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SCHOOL OF BIO & CHEMICAL ENGINEERING
DEPARTMENT OF BIOMEDICAL ENGINEERING

UNIT – I – Analytical Instrumentation – SBM1302

UNIT I INTRODUCTION TO ANALYTICAL INSTRUMENTS

Spectroscopy

It is the branch of science which deals with study of interaction of electromagnetic radiation with matter.

Energy is absorbed or emitted by matter in discrete amount called Photons. It is one of the powerful tool available for the study of atomic and molecular structure.

Atomic Spectroscopy:

Interaction of Electromagnetic radiation with atom is ground state is called Atomic Spectroscopy.

Molecular Spectroscopy

Interaction of EMR with molecules is called molecular spectroscopy.

Basic Definitions Electromagnetic Radiation

It is the form of energy that is transmitted through a space at enormous velocity. It does not requires any supporting medium.

Wavelength:

The distance between two thrust and through on an electromagnetic wave metre, millimeter, micrometer, centimeter.

Frequency

Number of wavelength unit in a passing through a given point in an unit time (cycles per second).

Velocity

It depends upon the medium through which the radiation is passed (m/s)

Polychromatic Beam

Mono: It carries only one particular wavelength whereas, Poly: Polychromatic possess several wavelength.

Spectrometer

It is an instrument which is used to record the variation in the intensity of radiation. Two types of spectra are obtained: i) Emission and ii) Absorption.

Emission

The sample is thermally or electrically excited which starts giving out radiation or this radiation are recorded in the spectrometer, finally given to the detector and a graph is obtained.

Absorption

Light source is given to the sample, and the sample has absorbed the radiation which is recorded by the spectrophotometer and a graph is obtained. **Electromagnetic radiation**

EMR is the type of energy that is transmitted through a space at a speed 8approximately 2 / 10m/s. It does not require any supporting medium for propagation.

EMR may considered as a discrete packet of energy called photons.

Energy (E) = hυ Photos consists of oscillating electric field component (E) and a magnetic field (M).

Electric and magnetic field are perpendicular to each other to the direction and propagation of photons.

The distance along with the direction and propagation of one complete cycle is known as wavelength (λ).

The number of flip on oscillation of the photos that occurs is second is known as frequency which is expressed is hertz (Hz).

Relationship between the photons and the frequency of its propagation.

$$E=h\,O \qquad (1)$$

E = Energy in ergs

υ = Frequency in cycles

h = Plank's constant

C = Velocity of light is vacuum

V C (1) Frequency is inversely proportional to wavelength Energy is directly proportional do the velocity of light and inversely proportional to the wavelength of light.

$$V \propto Y\lambda \qquad (2)$$

Comparing (1) and (2) equation

$$V \propto C / 5 \qquad (3)$$

Electromagnetic Spectrum

7 different waves in an electromagnetic radiation is called electromagnetic radiation is produced by events at the molecular, atomic or nuclear level.

Oscillation of nuclei, electrons in electric or magnetic field excitation of orbital electrons, rearrangement of other electrons (or) nuclear breakup which may gives rise to electromagnetic

radiation.

Since each of these events differs in term of the energy involve, the radiation they exit will have different wavelength. Thus a complete spectrum of EMR will be produced. **Electromagnetic spectrum** -The main regions and their wavelength, physical events involved in the production are indicated.

Interaction of radiation with matter

When a beam of radiant energy strikes the surface of a substance, the radiation interacts with the atoms and molecules of the substance. a) The radiation may be absorbed, transmitted scattered or reflected even it excites fluorescence depending upon the properties of the substance (a). The radiation may be transmitted with little absorption taking plate and therefore without much energy loss. b) The direction of propagation of beam may be altered by reflection, refraction, scattering. c) The radiant energy may be absorbed a part or entirely by the substance.

Absorption spectra

The pattern of any energy absorption by a substance by light of varying wavelength passes through it, is a unique characteristics of the substance.

This pattern is known as absorption spectra.

To establish the absorption spectrum for a given substance, transmittance or optical density of a particular concentration of the substance is measured at different wavelength.

The application of absorption spectra, is not limited to the visible region of the spectrum but also to the UV, infra red absorption of many substance.

Laws of Absorption

The absorption of light by any absorbing material is governed by two laws. The first law of these laws is known as

Bouger's Lambert Law

It states that, the amount of light absorbed is proportional to the thickness of absorbing material and is independent of intensity of incident light. The pattern of light absorption by successive equal path length."b" absorbing solution. Let us assume that a thickness beam has the ability to absorb 50% of the incident intensity of the light passing through it. If the intensity of radiation, incident upon such thickness is assigned a value of 1, the out coming that is transmitted will have value of 0.5.

Now, we place a second equal thickness „b' it will absorb 50% of the transmitted light and the second transmitted „b', will then have the value of 0.5. Exponential function may be expressed as -Kb I / I0 = e I -Intensity of the transmitted light I0 -Intensity of the incident light b -Absorbing thickness K -Linear absorption co-efficient of the absorbing material Power term in the above relationship can be removed by converting into logarithmicform.

LnI/ I0= -Kb (or) Ln= I0/I =Kb Changing to common logarithms, we get 2.303 log10 I0/I

= Kb (1)

Beer's law

This law states that the amount of light absorbed by a material is proportional to the number of absorbing material (i.e.) concentration of absorbing solution. This can be mathematically expressed in the form of an equation similar to the above. 2.303 log10 =I0 / I = K'C

(2) K' absorptivity constant CConcentration of the absorbing material Now combine the two equation for the Barger-Lambert and Beer's law. K and K' merge to become a single constant 'a'. The combined equation is written as

log10= I0/ I0 = abc

The equation is referred as Beer's Lambert Law. This combined law states that the amount of light absorbed is proportional to the concentration of the absorbing substance and to the thickness of the absorbing material.

The essential components of the spectrophotometer includes

- The stable and cheap radiant energy source
- Monochromator
- Transparent vessel (cuvettes) to hold the samples
- Photosensitive detector
- Amplifier and recorder

Radiant Energy Source

Materials which can be excited to high energy state by a high voltage electric discharge or by the electrical heating serves as the excellent radiant energy source.

Sources of ultraviolet radiation

Most commonly used UV radiation are hydrogen lamp and deuterium lamp.

Sources of visible radiation

Tungsten filament lamp is used as source of visible radiation.

Sources of IR Radiation

Globar source are the most satisfactory source of IR radiation.

Filter and monochromators

It selects narrow band of wavelength from the continuous spectra. There are two types of filter. They are -Absorbance filter -Interference filter

Absorbance filter

They allow the light of required wavelength to pass through and absorbs selectively the unwanted wavelength. Solid sheet of glass that has been coloured by pigment which is dissolved or dispersed in the glass.Eg: Dyed gelatin is used as a filter.

Interference filter

Semitransparent metal film is deposited on a glass plate (i.e.) a thin layer of electric material like magnesium fluoride, again another coating of metal film is coated over a glass plate for protection. When a ray of light is incident, a part of light is reflected back and a rest is transmitted out.

The outgoing rays are called constructive inference.

Monochromator

Monochromator resolves polychromatic radiation into its individual wavelength. It has three components.

i) Entrance slit

ii) Dispersing element (prism / grating)

iii) Exit slit.

Entrance slit

It provides a narrow source of light, so that there is no overlapping of monochromatic images. **Prism**

Electromagnetic radiation is dispersed to its component wavelength – or – prism is made up of quartz. –visible – made up of glass – Infrared – made up of alkali halides.

Prism undergoes dispersion the wavelength will not be overlapping but non-linear.

Grating

Whenever the light rays falls on a ruled line, it will disperse. They produce more than one order of diffraction.

Large number of parallel lines ruled on a highly polished surface such as Alumina – Eg. 15,000 – 30,000 per square inch. When light rays are impinged on these grooves, it will scatter light but it will be a linear dispersion but slightly overlapping.

Exit Slit

It selects the narrow band of dispersed spectrum for the observation by the detector.

Sample hold

Eg:Cuvette

It should be transparent to the wavelength region being recorded, thickness is usually 1cm, and uniform. It can be rectangular or cylindrical in shaft.

- UV the cuvette is made up of quartz or silica
- Visible the cuvette is made up of colouredcorrected fused glass.
- Corrosive / volatile solvents: The stoppered cuvette should be used.
- Contents should be homogeneous in nature
- Sample volume is about 2.5 to 3.0 cm 3
- For micro cuvette, the sample volume is 0.3 to 0.5 cm 3

Detector

It is used to measure the radiant energy through the sample. Detector maybe associated with read out system.

The base plate like aluminium and iron acts as one electrode. The layer of silver or gold which act as a collector electrode and a layer of semiconductor like selenium is deposited.

When the light rays is passed to the selenium layer, it generates electrons which is collected by the collector give to circuit where the current is developed.

The amount of current produced is proportional to the intensity of transmitted light which inturn depends upon the wavelength of incident monochromatic light.

The components of phototube includes (a) evacuated glass envelope (quartz window) (b) semi-cylindrical cathode inner surface wall is coated with alkali (or) alkaline earth oxide (c) centrally located metal wire anode.

The potential difference of approximately 90 V is applied across the electrode. The Quartz window allows the passage of radiation which strikes the photoemissive surface of the cathode.

Cathode will generate electrons because of presence of light sensitive material. The electrons are attracted by anode, so there will be flow of current in the external circuit.

Signal is detected and a current is amplified and given to the galvanometer. If the electron collection is 100% efficient, the phototube current should be proportional to the light intensity.

Photomultiplier tube

It considers of electrode covered with photo emissive material (cathode), collector electrode (anode). Large number of plate called dynodes.

When light rays falls on the cathode which is coated with photoemissive material, it generates electron. These electrons falls to the dynode, one electron falling on it gives four on five electrons. And this will fall on the next dynode, there will be again multiplication of electrons. Electrons finally reaching the collector measures the intensity of incident light.

Dynode is maintained at 70 – 100 V.

Amplifier and Recorder

Radiation detector generate electron signals which by the amplifiers, ammeter, potentiometer recorders.

Basic Components of the Instrument

The source of light is the tungsten filament lamp which passes the light on the sample through a filter. The filter may be either absorption or interference filter.

The sample holder is the cuvette, the light after passing through the sample holder falls on the surface of the photo cell. The output of the cell is measured on a micrometer.

Operation of the Instrument

The photocell is darkened by not passing light the sample. The meter is adjusted mechanically to read zero. The blank or a pure solvent is inserted in the path of the light beam and the incident light intensity is regulated.

This can be done either by rotating the photo cell about an axis perpendicular to the light beam or adjusting a diaphragm in the light beam.

Solution of both standard and unknown are inserted in place of a blank and the reading of the specimen relative to the blank is recorded.

The metre scale gives 100% transmittance Eg:spectronic-20 (reading type)

Double beam instrument

It operates like single beam spectrophotometer except that they have design to component possible variation in intensity of the light source.

Splitting the light beam from the lamp and directing one portion to the reference curvette and the other to the sample cuvette simultaneously and does the variation in light effect gets cancelled out.

Double beam instrument are ideally a spectral scan because the instrument automatically corrects for change in transmission through the reference cuvette as the wavelength is changed.

If a single beam instrument is used, it will be necessary at each change in wavelength to adjust the instrument to zero absorbance for a reagent blank before absorbing the recording the absorbance of the sample. Eg: Beckmann's model DB-G Carry model 16 – recording type

Carry model – 14 (recording type) Application of UV, visible spectrophotometer.

1. Quantitative analysis Identify classes of compound in both pure and
2. Qualitative analysis biological preparation.
3. Enzyme Assay
4. Molecular weight determination

5. Study of cis-trans isomers
6. Physico chemical studies
7. It is used to study the dissociating of acid and bases
8. To study structural elucidation of any compound.

Difference between UV and visible spectrophotometer Principle

UV spectrophotometer	Visible spectroscopy
1. Source of electromagnetic radiation: EMR in the region of 180 – 400 cm is used for the study	Source of EMR: EMR is the region of 400 700 nm is used for the study
2. Source of light: Radiation source is used are hydrogen lamp and deuterium lamp	Source of light: Radiation source is used are tungsten filament
3. Monochromator: Uses Prism/Grating to select the desired wavelength of the source	Monochromator: Uses filters for selecting the desired wavelength of light.
4. Sample container: Cuvette made up of quartz are used because glass may absorb some light radiation	Sample Container: Coloured corrected fused glass is used for making the cuvette.
5. Detector: Photomultiplier tube is used as a detector	Detector: Photoemissive cells are used as detector.
6. Type of instrument: It is mostly double beam type	Type of instrument: It can be either single or double beam type.
7. Nature of sample: Sample may be colourless but optically active.	Nature of sample: Sample is converted to a coloured product by a set of reaction.
8. Recovery of sample: Sample can be recovered after analysis	Recovery of Sample: Sample analysis cannot be recovered because of destruction
9. Reading and recording type: Usually recording type is used widely	Reading and recording type: Both reading and recording type is used.
10. Solvents: Less polar solvents are used	Solvents: Both polar and nonpolar solvents are used.

When a molecule at ground state level „G' is excited by light absorption it reaches a higher excited energy level (E2). Due to vibrational energy loss, the molecules looses some energy and reaches the still excited but lower energy level (E1), no light emission takes place during this step. As the molecule quickly returns to the more stable ground state level, light -8 is emitted. If this process is completed within 10 seconds, it is known as fluorescence.

Instrumentation

The light source is mercury arc discharge lamp (or) xenon arc tube. So that the light in the UV range is provided.

Both the monochromator present in aspectrofluorimeter are diffraction grating.

The primary grating allows the passage of light of the proper wavelength for absorption by the molecule.

The secondary grating transmit light of the specific wavelength emitted by the sample. Since the light is emitted is all the directions, the detector is placed at right angle to the beam of light from the source lamp to the sample.

This position prevents transmitted light originating in the lamp from reaching the detector.

Quartz or silica cuvette are used as sample holder. Photomultiplier tube is used as detector.

A spectroflurometer has a device to maintain the temperature from 25 -30 ^{o}C, since the fluorescence is maximum at this temperature.

Applications

It is used for the quantitative analysis of Ribifluorine, thyamine, estrogen, etc.

By using fluorescent probe intracellular force calcium concentration can be determined.

Fluorescent probes are used to study the membrane structure i.e. changed in mitochondrial membrane during oxidative phosphorylation.

By using acridine orange in spectrofluorometer visualization of nucleic acid within cellular organelles can be done.

Flame photometry

It is similar to that of spectrophotometer, the only exception is the replacement of the sample cell by a flame.

The general method of flame photometry can be applied in two complementary ways.

i) Flame emission photometry (or) emission flame photometry\

ii) Atomic absorption spectrometer.

Principle

In E.F.photometry, voltalization of a molecule in a flame produces free atom and then excited to higher energy level when the excited atom returns to the ground state, a characteristic emission of spectrum of the element is produced.

This is the principle of emission flame photometry.

Working

The sample is aspirated into a flame, after evaporation of the water. The molecules are dissociated by the heat to atomic vapour.

A small percentage of the atom is transforms to an excited state by the absorption of discrete packets of energy that displays orbital electrons to higher energy level.

The excitation is temporary. The atom immediately return to the ground state and in the process, the absorbed packets of energy is in the form of light. The emitted light is of wavelength specific for each element and can be quantitated under carefully controlled condition.

Atomic Absorption Spectrometer Principle

In A.A. spectrophotometer, the absorption of a beam of monochromoatic light by atom in a flame is measured.

Working

In A.A. spectroscopy a monochromatic light of a particular element is product by means of a hollow cathode lamp. A monochromator light is beamed through a long flame into which is aspirated the solution to be analysed. The heat energy dissociates the molecules and converts the components to atom. Although some atoms are activated, most remain in the ground state. The ground state atom of the same element as in the hollow cathode absorb their an resonance line. The amount of light absorbed varies directly with their concentration in the flame. The transmitted light that is not absorbed reaches the monochromator which passes only the wavelength close to the resonance line of the particular element to be analysed. The transmitted light strikes the detector and the light intensity is measured.

Applications

- Emission flame spectrophotometer are used to assay about 20 elements in the biological sample.
- Both the techniques are widely used, in clinical laboratories for the determination of trace elements in body fluids.
- In physiological and pharmacological research sodium, potassium, calcium, magnesium, cadmium and zinc are measured directly.
- It is useful in monitoring many therapeutic regimes.

Raman Spectrophotometer

Raman spectra can be absorbed by illuminating the sample width monochromoatic light and absorbing the light get scattered at right angle to the incident radiation.

Raman spectrometer consists of

i) A source of monochromator radiation

ii) Sample compartments

iii) Monochromator

iv) Detector system

v) Computer

Source: Helium – Neon laser which emits monochromatic light at 632.8 mm is the commonly used excitation source, in the modern Raman's spectrophotometer.

The wavelength being in the red region of the spectrum, there is a loss of scattering intensity associated with the use of longer wavelength and the photoelectric detectors are less sensitive.

This can be overcome by using Argon laser is the most commonly used source in the Raman spectrometer.

Sample chamber

Sample may be examined as solid, liquid solution or in the gas phase. Liquid-laser beam can be focused into a capillary tube, containing the liquid.

Solid:Laser beam is focused into a capillary tube, containing the powdered solid (few mg) the sample are adequate to give a good spectra.

Gas: Gas sample can be placed inside the laser cavity.

Detector: Photomultiplier tube is commonly used detector which provides excellent sensitivity, low noise and large dynamic range.

Single channel detector

It requires point by point spectral data acquisition and long scan time.

Multi channel detector

One dimensional diode array (or) two dimensional type (charged coupled device). These detector permits the collection of large portion of a vibrational spectrum in seconds.

Computer

Computer incorporated in the modern instruments, are essential for spectral manipulation (addition, subtraction etc.).

Note: The scattering of light at the same frequency as the incident radiation is called Rayleigh scattering.

The light scattered from a sample eliminated with the monochromatic beam frequency that are different from the incident spectroscopy is called Raman spectra.

Stokes line

If the photon strikes an atom or molecule in a liquid a part of energy of incident photon may be used to exit the atom of the liquid and the rest is scattered. Thus spectral line will have low frequency and it is called Stokes line.

Antistokes line

If the photon strikes an atom or molecule in a liquid which is in an excited state, the scattered photon gains an energy.

Thus spectral line will have higher frequency and it is called Antistokes line.

Raman spectrum can be recorded in two different ways.

By focusing the spectrum from the prism or gration on by photographic plate and measuring the line frequency and intensity.

By focusing the spectrum, produced by the monochromator on by a multiplier tube amplifying the detected signal and recording it.

Application

This technique is used to study, the changes in chemical bonding when a substance is added to an enzyme.

Raman Gas Analyserhave many practical application. For eg: They are used in medicine for real time monitoring of Anaesthetic and respiratory gas picture during surgery.

Raman spectroscopy is being investigated to detect explosives for airport security.

It is used to study about the proteins, DNA and the biological function.

With a Raman spectrophotometer, it can determine molecular structure, chemical constituents and properties of material.

FTIR – Fourier Transform Infrared Spectroscopy

FTIR is the two beam interferometer most commonly of Michelson type.

Principle of Michelson Interferometer

FTIR radiation entering the interferometer is split into two beam by a beam splitter. Beam "A" follows the straight path, before returning to the beam splitter, whereas the distance travelled by beam "B" can be varied before it recombines with beam splitter.

When the Beam "A" and "B" recombines and interference pattern is produced, which is incident on detector.

When the two beams are in face at the beam. The maximum intensity will reach the detector. If intensity will be minimum, in case the beams are out of phase. If provision is made in such a way that, mirror M2 is displaced uniformly, the detector output will be a sine wave whose frequency is determined by the translation velocity of M2 and a wavelength of the monochromatic radiation, the amplitude of the signal will depend upon the intensity of the incoming radiation.

Working

In FTIR, the IR radiation is from globar source. The IR rays is given to the interferometer, which contains beam splitter which splits the beam into beam "A" and Beam "B".

Both the beams are first passed to the reference where the error is nullified and then it is passed through the sample.

Both the beam passes through the sample, reach the mirror and are reflected back. The

reflected beam recombines and reaches the pyroelectric detector. This detector detects the interference pattern and allows only that frequency waves to pass through.

Analog to digital converter converts analog data into digital data which is given to the computer from where it is send to the scan control.

Only the interference pattern is collected, and send back to the computer from where it is given to the display, or to the high speed digital plotter.

Applications

- It is used to test the raw material.
- Qualitative analysis
- Quantitative analysis
- It is used to detect contamination even some trace amount.

Application of all the spectrophotometer Advantage Speed: All the frequencies are measured simultaneously most measurements of FTIR is mostly in seconds rather in than minutes.

Sensitivity: The detector employed are much more sensitive. It makes lower noise level.

Internally calibrated

The instrument is self calibrated and never need to be calibrated by user. Thus FTIR technique has brought significant technological advantages in IR spectroscopy.

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UNIT –II – Mass Spectrometer – SBM1302

UNIT II – MASS SPECTROMETER

The sample to be analysed is first bombarded with an electron beam to produce ionic fragment of the original molecule. These ions are then sorted out by accelerating then through electric and magnetic field according to their mass charge ratio. A record of the number of different kinds of ion is called the mass spectrum.

The molecules in the gas sample A to be analysed are bombarded with electrons to produce ions (B). These ions are accelerated in a high vaccum into a magnetic field (c) which deflects them into circular path D.

Since the deflection of lighter ion is greater than for heavy ions, ions streak separates into beam of different molecular weight. A suitably placed slit (F) allows a beam of a selected molecular weight to pass through a collection electrode (E). As the accelerating voltage and ion source is gradually reduced, the ion beams greater mass pass through the slit. When these ions fall on the collector electrodes, they produce minute electric current which may be measured after suitable amplification.

This amplitude will indicate the number of ions is each beam. The proportion of molecules of different masses in the gas sample, may this found and a complete analysis of gas sample may be found provided all the constituent gases have different molecular weight.

This is usually done for respirations gas analysis.

Instrumentation of Mass Spectrometer

In a mass spectrometer the starting point is a mixture of ions having different mass change ratio. The slit is used to select which mass to change ratio has to reach the detector. The different change to mass ratio across the detector. Slit and ion current is recorded as the function of time (mass).

The heated tungsten filament produces an electron beam which passes between the plate, a difference in electrical potential between then pull ion out of the beam.

The ion beam then enter the space between trapezoid shape magnetic poles which is deflected through an angle 60^o, 90^o, 120^o or 180^o.

When ions of mass ‘m’ and chare ‘e’ passes through an accelerating electric field, they would attain a velocity ‘v’ which can be expressed in terms of the accelerating voltage ‘V’

$$\frac{1}{2} mv^2 = eV \qquad (1)$$

Where $\frac{1}{2} mv^2$ (is the kinetic energy of the ion)

Equation (1) V can be written as 2 V, h are constant.

This equation shows that the radius of the orbit is a function of the mass by charge ratio of the particles.

In time of flight – mass spectrometer, ions of different mass by charge ratio are separated the difference in time that take travel over an identical part from the ion source to the collector.

This requires the starting time (the time at which the ion leaves the ion source) by using a gate to release the ions from the ion source in a very short time.

The ion transfers an evacuated tube caused the drift tube to reach the detector. The detector sensitivity the ion, since ions of different masses arrives at the detector at different time.

The accurate measurement of the time between activating the source and sensitizing the detector gives information about the mass of the ion.

The signal from the ion reaching the detector is amplified and given to oscilloscope, which gives the mass-spectrum in a very short time.

Instrumentation

Time of flight consists of

i) The electron gun for the production of ion

ii) A grid system for accelerating ion to uniform velocity

iii) A evacuated tube called the drift tube

iv) An ion detector

It "L" is the length of the drift tube in centimeters, "t" is the transit time in microseconds charged ions of mass "m" and constant energy Ve

Time (t)

$t = L/V$

Taking square on both sides

$T^2 = L^2$

Quadruple Mass Spectrometer

Quadruple mass spectrometer are simple in construction and light weight as compared to the other mass spectrometer. It has high speed electronic scanning and low pass.

Construction

A quadruple mass spectrometer consists of an ion source, a quadruple mass filter, a lens system to focus the ions into a quadruple filters. The arrangement consists of four cylindrical rod shaped electrodes, which provides a potential field distribution, periodic in time, and symmetric with respect to the axis which will transmit a select mass group and causes ions of improper

mass to be deflected away from the axis. This mass selection was a combination of dc potential and radio frequency potential.

By proper selection of potential and frequency, an ion of the desired mass can be made to pass through the system while unwanted mass will be collected on one of the electrode.

Operating principle of a quadruple mass spectrometer

i) Ion created through a bombardment in the ion source.

ii) Ions separated by the m/e ratio in the rod system

iii) Ions detected in the ion detector.

Working

During operation, opposite electrodes of the filters connected together and to one pair of potential.

is applied

ω = 2πt

u = dc voltage

v = peak amplitude of the rf voltage at a frequency ftv = with respect to angle and time

ϕt = u + v cos(2πft)

To the other pair of electrodes, same potential but of the opposite sign is applied ions emitted out of the ion source and focuses into the quadruple filters are made to undergo transverse nation by the rf and dc field perpendicular to the z-axis. With proper selection of u and v, ions of given mass / charge (m/c) ratio will have stable trajectories. These ions will oscillate about the z-axis and ultimately emerge from the exit of mass filter assembly.

Ions with other values of m/c (mass by charge) will have unstable solution, they will move away from the z-axis and ultimately strike the electrodes thus being removed.

Applications of mass spectrophotometer

i) It is used for studying respiratory, physics, in routine clinical investigation. Eg: continuous analysis of gas flow in a patient breathing cycle.

ii) It is used for quantitative analysis of mixture of the hydrocarbon

iii) There are used in refineries for trace element investigation i.e. analysis of lubricating oil.

iv) There are used for detecting and measuring the concentration of the pollutant in air and water.

Resolution in mass spectrometer

i) The difference between each mass from next integer is defined as a unit resolution.

ii) The capability of the equipments to distinguish the mass i.e. mass 500 from mass 50%

For eg: When describing resolution in mass spectrometer and quadruple mass spectrometer the peak shapes are usually flat, but in the case of time of flight, the peak height is 50% less than quadruple mass spectrometer and mass spectrophotometer.

Radiochemical instruments

Radiology is defined as the spontaneous disintegration of unstable atomic nucleus accompanied by the emission of ionizing radiation caused radioactivity.

Eg: Uranium.

α, β, and γ-rays are the radioactive emission. Radioactivity is irreversible and self-disintegrating. Artificial radio activity (or) induced radioactivity is the emission of α, $\beta$and γrays when radio active substances are bombarded with neutrons.

Laws governing radioactive disintegration

1. Atoms of all radioactive elements undergoes spontaneous disintegration to form fresh radioactive product with the emission of α, $\beta$and γ-rays.
2. The rate of radioactive disintegration is not affected by the environment factor but it depends on the number of atom of the original time present at any time.

Properties of α, $\beta$and γ-rays

S.No.	Parameters	α-ray	β-ray	γ-ray
1.	Charge	Positive	Negative	No charge
2.	Ionising power	Very high	High	Low
3.	Penetrating power	Shortest	100 times > than alpha	Very high
4.	Energy range	2 – 10 µeV	0 – 3 µeV	Higher than both

α-emission: αparticles composed of two protons and two neutron. They are the most harmful to the human tissues.

β-emission: They are positively or negatively charged and are high speed particles originating in the nucleus. They are not harmful have less ionizing property.

γ-emission: γ-rays are also called as photons or packets of energy. They have greatest penetrating capability and low ionizing property.

α, β, and γ-rays are expressed in the terms of the electron volt.

Interaction of radiation with matter

It can be divided into two categories.

1. Excitation
2. Ionisation

Excitation: Radiations can interact with an orbital electrons of the intervening matter that passing through. This interaction may be weak, capable of lifting an electron to a higher energy orbitals from its ground state. This electron eventually descents to its ground state andthe energy difference between the ground state and the higher energy orbital is emitted as photon. This type of interaction is known as excitation.

Ionisation: A closure interaction of radiation with matter can impart so much of energy to the orbital electrons that leaves the atom completely. This is in the formation of an ion pair (a positive atom ion and a negative electron). This process is termed as ionization.

Basic definitions

Radio Isotopes: They are defined as atomic species with same atomic number but different mass number. Eg: Uranium 238.

Isobar: They are defined as the atomic species with same mass number but difference atomic number. Eg:$_{90}Ux_1^{234}$, $_{90}Ux^{234}$.

Isotopes: It is defined as an atom with same number of neutron. Eg:80^{16}.

Isomers: They possess a same atomic number, same mass number but different half life period.

Half life period: It is equal to the time during which gives a amount of radioactive element is reduced by disintegration to half its initial amount.

Decay time

The half period of a radioactive isotope is the time required for half the initial stock of atom to decay. Thus, after one half period has elapsed, over gone the total activity of any single radioactive isotope will have fallen to half its initial value after two period, the activity will be ¼, to its initial value and so on. The half life period of radioactive isotope is given by half = $0.693/\lambda$.

Principle

The interaction of radioactivity with matter gives rise to ionization makes it possible to detect and measure the radiation. When an atom is ionised, it forms an ion pair, if the electrons are attracted towards on positively charged electrode and the positive ions to a negatively charged electrode a current would flow in an external circuit.

The magnitude of the current would be proportional to the amount of radioactivity present between the electrodes.

Instrumentation

An ionization chamber consists of a chamber which is filled with gas and is provided with two electrodes. A material having a very high insulation resistance such as polytetrafluoroethylene is used as an insulation between the inner and outer electrode of the ion chamber.

A potential difference of a few hundred volt is applied between the two electrodes. The radioactive source is placed inside or very near to the chamber. The charged particles moving through the gas undergo pollution to form in pair.

The chamber current will be proportional to the amount of radioactivity in the sample. Ionisation chamber are either operated in the counting node in which they response separating to each ionizing current. The current is usually in the order of 10^{-10} amps. It is measured by using a very high input impedance voltmeter. The current is indicated on a moving coil type ammeter.

The potentiometric recorder of the self balancing type can be used to record thesignal.

Applications

Liquid samples are usually counted by putting then in Ampoules and placing the ampoules inside the chamber.

Gaseous compound containing radioactive sources may be introduced directly into the chamber.

Geiger Muller Counter

Figure shows the variation of the count rate recorded by a typical GM-counter.

The GM counter is commonly called Geiger Muller tube or scintillation counter. This tube consists of a metal cylinder which act as a cathode and is about 1 – 2 cm in diameter. It has an axial insulated wire as an anode and is capable of being maintained at a high positive potential of 800 – 2500 V.

This assembly is placed in a tubular envelope containing a gas or mixture of gases which is easily ionisible. The envelope is gas type and it is typically filled to a pressure of 80 mm of Argon gas and 20 mm of alcohol. Alcohol and halogen act as a quenching gas and Argon act as a ionizing as.

The tube contains a window of thin mica or other suitable material which permits effective passage $\beta$and γradiations but not of α-radiation.

As the gas is ionized in the counting tube migration of ions takes place towards the appropriate electrode under the voltage gradient.

They soon acquire sufficient velocity which causes further ionization and gives rise to an avalanche of electrons travelling towards the central anode.

As a result, ion multiplication spreads to a complete sheath around the anode. The process infact, produces a continuous discharge, which fills the whole active volume of the counter, in less than a microseconds.

Each discharge builds up to a constant pulse of 1 – 10 volt. This pulse amplitude is sufficient to operate a scalar or rate meter without using any amplifier when the polarizing voltage is altered, the tube works in the voltage range exhibited by plateau i.e. starting voltage no counts are recorded.

Between the starting and beginning of the plateau the voltage is too low to produce constant pulse size. Also, beyond the plateau the count rate increase because of breakdown and spurious discharges through the tube.

The plateau is observed between 800 voltage and 1400 voltage for commercial tube. The slope of the plateau is generally expressed in terms of percentage of the count rate per volt.

Since the positive ions are produced by ionization which sheath around the anode wire which effectively lowers the potential gradient and the counter becomes insensitive to the entry of fresh ionizing particle. This is called dead time of the counter.

This dead time can be overcome by using quenching circuit, which reduces counted dead time and by reducing the number of organic molecules dissociated in each discharge.

The proportional counter is an ionization chamber that is operated at voltage beyond the ordinary ionization chamber region but below that of the Geiger region.

These counters are called proportional counter because the output pulse from the chamber stalls to increase with the increase in the electric field strength at the electrode but in still proportional to the initial ionization.

The radius of cathode is about ion and anode is about 0.001 cm with a polarizing voltage of 1000 V a polarising voltage of 1000 V is applied across these electrodes and when the radiation enters the chamber, they ionize the gas inside and the ions thus formed are attracted towards opposite polarity electrode.

The radiation source may be placed inside or outside the counter, there can be a continuous gas flow type counter where an argon methane mixture flows at atmospheric pressure from compressed gas tank at a rate of 200 million.

The output pulse from a proportional counter is only a few volt in magnitude therefore, a proper amplification is necessary.

The output signal from a proportional counter is given to a pre-amplifier. The preamplifier is the voltage follower gives very high input impedance. Then the next stage is the main amplifier which is a linear amplifier with low noise and very stable gain of the order of 500 -1000.

The signals are then given to a pulse height analyzer on pulse discriminator, which allows the pulses having amplitudes between the two level to pass through.

Then these pulses are counted to display as a direct indication of radio activity by using scalar.

Most of the scalar incorporate a counter / timer which displays the time taken to record a definite number of count the number of count which occurs within a definite time internal.

Biomedical applications of radio isotopes

Iodine 131: It helps in the detection of thyroid diseases and helps in identifying the

function of various organ like liver, spleen, kidney etc.

Iron 59: It helps in distinguishing many diseases caused by the deficiency of RBC's in the human body.

Sodium and Iodine: It helps in pumping action of heart and study of cases of restricted blood circulation.

Cobalt 60: It helps in the treatment of cancer.

Radio carbon dating C_{14}: It helps in the study of metabolism.

Tritium - H_3 – It helps in the study of effect of antibodies and also in metabolic studies.

Rhosphonis: It helps in the study of bone metabolism

FLAME PHOTOMETRY

Principle When a molecule at ground state level „G' is excited by light absorption it reaches a higher excited energy level (E2). Due to vibrational energy loss, the molecules looses some energy and reaches the still excited but lower energy level (E1), no light emission takes place during this step. As the molecule quickly returns to the more stable ground state level, light is emitted. If this process is completed within 10-8 seconds, it is known as fluorescence. Instrumentation The light source is mercury arc discharge lamp (or) xenon arc tube. So that the light in the UV range is provided. Both the monochromator present in a spectrofluorimeter are diffraction grating

The primary grating allows the passage of light of the proper wavelength for absorption by the molecule. The secondary grating transmit light of the specific wavelength emitted by the sample. Since the light is emitted is all the directions, the detector is placed at right angle to the beam of light from the source lamp to the sample. This position prevents transmitted light originating in the lamp from reaching the detector. Quartz or silica cuvette are used as sample holder. Photomultiplier tube is used as detector. A spectroflurometer has a device to maintain the temperature from 25 - 30oC, since the fluorescence is maximum at this temperature. Applications It is used for the quantitative analysis of Ribifluorine, thyamine, estrogen, etc. By using fluorescent probe intracellular force calcium concentration can be determined. Fluorescent probes are used to study the membrane structure i.e. changed in mitochondrial membrane during oxidative phosphorylation. By using acridine orange in spectrofluorometer visualization of nucleic acid within cellular organelles can be done.

Flame photometry

It is similar to that of spectrophotometer, the only exception is the replacement of the sample cell by a flame. The general method of flame photometry can be applied in two complementary ways. i) Flame emission photometry (or) emission flame photometry\ ii) Atomic absorption spectrometer

Principle

In E.F.photometry, voltalization of a molecule in a flame produces free atom and then excited to higher energy level when the excited atom returns to the ground state, a characteristic emission of spectrum of the element is produced. This is the principle of emission flame photometry. Working The sample is aspirated into a flame, after evaporation of the water. The molecules are dissociated by the heat to atomic vapour. A small percentage of the atom is transforms to an excited state by the absorption of discrete packets of energy that displays orbital electrons to higher energy level. The excitation is temporary. The atom immediately return to the ground state and in the process, the absorbed packets of energy is in the form of light. The emitted light is of wavelength specific for each element and can be quantitated under carefully controlled condition. Atomic Absorption Spectrometer Principle In A.A. spectrophotometer, the absorption of a beam of monochromoatic light by atom in a flame is measured.

Working In A.A. spectroscopy a monochromatic light of a particular element is product by means of a hollow cathode lamp. A monochromator light is beamed through a long flame into which is aspirated the solution to be analysed. The heat energy dissociates the molecules and converts the components to atom. Although some atoms are activated, most remain in the ground state. The ground state atom of the same element as in the hollow cathode absorb their an resonance line. The amount of light absorbed varies directly with their concentration in the flame. The transmitted light that is not absorbed reaches the monochromator which passes only the wavelength close to the resonance line of the particular element to be analysed. The transmitted light strikes the detector and the light intensity is measured. Applications Emission flame spectrophotometer are used to assay about 20 elements in the• biological sample. Both the techniques are widely used, in clinical laboratories for the• determination of trace elements in body fluids. In physiological and pharmacological research sodium, potassium, calcium,• magnesium, cadmium and zinc are measured directly. It is useful in monitoring many therapeutic regimes.•

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UNIT –III – Automated Biochemical Analysis – SBM1302

UNIT III - AUTOMATED BIOCHEMICAL ANALYSIS

Electrophoresis:

It is defined as the migration of charged particles under the influence of electric field Jiselius in 1937popularised electrophoresis which become very useful analytical tool in biochemical technique.

It is relatively rapid and convenient technique capable of analyzing and purifying several different types of molecules especially proteins and nucleic aids.

Principle

It is a molecular reparation technique reparation technique that involves the use of high voltage electric current for inducing the movement of charged molecules like protein, DNA, nucleic acid in a supporting medium.

Any charged ion or molecule migrates when placed in electric field.

The rate of migration of compound depends on the net charge, size, shape and applied current.

This can be represented by the following equation

$$V = \frac{E \times q}{f}$$

Where V = velocity of migration of the particle

E = electric field in volt

q = net charge of the molecule

f = mass and shape of the molecule

Electrophoretic mobility in gels

The movement of charged molecule is called mobility (μ). The mobility of molecule towards the opposite charge for instance the protein molecule with a positive charge move towards the negative pole of the supporting medium.

The medium may be a paper, gel or a capillary tube. The movement of this charged molecule is often expressed in terms of electrophoretic

$$\mu = \frac{Exq}{Exf}$$

$$\mu = \frac{q}{f}$$

The rate of migration is directly proportional to the charge and size and shape of the molecule with different charge/mass ratio migrate under the influence of electric field at different rate and hence gets separated.

Types of Electrophoresis

Paper electrophoresis is very useful in study of normal and abnormal plasma.

- The equipment required for electrophoresis consist two units, a power pack and an electrophoresis.
- The serum is mixed with bramophenol blue a spotted at the centre of a str9p of a specially.
- When an electric field of proper voltage is pass through the paper charged protein fraction bearing different charges move at different rate.
- After a run of 5-6 hours it is dried and observed. In human serum five different band can be identified on paper electrophoresis.
- They are in the order of albumin, α_1 – globulin, α_2-globulin, β-globulin and γ-globulin.
- Albumin being the fastest moving fraction on the protein of plasma forms the last band of the paper.
- γ-globulin which is the slow moving protein forms a band on the other end.
- The rest of the protein takes their position in between these -2 bands.

Cellulose acetate paper

- They are commercially available as stripped which are thin with uniform micropore structure
- The are less hydrophile and thus hold title buffer which results in better resolution in a shorter time
- This paper shows minimum absorption and give a dear separation with distinct zone.
- Hence the compound can be easily eluted with good recovery and the process is fast completed within 1 hour.
- Other advantages are:

The strips are chemically pure and do not contain lignin hemicellulose.

Polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis in acrylamide gel is frequently referred to SDS PAGE (sodium diodecylsulphate polyacrylamide gel electrophoresis). The components used in PAGE are

acrylamide, bisacrylamide, TEMED (tetra methyl ethylene diamde). Cross linked polyacrylamide gel are formed from the polymerization of acrylamide monomer in the presence of small amount of bisacrylamide. Several proteins of biological importance contain more than one polypeptide chain. These proteins are referred to as oligomeric proteins.

The structure of these proteins is stabilized by hydrogen bond, disulphate band, disulphide linkage, hydrophobia interaction,mercapto ethanol breaks disulphide bridges present in the oligomericproteibn.

SDS is anionic detergent which disruput macromolecule whose structure has been stabilized by hydrophobic interaction.

SDS bindings imparts a large negative charge to denature protein.

PAGE combined with SDS is most widely used method for analyzing protein mixture quantitatively.

It is particularly useful for monitoring protein purification.

The molecular weight of most protein could be determined by measuring the mobility of most protein in polyacrylamide gel containing SDS.

Sample to be separated is boiled for 5 mins in a sample buffer containing SDS and mercapto ethanol.

This treatment completely denature protein present it the sample and imparts negative charge to the polypeptide chain.

The sample buffer also contains a tracking dye which is to monitor the electrophoretic tank.

Process

In SDS-PAGE, the gel column consist of 2 parts (stacking gel and separating gel).

The separating gel is poured into a glass tube and allows to set. Then stacking gel is poured at the top of the separating gel. The stacking gel has large pore size and separating gel has comparatively small pore size.

Samples of protein known and unknown molwt are layered on top of each column separately.

The stacking gel allows the protein to move free and concentrate over the separating gel under the influence of electric field.

Protein continues the movement towards anode. Therefore protein have same charge. All proteins travel with same mobility. However it passes through separating gel, the protein separate owing to molecular sieving properties of gel. The small protein move fast as they can pass through the pour of the gel but large protein move slowly.

A plot of distance migrated vs log of the molecular weight gives the straight line hence if the protein of unknown molecular weight is electrophoresed with 2 or more protein of known molecular weight, the molecular weight of unknown protein can be calculated with an accurately ranging from 90 – 95%/

This is the most common way of estimating the molecular weight of molecular subunit.

Applications

Fine resolution of protein mixture is possible.

Pore size can be controlled and separation depends upon the size shape and charge of the molecule.

Highly suitable for histochemical and quantitative analysis.

The method of separating protein according to the isoelectric point in a pH gradient is called electric focusing.

This method has high resolution power because ordinary paper electrophoresis resolve focusing resolve into 40 band.

In conventional electrophoresis, the pH between anode and cathode is constant.

The positive charge ion migrate towards cathode and the negative charge ions migrate towards anode.

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 ionic point, it will possess a net positive charge and migrate in the direction of cathode.

One to the presence of pH gradient, the net charge of the molecule encounters a pH where net charge is zero, it will stop migrating.

This is the isoelectric point of protein. Each protein present in the mixture migrate to its isoelectric point and stops migration at that point.

Thus once the final stable focusing is reached, the resolution will be retained for longer time.

Applications

It is widely used for separation and identification of serum protein.

It is widely used in food and agriculture industry, forensic and genetic lab.

It is used in the research for reading enzymology, immunology, biochemistry, etc.

A standard gel electrophoresis provide huge advantage for molecular biology research. However it was unable to separate large molecules of DNA effectively.

This technique of pulse gel electrophoresis is used for the separation of large DNA molecules more than 15 – 20 kb.

Principle

Electric field is not constant and changes repeatedly pulse in direction and strength during the separation such that DNA molecular are forced to continuously orient and reorient themselves.

Larger the DNA molecules, longer is the time required for its orientation.

Process

The process is relatively similar to performing a standard gel electrophoresis except instead of constantly running the voltage in one direction, the voltage is periodically fixed among 3 directions that one4 runs in the central axis and other at the angel of 60^{o} either side.

This electric abrupt pulses changes the conformation of DNA molecules. They would be oriented by the influence of current in one direction and then reorient as a new electric field at a different angle to the first one was turned on.

DNA molecules tends to shutter through the gel matrix and the gel becomes fluidly during the electrical pulsing.

The pulse time are equal to each direction resulting in a net forward direction by DNA.

This process also ensures the migration of DNA on absolutely straight trace. The process was easier to dissolve DNA fragment upto 2000 kB with good resolution.

Applications

i) It is significant tool for characterization of larger molecules.

ii) The pore size can be controlled and the separation depends upon the size, shape and charge of the molecule.

iii) It is suitable for histochemical and quantitative analysis.

Agarose gel

Agarose is a natural product purified from the seaweed Rhodophyta.

It is a linear polysaccharide, agarose dissolve well and boiling and forms a gel when cooled.

1-3% of agarosegel are prepared by suspending dry agarose in a aqueous buffer (tris acetate buffer, tris buffer) and then boiling the mixture allowed to under a room temperature to form a rigid gel.

The pore size is determined by the concentration of agarose.

Low concentration produces larger pore size while high concentration produces small pore size.

Agarosegel are mostly used to study DNA molecules having high molecular weight

which cannot penetrate through polyacrylamide gel but can penetrate through 0.8% of agarose.

Procedure

Agarosegel are casted by melting the agarose in the presence of a desired buffer until a clean transparent solution is achieved.

The melted solution is then powered into a mould and allowed to harden.

Upon hardening, the agarose forms a matrix and the components are separated.

The gel is connected to a anode and cathode to the buffer reservoir through the paper wick.

The sample is applied in the well made in the gel.

After sometime the gel is washed to remove excess dye from the agarose and it is kept in an UV illumination which exists fluorescence if the DNA is present.

The gelis connected to a anode and cathode to the buffer reservoir through the paper wick.

Applications

1. It is used to isolate large number of protein and identity the molecular weight and purity.
2. It is convenient method to study DNA / RNA fragment.
3. The sequence of the DNA and molecular weight can be determined.
4. The location of a DNA in the gel can be directly determined by the use of fluorescent dye like ethidium bromide.

System Concept

The chemical analysis of blood and other body fluids is one of the earliest forms of diagnostic criteria leading to the investigations of the diseases.

In the early years of clinical biochemistry most of the required analyse were performed on urine since it available easily in large quantities.

With the development of semimicro and micro analytical techniques, analysis can be carried out with minute quantities of samples.

Therefore, the analysis of the blood serum (or) plasma is becoming more frequent. A majority of the blood analysis can now be performed by an automated system.

Advantages of Autochemical analysis

1. Precision (increased)
2. Minute quantities of samples are enough for analysis.

3. Repetitive monotonous work load of humans can be avoided and hence the error.
4. More reliable and accurate.

Types of analysers

1. **Electrolytic analysis**: It includes the detection of ions such as potassium, sodium, chloride and bicarbonate ions.
2. **Protein analysis**: It detects the presence of proteins, biolirubins (enzyme secreted by bilejuices), alkaline phosphatase.

Steps involved in automated analysis

1. Specimen identification
2. Specimen transport and delivery
3. Removal of proton and other interfering components.
4. Reagent handling and delivery
5. Chemical reaction phase
6. Measurement procedure
7. Signal processing and data handling
8. Report preparation.

These steps are carried out sequentially under the control of microprocessors.

System details

The automated system is usually a continuous, flow system, in which individual operations are performed on the flowing stream as it moves through the system.

The end product passes through the colorimeter to measure concentrations of various constituent. The final results are recorded on a strip chart recorder along with a calibration curve. So that the concentration of the unknowns can be calculated.

The automated system consists of a group of modular instruments.

1. Sampling unit
2. Proportioning pump
3. Manifold
4. Dialyser
5. Heating bath
6. Colorieter (flame photometer /fluorometer)

7. Recorder
8. Function monitor

System components

Sampling unit: The sampling unit enables to an operation to introduce unmeasured samples and standards into the autoanalyser system.

It consists of a circular turn table carrying around its rim 40 disposable polystyrene cups of 2 ml capacity.

The sample plate carrying these cups rotates at a predetermined speed.

The movement of the turntable is synchronized with the movements of a samplingcrook.

The hinged tubular crook is fitted at a corner of the base.

The crook carries a thin flexible polythene tube which can dip into a cup and allowthe contents water, standard (or) test solution to be aspirated. At regular intervolts, the crook is raised, so that the end of the sample tube is lifted clear of the cup.

Between each sampling, the crook enters a receptacle of water (or) suitable wash fluid to reduce cross – contamination of one sample with another.

The plate then rotates a distance sufficient to allow the tube, when it next moves down to dip into the nextcup.

One complete rotation of the plate thus presents 40 samples. Volume of the sample ranges from 0.2 to 1.0 ml.

In new version, thus successive samples are separated by a column of water instead of air. This provides a better separation.

Sample size : 0.1 to 8.5 ml

A programming can will help in rotational movement of the sampler.

Proportioning pump

The function of the proportioning pump is to continuously and simultaneously push fluids, air and gases through the analytical chain.

It is the heart of the automatic analysis system.

Samples and reagent stream is are driven by a single peristaltic pump which consists of two parallel stainless steel roller chains.

A series of flexible plastic tubes, one from the sampler, the other from reagent bottles (or) simply drawing in air is placed length wire along the platen.

The roller head assembly is driven by a constant speed gear motor.

When the rollers are pressed down and the motor switched on the compress the tubes containing, the liquid stream (sample, standard and reagents) against the platen.

Roller head rotates at a constant speed proportioning pumps are available either for single speed (or) for two-speed operation.

The single speed pump has the capacitor synchronous gear head utilizing 10 rpm output shaft at 50 Hz.

The two speed has a nonsynchronous 45 rpm motor.

In two speed operation, the slow speed helps in filling the system and washing draining.

High speed is not used for analysis.

Heavy duty pump is also available, which enables 23 pumps tubes to be used simultaneously.

Tubes are stretched before run

- Constant use causes

a) Loose – elasticity

b) Reduction in pump efficiency

Manifold

A manifold mainly consists of a platter, pump tubes, coils, transmission tubings, fittings and connections.

A separate manifold is required for each determinations.

The pump tubing and the connected coils are placed on a manifold platter, which keeps them in proper order for each test.

The pumping tubing are specially made they are of premeasured length and are meant to introduce all constituents of an analysis into the system.

Physical, chemical properties of the tubing are extremely important in the correct functioning of the pump.

It must be so flexible to maintain the flow rate.

- Standard transmission tubing
- Solva flex tubing
- Acid flex tubing
- Polyethyelene tubing
- Glass tubing

Size - 0.005 – 0.110 inch

Flow rate – 0.015 µl/min -3.90 µl/min

1) Mixing coil [2 types of coils]

Mixing coil are used to mix the sample (or) reagents. As the mixture rotate through a coil, the air bubble along with the rise and fall motion produces a completing homogeneous mixture.

Mixing coils are placed in a horizontal position to permit proper mixing

2) Delay coil

- Used to delay specimen for chemical reactions
- 40 ft long 1.6 mm in diameter Volume: 28ml

Dialyser

In analytical chemistry it is necessary to remove protein cells to obtain an interference free analysis.

This is accomplished by dialysis in the autoanalysis.

The dialyser module consists of a pair of Perspex plates, which are mirror grooved in continuous channel.

A semipermeable cellophane membrane is placed between the two plates and the assembly is clamped together.

The continuous groove channel thus get divided into two halves and the dialysis occur across the membrane.

A solution containing substance to be analysed passes along one half usually the upper one of the channel, while the solvent that is receptive to the substance to the substance to be removed enter the other half.

The substance to be separated from the sample diluents stream, will diffuse through the semipermeable membrane by armotic pressure into the recipient stream and the non- diffusable particles will be left behind.

The cellophane membrane usually used in the dialyser has a pore size of 40-60 Å. The rate of dialyser is stated to be dependent upon temp. 37^{o}C.

The temp is kept constant with a thermostatically controlled heater and a stirrer.

The channel path is 87 inch along which provides a large surface presentation to the dialysis membrane.

The plates of the dialyses most be matched set if the plates are not matched set the channels may be slightly off, causing leakage loss of dialyzing area, which would ultimately result in loss of sensitivity.

Quality of solute, concentration gradient, duration, area temperature, thickness and porosity determines the separation of protein during dialysis via semipermeable membrane.

Heating Bath

It is then passed to a heating. The heating bath is a double walled insulated vessel, in which a glass heating coil is immersed in a mineral oil. A thermostatically controlled heater maintain a constant temperature 0.1^{o}C, which can be read on thermometer.

Measurement of Techniques

Almost all automated analyser uses absorption as the major measurement.

Colorimeter:

Colorimeters used in the automated system continuously monitor the amount of light transmitted through the sample.

They employ flow through cuvettes. Eg: dual beam type colorimeter.

Recorders

The most common type of records used with automated system is the dc voltage nullbalance potentiometric recorder.Initially the setting of recorder pen is made to100% transmission.

Reagent blank kept running for sometime unit a smooth baseline is established on theafter obtaining a good baseline the run can be begun with a series of standardfollowed by the samples.

Since concentration of substance in a sample is related logarithmically to the percent transmission when this is plotted on a graph, the curve will not be linear.

Actual measurement are made only when it reaches steady state plateau.

Function monitor

Recording at steady state levels, the electrical output of the phototube is given to an oscilloscope called the function monitor.

Each sample curve is recorded entirely this enabling the operator to see all curves for each sample at all times.

SAMAC – II

The advanced version based on the auto-analyser concept is the Samac II (sequential multiple analysis plus computer).

This is a multichannel analyser which can perform 23 simultaneous analyser on the specimen.

168 specimen can be loaded on the system thereby SAMAC carries out about 2500 test results per hour.

The system is completely computer controlled usually two host computers and 10 microprocessors.

The system has two main subsystems.

- Analytical Processor: For instructing and monitoring analysis
- Results Processor: To provide data handling of results and preparation of edited report.

Approximately 600 µl of specimen is required for complete analysis. After the specimen

is sampled, diluted with distilled H_2O and segmented with air bubbles.

It is passed through a series of analytical cartridges (which contains reagent).

Each cartridge is a single channel analyser and includes peristaltic pump, pump tubes, mixing tubes and flow cells, pump tubes usually need 200 h of operation, dialyser for protein separation, heating bath for color development, spectrophotometry for quantitation for most analytes, Wavelength for each flow cell is selected through a computer, transmittance signalfrom the photomultiplier is converted to absorbance by a logarithmic amplifier, analogue signal is digitized in an A – D converter, Digital data are processed for peak detection, which is checked and tested for abnormal waveform, stored in memory.

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UNIT –IV – Microscopy – SBM1302

UNIT IV MICROSCOPY

Introduction

Microscopy is a study of different types of microscope and its clinical and functional features.

Microscopy is derived from a Greek word micro – too small, scope-too see.

Microscopes are the device (or) instrument used to see very minute organism which cannot be observed by our naked eye.

The study of different types of microscopes and their functional features can be termed microscopy.

Antony Von leewenhoek (1683), introduced microscope and he was known as father of microscopy.

Microscope is an instrument (or) device which magnifies the image that is too small to see in a naked eye.

Classification: Microscopes are of different types, classified based on two types:

i) Complexity of lens system

ii) Source of illumination

Basic definitions in microscope

Magnification: The optical system and the light rays magnifies the object.

Magnification varies from 100 – 4 lakhs. Magnification is obtained by the lens system. The lens near to the object is called objective lens and the lens near to the eye called eye piece

The rays from the object converged by the condenser lens it is magnified by the objective lens. Forming a real image which is further magnified by ocular lens and the form virtual image.

Magnification: Magnification Power of eye piece × Magnification power of objective lens.

Resolving power

It is the ability of the microscope to distinguish 2 adjacent point as distinct and separate is called resolving power.

Numerical aperture

It is a ratio of the diameter of the lens to the focal plane (or) focal length.

The distance between the centre of the lens to the focal point is called as the focallength.

The light rays will emerge and a meet at a point called focal point. NA = Diameter of lens /

focal length

The angle formed from the lens to the extreme rays to the objective lens is called as a aperture or 2 θ rays coming from edge of lens.

Limit of resolution

It is a smallest distance by which 2 object can be separated and still it can be viewed by the microscope as 2 separate objects.

It is based on the wavelength of source of illumination and NA. $d = \lambda / NA$

d - limit of resolution

λ - wavelength

2NA - Numerical aperture

When the limit of resolution is low the resolving power will be high.

Refractive Index

Refraction is a bent in the light rays that passes through one medium to another oil medium. Refractive index is calculated as RI = sin i /sin r (incident ray) / refracted ray R.Index varies within the medium

For eg.Oil =1.51 Water = 1.33

Air = 1

NA – The NA may be defined as a ratio of the diameter of the lens to the focal length (or) NA is the produce of the RI of the medium between the object and the objective lens.

NA = RI of the medium × (sin A – aperture) / 2 NA - n sin Θ

N - refractive index of the medium between objective × objects Θ - angle of aperture.

Oil immersion : To achieve high magnification with good resolution the lens must be small. The light rays should not be lost after they have passed through the stained specimen.

To preserve the direction of light rays at the highest magnification. Immersion oil is placed between the glass slides the oil immersion objective lens.

Light rays are refracted as the enter from the slide and the objective lens would have to be increased in diameter to capture them.

The oil has the same effect as increasing the objective diameter therefore it improves the resolving power of the lenses eg. Cedor wood oil.

Electron Microscope

Electron microscope are recent in origin and more sophisticated and useful than light

microscope. Knoll and Ruska developed in 1932.

Electron microscope differ in many aspect from optical microscope. Electron microscope uses a beam of electron as light source.

Electron microscope operated in a high vacuum condition.

Electron beam has a extremely shorter wavelength around 0.005 nm.

It provides greater resolving power that is more than 100 times than that of light microscope.

It provides magnification upto 4000 times.

Transmission Electron Microscope

It is a sophisticated microscope

Resolution 100 times better than other microscope.

Requirement:

- Electron gun
- Microscope column
- Electromagnetic lenses
- Fluorescent screen
- Transformer
- Vacuum pump
- Water cooling system

Electron gun

- It is present at the top of the microscopic body
- It acts as a source of illumination
- It contains the tungsten gun guarded by a cathode sheet and anode plate.
- Tungsten filament heated at the pressure electricity it releases electron.
- Electron get scattered and scattered electron are guarded by cathode sheet
- Anode plate helps in the pointing out the electrons to pass through a perfect path.

Electromagnetic lenses

- Electron cannot pass through the glass lens.

- Nut shaped electromagnetic lenses are used to flow the electron beam
- They are electromagnetic coils, that are coiled around a hollow metallic cylinder.
- Coils are connected to the high voltage current
- Current when pass through the coils, creates a magnetic field at the centre of hallow cylinder.
- Magnetic field helps for reflecting the electrons and magnifies the image.
- Magnetic objective lens
- Magnetic condenser lens
- Magnetic projector lens

Fluorescent Screen

It is made up of a fluorescence plate coated with flurophore that exits visible light. Electron beam falls on the fluorescent plate it gives the magnified image on the screen Image are recorded on the photographic plate.

These are used, since the electrons are harmful to our eyes.

Transformer

Electromagnetic coils requires high voltage. So it is connected to the transformer of a voltage about 220 - 500 kilovolts.

Vacuum pump

Electrons get deflected by the particles in air hence air must be vacuumised. So a clear image is obtained with high vacuum. Instrument is vacuumised with diffusion pump.

Water Cooling system

- Circulating pump
- Refrigerator plant and filter plants to prevent over heating.

Working

- Tungsten filament is heated
- It releases beam of electrons
- Beam of electrons passes through the condenser to specimen
- Since electron cannot pass through a glass lens. Electromagnetic lens Called magnetic lens are used to focus the beam of electrons
- When electron reaches the specimens scatters the electron

- Primary image is formed
- Projector lens latter magnifies the first primary image
- Final enlarge image of specimen is formed on screen (fluorescent)
- Magnified final can be recorded on a image photographic plate

Disadvantage

If the specimen is dry, external morphology cannot be viewed. Electrons have low penetration power on dry substances.

Very high vaccum condition can disturb the image formation.

Advantage

It helps to study, algae, bacteria viruses and fungi

Minute anatomical structure, internal structure can be visualized.

Scanning Electron Microscope

It operates from the difference principle, scattered (or) secondary electrons used I image formation.

It was discovered in 1960.

Principle

The specimen is coated with a thin film of metal. The beam of electron moves from one end of the surface to the other end and this electron are scattered back of the specimen and the image is formed the photographic plate.The specimen is usually coated with the gold because it increases the electrical conductivity and decreases the blurring of image

Construction

- Electron gun Electromagnetic lenses
- Detector and collector
- Amplifier (or) photomultiplier tube
- Cathode ray tube

Electron gun

- It is present at the top of the microscopic body
- It acts as a source of illumination
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- Magnetic objective lens
- Magnetic condenser lens
- Magnetic projector lens

Collector and detector

The secondary electron produced from the surface of the specimen arecollectedand transmitted to detector.

The secondary entering the detector then emits flashes or light. The emitted light rays converted into electrical signals.

Photomultiplier tube (or) amplifier

The signal is amplified and sent to cathode ray tube.

Cathode ray tube

It receives the amplified signal and forms a image on the screen.

Working

- Tungsten filament is heated
- It liberates electron
- These e^- more back and that is surface of object
- Primary image is formed
- Electrons generated, creates signals
- Electrons are amplified

- Later they are deducted by detector
- Electrons are passed through cathode ray tube
- Final image is formed
- Viewed and recorded on television receiver

Advantage

- Morphology of fungi, virus can be studied
- Dry specimen will not alter the morphology of specimen

Realistic 3 image of micro organism with great depth of focus and microorganism is in natural can be viewed. Eg: Bacteria in colon.

Disadvantage:

It has low resolving power internal components of the organism cannot be studied.

Scanning Tunneling Microscope (STM)

STM is a powerful technique for viewing surfaces at the atomic level. STM probes the density of states of a material using tunneling current. STM resolution is 0.1 nm - 0.01 nm.

STM can be used not only in ultra highvaccum but also in air, various other liquid (or) gas, ambient, at temperature ranging from 0 to a few hundred degree Celsius.

STM is based on the concept of quantum tunneling when a conducting tip is bought very clear to a metallic (or) semiconducting surface, a bias between the two can allow electrons to tunnel through the vaccum between them.

Variations in current as the probe passes over the surface are translated into an image.

STM can be challenging techniques, as it requires extremely clean surfaces and sharp tips.

Working

First the tip is brought into close proximity of the sample, coarse sample - to tip control.

- One tunneling is established piezoelectric transducers are implemented to move the tip in 3 directions
- As the tip is rastered across the sample in X-Y plane, due to the density State and therefore the tunnel current changes
- This change in current with respect to position can be measured

Itself (or) the height, z of the tip corresponding to a constant current can be measured These two modes are called

- Constant height mode
- Constant current mode

Constant current mode

Feed back electrons adjust the height by a voltage to the Piezoelectric height control mechanism.

This leads to height variation and thus the image comes from the tip topography across the sample and gives a constant charge density surface, this means contrast on the image is due to variation in charge density.

Constant height mode

In constant height the voltage and height are both held constant while the current changes to keep the voltage from changing, this leads to an image made current changes over the surface

which related to charge density.

Instrumentation

The components of an STM includes: Scanning tip, piezoelectric controlled height, XY scanner, coarse sample to tip control, vibration isolation system and computer. The resolution of a image is limited by the radius of curvature of the scanning tip of the STM.

The tip is often made up of tungsten platinum – iridium, tough gold is also used.

Magnetic levitation, was used to keep the STM free from vibrations, however, nowadays spring system are used.

Additionally mechanism for reducing eddy, currents are also implemented.

Maintaining the tip position with respect to the sample, scanning the sample in raster fashion and acquiring the data is computer controlled.

The computer is also used for enhancing the image with the help of image processing as well as performing quantitative morphological measurements control voltage for piezo tube.

Atomic Force Microscopy

AFM (or) scanning force microscope (SFM) is a very high resolution type of scanning probe microscope with resolution of fractions of nanometer.

Principle

AFM consists of a microscale cantilever with a sharp tip (probe) at its end that is used to scan the specimen surface. The cantilever tip is typically made up of Si (or) silica nitride. When the tip is bought into proximity of a sample surface, forces between the tip and the sample lead to a deflection of the cantilever according to Hoonke's law and describes about sample characteristics.

Working

- When the cantilever tip is bought into close proximity of a sample surfaces
- Forces between the tip and the sample lead to deflection of the cantilever
- Depending on the situations, forces that are measured in AFM include (mechanical contact forces, Vanderwaals force capillary forces, chemical Bonding electrostatic and magnetic forces)
- Deflection is measured using a laser spot reflected from the top of the cantilever
- Photodiodes.

Imaging Modes

AFM can be operated in number of modes depending on the application.

Contact Mode Operation

Force between the tip and the surface is kept constant during scanning by maintaining a constant deflection.

Dynamic mode operation

In dynamic mode, the cantilever is externally oscillated at close to its resonance frequency.

The oscillation amplitude, phase and resonance frequency are modified by tip sample interaction forces, these changes in oscillation with respect to the external reference oscillation. Provide information about the sample characteristics.

Advantages

- It provides 3D surface profile
- Sample do not require any special treatment that can change / damage the sample.
- Higher resolution
- Can wash in ambient air / liquid environment.

Disadvantages

- Can only image a maximum height on the order on micrometres and a maximum scanning area of 150 µms
- Incorrect choice of tips can leads to image artifacts.

Applications

- AFM helps in the study of biological membrane because it can resolve individual head region of the lipids.
- It is used to study membrane bounded proteins channels and receptor
- It has even been used to study DNA molecule and DNA – protein complexes.

Specimen Preparation

- Specimen used in electron microscope is very thin around 20 -100 nm
- Ultrathin sectioning is otherwise called as microtomy.

Microtomy

The process of cutting the cells in to very thin section is called microtomy. The specimen to be cut into slice must have a support and the support is provided a plastic by the process called fixation.

Fixation

Chemicals like glutarldehyde (or) osmium tetraoxide is used to stabilize the cell structure.

Dehydration

The specimen is dehydrated completely by organic solvent. Organic solvent like acetone (or) methanol is used.

Embedding

The specimen is soaked in unpolymerised liquid epoxy plastic until its completely permeated.

Plastic is harden to form a solid state.

Shadow casting

This technique involves depositing an extremely thin layer of metal eg. Platinum on the specimen.

Then specimen tired in vacuum jar.

Atom of heavy metals are projected on specimen producing a shadow behind the positive.

Examination of shadow image provides information as to the shape of specimen.

Negative staining

The specimen is cut into thin film and stained with phosphotungstic acid. These are heavy metal do not penetrate into the specimen and renders dark background.

The specimen appear bright against dark back it is method to study virus and cell inclusion.

Applications of electron microscope

1. It is used to examine the shape and size of the viruses a viral load.
2. It is used to measure the nanomaterials present in the any sample.
3. It is used to diagnose the human tissues at high magnification (ultrastructure level).
4. It is used to identifying the marker of cell differentiation to identify tumours and renal disease.
5. It is used to read about the biological materials (cryobiology) at low temperature eg. Proteins, cells, tissues, organs.
6. Electron microscope is used to study the toxic level of any substance.
7. Forensic study can be done with help of electron microscope.

Limitations of electron microscope

- Specimen being examined under a very high vacuum this cells cannot be examined in a

living state.

- Drying process may later some morphological characteristics.
- Low penetration power of the electron beam necessitating the use of thin sections to reveal the internal structure of the cell.

GAS ANALYSER

Paramagnetic Oxygen Analyser

Oxygen has the property of being paramagnetic in nature that is it does not have as strong magnetism as permanent magnets but at the same time it is attracted into a magnetic field. Nitric oxide and nitrogen dioxide are paramagnetic in nature, most gases are however slightly diamagnetic they are repelled out of a magnetic field. Paramagnetic oxygen analyser was first described by Pauling et al.(1946).

Principle The arrangement incorporates a small glass dumb bell suspended from a quartz• thread between the poles of a permanent magnet Pole places are wedge shaped in order to produce a non-uniform field.• When a small sphere is suspended in a strong non-uniform magnetic field, it is• subject to a force proportional to the difference between the magnetic susceptibility of his sphere.

The magnitude of this force can be F = C(K – K0)

C – magnetic field strength gradients,

K0 – Magnetic susceptibility of the sphere,

K – Magnetic susceptibility of the surrounding gas.

Forces exerted on the two spheres of the test body are thus a measure of the magnetic susceptibility of the sample and its O2 content is determined.

Construction of Instrument

The magnetic forces are measured by applying to one sphere an electrostatic force equal and opposite to the magnetic forces. The electrostatic force is exerted by an electrostatic field established by two charged vanes mounted adjacent to the sphere one vane is held at a higher potential than the test body, the other at a lower potential. Test body is connected electrically to the slider null adjust potentiometer – R2O. Potentiometer is a part of a voltage – dividing resistor network connected between ground and B+ potential to the test body can be adjusted over a large range. Exciter lamp directs a light beam onto the small mirror attached to the test body. From the mirror, the beam is reflected to a stationary mirror, onto a translucent screen, mounted on the front panel of the instrument, zero control – of the instrument is provided by ganged R13 – R15 which change the voltage present on each vane with respect to ground but does not change the difference in potential existing between them. This adjustment alters the electrostatic field. R19 –

provides span (or) sensitivity control. Working When no O2 is present the magnetic forces exactly balance the torque of the fibre O2 is present in the gas sample drawn in the chamber surrounding the dumb bell. It would displace dumb bell spheres. They would move away from the region of maximum magnetic flux density. Resulting rotation of the suspension Turns the small mirror and deflects the beam of light over a scale of the instruments. Scale is calibrated in % of volume of O2 (or) partial pressure of O2. Only a few improvement have been suggested and carried out in the development of O2 analyser. The original quantizer suspension has been replaced largely with a platinum – iridium suspension. Instead of measuring the deflection of the dumb bell a null balance system is preferred. Where the deflection is off-set by passing a current through a coil of wire attached to the dumb bell. The current required is proportional to the deflecting couple and thus to the O2 tension of the gas. Displacement of the dumb bell results in balancing the output from a pair of photocells. The difference in their output signals is fed to a differential amplifier which supplies HS o/p current to the dumb bell coil to null the deflection. Coil current is indicated on a meter. Oxygen analysers are available with continuous lead out 0 - 25% (or 0-100%) O2 Instruments are calibrated with the reference gas specified. Standard cell volume is 0 -10ml and response time is about1-S 9 Flow rate -60 – 250cc min (if sample enters through porous diffusion disc). If sample enters directly the flow rate is 40 – 60 cc/min.

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DEPARTMENT OF BIOMEDICAL ENGINEERING

UNIT –V – Chromatography – SBM1302

UNIT V CHROMATOGRAPHY

Definition

Chromatography is a physical method of separation of the components of a mixture by distribution between two phases of which one is stationary bed and other a fluid phase that percolates though along the stationary phase.

Chromatography was first reported by Russian Botanist classification.

Retention time (tr): The total time that o compound spends in both a mobile phase and stationary phase. In other words, the time between sample injection and aanalyte peak reaching detector at the end of the column is termed as retention time. It is generally expressed in mts.

Dead time (tm): The dead time is a time that anon retained compound spends in a mobile phase, which is also the amount of the time the non-retained compounds spend in the column.

Adjusted retention time (t'R): The adjusted retention time is the time that a compound spend in the stationary phase. The adjusted retention time is the difference between dead time and the retention time for a compound retention time for a compound.

$$T_t' = t_t - t_M$$

Capacity Factor / Partition Ratio (k′)

The capacity factor is the ratio of the mass of the compound in the stationary phase relative to the mass of the compound in the mobile phase.

$$K = (t_r - t_M) / T_M$$

Distribution Constant

Ratio of the concentration of a compound in the stationary phase relative to the concentration of the compound in the mobile phase.

$$KD = \frac{\text{Concentration of a CPD in stationary phase}}{\text{Concentration of a compound in mobile phase}}$$

The linear velocity is the speed not which the carried gas (or) mobile phase travels through the column. Linear velocity expressed in centimeters per second.

$$\mu = L/tM$$

Definition

Paper chromatography is defined as the technique in which the analysis of unknown substances is carried out mainly by the flow of solvents on specially designed filter paper.

Linear velocity (μ)

The linear velocity is the speed not which the carried gas (or) mobile phase travels through the column. Linear velocity expressed in centimeters per second.

$\mu = L / t_M$

Definition

Paper chromatography is defined as the technique in which the analysis of unknown substances is carried out mainly by the flow of solvents on specially designed filter paper.

Types of Chromatography

i) Paper adsorption, ii) Paper partition.

Paper adsorption: Paper impregnated with silica act as adsorbent (stationary phase) and solvent as mobile phase.

Paper partition: Moisture / water present in the pores of cellulose fibres present in filter paper act as stationary phase and solvent as mobile phase.

Principle

The principle of separation is mainly partition rather than adsorption. Cellulose layers in filter paper contains moisture which act as stationary phase. Organic solvents (or) buffers are used as mobile phases.

Requirements

- Stationary phase and paper used.
- Application of sample
- Mobile phase
- Development technique
- Detecting and visualizing agents

Stationary phase and paper used

Whattman filter paper of different grade like no.1, 2, 3. Choice of filter paper depends upon thickness, flow rate and purity. Eg: Modified filters: Acid (or) base washed paper, filter paper.

Application of Sample

The sample to be applied is dissolved in the mobile phase and applied using capillary (or micropipette.

Concentration of sample must be low.

Mobile Phase

Pure solvents, buffer solutions (or) mature of solvents are used. Eg:Hydropholic mobile phases, n-butanol, glacial acetic acid.

Development technique

Ascending development: The solvent flour against gravity. The spots are kept at the bottom portion of paper and kept in a chamber with mobile phase solvent at the bottom.

Detecting (or) visualizing agents: After the development of chromatogram the spot should be visualized.

Detecting colored spots can be done visuality.

But for detecting colorless spot any one of the technique can be used.

A) Non specific methods

1) Iodine chamber (brown colored spots are observed. When iodine crystals are applied. 2) UV – chamber for fluorescent compounds.

B) Specific methods

Specific spray reagents (or) detecting agents (or) visualizing agents are used to find out the nature of compound. Eg: ninhydrin in acetone for aminoacids, radioactive compound – autoradiography is used.

Quantitative analysis

1) **Direct method**: Densitometer is an instrument which measures the density of spots.
2) **Indirect method**: In this technique the spots are cut in to portions and eluted with solvents – solutions can be analysed by spectrophometry.

Rf value is the ratio of distance travelled by the solute to the distance travelled by the solvent front

$$R_f = \frac{\text{Distance travelled by salute}}{\text{Distance travelled by solvent front}}$$

$$R_x = \frac{\text{Distance travelled by sample}}{\text{Distance travelled by standard}}$$

Applications

- Paper chromatography is more useful for the analysis of polar compounds like aminoacids, sugars and natural products
- Separation of mixtures of drugs of chemical (or) biological origin, plant extracts.
- Separation of vitamins, antibiotics and proteins.
- Identification of foreign substances in drugs.
- Identification of decomposition products.
- Analysis of metabolites of drugs in blood, urine etc.

Identification of drug

- Drug
- Mobile phase
- Detecting agent
- Erythromycin
- Isobuttyl methyl ketone
- Nutrient agar containing
- Bacillus spp

Identification of related compounds

- Drug
- Mobile phase
- Detection Agent
- Vitamin A
- Diaxn Methanol 70 : 15
- UV – 366 nm

Thin Layer Chromatography (TLC)

Principle:

The principle of separation is adsorption one or more compounds are spotted on a thin layer of adsorbent coated on a chromatographic plate. The mobile phase solvent flows through because of capillary action (against gravitational force). The components move according to their affinities towards the adsorbent. The component with more affinity towards the stationary phase travels slower. The component with lesser affinity towards the stationary phase travels faster.

Thus the components are separated on a TLC based on the affinity of the components towards the stationary phase.

Practical requirements

- Stationary phase (silica gel – G)
- Glass plates
- Preparation and activation of TLC plates
- Application of sample
- Development tank
- Mobile phase
- Development tank
- Mobile phase
- Development technique
- Detecting (or) visualizing agents

Stationary phases

Several adsorbents which can be used as stationary phases, their composition and ratio in which they have to be mixed with H2O (or) solvents to form a slurry has to be calculated. Eg. Silica gel – G, Silica +CaSO4 1: 2

Glass Plates

Size -20 cm × 20 cm or 20 cm × 10 cm

Glass plates should be of good quality to withstand temperature for drying the plats.

Preparation and activation of TLC plates

After preparing the slurry

↓

By spreading (TLC spreader) technique

↓

Slurry is poured inside the reservoir of TLC spreader

↓

The thickness of the adsorbent layer is adjusted by using a knob in spreader

↓

Spreader is rolled only once on the plates

↓

Plates are allowed for setting (air drying)

↓

Plates are activated by keeping in an oven at 100^{o}C for 1 hour

Application of sample

Sample concentration 2 – 5µl

Sample is applied by using capillary tube (or) micropipette.

Spot should be kept at least 2 cm above the base of the plate and spotting area should not be immersed in the mobile phase in the development tanks.

Development tank

Developing tank or chamber of different sizes to hold. The plates of standard dimensions are used.

Mostly glass beakers, jars are used to avoid wastage of solvents.

It is better to use development tank.

Mobile Phase

Mobile phases depends upon various factors.

1) Nature of substances to be separated

2) Nature of stationary phase used. Eg: Petroleum ether, ethylacetate alcohols, cyclohexane, chloroform.

Development technique

One dimensional development (vertical)

In this technique the plates are kept vertical and the solvent flows against gravity, because of capillary action. Most separations done practically are of this type only.

Detecting (or) visualizing agents

After the development of TLC plates the spots should be visualized.

Detecting coloured spots can be done visuality

For colorless spots any one of the technique can be used.

Iron specific method

Iodine chamber method: where brown (or) amber spots are observed when the TLC plates are kept in a tank with few iodine crystals at the bottom.

Eg: 1. Sulphuric acid spray reagent, 2. UV chamber for fluorescent cpds.

Specific method

1) Ferric chloride for phenolic cpdsninhydrin – for aminoacids.

Qualitative analysis

R_f – retardation factor

$$R_f = \frac{\text{Distance travelled by salute}}{\text{Distance travelled by solvent front}}$$

R_f value ranges from 0 to 1.

When the R_f value of a sample and reference compound is same, the compound is identified by its standard.

Quantitative analysis

Direct method – the quantity of the individual spots can be determined by using denistomeric method.

Indirect method

Quantitative analysis can be done after eluting the individual spots with solvent and filtering off the phase and concentration is measured by using UV spectrophotometry.

Applications of TLC

1. Separation of mixtures of drugs of chemical plant extract etc.
2. Separation of carbohydrates, vitamins, antibiotics proteins.
3. Identification of drugs. Drugs, Stationary Phase, Mobile Phase, Detecting AgentmChloropromazine, Silica Gel G, Ether : Ethtylacetateand UV -254 nm

Column chromatography

Principle: When a column of stationary phase is use of the technique is called as column chromatography. Based on the nature of stationary phase that is whether it is solid (or) liquid it is called as column adsorption chromatography or column partition chromatography.

A solid stationary phase and a liquid mobile phase is used and the principle of separation is adsorption. When a mixture of components dissolved in the mobile phase is introduced into the column. The individual components move with different rates depending upon their relative affinities.

The compound with lesser affinity towards the stationary phase (adsorbent) moves faster and hence it is eluted out of the column first. One with greater affinity towards the stationary phase (adsorbent) moves slower down the column and hence it is eluted later. Thus the Cpds are separated.

$$R = \frac{\text{Distance moved by the solute}}{\text{Distance travelled by solvent front}}$$

(rate of movement of movement)

Practical Requirements

1. Stationary phase (adsorbents)

   i) Particles should have uniform size and distribution spherical shape

   ii) Should have mechanical stability

   iii) Should be inert should not react with the solute and other components.

   iv) Insoluble in mobile phases

   v) In expensive and should separate wide variety of compounds.

Eg:

Weak	**Medium**	**Strong absorbent**
Sucrose	$CaCO_3$	Activated Mg silicate
Starch	$Ca_3 (PO_4)_2$	Activated alumina

Silica gel is commonly used as stationary phase

Mobile Phase

Mobile phase is very important and they act as solvent, developer and as eluent. Eg.Petroleum ether, carbon tetra chloride, ether acetone, chloroform, alcohol and H_2O.

Column Characteristics

The material of the column is mostly good quality neutral glass since it should not be affected by solvents, acids (or) alkalies.

An ordinary burette can also be used as column for separation.

Size -100 : 1(length : diameter for more efficiency).

Column depends upon

1. Affinity of compounds towards the adsorbent used.
2. Type of adsorbent used
3. Quantity of the sample

Preparation of the column

The bottom portion of the column is packed with cotton wool (or) glass wool, above which the column of adsorbent is packed.

After packing the column with the adsorbent, a similar paper disc is kept on the top so that the adsorbent layer is not disturbed, which will leads to the formation of irregular bands in separation.

Column is prepared by wet packing technique.

Wet packing technique: This is the ideal technique. The required quantity of the adsorbent is mixed with the mobile phase and poured into the column.

The stationary phase settles uniformly in the column and there is no entrapment of air bubbles.

There will not be any crack in the column of adsorbent.

Thus the bands eluted from the column will be uniform and ideal for separation.

Sample application

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

The entire sample is introduced into the column at once and gets adsorbed onto the top portion of the column.

Development technique (elution)

The individual components are separated out from the column. The two techniques are:

i) Isocratic elution technique

In this elution technique the same solvent composition (or) solvent of same. Polarity in used throughout the process of separation. Eg.Chloroform.

ii) Gradient elution technique

In this elution technique, solvents of gradually increasing polarity (or) increasing elution strength are used during the process of separation.

Initially low polar solvent is used followed by gradually increasing the polarity, to a more polar solvent. Eg: Initially benzene, then chloroform, ethyl acetate.

Detection of components

The detection of colouredcomponents can be done visually. Different coloured bands are seen moving down the column which can be collected separately.

For detecting colourless compounds

i) UV-vis-detector

ii) Fluorescence detector.

Recovery of components

The best technique is to recover the components by a process called as elution. The components are called as eluate, the solvent called as eluent and the process of removing the components from the column is called as elution.

Recovery is done by collecting as different fractions of mobile phase of equal volume like 10 ml, 20 ml etc. (or) unequal volume. They can also be collected time wise fraction ever 10 to 20 mts. Fractions are measured by using UV-spectrophotometer.

Applications

1. Separation of mixture of Cpds
2. Isolation of bioactive constituents (components)
3. Isolation of metabolites from biological fluids
4. Estimation of drugs in formulations
5. Purification process

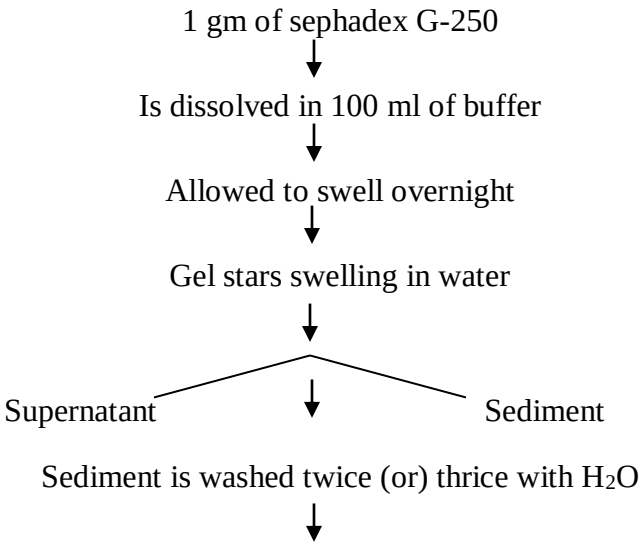
Principle

Gel permeation chromatography, the separation is based on molecular size and shape. The gel permeation column is packed with a stationary phase in the form of a gel which contains pores of a specific size.

As the sample is carried through the column bed by the carried liquid, the sample molecules penetrates through the pores in the gel depending upon the size and shape of the molecules it is separated

Large molecules do not penetrates the gel and are consequently quickly eluted.

Preparation of Sephadex G-20



Sediment act as gel and packed in column

Packing of column

Column size	-	75 cm
Gel	-	Sephadex G-250 (3 – 5)
Buffer	-	Tris 10.5 N, pH 7.5 + 0.15 M NaCl
Sample	-	Serum

Sample Applications

Sample is applied carefully over the buffer which drags the sample to the gel bed to enhance polymerization.

Since ptn has larger molecular weight it soon eluted out and other impurities with low molecular weight will enters the gel beads and are later eluted out.

After elution is completed by using elution buffer the fractions is collected by using test tube subjected to UV – spectrophotometer and OD valve is taken at 280 nm.

To determine the concentration of ab's.

Applications refer ion exchange and column

Ion Exchange resins (classification of resins)

Ion exchange chromatography is the process by which a mixture of similar charged ions can be separated by using an ion exchange resin which exchanges ions according to their relative affinities.

Principle

The principle of separation is by reversible exchange of function groups between the ions present in the solution and those present in the ion exchange rein.

Cation exchange: (separation of cations using CER). The cations to be separated are present in solution and exchange for similar ions present in cation exchange resin a solid matrix.

It is represented solid – $H^+ + M^+ \rightarrow$ solid – $M^+ + H^+$ (solution)

The cations retained by the solid matrix of IER can be eluted by using buffers of different strength and hence separation of cations can be effected.

Anion exchange (Separation of anions using anion exchange resin)

The anions to be separated are present in solution and exchange of similar ions present in anion exchange resin – a solid matrix.

Solid OH + A (Solution) $\rightarrow$ Solid – A + OH(solution)

The anions retained by the solid matrix of IER can be eluted by using buffers of different strength and hence separation of anions can be effected.

Ion exchange resins (classification of resins)

1. Strong cation exchange resin
2. Weak cation exchange resin
3. Strong anion exchange resin
4. Weak anion exchange resin

} According to chemical nature

Eg: Organic ion exchange resin are widely used organ (IER) are polymeric resin matrix containing exchange sites.

The resin is composed of polystyrene and divinnyl benzene.

Poystyrene→contains sites for exchange function groups

Divinyl benzene→act as cross linking age (mechanical stability)

Eg: Functional groups present in different IER

Strength cation exchange resin	-	SO_3H – sulphonic acid group
Weak cation exchange resin	-	COOH – Carboxnyl
Strong anion exchange resin	-	NR2 – 2^oAmine
Weak anion exchange resin	-	NH_2 Amine

Properties of rein

Particle size – 50 – 200 mesh, should allow free and uniform flow of mobile phase

Cross linking and swelling – optimum quantity of cross linking agent is required for effective separation.

Practical requirements

Column material and dimensions

Column are made up of glass

Which are resistant to strong acids and alkalies

Size – 20 : 1 to 100 : 1 for higher efficiency can be used.

Type of ion exchange resin

Packing of the column – Wet packing method is used.

The resin is mixed with the mobile phase and packed in the column uniformly. The ample to be separated is dissolved in the mobile phase and introduced all at once in to the column.

Mobile Phase

Only different strengths of acids, alkalis and buffers are used as eluting solvents. Eg: 0.1 N HCl, 1 N NaOH, acetate buffer, PoA buffer.

Development of the chromatogram and elution

After introduction of sample

↓

Using acids, alkalis and buffers of different pH are used

↓

To separate the compounds.

Elution: Isocratic elution technique, Gradient elution technique.

Isocratic elution technique – Same solvent composition is used. Same strength of acid, alkali or buffer.

Gradient elution technique – Initially less acidic or basic character is used followed by increasing the acidity or basicity of mobile phase.

Gradient elution is used for complex mixture fractions of the eluent is collected. Volume wise or time wise and analysed.

Analysis of the elute

Fracture collection analysed by using spectrophotometric method and radiochemical method.

Regeneration of the ion exchange resin

Regeneration refers to the replacement of the exchangeable cations (or) anions present in the original resin.

Regeneration of cation exchange resin

By charging the column with strong acid line HCl.

Regeneration of anion exchange resin

By using strong alkali like NaOH.

Applications

1. Softening of H_2O
2. Demineralisation (or) deionization of H_2O
3. Purification of some solutions to be free from ionic impurities.
4. Separation of inorganic ions (cations and anions).

5. Organic separation Eg: aminoacids, proteins antibiotics vitamins fatty acids etc.

6. Biochemical separations like isolation and some drugs (or metabolites of blood, urine etc.

Gas Chromatography

Gas chromatography consists of a gas solid chromatography (GSC) and gas liquid chromatography (GLC)

In both types gas is used as mobile phase and either solid (or) liquid is used as stastionary phase.

Principle

The principle of separation in GLC is partition gas is used as mobile phase. Liquid which is coated onto a solid support is used as stationary phase. The mixture of components to be separated is converted to vapour and mixed with gaseous mobile phase.

The component which is more soluble in the stationary phase travels slower and eluted later. The component which is less soluble in the stationary phase travels faster and eluted to out first.

The components are separated according to their partition coefficient.

Partition coefficient is the ratio of solubility of a substance distributed between two immiscible liquids at a constant temperature. Criteria for compounds to be analysed by GC>

Two important criteria are

1) Volatility : Less a compound is volatile it cannot be mixed with mobile phase. Hence volatility is important.

2) Thermostability: All the compounds will be in the form of vapour. There will be solid as well as liquid samples.

Hence to convert them to a vapour form they have to be heated at a higher temperature. At that temperature the compounds have to be thermostable they are not thermostable the compounds can be analysed by GC. Since they will be decomposed.

Practical requirements

1. Carrier gas
2. Flow regulators and flow meters
3. Injection device
4. Columns
5. Temperature control devices
6. Detectors

7. Recorders and integrators.

Carrier gas

The choice of carried gas determine the efficient of chromatographic separation

Most widely used carrier gases are : hydrogen, helium, nitrogen an argon.

Hydrogen: Better thermal conductivity, it is useful in cases of thermal conductivity detector a flame ionization detector.

Helium: Excellent thermal conductivity, thermal conductivity detector expensive.

Flow regulators and flow meters

As carrier gases are stored under high pressure flow regulators are used to deliver the gas with uniform pressure (or flow rate.

Flow method are used to measure the flow rate of carrier gas. Eg.Rotameter and soap bubble flow meter.

Gases – introduced into the column by valve devices.

Liquid – most GS instruments have a high quality rubber septum through which sample is injected.

Rubber is made up of good quality silicone rubber which can withstand high temperature of preheating device and withstand repeated injections over a period of time.

Solid – Samples are dissolved in a suitable solvent and then they are injected through a septum.

Columns

Column is one of the important part of GC which decides the separation of efficiency. Column are made up of glass $\rightarrow$ inert – do not react with sample, high fragile difficult to handle, or stainless steel $\rightarrow$ long file no fragility, but react with sample.

Column can be i) Analytical, ii) preparative

Analytical – 1 – 1.5 m, 3 – 6 mm (diameter) – only small amount of sample can be loaded.

Preparative – 3 – 6 M – 6 – 9 mm – High amount of sample can be loaded.

Eg. Open tubular column (or) capillary (or) go lay column

30 – 90 m in length, 0.025 – 0.075 cm. Made up of stainless steel in the form of coil.

Inner wall of capillary is coated with stationary phase liquid in the form of thin film

Column more resistance to the flow of carrier gas.

Temperature control device

Pre-heaters: are used in GC to converter the sample into its vapour form and mix them with the mobile phase (or) carrier gas.

Preheaters are present along with injecting device. As soon as liquid samples are injected they are converted into vapour form.

Thermostatically controlled oven

Detectors which can detect the difference between a pure carrier gas and a eluted component. Eg.Flame ionization detection.

The carrier gas used with this type of detector can be hydrogen.

This ionization detectors are based upon the electrical conductivity of carrier gases.

When pure carrier gases along passes there is not ionization and no current flows, when a component emerges from the column number of samples converted.

Ions are produced because of ionization of the thermal energy of the flame.

This causes a potential difference and causes a flow of current which is amplified and recorded as signal.

Advantages

This detector is extremely sensitive and back ground noise is low hence µg quantities of the solute can be detected. Linearity is excellent.

Stable and insensitive to small changes in the flow rate of carrier gas and water vapour.Responds to most of the organic compounds.

Recorders: are used to record the responsive obtained from detectors amplification, they record the baseline concentration and all the peaks obtained with respect to time.

Retention time for all the peaks can be found out from such recordings.

Integrators: They are improved various of with data processing capabilities.

They can record the individual peaks with RT, height and width of peak, peak area, percentage of area.

Applications

1. Assay of drugs
2. Presence of foreign (or) related substance
3. Purity of compounds
4. Isolation and identification of drugs (or) metabolites in urine, plasma and serum.

5. Isolation and identification of mixture of components like amino acids, plant extract etc.

High performance liquid chromatography (HPLC)

Introduction: The technique of HPLC is so called because of its improved performance when compared to classical column chromatography.

It is also called as HPLC since high pressure is used when compared to classical column chromatography. The development of HPLC from classical column chromatography can be attributed to development of small particle sizes. Smaller particle size is important since they offer more area over the conventional larger size particle.

Types of HPLC technique

Based on modes of chromatography – normal phase mode: In normal phase technique, a polar charge is used and nonpolar mobile phase is used.

Reverse phase mode: In reverse phase technique, a non-polar stationary phase and polar mobile phase is used. Hence polar components gets eluted. First and nonpolar components are retained for longer time. Since most of the drugs and pharmaceuticals are polar in nature they are not retained for a longer time and eluted faster which is advantageous.

Based on principle of separation

1. **Adsorption chromatography**: The principle of separation is separation of components takes place because of the difference in affinity of compounds towards stationary phase.

2. **Ion-exchange chromatography**: The principle of separation is ion exchange which is reversible exchange of functional groups. ion-exchange chromatography, an ion exchange resin is used to separate a mixture of similar charged ions for cations, a cation exchange resin is used. For anions, an anion exchange resin is used.

3. **Gel permeation chromatography**: In this type of chromatography, a mixture of components with different molecular sizes are separated by using gels. The gels used acts as molecular sieve and hence a mixture of substances with different molecular sizes are separated.

4. **Affinity chromatography**: uses the affinity of the sample with specific stationary phases.

Based on elution technique

Isocratic technique: In this technique the same mobile phase combination is used throughout the process of separation. The same polarity (or) elution strength is maintained throughout the process.

Gradient separation: In this technique, a mobile phase combination of lower polarity (or) elution strength is used followed by gradually increasing the polarity or elution strength. **Based on the scale of operation** : 1) analytical HPLC (2) preparative HPLC.

Based on the type of analysis: Qualitative analysis – which is used to identity the compound, detect the presence of impurities in this done by using retention time values.

Quantitative analysis: which is done to determine the quantity of the individual (or) several components in a mixture this is done by comparing the path area of the standard of sample.

Principle of separation in HPLC

The principle of separation in normal phase made and reverse phase mode is adsorption. When a mixture of components are introduced into a HPLC column, they travel according to their relative affinities towards the stationary phase. The components which has more affinity towards the adsorbent travels slower. The component which has less affinity towards the stationary phases travels faster. Since no two components have the same affinity towards the stationary phase, the components are separated.

Instrumental requirements

1. Pumps – solvent delivery system
2. Mixing unit, gradient controller and solvent degassing
3. Injector – manual (or) auto injection.
4. Guard column
5. Detectors
6. Recorders and integrators

Solvent delivery system: The solvents (or) mobile phases used must be passed through the column oil high pressure at about 1000 – 3000 psi. This is because as the particle size of stationary phase (sp) is few µ (5 – 10 µ) the resistance to the flow of solvent is high. Hence such high pressure is recommended. The solvent used must be of high purity preferably HPLC grade and filtered through 0.45 µ filter.

Pump: There are different types of pump available they are mechanical pump and pneumatic pumps. Mechanical pumps operate with constant flow rate this is used in analytical scale. Pneumatic pumps operate with constant pressure and use highly compressed gas.

Mixing unit: Mixing unit is used to mix solvents in different proportions and pass through the column mixing of solvents is done either with a static mixes which is packed with beads (or) magnetic stirrer and operates under high pressure.

Gradient controller: (refer) – gradient elution. Gradient controller is used when two (or) more solvent pumps are used for such separations.

Solvent degassing: When solvents are pumped under high pressure, gas bubbles are formed which will interfere with the separation process, steady baseline and the shape of the peak. Hence degassing of solvent is important which is done by using ultrasonication method.

Injector (Manual (or) autoinjector) eg.Rheodyne injects. It is the most popular injector. This has a fixed volume loop like 20 µl (or) 50 µl or more. Injector has two modes that is load position when the sample is loaded in the loop and inject mode when the sample is injected.

Analytical column: Analytical column is the most important part of the HPLC technique which decides the efficiency of separation.

Column material: The column are made up of either stainless steel, polyethylene, peek (polyether ether) all these withstand high pressure.

- Column length – 5 cm – 30 cm
- Column diameter -2mm – 50 mm
- Particle size – 1 – 20 µ
- Particle nature – spherical, uniform size, porous material.

Surface area : 1 gm of S.P provides surface area ranging from 100 - 800 sq.m with an average of 400 sq.m. eg: Silanol group, C_{18}octadecylsilane (ODS – column)

Detectors : used depends upon the property of the Cpds to be separated.

Eg. UV detector, this detector is based upon the light adsorption characteristics of the sample. Two types of this detector are available one is the fixed wavelength detector which operates at 254 nm where most drugs compound absorb. The other is variable wavelength detector which can be operated from 190 nm – 600 n.

Records and Integrators : Refer – GC

Application of HPLC: HPLC is being more widely used in several fields. Apart from the use an pharmaceutical field. It is used in chemical and petrochemical industry, environmental applications, forensic applications, biochemical separations, biotechnology, food analysis etc. In fact there is not field where HPLC is not being used. It is a versatile and sensitive technique which can be used in several techniques.

1. Qualitative and quantitative analysis of a given sample.
2. Checking the purity of a CPD and presence of impurities.
3. Isolation of identification of drugs or metabolites in urine, plasma, serum etc.
4. Isolation and identification of mixture of Cpds.
5. Multicomponent analysis (or) determination of mixture of drugs.
6. Identification of related Cpds
7. Assay of drugs