

Chromatographic media:

A matrix in its use here is a substance, usually in bead form to which a specific ligand is covalently bound. In order to form the matrix to be effective it must have certain characters such as,

- It must be insoluble in solvents and buffers employed in the process. It must be chemically and mechanically stable.
- It must be easily coupled to a ligand or spacer arm onto which the ligand can be attached. It must exhibit good flow properties and have a relatively large surface area for attachment.

Immobilized ligand:

The ligand can be selected only after the nature of the macromolecule to be isolated is known.

- When a hormone receptor protein is to be purified by affinity chromatography, the hormone itself is an ideal candidate for theligand.

  For antibody isolation, an antigen or hapten may be used as ligand.

- Ifanenzymeistobepurified,asubstrateanalog,inhibitor,cofactororeffectormaybe used as the immobilized ligand.

Attachment of ligand to matrix:

Several procedures have been developed for the covalent attachment of the ligand to the stationary

phase. All procedures for gel modification proceed in two separate chemical steps:

Activation of the ligand to the functional group on the matrix.Joining of the ligand to the functional group on the matrix.

Steps:

1. Loading affinitycolumn
2. Proteins sieve through matrix of affinitybeads.
3. Proteins interact with affinity ligand with some binding loosely and otherstightly.
4. Wash off proteins that do notbind.
5. Wash off proteins that bindloosely.
6. Elute proteins that bind tightly to ligand and collect purified protein ofinterest.

Applications:

1. It is used for isolation and purification of all biologicalmacromolecule.
2. It is used to purify nucleic acid, antibodies, enzymesetc.
3. To notice which biological compounds bind to a particularsubstance.
4. To reduce an amount of substance in amixture.

High-performance liquid chromatography

High-performance liquid chromatography (**HPLC**; formerly referred to as **high-pressure liquid chromatography**), is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbentmaterial. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out thecolumn.

Instrumentation: The schematic of an HPLC instrument typically includes a sampler, pumps, and a detector. The sampler brings the sample mixture into the mobile phase stream which carries it into the column. The pumps deliver the desired flow and composition of the mobile phase through the column. The detector generates a signal proportional to the amount of sample component emerging from the column, hence allowing for quantitative analysis of the sample components. A digital

microprocessor and user software control the HPLC instrument and provide dataanalysis.

Operation

The sample mixture to be separated and analyzed is introduced in a discrete small volume (typically microliters), into the stream of mobile phase percolating through the column. The components of the sample move through the column at different velocities, which are function of specific physical interactions with the sorbent (also called stationary phase). The velocity of each component depends on its chemical nature, on the nature of the stationary phase (column) and on the composition of the mobile phase. The time at which a specific analyte elutes (emerges from the column) is called its retention time. The retention time measured under particular conditions is considered an identifying characteristic of a given analyte.

Common mobile phases used include any miscible combination of water with various organic solvents (the most common are acetonitrile and methanol). Some HPLC techniques use water- free mobile phases. The aqueous component of the mobile phase may contain acids (such as formic, phosphoric or trifluoroacetic acid) or salts to assist in the separation of the sample components. The composition of the mobile phase may be kept constant ("isocratic elution mode") or varied ("gradient elution mode") during the chromatographic analysis. Isocratic elution is typically effective in the separation of sample components that are not very different in their affinity for the stationary phase. In gradient elution the composition of the mobile phase is varied typically from low to high eluting strength. The eluting strength of the mobile phase is reflected by analyte retention times with high eluting strength producing fast elution (=short retention times). A typical gradient profile in reversed phase chromatography might start at 5% acetonitrile (in water or aqueous buffer) and progress linearly to 95% acetonitrile over 5–25 minutes. Periods of constant mobile phase composition may be part of any gradient profile. For example, the mobile phase composition may be kept constant at 5% acetonitrile for 1–3 min, followed by a linear change up to 95%acetonitrile.

The chosen composition of the mobile phase (also called eluent) depends on the intensity of interactions between various sample components ("analytes") and stationary phase (e.g. hydrophobic interactions in reversed-phase HPLC). Depending on their affinity for the stationary and mobile phasesanalytes partition between the two during the separation process taking place in the column. This partitioning process is similar to that which occurs during a liquid–liquidextraction but is continuous, not step-wise. In this example, using a water/acetonitrile gradient,more hydrophobic components will elute (come off the column) late, once the mobile phase gets more concentrated in acetonitrile (i.e. in a mobile phase of higher eluting strength).

The choice of mobile phase components, additives (such as salts or acids) and gradient conditions depends on the nature of the column and sample components. Often a series of trial runs is

performed with the sample in order to find the HPLC method which gives adequate separation.

Fast protein liquid chromatography (FPLC), is a form of liquid chromatography that is often used to analyze or purify mixtures of proteins. As in other forms of chromatography, separation is possible because the different components of a mixture have different affinities fortwo materials, a moving fluid (the "mobile phase") and a porous solid (the stationary phase). In FPLC the mobile phase is an aqueous solution, or "buffer". The buffer flow rate is controlled by a positive-displacement pump and is normally kept constant, while the composition of the buffer can be varied by drawing fluids in different proportions from two or more external reservoirs. The stationary phase is a resin composed of beads, usually of cross-linked agarose, packed into a cylindrical glass or plastic column. FPLC resins are available in a wide range of bead sizes and surface ligands depending on theapplication.

In the most common FPLC strategy, ion exchange, a resin is chosen that the protein of interest will bind to the resin by a charge interaction while in buffer A (the running buffer) but become dissociated and return to solution in buffer B (the elution buffer). A mixture containing one or more proteins of interest is dissolved in 100% buffer A and pumped into the column. The proteins of interest bind to the resin while other components are carried out in the buffer. The total flow rate of the buffer is kept constant; however, the proportion of Buffer B (the "elution" buffer) is gradually increased from 0% to 100% according to a programmed change in concentration (the "gradient"). At some point during this process each of the bound proteins dissociates and appears in the effluent. The effluent passes through two detectors which measure salt concentration (by conductivity) and protein concentration (by absorption of ultraviolet light at a wavelength of 280nm). As each protein is eluted it appears in the effluent as a "peak" in protein concentration and can be collected for furtheruse.

FPLC was developed and marketed in Sweden by Pharmacia in 1982 and was originally called fast performance liquid chromatography to contrast it with HPLC or high-performanceliquid chromatography. FPLC is generally applied only to proteins; however, because of the wide choice of resins and buffers it has broad applications. In contrast to HPLC the buffer pressure used is relatively low, typically less than 5 bar, but the flow rate is relatively high, typically 1-5 ml/min. FPLC can be readily scaled from analysis of milligrams of mixtures in columns with a total volume of 5ml or less to industrial production of kilograms of purified protein in columns with volumes of many liters. When used for analysis of mixtures the effluent is usually collected in fractions of 1-5 ml which can be further analyzed, e.g. by MALDI mass spectrometry. When used for protein purification there may be only two collection containers, one for the purified product and one for waste.

A typical laboratory FPLC consists of one or two high-precision pumps, a control unit, a column, a detection system and a fraction collector. Although it is possible to operate the system manually,

the components are normally linked to a personal computer or, in older units, a microcontroller.

1. Pumps: GE/Pharmacia systems utilize two two-cylinder piston pumps, one for each buffer, combining the output of both in a mixing chamber. Waters systems use a single peristaltic pump which draws both buffers from separate reservoirs through a proportioning valve and mixing chamber. In either case the system allows the fraction of each buffer entering the column to be continuously varied. The flow rate can go from a few milliliters per minute in bench-top systems to liters per minute for industrial scale purifications. The wide flow range makes it suitable both for analytical and preparativechromatography.

2. Injection loop: A segment of tubing of known volume which is filled with the sample solution before it is injected into the column. Loop volume can range from a few microliters to 50ml or more.

3. Injection valve: A motorized valve which links the mixer and sample loop to the column. Typically the valve has three positions for loading the sample loop, for injecting the sample from the loop into the column, and for connecting the pumps directly to the waste line to wash them or change buffer solutions. The injection valve has a sample loading port through which the sample can be loaded into the injection loop, usually from a hypodermic syringe using a Luer-lock connection.

4. Column: The column is a glass or plastic cylinder packed with beads of resin and filled with buffer solution. It is normally mounted vertically with the buffer flowing downward from top to bottom. A glass frit at the bottom of the column retains the resin beads in the column while allowing the buffer and dissolved proteins toexit.

5. Flow Cells: The effluent from the column passes through one or more flow cells to measure the concentration of protein in the effluent (by UV light absorption at 280nm). The conductivity cell measures the buffer conductivity, usually in millisiemens/cm, which indicates the concentration of salt in the buffer. A flow cell which measures pH of the buffer is also commonly included. Usually each flow cell is connected to a separate electronics module which provides power and amplifies thesignal.

6. Monitor/Recorder: The flow cells are connected to a display and/or recorder. On older systems this was a simple chart recorder, on modern systems a computer with hardware interface and display is used. This permits the experimenter to identify when peaks in protein concentration occur, indicating that specific components of the mixture are beingeluted.

7. Fraction collector: The fraction collector is typically a rotating rack that can be filled with test tubes or similar containers. It allows samples to be collected in fixed volumes, or can be controlled to direct specific fractions detected as peaks of protein concentration, into separate containers.

Many systems include various optional components. A filter may be added between the mixer and

column to minimize clogging. In large FPLC columns the sample may be loaded into the column directly using a small peristaltic pump rather than an injection loop. When the buffer contains dissolved gas, bubbles may form as pressure drops where the buffer exits the column; these bubbles create artifacts if they pass through the flow cells. This may be prevented by degassing the buffers, or by adding a flow restrictor downstream of the flow cells to maintain a pressure of 1-5 bar in the effluent line.

The columns used in FPLC are large [mm id] tubes that contain small [µ] particles or gel beads that are known as stationary phase. The chromatographic bed is composed by the gel beads inside the column and the sample is introduced into the injector and carried into the column by the flowing solvent. As a result of different components adhering to or diffusing through the gel, the sample mixture getsseparated.

Columns used with an FPLC can separate macromolecules based on size, charge distribution (ionexchange), hydrophobicity, reverse-phase or biorecognition (as with affinity chromatography). For easy use, a wide range of pre-packed columns for techniques such as ion exchange, gel filtration (size exclusion), hydrophobic interaction, and affinity chromatography are available.[6]FPLC differs from HPLC in that the columns used for FPLC can only be used up to maximum pressure of 3-4 MPa (435-580 psi). Thus, if the pressure of HPLC can be limited, each FPLC column may also be used in an HPLCmachine.

Optimizing protein purification

Using a combination of chromatographic methods, purification of the target molecule is achieved. The purpose of purifying proteins with FPLC is to deliver quantities of the target at sufficient purity in a biologically active state to suit its further use. The quality of the end product varies depending the type and amount of starting material, efficiency of separation, and selectivity of the purification resin. The ultimate goal of a given purification protocol is to deliver the required yield and purity of the target molecule in the quickest, cheapest, and safest way for acceptable results. The range of purity required can be from that required for basic analysis (SDS-PAGE or ELISA, for example), with only bulk impurities removed, to pure enough for structural analysis (NMR or X-ray crystallography), approaching >99% target molecule. Purity required can also mean pure enough that the biological activity of the target is retained. These demands can be used to determine the amount of starting material required to reach the experimental goal. If the starting material is limited and full optimization of purification protocol cannot be performed, then a safe standard protocol that requires a minimum adjustmentandoptimizationstepsareexpected.Thismaynotbeoptimalwithrespectto experimental time, yield, and economy but it will achieve the experimental goal. On the other hand, if the starting material is enough to develop more complete protocol, the amount of work to reach the separation goal depends on the available sample information and target molecule properties. Limits to

development of purification protocols many times depends on the source of the substance to be purified, whether from natural sources (harvested tissues or organisms, for example), recombinant sources (such as using prokaryotic or eukaryotic vectors in their respective expression systems), or totally syntheticsources.

No chromatographic techniques provide 100% yield of active material and overall yields depend on the number of steps in the purification protocol. By optimizing each step for the intended purpose and arranging them that minimizes inter step treatments, the number of steps will be minimized.

A typical multistep purification protocol starts with a preliminary capture step which many times utilize ion exchange chromatography (IEC). The media (stationary phase) employed range from large bead resins (good for fast flow rates and little to no sample clarification at the expense of resolution) to small bead resins (for best possible resolution with all other factors being equal). Short and wide column geometries are amenable to high flow rates also at the expense of resolution, typically because of lateral diffusion of sample on the column. For techniques such as size exclusion chromatography to be useful, very long, thin columns and minimal sample volumes (maximum 5% of column volume) are required. Hydrophobic interaction chromatography (HIC) can also be used for first and/ or intermediate steps. Selectivity in HIC is independent of running pH and descending salt gradients are used. For HIC, conditioning involves adding ammonium sulphate to the sample to match the buffer A concentration. If HIC is used before IEC, the ionic strength would have to be lowered to match that of buffer A for IEC step by dilution, dialysis or buffer exchange by gel filtration. This is why IEC is usually performed prior to HIC as the high salt elution conditions for IEC are ideal for binding to HIC resins in the next purification step. Polishing is used to achieve the final level of purification required and is commonly performed on a gel filtration column. An extra intermediate purification step can be added or optimization of the different steps is performed for improving purity. This extra step usually involves another round of IEC under completely different conditions.

Although this is an example of a common purification protocol for proteins, the buffer conditions, flow rates, and resins used to achieve final goals can be chosen to cover a broad range of target proteins. This flexibility is imperative for a functional purification system as all proteins behave differently and often deviate frompredictions.

Gas chromatography–mass spectrometry (**GC-MS**) is an analytical method that combines the features of gas-chromatography and mass spectrometry to identify different substances within a test sample. Applications of GC-MS include drug detection, fire investigation, environmental analysis, explosives investigation, and identification of unknown samples, including that of material samples obtained from planet Mars during probe missions as early as the 1970s. GC-MS can also be used in

airport security to detect substances in luggage or on human beings. Additionally, it can identify trace elements in materials that were previously thought to have disintegrated beyond identification.

Instrumentation

The GC-MS is composed of two major building blocks: the gas chromatograph and the massspectrometer. The gas chromatograph utilizes a capillary column which depends on the column's dimensions (length, diameter, film thickness) as well as the phase properties (e.g. 5% phenyl polysiloxane). The difference in the chemical properties between different molecules in a mixture and their relative affinity for the stationary phase of the column will promote separation of the molecules as the sample travels the length of the column. The molecules are retained by the column and then elute (come off) from the column at different times (called the retention time), and this allows the mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately. The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments using their mass-to-chargeratio.

These two components, used together, allow a much finer degree of substance identification than either unit used separately. It is not possible to make an accurate identification of a particular molecule by gas chromatography or mass spectrometry alone. The mass spectrometry process normally requires a very pure sample while gas chromatography using a traditional detector (e.g. Flame ionization detector) cannot differentiate between multiple molecules that happen to take the same amount of time to travel through the column (*i.e.* have the same retention time), which results in two or more molecules that co-elute. Sometimes two different molecules can also have a similar pattern of ionized fragments in a mass spectrometer (mass spectrum). Combining the two processes reduces the possibility of error, as it is extremely unlikely that two different molecules will behave in the same way in both a gas chromatograph and a mass spectrometer. Therefore, when an identifying mass spectrum appears at a characteristic retention time in a GC-MS analysis, it typically increases certainty that the analyte of interest is in the sample.

Types of mass spectrometer detectors

The most common type of mass spectrometer (MS) associated with a gas chromatograph (GC) is the quadrupole mass spectrometer, sometimes referred to by the Hewlett-Packard (now Agilent) trade name "Mass Selective Detector" (MSD). Another relatively common detector is the ion trap mass spectrometer. Additionally one may find a magnetic sector mass spectrometer, however these

particular instruments are expensive and bulky and not typically found in high- throughput service laboratories. Other detectors may be encountered such as time offlight (TOF), tandem quadrupoles (MS-MS) (see below), or in the case of an ion trap MS^n where n indicates the number mass spectrometry stages.

GC-tandem MS

When a second phase of mass fragmentation is added, for example using a second quadrupole in a quadrupole instrument, it is called tandem MS (MS/MS). MS/MS can sometimes be used to quantitate low levels of target compounds in the presence of a high sample matrix background.

The first quadrupole (Q1) is connected with a collision cell (q2) and another quadrupole (Q3). Both quadrupoles can be used in scanning or static mode, depending on the type of MS/MS analysis being performed. Types of analysis include product ion scan, precursor ion scan, selected reaction monitoring (SRM) (sometimes referred to as multiple reaction monitoring (MRM)) and neutral loss scan. For example: When Q1 is in static mode (looking at one mass only as in SIM), and Q3 is in scanning mode, one obtains a so-called product ion spectrum (also called "daughter spectrum"). From this spectrum, one can select a prominent product ion which can be the product ion for the chosen precursor ion. The pair is called a "transition" and forms the basis for SRM. SRM is highly specific and virtually eliminates matrixbackground.

Ionization

After the molecules travel the length of the column, pass through the transfer line and enter into the mass spectrometer they are ionized by various methods with typically only one method being used at any given time. Once the sample is fragmented it will then be detected, usually by an electron multiplier diode, which essentially turns the ionized mass fragment into an electrical signal that is then detected.

The ionization technique chosen is independent of using full scan or SIM.

Electron ionization

By far the most common and perhaps standard form of ionization is electron ionization (EI). The molecules enter into the MS (the source is a quadrupole or the ion trap itself in an ion trap MS) where they are bombarded with free electrons emitted from a filament, not unlike the filament one would find in a standard light bulb. The electrons bombard the molecules, causing the molecule to

fragment in a characteristic and reproducible way. This "hard ionization" technique results in the creation of more fragments of low mass to charge ratio (m/z) and few, if any, molecules approaching the molecular mass unit. Hard ionization is considered by mass spectrometrists as the employ of molecular electron bombardment, whereas "soft ionization" is charge by molecular collision with an introduced gas. The molecular fragmentation pattern is dependent upon the electron energy applied to the system, typically 70 eV (electron Volts). The use of 70 eV facilitates comparison of generated spectra with library spectra using manufacturer- supplied software or software developed by the National Institute of Standards (NIST-USA). Spectral library searches employ matching algorithms such as Probability Based Matching and dot-product matching that are used with methods of analysis written by many method standardization agencies. Sources of libraries include NIST, Wiley, the AAFS, and instrument manufacturers.

Cold electron ionization

The "hard ionization" process of electron ionization can be softened by the cooling of the molecules before their ionization, resulting in mass spectra that are richer in information. In this method named cold electron ionization (Cold-EI) the molecules exit the GC column, mixed with added helium make up gas and expand into vacuum through a specially designed supersonic nozzle, forming a supersonic molecular beam (SMB). Collisions with the makeup gas at the expanding supersonic jet reduce the internal vibrational (and rotational) energy of the analyte molecules, hence reducing the degree of fragmentation caused by the electrons during the ionization process. Cold-EI mass spectra are characterized by an abundant molecular ion while the usual fragmentation pattern is retained, thus making Cold-EI mass spectra compatible with library search identification techniques. The enhanced molecular ions increase the identification probabilities of both known and unknown compounds, amplify isomer mass spectral effects and enable the use of isotope abundance analysis for the elucidation of elemental formulae.

Chemical ionization

In chemical ionization a reagent gas, typically methane or ammonia is introduced into the mass spectrometer. Depending on the technique (positive CI or negative CI) chosen, this reagent gas will interact with the electrons and analyte and cause a 'soft' ionization of the molecule of interest. A softer ionization fragments the molecule to a lower degree than the hard ionization of EI. One of the main benefits of using chemical ionization is that a mass fragment closely corresponding to the molecular weight of the analyte of interest isproduced.

In positive chemical ionization (PCI) the reagent gas interacts with the target molecule, most often with a proton exchange. This produces the species in relatively high amounts.

In negative chemical ionization (NCI) the reagent gas decreases the impact of the free electrons on the target analyte. This decreased energy typically leaves the fragment in great supply.

Analysis

A mass spectrometer is typically utilized in one of two ways: full scan or selected ion monitoring (SIM). The typical GC-MS instrument is capable of performing both functions either individually or concomitantly, depending on the setup of the particularinstrument.

The primary goal of instrument analysis is to quantify an amount of substance. This is done by comparing the relative concentrations among the atomic masses in the generated spectrum. Two kinds of analysis are possible, comparative and original. Comparative analysis essentially compares the given spectrum to a spectrum library to see if its characteristics are present for some sample in the library. This is best performed by a computer because there are a myriad of visual distortions that can take place due to variations in scale. Computers can also simultaneously correlate more data (such as the retention times identified by GC), to more accurately relate certaindata.

Another method of analysis measures the peaks in relation to one another. In this method, the tallest peak is assigned 100% of the value, and the other peaks being assigned proportionate values. All values above 3% are assigned. The total mass of the unknown compound is normally indicated by the parent peak. The value of this parent peak can be used to fit with a chemical formula containing the various elements which are believed to be in the compound. The isotope pattern in the spectrum, which is unique for elements that have many isotopes, can also be used to identify the various elements present. Once a chemical formula has been matched to the spectrum, the molecular structure and bonding can be identified, and must be consistent with the characteristics recorded by GC-MS. Typically, this identification done automatically by programs which come with the instrument, given a list of the elements which could be present in thesample.

A “full spectrum” analysis considers all the “peaks” within a spectrum. Conversely, selective ion monitoring (SIM) only monitors selected ions associated with a specific substance. This is done on the assumption that at a given retention time, a set of ions is characteristic of a certain compound. This is a fast and efficient analysis, especially if the analyst has previous information about a sample or is only looking for a few specific substances. When the amount of information collected about the ions in a given gas chromatographic peak decreases, the sensitivity of the analysis increases. So, SIM analysis allows for a smaller quantity of a compound to be detected and measured, but the degree of certainty about the identity of that compound is reduced.

Full scan MS

When collecting data in the full scan mode, a target range of mass fragments is determined and put into the instrument's method. An example of a typical broad range of mass fragments to monitor would be *m/z* 50 to *m/z* 400. The determination of what range to use is largely dictated by what one anticipates being in the sample while being cognizant of the solvent and other possible interferences. A MS should not be set to look for mass fragments too low or else one may detect air (found as *m/z* 28 due to nitrogen), carbon dioxide (*m/z* 44) or other possible interferences. Additionally if one is to use a large scan range then sensitivity of the instrument is decreased due to performing fewer scans per second since each scan will have to detect a wide range of massfragments.

Full scan is useful in determining unknown compounds in a sample. It provides more information than SIM when it comes to confirming or resolving compounds in a sample. During instrument method development it may be common to first analyze test solutions in full scan mode to determine the retention time and the mass fragment fingerprint before moving to a SIM instrumentmethod.

Selected ion monitoring

In selected ion monitoring (SIM) certain ion fragments are entered into the instrument method and only those mass fragments are detected by the mass spectrometer. The advantages of SIM are that the detection limit is lower since the instrument is only looking at a small number of fragments (e.g. three fragments) during each scan. More scans can take place each second. Since only a few mass fragments of interest are being monitored, matrix interferences are typically lower. To additionally confirm the likelihood of a potentially positive result, it is relatively important to be sure that the ion ratios of the various mass fragments are comparable to a known referencestandard.

Applications

Environmental monitoring and cleanup

GC-MS is becoming the tool of choice for tracking organic pollutants in the environment. The cost of GC-MS equipment has decreased significantly, and the reliability has increased at the same time, which has contributed to its increased adoption in environmental studies.

Criminal forensics

GC-MS can analyze the particles from a human body in order to help link a criminal to a crime. The analysis of fire debris using GC-MS is well established, and there is even an established American Society for Testing and Materials (ASTM) standard for fire debris analysis. GCMS/MS is especially useful here as samples often contain very complex matrices and results, used in court, need to be highlyaccurate.

Law enforcement

GC-MS is increasingly used for detection of illegal narcotics, and may eventually supplant drug-sniffing dogs. It is also commonly used in forensic toxicology to find drugs and/or poisons in biological specimens of suspects, victims, or the deceased.

Sports anti-doping analysis

GC-MS is the main tool used in sports anti-doping laboratories to test athletes' urine samples for prohibited performance-enhancing drugs, for example anabolic steroids.

Security

A post–September 11 development, explosive detection systems have become a part of all US airports. These systems run on a host of technologies, many of them based on GC-MS. There are only three manufacturers certified by the FAA to provide these systems, one of which is Thermo Detection (formerly Thermedics), which produces the EGIS, a GC-MS-based line of explosives detectors. The other two manufacturers are Barringer Technologies, now owned by Smith's Detection Systems, and Ion Track Instruments, part of General Electric Infrastructure SecuritySystems.

Chemical warfare agent detection

As part of the post-September 11 drive towards increased capability in homeland security and public health preparedness, traditional GC-MS units with transmission quadrupole mass spectrometers, as well as those with cylindrical ion trap (CIT-MS) and toroidal ion trap (T- ITMS) mass spectrometers have been modified for field portability and near real-time detection of chemical warfare agents (CWA) such as sarin, soman, and VX. These complex and large GC- MS systems have been modified and configured with resistively heated low thermal mass (LTM) gas chromatographs that reduce analysis time to less than ten percent of the time required in traditional laboratory systems. Additionally, the systems are smaller, and more mobile, including units that are mounted in mobile analytical laboratories (MAL), such as those used by the United States Marine

Corps Chemical and Biological Incident Response Force MAL and other similar laboratories, and systems that are hand-carried by two-person teams or individuals, much ado to the smaller mass detectors. Depending on the system, the analytes can be introduced via liquid injection, desorbed from sorbent tubes through a thermal desorption process, or with solid-phase micro extraction (SPME).

Chemical Engineering

GC-MS is used for the analysis of unknown organic compound mixtures. One critical use of this technology is the use of GC-MS to determine the composition of bio-oils processed from raw biomass.

Food, beverage and perfume analysis

Foods and beverages contain numerous aromatic compounds, some naturally present in the raw materials and some forming during processing. GC-MS is extensively used for the analysis of these compounds which include esters, fatty acids, alcohols, aldehydes, terpenesetc. It is also used to detect and measure contaminants from spoilage or adulteration which may be harmful and which are often controlled by governmental agencies, for examplepesticides.

Astrochemistry

Several GC-MS have left earth. Two were brought to Mars by the Viking program. Venera11 and 12 and Pioneer Venus analysed the atmosphere of Venus with GC-MS. The Huygensprobe of the Cassini-Huygens mission landed one GC-MS on Saturn's largest moon, Titan. The material in the comet 67P/Churyumov-Gerasimenkowill be analysed by the Rosetta mission with a chiral GC-MS in2014.

Medicine

Dozens of congenital metabolic diseases also known as Inborn error of metabolism are now detectable by newborn screening tests, especially the testing using gas chromatography–mass spectrometry. GC-MS can determine compounds in urine even in minor concentration. These compounds are normally not present but appear in individuals suffering with metabolic disorders. This is increasingly becoming a common way to diagnose IEM for earlier diagnosis and institution of treatment eventually leading to a better outcome. It is now possible to test a newborn for over 100 genetic metabolic disorders by a urine test at birth based onGC-MS.

In combination with isotopic labeling of metabolic compounds, the GC-MS is used for determining

metabolic activity. Most applications are based on the use of ^{13}C as the labeling and the measurement of ^{13}C-^{12}C ratios with an **isotope ratio mass spectrometer** (**IRMS**); an MS with a detector designed to measure a few select ions and return values as ratios.

LCMS

Liquid chromatography–mass spectrometry (LC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS). Coupled chromatography - MS systems are popular in chemical analysis because the individual capabilities of each technique are enhanced synergistically. While liquid chromatography separates mixtures with multiple components, mass spectrometry provides structural identity of the individual components with high molecular specificity and detection sensitivity. This tandem technique can be used to analyze biochemical, organic, and inorganic compounds commonly found in complex samples of environmental and biological origin. Therefore, LC-MS may be applied in a wide range of sectors including biotechnology, environment monitoring, food processing, and pharmaceutical, agrochemical, and cosmetic industries

MALDI, TOF mass spectrometry, MALDI-TOF

Matrix-assisted laser desorption ionisation (MALDI) produces gas phase protonated ions by excitation of the sample molecules from the energy of a laser transferred via a UV light-absorbing matrix. The matrix is a conjugated organic compound (normally a weak organic acid such as a derivative of cinnamic acid and dihydroxybenzoic acid) that is intimately mixed with the sample. These are designed to maximally absorb light at the wavelength of the laser, typically a nitrogen laser of 337 nm or a neodymium/yttrium-aluminium-garnet (Nd-YAG) at 355 nm. The sample (1–10 pmol mm^{-3}) is mixed with an excess of the matrix and dried on to the target plate, where they co-crystallise on drying. Pulses of laser light of a few nanoseconds duration cause rapid excitation and vaporisation of the crystalline matrix and the subsequent ejection of matrix and analyte ions into the gas phase. This generates a plume of matrix and analyte ions that are analysed in a TOF mass analyser. The particular advantage of MALDI is

the ability to produce large mass ions, with high sensitivity. MALDI is a very soft ionisation method that does not produce abundant amounts of fragmentation compared with some other ionisation methods. Since the molecular ions are produced with little fragmentation, it is a valuable technique for examining mixtures TOF is the best type of mass analyzer to couple to MALDI, as this technique has a virtually unlimited mass range. Proteins and other macromolecules of M_r greater than 400 000 have been accuratelymeasured.

Sample concentration for MALDI

Maximum sensitivity is achieved in MALDI–TOF if samples are diluted to a particular concentration range. If the sample concentration is unknown a dilution series may be needed to produce a satisfactory sample/matrix spot of suitable concentration on the MALDI plate. Peptides and proteins seem to give best spectra at around 0.1 to 10 pmolmm^{-3}. Some proteins, particularly glycoproteins, may yield better results at concentrations up to 10 pmol mm^{-3}. Oligonucleotides give better spectra at around 10 to 100 pmol mm^{-3} while polymers require a concentration around 100 pmol mm^{3}.

Applications

MALDI-IMS involves the visualization of the spatial distribution of proteins, peptides, lipids, and other small molecules within thin slices of tissue, such as animal or plant. The application of this technique to biological studies has increased significantly since its introduction. MALDI- IMS is providing major contributions to the understanding of diseases, improving diagnostics, and drug delivery. Significant studies are of the eye, cancer research, drug distribution and neuroscience.

MALDI-IMS has been able to differentiate between drugs and metabolites and provide histological information in cancer research, which makes it a promising tool for finding new protein biomarkers. However, this can be challenging because of ion suppression, poor ionization, and low molecular weight matrix fragmentation effects. To combat this, chemical derivatizationis used to improvedetection.

POSSIBLE QUESTIONS

UNITIII

PART-A (20 MARKS)

(Q.NO 1 TO 20 Online Examination)

PART-B (2 MARKS)

1. Define chromatography?
2. Explain in detail about applications of chromatography
3. What is FPLC?
4. Applications of affinity chromatography?
5. Write Full form of GC-MS.
6. Give an outline on the system components of FPLC

PART-C (8 MARKS)

1. Write a detail note on LPLC.
2. Elaborate on principle and applications of ion-exchange chromatography.
3. Write an account on operation and applications of GC-MS.
4. Briefly explain the types of chromatography and its uses.
5. Explain the applications of high performance liquid chromatography

Unit II

Questions	Option A	Option B	Option C	Option D	Answer
Thin layer chromatography is	partition chromatography	electrical mobility of ionic species	adsorption chromatography	migration of charged ions in electric field	**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 substances	is not a separation technique	**separate mixtures into pure substances**
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 weight	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 myoglobin migrates to the cathode	histones migrate to the cathode and myoglobin migrates to the anode	both proteins migrate to the cathode	**histones migrate to the cathode and myoglobin migrates to the anode**

In isoelectric focusing, proteins are separated on the basis of their	relative content of positively charged residue only	relative content of negatively charged residue only	size	relative content of positively and negatively charged residue	**relative content of positively and negatively charged residue**
In SDS-PAGE, the protein sample is first	treated with a reducing agent and then with anionic detergent followed by fractionation by electrophoresis	fractionated by electrophoresis then treated with an oxidizing agent followed by anionic detergent.	treated with a oxidizing agent and then with anionic detergent followed by fractionation by electrophoresis	treated with acetic acid	**treated with a reducing agent and then with anionic detergent followed by fractionation by electrophoresis**
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 unknowns using comparison techniques.	To fill in all the slots on the gel so you can run it	To practice loading the DNA before you get to the important DNA	So you know how long the gel needs to run	**It makes it easier to determine sizes of unknowns using comparison techniques.**
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 constituent of	Lactose	Agarose	lactose and agarose	not a constituent of lactose and agarose	**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	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**

Mercapto ethanol reduces	peptide bond	disulfide bond	Hydrogen bond	vander waals force	**disulfide bond**
Which causes decompo sition of persulpha te ion to give free radical during SDS PAGE	APS	TEMED	SDS	acrylamid e	**TEMED**
Urea at 3 to 12 M concentra tion disrupts	peptide bond	disulfide bond	Hydrogen bond	vander waals force	**disulfide bond**
EtBr intercalat es DNA and is visible under	dark condition	UV	visible light	IR	**UV**
Glycinate ion in stacking gel is	poorly ionized	completel y 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**

UNIT-III

SYLLABUS

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

Agarose gel electrophoresis is a method of gel electrophoresis used in biochemistry,molecularbiology, genetics, and clinical chemistry to separate a mixed population of DNA or proteins in a matrix of agarose. The proteins may be separated by charge and/or size (isoelectricfocusing agarose electrophoresis is essentially size independent), and the DNA and RNA fragments by length. Biomolecules are separated by applying an electricfield to move the charged molecules through an agarosematrix, and the biomolecules are separated by size in the agarose gelmatrix.

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

Principle

Principles of Gel Electrophoresis Electrophoresis are a technique used to separate and sometimes purify macromolecules - especially proteins and nucleic acids - that differ in size, charge or conformation. As such, it is one of the most widely-used techniques in biochemistry and molecular biology.

Casting ofgel

The gel is prepared by dissolving the agarose powder in an appropriate buffer, such as TAE or TBE, to be used in electrophoresis. The agarose is dispersed in the buffer before heating it to near-boiling point, but avoid boiling. The melted agarose is allowed to cool sufficiently before

pouring the solution into a cast as the cast may warp or crack if the agarose solution is too hot. A comb is placed in the cast to create wells for loading sample, and the gel should be completely set beforeuse.

The concentration of gel affects the resolution of DNA separation. For a standard agarose gel electrophoresis, a 0.8% gives good separation or resolution of large 5–10kb DNA fragments, while 2% gel gives good resolution for small 0.2–1kb fragments. 1% gels is often used for a standard electrophoresis. The concentration is measured in weight of agarose over volume of buffer used (g/ml). High percentage gels are often brittle and may not set evenly, while low percentage gels (0.1-0.2%) are fragile and not easy to handle. Low-melting-point (LMP) agarose gels are also more fragile than normal agarose gel. Low-melting point agarose may be used on its own or simultaneously with standard agarose for the separation and isolation of DNA. PFGE and FIGE are often done with high percentage agarose gels.

Loading of samples

Once the gel has set, the comb is removed, leaving wells where DNA samples can be loaded. Loading buffer is mixed with the DNA sample before the mixture is loaded into the wells. The loading buffer contains a dense compound, which may be glycerol, sucrose, or Ficoll, that raises the density of the sample so that the DNA sample may sink to the bottom of the well. If the DNA sample contains residual ethanol after its preparation, it may float out of the well. The loading buffer also include colored dyes such as xylene cyanoland bromophenol blue used to monitor the progress of the electrophoresis. The DNA samples are loaded using apipette.

Electrophoresis

Agarose gel electrophoresis is most commonly done horizontally in a submarine mode whereby the slab gel is completely submerged in buffer during electrophoresis. It is also possible, but less common, to perform the electrophoresis vertically, as well as horizontally with the gel rose on agarose legs using an appropriate apparatus. The buffer used in the gel is the same as the running buffer in the electrophoresis tank, which is why electrophoresis in the submarine mode is possible with agarosegel.

For optimal resolution of DNA greater than 2 kb in size in standard gel electrophoresis, 5 to 8 V/cm is recommended (the distance in cm refers to the distance between electrodes, therefore this recommended voltage would be 5 to 8 multiplied by the distance between the electrodes in cm). Voltage may also be limited by the fact that it heats the gel and may cause the gel to melt if it is run at high voltage for a prolonged period, especially if the gel used is LMP agarose gel.

Too high a voltage may also reduce resolution, as well as causing band streaking for large DNA molecules. Too low a voltage may lead to broadening of band for small DNA fragments due to dispersion anddiffusion.

Since DNA is not visible in natural light, the progress of the electrophoresis is monitored using colored dyes. Xylene cyanol (light blue color) comigrates large DNA fragments, while Bromophenol blue (dark blue) comigrates with the smaller fragments. Less commonly used dyes include Cresol Red and Orange G which migrate ahead of bromophenol blue. A DNA marker is also run together for the estimation of the molecular weight of the DNA fragments. Note however that the size of a circular DNA like plasmids cannot be accurately gauged using standard markers unless it has been linearized by restriction digest, alternatively a supercoiled DNA marker may beused.

Staining and visualization

DNA as well as RNA is normally visualized by staining with ethidium bromide, which intercalates into the major grooves of the DNA and fluoresces under UV light. The ethidium bromide may be added to the agarose solution before it gels, or the DNA gel may be stained later after electrophoresis. Destaining of the gel is not necessary but may produce better images. Other biological macromolecules, usually proteins or nucleic acids, according to their electrophoreticmobility. Mobility is a function of the length, conformation and charge of the molecule.

As with all forms of gel electrophoresis, molecules may be run in their native state, preserving the molecules' higher-order structure, or a chemical denaturant may be added to remove this structure and turn the molecule into an unstructured linear chain whose mobility depends only on its length and mass-to-charge ratio. For nucleic acids, urea is the most commonly used denaturant. For proteins,sodium dodecyl sulfate (SDS) is an anionic detergent applied to protein samples to linearize proteins and to impart a negative charge to linearized proteins. This procedure is called **SDS-PAGE**. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis. Proteins that have a greater hydrophobic content, for instance many membrane proteins, and those that interact with surfactants in their native environment, are intrinsically harder to treat accurately using this method, due to the greater variability in the ratio of boundSDS.

Sample preparation

Samples may be any material containing proteins or nucleic acids. These may be biologically derived, for example from prokaryotic or eukaryotic cells, tissues, viruses, environmental samples, or purified proteins. In the case of solid tissues or cells, these are often first broken down mechanically using a blender (for larger sample volumes), using a homogenizer (smaller volumes), by sonicatoror by using cycling of high pressure, and a combination of biochemical and mechanical techniques – including various types of filtration and centrifugation – may be used to separate different cell compartments and organelles prior to electrophoresis. Synthetic biomolecules such as oligonucleotides may also be used asanalytes.

The sample to analyze is optionally mixed with a chemical denaturant if so desired, usually SDS for proteins or urea for nucleic acids. SDS is an anionic detergent that denatures secondary and non–disulfide–linked tertiary structures, and additionally applies a negative charge to each protein in proportion to its mass. Urea breaks the hydrogen bonds between the base pairs of the nucleic acid, causing the constituent strands to separate. Heating the samples to at least 60 °C further promotesdenaturation.

In addition to SDS, proteins may optionally be briefly heated to near boiling in the presence of a reducing agent, such as dithiothreitol (DTT) or 2-mercaptoethanol (beta-mercaptoethanol/BME), which further denatures the proteins by reducing disulfide linkages, thus overcoming some forms of tertiary protein folding, and breaking up quaternary protein structure (oligomeric subunits). This is known as reducing SDS-PAGE.

A tracking dye may be added to the solution. This typically has a higher electrophoretic mobility than the analytes to allow the experimenter to track the progress of the solution through the gel during the electrophoretic run.

Preparing acrylamide gels

The gels typically consist of acrylamide,bisacrylamide, the optional denaturant (SDS or urea), and a buffer with an adjusted pH. The solution may be degassed under a vacuum to prevent the formation of air bubbles during polymerization. Alternatively, butanol may be added to the resolving gel (for proteins) after it is poured, as butanol removes bubbles and makes the surface smooth. A source of free radicals and a stabilizer, such as ammoniumpersulfateand TEMED are added to initiate polymerization. The polymerization reaction creates a gel because of the added bisacrylamide, which can form cross-links between two acrylamide molecules. The ratio of bisacrylamide to acrylamide can be varied for special purposes, but is generally about 1 part in 35. The acrylamide concentration of the gel can also be varied, generally in the range from 5% to 25%. Lower percentage gels are better for

resolving very high molecular weight molecules, while much higher percentages are needed to resolve smaller proteins.

Gels are usually polymerized between two glass plates in a gel caster, with a comb inserted at the top to create the sample wells. After the gel is polymerized the comb can be removed and the gel is ready for electrophoresis.

Electrophoresis

Various buffer systems are used in PAGE depending on the nature of the sample and the experimental objective. The buffers used at the anode and cathode may be the same or different.

An electric field is applied across the gel, causing the negatively charged proteins or nucleic acids to migrate across the gel away from the negative electrode (which is the cathode being that this is an electrolytic rather than galvanic cell) and towards the positive electrode (the anode). Depending on their size, each biomolecule moves differently through the gel matrix: small molecules more easily fit through the pores in the gel, while larger ones have more difficulty. The gel is run usually for a few hours, though this depends on the voltage applied across the gel; migration occurs more quickly at higher voltages, but these results are typically less accurate than at those at lower voltages. After the set amount of time, the biomolecules have migrated different distances based on their size. Smaller biomolecules travel farther down the gel, while larger ones remain closer to the point of origin. Biomolecules may therefore be separated roughly according to size, which depends mainly on molecular weight under denaturing conditions, but also depends on higher-order conformation under native conditions. However, certain glycoproteins behave anomalously on SDSgels.

Further processing

Following electrophoresis, the gel may be stained (for proteins, most commonly with CoomassieBrilliant Blue R-250; for nucleic acids,ethidium bromide; or for either, silver stain), allowing visualization of the separated proteins, or processed further (e.g. Western blot). After staining, different species biomolecules appear as distinct bands within the gel. It is common to run molecular weight size markers of known molecular weight in a separate lane in the gel to calibrate the gel and determine the approximate molecular mass of unknown biomolecules by comparing the distance traveled relative to the marker.

For proteins, SDS-PAGE is usually the first choice as an assay of purity due to its reliability

and ease. The presence of SDS and the denaturing step make proteins separate, approximately based on size, but aberrant migration of some proteins may occur. Different proteins may also stain differently, which interferes with quantification by staining. PAGE may also be used as a preparative technique for the purification of proteins. For example, **q**uantitative **p**reparative **n**ative **c**ontinuous **p**oly**a**crylamide **g**el **e**lectrophoresis (QPNC-PAGE) is a method for separating native metalloproteinsin complex biologicalmatrices.

Polyacrylamide gel (*PAG)* had been known as a potential embedding medium for sectioning tissues as early as 1964, and two independent groups employed PAG in electrophoresis in 1959. It possesses several electrophoretically desirable features that make it a versatile medium. It is a synthetic, thermo-stable, transparent, strong, chemically relatively inert gel, and can be prepared with a wide range of average pore sizes. The pore size of a gel is determined by two factors, the total amount of acrylamide present (%T) (T = Total concentration of acrylamide and bisacrylamide monomer) and the amount of cross-linker (%C) (C = bisacrylamide concentration). Pore size decreases with increasing %T; with cross-linking, 5%C gives the smallest pore size. Any increase or decrease in %C from 5% increases the pore size, as pore size with respect to %C is a parabolic function with vertex as 5%C. This appears to be because of non-homogeneous bundling of polymer strands within the gel. This gel material can also withstand highvoltagegradients, is amenable to various staining and destaining procedures, and can be digested to extract separated fractions or dried for autoradiography and permanent recording.

Components

- **Chemical buffer** Stabilizes the pH value to the desired value within the gel itself and in the electrophoresis buffer. The choice of buffer also affects the electrophoretic mobility of the buffer counterionsand thereby the resolution of the gel. The buffer should also be unreactive and not modify or react with most proteins. Different buffers may be used as cathode and anode buffers, respectively, depending on the application. Multiple pH values may be used within a single gel, for example in DISC electrophoresis. Common buffers in PAGE include Tris,Bis-Tris, orimidazole.
- **Counterion**balance the intrinsic charge of the buffer ion and also affect the electric fieldstrength during electrophoresis. Highly charged and mobile ions are often avoided in SDS- PAGE cathode buffers, but may be included in the gel itself, where it migrates

ahead of the protein. In applications such as DISC SDS-PAGE the pH values within the gel may vary to change the average charge of the counterions during the run to improve resolution. Popular counterions are glycine and tricine. Glycine has been used as the source of trailing ionor

slow ion because its pKa is 9.69 and mobility of glycinate are such that the effective mobility can be set at a value below that of the slowest known proteins of net negative charge in the pH range. The minimum pH of this range is approximately8.0.

- **Acrylamide (C_3H_5NO; mW: 71.08).** When dissolved in water, slow, spontaneous autopolymerizationof acrylamide takes place, joining molecules together by head on tail fashion to form long single-chain polymers. The presence of a free radical-generating system greatly accelerates polymerization. This kind of reaction is known as Vinyladditionpolymerisation. A solution of these polymer chains becomes viscous but does not form a gel, because the chains simply slide over one another. Gel formation requires linking various chains together. Acrylamide is a neurotoxin. It is also essential to store acrylamide in a cool dark and dry place to reduce autopolymerisationandhydrolysis.

- **Bisacrylamide (*N*,*N*′-Methylenebisacrylamide) ($C_7H_{10}N_2O_2$; mW: 154.17).** Bisacrylamide is the most frequently used cross linking agent for polyacrylamide gels. Chemically it can be thought of as two acrylamide molecules coupled head to head at their non-reactive ends. Bisacrylamide can crosslink two polyacrylamide chains to one another, thereby resulting in agel.

- **Sodium Dodecyl Sulfate (SDS) ($C_{12}H_{25}NaO_4S$; mW: 288.38).** (only used in denaturing protein gels) SDS is a strong detergent agent used to denature native proteins to unfolded, individual polypeptides. When a protein mixture is heated to 100 °C in presence of SDS, the detergent wraps around the polypeptide backbone. It binds to polypeptides in a constant weight ratio of 1.4 g SDS/g of polypeptide. In this process, the intrinsic charges of polypeptides become negligible when compared to the negative charges contributed by SDS. Thus polypeptides after treatment become rod-like structures possessing a uniform charge density that is same net negative charge per unit weight. The electrophoretic mobilities of these proteins are a linear function of the logarithms of their molecularweights.

  Without SDS, different proteins with similar molecular weights would migrate differently due to differences in mass-charge ratio, as each protein has an isoelectric point and molecular weight particular to its primary structure. This is known as Native PAGE. Adding SDS solves this problem, as it binds to and unfolds the protein, giving a near uniform negative charge along the length of thepolypeptide.

- **Urea (CO $(NH_2)_2$; mW: 60.06).** Urea is a chaotropic agent that increases the entropy of the system by interfering with intramolecular interactions mediated by non-covalentforces such as hydrogen bonds and Vander Waals forces. Macromolecular structure is dependent on the net effect of these forces, therefore it follows that an increase in chaotropic solutes denaturesmacromolecules,
- **Ammonium persulfate(APS) ($N_2H_8S_2O_8$; mW: 228.2)**. APS is a source of free radicals and is often used as an initiator for gel formation. An alternative source of free radicals isriboflavin, which generated free radicals in a photochemicalreaction. • **TEMED (*N*, N, N′, N′-tetramethylethylenediamine)** ($C_6H_{16}N_2$; mW: 116.21). TEMED stabilizes free radicals and improves polymerization. The rate of polymerization and the properties of the resulting gel depend on the concentrations of free radicals. Increasing the amount of free radicals results in a decrease in the average polymer chain length, an increase in gel turbidity and a decrease in gel elasticity. Decreasing the amount shows the reverse effect. The lowest catalytic concentrations that allow polymerization in a reasonable period of time should be used. APS and TEMED are typically used at approximately equimolar concentrations in the range of 1 to 10mM.

Chemicals for processing and visualization

The following chemicals and procedures are used for processing of the gel and the protein samples visualized in it:

- **Tracking dye**. As proteins and nucleic acids are mostly colorless, their progress through the gel during electrophoresis cannot be easily followed. Anionic dyes of a known electrophoretic mobility are therefore usually included in the PAGE sample buffer. A very common tracking dye is Bromophenol blue (BPB, 3', 3", 5', 5" tetrabromophenolsulfonphthalein). This dye is coloured at alkali and neutral pH and is a small negatively charged molecule that moves towards the anode. Being a highly mobile molecule it moves ahead of most proteins. As it reaches the anodic end of the electrophoresis medium electrophoresis is stopped. It can weakly bind to some proteins and impart a blue colour. Other common tracking dyes are xylene cyanol, which has lower mobility, and Orange G, which has a highermobility.
- **Loading aids**. Most PAGE systems are loaded from the top into wells within the gel. To ensure that the sample sinks to the bottom of the gel, sample buffer is

supplemented with additives that increase the density of the sample. These additives should be non-ionic and non-reactive towards proteins to avoid interfering with electrophoresis. Common additives are glycerol and sucrose.

- **Coomassie Brilliant Blue R-250 (CBB) ($C_{45}H_{44}N_3NaO_7S_2$; mW: 825.97)**. CBB is the most popular protein stain. It is an anionic dye, which non-specifically binds to proteins. The structure of CBB is predominantly non-polar, and it is usually used in methanolic solution acidified with acetic acid. Proteins in the gel are fixed by acetic acid and simultaneously stained. The excess dye incorporated into the gel can be removed by destaining with the same solution without the dye. The proteins are detected as blue bands on a clear background. As SDS is also anionic, it may interfere with staining process. Therefore, large volume of staining solution is recommended, at least ten times the volume of thegel.

- **Ethidium bromide (EtBr)** is the traditionally most popular nucleic acidstain.

Silver staining. Silver staining is used when more sensitive method for detection is needed, as classical Coomassie Brilliant Blue staining can usually detect a 50 ng protein band, Silver staining increases the sensitivity typically 50 times. The exact chemical mechanism by which this happens is still largely unknown. Silver staining was introduced by Kerenyi and Gallyas as a sensitive procedure to detect trace amounts of proteins ingels. The technique has been extended to the study of other biological macromolecules that have been separated in a variety of supports. Many variables can influence the colourintensity and every protein has its own staining characteristics; clean glassware, pure reagents and water of highest purity are the key points to successful staining. Silver staining was developed in the 14th century for colouring the surface of glass. It has been used extensively for this purpose since the 16th century. The colour produced by the early silver stains ranged between light yellow and an orange-red. Camillo Golgiperfected the silver staining for the study of the nervous system.Golgi's method stains a limited number of cells at random in their entirety.

- **Western Blotting** is a process by which proteins separated in the acrylamide gel are electrophoretically transferred to a stable, manipulable membrane such as anitrocellulose,nylon, or PVDF membrane. It is then possible to apply immunochemical techniques to visualise the transferred proteins, as well as accurately identify relative increases or decreases of the protein of interest. For more, see WesternBlot.

Isoelectric focusing takes place in a pH gradient. The charged molecules move towards the anode or the cathode until they reach a position in the pH gradient where their net charges are

zero. This pH value is the "**isoelectric** point" (pI) of the substance.

Principle of IsoelectricFocusing

The use of isoelectric focusing is limited to molecules which can be either positively or negatively charged. Proteins, enzymes and peptides are such amphoteric molecules. The net charge of a protein is the sum of all negative and positive charges of the amino acid side chains, but the three-dimensional configuration of the protein also plays a role.

Isoelectric focusing takes place in a pH gradient. The charged molecules move towards the anode or the cathode until they reach a position in the pH gradient where their net charges are zero. This pH value is the isoelectric point of the substance. Since it is no longer charged, the electric field does not have any influence onit.

The method of separating proteins according to their isoelectric points in a gradient is called isoelectric focusing. This technique was discovered by H. Svensson and Sweden. This method has a high resolution power because ordinary paper electrophoresis resolves plasma proteins into six bands whereas isoelectric focusing resolves them into 40 bands.

In conventional electrophoresis, the pH between anode and cathode is constant and the positively charged ions migrate towards the cathode and negatively charged ions migrate towards the anode. But in isoelectric focusing, a stable pH gradient is arranged. The pH gradually increases from anode to cathode. When a protein is introduced at a pH which is lower than its isoionic point, it will possess a net positive charge and will migrate in the direction of the cathode. Due to the presence of pH gradient, the net charge of the molecule changes due to ionization as it moves is zero, it will stop migrating. This is the isoelectric point of protein. Each protein present in the mixture will migrate to its isoelectric point and stop its migration at that point. Thus, once a final, stable focusing is reached, the resolution will be retained for a longtime.

Uses

Isoelectric focusing is widely used for the separation and identification of serum proteins. It is used in the food and agriculture industry, forensic and human genetic laboratories, for research in enzymology, immunology and membrane biochemistry, etc.

Immuno electrophoresis

Immunoelectrophoresisis a general name for a number of biochemical methods for separation and characterization of proteins based on electrophoresis and reaction with antibodies. All variants of immunoelectrophoresis require immunoglobulins, also known as antibodies, reacting with the proteins to be separated or characterized. The methods were developed and

used extensively during the second half of the 20th century. In somewhat chronological order: Immunoelectrophoretic analysis (one-dimensional immunoelectrophoresis*ad modum*Grabar), crossed immunoelectrophoresis (two-dimensional quantitative immunoelectrophoresis*admodum*Clarke and Freeman or *ad modum*Laurell), rocket-immunoelectrophoresis (one- dimensional quantitative immunoelectrophoresis*admodum*Laurell), fused rocket immunoelectrophoresis*admodum*Svendsen and Harboe, affinity immunoelectrophoresis*admodum*Bøg-Hansen.

Agaroseas 1% gel slabs of about 1 mm thickness buffered at high pH (around 8.6) is traditionally preferred for the electrophoresis as well as the reaction with antibodies. The agarose was chosen as the gel matrix because it has large pores allowing free passage and separation of proteins, but provides an anchor for the immunoprecipitates of protein and specific antibodies. The high pH was chosen because antibodies are practically immobile at high pH.An electrophoresis equipment with a horizontal cooling plate was normally recommended for the electrophoresis.

Immunoprecipitates may be seen in the wet agarose gel, but are stained with protein stains like Coomassie Brilliant Blue in the dried gel. In contrast to SDS-gel electrophoresis, the electrophoresis in agarose allows native conditions, preserving the native structure and activities of the proteins under investigation, thereforeimmunoelectrophoresis allows characterization of enzyme activities and ligand binding etc. in addition to electrophoreticseparation.

Crossed immunoelectrophoresisis also called two-dimensional quantitative immunoelectrophoresis*admodum*Clarke and Freeman or*admodum*Laurell. In this method the proteins are first separated during the first dimension electrophoresis, then instead of the diffusion towards the antibodies, the proteins are electrophoresed into an antibody-containing gel in the second dimension. Immunoprecipitation will take place during the second dimension electrophorsis and the immunoprecipitates have a characteristic bell-shape, each precipitate representing one antigen, the position of the precipitate being dependent on the amount of protein as well as the amount of specific antibody in the gel, so relative quantification can be performed. The sensitivity and resolving power of crossed immunoelectrophoresis is than that of the classical immunoelectrophoretic analysis and there are multiple variations of the technique useful for various purposes. Crossed immunoelectrophoresis has been used for studies of proteins in biological fluids, particularly human serum, and biologicalextracts.

Rocket immunoelectrophoresisis one-dimensional quantitative immunoelectrophoresis. The method has been used for quantitation of human serum proteins before automated methods

became available.

Fused rocket immunoelectrophoresisis a modification of one-dimensional quantitative immunoelectrophorsis used for detailed measurement of proteins in fractions from protein separation experiments.

Affinity immunoelectrophoresisis based on changes in the electrophoretic pattern of proteins through specific interaction or complex formation with other macromolecules or ligands. Affinity immunoelectrophoresishas been used for estimation of binding constants, as for instance with lectinsor for characterization of proteins with specific features likeglycancontent or ligand binding. Some variants of affinity immunoelectrophoresis are similar to affinitychromatography by use of immobilizedligands.

The open structure of the immunoprecipitate in the agarose gel will allow additional binding of radioactively labeled antibodies to reveal specific proteins. This variation has been used for identification of allergens through reaction with IgE.

Two factors determine that immunoelectrophoretic methods are not widely used. First they are rather work intensive and require some manual expertise. Second they require rather large amounts of polyclonal antibodies. Today gel electrophoresis followed by electroblottingis the preferred method for protein characterization because it's ease of operation, its high sensitivity, and its low requirement for specific antibodies. In addition proteins are separated by gelelectrophoresis on the basis of their apparent molecular weight, which is not accomplished by immunoelectrophoresis, but nevertheless immunoelectrophoretic methods are still useful when non-reducing conditions are needed.

Pulsed field gel electrophoresis is a technique used for the separation of large deoxyribonucleicacid (DNA) molecules by applying to a gel matrix an electric field that periodically changes direction.

The procedure for this technique is relatively similar to performing a standard gel electrophoresis except that instead of constantly running the voltage in one direction, the voltage is periodically switched among three directions; one that runs through the central axis of the gel and two that run at an angle of 60 degrees either side. The pulse times are equal for each direction resulting in a net forward migration of the DNA. For extremely large molecules (up to around 2 Mb), switching-interval ramps can be used that increases the pulse time for each direction over the course of a number of hours—take, for instance, increasing the pulse linearly from 10 seconds at 0 hours to 60 seconds at 18hours.

This procedure takes longer than normal gel electrophoresis due to the size of the fragments being resolved and the fact that the DNA does not move in a straight line through thegel.

Applications

PFGE may be used for genotyping or genetic fingerprinting. It is commonly considered a goldstandard in epidemiological studies of pathogenic organisms. Subtyping has made it easier to discriminate among strains of *Listeria monocytogenes*and thus to link environmental or food isolates with clinical infections.

Capillary electrophoresis

Capillary electrophoresis (**CE**) is a family of electrokinetic separation methods performed in submillimeter diameter capillaries and in micro- and nanofluidic channels. Very often, CE refers to capillary zone electrophoresis (CZE), but other electrophoretic techniques including capillarygel electrophoresis (CGE), capillaryisoelectric focusing (CIEF), capillary isotachophoresisand micellarelectrokinetic chromatography (MEKC) belong also to this class of methods.[1]In CE methods, analytes migrate through electrolytesolutions under the influence of an electric field. Analytes can be separated according to ionic mobility and/or partitioning into an alternate phase via Non-covalent interactions. Additionally, analytes may be concentrated or "focused" by means of gradients inconductivityandpH.

The instrumentation needed to perform capillary electrophoresis is relatively simple. A basicschematic of a capillary electrophoresis system. The system's main components are a sample vial, source and destination vials, a capillary, electrodes, a high voltage power supply, a detector, and a data output and handling device. The source vial, destination vial and capillary are filled with an electrolyte such as an aqueous buffer solution. To introduce the sample, the capillary inlet is placed into a vial containing the sample. Sample is introduced into the capillary via capillary action, pressure, siphoning, or electrokinetically, and the capillary is then returned to the source vial. The migration of the analytes is initiated by an electric field that is applied between the source and destination vials and is supplied to the electrodes by the high-voltage power supply. In the most common mode of CE, all ions, positive or negative, are pulled through the capillary in the same direction by electroosmotic flow. The analytes separate as they migrate due to their electrophoretic mobility, and are detected near the outlet end of the capillary. The outputofthedetectorissenttoadataoutputandhandlingdevicesuchas

an integrator or computer. The data is then displayed as an electropherogram, which reports detector response as a function of time. Separatedchemical compounds appear as peaks with different retention times in an electropherogram. Capillary electrophoresis was first combined with mass spectrometry by Richard D. Smith and coworkers, and provides extremely high sensitivity for the analysis of very small sample sizes. Despite the very small sample sizes

(typically only a few nanoliters of liquid are introduced into the capillary), high sensitivity and sharp peaks are achieved in part due to injection strategies that result in concentration of analytes into a narrow zone near the inlet of the capillary. This is achieved in either pressure or electrokinetic injections simply by suspending the sample in a buffer of lower conductivity (*e.g.* lower salt concentration) than the running buffer. A process called field-amplified sample stacking (a form of isotachophoresis) results in concentration of analyte in a narrow zone at the boundary between the low-conductivity sample and the higher-conductivity runningbuffer.

To achieve greater sample throughput, instruments with arrays of capillaries are used to analyze many samples simultaneously. Such capillary array electrophoresis (CAE) instruments with 16 or 96 capillaries are used for medium- to high-throughput capillary DNA sequencing, and the inlet ends of the capillaries are arrayed spatially to accept samples directly from SBS-standard footprint 96-wellplates.

Separation by capillary electrophoresis can be detected by several detection devices. The majority of commercial systems use UV or UV-Vis absorbance as their primary mode of detection. In these systems, a section of the capillary itself is used as the detection cell. The use of on-tube detection enables detection of separated analytes with no loss of resolution. In general, capillaries used in capillary electrophoresis are coated with a polymer (frequently polyimide or Teflon) for increased flexibility. The portion of the capillary used for UV detection, however, must be optically transparent. For polyimide-coated capillaries, a segment of the coating is typically burned or scraped off to provide a bare window several millimeters long. This bare section of capillary can break easily, and capillaries with transparent coatings are available to increase the stability of the cell window. The path length of the detection cell in capillary electrophoresis (~ 50 micrometers) is far less than that of a traditional UV cell (~ 1 cm). According to the Beer-Lambert law, the sensitivity of the detector is proportional to the path length of the cell. To improve the sensitivity, the path length can be increased, though this results in a loss of resolution. The capillary tube itself can be expanded at the detection point, creating a "bubble cell" with a longer path length or additional tubing can be added at the detection point. Both of these methods, however, will decrease the resolution of the separation. Post-column detection utilizing a sheath flow configuration has also beendescribed.

Fluorescence detection can also be used in capillary electrophoresis for samples that naturally fluoresce or are chemically modified to contain fluorescent tags. This mode of detection offers high sensitivity and improved selectivity for these samples, but cannot be utilized for samples that do not fluoresce. Numerous labeling strategies are used to create fluorescent derivatives or conjugates of non-fluorescent molecules, including proteins and DNA. The set-upforfluorescence detection in a capillary electrophoresis system can be complicated. The

method requires that the light beam be focused on the capillary, which can be difficult for many light sources. Laser-induced fluorescence has been used in CE systems with detection limits as low as $10^{-18}$to 10^{-21} mol. The sensitivity of the technique is attributed to the high intensity of the incident light and the ability to accurately focus the light on the capillary.[2]Multi-color fluorescence detection can be achieved by including multiple dichroic mirrors and bandpass filters to separate the fluorescence emission amongst multiple detectors (*e.g.*, photomultipliertubes), or by using a prism or grating to project spectrally resolved fluorescence emission onto a position-sensitive detector such as a CCD array. CE systems with 4- and 5-color LIF detection systems are used routinely for capillary DNA sequencing and genotyping ("DNAfingerprinting")applications.

In order to obtain the identity of sample components, capillary electrophoresis can be directly coupled with mass spectrometers or Surface Enhanced Raman Spectroscopy (SERS). In most systems, the capillary outlet is introduced into an ion source that utilizes electrosprayionization (ESI). The resulting ions are then analyzed by the mass spectrometer. This set-up requires volatile buffer solutions, which will affect the range of separation modes that can be employed and the degree of resolution that can be achieved. The measurement and analysis are mostly done with a specialized gel analysissoftware.

For CE-SERS, capillary electrophoresis eluantscan be deposited onto a SERS-active substrate. Analyte retention times can be translated into spatial distance by moving the SERS-active substrate at a constant rate during capillary electrophoresis. This allows the subsequent spectroscopic technique to be applied to specific eluants for identification with high sensitivity. SERS-active substrates can be chosen that do not interfere with the spectrum of the analytes.

Application

Capillary electrophoresis may be used for the simultaneous determination of the ions NH_4^+, Na^+, K^+, Mg^{2+} and Ca^{2+} in saliva

Polyacrylamide gel electrophoresis (PAGE)

About the PAGE method in general

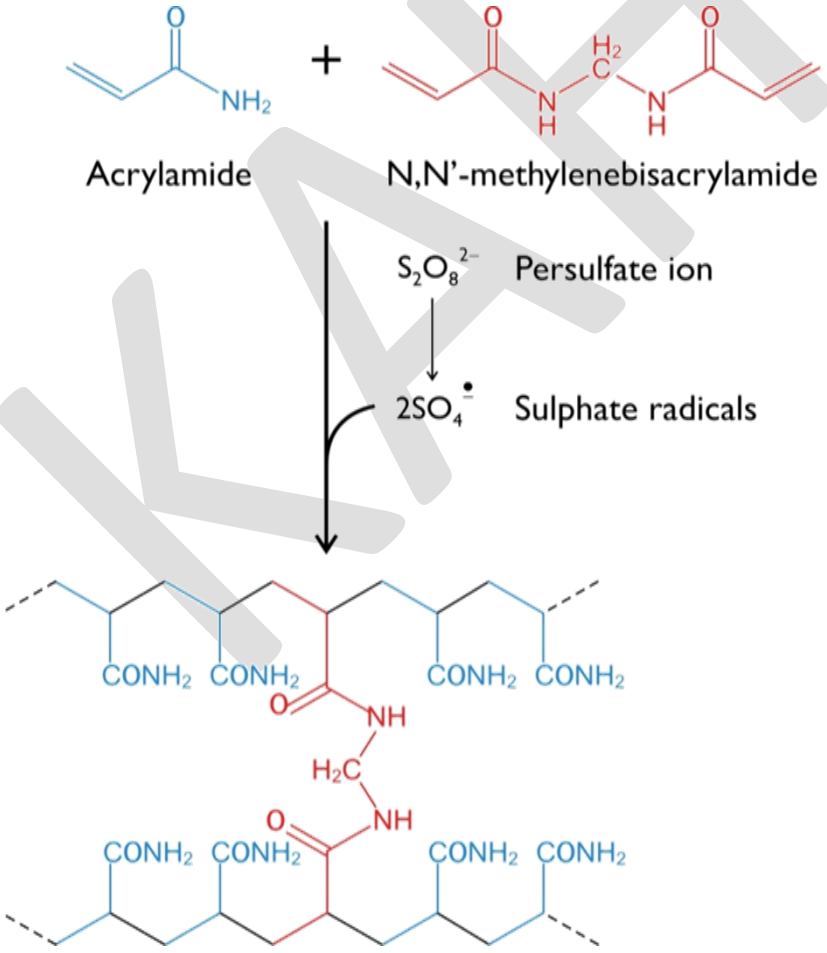
As mentioned previously, polyacrylamide gels can be used for the separation and analysis of proteins and relatively small nucleic acid molecules. For example, when it was first invented, Sanger's DNA sequencing method (see in details in Chapter 10) applied PAGE to separate linear single-stranded DNA molecules based on their length. The resolution of the PAGE method is so

high that, in the size range of about 10-1000 nucleotide units, it is capable of separating DNA molecules that differ in length only by a single monomer unit. In the case of single-stranded DNA, individual molecules are separated solely based on their length. This is due to the fact that, in the case of DNA (or RNA), the number of negative charges is a simple linear function of the number of monomer units (i.e. the length of the molecule). In other words, the specific charge (number of charges per particle mass) is invariant, i.e. it is the same for all DNA molecules. It is so because each monomer unit has one phosphate moiety that carries the negative charge. When an appropriate denaturing agent, such as urea, is added to the DNA sample and the gel is heated, the shape of the varying-length linear DNA molecules becomes identical. As a consequence, denatured molecules will be separated exclusively based on their size. (We will see the same principle at the SDS-PAGE method that separates denatured proteins almost exclusively based on their size (molecular weight)). There are several PAGE methods (SDS-PAGE, isoelectric focusing, 2D PAGE) that can be applied mostly for the separation of proteins based on distinct molecular properties.

At a given pH, different proteins carry different amounts of electric charge. Moreover, different proteins have different shapes and sizes, too. Consequently, during electrophoresis, proteins are separated by a complex combination of their charge, shape and size. PAGE separation of proteins provides high resolution. However, as three independent molecular properties simultaneously influence electrophoretic mobility, it will provide limited room for precise interpretation. For example, when two proteins are compared, it remains hidden what makes one of them migrate faster: a larger number of electric charges, a smaller size, or a more spherical shape. Nevertheless, even the simplest PAGE method, which will be referred to as native PAGE, provides many particular advantages (see below).

In order to increase the analytical applicability of the PAGE technology, several variations of the method have been established to separate proteins based on a single molecular property. As we will see, SDS-PAGE separates proteins based primarily on molecular weight, while isoelectric focusing separates proteins exclusively based on isoelectric point.

In the presence of suitable initiator and catalyst compounds, acrylamide can readily polymerise in a radical process. (Acrylamide is harmful by inhalation or skin contact, and thus it should be handled with care.) This reaction would lead to very long polyacrylamide chains, yielding a highly viscous liquid instead of a gel. As already mentioned, these long chains need to be cross-linked to form a three-dimensional network. This is achieved by mixing *N*,*N*′-methylenebisacrylamide into the acrylamide solution. In essence, *N*,*N*′-methylenebisacrylamide is composed of two acrylamide molecules covalently interconnected via a methylene moiety. When, during the polymerisation reaction, the acrylamide groups of *N*,*N*′-methylenebisacrylamide molecules become incorporated in the long polyacrylamide chains, cross-links are formed between the polyacrylamide chains leading to a gel (Figure 7.2). In the course of electrophoresis, ions (proteins or nucleic acids) are separated in this gel.



Molecular structure of the polyacrylamide gel. The three-dimensional molecular network comes into being by a radical polymerisation of acrylamide monomers and cross-linking *N*,*N*'-methylenebisacrylamide components.

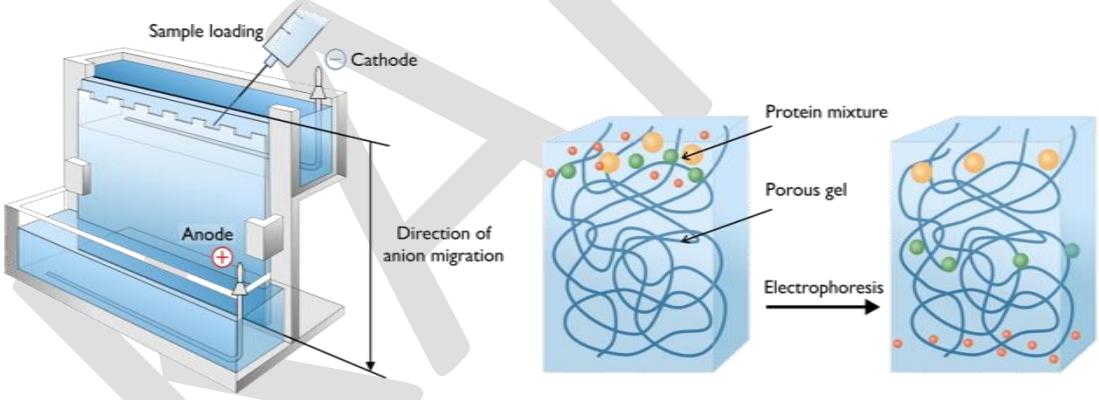
Without any modification, polyacrylamide electrophoresis separates macromolecular ions based on a combination of charge, size and shape. Size (and shape) separation is due to the molecular sieving property of the gel. The size range in which molecules can be separated is dictated by the average pore size of the gel. In the case of polyacrylamide gels, this can be controlled through the concentration of the acrylamide monomer and the proportion of the cross-linking *N*,*N*'-methylenebisacrylamide. The acrylamide concentration can be set in the range of about 4-20 % as this is the range in which the mechanical properties of the gel are appropriate. Below this range the gel will be too soft and it will not keep its shape, while above this range it will be too rigid and prone to break. The optimal proportion of the *N*,*N*'-methylenebisacrylamide component is 1-3 % relative to the acrylamide component. The polyacrylamide gel possesses all advantageous properties necessary for a good electrophoresis medium, i.e. it is hydrophilic, free of electric charges and chemically stable. A further very important property of the polyacrylamide gel is that it does not participate in any non-specific or specific binding interaction with proteins. Furthermore, the polyacrylamide gel does not interfere with common protein staining reactions.

When electrophoresis is performed under native (non-denaturing) conditions, such as near neutral pH and ambient or lower temperature, many enzymes retain their native conformation and, in turn, their enzymatic activity. This way, many enzymes can be separated and specifically detected in the gel after electrophoretic separation.

In the course of creating the gel, a buffer with a properly chosen pH is mixed into the acrylamide/*N*,*N*'-methylenebisacrylamide solution. Radical polymerisation is subsequently triggered by suitable catalyst and initiator compounds. The catalyst is usually ammonium persulfate, which spontaneously decomposes in aqueous media, thereby generating free radicals. These free radicals in themselves cannot efficiently cleave the double bonds of the acrylamide

molecule, but are able to excite the electrons of the initiator molecules. This leads to the generation of free radicals, originating from the initiator molecules, that are able to trigger radical polymerisation of acrylamide monomers. The most frequently used initiator is tetramethylethylenediamine (TEMED).

There are two types of gels according to their geometry. In early gel electrophoretic applications, gel tubes were used that allowed only a single sample to be run. Gel slabs were later introduced, allowing for many samples to be run at the same time in the same gel in parallel. Gel slabs became much more common than gel tubes. Gel slabs are created by pouring the gel-forming solution between two parallel glass sheets prior to polymerisation (Figure 7.3). Besides its higher throughput, this gel geometry provides another important advantage over gel tubes: samples are loaded side by side on such slabs and are run in the same gel at the same time. This allows for a more reliable comparison of the samples, facilitating the interpretation of experimental results.



. Separation of proteins in a polyacrylamide gel. As illustrated in the left panel, several samples can be run in parallel in a slab gel. Ions can move between the two electrodes only through the gel interconnecting the two chambers. The gel acts as a molecular sieve. The larger the molecule, the larger the drag force exerted on it by the gel.

Proper selection of pH and acrylamide concentration is instrumental for successful electrophoresis. For protein electrophoresis, the pH is set usually higher than the pI value of the

proteins in the sample. At such a pH, all proteins will be negatively charged and will move towards the anode. The buffer in the medium serves two purposes. One is to set and maintain the proper pH during electrophoresis. The other function of the buffer is to establish the electric current in the medium.

The majority of the electric current is carried by the ions of the buffer. Normally, the protein-ions that are separated by electrophoresis have only a negligible contribution to the current. In other words, proteins have a low ion transport number. However, if the buffer concentration is set too low, the contribution of proteins to carrying the current will increase, and the protein molecules will migrate rapidly. This usually leads to smearing of the bands of migrating proteins. On the other hand, if the buffer concentration is set too high, the mobility of the proteins will be too low. In this case the electrophoresis process would take a very long time. Unnecessary lengthening of the process provides excess time for diffusion, which lowers the resolution of separation.

According to the applied buffer system, gel electrophoretic methods can be classified into two types: continuous and discontinuous. Continuous methods apply the same buffer in the gel and in the two buffer chambers containing the electrodes. The only advantage of this method lies in its simplicity. More complex discontinuous methods were introduced to provide higher resolution. SDS polyacrylamide gel electrophoresis (see later) is usually associated with such a discontinuous system.

The discontinuous system applies two gels of different pore size and three different buffers. One of the gels, the resolving gel, is polymerised at a higher acrylamide concentration. The pore size of this gel is set according to the size range of the proteins to be separated. Another gel, the stacking gel is created on top of the resolving gel. (The gels are mounted in a vertical format.) The stacking gel is polymerised from a more dilute acrylamide solution to provide larger pores. This pore size does not provide a molecular sieving effect.

As mentioned above, there are three buffers: different ones in each of the two gels and a third one, the so-called ‘running buffer’ in the buffer chambers containing the electrodes. In the gel

buffers, the anion originates from a strong acid; it is usually chloride ion. Dissociation of strong acids does not depend on the pH: these acids always fully dissociate. Consequently, chloride ion is never protonated in the solution: its ionisation state is independent of the pH. On the other hand, the anion component of the running buffer is the conjugate base of a weak acid. Consequently, the ionisation state of this ion depends on the pH of the buffer. Glycinate ion is one of the most frequently used compounds for this purpose. The pH in the running buffer is set to 8.3.

The protein sample is layered on the top of the stacking gel. When an electric field is generated by the power supply, the protein ions and the ions of the running buffer enter the stacking gel. The pH in the stacking gel is set to 6.8. This value is only slightly higher than the pI value of glycine (6.5). At this pH, most glycine molecules are in a neutral zwitterionicstate, and only a small portion of the molecules carry a net negative charge. In this state, glycine has a low electrophoretic mobility and a corresponding low transport number. The local sparsity of ions elevates the local electric resistance of the medium. As the electric current must be of the same magnitude at any segments of the electric circuit (there is no macroscopic charge separation), the voltage will increase according to Ohm's law. Due to this effect, the migration speed of the proteins will be relatively high and the protein front will reach the chloride front in the stacking gel. The ion concentration in the chloride front is high and, therefore, here the electric resistance and the voltage are low. This slows down the protein front. This effect results in a very sharp protein front, with the protein molecules being crowded right behind the chloride ion front.

The protein sample will thus enter the resolving gel in a sharp band. The pH in the resolving gel is set to about 8.8. At this pH, almost all glycinate molecules are in the anionic state. Thus, the electric mobility of glycinate increases, and the concentrating effect applied by the stacking gel ends in the resolving gel. Different proteins will be separated in the resolving gel according to their charge, size and shape.

In most electrophoretic methods, a tracking dye is mixed in the sample. Usually, this dye is chosen to have a higher electrophoretic mobility than any of the components of interest (proteins

or nucleic acids) in the sample. The function of the tracking dye is to visualise the running front and, in turn, the completeness of the run. The most popular tracking dye is bromophenol blue.

The following sections review the various PAGE methods listed from the simplest to the most complex one.

Native PAGE

Native PAGE is an electrophoresis method to separate native proteins. The conditions are set such that the migrating proteins are kept in their native state. The buffers provide a non-denaturing, native-like milieu, and the electrophoresis is performed at low temperature in order to dissipate heat. Many enzymes retain their native conformation and their enzymatic activities while running in the gel. If certain conditions apply, these enzymes can be highly selectively detected within the gel through a specific 'staining' reaction even in the presence of a large excess of 'contaminating' proteins. After completion of electrophoresis, the gel is soaked in a solution containing the substrate of the enzyme. As the substrate is usually a small molecule, it quickly diffuses into the gel while the large enzyme molecules do not diffuse out. In an optimal case, the natural product of the enzymatic reaction is a coloured and insoluble compound that precipitates inside the gel and marks the exact location of the enzyme. Of course, most enzymes do not have such natural substrates. However, once the molecular mechanism of catalysis is revealed, synthetic substrates can be designed that, on the one hand, mimic natural substrates and, on the other hand, lead to colourful insoluble products.

Native PAGE is also a useful method for checking the uniformity of the isolated protein. Even if the purified protein sample contains only a single type of protein, the sample might not be uniform. Some of the molecules might be unfolded or have undergone chemical modifications. Unfolding changes the overall shape of the molecule, while most chemical modifications change the electric charge of native molecules. These alterations can be detected after traditional staining of the purified sample. If no such side products are present, protein molecules will run in a single sharp band. Otherwise, multiple bands or smearing of the band is expected.

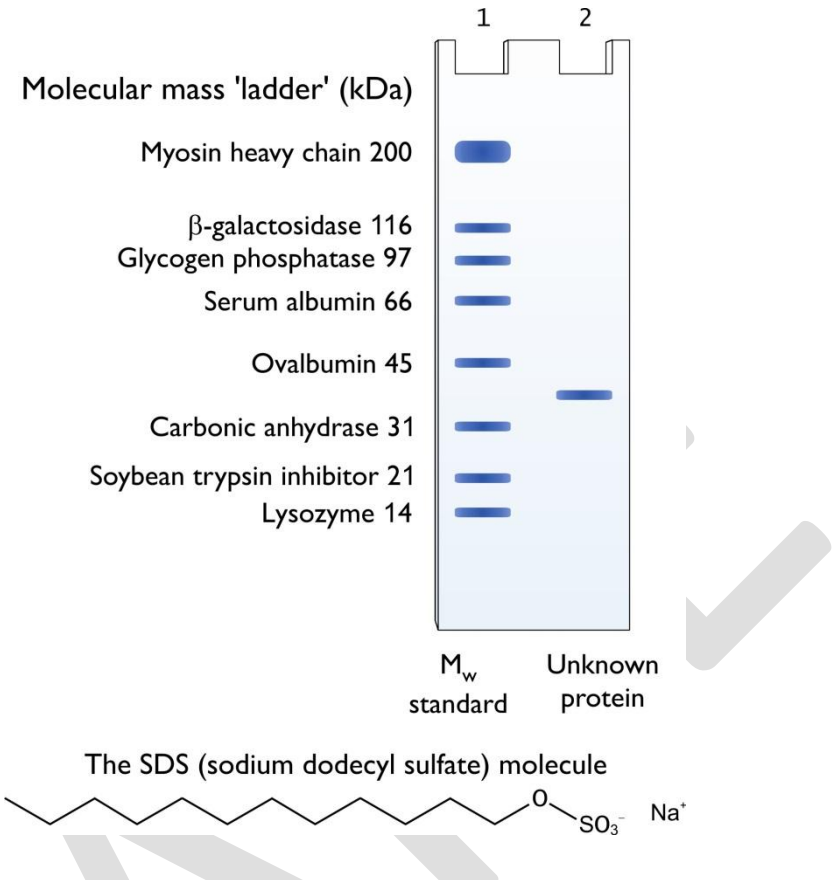
In addition, native PAGE can also be used to detect complex formation between proteins. If two (or more) proteins (or proteins and non-proteinous ligands) form a complex, the complex can be detected as an extra band in the gel. This is because in native-like conditions, many non-covalent (subunit-subunit, receptor-ligand, enzyme-inhibitor) interactions are maintained and the complex migrates apparently as a single molecule.

In the course of native PAGE, it is highly important to pay attention to the relationship of the pI values of the proteins or protein complexes and the pH of the gel buffer, as this will determine where individual proteins will migrate in the gel.

SDS-PAGE

SDS-PAGE is an electrophoresis method to separate proteins. However, unlike in the case of native PAGE, here the proteins migrate in their denatured state. As it was mentioned in the general introduction to traditional (native) PAGE, the migration velocity of proteins is a function of their size, shape and the number of electric charges they carry. As the velocity is a complex function of these properties, native PAGE cannot be used to estimate the molecular mass of proteins. The traditional native PAGE method is similarly unable to assess whether a purified protein is composed of a single subunit or multiple subunits. Even a multi-subunit protein may migrate in a single sharp band.

SDS-PAGE (Figure 7.4) was introduced to analyse such cases and to allow the estimation of the molecular mass of single-subunit proteins or those of individual subunits of multi-subunit proteins. SDS-PAGE is the most prevalent PAGE method currently in use.



SDS polyacrylamide gel electrophoresis. SDS (sodium dodecyl sulphate) is an anionic detergent that unfolds proteins and provides them with extra negative charges. The amount of the associated SDS molecules—and therefore the number of charges—is proportional to the length of the polypeptide chain. The SDS gel separates individual polypeptide chains (monomeric proteins and subunits of multimeric proteins) according to their size. The velocity of the proteins is an inverse linear function of the logarithm of their molecular mass. Proteins of known molecular mass can be used to establish a calibration curve (a descending line) along which the unknown molecular mass of other proteins can be estimated.

SDS (sodium dodecyl sulphate) is an anionic detergent. When proteins are treated with SDS at high temperature, radical conformational changes occur. The treatment breaks all native non-covalent intermolecular (inter-subunit) and intramolecular interactions. The subunit structure of multi-subunit proteins disintegrates and the proteins unfold. If the native structure is stabilised by

disulfide bridges, reducing agents are also added to open up these connections. SDS molecules bind to unfolded proteins in large excess, providing extra negative charges to the molecules.

The amount of the bound SDS molecules is largely independent of the amino acid sequence of the polypeptide chain and it is roughly a linear function of polypeptide length—i.e. the molecular mass of the protein. Therefore, upon SDS-treatment, the specific charge (the charge-to-mass ratio) of different proteins will become roughly identical. Another result of the treatment is that the shape of the different proteins becomes similar. The negatively charged SDS molecules repel each other, which lends a (presumably) rod-like shape to the SDS-treated proteins. These factors together result in a situation analogous to the one already discussed in this chapter for the PAGE separation of linear single-stranded (denatured) DNA molecules. Instead of being separated simultaneously by charge, shape and size, SDS-treated proteins—just like denatured linear DNA molecules—will be separated solely based on their size. As size is a linear function of mass, SDS-PAGE ultimately separates proteins based on their molecular mass.

SDS-PAGE is the most popular cost-effective method to estimate the molecular mass of protein subunits with considerable accuracy. The relative mobility (i.e. the running distance of the protein divided by the running distance of the tracking dye) of the SDS-treated protein is in inverse linear proportion to the logarithm of the molecular mass of the protein. By running several proteins of known molecular mass simultaneously alongside the protein of interest, a log molecular mass – relative mobility calibration curve (a descending linear graph) can be created. Based on the calibration curve, the estimated molecular mass of the protein in question can be easily calculated.

Table below shows the useful separating range of polyacrylamide gels as a function of acrylamide concentration. In the useful range, the log molecular mass – relative mobility relationship is linear.

Acrylamide concentration	Linear range of separation

(%)	(kDa)
15	12-43
10	16-68
7.5	36-94
5.0	57-212

Table Relation between acrylamide concentration and the molecular mass of optimally separated molecules

SDS-PAGE is a standard method for assessing whether the sample of an isolated protein is homogeneous. Besides that, SDS-PAGE is a robust method for the analysis of large supramolecular complexes such as multi-enzyme complexes or the myofibril, as discussed below. SDS-PAGE separates and denatures individual subunits of these complexes. Thus, all polypeptide chains will migrate separately in the gel. Via various staining procedures, all subunits can be visualised and the relative amounts of these proteins (subunits) can also be determined. This allows for the identification of each subunit of a complex and provides a good estimate of the stoichiometry of subunits, too.

Isoelectric focusing

In the course of isoelectric focusing, the conditions are set in a way that proteins will be separated exclusively based on their isoelectric point (Figure 7.5). The two termini and many side chains of proteins contain dissociable groups (weak acids or bases). The dissociation state of these groups is a function of the pH of the environment (as described quantitatively by the Henderson-Hasselbalch equation, see Chapter 3). Isoelectric focusing is based on the pH-dependent dissociation of these groups. Due to this pH-dependent phenomenon, the net electric charge of a protein molecule will be a function of the pH of the medium. If, in a given protein, the number of acidic residues (Asp, Glu) exceeds that of the basic ones (Arg, Lys, His), the protein will have a net negative charge at neutral pH. The isoelectric point (pI) of the protein—

i.e. the pH at which the net charge of the protein is zero—will be in the acidic pH range. Such proteins are often denoted as acidic proteins. If the number of basic residues exceeds that of the acidic ones, the protein will be positively charged at neutral pH, and its pI value will be in the basic pH range. These proteins are often called basic proteins.

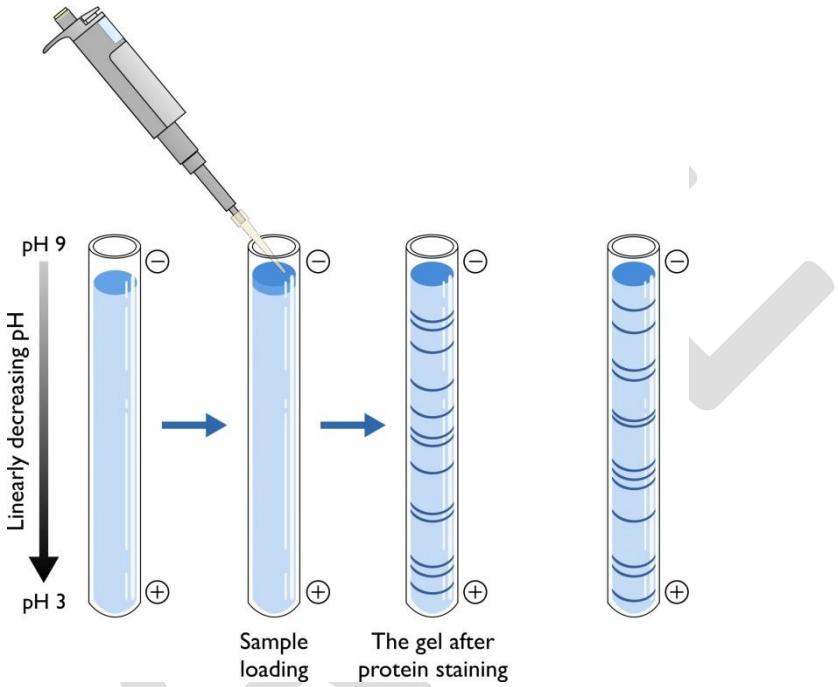


Figure Isoelectric focusing. In the course of isoelectric focusing, a pH gradient is created in the gel (usually made of polyacrylamide, less frequently agarose). Upon electrophoresis, various proteins will accumulate in different narrow regions of the gel where the pH equals their individual pI value. At this pH, the number of positive charges equals that of the negative charges on the protein—the net charge will thus be zero. Consequently, no resultant electric force is exerted on the protein.

Isoelectric focusing is an efficient high-resolution method because the pI values of various proteins are spread across a broad range. If the pH is lower than the pI of the protein, the protein will be positively charged and will move towards the cathode during electrophoresis. If the pH is higher than the pH of the protein, the protein will be negatively charged and will migrate towards

the anode. If the pH equals the pI value, the net charge of the protein will be zero and the protein will not migrate in the gel any further.

In the course of isoelectric focusing, proteins are placed in a gel representing a special medium in which the pH gradually decreases by going from the negative cathode towards the positive anode. As the protein migrates, it encounters a gradually changing pH and its net charge will also change accordingly. If it has a net negative charge and therefore moves towards the cathode, it will encounter a gradually decreasing pH, i.e. a more and more acidic environment. Consequently, the protein will take on more and more protons—up to a level where its net charge will be zero. This state is reached when the protein reaches a location where the pH equals its pI value. At this point, the protein will stop moving because no electric force will be exerted on it. If it spontaneously diffused further towards the anode, it would take on more protons, would become positively charged and would turn back to migrate towards the cathode. Following the same line of thinking, if a positively charged protein moves towards the cathode, it will encounter increasing pH and lose more and more protons. It will migrate to the place where the pH equals its pI value and will thus stop. If it diffused further towards the cathode, it would become negatively charged and would turn back towards the anode. As one can see, by performing electrophoresis in a medium in which the pH decreases from the cathode towards the anode, each protein will "find its place" according to its pI value and will become sharply focused at that location. In addition, it does not matter where exactly the proteins were introduced in the medium between the cathode and the anode.

A decisive component of this method is the usually linear pH gradient created inside the gel. There are two methods to create such a gradient. One of them applies carrier ampholytes (ampholyte is an acronym from the words amphoteric and electrolyte). Ampholytes or zwitterions are molecules that contain both weakly acidic and weakly basic groups. Just like in the case of proteins, the net charge of ampholytes is a function of the pH. In the course of isoelectric focusing, a mixture of various ampholytes is used such that the pI of the various ampholyte components will cover a range in which the pI values of the "neighbouring" ampholytes differ only slightly. This ampholyte mixture is soaked in the gel and an appropriate

electric field is generated by a power supply. This leads to a process analogous to the one already explained for proteins. Each ampholyte will migrate to the location where its net charge becomes zero. As soon as this steady-state is achieved, ampholytes will function as buffers and keep the pH of their immediate environment constant. This establishes the pH gradient in which the proteins can be separated.

The other, more sophisticated method applies special ampholytes that can be covalently polymerised into the polyacrylamide gel. The appropriate ampholyte gradient is created before the gel is polymerised. This way, the gradient will be covalently fixed in the gel, providing an immobilised pH gradient. The appropriate pH range provided by the ampholyte mixture should be selected based on the pI values of the proteins to be separated.

Regardless of how the pH gradient was created, once the proteins reach the location in the gel where the pH equals their pI, they finally stop moving and the system reaches a steady-state.

One of the potential technical difficulties encountered during isoelectric focusing originates from the fact that the solubility of proteins is lowest at their pI value (see Chapter 5). This can lead to the precipitation of some proteins in the gel. To prevent this unwanted process, urea is most often applied in the gel as an additive. Urea denatures proteins and keeps denatured proteins in solution. As the pI value of proteins is largely independent of their conformational state, this modification does not compromise the method. The solubility of membrane proteins can be further promoted by the addition of non-ionic detergents.

Isoelectric focusing is aimed at separating proteins based exclusively on their pI value—thus, independently of their size. Therefore, the molecular sieving property of the gel in this method should be avoided. The only function of the gel is to prevent free convectional flows in the medium. Accordingly, for isoelectric focusing, polyacrylamide gels are made at very low acrylamide concentrations, and sometimes even agarose gels are applied when very large pores are needed. Isoelectric focusing is usually performed in a horizontally-mounted electrophoresis apparatus and by applying intense cooling.

Two-dimensional (2D) electrophoresis

The various separation methods are all aimed at separating complex systems to individual components. Separation is always based on at least one physicochemical property that shows diversity among the components. The general problem encountered in the case of complex mixtures is that not all components differ significantly from all other components when only one property is considered. Accordingly, separation based on a single property rarely results in single-component fractions. Some components will be efficiently separated from all others, while some other components will remain in the mixture.

The remaining mixtures can be further fractionated by another separation technique that relies on a different physicochemical property. The most effective separation can be achieved if the combined consecutive separation steps rely on absolutely independent physicochemical properties. A good example of this is the very high-resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) that combines two already discussed electrophoresis methods, isoelectric focusing and SDS-PAGE.

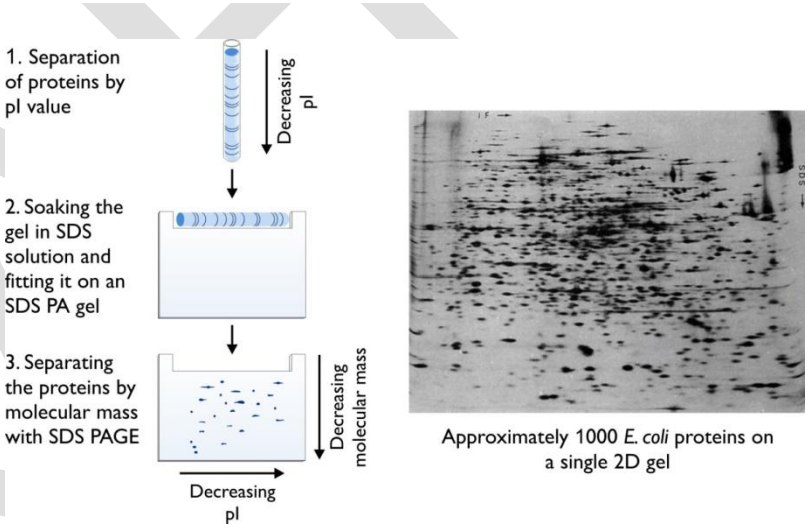


Figure Two-dimensional (2D) electrophoresis. 2D electrophoresis is the combination of isoelectric focusing and SDS-PAGE. Proteins are first separated based on their pI values and then based on their molecular mass. As these properties are completely independent, the combination of the two separation methods provides much higher resolution than either of the two methods alone.

As the first step of 2D gel electrophoresis, isoelectric focusing is performed to separate proteins based on their pI values. Only a single sample is loaded on a gel strip in this step. The

sample is separated in one dimension both in a primary and in a figurative sense. In a primary sense because the components are separated along a single line, and in a figurative sense as the separation is based on a single well-defined property, the pI value.

After the first separation step has been completed in the first dimension, the gel strip is soaked in an SDS solution and is fitted tightly to one side of a "classical" SDS polyacrylamide gel. The second separation step is traditional SDS-PAGE, which separates proteins based on their molecular mass. This second step represents a second dimension in both a primary and a figurative sense. The second separation is performed in a second dimension in a direction rectangular to that of the first separation, and the property utilised in the second step (molecular mass) is completely independent of the one utilised in the first step (pI).

If, after the first step, some gel regions contain different proteins that coincidentally have identical pI values, these proteins will be separated from each other in the second step if their molecular mass is different. Note that every aspect discussed for SDS-PAGE also applies to the second separation step of 2D-PAGE. Van der Waals interactions that might have held protein subunits together in the course of isoelectric focusing will break and individual subunits will become separated. If disulfide bridges need to be opened up, some kind of reducing agent needs to be added. Accordingly, in the second separation step, single polypeptide chains will migrate in the gel. If isoelectric focusing collects a multimeric protein at a certain gel location, the second electrophoresis step will dissect it into individual chains. If the multimer contains subunits of different sizes, these subunits will be separated from each other in the second separation step.

POSSIBLE QUESTIONS

UNIT-IV

PART-A (20 MARKS)

(Q.NO 1 TO 20 Online Examination)

PART-B (2 MARKS)

1. What are the factors affecting the electrophoresis techniques
2. What are the uses of SDS-PAGE?
3. Write about PAGE.
4. Comment on gel documentation.
5. How are DNA and RNA separated?

PART-C (8 MARKS)

1. Explain separation of DNA using agarose gel electrophoresis
2. Explain the principle of gel formation in polyacrylamide gel electrophoresis
3. How do proteins get separated in SDS-PAGE?
4. Explain capillary electrophoresis
5. Explain the separation of molecule based on its isoelectric point.
6. Write the applications of pulse field gel electrophoresis and capillary electrophoresis.

Unit III

Questions	Option A	Option B	Option C	Option D	Answer
Differential centrifugation is based on the differences in _____ of biological particles of different _____	Size,density	Sedimentation rate, sizes and density.	Size,structure	mass,size	Sedimentation rate, sizes and density.
Which of the following statements about the basic principle of sedimentation is False?	The denser a biological structure is, the faster it sediments in a centrifugal field.	The more massive a biological particle is, the slower it moves in a centrifugal field.	The denser the buffer system is, the slower the particle will move in a centrifugal field.	a. The greater the centrifugal force is, the faster the particle sediments	The more massive a biological particle is, the slower it moves in a centrifugal field.
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?	The use of tracers in medicine and industry.	The use of X-rays for medical examinations.	Sterilization of medical equipment.	To measure thickness in paper manufacture.	The use of X-rays for medical examinations.
Solute used in density gradient centrifugation is /are _____.	ammonium sulphate	caesium chloride	arabinose	dextrin	caesium chloride
Operating force of analytical ultra centrifuge is -------	6000000 g	60000g	600000g	6000g	600000g
Microfuge comes under the category of_____ speed centrifuge	medium	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.	alpha emitter	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_____	phosphorescence.	fluorescence.	alpha emitter	beta emitter	phosphorescence.
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 radioactivity to disappear from sample.	The length of time it takes for the radioactivity of a sample to increase by half	The length of time it takes for the radioactivity of a sample to decrease by half.	Half the time it takes for the radioactivity of the sample to decrease by half.	The length of time it takes for the radioactivity of a sample to decrease by half.
What is alpha radioactivity?	A proton	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-life to the rate of decay	a relationship between the half-life of a nuclide and the age of the earth	a process by which a non-radioactive decays into a radioactive nuclide	a series of steps that shows how a radioactive nuclide decays into a non-radioactive nuclide.	a series of steps that shows how a radioactive nuclide decays into a non-radioactive nuclide.
A nuclide with a long half-life_____.	has fewer atoms left after one hour than a nuclide with a small half-life	decays at a faster rate than a nuclide with a small half-life.	has a smaller decay constant than a nuclide with a short half-life.	is a non-radioactive nuclide.	has a smaller decay constant than a nuclide with a short half-life.
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 outside the body?	alpha rays and X-rays are hazardous because the rays are highly penetrating, causing damage inside the body quite easily.	alpha particles are very high in energy and can pass through living tissue without harming it.	Less alpha-particle emission occurs naturally than γ-ray or X-ray emission.	alpha-particles are less hazardous because they are electrons, while γ-rays and X-rays have much more mass.	alpha rays and X-rays are hazardous because the rays are highly penetrating, causing damage inside the body quite easily.
Which of the following emissions is common in carbon-14?	Beta - emission	lambda - ray emission.	Electron capture	alpha-emission	Beta - emission
Self-absorption would least likely effect the efficiency of counting _____.	alpha radiation	beta radiation	X-rays	gamma-rays	gamma-rays
A GM detector is used for counting ___	beta particles	gamma rays	alpha particles	neutron	gamma-rays

The spontaneous disintegration of the nuclei of some of the isotopes of certain elements are called---	Radioactivity	Radiowaves	Radioactive decay	Radiation	Radioactive decay
Geiger Muller counter is related to ----------	Chromatography	Electrophoresis	Radioactivity	Centrifugation	Radioactivity
Ultra centrifuges are run under vacuum to avoid_____.	heat	cold	air circulation	speed	heat
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	Molybdenum-99.	Rhenium-186.	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_____	ethyl formate.	chloroform	benzene	acetic acid	ethyl formate.
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?	Breakdown of an organ into tissues	Breakdown of a tissue to separate cells from intercellular substance	Separation of different cellular components	Breaking of cells and suspending them in 0.25M sucrose solution	Breaking of cells and suspending them in 0.25M sucrose solution
Which one is a structure which is not present in intact cell but obtained during cell fractionation?	Microbodies	Endoplasmic reticulum	Ribosomes	Microsomes	Microsomes
Which of the following represents the various fractions obtained during differential centrifugation?	Plastidal, microsomal and cytosol	Nuclear, mitochondrial and microsomal	Nuclear, plastidal and cytosol	Plastidal, mitochondrial and microsomal	Nuclear, mitochondrial and microsomal

Radioactive isotopes like C^{14}, H^{3}, I^{131}, P^{32}, S^{35} are used in one of the following techniques:	cell fractionation	Microspectrophotometry	Autoradiography	chromatography	Autoradiography
Pure fractions of cellular components can be obtained by	X-ray diffraction	Differential centrifugation	Autoradiography	Microspectrophotometry	Differential centrifugation
Autoradiography will produce:	Metallic silver grains	Intense blue color	Bright fluorescence	Red and blue contrast	Metallic silver grains
The order of sedimentation of subcellular structures during differential centrifugation is:	Lysosome-ribosome-mitochondria-nucleus	Lysosome-mitochondria-nucleus-ribosome	Mitochondria-nucleus-lysosome-ribosome	Nucleus-mitochondria-lysosome-ribosome	Nucleus-mitochondria-lysosome-ribosome
Radioactivity was discovered by	Henri Becquerel	Wilhelm Roentgen	Watson and Crick	Albert Einstein	Henri Becquerel

The International System of Units (SI) unit of radioactive activity is the	becquerel	curie	micro	milli	becquerel
An older unit of radioactivity is the	becquerel	curie	micro	milli	Curie
_____ refers to the particles which are emitted from nuclei as a result of nuclear instability.	Radioactivity	Radiowaves	Radioactive decay	Radiation	Radioactivity
_____ occurs when an atomic nucleus breaks down into smaller particles	Radioactivity	Radiowaves	Radioactive decay	Radiation	Radioactivity

One _____ __ is defined as the activity of a quantity of radioactive material in which one nucleus decays per second	becquerel	Curie	micro	milli	becquerel
The _____ is a non-SI unit of radioactivity	becquerel	Curie	micro	milli	Curie
An _____ ___ is an image on an x-ray film or nuclear emulsion produced by the pattern of decay emissions from a distribution of a radioactive substance.	cell fractionation	Microspectrophotometry	Autoradiography	chromatography	Autoradiography

_____ is any technique used to produce an image of the 2D distribution of a radioactive substance	cell fractionation	Microspectrophotometry	Autoradiography	chromatography	Autoradiography
Which one of the following can be used to confirm whether drinking water contains a gamma emitting isotope or not?	Spectrophotometer	Microscope	Scintillation counter	lead plate	Scintillation counter
Which of the following techniques would be used to detect a metabolite labelled with ^{3}H?	Infra red spectroscopy	Nuclear magnetic resonance spectroscopy	Scintillation counting	Mass spectrometry	Scintillation counting

Which of the following techniques would be used to detect a metabolite labelled with ^{14}C?	Infra red spectroscopy	Nuclear magnetic resonance spectroscopy	Scintillation counting	Mass spectrometry	Scintillation counting
_____ is a common procedure used to separate certain organelles from whole cells for further analysis of specific parts of cells	chromatography	Differential centrifugation	X-ray diffraction	Auto-radiography	Differential centrifugation

UNIT-IV

SYLLABUS

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**.**

Radio isotopes

An atom consists of an extremely small; positively charged nucleus surrounded by a cloud of negatively charged electrons. Nuclei consist of positively charged protons and electrically neutral neutrons held together by nuclear force.

Units of radioactivedecay

The International System of Units (SI) unit of radioactive activity is the becquerel(Bq), named in honour of the scientist Henri Becquerel. One Bq is defined as one transformation (or decay or disintegration) persecond.

An older unit of radioactivity is the curie, Ci, which was originally defined as "the quantity or mass of radium emanation in equilibrium with one gram of radium (element)". Today, the curie is defined as 3.7×10^{10} disintegrations per second, so that 1 curie (Ci) = 3.7×10^{10}Bq. For radiological protection purposes, although the United States Nuclear Regulatory Commission permits the use of the unit curiealongside SI units, the European Union European units ofmeasurement directives required that its use for "public health ... purposes" be phased out by 31 December1985.

Radioactive decay (also known as **nuclear decay** or **radioactivity**) is the process by which the nucleus of an unstable atom loses energy by emitting radiation, including alpha particles, betaparticles, gamma rays, and conversion electrons. A material that spontaneously emits such radiation is considered**radioactive**.

Radioactive decay is a stochastic (i.e. random) process at the level of single atoms, in that, according to quantum theory, it is impossible to predict when a particular atom will decay, regardless of how long the atom has existed. For a collection of atoms however, the

collection's decay rate can be calculated from their measured decay constants or half-lives. This is the basis of radiometric dating. The half-lives of radioactive atoms have no known lower or upper limit, spanning a time range of over 55 orders of magnitude, from nearly instantaneous to far longer than the age of the universe. A radioactive source emits its decay products isotropically(all directions and without bias) in the absence of externalinfluence.

Types of radioactive decay

Early researchers found that an electric or magnetic field could split radioactive emissions into three types of beams. The rays were given the names alpha, beta, and gamma, in order of their ability to penetrate matter. While alpha decay was observed only in heavier elements of atomic number 52 (tellurium) and greater, the other two types of decay were produced by all of the elements. Lead, atomic number 82, is the heaviest element to have any isotopes stable (to the

limit of measurement) to radioactive decay. Radioactive decay is seen in all isotopes of all elements of atomic number 83 (bismuth) or greater. Bismuth, however, is only very slightly radioactive, with a half-life greater than the age of the universe; radioisotopes with extremely long half-lives are considered effectively stable for practical purposes.

In analysing the nature of the decay products, it was obvious from the direction of the electromagnetic forces applied to the radiations by external magnetic and electric fields that alpha particles carried a positive charge, beta particles carried a negative charge, and gammarays were neutral. From the magnitude of deflection, it was clear that alpha particles were much more massive than beta particles. Passing alpha particles through a very thin glass window and trapping them in a discharge tube allowed researchers to study the emission spectrum of the captured particles, and ultimately proved that alpha particles are helium nuclei. Other experiments showed beta radiation, resulting from decay and cathode rays, were high- speed electrons. Likewise, gamma radiation and X-rays were found to be high-energy electromagneticradiation.

The relationship between the types of decays also began to be examined: For example, gamma decay was almost always found to be associated with other types of decay, and occurred at about the same time, or afterwards. Gamma decay as a separate phenomenon, with its own half-life (now termed isomeric transition), was found in natural radioactivity to be a result of the gamma decay of excited metastable nuclear isomers, which were in turn created from other types of decay.

Although alpha, beta, and gamma radiations were most commonly found, other types of emission were eventually discovered. Shortly after the discovery of the positron in cosmic ray products, it was realized that the same process that operates in classical beta decay can also

produce positrons (positron emission), along with neutrinos (classical beta decay produces antineutrinos). In a more common analogous process, called electron capture, some proton-rich nuclides were found to capture their own atomic electrons instead of emitting positrons, and subsequently these nuclides emit only a neutrino and a gamma ray from the excited nucleus (and often also Augerelectrons and characteristic X-rays, as a result of the re-ordering of electrons to fill the place of the missing captured electron). These types of decay involve the nuclear capture of electrons or emission of electrons or positrons, and thus act to move a nucleus toward the ratio of neutrons to protons that have the least energy for a given total number of nucleons. This consequently produces a more stable (lower energy)nucleus.

(A theoretical process of positron capture, analogous to electron capture, is possible in antimatter atoms, but has not been observed, as complex antimatter atoms beyond antiheliumare not experimentally available. Such decay would require antimatter atoms at least as complex as beryllium-7, which is the lightest known isotope of normal matter to undergo decay by electroncapture.)

Shortly after the discovery of the neutron in 1932, Enrico Fermi realized that certain rare beta-decay reactions immediately yield neutrons as a decay particle (neutron emission). Isolated proton emission was eventually observed in some elements. It was also found that some heavy elements may undergo spontaneous fission into products that vary in composition. In a phenomenon called cluster decay, specific combinations of neutrons and protons other than alpha particles (helium nuclei) were found to be spontaneously emitted fromatoms.

Other types of radioactive decay were found to emit previously-seen particles, but via different mechanisms. An example is internal conversion, which results in an initial electron emission, and then often further characteristic X-rays and Auger electrons emissions, although the internal conversion process involves neither beta nor gamma decay. A neutrino is not emitted, and none of the electron(s) and photon(s) emitted originate in the nucleus, even though the energy to emit all of them does originate there. Internal conversion decay, like isomeric transition gamma decay and neutron emission, involves the release of energy by an excited nuclide, without the transmutation of one element into another.

Rare events that involve a combination of two beta-decay type events happening simultaneously are known (see below). Any decay process that does not violate the conservation of energy or momentum laws (and perhaps other particle conservation laws) is permitted to happen, although not all have been detected. An interesting example discussed in a final section, is bound statebeta decay of rhenium-187. In this process, beta electron-decay of the parent nuclide is not accompanied by beta electron emission, because the beta particle has been captured into the K- shell of the emitting atom. An antineutrino is emitted, as in all negative betadecays.

Radionuclides can undergo a number of different reactions. These are summarized in the following table. A nucleus with mass number *A* and atomic number *Z* is represented as (*A*, *Z*). The column "Daughter nucleus" indicates the difference between the new nucleus and the original nucleus. Thus, (*A* − 1, *Z*) means that the mass number is one less than before, but the atomic number is the same as before.

If energy circumstances are favorable, a given radionuclide may undergo many competing types of decay, with some atoms decaying by one route, and others decaying by another. An example is copper-64, which has 29 protons, and 35 neutrons, which decays with a half-life of about 12.7 hours. This isotope has one unpaired proton and one unpaired neutron, so either the proton or the neutron can decay to the opposite particle. This particular nuclide (though not all nuclides in this situation) is almost equally likely to decay through positron emission (18%), or through electroncapture (43%), as it does through electron emission (39%). The excited energy states resulting from these decays which fail to end in a ground energy state, also produce later internalconversion and gamma decay in almost 0.5% of thetime.

More common in heavy nuclides is competition between alpha and beta decay. The daughter nuclides will then normally decay through beta or alpha, respectively, to end up in the same place.

The **Geiger counter** is an instrument used for measuring ionizing radiation used widely in such applications as radiation dosimetry,radiological protection, experimental physics and the nuclearindustry.

It detects ionizing radiation such as alpha particles, beta particles and gamma rays using the ionization effect produced in a Geiger–Müller tube; which gives its name to the instrument. In wide and prominent use as a hand-held radiation survey instrument, it is perhaps one of the world's best-known radiation detection instruments.

The original detection principle was discovered in 1908 at the Cavendish laboratory, but it was not until the development of the Geiger-Müller tube in 1928 that the Geiger-Müller counter became a practical instrument. Since then it has been very popular due to its robust sensing element and relatively low cost. However, there are limitations in measuring high radiation rates and the energy of incident radiation.

A Geiger counter consists of a Geiger-Müller tube, the sensing element which detects the radiation, and the processing electronics, which displays the result.

The Geiger-Müller tube is filled with an inert gas such as helium, neon, or argon at low pressure, to which a high voltage is applied. The tube briefly conducts electrical charge when a particle or photon of incident radiation makes the gas conductive by ionization. The ionization is considerably amplified within the tube by the Townsend discharge effect to

produce an easily measured detection pulse, which is fed to the processing and display electronics. This large pulse from the tube makes the G-M counter relatively cheap to manufacture, as the subsequent electronics is greatly simplified. The electronics also generates the high voltage, typically 400– 600 volts that has to be applied to the Geiger-Müller tube to enable itsoperation.

Readout

There are two types of radiation readout; counts or radiation dose. The counts display is the simplest and is the number of ionizing events displayed either as a count rate, commonly "counts per second", or as a total over a set time period (an integrated total). The counts readout is normally used when alpha or beta particles are being detected. More complex to achieve is a display of radiation dose rate, displayed in a unit such as the sievertwhich is normally used for measuring gamma or X-ray dose rates. A G-M tube can detect the presence of radiation, but not its energy which influences the radiation's ionising effect. Consequently, instruments measuring dose rate require the use of an energy compensated G-M tube, so that the dose displayed relates to the counts detected. The electronics will apply known factors to make this conversion, which is specific to each instrument and is determined by design andcalibration.

The readout can be analog or digital, and increasingly, modern instruments are offering serial communications with a host computer or network.

There is usually an option to produce audible clicks representing the number of ionization events detected. This is the distinctive sound normally associated with hand held or portable Geiger counters. The purpose of this is to allow the user to concentrate on manipulation of the instrument whilst retaining auditory feedback on the radiationrate.

Limitations

There are two main limitations of the Geiger counter. Because the output pulse from a Geiger-Müller tube is always the same magnitude regardless of the energy of the incident radiation, the tube cannot differentiate between radiation types. A further limitation is the inability to measure high radiation rates due to the "dead time" of the tube. This is an insensitive period after each ionization of the gas during which any further incident radiation will not result in a count, and the indicated rate is therefore lower than actual. Typically the dead time will reduce indicated count rates above about 10^4 to 10^5 counts per second depending on the characteristic of the tube being used.[2]Whilst some counters have circuitry which can compensate for this, for accurate measurements ion chamber instruments are preferred for high radiationrates.

The application and use of a Geiger counter is dictated entirely by the design of the tube, of which there are a great many, but they can be generally categorised as "end-window", or windowless "thin-walled" or "thick-walled", and sometimes hybrids of these types.

Types and application of Geiger Muller counter Particle detection

The first historical uses of the Geiger principle were for the detection of alpha and beta particles, and the instrument is still used for this purpose today. For alpha particles and low energy beta particles the "end-window" type of G-M tube has to be used as these particles have a limited range even in free air, and are easily stopped by a solid material. Therefore, the tube requires a window which is thin enough to allow as many as possible of these particles through to the fill gas. The window is usually made of mica with a density of about 1.5 - 2.0 mg/cm^2.

Alpha particles have the shortest range, and to detect these windows should ideally be within 10mm of the radiation source due to alpha particle attenuation in free air. However, the G-M tube produces a pulse output which is the same magnitude for all detected radiation, so a Geiger counter with an end window tube cannot distinguish between alpha and beta particles.[2]A skilled operator can use distance to differentiate alpha and high energy beta, but with the detector in close contact with the radiation source the types are indistinguishable. The "pancake" Geiger- Muller detector is a variant of the end window probe, but designed with a larger detection area to make checking quicker. However the pressure of the atmosphere against the low pressure of the fill gas limits the window size due to the limited strength of the windowmembrane.

High energy beta particles can also be detected by a thin-walled "windowless" G-M tube, which has no end window. Although the tube walls have a greater stopping power than a thin end window, they still allow these more energetic particles to reach the fill gas.

End-window G-M detectors are still used as a general purpose portable radioactivecontamination measurement and detection instrument, owing to their relatively low cost, robustness and their relatively high detection efficiency; particularly with high energy beta particles. However, for discrimination between alpha and beta particles or provision of particle energy information, scintillation counters or proportional counters should be used. Those instrument types are manufactured with much larger detector a area, which means that checking for surface contamination is quicker than with a G-Minstrument.

Gamma and X-raydetection

Geiger counters are widely used to detect gamma radiation, and for this the windowless tube is

used. However, efficiency is generally low due to the poor interaction of gamma rays compared with alpha and beta particles. For instance, a chrome steel G-M tube is only about 1% efficient over a wide range ofenergies.

The article on the Geiger-Muller tube carries a more detailed account of the techniques used to detect photon radiation. For high energy gamma it largely relies on interaction of the photon radiation with the tube wall material, usually 1–2 mm of chrome steel on a "thick-walled" tube, to produce electrons within the wall which can enter and ionize the fill gas. This is necessary as the low pressure gas in the tube has little interaction with high energy gamma photons. However, for low energy photons there is greater gas interaction and the direct gas ionisation effect increases. With decreasing energy the wall effect gives way to a combination of wall effect and direct ionisation, until direct gas ionisation dominates. Due to the variance in response to different photon energies, windowless tubes employ what is known as "energy compensation" which attempts to compensate for these variations over a large energyrange.

Low energy photon radiation such as low energy X rays or gamma rays interacts better with the fill gas. Consequently, a typical design for low energy photon detection for these is a long tube with a thin wall or with an end window. The tube has a larger gas volume than a steel walled tube to give an increased chance of particleinteraction.

Neutron detection

A variation of the Geiger tube is used to measure neutrons, where the gas used is borontrifluorideor helium-3 and a plastic moderator is used to slow the neutrons. This creates an alpha particle inside the detector and thus neutrons can becounted.

Gamma measurement—personnel protection and process control

The term "Geiger counter" is commonly used to mean a hand-held survey type meter; however the Geiger principle is in wide use in installed "area gamma" alarms for personnel protection, and in process measurement and interlock applications. A Geiger tube is still the sensing device, but the processing electronics will have a higher degree of sophistication and reliability than that used in a hand held surveymeter.

Physical design

For hand-held units there are two fundamental physical configurations: the "integral" unit with both detector and electronics in the same unit, and the "two-piece" design which has a separate

detector probe and an electronics module connected by a short cable.

In the 1930's a mica window was added to the cylindrical design allowing low-penetration radiation to pass through with ease.

The integral unit allows single-handed operation, so the operator can use the other hand for personal security in challenging monitoring positions, but the two piece design allows easier manipulation of the detector, and is commonly used for alpha and beta surface contamination monitoring where careful manipulation of the probe is required or the weight of the electronics module would make operation unwieldy. A number of different sized detectors are available to suit particular situations, such as placing the probe in small apertures or confined spaces.

Gamma and X-Ray detectors generally use an "integral" design so the Geiger–Müller tube is conveniently within the electronics enclosure. This can easily be achieved because the casing usually has little attentuation, and is employed in ambient gamma measurements where distance from the source of radiation is not a significant factor. However, to facilitate more localised measurements such as "surface dose", the position of the tube in the enclosure is sometimes indicated by targets on the enclosure so an accurate measurement can be made with the tube at the correct orientation and a known distance from the surface.

There is a particular type of gamma instrument known as a "hot spot" detector which has the detector tube on the end of a long pole or flexible conduit. These are used to measure high radiation gamma locations whilst protecting the operator by means of distance shielding.

Particle detection of alpha and beta can used in both integral and two-piece designs. A pancake probe (for alpha/beta) is generally used to increase the area of detection in two-piece instruments whilst being relatively light weight. In integral instruments using an end window tube there is a window in the body of the casing to prevent shielding of particles. There are also hybrid instruments which have a separate probe for particle detection and a gamma detection tube within the electronics module. The detectors are switchable by the operator, depending the radiation type that is beingmeasured.

Guidance on application use

In the United Kingdom the HSE has issued a user guidance note on selecting the best portable instrument type for the radiation measurement application concerned. This covers all radiation protection instrument technologies and is a useful comparative guide to the use of G-M detectors.TheguidedoesnotrecommendtheG-Mdetectorformixedalphaandbeta

contamination detection, and they are only recommended as "satisfactory" for beta-only contamination. However, for gamma and low-voltage X-rays they are recommended as the best

instrument type.

In 1908 Hans Geiger, under the supervision of Ernest Rutherford at the Victoria University ofManchester (now the University of Manchester), developed an experimental technique for detecting alpha particles that would later be used in the Geiger-Müller tube. This early counter was only capable of detecting alpha particles and was part of a larger experimental apparatus. The fundamental ionization mechanism used was discovered by John Sealy Townsend by his work between 1897 and 1901, and is known as the Townsend discharge, which is the ionization of molecules by ionimpact.

It was not until 1928 that Geiger and Walther Müller (a PhD student of Geiger) developed the sealed Geiger-Müller tube which developed the basic ionization principles previously used experimentally. This was relatively small and rugged, and could not only detect alpha and beta radiation such as prior models but also gamma radiation. Now a practical radiation instrument could be produced relatively cheaply, and so the Geiger-Muller counter was born. As the tube output required little electronic processing, a distinct advantage in the thermionic valve era due to minimal valve count and low power consumption, the instrument achieved great popularity as a portable radiationdetector.

Modern versions of the Geiger counter use the halogen tube invented in 1947 by Sidney H.Liebson. It superseded the earlier Geiger tube because of its much longer life and lower operating voltage, typically 400-600volts.

Scintillation counter

A scintillation counter is an instrument for detecting and measuring ionizing radiation by using the excitation effect of incident radiation on a scintillator material, and detecting the resultant light pulses.

Scintillation counters are used to measure radiation in a variety of applications including hand held radiation survey meters, personnel and environmental monitoring for radioactivecontamination, medical imaging, radiometric assay, nuclear security and nuclear plant safety.

Several products have been introduced in the market utilizing scintillation counters for detection of potentially dangerous gamma-emitting materials during transport. These include scintillation counters designed for freight terminals, border security, and ports, weigh bridge applications, scrap metal yards and contamination monitoring of nuclear waste. There are variants of scintillation counters mounted on pick-up trucks and helicopters for rapid response in case of a security situation due to dirty bombs or radioactive waste. Hand-held units are also

commonly used.

Solid scintillation counter

Scintillation counter consists of a scintillation chamber connected by a light tube to a photomultiplier tube. In solid scintillation counter the sample is placed close to the flour crystal which in turn is placed adjacent to a photomultiplier. The photomultiplier is connected to a high voltage supply and a scaler. Radiations entering the chamber activate the scintillator to emit visible light photons, which pass through the light tube to fall on the photoelectric cathode surface of the photomultiplier. The photomultiplier converts the optical signal to an electrical one and provides a large degree of amplification. This consists of an evacuated coated at one end with a photocathode made of alkali metals, which is maintained at a large negative potential. Photons liberate electrons from the cathode which are accelerated towards a dynode and here knock out a number of secondary electrons. This process continues down the dynode chain until eventually a large signal is collected. The vast number of electrons finally impinges on a collector plate to generate a current pulse, which can be visually observed and electronically recorded. Solid scintillation counting is useful for measurement of gamma emittingisotopes.

Liquid scintillation counter

Liquid scintillation counting is the measurement of activity of a sample of radioactive material which uses the technique of mixing the active material with a liquid scintillator, and counting the resultant photon emissions. The purpose is to allow more efficient counting due to the intimate contact of the activity with the scintillator. It is generally used for alpha and beta particle detection.

Samples are dissolved or suspended in a "cocktail" containing a solvent (historically aromatic organics such as benzene or toluene, but more recently less hazardous solvents are used), typically some form of a surfactant, and small amounts of other additives known as "fluors" or scintillators. Scintillators can be divided into primary and secondary phosphors, differing in their luminescenceproperties.

Beta particles emitted from the isotopic sample transfer energy to the solvent molecules: the πcloud of the aromatic ring absorbs the energy of the emitted particle. The energized solvent molecules typically transfer the captured energy back and forth with other solvent molecules until the energy is finally transferred to a primary scintillator. The primary phosphor will emit photons following absorption of the transferred energy. Because that light emission may

be at a wavelength that does not allow efficient detection, many cocktails contain secondary phosphors that absorb the fluorescence energy of the primary phosphor and re-emit at a longer wavelength.

The radioactive samples and cocktail are placed in small transparent or translucent (often glass or plastic) vials that are loaded into an instrument known as a liquid scintillation counter. Newer machines may use 96-well plates with individual filtersineachwell.Manycountershavetwophotomultipliertubesconnectedinacoincidence circuit. The coincidence circuit assures that genuine light pulses, which reach both photo multiplier tubes, are counted, while spurious pulses (due to line noise, for example), which would only affect one of the tubes, areignored.

Counting efficiencies under ideal conditions range from about 30% for tritium (a low-energy beta emitter) to nearly 100% for phosphorus-32, a high-energy beta emitter. Some chemical compounds (notably chlorine compounds) and highly colored samples can interfere with the counting process. This interference, known as "quenching", can be overcome through data correction or through careful samplepreparation.

High-energy beta emitters, such as phosphorus-32, can also be counted in a scintillation counter without the cocktail, instead using an aqueous solution. This technique, known as **Cherenkov counting**, relies on the Cherenkov radiation being detected directly by the photomultiplier tubes. Cherenkov counting in this experimental context is normally used for quick, rough measurements, since the geometry of the sample can create variations in the output.

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 or gamma 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 phosphor imaging 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 historadiographyor micro radiography, in which the sample is X-rayed using an external source. Some autoradiograph 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 theplant.

Applications

In biology, this technique may be used to determine the tissue (or cell) localization of a radioactive substance, either introduced into a metabolic pathway, bound to a receptor or enzyme, or hybridized to a nucleicacid.

The use of radiolabeled ligands to determine the tissue distributions of receptors is termed either *in vivo* or *in vitro* **receptor autoradiography** if the ligand is administered into the circulation (with subsequent tissue removal and sectioning) or applied to the tissue sections, respectively. The ligands are generally labeled with ^{3}H (tritium) or ^{125}I(radioiodine). The distribution of RNA transcripts in tissue sections by the use of radiolabeled, complementary oligonucleotidesorribonucleicacids("riboprobes")iscalledinsituhybridizationhistochemistry.
Radioactive precursors of DNA and RNA, [^{3}H]-thymidine and [^{3}H]- uridinerespectively, may be introduced to living cells to determine the timing of several phases of the cell cycle. RNA or DNA viral sequences can also be located in this fashion. These probes are usually labeled with ^{32}P, ^{33}P, or ^{35}S. In the realm of behavioral endocrinology, autoradiography can be used to determine hormonal uptake and indicate receptor location; an animal can be injected with a radio labeled hormone, or the study can be conducted *invitro*.

Biosafety methods in radioactive laboratory

All ionizing radiations can have effect on the human body. When radiations damages living cells, it can destroy or mutate the cells, possibly causing a cancerous growth. There is a great difference in the penetrating powers of alpha particles, beta particles and gamma particles. Of the three types of radiation, alpha particles are easiest to stop. A sheet of paper is all that is needed for their absorption. However, it may require a material with a greater thickness and density to stop beta particles. Gamma rays have the most penetrating power of the three radiation types. Hence the most important safety aspect when dealing with radioactive sources is that of shielding often simply keeping a reasonable distance from the source will be sufficient as the air act as a shield. When working with more intense sources, some form of shielding may be required. Sealed sources should be handled with tongs or a special source holder never with the fingers. One should not probe inside sealed sources or allow them to come into contact with any substance, which might attack or dissolve the source or its container. When not in use sealed sources should always be returned to their lead lined storage boxes. Radioactive

substances should be handled with the same care and respect as concentrated acids. Washing of hands thoroughly after radioactive source is veryessential.

Flow cytometer

Modern flow cytometers are able to analyze several thousand particles every second, in "real time," and can actively separate and isolate particles having specified properties. A flow cytometer is similar to a microscope, except that, instead of producing an image of the cell, flow cytometry offers "high-throughput" (for a large number of cells) automated quantification of set parameters. To analyze solid tissues, a single-cell suspension must first be prepared.

A flow cytometer has five main components:

- a flow cell - liquid stream (sheath fluid), which carries and aligns the cells so that they pass single file through the light beam forsensing
- a measuring system - commonly used are measurement of impedance (or conductivity) and optical systems - lamps (mercury,xenon); high-power water-cooled lasers (argon, krypton, dyelaser);low-powerair-cooledlasers(argon(488nm),red-HeNe(633nm),green-HeNe, HeCd (UV)); diode lasers (blue, green, red, violet) resulting in light signals
- a detector and Analogue-to-Digital Conversion (ADC) system - which converts analogue measurements of forward-scattered light (FSC) and side-scattered light (SSC) as well as dye- specific fluorescence signals into digital signals that can be processed by a binarycomputer
- an amplification system - linearorlogarithmic
- a computer for analysis of thesignals.

The process of collecting data from samples using the flow cytometer is termed 'acquisition'. Acquisition is mediated by a computer physically connected to the flow cytometer, and the software which handles the digital interface with the cytometer. The software is capable of adjusting parameters (e.g., voltage, compensation) for the sample being tested, and also assists in displaying initial sample information while acquiring sample data to ensure that parameters are set correctly. Early flow cytometers were, in general, experimental devices, but technological advances have enabled widespread applications for use in a variety of both clinical and research purposes. Due to these developments, a considerable market for instrumentation, analysis software, as well as the reagents used in acquisition such as fluorescently labeled antibodies has developed.

Modern instruments usually have multiple lasers and fluorescence detectors. The current record for a commercial instrument is ten lasers and 18 fluorescence detectors. Increasing the

number of lasers and detectors allows for multiple antibody labeling, and can more precisely identify a target population by their phenotypic markers. Certain instruments can even take digital images of individual cells, allowing for the analysis of fluorescent signal location within or on the surface ofcells.

Applications

The technology has applications in a number of fields, including molecularbiology,pathology, immunology,plant biology and marine biology. It has broad application inmedicine(especially in transplantation, hematology, tumor immunology and chemotherapy, prenatal diagnosis, genetics and sperm sorting for sex preselection). Also, it is extensively used in research for the detection of DNA damage, caspase cleavage and apoptosis. In marine biology, the autofluorescent properties of photosynthetic planktoncan be exploited by flow cytometry in order to characterise abundance and community structure. In protein engineering, flow cytometry is used in conjunction with yeast displayandbacterial display to identify cell surface-displayed protein variants with desiredproperties.

POSSIBLE QUESTIONS

UNIT-V

PART-A (20 MARKS)

(Q.NO 1 TO 20 Online Examination)

PART-B (2 MARKS)

1. Explain the types of radioactivity decay.
2. What is liquid scintillation counter?
3. Write a detail note on safe handling of radioisotopes.
4. How will you measure radioactivity?
5. How will you detect radioactivity?

PART-C (8 MARKS)

1. Give a note on solid scintillation counter.
2. Discuss radioisotopes with their applications.
3. Discuss in detail the biosafety methods in radioactive laboratory.
4. Write the principle and instrumentation of Geiger-Muller counter.
5. List out the application of Geiger-Muller counter, solid and liquid scintillation counter.

6.Explain in detail on flow cytometry.

Unit IV

Questions	Option A	Option B	Option C	Option D	Answer
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, Blue	Red, Green, Blue	Red, Green, Blue
A colorimeter is used to _____.	determine the heat of a reaction	determine the heat given off/absorbed during some process	store the heat from a chemical reaction	determine the heat of the product	determine the heat given off/absorbed during some process

The amount of light is absorbed is directly proportional to the length of the medium through which the light passes is --------	Beer's law	Lambert's law	Beer and Lambert's Law	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 Law	Planck's law	Beer's law
The correct order for the basic features of a mass spectrometer is_____	acceleration, deflection, detection, ionisation	ionisation, acceleration, deflection, detection	acceleration, ionisation, deflection, detection	acceleration, deflection, ionisation, detection	ionisation, acceleration, deflection, detection

Which one of the following statements about ionisation in a mass spectrometer is incorrect ?	gaseous atoms are ionised by bombarding them with high energy electrons	atoms are ionised so they can be accelerated	atoms are ionised so they can be deflected	it doesn't matter how much energy you use to ionise the atoms	it doesn't matter how much energy you use to ionise the atoms
Beer's Law states that	absorbance is proportional to both the path length and concentration of the absorbing species	absorbance is proportional to the log of the concentration of the absorbing species	absorbance is equal to P0/P	absorbance is equal to P/P0	absorbance is proportional to both the path length and concentration of the absorbing species
A UV-VIS spctrophotometer has ------ light sources.	Two	Three	One	Four	Two
In colorimetry, Beer-Lamberts law is used to evaluate_____.	quantitative measurements	qualitative measurements	absorbance measurements	adsorption spectrum	quantitative measurements

According to the Beer-Lambert law, on which of the following does absorbance NOT depend?	Colour of the solution	Solution concentration	Distance that the light has travelled through the sample	Extinction coefficient of the sample	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 and dilute.	concentrated
Colorimetry is a form of _____.	Photometry	Electrophoresis	Chromatography	Spectrofluorimetry	Photometry
_____ _____ is a spectroanalytical procedure for the quantitative determination of chemical elements	Atomic absorption spectroscopy	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 complexing agents	Addition of KCl to the matrix	Addition of oxyanions such as sulfate or phosphate	Internal standards methods	Addition of KCl to the matrix
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 possible	the highest wavelength possible	the wavelength at which the chemical's absorption value is the lowest	the wavelength at which the chemical's absorption value is the highest	the wavelength at which the chemical's absorption value is the lowest
Using a standard curve, if you know the absorbance of an unknown sample, what else can be determined about the unknown?	the wavelength of maximum absorbance	the molecular weight of the sample	the concentration of the samples	the identify of the sample	the concentration of the samples
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 into electrical	It is a graph of a chemical relating the absorbance to wavelength	It is a graph of a chemical relating the absorbance to concentration	It is the amount of radiation retained by a sample	It is a graph of a chemical relating the absorbance to wavelength
Basically, what is the path of light through a spectrophotometer ?	meter, photodetector, filter, sample, light source	meter, filter sample, photodetector, light source	light source, filter, sample, photodetector, meter	light source, sample, filter, photodetector, meter	light source, filter, sample, photodetector, meter
The ratio of transmitted light (l) 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 Law	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 ^{2}H?	Infra red spectroscopy	Nuclear magnetic resonance spectroscopy	Scintillation counting	Mass spectrometry	Mass spectrometry
_____ is used to determine the structural formula of an unknown chemical compound	Infra red spectroscopy	Nuclear magnetic resonance spectroscopy	Scintillation counting	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 spectrum	Ultraviolet radiation
_____ has a longer wavelength than visible red light	Ultraviolet radiation	Infrared radiation	visible spectrum	electromagnetic spectrum	Infrared radiation
Sunlight consists of the entire	Ultraviolet radiation	Infrared radiation	visible spectrum	electromagnetic spectrum	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, yellow, orange, and red	blue, violet, green, yellow, orange, and red	green, violet, blue, orange, yellow, and red	red, blue, green, yellow, orange, and violet	violet, blue, green, yellow, orange, and red
Light with such short wavelengths is called	Ultraviolet light	Infrared light	visible spectrum	electromagnetic spectrum	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

UNIT-V

SYLLABUS

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.

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 certainbounds.

Basic Elements in a Quality System

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

- ***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 anydeficiencies.

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

  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 qualityrequirements.

- ***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 manufacturingoperation.

personal protective equipment

Always wear proper eye protection in the lab.

– safety glasses or goggles

- Do not wear contact lenses in thelab.
- Wear proper gloves.
- Wear aprons or labcoats.
- Wear closed-toeshoes.
- Wear hearing protection if the noise level isgreater than 85dBA.
- Wash arms and hands immediately afterworking with allergens, carcinogens,pathogenic organisms, or toxicchemicals.

General safety-biological safety, chemical safety and fire safety

- Avoid workingalone.
- Clean upspills.
- Do not store or consume food or beverages in thelab.
- Use proper guards – rotating parts– sharp edges– hot surfaces– machine belts,pto's
- Do not use defectiveequipment.

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 therelatedactivities.

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 datamanagement principles to the current quality management system to integrate those elementsthatassure 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 theirwork;

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

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

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 asnecessary.

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 andsystems 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 andreviewed 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 continualimprovement.

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 todata

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, andreported."*

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

-improvesafety

- 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 havedone

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 performedtherein.

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 iteminformationshouldexist:Adescriptivetitle;astatementwhichrevealsthenatureand 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 begiven.

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. TheGLP

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 thedata.

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 theOECD 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.

POSSIBLE QUESTIONS

UNIT-I

PART-A (20 MARKS)

(Q.NO 1 TO 20 Online Examination)

PART-B (2 MARKS)

1. Write about the Basic Terminology of Sedimentation rate?
2. Define Absorbance with example.
3. What is the difference between settling and sedimentation?
4. What are the two types of rotors found in high-powered centrifuges?
5. What is the meaning of the word "derated"?
6. What is the beer lambert law? What are the correlations derived from it.]
7. What is chemiluminescence? What are its uses in diagnostic?
8. What is fluorescence? How is it used in diagnostics?

PART-C (6 MARKS)

1. Define Svedberg equation. Explain in detail about the principles behind centrifugation.
2. Explain in detail on Density gradient centrifugation.
3. Explain in detail on isopycnic centrifugation.
4. Explain in detail on rate zonal centrifugation.
5. What is the principle of colorimetry? Draw a simple labelled diagram of a colorimeter
6. What is the principal of a flame photometerr?

7. Write a short note on spectrophotometry?

8. Write in detail on FTIR.

9. Explain about NMR.

10. What is the process involved in flow cytometer?

Unit V

Questions	Option A	Option B	Option C	Option D	Answer
The desire to maintain a safe laboratory environment for all begins with	A. prevention	B. ubiquity	C. microbiology	D. accidents	A. prevention
When a chemical splashes in the eye rinse for	A. 10 seconds	B. 30 seconds	C. 5 minutes	D. 15 minutes	D. 15 minutes
Which of the following types of personal protective equipment (PPE) is frequently used	A. safety glasses	B. lab coats	C. face shields	D. all of the above	D. all of the above
Chemical, reagents, broth cultures should be pippeted by	A. mouth	B. ear	C. pipette	D. nose	C. pipette
Good work practices include	A. smelling and tasting chemicals	B. not washing hand before and after lab	C. confining long hair and loose clothing	D. using damaged equipment and glassware	C. confining long hair and loose clothing

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	A. sterilization technique	B. aseptic technique	C. disinfectal technique	D. pathogen technique	B. aseptic technique
After a biohazard spill is covered with paper towels and disinfectant solution, it must sit for	A. 5 minutes	B. 30 minutes	C. 60 minutes	D. 20 minutes	B. 30 minutes
What is needed for the source of nutrient for the growth and reproduction of microbes	A. pathogens	B. bacteria	C. reagents	D. media	D. media

To prevent the contamination of microscopes and surrounding areas disinfect /clean used slides prepared by students with	A. 70% ethanol and lens paper	B. acetone and lens paper	C. 5% methylene blue and lens paper	D. water and lens paper	A. 70% ethanol and lens paper
Which of the following extinguishers is suitable for a fire involving flammable liquids	A. carbon dioxide extinguisher (black)	B. powder extinguisher (blue)	C. foam extinguisher (cream)	D. polka dot extinguisher (dotty)	A. carbon dioxide extinguisher (black)
GLP is an	A. Glass ware	B. FDA regulation	C. Analytical laboratory	D. Safety rules	B. FDA regulation
Which of the following is the principles of GLP?	A. Test systems	B. Reporting of study results	C. Test and reference substances	D. All the above	D. All the above
How many types of inspection	A. 2	B.4	C.3	D.5	C.3

SOP is otherwise known as	A. Standard operating procedures	B. System operating procedures	C. Safety operating procedures	D. Stationary operating procedures	A. Standard operating procedures
What good laboratory must contain?	A. Area should be free from smoke, smell, dust	B. Maintenance and calibration data	C. Air conditional the lab with humidity control	D. Both A and C	D. Both A and C
The prevention of large scale loss of biological intergrity is	A. Fire safety	B. Bio safety	C. Chemical safety	D. Test systems	B. Bio safety
Which of the following is not a laboratory safety rule?	A. You should never mix acids with bases	B. You should tie back your long hair	C. You should never add water to acid	D. All the above	A. You should never mix acid with bases
Which piece of laboratory equipment is best-suited for accurately measuring the volume of a liquid?	A. Graduated cylinder	B. Beaker	C. Erlenmeyer flask	D. More than one of the above	A. Graduated cylinder

Which piece of laboratory equipment can be used to store chemicals for long periods of time?	A. Burette	B. Evaporating dish	C. Beaker	D. More than one of the above	C. Beaker
The independent variable in an experiment is:	A. The variable you hope to observe in an experiment	B. The variable you change in an experiment	C. The variable that isn't changed in an experiment	D. None of these is correct	B. The variable you change in an experiment
Qualitative results refers to	A. Results that can be observed during an experiment	B. Results those are difficult to observe during an experiment	C. Results that require numerical data	D. None of these is correct	D. None of these correct
. When drawing a graph that measures family average income over a period of 50 years , the independent variable is	A. Income	B. Average	C. Years	D. It is impossible to say	C. Years

Accuracy is defined as	A. A measure of how often an experimental value can be repeated	B. The closeness of a measure value to the real value	C. The number of significant figures used in a measurement	D. None of these	B. The closeness of a measure value to the real value
How many significant figures are present in the number 10,450?	A. Three	B. Four	C. Five	D. None of these	B. Four
. The key component of GLP system of quality is	A. Quality unit	B. Quantity unit	C. Quality reading unit	D. Quality assurance unit	D. Quality assurance unit
Microscope is wiped by using	A. 90% isopropyl alcohol +30% water	B. distilled water	C. 75 % ethanol	D. only with water	A. 90% isopropyl alcohol +30% water
Which one of the following is correct?	A. acid can be added to water	B. water can be added to acid	C. both a and b	D. none of these	A. acid can be added to water
. Before operating inoculation chamber the palm should be wiped with	A. Ethanol	B. distilled water	C. sanitizer	D. all of the above	A. Ethanol

Which one of the following are GLP regulations on requirements	A. 21CFR58	B. 40CFR160	C. 21CFR211	D. a and b only	D. a and b only
A “class –D” fire extinguisher can be used to treat fires involving which as fuel sources	A. ordinary combustibles (wood and plastics)	B. electrical equipment	C. combustible metals	D. flammable to combustible liquids	C. combustible metals
Which of the following id not a type of firefighting equipment	A. fire blanket	B. hose reel	C. sprinkler	D. ice cubes	D. ice cubes
Why shouldn’t carbon dioxide extinguishers be used in confined spaces	A. they might explode	B. harmful fumes may be inhaled	C. they could cause claustrophobia	D. they might not show up if its dark	B. harmful fumes may be inhaled
What is the correct definition of fire	A. a chemical reaction from which heat and light are emitted	B. hot orange stuff	C. mixture of carbon dioxide and nitrogen	D. a yellow coloured solution	A. a chemical reaction from which heat and light are emitted

What is the extraction as practiced in the organic chemistry laboratory	A. the removal of one solid material from other	B. the separation of one substance from the another based on solubility	C. the removal of painful or impacted teeth	D. none of these	B. the removal of one substance from the another based on solubility
Latex gloves	A. may be reused only if they have not be been permeated	B. may be reused as long as they are clean	C. should never be reused	D. both a and b only	C. should never be reused
What is distillation?	A. distillation is when a liquid is evaporated and then recondensed in another container	B. distillation is when material heated to melting and then separated	C. distillation is when a substance is dissolved, heated and then precipitated	D. none of these	A. distillation is when a liquid is evaporated and then recondensed in another container
. What piece of laboratory equipment is best suited for accurately measuring the volume of a liquid	A. graduated cylinder	B. beaker	C. Erlenmeyer flask	D. more than one of the above	A. graduated cylinder

What piece of laboratory equipment can be used to store chemical for log periods of time	A. burette	B. evaporating dish	C. beaker	D. more than one of the above	C. beaker
Qualitative results refer to	A. results that can be observed during an experiment	B. results that is difficult to observe during an experiment	C. results that require numerical data	D. none of these is correct	D. none of these is correct
. Accuracy is defined as	A. a measure of how often an experimental value can be repeated	B. the closeness of a measured value to the real value	C. the number of significant figures used in a measurement	D. none of these	B. the closeness of a measured value to the real value