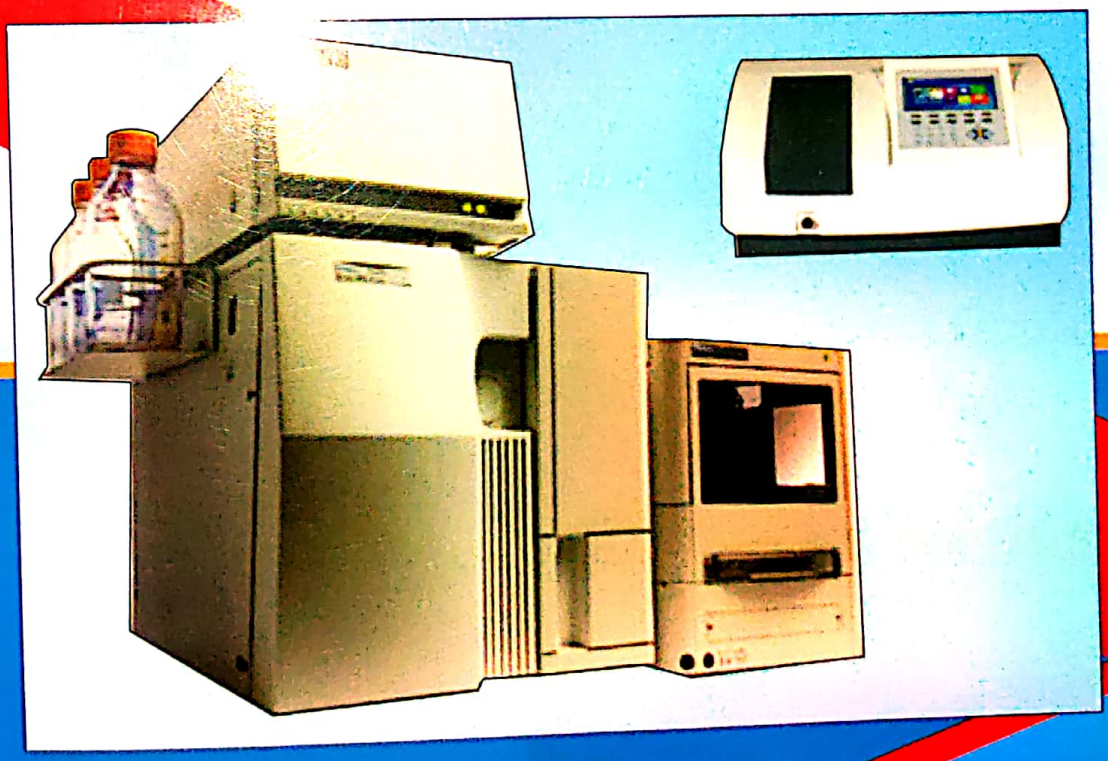
AS PER PCI REGULATIONS
FINAL YEAR B. PHARM.

SEMESTER
VII

INSTRUMENTAL METHODS OF ANALYSIS

Dr. K. R. MAHADIK Dr. L. SATHIYANARAYANAN



NIRALI PRAKASHAN
ADVANCEMENT OF KNOWLEDGE

A Text Book of

INSTRUMENTAL METHODS OF ANALYSIS

As Per PCI Regulations

FINAL YEAR B. PHARM.

Semester – VII

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Syllabus

Unit I

(10 Hours)

UV Visible spectroscopy

Electronic tranhsitions, chromophores, auxochromes, spectral shifts, solvent effect on absorption spectra, Beer and Lambert's law, Derivation and deviations. **Instrumentation:** Sources of radiation, wavelength selectors, sample cells, detectors Photo tube, Photomultiplier tube, Photo voltaic cell, Silicon Photodiode.

Applications: Spectrophotometric titrations, Single component and multi component analysis.

Fluorimetry:

Theory, Concepts of singlet, doublet and triplet electronic states, internal and external conversions, factors affecting fluorescence, quenching, instrumentation and applications.

Unit II

(10 Hours)

IR spectroscopy:

Introduction, fundamental modes of vibrations in poly atomic molecules, sample handling, factors affecting vibrations

Instrumentation - Sources of radiation, wavelength selectors, detectors - Golay cell, Bolometer, Thermocouple, Thermister, Pyroelectric detector and applications

Flame Photometry: Principle, interferences, instrumentation and applications

Atomic absorption spectroscopy: Principle, interferences, instrumentation and applications

Nepheloturbidometry: Principle, instrumentation and applications

Unit III

(10 Hours)

Introduction to chromatography

Adsorption and partition column chromatography: Methodology, advantages, disadvantages and applications.

Thin layer chromatography: Introduction, Principle, Methodology, Rf values, advantages, disadvantages and applications.

Paper chromatography: Introduction, methodology, development techniques, advantages, disadvantages and applications.

Electrophoresis: Introduction, factors affecting electrophoretic mobility, Techniques of paper, gel, capillary electrophoresis, applications.

Unit IV

(08 Hours)

Gas chromatography: Introduction, theory, instrumentation, derivatization, temperature programming, advantages, disadvantages and applications

High performance liquid chromatography (HPLC)-Introduction, theory, instrumentation, advantages and applications.

Unit V

(07 Hours)

Ion exchange chromatography: Introduction, classification, ion exchange resins, properties, mechanism of ion exchange process, factors affecting ion exchange, methodology and applications

Gel chromatography: Introduction, theory, instrumentation and applications

Affinity chromatography: Introduction, theory, instrumentation and applications.

Contents

UNIT - I

Chapter ... 1

INTRODUCTION TO INSTRUMENTAL TECHNIQUES

Objectives:

Upon completion of this section, the student should be able to

- Understand the basics of instrumental analytical techniques.
- Explain the types and physical properties involved in various instrumental techniques.
- Explain the function, advantages and disadvantages of instrumental techniques.

1.1 INTRODUCTION

The term **Pharmaceutical analysis** can be explained as qualitative and quantitative determination of the ingredients used in a pharmaceutical formulation using a well-defined methodology. The method ranges from simple qualitative colour reaction tests to analytical methodologies using sophisticated analytical equipments. The qualitative analysis gives the information about the quality of the sample or in simple terms, it reveals the identity of the substances to be analysed while the quantitative analysis reveals the amount or quantity of ingredients present in the pharmaceutical product. Conventional analytical methodologies involved manual procedures using simple glassware such as burette, pipette, conical flask and beakers whereas the introduction of various analytical instruments resulted in more accurate and reproducible results. Such analytical methods using instruments are called as instrumental analytical techniques. This chapter focuses on such instrumental techniques.

Analytical methods are generally classified into instrumental and non-instrumental techniques. In the former, measurement of some physical property is made to determine the contents or composition of a substance, while in non-instrumental, the conventional physico-chemical properties are used to analyze the sample. The non-instrumental methods which are primarily based upon the measurement of mass and/or volume are also called as chemical methods; and include the techniques of volumetric and gravimetric analysis. These even now continue to occupy their place in analytical technique because of simplicity, ease and reproducibility. The instrumental methods of analysis are based upon the measurement of some physical property of substance using instrument to determine its chemical

composition. Table 1.1 gives the list of measurement of physical properties involved and the respective instrumental method adopted.

Table 1.1: Physical Property and Instrumental Method

Physical Property involved	Instrumental Method adopted
Electrical potential	Potentiometry
Electrical conductance	Conductometry
Quantity of electricity	Coulometry, Electrogravimetry
Electrical current	Polarography, Amperometry, Coulometry
Absorption of radiation	UV, visible, IR spectrophotometry, Atomic absorption spectrophotometry, Nuclear magnetic resonance spectrophotometry.
Emission of radiation	Emission spectroscopy, Flame photometry, Fluorometry, Radiochemical methods.
Scattering of radiation	Turbidimetry, Nephelometry, Raman spectroscopy.
Refraction of radiation	Refractometry, Interferometry.
Rotation of radiation	Polarimetry, Optical rotatory dispersion, Circular dichroism.
Diffraction of radiation	X-ray diffraction method.
Thermal properties	Thermo Gravimetric Analysis (TGA), Differential Scanning Colorimetry (DSC).
Mass to charge ratio	Mass spectrometry.

Physical property of a substance may be specific or non-specific. In both cases, appropriate measurements are made and their signals are converted into units which are used for determination of structure or concentration.

Instruments used in chemical analysis are unique in their function. They do not give direct quantitative data but supplies information which can be converted into a suitable form which correlates with the structure or the content. Thus, it acts as a communicative device. It consists of the following steps:

1. **Signal Generation:** Signal denotes the response from the analyte which is to be analysed in the experiment. A signal generator produces the signal which is indicative of the analyte and its concentration. In case of pH meter, the hydrogen ion concentration from the solution acts as a signal generator. In colorimeter, the colour of the solution and the radiation source are the signal generator.
2. **Signal Transformation:** Generally, the signal generated is not in the measurable form. Hence, it has to be converted into a more conveniently measurable unit. This work is done by the use of transducers. In pH meter, the glass calomel electrode

converts the signal into electrical potential while in the case of colorimeters and spectrophotometers, the radiant energy is converted into electrical energy with the help of the thermocouples, phototubes, photo multiplier tubes etc.

3. **Amplification:** Sensitivity of the instrument is more important. The signal which is generated should be sufficient enough to be sensed by the detector. The transducer signal is generally modified by signal processors to make it more convenient for operation. Generally, it involves amplification of signal. Most of the time amplification is done electronically to increase the sensitivity.
4. **Readout system:** This is the system which reads the amplified signal for the purpose of documentation. The processed signals are converted into observable signals. The transducer and amplified signal is presented as a displacement along the scale or on the chart of the recorder. It involves the deflection of needle of galvanometer, deflection of light from mirror of the galvanometer, or displacement of meniscus of burette or blackening of photographic phase.

There is a tremendous development in the field of instrumentation analysis, with the development of electronics. Modern analytical instruments employ microprocessors, computers, amplifiers and integrated circuits to get a rapid and reproducible signal every time.

It is essential for every instrument that it should give rapid response and the response should be quantitative and proportional to the information it receives. From the generation of signal, its amplification and converting into display signals, etc., a great deal of electronic circuits is involved in instruments. Instrumental methods have their advantages and limitations in analytical field. Considering the pros and cons of an instrument, it is employed in analytical studies.

1.2 ADVANTAGES OF INSTRUMENTAL METHODS

(a) Amount of sample requirement for analysis is very less than conventional methods.
(b) Time required for analysis is much less thus results can be obtained quickly.
(c) Complex mixture can be analyzed either with or without their separation depending on method of analysis.
(d) More accurate and reliable results are obtained with instrumental analysis.
(e) Free from manual errors.
(f) Instruments reduce the more human efforts.
(g) Devoid of variation of results,

1.3 DISADVANTAGES OF INSTRUMENTAL METHODS

(a) Skilled persons are needed to handle equipments in case of sophisticated equipments. Even for basic equipments training is needed for handling.
(b) Generally, cost of instrumental methods is high than non instrumental methods since instrumental methods involve expenses including equipment cost, maintenance of equipments, specialized reagents and chemicals.

(c) The sensitivity and accuracy depends upon the type of instrument.
(d) Fluctuations in results are common depending upon the quality of instruments.
(e) Calibration needs to be performed every time before use of instrument or periodically to get reproducible results.
(f) Besides instrumental methods, other methods are required for checking results.
(g) In some cases, instrumental method may not be specific.

Despite the above mentioned limitations, there is no doubt that instrumental methods of analysis have achieved a prominent place in the field of analysis. Every new edition of pharmacopoeia IP, BP or USP and a number of books in analytical chemistry is a testimony to the importance of instrumental methods. British Pharmacopoeia and the USP have introduced the use of Infrared spectroscopy as one of the tests for identification of compounds. In quantitative analysis, more than fifty percent official compounds are now analyzed by instrumental methods.

Instrumental methods are not only important in pharmaceutical analysis for analyzing basic drugs or chemicals or its formulations but play significant role in other fields also. Various instruments are used as diagnostic tool in medical profession without which diagnosis of disease and its treatment would not have been possible e.g. X-rays, ultrasound, NMR, scanning instruments, laser beam, etc. Furthermore, in clinical analysis of biological fluids, tissue and organ analysis, toxicological and forensic analysis, instruments are widely used.

In pharmaceutical field, instruments are not only used for qualitative and quantitative analysis but they find extensive use in manufacture of various drug formulations from their controlled release and therapeutic value. The role and usefulness of instruments in other areas and fields are endless.

REVIEW QUESTIONS

1. Explain instrumental method of analysis and its importance.
2. What are the types of various instrumental techniques and mention the physical properties involved in each technique?
3. Explain the general functions involved in an instrumental technique.
4. Write the advantages and disadvantages of instrumental techniques.

Chapter ...2

ELECTROMAGNETIC RADIATION AND ABSORPTION SPECTROSCOPY

Objectives:

Upon completion of this section, the student should be able to

- Understand the basics of electromagnetic radiation and its properties.
- Explain the various regions of electromagnetic radiation.
- Explain the interaction of electromagnetic radiation with matter.
- Understand the process of absorption and explain absorption spectra.

2.1 ELECTROMAGNETIC RADIATION

Electromagnetic radiation (EMR) is a form of energy, which consists of electric and magnetic vectors. EMR is produced by oscillating electric and magnetic disturbance, or by the movement of electrically charged particles traveling through a vacuum or matter. EMR exhibits both wave and particle properties. The wave properties of radiation source include the phenomena of reflection, refraction, interferences etc. while the discrete particles called as photon have definite energies. Both these properties are inseparable and are distinctive feature of electromagnetic radiation.

Nature of Electromagnetic Radiation:

The electromagnetic radiation is propagated as a transverse wave. The electric and magnetic fields of EMR come at right angles to each other and combined wave moves perpendicular to both magnetic and electric oscillating fields thus the disturbance. Electron radiation is released as photons. Photons are considered as bundles of energy that travel at the speed of light as quantized harmonic waves. This energy is categorized based on its different wavelength into the electromagnetic spectrum as various regions. These electric and magnetic waves travel perpendicular to each other and have certain characteristics, including amplitude, wavelength, and frequency. (Fig. 2.1).

Wavelength:

The wave is described either in terms of wavelength or in terms of the frequency.

Wavelength is described as the distance of one complete cycle or from one crest to another. Otherwise wavelength is defined as the distance between two successive maxima or minima of a wave. The symbol of wavelength is λ (Lambda).

Frequency: It is the number of cycles passing through a fixed point per unit time and the symbol of frequency is υ (hertz).

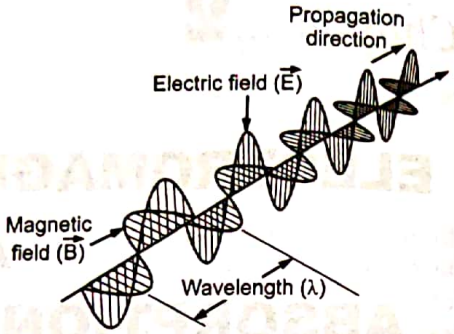


Fig. 2.1: Electromagnetic Wave

The wavelength and the frequency are related to the velocity of light by the formula

$$\lambda\upsilon = \frac{c}{n} \quad \text{... (2.1)}$$

Where c = Velocity of light in vacuum (2.99×10^{10} cm/sec) and n is the refractive index (the ratio of the velocity of light in vacuum to its velocity in the medium of study). For many purposes n is considered as uniform and hence,

$$\lambda\upsilon = c \quad \text{... (2.2)}$$

Or

$$\lambda = \frac{c}{\upsilon} \quad \text{... (2.3)}$$

The reciprocal of the wavelength is called the wave number. It is a number of waves in a unit length and is represented by $\bar{\upsilon}$ in cm^{-1}.

The wavelength of electromagnetic radiation varies from a few angstroms to several metres. The units and symbols used to describe the electromagnetic radiation are shown in Table 2.1.

Table 2.1: Relation of units and symbols in electromagnetic radiation

Nature	Unit	Symbol	Conversions
Wavelength λ	Angstrom	A°	$1\ A = 10^{-8}$ cm
	Nanometer	nm	$1\ nm = 10^{-7}$ cm
	Millimicron	mμ	1 mμ = 1 nm
	Micron	μm	$1\ \mu m = 10^{-4}$ cm
Frequency υ	Cycles per sec	CPs	1 Hz = 1 cps
	Hertz	Hz	
	Megahertz	MHz	$1\ MHz = 10^{6}$ cps
		cm^{-1} or $\bar{\upsilon}$	$cm^{-1} = \frac{1}{l}$
Intensity of radiation	Energy per sec.	I	

Electromagnetic radiation possesses a quantum of energy. The energy unit of light is called as photon, and is related to the frequency by,

$$E = h\upsilon = hc/\lambda$$

Where, E is the energy of photon in erg

H is Planck's constant = 06.62×10^{-27} erg/sec.

It is apparent thus, that the shorter the wavelength greater the energy.

The various regions of the electromagnetic spectrum of use and importance are shown in the Fig. 2.2.

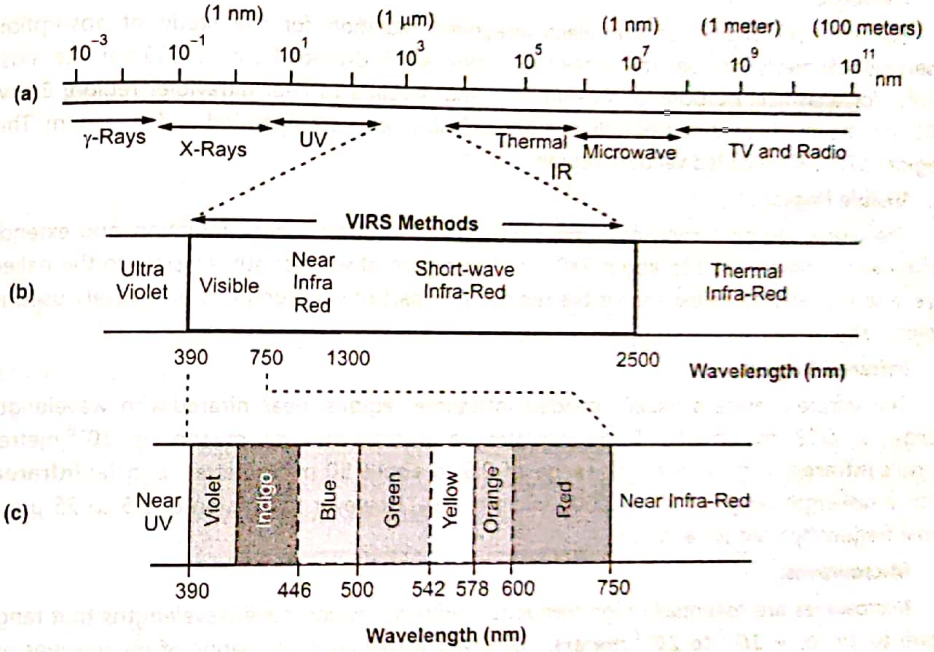


Fig. 2.2: Various Regions of EMR

The following are the major regions of EMR.

1. Gamma rays, 2. X-Rays. 3. Ultra Violet, 4. Visible, 5. Infra red, 6. Micro waves, 7. Radio waves.

1. Gamma Rays (Wavelength):

Gamma rays have the shortest wavelengths (< 0.01 nanometers or < 10^{-12} meters). This is the region which has got highest energy than any region of the electromagnetic spectrum. Gamma rays are created by the nuclear explosions, neutron stars, pulsars in space and are biologically hazardous. X-Rays range in wavelength from 0.01 – 10 nm and are primarily generated from by super-heated gas from exploding stars and quasars.

2. X-Rays:

The X-rays region in EMR is placed next to the gamma rays and has the longer wavelength than gamma rays. The wavelength of this region falls in a range of 0.01 – 10 nm or 10^{-8} to 10^{-12} meters. X-rays are roughly classified into soft X-rays and hard X-rays. Soft X-rays have relatively short wavelengths of about 10 nanometers whereas Hard X-rays have wavelengths of about 100 picometers. X-rays are produced by accelerating electrons and are able to penetrate many different types of materials. X-rays are commonly used for medical imaging and for inspecting cargo and luggage.

3. UV-Rays:

The principal region of the electromagnetic radiation for the study of absorption spectrophotometry includes the ultraviolet region which extends from 10–380 nm, the most useful for analytical purpose is 200–380 nm, and is called as near ultraviolet region. Below 200 nm, the air absorbs appreciably and hence instruments are operated under vacuum. This region (120–180) is called vacuum region.

4. Visible Region:

The visible region comprises a very small part of electromagnetic radiation, and extends from near ultraviolet 380 to about 780 nm. This region of wavelength is visible to the naked eye, and is therefore called the visible region. This part of wavelength is extensively used in colorimetry.

5. Infrared Region:

The infrared range is usually divided into three regions: near infrared with wavelength range of 0.78 to about 2.5 micrometres (a micrometre, or micron, is 10^{-6} metre); middle **infrared**, with wavelength range of 2.5 to about 50 micrometres; and far **infrared**, with wavelength range of 50 to 1,000 micrometres.. However the region of 2.5 to 25 μm is most frequently used for analysis.

6. Microwaves:

Microwaves are essentially high frequency radio waves and have wavelengths that range 1mm to 1m or ***~ 10^{-3} to 10^{-1} meters.*** Different wavelengths or bands of microwaves are used for different applications. Mid-wavelength microwaves can penetrate haze, light rain and snow, clouds, and smoke are beneficial for satellite communication and studying the Earth from space. Radar technology sends pulses of microwave energy and senses the energy reflected back.

7. Radio Waves :

Radio waves have the longest wavelengths in the electromagnetic spectrum with wavelengths ranging from approximately 1mm to several hundred meters (***> 10^{-1} meters***).

Radio waves are used to transmit a variety of data. Wireless networking, television and amateur radio all use radio waves. The uses of radio frequencies are usually regulated by governments.

When a molecule is exposed to electromagnetic radiation, a certain amount of energy associated with the particular radiation is absorbed by the molecule. There is a transfer of energy from the beam of radiant energy to the molecule. This is called 'absorption' and the study of this is called as absorption spectrophotometry. When the process is reverse, in which the internal energy of molecule is converted into radiant energy, it is called 'emission'. There are other ways of energy instead being absorbed, scattered or re-emitted, which is of different consideration.

When a molecule is at rest, it is considered to be in ground state of energy level E_O. There are three basic internal energy levels in a molecule. First is the rotational energy that allows molecule to rotate about various axes. This energy is very small. The second is the vibrational energy level, which allows atoms or groups of atoms within a molecule vibrate relative to each other. Third, the electrons of molecule may be raised to higher electron energy, known as electronic transition. Thus, a total energy of molecule at ground state can be shown as

$$E_O = E_{electronic} + E_{vibration} + E_{rotational}$$

When a molecule at a ground state of energy level E_O is exposed to a radiation in ultraviolet to infrared region, a certain quantity of energy is absorbed by the molecule and it is raised to the excited state of energy E_1, E_2 etc. The quantity of energy associated with the wavelength of ultraviolet is more visible is small and with infrared it is very small. If radiation of wavelength corresponding to one of the natural frequencies of a molecule strikes it, the radiant energy may be absorbed to raise the molecule at excited state level. The amount absorbed would be

$$\Delta E = 286{,}000 \text{ kcal mole}^{-1}$$

A schematic energy level diagram for a simple diatomic molecule is shown in Fig. 2.3.

A given electronic transition may involve vibration as well as rotational changes, resulting in several photon energies being absorbed.

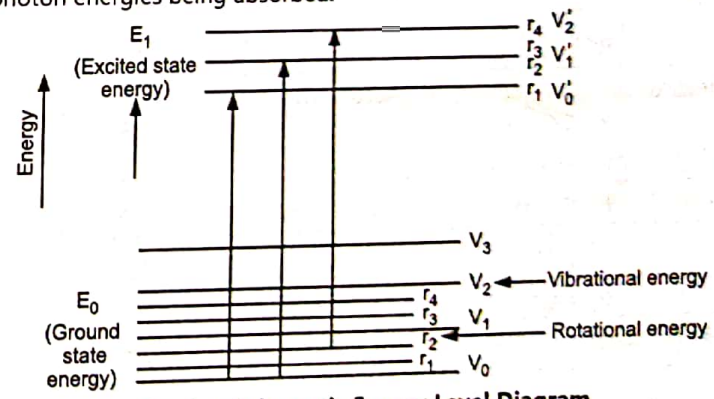


Fig. 2.3 : Schematic Energy Level Diagram

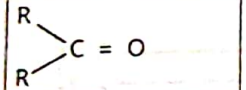
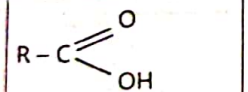
Molecules from excited state of energy return to ground state in 10^{-9} to 10^{-8} seconds, and in the process the absorbed energy is released, if molecule returns to a ground state

directly, heat is evolved, and if it returns by a way of second excited state then the energy is released in the form of heat and light.

2.2 ABSORPTION SPECTRA

Absorption of light in ultraviolet and visible regions gives rise to the absorption spectra. These spectra of molecules and ions are due to the transitions between electronic energy levels of certain types of groups present in the molecule. A group that gives rise to absorption in visible and near ultraviolet is known as a **chromophore**. The term chromophore was originally applied for unsaturated groups/atoms which were thought essential for colour (chrome = colour). Most unsaturated groups, heteroatoms carrying lone pair of electrons are potential chromophore. Now-a-days, the term chromophore is given to a group which when attached to a molecule shows absorbance in ultraviolet-visible region at a specified wavelength. Table 2.2 lists some common chromophores and their approximate wavelengths of maximum absorption. Although a chromophore shows absorption in a certain region of spectrum, the maximum absorption will depend on the particular molecule and the influence of other groups in the molecule.

Table 2.2: Absorption bands and electronic transitions for common chromosphores

Chromophore	System	Example	Maximum	Electronic Transition
Alkane	R – CH = CH – R	Ethylene	165 193	$\pi \rightarrow \pi^*$
Amine	R – NH_2	Aniline (cation)	203 254	$\pi - \pi^*$
Amide	R – $CONH_2$	Acetamide	220	–
Azo	R – N = N – R	Azomethane	338	–
Carbonyl (ketone)		Acetone	188 279	$\pi - \pi^*$ $n - \pi^*$
Carbonyl (aldehyde)		Acetaldehyde	180 290 230	$\pi - \pi^*$ $n - \pi^*$ $\pi - \pi^*$
Carboxyl	R – C – OH	Benzoic acid	270	
Nitro Nitroso Nitrate	R – NO_2 R – N = O R – ONO_2	Nitromethane Nitrosobutane n-Butylnitrate	201 302 270	

Changes in the absorption spectra can be brought out by certain groups attached to a fully saturated system. These groups do not absorb radiation at wavelengths > 200 nm. They do modify and shift the absorption bands. Such groups are called Auxochromes. The groups like – OH, – NH_2, – Cl etc. have non-bonding valency electrons. They show intense absorption in far ultraviolet region.

When an auxochrome is attached to a chromophore, the chromophore absorption band is shifted to longer wavelength. This is called bathochromic shift (Red shift), when there is increase in intensity of absorption it is termed as hyperchromic effect (The molar absorptivity increases). When a shift of absorption band is to a shorter wavelength the effect is termed as hypochromic shift (blue shift) and reduction in intensity is called hypochromic effect. (Fig. 2.4)

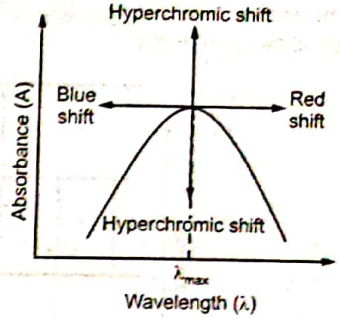


Fig. 2.4 : Spectral Shifts

The types of electrons involved in absorption of energy in the ultraviolet-visible regions are mainly of three types:

(a) σ (sigma) Electrons: These are usually found in fully saturated system like alkane. These electrons require very large amount of energy for their excitation and hence do not show absorption in ultraviolet region. The absorption band given in some cases is due to their interaction with π^* (pie excited) electrons to give hyper conjugation. The absorption band is encountered in vacuum ultra-violet region.

(b) n-electrons: The valence electrons which do not participate in chemical bonding in molecule are called as n-electron or non-bonding electrons. These are located principally in the atomic orbital of nitrogen, oxygen, sulphur and halogens as lone pair electrons. Their transition from n to π^* gives absorption in ultraviolet region.

(c) π-electrons: These are found in unsaturated compounds in multiple bonds. The π electrons are generally 'mobile electrons'. The outer shell electrons, the atomic p-orbital electrons are responsible for pi-bonds. The basic absorption occurs due to transition of π–π^* which gives band in ultraviolet-visible region.

Transition of electrons to their excited higher energy levels gives rise to mainly four types of absorption bands.

K-band spectra arise from π–π structures due to π–π^* transitions. These are characterized by high molar absorptivities (ε more than 100,00). In diene, for example, K-band results due to resonance transition:

$$C = C - C = C \text{ to } C^+ - C = C - C^-$$

It also results in aromatic compounds possessing chromophore groups.

R-bands arise from n–π^* transitions and show intermediate molar absorptivity. The weak intensity bands are found in non-aromatic compounds having chromophore and auxochrome groups like OH, NH_2, – SH, – CHO, – C=) etc.

B-band spectra are characteristics of aromatic and hetro aromatic molecules. These give weak absorption bands and show very low molar absorptivity (250).

E-band spectra arises from oscillations of electrons in aromatic ring systems.

The energy level diagram (Fig. 2.5) shows the ΔE values for transitions from ground to excited state in the order.

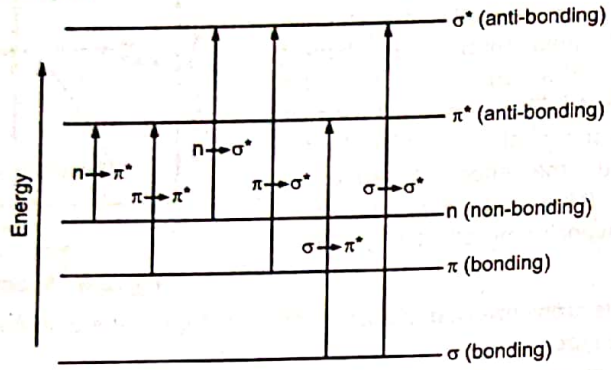


Fig. 2.5: Diagram showing electronic transitions

Many molecules contain more than one chromophore and/or auxochromes. Interaction of radiant energy with such molecules and the resultant spectra depends upon the relative positions of two chromophores. In general it can be said that:

1. When two chromophores are separated by more than one carbon unit, then total absorption is sum of the absorption of each chromophore.
2. When two chromophores are adjacent to each other, absorption shifts to longer wavelength (bathochromic shift) and intensity of absorption (hyper-chromic effect) increases.
3. When two chromophores are attached to same carbon atom, there results in summation of absorption and shift are towards longer wavelength. However, the degree of shift is less than shown by conjugated chromophore.
4. Auxochrome shifts and modifies absorption. The auxochromes encountered in covalently unsaturated moieties such as $-NH_2$ are more effective in modifying absorption.
5. The other auxochrome like –OH, $-\overset{+}{N}H_3$ or – S also shifts absorption spectra to higher wavelength.

Some examples of chromophore showing basic absorption and shift of spectra are given below:

1. Dienes and Polyenes:

These show shift of absorption to longer wavelength. Butadiene ( ) shows λ_{max} at 217 nm while hexatriene ( ) has λ_{max} at 256 nm. Woodward observed that the diene absorption is influenced by the groups and molecules and he worked out a rule (known as Woodward rule) for predicting the expected λ_{max} by calculations. Table 2.3 shows the rules for calculation of absorption.

Table 2.3: Absorption spectra calculations

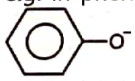
Parent structure not having diene in ring	214
Parent structure having diene in ring	253
For each substituent double bond in conjugation	30
Alkyl substituent in ring	5
N-alkyl	60
S-alkyl	30
λ_{max} of Total	592 nm

2. Aldehydes and Ketones:

Simple aldehydes and ketones show absorption around 280 nm (weak) and 190 nm (medium) e.g. acetone, due to π–π*, λmax is at 188 nm and n – π* transition shows λmax at 276 nm. Similarly, acetaldehyde π–π* show λmax at 194 and due to n – π* 292 nm.

3. Aromatic and Heterocyclic:

Benzene exhibits three bands at 255 nm (low absorptivity), 200 nm and at 185 nm (high absorptivity). The substitution shows marked effect e.g. alkyl substituent —CH_3 in toluene by replacing –H of benzene shows bathochromic shift from 255–261 nm. The other groups like – OH, – NH2 also cause bathochromic shift. The effect is more pronounced in polar solvents, e.g. in phenol, the – OH substitution shows λmax at 210 and 270 in water. The phenolate



ion (in alkaline pH) shows λmax at 235 and 287 nm.

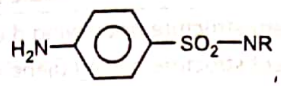
Amino group is a powerful auxochrome and when attached directly to benzene ring shows shift in benzene λmax from 200, 255–230, 280 nm, in Aniline. Aniline in acidic media forms aniline cation which is less effective as auxochrome (λmax = 203 and 254 nm). In the case of heterocyclic compounds, it is necessary to have spectrum of basic ring for comparison and interpretation.

4. Fusion of Two Rings:

Fusion of two or more benzene rings causes bathochromic shift. This results because of conjugation of chromophore which modifies basic spectra. For example:

	Number of rings	λ_{max}
Benzene	1	255
Naphthalene	2	312
Anthracene	3	375
Naphthacene	4	471 (Yellow coloured)
Pentacene	5	575 (Blue coloured)
Pyridine	1	195, 250
Quinoline	2	275, 311
Isoquinoline	2	262, 317

5. pH effect:



The effect of pH is very striking, e.g. in Sulpha drugs , the intense λ_{max} is observed at 251 nm in 0.1 N sodium hydroxide solutions. This is due to $-NH_2$ group which shows its powerful auxochrome. The peak is lost in 0.1 N hydrochloric solutions for the same sulpha.

Fig. 2.6 shows the effect of pH on the absorption spectra of sulpha drugs.

6. Solvent effects:

Solvents play a striking role on the absorption spectra of compounds. Many substances show fine structure bands when measured in solvents of low dipole moments. Thus, benzene shows number of bands in petroleum ether (low dipole) whereas in aqueous solutions the fine structures are lost. The electronic transitions of $n - \pi^*$ are more pronounced in certain solvents. For example, the spectra of iodine in polar solvents like ethanol shows characteristic shift to shorter wavelength (showing brownish colour), whereas it appears as purple in chloroform. Solvents begin to absorb ultraviolet energy at some specified wavelength. Thus, those solvents which show λmax at particular wavelength cannot be used as solvent for absorption studies of compound having λmax near the solvent λmax. Impurities in solvents show that it is essential to use good grade (spectroscopic grade) solvents for absorption studies. Some solvents are acidic or basic in nature and they do affect the λmax due to their pH effect.

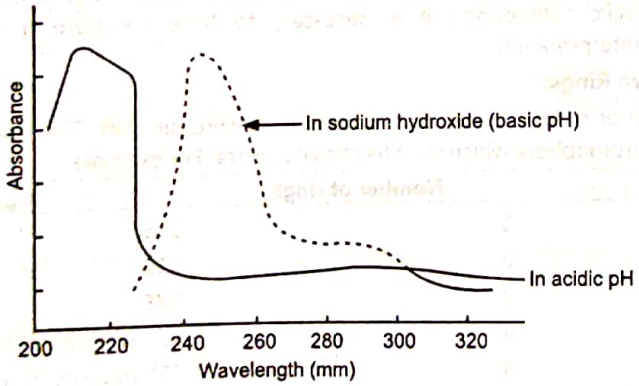


Fig. 2.6 : Effect of pH on the Absorption of Sulpha Drugs

Wavelength of absorption of common solvents is given in Table 2.4.

Table 2.4

Solvents	λ_{max} in nm
Acetone	188, 276
Benzene	200, 255
CCl_4	265
$CHCl_3$	245
Cyclohexane	210
DMF	270
Diethyl ether	220
Ethanol	210
N-Hexane	210
Toluene	261
Water	200
Xylene	266

7. Temperature Effects:

At low temperatures the absorption bands of most substances are sharper than at room temperature. The solute-solvent interaction is decreased at low temperature and hence studies are carried out at 15–25°C.

REVIEW QUESTIONS

1. What do you mean by EMR? Explain the properties of EMR.
2. What are various shifts and effects in absorption spectroscopy?
3. Explain the electronic transitions in absorption spectroscopy.
4. What do you mean by chromophore? Explain the various factors that shift absorption of chromophores with suitable examples.

Chapter ...3

UV-VISIBLE SPECTROSCOPY

Objectives:

Upon completion of this section, the student should be able to

- Describe the various components and their working of spectrophotometer.
- Explain the types of commercial instruments used in photometry and their working.

3.1 INTRODUCTION

UV-Visible region in EMR extends from 200-800 nm. Any compound which has absorption at this region can be conveniently analysed by UV-Visible spectroscopy. The instrument which is designed to determine the absorption by analytes in the UV region is called as UV-Vis spectrophotometer.

A colorimeter or spectrophotometer consists of the following essential components:

1. A source of radiant energy to emit radiation.
2. A collimating system consisting of lenses, mirror, slits etc. which collimate and focus the beam on sample.
3. A monochromator system that isolates the wavelengths.
4. A sample holder or container to hold sample.
5. A detector system of collecting transmitted radiation and
6. A suitable amplifier or readout galvanometer. A block diagram of any model of spectrophotometer will represent the features as shown in Fig. 3.1.

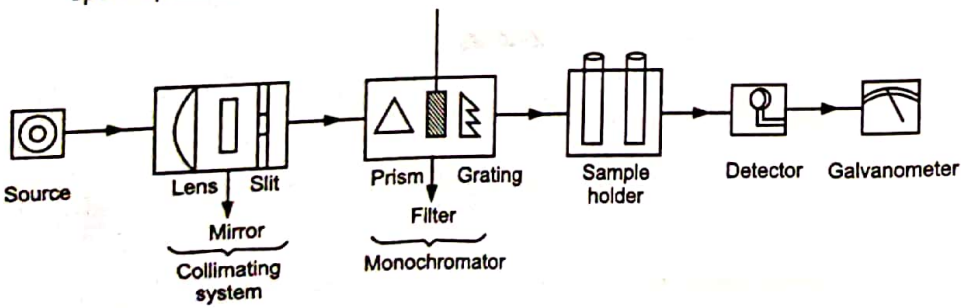


Fig. 3.1: Block diagram of spectrophotometer

The selection of components in fabrication of instrument involves many factors. A source of radiant energy is selected on the basis of nature of work, stability, wide range and operating temperature. The associated optical system including the selection of device for obtaining monochromatic radiation is based upon sensitivity sophistication and cost of instrument. The detector and galvanometer for readout are now-a-days developed with wide sensitivity and digital reading with recording device.

3.2 SOURCES OF RADIANT ENERGY

Wide ranges of sources which provide radiant energy are available. A source of radiant energy should have the following features:

1. It should be stable and should show no fluctuations.
2. It should provide incident light of sufficient intensity.
3. It should emit a continuous spectrum of high and uniform intensity.
4. It should be appropriate for the purpose of work.
5. It should not show fatigue on continued use.

As almost all sources of radiant energy operate on electricity, a power supply of these sources should be of stable type resisting voltage fluctuations. A power supply stabilizer unit is helpful in this respect.

For ultraviolet radiation:

The hydrogen lamp and deuterium lamp are the most common sources of ultraviolet radiation. In hydrogen lamp, a pair of electrodes is enclosed in a glass tube provided with silica or quartz window (for ultraviolet radiation to transmit through) and is filled with hydrogen gas at low pressure. When current is passed through these electrodes maintained at high voltage, discharge of electron occurs which excites hydrogen gas to high energy states. The electrons return to their ground state and emit radiation in the region of 180–350 nm. When deuterium replaces hydrogen, similar excitation and relaxation of gas results in emission of radiation of ultraviolet region, but the intensity is of high order. Similar to hydrogen or deuterium lamp, a xenon discharge lamp is used as a source of continuous plus additional intense radiation. The lamp since operates at high voltage becomes very hot during operation and hence needs thermal insulation. Emission of visible region radiation also occurs along with ultraviolet radiation. A heat absorbing filter is often inserted between the lamp and sample holder to absorb the heat radiation.

For visible radiation:

A glass enclosed tungsten filament incandescent lamp is most widely used source for visible and near infrared region. The filament is heated by a stabilized power supply or by storage battery. It is available in various sizes and shapes suitable for fixing in the instrument and operates from 3-220 volts. The tungsten lamp emits continuous radiation in the region of 350-2500 nm. About 15% of radiant energy falls within visible region. This source of radiation being inexpensive is very common in most spectrophotometers.

A mercury discharge lamp in glass tube for visible and in fused silica envelope for ultraviolet-visible region is also employed. The lamp on excitation emits continuous radiation in 350-800 nm. A very high intense radiation given out at 366, 405, 436, 546, 578 nm can be obtained using appropriate filter.

3.3 COLLIMATING SYSTEM

The radiation emitted by the source is collimated (made parallel) by lenses, mirrors and slits. The lenses and mirrors are seldom used in combination to have radiation collimated. Materials used for lenses must be transparent to the radiation being used. The absorbance of such material should be less than 0.2 per cent at the wavelength of use. Ordinary silicate glass transmits between 350–3000 nm and is suitable material for visible and near infrared radiation. Quartz or fused silica is used as a material for lenses for work below 300 nm. The limit of quartz is about 210 nm, which is satisfactory for the purpose.

Similar to lenses, prisms are used for collimating purpose. Prisms made from glass are used in visible region while those made from quartz or fused silica is used in UV visible region.

Mirrors are used to reflect, focus or collimate light beams in spectrophotometer. Front surfaced mirrors are used to minimize light losses. For this, mirrors are aluminized on their front surfaces. Half-silvered mirrors are invariably employed in double beam instruments. To reduce the scattering by reflections, glass surfaces are coated with magnesium fluoride in a thin film. In infrared regions, mirrors are used because most materials are not sufficiently transparent and cause significant energy losses.

Slit width is an important device in resolving polychromatic radiation into its individual wavelength or into monochromatic radiation. To achieve this entrance slit and exit slit are used. The width of slit plays an important role in resolution of polychromatic radiation. Narrow slit widths isolate narrow bands; however, it limits the intensity which reaches the detector. Fig. 3.2 depicts the distribution of wavelengths leaving the slit. The nominal wavelength is that set on the instrument and is the wavelength of maximum intensity passed by the slit. The intensity of radiation at wavelengths on each side decreases. The effective band width is defined as the range of wavelength over which the transmission is at least one half of its maximum value. The spectral slit width is theoretically twice the nominal band width and is a measure of the total wavelength spread that is passed by the slit.

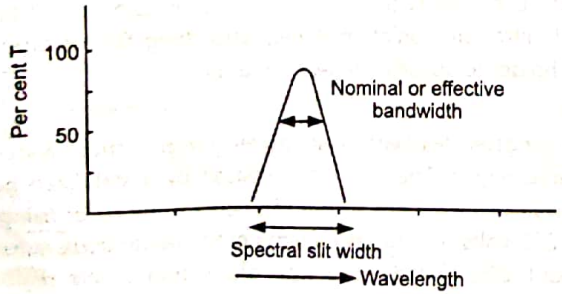


Fig. 3.2 : Distribution of wavelengths leaving the slit

If the intensity of the source of radiation and sensitivity of the detector is satisfactory, the spectral purity can be improved (i.e. the band pass decreased) by decreasing the slit width. However, at very narrow slit openings the reduction in the slit width does not cause a proportional reduction in the spectra isolated.

3.4 MONOCHROMATOR

The purpose of employing devices is to resolve wide band of polychromatic radiation into a narrow band of monochromatic radiation. There are many types of dispersing devices employed for this purpose.

Filters:

Filters are used for isolation of narrow band of radiant energy of desired spectral region. Filters allow transmission of only limited wavelength regions while absorbing most of the radiation of other wavelengths. The filters are of many types like glass filters, gelatin filters and interference filters etc. Selection of filter is usually a compromise between peak transmittance and band pass width, in which the former should be as high as possible and latter as narrow as possible.

Glass filters:

These are the pieces of coloured glass which transmit limited wavelengths. Glass filters transmit radiation with an effective band width between 20–50 nm. The colours in the glass filters are produced by incorporating oxides of metals like V, Cr, Mn, Fe, Ni, Co, Cu etc. The colours produced are with Co-blue, Cu-blue-green, Mn-purple, Fe-green, and Cd-yellow. The glass filters are unaffected by heat and light. The only disadvantage of glass filters is that they allow broadband transmission through it. The filters are manufactured by many companies: Kodak, Alford, Corning and so forth and carry numbers on them. The transmission wavelength of the Corning brand filters is as given below in Table 3.1.

Table 3.1: Filters used indicating wavelength in nm

Number	Spectrum	Transmitted wavelength in nm
601	Violet	380 – 470
602	Blue	440 – 490
603	Blue-green	470 – 520
604	Green	500 – 540
605	Yellow-green	530 – 570
606	Yellow	560 – 610
607	Orange	570

Selection of filter is based upon constructing a calibration curve of optical density or transmission versus concentration using each filter. Best filter is the one that gives maximum absorption or minimum transmittance for the given concentration of absorbing solution. Alternatively, a concept of colour wheel is followed. In the colour wheel as shown in Fig. 3.3, main six colours found in rainbow are arranged. The main colour and its complementary colour are just opposite. Thus for example, solution to be analysed is blue in colour, a filter having a complementary colour orange is used in the analysis.

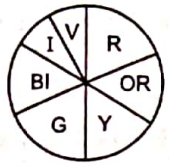


Fig. 3.3 : Colour wheel

Gelatin filters:

Gelatin filters are manufactured from thin gelatin sheets, which are coloured with organic dyes during their manufacture. The thin sheets of gelatin filters transmit 10–30 nm band of wavelength which is slightly superior over glass filters. The gelatin sheet is sandwiched between a pair of clear glass to obtain the filter. Since, gelatin gets affected by heat radiation, heat absorbing filters are used along with gelatin filters.

The gelatin filters are now-a-days outdated because

(i) They tend to deteriorate with time

(ii) They get affected by heat and moisture and

(iii) The colour of dye gets bleached.

Interference filters:

Narrow bandwidths are obtained with interference filters. Interference filters are constructed by using two parallel glass plates which are silvered internally and separated by thin film of transparent dielectric spacer of low refractive index. Magnesium fluoride (n_D = 1.38) is commonly used as dielectric material. Light incident upon the face of filter at 90° is reflected back and forth between the metal films. Constructive interference between different pairs of light rays occurs. The transmission of a spectral band by interference when light is incident sin 90° = 1 is given by equation:

$$m\lambda = 2d\,(n)\,(\sin\theta) \qquad \ldots (3.1)$$

Where, d is the thickness of dielectric spacer whose refractive index is n. Since sin θ = sin 90° = 1, the equation is

$$m\lambda = 2\,dn \qquad \ldots (3.2)$$

Where, m is integer. By adjusting the suitable thickness between the plates the suitable wavelength for transmission is obtained. These filters have a bandwidth of 10–15 nm with peak transmittance of 40–60 per cent.

Prisms:

Next to filters, prisms made from glass, quartz or fused silica is employed as dispersing devices in spectrophotometer. Since, glass has dispersing power about three times that of quartz, it is used in the visible portion of the spectrum. Quartz or fused silica prisms are the choice materials for ultraviolet spectrum. The mechanism in dispersing polychromatic beam of radiation into small bands of wavelengths by prism depends on the variation of the index of refraction with wavelength. Dispersion is defined as $\frac{d\theta}{dx}$ i.e. the change in angle of deviation with respect to the change in wavelength. When a beam of radiation passes from air into the dense material of prism, it will be refracted and bend towards perpendicular and further will show opposite effect when it emerges into air. Fig. 3.4 shows ∠ i as incident angle; ∠ r as reflected from air to prism, ∠i' as incident in prism and ∠r' as refracted from prism to air.

Fig. 3.4

Since, the index of refraction depends on the wavelength; the shorter wavelengths are refracted more than longer wavelengths. It will thus be seen that when white light from electric bulb is passed through glass prism, dispersion of polychromatic light (white) in rainbow occurs as shown in Fig. 3.5 (a).

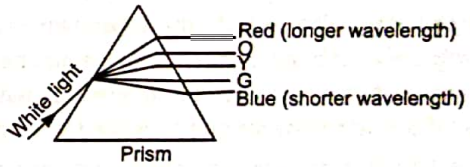


Fig. 3.5 (a)

Now, by rotation of the prism, different wavelengths of the spectrum can be made to pass through an exit slit on the sample. A prism monochromator mechanism is shown in Fig. 3.5 (b).

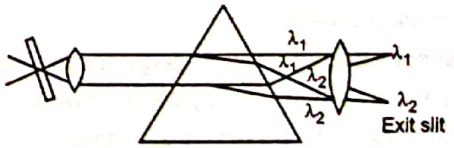


Fig. 3.5 (b)

The effective separation of wavelengths depends on the dispersive power of the prism material and the apical angle of the prism. For most satisfactory work it is 60°. There are two types of mounting of the prisms in an instrument. One type is called as Cornu-type [Fig. 3.6 (A)] which allows the light beam to pass through the prism in the cornu-type mounting prism has an apical angle of 60° and it is so adjusted that on rotation the emerging light is allowed to fall on exit slit. The other type is called as littrow-type [Fig. 3.6 (B)]. In this the apical angle of prism is 30° and its one surface is aluminized which reflects light back to pass through prism and to emerge on the same side of light source, i.e. light does not pass through the prism on the other side.

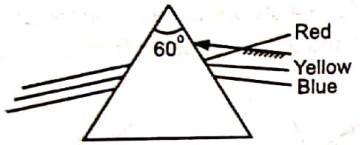


Fig. 3.6 (A) : Cornu-type

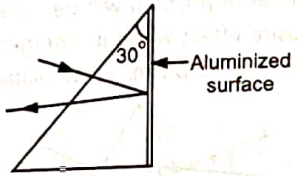


Fig. 3.6 (B) : Littrow-type

In both these types of mounting of prisms, the two surfaces of prisms must be carefully polished and cleaned.

Diffraction Gratings:

More refined nature of dispersion of light is obtained by means of a diffraction grating. These consist of large number of parallel lines or grooves about 15000–30,000 per inch are ruled on highly polished surface of aluminium. The lines or grooves drawn on plate are the scattering centers for light beam impinging on it. Because of constructive interference the light rays are dispersed and employing exit slit the separation of desired wavelength is accomplished. The resolving power of grating depends on the number of lines ruled per inch on the surface and increases with increasing number of lines. Generally, resolving power of grating is better than that of prisms, and hence grating is used in all regions of the spectrum.

Generally, gratings are difficult to prepare and original gratings are expensive. The master grating is used as a mould to prepare replica gratings. On to the master grating of aluminium, an epoxy resin is applied. When the epoxy resin has hardened and set, the replica is taken out and its surface made reflective by aluminizing. The replica gratings are less expensive. Advantages of gratings are:

1. It provides a light of narrow wavelength.
2. With reflection gratings there is no loss of energy due to absorption by the material.
3. Gratings are sturdy and are less affected by water vapours.
4. Gratings are employed for calibration of wavelength dials of the instrument. A simple line diagram of grating monochromator is shown in Fig. 3.7.

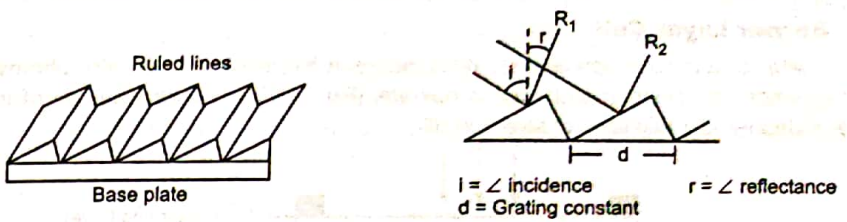


Fig. 3.7 : Grating Monochromator

3.5 SAMPLE HOLDER

The cells or cuvettes or test tubes are used for handling the liquid samples. For the use in visible region, the cell may either be rectangular or cylindrical in nature. These are prepared from good quality glass like corning which shows uniform transmission. Test tube types are employed for routine use. The rectangular types are either 1 cm, 4 cm in internal diameter. For study in the ultraviolet region the cells or cuvettes are prepared from quartz or fused silica. Larger size cells are prepared from good quality glass with quartz window. The internal diameter of cells is either 0.5 cm, 1 cm or 2 cm. Microcells are employed for small samples. The cuvettes with lid are used for handling volatile type solvents and solutions.

The surfaces of absorption cells must be kept scrupulously clean. No fingerprints or blotches should be present on cells. Cleaning of the cells can be carried out by washing with distilled water or with dilute alcohol, acetone or detergent solutions.

3.6 DETECTOR DEVICES

The light or the intensity of transmitted radiation by a sample is collected on a detector device. This is to measure the amount of transmitted radiation. Most modern detectors generate an electrical current after receiving the radiation. The generated current is often amplified and passed on to a meter, a galvanometer or a recorder. The detectors are of many types. They give different signals. Some important requirements of a good type of detector are:

1. It should give quantitative response.
2. It should have high sensitivity and low noise level.
3. It should have a short response time.
4. It should provide signal or response quantitative in wide spectrum of radiation received.
5. It should generate sufficient signal or electrical current which can be measured or easily amplified for detection by meter.

The following types of detectors are employed in instrumentation of absorption spectrophotometry.

3.6.1 Barrier Layer Cell

This detector is most simple and sturdy in nature. A barrier-layer cell is also photovoltaic cell. It is simple to construct and easy to operate. [Fig. 3.8] On a base plate (A) of iron or copper is deposited a thin layer of selenium (B).

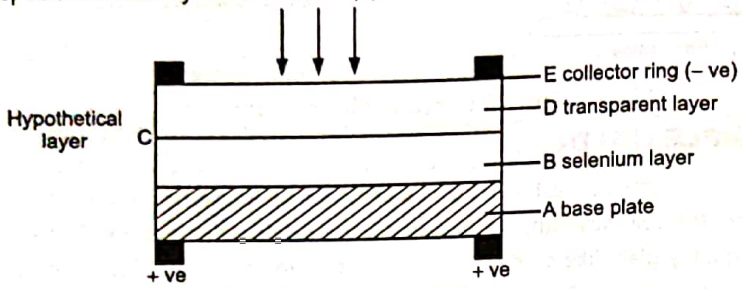


Fig. 3.8 : Barrier Layer Cell Detector

Over this layer is a very thin transparent layer of silver (D) is spread. It has over it, a collecting ring (E) to collect electrons. Between the layer of selenium (B) and silver (D) is a hypothetical barrier layer (C). When light or a radiant energy falls on the surface of barrier layer cell, the light passes through transparent metal layer D and through hypothetical layer C to delay the progress of action on selenium layer B. On striking selenium layer it excites electrons from selenium which pass through hypothetical barrier layer and are collected on collector ring E. As a result of giving out of electrons from selenium layer spread on base plate, the latter possess a positive charge. Thus, the barrier layer cell generates its own electromotive force. If the cell is connected to a galvanometer, a flow of current is observed. This cell is simple, sturdy and does not require any external power supply. At low level of illumination it produces photo-current proportional to the radiant power received on it. The current produced by barrier-layer cell is small (in μA) and hence amplification is carried out to read on galvanometer. Barrier layer cell has disadvantages of (a) slow response, (b) fatigue effect and (c) poor modulation and adaptability.

Photo-tubes:

Photo-tube detector is also known as photo-emissive tube. This consists of spherical shaped vacuum bulb containing photo-emissive cathode and an anode. The inner surface of semi-cylindrical cathode mounted inside the bulb is coated with photo-sensitive material like cesium oxide. A metal wire nearby is an anode. A high voltage is impressed (90–100 V) between them. When radiant energy falls on the surface of photo-sensitive cathode, electrons are emitted which are attracted to the anode causing current to flow. This current is amplified and measured. The response of phototube is dependant on the material used for coating and wavelength of light striking it. Different photo-tubes are used for different regions of spectrum of light. A construction of phototube is shown in Fig. 3.9. Current produced by photo-tube is generally small, and hence requires amplification. A small current

known as **dark current** is usually observed when phototubes are used as detectors in the instrument. This is attributed to the scattered electron emission from photosensitive cathode due to stray radiation striking its surface. Naturally, the magnitude of it increases as the surface area of cathode is more. A compensating device to eliminate this 'Dark current' is usually employed in many instruments.

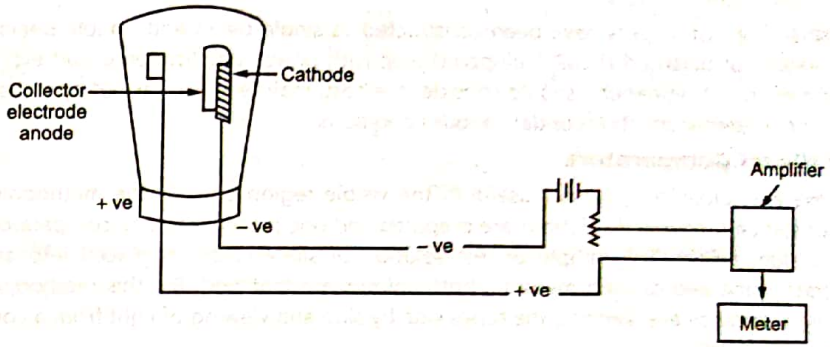


Fig. 3.9 : Phototube detector

Photo-multiplier Tubes:

This is more refined and sensitive device developed over the concept of photo-tube. In the mechanism of photo-tube it is seen that on striking the photo-sensitive material of cathode, electrons are released. If the released electrons are under the accelerated electric potential brought to another electron-active surface, it will release more electrons. These in turn, by acting on another electron-active surface will produce more and more electrons. Thus, a large current is generated by photo-multiplication. This mechanism is utilized in photomultiplier tubes. A schematic diagram is shown in Fig. 3.10. In a vacuum tube, a primary photo-cathode is fixed which receives radiation from the sample. Some eight to ten dynodes are

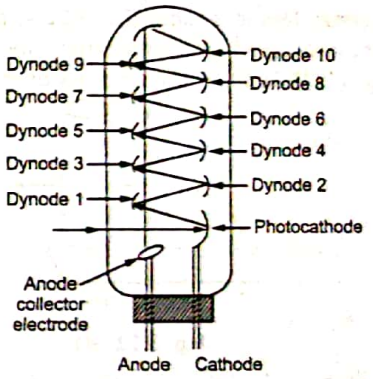


Fig. 3.10 : Photomultiplier tubes detector

fixed each with increasing potential of about 90 V. Near the last dynode is fixed an anode or electron collector electrode. A high voltage is maintained between the cathode and anode.

The light received by cathode releases electrons which through series of dynode produce more electrons. These released electrons by last dynode are collected by anode and photo-current is produced. Photo-multiplier is extremely sensitive to light and is best suited where weaker or low radiation is received.

Commercial Instruments:

The instruments used in absorption photometry can be classified into three categories:

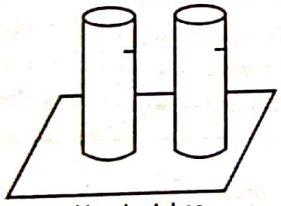
1. Visual comparators
2. Simple filter photometer and
3. Spectrophotometers.

Further, the instruments have been constructed as single beam and double beam type, direct reading or balanced circuit (null point) type with power stabilization circuit etc. In the selection of an instrument one should consider the cost, maintenance, ease of operation and adaptation to special situations under various conditions.

3.6.2 Visual Comparators

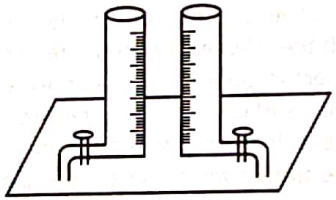
These are colour comparators useful in the visible region only. In the methodology, a series of standard coloured solutions are prepared and one placed into one comparator tube to a constant height. The sample or test solution of same colour is placed into another comparator tube and dilution made till both colours are matched. For this method, colour matching is done by eye, keeping the tubes side by side and viewing of light from a common source (or daylight).

The comparator tubes are made from good quality pyrex or corning glass of same thickness and are usually spherical in shape (like test tube). They are of same size and diameter. Nessler's tubes (Fig. 3.11 A) of 50 or 100 ml capacity with gravitation marking are commonly used as comparator tubes. The other types of tubes are Hehner cylinder [Fig. 3.11 (B)] which have stopcock on the side of the base.



Nessler tubes

Fig. 3.11 (A)



Hehner cylinder

Fig. 3.11 (B)

When the colour between the unknown (test) and the standard is matched, the concentration of unknown is found by noting the depth of two solutions (height) and from concentration of standard by a formula

$$C_u h_u = C_s h_s$$

$$C_u = C_s \times \frac{h_s}{h_u}$$

Where, C_u and C_s denote the concentration of unknown and standard solutions and h_u and h_s refers to respective height of solutions. For more details of various methods in visual comparator refer colorimetric analysis.

3.6.3 Filter Photometer

A line diagram of single beam, direct reading filter photometer is given in Fig. 3.12.

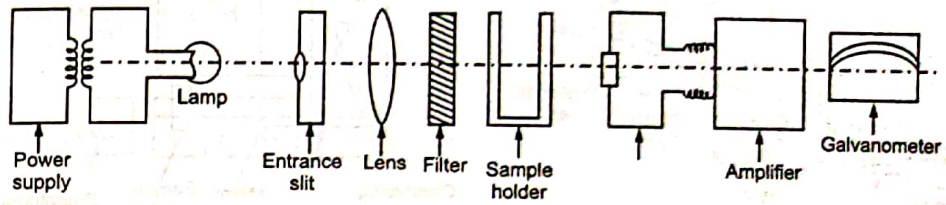


Fig. 3.12 : Line diagram of single beam, direct reading filter photometer

The essential components of filter photometer are :

(a) light source

(b) lens/entrance slit

(c) filter

(d) sample holder

(e) detector and

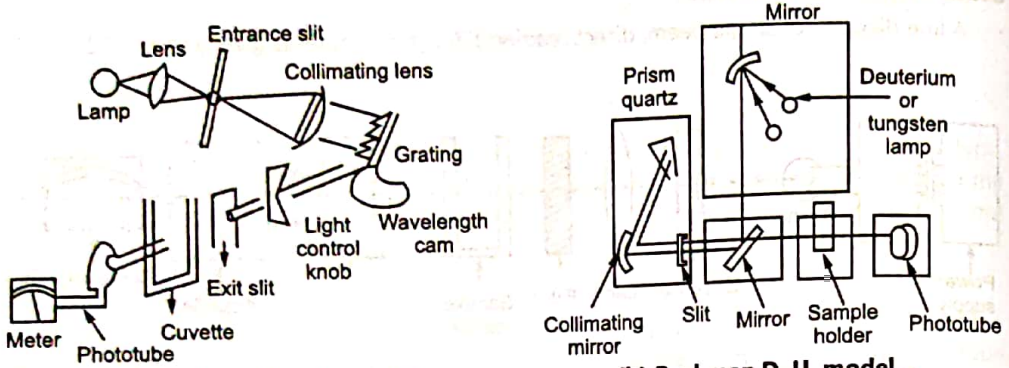
(f) galvanometer or readout meter.

Light source can be ordinary electric bulb or a lamp of 6 or 12 V storage battery by regulated voltage transformer to minimize voltage fluctuations.

Light from the source is carried through lens and/or through aperture to pass through a suitable filter. The type of filter to be used is governed by the colour of the solution. The sample solution to be analysed is placed in a test tube or cuvettes known as sample holder. After passing through the solution, the light strikes the surface of detector (barrier-layer cell or phototube) and produces electrical current. The output of current is measured by the deflection of needle of light-spot galvanometer or microammeter. This meter is calibrated in terms of transmittance as well as optical density. The readings of solution of both standard and unknown are recorded in optical density units after adjusting instrument to a reagent blank.

3.6.4 Spectrophotometer

Several models of various instrument manufacturers are available in market. A line diagram of single beam spectrophotometers is given in Fig. 3.13 (a) and 3.13 (b) while a schematic diagram for double beam instrument is shown in Fig. 3.14.



(a) Bausch and Lamb model **(b) Beckman D. U. model**

Fig. 3.13 : Schematic diagram of single beam spectrophotmeter

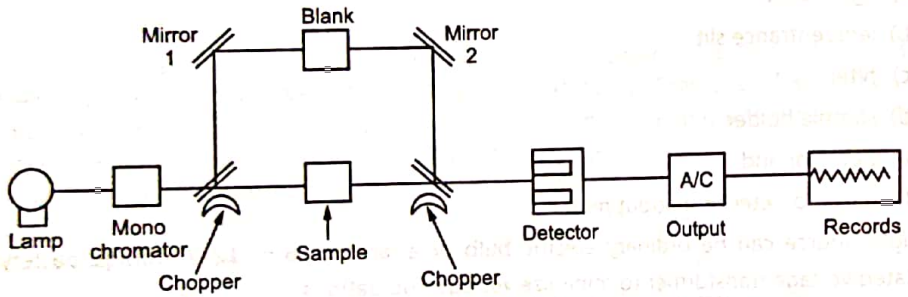


Fig. 3.14 : Schematic diagram of double beam spectrophotmeter

The essential features of a spectrophotometer are:

- source radiation
- collimating system
- monochromator (mainly prisms or gratings)
- sample holder
- detector
- read out meter.

(a) Source of radiation:

For the visible region, tungsten lamp is commonly employed in an instrument. It emits continuous, incandescent radiations. For UV region, a hydrogen (or deuterium) discharge lamp is used. Some instruments also provide mercury vapour lamps to give intense radiation of specific wavelength both in UV-visible region. Commonly, a voltage establisher is incorporated in instrument to give radiations without fluctuations or fatigue.

(b) Collimating system:

It consists of lens, mirrors and/or aperture of entrance slit in spectrophotometer. This allows a narrow beam of collimating light and directed either on quartz or silica prisms or a grating to render monochromatic radiations.

(c) Monochromator:

Some instruments make use of prism in a form of 30°–60°–90° triangle with apical angle of 30°. This prism has its back aluminized which reflects the refracted ray through Littrow mounted prism back to the same collimating mirror at a different height (so to give different wavelength). Alternately, a grating device having aluminized back surface is employed to obtain monochromatic radiation.

(d) Sample holder:

It is a slot in instrument which holds test-tubes, cuvettes of different size and capacity. The sample tubes are made from good quality glass having uniform transmittance. The tubes or cells are of uniform size, shape and internal diameter. For study in UV region the cells or cuvettes are made from quartz or fused silica. For handling volatile solvents or solutions, the cells have cover or lid.

(e) Detector:

Detector unit is usually a barrier layer cell or a photo-tube. In some instruments two interchangeable photo-tubes are employed to be useful in red region (625–1000 mμ) and blue region (210–625 mμ) of wavelength. In double beam instruments two photo-tubes or photomultiplier tubes are employed.

(f) Galvanometer or meter:

It is to record the current generated and amplified by detector. These have coarse and fine adjustment knobs through which adjustment of dark photo-current between zero and 100 per cent transmittance with respect to blank or solvent is adjustable. The meter is generally calibrated in transmittance and absorbance (optical density) units. In some instruments (double beam type), a null point technique is used in readout meter.

The assay of following drugs can be performed by UV-visible spectrophotometry:

		Solvent used	Wavelength in nm
1.	Amodiaquine hydrochloride	HCl 0.1 N	343
2.	Amoxicillin trihydrate capsules	Water	325
3.	Ampicillin capsules	Water	325
4.	Carbimazole	Water	291
5.	Chloramphenicol	Water	278
6.	Digitoxin tablets	Water	550
7.	Dithranol	Glacial acetic acid	450

		Solvent used	Wavelength in nm
8.	Folic acid	0.1 M NaOH	550
9.	Furazolidine	DMF	367
10.	Griseofulvin	Ethanol	291
11.	Hydrochlorothiazide tablets	0.1 M NaOH	273
12.	Isoxsuprine hydrochloride	0.1 M HCl	274
13.	Labetalol hydrochloride tablets	0.05 M H_2SO_4	302
14.	Methandienone	Ethanol	287
15.	Nifedipine capsules	Methanol	350
16.	Phenformin hydrochloride	Water	520
17.	Psoralen	Methanol	247
18.	Quinidochlor tablets	Methoxyethanol	650
19.	Rifampicin	Methanol	475
20.	Reserpine	Ethanol	388
21.	Spironolactone	Methanol	238
22.	Stilbesterol	Ethanol	418
23.	Triamcinolone acetamide	Ethanol	240
24.	Imidazole tablets	Methanol	310
25.	Tubocuranine chloride	Water	280
26.	Tomfool maleate tablets	0.05 M H_2SO_4 + Carbonate Buffer	295
27.	Verapamil hydrochloride tablets	0.1 M hydrochloric acid	278
28.	Protryptalline Tablets	1M HCL + Methnol	292

REVIEW QUESTIONS

1. Discuss in detail the construction and working of instrumentation of a spectrophotometer.
2. What are the types of commercial instruments of photometry?
3. Discuss monochromators.
4. Write a detailed note of detectors used in UV visible spectroscopy.
5. Explain the instrumentation of a double beam spectrophotometer with suitable diagram.
6. List out the components of a spectrophotometer.
7. Give examples of drugs which are assayed by UV-Vis spectroscopic technique.

Chapter ...4

FLUORIMETRY

Objectives:

Upon completion of this section, the student should be able to

- Understand the principle of Fluorimetry.
- Explain the process of fluorescence and factors influencing fluorescence.
- Describe the instrumentation of Fluorimeter and its functioning.
- Explore the applications of Fluorimetry.

4.1 INTRODUCTION

When certain molecules are exposed to the electromagnetic radiation they exhibit fluorescence. Fluorescence is a process of re-emission of radiant energy absorbed in the form of visible light. In this process, the light emitted is always of higher wavelength than that absorbed. In fluorescence, absorption and emission of light takes place in very short time (10^{-12} to 10^{-9} seconds). If there is delay in the emission of light then the phenomenon is called phosphorescence. The delay period may range from fraction of a second to few days. Both these processes of re-emission are generally designated as luminescence. The quantitative determination of fluorescence is the base of fluorimetry analysis.

4.2 PRINCIPLE OF FLUORESCENCE

A molecule at rest or in ground state has three energy levels, i.e. rotational, vibrational and electronic. When an electromagnetic radiation falls on the molecule, it brings changes into its energy levels during the process of absorption. This is illustrated in Fig 4.1. Within each electronic level of the ground state, there is large number of vibrational energy levels (v_0, v_1, v_2 ...). Electrons of a molecule prefer to remain at the lowest vibration level of the ground electronic state. Absorption of ultraviolet or visible light by a molecule brings it into its excited electronic state. Each of the excited electronic states of molecule has many different vibrational energy levels $\left(v_0', v_1', v_2' \ldots\ldots\right)$. During the absorption of radiation, electrons are promoted to an excited electronic energy in one of these vibration levels ($h\upsilon_1$). Most usually and common is a singlet state, i.e. one in which all electrons are paired and in each pair two electrons spin about their axis in opposite directions. Average lifetime of a molecule in excited state is short, usually 10^{-8} to 10^{-6} sec. During this period molecule at each vibrational level of excited state would lose energy by other two processes. Molecule may

lose energy by emitting photon or radiation and fall to the original ground state. The energy of emitted light would be exactly the same as that absorbed. (This is shown by usual absorption). However, the other process may be that usually some molecules initially undergo radiation loss of energy because of collision and fall to lowest vibrational energy of the excited state. The vibrational energy is considered to be lost to the solvent molecule ($h\upsilon_3$). From lowest vibration level of excited state, molecules usually return to ground state by photoemission of energy $h\upsilon_2$. This energy is lower than the energy absorbed and so the emitted radiation is of longer wavelength. This emitted light is called fluorescence. Thus, fluorescence is defined as a radiation emitted in transition of a molecule from lowest level of singlet excited state to singlet ground state.

In certain cases, the electrons cross over from excited vibrational singlet state to excited vibrational triplet state. Triplet state is a metastable electronic level lower than singlet state. When this occurs, the lifetime is much longer, often from fraction of a second to seconds. The energy emitted from triplet state to vibrational ground level ($h\upsilon_4$) is called phosphorescence.

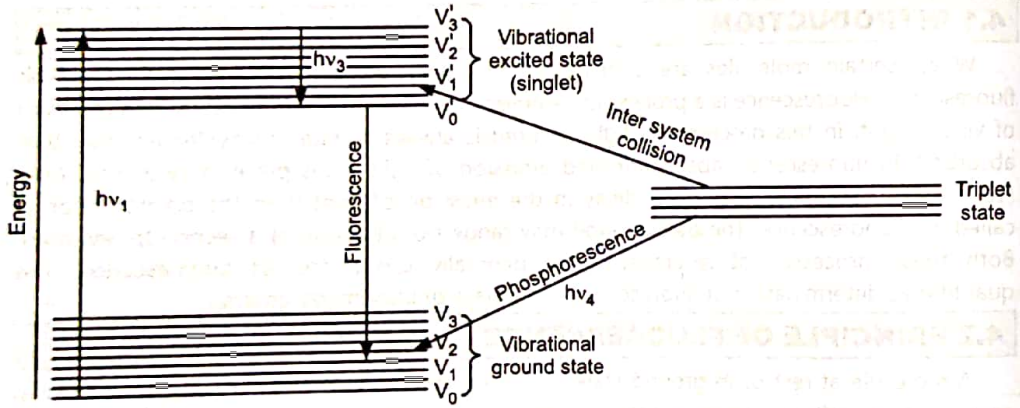


Fig. 4.1: Energy level

Fig. 4.1 shows Energy level diagram illustrating energy changes on absorption of electromagnetic radiation showing fluorescence and phosphorescence phenomena.

4.3 MOLECULAR STRUCTURE AND FLUORESCENCE

It is observed that all substances do not show fluorescence. Some exhibit fluorescence under suitable conditions of excitation by themselves while others show it only when converted into suitable fluorogenic moiety. Thus, there appears a certain requirement in molecular structure for exhibiting fluorogenic activity. A generalization is as under:

1. Fluorescence is shown by those molecules which have absorbancy. Certain types of electrons present in the molecule show high absorbancy value and hence fluorescence.

2. Compounds with multiple double bond and conjugated bonds show fluorescence. A high degree of resonance stability is necessary. Any group or substituent that alters resonance stability affects fluorescence.
3. In many aromatic and heterocyclic compounds, fluorescence is shown by the presence of certain substituted groups. There should be at least one or more electron donating groups such as – NH_2, – OH, – OCH_3 on the resonating nucleus.
4. Electron withdrawing groups like – COOH, – NO_2, – N = N–, –Cl, –Br, –I diminishes or destroys fluorescence.
5. Ring closure is conducive to the fluorescence in aromatic compounds, e.g. fluorescin, eosin etc.
6. Polycyclic compounds such as vitamin K, nucleosides, purine and the polyene such as vitamin A exhibit fluorescence.
7. Formation of metal chelates also promotes fluorescence. This is because of rigidity of molecule preventing excitation energy being dissipated in other ways than fluorescence.
8. Position of chromophore has important influence on fluorescence. Sharp changes of fluorescence occur both in intensity and degree with change in pH and solvent.
9. Ionization and dissociation of molecule which leads to increased resonance energy enhances fluorescence. The ionized or unionized form may be fluorescent and this is affected by pH changes, e.g. the violet fluorescence shown by aniline base in neutral or alkaline solution is lost in acidic solution.

4.4 FACTORS WHICH AFFECT FLUORESCENCE

Fluorescence is a very sensitive phenomenon exhibited by a substance in a given solution. There are number of factors which directly and indirectly affect fluorescence and decrease its intensity and sensitivity which is termed as 'quenching'. There are substances which by their presence compete for the electronic excitation energy and decrease the fluorescence. Some factors which affect fluorescence are:

(i) Concentration of substance:

Fluorescence is best given in dilute solution. In concentrated solution, the intensity of fluorescence is reduced and is not quantitative. This is called concentration quenching.

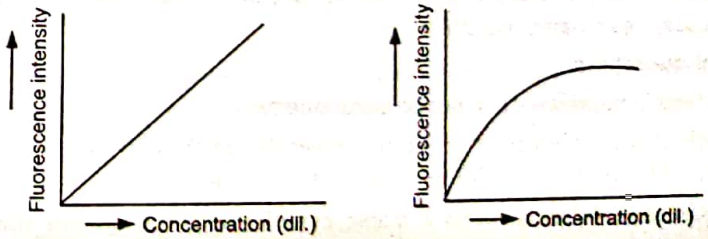


Fig. 4.2 : Effect of Concentration on fluorescence intensity

The reason is that in dilute solutions, the absorbed light is distributed equally through the entire path length of the solution. But at higher concentrations the first part of the solution in the path absorbs more of radiation and less is available for the remaining path. Thus, in concentrated solution the fluorescence decreases because of

(a) Vibration loss due to intramolecular collision, and

(b) By reabsorption of the emitted fluorescence.

(ii) Oxygen:

Presence of oxygen causes decrease in fluorescence. The interference is due to direct oxidation of fluorogenic material into non-fluorogenic and also indirectly due to quenching of fluorescence.

(iii) Photodecomposition:

In Fluorimetry, generally a high-intensity of radiation is required for excitation. This high intensity radiation may bring irradiation changes or photochemical changes in a substance destroying fluorescence. The light used must be of such suitable wavelength that it is not strong enough to cause photodecomposition. The measurement of fluorescence should be done rapidly.

(iv) pH:

Alteration of pH affects fluorescence significantly since it brings changes in the ionized and non-ionized form of fluorogenic material, e.g. phenol shows fluorescence in both ionized and unionized forms. In neutral and alkaline solution, it ionizes and gives weak fluorescence. While in strongly acidic pH, phenol shows intense fluorescence.

(v) Temperature and viscosity:

Increase in temperature and decrease in viscosity is likely to cause collision between the molecules and thus decrease in fluorescence by deactivation of excited molecule. Low temperature and appropriate dilute solution are necessary for quantitative results.

(vi) Impurities and other substances:

Certain substances act as impurity and show fluorescence quenching, e.g. iodide ion is an extremely effective quencher. Organic substances, especially aromatic type in dilute solutions (1-2 ppm) have tendency of adsorption on the surface of cell. Addition of small amount of polar solvent usually eliminates this effect.

(vii) Chemical quenching

The quenching in fluorescence may also occur due to:

(a) Collision of excited molecule with other molecule/ion or impurity which results in the transfer of fluorescent intensity from excited molecule to other molecules or

(b) The quencher molecule forms a stable complex with the ground state molecule known as static quenching.

(viii) Inter filter effect:

There may be presence of non-fluorogenic material in the sample which absorbs light and thus shows filter effect. The presence of such materials should be kept minimal and constant both in standard and test sample.

4.5 RELATIONSHIP BETWEEN CONCENTRATION AND FLUORESCENCE

For the quantitative studies there should be a definite relationship (linear) between the concentration of species and the fluorescent intensity which is emitted. Such a relationship exists for very dilute solutions and fluorescent intensity. The Beer's law can be applied and thus,

$$F = K (I_o - I_t) \quad \text{... (4.1)}$$

Where,

I_o = Intensity of incident radiation

I_t = Intensity of transmitted radiation

$I_o - I_t$ = Intensity of radiation absorbed by the solution

F = Intensity of fluorescent radiation

K = Proportionality constant

F is assumed proportional to the intensity of radiant energy absorbed $(I_o - I_t)$

Now, according to Beer-Lambert's law

$$I_t = I_o . 10^{-abc} \quad \text{... (4.2)}$$

a = Absorptivity

b = Path length in cm

c = Concentration in gm/lit.

And $$(I_o - I_t) = I_o (1 - 10^{-ABC}) \quad \text{... (4.3)}$$

So $$F = K I_o (1 - 10^{-ABC}) \quad \text{... (4.4)}$$

If $$KI_o = F_o \quad \text{... (4.5)}$$

Then $$F = F_o - F_o 10^{-abc} \quad \text{... (4.6)}$$

$$\therefore \quad \text{Log} \frac{F_o}{F_o - F} = abc \quad \text{... (4.7)}$$

If abc is small > 0.01 then F = Kc. Thus, for low concentrations fluorescence intensity is directly proportional to the concentration and it is also proportional to the intensity of the incident radiation.

This equation holds for concentrations of the order of a few parts per million or less, depending on the substance. At higher concentrations, the fluorescent intensity generally decreases. It is noted that the fluorescence is concentrated near the entrance of cell and there is less and less in the remainder of the cell. Although the exciting light does penetrate through the solution it is not evenly distributed along its path and hence the fluorescence emission is not related to the absorption phenomenon.

According to equation it is assumed that the fluorescence is measured in the same path as the incident radiation. In practice, however, the fluorescence is measured at right angles to the incident light. The equation still holds true when b is replaced by b', the depth of cell in the direction of the detector.

4.6 INSTRUMENTATION

An instrument used for measuring fluorescence is called as fluorimeter. In fluorescence measurement, it is necessary to separate the emitted radiation from the incident and this is done by measuring the fluorescence at right angles to the incident radiation. There are different types of instruments depending upon the sophistication employed in them. However, fluorimeter in general are of two types;

1. filter fluorimeter and
2. spectrofluorimeter

In the filter type, two filters are used, one to isolate exciting wavelength from its source and another for emission wavelength. In the spectrofluorimeter, instead of using filters, the instrument incorporates two monochromators, one to select wavelength of excitation and other to select the wavelength of fluorescence.

A line diagram of a simple filter type fluorimeter is given in Fig. 4.3. The components of a typical fluorimeter are given in Fig. 4.3.

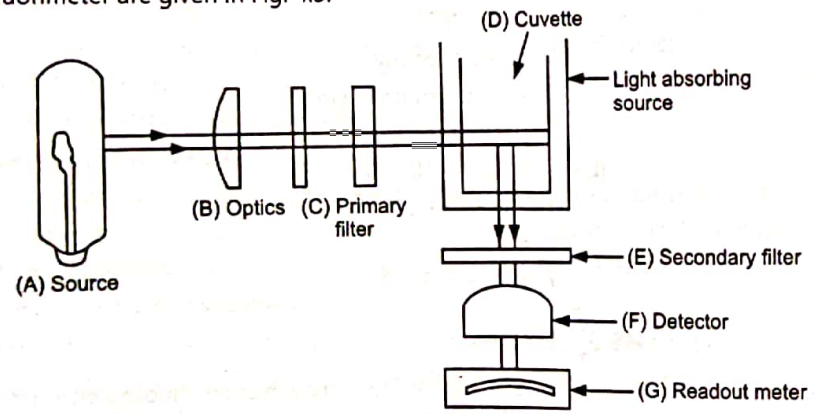


Fig. 4.3 : Line diagram of Fluorimeter

In fluorimeter, an ultraviolet light is required as a source of illumination (A). A mercury vapour lamp with glass or fused silica envelope is commonly used as a source. When a spark is passed through mercury vapour at low pressure principle lines of wavelength are emitted. By isolating one of the principle lines at 366, 405, 436, 520 or 580 nm high intensity radiation can be isolated using suitable filter (primary filter). In more sophisticated instruments, a high pressure xenon lamp is used as a source of radiation.

The radiation from the source is collected on lenses, which are made of either quartz or glass and passed through a slit (B). The radiation is then passed through filter termed as primary filter (C). The primary filter allows the passage of wavelength required for excitation. In simple instruments, glass filters are used as they allow appreciable amount of wavelength to pass through. Interference filters are superior as they allow narrow band of wavelength to pass through.

The cuvette or cell (D) is usually 1 cm^2 type and is made of good quality glass for routine work. The cuvettes have a lid to prevent vapourization of volatile material. The cuvette unit is mounted in a chamber having light absorbing surface.

The fluorescent radiation is emitted in all directions. The radiation at right angles to the incident is allowed to pass through second filter known as secondary filter (E). The secondary filter allows the passage of wavelength of emission and absorbs the excitation wavelength. The fluorescent beam is collected on detector (F) which is usually a photomultiplier tube. This detects and measures even a weak fluorescence. To protect detectors from measuring reflected or scattered exciting radiation, secondary filters are placed just in front of photodetector. The signals from detector are fed to the readout meter (G) which is a galvanometer.

It is to be borne in mind that since it is difficult to measure absolute fluorescence intensity, measurement of fluorescence of sample is made with reference to a fluorescence produced by a standard substance. Frequently, instrument is calibrated with various concentrations of standard solutions, including for solvent and cuvettes to find true value of fluorescence. The sample solution is treated under identical conditions for recording the fluorescence. From the plot of fluorescence concentration curve, concentration of sample solution is determined.

Commercial fluorimeter is more sophisticated and are of filter or spectrometer type. Further, these may be a single beam or double beam in design. A number of popular name like Colem, Klett, Lumetron, Hilger-sppekker, Hitachi, Beckman are available in the market.

4.7 APPLICATIONS

One of the important features of fluorescence analysis is its sensitivity. And in this respect it is considered to be superior to absorption spectrophotometry. In absorption spectrophotometry, the difference between the two final signals I_o and I_t is measured. The sensitivity is thus governed by the ability to distinguish between these two, which is dependent on the strength and nature of the instrument, besides other factors. In Fluorimetry, however, we measure the difference between zero and finite number. Thus in principle the limit of detection is governed by the intensity of source and sensitivity of detector. It is thus possible to measure fluorescence in very dilute (10^{-8} M) solutions.

Fluorimetric analysis is possible for wide variety of drugs. Those substances which are intrinsically fluorescent are readily determined simply by dissolving in appropriate solvent or media, e.g. Aminocrine in 0.1 N hydrochloric acid, Ergometrine in 1 per cent tartaric acid, Riboflavine in aqueous buffer of pH 6, quinine in 0.1 N sulphuric acid etc.

Substances which by themselves are non-fluorescent can be converted into fluorogenic by chemical change. Such change can be carried out both in organic and inorganic compounds, e.g. Thiamine (vitamin B_1) is oxidized to thiochrome adrenaline to adrenochrome.

In other cases, fluorogenic reagent is allowed to react with organic or inorganic compounds to form fluorogenic complexes. Thus, substances like allyl morphine, para-amino salicylic acid, chloroquine; folic acid, menadione, phenobarbitone; procaine, thymol, aluminium, selenium, etc. can be analysed by coupling with suitable reagent.

It is possible to estimate one drug in presence of other fluorimetrically by adopting suitable technique; e.g. by changing pH, thus converting ionic to non-ionic and vice-versa. Morphine, codeine, atropine can be estimated by preparing derivative which are fluorogenic in nature.

REVIEW QUESTIONS

1. Explain the term Fluorescence and phosphorescence.
2. Write the principle of fluorimetry.
3. Why all molecules are not exhibiting fluorescence? Is there any requirement of molecular structure for exhibiting fluorogenic activity? Explain.
4. Discuss the instrumentation and working of fluorimeter.
5. What do you mean by quenching?
6. What are the factors affecting fluorescence? Explain each factor.
7. Write the applications of fluorimetry.

UNIT - II

Chapter ...5

INFRARED SPECTROSCOPY

Objectives:

Upon completion of this section, the student should be able to

- Understand the basics of Infrared spectroscopy including its principle, theory and applications.
- Explain the origin of IR spectra, its significance and types of molecular vibrational modes.
- Explain the pharmaceutical applications of IR spectroscopy.

5.1 INTRODUCTION

Infrared (IR) spectroscopy is one of the most important analytical techniques among the spectroscopic techniques. The main use of this technique is in organic and inorganic chemistry to determine functional groups in molecules. It is known for its great advantages that almost any sample in any state may be studied. Liquids, solutions, pastes, powders, films, fibres, gases and surfaces can all be examined with a judicious choice of sampling technique. Infrared spectrometers have been commercially available since the 1940s. At that time, the instruments relied on prisms to act as dispersive elements, but by the mid 1950s, diffraction gratings had been introduced into dispersive machines. IR spectroscopy is a technique based on the vibrations of the atoms of a molecule. IR spectroscopy measures the vibrations of atoms, through which it is possible to determine the functional groups.

An infrared spectrum is commonly obtained by passing infrared radiation through a sample and determining the fraction of the incident radiation which is absorbed at a particular energy. The energy at which any peak in an absorption spectrum appears corresponds to the frequency of a vibration of a part of a sample molecule. The term "infrared" covers the range of the electromagnetic spectrum between 0.78 and 1000 µm. In the context of infrared spectroscopy, wavelength is measured in "wave numbers", which have the units cm^{-1}.

$$\text{Wave number} = \frac{1}{\text{Wavelength in centimeters}}$$

Infrared region has been divided into three sections;

1. Near infrared region
2. Middle infrared region
3. Far infrared region.

The wavelength ranges for the above regions are given below.

Region	Wavelength (λ) range μ	Wave number $(\bar{u})$ range cm^{-1}
Near IR	0.78–2.5	12800–4000
Middle IR	2.5–50	4000–200
Far IR	16–200	625–10
(Most useful Infrared region)	2.5–16	4000–625

We know that wave number is related to wavelength by the expression

$$\therefore \quad \bar{\upsilon} = \frac{1}{\lambda} \text{ and } \lambda = \frac{c}{v} = \frac{\text{Velocity of light}}{\text{Frequency}} \text{ cm/sec.}$$

$$= \bar{\upsilon} = \frac{v}{c}$$

Further, energy of quantum E corresponding to radiation of frequency υ is given by

$$E = h\upsilon, \quad \therefore E = h\,\bar{\upsilon}\,c; \bar{\upsilon} = \frac{E}{hc}$$

In the above equation, both h and c are constant. Thus, the energy of radiation is directly proportional to the wave number and inversely proportional to the wavelength.

As the IR radiation is of longer wavelength, it is associated with much lower energy than visible and UV radiation.

A chemical substance, when exposed to radiation in IR region, shows marked selective absorption bands. This is because after absorption of IR radiations, molecules of substance vibrate at many rates of vibrations giving rise to closely packed absorption bands, commonly known as IR absorption spectrum. The various bands seen in IR spectrum are due to characteristic functional groups and bonds present in a substance. Thus, an IR spectrum of a chemical substance is considered as a fingerprint and used for its identification. Band positions in an IR spectrum may be expressed conveniently by the number $\bar{\upsilon}$ where unit is cm^{-1}. The band intensities may be expressed either as Transmittance (T) or Absorbance (A) on ordinate and wave number on abscissa.

5.2 THE ORIGIN OF IR SPECTRA

The infrared spectrum is formed as a consequence of the absorption of electromagnetic radiation at frequencies that correlate to the vibration of specific sets of chemical bonds from within a molecule. The total energy of a molecule at any given moment is defined as the sum of the contributing energy terms:

$$E_{Total} = E_{Electronic} + E_{Vibrational} + E_{Rotational} + E_{Translational}$$

Among these energies, the vibrational energy component is a higher energy term and corresponds to the absorption of energy by a molecule as the component atoms vibrate about the mean center of their chemical bonds. For a molecule to show infrared absorptions it must possess a specific feature, i.e. an electric dipole moment of the molecule must change during the vibration. This is the *selection rule* for infrared spectroscopy.

Simple harmonic oscillator and anharmonicity are sufficient to explain the origin of many of the characteristic frequencies. However, the fundamental vibrational frequency of a molecule can be expressed by Hooks Law.

To understand these vibrations let us consider a diatomic molecule AB held by a covalent bond with mass m_1 and m_2 of the two atoms A and B. Each atom consists of nucleus corresponding to the atoms A and B, accompanying their electrons. The two atoms are connected by a covalent bond as a spring with two atoms at either end. The stiffness of spring is taken as a force constant k (Fig. 5.1).

The frequency of motion is

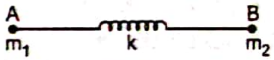


Fig. 5.1

$$\bar{\upsilon} = \frac{1}{2\pi c}\sqrt{\frac{k}{c}} \quad \text{... (5.1)}$$

Where, υ = frequency, μ is the reduced mass of individual atoms.

As $$\mu = \frac{m_1 m_2}{m_1 + m_2} \quad \therefore \quad \frac{1}{\mu} = \frac{1}{m_1} + \frac{1}{m_2} \quad \text{... (5.2)}$$

The force constant for a bond varies slightly from one compound to another. However, it can be calculated, e.g. C–O bond in methanol. It has $f = 5 \times 10^5$ dynes per cm and μ = 6.85. It shows V = 1110 cm^{-1}. In spectrum, a strong band for methanol is observed around wave number 1034 cm^{-1}. It is thus seen that if force constant is known, approximate frequency of band can be calculated.

For vibrational mode to occur on striking IR radiation, it is essential that there is a change in dipole moment during vibration. In diatomic molecules, if there is no change in dipole, no vibration is seen in IR region. Polyatomic molecules having no dipole moment of their own may be made to undergo vibrational rotational transition.

The vibrational energy of a chemical bond is quantized and is given by

$$E_{Vib} = \left(v + \frac{1}{2}\right)h\upsilon \quad \text{... (5.3)}$$

Where, v = Number of vibrational levels as 0, 1, 2

h = Planck's constant

υ = Vibrational frequency of the bond

The energy difference between two vibrational levels is given by

$$\Delta E_{Vib} = h\upsilon \quad \text{... (5.4)}$$

In summary, the absorption of IR radiation causes the excitation of molecule to higher vibrational levels and is quantized when transitions occur from lowest level (v = 0) to first level (v = 1). The frequency of that radiation is given by $h\upsilon = E_1 - E_o$ and is called fundamental vibration frequency; and when transition from (v = 0) to (v = 2) occurs it is called **overtone frequency.**

The Hook's law provides link between the strength of the covalent bond, masses of atoms and vibrational frequency. The greater the masses of attached atoms, the lower the IR frequency at which the bond will absorb.

1. **For a stronger bond (larger k value), u increases:**

As examples of this, in order of *increasing bond strength* compare:

- **C–C bonds:** C–C (1000 cm^{-1}), C = C (1600 cm^{-1}) and C ≡ C (2200 cm^{-1}),
- **C–H bonds:** C–C–H (2900 cm^{-1}), C = C-H (3100 cm^{-1}) and C ≡ C – H (3300 cm^{-1}),

2. **For heavier atoms attached (larger m value), μ decreases:**

As examples of this, in order of *increasing reduced mass* compare:

- C–H (3000 cm^{-1})
- C–C (1000 cm^{-1})
- C–Cl (800 cm^{-1})
- C–Br (550 cm^{-1})
- C–I (about 500 cm^{-1})

Thus Hooke's Law states:

- The vibrational frequency is proportional to the strength of the spring; the stronger the spring, the higher the frequency.
- The vibrational frequency is inversely proportional to the masses at the ends of the spring; the lighter the weights, the higher the frequency.

As per the Hooke's Law:

- Stronger bonds absorb at higher frequencies.
- Weaker bonds absorb at lower frequencies.
- Bonds between lighter atoms absorb at higher frequencies.
- Bonds between heavier atoms absorb at lower frequencies.

We can describe the molecular vibrational model in terms of a minimum set of fundamental vibrations, based on a threefold set of coordinate axes, which are known as the normal modes of vibration. A molecule composed of n-atoms has 3n degrees of freedom, six of which are translations and rotations of the molecule itself. This leaves 3n-6 degrees of

vibrational freedom (3n-5 if the molecule is linear). The number of normal modes of vibration for a given molecule can be determined from the following Equations.

1. Number of normal modes = 3N - 6 (For non-linear molecules).
2. Number of normal modes = 3N - 5 (For linear molecules).

Note : N = Number of atoms in the molecule.

3 N = The degrees of freedom in three fold of coordinate axes.

The functional group of the molecules gives rise to infrared absorption frequencies, and each group has its own unique contribution based on its extinction coefficient. Theoretically, the absorption frequencies are assigned in the infrared spectrum to much more that just simple harmonic (or anharmonic) stretching vibrations. In practice, various other deformation motions (angular changes), such as bending and twisting vibrations also contribute to the overall absorption spectrum. The stretching absorptions of a vibrating chemical bond occur at higher frequencies (wave numbers) than the corresponding bending or bond deformation vibrations, which denote that energy and frequency are proportionally related. Many other spatially related scenarios such as cis and trans spatial relationship differences, in-plane, out-plane, twisting, and rocking vibrations contribute specific frequencies in IR spectrum. Many of these vibrations exhibited as C-H vibrations. Molecular symmetry of the molecule, relative electronegativity, bond order and relative mass of the atoms also has impact in the IR spectrum. Transitions to higher energy levels give rise to overtone bands in the mid-infrared region occur at approximately twice the fundamental frequency for the first overtone. Higher overtones are also occurs at lower intensity in the near-infrared spectral regions, between 12500 and 4000 cm^{-1}.

In addition to these, combination bands (sum and difference), bands due to transitions from energy states higher than the ground state or "hot bands", and bands due to interactions between a weaker overtone or combination band and Fermi resonance bands also contribute the IR spectrum. The additional functional groups in the molecule make IR spectrum more complex with their vibrational patterns.

Molecular Vibrations:

The positions of atoms in molecules are not fixed; they are subject to a number of different vibrations.

There are two simplest modes of molecular vibrations in a molecule which are infrared active and give rise to absorptions as given below.

- Stretching vibrations
- Bending vibrations.

The fundamental modes of vibrations are represented by stretching vibrations.

(a) Stretching vibrations:

The stretching vibrations are those in which two bonded atoms oscillate continuously, without altering bond axis or bond angles. They are of two types: (a) asymmetric and (b) symmetric.

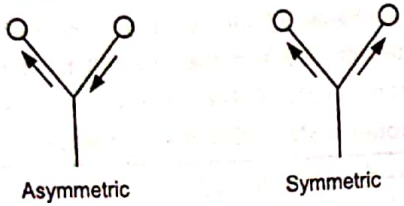


Fig. 5.2 : Stretching vibrations

In symmetric, both atoms move away from central atom while in asymmetric one atom moves away from central atom while other moves towards it. Stretching vibrations generally require higher energies than bending.

(b) Bending (or deformed) vibrations:

These are characterised by continuously changing bond angle and axis with common atom. These are of various types as (positive indicates movement out of plane above and negative indicates movement back of plane).

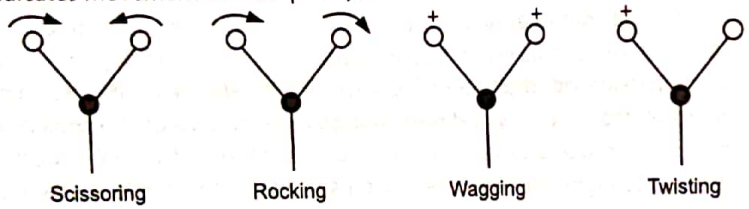


Fig. 5.3 : Bending vibrations

1. Scissoring vibrations occur when two atoms move back and forth towards each other.
2. Rocking vibrations occur due to oscillations of atoms back and forth out of equilibrium plane.
3. Wagging vibrations result when the unit oscillates in equilibrium plane-formed by the atoms.
4. Twisting vibrations occur when structural unit rotates around the bond which joins to the molecule.

Factors Influencing Vibrational Frequency:

From the discussion above we know that the probable frequency of absorption can be calculated by the Hook's law. However, it has been observed that the calculated value of frequency of absorption is not exactly equal to the experimental value. There are many factors which are responsible for shifts in vibrational frequencies.

1. The frequency shift may occur due to the effect of molecule in the immediate neighborhoods of bond,
2. Change in force constant of bond due to electronic structure and
3. Due to different states of the same substance, e.g. solid, liquid or gas (vapour).

The energy of vibration and thus the wavelength of its absorption peak are influenced by other vibrations in a molecule. The influence and extent of coupling of vibrations plays significant role.

5.3 EXAMINATION OF INFRARED SPECTRUM

Recorded infrared spectrum shows number of absorption peaks, some fine, some broad with varying intensities in its complete region (4000–650 cm^{-1}). The identification of unknown organic compound is usually done by examining certain region of the spectrum to get clues about the presence or absence of certain group frequencies. From the number of absorption peaks, some show distinctly the presence of particular group which helps in identification of a compound. While reading and interpreting spectra, it is to be remembered that no attempt to be made to assign for all peaks. Some important regions which are examined are described below:

(a) The region between 4000-1400 cm^{-1}:

This broad region shows presence or absence of many groups in the molecule. The important groups accounted for include NH, OH, C = O, C = C, C = N etc. The presence of aromatic nucleus (2000–1670) and hydrogen bonding O–H, N–H etc. are also encountered in this region.

(b) The region between 1400-900 cm^{-1}:

This is commonly known as finger print region. This region accounts for many absorption bands characteristic of functional groups. Since, location of different functional groups can be attributed in this region it is termed as fingerprint region. Absorption bands due to bending vibrations of different groups as well stretching vibrations of C – C, C – O, C – N are observed. Since, number of sharp bands of varying intensities is encountered in this regional close examination of bands for establishing identity is needed.

(c) The region below 900 cm^{-1}:

This region from 900–650 or 450 cm^{-1} gives few but sharp bands which can be accounted for the presence of specific groups. The presence of an aromatic nucleus is indicated in 1000–625 cm^{-1} region. The N–H, C–H rocking is also seen in this region.

Group frequencies:

Various authors have studied and tabulated frequencies of absorption of characteristic group, which information is available in number of standard text books. A very brief abstract of some principle absorption bands of selected functional groups is given in Table 5.1.

Table 5.1: Absorption peaks with intensity of some selected functional groups

Type of compound C–H stretching	Frequency range (cm^{-1})	Intensity
C – H stretching		
Alkane	2850–2970	s
Alkenes	3010–3095	m
Alkynes	3320–3310	s
Aromatic rings	3310–3100	m
Aldehyde	2900–2500	s

C = C and C ≡ C bond stretching		
Alkene	1680–1620	s – m
Alkynes	2300–2100	s – m
Carbonyl C = O stretching		
Saturated aliphatic ketone	1750–1700	s
α, β unsaturated aliphatic ketone	1685–1660	s
Saturated aliphatic aldehydes	1740–1720	s
α, β unsaturated aldehyde	1705–1680	s
Aryl aldehyde	1700–1680	s
Saturated esters	1750–1735	s
Unsaturated esters	1730–1715	s
Aryl aldehyde	1730–1715	s
Saturated carboxylic acids	1725–1700	s
Unsaturated carboxylic acids	1715–1690	s
Aryl carboxylic acid	1700–1680	s
Amide	1680–1630	s
Imide	1700–1670	s
Lacto	1720–1660	s
Thiocarbonyl C = S	1200–1050	s
Sulphone S = O	1180–1140	s
Sulphonamide	1350–1300	s
O–H (stretching)		
Alcohol (O–H) stretching free	3650–3450	s
Hydrogen bonded	3570–3450	s
Sec. and ter. (O–H) bending alcohol	1100–1050	s
N–H (stretching)		
Prim., Sec., Ter. Amines	3500–3400	M
N–H bending		
Prim., Sec. amine	1650–1550	M
C–N Stretching		
Aliphatic	1200–1000	w
Aromatic	1350–1250	m
	and 860	m
C ≡ N in nitrile	2280–2200	s

Halogen compounds		
C – F	1400–1000	s
C – Cl	800–600	s
C – Br	650–500	s
C – I	600–500	s

Aromatic compound:

The presence of aromatic nucleus in a compound is indicated in three regions: (a) 2000–1670 cm^{-1}, (b) 1670–1430 cm^{-1} and (c) 1000–625 cm^{-1}. Very sharp and fine bands due to C–H bending occur in 1000–625 cm^{-1} region. Compared to parent benzene ring, monosubstituted compounds show absorption at 700 cm^{-1}, while disubstituted around 750 cm^{-1}, and para substituted around 860–800 cm^{-1}. Though presence of aromatic nucleus could be indicated by absorption bands in this region, the absence of it is more indicative of substance being non-aromatic.

Hydrogen bonding:

Hydrogen bonding between O–H, N–H groups is usually shown by strong absorption bands in 3700–2700 cm^{-1} region in stretching vibrations between hydrogen and other atom occurs. In OH, the O–H stretching vibrations appear at higher wave numbers and are broad than N–H band. Usually, hydrogen bonding tends to broaden the peaks and shows absorption at lower wave number.

Unstauration:

The unstauration in a compound could be due to triple bond which is indicated by peak for C ≡ C at 2250–2275 cm^{-1} for C ≡ N at 2180–2120 cm^{-1}. The double bond due to (C=O) carbonyl group show stretching around 1700 cm^{-1}. If absorption peak occurs in 1770–1725 cm^{-1}, it could be due to carboxyl stretching in esters, acid chloride, anhydrides etc.

Absorption peak around 1690–1600 cm^{-1} arises due to C=C and C=N stretching vibrations. Conjugation usually results in a lower absorption peak by 20 cm^{-1}. In case of carbonyl function (C=O) peak occurs at 1720 cm^{-1}. This may be attributed to the following chemical class of compounds (a) Ketone, (b) aldehyde, (c) ester, (d) lactone, (e) anhydride and (f) carboxylic acid. To differentiate these, examination for presence or absence of peaks at other region is carried out.

Fermi Resonance:

When a fundamental vibration couples with an **overtone or combination band** it results in different vibration. This coupled vibration is called Fermi resonance. Fermi resonance results in the splitting of two vibrational bands that have nearly the same energy and symmetry. The wave functions for the two resonant vibrations mix according to the harmonic oscillator approximation, and the result is a shift in frequency and a change in intensity in the spectrum. As a result, two strong bands are observed in the spectrum, instead of the expected strong and weak bands. It is not possible to determine the contribution from each

vibration because of the resulting mixed wave function. Example: In the given figure, the bands shown in the top represent two fundamental vibrations without Fermi resonance whereas the bands shown in the bottom represent change in bands as a result of Fermi resonance. The Fermi resonance resulted in increase in energy of first band and decrease in energy of second band thus resulted in "Fermi doublet".

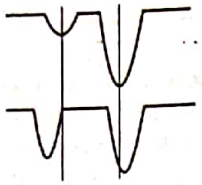


Fig. 5.4 : Fermi resonance

Overtones:

Overtones occur when a vibrational mode is excited from $v = 0$ to $v = 2$, which is called the first overtone, or $v = 0$ to $v = 3$, the second overtone. The fundamental transitions, $v = \pm 1$, are the most commonly occurring, and the probability of overtones rapidly decreases as $v = \pm n$ increases. Based on the harmonic oscillator approximation, the energy of the overtone transition will be about n times the fundamental associated with that particular transition. The anharmonic oscillator calculations show that the overtones are usually less than a multiple of the fundamental frequency. Overtones are generally not detected in larger molecules.

Combination bands:

Combination bands are observed when more than two or more fundamental vibrations are excited simultaneously. One reason a combination band might occur is if a fundamental vibration does not occur because of symmetry. This is comparable to **vibronic coupling** in electronic transitions in which a fundamental mode can be excited and allowed as a "doubly excited state." Combination implies addition of two frequencies, but it also possible to have a difference band where the frequencies are subtracted.

Vibrational coupling:

In addition to the vibrations mentioned above, interaction between vibrations can occur (*coupling*) if the vibrating bonds are joined to a single, central atom. This is called as vibrational coupling. Vibrational coupling is influenced by a number of factors.

- When a common atom is present between two bonds and during their vibration coupling of stretching vibrations occurs. When a bond is common between two vibrating groups then coupling of bending vibrations occurs.
- Coupling between a stretching vibration and a bending vibration occurs if the stretching bond is one side of an angle varied by bending vibration.
- Coupling is greatest when the coupled groups have approximately equal energies.
- No coupling is seen between groups separated by two or more bonds.

5.4 INSTRUMENTATION

Infrared spectrometers are composed of the same basic components as in the ultraviolet and visible regions, although the source of radiation, detectors and the materials used in the fabrication of the optical components are different. The standard infrared spectrophotometer is a filter-grating or prism-grating instrument covering the range from 4000–650 cm^{-1} (02.5–15.4 μ). The grating instruments offer high resolution that permits separation of closely spaced absorption bands, accurate measurements of band positions and intensities and high scanning speeds for a given resolution and noise level.

The radiation from a source emitting in the infrared region is interrupted (i.e. chopped, pulsed or modulated) at a low frequency level (10–26 Hz) and is passed alternately through the sample and the reference. This minimizes the effect of stray radiations emerging from the sample and cell before it reaches the detector. The temperature and humidity affects the performance of infrared spectrophotometer.

The following are the essential components of an infrared spectrophotometer:

1. Light source
2. Monochromator and optical materials
3. Sample holder
4. Detector and
5. Instrument for recording the response (Recorder)

5.4.1 Infrared Radiation Sources

The infrared radiation sources are the hot bodies, continuously emitting the radiations, which approximate a black body radiator in their emission properties.

(a) Incandescent lamp:

A closed wound nichrome coil can be raised to incandescence by resistive heating. A black oxide film formed on the coil give acceptable emissivity. In this, the temperature can be reached up 1100°C. The nichrome coil does not require water cooling. It requires little or no maintenance and gives long service. This source is recommended where reliability is essential. Though this source is simple and rugged, it is less intense than some other infrared radiation sources.

A rhodium wire heater sealed in a ceramic cylinder has also been used as a source of infrared radiations.

(b) Nernst glower:

In IR spectroscopy, Nernst glower is the most commonly used source of radiation. It is constructed by fusing a mixture of oxides of metals like zirconium, yttrium and thorium. They are moulded in the form of hollow tubes or rods about 1–3 mm in diameter and 2–5 cm in length. The ends of the rods are cemented to short ceramic tubes for mounting and short platinum leads are provided for power connections.

Nernst glowers are fragile. They have negative coefficient of resistance and they are preheated to be conductive. Thus, they are provided with auxiliary heaters. To prevent overheating they are provided with ballast, but they should also be protected from draught even as ventilation is needed to remove surplus heat.

The energy output of Nernst glower is predominantly concentrated between 1–10 μ with relatively low energy beyond 10 μ. Radiation intensity is approximately thrice that of nichrome and globar sources, except in the near infrared region.

The main advantages of Nernst glower are that it emits infrared radiations over wide wavelength range and the intensity of radiation remains steady and constant over a long period of time; second, it can be used in air as it is not oxidized.

(c) Globar Source:

It is a rod of sintered silicon carbide 6–8 mm in diameter and 50 mm in length. It is self starting and is electrically heated. The operating temperature is about 1300°C. It has a positive coefficient of resistance and can conveniently be controlled with a variable transformer. It is often enclosed in a water cooled brass tube, with a slot provided for the emission of radiations. It emits maximum radiation at 5200 cm^{-1}. In comparison with Nernst glower the Globar is a less intense source below 10 μ. The two sources are comparable to about 15 μ, and the Globar is superior beyond about 15 μ.

(d) Mercury Arc:

In the very far infrared region; i.e. beyond 50 μ (200 cm^{-1}), black body type sources lose effectiveness as their radiations decrease with the fourth power of wavelength. Mercury arc gives intense radiation in this region. It is enclosed in a quartz jacket to reduce loss. The output from mercury arc is similar to that of black body sources, but additional radiation is emitted from plasma which enhances the long wavelength output.

(e) Tungsten Filament Lamp:

This source is useful for near infrared region only.

5.4.2 Monochromator

The radiation source emits radiations of various frequencies. As the sample in IR spectroscopy absorbs only at certain frequencies, it is therefore necessary to select desired frequencies from the radiation source and reject the radiations of other frequencies. This selection is achieved by means of monochromator. The monochromators are of two types: (a) Prism monochromator and (b) Grating monochromator.

(a) Prism Monochromator:

These are favoured because of greater range and simplicity. Neither glass nor quartz is sufficiently transparent to infrared radiations and therefore other materials like halogen salts are used in prism monochromators as they are transparent to infrared radiations.

Quartz prisms are used only in the near infrared region (0.8–3 μ). It is absorbed strongly beyond 4 μ.

The bulk of analytical work in the infrared region is done using crystalline sodium chloride as the prism material. It has high dispersion in the region between 5–15 μ and adequate up to 2.5 μ crystalline potassium bromide and cesium bromide are satisfactory for far infrared region (15 μ–40 μ). In the near infrared region (1–5 μ), lithium fluoride is used as prism material.

All the commonly used prism materials except quartz are water soluble and are easily scratched. These materials must be protected from moisture either by using desiccants or by placing in a sealed housing which is evacuated.

In the infrared spectrometers the focusing of the radiations is achieved by using concave mirrors rather than prisms. These mirrors can be prepared from various materials like metals or glass coated aluminium. The main advantage of these materials is that materials have no chromatic aberration and are sturdy. Besides concave mirrors plane reflecting mirrors are also used.

The prism monochromator may be a single pass monochromator or a double pass monochromator as shown in Figs. 5.5 and 5.6 respectively.

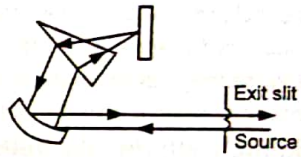


Fig. 5.5: Single pass monochromator

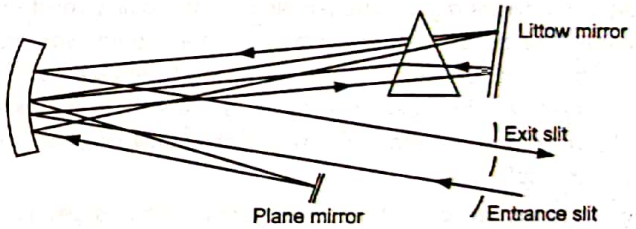


Fig. 5.6: Double pass monochromator

(i) Single pass monochromator:

The sample is kept at or near the focus of the beam, just before the entrance slit A to the monochromator. The radiation from the source after passing through the sample and the slit strikes the off-axis parabolic Littrow mirror 3. This renders the radiation parallel and is transmitted to the prism 'C'. The dispersed radiation after reflecting from a plane mirror 'D' returns through the prism second time and focuses into the exit slit of the monochromator and then to the detector part of the instrument.

(ii) Double pass monochromator:

In the double pass monochromator, there occurs a total of four passes of radiation through prism as shown (1) (2) (3) and (4) in the Fig. 5.5. The double pass monochromator produces more resolution of radiation than single pass monochromator.

In both single and double pass monochromators, sodium chloride (rock salt) prisms are employed for the entire region from 4000–650 cm^{-1} (2.5 to 15.4 μ).

Prisms of lithium fluoride and calcium fluoride give more resolution in the region where the significant stretching vibrations are located.

(b) Grating monochromator:

The grating is essentially a series of parallel straight lines cut out into a plane surface. It is usually constructed from glass or plastic which is coated with aluminium. To minimize greater amounts of scattered radiations and the unwanted radiations of other spectral orders, the gratings are blaze to concentrate the radiation into a single order. A grating is generally used in combination with a small prism which acts as order sorter. Sometimes filters transparent over a limited wavelength range are incorporated with gratings. Grating monochromator has certain advantages over prism monochromator as (a) the grating construction material is not attacked by moisture and is not subjected to etching where on the salt prisms are affected by moisture and can be subjected to etching; (b) grating mono-chromator can be used over considerable wavelength range and (c) grating monochromators are sturdy and long lasting.

5.4.3 Sample Holders (Sample Cell) and Sampling of Substances

As solvents used to prepare sample solutions have the tendency to absorb the infrared radiation, sample cells or sample holders are usually of much narrower (0.1–01 mm) than the one used in visible or ultraviolet region. The sample cells are usually constructed using pickle salt (sodium chloride). The sample cells are demountable and teflon spacers are used along with sample cell to adjust the path lengths. Fixed path length cells are also available and they can be filled or emptied with hypodermic syringe. As the sample cells are made of alkali metal salts, they become foggy due to moisture and thus they need polishing with buffing powder to render them useful again.

The sampling of the substance in infrared spectrophotometry depends upon the state of the sample, i.e. whether it is gas, liquid or solid. Depending upon the nature, various sampling techniques have been developed and used. The inter-molecular forces of attraction are more operative in solid phase than in gases. The sample of the same substance shows shift in the frequencies of absorption as it passes from the solid to the gaseous state. In some cases, additional bands are also observed with the change in the state of the sample. Therefore, it is always important to mention the state of the sample and the solvent to be employed for scanning in the infra region for correct interpretation of spectra. The samples whose spectra are to be recorded must be pure and free of water.

Sampling of solids:

Solid whose infrared spectra are to the recorded can be sampled in various ways:

1. Solid dissolved in solvent:

The solid samples are usually dissolved in a suitable solvent and this solution is used in one of the cells. This method cannot be used for all solids because suitable solvents are limited in number and generally no single solvent is transparent throughout the infrared region. The commonly used solvents are carbon tetrachloride, chloroform, alcohols, acetone, cyclohexane and carbon disulphide. Sometimes two solvents have complementary absorption region are used to cover the complete wavelength region. When the solutions of solids are used for scanning in the infrared region, the absorption due to solvent has to be compensated by keeping the solvent in a cell of same thickness as that of sample in that path of reference beam of a double beam spectrophotometer.

2. As solid film:

In this technique, sample solution is placed on the surface of a potassium bromide or sodium chloride and the solvent is allowed to evaporate. Thus, the solid sample forms a thin film on the surface of cell. This technique is useful for rapid qualitative analysis but not for quantitative analysis.

3. Mull technique:

In this technique, the solid sample is mixed with heavy mineral oil (Nujol) to form a paste. This paste is then sandwiched between two salt plates and then used for spectral measurement. Although Nujol is transparent in most parts of the infrared region but it has absorption maxima at 2915, 1462, 1376 and 719 cm^{-1}. This is the drawback in using Nujol for certain compounds which may have absorption in the region similar to Nujol. This technique is mostly used for qualitative work and not for quantitative estimations.

4. Pressed pellet technique (Disk method):

This technique is frequently used for the qualitative work. In this, a small amount of finely ground solid sample (dried) is intimately mixed with about 100 times its weight of powdered potassium bromide (IR grade and thoroughly dried) in a small agate pestle mortar. This mixture is pressed under a high pressure (25000 psi/g) in an IR tablet press to form a small pellet or tablet. The resulting pellet is transparent to infrared radiation and can be used as such.

This technique has some advantages over the Nujol Mull method. (i) It eliminates the problem of bands which appear in IR spectrum due to use of Nujol; (ii) The potassium bromide pellet if preserved properly can be reused for recording the spectra if required again; (iii) The resolution of spectrum in potassium bromide pellet is superior to the one obtained in Nujol mull technique. One disadvantage associated with this technique is that as high pressure is involved in the preparation of pellet, there could be polymorphic changes in the crystalline of samples like inorganic complexes that can cause complications in the IR spectrum.

Sampling of Liquids:

A diagrammatic representation of sample cell is given in Fig. 5.7.

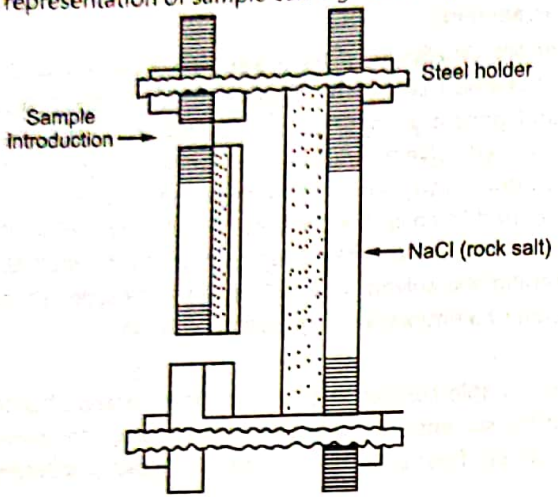


Fig. 5.7 : Sample cell

The samples that are liquids at room temperature are usually handled in the pure form and free from moisture in the form of thin layers in variety of absorption cells. Various types of cells like sandwich cell, demountable cell and cavity cells are available for handling liquid samples. These cells are made up of sodium bromide, potassium bromide or thallium bromide. In demountable cell the salt plates are usually separated by a gasket and held together by a clamp. The thickness of liquid layer can be adjusted by spacers. The sample thickness should be such that the transmittance lies between 15–70 per cent. Usually for most of the liquids layer thickness of 0.01–0.05 mm is quite satisfactory.

Sometimes the liquid samples can be dissolved in a suitable solvent and scanned in the infrared region using any suitable cell. In double beam spectrophotometer, 'matched cells' are generally employed. In one cell, sample solution is placed while in other the solvent employed is placed. The cells used in this must have the same thickness. These sample cells must be protected from moisture.

Sampling of gases:

Gas samples are examined in infrared region after removing the moisture or water vapours. The dried gases are introduced via a stopcock and a system whereby a partial pressure of about 5–50 mm of mercury can be applied. The gas sample is introduced into the gas cell which is made up of glass or a metal cylinder of about 10 cm long. The end walls of the gas cell are made of sodium chloride. For measuring very low concentration of gases long path cells are required. However, the sampling area of most spectrophotometers is restricted in length. The gas cell is equipped with mirrors and used to bring about multiple reflections to increase the effective path length.

- Sometimes the GLC is coupled with IR spectrophotometers to analyse elutes from GLC, for this purpose special cells are designed.

5.4.4 Detectors

There are two types of detectors used in infrared spectrophotometry: (a) thermal detectors and (b) photo-detectors.

(a) Thermal detectors:

When the infra radiations falls on these detectors, they cause heating which gives rise to a potential difference which is measured. This potential difference depends upon the amount of radiation. The thermal detectors commonly used are thermocouples, bolometer and thermistors and Golay cell or Golay detector.

(i) Thermocouple: It is the most commonly used detector in infrared spectrophotometry. Thermocouples are basically the dissimilar strips of metals joined together at one end. Thermocouples are constructed in various ways. In one of the thermocouple detectors two fine wires of metals which have different thermoelectrical properties are welded with blackened gold foil, and which absorbs the radiations. One welded joint (cold junction) is kept at constant temperature and the other welded joint (hot junction) is exposed to radiations. This exposure of hot junction causes a rise in its temperature. Thus, as the two junctions are at different temperatures, it causes a potential difference which is proportional to degree of heating of hot junction (or amount of radiations falling on the hot junction).

(ii) Bolometer: They are constructed from metals or semiconductors. In this, large change of electrical resistance depends on temperature. When the radiations fall on bolometer, there is temperature change which causes change in the resistance of the conductor. This change in resistance depends upon the amount of radiations falling on the bolometer.

Bolometer is made in one arm of the Wheatstone bridge and a similar strip of metal is used as balancing arm of the bridge, which is not exposed to infrared radiations. When no infrared radiations fall on the bolometer, the bridge remains balanced. As the radiations fall on the bolometer, the bridge becomes unbalanced due to change in electrical resistance and thus the electrical current flows through galvanometer G. The amount of current flowing through galvanometer is a measure of the intensity of the radiations falling on the detector. The response time for bolometer is 4 m sec. The schematic representation is given below.

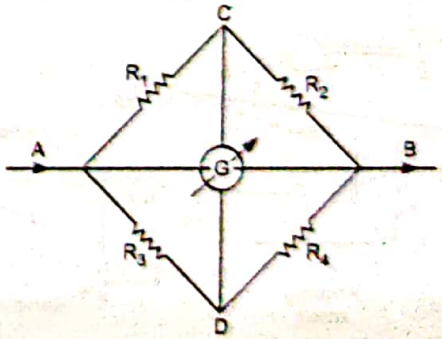


Fig. 5.8 : Bolometer

Either thermocouples or bolometer is fitted in steel housing having potassium bromide or cesium iodide window and it is evacuated, which decreases the noise and increases sensitivity.

(iii) Thermistors: These function similar to bolometer. They are the resisters made by fusing several metallic oxides. These show a negative thermal coefficient of electrical resistance.

(iv) Golay cell or Golay detectot: Golay cell is now-a-days used in several commercial spectrophotometers. It consists of a small metal cylinder, one end of which closed by blackened metal plate and the other with a metalized diaphragm. A light beam falls on the diaphragm which reflects to phototube. The cylinder is filled with non-absorbing gas like xenon. When the radiations fall on blackened metal plate, it is heated, which causes the expansion of gas; this in turn affects the diaphragm (motion of the diaphragm). This causes the change in the output of cell received by the phototube, which can be modulated according to the power of the falling radiations on Golay cell. Thermocouples and Golay detectors possesses similar sensitivity in the mid infrared region.

(b) Photon detectors:

Photon detectors are widely used in near infrared region. They consist of suitable semiconductors like lead sulphide, lead telluride or germanium which are non-conducting at lower energy state. When the radiations fall on these they are raised to higher level which can conduct and produce a signal which is proportional to the amount of radiation. In these there is a drop of electrical resistance and if small voltage is applied there is a large increase in current which can be amplified and indicated on a meter or recorder.

5.4.5 Recorder

In infrared recording spectrophotometers as the sample absorbs some energy, the sample beam and reference beam differ in their radiant energies. Then detector system generates the signal which is normally amplified and goes to servometer. The servometer which is connected to attenuator comb blocks the part of reference beam till energies of reference and sample beams are equal and thus beam balance is achieved (i.e. optical null). The attenuator comb is tied mechanically to the pen of the recorder and paper driver. They are synchronized with the automatic rotation of wavelength mirror. The transmittance of the sample is recorded as a function of wavelength.

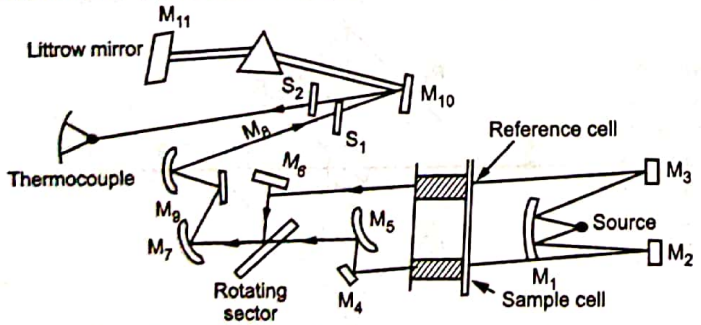


Fig. 5.9: Block diagram of Infrared IR spectrophotometer

A schematic diagram of a double beam IR spectrophotometer is shown in Fig. 5.9. In the instrument 'S' is source (Nernst glower) of infrared radiation. Two beams of light of identical intensity are reflected by M_1 plane mirror and are picked up and reflected by mirrors M_2 and M_3. One beam passes through sample reflected by M_4 and M_5 to rotating sector mirror which reflects sample beam during one half of its rotation. The reference beam reflected by M_6 is reflected by rotating sector during other half of its rotation. Any difference between the two beams causes an out of balance signal. The rotating sector passes the reference beam and reflects the sample beam by mirror M_7, M_8 and M_9 and passes through slit-1 into the monochromator where it is dispersed by double passing through prism after reflector Littrow mirror M_{11}. The selected beam by M_{10} is allowed to fall on exit slit S_2 and then to the detector. An alternating potential signal from the detector is converted into frequency. The frequency of the alternating potential can be determined by rotation of rotating sector mirror. The magnitude of signal becomes zero when beams are equal. The output of the amplifier is used to rotate a small motor which drives a comb shaped attenuator. The comb is connected mechanically to the recording pen which records on paper. The rate of movement of paper is synchronized with the automatic rotation of the wavelength mirror.

5.5 PHARMACEUTICAL APPLICATIONS OF INFRARED SPECTROSCOPY

Infrared spectrophotometer is a very important tool used in qualitative identification and quantitative estimation of many drugs and chemicals. The instrument is particularly useful in pharmaceutical industry in identification of drugs and detection of impurities.

Qualitative analysis:

It is clear from earlier discussion that each compound or substance gives a characteristic IR spectrum. Thus, for identification, IR spectrum of a substance is compared with the IR spectrum of the authentic sample of the same substance. The sample spectrum is superimposed on the spectrum of authentic sample and if the spectra of both are identical then substance under examination and the authentic sample are the same.

Various pharmacopoeias like IP, BP and USP have included "IR spectra" as one of the test for identification of many drugs and substances.

Detection and identification of impurity in pharmaceutical substances can be ascertained by IR spectrophotometry. When a compound contains impurity, it reduces sharpness of individual bands, causes appearance of extra band or peak. Conditions for detection of impurity are most favourable when impurity possesses a strong band in IR region where main substance does not possess absorption band in that region, e.g. small quantity of ketone in hydrocarbon can be detected as a band near 1720 cm^{-1}, characteristic of ketone.

IR spectrophotometer is also useful in determining shape or symmetry of molecule, e.g. NO_2 (nitrogen dioxide) if linear, should show two bands and if non-linear three bands. IR spectra of NO_2 gives three bands in 750, 1323, 1616 cm^{-1} region showing it is a bend structure and not a linear.

Presence of water in a sample can be readily detected by IR spectrophotometer. Small quantity water held will show three characteristic bands in 3600–3200 cm^{-1}, 1650–1620 cm^{-1} and 600–450 cm^{-1} regions. If water is held coordinated to metal ion additional band in 880–650 cm^{-1} region is observed. IR spectrophotometer is also widely used to analyze air contaminants in various fields.

Quantitative analysis:

In quantitative analysis studies by IR spectrophotometer records are obtained as percentage transmittance 'T', as a function of wavelength or frequency exposed. Concentration of substance, in solution or in solid (for disk method), cell path length, and slit width are selected such that 20–60% transmittance is recorded.

Generally, all quantitative spectrophotometry measurements are governed by Beer-Lambert law. In IR spectrophotometry deviations to Beer's law are more due to (a) weak intensity of light source, (b) weak detection by detectors and (c) by employing wider slit width as against UV-visible spectrophotometer. It is therefore necessary to check the plot of per cent transmittance *vs* concentration which should be a straight line before proceeding for IR spectrophotometric recording at a selected wavelength.

Another error usually encountered in quantitative methodology is fixing the base line. In most cases organic substances giving complex spectra the determination is a serious concern and a baseline technique (drawing a tangent to provide base line) is used.

For quantitative determination selection of suitable solvent for liquid cell method is most essential. Usually chloroform is considered as the best solvent. Carbon tetrachloride, carbon-disulphide, pyridine can also be used. The mull technique can also be used provided the sample and standard substance disks are prepared carefully.

In quantitative analysis, the substance used for obtaining spectra and the authentic sample are analysed simultaneously at a selected wavelength.

Detection and determination of complexes, polymorph, isomers and impurities are also carried out by IR spectroscopy.

In the research field, identification of unknown compound is mainly done by examining absorption peaks in various regions of IR spectrum and comparing those with the correlation charts of absorbance given in the books. In synthetic studies, completion of chemical reaction can be studied by recording IR of reactants and reaction products at a suitable interval, e.g. in reduction reactions of carbonyl group nitro group or instauration, the absence of characteristic peak will be evidenced.

Analysis and Interpretation of Organic Compounds Based on FTIR Spectra:

For interpretation of an IR spectrum the following simple ways are to be followed. At the initial stage one should not attempt to concentrate on all bands appeared in the IR spectrum. Instead, the students should concentrate on the major peaks. The authors suggest the students to practice with several IR spectrums for the typical shape of the peaks and the regions where they appear. Some of the tactics for interpretation is explained below.

1. While looking at the IR spectrum one should concentrate on determining the presence and absence of major functional groups. It is necessary to look for the basic compounds. The base values are given below.

Group	Frequency
O – H	3400
N – H	3400
C – H	3000
C ≡ N	2250
C ≡ C	2150
C = O	1715
C = C	1650
C – O	1100

2. Initially look at the spectrum for the presence of a carbonyl group C = O. It will give absorption at 1820-1660 cm^{-1}.
3. If C = O is present, then check for the following assumptions. As there is 'O' there may be possibility of groups that contain 'O' including COOH, CHO, CONH, COO –, anhydrides or ketones.
   - **Acids:** Is OH group is present or not? If OH is present then it will give a broad absorption near 3400-2400 cm^{-1}. It will be usually overlaps C – H.
   - **Aldehydes:** Is aldehyde CH present? If CH of aldehydes present then there will be two weak absorptions near 2850 and 2750 cm^{-1} on right side of the aliphatic C – H absorptions.
   - **Amides**: Is N – H present? If N – H present then there will be medium absorption near 3400 cm^{-1}; NH may give two peaks (doublet) with equal halves.
   - **Esters:** If esters C – O then a strong intensity near 1300-1000 cm^{-1} may be present.
   - Anhydrides will show two C = O absorptions near 1810 and 1760 cm^{-1}.
   - **Ketones**: Ketones gives a strong peak at 1715 cm^{-1}.

If C = O peak is absent then the following should be observed:

- **Alcohols and Phenols:** O – H peaks should be observed which will give broad absorption near 3400-3300 cm^{-1}.
- **For Amines:** N – H peaks can be observed near 3400 cm^{-1} as medium intensity.
- **For Ethers**: C– O peaks near 1300 -1000 cm^{-1}.
- **Double bonds and /or aromatic rings:** –C = C– Weak absorption near 1650 cm^{-1}; 1600-1450 cm^{-1}. Medium or strong absorption denotes an aromatic ring.

  The double bond or aromatic ring can be confirmed by correlating with the C–H region. Generally vinyl and aromatic C – H can be observed on the left side of the 3000 cm^{-1} whereas aliphatic C – H is observed on the right side to 3000 cm^{-1}

- **Triple Bonds:** C ≡ N gives a medium and sharp absorption near 2250 cm^{-1} whereas C = C bands can be observed as weak, sharp absorption near 2150 cm^{-1}. C ≡ H can be observed near 3300 cm^{-1}.
- **Nitro groups:** Nitro groups give two strong bands at the ranges of 1600-1530 cm^{-1} and 1390-1300 cm^{-1}.
- **Hydrocarbons:** If hydrocarbons are assumed then any of the above bands are not observed. Instead there may be C – H bands near 3000 cm^{-1}. The hydrocarbons spectrum will not be complex. There may be additional bands in the region of 1460 and 1375 cm^{-1}.

From the above tactics one can assume approximately the functional groups. Then with the knowledge of different group frequencies the interpretation can be done. The following IR spectrums are some examples of each category. Please observe the bands considering the above points.

In addition to these above points please consider the following points. If the spectrum is simple, as defined, then the compound may be a low-molecular-weight organic or inorganic compound, such as a simple salt of a common molecular ion (carbonate, sulfate, nitrate, ammonium, etc.) or a covalent species (chloroform, dichloromethane, methanol, water, etc.).

If the spectrum has well defined bands then look for specific peaks and follow the above tactics. If absorptions above 3000 cm^{-1}, then the compound is likely to be unsaturated (contains C=C) or aromatic. If isolated absorptions occur at 3010 and/or 3040 cm^{-1}, then the absorbing species is mostly simple olefinic unsaturation. Is the main absorption below 3000 cm^{-1}. If so, the compound is probably aliphatic. If the main absorptions are approximately 2935 and 2860 cm^{-1}, and there are also absorptions at 1470 and 720 cm^{-1}, then the compound probably contains a long linear aliphatic chain.

In the following section the IR spectrum of different categories are compounds are discussed. The main peaks for each category should be noted.

Alkane:

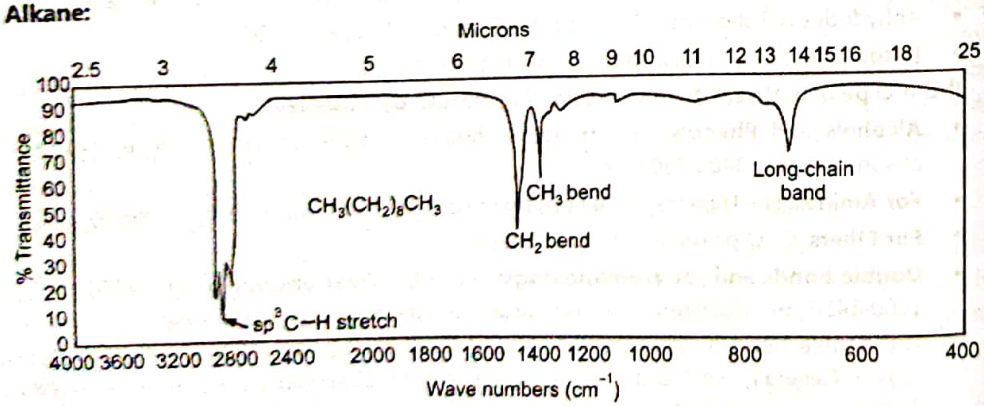


Fig. 5.10: The infrared spectrum of Decane (neat liquid, KBr plates)

In Decane, each band in the spectrum can be assigned:

- C – H stretch : 2961, 2928 and 2868 cm^{-1}
- C – H bending : 1385, 1461 cm^{-1}
- Observe long chain bending around 725 cm^{-1}

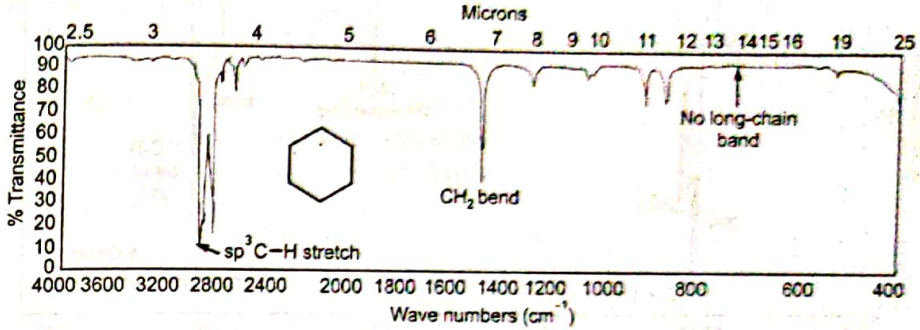


Fig. 5.11: The infrared spectrum of cyclohexane (neat liquid, KBr plates)

Cyclohexane, each band in the spectrum can be assigned:

- C – H stretch : 2938, and 2856 cm^{-1}
- CH_2 bending : 1455 cm^{-1}
- Note the absence of long chain bending.

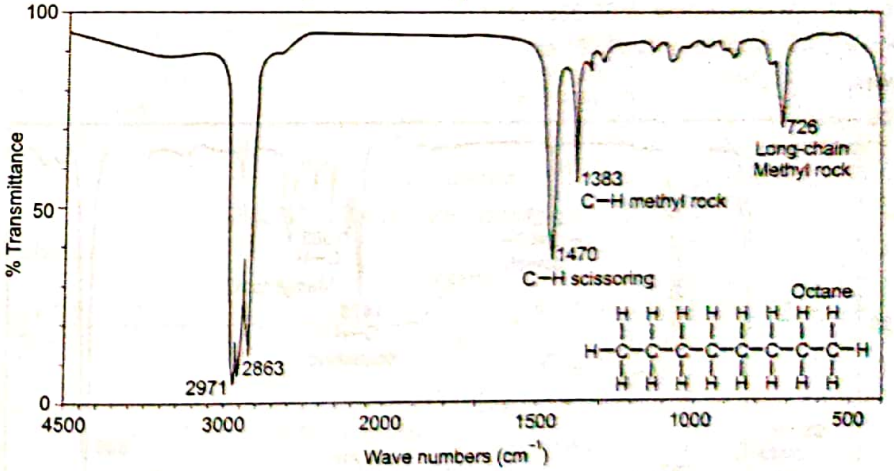


Fig. 5.12: The infrared spectrum of Octane

Octane, each band in the spectrum can be assigned:

- C – H stretch : 2971, 2863 cm^{-1}
- C – H bending : 1383, 1470 cm^{-1}
- Observe long chain bending around 726 cm^{-1}

Alkene:

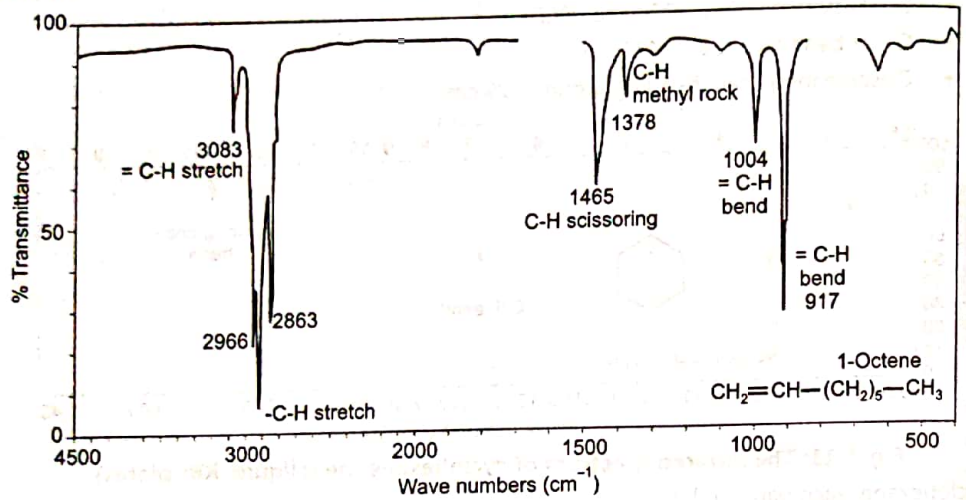


Fig. 5.13: The infrared spectrum of octene

Octene, each band in the spectrum can be assigned:

- C = C stretch from 1680-1640 cm^{-1}
- = C – H stretch from 3100-3000 cm^{-1}
- = C – H bend from 1000-650 cm^{-1}

Alkynes:

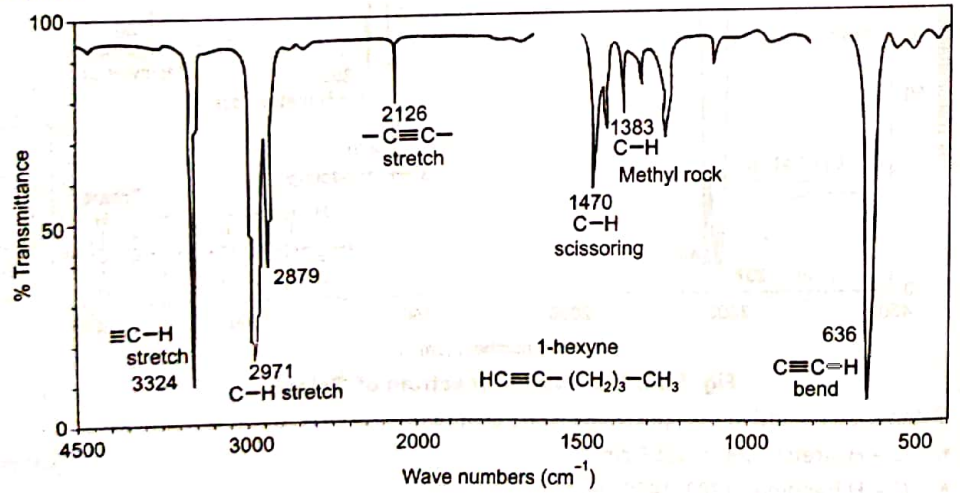


Fig. 5.14: The infrared spectrum of n alkynes (1- hexyne)

n alkynes (1-hexyne), each band in the spectrum can be assigned :

- – C ≡ C– stretch from 2260-2100 cm^{-1}
- – C ≡ C – H : C–H stretch from 3330-3270 cm^{-1}
- – C ≡ C – H : C–H bend from 700-610 cm^{-1}

Aromatic Compound:

In aromatic compounds, each band in the spectrum can be assigned:

- C – H stretch from 3100-3000 cm^{-1}
- Overtones, weak, from 2000-1665 cm^{-1}
- C – C stretch (in-ring) from 1600-1585 cm^{-1}
- C – C stretch (in-ring) from 1500-1400 cm^{-1}
- C – H "oop" from 900-675 cm^{-1}

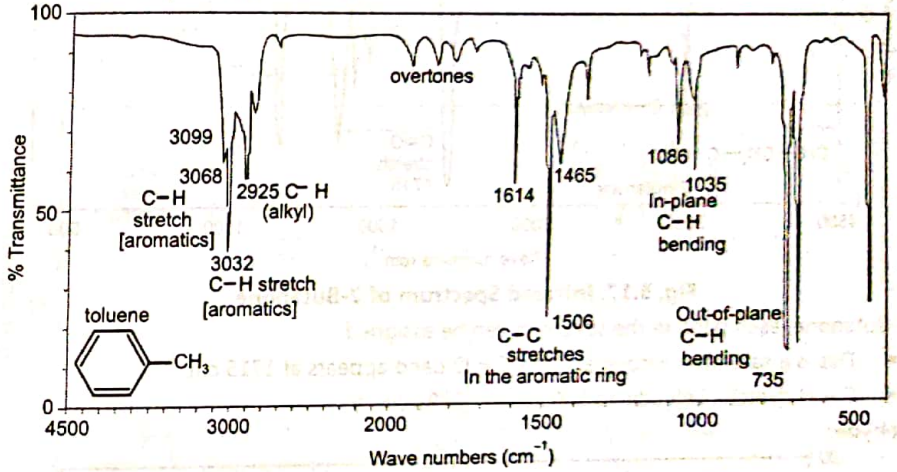


Fig. 5.15: The infrared Spectrum of Toluene

Alcohols:

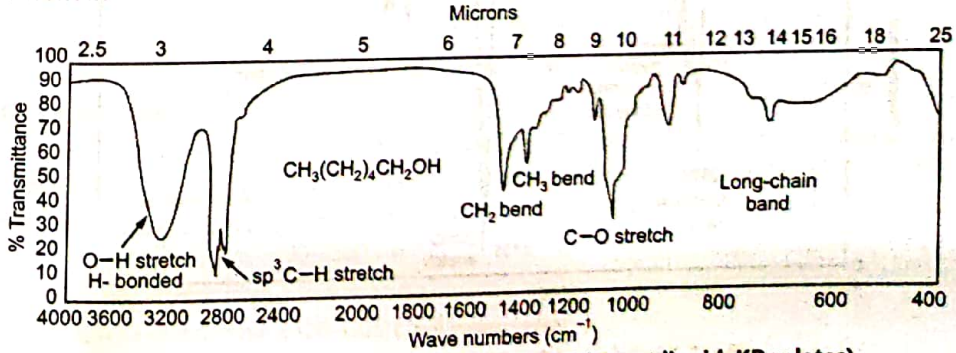


Fig. 5.16: The infrared Spectrum of 1-hexanol (neat liquid, KBr plates)

In alcohol, each band in the spectrum can be assigned :

- C = O stretch - Aliphatic ketones 1715 cm^{-1}
  - Unsaturated ketones 1685-1666 cm^{-1}
- O – H – Broad band 3390 cm^{-1}

Ketone:

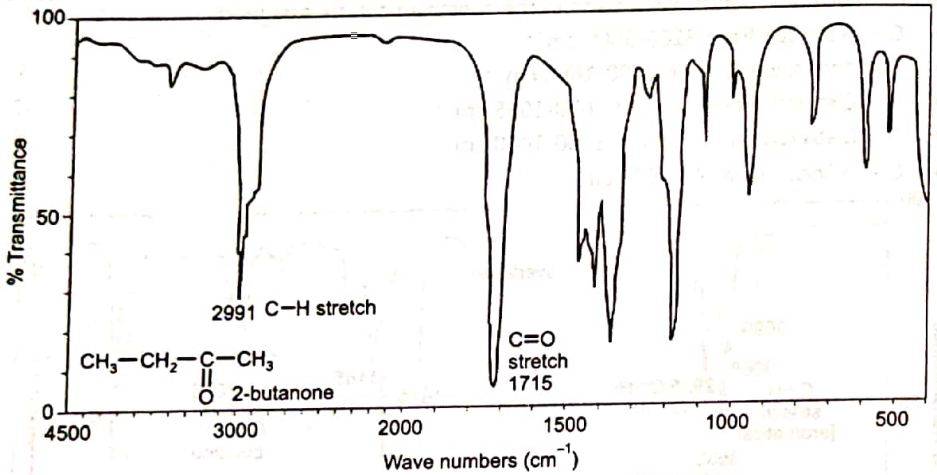


Fig. 5.17: Infrared Spectrum of 2-Butanone

In 2-Butanone, each band in the spectrum can be assigned :

- This is a saturated ketone and the C = O band appears at 1715 cm^{-1}.
- C – H stretch : Left side to 3000 cm^{-1} (2991 cm^{-1})

Aldehyde:

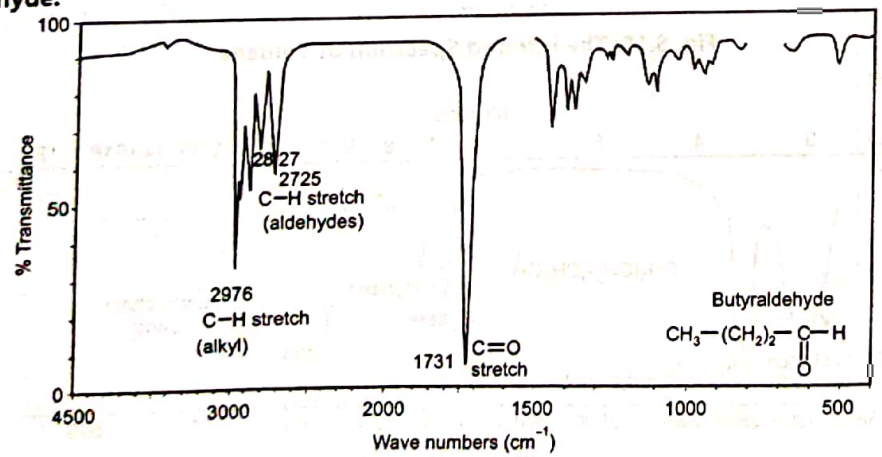


Fig. 5.18: Infrared Spectrum of Butyraldehyde

In aldehyde, each band in the spectrum can be assigned:

- C – H stretch regions gives two peaks with weak intensity ~ 2850 and 2750 cm^{-1}
- C = O stretch :
  - Aliphatic aldehydes 1740-1720 cm^{-1}
  - Alpha, beta-unsaturated aldehydes 1710-1685 cm^{-1}

Ester:

In Ester, each band in the spectrum can be assigned:

The carbonyl stretch C = O of esters appears:

- C = O stretch :
  - Aliphatic from 1750-1735 cm^{-1}
  - Alpha, beta unsaturated from 1730-1715 cm^{-1}
- C – O stretch from 1300-1000 cm^{-1}

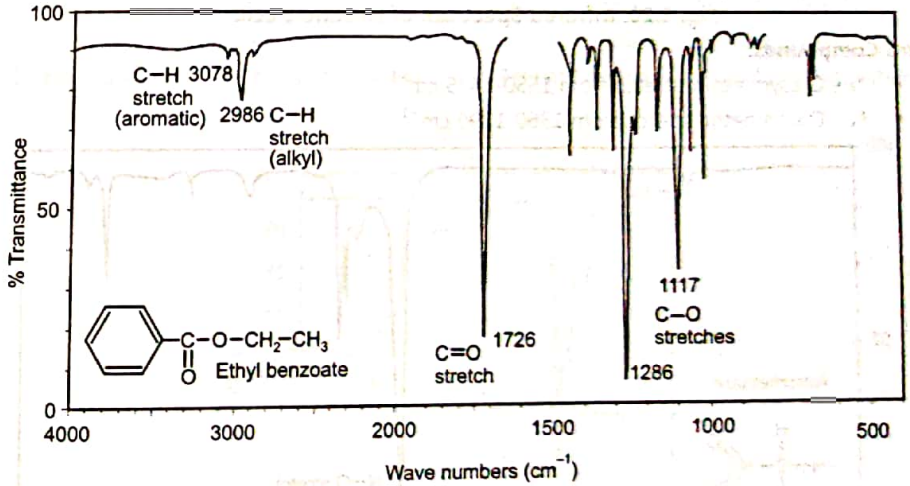


Fig. 5.19: Infrared Spectrum of Ethyl benzoate

Carboxylic acid:

The carbonyl stretch C = O of a carboxylic acid appears as an intense band from 1760-1690 cm^{-1}. The exact position of this broad band depends on whether the carboxylic acid is saturated or unsaturated, dimerized, or has internal hydrogen bonding.

- – H stretch from 3300-2500 cm^{-1}
- C = O stretch from 1760-1690 cm^{-1}
- C – O stretch from 1320-1210 cm^{-1}
- O – H bend from 1440-1395 and 950-910 cm^{-1}

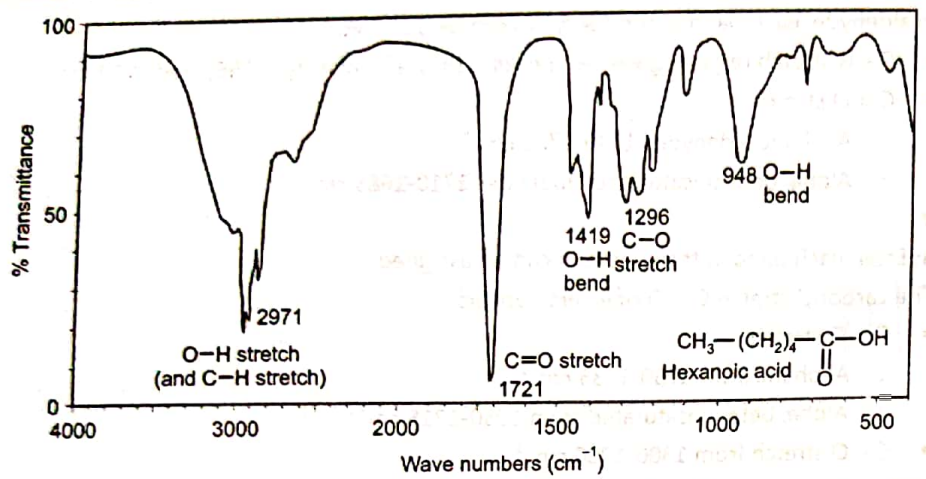


Fig. 5.20: Infrared Spectrum of Hexanoic acid

Nitro Compounds:

- N – O asymmetric stretch from 1550-1475 cm^{-1}
- N – O symmetric stretch from 1360-1290 cm^{-1}

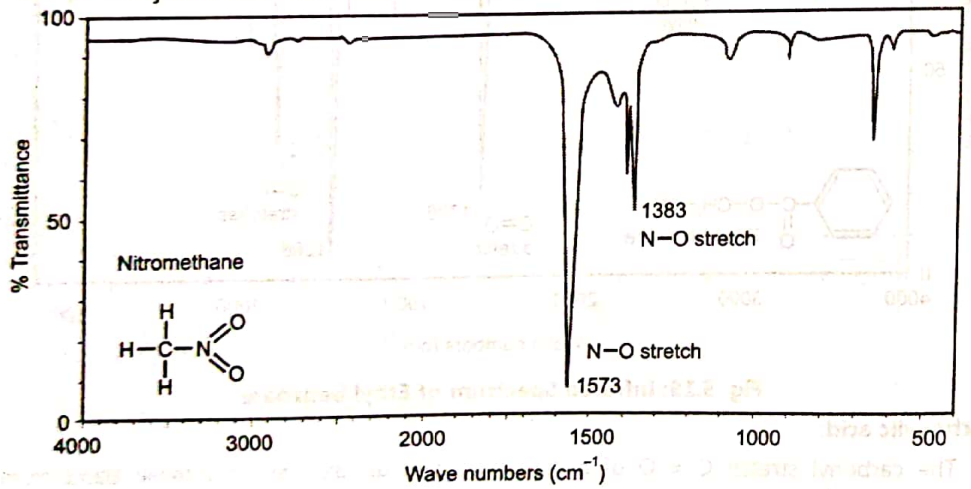
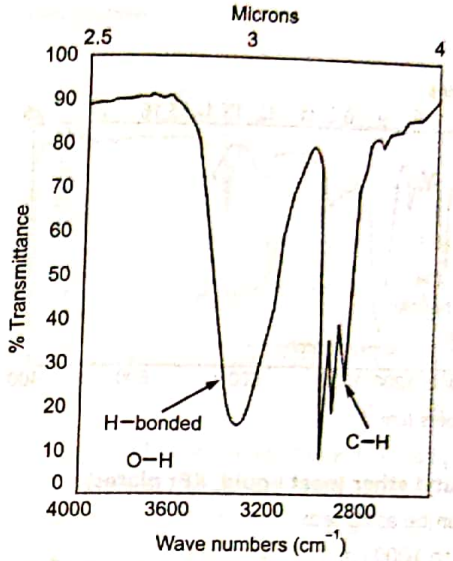


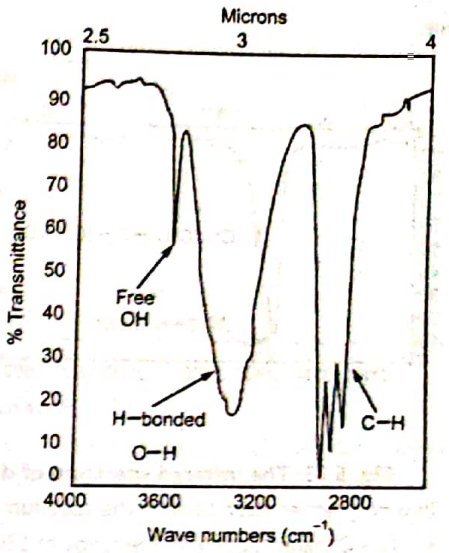
Fig. 5.21: Infrared Spectrum of Nitomethane

OH bands:

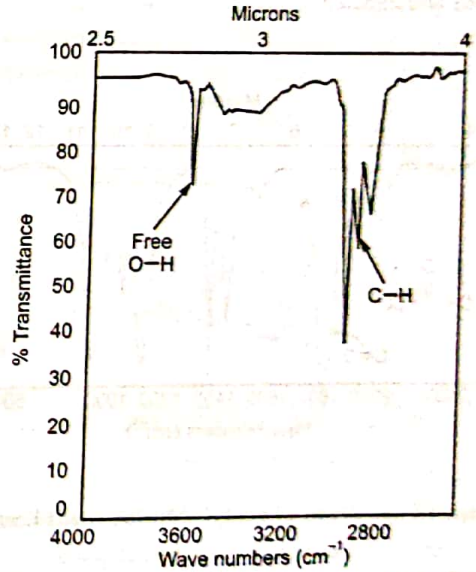
- OH bands usually appear around 3600 cm^{-1} for free OH and alcohol OH groups.
- OH groups with Hydrogen bonding, acids appear at 3300 cm^{-1}. The effect of Hydrogen bonding in the frequency of OH group is shown below. Free OH at 3600 cm^{-1} is sharp and shifted to 3300 cm^{-1} with broad shape.



(a) Hydrogen bonded O–H only (neat liquid)



(b) Free and hydrogen bonded O–H (dilute solution)



(c) Free and hydrogen bonded O–H (very dilute solution)

Fig. 5.22: The O-H stretch region

Ether:

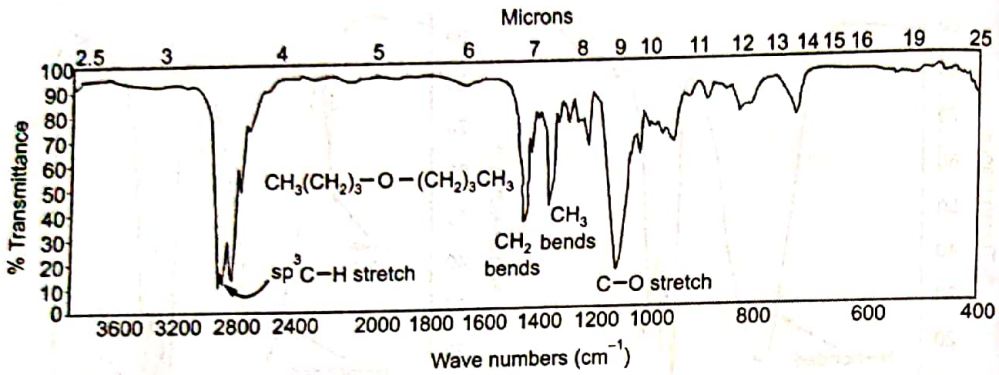


Fig. 5.23: The infrared spectrum of dibutyl ether (neat liquid, KBr plates)

In dibutyl ether, each band in the spectrum can be assigned:

- C – O bands appear in the range of 1300 to 1000 cm^{-1}
- CH_2 and CH_3 bending : 1467 and 1375 cm^{-1} respectively.
- C – H stretch : 2868 and 2962 cm^{-1}

Acid chloride:

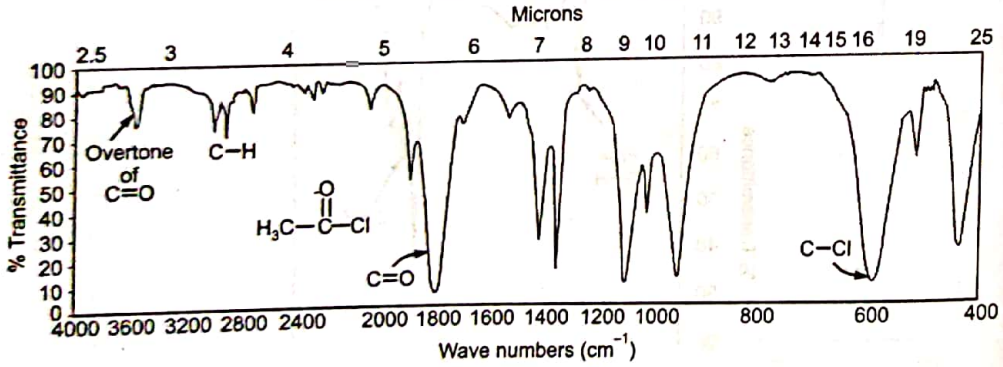


Fig. 5.24: The infrared spectrum of acetyl chloride (neat liquid, KBr plates)

In acetyl chloride, each band in the spectrum can be assigned:

- C = O – 1800 cm^{-1}
- C – Cl – 595 (Range 785 to 540 cm^{-1})
- Overtone 3590 cm^{-1}

Anhydride:

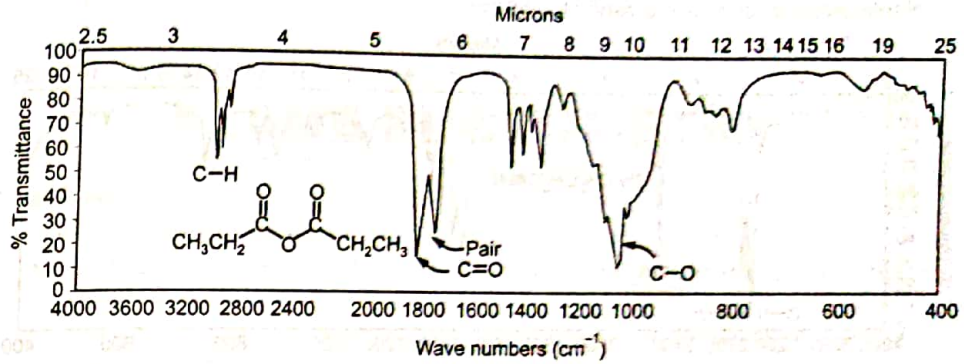


Fig. 5.25: The infrared spectrum of propionic anhydride (neat liquid, KBr Plates)

In propionic anhydride, each band in the spectrum can be assigned :

- Anhydride C = O – Two peaks 1810 and 1760 cm^{-1}
- C – O – 1090 cm^{-1}

Amines:

Two bands are observed with primary amines; one band is observed with secondary amine whereas no peak is the indication of tertiary amine in the 3300-3400 cm^{-1} region.

NH– peaks are observed between 3300-3400 cm^{-1}.

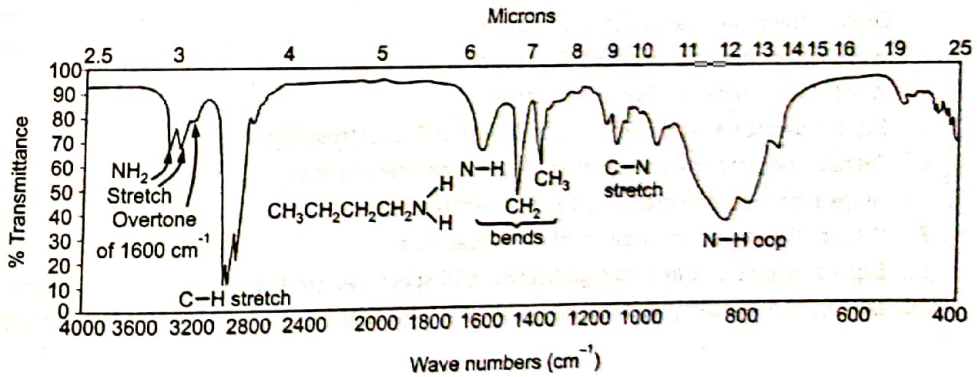


Fig. 5.26: The infrared spectrum of butylamine neat liquid, KBr Plates)

In butylamine, each band in the spectrum can be assigned :

- NH – stretch two peaks in the region 3350 and 3390 cm^{-1}
- NH_2 bending at 1600 cm^{-1}
- CH_2 and CH_3 bending at 1465 and 1375 cm^{-1} respectively.

Nitriles:

Nitrile group gives a strong band at 2250 cm^{-1}.

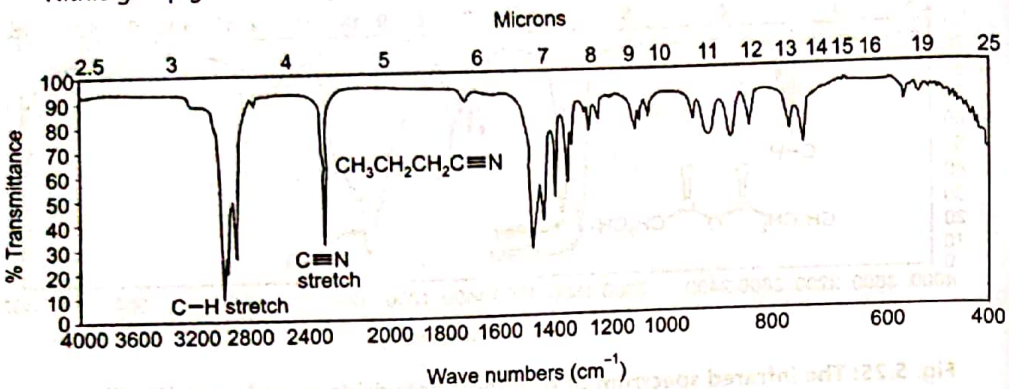


Fig. 5.27: The infrared spectrum of butyronitrile neat liquid, KBr Plates)

In butyronitrile, each band in the spectrum can be assigned :

- C ≡ N 2249 cm^{-1}
- C – H stretch 2989 and 2965 cm^{-1}

REVIEW QUESTIONS

1. Write the principle and theory of IR spectroscopy.
2. Discuss the molecular vibrations in detail.
3. Write a brief note on origin of IR spectra.
4. What do you mean by Fermi resonance?
5. Explain overtones and combination bands in IR spectroscopy.
6. Discuss the instrumentation of an IR spectrometer in detail.
7. Write a note on detectors used in IR spectrometers.
8. Discuss the important regions of an IR spectrum.
9. Explain Hook's Law and its significance in IR spectroscopic theory.
10. Discuss the sampling techniques involved in IR spectroscopy.

Chapter ...6

FLAME PHOTOMETRY

Objectives:

Upon completion of this section, the student should be able to

- Understand the basics of Flame photometry, instrumentation involved and its applications.
- Explain the various components of Flame photometer and their functions.
- Describe the applications of Flame photometry.

6.1 INTRODUCTION

Flame photometry or flame emission spectroscopy is a technique involved in detection of characteristic radiation emitted in flame by individual element and the correlation of emitted intensity with the concentration of that element. It is a well-known observation that several elements like sodium or potassium when burned in Bunsen flame emits a characteristic colour. The brightness of the colour varies with the amount of element introduced into the flame. A method of quantitative determination of a particular element using flame has been developed and is known as flame photometry.

The method of excitation of sample in flame is simple and is carried out as under.

A small volume of sample, dissolved in water or in a suitable organic solvent is placed in a cup of atomizer. Air, oxygen and combustible gas is fed to atomizer under controlled conditions. This allows solution of sample to be sprayed into flame. Vapourization of solvent as well as excitation of element takes place in flame and radiations are emitted. The radiation from flame is allowed to pass through dispersing device to isolate the desired region of the spectrum. The intensity of isolated radiation is allowed to fall on photocell and after amplification; the intensity of isolated radiation is measured. After calibrating the instrument for different concentrations of known element, the intensity of unknown is measured and the amount of element is found out.

6.2 PRINCIPLE AND THEORY

The principle of flame photometer involves the emission process. Compounds of the alkali and alkaline earth metals can be thermally dissociated in a flame and that some of the atoms produced will be further excited to a higher energy level. When these atoms return to the ground state they emit radiation which lies mainly in the visible region of the spectrum.

Each element will emit radiation at a wavelength specific for that element. The whole process can be categorised as below.

(i) Desolvation:

(a) The metal particles in the flame are dehydrated by the flame and the process of desolvation takes place. Water or other solvent is vapourized leaving minute particles of dry salt.

(ii) Vapourisation:

(a) Evaporation of the solvent occurs as the second step at the high temperature of the flame.

(iii) Atomization:

Flame heat converts a part of all of the gaseous molecules/metal particles to give neutral atoms.

(iv) Excitation:

Some of the free metal atoms react with other radicals or atoms present in the flame gases. The vapours of neutral metal atoms or molecules containing the metal atoms are excited by the thermal energy of flame resulting into ionization and excitation of neutral atoms. The electrostatic force of attraction between the electrons and nucleus of the atom helps them to absorb a particular amount of energy. The atoms then shifted to the exited energy state.

(v) Emission process:

The excited higher energy state is unstable and the atoms return to the initial stable low energy state with the emission of energy in the form of radiation of characteristic wavelength, which is measured by the photo detector.

To obtain emission spectra, suitable flames are required. There are some important requirements of flames:

1. It should have proper temperature to carry out function.
2. The temperature should remain constant throughout operation.
3. There should not be any fluctuation during its burning.
4. The spectrum of flame should not interfere with the observations when emission is being measured.

Thus for example, when a solution of calcium chloride ($CaCl_2$) is sprayed into the flame, the following steps occur:

$$\underset{\text{(Solution)}}{CaCl_2} \xrightarrow{\text{Aspiration}} \underset{\text{(Solid)}}{CaCl_2} \xrightarrow{\text{Vapourization}} \underset{\text{(Gas)}}{CaCl_2}$$

$$\underset{\text{(g)}}{CaCl_2} \xrightarrow[\Delta]{\text{Dissociation by}} \underset{\text{(g)}}{Cl_2} + \underset{\text{(g)}}{Ca}$$

$$\underset{\text{(g)}}{Ca} \xrightarrow[\text{Flame}]{\Delta} \begin{cases} \xrightarrow{\text{Ionization}} Ca^{+} \text{ (ion)} \\ \xrightarrow{\text{Excitation}} Ca^{0} \text{ neutral atom} \\ \xrightarrow{+ O \text{ from gases}} CaO \\ \xrightarrow{+ OH \text{ from gases}} CaOH\ (g) \end{cases}$$

All the species, i.e. ionized atom and neutral atom of Ca as well as molecules of CaO and CaOH are excited by the thermal energy of flame. From this excited state when these ions, neutral atoms of Ca and their molecular species fall to the ground state emission spectra are produced.

The spectra fall into two main categories:

1. Those consisting of lines originating from excited atoms or ions.
2. The band spectra resulting from molecule.

When an electron falls to ground level from excited in neutral atom, emission occurs as discrete lines. The relation between the frequency 'υ' of radiation emitted to the energies E_1 and E_2 of two states of atoms is given as $E_1 - E_2 = h\upsilon$ (h = Planck's constant). The emission does not occur as single line spectra. If electrons fall to ground level through intermediate orbitals, then the emission of several spectral lines occurs. Spectra of ion of atom are different from the spectra of neutral atom. Due to high temperature of flame, atom from high excited energy gives emission because of transition of excited electrons of an atom to lower energy level. Due to high temperature of flame ion spectra is also given out as seen in alkaline earth metals.

The excited energy given out in case of neutral atoms is observed at (red) 671 nm for lithium, (yellow) 590 nm for sodium, (red) 767 and 769 nm for potassium and blue 422 nm for calcium etc.

Band spectra arise from electronic transitions involving molecules. Since molecules have internal vibrational, rotational and electronic excitation levels, the emitted energy is spread over wide band of spectrum known as band spectra.

6.3 INSTRUMENTATION

Flame photometer as measures the emitted radiation, the optical and electronic system used in spectrophotometer is similar. Any model of flame photometer consists of the following parts:

1. Fuel gas, pressure regulator and flow meters
2. The atomizer
3. The optical system
5. Photosensitive detectors
6. Recording or reading meter.

1. Fuel gas, pressure regulators and flow meters:

In order to supply suitable thermal energy through flame, fuel gas is burnt in the burner. A simple structural diagram of a flame is shown in Fig. 6.1 and 6.2. The unburnt fuel gas emerging from region A, mixes with air/oxygen at outlet of burner comes into pre-heating region B.

Gases emerging from this mainly consist of CO, H_2, CO_2 and hydrocarbons. On burning, a steady temperature is provided throughout the outer mantle of flame.

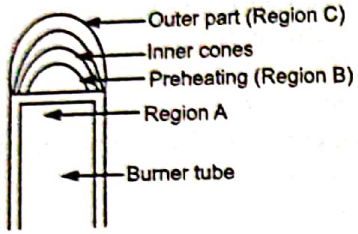


Fig. 6.1 Regions of Flame with burner tube

To supply sufficient temperature between 1000–3000°C, various fuel gases like coal gas, illuminating gas, cooking gas and various hydrocarbon gases are employed. Using various mixtures of fuels with air or oxygen a sufficiently high temperature is obtained.

Table 6.1 Records the temperature given for various fuels in air and in oxygen.

Table 6.1: Flame temperature with oxidants

Fuel	Oxidant	Temperature of Flame
Propane	Air	1925°C
Propane	Oxygen	2800°C
Hydrogen	Air	2100°C
Hydrogen	Oxygen	2780°C
Acetylene	Air	2000°C
Acetylene	Oxygen	3050°C

An ordinary air-gas flame gives about 1700°C which is sufficient to excite some alkali and alkaline earth metals. Higher temperature is obtained by using mixture of hydrocarbons and acetylene.

Fuel gas is generally obtained from cylinders where they are stored under pressure.

To obtain steady flame for giving emission, it is necessary that the gas pressure and gas flow is maintained constant. Usually 10 lb / in^2 of fuel gas and 30 lb / in^2 of air or oxygen are supplied. Flow rate ranges from 2-10 ft^3 / hr. This is adjusted by operating capillary flow rate meter or rotameter.

2. Atomizer:

Atomization and Excitation:

Atomization is the process of converting a solid, liquid, or solution to be analyzed into a free gaseous atom. This requires usually a thermal energy using various sources. Excitation is the process that the outer most electrons of atoms absorb energy and shifted to higher energy state. The same source of thermal energy usually serves as the excitation source. Atomization and excitation is the process involved in all atomic spectroscopic techniques.

The common sources used for atomization are:

- Flames
- Burners
- Furnaces
- Electrical discharges
- Plasmas.

This is a very integral and important part of flame photometer. The purpose of atomizer is to introduce liquid sample into flame at stable and reproducible rate. Atomizer should remain unaffected by solutions and solvents. Further, atomizer can be readily cleaned and should be sturdy in nature.

Atomizers are classified into two types:

Flame Atomiser:

(I) Flames:

Most of molecules are dissociated into free atoms by heat energy produced by flames and furnaces many elements can be excited by this energy. The flames can be categorized based on the mixing way of the combustible gases and the combustion feeding gases. When the gases are mixed before the combustion as in the case of Bunsen burner it is called as premixed flame. When the oxygen feeding the combustion gets to the flame from air, the flame is called diffusion flame as in the case of the flame of a candle.

- **Fuel and oxidants:**

  Fuel and oxidant are required to produce flame. The combination of fuel and oxidants is so important that it should be suitable for conversion of samples to neutral atoms followed by excitation of atoms. Thus the temperature of flame plays a major role. With high temperature of flame the elements in sample may get convert into ions instead of neutral atoms. Similarly with low temperature there may not be excitation of atoms occur. So a combination of fuel and oxidants is used such that there is desired temperature to facilitate atomisation and excitation. The commonly used combination of fuel and oxidants are given in Table 6.1.

- **Structure of Flame:**

Flame consists of three important regions. These are the primary combustion zone, interconal region and outer cone (secondary combustion zone). Fuel oxidant ratio has influence over the appearance and the relative sizes of these regions. The primary combustion zone does not take part in the flame emission process as there is no thermal equilibrium. It is usually blue in colour. The most important region which takes part in the emission process is the interconal region. It is rich in free atoms and is involved in the excitation process. In The outer cone the products of the inner core are converted to stable molecular oxides. Regions of the flame are shown in the Fig. 6.2.

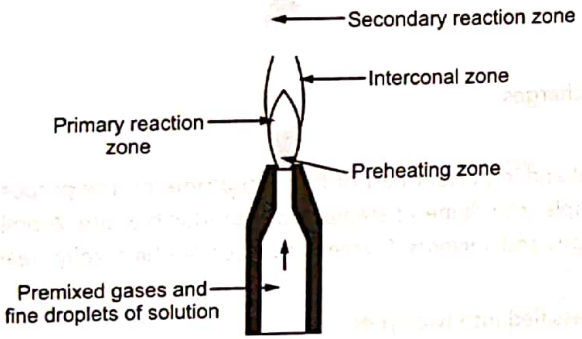


Fig. 6.2: Structure of Flame

(II) Burners:

Main requirement of burner is that when fuel gas is supplied along with air/oxygen at a constant pressure, it should produce a steady flame. For low temperature flame Meeker burner is used. It carries a metal grid across the burner open tube which prevents flame from striking back down. There are two common types of atomizer burner employed in flame photometer. These are:

(a) Total consumption burner.

(b) Pre mixed burner.

(a) Total consumption burner:

It consists of inlets for fuel and oxidants at the base of the apparatus. Sample is also kept at the base to be aspirated. Acetylene which is commonly used as fuel and air the most used oxidant are forced, under pressure, into the flame. The sample is drawn into the flame by aspiration by the vacuum created because of the movement of fuel and oxidant. The aspirated sample reaches the burner head with a nebulizing effect. It is mixed with fuels and oxidant at the base of the flame. The resulting flame is turbulent and non-homogenous which is not suitable for atomic absorption spectrometers.

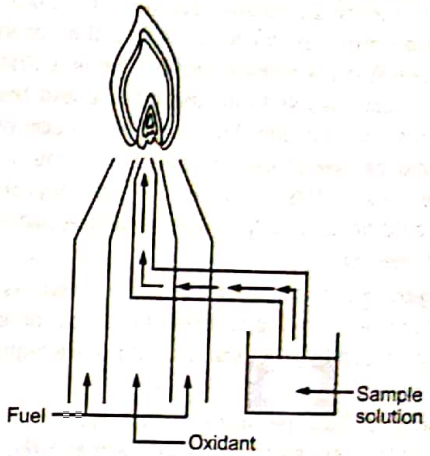


Fig. 6.3: Total Consumption burner

(b) Premix burner:

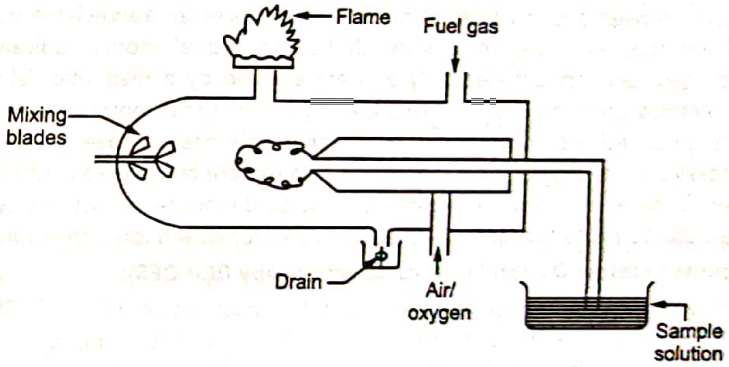


Fig. 6.4: Premix burner (Any one Figure can be used)

The premix burner is typically used in atomic absorbance spectrometer and in emission spectrometer also. The sample is nebulized and mixed with the fuel and oxidant prior to introduction into the flame, with the use of a series of baffles. The sample is drawn from the sample container via the vacuum created by the rushing fuel and oxidant (aspiration). A drain line is required in this design in order to remove sample solution droplets that do not make it all the way to the flame.

Non-Flame Atomizers:

(i) **Graphite Furnace:** This furnace is a small cylindrically shaped with a sample injection port at the top. The light to be absorbed enters one end of the cylinder and emerges through the other end. The sample solution (from 1-100 uL) is syringe-injected into the furnace through the injection port. The high temperature of the furnace (about

2500°C) is reached in stages, ultimately resulting in atomization as in the flame. The atomized metal species then absorbs the light, and the absorption is measured. One obvious difference between the furnace and the flame is that, contrary to the flame, the sample is not continuously fed into the furnace and the sample distribution is neither homogeneous nor reproducible. Thus, a furnace offers greater sensitivity (because more atoms can be placed in the path of the light) and requires fewer samples, but sometimes suffers from lack of accuracy and precision. Thus, the graphite furnace should be used only when the sample size is small and/or when the greater sensitivity is needed.

(ii) Electrical discharges: Electrical discharges are used as sources for emission spectroscopy using applied currents or potentials across an electrode in an inert gas. The temperatures produced by electrical discharges are higher than traditional flame systems.

(iii) Plasmas: A plasma is a hot, partially ionized gas that contains an abundant concentration of cations and electrons. The plasmas used in atomic emission are formed by ionizing a flowing stream of argon gas, producing argon ions and electrons. Plasmas have been used as source of atomization/excitation in emission spectroscopy. A plasma is any form of matter that contains an appreciable fraction (>1%) of electrons and positive ions in addition to neutral atoms, radicals and molecules. They can conduct electricity and are affected by a magnetic field. The electrical plasmas used for analytical OES are highly energetic, ionized gases. They are usually produced in inert gases, although some work has also been done using reactive gases such as oxygen. These plasma discharges are considerably hotter than flames and furnaces and, thus, are used not only to dissociate almost any type of sample but also to excite and/or ionize the atoms for atomic and ionic emission.

(a) Inductive Coupled Plasma Optical Emission Spectroscopy (ICP OES):

It is an exclusive emission spectroscopic instrument. It consists of an ICP torch. The ICP torch consists of three concentric quartz tubes, surrounded at the top by a radio-frequency induction coil. The sample is mixed with a stream of Ar using a nebulizer, and is carried to the plasma through the torch's central capillary tube. When RF power (typically 700 - 1500 watts) is applied to the load coil, an alternating current moves back and forth within the coil, or oscillates, at a rate corresponding to the frequency of the generator. In most ICP instruments this frequency is either 27 or 40 megahertz (MHz). This RF oscillation of the current in the coil causes RF electric and magnetic fields to be set up in the area at the top of the torch. With argon gas being swirled through the torch, a spark is applied to the gas causing some electrons to be stripped from their argon atoms. These electrons are then caught up in the magnetic field and accelerated by them. Adding energy to the electrons by the use of a coil in this manner is known as inductive coupling. These high-energy electrons in turn collide with other argon atoms, stripping off still more electrons.

This collisional ionization of the argon gas continues in a chain reaction, breaking down the gas into plasma consisting of argon atoms, electrons, and argon ions, forming what is known as Inductively Coupled Plasma (ICP) discharge. The ICP discharge is then sustained within the torch and load coil as RF energy is continually transferred to it through the inductive coupling process.

A schematic diagram of the inductively coupled plasma source (ICP) is shown in Fig. 6.5.

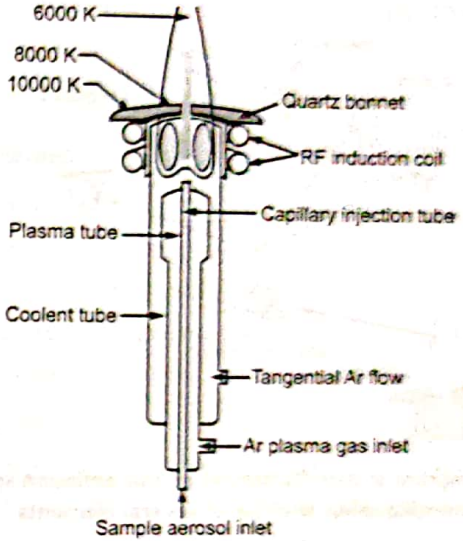


Fig. 6.5: Schematic diagram of an inductively coupled plasma torch.

(b) Arc or Spark Emission Spectrography:

The technique is spark or arc emission spectrography. In this technique, a high voltage is used to excite a solid sample held in an electrode in such a way that when a spark jumps from this electrode to another electrode in the arrangement, atomization, excitation, and emission occur, and the emitted light again is measured. The usual configuration is such that the emitted light is dispersed and then detected with the use of photographic film. The "picture" that results is that of a combined line spectrum of all the elements in the sample. Identification (qualitative analysis) is then possible by comparing the locations of the lines on the film to the locations of lines on a standard film.

(c) Miscellaneous:

Multi-elemental analysis:

Atomic emission spectroscopy is ideally suited for multielemental analysis because all analytes in a sample are excited simultaneously. If the instrument includes a scanning monochromator, we can program it to move rapidly to an analyte's desired wavelength,

pause to record its emission intensity, and then move to the next analyte's wavelength. This sequential analysis allows for a sampling rate of 3-4 analytes per minute.

Another approach to a multielemental analysis is to use a multichannel instrument that allows us to simultaneously monitor many analytes. A simple design for a multichannel spectrometer couples a monochromator with multiple detectors that can be positioned in a semicircular array around the monochromator at positions corresponding to the wavelengths for the analytes.

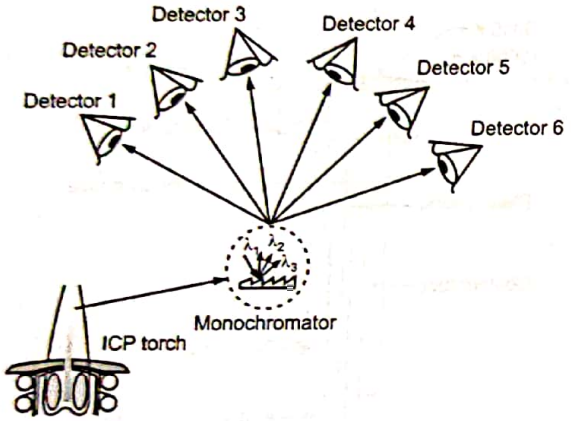


Fig. 6.6: Schematic diagram of a multichannel atomic emission spectrometer for the simultaneous analysis of several elements

3. The Optical System:

Function of optical system is to collect the light from steady fast flame, render it monochromatic by means of lens, prisms and focus on to the phototube. Concave mirror is frequently placed behind flame to collect scattered radiations and focus back into flame. By this mechanism intensity of emitted light is nearly doubled.

Filters and monochromators are needed to isolate the light of specific wavelength from remaining light of the flame. For this simple filters are sufficient in case of few elements like Ca, Na, K and Li. So a filter wheel with filter for each element is taken usually with instrument such as flame photometer. When a particular element is analyzed, the particular filter is used so that it filters all other wavelengths. In most simple flame photometers absorption or interference filters are used. Absorption filters of glass transmit rather wide spectral band. These are not suitable for analyzing samples giving lines to those which lie in close proximity to the analytical line. Interference filters are better in giving good resolution. These filters transmit the desired emitted wavelength energy and absorb the light of flame. Dispersion media can be quartz for operating in ultraviolet region. For finer separation of emitted light diffraction grating and/or prism is employed in an instrument which is commonly known as flame spectrophotometer.

4. Photo-detectors:

Most flame photometers or flame spectrophotometers mainly employ either Barrier layer cell or phototube or photo-multiplier as a detector. The latter provides a maximal signal for weak emission lines. RCA tubes operating in 200–700 nm are used while EM_1 tube in visible and above 800 nm are used. Usually photovoltaic cell or photoemissive cell or photomultiplier tube are used as detectors.

5. Recorder or Readout meters:

Recording System devices is usually a computing system with suitable software. Data collection has greatly advanced with the aid of computer technology that has replaced the strip charts of the decades before. The photo-current produced by the detectors is often amplified and fed to a light spot moving coil galvanometer. The sensitivity of this is adjusted through coarse and fine knobs, which adjust sensitivity ranging from 0.007–0.004 μ/mm scale divisions. The double beam instruments usually are fitted with a strip chart recorder.

Commercially, flame photometers are available as:

(a) Single beam instruments

(b) Double beam instruments.

Besides, these are either filter type (flame photometer) or spectrometer-grating type (flame spectrophotometer). A schematic line diagram for both types is shown in Figs. 6.8 and 6.9.

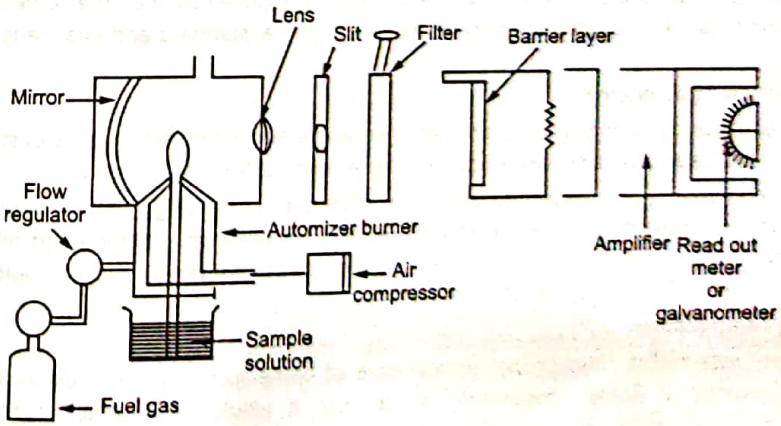


Fig. 6.8 Filter type (flame photometer)

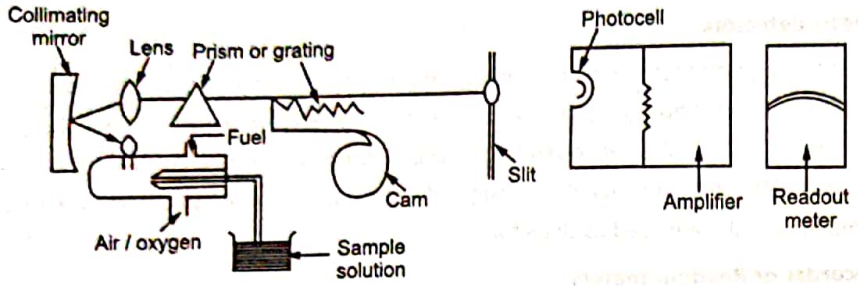


Fig. 6.9: Grating type (flame spectrophotometer)

Working:

Fuel gas is fed to burner at a steady rate of flow and air/oxygen is supplied to flame via air compressor. Distilled water or solvent is placed in cup of automizer-burner and it is allowed to be sprayed in flame. The radiation emitted in flame is collected via lens, slit and appropriate filter on photodetector. Adjusting coarse knob and fine knob, the galvanometer spot is adjusted to zero reading. Highest concentration of solution to be examined is now placed in cup and reading of galvanometer adjusted to 100. Intermediate concentrations are then atomized and readings are recorded (between 0–100). From the readings of galvanometer a calibration graph is plotted. Solution under test is run through automizer and reading of galvanometer is recorded. The concentration of unknown is found from the graph.

Double beam instruments:

A single beam instrument contains only one set of optics and detector. In double beam instruments a second light path is obtained from flame giving radiations by the added internal standard. The signal provided by one detector is opposed by the other detector. By adjusting indicator devices, ratio of concentrations of internal standard and analyte is found out.

Errors in Flame Photometry:

Flame emission photometry being an instrumental method, some errors does exist in this method. The cause and possible remedies for their removal is discussed as under.

Flame photometry is concerned about a molecule containing metal to undergo vapourization, dissociation into neutral atoms and ions, followed by excitation and releasing energy in the form of radiation. For good results, all the steps involved as above should occur in proper sequence. Some possible sources of error are:

1. Flame Temperature:

It is very essential to have proper temperature of flame to bring about excitation and release of energy. If flame temperature is too low, it would be insufficient to cause vapourization, dissociation and excitation of atom. No lines or few weak lines would be obtained. The temperature should not be too high to have deteriorating effect.

2. Chemical Interference:

When another component is present in the sample as impurity, error occurs. The magnitude of error depends upon the ratio of concentration of contaminant with the element under examination. In determination of certain metal ions (cations), anions present in the solution have depressant effect on the intensities of number of cation lines, e.g. more than 5 per cent oxalate, sulphate, phosphate ions bring decrease in emission intensities of alkaline earth cations. Thus, when contaminants are present, they need to be eliminated by precipitations/complexation or by other methods.

3. Radiation Interference:

Sometimes, presence of certain element causes radiation interference by emitting light of wavelength identical to that of element under examination. The detector is thus unable to distinguish the lines and their intensities. This difficulty is generally eliminated by removing the interfering element or by adding an identical amount to the solution for constructing calibration curve.

6.4 APPLICATIONS

Flame photometry has been widely used in various industries like chemicals and pharmaceuticals, soils and agriculture, ceramics and glass, plant materials and water, oceanography, and in biological and microbiological laboratories.

1. Determination of sodium, potassium, calcium and magnesium in biological fluids like serum, plasma, urine etc. is routinely carried out by flame photometer.
2. Analysis of industrial water, natural water for determining elements responsible for hard water (like calcium, magnesium, barium etc.) is standard procedure in many laboratories.
3. Soil samples are routinely analysed mainly for sodium and potassium and also for calcium and magnesium (after removing other interfering elements) by flame photometer.
4. Some important elements which are commonly determined by this method are aluminium, barium, calcium, cesnium, chromium, copper, iron, lead, magnesium, manganese, potassium, sodium, strontium and zinc.
5. In glass industry, flame photometry is used in determination of sodium, potassium, boron, lithium etc.
6. In cement industry, this method is used in estimation of sodium (Na_2O), potassium (K_2O), calcium (CaO), magnesium (MgO), manganese (MnO_2) and lithium (Li_2O).
7. Flame photometry is extensively used in estimation of alkali-alkaline earth metals besides other metals present in metallurgical products, catalysts, alloys etc.
8. Flame photometry has also been used in determination of certain metals like lead, manganese, in petroleum products like gasoline, lubricating oils and organic solvents.
9. Analysis of ash by flame photometer is routinely carried out in various industries for estimating alkali and alkaline earth metals as their oxides.

REVIEW QUESTIONS

1. Write the principle of flame photometry.
2. Discuss the instrumentation involved in flame photometry.
3. What do you mean by atomizers? What are the types of atomizers and their functions?
4. What are the fuel and oxidants used in flame photometry? Explain their properties and role in Flame photometry.
5. Draw the structure of flame and explain parts of flame.
6. Write a note on errors or interferences in flame photometry.
7. Write the applications of flame photometry.

Chapter ...7

ATOMIC ABSORPTION SPECTROPHOTOMETRY

Objectives:

Upon completion of this section, the student should be able to

- Understand the basic principle and theory involved in atomic absorption spectroscopy.
- Explain the construction and working of Atomic absorption spectroscopic instrument
- Describe the applications of atomic absorption spectroscopy.

7.1 INTRODUCTION

Atomic spectroscopy is thought to be the oldest instrumental method for the determination of elements. These techniques are introduced in the mid of 19th century during which Bunsen and Kirchhoff showed that the radiation emitted from the flames depends on the characteristic element present in the flame.

Atomic emission spectroscopy has a long history. Qualitative applications based on the color of flames were used in the smelting of ores as early as 1550 and were more fully developed around 1830 with the observation of atomic spectra generated by flame emission and spark emission. Norman Lockyer (1836–1920) in the early 1870s developed the quantitative applications based on the atomic emission from electrical sparks were. However the technique became more useful after the applications based on flame emission by H. G. Lundegardh in 1930. Atomic emission based on emission from plasma was introduced in 1964.

Atomic absorption spectroscopy involves the study of the absorption of radiation by neutral atoms in gaseous state. For this the sample is first converted into atomic vapour and then absorption of atomic vapour is measured at the selected wavelength which is characteristic of each element under study. For quantitative studies the measured absorbance is proportional to the concentration of element in vapour state. Since, the technique involves spraying of a solution into a flame, it is also called absorption flame photometry.

This technique appears similar to flame photometry, the difference being, this method is based on absorption from the flame rather than emission into the flame. The principle of

atomic absorption is similar to the absorption spectrophotometry of UV visible type; however, the instrumentation is of different types.

In the technique of atomic absorption analysis, the solution of sample in a suitable solvent is sprayed in a form of fine mist into a flame. Due to high temperature of flame, the element to be determined is reduced to element state in a vapour form. A suitable source of radiation is allowed to fall and pass through flame to the detector. Absorption of energy occurs by the vapour phase of element in flame and remainder is transmitted to the detector. The flame not only serves to convert element into its gaseous atomic form but also acts as a cell in the usual type of spectrophotometer.

In the absorption process by the gaseous atomic form, a series of well-defined lines occur due to electronic transition of outermost shell electrons of the element. In the absorption process the number of atoms capable of absorbing any transmitted light of characteristic wavelength is proportional to the product of concentration of atoms in flame and the path length in flame. In most determinations, concentration of sample solution, wavelength of radiation used for absorption studies, the temperature and height of flame is so adjusted to give accurate and quantitative results.

Atomic spectroscopy involved three major techniques.

1. Atomic emission spectroscopy
2. Atomic absorption spectroscopy
3. Atomic fluorescence spectroscopy

Emission spectroscopy can be applied to both atoms and molecules. Although the molecular UV-visible emission spectroscopy is used for sample analysis its use is limited due to the requirement of thermal energy which may lead to decomposition of sample. The limitations of molecular IR emission and molecular UV-Visible emission concepts made atomic emission spectroscopy as a successful technique.

7.2 PRINCIPLE

All atoms have certain electrons around its nucleus. These electrons are occupied in the orbitals of ground state with lower energy and stable configuration. In atomic emission, a sample is subjected to a high energy, using a thermal source such as an electrical arc, a flame, or plasma. Due to the induced thermal energy the outermost electrons of the atoms are exited and absorb energy thus shifted to excited state with higher energy. However this state is unstable and the electrons return to their initial original orbital position by loosing energy in the form of emission of radiation. This energy is equivalent to the energy that was absorbed during absorption process. This process is said to be atomic emission. (Fig. 7.1) The intensity of the light emitted at specific wavelengths is measured and used to determine the concentrations of the elements of interest.

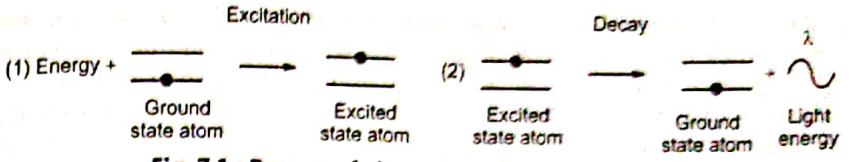


Fig. 7.1 : Process of absorption (1) and Emission (2)

The wavelength of the emitted radiant energy is directly related to the electronic transition which has occurred. Since every element has a unique electronic structure, the wavelength of light emitted is a unique property of each individual element. The emission spectrum of an element exposed to such an energy source consists of a collection of the allowable emission wavelengths, commonly called emission lines, because of the discrete nature of the emitted wavelengths. Emission techniques can also be used to determine how much of an element is present in a sample. For a "quantitative" analysis, the intensity of light emitted at the wavelength of the element to be determined is measured. The emission intensity at this wavelength will be greater as the number of atoms of the analyte element increases.

The technique of flame photometry is an application of atomic emission for quantitative analysis.

7.3 INSTRUMENTATION

An atomic emission spectrometer is similar in design to the instrumentation for atomic absorption. The only difference is the atomic absorption spectrometer uses an additional lamp source such as hallow cathode lamp. Most flame atomic absorption spectrometers can be used for atomic emission by turning off the hollow cathode lamp and monitoring the difference in the emission intensity when analyzing the sample. However equipments exclusively for atomic emission are also available which use plasmas, arcs, sparks, and lasers as atomization and excitation sources.

A schematic line diagram of single beam atomic absorption spectrophotometer is shown in Fig. 7.2.

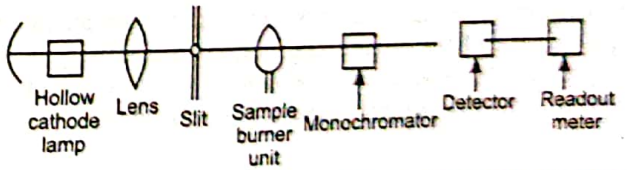


Fig. 7.2 : Schematic diagram of single beam atomic absorption spectrophotometer

The source of radiation is exceedingly important because the width of absorption line used in analysis is very small. The conventional sources such as tungsten lamp, xenon discharge lamp or mercury vapour lamp are not satisfactory because of broad-bandwidth emerging from those sources. The radiation source should give monochromatic light and it should not exhibit fluctuations. Most commonly, the hollow cathode discharge tube is used. The cathode discharge tube is now available for estimation of many elements. It consists of

thick walled glass tube with a transparent window at one end. Inside the tubes are two tungsten wires one with hollow metal cylinder acting as cathode and the other as anode. The tube is filled with pure helium or argon gas at 1-2 mm pressure and potential of 600–1000 volts is applied to it. When the tube is on, the gas inside ionizes and current starts flowing. With an applied potential, gaseous cations acquire sufficient energy to dislodge some metal from the surface of cathode to give atomic cloud. This when excited emits characteristic radiation. During operation, some metal atoms diffuse back and redeposit on the surface of cathode. With proper applied potential desirable radiations of intensity can be obtained.

The optic system for separating the emitted radiation from hollow cathode discharge lamp consists of lens and/or entrance slit. The beam is focused on the centre of the flame.

The flame and sample burning unit are characteristics in atomic absorption spectroscopy. The burner has two main functions to perform:

1. It must introduce the sample in a fine mist at a constant and reproducible rate.
2. It must reduce the metal (element) to its atomic state in vapour form.

The flame shape is also important as it should give long path length so that more number of atoms is possible to come into path for absorption. Various types of atomizers or nebulizers are commercially available. Furthermore, non-flame atomizer involving introduction of heating devices to replace flame have also been marketed. Monochromator is another important device in atomic absorption spectrophotometry. Since, the radiation from the source consists of a line spectrum and the radiation emerging from gas is also given out, isolation of required line radiation is obtained by using grating monochromator. Separation of line from undesirable lines (from flame) is necessary to obtain accuracy and sensitivity. A glass filter can also be used for the same purpose.

The detector device, like UV-visible spectrophotometer is mostly a phototube or photomultiplier tube. The radiant energy received by detector is converted into electric signal. Photomultiplier tube is particularly useful when lines used for study lie in ultraviolet or blue region of visible light.

Amplification of current so produced is carried by amplifier and brought to the read out meter. Now-a-days in most of the models chart recorders are used as readout devices. A chart recorder is a potentiometer using a servometer to move the recording pen. The displacement of pen showing lines is proportional to the input voltage.

Operational procedure:

The operation of instrument will vary from one instrument to another. However, there are some common operational steps as:

(a) Preparation of samples:

A sample to be analysed needs to be in the form of solution. In most cases material is dissolved in suitable solvent (using acid/alkali) and resulting solution is diluted with water. This diluted solution is then sprayed directly into the flame. For substances which are not

water soluble solutions are made in oil or other non-aqueous solvents and then diluted with semi-polar solvents. Usually, contamination and corrosion of the nebulizer burner results if the solution used earlier is not removed. Washing/spraying of water between the readings of each solution should be routinely carried out.

(b) Selection of radiant wavelength:

Light source generally gives out several emission lines. However, the line which shows greater sensitivity to the element under study should be selected. The lines or wavelength which are undesirable and which interfere are easily removed by chopping the exciting beam before entering into the flame.

(c) Fuel and oxidants:

Natural gas, cooking gas, propane, butane, acetylene is very common fuel used in burner cum atomizer. With the supply of air higher temperature can be obtained. Low temperature flames are satisfactory for certain elements like copper, lead, cadmium for which natural gas and air is satisfactory. The non-luminous acetylene-air flame which gives higher temperature (about 2200–2400°C) is best for elements like magnesium, calcium, iron and certain transition metals. Luminous acetylene-air flame which provides reducing conditions is used for barium, chromium etc. The total consumption burner and premix burner as used in flame photometry are also used in atomic absorption spectroscopy.

(d) Spraying technique:

The same technique as used in flame photometry is adopted in this method. The concentration of sample is so adjusted as to give absorption between 20-80 per cent (i.e. absorbance between 0.1–0.2).

(e) Standard solutions and calibration graph:

The concentrations for standard solutions vary according to the ppm and type of instrument employed. Standard solutions are prepared as per the instructions given. The calibration curve is obtained by the same way as per spectrophotometry method. Instrument is adjusted to zero (with water) and 100 or infinite with highest concentration of standard. The remaining intermediate concentration solutions are then sprayed and the readings are noted. The test solution is then analysed and its concentration found from the calibration graph.

The other method described by various pharmacopoeias is known as standard addition method. In this technique, known volumes (amounts) of standard solution are added to a fixed volume of sample solution and diluted to volume. The readings of these solutions are plotted in the normal way. Extrapolation of curve gives concentration of sample solution.

7.4 APPLICATIONS

1. Atomic absorption spectroscopy is very widely used in metallurgy, alloys and in inorganic analysis. Almost all important metals have been analysed by this method. It is an ideal method for analysis of many ores, minerals and alloys.

2. Biochemical analysis: A number of elements present in biological sample can be analysed by atomic absorption method. These include estimation of sodium, potassium, lead, zinc, mercury, cadmium, calcium, magnesium and iron.
3. Pharmaceutical analysis: Estimation of zinc in insulin preparations, oils, creams and in calamine, calcium in number of calcium salts; lead in calcium carbonate and also as impurity in number of chemical salts have been reported.
4. Sodium, potassium and calcium in Saline and Ringer solutions are estimated by this method.
5. In petroleum industry, metallic impurities in petrol, lubricating oils have been determined.
6. Analysis of ash for determining the contents of sodium, potassium, magnesium, calcium and iron is carried out in boiler deposits.
7. In cement industry, estimation of sodium, potassium, calcium, magnesium is carried out to determine the quality of cement.
8. Besides, atomic absorption, spectroscopy finds wide applications in various industries like agriculture, soil, forestry oceanography, fertilizer etc.
9. Atomic absorption spectrometry is used in the assay of (a) Intraperitoneal dialysis fluid (for calcium, magnesium), (b) Activated charcoal (for zinc), (c) Cisplatin (for silver).

REVIEW QUESTIONS

1. Write the principle and theory involved in atomic absorption spectroscopy.
2. Discuss the instrumentation involved in atomic absorption spectroscopy.
3. Draw a schematic diagram of atomic absorption spectroscopic instrumentation.
4. Write the differences between absorption and emission spectroscopy.
5. Write the applications of atomic absorption spectroscopy.
6. Compare flame emission spectroscopy with atomic absorption spectroscopy.

Chapter ...8

NEPHELOTURBIDIMETRY

Objectives:

Upon completion of this section, the student should be able to

- Understand the basic concepts of nepheloturbidimetry including its principle and theory.
- Explain about the instrumentation and working of nepheloturbidimetry.
- Describe the applications of nepheloturbidimetry.

8.1 INTRODUCTION

The techniques of nephelometry and turbidimetry are concerned about the analysis of collidal or fine suspension system. When a light of incident radiation is allowed to fall on a fine dispersed suspension, a part of light is absorbed, a part is reflected and refracted and remainder is transmitted out. The optical property of a fine, stable suspension will depend upon the concentration of dispersed phase. The measurement of the intensity of transmitted light of a suspension in relation to the concentration of dispersed phase is the basis of Turbidimetric analysis.

If the suspension or colloidal sample is viewed at right angles to the direction of incident light, the system appears cloudy because of scattering of light from the particles of suspension. This scatter is due to Tyndall effect. Because of the scattering the colloidal system appears milky, hazy or cloudy. The measurement of the intensity of scattered light of suspension in relation to the concentration of dispersed phase forms the basis of Nephelometry analysis.

The Nephelometry and Turbidimetric are meant for determination of concentration of dispersed phase of a suspension. They differ in that in nephelometry, the amount of light scattered by the particles is measured by viewing at right angles to the incident light while in turbidimetric the amount of light transmitted by the suspension is measured.

Turbidimetry is similar to colorimetry since both methods measure the amount of transmitted light.

For the quantitative determination by Nephelometry or Turbidimetry the following conditions are necessary:

1. The suspension should be dilute.
2. The suspension should be stable and not allow the particles to settle down.

3. The particles of suspension should be small and fine.
4. The particles should have a uniform shape and size to allow uniform scattering of light.
5. For the stability of suspension addition of other salt and protective colloid should be used.
6. Viscosity and temperature should be such as to maintain stable suspension.

Both the Turbidimetry and Nephelometry are very sensitive in dilute solutions. For the determination of concentration of dispersed particles a calibration graph is plotted. After a particular concentration the optical property is not very linear.

8.2 THEORETICAL PRINCIPLE

Theoretical principle of Nephelometry and Turbidimetric analysis is similar to colorimetry. The intensity of light scattered or transmitted by a suspension is a function of the concentration of scattering particles of uniform shape and size. In dilute suspensions the amount of scatter depends upon the number of particles intersecting the beam of light. Provided no multiple scatter occur, the total scatter is simply the sum of the individual scatters by the particles. Thus, the Beer's law as applied in colorimetry can be applied.

$$\frac{I_t}{I_o} = 10^{-rbc}$$

Where 'r' is the turbidimetric coefficient, b is path length; c is the concentration in g/litre. The correlation of concentration of dispersed particle to that of transmission or scattered light will hold provided that

1. there is no settling of particles
2. no coagulation of dispersed particles
3. no nucleation and crystal growth and
4. absence of interfering ions, etc.

The Nephelometry or Turbidimetric analysis is carried out for a system which gives turbidity by itself. The sample material in a fine particle form is dispersed in a suitable solvent to get a stable suspension. Alternatively, turbidity is produced by addition of suitable reagent into a solution. The turbidity so produced is compared with standard turbidity produced in similar manner under similar conditions.

8.3 INSTRUMENTATION

Usual type of photoelectric colorimeter can be used for turbidimetric analysis. The sample holder allows large size cuvettes or cells to accommodate in instruments. Ordinary electric bulb or a light source of 6 volts or 6 watt bulb can be used in the instrument. A phototube or Barrier layer cell can be employed as a detector. A line diagram of simple type of turbidimeter is shown in the Fig. 8.1.

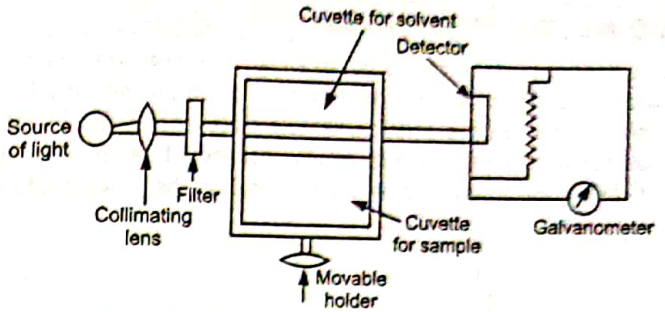


Fig. 8.1 : Schematic diagram of Turbidimeter

Nephelometer (Fig. 8.2) is designed on the Tyndall effect. In an instrument, a suitable source of light 6-8 volts tungsten lamp is focused by lens and/or prism on the side of test tube or cuvettes containing a sample solution under test. The scattered light at 90° to the path of incident light is collected on a photocell. The current so produced is amplified and supplied to an indicator meter graduated from 0–100 divisions. The meter is also calibrated with corresponding standard solutions in Nephelometry-Turbidimetric Units (NTU) of sulphate or phosphate standards. The calibration is done from 0–40 or 50 divisions on meter with respect to NTU. The sensitivity of meter is adjusted to fine or coarse in various ranges from 04–100 NTU. The line diagram of simple Nephelometry is shown in Fig. 8.2.

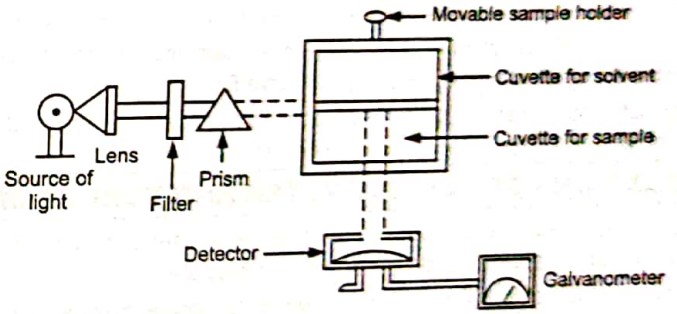


Fig. 8.2 : Schematic diagram of Nephelometer

Working of the Instrument:

Light source is started on and an appropriate filter (in case of filter type instrument) is inserted in the instrument. With pure solvent or distilled water zero setting is adjusted to '0' of galvanometer. With the standard turbidity or highest expected turbid solution, instrument is set to 100 divisions. Various solutions/ suspensions of intermediate turbidity are put in the sample tubes and readings recorded. A calibration graph is drawn for the various concentrations of turbid solutions. Now a sample of unknown or test is inserted in the instrument and turbidity reading is recorded. The concentration of unknown is found from the graph.

8.4 TURBIDIMETRIC TITRATIONS

Turbidimetric titrations can be carried out in a manner similar to photometric titrations. A sample solution which forms a finely colloidal type of precipitate on addition of titrant (reagent) can be analysed. In the titration, sample material is taken in cell (50–100 ml capacity having stirring mechanism) and regular volume of titrant is added by using microburette. A graph of absorbance is plotted against the volume of titrant added. [Fig. 8.3].

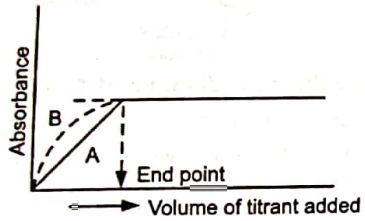


Fig. 8.3 : Turbidimetric titration plot (Absorbance Vs Volume of titrant)

In an ideal situation, absorbance increases linearly as the precipitate forms on addition of reagent to a point and then remains same. The intersection of the two straight lines gives an equivalence point.

This holds true only if the number of particles increase linearly to the end point and size and shape of particles remain same throughout. In practice, however, it is found that the added reagent may simultaneously form some new particles or get added to those previously formed giving distortion of lines (Fig. 8.3 curve B).

Some important determinations by this method include carbonates as $BaCO_3$, chloride as AgCl, calcium as calcium oxalate, zinc as ferricyanide, etc.

8.5 APPLICATIONS OF NEPHELOMETER AND TURBIDIMETER

There are numerous applications of the use of nephelometer or turbidimeter in various industries including pharmaceutical industry. Some examples are:

1. For determination of ions like sulphate, chloride, carbonate, magnesium, calcium etc.
2. In determination of impurities in pharmacopoeial substances.
3. In determination of growth of bacteria in culture media and nutrient media.
4. In determination of growth of micro-organism in vitamin and antibiotic assays.
5. In determination of particles or solids in aerosols, injections and liquid preparations.
6. It is employed in soap and detergent industry in determination of cloud point.
7. It is used in determination of sediment and particles in water treatment tank and sewage tank.
8. The above instruments are used for various purposes in other industries like paper, pulp making, beverages, an oil refinery, petroleum, dye and paints etc.

REVIEW QUESTIONS

1. Differentiate Nephelometry or Turbidimetry.
2. Write the principle of Nepheloturbidimetry.
3. Draw a schematic diagram of nephelometer and turbidimeter, discuss various components and their working.
4. What are the basic conditions for analytes to be analysed by Nephelometry or Turbidimetry?
5. Write a note on turbuidometric titrations.
6. Write the applications of Nepheloturbidimetry.

UNIT - III

Chapter ...9

INTRODUCTION TO CHROMATOGRAPHY

Objectives:

Upon completion of this section, the student should be able to

- Understand and define the process of Chromatography.
- Describe the classification of chromatography.
- Explain various elution techniques used for separation and choice of techniques depending upon the nature of analytes.

9.1 INTRODUCTION

A variety of methods is available for the separation of components from the mixture and to analyse them. There are two methods for analysis:

1. Chemical Methods
2. Physical Methods.

The physical methods include:

(a) Fractional distillation (b) Extraction
(c) Counter current distribution (d) Fractional precipitation
(e) Crystallization etc.

These methods are effective in separation, purification and identification of many compounds. However, difficulty arises in case of compounds where individual components have similar physical and chemical properties i.e. mixture of liquids having very close boiling points, etc. However, these methods are not satisfactory in biological materials.

Chromatographic methods represent the most useful and powerful technique for these problems. This technique was first introduced by a Russian scientist, Tswett in 1906. He demonstrated chromatography using separation of different coloured components of leaf extract by pouring it into a glass column packed with calcium carbonate, as adsorbent. The solvent (water) was passed through the top of the column and some chlorophyll pigments of mixture were separated. They are used for the separation of components of a complex mixture. Because of the rapidity and effectiveness, chromatography has been used in all the fields e.g. chemistry, biology, medicine, dyes, forensics and clinical studies with advantages over other methods.

A salient advantage of chromatographic methods is that they are relatively 'gentle' and disallow the decomposition of substances. This is important especially for labile substances and substances of biological origin.

Another advantage is that the separations can be carried out on micro or semi-micro scale, i.e. a small quantity of mixture is required for analysis.

Chromatographic techniques are simple, rapid and require simple apparatus. The complex mixtures can be handled with comparative ease.

Chromatographic separation relies on relative movement of two phases, similar to fractional distillation or countercurrent distribution, but in chromatography one phase is stationary phase and the other is mobile. The mobile phase passes over the stationary phase and transports components of the mixture at different speeds in the direction of the flow of mobile phase. The separation of components is a result of the differential affinity of the components for the mobile phase and a stationary phase.

Definition: Chromatography is a separation technique, used to separate components of a mixture using a fixed/stationary phase and moving/mobile phase. The term 'chromatography' is derived from the Greek words the 'chrome' (colour) and 'to write' (graph). Recently, the IUPAC has defined Chromatography as "A method used primarily for the separation of the components of a sample, in which the components are distributed between two phases, one of which is stationary while the other mobile. The stationary phase may be a solid or a liquid supported on a solid or a gel, and may be packed in a column, spread as a layer or distributed as a film. The mobile phase may be gaseous or liquid."

9.2 HISTORY

The study of chromatography started in the eighteenth century when Runge studied with great interest the nature of inorganic compounds on filter papers. He separated inorganic salts and observed that inorganic salts travel to different extent producing attractive pattern. In the year 1898, Day in the USA forced crude petroleum through a column of limestone and fuller's earth. He observed that first portion was of light hydrocarbons, followed by hydrocarbons of aromatic nature, unsaturated type, heterocyclic and nitrogen-sulphur containing high molecular weight hydrocarbons.

The Russian botanist, Michael Tswett discovered the chromatographic principle in 1906, he used a glass column of calcium carbonate for separation of chlorophyll pigments from plant by using petroleum ether. The pigments, according to their adsorption patterns, were resolved into various coloured zones; he then separated and estimated them.

Between 1910 and 1930, very little work was published about chromatography. The major development occurred around 1930 when Lederer and co-workers in 1931, separated lutein and xanthenes on a column of calcium carbonate powder. Further developments soon followed when Kuhn, Karrer, and Ruzicka, separated plant carotenes into several components by adsorption chromatography. This helped in resolution of naturally occurring mixture of pigments, sugars, amino acids, proteins, vitamins and hormones. This led to the development of absorption and partition column chromatography for identification, separation, isolation, both on preparative and analytical scale.

In 1935, Adams and Holmes observed some synthetic ion exchange resins capable of exchanging ions and thus ion exchange chromatography came into existence.

Tiselius (1940) and Claesson (1946) studied the properties of solutions in the chromatographic process classified these into three groups based on the principle of separation:

(a) Frontal analysis (b) Displacement analysis and

(c) Elution analysis.

Tiselius was awarded the Nobel Prize for this achievement in the year 1948.

Another broad type of chromatography, involving partition between two liquids was proposed by Martin and Synge in 1941. They used chromatographic column, filled it with silica gel particles with water retained on silica gel and passed chloroform flowing through the column. This system successfully separated the acetylated amino acids according to their partition coefficient.

In 1944, Martin, Consden, Gorden replaced silica gel column by strips of filter paper and developed Paper Chromatography. For this achievement, they were awarded the Nobel Prize in 1952.

Thin layer chromatography though discovered first by Izmailer and Shraiber, was further developed by Stahl and co-workers using silica gel on glass plates. They successfully demonstrated the usefulness of TLC in separation of a wide variety of substances.

Reversed phase paper chromatography was then devised wherein the paper is impregnated with a hydrophobic liquid and aqueous phase (or polar) liquid is used as mobile phase. This technique is used for separation of materials having poor solubility in water.

Amongst the latest and the most effective chromatographic technique is Gas Chromatography. It was introduced by Martin and James in 1952. The components of the mixture migrate at different speeds when carried along by an inert gas which acts as mobile phase. This method is more advantageous than other methods in speed, accuracy, sensitivity and versatility. The instrumentation of gas chromatography followed in the wake; presently this technique is used in routine separation and identification of compounds.

The theoretical aspects of chromatography were first studied by Wilson in 1949, he discussed the quantitative aspects in terms of diffusion, rate of adsorption and isotherm, non-linearity, etc. Glueckauf in 1949, described the column performance in terms of stationary phase, particle size and diffusion. However, it was Van Deemter and co-workers, who in 1956 developed the rate theory to describe the separation process. Giddings in 1963, pointed out that if the efficiencies of gas chromatography were to be achieved in liquid chromatography, and then particle sizes of 2.20 μm were required, this would entail high mobile phase inlet pressure. This led to the discovery of High Pressure (performance) Liquid Chromatography (HPLC).

There has been continuous development in chromatography particularly in techniques, materials and requirement of instrumentation which has resulted in the efficient, reliable and sensitive chromatographic methods in use today. The latest development in chromatography is HPTLC technique.

9.3 CLASSIFICATION

In all types of chromatography, separation of the components of a mixture results either by adsorption or partition for the column material. Binding of a compound to the surface of solid phase takes place in adsorption while in partition, a compound gets distributed into two liquid phases. The usual type of chromatography involves movement of liquid phase over stationary phase carrying with it, a solute which has varying degree of affinity for stationary phase. Thus, depending upon two phases, the chromatographic methods are classified as:

I. Partition chromatography:

This involves liquid or gas as mobile phase and another liquid or solid as a stationary phase.

The operations include:

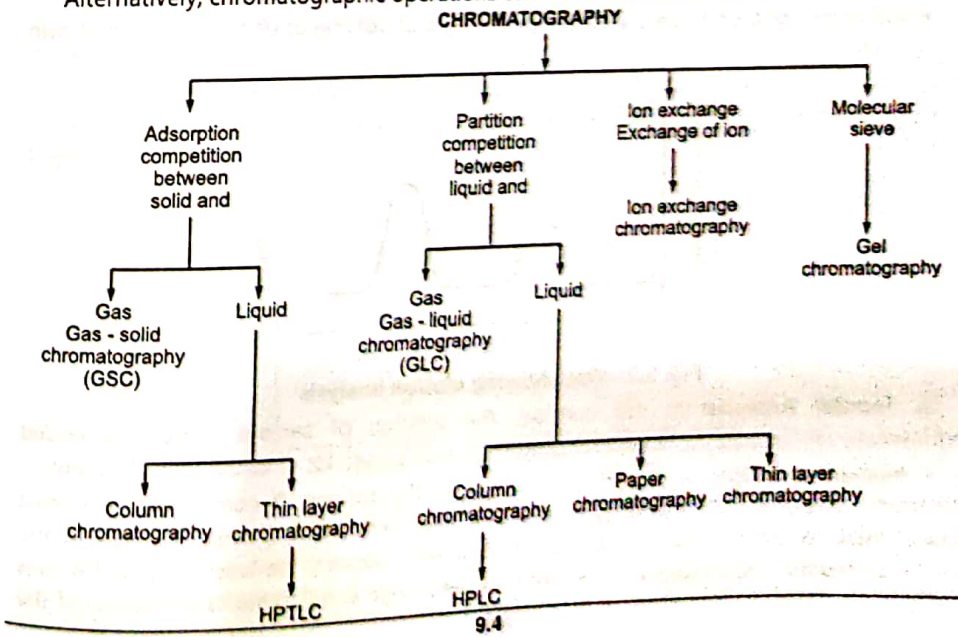
1. Partition column chromatography.
2. Paper chromatography.
3. Thin layer chromatography.
4. Gas-liquid chromatography.
5. High performance liquid chromatography.

II. Adsorption chromatography:

This involves liquid or gas as mobile phase and adsorbent solid as stationary phase. The types under this category include:

1. Adsorption column chromatography
2. Thin layer chromatography
3. Gas-solid chromatography.

Alternatively, chromatographic operations can be classified as follows:



9.4 SEPARATION TECHNIQUES

All chromatographic methods are meant to separate two or more components from the mixture, which methods are executed by distribution of components between stationary and mobile phase. Chromatographic separation can be effected by the following techniques:

1. Elution Analysis: It is a very common method used in column chromatography. In this method, a small volume of mixture to be separated is added on the top of column and mobile phase is allowed to flow through column. As mobile phase moves down the column, the mixture introduced on the column gets separated into zones as the components of mixture are adsorbed to the column material to different extent (Fig. 9.1 (a), (b)). On further passage of mobile phase each component of mixture is eluted out as separated component.

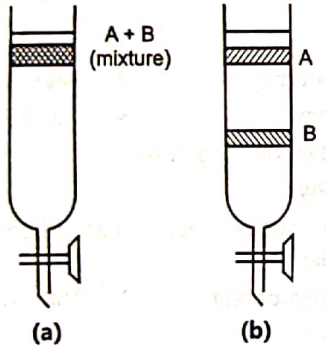


Fig. 9.1 : Elution analysis - Separation of mixture through column

A plot of the amount of separated compound against volume of elute fractions is shown in (Fig. 9.2).

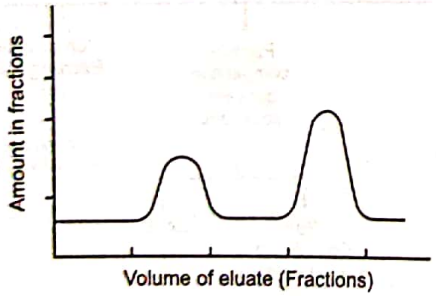


Fig. 9.2 : Plot showing elution analysis

2. Frontal Analysis: In this method, the solution of sample mixture is added continuously on the column. No mobile phase (solvent) is used for development of column.

A mixture containing *A*, *B* and *C* is added on the column. If component *A* is least adsorbed; component *B* to intermediate extent and component *C* most strongly to the column material, then as the mixture flows through the column, the least adsorbed *A* runs down the column fast; component *B* to intermediate stage and *C* remains at the top of the

column. When more sample mixture passes through column, first few fractions of eluate will contain *A*, subsequent fractions will contain *A* + *B* and the final or the last *A* + *B* + *C*. Only partial separation of *A* from *B* and *C* occurs. A complete separation of *A*, *B* and *C* cannot be achieved unless the fractions are again developed on another column.

A plot of amount of substance against volume of eluate is shown in Fig. 9.3.

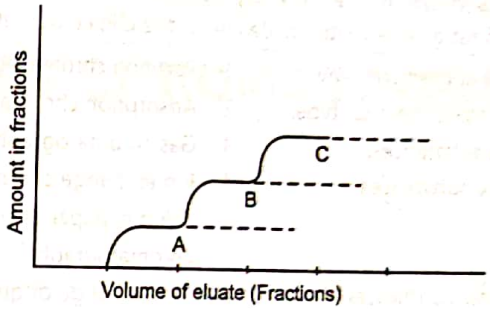


Fig. 9.3 : Plot of frontal analysis

Tiselius developed this technique in 1940.

3. Displacement Analysis: In displacement analysis, a small volume of mixture is added to the column and elution is carried out by a solvent containing a solute which has high absorptivities for column material. The adsorbed constituents of mixture are displaced by the solute from mobile phase. Each solute in the mixture in turn displaces another solute which is less firmly adsorbed. The least adsorbed constituent is pushed out of the column. The substance used in mobile phase is called as displacer; hence, the technique is known as "displacement analysis".

In separation of mixture containing *A*, *B* and *C* (with *A* < *B* < *C* adsorption), if *D* is used as displacer, then the plot of amount of substance against volume of eluate will be as shown in Fig. 9.4.

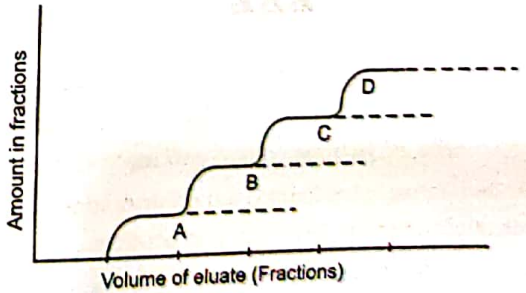


Fig. 9.4 : Plot of displacement analysis

This technique is mainly used in preparative work; hence, not suitable for analysis since there are chances of overlapping for as much as zones are not distinctly separated.

9.5 CHOICE OF METHOD

The choice of the method is by and large empirical as there is no way of predicting the best procedure for separation, except in some simple cases. It is useful to try simpler techniques as paper or TLC first as they can often provide a useful guide to the type of system to be used for separation. The more sophisticated technique can then be applied or adopted. The following list gives a rough guideline for the choice of method:

1. Substances of similar chemical type.	1. Partition chromatography.
2. Substances of different chemical type.	2. Adsorption chromatography.
3. Gases and volatile substances.	3. Gas chromatography.
4. Ionic and inorganic substances.	4. Ion exchange chromatography, or column, paper or thin layer chromatography.
5. Ionic from non-ionic substances	5. Ion exchange or gel chromatography.
6. Biological materials, compounds of high molecular weight.	6. Gel chromatography, electrophoresis.

In the event of difficult separations, when simpler methods are inadequate, High Performance Liquid Chromatography (HPLC) may provide the results.

REVIEW QUESTIONS

1. Define chromatography.
2. Classify chromatography.
3. Write the elution techniques involved in chromatographic separation.
4. Write a note on choice of methods of chromatography.

Chapter ... 10

ADSORPTION AND PARTITION COLUMN CHROMATOGRAPHY

Objectives:

Upon completion of this section, the student should be able to

- Understand the basic principle of adsorption and partition column chromatography.
- Understand the operational techniques of adsorption and partition column chromatography.
- Describe the applications of adsorption and partition column chromatography.

10.1 INTRODUCTION

Chromatography is a technique employed for separation of the components of a mixture by continuous distribution of the components between two phases: mobile and stationary. The mobile phase moves over the stationary phase continuously. When the stationary phase is a solid support of adsorptive nature and mobile phase is liquid or gaseous phase, it is called adsorption chromatography and when the stationary phase is liquid with the mobile phase as liquid or gaseous, it is called as partition chromatography. When the chromatographic operations are in progress using a column, it is called column chromatography.

There are four types of column chromatography:

1. Adsorption chromatography in which the components of a mixture are selectively adsorbed on the surface of packing column material, i.e. adsorbent.
2. Partition chromatography in which the component is partitioned between the mobile phase and their stationary phase held stationary on inert solid support.
3. Ion exchange chromatography in which the constituent of a sample is selectively retained by exchange resin by replacing ion/s on packing material.
4. Gel chromatography is the method in which the column is packed with a permeable gel which accomplishes separation by sieving or molecular filtration action.

10.2 ADSORPTION COLUMN CHROMATOGRAPHY

American geologist Day and Russian botanist Tswett are credited with the history of adsorption chromatography. Tswett separated leaf pigments into different coloured bands on calcium carbonate column, while Day achieved fractionation of crude petroleum on limestone. It appears that neither Tswett nor Day was aware of the work of the other pioneers. It was the work of Kuhn and Lederer in 1931 on polygene pigments that renewed interest in chromatography.

Theoretical Principle: The basic principle of adsorption chromatography is adsorption of a component at the solid-liquid interface. For good separation, the component of the mixture should have different degree of affinity for the solid support, i.e. adsorbent and the interaction between adsorbent and component should be reversible. The component which has strong adsorption for column material stays up while that component which has less affinity moves down the column at a faster rate as the eluate passes through the column.

Component of mixture is bound to solid surface by specific interaction between polar groups of molecule on the adsorbing surface. The exterior properties of atoms, ions or molecules of adsorbents differ from the interior. The bonds at the surface layer are perturbed and have higher energy level. This is called surface activity. The attractive forces may be ionic (electrostatic), dipole-dipole, dipole-induced dipole or simple London forces. Thus, the solute from the solution when comes into contact, gets adsorbed on the surface. Usually, the surface loses activity when it is covered by a monolayer of adsorbed species.

Adsorption isotherm, similar to partition coefficient, can be determined for a solute. A plot of equilibrium concentration of species in a solution and that of amount adsorbed is called adsorption isotherm. It can be shown as a graph of amount adsorbed (C_s) *vs.* amount in mobile phase (C_m) at a constant or fixed temperature. Usually, three types of adsorption isotherms are observed.

1. **Linear adsorption isotherm:** It is a linear graph indicating the amount of substance adsorbed per gram of adsorbent proportional to the concentration of solution, i.e. $k = C_S/C_m$.
2. **Convex adsorption isotherm:** This is due to variation in activity of adsorbent and shows that system is not linear.
3. **Concave adsorption isotherm:** In this, the adsorption from strong solution is greater than the weak solutions. The additional reaction that takes on an adsorption enhances overall adsorption process.

For satisfactory separation technique, the linear adsorption isotherm is ideal.

10.3 OPERATIONAL TECHNIQUE

Generally, in this technique, the mixture to be separated is taken in a suitable solvent and a small amount of the solution is added to the uniformly packed column with an adsorbent. The sample is allowed to enter the column. A suitable developing solvent is added on the top of the column and the solvent allowed to run down.

During the process, the component with higher adsorption is retained at the top while the one having less adsorption runs down the column. The procedure is continued till the components are successfully separated out. The ease of displacement of the solute molecule will depend on the absorptivity's of the solute and polarity of developing solvents. Either the material is separated as bands on the column or eluted out of column.

The practical considerations of a successful technique can be considered as:

1. Columns:

Chromatographic columns can be obtained commercially with spring loaded stopcocks with a sintered glass disc at the bottom. Generally a length to breadth ratio of 5:1 or 8:1 is good enough for optimal separation. A stopcock fitted to a column is desirable as it allows control of flow rate of solvent. A small plug of glass wool or a porous plate is used at the bottom to support the adsorbent used in the column (Fig. 10.1). In those cases where the substance undergoes oxidation in the presence of air, a flow of neutral gas such as nitrogen is maintained during the operation. The small size columns are only few mm in diameter and few cm in length while large columns are several cm in diameter and correspondingly greater in length.

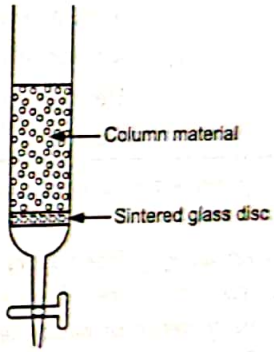


Fig. 10.1 : Column chromatography

2. Adsorbents:

Many solid organic and inorganic substances are used as adsorbents such as alumina, silica gel, kieselguhr, magnesium oxide, calcium carbonate, calcium phosphate, carbon, starch, sugar, cellulose etc. The selection cannot be made at random as there are various factors which guide their choice. The ideal properties for the adsorbent materials are:

1. There should not be any reaction with the substance to be separated.
2. It should be insoluble with the solution under test and solvents used for elution.
3. It should not catalyze the decomposition of substance.
4. It should be a colourless, uniform in size and shape.
5. Its properties should be uniform and remain so during experimental operations.
6. It should have high mechanical stability.

Adsorbents can be classified according to their activity:

1. Weak adsorbent – Sucrose, Cellulose, Starch, Talc etc.
2. Intermediate adsorbent – Calcium carbonate, Calcium phosphate, Magnesia, Slaked lime, Silica gel etc.
3. Strong adsorbent – Alumina, Fuller's Earth, Charcoal.

The commonly used adsorbents are listed in Table 10.1 below with the types of compounds separated with their aid.

Table 10.1: Adsorbents used in column chromatography

Adsorbent	Used to separate
Alumina, magnesia	Sterols, vitamins, esters, alkaloids
Silica gel	Sterols, amino acids
Carbon	Amino acids, peptides, carbohydrates
Magnesium carbonate	Porphyrins
Magnesium silicate	Alkaloids, esters, glycerides, sterols
Calcium carbonate	Xanthophylls, carotenoid
Aluminium silicate	Sterols
Starch	Enzymes

Alumina is preferred for separation and analytical work, its advantage being that it can be used many times and again by regeneration.

Alumina surface is capable of exhibiting different types of solute-sorbent interaction because of its strong positive fields surrounding the Al^{3+} which allow interaction with easily polarizable molecules, or due to the presence of basic sites (probably O^{2-}) which allow interaction with proton donors. Charcoal and magnesia can be used successfully in chromatography.

3. Preparation of Adsorbents:

Since, adsorbents need activation before use, adsorbents such as alumina and carbon can be activated by heating in vacuum for the loss of water and other adsorbed material. Generally, there is an optimum temperature for activation, e.g. alumina (about 400°C). The period of heating is also important, as long time heating will lose its activity. Three to four hours is usually sufficient. In most cases, heating at 200°C for four hours is safe and desirable for most solids.

If freshly activated solid proves too active for separation, deactivation may be carried out by controlled addition of water. Powdered sugar, sieved and dried in a desiccator has been used for the separation of colouring matters.

4. Developers:

These are the compounds or reagents used for the production of colour for colourless substances. These substances should have less affinity for adsorbents than the components to be separated on column. The general reagents such as hydrogen sulphide, ammonium sulphide, potassium ferricyanide, potassium thiocyanate etc. are used as developers.

5. Solvents:

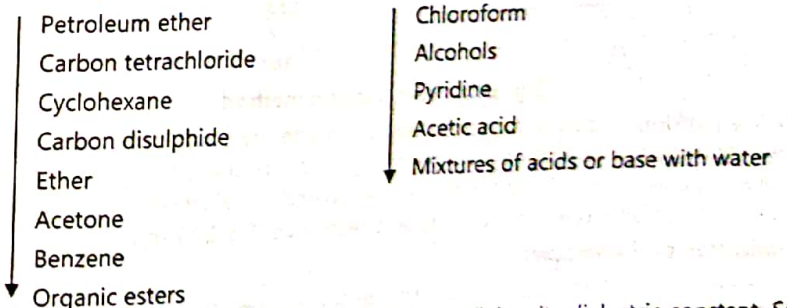
The success of chromatographic analysis depends upon the solid adsorbent and the solvent mobile phase. Solvent plays an important role. Solvents are used:-

1. To transfer the mixture to the column,
2. To effect the process of the development of chromatogram by which the zones are separated to the highest possible extent and,
3. For the complete elution.

The solvent plays an active part in the adsorption process and competes with the sample molecules for active sites on the adsorbent. Thus, the stronger the binding of solvent molecules, greater the amount of time the solute molecules spend in the mobile phase and hence they are eluted faster.

Solvent employed in elution may be single pure solvent or a mixture of solvents or the different solvents at different stages.

The choice of solvent is mainly influenced by the nature and solubility of the mixture. It is always better to choose a solvent of indifferent eluting power so that strongest eluting solvent can be tried at the end. Generally for polar adsorbents such as alumina and silica gel, the strength of adsorption increases with polarity of adsorbate. For carbon, the order is reversed. The sequence of solvents used is in the following sequence:



The eluting power of the solvent is practically parallel to its dielectric constant. Solvents should satisfy the practical factors such as viscosity, stability, compatibility, solubility and purity. Significant factor is that the solvent should provide maximum separation in minimum time.

6. Packing the columns:

Packing the column uniformly is one of the important ingredients for successful chromatography. This will enable minimizing the distortion of the chromatographic boundaries. For open tubular chromatography, the size of the packing particles should be > 150 μm to obtain acceptable flow rates. Channeling is usually caused by the inclusion of air bubbles during packing.

There are two methods used for packing the columns. They are: (a) Wet Packing and (b) Dry Packing.

(a) Wet packing: (Fig. 10.2) Wet packing is common with adsorbents like alumina, magnesia etc. In this case, the slurry of adsorbent with the solvent is prepared and is poured into the glass column having glass wool or a sintered disc at the lower end; the cotton plug too can be used. The slurry with the solvent is poured until the desired height of column is achieved. When adsorbent settles down, a filter paper disc and washed sand is placed at the top and the solvent is allowed to run down until the liquid level is about 1 cm above the top level of the column. The filter paper and sand prevent the disturbances in column when fresh mobile phase is added. The liquid level should never be below the adsorbent; otherwise cracks will develop rendering the column useless.

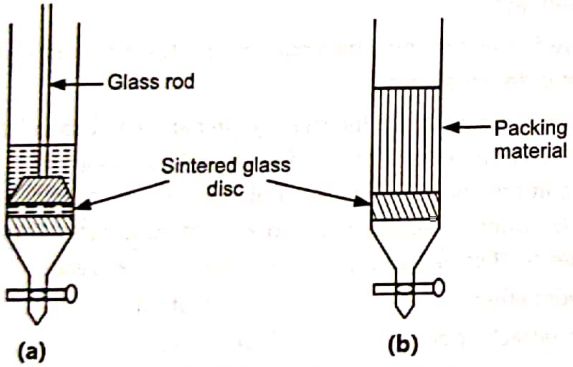


Fig. 10.2 : Wet packing method

(b) Dry packing: In this, adsorbent is poured as fine dry powder in column. The column is tapped regularly and carefully during the filling. This is continued until columns of desired height are available. Solvent is added to the column and it is allowed to run down. Air which is trapped in the column is removed by tapping technique (Fig. 10.2 (b)).

7. Application of the sample:

It is important to apply the sample to the top of the column as evenly as possible. Application of the sample can be made by a small pipette whose tip is placed against the column wall just above the surface of the adsorbent (Fig. 10.3). Another method is to use one or two small discs of volatile solvent soaked filter paper. The discs are placed on the top of column.

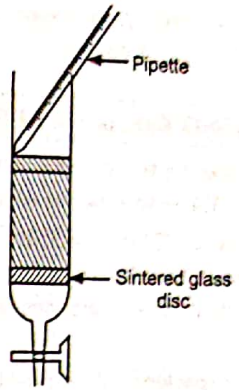


Fig. 10.3 : Applications of sample in column

Elution Procedures:

There are three principle elution procedures commonly employed: (a) isocratic, (b) stepwise elution and (c) gradient elution.

(a) Isocratic elution: In this, a solvent mixture of unvarying composition is allowed to run through the column until separation is complete. The process is terminated when different coloured bands are observed on the column. The contents of the column can now be extruded and the separated constituents extracted by means of suitable solvent. Isolation of the bands is facilitated by the use of transparent nylon tube as the column container. The contents of the column after completion are separated by cutting the tube into sections. An alternative and more commonly used method is to allow the column to run until the separated components can be detected in the column effluent (eluate).

(b) Stepwise or fractional elution: In fractional elution, only one solvent is used; this will result into elution of only some of the components of the mixture. Hence, to remove components which are firmly held, a stronger eluting solvent will be required. Sometimes it becomes necessary to use several different solvents to increase polarity for the successive displacement of different components. This is stepwise elution. The advantage of this technique is that sharper separations are obtained. However, the disadvantage is that a given compound may give rise to more than one peak in the successive steps.

(c) Gradient elution: This technique was first described by Williams and Tiselius which involves the use of a continuously changing eluting medium. The effect of this gradient is to elute successively the more strongly adsorbed substances at the same time to reduce tailing. Thus, the chromatographic bands will become more concentrated and occupy less of the column. Currently, microprocessor controlled solvent delivery modules are available which can generate the required gradient profile for stepwise, linear or isocratic development.

8. Detectors:

For quantitative determination of the dissolved substances in an eluate, there are various detectors used: refractometer, colorimeter, spectrophotometer, flame ionization detectors,

conductivity detectors etc. The eluates are collected separately. Substances which are colourless in ordinary light but fluoresce strongly in UV light are detected by UV lamps and fluorimeter.

10.4 FACTORS AFFECTING COLUMN EFFICIENCY

It is frequently necessary to separate two or more very closely related substances having close chromatographic properties. The various factors which affect the column efficiency are:

1. **Dimensions of the column:** Column efficiency is improved by increasing the length/width ratio of column. Recently length/width ratio of 10:1 to 100:1 is found to be most satisfactory. For successful separations sample/column packing ratio ranges from 1:20 to 1:100.
2. **Particle size of column packing:** It is possible to improve the efficiency by decreasing the particle size. This may decrease the flow rate. Particles from 100–200 mesh are generally satisfactory.
3. **Pore diameter of column packing:** Polar adsorbents have been found to have a pore diameter ≤ 20 A°. The decrease in average pore diameter from 17–20 A° doesn't impair the efficiency.
4. **Temperature:** The speed of elution increases at high temperature as adsorption is highly reduced. However, column chromatography is preferably carried out at room temperature. Recovery of sample can be increased by decreasing the temperature. Difficultly soluble samples are generally separated at higher temperature.
5. **Quality or Nature of Solvents:** Usually, solvents having low viscosity are chosen for higher efficiency as the rate of flow is inversely proportional to the viscosity. Solvents of proper viscosity, good elution power and good quality are selected.
6. **Packing the column:** The packing of the column should be uniform. It should neither be too firm nor too loose. There should not be air bubbles or cracks in column, otherwise the separation will not take place properly or tailing will occur.

Applications:

1. **Separation of mixture of geometrical isomers:** The separation of cis/trans isomers is possible by this technique e.g. separation of cis/trans carotenoid is possible on calcium carbonate column and other adsorbents. The separation is mainly based on steric factors. Isomers whose functional groups can approach the surface of adsorbent more easily are more strongly adsorbed.
2. Determination of amino acids from protein hydrolyzates.
3. Separation of diastereomers, racemates and tautomeric mixtures.
4. Separation of 17-ketosteroids.
5. Separation and analysis of binary drugs in combinations.

10.5 PARTITION COLUMN CHROMATOGRAPHY

In partition column chromatography, the solid adsorbent is replaced by a packing material comprising a support material coated with a stationary phase. The stationary phase should be immiscible or at the most sparingly miscible with mobile phase. In this technique, the solute gets distributed between the two phases, depending on its partition coefficient.

The solid support used for stationary phase should be inert to the substances to be separated. The coated solid is packed in columns as in adsorption column chromatography. Desirable properties of satisfactory solid support are as follows:

1. The support material must adsorb and retain the stationary phase.
2. It should expose large surface areas to the mobile phase.
3. It should be mechanically stable and easy to pack into the column when loaded with stationary liquid.
4. It must not obstruct the solvent flow.
5. It should be chemically inert and have uniform size and shape. Practically, there is no support which has all these ideal properties, barring exceptions.

Commonly used supports are:

(a) Silica gel (silicic acid).
(b) Diatomaceous earths (Kieselguhr, Celite etc.) and cellulose.
(c) Starch.
(d) Purified sand.
(e) Brick-powder or glass beads may be used to a limited extent.

Silica gel is most commonly used material with water or a buffered aqueous solution as a stationary phase. The amount of liquid held is about 0.6 cm^3/gm of gel. Silica gel used for chromatography is in the form of a fine white powder with a fairly narrow range of uniform particle size.

Diatomaceous earths are available commercially as Kieselguhr, Celite etc. The amount of liquid stationary phase used with these solids is about 0.8 cm^3/gm. Kieselguhr has very little adsorptive capacity and therefore makes an ideal support for partition chromatography.

Cellulose powder is available, ready for use and usually requires no further treatment; not even the addition of the stationary phase, since this is acquired from the aqueous solvent.

The mobile phase in partition chromatography may be a liquid or a gas. The partition principle is involved in separation. It is normal to choose a solvent system so that there is a considerable difference between the solvent strength parameters of the mobile and stationary phases e.g. pentane would be the optimum choice as eluent with water as stationary phase. However, solvent stripping, i.e. stripping/washing off the stationary phase from the column may result over a long period of time. This problem can be overcome by pre-saturating the eluent with the stationary phase before it contacts the packing or by placing a pre-column at the chromatographic-column inlet.

Some typical applications and separations effected with partition columns are given below:

Table 10.2: Applications and Separations with Partition Columns

Support	Stationary phase	Mobile phase	Separation
Silica gel or Kieselguhr	Water	Chloroform, butanol	Acetylated amino acid
Silica gel	Aniline	Iso-propanol/benzene	Alkenes and Cycloalkanes
Cellulose	Water	Methanol, butanol, chloroform	Phenols
Starch	Water	Propanol, hydrochloric acid	Purines
Silica gel	Water (buffered)	Chloroform/butanol	$C_2 - C_8$ fatty acid.

REVIEW QUESTIONS

1. Write the principle of adsorption and partition column chromatography.
2. Write a note on adsorbents used in column chromatography.
3. Explain the methods of column packing.
4. Write the factors affecting column efficiency.
5. Explain the types of elution.
6. Write a note on partition column chromatography.
7. What are the ideal properties of adsorbents used in column chromatography?
8. What are the ideal properties of solid support used in partition chromatography?

Chapter ... 11

THIN LAYER CHROMATOGRAPHY

Objectives:

Upon completion of this section, the student should be able to

- Understand the principle of Thin layer chromatography (TLC) technique.
- Understand the step involved in the practice of TLC technique.
- Explore various materials used in the TLC, perform TLC and describe the applications of the technique.
- Understand the concept, steps involved in practice and applications of High performance TLC.

11.1 INTRODUCTION

Thin layer chromatography (TLC) uses the principle of either adsorption or partition to accomplish the separation and purification of compounds. The separation of compounds is based on differences in solubility of compounds in the two phases. In the case of TLC, the mobile phase is a liquid solvent phase and stationary phase is a solid phase with a high surface area. The **stationary phase** normally consists of a finely divided **adsorbent**, silica (SiO_2) or alumina (Al_2O_3) powder, used in the form of a thin layer (about 0.25 mm thick) on a supporting material. The support is usually a sheet of glass or metal foil. The **mobile phase** consists of a volatile organic solvent or mixture of solvents.

TLC is often used as an analytical technique rather than a preparative method. However thicker layers (about 2 mm) and large plates with a number of spots or a stripe of sample, can be used as a preparative method. TLC is a sensitive technique; microgram (0.000001 g) quantities can be analyzed by TLC and it takes less time for an analysis (about 5-10 minutes).

In 1938, Izmailov and Shraiber described the basic principle underlying the process and used it for separation of plant extracts. Consden, Gorden and Martin (1944) started using filter papers instead of open column. Attempts were made using adsorption chromatography on impregnated filter paper and later on glass-fiber, paper coated with silicic acid or alumina. It is Stahl (1958) who is mainly credited with bringing out the work on preparing plates and separation of wide variety of compounds.

Thin layer chromatography is a simple and rapid method carried out using thin layer of adsorbents on plates. TLC not only combines the advantages of paper and column

chromatography but in certain aspects it is found to be superior to either method. The advantages are:

1. It requires little equipment.
2. It requires less (less than 1 hour) time for separation while in the case of column and paper, it requires several hours or days.
3. It is more sensitive, i.e. separation effects are usually superior to those of other methods.
4. Lower detection limit of analytical sample in TLC is approximately one decimal lower than that in paper chromatography and very small quantities of sample is sufficient for analysis.
5. Spraying with corrosive agents for identification is permissible, which is not possible in paper chromatography as cellulose gets destroyed.
6. Individual samples do not get diffused unlike paper chromatography; hence, sensitivity of detection is more.
7. The method is used for adsorption, partition, ion exchange chromatography as there is wide range of adsorbents available.
8. The components which are separated can be recovered easily by scratching the powdery coating of the plate and quantitative separation of spots or zone is possible.
9. It is possible to visualize the components for identification by UV light as the inorganic adsorbent background does not fluoresce.
10. This method can be applied to preparative separation with the aid of thicker layers of adsorbents.

11.2 PRINCIPLE OF THIN LAYER CHROMATOGRAPHY

As already stated TLC follows the principle of either adsorption or partition depending upon the stationary phase used. If solid phase is used as stationary phase then the adsorption will be the principle and if liquid coated in solid support is used then partition will be the principle. Generally, in thin-layer chromatography, the stationary phase is a polar absorbent, usually finely ground alumina or silica particles. This absorbent is coated on a glass slide or plastic sheet creating a thin layer of the particular stationary phase. Almost all mixtures of solvents can be used as the mobile phase. By manipulating the mobile phase, organic compounds can be separated.

A solution of the sample containing a mixture of compounds is applied to the layer of adsorbent, near one edge, as a small spot. When the plate comes in contact with mobile phase in a container under closed condition, the solvent, travels up the layer of adsorbent by capillary action. Depending upon the solubility and rate of migration, compounds in the mixture move up on the plate at different rates resulting in separation of the compounds.

This process of moving the compounds with the solvent is referred to as **elution** and the solvents used are **eluting solvents**. This overall procedure is referred to as "developing" the TLC plate.

11.3 TECHNIQUE

In thin layer chromatography, the separation is carried on a glass or plastic plate, which is coated with a thin uniform layer of finely divided inert adsorbent such as silica gel or alumina. The plates are activated, the solution of the sample in a volatile solvent is applied by using a capillary tube or a micropipette to a spot keeping 1-2 cm from the bottom of TLC plate. The position of the sample spot is indicated by marking an 'origin line' on the plate with the lead pencil. When the spot has dried the plate is placed vertically in a suitable tank with its lower end immersed in selected mobile phase. The solvent rises by capillary action, resolving the sample mixture into discrete spots. At the end of the run the solvent is allowed to evaporate from the plate and the separated spots are located and identified by various physical and chemical methods.

(A) Adsorbents:

In the beginning of TLC method, only few coating materials were used as adsorbents such as alumina, silica gel, Kieselguhr etc. However, currently, there is a variety of adsorbents which can be selectively utilized. While choosing the adsorbents, factors to be considered are: (a) characteristic of compounds to be separated, (b) solubility of compounds, (c) nature of substance to be separated i.e. acidic, basic, amphoteric, and (d) to see whether compound is liable to react chemically with adsorbent/solvent, or not. Besides, the two general properties that decide its application are particle size and the homogeneity as the adhesion to the support depends upon them. A particle size of 1-25 μm is preferred.

Adsorbents do not generally adhere to the glass plates satisfactorily; hence, binders like gypsum, starch are added. Gypsum (Calcium sulphate) in 10-15% w/w is widely used as binder for the plates.

(I) Inorganic Adsorbents:

Silica gel is prepared by the hydrolysis of sodium silicate to polysilicic acid which on further condensation and polymerization yields silica gel. Binder (10% w/w) is added to complement mechanical strength to the layer and enhance adhesion to the plate. Silica gel G indicates silica gel with a gypsum binder, i.e. calcium sulphate hemi-hydrate. The presence of calcium ions does not affect most of the separations. Starch is another binder, but it does not allow the use of corrosive locating agents. The silica gel commonly used in TLC studies has a mean particle size of 15 μm with a particle size range of 05–40 μm. The adsorption properties of the silica gel can be modified by incorporating substances such as bases or buffers to prepare coatings with accurately defined pH. Silica gel is most commonly used to separate amino acids, alkaloids, fatty acids, lipids, steroids, essential oils, sugars, terpenoids etc.

Alumina: Alumina (Al_2O_3) is the choice next to silica gel. It often contains sodium carbonate and bicarbonate whose presence affects its adsorptive properties. Alumina can be produced with its Retention factor: acidic, basic or neutral. Neutral alumina is principally used with organic eluent. It is suitable for use with a substance that is either liable or bound to strong alkalis. Acidic alumina is used for separation of neutral or acid materials while basic alumina is used to separate steroids, alkaloids and aromatic, and unsaturated hydrocarbons.

Kieselguhr: Kieselguhr [Diatomaceous earth] has neutral pH. It is available with or without binder. It has less capacity of resolution than alumina and silica gel.

Magnesia: Magnesia (MgO) often replaces alumina. It is finely divided to allow filtration and can be mixed with filter aid. Active magnesia is obtained by dehydration of the hydroxide.

Magnesium Silicate, Calcium Silicate: These are utilized for separation of sugars and its acetates, phenylosazones. In this, the adsorptive power increases with decreasing water content.

Others: Various other inorganic materials are utilized for TLC such aluminium silicate, bauxite, bentonite, barium sulphate, calcium carbonate, calcium hydroxide, calcium sulphate, dicalcium phosphate, Fuller's earth and zinc carbonate etc.

(II) Organic Adsorbents:

Some organic adsorbents used in TLC are:

(a) Cellulose and its Acetylates: These adsorbents are fibrous and can be used with relative advantages over paper as the flow is more even and there is less diffusion of the dissolved substances. Modified cellulose powders are used to obtain ion exchange separations in TLC and can be used with or without binder.

Cellulose contains adsorbed water which brings separation by partition mechanism. These materials are commonly used for separating hydrophilic substances like amino acids, sugars etc.

(b) Charcoal and Activated Carbon: Charcoal has the specific property of adsorbing strongly aromatic substances. Adsorptive property of activated carbon can be modified by depositing on it a film of a non-electrolyte or a fatty acid.

Others: Similarly other adsorbents such as Dextran gels, ion-exchange resins, polyamides, polyethylene powder, sucrose etc. are used for variety of separations.

Sometimes mixed adsorbents are also used. In such cases, mixture behaves either as one of the two adsorbents and second as a diluent or adsorption is shared between two adsorbents, almost linearly with the percentage composition of the mixture.

(B) Preparation of Chromatoplates:

Glass plates or flexible plates are used for spreading the adsorbent. The size used depends on the type of separation to be carried out, the type of chromatographic tank and spreading apparatus available. The standard sizes are 20 × 5 cm, 20 × 10 cm or 20 × 20 cm. The surface of the plate should be flat and without irregularities. The standard film thickness is 250 µm; thicker layers 0.5-2.00 mm are used for preparative separations.

There are various methods for the application of thin layers of adsorbent on the plates. Suspension or slurry of the coating material is used to give uniform thickness of layer throughout the length of the plate. There are four methods of applying the thin layer of the adsorbent on its support.

(a) Pouring: Slurry of finely divided and homogeneous particle size is poured on a plate and allowed to flow to cover it evenly. To ensure reproducible thickness of layer, exact quantities of adsorbent should be used.

(b) Dipping: This technique can be used for small plates by dipping the two plates at a time, back to back in slurry of adsorbent in chloroform or chloroform-methanol or other volatile solvents. It is the most convenient method for making a number of plates for rapid qualitative separations. But in this case, the exact thickness of layer is not known and evenness of the layer may not be good.

(c) Spraying: This technique is out of vogue.

(d) Spreading: All the above methods fail to give thin and uniform layers. Modern methods utilize the spreading devices for preparation of uniform thin layers on glass plates. Currently, two types of spreaders are used:

1. Moving spreader, (Fig. 11.1) and
2. Mobile spreader

A modified spreader gives the layer thickness from 0.2–02.0 mm.

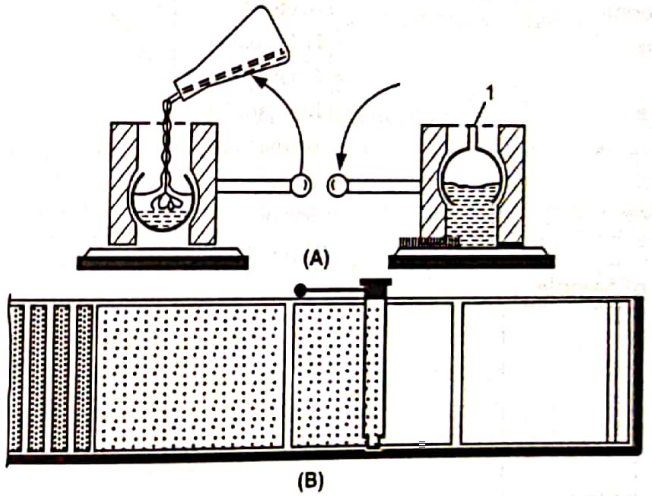


Fig. 11.1 : Moving spreader

Pre-coated plates ready for use of different adsorbents are available in uniform and optimal layer thickness for intended purposes and are abrasive-resistant. They can be sprayed even with corrosive agents.

(C) Activation of Plates:

After spreading, the plates are air-dried for 5-10 minutes; then further dried and activated by heating at about 100°C for 30 minutes. Plates made with volatile organic liquids may not require this further drying. By removing the liquids associated with layer completely, the adsorbent layer is activated. Plates may be kept for short periods in desiccator but long storage is not recommended.

(D) Solvent System:

The choice of the mobile phase depends on the same factors as that of adsorption column, i.e. the nature of substance to be separated and the adsorbent material to be used. It is preferable to use an organic solvent mixture of as much low polarity as possible. Polarity of solvent and substance to be separated plays an important role in selection. Highly polar solvents are generally avoided to minimize adsorption of any components of the solvent mixture. Use of water as a solvent is avoided as it may loosen the adhesion of a layer on a glass plate and give rise to mechanical retention factor in separation.

A good guide for choosing a solvent system is to consult "eluotrophic series". The suitable mixing gives mobile phases of intermediate eluting power. By and large, it is better to avoid mixture of more than two components for as much as complex mixtures readily undergo phase change with changes in temperature. When mixtures are used, equilibrium is to be taken care of besides the purity of solvents, which is equally important. The following solvents are commonly used:

Petroleum ether	Pyridine
Carbon tetrachloride	Acetone
Trichloroethylene	N-Propanyl
Benzene	Ethanol
Dichloromethane	Methanol
Chloroform	Formamide
Diethyl ether	Water
Diethyl formamide	Glycol
Ethyl acetate	Glycerine

(E) Application of Sample:

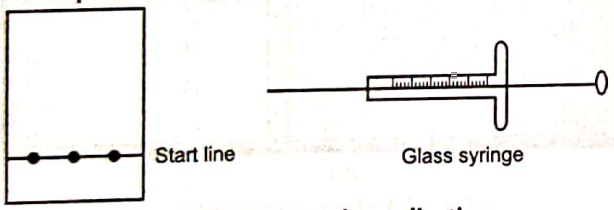


Fig. 11.2 : TLC sample application

The sample solution in a non-polar solvent is applied, which solvent has the tendency to spread out from the starting spot and in turn affect the Retention factor value. The solvent used should be relatively volatile one. The area of application should be kept as small as possible for sharper and greater resolution. For the preparative work, the sample is applied in a narrow band. The pipette, loop or syringe can be used for applying the sample.

Before the sample application, (Fig. 11.2) the starting point and finish line is usually marked. Commercial spotting plates are available for marking the starting line, finish line and for uniformly spacing of spots on the starting line. The spots should be within 2-5 mm in diameter. For the preparative work, sample up to 4 mg is applied on starting line as a streak.

(F) Development Chambers:

The TLC plate is placed vertically in a rectangular chromatography tank or chamber (Fig. 11.3). The type and size of chamber decides the success and Retention factor value. They are classified according to the separation technique used as follows:

(a) Tanks for ascending development.
(b) Tanks for descending development.
(c) Tanks for horizontal development.
(d) Tanks for thin-layer electrophoresis.

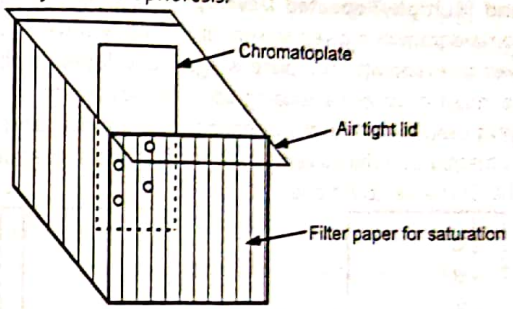


Fig. 11.3 : TLC chamber

Glass or stainless steel is most suitable for first three methods. Degree of saturation condition of tank atmosphere is one of the very important factors. If the tank is not saturated, solvent will climb up and evaporate, affecting the retention factor value. To ensure saturation, put a sheet of filter paper along the broad side of tank and dipping in the solvent mixture. The development should be carried out at room temperature in diffused daylight. Sunlight should be avoided by covering the chamber with glass plate.

(G) Development of Chromatograms:

Besides ascending method which is common, other methods used are:

(i) Ascending Development: The plates after spotting of the sample are placed in chromatography chamber containing solvent at the bottom. The flow of solvent is from bottom to top [Fig. 11.4 (a)].

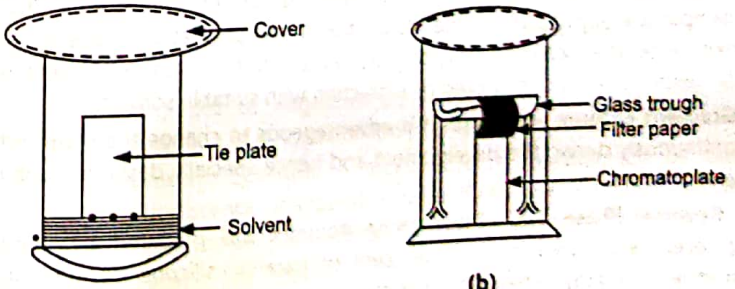
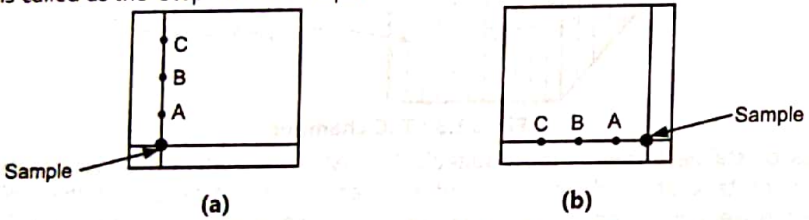


Fig. 11.4 : Ascending development

(ii) Descending: In this method, flow of the solvent from reservoir to the plate is through a filter paper strip. Solvent moves from top to bottom of the plate as illustrated in [Fig. 11.4 (b)].

(iii) Horizontal Development: In this case, loose and non-sticky layer plates are used with a of shallow dish and a ground-glass cover. The plate is supported on a T-shaped glass piece and the end of the thin layer plate is pressed against a filter paper strip of same width. This arrangement allows the solvent in the bottom of the dish to be transported up to thin layer film.

(iv) Stepwise and Multiple/Repeated Development: This technique is carried out by developing the chromatogram in a given solvent. It is then removed from the chamber and the solvent is allowed to evaporate. The plate is again developed in the same solvent. This can be repeated a number of times depending upon the separation to be achieved. Thus in this case, if the solvent used for first and subsequent runs are the same, it is called 'multiple or repeated' development. But if the solvents used for first and subsequent runs are different then it is called as the 'Stepwise' technique.



(a) (b)

Fig. 11.5: Two dimensional developments

(v) Two-Dimensional Development: If the components of the mixture are not completely separated by development in a single direction, it is possible to resolve by developing in second solvent in direction perpendicular to the first development. In this technique, the sample spot is applied at corner of plate. First development is carried out by ascending method in one solvent system. The plate is taken out, solvent allowed to evaporate and second development is carried out in another solvent system by changing the edge of the plate at 90° (Fig. 11.5).

(vi) Preparative TLC: It is an important method for preparative separations. In this method, sample is always applied in bands or streaks and separation is affected by multiple development. After the localization of spot (UV visualization) the band or streak is scrapped out and the resolved sample recovered by extraction with suitable solvent.

(vii) Gradient Elution: Sometimes it is advantageous to change the composition of the solvent continuously during the development and hence specially designed tank is used for gradient elution work.

(viii) Reverse Phase TLC: These chromatoplates are prepared by immersing the adsorbent layer very slowly in 5-10 per cent of paraffin, silicone oil, and undecane in petroleum ether or diethyl ether. After removing the plate and evaporating the solvent, the

plate is ready for chromatography. Paraffin and silicon oil provides the permanent impregnation whereas undecane can be removed after development after heating the plate at 120°C.

(H) Location of Spots:

The method to locate colourless substances is similar to those used in paper chromatography. Physical methods include the ultraviolet, fluorescence or radioactive counting. Many a time the whole plate may be made fluorescent at the beginning by inclusion of a suitable dye.

In case of chemical methods, locating agents are applied by spraying and not by dipping. During spraying, care should be taken to prevent disturbing the layers. Concentrated sulphuric acid can be used as locating agent as it produces coloured spots which are visible in daylight as well as UV light. One more locating agent for organic substances is iodine vapour. Plate is exposed to iodine vapours, by placing in closed vessel containing a few iodine crystals. In addition most of the locating agents used for paper for specific compounds are also applicable to TLC.

(I) Evaluation of the Chromatogram:

After locating the spots on plates and marking their position and size, they are evaluated either qualitatively or quantitatively.

(a) Qualitative: In this case, the retention factor value of standard or authentic sample for the same mobile phase is known and is recorded in literature. The retention factor value of the sample is calculated and on comparison of Retention factor values for known and unknown qualitative identification of sample is made.

(b) Quantitative: The methods for quantitative analysis are as follows:

1. Direct Methods:

(a) Visual Comparison: For a quick semi-quantitative analysis of the amount of components, visual comparison of spot size, intensity of spot or the combination of two with the known standard spots is made.

(b) Spot Areas and Weight Relationship: From the area of spot, amount of substance present is calculated. Usually, a linear relationship is found between the spot area and weight of the compound present in it.

(c) Spot Densitometry: After development of the chromatogram, the plate is sprayed with specific reagent and colour developed is measured directly in densitometer or spots on photographs or negatives are used in densitometer. This method is applicable for detection of all types of compounds.

(d) Direct Spectrometry: Quantitative measurements are obtained by reading the absorption or fluorescence of separated zones directly on TLC plates at wavelength of maximum absorption of substance by chromatogram spectrometer.

(e) Spectral Reflectance: The spectral reflectance of dyes adsorbed on adsorbent has been investigated as a quantitative technique for TLC.

2. Indirect Methods:

These involve the separation by TLC, recovery by the quantitative elution and subsequent estimation with suitable method; this is elution technique. In this method, the areas containing the substance after localization are marked and then scooped out with the help of vacuum cleaner without any loss. Then solute from adsorbent is eluted by simple agitation with proper solvent after removing the adsorbent. The eluate is then analyzed by any technique like Colorimetry, Spectrophotometry, Fluorimetry, Radiometry, Flame photometry etc.

R_f Values:

The R_f value is the "retardation factor" or the "ratio-to-front" value expressed as a decimal fraction.

The R_f value can be calculated as;

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}} \quad \ldots (11.1)$$

This number can be calculated for each spot observed on a TLC plate. Essentially, it describes the distance traveled by the individual components. If two spots travel the same distance or have the same R_f value then it might be concluded that the two components are the same molecule. For R_f value comparisons to be valid; however, TLC plates must be run under the same exact conditions. These conditions include the stationary phase, mobile phase, and temperature. Just as many organic molecules have the same melting point and colour, many can have the same R_f value, so identical R_f values doesn't necessarily mean identical compounds. Additional information must be obtained before this conclusion can be made. It is important to restate that this number is only significant when the same chromatographic conditions are used.

Fig. 11.6 shows a diagram of a typical TLC plate and how the distances are measured to calculate the R_f value.

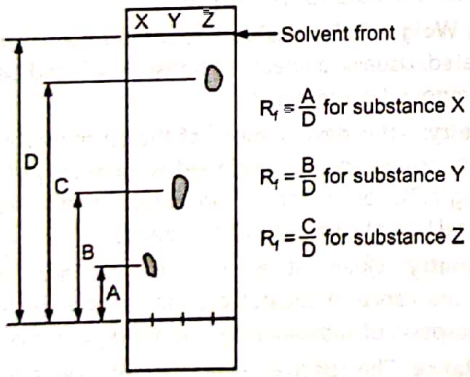


Fig. 11.6: Calculation of R_f value

R_f value is a constant for each component only under identical experimental conditions. It depends upon several factors:

(a) **Nature of Adsorbent:** Different adsorbents will give different retention factor value for same solvent. Reproducibility is only possible for given adsorbent of constant particle size and binder.

(b) **The Mobile phase:** The purity of solvents and quantity of solvent mixed should be strictly controlled.

(c) **Activity:** Rigorous control on the temperature of activation and the storage conditions of adsorbent.

(d) **Thickness of Layer:** In standard plates approximately 250 µm is preferred thickness of layer. Below 200 µm the retention factor values vary considerably. The layers may be of higher or lower thickness in individual compounds.

(e) **The Temperature:** Generally, separations should be effected at constant temperature to avoid changes in solvent composition.

(f) **Equilibration:** Equilibrium of chamber used for development is more important in TLC than in paper chromatography; hence, saturation of atmosphere with the solvent vapour is important.

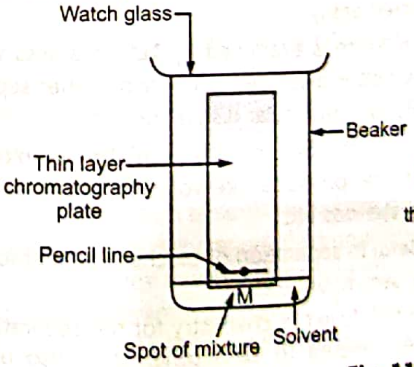
(g) **Loading:** The best results are obtained with a loading of about 10 µg per spot on 250 µm plate. If loading is more, spreading of spot and tailing may occur.

(h) **Dipping zone:** Distance of starting point from the solvent surface is also a important factor.

(i) **Chromatographic Technique:** Depending upon the technique used i.e. ascending, descending, horizontal etc. the Retention factor value will change for the same solvent system.

Diagram showing entire process of TLC:

1. Initial development



2. During development

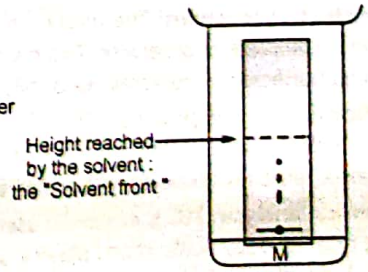
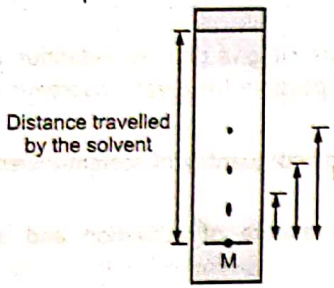


Fig. 11.7

3. After development



4. Visualisation by UV light

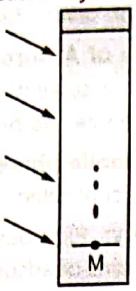


Fig. 11.8

5. Visualisation by iodine vapour method.

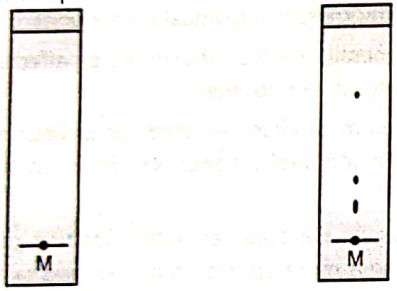


Fig. 11.9

11.4 APPLICATIONS

Thin Layer Chromatography has wide applications. TLC has applications in different field of study such as in the pharmaceutical industry, insecticides and pesticides industry, medicine and other industries. It is applied as an important tool in various ways which are as follows.

Purity of sample: Purity of sample can be carried out with TLC. Direct comparison is done between the sample and the standard or authentic sample; if any impurity is detected, then it shows extra spots and this can be detected easily.

Examination of reactions: The reaction mixture is examined by TLC to assess whether the reaction is complete or otherwise. This method is also used in checking other separation processes and purification processes like distillation, molecular distillation etc.

Identification of compounds: Thin layer chromatography can be employed in purification, isolation and identification of natural products like volatile oil or essential oil, fixed oil, waxes, terpenes, alkaloids, glycosides, steriods etc.

Biochemical analysis: TLC is extremely useful in separation of biochemical metabolite or constituent from its body fluids, blood plasma, serum, urine etc.

In chemistry: TLC methodology is increasingly used in chemistry for the separation and identification of compounds which are closely related to each other. It is also used for identification of cations and anions in inorganic chemistry.

In Pharmaceutical Industry: Various pharmacopoeias have adopted TLC technique for detection of impurity in a pharmacopoeial drug or chemical. More than 130 drugs are tested by TLC method for detecting impurity as per BP.

Various drugs like hypnotics, sedatives, anticonvulsant tranquillizers, antihistaminic, analgesics, local anaesthetics; steroidal drugs have been tested qualitatively by TLC method.

One of the most important applications of TLC is in separation of multicomponent pharmaceutical formulations.

In food and cosmetic industry, TLC method is used for separation and identification of colours, preservatives, sweetening agent, and various cosmetic products.

In Plant Extracts: It is used in identifying the compounds present in the plant extracts qualitatively and quantitatively. The TLC fingerprints are useful mean for the identification purpose

TLC assays: TLC assays are used for determining the biological activities of compounds. Such as anti oxidant, anti-fungal and antibacterial activities. For example, the compounds after separation in TLC plate are sprayed with DPPH reagent in case of antioxidant assay and the colour change is observed.

11.5 HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

High Performance Thin Layer Chromatography (HPTLC) is an advanced version of conventional Thin Layer Chromatography (TLC) in which automation plays a major role. It is also called as flat bed chromatography. Compare to TLC, HPTLC provides efficient separation. The basic principle of both TLC and HPTLC is similar. But HPTLC differs in various aspects such as wide variety of pre-coated stationary phases, automated sample application, densitometric detection using various detectors and photodocumentation. These advances make HPTLC, a superior technique when compared to TLC.

Principle:

The principle involved in HPTLC is similar to TLC. The principle follows the category of adsorption, when the stationary phase is a solid and the mobile phase is liquid; and partition, when the stationary phase is a liquid or a solid coated with liquid and mobile phase is a liquid. Separation process may follow either adsorption or partition or both depending upon the nature stationary phases and mobile phase used.

Technique:

In brief, the sample is applied on the pre coated plates coated with stationary phases using applicators. These plates will be developed in the premixed mobile phases after proper saturation in a saturation tank. When mobile phase moves up through the layer of stationary phase, against gravity, separation of compounds takes place due to differences in the affinity of various compounds towards stationary phase. Separation is possible as no similar affinity is possible for different compounds. After chromatographic development the plates are scanned densitometrically in the UV-Visible region using their appropriate wavelength. The R_f value of the compounds are calculated using the software. The R_f value of sample

compounds is compared with standards for identification of compounds. In case of quantitative analysis calibration curve method is generally used to quantify the substances from the plot of concentration versus area of peaks. The chromatogram is obtained from the computing data.

The following steps are involved in the practice of HPTLC:

- Sample and standard preparation
- Selection of stationary phases
- Selection of chromatographic layer
- Layer pre-washing
- Layer pre-conditioning
- Application of sample and standard
- Chromatographic development
- Detection of spots
- Densitometric scanning
- Documentation of chromatographs

1. Sample Preparation:

For normal phase chromatography using Silica gel/Alumina pre-coated plates, solvent generally should be non-polar and volatile type. Since, polar solvents tend to give circular shape at origin. For reversed phase chromatography, usually polar solvents are used for dissolving the sample.

2. Selection of Stationary Phase:

The stationary phase is coated either on aluminium foil or glass plates of various sizes. There are different sizes of plates are commercially available which are given below.

20 × 20 cm, 10 × 20 cm, 5 × 10 cm and 5 × 7.5 cm. Depending on the requirement plate size is decided. The edges of the plates should be uniform to obtain constant R_f values.

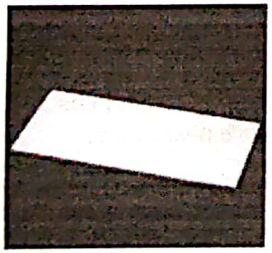


Fig. 11.10 : Precoated TLC plate

3. Selection of chromatographic layer:

The selection of layer depends on the nature of material to be separated. Commonly used materials are Silica gel 60F, Alumina, Cellulose (micrystalline), PEI impregnated cellulose etc. These materials can be coated on handmade plates with or without binders. Pre-coated

plates are commercially available and are commonly used. However, these are costlier than the handmade plates.

4. Prewashing:

Plates need to be prewashed to remove water vapours or other volatile impurities, which might get trapped in the plates. These give dirty zones and spots on the plates. To avoid this, plates are cleaned by using methanol as solvent by ascending or descending or by dripping continuous mode.

5. Pre-Conditioning:

The prewashed plates or plates exposed to humidity and surroundings are need to be activated by placing them in oven at 120°C for 15-20 minutes. This process is known as conditioning; this allows the active centers of coating materials attenuated for better separation of sample material.

6. Sample application:

It is the most important step for obtaining good resolution and results. Application of 1.0–05 µl is most satisfactory for HPTLC application of the sample and standard as a band gives better separation, equal Retention factor values, and less spot broadening. The sample application is carried out by Linomat type applicator on the plates which give uniform, safe and standard results.

7. Chamber Saturation:

This affects the effective separation of sample. For low polarity mobile phase there is no need of saturation; however, saturation is expedient for highly polar mobile phases. Partial saturation is recommended for mobile phase composition leading to phase separation. For reverse phase chromatography it is essential to saturate the chamber with methanol or polar solvent.

8. Mobile phase:

The selection of appropriate mobile phase is based on the trial and error wherein chemical properties of solute and solvent, solubility of analyte absorbent layer etc. are considered at analyst's discretion. The eluotrophic series of various solvent based on the adsorption energies can be used as a guide for selection of mobile phase composition.

9. Chromatographic development:

Various forms of chromatographic development like ascending, descending, horizontal, continuous, gradient and multidimensional, can be tried. For HPTLC plates, migration distance of 5-6 mm is sufficient. After development, plates are removed from the chamber and dried to remove traces of mobile phase. Common problems encountered during chromatographic development are as follows:

(a) Tailing: This may occur due to the presence of traces of impurities or more than one ionic species of substances under chromatography. This can be reduced by buffering the mobile phase system with acidic (1–2 per cent acetic acid) or basic (ammonia) solution. It keeps the materials to be separated in non-ionic forms. Sometimes, tailing may be due to overloading of sample plates.

(b) Diffusion: This is seen as zones on chromatographic plates. This may arise due to non-uniformity of mobile phase, longitudinal diffusion between mobile phase and stationary phase or due to non-equilibrium of stationary phase.

10. Detection of spots:

Immediately after the development process is complete, the plates are removed from the chamber and dried to remove traces of mobile phase. Generally, detection can be done by iodine vapour in iodine chamber or by visual inspection at 254 nm of ultraviolet region in UV cabinet.

11. Scanning and documentation:

Currently, HPTLC equipments are supplied with computer equipped with data recording and storing devices. The development of HPTLC plates is scanned at selected UV regions wavelength by the instrument and the detected spots are seen on computer in the form of peaks. The scanner converts band into peaks and peak height or area is related to the concentration of the substance on the spot. The peak height and area under the spot (curves) are measured by the instruments and are recorded as per cent on the printer.

Furthermore, the plates carry supplier's name, batch number, chemical code etc. on the edge of the pre-coated plates. This helps in storing the data of individual plates for further use as well as for photo documentation and storage.

Factors Influencing TLC/HPTLC separation are:

1. Type of stationary phase.
2. Layer thickness and binders in the layers.
3. Mobile phase.
4. Solvent purity.
5. Size and shape of developing chamber.
6. Amount of sample to be spotted.
7. Chamber saturation.
8. Size of spot applied.
9. Solvent level and development level in the chamber.
10. Relative humidity and temperature.
11. Development distance.
12. Mode of development.

Instrumentation:

The instrumentation of HPTLC involves the following components.

1. Plate coaters
2. Drying racks
3. Plate cutters
4. Immersion device
5. Plate heater
6. Sample applicator
7. Development chamber
8. Derivatisation devices
9. Scanning densitometer

1. Plate Coaters:

These are the devices which are used to coat the plates. The sorbents are coated on the selected plates of glass or aluminium or suitable support. The process can be done either manually or using automatic coater.

(i) Automatic Plate coater: The adsorbent suspension is filled in a hopper under which the plates to be coated are placed. A motor is used to move the plates at a uniform speed and the coating is done uniformly.

(ii) Manual coater: Using this coater, coating is done similar to the automatic coating. But instead of the motor driven mode, the plates are pushed by hand one after the other.

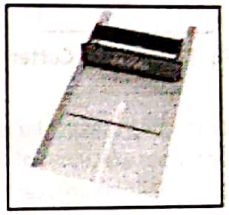


Fig. 11.11: Hand operated Plate corner

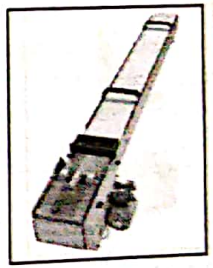


Fig. 11.12: Automatic Plate coater

2. Drying Rack:

It is used to dry the plates after coating. It consists of 10 individual aluminium trays. A tin box for storing the trays and two wire handles, to move the stack while hot, are supplied.

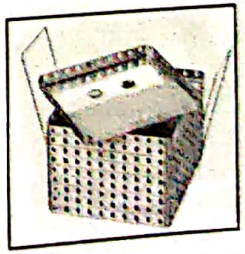


Fig. 11.13: Drying Rack

3. Plate Cutter:

Cutting of HPTLC plate is important. As the edges of the plate during manual cutting become irregular, it affects the separation efficient. Hence plate cutters are used to cut HPTLC plates easily and more precisely. It can cut plates with a thickness up to 3 mm.

It does not damage the sensitive layer. It is easy to handle.

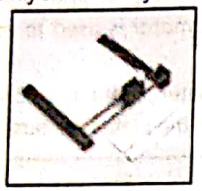


Fig. 11.14: Plate Cutter

4. Immersion device:

For dipping technique this device is more useful. The chromatogram must be immersed and withdrawn at a uniform speed for proper execution of the dipping technique. This device is designed for that purpose that it has uniform vertical speed and the immersion time is selectable between 1 and 8 seconds. The device can be set to accommodate 10 cm and 20 cm plate height.

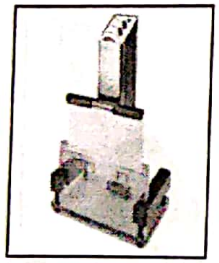


Fig. 11.15: Immersion device

5. Plate Heater:

It is used for heating TLC plates to a given temperature between 25 and 200°C. It has a heating surface which is resistant to all common reagents. It ensures the homogenous heating across the plate.

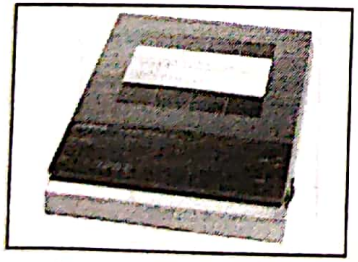


Fig. 11.16: Plate Heater

6. Sample application:

This is the critical step in HPTLC practice. Sample applicators of manual, semi-automatic and automatic system are available. Recent days manual applicators are completely replaced by either semi-automatic or automatic applicators. The semi-automatic applicators uses a micro syringes in which the sample is loaded. The parameters are set through the software system. The plates are kept at a stage provided in the applicators. When the switch is on the required volume of sample is spotted onto the plate as band.

(a) Manual Sample Applicator: The Nanomat serves for easy application of samples in the form of spots onto TLC and HPTLC layers.

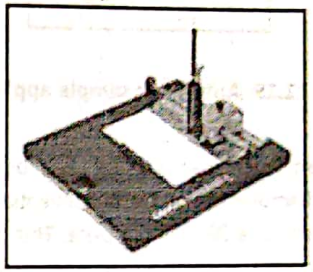


Fig. 11.17: Manual Sample Applicator

(b) Semi automatic sample applicator (Linomat): This device applies the sample through a syringe. The parameters of the sample application including size of the band, space between bands, volume of sample and speed of application can be set through the software. The applicator then apply samples in the form of fine bands on the TLC plate.

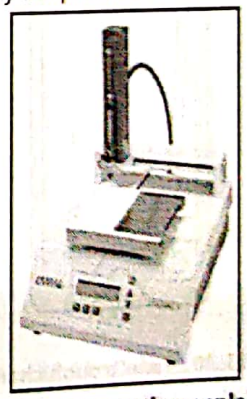


Fig. 11.18: Semi-automatic sample applicator

(c) Automatic sample applicator (ATS): Samples are either applied as spots (0.1-5 micro lit) or as bands or rectangles (0.5 - >50 micro lit). Large volume of samples can be applied as rectangles so the spreading on the plates is avoided.

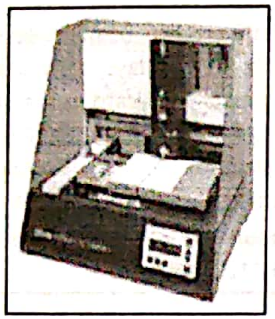


Fig. 11.19: Automatic sample applicator

7. Developing Chamber:

(a) Twin trough chamber: These chambers are used for development. The resolution is improved using twin trough chambers compared to conventional chambers. 20 mL of solvent is sufficient for the development of a 20 × 20 cm plate. This not only saves solvent, but also reduces the waste disposal problem.

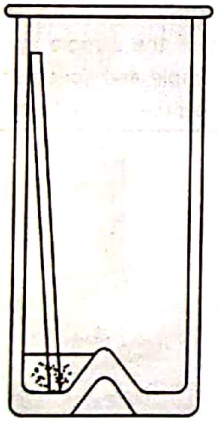


Fig. 11.20 : Twin trough chamber

- **Reproducible pre-equilibrium with solvent vapor:** For pre-equilibration, the TLC plate is placed in the empty trough opposite the trough which contains the pre-conditioning solvent. Equilibration can be performed with any liquid and for any period of time.

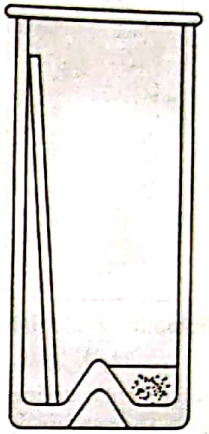


Fig. 11.21 : Pre-equilibration

- **Start of development:** It is started only when developing solvent is introduced into the trough with the plate.

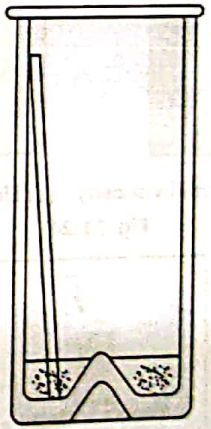


Fig. 11.22 : Development

(b) Automatic developing chamber (ADC): ADC is used for a programmed development. This is fully automatic and independent of environmental effects. The activity and pre-conditioning of the layer, chamber saturation, developing distance and final drying can be pre-set and automatically monitored by ADC.

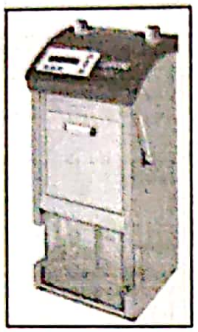
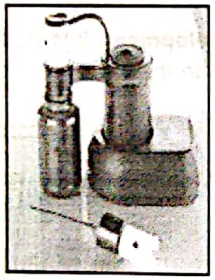


Fig. 11.23: Automatic Developing Chamber (ADC)

8. Derivatization device :

For the purpose of derivatization different devices are available. The usual methods of derivatization or Spraying and Dipping. For spraying usual spray containers are used whereas for dipping, immersion device is used which is mentioned above.



(a) Spray solutions of normal viscosity (b) Liquids of higher viscosity

Fig. 11.24

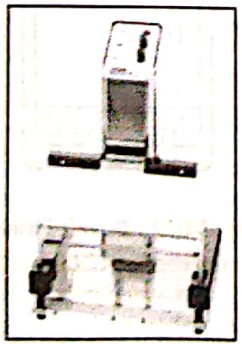


Fig. 11.25: Dipping device

9. Scanning Densitometer:

The densitometry scanner connected to a computer can be fully controlled by software. The scanner features three light sources, a deuterium lamp, a tungsten lamp and a high pressure mercury lamp. The scanning speed is selectable between 1 and 100 mm/s.

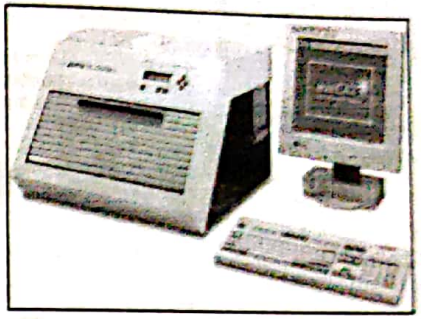


Fig. 11.26: Scanning Densitometer

It consists of the following components:

- Lamp selector
- Entrance lens slit
- Monochromator entry slit
- Grating
- Mirror
- Slit aperture disc
- Mirror
- Beam splitter
- Reference photo multiplier
- Measuring photo multiplier
- Photo diode for transmission measurements

Lamp selector is used to select the lamp. The light radiation from the selected source is passed through the entrance slit and the desired wavelength is isolated using the monochromators. The monochromatic radiation is allowed to pass through the selected slit aperture as per the size of the band applied, and then reaches the plate with the help of lens and mirrors. The radiation impinges on the spot of the plate and the absorption/reflectance is measured by the photomultiplier. The signals are amplified and converted as peaks and appear in the computing system.

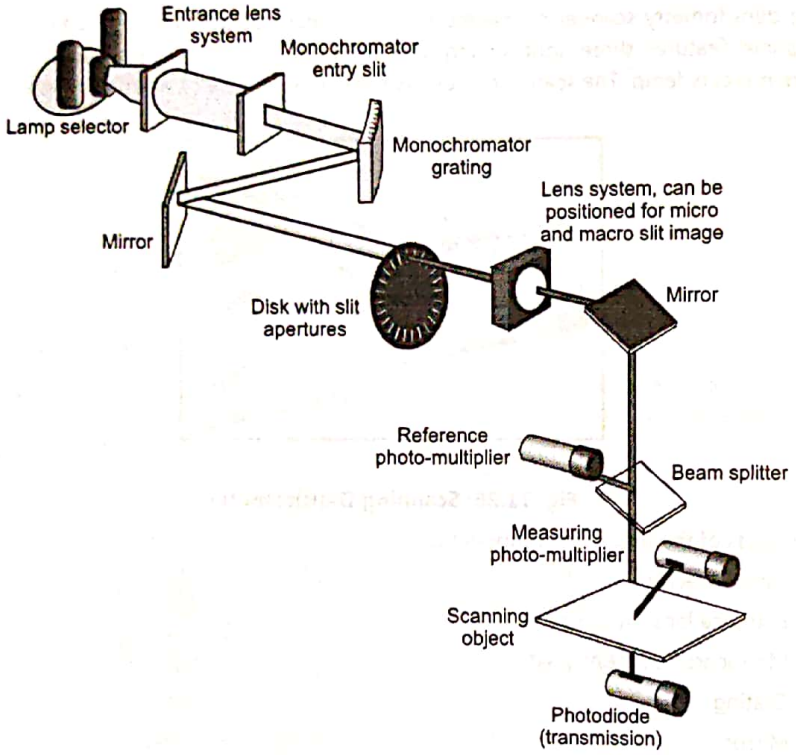


Fig. 11.27: Spectrodensitometer optical arrangements

Photo documentation:

This device is used to document the chromatographic plate after development. It is connected with computer and during scanning; the plate can be photographed at a wavelength of 254 or 366 nm and can be documented.

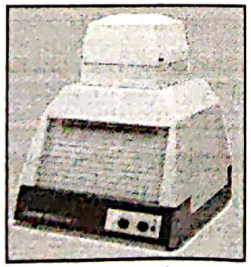


Fig. 11.28: Photo-documentation with digital camera

11.5.1 Automated Multiple Development

This is an advanced version in HPTLC. The Automated Multiple Development procedure allows thin-layer chromatography to be utilized for tasks that could not be performed by TLC in the past. In column liquid chromatography, gradient elution is common on reversed phases. In Thin-Layer chromatography this is not relevant. However AMD procedure can be successfully employed for reproducible gradient elution with silica gel as the stationary phase.

The principle of the AMD procedure:

- The HPTLC plate is developed repeatedly in the same direction.
- Each successive run extends over a longer solvent migration distance and uses a solvent of lower elution strength than the one before.
- Between runs, the solvent is completely removed from the developing chamber and the layer is dried under vacuum.
- The combination of focusing effect and gradient elution results in extremely narrow bands. Their typical peak width is about 1 mm. This means that, with the available separation distance of 80 mm, up to 40 components can be completely resolved, i.e. with base line separation.

In another version of AMD controlled by software, the gradient, made from up to five solvent bottles, is defined by software. Gradient and developing distance for each run can be shown graphically for verification. Then all individual runs of the developing program are performed fully automatic and monitored by software.

Key features:

- Multiple development and gradient elution.
- Separation power improved over regular HPTLC by a factor 3.
- Data input and monitoring through software.
- Utilizing time also after working hours.

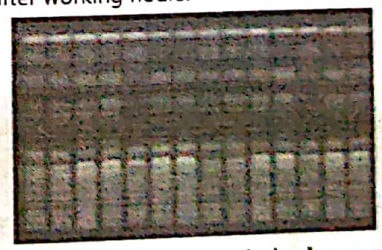


Fig. 11.29: Separation of various rhubarb samples by AMD

Example: Separation of various rhubarb samples by AMD.

Detection: UV 366 nm Mobile phase: gradient in 10 steps: methanol - dichloromethane (40:60) to (10 : 90) in nine steps, 40 mm developing distance, then one isocratic step methanol - dichloromethane (10 : 90) over 70 mm developing distance.

11.5.2 Automatic Developing Chamber

Automatic Developing Chamber is the heart of an HPTLC system. It performs the development step fully automatically, reproducibly, and independent of environmental effects. The activity and pre-conditioning of the layer, chamber saturation, developing distance and final drying can be pre-set and automatically monitored by the ADC. Two modes of operation are possible: stand-alone with input of parameters via keypad, or remote operation from win CATS with process monitoring, documentation of operating parameters, and reporting.

11.5.3 Horizontal TLC

In the Horizontal Developing Chamber, a plate can be developed in the sandwich as well as in the tank configuration. The chamber is suitable for all kinds of solvents.

In the Horizontal Developing Chamber, the HPTLC plate is developed from both opposing sides towards the middle. This permits the number of samples to be doubled as compared with development in a tank, provided the separation distance of 45 mm, i.e. 50 mm minus 5 mm distance from the edge, is sufficient.

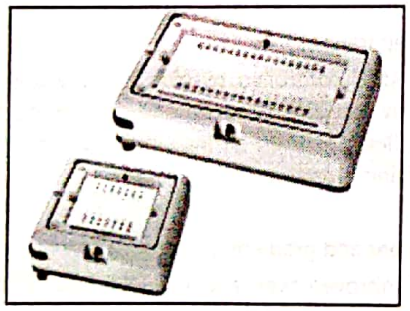


Fig. 11.30 : Horizontal TLC

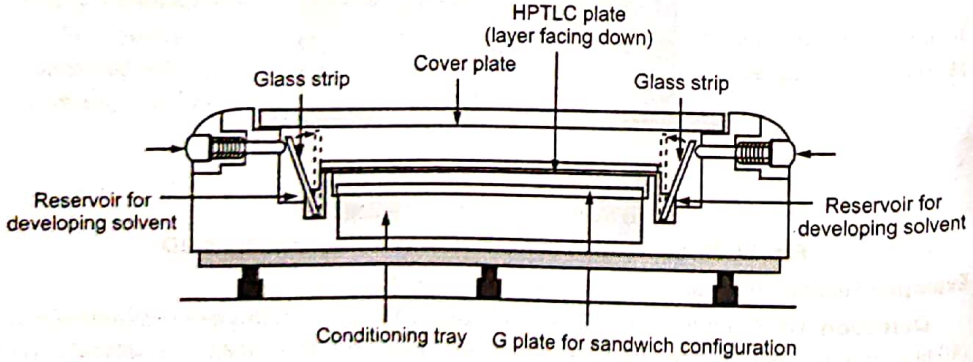


Fig. 11.31: Product Description Scheme

11.5.4 Any other Development Modes

Development with six different solvents can be tested side by side

Key features:

- Development with six different solvents can be tested side by side.
- Sandwich as well as tank configuration can be simulated side by side, making results directly comparable.
- Six different conditions of pre-equilibration, including relative humidity, can be tested simultaneously.
- These variations of developing conditions can be freely combined.

11.5.5 Applications of HPTLC

HPTLC is one of the most widely applied methods for the analysis in pharmaceutical industries, clinical chemistry, forensic chemistry, biochemistry, cosmetology, food and drug analysis, herbal analysis and environmental analysis. Some of the important areas of applications are given below.

1. **Herbal analysis:**

   HPTLC is widely used for the herbal analysis. Herbal extracts which are known to contain more chemical constituents are difficult to be analysed by other techniques. HPTLC is a reliable technique to analyse herbal extracts and poly herbal formulations. Multidimensional chromatography is often a good choice in case of very complex samples, offering many advantageous features in the analysis of medicinal plants.

   - **Fingerprint analysis:** Fingerprint analysis approach using high-performance thin-layer chromatography (HPTLC) has become the most potent tool for quality control of herbal medicines because of its simplicity and reliability. It can serve as a tool for identification, authentication, and quality control of herbal drugs.
   - **Herbal drug standardisation:** The active phytoconstituents present in the plant can be identified and quantified in the plant extracts and formulations thus useful in standardisation of herbal products. Example for herbal analysis includes analysis of Andrographolide from *Andragraphis paniculata*, Bacosides from *Bacopa monnierra* Linn, withanolides from *Withania somnifera*.

2. **Pharmaceutical Analysis:**

   HPTLC is useful in analysis of pharmaceutical drug as bulk and in formulations. Numbers of reports are available for the simultaneous analysis of drug in formulations such as tablets, capsules, ointments, creams and other formulations. Examples, Fexofenadine hydrochloride and montelukast sodium, Lamivudine and Tenofovir Disoproxil Fumarate, ofloxacin and ornidazole etc. HPTLC is also used in analyzing the purity and efficacy of many pharmaceutical preparations and dosage forms.

3. **Quality Control:**

HPTLC has been used for routine quality control of drugs in pharmaceutical formulations. Validated sensitive and highly selective stability indicating methods were reported for simultaneous quantitative determination of many drugs such as sulpiride and mebeverine hydrochloride in presence of their reported impurities.

4. **Stability studies:**

Stability indicating assay is one of the important areas of pharmaceutical analysis. The specificity is confirmed by forced degradation studies. HPTLC finds its application in this area. The stability of the active pharmaceutical Ingredient and its formulations can be conveniently studied using HPTLC. Examples: Trandolapril, Emtricitabine, Febuxostat etc.

5. **Forensic science:**

HPTLC is much useful in identifying the drugs such as Morphine in urine thus useful in forensic sciences. Evaluation of thiopental levels in the post-mortem blood by simple and rapid HPTLC method has been reported.

6. **To Identify Adulterants:**

HPTLC is useful in identifying the adulterants using the fingerprinting analysis. For example, the general practice of quality assessment of extracts of *Emblica officinalis* fruit and *Cassia angustifolia* leaves with their adulterants, *Impoea batata* and *Cassia tora*, respectively, were distinguished by the help of HPTLC fingerprint analysis.

7. **Preparative studies:**

It is a useful technique for the preparative isolation of chemical constituents from medicinal plant extracts. The 190 mm band is applied to the 20 × 10 plate and after development the desired band is scrapped to isolate the particular constituent.

8. **Bioequivalence studies:**

HPTLC is applied for Bioequivalence studies also. Example bioequivalence study on Azithromycin has been done using HPTLC.

9. **Biomarker analysis:**

HPTLC method has been used for detection, and quantification of biomarkers. Example Analysis of quercetin in *Michelia champaca* L (Magnoliaceae), and the estimated values indicate that the leaves are the richest source of the quercetin.

11.5.6 Advantages of HPTLC

HPTLC has many advantages compared to TLC, HPLC and other chromatographic techniques.

1. It assures more accuracy, precision and rotates than TLC due its involvement of automatic application, controlled developments and densitometric scanning.
2. Sample application in the form of band to improved efficiency of separation (Fig. 11.32).

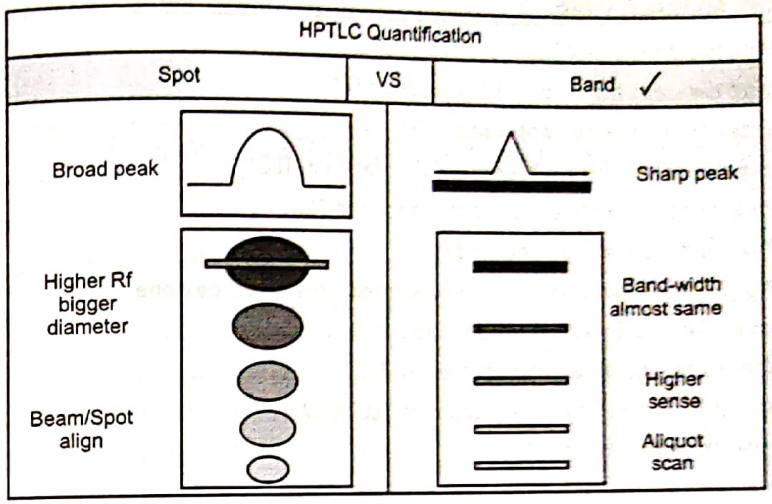


Fig. 11.32: Comparison of band and spot application

3. High through put screening makes the technique more versatile.
4. Sample requirement is very less.
5. No risk of involvement of costlier stationary phases like HPLC columns.
6. Less amount of mobile phase is required (around 10-15 ml of solvent mixture).
7. Skilled persons are not required like HPLC.
8. More suitable for analysis of complex mixtures such as plant extracts for which HPLC may need special requirements.
9. No tedious procedure is involved in practice of HPTLC.
10. Confirmation of compounds can be done using *in situ* UV spectra.

11.5.7 Limitations of HPTLC

1. It does not obey Beer's law thus the linearity should be confirmed by residual analysis.
2. Smiling effects are possible due to improper salvation thus affecting reproducibility.
3. Limitations of separation of more compounds in small plates.
4. Struggling to get incorporated in the official monographs.
5. Blockage of needle occurs when concentrated sample is used.
6. The whole setup of equipment cost is more.
7. Low sample capacity application in case of preparative HPTLC.

REVIEW QUESTIONS

1. Write the principle of TLC.
2. Write the steps involved in the practice of TLC.
3. Write a note on adsorbents used in TLC.
4. How stationary phase influence the separation in TLC?
5. Write a note on mobile phase optimization in TLC.
6. Why pre-conditioning is required?
7. Why chamber saturation is to be performed? How it can be done?
8. Explain various development chambers in TLC.
9. Write a note on visualization process in TLC.
10. What do you mean by derivatization in TLC? Explain.
11. Write the applications of TLC.
12. Explain the steps involved in the practice of HPTLC.
13. Compare TLC and HPTLC.
14. What are the advantages of band applications than spot application?
15. What is automated development chamber? Explain its functions.
16. What is R_f value? How to calculate R_f value? What is its significance?
17. Write the applications of HPTLC.

Chapter ... 12

PAPER CHROMATOGRAPHY

Objectives:

Upon completion of this section, the student should be able to

- Understand the concept, principle and technique of paper chromatography.
- Describe the method of performing paper chromatography of various types.
- Describe the materials required, reason to use those materials and to demonstrate the technique of paper chromatography.
- Describe the applications of paper chromatography.

12.1 INTRODUCTION

Paper chromatography was introduced by Martin and Synge in the late 1940s in England. Martin's research group developed this first microanalytical chromatography. It was developed to analyse the structure of proteins for which Martin and Synge received the prestigious Nobel Prize in chemistry in 1952. They applied partition chromatographic technique for the analysis of mixture of products after breaking down the fiber of wool protein which was a complex mixture. They could separate the mixture into the individual amino acid and peptide products thus established the structure of wool protein.

Paper chromatography has a wide and versatile field of applications. It is used in almost all areas to solve the complicated problems in chemistry, biology and biochemistry, etc. It has many advantages:

1. The equipment is very simple and is easily available.
2. It has high efficiency of separation.
3. Separation can be effected on macro, micro or semi-micro scale.
4. Closely related homologues, isotopes, isomers and very labile and reactive substances can be separated readily and satisfactorily.

Thus, paper chromatography is the technique wherein the separation of an unknown substance is accomplished by the flow of solvents on the specially designed chromatographic paper, the solvent goes up by capillary action and the separation is effected by differential migration of the substance due to difference in distribution coefficients.

12.2 THEORETICAL PRINCIPLE

The principle involved in the paper chromatography is partition. It means the analyte mixture is partitioned between two immiscible liquids in which one liquid is a stationary phase and another liquid is a mobile phase. Paper is made up of cellulose which consists of hydroxyl group. The structure of cellulose is shown in Fig. 12.1. The OH group in the cellulose are responsible for the hydrogen bonding of water. This set up contribute the 6% weight of the paper. The water molecules held on the cellulose fibers of the paper act as the stationary phase.

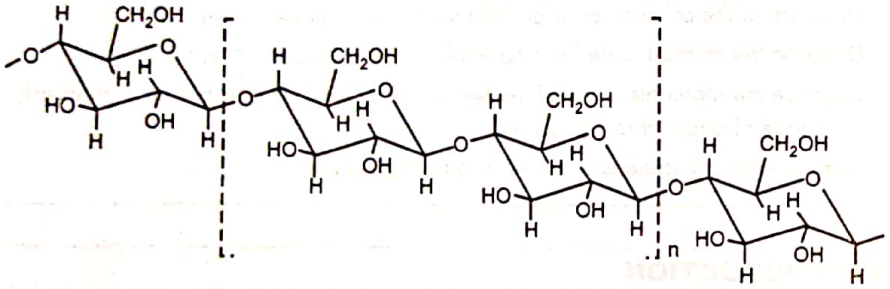


Fig. 12.1: Structure of cellulose

Thus the technique is called as liquid-liquid chromatography. When this paper is placed in a liquid which is single solvent or mixture of solvents, the solvent is pulled through the paper by capillary action. The mobile phase travels up through the paper thus carries the analytes upward according to their partition behaviour thus separation occurs. The partition coefficient, k, is the equilibrium constant for the distribution of molecules between the mobile phase and the stationary phase. It is this equilibrium that separates the components.

It is difficult to visualize theoretical plate concept in paper chromatography, it is well known that separation is achieved by successive equilibrations of sample between two phases; one of which moves over the other. The stationary phase is made up of the solvent held by the paper and mobile phase is the irrigating eluent. Both these phases are in contact over a very large interface on filter paper.

In case, water is used as stationary phase, the water cellulose complex concept is involved. The water absorbed in amorphous regions of cellulose is distinct from the bulk of mobile water. The water absorbed may be regarded as chemically bound and not as a liquid; thus various solvent systems containing water or polar solvent act as distinct stationary phase. It is to be noted that cellulose (of Paper) because of its structure can play a dual role of adsorption and partition in paper chromatography. Thus, either partition or adsorption or both may play a major role depending of course on conditions during analysis.

12.3 R_f VALUE AND VARIATION

In paper chromatography, the results are represented by R_f value, which represents the movement or migration of solute relative to the solvent front. This indicates position of migrated spots on chromatogram.

The R_f value is calculated as:

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

In other words, R indicates the fraction of the solute molecules in a solvent at any specified time. Thus, R_f is a function of partition coefficients and is a constant for a given substance, provided the conditions of chromatographic system are kept constant.

However, for greater reliability, reference standards are used in parallel runs or as internal standards. Sometimes the solvent front runs off the paper (Fig. 12.2) then the position of individual spot is measured relative to position of standard substances (Say x).

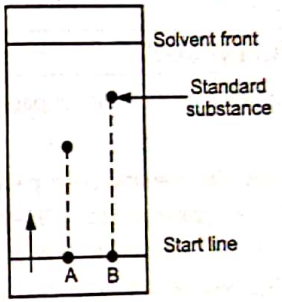


Fig. 12.2 : Diagram showing chromatographic run

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

It should be noted that R_f value is always less than unity but R_x can be greater than 1.

R_f values of two different compounds are different as other physico-chemical constants. R_f values of various compounds are recorded in literature with the mention of solvent system used. It is from the R_f value comparison identification of compounds is possible. The value varies with the solvent used; hence, it is represented with reference to the solvent.

Some of the factors which affect R_f value are:

1. The solvent system and its composition
2. Temperature
3. The quality of the paper
4. Distance through which the solvent runs
5. The quality and nature of solvents used
6. The direction of the fibres of the paper
7. The method of development.

12.4 TYPES OF PAPER CHROMATOGRAPHY

Depending upon the migration forces that are involved in separation, paper chromatography is classified into two types:

1. Paper Partition Chromatography:

In this standard method of analysis, the paper is utilized as a support with one solvent as mobile phase and other as a stationary phase. The migration of substances is due to differences in partition coefficients.

2. Paper Adsorption Chromatography:

In some cases, the paper to be used is coated or impregnated with adsorbents like silica gel or alumina. Thus, the modified paper is used as an adsorbent and the solvent is allowed to flow over the unknown components. Thus, migration of substances is due to the difference in adsorptive powers of substances to be separated. However, as this technique is not much used, it is only of academic interest.

12.5 OPERATIONAL TECHNIQUE

The following points should be taken into account in paper chromatography.

1. Choice of Filter Paper:

Chromatography paper is a specially manufactured paper. Whatman filter papers are used extensively. In general, this filter paper contains 98–99 per cent of α-cellulose. The mineral content may vary from 0.07–0.01 per cent. Besides this, β-cellulose, ether soluble matter, ammonia and Lyophillic substances (waxes, fats, etc.) are also present. Chromatographic papers are available in packs of 100 and 500 sheets of 46 × 57 cm or 58 × 68 cm and are cut to required size. The rectangular or square papers are cut from the sheet for separation of substances. There are various grades and types of paper available for separation of a sample. The proper choice of paper depends upon the sample and solvent system used. Another important factor that governs the choice of paper is whether the paper is to be used for quantitative, qualitative or preparative chromatographic analysis. The choice of paper is also based on the thickness, flow rate, purity and net strength.

To speed up chromatographic analysis, coarser and faster papers are used, i.e. Whatman paper number 31 is about four times faster than Whatman paper number 1. Slow papers are used rarely but they are important for separation of substances having close R_f value.

2. Modified Filter Papers:

For efficient separation of certain substances, specially treated or modified filter papers are used, i.e. buffered or treated papers like Whatman -Phosphate, Whatman -citrate or paper treated with alumina, silicic acid etc. In case of reversed phase chromatography, paper is impregnated in mobile phase (non-polar solvent), dried and then used.

Following section details the types of chromatographic papers.

Types of Chromatographic Papers:

Whatman chromatography papers:

Whatman chromatography papers are widely used across the world, reflecting their purity, high quality and consistency. They are made from specially selected cotton cellulose and rigorously quality controlled to ensure uniformity within the grade. These papers are manufactured and tested specifically for chromatographic techniques to ensure the wicking capability and uniformity of capillary action that is important in obtaining clean and even transfers during blotting.

Whatman cellulose filters are manufactured from high-quality cotton linters which have been treated to achieve a minimum alpha cellulose content of 98%. Cellulose chromatography papers are available in different grades with different thickness and flow rate characteristics, optimized for specific application. The grades come in both sheet and roll formats. E.g. Grade 1, 2, 3, 4, 17, 20 etc. Some papers were available in slow, standard and fast grades with the speed of development controlled by the coarseness of the cellulose fibres and the packing density. In general, the standard papers gives the best results for rapid analysis. Fast papers are more suitable for simple separations and the slow papers used where the greatest resolution is required.

Whatman polypropylene membrane filters are ideal for numerous applications in chromatography and biotechnology laboratories. They come in a range of diameters and pore sizes from 0.2 µm to 1.0 µm.

Ion exchange papers for ion exchange chromatography are available in different grades. These papers come in a variety of different diameter circles and sheet formats. Some of the commercially available papers are given below.

(a) DE81 weak anion paper: It is a thin (0.20 mm) DEAE cellulose paper – a weakly basic anion exchanger with diethylaminoethyl functional groups. The ion exchange capacity is 1.7 µeq/cm^2 and wicking flow rate is 95 mm/30 min. It is used for reverse transcriptase assays and DNA polymerase.

(b) P81 strong cation paper: It is a 0.23 mm cellulose phosphate paper with a strong cation exchanger for use with protein kinase assays with peptide substrates.

(c) SG81 non-charged silica gel cellulose paper: SG81 is a 0.27 mm non-charged paper combining cellulose and large pore silica gel that is used for separation of phospholipids, steroids, phenols, and dyes.

In addition, acetylated or benzoylated papers, silicone oil-impregnated papers, as well as silica and alumina impregnated papers are also used.

In preparative applications more specialized materials such as Whatman No. 3 MM and 31ET or Schleicher and Schull 2071 are used.

3. Preparation of Paper:

Once the type of paper is decided, it is cut in desired size and shape depending upon the work to be carried out. Generally, rectangular and square shapes are used. After noting the direction of run on paper, start line is marked as shown in Fig. 12.2.

While storing, paper should be kept away from any fume (especially ammonia) and should not be subjected to large changes in humidity.

4. Preparation of Sample:

The mixture to be separated is applied to the paper as a solution. It is important to choose proper solvent for making solution. Generally, a weighed amount of mixture is dissolved in volatile solvent and a minimum volume of concentrated solution is applied on the paper avoiding diffusion of spot. Extracts from soil, biological cells or tissue materials are taken out with the help of some solvent which is directly applied on the paper.

Aqueous biological extracts, urine, neutralized protein hydrolyzates and other solutions which may have to be examined for amino acids and sugars will always contain appreciable amounts of inorganic material. Removal of these is called "desalting" and should always be carried out without affecting organic compounds. Desalting is carried out by electrolytic method, electro-dialysis, ion-exchange membrane, column techniques etc.

No standard procedure for preparation of samples is exemplified, as several factors affect sample volumes of 10–20 μl containing as many μg of substance is spotted.

5. Application of the Sample:

The starting line is marked on the paper with an ordinary pencil some 5 cm from the bottom edge. On the starting line marks are made about 2 cm apart from each other. Micropipette or glass capillary or platinum loop is used for application of sample. The sample may be applied as spots or bands. Generally, size of spot should be as small as possible. Diameter of spot should not exceed 5 mm. The quantity of sample applied to the paper is important rather than volume. When solution is very dilute, it can be concentrated on paper by applying a series of drops to the same spot, allowing each drop to evaporate before applying the next. The micro-syringe is also used for application of sample of proper size. For quantitative work the sample is applied by capillary in the form of narrow strip 5-10 mm long, 25 mm apart or as a single spot at least 10 mm apart. Amount of sample applied to the paper depends on the capacity of solvent. Optimum concentration required for quantitative separation, time required for development etc. vary considerably.

Drying of the spotted chromatogram should be carried out carefully in air. Hot air is not advisable particularly for acid solution as it may cause blackening of paper.

6. Solvents:

A number of solvents can be used in the paper chromatography. The selection of proper solvent depends mainly on nature of substance to be separated. Factors which affect the selection are viscosity, surface tension, polarity etc.

In general, one-phase system is used for development in paper chromatography, avoiding the two-phase system. The solvent should be inexpensive and very pure.

The solvents used in paper chromatography are given in increasing order of polarity as under:

n - Hexane	Ethyl acetate
Cyclohexane	n-butanol
Carbon tetrachloride	n-propanyl
Benzene	Acetone
Toluene	Ethanol
Trichloroethylene	Methanol
Diethyl ether	Water
Chloroform	

If pure solvent is not satisfactory, solvent system of suitable polarity is obtained by trying out mixture of solvents.

Some well-known solvent systems recorded in standard reference books are:

7. Chromatographic Tank or Chambers:

The chromatographic tanks are made from many materials like glass, plastic or stainless steel. Glass tanks are preferred and are most commonly used. They are available in various sizes depending upon the length and breadth of paper and type of development. The chambers or tanks have a lid with a hole (closed during development) for introducing solvent through it. Equilibrium of chamber with solvent is carried out by using solvent-dipped filter paper.

8. Development of Chromatogram:

For proper development following points are taken into consideration:

1. Sufficient amount of solvent should be present in the chamber.
2. During development, paper should be freely suspended and should be vertical.
3. Large temperature changes and exposure to light should be avoided.
4. The atmosphere of the chamber should be saturated with the solvent's vapours.

The paper is so dipped in the solvent that the spots will not dip completely into the solvent. The solvent will run over the paper by capillary action. It is allowed to run maximum distance not exceeding two-third of total height of paper for better and efficient resolution. After development is complete, paper is taken out of the chamber carefully.

9. Drying of Chromatogram:

The wet chromatogram after development is dried in special cabinet heated electrically with temperature controls. The drying can also be carried out by transferring chromatograms to racks and putting in drying cabinets. They are dried by cold or hot air depending on volatility of solvents. A simple hairdryer is a convenient device to dry chromatograms.

10. Location of Spots:

Once the developed chromatogram is dried, next step is to locate the spots. If the substances are coloured, they are visually detected easily, but for colourless substances, various methods are used:

1. Physical and
2. Chemical methods.

1. **Physical Methods:** Physical methods have the advantage that substances on paper are not converted into other compounds and can be recovered for further studies.
2. **Chemical Methods:** In this, chemical treatment is used to develop a colour. Chromatograms are exposed to vapours or gases of chemicals or sprayed with reagent. The chemicals and reagents so used are called "locating agents".

Various colouring or locating agents are available and used depending upon the chemical nature of substance under examination. For spraying a reagent glass atomizers are used (Fig. 12.3).

Physical methods used are observation under UV light, detection of fluorescence and radioisotope (for radioactive compounds) measurements.

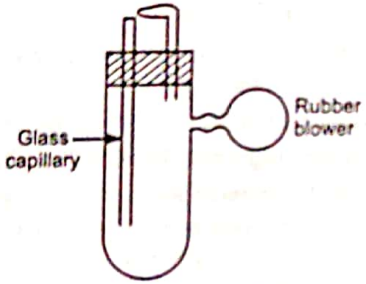


Fig. 12.3 : Glass atomizer

After spraying, the paper is allowed to dry (in air), or heated at specified temperature. The spots developed are marked by pencil; the centres determined and R_f values are calculated.

12.6 QUANTITATIVE ANALYSIS

In general, for qualitative analysis the simple measurement of R_f value either by the comparison with the reference substances or with the standard values in literature is done. But for quantitative use the technique requires not only a quantitative separation but also quantitative location and evaluation of the substance present. The quantitative method can be either by estimation of the amount of the substance in the spot on the paper or by removal of the substance from the paper and analysis of the separate fractions by conventional quantitative techniques. All the available methods can be divided into two main groups:

1. Evaluation of substance on the paper directly.
2. Removal of substance from the paper [Elution method].

The proper choice of method depends mainly upon the factors like physical and chemical properties of the compound, composition and capacity of the substance and degree of desired accuracy.

(a) Evaluation of Substance on Paper (Direct measurement methods):

(i) Visual Comparison of Spots: In this, a number of chromatograms are run on same sheet with the reference solution containing known amount of substance. Several reference solutions containing different concentrations are needed and the spot is compared for its size and intensity of colour by fluorescence or UV absorbance. The unknown compound is estimated by comparison.

(ii) Measurement of Area of Spot: When the outlines of the spot or zones are well defined, the size of the spot may serve for determining the quantity of substance. Frequently, a linear relationship is obtained between the logarithms of the quantity and area of the spot. The area can be measured with a planimeter or a graph paper.

(iii) Radiotracer Analysis: The radioactive element, which is labelled, is used to locate/determine the quantity of material on the chromatogram. The compound on chromatogram is identified by subjecting to neutron bombardment. The activity and location are measured by passing the paper either in a gas flow counting chamber or a thin window Geiger Muller tube.

(iv) Removal of Substance from the Paper (Elution Method): For quantitative analysis the substance is removed by elution from paper. The simplest procedure is to cut out the appropriate part of filter paper and soak it in optimal amount of the solvent. Extractions may be hastened by shaking or by warming. The eluates so obtained from the chromatogram may be diluted or concentrated and then analysed by any suitable technique.

12.7 DEVELOPMENT TECHNIQUES

There are various development methods which can be used in paper chromatography. The proper choice mainly depends upon the class of compounds to be investigated. They are of following types:

(a) Descending Chromatography: The apparatus consists of a well-sealed glass tank of suitable size and shape provided with a trough for the mobile phase in the upper portion. The paper with the sample spotted is inserted in trough containing the mobile phase, the jar itself having been equilibrated with the mobile phase prior to elution [Fig. 12.4 (b)]. Since, the movement of mobile phase is descending; it is referred as descending chromatography. The advantage of this is that the development can be continued even though the solvent runs off at the other end of the paper.

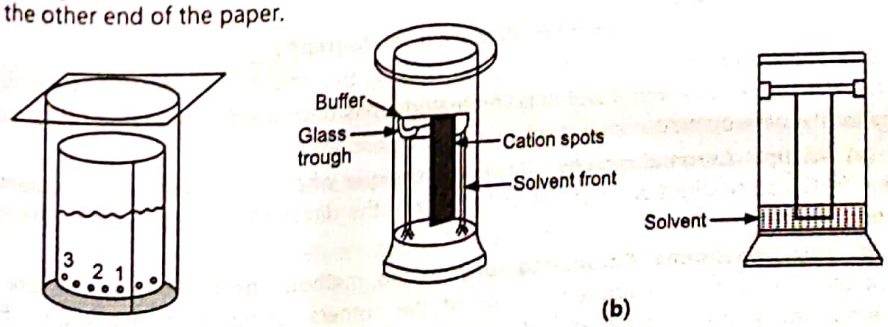


Fig. 12.4 : Descending chromatography

(b) Ascending Chromatography: In this case, the solvent stream moves upwards. The mobile phase is placed in suitable container at the bottom of the chamber or in the chamber itself. The samples are applied a few centimeters from the bottom edge of the paper suspended from a hook [Fig. 12.4 (a)]. The paper may be rolled into a cylinder, held together by staples or plastic clips.

(c) Ascending-Descending Chromatography: A hybrid of the above two techniques is called ascending-descending chromatography. Here the upper part of the ascending chromatogram can be folded over a glass to change over into the descending after crossing the glass rod (Fig. 12.5).

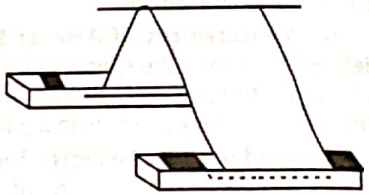


Fig. 12.5 : Ascending and descending chromatography

(d) Radial or Disk Chromatography: This is rarely used in extraordinary cases. In this case, a circular piece of paper is taken which has a wick cut parallel to the radius from the edge to the centre. The sample is deposited at the centre of the paper and at the upper end of the wick. The paper is then laid on the edge of a circular disk with the wick dipping into the solvent at the bottom of the dish, as shown in Fig. 12.6.

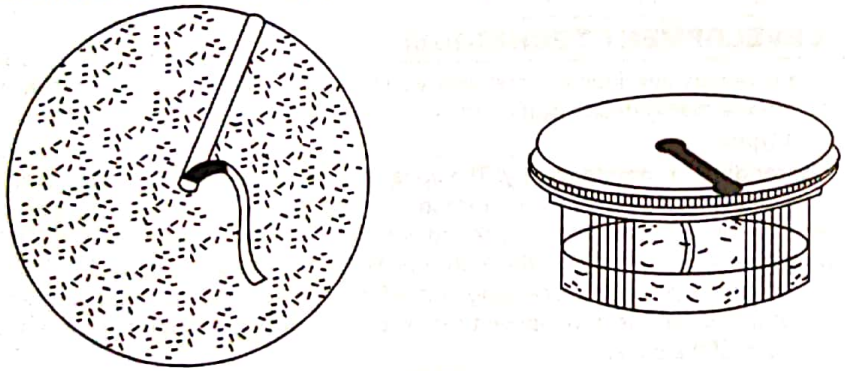


Fig. 12.6 : Radial chromatography

The liquid ascends by the wick and flows radically through the paper. While moving, it carries the compounds with it and thus chromatogram is developed. However, it takes more time for the development.

(e) Multiple Chromatography: This is a technique wherein the irrigation is repeated either in the same direction as in the first run or in the direction perpendicular to the first system.

(f) Two-Dimensional Chromatography: In this method, the paper is cut/square or rectangle. The sample is applied to one of the corners. Using a solvent system first development is carried as per ascending method. The paper is taken out and dried. The

second development is performed at the right angle to the direction of the first run using another solvent system. The larger the area of paper, more complete the resolution of complex mixtures.

Applications of Paper Chromatography:

1. Among all the chromatography methods, paper chromatography is an inexpensive and rapid method that provides reliable results. It is used as a qualitative method for identifying the components in a mixture.
2. It is widely used for the separation of amino acids. In addition to qualitative analysis, the quantitative estimation of amino acids such as glutamic acid, aspartic acid, glycine, and alanine can be performed.
3. It has been proven in analysis of substances including proteins, peptides, amino acids, poly-, oligo-, di- and monosaccharides, natural products, sterols, steroids, bile acids, pigment, dyes and inorganic species.
4. Separated compounds can be recovered by cutting the spot, redissolving in suitable solvent and evaporating the solvent.
5. Used in several scientific studies in identification of unknown organic and inorganic compounds from a mixture.
6. Phenolic substances such as anthocyanins, flavones and other polyphenolic substances can be separated and identified.
7. In forensic studies paper chromatography is used in crime scene investigation and DNA and RNA sequencing along with other studies.
8. Paper chromatography is used as an analytical chemistry technique for identifying and separating colored mixtures like pigments.
9. Sugars, amino acids, lipids and nucleic acids and other biomolecules can be easily identified by spraying with appropriate reagents to detect these specific compounds.
10. It has been used for the taxonomical and genetic study example, Taxonomic study in the molluscan genus *lymnaea*.
11. It is used for the study of structures of steroids.

REVIEW QUESTIONS

1. Paper chromatography is a liquid-liquid chromatography. Justify.
2. Write a note on stationary phases used in paper chromatography.
3. Explain the various development modes in paper chromatography with suitable diagram.
4. What is circular paper chromatography? Explain.
5. Write the applications of paper chromatography

Chapter ... 13

ELECTROPHORESIS

Objectives:

Upon completion of this section, the student should be able to

- Understand the basic concept of electrophoretic separation.
- Describe the technique of electrophoresis and its various types.
- Explain the instrumentation involved in electrophoresis.
- Describe the applications of electrophoresis technique.

13.1 INTRODUCTION

Separation of charged particles in a solution was effectively demonstrated under the influence of electric field supplied externally through a process called Electrophoresis. The separation in electrophoresis is based on differential migration rates of charged particles within an electric field. This process was established by Tiselius in the 1930s, for which Nobel Prize was awarded to Tiselius in 1948. In 1981 Jorgenson and Lukacs reported an electrophoretic separation technique within open, fused-silica capillaries rather than the typical application of electrophoresis to protein separation within gel slabs; a remarkable aspect of their work was the outstanding separation efficiency and fast separation times.

When a potential difference is applied between the two electrodes in a colloidal solution, it has been observed that the colloidal particles are carried to either the positive or the negative electrode. In other words, they behave as if they have electrical charge present within them with respect to the dispersion medium. This phenomenon is known as electrophoresis and may be defined as the migration of the colloidal particles through a solution under the influence of the electric field.

13.2 THEORY

The basic theory of electrophoresis can be explained as follows. Electrophoresis is a physical method of analysis which involves separation of the compounds that are capable of acquiring electric charge in conducting electrodes. The basic principle of electrophoresis involves the tendency of ions with positive or negative charges that are suspended between two electrodes to travel towards the electrodes of opposite charges.

The rate of migration of particle depends upon the following factors:

(a) Characteristics of the particle

(b) Net charge

(c) Charge/mass ratio

(d) Nature of the suspended medium

(e) Molecular shape

(f) Temperature

(g) Porosity and viscosity of the matrix

If a mixture of electrically charged molecules is placed in an electric field of field strength E, charged molecules move towards the electrode of opposite charge due to the phenomenon of electrostatic attraction. However, different molecules will move at quite different and individual rates depending on the physical characteristics of the molecule and on experimental system used. The rate of migration of molecule or the velocity of movement,

$$v = \frac{(E \times q)}{f} \quad \text{... (13.1)}$$

Where;

E = Applied voltage

q = Net charge on the molecule

f = Friction of the molecule

Rate of migration of a molecule increases with increased applied voltage and increased net charge on the molecule. The net charge is determined by the number of positive and negative charges in the molecule. Conversely, the mobility of a molecule decreases with increased molecular friction. The frictional coefficient describes frictional resistance to mobility and depends on a number of factors such as mass of the molecule, its degree of compactness, buffer viscosity and the porosity of the matrix. Molecules of similar net charge separate due to differences in frictional coefficient while molecules of similar mass/shape may differ widely from each other in net charge. Consequently, it is often possible to achieve very high resolution separation by electrophoresis.

Current (e.g., mA) is often used to define the voltage requirements of an Electrophoretic separation as voltage is the function of current. Heating of the separation matrix must be controlled since electrophoretic mobility is also a function of temperature. When larger molecules are to be separated then molecular size may prove to be the most important determinant of mobility. If the charge to mass ratio on the macromolecules being separated is approximately equal molecular size becomes the sole determinant of electrophoretic mobility.

Instrumentation:

The genereal components of instrumentation of electrophoresis consists of electrophoretic chamber which is usually the chamber that contains the electrolytic solution, electrodes, source for electric potential and diffusion barriers. The instrumentation is discussed in detail for individual techniques in the further sections.

Various Types of Developments:

Techniques Employed in Electrophoresis:

1. Zone Electrophoresis
   (a) Paper Electrophoresis
   (b) Gel Electrophoresis
   (c) Thin Layer Electrophoresis
   (d) Cellulose acetate Electrophoresis
2. Moving Boundary Electrophoresis
   (a) Capillary Electrophoresis
   (b) Isotachophoresis
   (c) Isoelectric Focusing
   (d) Immuno Electrophoresis

13.3 ZONE ELECTROPHORESIS

In zone electrophoresis technique charged particles migrate in supporting media. Different supporting media are used which include paper, starch gel, cellulose acetate membrane and polyacrylamide. Depending upon the supporting media used, there are different types of zone electrophoresis techniques. The migrated particles are distributed into discrete zone on the support media. These components are then easily isolated and analysed further.

In brief, the process involved in zone electrophoresis is explained as follows. Prior to the application of sample the supporting media is saturated with buffer solution. Thus, the interference of media is prevented with the process of separation. The ends of the media are connected with electrodes through a connecting bridge. The sample is applied as narrow band across the media block midway between the ends. To get a good result of separation, the sample is applied in limited amount and as a narrow band. When current is passed, components present in the sample band migrate with velocities that depend on their electric charge, molecular shape and size. The charged particles moved towards electrodes of opposite charges. This result in a series of separated bands or zones of sample components.

Advantages:

1. Requirement of sample is less.
2. Largely used in clinical chemistry and biochemistry for separation of amino acids and proteins.
3. Inexpensive technique and easy to maintain.

Disadvantages:

1. Unsuitable for accurate mobility and isoelectric point determination.
2. Interferences of supporting medium, such as capillary flow, electro osmosis, adsorption and molecular sieving may lead to poor resolution.

Instrumentation:

1. **Electrophoretic chamber:** Electrophoretic chamber basically denotes the two chambers containing the electrolyte solution where the potential difference can be applied.
2. **Electrodes:** Electrodes consists of two conducting rods applied at two electrophoretic chamber in order to apply the potential difference across them.
3. **Diffusion barriers :** Diffusion barriers are the components used in supporting media in which it assists in the distinct segregation of the components and rectify the results in the more precise way.
4. **Supporting/Stabilizing media (inert to sample and any developing reagents):** Supporting media is the major platform for the separation and during the process, they are basically saturated with buffer in order to avoid the separation errors.

13.3.1 Paper Electrophoresis

In this technique paper or cellulose is used as the separation media or the supporting media and hence the technique is termed as paper electrophoresis. It is commonly used technique for separation of small molecule and this technique is not recommended for separation of large molecules. The reason is the two main properties, adsorption and surface tension influence the result of separation in paper electrophoresis. In case of macromolecules such as protein, the technique results in poor resolution. Sample application in paper electrophoresis can be done in two ways namely Dry application and Wet application.

Dry application:

In dry application, a sample of solutes which is dissolved in distilled water or a volatile buffer is applied on the paper as small spot. Then the paper is dried for evaporation of solvents. Now the paper is dampened with the electrophoresis buffer. Dampening is done by dipping or spraying and blotting the ends of the paper so that wetting of the paper from both ends meets at the origin line where the sample is spotted simultaneously.

Wet application:

In this method papers are initially dampened with electrophoresis buffer. Then the, samples are applied to paper.

The paper electrophoresis technique can be carried out in the following ways:

1. Horizontal Paper Electrophoresis (Horizontal technique).
2. Vertical Paper Electrophoresis (Vertical technique).
3. Continuous Electrophoresis.

Vertical technique whereby separation is supported by gravity too. The two methods of separation are shown in the given figures.

1. Horizontal Paper Electrophoresis:

In this technique, the paper strip is placed in horizontal position as shown in the figure. Both ends of the paper strip is connected with the electrophoresis buffer solutions

separately. The separation is bound within the filter paper strip. When potential difference is applied across the two ends, components of the mixture get separated on the basis of their electrophoretic ability.

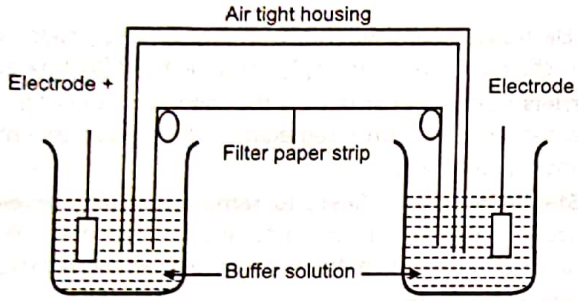


Fig. 13.1: Horizontal paper electrophoresis

2. Vertical Paper Electrophoresis:

This technique slightly varies from the horizontal technique. The variation is the arrangement of filter paper strip. The sample application point is in the midpoint of the paper strip, In addition to the electrophoretic affinity of the component the separation is achieved by the aid of the gravity also. Compare to horizontal technique, the separation efficiency is slightly enhanced in this technique.

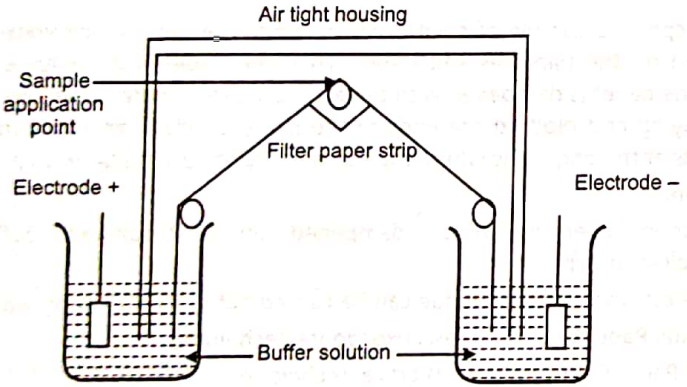


Fig. 13.2: Vertical paper electrophoresis

Filter papers such as No. 1 and No. 3 mm in strip of 3 or 5 cm wide have been used to good effect. Separation takes place in 12 to 14 hours in both cases.

Advantages:

1. Simplicity of the technique
2. Handling and maintenance is quite easy.

Disadvantages:

1. There may be possibilities of tailing and distortion of the component bands.
2. Compounds that have adsorptive and ionogenic properties cannot be separated.

3. Continuous Electrophoresis:

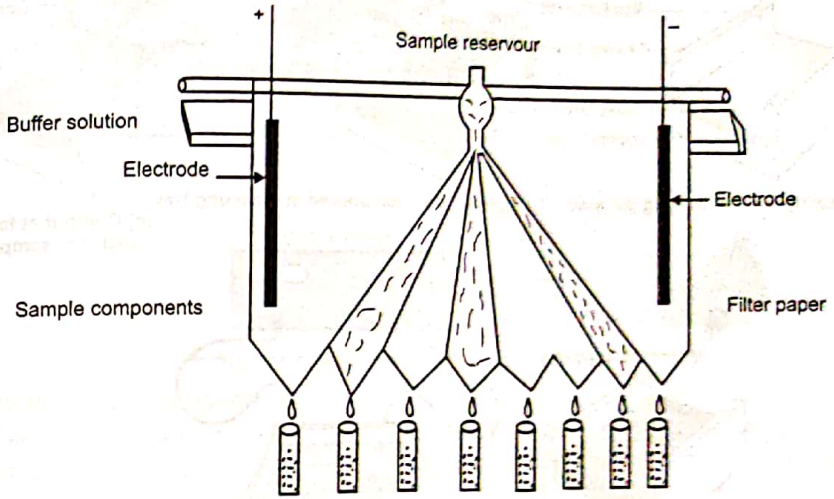


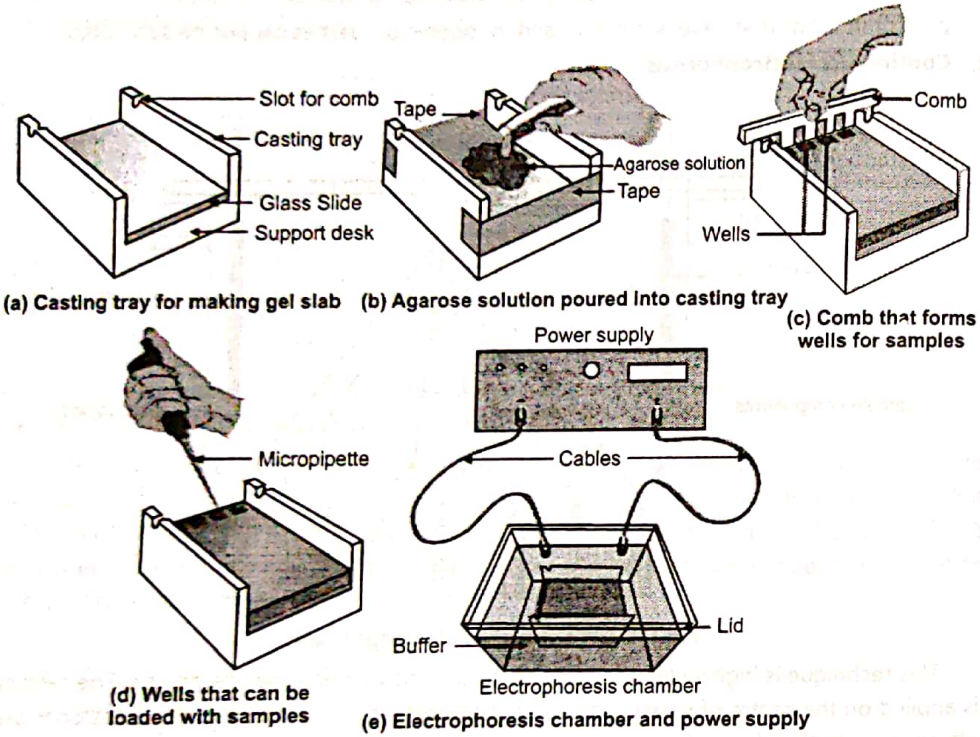
Fig. 13.3: Continuous electrophoresis

This technique is highly efficient compare to the above mentioned technique. The sample is applied on the centre of paper but in a continuous fashion from a reservoir. Electrodes are attached on both sides and when the potential is applied (about 500 V) the sample mixture gets separated. The separated compounds are collected in containers, solvent is evaporated to obtain pure fractions which can be reused. Finally the paper is stained to locate the components. This method of continuous electrophoresis is used for preparative scale purpose.

13.3.2 Gel Electrophoresis

Gel electrophoresis is a separation technique which uses the gel as a separating pocket. Molecules are separated in aqueous buffers supported within a polymeric gel matrix. Based on the molecular size of the substances molecular sieving technique is employed to facilitate the separation. Molecular sieving technique is the one in which electrophoretic mobility and migration of solutes is purely depends on viscosity and pore size. Hence, the molecular weight decides the migration of macromolecules in the system. The macromolecules are retarded or obstructed in the colloidal Gel material which is in a solid form (99% is water) and smaller molecules are allowed to migrate freely. The support media is electrically neutral. Examples for gels include Agar and Agarose gel, Starch, Sephadex, and Polyacrylamide gels.

Instrumentation:



(a) Casting tray for making gel slab (b) Agarose solution poured into casting tray (c) Comb that forms wells for samples

(d) Wells that can be loaded with samples (e) Electrophoresis chamber and power supply

Fig. 13.4: Gel electrophoresis

Gel Electrophoresis is carried out in two methods:

(i) Horizontal starch gel electrophoresis

(ii) Vertical starch gel electrophoresis

(iii) Polyacrylamide Gel Electrophoresis (PAGE)

(i) Horizontal Gel Electrophoresis:

The technique behind the horizontal gel electrophoresis is same as that of the horizontal paper electrophoresis with the difference of the use of gel in the separation media or the supporting media. The gel acts as the pocket in which the components with the smaller molecular size are trapped and it becomes easy to separate some of the specific components.

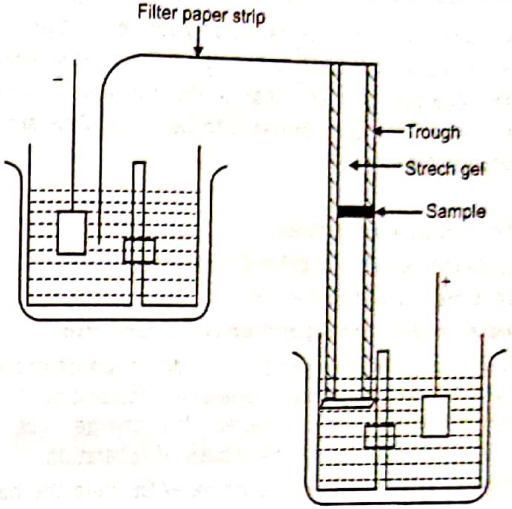


Fig. 13.5: Horizontal gel electrophoresis

(ii) Vertical Gel Electrophoresis:

The technique employed here is also as similar as the above technique in case of principle but the arrangement of the experiment is differing in this case. It is not even similar to the vertical paper electrophoresis in the case of the arrangement. In this case, the sample is kept in the midpoint of the separation plate which is at 90 degrees with the ground. The separation is aided by the gravity and the efficiency of the separation is enhanced.

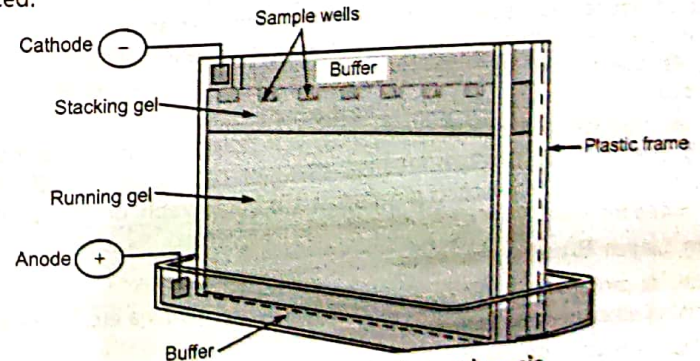


Fig. 13.6: Vertical gel electrophoresis

(iii) Polyacrylamide Gel Electrophoresis (PAGE):

In this method, Proteins are separated on the basis of charge to mass ratio and molecular size, a phenomenon called Molecular sieving. In this technique, the molecular sieve used is polyacrylamide. It is prepared by polymerizing acryl amide (CH_2=CH-CO-NH_2)

monomers in the presence of methylene-bis-acrylamide to cross link the monomers. Polyacrylamide gel structure is held together by covalent cross-linking. Polyacrylamide gel is tougher than agarose gels and also is thermostable, transparent, strong and relatively chemically inert if the comparison is to be made. Gels are uncharged and are prepared in a variety of pore sizes.

Advantages:

1. It can be used for preparative purpose.
2. Gels are stable over wide range of pH and temperature.
3. Gels of different pore size can be formed.

PAGE can be classified according the separation conditions into:

(i) Native-PAGE: This is generally used for the separation of protein mixtures. In this method, native gels are run in non-denaturing conditions. Thus the analytes for example protein's structure is unaffected. The charge, size and shape of the macromolecules to be separated form the basis of separation.

(ii) Denatured-Page or SDS-PAGE: Unlike native PAGE, here the basis for separation is the molecular weight of proteins. It can also be employed as the common method for determining molecular weight of proteins. It can be a very useful tool for checking purity of protein samples.

General Procedure of PAGE:

A column is filled with the gel of different pore sizes. Large pore gels are placed at the top and small pore gels are at the bottom. Sample is placed over the top of the gel column covered by a buffer solution. The pH of the buffer solution is in such a way to convert sample components into anions. The foot of the gel column is made to dip in the same buffer in the bottom reservoir. The electric field is imposed through the column by cathode and anode which are placed above and below the column. Macromolecular anions move towards the anode down the gel column through the gel pores. Rate of migration depends on the charge to mass ratio. On the basis of electrophoretic mobility and gel filtration effect separation of components occur.

After the electrophoresis is complete, visualization is done by staining the molecules in the gel using dyes such as ethidium bromide, silver, or coomassie blue. If the analyte molecules fluoresce under ultraviolet light then a UV photograph or if the analyte has radioactivity added for visibility, an autoradiogram can be recorded.

13.3.3 Thin Layer Electrophoresis

Studies can be carried out in thin layer of silica, keisulguhr, alumina etc. It has advantages of being less time consuming and producing good resolution.

This method is widely used in combined electrophoretic-chromatography studies in two dimensional studies of proteins and nucleic acid hydrolysates.

13.3.4 Cellulose Acetate Electrophoresis

In this case, cellulose acetate is used as the electrophoretic medium for the separation. It contains 2-3 acetyl groups per glucose unit and its adsorption capacity is less than that of paper. It gives sharper bands. It also provides a good background for staining glycoproteins.

Advantages:

1. No tailing of proteins or hydrophilic materials.
2. Available in wide range of particle size and layer thickness.
3. Give sharp bands and offer good resolution.
4. High voltage can be applied which will enhance the resolution.

Disadvantages:

1. Expensive.
2. Presence of sulphonic and carboxylic residue causes induced electroosmosis during electrophoresis.

Application:

1. Widely used in analysis of clinical and biological protein samples like albumin and globulins. It can be a great alternative to paper electrophoresis.

13.4 MOVING BOUNDARY ELECTROPHORESIS

Principle:

The moving boundary method allows the charged species to migrate in a free moving solution without the supporting medium.

Instrumentation:

The instrumentation is a simple set up that consists of a U shaped glass cell of rectangular cross section. Both the sides of the U cell are provided with. An inlet for sample introduction is available at the bottom or sides of the U cell. The sample solution is introduced through the inlet, and the apparatus is placed in a bath maintained at 40°C. Detection is done by measuring refractive index throughout the solution (Schlieren optical system).

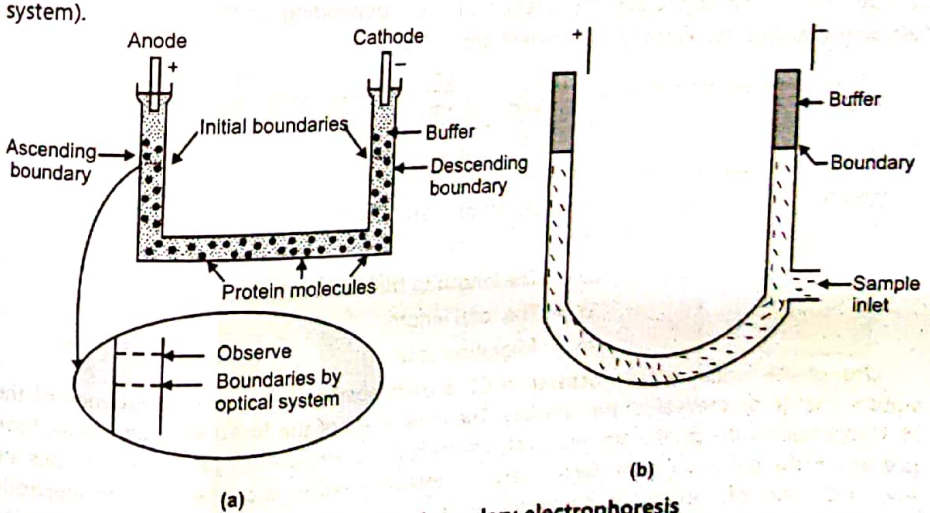


Fig. 13.7: Moving boundary electrophoresis

Advantages:

1. Biologically active fractions can be recovered without the use of denaturing agents.
2. A reference method for measuring electrophoretic mobilities.
3. Minute concentrations of the sample can be detected (0.05 mg/ml by Interferometric optical system).

Disadvantages:

1. It is relatively costlier.
2. Elaborated optical system is required.

Application:

1. It can be used to study homogenecity of a macromolecular system. It can also be used in analysis of complex biological mixtures.

13.4.1 Capillary Electrophoresis

Capillary electrophoresis (CE) employs narrow-bore (20-200 µm i.d.) capillaries to perform high efficiency separations of both large and small molecules. These separations are facilitated by the use of high voltages, which may generate electroosmotic and electrophoretic flow of buffer solutions and ionic species, respectively, within the capillary. The properties of the separation and the ensuing electropherogram have characteristics resembling a cross between traditional polyacrylamide gel electrophoresis (PAGE) and modern high performance liquid chromatography (HPLC).

Theory:

In CE, the analyte ions are separated under the influence of an electric field of an applied voltage. The mobility or the electrophoretic mobility is depending on the net charge, electric field strength, drag force, friction coefficient and velocity.

$$\mu EP = \frac{Vep}{E} = \frac{\left(\frac{Ld}{tm}\right)}{\left(\frac{V}{Lt}\right)}$$

Where,

Vep = Electrophoretic velocity
E = Electric field strength
Ld = The length to the detector,
Lt = The total length,
tm = Migration time

One of the fundamental processes in CE is electroosmosis. It is a consequence of the surface charge on the wall of the capillary. The inner walls of the fused silica capillaries have ionizable silanol groups and are negatively charged. Equal amounts of cations and anions are present in the buffer solution. Each reservoir containing buffer is connected with electrode and both end of capillary is connected to each reservoir. When a voltage is applied

negatively-charged wall attracts positively-charged ions from the buffer creating an electrical double layer. When a voltage is applied across the capillary, cations in the diffuse portion of the double layer migrate in the direction of the cathode, carrying water with them. The result is a net flow of buffer solution in the direction of the negative electrode. This process is called as electrosmotic flow. The mobility of this EOF is calculated. The higher the pH, the more negative charges on the capillary wall and the more positive charges in the fluid. This will generate a stronger EOF.

Instrumentation:

The basic instrumental configuration of CE includes the following components:

1. A fused-silica capillary with an optical viewing window
2. A controllable high voltage power supply
3. Two electrode assemblies
4. Two buffer reservoirs
5. An ultraviolet (UV) detector

Narrow-bore capillaries range normally from 25 to 100 mm in internal diameter (ID). Capillaries are typically of 50 µm inner diameter and 0.5 to 1 m in length. The capillary is filled with electrolyte solution which conducts current through the inside of the capillary. The ends of the capillary are placed in the buffer reservoirs and the optical viewing window is aligned with the detector. After filling the capillary with buffer, the sample can be introduced by dipping the end of the capillary into the sample solution and elevating the immersed capillary a foot or so above the detector-side buffer reservoir.

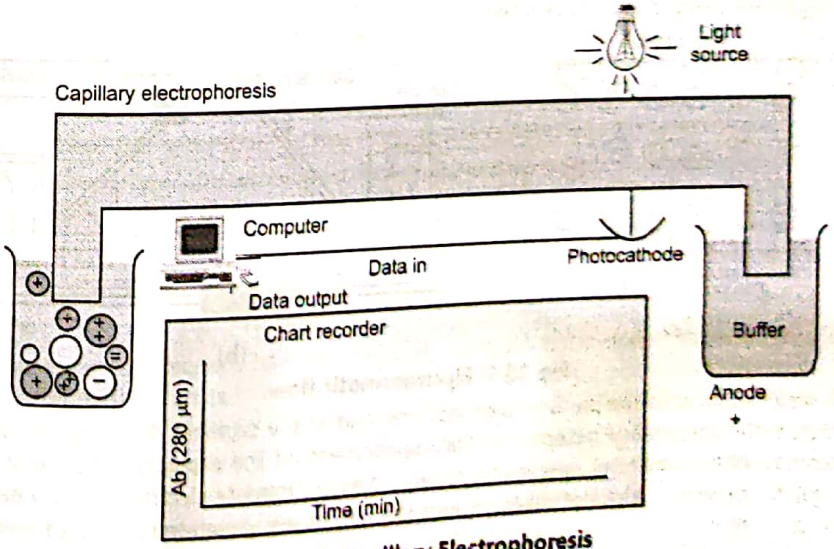


Fig. 13.8: Capillary Electrophoresis

A high voltage (typically 10-30 kV) is applied. Due to electroosmotic flow, all sample components migrate towards the negative electrode. The capillary can also be filled with a gel, which eliminates the electroosmotic flow. Separation is accomplished as in conventional gel electrophoresis but the capillary allows higher resolution, greater sensitivity, and on-line detection.

Sample application is done by either:

(a) High voltage injection: Potential is applied causing the sample to enter capillary by combination of ionic attraction and electroosmotic flow.

(b) Pressure injection: Pressure difference is used to drive the sample into capillary by applying vaccum.

When potential difference is applied, net migration occurs in the direction of cathode. Even substance with net negative charge migrates in the direction of cathode due to the phenomenon called as Electro Osmotic Flow. Neutral molecule moves at the same speed as the electroosmotic force. Positively charged species move faster, speed is sum of electroosmotic force and electrophoretic mobility. Negatively charged molecules lag behind.

Electroosmotic Flow:

One of the fundamental processes that drive CE is electroosmosis. The positively-charged counterions are attracted towards the surface of the silicate glass capillary containing negatively charged functional groups. This process carries solvent molecules in the same direction which is called as electroosmotic flow. During a separation, uncharged molecules move at the same velocity as the electroosmotic flow. Positively charged ions move faster and negatively charged ions move slower.

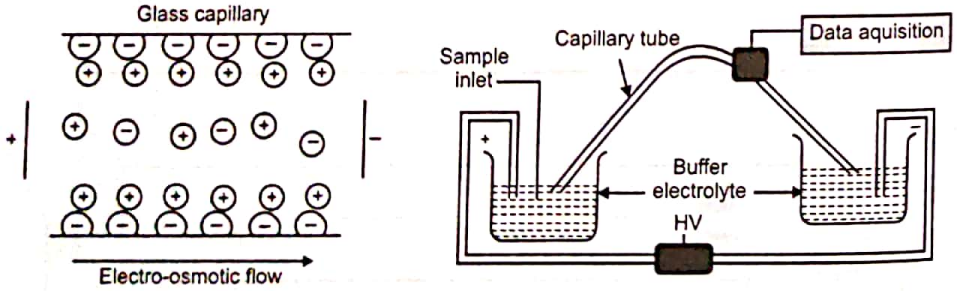


Fig. 13.9: Electroosmotic flow

A small volume of sample is moved into one end of the capillary. The capillary passes through a UV absorbance detector, at the opposite end of the capillary. Application of a voltage causes movement of sample ions towards their appropriate electrode usually passing through the detector. A plot of detector response with time is generated which is termed an electropherogram.

13.4.2 Isotachophoresis

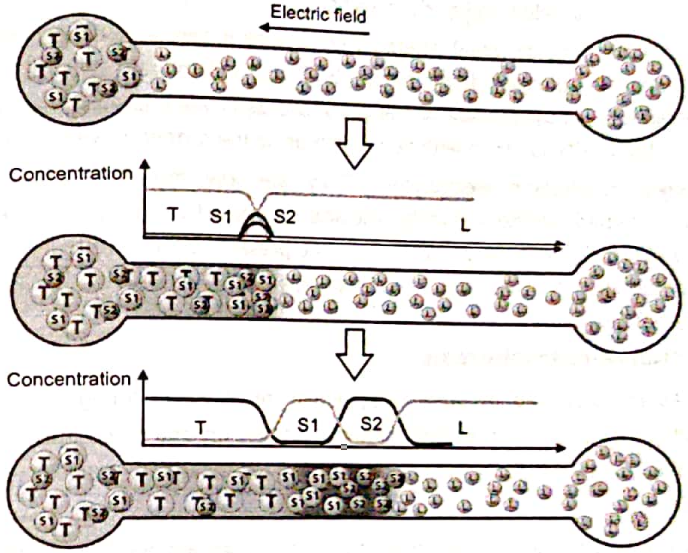


Fig. 13.10: Isotachophoresis

The technique of isotachophoresis depends on the development of potential gradient.

Principle:

It is based on principle of moving boundary electrophoresis. A leading electrolyte (e.g. chloride) with a higher mobility than the analytes, and a trailing electrolyte (e.g. glycinate) with a lower mobility are used. Solution in which the separation takes place is normally an aqueous medium, which contains sucrose to provide a higher density to the solution. Where the separation by isoelectric focusing depends on the existence of a pH gradient in the system. The technique of isotachophoresis depends on the development of a potential gradient. Separation of the ionic components of the sample is achieved through stacking them into discrete zones in order of their mobilities, producing very high resolution.

13.4.3 Isoelectric Focusing

Principle:

As we know that proteins have an isoelectric point pH, when electrophoresis is run in a solution buffered at constant pH, proteins having a net charge will migrate towards the opposite electrode so long as the current flows. The use of pH gradient across the supporting medium causes each protein to migrate to an area of specific pH. The pH of the protein equals the pH of the gradient, thus resulting in sharp well defined protein bands. A procedure to determine the isoelectric point (PI) of proteins thus, a mixture of proteins can be electrophoresed through a solution having a stable pH gradient in from the anode to the

cathode and a each protein will migrate to the position in the pH gradient according to its isoelectric point. This is called isoelectric focusing.

Protein migrates into the point where its net charge is zero – isoelectric pH. Protein is positively charged in solutions at pH below its PI and will migrate towards the cathode. Protein is negatively charged in solution at pH above its PI will migrate towards the anode. They will be in the Zwitterion form with no net charge so the further movement will cease.

Ampholytes (amphoteric electrolytes)- They are low molecular mass (600-900D) oligomers with aliphatic amino and carboxylic acid groups with a range of isoelectric points. Ampholytes help to maintain the pH gradient in the presence of high voltage. It can also use gels with immobilized pH gradients - made of acrylamide derivatives that are covalently linked to ampholytes.

13.4.4 Immunoelectrophoresis

Antibodies are produced by immune system in response to foreign macromolecules. Each antibody binds specifically to one feature (epitope) on one macromolecule (antigen). This allows the use of antibodies for detection and quantitation of specific proteins in a complex mixture.

When Electrical potential is applied to study of antigen-antibody reactions, it is called Immunoelectrophoresis. The antibodies are electrophoretically separated and antigens diffuse towards each other resulting in precipitin arcs where antigen antibody complexes form. This technique has been referred to as immune electrophoresis.

Antibody is placed in trough cut parallel to the direction of the electrophoresis. Run the electrophoresis as a result precipitin arcs will be formed due to Ab-Ag complex formation. A fluid containing proteins antigens is placed in a well in a thick layer of buffered agar and an electric current is applied, antigens will be distributed in separate spots along a line passing through the well and parallel to the direction of current flow.

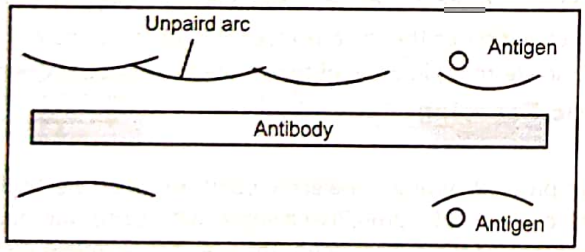


Fig. 13.11: Immunoelectrophoresis

Method:

It is usually carried out in 2% agar gel medium. The antigen mixture is applied into a small circular wells cut out of agar and the initial electrophoretic separation is carried out

depending on their charge and molecular weight. After the initial separation; the antibody mixture is then introduced into a narrow slot in the gel about 0.5 to 1.0 cm from the separated antigens. During this period, the antigen components diffuse radially outwards, towards the diffusing antibody and precipitation takes place in elliptical arcs as related antigens and antibodies diffuse into one another.

Advantages:

1. Spreading of bands is minimized due to the application of the applied field and the pH gradient, high resolution can be achieved.

Disadvantages:

1. Carrier ampholytes generally are used in relatively high concentration, a high voltage power source (up to 2000 V) is necessary and power is in the vicinity of 2 to 50 W. As a result the electrophoretic matrix must be cooled.

Application:

1. It is mainly used for separating protein and peptides. It is used in clinical, forensic and human genetics laboratories for the separation and identification of serum protein in research in enzymology, membrane biochemistry, microbiology and immunology.

13.5 GENERAL APPLICATIONS OF ELECTROPHORESIS

General applications of electrophoresis are as follows:

1. It has a wide application in analysis of proteins thus help in understanding the structure and function of proteins. Useful for separation of proteins and amino acids in biochemistry and clinical chemistry.
2. Useful in DNA Sequencing. Through electrophoresis, specific DNA sequences can be analyzed, isolated and cloned. The analyzed DNA may be used in forensic investigations and paternity tests.
3. Useful in analysis of new antibiotics and which types of bacteria are antibiotic-resistant.
4. Separation of organic acid, alkaloids, carbohydrates, amino acids, alcohols, phenols, nucleic acids, insulin.
5. Vaccine analysis is one of the many important applications of electrophoresis. There are several vaccines that have been purified, processed and analyzed through electrophoresis, such as the influenza vaccine, hepatitis vaccine and polio vaccine.
6. It has applications in food industry and agricultural testing.
7. It is employed in biochemical and clinical fields i.e. in the study of protein mixtures such as blood serum, haemoglobins and in the study of antigen-antibody interactions.

8. Electrophoresis in combination with autoradiography is used to study the binding of iron to serum proteins.
9. Used for analysis of terpenoids, steroids and antibiotics.
10. For testing purity of thyroid hormones by zone electrophoresis.
11. Quantitative separation of all fractions of cellular entities, antibiotics, RBC, Enzymes etc. is possible.

REVIEW QUESTIONS

1. Write the principle of electrophoresis.
2. Discuss the types of electrophoresis in detail.
3. Discuss the zone electrophoresis and its types.
4. Write a note on immunoelectrophoresis.
5. Write an exhaustive note on various developments of electrophoresis.
6. Write the applications of electrophoresis.

UNIT - IV

Chapter ...14

GAS CHROMATOGRAPHY

Objectives:

Upon completion of this section, the student should be able to

- Understand the basic concept of gas chromatography including its principle and theory.
- Understand the instrumentation of gas chromatography.
- Explain the applications of gas chromatography.

14.1 INTRODUCTION

Gas chromatography is one of the widely used chromatographic techniques, which uses inert gas as the mobile phase. In gas chromatography the components of a sample, after vaporisation, are separated by being partitioned between gaseous mobile phase and solid or liquid stationary phase. The inert gas does not interfere with the analyte but transport the components through the column and facilitate the separation. Gas chromatography is a widely used technique for separation of gaseous and volatile substances which are difficult to separate and analyse. It is a rather simple and inexpensive method, generally efficient in regard to separation. In gas chromatography, since gas as a moving phase is passed through a column containing adsorbent or a liquid adsorbent supported on an inert solid, adsorption or partition is possible. In 'gas solid adsorption chromatography' (GSC), the components of the mixture distribute themselves between the gas phase and the adsorbent and the separation is due to the differences in adsorptive behaviour. While in 'gas liquid-chromatography' (GLC), the components of mixture distribute themselves between gas phase and the stationary liquid phase according to their partition coefficients, the solid functions only as a support for the liquid stationary phase, enabling it to present a large surface for the gas. Thus, GLC has a much greater application in analysis of most of the organic compounds, which have a measurable vapour pressure at the temperature employed.

Though fundamentally it is a separation technique, it provides identification of compounds and the quantitative estimation also. It finds application in analysis of varied type gases and pollutants, petroleum and petrochemicals, oils and fats, food and flavours, drugs and vitamins, steroids and alkaloids, blood and serum, pesticides and fungicides, radioactive isotopes and a number of miscellaneous purposes.

History:

Gas chromatography is a very similar technique to column chromatography except the gas is used as mobile phase instead of liquid. Gas solid chromatography was initially developed by G. Damkohler and H. Thiele (1943), but gas-liquid chromatography was originated by Nobel Laureate: A. J. P. Martin with A. T. James who announced this technique in 1952. Martin has theoretically predicted feasibility of GLC in separation analysis. GLC was further developed with improved instrumentation and applications by various workers.

14.2 PRINCIPLE AND THEORY

Gas chromatography requires a mobile phase and a stationary phase like all other chromatography techniques. However, the mobile phase is comprised of an inert gas such as helium, argon, or nitrogen. The stationary phase consists of a packed column in which the packing or solid support, or a liquid coat act as stationary phase. The main principles involved are adsorption and partition for gas solid chromatography and gas liquid chromatography, respectively. In gas solid chromatography the analytes of the mixture distribute themselves between the gas phase and the adsorbent.

The difference in the adsorptive behaviour causes separation, whereas in gas liquid chromatography, the partition co-efficient is the main factor for the separation. The analytes of mixture distributes themselves between gas phase and liquid phase according to their partition co-efficient. The separation of compounds is based on the different strengths of interaction of the compounds with the stationary phase.

The stronger the interaction is, the longer the compound interacts with the stationary phase, and the more time it takes to migrate through the column thus lead to longer retention time. The separation of the analytes depends upon the vapour pressure of the compound, polarity of the compound and stationary phase, column temperature and flow rate of mobile phase. The above factors decide the retention time of the analytes.

Gas solid chromatography technique follows the laws of Freundlich or Langmuir and Gas liquid chromatography follows the Henry's law of partition. According to Freundlich or Langmuir law when a gas comes in contact with an adsorbent certain amount of the gas is adsorbed on the surface of the adsorbent. This phenomenon is explained by the following expressions.

Freundlich's Law states:

$$x/m = KC^{1/n}$$

Langmuir's Law states:

$$x/m = K_1C + K_2C$$

Where;

x = Mass of the gas absorbed

C = Concentration of vapour in the gas phase

K, K_1, K_2 = Constants

Henry's law states:

"At constant temperature, the mass of a gas dissolved in a given volume of a solvent is directly proportional to its partial pressure in the gas phase in equilibrium with the solution". Thus when gas comes in contact with liquid certain amount of gas is dissolved in the liquid according to;

$$x/m = KC$$

14.3 TECHNIQUE OF GAS CHROMATOGRAPHY

Gas chromatography is a special form of chromatography wherein the moving phase is a gas and the stationary phase may be either liquid or solid. The technique is suitable for separation of materials which are volatile without decomposition.

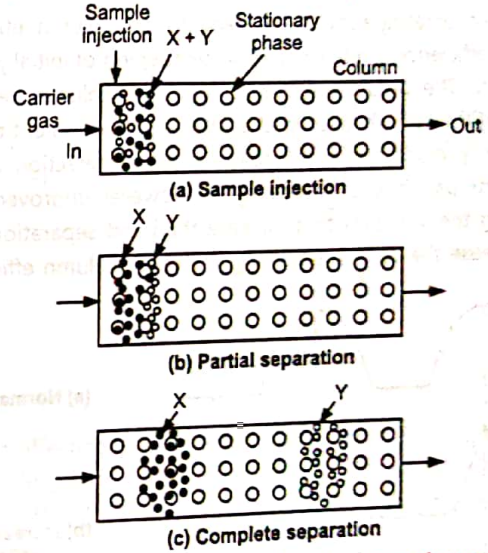


Fig. 14.1 : Separation process in gas chromatography

In this method, the sample is introduced into the moving carrier gas stream and is carried by it through the column. The column contains either the active solid (GSC) or a liquid of low vapour pressure held upon an inert solid (GLC). The active solid or non-volatile liquid acts as stationary phase whereas the carrier gas acts as mobile phase. The components of mixture sample distribute between two phases. The adsorption or solubility properties may differ from component to component and therefore the components are carried along the column at different rates, and finally they emerge at the outlet of the column in distinct zones (peaks) separated by the carrier gas. On emerging, the vapours of the components are detected by suitable detector accompanied by an automatic recording.

When the sample in the vapour phase is introduced into the column by carrier gas, some molecules of the sample get rapidly dissolved in liquid and a dynamic equilibrium is

established. At equilibrium the concentration of molecules of each type is a constant ratio in two phases e.g. molecules of compound *X* distribute equally between the two phases and molecules of compound *Y* on other hand may be highly soluble in the liquid phase therefore a few molecules will be in the vapour phase when the equilibrium is attained. In this position, the carrier gas will drag molecules of *X* leaving those of *Y* behind in the column. Molecules of *X* will be carried to the region containing fresh liquid and some of them get dissolved until new equilibrium is reached. In the meanwhile, a fresh mobile gas comes over the liquid containing *Y*; some of molecules *Y* will again enter the gas to reach equilibrium. Thus, the volatile molecules are continuously dragged to the head while the less volatile molecules fall back, they too are picked up and forwarded by continuous gas stream and if conditions are right, a clean separation is achieved. [See Fig. 14.1].

The resolution of chromatography peaks depends on: column efficiency and solvent efficiency. The column efficiency is related to peak broadening of initially compact band as it passes down a column. The broadening results from the column design and operating conditions and can be quantitatively described by the height equivalent to a theoretical plate (HETP). Solvent efficiency results from the solute-solvent interaction and determines the relative position of solute bands on a chromatogram. However, improved separations can be obtained by controlling the variables that increase the band separation (increased solvent efficiency) and/or decrease the band broadening (increased column efficiency) as illustrated in Fig. 14.2.

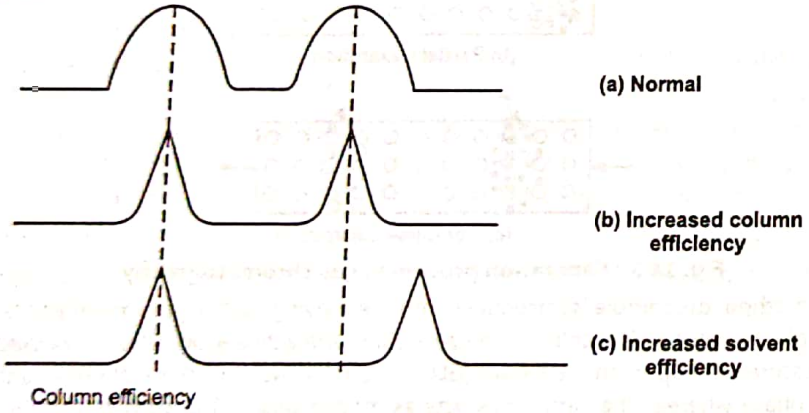


Fig. 14.2 : Effect of column efficiency in separation

Column efficiency:

Column efficiency is measured by the number of theoretical plates. The original theory of chromatography, i.e. plate theory was able to describe the effects of variables that influence the migration rates in quantitative terms. However, the plate theory is unable to describe the effects of factors which are responsible for band broadening. Hence, plate theory is now supplemented by rate theory which accounts for the latter variables also.

(a) Plate Theory:

The plate concept is adapted from the theory of distillation columns. According to the theory a chromatographic column is composed of discrete, but continuous, narrow, horizontal plates. It is assumed that, during chromatographic process the equilibration of the solute between mobile and the stationary phase takes place at each theoretical plate with the step-wise transfer of solute and solvent from one plate to the next.

As in distillation, in gas chromatography, the discrete plate is an artificial concept. The separation efficiency of chromatographic column increases with increasing number of theoretical plates. Thus, the number of theoretical plates *N* is used as measure of column efficiency. Theoretical plates can be easily measured from the chromatogram. The number of theoretical plates *N*, is given by

$$N = 16\left(\frac{t}{w}\right)^2$$

Where, 't' is the distance from injection to peak maximum (retention time) and 'w' is the peak width in units of time which can be determined by drawing the tangents about 2/3 of the height to the peak at the points of inflection. This is illustrated in Fig. 14.3.

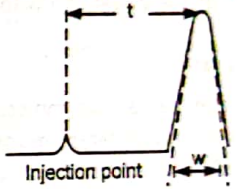


Fig. 14.3

The second term affecting column efficiency is Height Equivalent to Theoretical Plates (HETP); which is that length of column necessary for the attainment of solute equilibrium between mobile and stationary phase. HETP is related to the number of theoretical plates *N* by

$$HETP = \frac{L}{N} = \frac{L}{16}\left(\frac{w}{t}\right)^2$$

Where, 'L' is the length of chromatographic column, usually in centimeters. HETP calculations are useful in comparisons between columns of different lengths. Thus, HETP and *N* are the preferred measures of column efficiency.

(b) Rate Theory:

Several chromatographic theories have been developed to account for the shape of elution curves from chromatographic columns. The rate theory developed by Van Deemter et al. successfully describes the influence of variables that affect the band separation (retention time) and band broadening. Van Deemter equation is useful in optimizing the chromatographic performance and can be expressed as:

$$HETP = A + B/u + Cu$$

Where A, B and C are coefficients of Eddy diffusion, longitudinal diffusion and mass transfer respectively and 'u' the linear gas velocity (flow rate) through the chromatographic column. The linear gas velocity is measured by

$$\mu = \frac{\text{Length of column, (cm)}}{\text{Retention time of air, (second)}}$$

When HETP is plotted against u, we get a hyperbola with a minimum HETP. This minimum is the optimum flow rate (u) whereas the column efficiency is at the peak. (Fig. 14.4)

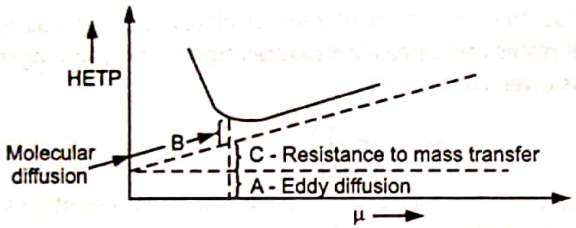


Fig. 14.4 : Van Deemter plot

The influence of the parameters of the equation on the separation efficiency has been discussed by Keulemans et al.

(a) Eddy diffusion (Multiple paths) 'A':

In packed columns, the solute and carrier gas molecules travel along many paths of different length, thus solute molecules have different residence time. This results into peak broadening which broadening depends upon the size of packing particles, the shape and the manner of packing and on the column diameter. (Fig. 14.5) The term *A*, i.e. 'Eddy diffusion' can be decreased by using smaller particle size but it is easier to obtain regular packing with larger particles; hence, the particle size should be optimum. Smaller particles also increase the pressure drop across the column leading to disturbance in linear gas velocity, which ultimately decreases column efficiency. Thus, the Eddy diffusion can be minimized by using small particles of uniform size and smaller diameter columns. Generally, the particle size up to 100-120 mesh range and the columns with 1/8 inch inner diameter are used for good resolution.

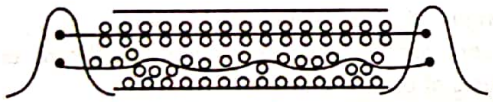


Fig. 14.5 : Eddy's diffusion

(b) Molecular Diffusion:

Molecules from solution of high concentration tend to move to low concentration by diffusion. This phenomenon of broadening by diffusion occurs during the travel of band of solute on column. The diffusion by concentration gradient occurs on both upper and lower side of the band. This is known as longitudinal diffusion, this is proportional to the solute diffusivity in the carrier gas. High solute diffusivity leads to band broadening with

consequent loss of efficiency. Solute diffusion in the liquid phase is negligibly smaller than the gas. Diffusivity is the property of both solute and carrier gas and may be reduced by increasing the density of the carrier gas. This can be done by increasing the pressure or molecular weight of the gas. Thus, the molecular diffusion can be decreased by using the optimum linear gas velocity (flow rate) and using high molecular weight of carrier gas, e.g. nitrogen, or argon than hydrogen or helium.

(c) Resistance to mass transfer 'C' term:

This term describes the effect of the amount and viscosity of liquid on the solid support. A low viscosity low vapour pressure solvent with good absolute and differential solubility for the sample should be used. Also, low liquid loadings (1-10 per cent) have the advantage of fast analysis and lower temperature operation. Low liquid loadings, however, reduce the sample capacity and may require highly inactive solid supports. Lowering the temperature improves the resolution and decreases decomposition of compounds but at the same time may increase the adsorption and time of analysis. Simultaneous reduction of liquid loading and temperature is generally beneficial.

Solvent efficiency: The major advantage of Gas chromatography over distillation is that one can use selective solvent or liquid phase and thus the substances having same vapour pressure can be easily separated. The selection of proper solvent can be facilitated by considering the following factors:

(I) Interaction forces and partition coefficient:

There are four interaction forces that can aid gas chromatographic separation viz. orientation, induced dipole, non-polar and specific interaction forces. Orientation forces result from the interaction between two permanent dipoles, i.e. hydrogen bond. Induced dipole or Debye forces result from the interaction between permanent dipole in one molecule and induced dipole in a neighbouring molecule. These forces are usually small. Non-polar forces result from synchronized variations in the instantaneous dipoles of the two interacting species. They are weaker than the first two types of forces. Specific interaction forces result from chemical bonding by complex formation between solute and solvents.

All these forces determine the solubility of solute and thus separation in chromatography. The combined effect of these forces is expressed by partition coefficient (K).

$$K = \frac{\text{Amount of solute/unit volume of liquid phase}}{\text{Amount of solute/unit volume of carrier gas}}$$

The higher value of 'K' indicates that most of the substance is retained in the liquid phase and only a small fraction of the substance will be eluted in the carrier gas, thus leading to slow movement of the substance down the column. The separation of two compounds is possible if their partition coefficients are dissimilar. The greater the difference in these partition coefficients, shorter is the length of column required for their separation.

(II) Solvent efficiency and temperature:

The solvent efficiency is measured by the relative retention (α) which is the ratio of adjusted retention times or partition coefficients. The relative retention differs from the separation factor (SF) which is the ratio of uncorrected retention times (Fig. 14.6).

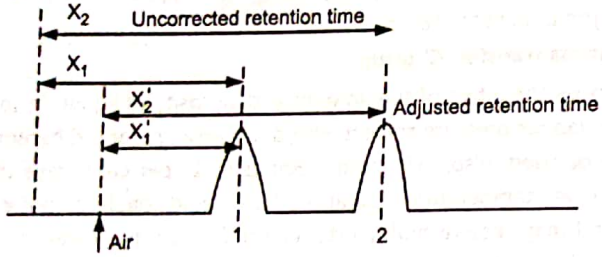


Fig. 14.6

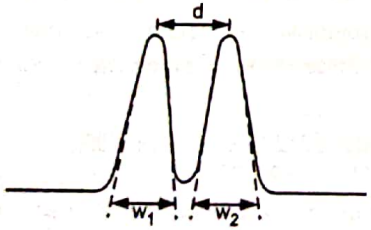
$$\text{Solvent efficiency } \alpha = \frac{X_2'}{X_1'} = \frac{K_2}{K_1}$$

$$\text{Separation factor SF} = \frac{X_2}{X_1}$$

Both 'α' and 'K' are temperature dependent. The α is constant over a limited range of temperature while K decreases with increasing temperature. This will lead to decreased elution time and decreased separation since the substance will remain in gas phase for more time than in liquid phase which is responsible for separation. Thus to achieve better separations, lower temperature should be used. This will lead to more liquid phase interaction, more separation and longer analysis time. As a minimum, the solute should spend 50 per cent of the time in liquid phase.

(III) Resolution (R):

The true separation of two consecutive peaks on a chromatogram is measured by resolution. It is the measure of both the column and solvent efficiencies and accounts for both the narrowness of the peaks and the separation between maxima (Fig. 14.7).



$$R = \frac{2d}{W_1 + W_2}$$

Fig. 14.7: Calculation of resolution

If R = 1, the resolution of two equal-area peaks is approximately 98 per cent complete.

If R = 1.5, baseline separation (99.7 per cent resolution) is achieved.

(IV) Number of plates for required separation:

The number of plates and thus the length of column required can be calculated by using the following equation.

$$N_{red} = 16\,R^2\left(\frac{\alpha}{\alpha - 1}\right)^2\left(\frac{K_2' + 1}{K_2'}\right)^2$$

Where, K_2' = Capacity factor for peak two

$$= \frac{\text{Adjusted retention time } X_2'}{\text{Retention time in air}}$$

R = Resolution

And α = Solvent efficiency

14.4 INSTRUMENTATION OF GAS CHROMATOGRAPH

The modern gas chromatograph consists of the following basic components illustrated in Fig. 14.8.

1. Carrier Gas:

The main purpose of the carrier gas is to transport sample components through the column. For selection of carrier gas, the factors should be ordered as follows:

(a) Chemically inert not to interact with sample or stationary phase.

(b) Suitable for the detector to be utilized and the type of sample analysed.

(c) Optimum column performance consistent with the desired speed of analysis.

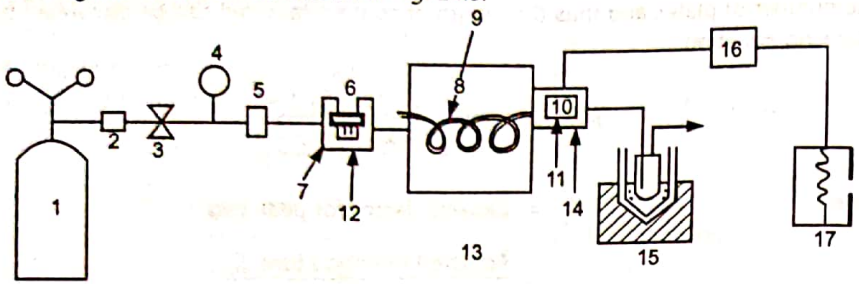
(d) Readily available, cheap and of high purity.

(e) Risk-free of fire or explosion hazard.

The carrier gases commonly used are hydrogen, helium, argon and nitrogen. For most applications with thermal conductivity detector either hydrogen or helium is used. Between hydrogen and helium, the latter should be preferred for safety reasons. Hydrogen is used because it is cheap and when Helium is not available. With flame ionization, hydrogen is used in producing a flame in the detection system and hence nitrogen or helium is used as a carrier gas. For electron capture detector argon is used as carrier gas but argon is not readily available in India.

Purity of carrier gas is very important in gas chromatography. Usually, more than 99.9 moles per cent purity is desirable. Moisture and other gases contaminations are removed by using filter, drier and absorbing tubes. The commonly employed source of carrier gas is a compressed gas cylinder. Sometimes commercially available small units of continuous gas generators are also used. To the compressed gas cylinder, is fixed pressure regulator preferably double stage and a pressure gauge to read the pressure. It is recommended that a molecular sieve, filter drier, flow meter, needle valve should be incorporated in the carrier gas supply line.

A line diagram of GLC unit is shown in Fig. 14.8.



1. Carrier gas
2. Filter drier
3. Pressure control valve
4. Pressure gauge
5. Flow meter
6. Sample injector port
7. Vaporizer heat
8. Column
9. Column oven
10. Detector
11. Detector oven
12. 13. 14. Heater controls
15. Fraction trap 1
16. Detector electronic
17. Recorder

Fig. 14.8 : Schematic diagram of GLC unit

2. Sample Injection System:

The sample is introduced in the column in the form of a sharp plug through an injection port, which contains a gas tight self-sealing type rubber septum through which the sample is injected by a syringe (Fig. 14.9). Immediately after injection, the sample has to be vaporized instantly and flown into the column with minimum pressure change, flow rate and back diffusion. For this purpose injection ports are heated and specially designed. The temperature of sample injection port is kept 20–50°C above the column temperature.

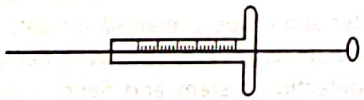


Fig. 14.9 : Sample injecting syringe

To guard against sample deposition after long use narrow base glass or metal inserts are provided in the injection port.

The sample deposition on insert can be taken out and cleaned periodically. Sometimes, the sample is released just in the column using long needle syringe. Liquid samples are generally injected in μl quantities (0.1–10 μl) with a hypodermic syringe. Gases can also be injected by similar syringes which have gas tight (Teflon tipped) plunger, but are of larger capacity (01–10 ml). Material of construction of the injection port should not have any catalytic effect on the system.

(a) Gas Samples: Gases are most conveniently introduced by a typical export gas sampling valve which is installed on the gas chromatograph. In these, only carrier gas flows through the column at a specific position while the sample gas flows through the standard volume loop and the gas trapped within the loop is carried into the column. Use of these sampling valves is particularly important to handle gases that contain lighter components where the syringe technique is not very satisfactory. The sample gas can also be injected at the top of the column by a hypodermic syringe. The Hamilton Teflon coated gas syringe is particularly suitable.

(b) Liquid sample: Liquids are most conveniently introduced by a micro-syringe which is of different size. Sometimes, the syringe needle will take minute cores from the disc or cap and deposit them on the top of the column where they may give distorted peaks as they absorb the components. Small samples can also be introduced by micropipette. They may be made by fusing short lengths of very fine capillary into wider tubing. They can introduce small volumes up to 0.01 micro-litres.

(c) Solid samples: Solid samples should be made to vaporize as quickly as possible by heating the injection port by means of a small coil. Generally, samples can be weighed into thin glass ampoules, sealed and placed in the gas stream and then crushed in the ancillary tube which is heated by heating coils to vaporize the sample. An alternative method is to dissolve the solid sample in a volatile solvent and inject like liquid sample. But in this case the sample size cannot be measured accurately.

3. Column Technology:

Column is the very important unit of GC, where the separation takes remarkably. The unit parts comprising the complete chromatographic column are the column, the support medium, and the liquid phase.

(a) The column: Depending upon the separation, the columns may vary in shape and dimensions. There are two types of column shapes as coiled helix and U-tube type. Coiled helical shape is most efficient shape but here the problem arises in uniform, even packing. U-tube columns are advantageous due to their short length and easy, even packing. The columns may be made up of glass, aluminium, copper, steel cupronickel or stainless steel. Nylon and other synthetic plastic tubings are also used but their use is prohibited at high temperature. Tubes may be 2-10 mm in diameter and from 2-4 metres in length. Glass is frequently used in U-tubes. W-tube columns are used for separation of compounds which are sensitive to catalytic action, e.g. biological products.

(b) Support medium: The purpose of solid support is to provide large and inert surface area for holding the liquid phase in thin and uniform film. It must be poor adsorbent and must be finely divided porous substance having a large surface area. It should also be chemically inert, heat stable and having sufficient mechanical strength to prevent fractionating with normal handling and be uniformly wetted by the liquid phase. No substance meets all these requirements perfectly. Most common supports

are available from diatomaceous earth. Two types are available namely firebrick and Kieselguhr. The firebrick is commercially available as chromosorb P. Kieselguhr is more fragile than fire brick and sold under the name chromosorb W., Celite, Embacel and Celatom. Glass beads, porous polymers, unglazed tiles, sand, fluorinated resins etc. are also used as support medium.

(c) Liquid phase: There is no well accepted method for selecting the best liquid phase for a particular separation. The right selection is based on mainly experience and/or trial and error. The main requirements in choosing a liquid phase are:

(i) **Non-Volatility:** It should be practically non-volatile and stable at the operating conditions.

(ii) **Selectivity:** It should show selectivity for the components to be separated.

(iii) **Compatibility:** It should have reasonable compatibility for the sample components.

(iv) **Low viscosity:** It should have low viscosity at the operating temperature.

(v) **Chemically inert:** It should be chemically inert towards the solutes at the column temperature.

(vi) It should dissolve in a volatile solvent and wettable on the support surface.

Generally, the upper temperature limit is set for a liquid phase, above which the liquid may start bleeding, causing the baseline disturbance. The liquid phase chosen mainly depends on the composition of the sample. For an efficient separation, the liquid phase should be similar in chemical structure to the components of mixture, e.g. hydrocarbons are best separated with hydrocarbon solvent, polar compounds with polar solvent.

Liquids may be classified as:

1. **Very polar:** Glycols, glycerols, amino alcohols, hydroxy acids, polyphenols, dibasic acids etc.
2. **Polar:** Alcohols, fatty acids, phenols, primary and secondary amines.
3. **Intermediate:** Ethers, ketones, aldehydes, esters, tertiary amines etc.
4. **Low polarity:** Chloroform, dichloromethane, 1, 2 dichloromethane, aromatic hydrocarbons etc.
5. **Islon-polar:** Saturated hydrocarbons, halo-hydrocarbons.

(d) Preparation of Chromatographic Columns: Three types of analytical columns are used in gas chromatography; packed, support coated open tubular, open tubular columns.

(i) **Packed columns:** These are prepared by packing metal or glass tubings with granular stationary phase. For gas solid chromatography the columns are packed with adsorbents or porous polymers, while in GLC columns are packed by coating the liquid phase over an inert solid support.

(ii) **Open tubular columns:** These columns are called capillary or Golay columns prepared from long (100–130 ft) capillary tubing having uniform and narrow internal diameter [0.01–0.03 inch]. The inside wall of capillary tubing is coated by liquid phase in the form of thin and uniform film. The carrier gas faces least resistance as there is no packing in the column. The sample capacity of this column is very low.

(iii) **Support coated open tubular columns:** SCOT columns are made by depositing a micron size porous layer of support material on the inside wall of a capillary column and then coating with a thin film or liquid phase. These columns have higher sample capacity and yield better resolution.

Generally, in the preparation of columns it is necessary to prepare the stationary phase by coating the liquid phase over the inert stationary support media. The coating is accomplished as follows:

1. Weigh out the required weight of solid support media of correct mesh size and place it in a rotary evaporation flask.
2. Calculate and weigh the appropriate amount of liquid phase to give the correct liquid loading.
3. Dissolve the liquid phase in sufficient volume of solvent to just wet the solid support media.
4. Add slowly the dissolved liquid phase to the support media in flask with stirring until an even slurry is formed and mixed properly.
5. Attach the flask to a rotary evaporator and allow the solvent to evaporate. Rotate the flask until uniform coating of liquid is confirmed.
6. Select a suitable column and plug one end with glass wool and to the open end, add the stationary phase. Uniformly packed columns are prepared with the help of an electric vibrator along the column or by constant tapping during the addition. U-tubes are packed by filling from one end towards the centre with vibrators.
7. The column is now fastened into the chromatograph and conditioned by passing carrier at about 25°C above the operating temperature for 24 hours or for fixed time. An ideally prepared column will maintain constant zero baseline on chromatogram.

(e) Equilibration of the column: Before introduction of the sample, complete equilibration or conditioning must be obtained. Column packed with stationary phase is attached to the instrument and desired flow rate of carrier gas is adjusted by flow regulators. The column temperature is set at desired temperature, but below the upper temperature limit of the liquid phase used. Conditioning is achieved by passing carrier gas for at least 6 hours or generally 24 hours. A properly conditioned column will show zero baseline on the recorder.

(f) Control of column temperature: Columns are usually operated above room temperature except for gaseous samples. A temperature programming is now used where the column is not kept at constant temperature but is subjected to controlled rise which reduces the retention time of the less volatile samples to be analysed more rapidly. For this, various methods have been used, i.e. vapour jackets, electrically heated air baths and liquid bath or metal block etc. Temperature control program in GC is explained as below.

Temperature Control Program:

Temperature plays a crucial role in Gas Chromatography. The temperature programmes regulate the increase of temperature during the analysis. In normal GC, separation of components differing widely in their boiling point under isothermal condition is not satisfactory. Programmed temperature can separate such mixtures effectively by choosing column temperature. In this system, a lower temperature is selected initially and it is used to resolve the early peaks. Then the temperature is increased to push out the higher boiling point components. Different methods are followed for temperature programming. The temperature is increased immediately after sample injection and kept constant at the program level until the high boiling components are eluted out. Then the temperature is returned to normal.

The initial temperature is maintained for 10 minutes and then temperature is increased. The third method involves to increase the temperature step by step to reach the final temperature. In general, linear temperature GC requires a dual column system to compensate the column bleeding. Separate heaters are needed for heating injection, column oven and detector systems. It also requires differential flow meters, low mass column oven for rapid heat transfer and pure gases.

4. Detectors:

After the resolution of solutes, each vaporized component emerges in turn from the column and is carried into the detector mixed with carrier gas. The detector receives impulse from the elute of the column in the form of solute. Vapour is sensed by the detector. It converts this impulse into an electrical signal proportional to the concentration of the solute in carrier gas. This signal is amplified and recorded as a peak on the chromatograph. Thus, the detector is considered to be the brain centre of the instrument. A good detector should (a) be stable against the effects of the extraneous noise in detector system, (b) give a rapid and linear response to changes in solute vapour as the column effluent passes through the detector, and (c) have concentration reproducibility and sensitivity to a wide range of solute vapours. There are numerous types of detectors used in GC, but only three types are in common use.

(a) Thermal Conductivity Detector: This is also known as Katharometer or Hot wire detector. The TCD is based on the fact that the rate of loss of heat from body depends upon thermal conductivity of the surrounding gas and that the thermal conductivity of the

surrounding gas is a function of its composition. Thus, the rate of loss of heat is related to the composition of the surrounding gas.

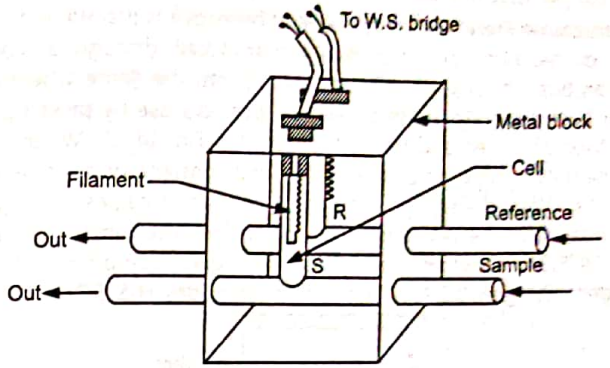
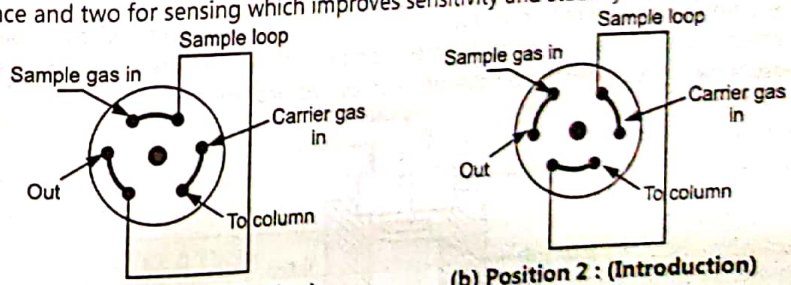


Fig. 14.10 : Thermal Conductivity Detector

TCD (Fig. 14.10) consists of two chambers of small volumes made within a metal block, each containing a resistance wire or a thermistor which have a high temperature coefficient of resistance. These resistances constitute the reference [R] and the sensing [S] elements respectively and are included in two arms of a Wheatstone bridge circuit. The carrier gas passes in both the cells and the arrangement is such that the column effluents are moved into the sensing side only, the filaments R and S which get heated due to passage of a small constant current, are quite matching when only a carrier gas is passing in both the cells. As the sample component enters the sensing cell, (Fig. 14.11) the temperature of filaments change due to widely different thermal conductivity of the sample component than that of the carrier gas. Consequently, the resistance of 'S' also varies and the bridge becomes unbalanced. This off-balance current is signaled to the recorder which is recorded on chromatogram. Now-a-days it is a common practice to provide four filaments TCD, two for reference and two for sensing which improves sensitivity and stability of the instrument.



(a) Position 1 : (Purging) **(b) Position 2 : (Introduction)**

Fig. 14.11

TCD filaments are made of platinum, tungsten or alloys having large temperature coefficient of resistance and corrosion resistant. Thermistors are made of oxides of manganese, cobalt or nickel to which some trace elements are added. It responds to all

substances except carrier gas and its sensitivity depends upon the type of carrier gas, filament current, temperature and flow rate of carrier gas.

(b) Flame Ionization Detector: A tiny flame of hydrogen is maintained at a capillary jet made of quartz or platinum, air or oxygen is introduced through a side by inlet for supporting the combustion. Column effluents are led into the flame wherein ionization of components may take place. An electrode system located close by picks up the ionization current which is then amplified and fed to the recorder (Fig. 14.12). When only carrier gas passes through the flame, there is no or very small and constant ionization current recorded as a steady baseline. When the sample component elutes and passes through the flame, its molecules are ionized and the resulting ionization current after amplification is fed to the suitable recorder. A FID is sensitive to almost all the organic compounds but insensitive to noble gases, oxygen, nitrogen, CO, CO_2, water, nitrogen oxides, H_2S, SO_2, CS_2 etc.

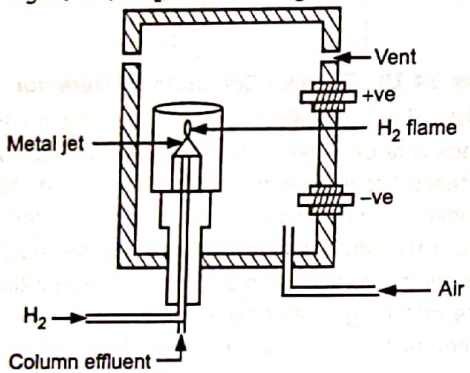


Fig. 14.12 : Flame Ionization Detector

The FID is not concentration sensitive but is rather mass sensitive, i.e. it gives the response proportional to total mass of component entering the detector and is therefore independent of carrier gas flow rate.

(c) Electron Capture Detector: It responds to only those compounds whose molecules have an affinity for electrons, e.g. chlorinated compounds, unsaturated compounds, etc. Nonetheless, it hardly responds to compounds such as hydrocarbons.

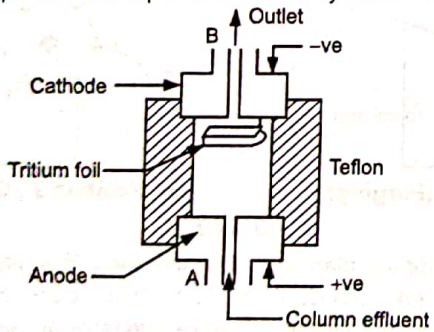


Fig. 14.13 : Electron Capture Detector

A tritium (titanium tritide) or Ni^{63} foil placed inside the cell ionizes the carrier gas molecules to form electrons that move slowly towards the anode under a fixed voltage (Fig. 14.13). Thus, a standing current is produced which is amplified by an electrometer. When a component having affinity for electrons elutes out of the column and enters the detector, it absorbs some electrons causing drop in standing current. This loss of current is traced by the recorder as a peak.

The disadvantage of this detector is its temperature limitation (220°C) and requirement of very pure/ultra pure nitrogen and argon-methane mixture as a carrier gas.

(d) β-ray Ionization Detector: The principle of ionization in this detector is reverse of flame ionization detector. In this detector, argon which is used as carrier gas is ionized by subjecting it to ionizing radiations. The ionizing radiations, radium 226, strontium 90 or tritium not only ionize it, but also raise a considerable proportion of it to an excited state. The energy of those excited atoms is sufficient to ionize vapour molecules when collision occurs. Thus, the presence of molecules of eluted vapour will give rise to an increase in the current passing through the chamber. Generally, the body of the detector forms one electrode. The other collecting electrode is insulated by a plug of Teflon. The body of the detector is made of brass.

The Argon detector fails to give linear response in case of inorganic substances viz. H_2, N_2, O_2 noble gases, CO, H_2O, H_2, CS_2, nitrogen oxides etc.

5. Recorder:

The signal from a gas chromatograph is continuously recorded as a function of time, generally by a potentiometric recorder. In a potentiometric recorder, the input response is continuously balanced by a feedback response; a pen connected to this system moves proportionately along the width of the chart paper, thus recording the signal, even as the chart paper moves at a fixed speed along its length. Before operating a recorder, its zero should be adjusted with the input zero otherwise the baseline will shift.

6. Integrator:

An integrator is employed for simultaneous measurement of areas under chromatographic peaks by mechanical/electronic means. Manual techniques for measurement of peak area are time consuming, tedious and are less precise. Electronic integrators print out of the peak area digitally and give highest precision but they are quite expensive.

14.5 DERIVATIZATION

When a compound is not suitable for analysis using GC, it is chemically modified to produce a new compound or derivative of the parent compound that is suitable for analysis. This process is termed as Derivatization. Derivatization is done prior to gas chromatography.

This process is often desirable for the following reasons:

1. To improve thermal stability of compounds, particularly compounds that contain polar functional groups.

2. The volatility of the compounds can be adjusted so that the separation properties can be changed and effective resolution can be achieved.
3. To convert the compound suitable for detection.

The most commonly used derivatization procedures involve the "substitution of active hydrogens" on the compound to be derivatized with a variety of functional groups. These functional groups impart the desired characteristics to the compound, while eliminating the adverse effects. Generally the compound is derivatized with suitable reagent to derivatize all relevant functional groups rapidly and the resultant mixture is injected directly into the Gas Chromatography.

For Example:

1. N, O-bis (trimethyl silyl) acetamide or N, O-bis (trimethyl silyl) trifluro acetamide are used to convert one active hydrogen in polar groups such as OH, COOH, NH_2, NH, and SH to silylated group. (-O-Si$(CH_3)_3$).
2. Androsterone exhibits poor peak shape and poor separation by GC. It contains a hydroxyl group and a carbonyl group. It is derivatised using trimethylsilylimidazole (TMSI) a strong silyl donor silylation, active hydrogen on OH, is replaced to produce a trimethylsilyl (TMS) derivative. The carbonyl group is reacted with another derivatizing reagent Methoxyamine to give forming an oxime derivative (CH_3ON). Oxime derivatives not only improve chromatographic performance, but also alter GC separations.

Advantages of Gas Chromatography:

1. This technique has a very high resolution power. Complex mixtures can be resolved into it's components by this method. The separation, identification and determination of compounds with negligible differences in boiling points are possible by this technique e.g. separation of methyl esters of stearic, oleic and linoleic acids is possible.
2. Sensitivity in detection is very high with thermal conductivity detectors. One can detect down to 100 ppm, while flame detectors, electron capture and phosphorus detectors can detect ppm, parts per billion (ppb) or pictograms (10^{-12} g) respectively.
3. It is a micro-method hence small sample size is required. Micro-litre of sample is sufficient for complete analysis.
4. The speed of analysis is fast. The use of gas as the moving phase has the advantage of rapid equilibrium between the moving and stationary phases and allows use of high carrier gas velocities, leading to fast analysis in seconds, minutes or in hours.
5. It involves relatively simple instrumentation operation of gas chromatograph and related calculations do not require highly skilled personnel and thus this technique is suitable for routine analysis.
6. It gives relatively good precision and accuracy.

7. Qualitative and quantitative analysis at a time is possible. The area produced for each peak is proportional to that concentration.
8. The cost of gas chromatograph is very low compared to the data obtained.

14.6 APPLICATIONS OF GAS CHROMATOGRAPHY

(I) Qualitative analysis:

Though gas chromatography is a potential method of separation, it does not yield characteristic symptoms as in case of the chemical or spectroscopic methods. Nevertheless having some data about the origin and the nature of the sample, gas chromatography can be used for the following types of qualitative analysis.

(a) The method can be used to confer identity of a suspected compound but it alone cannot establish the structure of unknown compound. It can provide evidence that two substances are not the same if they have different retention characteristics under identifiable conditions. But if two substances give coincident peaks, it indicates that two compounds may be same.

The retention characteristics of an unknown compound can be expressed mainly as relative retention.

$$r_{A/B} = \frac{V_{gA}}{V_{gB}}$$

Where, V_{gA} = Specific retention volume for 'A'

V_{gB} = Specific retention volume for 'B'

$r_{A/B}$ = Relative retention and

A = sample and

B = reference standard

Examples of reference standards include: ethanol, cholesterol, pentobarbital, Chlorpheniramine, Santonin, codeine etc.

Although relative retention is not highly reliable for identification of an unknown, it is useful for analysis of compounds in a mixture of known composition as in routine analysis of body fluids during therapeutic monitoring.

(b) To supplement identification of unknown by retention data, the suspected compound can be added to the unknown compound and to see if there is a concomitant increase in the peak height.

(c) Other approaches which aid in confirmation are: *(a)* derivative the unknown and see if the retention time of derivative compares with that of known derivative, *(b)* trap the material when it elutes and analyse it by standard procedure like UV, IR, NMR or chemical tests.

(II) Quantitative analysis:

The major application of gas chromatography is for quantitative analysis of individual components in a mixture. The method has advantages over titrimetric or spectrophotometry

because the separation as well as qualitative-quantitative evaluation of a mixture can all be performed at once.

The quantitative analysis can be done by the following methods:

1. Area normalisation method or area percent method
2. Corrected area normalisation method
3. Internal standard method
4. External standard method

1. Area normalisation method/ area percent method:

The normalization method is the easiest method which requires no reference standards or calibration solutions to be prepared. In this method, the ratio of individual peak area and the sum of all peak area is taken into consideration. The sum of all peak areas is assumed as 100%. Then the percentage of each peak is calculated in terms of 100%.

For example:

If a chromatogram consists of three peaks A, B and C with areas of 10, 30 and 50 respectively, and the sum of all peaks is 90, the concentration of A is (10/90) × 100 = 11.11%. Similarly Concentration of B = (30/90) × 100 = 33.33 %.

$$\% \text{ Concentration} = \frac{\text{Peak area}}{\Sigma \text{ Peak areas}} \times 100$$

2. Corrected area normalisation method:

In area normalisation method it is assumed that the detector response is same for all peaks. If the detector response is not same for all peaks then corrected area normalisation method is followed. Initially relative response factor (RRF) is determined using known amount of standard. This RRF is used to determine the corrected peak area for each peak of samples which is related to the concentration.

3. Internal-standard Method:

In this method, a reference standard is added in a constant amount to samples and calibration standards and analysed. This reference standard is termed as internal standard. The ratio of analyte peak area to internal standard peak area is used as analytical parameter. An internal standard (IS) is a compound that is similar in physical and chemical characteristics to the sample being analysed. It must be inert to the sample and must not react with the sample or any solvent used to dilute or prepare it for GC. The purpose of the internal standard is to behave similarly to the analyte but to provide a signal that can be distinguished from that of the analyte. The peak of the internal standard must not overlap with the peaks of the analytes.

4. External standard method:

In this method, a reference standard is chosen and it is chromatographed separately from the sample, maintaining the chromatographic conditions constant. The results of two chromatograms are compared

To reduce the effect of any changes in the operating conditions the sample and reference solutions can be chromatographed alternately. The data from the reference chromatograms run before and after the sample are then used for calculating results of each assay.

The reference standard (or standards) can be chosen to be the same as the solute (or solutes) in the sample. This eliminates the need for *response factors*. In addition, the external standard(s) can be made up to have concentration(s) closely similar to the component(s) of the sample, thus, errors is reduced. The sample must fall within a range bracketed by the calibration solution.

(III) Application in Pharmaceutical Analysis:

Gas chromatography plays an important role in the analysis of pharmaceutical products and drugs. It is used in quality control, analysis of new products and in monitoring metabolites in biological fluids.

Few of the applications include:

(a) **Antibiotics:** Penicillins and derivatives, gentamycin, kanamycin, neomycin, tetracycline, chloramphenicol,

(b) **Anti TB drugs:** Ionized, Ethambutol.

(c) **Antiviral:** Amantidine, Idoxuridine, cytarabin.

(d) **Antineoplastic:** Fluorouracil, 6-Mercaptopurine, Doxorubin etc.

(e) **General anaesthetics:** Ether, ethanol, chloroform.

(f) **Sedative hypnotics:** Barbiturates, Glutethimide.

(g) **Tranquillizers:** Diazepam, flurazepam, chlordiazepam etc.

(h) **CNS stimulants:** Nikethamide, caffeine, theophylline.

Similarly vitamins, sulpha drugs, sympathomimetics, alkaloids, steroids, antipyretic etc. are analysed by gas chromatography method.

The assay of the following drugs can be possible by gas chromatography.

	Drug	Column	Carrier gas	Detector
1.	Atropine sulphate eye ointment, injection, tablets	Glass column packed with acid washed silanised diatomaceous support coated with 3 per cent w/w of phenyl methyl silicone fluid.	Nitrogen	FID
2.	Chloroxylenol solution	Glass column packed with acid washed silanised diatomaceous support coated with 3 per cent w/w of polyethylene glycol.	Nitrogen	FID
3.	Clove oil	——"——		
4.	Econazole nitrate cream	Glass column acid washed silanised diatomaceous support coated with 3 per cent w/w of phenyl methyl silicone fluid glass column.	Nitrogen	FID

Drug	Column	Carrier gas	Detector
5. Ethosuximide syrup	Glass column acid washed silanised diatomaceous support coated with 3 per cent w/w of cyano-propyl methyl phenyl methyl silicone fluid.	Nitrogen	FID
6. Ethyloestrenol and its tablets and its tablets	Glass column acid washed silanised diatomaceous supported coated with 3 per cent w/w of phenyl methyl silicone fluid.	Nitrogen	FID
7. Fenfluramine hydrochloride tablets	Glass column acid washed silanised diatomaceous support coated with 10 per cent w/w of polyethylene glycol and 2 per cent w/w of potassium hydroxide.	Nitrogen	FID
8. Homatropine hydrobromide eye drops	Glass column packed with acid washed diatomaceous support coated with 3 per cent w/w of phenyl methyl silicone fluid.	Nitrogen	FID
9. Hyoscine hydrobromide injection and tablets	Glass column packed with acid washed diatomaceous support coated with 3 per cent w/w phenyl methyl silicone fluid.	Nitrogen	FID
10. Lincomycin Hydrochlaride	Glass column packed with acid washed silicanised diatomaceous support coated with 3 per cent w/w of phenyl methyl silicone fluid.	Helium	FID
11. Mianserin Hydrochloride tablets.	Glass column packed with acid washed silanised diatomaceous support coated with 3 per cent w/w of phenyl methyl silicone fluid.	Nitrogen	FID
12. Stearic acid	Glass column packed with acid washed silanised diatomaceous support coated with 15 per cent w/w of diethylene glycol succinate polyester.	Nitrogen	FID
13. Troxidone and its capsules	Stainless steel column packed with porous polymer beads.	Nitrogen	FID

- **Determination of Pesticides:**

  Gas Chromatography is a useful tool for the identification and determination of pesticides especially when combined with MS. The technique is much used to determine pesticidal residue in food products, aquaculture products, and agricultural products.

- **In Food Industry:**

  Gas Chromatography has been employed in food industry for separation and identification of lipids, carbohydrates, proteins, colorants, flavours and preservatives. Further the identification of vitamins, steroids and trace elements also can be done using Gas Chromatography.

The fatty acid contents, aldehydes and ketones and sugar contents in the dairy products can be analysed by GC. It is used in determining free cholesterol in milk fat.

- **In Forensic sciences:**

  GC is a useful tool for determination of steroid drugs in the blood samples, used in athletes and sports activities. In forensic sciences, the biological matrices such as blood plasma, serum and urine samples can be analysed for the drug content. However GC-MS is more sensitive in such studies.

- **In Air monitoring:**

  GC-FID is used for the determination of the volatile organic compounds. It is used for the analysis of Toluene, Ethylbenzene, o-xylene and Cumene in air. Volatile organic compounds are a cause of concern for human health due to their increased presence in the indoor environment. They are responsible for a phenomenon known as the sick building syndrome (SBS). Thus GC analysis becomes an important tool.

- **In natural products:**

  GC can also be used to determine the identity of natural products containing complex mixtures of similar compounds. For example, the geographic source of crude oil or natural gas can be determined by the GC "fingerprint", or relative distribution of major and trace compounds in each oil.

- **In volatile mixture separation:**

  Gas Chromatography is used for the separation of volatile mixtures. It can be used in many different fields such as pharmaceuticals cosmetics and even environmental toxins. Since the samples have to be volatile, human breath, blood, saliva and other secretions containing large amounts of organic volatiles can be easily analyzed using GC.

- **In Petroleum Industry:**

  Gas chromatography has been used in analysis of crude petroleum products, fractions gasoline, waxes, LPG, Sulphur and nitrogen compounds and reformats etc. Gas chromatography has been used to separate petroleum hydrocarbons into components as a preliminary to their identification by spectral studies.

- **In Biochemical and Clinical:**

  The technique is especially useful for applications involving body components of all types. Blood gases, estrogens, vanillin, mandelic acid etc. have been determined and analyzed in clinical medicine.

- **In Cosmetic and Perfume Fields:**

  Gas chromatography is helpful in determining the composition of various cosmetics, the quality of ingredients and the components of suitable fragrances.

REVIEW QUESTIONS

1. Write the principle of Gas chromatography.
2. Explain the rate and plate theories.
3. Write the superiorities of Gas chromatography.
4. Explain the instrumentation involved in Gas chromatography.
5. Write a note on detectors of Gas chromatography.
6. Write a note on temperature program in GLC.
7. Write a note on derivatization in GC.
8. Write the applications of Gas chromatography.

Chapter ...15

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Objectives:

Upon completion of this section, the student should be able to

- Understand the basic principle and theory of HPLC.
- Explore various components of HPLC instruments including pumps, injectors, columns and detectors and explain their functions.
- Describe the applications of HPLC.

15.1 INTRODUCTION

HPLC is one of the sophisticated analytical techniques of chromatographic separations. It is widely used in industries, research and development centers, quality control laboratories, food testing laboratories and in educational institutions. It is basically liquid chromatography in which the liquid mobile phase is mechanically pumped through a column that contains a stationary phase. Originally the term HPLC was referred as High Pressure Liquid Chromatography due to the involvement of high pressure but later on it is termed as High performance liquid chromatography due to its versatility and power.

Liquid chromatography though cumbersome has the distinct advantage of operating at low temperatures and is advantageous for separation of proteins, nucleosides which are thermolabile.

In conventional liquid chromatography, a dilute solution of sample is passed through a column packed with solid particles. Thus, liquid is passed through vertical columns under gravitational flow. This is passed with slow speed and especially if the packing granules were small enough to give efficient separation, then the delivery under gravity decreases even up to a few drops per minute.

The obvious way to increase the flow rate and get efficient separation is to force the liquid by a positive displacement pump or by gas pressure. This can be achieved by making certain modifications in columns like using smaller diameter and smaller surface area of column particles with the aid of other suitable packing structure. Such a technique is High Performance Liquid Chromatography (HPLC). HPLC is one of the sophisticated analytical techniques of chromatographic separations. It is widely used in industries, research and

development centers, quality control laboratories, food testing laboratories and in educational institutions. It is basically liquid chromatography in which the liquid mobile phase is mechanically pumped through a column that contains a stationary phase. Originally the term HPLC was referred as High Pressure Liquid Chromatography due to the involvement of high pressure but later on it is termed as High performance liquid chromatography due to its versatility and power. It has several times more resolving power than open column liquid chromatography, HPLC is used for speedy resolution of complex mixtures.

15.2 PRINCIPLE

The principle involved in HPLC can be either adsorption or partition. The chromatographic separation involved in HPLC is the result of interaction of sample with both stationary phase and mobile phase. The analytes are injected into the flow of mobile phase, just in front of the separation column. The outlet of the column is connected to a detector where the eluted substances are detected. The separation is achieved in the column packed with stationary phase material of low particle size and the liquid mobile phase is pumped through the column. The reliable flow rate of the mobile phase with appropriate pressure is applied. The sample mixture is interacted between the stationary phase and mobile phase. The interaction between these phases results in the separation.

The efficiency of separation in HPLC is depending upon many factors that include relative retention, the partition coefficient of the solute, plate count, mobile phase viscosity, type and characteristics of column packing, column length, particle size of stationary phase and temperature. Even the pressure drop can degrade the column performance. Pressures below 5000 psi are usually preferred in HPLC. The band broadening terms Eddy's diffusion, longitudinal and mass transfer explained by the Van deemter equation are to be considered for the efficient separation. The advantage of HPLC compared to conventional column chromatography is the reduced length of column, new lower particle size stationary phase, controlled flow rate and pressure that can be withdrawn by the column, thus the efficient separation.

Advantages of HPLC:

1. It provides a specific, sensitive and precise method for analysis of different complicated samples.
2. There is ease of sample preparation and sample introduction.
3. There is speed of analysis.
4. The analysis by HPLC is specific, accurate and precise.
5. It offers advantage over gas chromatography in analysis of many polar, ionic substances, metabolic products and thermolabile as well as non-volatile substances.

Classification:

HPLC can be broadly classified into two major types:

1. Normal Phase Liquid Chromatography
2. Reverse Phase Liquid Chromatography

1. Normal Phase Liquid Chromatography:

It is used much less frequently than reversed-phase chromatography. The main reason for this is that many separations can be accomplished using reversed-phase as reversed-phase is easier, and hence more common.

Stationary Phase: The stationary phase used in normal phase chromatography is polar in nature. Generally silica columns of the non-bonded phase are used as stationary phases in normal phase. In addition to this alumina columns are also used. Of the bonded phase, cyano columns, diol and amino columns are used.

Mobile Phase: Non-polar solvents such as hexane, heptane, iso-octane are generally used as mobile phases in combination with slightly more polar solvents such as isopropanol, ethyl-acetate or chloroform. Retention increases as the amount of non-polar solvent increases in the mobile phase.

2. Reversed Phase Liquid Chromatography:

This is widely used chromatographic technique compared to normal phase chromatography. The stationary and mobile phases are exactly opposite to the normal phase chromatography. That is the stationary phase is non-polar in nature whereas the mobile phase is polar in nature.

Stationary phase: Generally Octyl decayl silanol (C18/ODS) columns are most widely used as stationary phase for reverse phase chromatography. In addition C8, C4 columns are also used. Number of bonded phase columns are also used depending upon the nature of samples to be separated.

Mobile Phase: In reverse phase chromatography water is usually used as the base solvent. Other polar solvents such as Methanol, Acetonitrile or Tetrahydrofuran are added in fixed or varying proportions. pH is adjusted by buffers to modify separations of ionizable solutes. Ion-pairing reagents also enhance separation and selectivity of charged analytes by increasing retention on hydrophobic bonding phases.

The elution in HPLC can be classified into two categories:

1. Isocratic elution
2. Gradient elution

1. Isocratic Elution:

In this technique, the mobile phase composition is fixed and constant throughout the chromatographic procedure. For example, if a method consisting of mobile phase as methanol and water in the ratio of 70:30, the same ratio is maintained for the entire chromatographic procedure in isocratic method.

2. Gradient Elution:

In this technique, the composition of mobile phase is changed either stepwise or continuously as elution proceeds. For example, initially a composition (methanol : water, 70:30) for some time period (10 min), is maintained then the polarity is modified by changing the ratio to (80:20) for next 5 min and then to (90:10) for another 5 min.

Changing of composition to bring out the desirable separation is called as gradient elution method.

15.3 INSTRUMENTATION

A line diagram of HPLC unit is shown in Fig. 15.1. To attain reasonably high flow rates and yet keep particle size of packing very low (3-10 μm), pumping pressures of several hundred atmospheres (2000–8000 psi) are required. Thus, the equipment for HPLC is quite elaborate though simple.

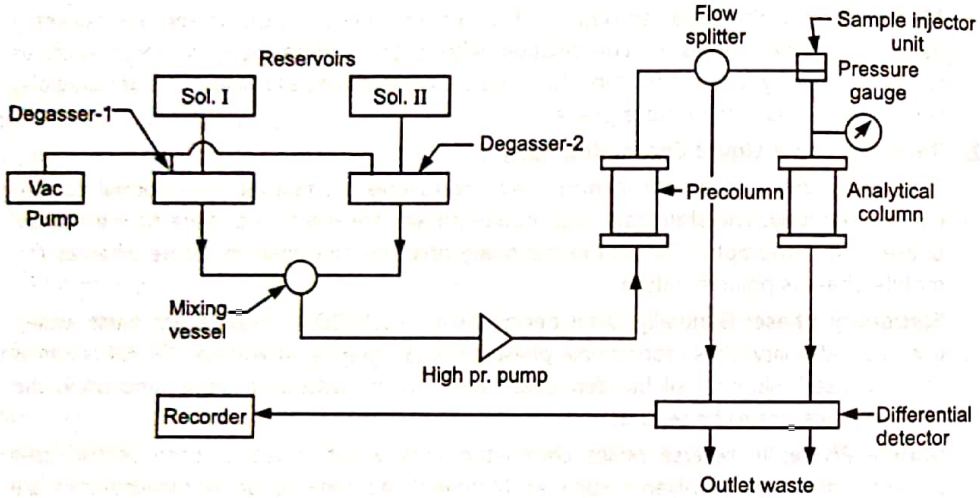


Fig. 15.1 : Line Diagram of HPLC

15.3.1 Mobile Phase Reservoir and Solvent Treatment Systems

A modern HPLC apparatus is equipped with one or more glass or stainless steel reservoirs, containing 500 ml or more of solvent. The reservoirs are often equipped by means of removing dissolved gases usually O_2 and N_2, that interfere by forming bubbles in the columns and detector systems. These bubbles cause band spreading; in addition, they interfere with the performance of the detector.

Degassing:

It is necessary to remove the dissolved gases present in the mobile phase solvent.

Different techniques are followed for degassing which are discussed below.

(a) External Vacuum Degassing: In this method, the solvent in a container is kept in an ultrasonic bath. Ultrasonication is done under vacuum using a vacuum pump. This process will remove the dissolved gases from the solvents and then can be used for HPLC. This technique is more useful for solvents, which can absorb gases like carbon dioxide and also useful for eluent blanketed with an inert gas like helium. This approach is explained in Fig. 15.2.

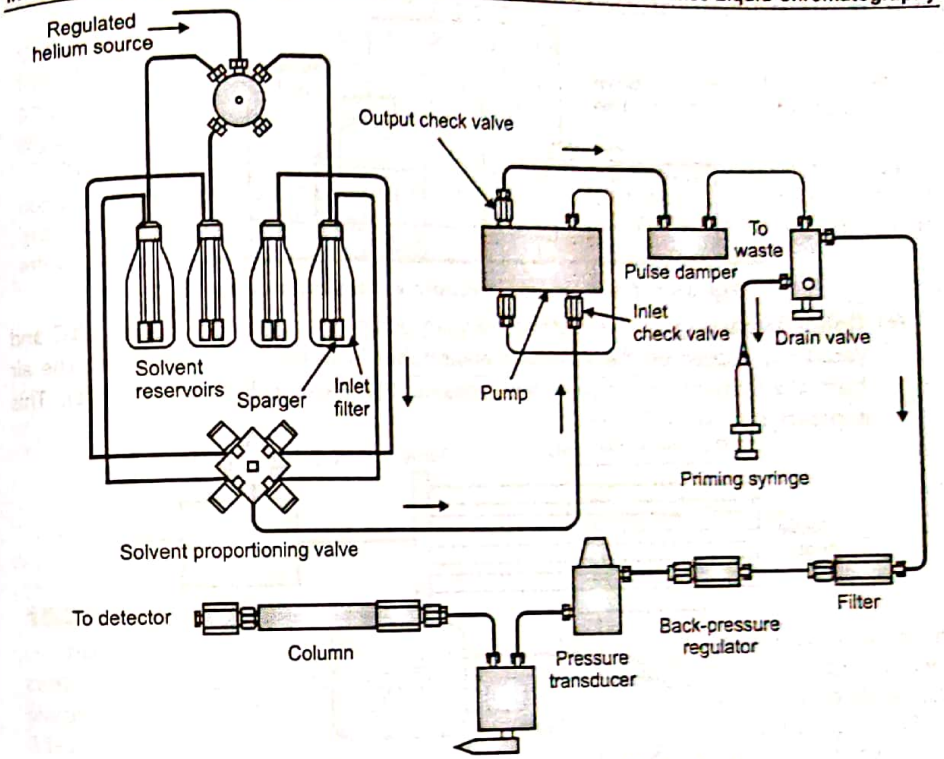


Fig. 15.2: Instrumentation of a typical HPLC unit

(b) Helium Sparging: In this technique, helium is bubbled into the solvent which will remove the other dissolved gases. The volume and time of application of helium should be decided. Helium is insoluble in the mobile phase so it escapes out without interfering with the chromatographic process. This process is called as helium sparging. This can be done online if the helium tank is equipped with the HPLC unit or the process can be done off line. However, the limitations are that Helium may selectively volatilize the more volatile solvent, thus changing the composition of the premixed solvents, more expensive and required in large quantity.

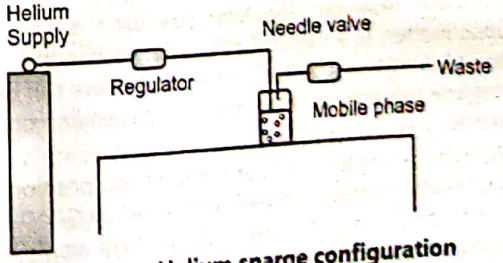


Fig. 15.3: Helium sparge configuration

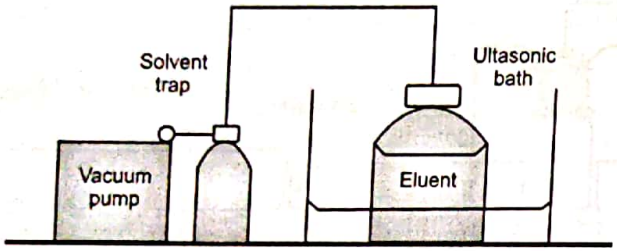


Fig. 15.4: Basic external vacuum degassing station

(c) Online Degassing: In this method, vacuum pump is equipped with the HPLC and vacuum is applied on the semi-permeable tubes in which the solvents run. The air from the solvents is removed and goes to the waste collecting container. This approach is shown in Fig. 15.5.

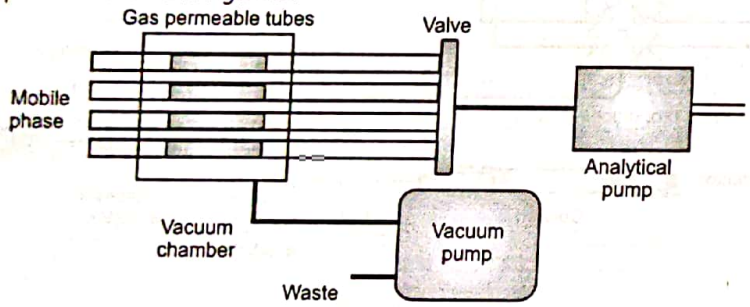


Fig. 15.5: On line degas schematic

(d) Filters: Other than the above methods filters are also used to remove the dust and other matters from the solvents. Membrane filters of 0.45μ are usually used. The mobile phase filtered through these filters using Buchner funnel under vacuum followed by ultrasonication.

(a) a vacuum pumping system or (b) a distillation system or (c) devices for heating and stirring the solvents or (d) device for sparing in which the dissolved gases are swept out of solution by fine bubbles of an inert gas of low solubility.

Often there is a filter for removing dust and particulate matter from the solvents. An alternative way would be to filter them through a Millipore filter under vacuum before introduction into the solvent reservoir. The filter size used is normally 0.2μm. This will eliminate the superfluous matter. In Analytical HPLC, the mobile phase is pumped through the column at flow rate of 1-5 ml/min. In HPLC, the mobile phase can be an aqueous organic mixture, a mixture of organic solvents or buffer solution, depending on the chromatographic method and on the detector used.

A separation that employs a single solvent of constant composition is termed *ISOCRATIC* elution. Frequently, separation efficiency is greatly enhanced by *GRADIENT* elution. Here two or more solvent systems that differ significantly in polarity are employed. The proportion of the two solvents is varied in a programmed way, sometimes continuously and sometimes

step-wise. Modern HPLC equipment is often equipped with devices that introduce solvents from two or more reservoirs into a mixing chamber at continuously varying rates; the proportioning values that are provided alter the volume ratio of the solvents linearly or exponentially with time.

The advantage of gradient over isocratic elution is that the separation of the same five components of a mixture, the speed of separation increases (retention time decreases) with gradient elution without any deletion in resolution. Thus, this gradient elution produces effects similar to temperature programming in gas chromatography.

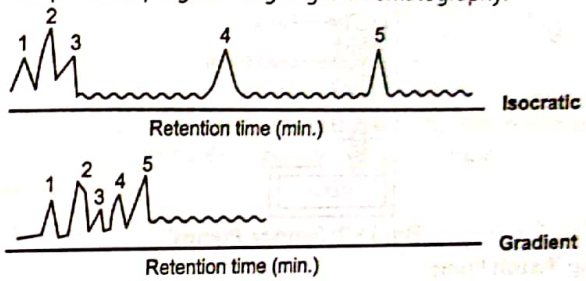


Fig. 15.6

15.3.2 Pumps

The pumps are used to pass mobile phase through the column at high pressure and controlled flow rate. Furthermore, the pumps used in HPLC should have the following features: (a) Generation of pressures up to 6000 psi. (b) Flow rates ranging from 0.1-10 ml/min. (c) Flow control and flow reproducibility of ± 0.5%. (d) It should be composition resistant and give a pulse free output. (e) It should be easy to change from one mobile phase to another. (f) The pump should be easy to dismantle and repair.

These pumps are necessary to force the liquid (mobile phase) through the column with finely packed particles. It should be noted that the high pressures generated by the pumps should not lead to an explosion hazard as liquids are not very compressible.

Different pumping systems are used in HPLC among which the three important types are discussed below.

Based on the mechanism of working the pumps can be classified into:

1. Syringe Pump/Displacement pumps
2. Reciprocating piston pumps
3. Constant pressure pumps

1. Syringe Pumps:

A syringe pump consists of a large barrel syringe with a plunger connected to a digital stepping motor or precision-screw drive. As the plunger moves forward, it drives the fixed volume of solvent through the chromatograph with a pulseless flow. These pumps are known for their pulseless flow of solvents. The flow rates are less than 100 μl/min. Flow is independent of viscosity and column back pressure. However, the run-time is limited by the volume of syringe and no flow occurs during the refill step. It has limitation

such as low solvent capacity (200-500 ml) and it is not easy to change solvent during gradient elution.

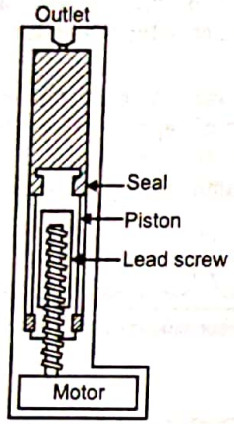
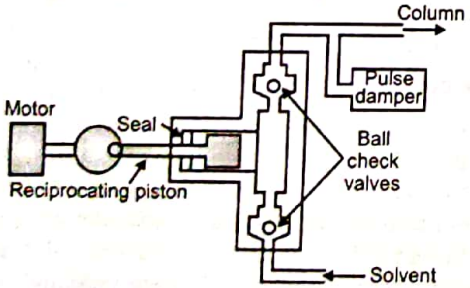


Fig. 15.7: Syringe Pumps

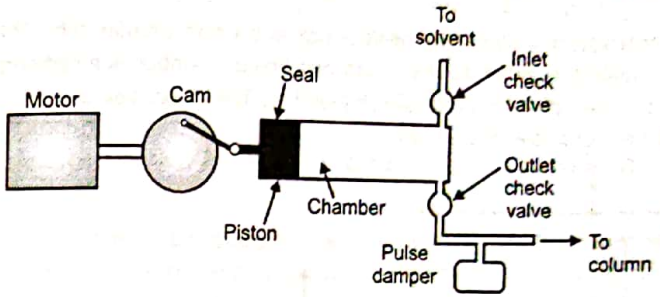
2. Reciprocating-Piston Pump:

A reciprocating pump is the most common design used in modern HPLC. The mechanism is similar to the constant displacement pump. The pump head consist of check valves and seal-piston assembly. The check valves regulate the flow of solvents from the reservoir to the pump chamber and further to the column. Two strokes namely fill stroke and delivery stroke are involved in the functioning. During fill stroke, the solvent is able to enter the liquid chamber from the solvent reservoir only. During the delivery stroke, the piston moves into the liquid chamber and pressurizes the liquid and the inlet check valve is forced to close. When the pressure inside the pump head exceeds the pressure on the column side of the pump, the outlet check valve opens and the mobile phase flows towards the column.

The advantages of this pump are the continuous solvent flow, no restriction on the reservoir size or operating time, easy and rapid solvent changes while doing gradient elution. These pumps are valuable for equipments used in automatic operations. The diagram of a reciprocating pump is as shown in Fig. 15.8.



(a)



(b)

Fig. 15.8 (a) Reciprocating-Piston Pump (b) Reciprocating piston pump head showing the check valves, piston, seal and damper

3. Constant Pressure Pumps:

In these pumps, high pressure from gas is introduced through a large piston which drives the solvent from the pump chamber to the column. The solvent chamber volume is around 70 ml (Fig. 15.9). Since the pressure on the solvent is proportional to the ratio of the area of the two pistons, usually between 30:1 and 50:1, a low-pressure gas source of 1 atm can be used to generate high liquid pressures (1-400 atm). An intermediate solvent can be used to reduce the interference of dissolved gas by entering to solvent. The rapid refill of the solvent chamber is facilitated by a valve. This system provides a continuous and pulseless pumping, and high flow rates for preparative applications. This type is used for packing columns; however it is inconvenient for gradient elution.

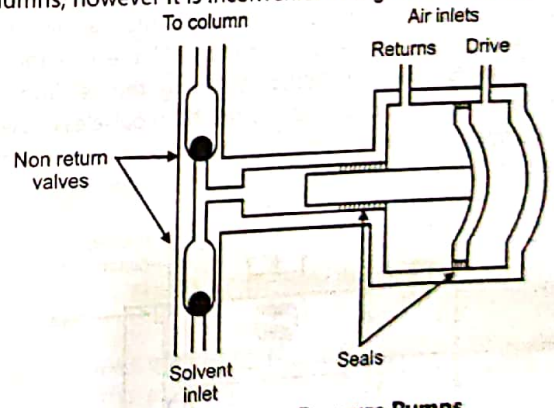


Fig. 15.9: Constant Pressure Pumps

Pump Pulsations:

Pump pulsations may be a problem during the trace analysis of analytes due to the baseline noise. It can be minimized by proper selection of pumps or by the use of cam design or pulse dampners. The flow delivered by a single-piston pump is relatively pulsating, so single piston-pumps are rarely used to deliver eluent.

Cam Design:

This set up consists of a two pump head design and a non-circular cam. The non-circular cam rotate and drive the piston so that when one liquid chamber is emptying, the other is refilling, thus, the two process occur simultaneously. This produces a pulseless flow. The pistons are 180° out of phase (Fig. 15.10).

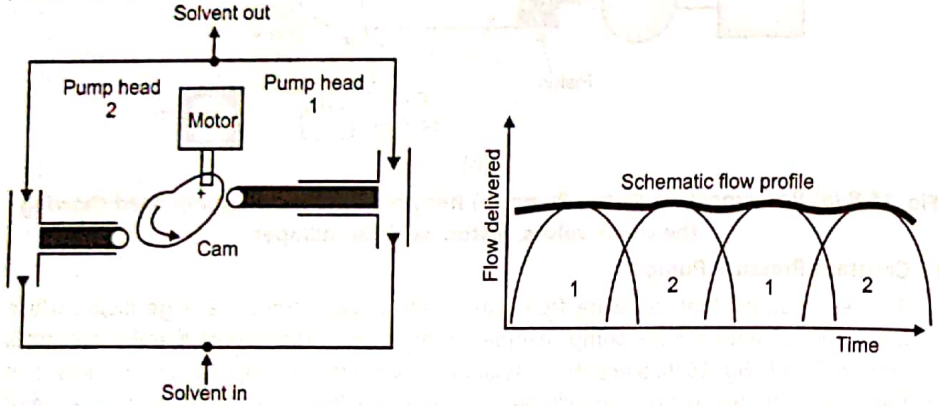


Fig. 15.10: Cam driven, dual head reciprocating piston pump capable of delivering constant flow with relatively low pulsation. Flow rate is controlled by cam rotation frequency

Pulse Dampers:

These are the useful set up in addition to cam designer. Most reciprocating pumps incorporate pulse dampers. Pulse dampers consist of long and narrow tubings folded back on them many times and placed between the pump and the injector. They receive the solvent during the delivery stroke and discharge during the refilling stroke through a restrictor. This set up minimize the fluctuations and results in pulseless flow. The limitation of setting up of pulse dampers is the increase in system volume which makes the solvent change over particularly in the gradient elution, making it inconvenient.

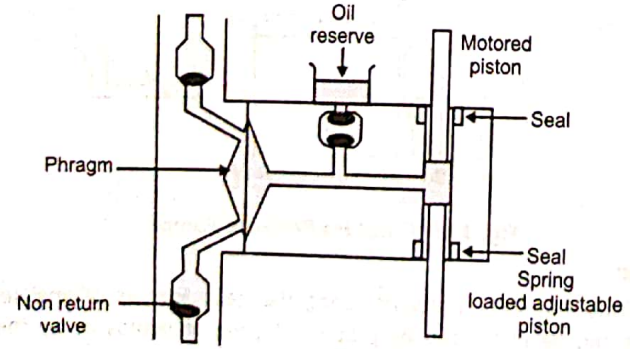


Fig. 15.11: Syringe pumps

Other than the above types of HPLC pumps there are other types of pumps classified according to their flow rates that include standard bore pumps, microbore pumps, and preparative pumping systems. Further on the basis of the materials used for construction of pumps they are classified as, metallic and non-metallic pumps. A metallic pump uses metals like titanium or steel may be used. In case of non-metallic pumps, Polyether ether ketone (PEEK), Polytetrafluoroethylene (PTFE or Teflon), ceramic etc. are used.

15.3.3 Precolumn

Some HPLC instruments are equipped with a precolumn, which contains a packing chemically identical to that in the analytical column. Particle size is large; hence the pressure drop across the precolumn is negligible with respect to the analytical column. The precolumn is mainly used to remove the impurities from the solvent and thus prevent contamination of the analytical column.

15.3.4 Sample Injectors

Often the limiting factor in the precision of liquid chromatographic measurements lies in the reproducibility wherewith samples can be introduced into the column packing. It must be noted that overloading of the sample causes band broadening. Therefore, minimum amount of sample must be introduced. It is convenient to introduce the sample without depressurizing the system. The sample is usually injected at the head of the column with minimum disturbance of the column material.

There are different types of sample injection systems in practice.

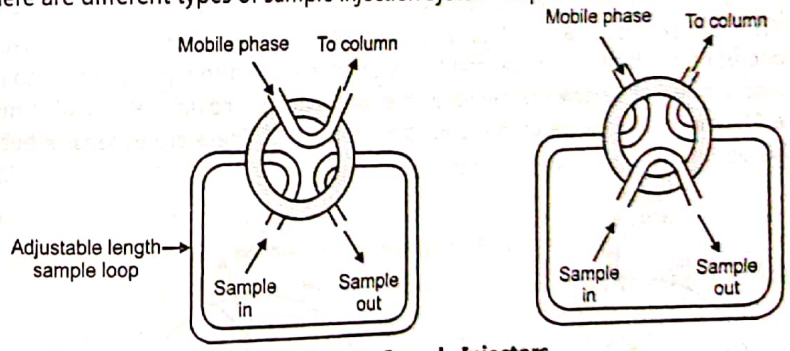


Fig. 15.12 : Sample Injectors

Earlier Methods:

In earlier days, samples were injected through a self-sealing rubber septum. This method is very simple method. For this purposes usually syringes are used. But the method lacks reproducibility.

The other method is called as "stopped flow method". In this method mobile phase flow is stopped for a while and the sample is injected through the opening at the top of the column packing. After injecting the sample the opening is closed and mobile phase flow is restarted. This is also simple method and both methods are still used in the home-made instruments.

Current Methods:

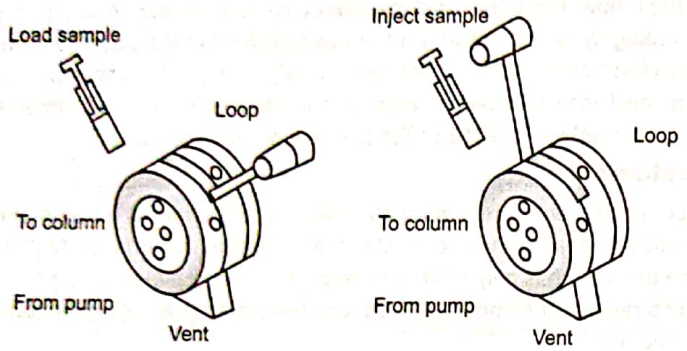


Fig. 15.13 : Loop injectors

Nowadays the above methods are replaced by the incorporation of valves for injection. There are two types of valves:

1. External loop valve injector
2. Internal loop valve injector

1. External Loop:

It has 6 ports, out of which 2 ports connected with a fixed volume sample-loop and other 4 ports are used to carry the mobile phase and sample in and out of the valve. The valve consists of two positions namely 'load' and 'inject'. At load position, sample is loaded into the loop using a microlitre syringe and the position is rotated to inject position to inject the fixed volume of sample contained in the loop into the column along with mobile phase. The minimum volume of this loop starts from 10 µl. These are expensive but give precise results.

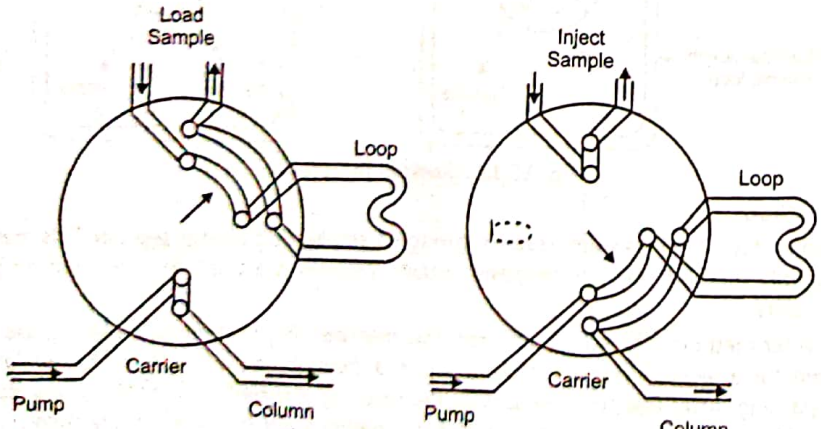


Fig. 15.14: External loop

2. Internal Loop:

This type of valves consists of 4 ports with an internal loop. In this, the sample loop is an engraved slot in the body of the valve. These types are used for small volume injection. This also has the same functioning as like external loop valve.

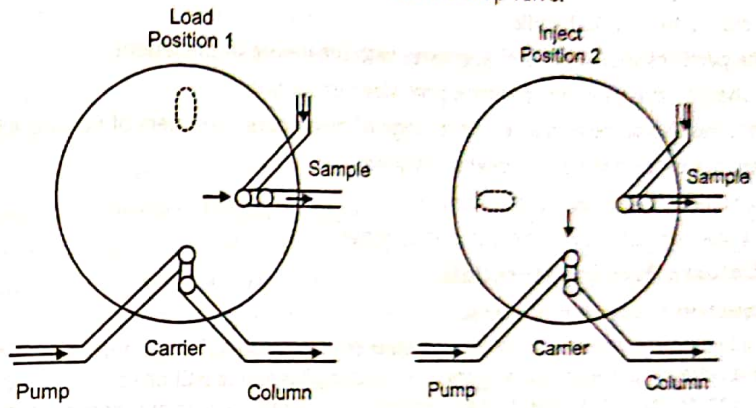


Fig. 15.15: Internal loop

15.3.5 Liquid Chromatographic Columns

They are usually constructed from smooth bore stainless steel tubing or heavy-walled glass tubing. If prepared from heavy walled glass tubing, then pressure is restricted to lower than 600 psi. Occasionally, you may come across coiled columns, but their use is very limited.

The columns are of two types: *(a)* Analytical *(b)* Preparative.

For analytical columns:

Size: Length: 25–100 cm with internal diameter of 2-6 mm.

For preparative columns:

Size: Length: 25-100 cm and internal diameter of 06 mm or more.

The common particle size is 5-10 µm; recently manufacturers have been producing high speed, high performance micro-columns which have smaller dimensions.

Length: 3-7.5 cm and internal diameter of 1-4.6 mm; particle size: 3 or 5 µm.

The main advantage of these columns is speed and minimum solvent consumption. These columns should be provided with a system for temperature control to withstand high pressure.

Requirements for an Ideal HPLC Column:

An ideal HPLC column should fulfill the following conditions.

1. It should have uniform column packing.
2. It should have spherical particles.
3. The particle diameters should be ranging from 3 to 10 µm.

4. The porosity of the particles should be in the range 50-70%, extending to 80% for the size-exclusion chromatography.
5. The column should withstand the pressure during operation.
6. It should give reproducible results.
7. It should be easy to handle.
8. The particles should not shrink or swell with the nature of the eluent.
9. It should have a uniform particles pore size distribution.
10. Particles should be available with a range of mean pore diameters of 60-100 A°.
11. Column packing should be chemically inert.
12. It should provide reproducible results.
13. It should be easily available and cost effective.

15.3.6 Column Packing Materials

Construction Materials of Column:

Most columns are constructed from 316 stainless steel, Glass, Teflon and PEEK. Columns are also available for use with more aggressive mobile phases like HCl or solutes like proteins that may adsorb to the stainless steel. Polymeric columns are more common for ion-exchange packings while glass is commonly used for protein separations.

Types of Columns:

(i) Guard Column: Guard column is the column placed before the analytical column. It contains a packing chemically identical to that in an analytical column with large particle size. The pressure drop across the precolumn is negligible as compared to that in the analytical column. These columns are otherwise called as precolumns. They are used to protect the analytical column from the impurities and other contaminants from solvent. It also removes the components that bind irreversibly to the stationary phase. The guard columns are compulsorily used during the bioanalytical studies to protect the analytical column from biological matrix.

(ii) Analytical Column: Analytical column is considered as the heart of an HPLC system. The reason is that it is the part where the separation of the mixture takes place. The efficiency of the separation purely depends on the column.

Types of Analytical Columns:

(a) Small-bore Columns: Small-bore or microbore is the term used for HPLC columns having the diameter less than about 2 mm. They are also known as 'microcolumns'. One of the advantages of using small-bore columns is that they are operated at much lower mobile phase flow rates than the 4.6 mm columns, so there is a large reduction in the solvent consumption.

(b) 3 × 3 Columns: Short (3.3 × 4.6 mm) columns packed with 3 µm bonded silica stationary phases have sufficient efficiency for many separations. They are commonly called as 3 × 3 columns and compared to the conventional columns, they offer the following advantages.

(c) Monolithic Columns: It is a type of column used in HPLC that has porous channels rather than being packed with beads. They have a structure different than that of the traditional columns. Their construction is more similar to rod with random channelling and outcroppings.

Types of Packing used in HPLC Columns:

Different types of packing used in HPLC are as follows:

(a) Porous
(b) Pellicular
(c) Bonded phases.

(a) Porous Packings: Porous microparticles are the most commonly used stationary phase particles in modern HPLC. They consist of fully porous particles that can be either irregular or spherical in shape. The diameters are ranging from 3 to 10 µm. The pores provide the surface with which the sample interacts. Particles with smaller pore size exhibit a larger surface area and therefore have greater retention. Large particles like proteins require a large pore size. The particles are composed of silica, alumina, the synthetic resin polystyrene-divinylbenzene, or an ion-exchange resin. Silica is by far the most common packing used in LC.

(b) Pellicular Packings: Pellicular materials consist of a solid spherical bead of relatively large inner diameter with a thin outer layer of stationary phase. The original pellicular particles were spherical, non-porous, glass or polymer beads with typical diameters of 30 to 40 µm. A thin, porous layer of silica, alumina, a polystyrene-divinyl benzene synthetic resin, or an ion-exchange resin is deposited on the surface of these beads. They are much useful for the ion-exchange chromatography. They give higher efficiencies (lower HETP) than the porous particles, but are restricted to small sample loadings due to their low active surface area. The advantages include ease of packing, long term stability and low cost.

(c) Bonded-Phases: The supports for majority of the bonded phase packings used in partition HPLC are prepared from rigid silica, or silica-based compositions. These solids are formed as uniform, porous, mechanically sturdy particles commonly having diameters of 1.5-10 µm, with 3 and 5 µm particles being the most common ones. The surface of fully hydrolyzed silica (hydrolyzed by heating with 0.1 M HCl for a day or two) is made up of chemically reactive silanol groups.

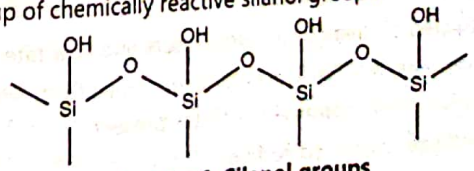


Fig. 15.16: Silanol groups

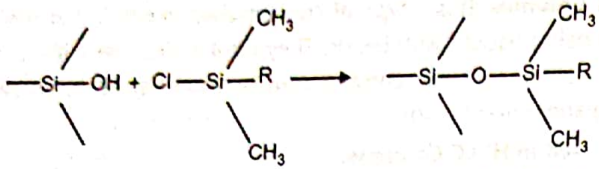


Fig. 15.17: Reaction for silanization (R is an alkyl or a substituted alkyl group)

The most useful bonded-phase coatings are siloxanes formed by the reaction of the hydrolyzed surface with an organochlorosilane, as shown in the above Fig. 15.17.

End Capping:

The unreacted SiOH groups, unfortunately, impart an undesirable polarity to the surface, which may lead to the tailing of the chromatographic peaks, particularly for basic solutes. To lessen this effect, siloxane packings are frequently capped by further reaction with chloromethylsilane that, because of its smaller size, can bond some of the unreacted silanol groups. This process is called as End capping. Bonded phase packings can be classified as reversed phase when the bonded coating is non-polar in nature and as normal phase when it contains polar functional groups.

Column Temperature Control:

For some applications, control of column temperature is not necessary and the columns are operated at room temperature. Often, however, better, more reproducible chromatograms are obtained by maintaining a constant column temperature. Most modern equipments are now equipped with column ovens to maintain the temperature.

7. Detectors:

A detector is required to sense the presence, and the amount of sample component in the column effluent. A detector that measures property possessed by both mobile phase and solute is called **bulk property detector**, e.g. Refractive Index detector.

If the solute possesses the property e.g. absorption of UV/visible light of electrochemical property, the detectors are called a **solute property detector**.

A good detector should have the following features:

(a) It should respond to all components of the mixture in a wide range of mobile phases.

(b) It should not respond to mobile phase.

(c) It should be unaffected by changes in temperature and flow rate.

(d) It should have high sensitivity, i.e. larger detector signal for smaller amount of solute. Low noise and a wide linear response to solutes present.

(e) It should not constitute to zone spreading.

(f) Non-destructive, inexpensive, reliable and easy to use.

Generally two types of detectors are used:

1. Refractive index monitors detectors:

Since, every compound has its own refractive index, this property becomes a universal indicator. A differential refractometer continuously monitors the difference in RI between the pure mobile phase (reference stream) and the column effluent. The advantages of these detectors are: (a) They respond to nearly all solutes. (b) They are reliable and unaffected by flow rate. (c) They do not require any double bond or aromaticity to be present in the structure for elucidating a response while disadvantage is that there must be a difference between the refractive index of the solutes and of the mobile phase. Besides, this is not a sensitive detector.

Construction of Refractive Index monitor detector:

1. Several different designs of a refractive index detector have been used in HPLC, one of which is deflection refractometer. [See Fig. 15.18].

Light from the source is focused on the cell, which consists of sample and reference chambers separated by a diagonal sheet of glass. After passing through the cell, the light is diverted to a beam splitter 'B' to two photocells P_1 and P_2. A change in the refractive index of the sample stream causes a change in the relative amounts of light falling on P_1 and P_2 and therefore a difference in their relative output. The difference is amplified, giving an error signal at the amplifier output that operates a servo-meter which rotates the beam splitter until the error is reduced to zero. The beam splitter movement is proportional to the difference in refractive index and is measured by the recorder.

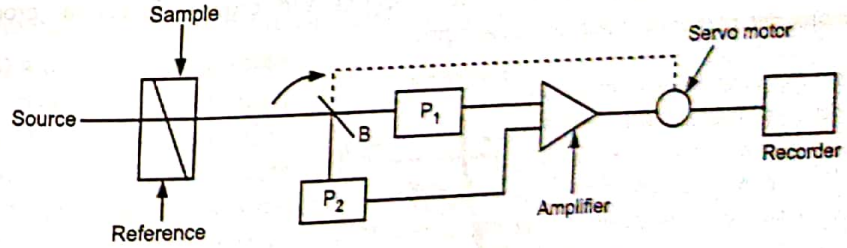


Fig. 15.18

2. UV-visible absorption detector:

The principle is that the mobile phase from the column is passed through a small flow cell held in the radiation beam of the UV/visible spectrophotometer. These detectors are selective, in that they detect only those solutes that absorb UV/visible radiation e.g. alkenes, aromatic compounds and compounds having multiple bonds between C and O, N or S.

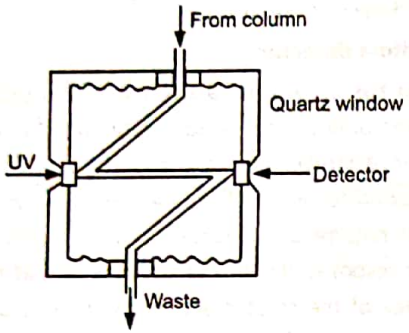


Fig. 15.19: Flow cell for UV/visible absorbance detector

This is 1000 times more sensitive than RI detectors. A low pressure mercury lamp acts as a source. Alternatively, a Deuterium lamp or a tungsten filament with intermediate filters can also act as a source.

Both fixed and variable wavelength UV/visible detectors are available, the latter can operate between 190–700 mm with number of absorbance ranges. Fixed wavelength detectors can operate at 254 nm, 280 nm or other wavelength where most organic compounds, double bonds/aromatic groups cause absorption. Since, most common solvents do not absorb in UV/visible region, the detector is sensitivity free from interferences.

UV Absorption Detectors with Filters:

These detectors use some additional substitution filters to use additional lines such as at 250, 313, 334 and 365 nm. For solute that absorb at one of these wavelengths, these detectors cannot be used. Several inorganic species and organic functional groups encompass one or more of these UV wavelengths.

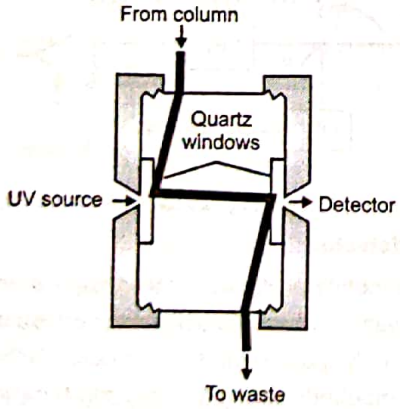


Fig. 15.20: A typical Z-shaped UV flow-through cell

Absorption Detectors with Scanning Capabilities:

These detectors are provided with scanning spectrophotometer with grating optics. This provides various benefits such as single wavelength analysis and multi-wavelength analysis. Best wavelength for each eluent can be selected in a mixture and analysed.

Photodiode Array (PDA):

These are the most powerful UV-visible detectors. The detection is carried out in the UV-visible region. A DAD has multiple photodiode arrays. Using this detector a wide range of wavelengths can be employed at a time. At an interval of 1 second or less the spectra of analytes are recorded. In addition to retention time the comparison of spectra will give the proper identification of the analyte. The light from the lamps directly approach the flow cell, dispersed by the diffraction grating, and the amount of the dispersed light is estimated for each wavelength in a photodiode array.

Disadvantages (compared to UV-VIS detectors):

(i) Noise is large as the amount of light is small,

(ii) DAD is susceptible to various changes like lamp fluctuations, because the reference light cannot be received.

However, DAD has recently been improved to reduce its difference from performance from UV detectors.

(ii) Fluorescence Detectors:

Fluorimetric detectors used In HPLC are similar in design to the fluorometers and spectrofluorometers used for fluorometry. In most of these, fluorescence is observed by a photoelectric transducer located at 90° to the excitation beam. Mercury excitation source or xenon source and monochromator to isolate the fluorescence radiation are used. The advantage of the fluorimetric detectors is their higher sensitivity.

(iii) Amperometric Detectors:

These detectors are based on measurement of electrochemical detection. The detector has three types of electrodes which include, reference electrode, working electrode and auxiliary electrode. The potential of working electrode is set relative to the reference electrode. When the analyte which is electroactive substance reaches the detector, it is subjected to reaction of either oxidation or reduction in the electrodes. This reaction creates some current flow in the electrode due to the transfer of electrons. The electrodes are connected to an electronic circuitry and electric current signal is amplified and measured as signal.

Other detectors:

(i) Infrared Absorption Detectors:

IR spectrophotometer and FTIR spectrophotometer have been used for HPLC. IR detector cells are similar in construction to those used in the UV instruments, except that the IR cuvettes are made up of Sodium chloride or Calcium fluoride. Cell path lengths range from

0.2 to 1.0 mm and volumes from 1.5 to 10 µL. A major limitation in the use of the IR detectors is the low transparency of many useful solvents. Also, the use of aqueous mobile phases is restricted.

(ii) Evaporative Light Scattering Detector (ELSD):

In this, the column effluent is, converted into a fine mist by a flow of nitrogen or air using a nebulizer. The fine-droplets are passed through a heated tube where the mobile phase evaporates, leaving fine particles of the analyte. This is then passed through a laser beam. The scattered radiation is then detected at the right angles to the flow by a silicon photodiode.

(iii) Mass Spectrometric Detectors:

Nowadays the highly sophisticated mass spectrometric detectors are widely used due to their sensitivity and reliability. When mass spectrometer is used as a detector for LC (LC-MS), it can greatly aid in identifying species as they elute from a chromatographic column. Various interfaces are used to couple LC with MS.

8. Recorders:

The signals from a detector are recorded as deviations from a baseline. Two pen recorders are used with instruments having two detectors. The peak position along the curve relative to the starting point denotes the particular component. With proper calibration, the peak is a measure of amount of component in a sample.

Applications of HPLC:

HPLC is widely used for various applications and widely accepted technique for separation.

1. **Pharmaceutical Industries:** HPLC has got a prime importance is the pharmaceutical industry. It is used in the departments such as R&D, quality control and F&D. It is used for the analysis of samples from initial step that is raw material analysis till the final product analysis.

   E.g. reverse phase HPLC is used for the analysis of polar compounds and polyphenols, steroids, vitamins etc. Normal phase HPLC is used for non-polar compounds.

   It is mainly used for the assay of pharmaceutical drugs. The chromatographic method is developed by optimizing to mobile phase, composition pH, flow rate, column temperature, and validated to ensure the applicability of the method. Multi component analysis can be done conveniently by HPLC.

2. **Stability Studies:** It is widely used for the stability study of pharmaceutical products. Stability of the products is comparatively analysed with standard. In addition the specificity of the analytical method is checked by doing formed degradation studies such as acid, base, oxidation and photo degradation.

3. **Bioanalysis**: HPLC is much more useful for the bioanalytical studies. It is used to determine the drug in biological matrices such as blood, plasma, urine, serum and faeces. Thus it is useful in pharmacokinetics and bioequivalence studies.

4. **Natural Product Analysis:** HPLC is helpful for the standardization of herbal extracts in terms of marker constituents. Example curcuminoids from turmeric extract; withanolides from Ashwagandha extracts. It is also used to standardize polyherbal formulations.

5. **Food Analysis:** HPLC has got a great role in food products analysis. Analysis of honey samples for methylglyoxal content, food formulations, and dairy products. Sugar analysis can be done in the food products using HPLC coupled with refractive index detector.

   The nutritional value of nutraceuticals can be done by HPLC by analysing their flavonoid contents, amino acid and polyphenol contents. Depending on the nature of the sample, the column is selected.

6. **Drug Interaction Studies:** It is a useful tool in drug-drug interactions and Herb-Drug interaction studies. The pharmacokinetic pattern of biosamples containing individual drug and combination of Herb-Drug and Drug-Drug are compared to predict possible interactions.

7. **Preparative Analysis:** Preparative HPLCs are used to isolate the separated component from a mixture. This HPLC has a major role in the isolation of phytoconstituents from herbal fractions after column chromatography.

8. **Forensic Sciences:** HPLC is much useful in the forensic analysis to identify and quantify the compounds in blood, plasma, serum and urine. For example: the presence of morphine can be analysed in biological matrices. During suicidal cases the identification of poisonous substances can be analysed by HPLC.

9. **Cosmetics:** HPLC is a useful tool in the cosmetics industries. Many cosmetic products can be analysed by various HPLC methods especially the herbal based cosmetic products are analysed by HPLC for the active contents. Both qualitative and quantitative analysis is carried out using HPLC.

Assay of the following drugs can be performed by high performance liquid chromatography.

Drug	Column	Mobile Phase
1. Alprazolam and it's tablets	Stainless steel column packed with porous silica particles (5-10 µm)	Acetonitrile, chloroform, 1 butanol, water, glacial, acetic acid (850:80:50:20:05)
2. Amitriptyline HCl tablets	Stainless steel column packed with octadecyl silica chemically bonded to porous silica or ceramic micro-particles (5-10 µm)	Acetonitrile: Water (Equal volume) + 0.03 M sodium hexane sulphonate pH adjusted to 4.5 by glacial acetic acid.
3. Betamethasone sodium phosphate tablets	Stainless steel column packed with octadecyl silane chemically bonded to porous silica or ceramic micro-particles (5-10 µm)	Citrophosphate buffer pH 5.0 + methanol (55:45)

Drug	Column	Mobile Phase
4. Captopril tablets.	Stainless steel column packed with octadecyl silane chemically.	Methanol + water containing 0.05 volume of phosphoric acid (55:45)
5. Cefadroxil and its tablets, capsules and oral suspension.	Stainless steel column packed with octadecyl silane chemically.	Phosphate buffer pH 5 + Acetonitrile (96:4)
6. Chlorambucil tablets	——— • ———	Acetonitrile + 0.02 M Potassium dihydrogen phosphate (60:40)
7. Ciprofloxacin	——— • ———	0.025 M phosphoric acid previously adjusted with thioethanolamine to pH 3 and Acetonitrile (87:13)
8. Diclofenac sodium injection.	Stainless steel column packed with octyl silane chemically bonded to totally porous silica particles (5 – 10 µm)	Methanol + 0.1 M sodium acetate solution (60:40)
9. Diltiazem HCl and its tablets.	Stainless steel column packed with octadecyl silane chemically bonded to porous silica or ceramic micro-particles (5-10 µm)	Buffer (0.116% w/v d- 10-Camphorsulfonic acid in 0.1 M Sodium acetate pH adjusted to 6.2 by 0.1 M sodium hydroxide) + Acetonitrile + Methanol (50:25:25)
10. Enalepril maleate and its tablets.	Stainless steel column packed with rigid spherical styrene Divinyl benzene copolymer (5-10 µm)	Phosphate buffer pH 6.8
11. Folic acid tablets.	Stainless steel column packed with octadecyl silane chemically bonded to porous silica or ceramic micro-particles (5-10 µm)	0.05 M potassium dihydrogen phosphate + Acetonitrile adjusted to pH 6 with sodium hydroxide (93:7)
12. Fusidic acid oral suspension	Stainless steel column packed with octadecyl silane chemically bonded to porous silica or ceramic micro-particles (5-10 µm)	Acetonitrile + 1% v/v solution of glacial acetic acid + methanol (60:30:10)
13. Guggulipids and its tablets.	——— • ———	Acetonitrile + water (65:35)

Drug	Column	Mobile Phase
14. Haloperidol tablets	——— • ———	1% w/v solution of ammonium acetate + Acetonitrile (55:45)
15. Insulin	——— • ———	0.1 M sodium dihydrogen phosphate adjusted to pH 2 with phosphoric acid + Acetonitrile (72.5:27.5)
16. Methotrexate and its injection	——— • ———	Phosphate buffer of pH 8 + Acetonitrile (92:8)
17. Norfloxacin tablets	——— • ———	0.1% v/v of phosphoric acid + Acetonitrile (85:15)
18. Omeprazole and its capsules	——— • ———	Phosphate buffer pH 7.4 + Acetonitrile (65:35)
19. Pyroxicam and its capsules	——— • ———	Methanol + Buffer solution prepared by diluting mixture of 7.72 g of anhydrous citric acid in 400 ml water + 5.35 g of sodium phosphate in 100 ml to 1000 ml with water (45:55)
20. Ranitidine HCl and its injection, tablets.	——— • ———	Methanol + 0.1 M Ammonium acetate (85:15)
21. Pyrimethamine and Sulphadoxine tablets	Stainless steel column packed with octadecyl silane chemically bonded to porous silica or ceramic micro-particles (5 to 10 µm)	0.1 % v/v glacial acetic acid + Acetonitrile (4:1)
22. Salbutamol sulphate tablets	Stainless steel column packed with spherical particles of silica, surface of which has been modified by chemically bonded nitrile groups.	Water + 0.5 M Ammonium acetate + 2 Propranolol, pH of mixture adjusted to 4.5 (65:30:5)
23. Thiamine HCl injection and tablets.	Stainless steel column packed with octadecyl silane chemically bonded to porous silica or ceramic micro particles (5 to 10 µm)	1 gm of sodium heptanes sulphonate dissolved in a mixture of 180 ml methanol and 10 ml of methyl amine diluting to 1000 ml with water and adjusting the pH to 3.2 with phosphoric acid

Drug	Column	Mobile Phase
24. Triamcinolone acetamide injection	———— • ————	Methanol + Water + (56:44)
25. Vinblastine sulphate	Stainless steel column packed with octyl silane chemically bonded to porous silica particles (5 to 10 µm)	Methanol + 1.5% v/v diethyl amine + adjusted to pH 7.5 with phosphoric acid + Acetonitrile (50:38:12)
26. Esmolol Tablets	Stainless steel column 30 cm x3.9mm ODS (5 µm)	Mixture of 3.0g potassium dihydrogen phosphate in 650 ml of water, acetonitrile and methanol (65:15:20)
27. Rabeprazol sodium	Stainless steel coloumn 25 cmx 4.6 mm C8 (5 µm)	0.1M Phosphate buffer (pH 7.0) and acetonitrile (72:28)
28. Ritonavir	Stainless steel coloumn 15 cmx 4.6 mm ODS(5 µm)	Acetonitrle and buffer (3.4g of sodium acetate and 0.94g of hexane sulhonate in 1000 ml water) adjusted to pH 4.0 with HCL (45:55)

REVIEW QUESTIONS

1. Write the principle of HPLC.
2. What do you mean by normal phase and reverse phase columns?
3. Write a note on HPLC columns.
4. Write a note on packings used in HPLC columns.
5. Discuss the instrumentation involved in HPLC in details.
6. Write a note on different types of pumps used in HPLC.
7. Write a note on injectors in HPLC.
8. What are the detectors used in HPLC. Explain any two in detail.
9. Write the applications of HPLC.

UNIT - V

Chapter ...16

ION EXCHANGE CHROMATOGRAPHY

Objectives:

Upon completion of this section, the student should be able to

- Understand the principle and theory of Ion exchange chromatography.
- Understand the technique used in Ion exchange chromatography.
- Describe the types of resins used, preparation of stationary phase and conduct of experiment of Ion exchange chromatography.
- Explain the applications of Ion exchange chromatography.

16.1 INTRODUCTION

Ion exchange is probably the most frequently used chromatographic technique for the separation and purification of proteins, polypeptides, nucleic acids, polynucleotides, and other charged biomolecules. This technique is based on the interaction between charged solute molecules and oppositely charged moieties covalently linked to a chromatography matrix. The method is known for its widespread applicability, high resolving power, high capacity, simplicity and controllability. Ion exchange starts with the equilibration of the exchanger using pH, and ionic strength. During equilibration, the exchangeable groups are associated with counter ions. Once equilibrium is reached and the sample is added, the molecules undergo addition and adsorption with an appropriate charge that displace the counter ions and bind reversibly to the matrix. The unbound materials will pass through the column with the void volume. In the third stage, substances are removed from the column by increasing the ionic strength of the eluting buffer.

Ion exchange material is water insoluble solid of complex structure comprising ions capable of exchanging with ions in the surrounding medium in a reversible process. Since, the process involves exchange of ions, the material is called exchanger.

Use of inorganic clays to soften water was recorded long ago. Soils, clays and zeolites (natural and synthetic) are used to soften water. The ion exchange capacity of these materials differs markedly. Zeolite has three dimensional network structure with regular pores through which water can pass. Thus, if water containing calcium or magnesium ions is passed through a bed of zeolite containing sodium ions, exchange takes place and water gets purified.

$$Ca^{2+} + 2\,Na^{+}Z \rightarrow 2Na^{+} + Ca^{2+}Z \quad \text{where, Z = zeolite}$$

Zeolite type ion exchanger besides being unstable towards acids and bases cannot be used over wide pH range.

Since the synthesis of sulphonic acid and polyamine resins by Adams and Holmes in 1935, a large variety of synthetic resins have been developed. Synthetic resins have larger ion exchange capacity than the resins of natural resources. The synthetic resins are prepared from styrene, Divinyl benzene (DVB), phenolsulphonic acid, phenol-formaldehyde, various amines, etc. (a cross linking agent imparting strength to the polymer by joining the chains of various position).

Principle:

The principle involved in the ion exchange chromatography is the ionic interaction or the attraction between the oppositely charged ions. The stationary phase is a polymer matrix in which charge bearing functional group is attached. Charged molecules bind electrostatically to oppositely charged functional groups that have been bound covalently on the matrix. Ion exchange chromatography is a type of adsorption chromatography so that, charged molecules adsorb to ion exchangers reversibly. This allows the molecules to be bounded or eluted by changing the ionic environment. Ion exchangers can be used in column chromatography to separate molecules according to charge; an ion exchanger is usually a three-dimensional network or matrix that contains covalently attached charged groups. It can be a positively charged group or negatively charged group and called as cationic exchanger and anion exchanger respectively. The negatively charged group will exchange positive ions. The positively charged group will exchange negative ions. The negative charge of the ion exchanger, e.g. COOH group results only when the pH is sufficiently high to permit dissociation of the COOH site. Then it will attract positive ions. Similarly the positive charge of ion exchange site (e.g. tertiary amine) results only when the medium is acidic. The association of protons with basic groups results in positive charge. Then the positive charge will attract negative ions.

16.2 THEORY

Separation in ion exchange chromatography depends upon the reversible adsorption of charged solute molecules to immobilized ion exchange groups of opposite charge.

The ion exchange process can be explained by the following five main stages. These steps are illustrated schematically below.

- In the first stage, the ion exchanger is in a state, which allows the binding of the desired solute molecules depending upon its pH and ionic strength. The exchanger groups are associated at this time with exchangeable counter-ions (usually simple anions or cations, such as chloride or sodium).
- In the second stage adsorption takes place. When sample is applied, the sample molecules due to their charges displace counter-ions and bind reversibly to the ion exchanger. Unbound molecules can be washed out using starting buffer.
- In the third stage, desorption occurs. The substances that are bound to the ion exchangers are removed by increasing the ionic strength of the mobile phase or changing its pH. This process is called as desorption. Introduction of an increasing salt concentration gradient results in elution of substances in the order of their strengths of binding. The strongly bound substances eluted later and weakly bound substances will be eluted first.
- In the fourth stage the strongly bound substances are completely removed from the column.
- In the fifth stage re-equilibration is performed for the next purification. Different degrees of interaction of substances with the ion exchangers due to differences in their charges, charge densities and distribution of charge on their surfaces lead to separation. Ionic strength and pH can be varied to control these interactions.

16.3 ION EXCHANGE MATERIALS

Various materials possess varying ion exchange capacity, which can be classified:

(a) Synthetic inorganic ion exchangers:

These materials have a relatively open three-dimensional framework structure with channels and interconnecting cavities e.g. alumina-silicate, TiO_2, ThO_2, zirconium oxide, phosphate etc.

The hydrous oxides of tri- and tetravalent metals are useful as cation exchangers. Besides, phosphate, molybdate, tungstate, vandate etc. of some metals act as cation exchanger materials.

(b) Natural organic ion exchangers:

Coal, paper, cotton and the like can be converted into cation exchange by reaction of sulphonation or phosphorylation. They act as cation exchangers since they carry sulphonic acid or carboxyl groups attached to them. These materials are less uniform in structure and get readily affected by other chemicals.

(c) Synthetic organic ion exchangers:

The synthetic ion exchanger resins are made of cross-linked polymer network to which are attached various functional groups. The nature of functional group determines whether it is a cation exchanger or an anion exchanger. In cation exchanger materials, the acid groups are sulphonic acid, carboxylic acid or phenolic, while in anion exchanger resins the groups are basic as amine, quaternary ammonium, etc. The number and type of functional group is the determiner to ascertain whether the resin is a strong or a weak exchanger.

There are many types of synthetic ion exchangers with different physical and chemical properties. Some are liquid in nature such as long chain water immiscible aliphatic amines which act as anion exchanger or diallyl phosphate of fatty acid as cation exchanger.

16.4 SYNTHETIC ION EXCHANGE RESINS

These may be regarded as polymers consisting of three dimensional hydrocarbon networks to which are bonded a large number of electrically charged groups. Originally, the term resin was applied to naturally occurring amorphous solids such as amber, shellac, rosin, copal etc. Many polymeric substances occur in nature such as cellulose, rubber, starch, proteins and resins. Currently, the term resin is used for synthetic polymers which are similar to natural resin in physical properties.

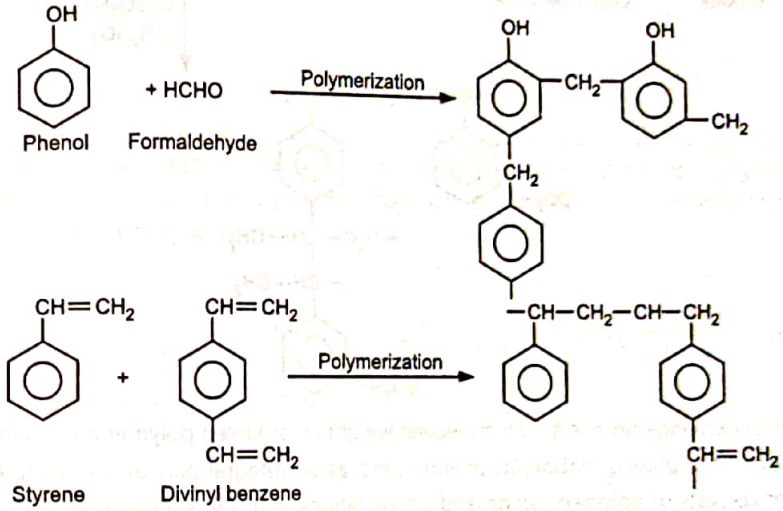
These polymers are cross-linked, show thermosetting properties (polymers which change irreversibly into hard and rigid materials on heating) due to three-dimensional network structure. These synthetic polymers are made from small units of chemicals by polymerization either by addition or condensation reactions.

The ion exchange resin should meet the specified standards:

1. It should have a sufficient degree of cross-linking (approximately 4-8%) for use in chromatography.
2. It should be insoluble in common solvents.
3. It must be chemically stable.
4. It should be sufficiently hydrophilic to permit diffusion of ions through its structure at a constant and finite rate.
5. The swollen resin must be denser than water.
6. It should have desired particle size and shape.
7. It must contain sufficient number of ion exchange groups.
8. The resin should have ability of regeneration and reuse.
9. The resins may exchange cation or anion therefore they are termed as cation or anion exchange resins.

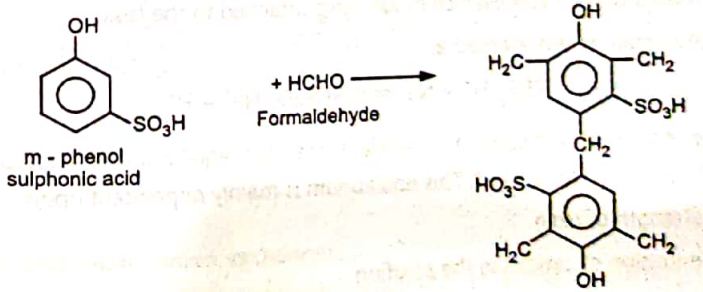
16.5 MANUFACTURE OF ION EXCHANGE RESINS

Ion exchange resins are manufactured by different chemical reactions of polymerization, condensation or by addition reactions. Generally, hydrocarbon network is built by polymerization using phenol-formaldehyde or styrene and divinyl benzene as:



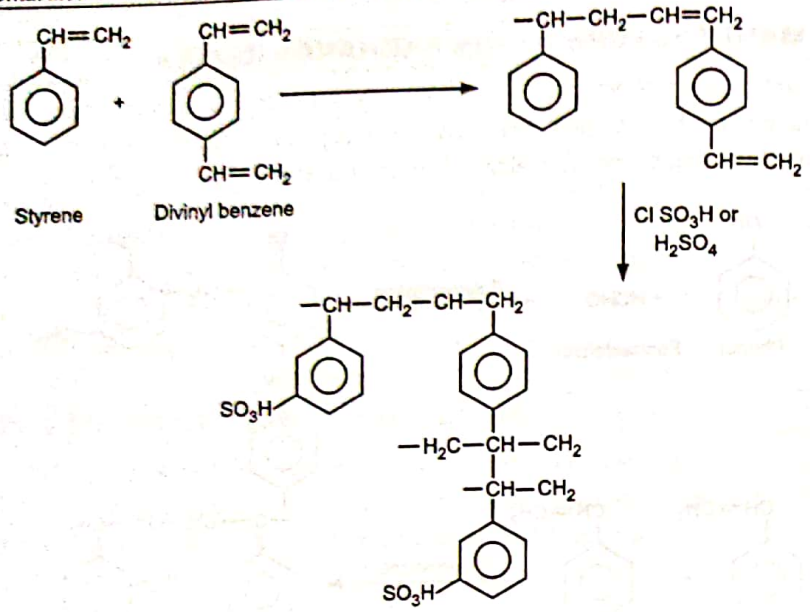
Suitable chemical reactions are now brought about on the polymerized material to convert them into cation exchange or anion exchange resin.

(a) Cation exchange resin: These resins are synthesized by using phenol or substituted phenol and formaldehyde by polymerization as



Or

From Styrene and Divinyl benzene as



A cation exchange resin is a high molecular weight cross linked polymer containing acidic groups such as sulphonic, carboxylic, phenolic, etc. as an integral part of the resin. A cation exchanger consists of polymeric anion and active cations. But the resin as such is electrically neutral. The resins containing sulphonic group are considered as strongly acidic cation exchange resins. Thus, when the resin is treated with a strong acid (5% HCl), entire sulphonate is converted to acid form (hydrogen form). This resin acts as a strong acid which can be represented as $RSO_3^-H^+$ where, R represents the resin network. Now, if the salt solution is passed through the hydrogen form of resin, the H^+ will be replaced by an equivalent amount of the cation, which in turn gets attached to the resin.

The reaction may be represented as:

$$RSO_3^- H^+ + Na^+ \rightleftharpoons RSO_3^- Na^+ + H^+$$

From the above equilibrium, it is evident that for equilibrium of sodium ion, one equivalent of hydrogen ion is freed. This equilibrium is mainly dependent upon:

I. Acid strength of resin.

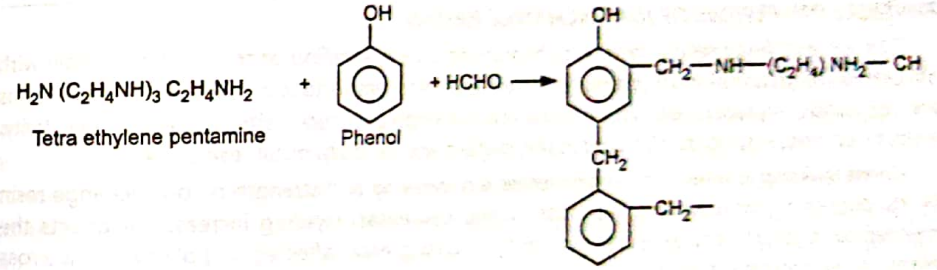
II. Concentration of cations in the solution

For a strong cation exchange resin, the exchange affinity for cations depends upon the charge of cations.

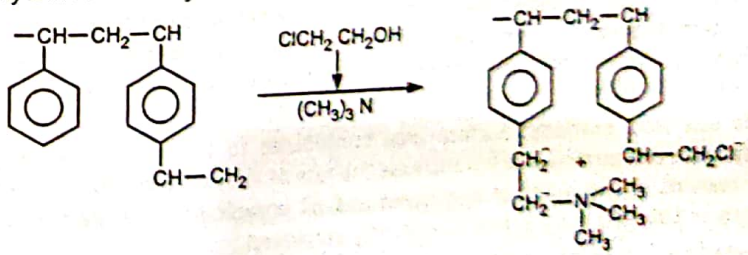
Some of the commercially available cation exchange resins are:

	Commercial Name	Functional Group
1.	Amberlite IR – 100 IR – 105 IR – 1019	– OH, – CH_2SO_2OH
2.	Amberlite – 200	– SO_3H
3.	Amberlite IRC – 50	– COOH
4.	Zeolite	– SO_3H
5.	SE-cellulose	– C_2H_4 – SO_3H

(b) Anion Exchange Resins: Like cation exchange resins, anion exchange resins are prepared by condensation and polymerization of various aliphatic or aromatic amines with phenol and formaldehyde as



Alternatively anion exchanger resins are prepared from the transparent beads obtained by the co-polymer of styrene and divinyl benzene. The polymer is treated with epichlorohydrin followed by treatment of trimethylamine.



Quaternary ammonium anion exchanger:

An anion exchange resin is a polymer containing an amine or quaternary ammonium groups as integral parts of the resin and an equivalent amount of anions. Anion exchange resin can be denoted by RNH_2. The anion exchange resin when treated with hydrochloric

acid, the substituted ammonium cation is obtained as RNH_3Cl^-. When it is treated with solutions of any ionized material, the exchange takes place as:

$$2RNH_3Cl^- + 2H^+ + SO_4^- \rightleftharpoons [RNH_3^+]_2 SO_4^{2-} + 2\,HCl$$

Anion exchange resin thus functions similar to cation exchange resins. Some of the anion exchange resins commercially available are:

Commercial Name	Functional Group
1. Amberlite IRA – 400	– OH
2. Amberlite IRA – 410	Quaternary ammonium
3. Zeolite Pf.IB	Quaternary ammonium – $CH_2\,N^+\,(CH_3)_3$
4. De-acidite	– $N\,(C_2H_5)_2$
5. Dowex A-1	Quaternary ammonium

PHYSICAL PROPERTIES OF ION EXCHANGE RESINS

The ion exchange resins behave as hygroscopic gels, swelling or shrinking reversibly with absorption or desorption of moisture/water. The most important properties of these resins are exchange capacity, density, mechanical strength, particle size, capacity, selectivity, amount of cross linking, swelling, porosity, surface area and chemical resistance.

Cross linking: It affects many properties e.g. swelling and strength of ion exchange resin by its degree of cross linking. As cross linking decreases, swelling increases; it affects the mechanical strength and swelling. Solubility is also greatly affected. If polystyrene is cross linked by incorporation of divinyl benzene, the mechanical strength is imparted to the resin thereby making it insoluble in common solvents.

Swelling: When resin swells, the polymer chain spreads apart forming a narrow passage throughout the resin bed. The weight of swelling of a styrene – DVB co-polymer to toluene can be measured by taking known weight of dry co-polymer in toluene, removing excess of toluene by centrifuging and then weighing the swollen co-polymer. In polar solvents, swelling occurs while in non-polar solvents, contraction occurs. Electrolyte concentration affects the degree of swelling.

Particle size and porosity: Surface area contributes to the rate of exchange. Large surface area and small particle size will increase the rate of ion exchange. Ion exchange resins are stable towards strong acids, strong bases and all organic solvents. Particle size range 50–100 mesh or 100–200 mesh is most commonly employed.

Regeneration: Ion exchange resins after use get deactivated as the replacement of ion takes place. In cation exchange resin, cations from the given solution get attached to the resin and deactivation results. So the cation exchange resins are regenerated by treatment with aqueous acid followed by washing with water. The resin gets converted to H^+ form and can be used for analytical purpose. Similarly, anion exchange resins are regenerated by treatment with sodium hydroxide or sodium carbonate solution followed by washing with

water until the washing is neutral. This regenerated ion exchange resin can be reused for the further separation of ions.

16.6 MECHANISM OF ION EXCHANGE PROCESS

The ion exchangers behave as a porous network which carries a surplus electric charge distributed over the surface and throughout the pores. The surplus charge is compensated by the ions of opposite charge. When the ionization takes place, they are exchanged with the ions which migrate into the solution. In this process, chemical bonds are not formed but the exchange occurs by the diffusion in two different stages:

1. **Film Diffusion:** Through an extremely thin film, there is a diffusion of counter-ions through a surface liquid which surrounds the ion exchanger. It is prominent in dilute solutions and has smaller counter-ions.
2. **Particle Diffusion:** It refers to diffusion of counter-ions within the pores of ion exchanger. It is predominant at high concentration and with large ions. This is increased by exchangers with low degree of cross linking, high exchange capacity, small particle size, and counter-ions with low valency and increasing temperature.

Non-electrolytes and weak electrolytes are usually absorbed by ion exchanger much more strongly than electrolytes. Absorption is enhanced by the formation of complexes between the ionic portions of the exchanger and the non-electrolyte solute. Sorption is decreased when the solute molecule becomes too large to enter the exchanger network. The exchanger then acts as a sieve or filter. In crystal lattice approach, the exchanger acts as a completely dissociated solid in which each ion is surrounded by a fixed number of ions of opposite charge. Since, the ions on the surface are less influenced by attractive forces, the surface ions are readily influenced for other ions, when exchanger is placed in highly polar solvent like water. The ion selectivity will depend on how strongly the surface ion is held by attractive forces within the crystal.

Ion Exchange Equilibrium:

Ion exchange resins contain fixed charge on matrix and counter balance the opposite replaceable charge. The free replaceable charge is readily exchanged with the charge or ion from solution. Thus, in case of cation exchange resin, cation or positive charge on resin is replaced by the positive ion from solution. It can be shown as

$$(Res^-)\,A^+ + B^+\,(solution) \rightleftharpoons (Res^-)\,B^+ + A^+\,(solution)$$

In running column chromatography, the equilibrium is completely displaced from left to right. If the solution contains several ions of cations like C^+, D^+, E^+ etc. the exchanger shows different affinity for the cations. The extent of one ion exchanged in preference to other ion is of fundamental importance.

The exchange of particular ion is governed by several factors: (a) nature of ion exchange resin, i.e. strong or weak type (b) nature and number of functional groups on resin (c) pH of solution (d) concentration of solution in contact with resin.

It is generally observed that the exchange of higher valent ion on the exchanger with lower valent ion in solution is favoured by increasing the concentration. While dilution favours the exchange of lower valent ion on exchanger then for higher valent ion from solution.

Ion Exchange Capacity:

Total ion exchange capacity of resin is dependent upon the total number of ion active groups per unit weight of resin. Greater the number, greater is the capacity of resin. This capacity is usually expressed as milliequivalents per gram of exchanger. The capacities of weak acidic cation exchange or resin also depends on pH. Good values are given about pH 9.0 for weak acidic or pH 5.0 or below for weak basic resins.

16.7 OPERATION TECHNIQUE

There are three methods of operation of column for ion exchange chromatography:

1. Displacement analysis
2. Frontal analysis
3. Elution analysis

The basis of all these operations is that the solute has some affinity for the substrate over which it flows. The affinity is due to partition, adsorption or ion exchange properties of the column. The operational procedures in ion exchange chromatography are as follows:

1. **Column:**

The columns are so designed that any kind of disturbance in the flow of liquid is avoided. All the operations are carried out in the down-flow direction. As the liquid moves down, the ions come in contact with unreacted resin with the result that all the ions are completely exchanged with the resin. The geometry of column depends completely on the separation factor. The separation is improved by increasing the length of the column but the length cannot be increased beyond a critical length. Uneven flow of liquid is possible in case of columns too wide or too narrow in size. Generally, the ratio of 10:1 or 100:1 of height to diameter is optimal for efficient separation.

2. **Packing the column:**

The resin is treated with the solvent to achieve equilibrium before packing the column. The slurry of the resin is poured into the column. The solvent which is to be used as an eluent should be used for making the slurry. The slurry is added in several portions allowing the resin to settle down. After packing, the solvent is passed for a certain time to achieve proper flow rate. The level of the solvent is adjusted.

3. **Application of the sample:**

After packing the column, the solution to be analyzed is added to the top of the column and allowed to pass through the bed of ion exchanger. For this purpose the syringe or pipette is utilized. Some time is allowed so that the ions in solution come in contact with ion exchanger.

4. **Elution:**

The components of mixture separate and move down the column individually at different rates depending upon the affinity of the ion for ion exchanger. The ions with least attraction will move most rapidly with the solvent and as they move downwards the distance between them increases. The eluates are collected at different stages. The efficiency of separation increases with increasing column lengths and lower flow rates.

5. **Analysis of the eluate:**

After passing through the ion exchange columns, the eluate is analyzed by various methods such as refractive index, pH, light absorption, etc. The readings are then plotted against the eluate volume to calculate the results.

16.8 APPLICATIONS

Ion exchange resins have wide range of applications in many industries.

1. Ion exchange chromatography represents one of the most efficient methods that provide accurate and rapid determination of ionic species in water samples. Basically, anions and cations can be independently separated and it can be expanded for the simultaneous determination of inorganic anions and cations. Example seven anions (F^-, $H_2PO_4^-$, NO_2^-, Cl^-, Br^-, NO_3^-, and SO_4^{2-}) and/or five common inorganic cations (Na^+, NH_4^+, K^+, Mg^{2+} and Ca^{2+}) have been separated using a single pump, a single eluent and a single detector.
2. It is applied for the determination of bromate in undiluted seawater samples using a two-dimensional approach comprising two columns connected in series.
3. Determination of perchlorate at the sub-µg/L level in pure water and in hardwater samples with high background ion concentrations are achieved using a standard anion exchange column as concentrator. This allows determination of perchlorate as low as 0.2 µg/L in low ionic strength matrices.
4. One of the problems of iodide estimation by conductivity detection is the expected interference from other ions and poor sensitivity of detection. Ion chromatography employing anion-exchange column with amperometric detection is demonstrated to be well suited for quantitative estimation of iodide and iodate in iodised salt.
5. Ion exchange chromatography has been proved to be useful in determination of the transition metal ions and determination of heavy metals in different solid matrices. Ion chromatography preceded by microwave-assisted acidic digestion of tissues samples in appropriate conditions is used for the determination of Co^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, and Zn^{2+} in human tissues.
6. Ion exchange chromatography is used for separation and purification of charged or ionisable molecules such as proteins, peptides, enzymes, antibiotics, vitamins, DNA etc.

7. It is used for softening the hard water by removal of Ca^{2+}, Mg^{2+} and other divalent ions which are responsible for the hardness of water by exchange with Na^{+} ions. In addition the technique is used to demineralise the water. The metallic cations are exchanged with H^{+} ions and anions are exchanged with OH^{-} ions by passing water through cationic and anionic exchangers. Finally H^{+} and OH^{-} ions combined to form the complete demineralised water.
8. Ion exchange chromatography has been applied for the determination of hydrogen cyanide in cigarette smoke through the development and application of electrochemical detection.
9. Hippuric acid (HA) is a kind of metabolite of toluene in human body, therefore, HA is a physiological component of human urine if toluene was inhaled. In order to diagnose patients who are suffering from a series of diseases caused by elevated HA levels, the determination of HA in human urine is necessary. Ion exchange chromatography is useful in determining the HA level in human urine.
10. Ion exchange chromatography is useful for the separation of sugars, amino acids, lanthanides, actinides. Phenolic compounds which cannot be detected by fluorescence detection due to weak fluorescent property can be determined by Ion exchange chromatography.

REVIEW QUESTIONS

1. Explain the principle and theory involved in Ion exchange chromatography.
2. Write a note on ion exchange resins.
3. What are the physical properties of ion exchange resins?
4. Discuss the mechanism of ion exchange process.
5. Explain the operational technique of Ion exchange chromatography.
6. Write the applications of Ion exchange chromatography.

Chapter ...17

GEL CHROMATOGRAPHY

Objectives:

Upon completion of this section, the student should be able to

- Understand the basic principle and theory of gel chromatography.
- Understand the technique of gel chromatography.

17.1 INTRODUCTION

In previous chapters, we have discussed about various chromatographic techniques which are based on different principles. In those techniques, it is rather difficult to define the variables which govern the method of separation. Apart from all those techniques, it was postulated by "Mould and Synge" in 1954, that separation can be achieved based on molecular sizes of analytes. They showed this concept of separation on uncharged substances during the migration through gels. This formed the basis for separations based on the relative sizes of the molecules. The systematic use of this principle was introduced in 1954 by "Porth and Flodin" who termed it as gel filtration for their method of separating large molecules of biological origin by means of polysaccharide gel (*Sephadex*). *Moore* used the term "Gel Permeation Chromatography". In 1964, "Determann" suggested that the "Gel Chromatography" is the most appropriate term for this technique. This method is mainly based on the differences in molecular dimensions and has different names like Gel Filtration, Molecular Sieve Filtration, Restricted Diffusion Chromatography, Exclusion Chromatography, Molecular Sieve Chromatography, etc.

Gel Chromatography method separates different substances depending on their molecular size. This technique differs from other partition chromatographic techniques. In this technique, the particles which come from stationary phase in the column are an uncharged gel. The gel swells in the same solvent which percolates through the bed.

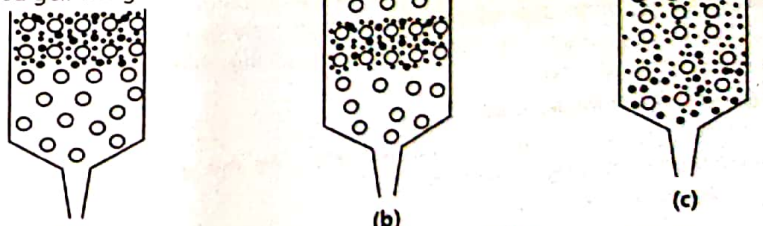


Fig. 17.1: Stages of separation

The stationary phase is a porous polymer matrix where the pores are completely filled with the solvent to be used as the mobile phase. The pore size is very important. The basis of the separation is the molecules above a certain size are totally excluded from entering and occupying the pores and the interior of the pores is accessible partly or wholly to smaller molecules. The flow of mobile phase will cause larger molecules to pass through the column unhindered without penetrating the gel matrix, whereas small molecules will be retarded because of their penetration in the gel (Fig. 17.1).

Thus, the components of the mixture emerge from the column in order of relative molecular mass, the largest first. The components which are completely excluded from the gel will not be separated from each other, and similarly small molecules which completely penetrate the gel will not be separated from the gel. The molecules of intermediate size will be retarded to a degree dependent on their penetration of the matrix. If the substances are of similar chemical type, they are eluted in order of relative molecular mass. Adsorption effects on the surface of gel particles are ignored and thus gel chromatography may be looked upon as a kind of partition chromatography.

17.2 MECHANISM

There are three mechanisms which have been proposed to describe the separation process. It is the process wherein solute molecules are distributed between two liquid phases (liquid in the gel pores and the liquid outside the gel).

(a) Steric Exclusion Effect:

In this, it is presumed that different fractions of the total pore volume are accessible to different size molecules because the gel particles contain a distribution of pore size. The large molecules get small number of pores into which they enter. Thus, small molecules can enter large number of pores.

The steric exclusion effect is more prominent when major particles are larger than many pores of the gel.

(b) Restricted Diffusion Mechanism:

The process is diffusion controlled, i.e. there is no diffusion equilibrium. Retention volume will be affected by changes in flow rates. The absence of diffusion equilibrium is most pronounced at very high linear velocities.

(c) Secondary Exclusion effect:

If the mixture of large and small molecules is placed on the gel, small molecules diffuse rapidly into the pores of gel and the diffusion of larger molecules in unoccupied pores is reduced. Thus, the larger molecules move further down, till they find unoccupied pores; this results in enhancement of separation.

Advantages of Gel Chromatography:

Gel Chromatography is based on the separation on the differences in molecular dimensions, it has the following advantages:

1. It is very simple to perform.
2. It is not sensitive to eluent composition and the temperature.

3. The gel matrices do not cause degeneration of biological materials and hence can be performed under very mild conditions.
4. The range of separation can be varied by varying the contents of gel matrix.
5. The gels are very stable and can be used again and again without any change in their properties.

17.3 TECHNIQUE

Apparatus for gel chromatography is similar to that used for other forms of liquid chromatography. This is discussed below:

1. Column:

The column (Fig. 17.2) is similar to that used in column chromatography and consists of a straight glass tube with a bed support at the bottom. The bed support is of such type that it allows the liquid to flow through while the bed material is retained.

Some glass wool or filter paper is put at the bottom of the tube which is then covered with quartz sand or glass beads. The diameter of the column is generally larger than those commonly used in partition and adsorption chromatography. The large column diameters and greater column lengths are preferred for high resolutions. The columns for gel chromatography are commercially available with diameters ranging from 1-20 cm.

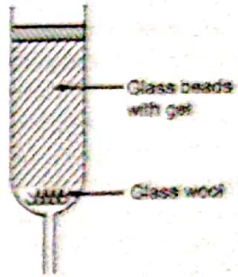


Fig. 17.2 : Column for gel chromatography

2. Gel:

There are many gels but only a few of them can be used in gel chromatography. In general, the gel should satisfy some of the requirements so that it can be used in this technique. The requirements are:

(i) The matrix of the gel should be inert chemically.
(ii) The gel should be stable mechanically.
(iii) The particle size should be uniform.
(iv) The content of the ionic groups should be low in the gel.
(v) The matrix of the gel should have the uniform porosity.
(vi) The degree of swelling should also be small, since soft materials may bleed down while in use resulting in the alteration of the flow characteristics of the column. Also, it is easier to pack non-swelling materials on the column.

(a) Classification:

The gels are generally:

1. Rigid 2. Semi-rigid 3. Soft gel

1. **Rigid gels:** Rigid gels have fixed, uniform pore volume, high column permeability. They also provide highly permeable columns with average capacity. Both hydrophilic and lyophilic ones have been prepared. Both silica gel and glasses, which are rigid substances, exhibit adsorption (retarding especially polar species) which interferes with size separation.
2. **Semi-rigid gels:** These gels swell to about 1.1-1.8 times their dry volume, are open caged spheres, can withstand high pressures and provide a range of pore sizes. Semi-rigid gels are used primarily with aqueous solvents. These gels have been chemically and physically modified to render them wettable. These include cross-linked polystyrene, ion-exchange resins and polyvinyl acetate gels.
3. **Soft gels:** The soft gels imbibe large quantities of solvents into their pores and swell to many times their dry volume. They gain their porosity in proportion to the volume of solvent imbibed. They get easily deformed in the inertial field of moving solvent, resulting in enhancement of column pressure drop. At high solvent velocity, the gel bed gets compressed giving rise to voids in the column. Besides this, soft gels become more fragile with increase in porosity and thus they become useful for small molecules. While employing these gels, the solvent velocities must be kept low, giving high efficiency and capacity. Soft gels consist of cross-linked dextrans (sephadex), starch, rubber, lightly cross linked polystyrenes and polyacrylamide gels.

In general, the inorganic stationary phases such as porous glass or silica gel are available in a range of closely controlled pore sizes which do not swell and hence packing the columns become easier and the solvent can be changed without affecting the efficiency. They are stable at high temperatures. However, they are less efficient than organic polymer gels. Xyrogels (obtained by the copolymerization of dextran with epichlorohydrin and subsequent cross linking of the dextran chains by glyceryl bridges) such as sephadex are three dimensional hydrophilic product that swell strongly in aqueous medium. It is stable in pH range 2-10, but is hydrolyzed by concentrated acids and attacked by oxidants. Columns can be stored in 5% formalin solution. Bacterial growth on it can be avoided by using buffers saturated with chloroform.

(b) Choice of Gel:

In gel chromatography, two types of separations are done:

1. The separation of high molecular weight substances from low molecular weight substances. This is called as group separations or desalting.
2. The second type of separation is called fractionation wherein the similar substances are eluted closer to another which may sometimes overlap.

The selection of column packing is generally based on the permeation range of the gel. In group separation, the groups are eluted with application of large volumes of samples, whereas the sample size is limited in the fractionation method. For group separation Sephadex G-25, Sephadex G-50, Bio-Gel P-6, Bio-Gel P-10 is used. In the fractionation method Sephadex G-25, Sephadex G-100, Sephadex G-500 are used.

(c) Particle size:

For routine work, the gel in the powder form with particle size of 70 μ in diameter is used, but the use of finer grade material gives further improvement in the resolution. The material with particle diameter less than 40 μ can be used in many cases. Sometimes, the commercially available gel may be improved by sieving.

(d) Gel preparation:

In gel preparation, the dry powder is allowed to swell in the liquid to be used as an eluent. The required weight of the dry powder is taken which is then mixed up with excess of liquid for swelling. This mixture is left as such till the equilibrium is achieved; this takes a very long time. The best way is to warm the gel slurry in a boiling water bath to about 100°C. By this method, the swelling will be complete in a couple of days and the bacteria and the fungus present in the suspension are killed and the dissolved air is also removed. The slurry is then cooled before packing.

(e) Drying of gels:

The gels can very well be stored in the wet state and thus there is no necessity of drying them. Agarose gels are not dried as it is difficult to bring them back to the original state. However, dextran gels (Sephadex), acryl amide gels (Biogel) can be dried without any damage and can be returned to the wet state without difficulty.

3. Packing of the column:

The general methods of packing the column as described in column chromatography are not used in gel chromatography. Dry packing of column followed by the liquid will result in the cracking of the column due to swelling of the gels. The most preferable method is to attach a large container to the top of the column. The column is filled with eluent and the thin slurry is poured from the top. The gel settles down in the column and packing is done; this however does not give an even packing.

The procedure for packing depends on the nature of the gel. Special efforts are needed to maintain uniform slurry. With soft gels more careful packing is needed while the hard gels do not require so many precautions. The gel is allowed to swell, and then deuterated under vacuum. The gel is then allowed to settle and the supernatural liquid is taken off. The gel is remixed and then poured in the column. Packing in many steps should be avoided as it gives uneven packing. Liquid should be carefully added; otherwise the gel surface will be disturbed.

With hard gels, the principle of the packing remains the same with slightly modified procedures. Agarose gels which are too thick to be packed directly into the column, are mixed with buffer solution, deuterated under vacuum and then packed.

Sometimes, the eluent is kept at 15–30°C above the column, which avoids the formation of bubbles. This is possible by placing an incandescent lamp just below the eluent reservoir.

4. Preparation of Sample:

The sample is dissolved in a proper solvent so that there will not be any solid particle or other substances which may be strongly adsorbed on the gel. The sample volume should be such that it will give the required separations. In analytical applications, sample of 1-3% of total bed volume is used. However, in group separation sample of 25-30% of total column volume is used. The smaller the sample volume, the greater will be reduction of the

component concentration in the eluate. The dilution effect must also be taken into account in deciding the column and sample sizes.

5. Application of the Sample:

In most cases, the sample is applied at the top of the gel bed and the flow is started. The sample solution is allowed to pass into the bed. A small amount of eluent is added to wash the traces of sample into the bed. The use of pipette with bent tip is preferable for the application. Viscous samples are introduced with the help of a valve loop. Commercial plunger type columns have special inlet arrangements for the sample.

6. Solvents:

The role of solvent in gel chromatography is less important than the other forms of the chromatography. The choice is often made based on the solubility characteristics of the sample and the type of detector used. In general the solvent should dissolve the sample and be sufficiently similar to the gel to wet it and prevent the adsorption. It must swell the soft gels. The important aspect is that the moving solvent, the trapped solvent and the gel must interact with the solute identically. The solvent viscosity restricts diffusion and affects the resolution. The solvent used must be compatible with the detector used and the system hardware. In many cases, the presence of salt as an electrolyte is important, as many macromolecules change their size when the solvent composition or the electrolyte concentration is changed. In addition, the pore size of soft gel changes with the changes in electrolyte concentration. Thus, the solvent composition will affect elution behaviour.

7. Detector:

There are varieties of commercially available detectors. They are mainly based on a variety of physical properties of molecules in the solution. Many times multiple detectors are used. One type is used to indicate the elution of all components of the sample and the other to detect the specific component in the eluate. Thus, with the help of two detectors, information of both the molecular weight and the relation of sample can be obtained.

The differential refractometer is very sensitive in some units detecting difference of 10^{7} or even 10^{8} refractive index units. They are mostly used as they are not based on presence of specific functional groups. These detectors are temperature sensitive.

The visible and UV photometer is also used in cases where the solutes absorb radiation of wavelength, but the solvent does not; or those solutes which have high extinction coefficients. The detectors based on flame ionization, heat of absorption, electrical conductivity; IR absorption, polarography, gravimetric etc. are also used.

REVIEW QUESTIONS

1. What do you mean by Gel chromatography? Write its principle.
2. What are the types of gels in Gel chromatography?
3. Explain the technique of Gel chromatography.
4. What is the mechanism involved in the separation process of Gel chromatography?

Chapter ... 18

AFFINITY CHROMATOGRAPHY

Objectives:

Upon completion of this section, the student should be able to

- Understand Principle of Affinity chromatography.
- Understand the basic principle and theory of Affinity chromatography.
- Understand Mechanism of Affinity Binding

18.1 INTRODUCTION

Affinity chromatography is one of the most powerful chromatographic methods for purification of a specific molecule or a group of molecules from complex mixtures. The basis of the technique is highly specific and reversible biological interactions between two molecules. This includes interactions between enzyme and substrate, receptor and ligand, or antibody and antigen. One of the interacting molecules is referred to as affinity ligand, is placed onto a solid matrix to create a stationary phase. The target molecule is in the mobile phase. Successful affinity purification requires a certain degree of knowledge and understanding of the nature of interactions between the target molecule and the ligand to help determine the selection of an appropriate affinity ligand and purification procedure. Biomulecules are purified using purification method that separates in accordance to difference in specific properties. Affinity chromatography is unique in purification since it enables purification based on is unique in purification since it enables purification based on biological function or individual chemical structure.

This technique offers high selectivity, high resolution and high capacity for proteins target proteins is collected in purified and concentrated form. The forces which act in interaction of ligand and target molecules are electrostatic, hydrophobic interactions, Vander waal forces / hydrogen bonding.

Affinity purification offers time saving over less selective multistep procedure. For high degree of purity, if this is no suitable ligand for affinity purification, multi-step process can be developed using purification strategy of capture, intermediate, purification and polishing (Cipp).

Successful affinity purification requires biospecific ligand which can covalently attach to matrix. The ligand and target molecule binding must be reversible to allow target molecules to be removed in an active form. The affinity chromatography is a type of liquid chromatography for analysis of sample components. Thus the interaction between dissociation constant Kd.

$$Kd = \frac{[A]\,[B]}{[AB]}$$

Where,

A = Molecule targeted

B = Ligand

C = Complex formed between them

Kd varies between 10^{-3} to 10^{-7} m for affinity binding.

18.2 PRINCIPLE OF AFFINITY CHROMATOGRAPHY

Principle of affinity chromatography based on the covalent attachment of an immobilized biochemical, an affinity ligand, to a solid support. When a sample is passed through the column, only the solute which is binds to the ligand is retained. The other components of sample elutes out. The separation mechanism is exactly like a lock and key model. The mobile phase condition is changed to elute the retained components. The steps are explained in Fig. 18.1.

1. In the first step affinity medium is initially equilibrated.
2. In the second step the adsorption of solute in the target as per the lock and key approach and elution of un bound solutes is takes place.
3. In the third step the bound solutes are eluted by changing the conditions of mobile phase
4. In fourth step the medium is re equilibrated for further use.

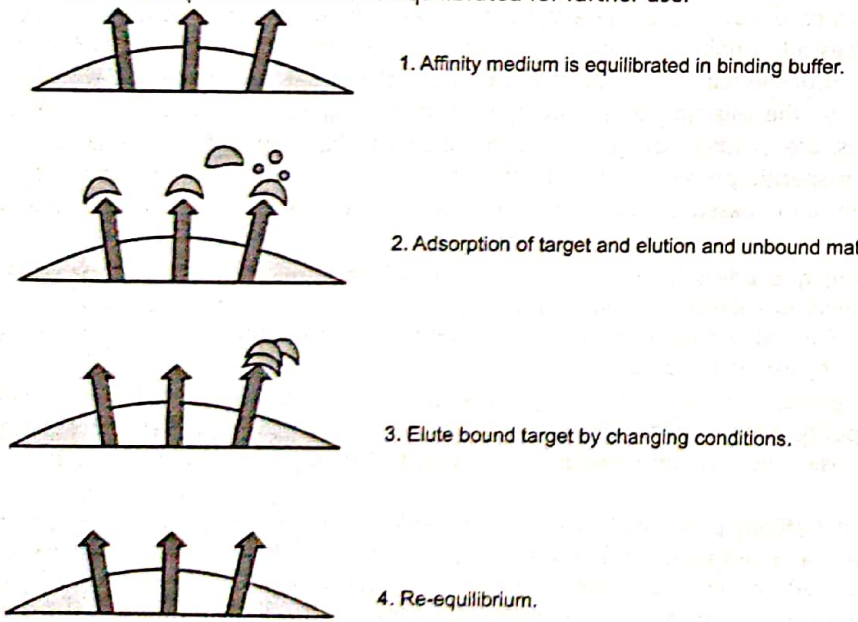


Fig. 18.1 : Key stages in affinity purification

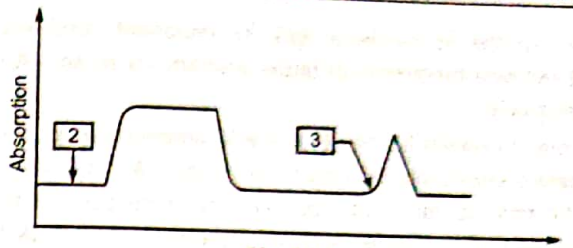


Fig. 18.2

18.3 MECHANISM OF AFFINITY BINDING

A commonly used model to illustrate affinity binding is the lock and key analogy. A unique structure present on the surface of a protein is the key that will only bind to the corresponding lock, a specific ligand on a chromatographic support.

Affinity-tagged Purification:

This process consists of two steps. A protein is first purified by affinity chromatography and then desalted.

Step 1:

In the first step, a recombinant protein mixture is passed over a chromatography support containing a ligand that selectively binds proteins that contain an affinity-tag sequence (typically His or GST). Contaminants are washed away, and the bound protein is then eluted in pure form.

Affinity tags have different advantages. In immobilized metal affinity chromatography (IMAC), Histidine binds with good selectivity to Ni2+ or other transition metals immobilized to the ligand; the tagged protein can be selectively eluted with imidazole. Proteins tagged with GST bind to glutathione as the ligand, and are eluted with solutions of glutathione. Proteins with an enzymatically active GST fusion tag can only be purified under native conditions. In contrast, polyhistidine-tagged proteins may be purified under native or denaturing conditions.

Step 2:

During the second step of desalting, affinity-purified samples can simultaneously undergo buffer exchange to remove salts in preparation for downstream applications. A number of desalting techniques, including size exclusion chromatography, dialysis, and ultrafiltration, also allow buffer exchange. Desalting often includes the removal not only of salt, but also of other foreign substances, such as detergents, nucleotides, and lipids.

Affinity chromatography can be broadly divided into two method types:

The first method uses a naturally occurring structure or sequence of amino acids on the protein as the binding site. Examples include the affinity of Affi-Gel Blue support binding for albumin's bilirubin-binding site and the binding of protein A in the Affi-Gel and Affi-Prep

protein A supports to the Fc region of IgG. An important consideration for antibody purification is to determine the affinity of target antibody for protein A/G chromatography media, which varies widely.

The second method involves binding to a special amino acid sequence engineered into the protein of interest, commonly referred to as a "tag". A number of different tags are available. Two of the most commonly used protein tags are the polyhistidine tag, which binds to certain metal-containing complexes such as those in Profinity™ IMAC resins, and the glutathione s-transferase (GST) sequence, which binds to glutathione. Theoretically, any protein can be purified using the tagging method; however, many factors must be considered to design a process to purify tagged recombinant proteins.

18.4 APPLICATIONS

1. This technique has great impact on molecular biology, biochemistry, biotechnology.
2. Affinity chromatography is used as a powerful tool for biologically active molecules purification process like proteins.
3. Affinity chromatography can be used whenever a suitable ligand is available for protein of interest.
4. This technique initially used for purification of enzymes but now it is used for various purposes like purification of nucleotides, nucleic acid, immunoglobin, membrane receptors etc.

REVIEW QUESTIONS

1. Explain the principle and applications of Affinity Chromatography.
2. Describe Mechanism of Affinity Binding.

Note:

ABOUT AUTHORS

[illegible] graduated in Pharmacy from Shivaji University in 1980, Masters [illegible] in 1982, Ph.D. in Pharmaceutical sciences from [illegible] and Ph.D. in Law from Bharati Vidyapeeth Deemed to be [illegible] Professor of Pharmaceutical Chemistry and Principal at [illegible] (Deemed to be University) Poona College of Pharmacy, Pune. He [illegible] in teaching and research. More than 85 M. Pharm. [illegible] awarded degrees and one post-doctoral research has been [illegible] guided under his able guidance. This has translated into 21 patents, [illegible] papers & 42 review articles in international journals, 81 [illegible] articles in national journals, 171 presentations in national & international conferences and 13 books to his credit. He has completed more than 10 research projects from various funding agencies. He is the recipient of "Best Teacher Award" by Government of Maharashtra in 2004, "Bharati Ratna Puraskar" by Bharati Vidyapeeth, Pune in 2009, "IPA Fellowship Award" in 2008, "Best Principal of the Year Award 2015" by APTI, Dewang Mehta National Education Award for Best Professor in Pharmacy for the year 2015, Dr. K. M. Purohit Award" by IAST&M in 2017, "Outstanding Services Award" 2018 by the IPA India and "Life Time Achievement Award" by APTI (MP) in 2014 and IPA Pune in 2020. He has been member of more than 20 professional bodies and scientific referee for more than 10 international journals in pharmaceutical sciences. He was Hon. Secretary and Hon. Treasurer of IPA, Pune branch. He has been the Member of Management Council, Academic Council, faculty and Board of Studies of several Universities. He has been the subject experts' scientific referee for various regulatory bodies.

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