Read to Lead

AS PER (PCI) SYLLABUS
B.Pharma
7th
Semester

Instrumental Methods of Analysis

Dr. S.Malathi
Dr. Pallavi Mangesh Patil
Dr. Sunil Kumar

THAKUR PUBLICATION PVT. LTD.
LUCKNOW

Contents

Chapter 1: UV Visible Spectroscopy

Chapter 2: Fluorimetry

Chapter 3: IR Spectroscopy

Chapter 4: Flame Photometry

Chapter 5: Atomic Absorption Spectroscopy

Chapter 6: Nepheloturbidometry

Chapter 7: Introduction to Chromatography

Chapter 8: Column Chromatography

Chapter 9: Thin Layer Chromatography

Chapter 10: Paper Chromatography

Chapter 11: Electrophoresis

Chapter 12: Gas Chromatography

Chapter 13: High Performance Liquid Chromatography (HPLC)

Chapter 14: Ion Exchange Chromatography

CHAPTER 1

UV Visible Spectroscopy

1.1. UV-VISIBLE SPECTROSCOPY

1.1.1. Introduction

Ultraviolet and visible spectroscopy deals with the recording of the absorption of radiations in the UV and visible regions of the electromagnetic spectrum. The UV region extends from **10-400nm**. It is sub-divided into the **near UV (quartz) region** (200-400nm) and the **far or vacuum UV region** (10-200nm). The visible region extends from **400-800nm**.

Absorption of electromagnetic radiations in the UV and visible regions induces the excitation of an electron from a lower to higher molecular orbital (electronic energy level). Since UV and visible spectroscopy involves electronic transitions, it is often called **electronic spectroscopy**. Organic chemists use UV and visible spectroscopy for detecting the presence and elucidating the nature of conjugated multiple bonds or aromatic rings.

1.1.2. Theory

Ultraviolet absorption spectra arise from transition of electron(s) within a molecule or of ions from lower to higher electronic energy levels. The **ultraviolet emission spectra** arise from the reverse type of transition. For radiation to cause electronic excitation, it must be in the UV region of the electromagnetic spectrum.

When a molecule absorbs UV radiation of frequency ν sec^{-1}, the electron in that molecule undergoes transition from a lower to a higher energy level or molecular orbital. The **energy difference** is given by:

$$E = h\nu \text{ erg}$$

The actual amount of energy required depends on the difference in energy between the ground state (E_0) and excited state (E_1) of the electrons. Thus, the above equation becomes:

$$E_1 - E_0 = h\nu$$

It is known that the total energy of a molecule is equal to the sum of electronic, vibrational and rotational energies. The magnitude of these energies decreases in the following order, $\mathbf{E_{elec} < E_{vib} < E_{rot}}$.

As UV energy is quantised, the absorption spectrum arising from a single electronic transition should consist of a single discrete line. But, a discrete line is not obtained because electronic absorption is superimposed upon rotational and vibrational sub-levels.

For this reason, the spectra of simple molecules in the gaseous state contain narrow absorption peaks, where each peak is representing a transition from a particular combination of vibrational and rotational levels in the electronic ground state to a corresponding combination in the excited state. This is shown in **figure 1.1**

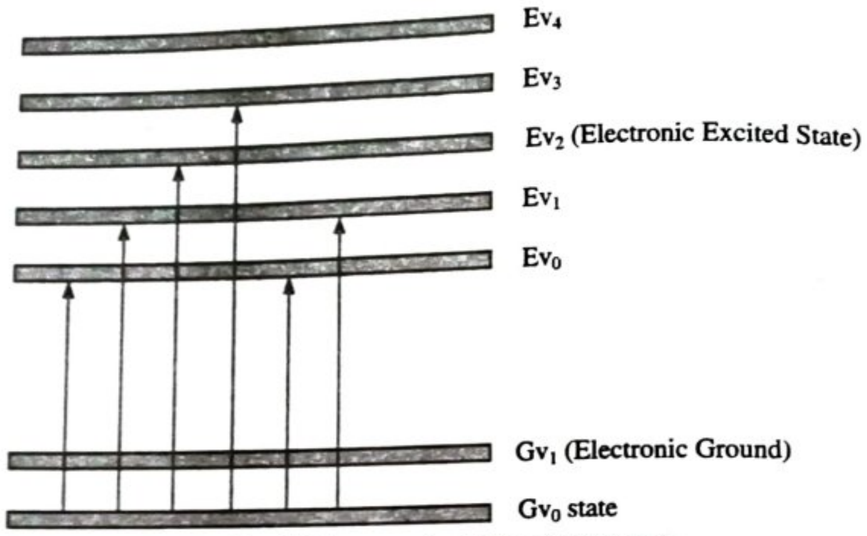


Figure 1.1: Energy Level Diagram of a Diatomic Molecule

In case of complex molecules having more than two atoms, discrete bands coalesce to produce **broad absorption bands** or **band envelopes**. Energy absorbed in the UV region produces changes in the electronic energy of the molecule resulting from transitions of valence electrons in the molecule. **Three types of electrons** involved in organic molecules are:

1) **σ-Electrons:** These electrons are involved in saturated σ-bonds, such as those between carbon and hydrogen in paraffin. As the amount of energy required for exciting the electrons in σ-bonds is much more than that produced by UV light, compounds containing σ-bonds do not absorb UV radiation. Thus, paraffin compounds are frequently useful as solvents.

2) **π-Electrons:** These electrons are involved in unsaturated hydrocarbons. Typical compounds with π-bonds are trienes and aromatic compounds.

3) **n-Electrons:** These electrons are not involved in the bonding between atoms in molecules. **Examples** of organic compounds containing these electrons are nitrogen, oxygen, or halogen. As n-electrons can be excited by UV radiation, any compound having nitrogen, oxygen, sulphur, halogen compounds, or unsaturated hydrocarbons may absorb UV radiation.

1.1.3. Principle

Absorption of electromagnetic radiation by substances in the visible and UV regions of the spectrum ranges from 200-700nm changes in the electronic structure of ions and molecules. UV and visible light are energetic enough to promote outer electrons to higher energy levels. UV-vis spectroscopy is usually applied to molecules and inorganic ions or complexes in solution. The UV-vis spectra have broad features that are of limited use for sample identification but are very useful for quantitative measurements. The analyte concentration in solution can be determined by measuring the absorbance at some wavelength and by applying the **Beer-Lambert (Beer's) Law**.

Many molecules absorb UV or visible light. Absorbance of a solution increases as attenuation of the beam increases. Absorbance is directly proportional to the path length (b) and the concentration (c) of the absorbing species. **Beer's Law** states that:

$$A = abc$$

Where, a = Absorptivity (a proportionality constant).

Different molecules absorb radiation of different wavelengths. An absorption spectrum will show a number of absorption bands corresponding to structural groups within the molecule. **For example,** absorption that is observed in the UV region for the carbonyl group in acetone matches with the same wavelength as the absorption from the carbonyl group in diethyl ketone.

1.1.4. Electronic Transitions

For most molecules, the lowest-energy occupying molecular orbitals are the σ-orbitals, which correspond to σ-bonds. The π-orbitals lie at somewhat higher energy levels; and the orbitals holding unshared pairs, i.e., the non-bonding (n) orbitals, lie at even higher energies. The unoccupied or anti-bonding orbitals (π* and σ*) are the orbitals of highest energy. **Figure 1.2** shows a typical progression of electronic energy levels.

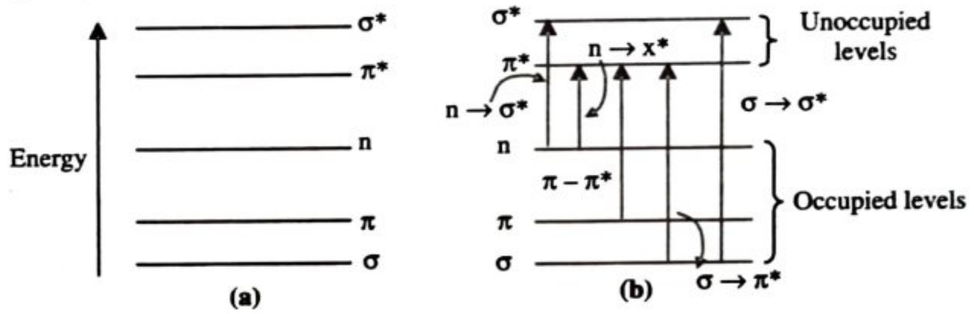


Figure 1.2: Electronic Energy Levels and Transitions

In all the compounds other than alkanes, the electrons may undergo several possible transitions of different energies. Some of the most important transitions are illustrated in **figure 1.2(b)**. Clearly, the energy required to bring about transitions from the **Highest Occupied Energy Level (HOMO)** in the ground state to the **Lowest Unoccupied Energy Level (LUMO)** is less than the energy required to bring about a transition from a lower occupied energy level. Thus, in **figure 1.3** an $n \rightarrow \pi^*$ transition would have a lower energy than a $\pi \rightarrow \pi^*$ transition. For many purposes, the transition of lowest energy is the most important.

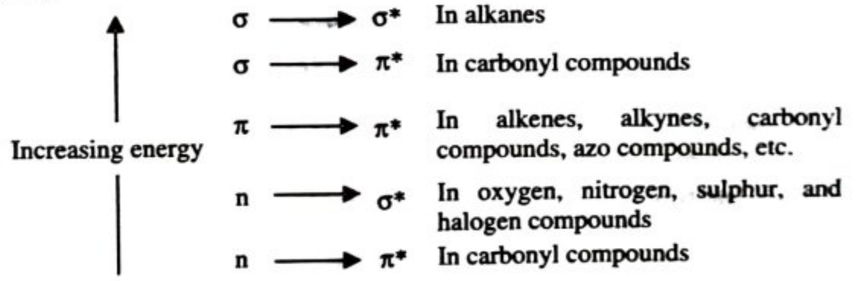


Figure 1.3: Electronic Transitions

1.1.4.1. Electronic Transitions of Organic Species

The different types of electronic transitions are:

1) **$\sigma \rightarrow \sigma^*$ Transition:** A transition of an electron from a bonding sigma orbital to the higher energy anti-bonding sigma orbital is designated as $\sigma \rightarrow \sigma^*$ (sigma to sigma star). In alkanes, this is the only transition available. Sigma bonds are very strong, therefore this transition is a high energy process that requires very short wavelengths, i.e., high energy UV light of ~150nm. A study of such transition is done in vacuum UV region, since below 200nm oxygen present in air begins to absorb. This can be shown in **figure 1.4**.

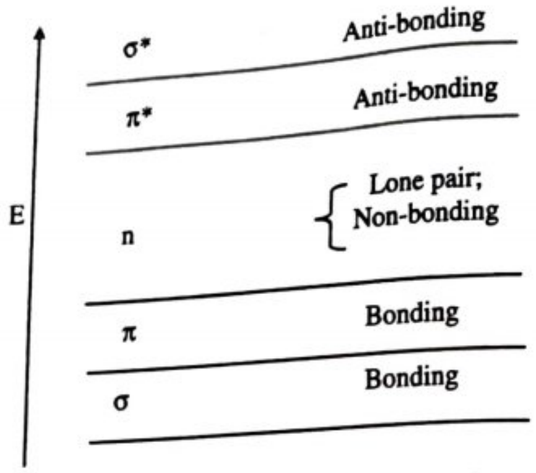


Figure 1.4: Electronic Energy Levels

2) **n → σ* Transition:** This transition involves saturated compounds with one heteroatom with unshared pair of electrons (n electrons), **e.g.**, saturated halides, alcohols, ethers, aldehydes, ketones, amines, etc. These transitions require comparatively less energy than σ → σ* transitions. The n → σ* transitions are sensitive to hydrogen bonding. **For example,** alcohols form hydrogen bonds with the solvent molecules. Such association occurs due to the presence of non-bonding electrons on the heteroatom and thus, this transition requires greater energy.

3) **π → π* Transition (K-Band):** This transition is available in compounds with unsaturated centres, **e.g.**, simple alkenes, aromatics, carbonyl compounds, etc. This transition requires lesser energy than n → σ* transition. In a simple alkene, although several transitions are available, the lowest energy transition is the π → π* transition and an absorption band around 170-190nm in unconjugated alkenes is due to this transition. **For example,** in saturated ketones, the most intense band around 150nm is due to π → π* transition.

4) **n → π* Transition (R-Band):** In this transition, an electron of unshared electron pair on a heteroatom is excited to π* anti-bonding orbital. This transition involves least amount of energy than all the transitions and therefore, gives rise to an absorption band at longer wavelengths. **For example,** in saturated aliphatic ketones, the n → π* transition at around 280nm is the lowest energy transition. This n → π* transition is forbidden by symmetry consideration, thus the band intensity due to this transition is low, although the wavelength is long (lower energy).

1.1.4.2. Electronic Transitions of Inorganic Species

A large number of inorganic salts containing atoms with electrons in d-orbitals give weak absorption bands in the visible range. The ions and complexes of the elements of the first two transition series belong to this group and are coloured. The colour of these species is due to transitions amongst d-orbitals. The complex formation of these ions with solvent molecules or with other ligands lifts the degeneracy of the five d-orbitals. As a consequence, these split into groups having different energies. The electronic transitions from the lower energy d-orbitals to higher energy d-orbitals are responsible for the observed colour. These transitions are called **d-d transitions**.

The blue colour of aqueous solutions of copper sulphate and the violet colour of potassium permanganate are some of the **examples**. The ions of lanthanides and actinides are also coloured, however, these involve **f-f transitions**. The nature of spectrum in these ions is different because the f-electrons are relatively less affected by external influences

due to the shielding effect by the occupied orbitals of higher principal quantum number. These ions absorb the radiation of UV and visible region in narrow bands. The representative visible spectra of the ions of transition and inner transition elements are given in **figure 1.5**.

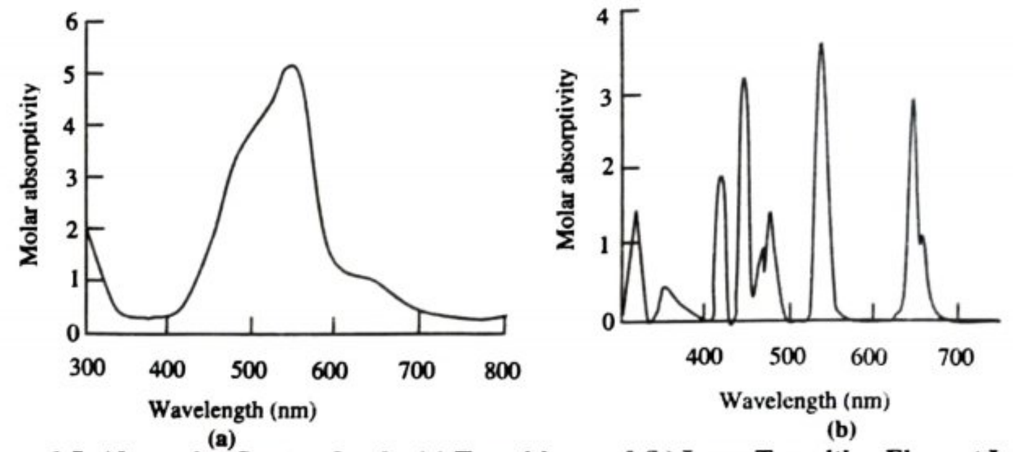


Figure 1.5: Absorption Spectra for the (a) Transition, and (b) Inner Transition Element Ions

1.1.5. Chromophores

Originally, a chromophore was considered a system responsible for imparting colour to the compound. Nitro-compounds are generally yellow in colour. Clearly, nitro group is the chromophore that imparts yellow colour. Similarly, aryl conjugated azo group is a chromophore for providing colour to azo dyes. Now, the term chromophore is defined as **any isolated covalently bonded group that shows a characteristic absorption in the UV or visible region**.

Absorption occurs irrespective of the fact whether colour is produced or not. Some of the important chromophores are **ethylenic, acetylenic, carbonyls, acids, esters, nitrile group**, etc. A carbonyl group is an important chromophore, although the absorption of light by an isolated group does not produce any colour in UV spectroscopy.

Types of Chromophores

1) Chromophores containing π-electrons and undergoing n → π transitions, **e.g.,** ethylenes, acetylenes, etc.
2) Chromophores containing both π-electrons and n (non-bonding) electrons, and undergoing π → π and n → π transitions, **e.g.,** carbonyls, nitriles, azo compounds, nitro compounds, etc.

The **other types of chromophores** are:

1) **Independent Chromophores:** A single chromophore imparts colour to the compound. **For example,** azo group, –N=N–, nitroso group, –NO, and *o*- and *p*-quinonoid groups, etc.
2) **Dependent Chromophores:** More than one chromophore is required to produce colour in the chromogen. **For example,** C=O group, C=C group, etc. Acetone having one ketonic group is colourless, diacetyl having two ketonic groups is yellow, and triketopentane having three ketonic groups is orange.

Identification of Chromophores

There are no set rules for the identification of a chromophore. The change in position as well as the intensity of absorption depends on a large number of factors. Following points may be useful:

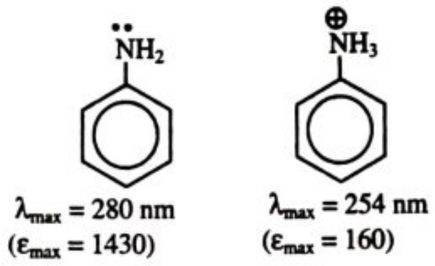
1) Spectrum having a band near 300mμ possess two or three conjugated units.

2) Absorption bands near 270-350mμ with very low intensity (ε_{max} 10-100) are because of n→n* transitions of the carbonyl group.
3) Simple conjugated chromophores, like dienes or α,β-unsaturated ketones, have ε_{max} values ranging from 10,000-20,000.
4) Absorption with ε_{max} value ranging from 1,000-10,000 reveals the presence of an aromatic system. If aromatic nucleus is substituted with groups that can extend the chromophore, absorption takes place at still higher values of extinction coefficients.

1.1.6. Auxochromes

An auxochromic group can be defined as **any group which does not itself acts as a chromophore but whose presence shifts of the absorption band towards the red end of the spectrum** (longer wavelength). Absorption at longer wavelength is due to the combination of a chromophore and an auxochrome to give rise to another chromophore.

An auxochromic group is called **colour enhancing group**. Auxochromic groups do not show characteristic absorption above 200mμ. Some common auxochromic groups are **—OH, —OR, —NH_2, —NHR, —NR_2, —SH**, etc. The auxochrome effect is due to its ability to extend the conjugation of a chromophore by the sharing of non-bonding electrons. Thus, a new chromophore has a different value of absorption maximum as well as extinction coefficient. **For example**, benzene shows an absorption maximum at 255mμ [ε_{max} 203], whereas aniline absorbs at 280mμ [ε_{max} 1430]. Hence, amino (—NH_2) group is an auxochrome.

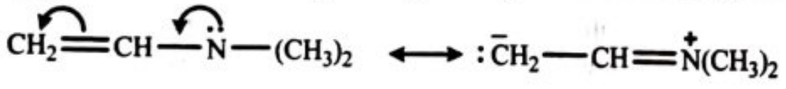


λ_{max} = 280 nm (ε_{max} = 1430) λ_{max} = 254 nm (ε_{max} = 160)

In aniline, —$\ddot{N}H_2$ group acts as a chromophore. But in anilinium ion, there is no lone pair of electron on nitrogen atom.

Mechanism

All auxochromic groups contain non-bonding electrons. Due to this, there is an extension of conjugation of the chromophore by sharing the non-bonding electrons.



The extended conjugation has been responsible for bathochromic effect of auxochrome.

1.1.7. Spectral Shifts

The position of absorption maximum and absorption intensity can be modified in different ways by some structural changes or solvent change as given below:

1) **Bathochromic Shift:** It is an effect in which the absorption maximum is shifted towards longer wavelength due to the presence of an auxochrome or by the change of solvent **(figure 1.6)**.

   Such an absorption shift towards longer wavelength is called **bathochromic** or **red shift**. The n → π* transition for carbonyl compounds experiences bathochromic shift when the solvent polarity is decreased.

2) **Hypsochromic Shift:** It is an effect in which the absorption maximum is shifted towards shorter wavelength. Such an absorption shift towards shorter wavelength is called **hypsochromic** or **blue shift**. It may be caused by the removal of conjugation and also by changing the solvent polarity.

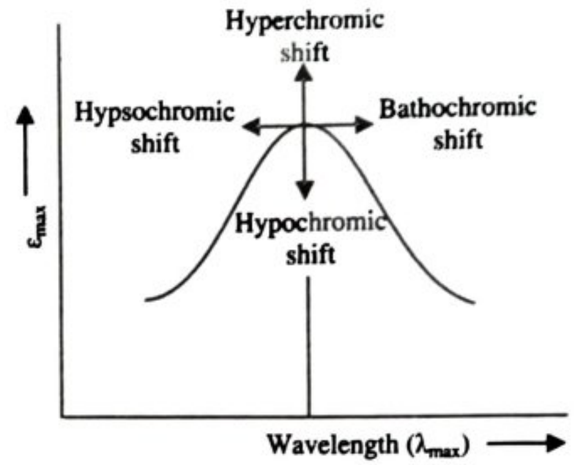


Figure 1.6: Absorption and Intensity Shifts

3) **Hyperchromic Shift:** It is an effect in which the intensity of absorption maximum increases, i.e., the value of ε_{max} increases. **For example,** the B-band for pyridine at 257mμ (ε_{max} 2750) is shifted to 262mμ (ε_{max} 3560) for 2-methyl pyridine (i.e., the value of ε_{max} increases). Introduction of an auxochrome usually increases the intensity of absorption.
4) **Hypochromic Shift:** It is an effect in which the intensity of absorption maximum decreases, i.e., the value of ε_{max} decreases. It may be caused by the introduction of group which distorts the geometry of the molecule. **For example,** biphenyl absorbs at 250mμ (ε_{max} 19000) whereas 2-methyl biphenyl absorbs at 237mμ (ε_{max} 10250) (i.e., the value of ε_{max} decreases) due to the distortion caused by the methyl group in 2-methyl biphenyl.

1.1.8. Solvent Effect on Absorption Spectra

A most suitable solvent is one that does not itself absorbs radiation in the region under investigation. A dilute solution of the sample is always prepared for spectral analysis. Most commonly used solvent is **95% ethanol**. Ethanol is a best solvent as it is cheap and is transparent down to 210mμ. Commercial ethanol should not be used because it is having benzene which absorbs strongly in the ultraviolet region. Some other solvents which are transparent above 210mμ are **n-hexane, methyl alcohol, cyclohexane, acetonitrile, diethyl ether**, etc. Some solvents with their upper wavelength limit of absorption are given in **table 1.1**:

Table 1.1: Solvents with Upper Wavelength Limit

Solvents	λ of Absorption (mμ)
Ethanol	210
Hexane	210
Methanol	210
Cyclohexane	210
Diethyl ether	210
Water	205
Benzene	280
Chloroform	245
THF (Tetrahydrofuran)	220
Carbon tetrachloride	265

Hexane and other hydrocarbons can be used because these are less polar and have least interactions with the molecule under investigation. For UV spectroscopy, ethanol, water, and cyclohexane serve the best purpose.

The positions as well as the intensity of absorption maximum get shifted for a particular chromophore by changing the solvent polarity. By increasing the solvent polarity, compounds such as dienes and conjugated hydrocarbons do not experience any appreciable shift.

Hence in general, the absorption maximum for non-polar compounds is usually shifted with the change in polarity of solvents. The **α,β-unsaturated carbonyl compounds show two different shifts**:

1) **n→π* Transition (Less Intense):** In this case, the absorption band moves to shorter wavelength by increasing the solvent polarity. In this transition, the ground state is more polar as compared to the excited state. The hydrogen bonding with solvent molecules occurs to a lesser extent with the carbonyl group in the excited state. **For example,** absorption maximum of acetone is at 279mμ in hexane as compared to 264mμ in water.

2) **π→π* Transitions (Intense):** In this case, the absorption band moves to longer wavelength by increasing the solvent polarity. The dipole-dipole interactions with the solvent molecules lower the energy of excited state more than that of the ground state. Hence the value of absorption maximum in ethanol will be greater than that observed in hexane. **Figure 1.7** depicts the absorption shift with change in solvent polarity.

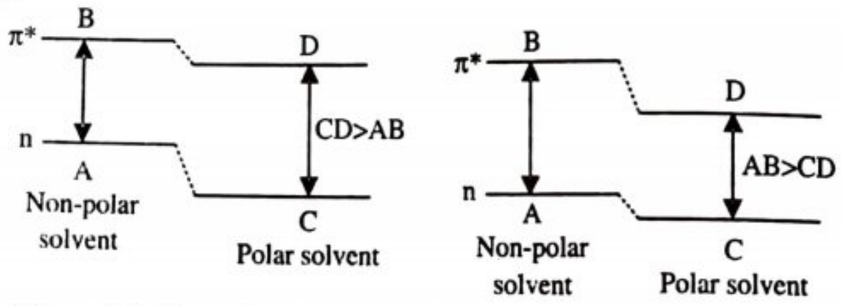


Figure 1.7: Absorption Shift with Change in Polarity of the Solvent

In short, π*-orbitals get more stabilised by hydrogen bonding with the polar solvents (like water and ethanol). It is because of greater polarity of π*-orbital compared to π-orbital. Thus, small energy will be needed for such a transition and absorption shows a red shift. The n→σ* transitions are also very sensitive to hydrogen bonding.

Alcohols as well as amines form hydrogen bonding with the solvent molecules. Such associations occur because of the presence of non-bonding electrons on the heteroatom and thus, transition requires greater energy. In general, it is said that:

1) When a group (say, carbonyl) is more polar in the ground state than in the excited state, increasing solvent polarity stabilises the non-bonding electrons in the ground state because of hydrogen bonding. Thus, absorption is shifted to lower wavelength.

2) When the group is more polar in the excited state, absorption gets shifted to longer wavelength with increase in solvent polarity, which helps in stabilising the non-bonding electrons in the excited state.

The increase in solvent polarity shifts n→π* and n→σ* bands to shorter wavelengths and π→π* bands to longer wavelengths.

1.2. BEER & LAMBERT'S LAW - DERIVATION

1.2.1. Introduction

In a typical absorption spectral measurement, a monochromatic radiation is made to fall on a sample taken in a suitable container, called **cuvette**. In such a situation a part of the radiation is reflected, a part is absorbed, and a part is transmitted. The **intensity of original radiation** (P_o) is equal to the sum of the intensities of reflected (P_r), absorbed (P_a), and transmitted (P_t) radiations, i.e.,

$$P_o = P_r + P_a + P_t$$

The effect of reflection can be compensated by passing equal intensities of beams through the solution and solvent contained in the same or similar container and comparing the transmitted radiations. Thus, the above equation can be rewritten as:

$$P_o = P_a + P_t$$

The intensity of transmitted light is measured and is found to depend on the thickness of absorbing medium and the concentration, besides the intensity of the incident radiation. This dependence forms the basis of spectrometric determinations and is given in terms of two fundamental laws. One is **Bouguer's law** or **Lambert's law**, which expresses the relationship between the light absorption capacity of the sample and the thickness of the absorbing medium; and the other is **Beer's law**, which expresses the relationship between the light absorptive capacity of the sample and its concentration. The two laws are combined together to give Beer-Lambert's law. These laws are individually discussed below:

1) **Lambert's or Bouguer's Law: Lambert** and **Bouguer** independently studied the decrease in radiation intensity when it passes through a substance and made the following **observations**:
   i) The amount of monochromatic light absorbed by a substance is proportional to the intensity of incident light, i.e., the ratio of the intensity of transmitted light to that of the incident light is constant.
   ii) The intensity of transmitted light decreases exponentially when the thickness of substance, through which the light is passing, increases linearly.

These observations, called Lambert's law can be translated into a mathematical expression as described below:

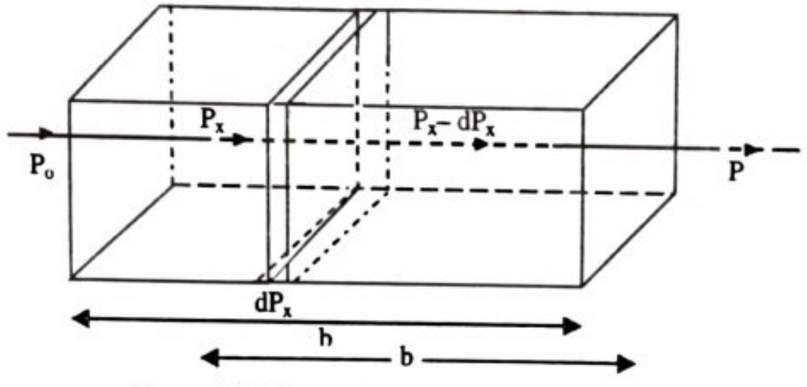


Figure 1.8: Illustration for the Lambert's Law

In **figure 1.8**, if P_o represents the radiant power of incident light and P represents the radiant power of transmitted light after passing through a slab of thickness b, consider a small slab of thickness dx, then the change in power (dP_x) is proportional to the power of incident light (P_x) multiplied by the change in thickness (dx) of the slab through which the light is passed.

$$dP_x \propto P_x dx \qquad \text{Or,} \quad dP_x = -kP_x dx \qquad \text{.....(1)}$$

Where, k is the proportionality constant and the negative sign indicates that radiant power decreases with absorption. **Equation (1)** can be re-arranged to:

$$\frac{dP_x}{P_x} = -kdx \qquad \text{.....(2)}$$

On integrating **equation (2)** within the limits of P_o to P for intensity and 0 to b for the thickness:

$$\int_{Po}^{P} \frac{dP_x}{P_x} = -k\int_{0}^{b} dx$$

$$\ln\frac{P}{P_o} = -kb \qquad \text{.....(3)}$$

Equation (3) is the mathematical expression for Bouguer-Lambert law or Lambert's law. On changing this equation to base 10 logarithms and re-arranging:

$$\log\frac{P_o}{P} = \frac{k}{2.303}b = k'b \qquad \text{..... (4)}$$

The ratio P/P_o has been inverted to remove the negative sign. Lambert's law applies to any homogeneous non-scattering medium, regardless of whether it is gas, liquid, solid, or solution.

2) **Beer's Law:** In **1852**, **Beer** and **Bernard** independently studied the dependence of intensity of transmitted light on the concentration of solution. It was observed that the relation between the intensity of transmitted light and concentration was exactly the same as found by **Lambert** for the intensity of transmitted light and the thickness of absorbing medium.

Let us assume a monochromatic radiation beam of intensity P_x traversing any thickness of solution of a single absorbing substance of concentration c. If c is changed by a small amount dc to c + dc, the change in transmitted power dP_x is proportional to the incident intensity P_x and dc.

Hence, $dP_x \propto P_x dc$

$$dP_x = -k''P_x dc \qquad \text{.....(5)}$$

Where, k″ is the proportionality constant and the negative sign indicates that radiant power decreases with absorption. On rearranging **equation (5)**:

$$\frac{dP_x}{P_x} = -k''dc \qquad \text{.....(6)}$$

On integrating **equation (6)** within the limits of P_o to P for intensity and 0 to c for concentration:

$$\int_{P_o}^{P} \frac{dP_x}{P_x} = -k''\int_{0}^{c} dc \qquad \text{Or,} \qquad \ln\frac{P}{P_o} = -k''c$$

$$\text{Or,} \qquad \log_{10}\frac{P_o}{P} = \frac{k''}{2.303}c \qquad \text{.....(7)}$$

Equation (7) is the mathematical expression for Beer's law. The Lambert's and Beer's laws are combined to obtain **equation (8)**:

$$\log\frac{P_o}{P} = abc \qquad \text{.....(8)}$$

In this expression, 'a' is a constant (combining two constants k′, k″ and the numerical factor) and is called **absorptivity** (extinction coefficient) whose value depends on unit of concentration (c) used and is a function of wavelength of the monochromatic light used. The concentration is generally expressed in terms of gm/dm^3 and 'b' in cm. Therefore, it has units of $\mathbf{cm^{-1}g^{-1}dm^3}$. However, if the concentration is expressed as mol/dm^3 and b in cm, then it is called **molar absorptivity** (molar extinction coefficient) and expressed as ε. Its unit is $\mathbf{cm^{-1}mol^{-1}dm^3}$. The **modified expression for the Beer-Lambert's law** is:

$$\log\frac{P_o}{P} = \varepsilon bc \qquad \text{.....(9)}$$

The term $\log P_o/P$ is called **absorbance** and is represented as 'A'. Thus:

$$A = \log\frac{P_o}{P} \qquad \text{.....(10)}$$

Now the expression for Beer-Lambert's law becomes:

$$\log\frac{P_o}{P} = A = abc \text{ or } \varepsilon bc \qquad \text{.....(11)}$$

1.2.2. Deviations

According to Beer's law, the absorbance (A) (for a monochromatic radiation beam) of a solution containing an absorbing compound (X), is proportional to its concentration (c), and is given by:

$$A = \varepsilon bc$$

Where, ε = Molar absorptivity of X at particular wavelength.
b = Optical path length.

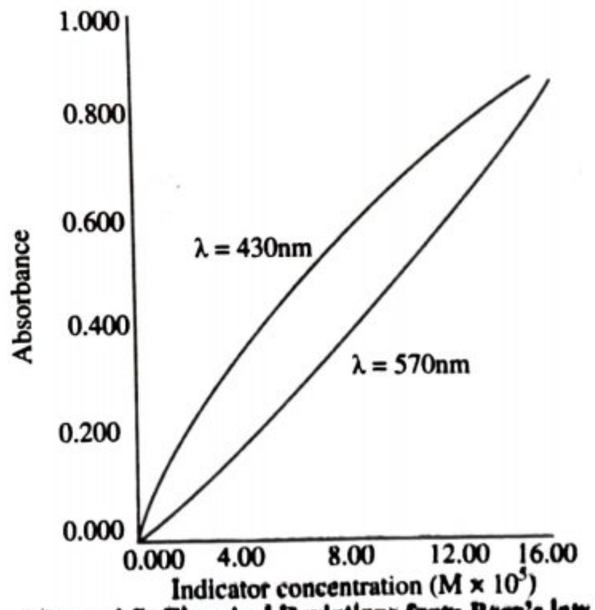


Figure 1.9: Chemical Deviations from Beer's law for Un-buffered Solutions of the Indicator

Beer's law is subjected to certain real and apparent deviations. Real deviations are most usually encountered in relatively concentrated solutions of the absorbing compound (>0.01M). These deviations are due to the interactions between the absorbing species and due to alterations of refractive index of the medium.

The **three types of deviations** that may occur from Beer-Lambert's Law are:

1) **Chemical Deviations:** These deviations appear when the absorbing species undergo association, dissociation, or reaction with the solvent to give products that absorb differently from the analyte. The extent of such departures can be predicted from the molar absorptivities of the absorbing species and the equilibrium constants for the equilibrium involved. Unfortunately, since the effects of such processes on the analyte are unknown, there is no opportunity to correct the measurement. Typical equilibrium that gives rise to this effect includes monomer-dimer equilibrium, metal complexation equilibria, acid-base equilibria, and solvent-analyte association equilibria.

   The plots of **figure 1.9** illustrate the kinds of deviations from Beer's law that occur when the absorbing system undergoes dissociation or association. Notice that the direction of curvature is opposite at the two wavelengths.

2) **Instrumental Deviations by the Stray Light:** Stray radiation, commonly called stray light, is defined as **radiation from the instrument that is outside the nominal wavelength band chosen for the determination**. This stray radiation often is the result of scattering and reflection of the surfaces of gratings, lenses or mirrors, filters, and windows. When measurements are made in the presence of stray light, the observed absorbance is given by:

$$A' = \log \frac{P_0 + P_s}{P + P_s}$$

   Where, P_s = Radiant power of the stray light. Stray light always causes the apparent absorbance to be lower than the true absorbance. The deviations due to stray light are most significant at high absorbance values. Because stray radiation levels can be as high as 0.5% in modern instruments, absorbance levels greater than 2.0 are rarely measured unless special precautions are taken or special instruments with extremely low stray light levels are used. Some inexpensive filter instruments can exhibit deviations from Beer's law at absorbance as low as 1.0 because of high stray light levels or the presence of polychromatic light.

3) **Deviations by the Polychromatic Radiation:** Strict adherence to Beer's law is observed only with truly monochromatic radiation. Monochromators are used to isolate portions of the output from continuum light sources; hence a truly monochromatic radiation never exists and can only be approximated, i.e., by using a very narrow exit slit on the monochromator.

   The incident radiation consists of just two wavelengths (λ' and λ"), with powers (P_0' and P_0''). Considering that $A = -\log(P/P_0)$, then the power of radiation (P) to come out from the cell for each wavelength would be:

$$P' = P_0 10^{-\varepsilon' bC}$$
$$P'' = P_0 10^{-\varepsilon'' bC}$$

   Where ε' and ε" = Molar absorptivity for each wavelength. Therefore, the measured absorbance (A_m) will be:

$$A_m = -\log\left(\frac{P' + P''}{P_0' + P_0''}\right) = \log\left(\frac{P_0' + P_0''}{P_0' 10^{-\varepsilon' bC} + P_0'' 10^{-\varepsilon'' bC}}\right)$$

1.2.3. Limitations

The linearity of Beer-Lambert's law is limited by chemical and instrumental factors. Causes of non-linearity include:

1) Deviations in absorptivity coefficients at high concentrations (>0.01M) due to electrostatic interactions between the molecules in close proximity,
2) Scattering of light due to particulates in the sample,
3) Fluorescence or phosphorescence of the sample,
4) Changes in refractive index at high analyte concentration,
5) Shifts in chemical equilibria as a function of concentration,
6) Non-monochromatic radiation deviations can be minimised by using a relatively flat part of the absorption spectrum such as the maximum of an absorption band, and
7) Stray light.

1.3. WOODWARD-FIESER RULE

1.3.1. Introduction

Woodward-Fieser Rules describe the **effect of alkyl substituents or ring residues**. From the study of UV absorption spectra of a large number of compounds, **Woodward** gave certain rules for correlating λ_{max} with molecular structure. Since then these rules have been modified by **Scott** and **Feiser** because of more experimental data.

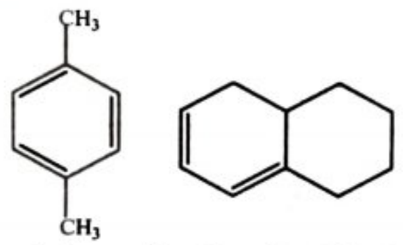
The modified rules, known as Woodward-Fieser rules, can be used to calculate the position of λ_{max} for a given structure by relating the position of λ_{max} with the position and degree of substitution of chromophore. Application of Woodward-Fieser Rules on different classes of compounds is discussed below:

1) Woodward-Fieser rules for calculating λ_{max} in conjugated dienes, trienes, and polyenes, and
2) Woodward-Fieser rules for α,β-unsaturated carbonyl compounds

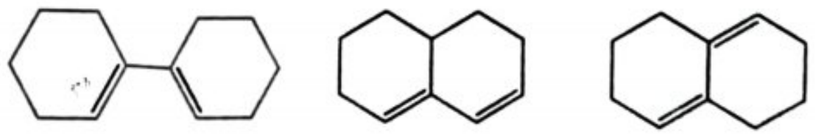
1.3.2. Woodward-Fieser Rules for Calculating λ_{max} in Conjugated Dienes, Trienes, and Polyenes

Before considering the rules for dienes, trienes, etc., it will be useful to discuss some terms involved in the rules:

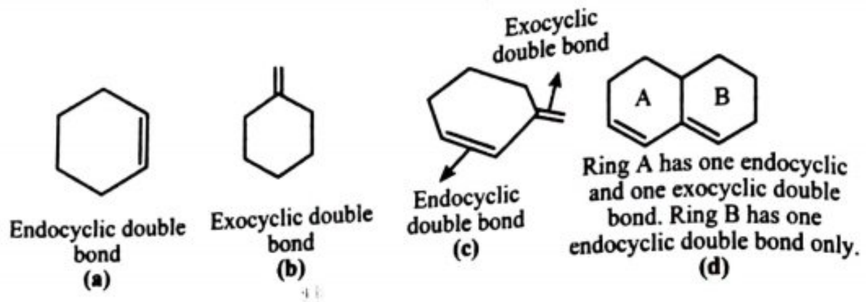
1) **Homoannular Diene:** It is a cyclic diene having conjugated double bonds in the same ring. **For example,**



2) **Heteroannular Diene:** It is a cyclic diene in which double bonds in conjugation are present in different rings. **For example,**



3) **Endocyclic Double Bond:** It is a double bond present in a ring as shown below in **structure (a)**.

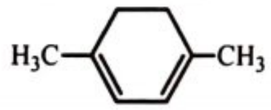


4) **Exocyclic Double Bond:** It is a double bond in which one of the doubly bonded atoms is a part of a ring system as shown above in **structure (b)**. According to these rules, each type of diene or triene system is having a certain fixed value at which absorption takes place. This constitutes the basic value or parent value. The contributions made by various alkyl substituents or ring residues, double bonds extending conjugation and polar groups (such as –Cl, –Br, and –OR) are added to the basic value to obtain λ_{max} for a particular compound. The parent values and contributions of different substituents/groups are given in **table 1.2**:

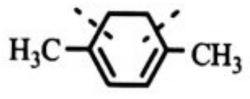
Table 1.2: Parent Values and Increments for Different Substituents/Groups

1) **Parent Values**	
i) Acyclic conjugated diene and heteroannular conjugated diene	215nm
ii) Homoannular conjugated diene	253nm
iii) Acyclic triene	245nm
2) **Increments**	
i) Each alkyl substituent or ring residue	5nm
ii) Exocyclic double bond	5nm
iii) Double bond extending conjugation	30nm
iv) **Auxochromes**	
a) –OR	6nm
b) –SR	30nm
c) –Cl, –Br	5nm
d) $-NR_2$	60nm
e) $-OCOCH_3$	0nm

Example 1: Calculate λ_{max} for 1,4-dimethylcyclohex-1,3,-diene.

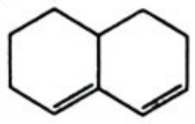


Solution: The given compound is a homoannular diene which is having two alkyl substituents and two ring residues (as depicted below by dotted lines).



∴ Parent value for homoannular diene = 253nm
Two alkyl substiuents = 2 × 5 = 10nm
Two ring residues = 2 × 5 = 10nm
∴ Calculated value = 273nm
Observed value = 265nm

Example 2: Calculate λ_{max} for



Solution: Base value = 214nm
Ring residue = 3 × 5 = 15nm
Exocyclic double bond = 1 × 5 = 5nm
∴ Calculated value = 214 + 15 + 5 = 234nm

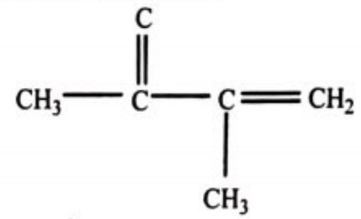
1.3.3. Woodward-Fieser Rules for α,β-Unsaturated Carbonyl Compounds

Woodward-Fieser rules for calculating λ_{max} for α,β-unsaturated carbonyl compounds modified by **Scott** may be summed-up in **table 1.3**:

Table 1.3: Parent Values and Increments for Different Substituents/Groups

1) **Parent Values**					
i) α,β-unsaturated acyclic or six-membered ring ketone					215nm
ii) α,β-unsaturated five-membered ring ketone					202nm
iii) α,β-unsaturated aldehyde					210nm
iv) α,β- unsaturated carboxylic acids and esters					195nm
2) **Increments**					
i) Each alkyl substituent or ring residue					
a) at α-position					10nm
b) at β-position					12nm
c) at γ and higher positions					18nm
ii) Each exocyclic double bond					5nm
iii) Double bond extending conjugation					30nm
iv) Homoannular conjugated diene					39nm
v) **Auxochromes**	**Positions**				
	α	**β**	**γ**	**δ**	
a) –OH	35	30	–	50	
b) –OR	35	30	17	31	
c) –SR	–	85	–	–	
d) $-OCOCH_3$	6	6	6	–	
e) –Cl	15	12	–	–	
f) –Br	25	30	–	–	
g) $-NR_2$	–	95	–	–	

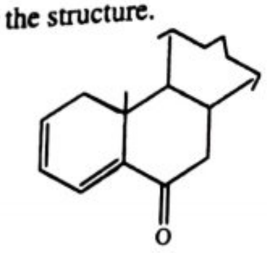
Example 3: Calculate λ_{max} for the compound.



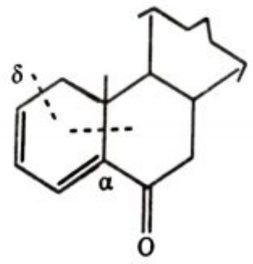
Solution: The given compound is an α,β-unsaturated acyclic ketone which is having an alkyl substituent to α-position.

∴ Parent value for α,β-unsaturated acyclic ketone = 215nm
One alkyl substitution in α-position = 10nm
∴ Calculated value = 225nm
Observed value = 220nm

Example 4: Compute λ_{max} for the structure.



Solution: The given structure represents an α,β-unsaturated 6-membered ring ketone having a ring residue at α-position and another ring residue at δ-position. It is having an exocyclic double bond, a double bond extending conjugation, and a homoannular diene component.



∴ Parent value	= 215nm
One α ring residue	= 10nm
One δ ring residue	= 18nm
One exocyclic double bond	= 5nm
One double bond extending conjugation	= 30nm
One homoannular conjugated diene	= 39nm
∴ Calculated value	= 317nm
Observed value	= 319nm

1.4. INSTRUMENTATION

1.4.1. Introduction

The essential parts of a spectrophotometer are:

1) **Radiation Source:** Both the tungsten and D_2 lamp are present in the UV-visible spectrophotometer.
2) **Wavelength Selector:** It constitutes three essential parts:
   i) **Filter:** Absorption and interference filters are mainly used.
   ii) **Monochromator:** It gives the desired wavelength in the entire UV or visible region.
   iii) **Slits:** There are two slits, i.e., entrance slit and exit slit.
3) **Cells or Cuvettes:** For holding the sample solution and the pure solvent (reference).
4) **Detector:** The most commonly used detectors are photo-emissive cells or phototubes and photomultiplier tubes.
5) **Recording System:** Recording is done by recorder pen.
6) **Power Supply**

Spectrophotometers are of **two types**:

1) Single-beam spectrophotometers, and
2) Double-beam spectrophotometers.

1.4.2. Sources of Radiation

The best source of light is the one which is more stable, more intense and which gives range of spectrum from 180-360nm (up to 400nm). The different sources available are:

1) **Hydrogen Discharge Lamp:** In these lamps, hydrogen gas is stored under relatively high pressure. When an electric discharge is passed through the lamp, excited hydrogen molecules will be produced which emit UV radiations. The high pressure in the hydrogen lamps causes the hydrogen to emit a continuum rather than a simple hydrogen spectrum.

   Hydrogen lamps cover the range of **3500-1200Å**. These lamps are stable, robust, and widely used. If deuterium (D_2) is used instead of hydrogen, the emission intensity is increased by as much as a factor of 3 at the short-wavelength end of the UV range.
2) **Deuterium Lamp:** It is similar to hydrogen discharge lamp, but filled with deuterium in the place of hydrogen. It offers 3-5 times more intensity than other types. Deuterium lamps are more expensive than hydrogen lamps but are used when higher intensity is required.
3) **Xenon Discharge Lamp:** In this lamp, xenon at 10-30 atmospheric pressure is filled in and it has two tungsten electrodes. The **intensity is greater** than the hydrogen discharge lamp.
4) **Mercury Arc:** This contains mercury vapour and offers bands which are sharp. The spectrum is not continuous. Hence, it is **not widely used**.
5) **Tungsten Lamp:** This lamp is similar in its functioning to an electric light bulb. It is a tungsten filament heated electrically to white heat. It has two shortcomings. The intensity of radiation at short wavelengths (<350nm) is small. Furthermore, to maintain a constant intensity, the electrical current to the lamp must be carefully controlled.

1.4.3. Wavelength Selectors

Wavelength selectors consist of three parts:

1) Filters,
2) Monochromators, and
3) Slits.

1.4.3.1. Filters

Filters provide high radiation throughout, approximately 50-80% efficiency. The two types of filters are:

1) **Absorption Filters:** These filters derive their effects from bulk interactions of radiation within the material. Absorption filters are produced in a variety of host materials, like gelatin, glass, liquid, and plastic. **Glass filters** are used in automated chemical analysis equipment and colorimetry. The scattering type depends on scattering crystals formed within the glass mass through a reduction and thermal treatment. Shorter wavelengths are scattered and absorbed, while longer wavelengths are unaffected. **Cut-on and cut-off (or sharp-cut) filters** are widely used as blocking filters to suppress unwanted spectral orders from interference filters and diffraction gratings.

   There are a wide variety of **plastic filters**, of both sharp cut and intermediate bandwidth types. Plastic filters may be produced either by bulk colourants introduced into the basic batch or through subsequent dye treatments of clear base stock. Cut-on types, unlike their glass counterparts, exhibit no fluorescence in the visible region.

2) **Interference Filters:** These filters, as the name implies, are based on the phenomenon of optical interference. A simple two-interface (Fabry-Perot) filter consists of a dielectric spacer film (CaF_2, MgF_2, or SiO) sandwiched between two parallel, partially reflecting metal films, usually of silver (**figure 1.10**). The thickness of dielectric film is controlled to be only one, two, or three half-waves thick. These are referred to as first-, second-, or third-order filters, respectively.

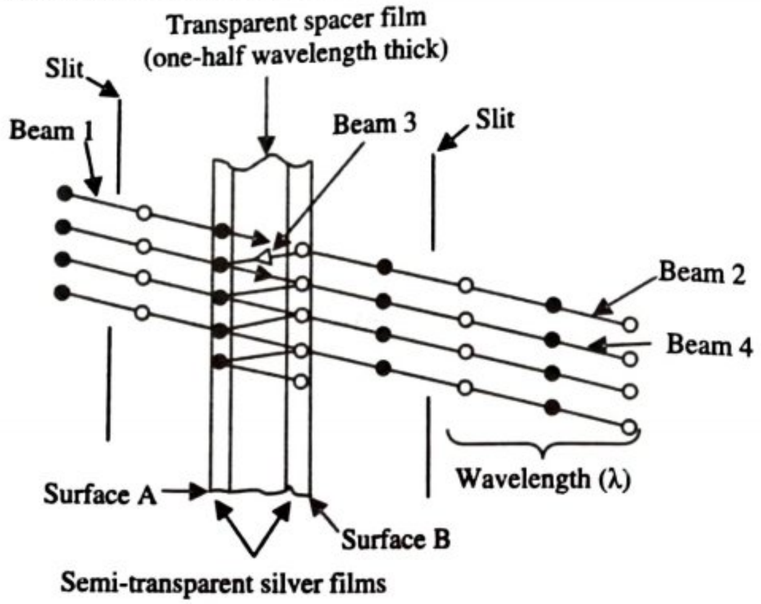


Figure 1.10: Path of Light Rays through Interference Filter

A portion of the incident radiation normal to the filter (beam 1) passes through beam 2, while another portion (beam 3) is reflected back from surface B to surface A. Portion of this reflected radiation is again reflected from surface A through the dielectric layer and exits as beam 4 parallel (actually coincident) to beam 2.

Thus, the **path travelled by beam 4 is longer than that of beam 2** by twice the product of the dielectric spacer thickness and its refractive index. When the layer thickness (b) is half the wavelength of the radiation to be transmitted in the refractive index (η) of the dielectric, beams 2 and 4 are in phase and interfere constructively.

The **expression for central wavelengths at which full reinforcement occurs** is:

$$\lambda = \frac{2\eta b}{m}$$

Where, m = Order number. Since partial reinforcement occurs for other path differences, the filter actually transmits a band of radiant energy. Furthermore, the angle of incidence of the radiation must be 90°. Any phase shifts on reflection are ignored. The bandwidth is 10-15nm, Full Width at Half Maximum (FWHM) transmission; the maximum transmission is usually 40% with this type of filter.

1.4.3.2. Monochromators (Prisms and Gratings)

The monochromator is used to disperse the radiation according to the wavelength. The essential elements of a monochromator are an **entrance slit**, a **dispersing element**, and an **exit slit**. The entrance slit sharply defines the incoming beam of heterochromatic radiation. The dispersing element disperses the heterochromatic radiation into its component wavelengths whereas exit slit allows the nominal wavelength together with a band of wavelengths on either side of it. Position of the dispersing element is always adjusted by rotating it to vary the nominal wavelength passing through the exit slit.

The dispersing element may be a **prism** or **grating**. The prisms are generally made of glass, quartz, or fused silica. Glass has the highest resolving power but it is not transparent to radiations having the wavelength between 2000-3000Å because glass absorbs strongly in this region. Quartz and fused silica prisms are transparent throughout the entire UV range and are widely used in UV spectrophotometers. Fused silica prisms are little more transparent in the short wavelength region than quartz prisms, and are used only when very intense radiation is required. The mirrors in the optical system are front surfaced because glass starts to absorb in the UV region.

Characteristics of a Monochromator

1) It allows the largest entrance slit width for the band pass required.
2) It has the highest dispersion.
3) The largest optics is affordable.
4) Longest focal length is affordable.
5) Highest groove density will accommodate the spectral range.
6) Optics and coatings are appropriate for specific spectral range.
7) Entrance optics will optimise endues.
8) If the instrument is to be used at a single wavelength in a non-scanning mode, it must be possible to adjust the exit slit to match the size of the entrance slit image.

Types of Monochromator

1) **Prism Monochromator:** A **single-pass monochromator** has been illustrated in **figure 1.11**. 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 entrance slit, strikes the off-axis parabolic Littrow mirror (B), which renders the radiation parallel and sends it to the prism (C). The dispersed radiation after reflecting from a plane mirror (D) returns through the prism a second time and focuses onto the exit slit of the monochromator, through which it finally passes into the detector section.

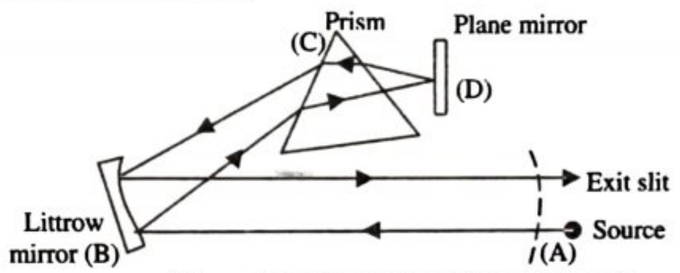


Figure 1.11: Single-Pass Monochromator

In **double-pass monochromator (figure 1.12)**, there occurs a total of four passes of radiations, i.e., (1), (2), (3) and (4) through the prism. The double pass monochromator produces more resolution than the monochromator in the radiation, before it finally passes on to the detector.

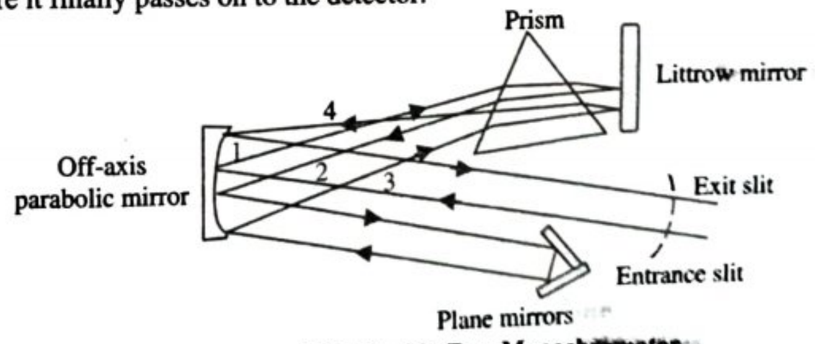


Figure 1.12: Double-Pass Monochromator

2) **Grating Monochromator:** Gratings provide an alternative means of producing monochromatic light. A diffraction grating (**figure 1.13**) consists of a series of parallel grooves (lines) on a reflecting surface that is produced by taking a replica from a master carefully prepared using a machine, or from one which is holographically generated. The grooves can be considered as separate mirrors from which the reflected light interacts with the light reflected from neighbouring grooves to produce interference, and so to select preferentially the wavelength that is reflected when the angle of the grating to the incident beam is changed.

When parallel radiation illuminates a reflecting diffraction grating, the multiple reflections from the mirror grooves will overlap and interfere with each other. If the reflected waves are in phase, interference is said to be **constructive** and the reflected light is not affected. If the reflected waves are out of phase, there is **destructive** interference and light of the wavelength at which such interference occurs will not be propagated.

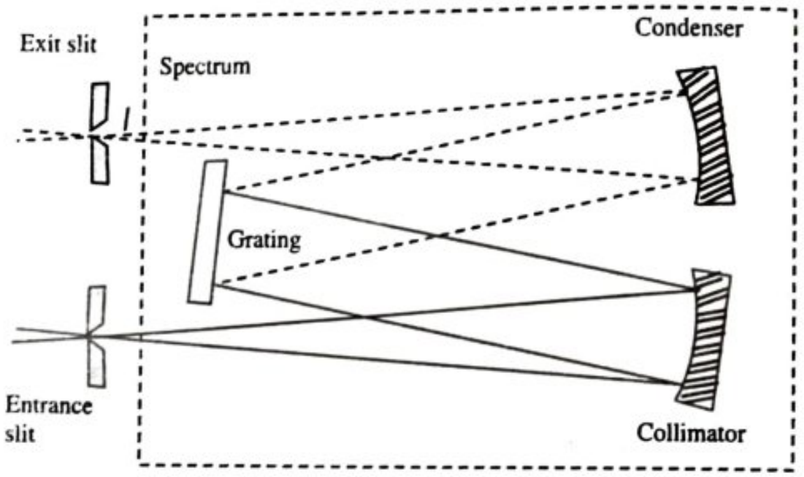


Figure 1.13: Grating Monochromator

The **relationship that determines the wavelength of reflected light** is expressed by:

$$n\lambda = 2d\sin\theta$$

Where, n = Order.
d = Separation of the reflecting surfaces (or lines).
θ = Angle of incidence of the radiation.

Grating monochromator possesses the following **advantages over prism monochromator**:

i) Grating can be made with materials which are not attacked by moisture like aluminium. On the other hand, metal salt prisms get subjected to etching from atmospheric moisture.
ii) Grating monochromators can be used over considerable wavelength ranges.
iii) Grating offers better resolution and energy transfer.
iv) Grating has constant bandwidth due to linear dispersion.
v) Grating requires less complicated wavelength drive mechanism.
vi) Stray light is limited to imperfections at the grating surface.

1.4.3.3. Slits

There are two slits, i.e., **entrance slit** and **exit slit**. The main function of the entrance slit is to provide a narrow source of light so that there should be no overlapping of monochromatic images. From this, the exit slit selects a narrow band of dispersed spectrum for observation by the detector. In practical spectrophotometry, the monochromator module is not capable of isolating a single wavelength of radiation from

the continuous spectrum emitted by the source. Rather, a definite band of radiation is passed by the monochromator. This finite band arises from the slit distributions. The entrance or aperture of a monochromator is a long, narrow slit whose width is generally adjustable. Inside the monochromator, the rays diverge from the entrance slit and illuminate the collimator mirror, which renders the rays parallelly and focuses them on the dispersing element. Leaving the collimator, the parallel set of rays is a broadened version of the entrance slit.

This rectangle of radiation must be large enough to illuminate the entire side of the prism or the length of the grating. In turn, the dispersing device separates the incident polychromatic radiation into an array of monochromatic rectangles, each of which leaves the dispersing device at a slightly different angle. The monochromatic rectangles overlap. The dispersed beam is intercepted by a second collimating mirror identical to the first (or a segment of the first collimator), which is used to focus and reduce each rectangle to an image of the entrance slit. These final images fall in a plane called the **focal plane**, in which a stationary exit slit is located. The distance between the second collimator and the exit slit is called the **focal length of the monochromator**.

1.4.4. Sample Cells or Cuvettes

Sample containers, which are usually called cells or cuvettes, must have windows that are transparent in the spectral region of interest. **Quartz** or **fused silica** is required for the UV region (wavelengths less than 350nm) and may be used in the visible region. **Silicate glass** is ordinarily used for 375-2000nm region because of its low cost compared to quartz. **Plastic cells** are also used in the visible region.

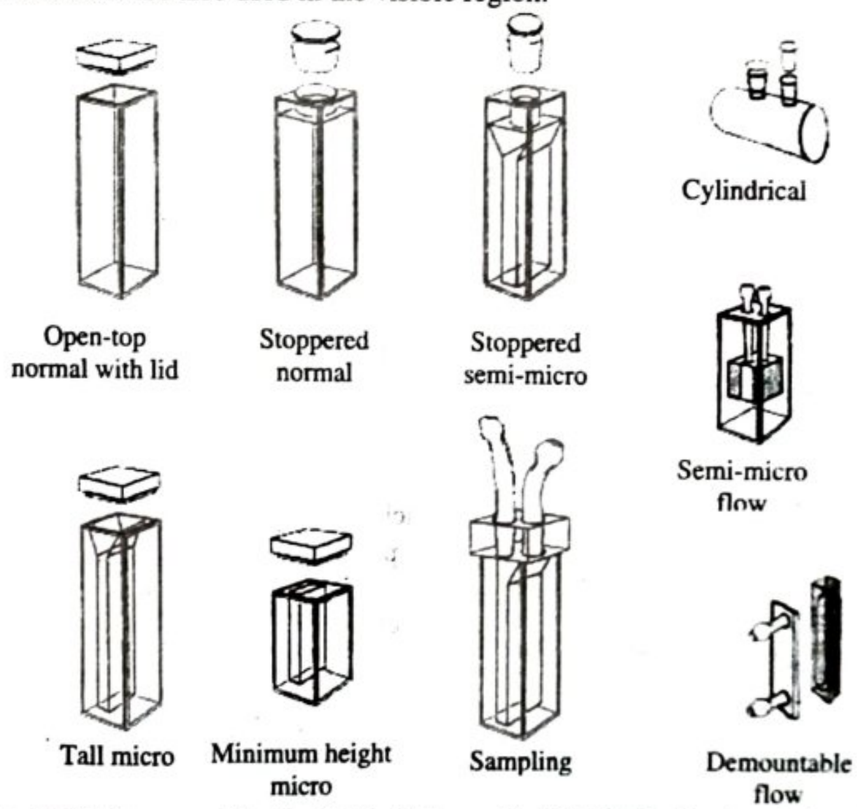


Figure 1.14: Commercially Available Cells used in UV-Visible Spectrophotometer

The best cells have windows that are perpendicular to the direction of the beam in order to minimise reflection losses. The most common cell path length for studies in the UV and visible regions is of 1cm; matched, calibrated cells of this size are available from several commercial sources. Many other cells with shorter and longer path lengths can be purchased.

For reasons of economy, cylindrical cells are sometimes used. Particular care must be taken to duplicate the position of such cells with respect to the beam; otherwise, variations in path length and reflection losses at the curved surfaces can cause significant error. Some typical UV-visible cells are shown in **figure 1.14**.

1.4.5. Detectors

Detectors used in UV-visible spectrophotometers can be called as **photometric detectors**. The most commonly used detectors are:

1) Phototubes or photo-emissive cells,
2) Photomultiplier Tubes (PMT),
3) Photovoltaic cell or barrier-layer cells, and
4) Photomultiplier Silicon photodiode array detector.

1.4.5.1. Photo Tubes or Photo-Emissive Cells

This detector is composed of an evacuated glass tube, which consists of a photocathode and a collector anode. The photocathode is coated with elements of high atomic volume (like caesium, potassium, or silver oxide), which can liberate electrons when light radiation falls on it. This flow of electrons towards anode produces a current proportional to the intensity of light radiation. Composite coatings like caesium/caesium oxide/silver oxide can also be used, which increases the sensitivity and range of wavelength in which the detector can be used (UV region).

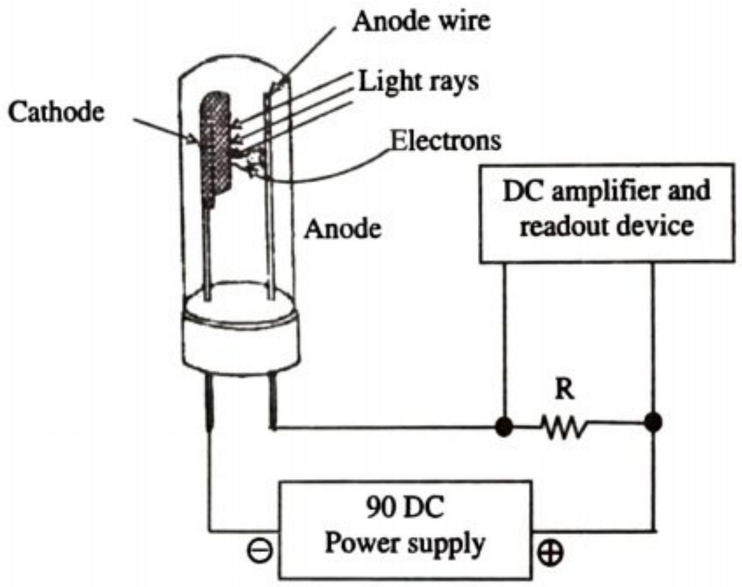


Figure 1.15: Photo Tube or Photo-Emissive Cell

The signal from the detector can also be amplified using an amplifier circuit (**figure 1.15**). Phototubes have **better sensitivity** when compared to photovoltaic cell, and hence are more widely used.

1.4.5.2. Photomultiplier Tubes (PMT)

This type of detector is the **most sensitive** of all the detectors, is expensive, and is used in sophisticated instruments. The principle employed in this detector is **multiplication of photoelectrons by secondary emission of electrons**. This is achieved by using a photocathode and a series of up to 10 anodes (dynodes).

Each dynode is maintained at 75-100V higher than the preceding one. At each stage, the electron emission is multiplied by a factor of 4 or 5 due to secondary emission of electrons; and hence an **overall factor** of 10^6 is achieved.

PMT can detect very weak signals. The sensitivity of photo-emissive cell (photocell) can be increased considerably by employing the photomultiplier tube (**figure 1.16**).

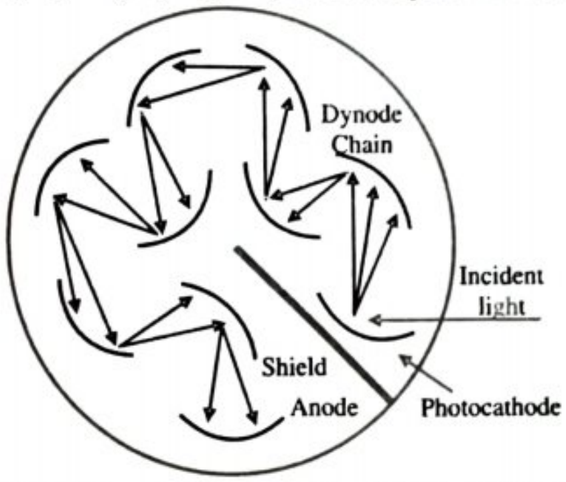


Figure 1.16: Schematic Representation of Photomultipier Tube

When the light radiation is incident upon the cathode surface, electrons are ejected. These are accelerated to the sensitive surface of the dynode where secondary electrons are emitted in greater number than initially striking the plate. These electrons in turn are accelerated to the surface of another dynode maintained at a higher potential, where the number of electrons is again increased by a factor of 4 or 5. The process is repeated several times until a large number of electrons arrive at a collector. The number of electrons falling on the collector measures the intensity of light incident on the cathode surface.

As the output of a photomultiplier tube is only several milli-amperes, it can be used to measure intensities about 200 times weaker than those measurable with a photoelectric cell and amplifier. The **response time of a photomultiplier tube is 10^{-9} s**. A photomultiplier tube must be carefully shielded from stray light.

1.4.5.3. Photovoltaic Cell or Barrier-Layer Cells

This cell is also known as **photronic cell** and operates without the use of a battery. It consists of a metal **base plate (of iron or aluminium), that acts as an electrode**. On its surface, a thin layer of a semiconductor metal (like selenium) is deposited. Then, the surface of selenium is covered by a very **thin layer of silver or gold that acts as the second collector electrode (figure 1.17)**.

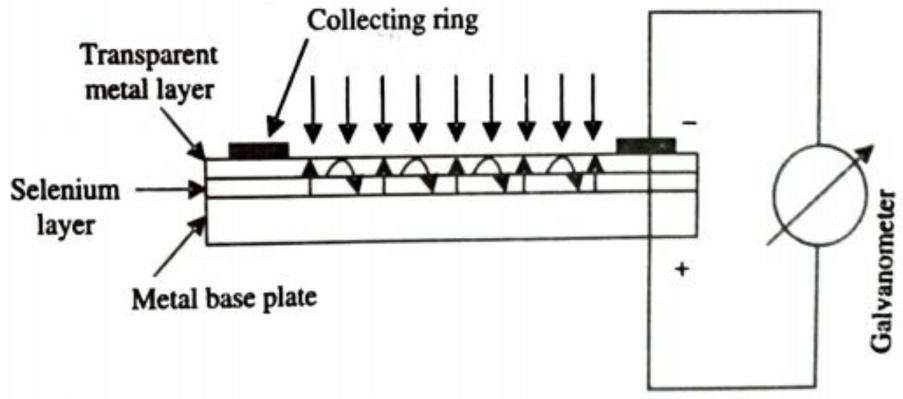


Figure 1.17: A Typical Photovoltaic Cell

When the radiation is incident upon the surface of selenium, electrons are generated at the selenium-silver interface. These electrons are collected by the silver. Accumulation of electrons on the silver surface creates an electric voltage difference between the silver

surface and the basis of the cell, and if the external circuit has a low resistance (abou 400Ω or less), a photocurrent will flow which is directly proportional to the intensity o the incident radiation beam. If this cell is connected to a galvanometer, a current wil flow that will vary with the intensity of the incident light.

These cells offer the **advantages** of being rugged and requiring no external powe supply; however, their use is generally **limited to the visible region**. The wavelengt response (450-650nm) is close to that of the human eye. High level of illumination i required to avoid the problems of amplifying the output which arise from the smal resistance of the external circuit.

1.4.5.4. Silicon Photodiode Array Detector

The principle involved in silicon photodiode detector is that **upon exposure to light th electrical properties of the detector change** (this is the internal photoelectric effect) Solar cells have the similar structure and work on the same principle.

The diode array is a multichannel detector that makes simultaneous measurement o wavelengths of dispersed radiation. It is made up of an array of silicon photodiode situated on a solo silicon chip (generally 1024). Each diode is scanned for response.

Though photomultiplier tube is more sensitive, diode array detector is beneficial fo simultaneous measurement of wavelengths. Since alignment problems are absent in diod array detector, it is more rugged than the photomultiplier tube. In diode array detector, optical performance variations with wavelength change are null, which are generally see in scanning monochromator instruments.

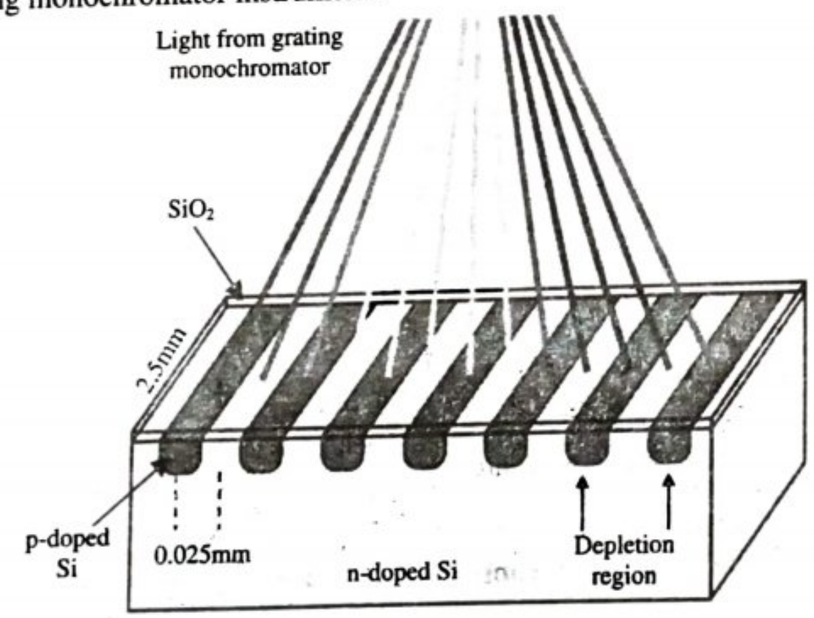


Figure 1.18: Diode Array Detector

Silicon photodiodes are **advantageous** over photomultipliers because of their low cost, low sensitivity in the light-receiving surface, and no need of special power supply. It obtains photometric data like that of photomultipliers if the light intensity is relatively large.

1.4.6. Recording System

The signal from the photomultiplier tube is finally received by the recording system. The recording is done by a **recorder pen**. This type of arrangement is only done in recording UV spectrophotometers.

1.4.7. Power Supply

The power supply serves **three** main functions:

1) It decreases the line voltage to the instruments operating level with a transformer.
2) It converts AC to DC with a rectifier if direct current is required by the instrument.
3) It smoothens out any ripple that may occur in the line voltage to deliver a constant voltage to the source lamp and instruments.

1.4.8. Single Beam UV Spectrophotometer

The **steps** involved in a single beam UV spectrophotometer (**figure 1.19**) are:

1) UV radiation is given off by the source.
2) A convex lens gathers the beam of radiation and focuses it on the inlet slit.
3) The inlet slit permits light from the source to pass, but blocks-out stray radiation.
4) The light then reaches the monochromator, which splits it up according to wavelength.
5) The exit slit is positioned to allow light of the required wavelength to pass through.
6) Radiation at all other wavelengths is blocked-out.
7) The selected radiation passes through the sample cell to the detector, which measures the intensity of the radiation reaching it.
8) By comparing the intensity of radiation before and after it passes through the sample, it is possible to measure how much radiation is absorbed by the sample at the particular wavelength used.
9) The output of the detector is usually recorded on graph paper.

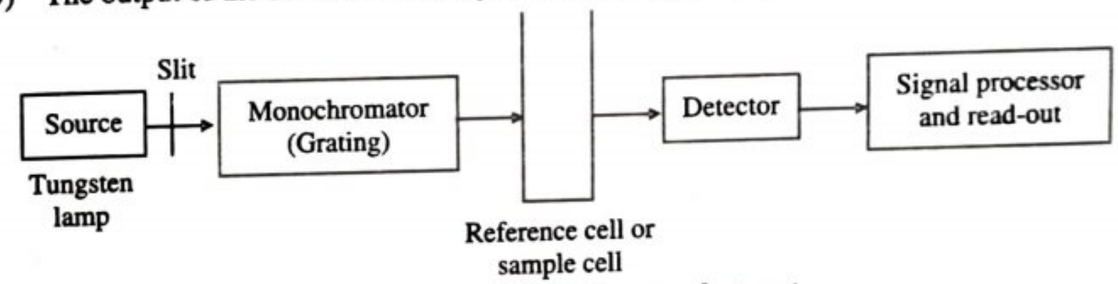


Figure 1.19: Single Beam Spectrophotometer

One **problem** with the single beam system is that it **measures the total amount of light reaching the detector**, rather than the percentage absorbed. Light may be lost at reflecting surfaces or may be absorbed by the solvent used to dissolve the sample. Furthermore, the source intensity may vary with changes in line voltage. **For example,** when the line voltage decreases, the intensity of the light coming from the source may decrease unless special precautions are taken. Consequently, the intensity of radiation may be constantly changing.

Another problem is that the **response of the detector varies significantly with the wavelength of the radiation falling on it**. Even if the light intensity is constant at all wavelengths, if the wavelength is steadily increased from 200-750nm, the signal from the detector starts at a low value, increases to a value that is steady over a wide range, and then decreases once more. This relationship between the signal from the detector and the wavelength of radiation is called the **response curve**.

1.4.9. Double Beam UV Spectrophotometer

A double beam spectrophotometer (**figure 1.20**), is also called a UV-visible spectrophotometer and it utilises **two sources**:

1) A tungsten lamp (400-800nm), and
2) A D_2 lamp (200-400nm).

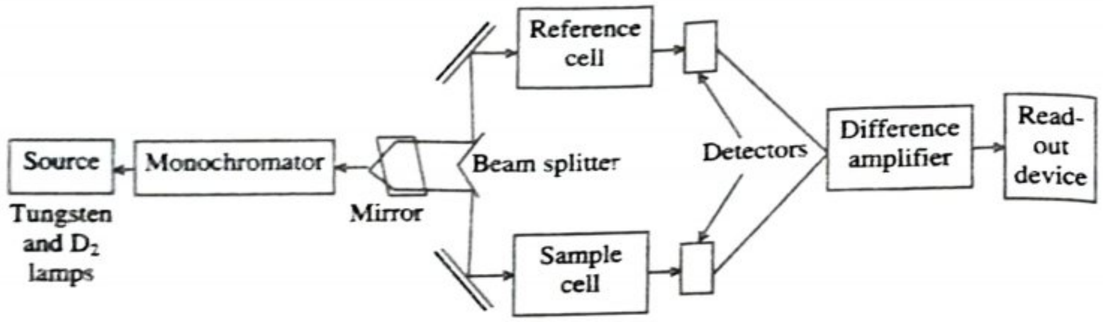


Figure 1.20: Double Beam Spectrophotometer

The **steps** involved in a double beam UV spectrophotometer are:

1) The radiation from the selected source passes through a fixed slit to the surface of reflection grating (monochromator), and from the diffracted radiation, the desired wavelength is selected.
2) This selected beam of light falls on a V-shaped mirror, called a **beam splitter** that splits the radiations into two beams; one of which passes through the reference cell containing pure solvent and the other simultaneously passes through the sample solution cell.
3) The transmitted light from the two cells go to the photoelectric detector alternatively, and the difference in the absorbance by the solvent and the sample solution is measured electronically with very high accuracy.

Although the double beam instruments are more complicated and expensive, they do offer the following **advantages**:

1) It is not necessary to continually replace the blank with the sample or to zero adjust at each wavelength as in the single beam units.
2) The ratio of the powers of the sample and reference beams is constantly obtained and used. Any error due to variation in the intensity of the source and fluctuation in the detector is minimised.
3) Because of the previous two factors, the double beam system lends itself to rapid scanning over a wide wavelength region and to the use of a recorder or digital readout.

1.4.10. Comparison of Single Beam and Double Beam Spectrophotometers

The essential features of single and double beam spectrophotometers are given in **table 1.4**:

Table 1.4: Difference between Single and Double Beam Spectrophotometers

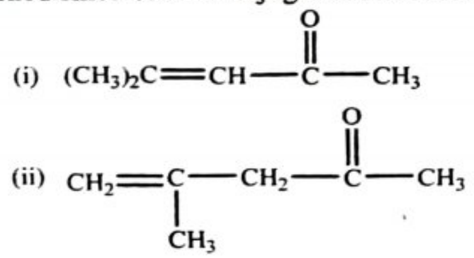
Instrumental Parameters	Single Beam Spectrophotometer	Double Beam Spectrophotometer
Common Name	Visible spectrophotometer	UV-visible spectrophotometer
Source	Tungsten lamp 400-800nm	Tungsten and D_2 lamps 200-400nm and 400-800nm, respectively
Wavelength Selection	Monochromator prism or grating	Monochromator prism or grating
λ_{max} Determination	Possible	Possible
Cells	Pyrex glass tube	Pyrex and quartz cells
Detection	Photoelectric effect	Photoelectric effect
Quantitative Analysis	Coloured solutions	Coloured and colourless solutions

1.5. APPLICATIONS

1.5.1. Introduction

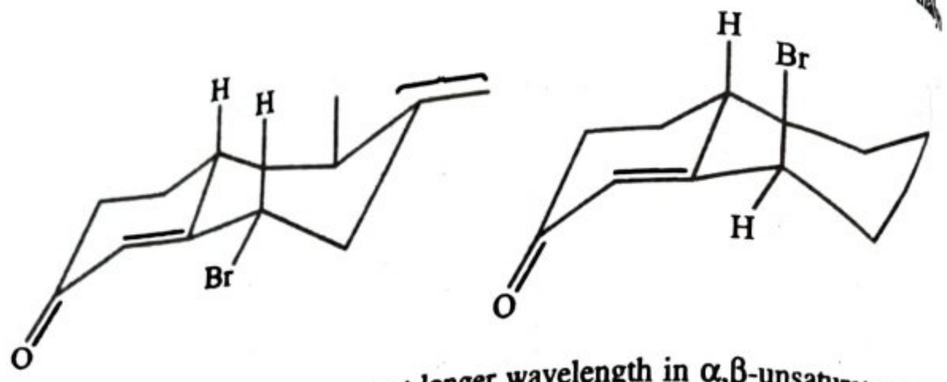
UV-Vis spectroscopy has been mainly applied for the detection of functional groups (chromophore), the extent of conjugation, detection of polynuclear compounds by comparison, etc. Some of the important applications are:

1) **Detection of Functional Groups:** The technique is applied to detect the presence or absence of a chromophore. The absence of a band at a particular wavelength may be regarded as an evidence for the absence of a particular group in the compound. A little information can be drawn from the UV spectrum if the molecule is very complicated. If the spectrum is transparent above 200mμ, it shows the absence of:
   i) Conjugation,
   ii) A carbonyl group (aldehydes and ketones),
   iii) Benzene or aromatic compounds, and
   iv) Bromo or iodo atoms.
2) **Extent of Conjugation:** The extent of conjugation in polyenes R—(CH=CH)$_n$—R can be estimated. Addition in unsaturation with the increase in the number of double bonds (increase in the value of n) shifts the absorption to longer wavelength. It is found that the absorption occurs in the visible region, i.e., at about 420mμ, if n = 8 in the polyene. Such an alkene appears coloured to the human eye.
3) **Distinction in Conjugated and Non-Conjugated Compounds:** It also distinguishes between a conjugated and a non-conjugated compound. The following isomers can be readily distinguished since one is conjugated and the other is not:



The forbidden n → π* band for the carbonyl group in the compound **(i)** will appear at longer wavelength compared to that for the compound **(ii)**. The alkyl substitution in an alkene causes a bathochromic shift. The technique is not much useful for the identification of individual alkenes.

4) **Identification of an Unknown Compound:** By comparing the spectrum of an unknown compound with that of a known compound, the former can be identified. If the two spectra coincide, the two compounds must be identical. If the two spectra do not coincide, the expected structure is different from the known compound.
5) **Identification of a Compound in Different Solvents:** Sometimes, the compound structure changes with the change in solvent. Chloral hydrate shows an absorption maximum at 290mμ in hexane while the absorption disappears in the aqueous solution. Clearly, the compound contains a carbonyl group in hexane solution and its structure is $CCl_3.CHO.H_2O$, whereas in aqueous solution it is present as $CCl_3.CH(OH)_2$.
6) **Distinguishes between Equatorial and Axial Conformations:** This technique also distinguishes between equatorial and axial conformations. Consider the following conformations:



The $n \rightarrow \pi^*$ (R-band) that appears at longer wavelength in α,β-unsaturated ketone is influenced by the presence of polar group in the γ-position. The effect of an axial substituent to displace the R-band to longer wavelength is greater compared to that observed in its equatorial isomer.

7) **Determination of Strength of Hydrogen Bonding:** Solvents like water, C_2H_5OH etc., form hydrogen bonds with the n-electrons of carbonyl oxygen. Due to this, the energy of n-electrons in the ground state is lowered depending on the strength of hydrogen bonds.

Thus, $n \rightarrow \pi^*$ transition of carbonyl compounds is shifted towards shorter wavelength. Hence, by measuring the λ_{max} of a carbonyl compound in a non-polar and polar protic solvent, the strength of hydrogen bond can be determined.

1.5.2. Spectrophotometric Titrations

In a spectrophotometric titration, the equivalence point is determined with a spectrophotometer. In this technique, the titration vessel is kept directly in the light path of the instrument. Then, the absorbance of the solution is determined after adding titrant and a plot of absorbance as a function of volume of titrant is prepared. Typical titration curves are shown in **figure 1.21**.

If the titration reaction is complete, the titration curve will consist of two straight lines intersecting at the equivalence point, similar to amperometric and conductometric titrations.

On the other hand, if the titration reaction is not complete, there occurs appreciable curvature in the equivalence point region but extrapolation of the two linear segments of the titration curve to their intersection gives the equivalence point volume.

From **figure 1.21**, it is concluded that:

1) **Curve (a)** is characteristic of a case where only the titrant absorbs. An interesting **example** of this is the titration of arsenic(III) with bromate-bromide, where the absorbance readings are taken at the wavelength where bromine absorbs.

   As long as arsenic(III) remains in the solution, the absorbance will not be changed because the product does not absorb in that region. As soon as arsenic(III) is consumed by the titrant, the absorbance will increase due to the colour of the titrant (bromine) alone.

2) **Curve (b)** is characteristic of a case where only the product of reaction absorbs. An **example** of this is the titration of Cu(II) with EDTA, carried out at 745nm wavelength. This wavelength is selected because at this position the EDTA-copper complex possesses a much greater absorbance compared to copper solution alone.

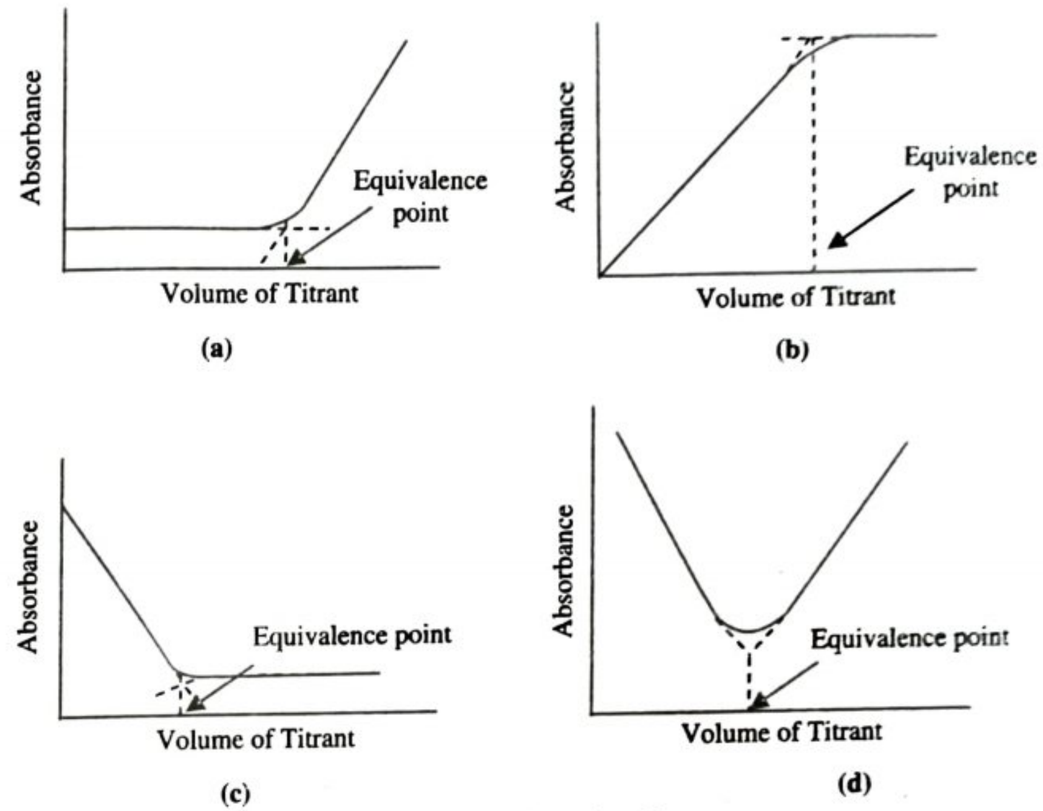


Figure 1.21: Titration Curves

3) **Curve (c)** is characteristic of a case where only the substance being titrated absorbs, while the titrant and the product do not absorb. **An example** is the titration of *p*-toluidine in butanol with perchloric acid at 290nm wavelength.

   This wavelength is selected because *p*-toluidine sharply absorbs at this wavelength; whereas the titrant, perchloric acid shows no absorbance in this region. As soon as the whole quantity of *p*-toluidine reacts with perchloric acid, the absorbance will become constant after the equivalence point.

4) **Curve (d)** is obtained when a coloured analyte is converted into a colourless product by a coloured titrant. When titrant is added, the analyte colour starts fading due to the formation of a colourless product. But after the equivalence point, absorbance again rises due to the colour of the titrant alone.

1.5.2.1. Choice of Wavelength

A number of criteria are considered in the choice of wavelength. It may be necessary to balance one against another:

1) The highest absorption peak will yield the maximum sensitivity.
2) A chosen wavelength should yield a minimum interference from other species that may be in the system.
3) Because of the errors that arise from sharply rising or sharply falling portions of the absorption curves, these sections should be avoided.
4) Care should be taken to avoid a sharp band if the slit width exceeds the bandwidth.

In **figure 1.22**, the **solid line represents the absorption curve for the sample** species and the **dashed line represents the absorption curve of interference**. This interference may be either caused by the necessary excess of one of the colour-forming reagents or is naturally occurring in the studied system.

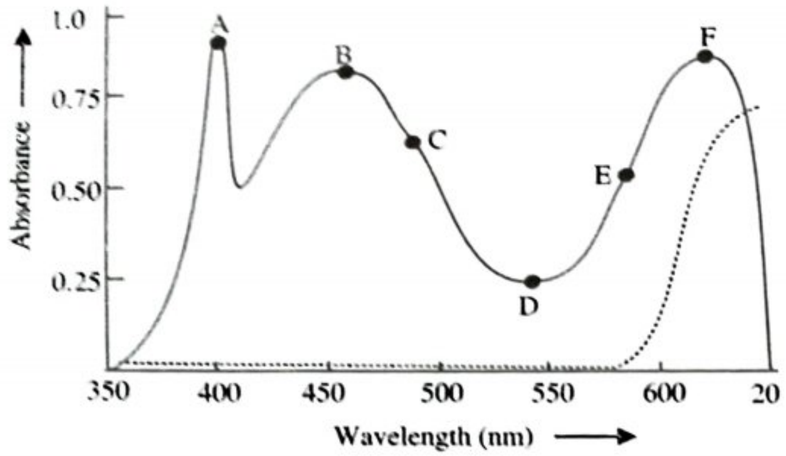


Figure 1.22: Choice of Experimental Wavelength

When all the factors are considered, point B would be the chosen wavelength when using a 20nm bandwidth. Whereas, the compound possesses a greater absorptivity, and thus a greater potential sensitivity is observed at both A and F than at B. This advantage is lost when the potential for interference (dashed line) as well as the slit width are considered.

Points C and E are avoided because of the error associated with absorbance measurements on sharply rising or falling curves. Point D would yield an accurate reading but its sensitivity is less than one-third of that at point B.

1.5.2.2. Apparatus

In order to carry out spectrophotometric titrations, a special titration cell (**figure 1.23**) of 5-100ml capacity is employed that fits in the cell compartment of the spectrophotometer. The cell is made up of perspex sheet. As the material perspex is opaque to UV light, two openings are made in the cell to accommodate cellular quartz windows.

These windows are inserted in such a way that the beam of monochromatic light passes through their centres to the photoelectric cell. The cell has two small openings, one for the tip of a microburette and the other for a micro stirrer. Except the quartz windows, the whole cell is covered with black paper to exclude all extraneous light.

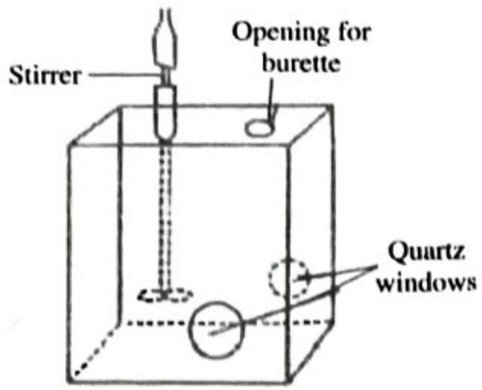


Figure 1.23: Titration Cell

1.5.2.3. Technique

The experimental technique is simple and involves the following steps:

1) The solution to be titrated is taken in the cell.
2) Then, the cell is kept in the light path of a spectrophotometer.
3) The spectrophotometer is adjusted to the wavelength at which experiment is to be carried out and the instrument is set at zero absorbance (if the reactant is colourless) or at some other starting value (if the reactant is coloured) that lies within the linearity range of the instrument.
4) After this a known volume of titrant is added to the stirred solution in the cell and the absorbance is read again.

5) This is repeated at several points before the end point and at several points after the end point.
6) Finally, absorbance is plotted against the volume of titrant added.
7) From this graph, the equivalence point is obtained.

1.5.2.4. Dilution Correction

Since absorbance depends upon concentration, the effect of dilution should be taken into consideration. This error can be overcome by one of the following **methods**:

1) Either the titrant is made much more concentrated than the solution to be titrated so that there is negligible change in volume, or
2) A simple **correction factor** is included in the calculation.

$$A = A' \frac{V + v}{V}$$

Where,

A and A' = Corrected and measured absorbances, respectively.
V = Original volume of solution.
v = Volume of titrant added.

For best results, appropriate dilution corrections must be made and the above equation becomes:

$$A_c = \left(\frac{V_i + V_a}{V_i} \right) (A_m)$$

Where,

A_c = Corrected absorbance.
V_i and V_a = Respective initial and added volumes.
A_m = Measured absorbance.

However, a negligible error will result from ignoring dilution corrections if the titrant concentration is much greater than the concentration of the solution being titrated.

1.5.2.5. Applications

Some typical methods of analysis by spectrophotometric titrations are:

1) **Acid-Base Methods:** Phenols can be titrated with NaOH. Absorbance occurs due to the formation of phenolate ion.
2) **Oxidation-Reduction Methods:** Ce(III) can be titrated with Co(III), forming Ce(IV).
3) **Complexometric Titrations:** Bi(III) can be titrated with thiourea; copper(II) is added, resulting in the formation of Cu-EDTA complex; or thiourea is added resulting in the disappearance of bismuth-thiourea complex. Fe(III) can be titrated with EDTA, resulting in the disappearance of Fe sulphosalicylic acid complex.

   Cu(II) can be titrated with EDTA, resulting in the formation of Cu-EDTA complex.

   Copper and bismuth in a mixture can be estimated by just a single titration with EDTA. Measurements are made at 745nm, where copper-EDTA complex absorbs strongly but bismuth complex does not.
4) **Precipitation Titrations:** SO_4^{2-} ions can be titrated with Ba(II) ions, resulting in the appearance of turbidity. F^- ions can be titrated with Th(IV) ions. Reaction of Th(IV) with the indicator SPADNS results in a precipitate.

1.5.2.6. Advantages

Spectrophotometric titrations have several advantages over direct spectrophotometric analysis:

1) It can be applied to a large number of non-absorbing constituents as only one absorber is to be present among the reactant, the titrant, or the reaction products.
2) Presence of other absorbing species at the analytical wavelength does not cause interference because only the change in absorbance is significant.
3) It can be applied to highly coloured solutions that could not be determined by the visual indicators.
4) It can be applied to such reactions that tend to be appreciably incomplete at the equivalence point.
5) It is quite accurate. One can obtain accuracy and precision of a few tenths per cent with comparative ease by spectrophotometric titrations.
6) In contrast to normal absorbance procedures, it is not necessary that the titration be performed in the region or wavelength of maximum absorbance. Thus, a greater choice of wavelengths is available.
7) Relatively large quantities of other absorbing species at the chosen wavelength are necessary before noticeable interference results.
8) Turbid solutions may be titrated in selected circumstances.
9) More dilute solutions may be employed than in other types of titrations.
10) A variety of equipment may be employed.

1.5.3. Single Component Analysis Methods

The methods involved in the calculation of single component analysis are:

1) **Direct Analysis:** Essentially all compounds containing conjugated double bond or aromatic rings, and many inorganic species absorb light in the UV-visible regions. In these techniques, the substance to be determined is dissolved in a suitable solvent and diluted to the required concentration by appropriate dilutions, and then absorbance is then measured.
2) **Indirect Analysis:** This method involves analysis after addition of some reagent. These methods are based on the conversion of analyte by a chemical reagent having different spectral properties. Chemical derivatisation may be adopted for any of the several reasons:
   i) If the analyte absorbs weakly in the UV-region.
   ii) The interference from irrelevant absorption may be avoided by converting the analyte to a derivative, which absorbs in the visible region, where irrelevant absorption is negligible.
   iii) This technique can be used to improve the selectivity of the assay in presence of other UV radiation absorbing substance.
   iv) Cost.

Methods of Calculating Concentration in Single Component Analysis

1) By using the relationship $A = abc$.
2) By using the formula $Cu = (A_u/A_s) \times C_s$.
3) By using the equations $y = mx + c$.
4) By using the Beer's curve.

1.5.4. Multicomponent Analysis Methods

UV spectrophotometric techniques are mainly used for multicomponent analysis, thus minimising the cumbersome task of separating interferents and allowing the determination of an increasing number of analytes, consequently reducing analysis time

and cost. Multicomponent UV spectrophotometric methods are based on recording and mathematically processing absorption spectra. The multicomponent analysis methods offer the following **advantages**:

1) Avoiding prior separation techniques, **e.g.,** extraction, concentration of constituents, and clean-up steps that might be required.
2) Spectral data are readily acquired with ease.
3) The process is fast, accurate, and simple.
4) Wide applicability to both organic and inorganic systems.
5) Typical detection limits of 10^{-4} to 10^{-5} M and moderate to high selectivity.

Different UV spectrophotometric multicomponent analysis methods include:

1) Simultaneous equation method,
2) Difference spectrophotometry,
3) Derivative Spectrophotometry (DS),
4) Absorbance ratio spectra method,
5) Derivative ratio spectra method,
6) Double divisor ratio spectra derivative method,
7) Successive ratio-derivative spectra method,
8) Q-absorbance ratio method,
9) Isosbestic "isoabsorptive" point method,
10) Absorptivity factor method,
11) Dual wavelength method,
12) Ratio Subtraction Method (RSM),
13) Mean centering of the ratio spectra,
14) Absorption Factor Method (AFM), and
15) Multivariate chemometric methods.

1.5.4.1. Simultaneous Equation Method (Vierordt's Method)

If a sample contains two absorbing drugs (x and y) each of which absorbs at the λ_{max} of the other, both the drugs can be determined by this method. The information required is:

1) The absorptivities of x at λ_1 and λ_2 (a_{x_1} and a_{x_2}, respectively).
2) The absorptivities of y at λ_1 and λ_2 (a_{y_1} and a_{y_2}, respectively).
3) The absorbance of diluted samples at λ_1 and λ_2 (A_1 and A_2, respectively).

Let C_x and C_y be the concentration of x and y respectively in the diluted samples. Two equations are constructed based upon the fact that at λ_1, the absorbance of the mixture is the sum of the individual absorbance of x and y:

$$A_1 = a_{x1}\, b\, C_x + a_{y1}\, b\, C_y \quad \text{.....(12)}$$

$$A_2 = a_{x2}\, b\, C_x + a_{y2}\, b\, C_y \quad \text{.....(13)}$$

For measurements in 1cm cells, b =1cm. On rearranging **equation (13)**:

$$C_y = \frac{(A_2 - a_{x2} C_x)}{a_{y2}} \quad \text{.....(14)}$$

On substituting **equation (14)** in **equation (12)** and rearranging:

$$C_x = \frac{(A_2 a_{y1} - A_1 a_{y2})}{(a_{x2} a_{y1} - a_{x1} a_{y2})} \quad \text{.....(15)}$$

$$C_y = \frac{(A_1 a_{x2} - A_2 a_{x1})}{(a_{x2} a_{y1} - a_{x1} a_{y2})} \quad \text{.....(16)}$$

1.5.4.2. Difference Spectrophotometry

The selectivity and accuracy of spectrophotometric analysis of samples containing absorbing interferents may be markedly improved by this technique. The essential feature of this method is that the measured value is the absorbance difference (ΔA) between two equimolar solutions of the analyte in different chemical forms exhibiting different spectral characteristics. The criteria for applying difference spectrophotometry to the assay of the substance in the presence of other absorbing substances are that:

1) Reproducible changes may be introduced in the spectrum of the analyte by adding one or more reagents.
2) The absorbance of the interfering substances is not altered by that reagent.

The simplest and the most commonly employed techniques for altering the spectral properties of the analyte is the adjustment of pH by means of aqueous solutions of acids, alkalis, or buffers.

1.5.4.3. Derivative Spectrophotometry (DS)

In this technique, the first or higher derivative of absorbance or transmittance with respect to wavelength is recorded *versus* the wavelength. In a derivative spectrum, the ability to detect and to measure minor spectral features is considerably enhanced. This enhancement of characteristic spectral detail can distinguish between very similar spectra and follow subtle changes in a spectrum. Moreover, it can be of use in quantitative analysis to measure the concentration of an analyte whose peak is obscured by a larger overlapping peak due to something else in the sample (and thus avoid prior separations) **(figure 1.24)**.

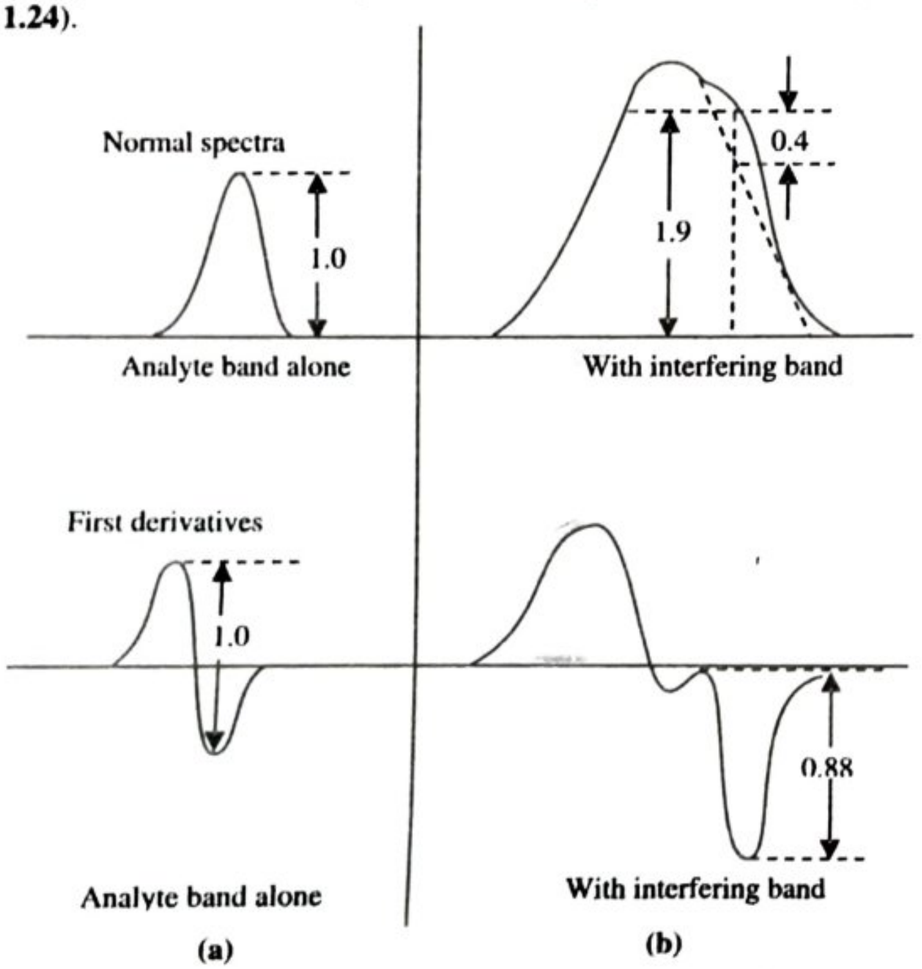


Figure 1.24: First-Derivative Spectrometry for Quantitative Measurement of the Intensity of a Small Band (a) Alone and (b) Obscured by a Broader Overlapping Band

In this particular **example**, if one draws the best guess of the tangent to the spectrum with an interfering band as shown by the broken lines, the reading of 0.4 for the absorbance of the analyte is far too low, whereas a guess at the maximum of the analyte peak gives an absorbance of 1.9, far too high. Referring to the derivative spectrum in the lower right of **figure 1.24**, one takes as the measure of the analyte absorbance the vertical distance between the adjacent maximum and minimum of the first derivative. Now the estimate of the analyte absorbance is low by only 12%. Whenever the interfering band is broader than the analyte band by atleast a factor of two, it is usually advantageous to base the measurement on the derivative spectra. If two substances (X and Y), absorb in the same spectral region, the absorbances are additive, i.e., $A = A_X + A_Y$, and each substance follows Beer's law, then $P = P_0 10^{-A}$. The **first derivative of P with respect to λ** becomes:

$$\frac{dP}{d\lambda} = 10^{-A}\frac{dP_0}{d\lambda} - 2.303P_0 10^{-A}\left[bC_X\left(\frac{d\varepsilon}{d\lambda}\right)_X + bC_Y\left(\frac{d\varepsilon}{d\lambda}\right)_Y\right] \quad .....(17)$$

If P_0 does not vary significantly with wavelength in the spectral region of interest, $dP_0/d\lambda$ can be neglected. If the molar absorptivity of one component (say ε_Y) varies only slightly with λ in the spectral region under investigation, $bC_Y(d\varepsilon/d\lambda)_Y$ can be neglected, and **equation (17)** reduces to:

$$\frac{dP}{d\lambda} = -2.303PbC_X\left(\frac{d\varepsilon}{d\lambda}\right)_X \quad .....(18)$$

Under the same conditions, the **differentiation of $A = A_X + A_Y$** with respect to λ yields:

$$\frac{dA}{d\lambda} = bC_X\left(\frac{d\varepsilon}{d\lambda}\right)_X \quad .....(19)$$

Thus, the first derivative is directly proportional to the concentration of X, provided that P_0 and ε_Y do not vary appreciably with wavelength in the spectral range measured. If the interfering substance (Y) has an absorption band close to X, the first derivative curves are distorted [**figure 1.24 (b)**] unless the two absorption bands are nearly identical in λ_{max} and bandwidth, in which case the first derivative curve is of no help.

Second-derivative spectra are also sometimes useful. At λ_{max}, the second derivative is directly proportional to concentration. For adequate sensitivity, $d^2\varepsilon/d\lambda^2$ must be large. This requires a narrow absorption band (4nm or less); thus the second-derivative method is most useful for atomic and gas molecular spectra.

A variety of different experimental techniques have been used to obtain derivative spectra. If the spectrum has been recorded digitally or is otherwise available in computer-readable form, the differentiation can be done numerically. Alternatively, the derivative spectra may be recorded directly in real time, either by wavelength modulation or by obtaining the time derivative of the spectrum when the spectrum is scanned at a constant rate. In the latter case, a quite simple electronic differentiator is used.

Most modern UV-vis spectrophotometers are capable of recording derivative spectra. Dual-wavelength spectrophotometers can obtain first-derivative spectra by scanning the spectrum with a small, constant difference between the two wavelengths.

1.5.4.4. Absorbance Ratio Spectra Method

The absorption spectrum of the mixture of two compounds (x and y) "measured in 1cm cell" is defined by the equation: .....(20)

$$A_M = a_x C_x + a_y C_y$$

Where,

A_M = Absorbance of the mixture.

a_x and a_y = Molar absorptivities.

C_x and C_y = Concentrations of x and y.

If the absorbance of the mixture is divided by the absorbance of a standard solution o (absorbance $A_x^0 = a_x C_x^0$), the following equation results:

$$\frac{A_M}{A_x^0} = \frac{C_X}{C_x^0} + \frac{A_y}{A_x^0} \quad \text{.....(2}$$

The ratio $\frac{C_X}{C_x^0}$ is a constant value, which can be eliminated by taking the difference absorbance ratio amplitudes between two wavelengths (λ_1 and λ_2, peak to p measurement).

$$\left[\frac{A_M}{A_x^0}\right]_{\lambda 1} - \left[\frac{A_M}{A_x^0}\right]_{\lambda 2} = \left[\frac{A_Y}{A_x^0}\right]_{\lambda 1} - \left[\frac{A_Y}{A_x^0}\right]_{\lambda 2} \quad \text{.....(2}$$

Equation (3) illustrates that the amplitude difference in the mixture absorbance n between two wavelengths (λ_1 and λ_2, termed peak to peak, peak to trough, maximum minimum measurement, or ratio difference spectrophotometric method) is equal to same amplitude difference for compound y after cancelling the constant interference to compound x.

1.5.4.5. Derivative Ratio Spectra Method

This simple spectrophotometric method developed by **Salinas *et al.***, is based on derivation of the ratio spectra for resolving binary mixtures. It permits the use wavelength of highest value of analytical signals with several maxima and mini which gives an opportunity for the determination of active compounds in the presenc other compounds and excipients that could possibly interfere in the assay.

Calculation of the first derivative will remove the constant value due to $\frac{C_X}{C_x^0}$ **equation (23)**, so concentration of y can be easily determined without any interfere from the drug x.

$$\frac{A_M}{A_x^0} = \frac{C_X}{C_x^0} + \frac{A_y}{A_x^0} \quad \text{.....(}$$

The difference between the two spectra $\frac{A_M}{A_x^0}$ and $\frac{A_y}{A_x^0}$ **(figure 1.25)** is due to constant interference value due to compound x $\left(\frac{C_X}{C_x^0}\right)$. Elimination of such interfer can be done by measurement of ratio spectra difference between two wavelengths calculating derivative of the ratio spectra. Second derivative of the ratio spectra ma also used to improve linearity, mean % recoveries, and decrease relative stan deviation. A derivative ratio spectrum was modified for the determination of ter mixtures using the derivative ratio spectra zero-crossing method. This metho realised by measurement of amplitudes at the zero-crossing points in the deriv ratio spectra.

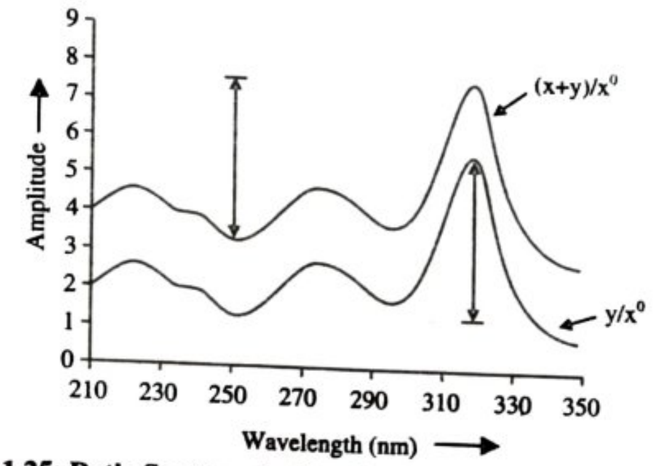


Figure 1.25: Ratio Spectra of a Standard Solution of y and a Mixture Solution (x and y) Containing the same Concentration of y, Using x^0 as a Divisor

1.5.4.6. Double Divisor Ratio Spectra Derivative Method

This method is based on the use of the derivative of the ratio spectrum obtained by dividing the absorption spectrum of the ternary mixture by a standard spectrum of a mixture of two of the three compounds in the mixture, and measuring at either the maximum or minimum wavelengths. It can only be used for the mixtures having known ratio of the concentrations of two interfering compounds (used as double divisor). If a mixture of three compounds (x, y, and z) is considered, if Beer's law is obeyed for all compounds over the whole wavelength range used, and if the path length is 1cm, the absorption spectrum of the ternary mixture at wavelength λ can be written as:

$$A_m = a_x + C_x + a_y C_y + a_z C_z \quad \text{.....(24)}$$

Where, A_m = Absorbance of the mixture.

a_x, a_y and a_z = Absorptivities of x, y and z, respectively.

C_x, C_y and C_z = Concentrations of x, y and z, respectively.

A similar equation for two compounds in the same ternary mixture as in a standard binary mixture can be written as:

$$A_m = a_x C_x^0 + a_y C_y^0 \quad \text{....(25)}$$

If **equation (24)** is divided by **equation (25)** corresponding to the spectrum of a standard solution of two of the components in the ternary mixture, the ratio spectrum is obtained as:

$$\frac{A_m}{a_x C_x^0 + a_y C_y^0} = \frac{a_x C_x + a_y C_y}{a_x C_x^0 + a_y C_y^0} + \frac{a_z C_z}{a_x C_x^0 + a_y C_y^0} \quad \text{....(26)}$$

The ratio of the sum of (a_x C_x + a_y C_y) to the sum of $(a_x C_x^0 + a_y C_y^0)$ is equal to a constant (k) with respect to λ. On replacing the above constant in **equation (26)**:

$$\frac{A_m}{a_x C_x^0 + a_y C_y^0} = k(\text{constant}) + \frac{a_z C_z}{a_x C_x^0 + a_y C_y^0} \quad \text{....(27)}$$

However, if the standard concentrations of C_x^0 and C_y^0 in **equation (28)** are equal or very close to each other: $C_x^0 = C_y^0$ or $C_x^0 \approx C_y^0$ ....(28)

$$a_x C_x^0 + a_y C_y^0 = C_x^0 (a_x + a_y)$$

On substituting **equation (28)** in **equation (27)**:

$$\frac{A_m}{(a_x + a_y)C_x^0} = k(\text{constant}) + \frac{a_z C_z}{(a_x + a_y)C_x^0} \quad \ldots.(29)$$

If the first derivative of **equation (29)** is taken, the derivative of a constant is zero:

$$\frac{d}{d\lambda}\left[\frac{A_m}{(a_x + a_y)C_x^0}\right] = \frac{d}{d\lambda}\left[\frac{a_z}{(a_x + a_y)}\right]\frac{C_z}{C_x^0} \quad \ldots.(30)$$

Equation (30) is the **mathematical foundation of multicomponent analysis**, whic permits the determination of the concentration of each of the active compounds i solution without interference from the other components of the ternary system.

1.5.4.7. Successive Ratio-Derivative Spectra Method

This method is used for simultaneous determination of the three compounds in terna mixtures without need to know the ratio of concentration of species. It is based on t successive derivative of ratio spectra in two steps.

A mixture of three compounds (x, y, and z) is considered. If Beer's law is obeyed in t whole wavelength range used and by considering the path length as 1cm, the absorban of ternary mixture at each wavelength can be written as:

$$A_m = a_x C_x + a_y C_y + a_z C_z \quad \ldots.(31)$$

Where,

A_m = Vector of the absorbance of the mixture.
a_x a_y and a_z = Absorptivity vectors of x, y and z, respectively.
C_x, C_y and C_z = Concentrations of x, y, and z, respectively.

On dividing **equation (31)** with a_z corresponding to the spectrum of a standard solutic of z in ternary mixture:

$$B = A_m/a_z = a_x C_x/a_z + a_y C_y/a_z + C_z \quad \ldots.(32$$

If the first derivative of **equation (32)** is taken, since the derivative of a constant (C_z) zero:

$$\frac{dB}{d\lambda} = \frac{d}{d\lambda}[a_x C_x / a_z] + \frac{d}{d\lambda}[a_y C_y / a_z] \quad \ldots.(33$$

On dividing **equation (33)** with $(d/d\lambda)(a_y/a_z)$, corresponding to the derivative of the ra of the spectra of standard solutions of y and z, the second ratio spectrum is obtained as:

$$D = \frac{dB/d\lambda}{(d/d\lambda)(a_y/a_z)} = \frac{(d/d\lambda)[a_x C_x/a_z]}{(d/d\lambda)(a_x/a_z)} + C_y \quad \ldots.(34$$

f the first derivative of **equation (34)** is taken, since the derivative of a constant (C_y) ero:

$$\frac{dD}{d\lambda} = \frac{d}{d\lambda}\left[\frac{(d/d\lambda)(a_x C_x/a_z)}{(d/d\lambda)\left(\frac{a_y}{a_z}\right)}\right] \quad \ldots.(35$$

Equation (35) is the **mathematical foundation of multi-component analysis** that permits the determination of concentration of each of the active compounds in the solution (x in this equation) without interference from other compounds of the ternary system (y and z in these equations).

1.5.4.8. Q-Absorbance Ratio Method

This method (also termed **absorption ratio method**) is a modification of the simultaneous equation method. According to this method, the ratio of absorbance at any two wavelengths for a substance, that obeys Beer's law, is a constant value independent of the concentration and path length. This constant is termed as **Hufner's Quotient** or **Q-value**. This method involves the measurement of absorbance at two wavelengths, one being the λ_{max} of one of the components (λ_2) and the other being a wavelength of equal absorptivity of the two components (λ_1), called the **iso-absorptive point**. The concentration of each component can be calculated as:

$$C_x = (Q_m - Q_y/Q_x - Q_y) * A/a_1 \quad \ldots.(36)$$

$$C_y = (Q_m - Q_x/Q_y - Q_x) * A/a_2 \quad \ldots.(37)$$

Where,

C_x and C_y = Concentrations of x and y, respectively.
A = Absorbance of sample at iso-absorptive wavelength.
a_1 and a_2 = Absorptivity of x and y at isoabsorptive wavelength, respectively.

$$Q_m = \frac{\text{Absorbance of the sample solution at } \lambda_{max} \text{ of one of the components } (\lambda_2)}{\text{Absorbance of the sample solution at isoabsorptive wavelength}} \quad \ldots.(38)$$

$$Q_x = \frac{\text{Absorbance of x at } \lambda_{max} \text{ of one of the components } (\lambda_2)}{\text{Absorbance of x at isoabsorptive wavelength}} \quad \ldots.(39)$$

$$Q_y = \frac{\text{Absorbance of y at } \lambda_{max} \text{ of one of the components } (\lambda_2)}{\text{Absorbance of y at isoabsorptive wavelength}} \quad \ldots.(40)$$

1.5.4.9. Isosbestic "Isoabsorptive" Point Method

Erram and **Tipnis** developed the isosbestic point method. This technique can be used only if the spectra of the same concentration of the two studied drugs cross at a **isosbestic** or **isoabsorptivity point**. At the isosbestic point, both drugs have equal absorptivities and their mixture acts as a single component and gives the same absorbance as pure drug. This theory can be confirmed experimentally by recording the absorbance spectra of a certain concentration of the two drugs and the absorbance spectra of a binary mixture containing the same concentration. The absorbance value at the isosbestic points (A_{iso}) was determined, and the total concentration of both drugs was calculated (**figure 1.26**).

Since the concentration of one of them in this mixture can be measured using other spectroscopic method (DS), the concentration of the other can be calculated by subtraction. A linear correlation was obtained between the absorbance values and the corresponding drug concentrations. A mixture of two drugs (x and y) is considered. The absorbance of each drug can be calculated at any wavelength (λ) from the equation:

$$A = A^{1\%}_{1cm} bc . \quad \ldots.(41)$$

For drug x: $A_x = A_x{}^{1\%}_{1cm}\ bc_x$ .....(42)

For drug y: $A_y = A_y{}^{1\%}_{1cm}\ bc_y$ .....(43)

Where,
A_x and A_y = Absorbance of x and y, respectively.
C_x and C_y = Concentrations of x and y, respectively.
$A_{x\ 1cm}^{1\%}$ and $A_{y\ 1cm}^{1\%}$ = Absorptivities of x and y, respectively. when the path length is 1cm and concentration is 1g/100ml.

If $C_x = C_y$ and $A_x = A_y$, this λ is called the **isosbestic point**, and at this λ:

$$A_{x\ 1cm}^{1\%} = A_{x\ 1cm}^{1\%} \quad \text{.....(4}$$

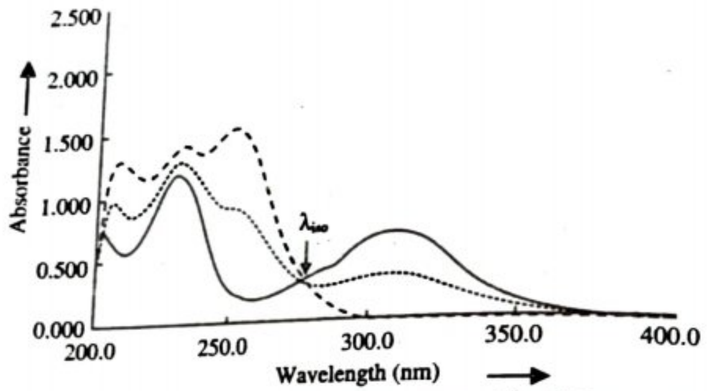


Figure 1.26: Zero Order Absorption Spectra of 20μgml^{-1} of Metronidazole (——), 20μgml^{-1} Diloxanide Furoate (- - - - -) and (1:1) Mixture Containing 10μgml^{-1} of each (·····) Using Methanol as a Blank

For a mixture of both drugs, the absorbance at this λ can be calculated as:

$$A_M = A_{x\ 1cm}^{1\%} C_{xM} + A_{y\ 1cm}^{1\%} C_{yM}$$

$$A_M = A_{x\ 1cm}^{1\%} (C_{xM} + C_{yM}) = A_{x\ 1cm}^{1\%} (C_{TM})$$

Where, A_M = Absorbance of the mixture of both drugs at isosbestic point.
C_{xM} and C_{yM} = Concentrations of x and y in the mixture, respectively.
C_{TM} = Concentration of the mixture of both drugs.

Therefore, $(C_{xM} + C_{yM}) = (C_{TM})$ .....(4

Thus, having the total concentration of both drugs, if the concentration of one of the can be determined separately by any other method, the concentration of the second dr can be calculated by subtraction. This method has been successfully applied for t **simultaneous determination of several binary mixtures, e.g.,** metronidazole a diloxanide furoate, ezetimibe and atorvastatin, and sitagliptin and metformin.

1.5.4.10. Absorptivity Factor Method

The absorptivity factor (**modification of the classical isoabsorptive method**) is appli for the analysis of binary mixture if only there is a large difference in absorptivity of bo the drugs, so there is no occurrence of an isoabsorptive point. In isoabsorptive techniq (**figure 1.27**), the spectra of the same concentration of the two studied drugs should cr at **isoabsorptivity point**, at which they have equal absorptivities; while in absorptiv factor method, the crossing point did not occur at equal concentration.

rossing point is obtained only between different concentrations of the two drugs at hich the absorptivities of the two drugs are not equal, but they are equal to the inverse the ratio of the used concentrations.

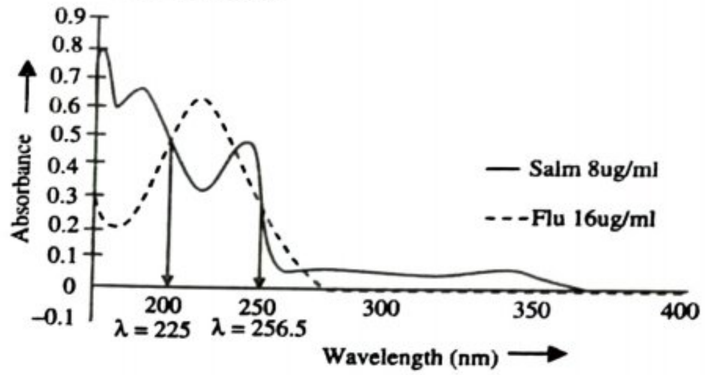


Figure 1.27: Zero Order Spectra of 8μgml^{-1} Salmeterol and 16μgml^{-1} Fluticasone Showing the Absorptivity Factor Points

or two drugs (x and y) in the mixture, the concentration of y can be determined by using y of the well-established spectrophotometric methods; drug x can be determined by the **sorptivity factor method**. This method depends on the calculation of the absorptivity ctor, which is the ratio between two absorptivities (a_x and a_y) at intersection point with e same absorbance value. This point is called the **absorptivity factor point (k_F)**. This summarised as follows:

$A_x = A_y$

$a_x b_x C_x = a_y b_y C_y$ (Where, $b_x = b_y = 1cm$)

$a_x C_x = a_y C_y$

$$\frac{a_x}{a_y} = \frac{C_y}{C_x} = F \quad \text{.....(4}$$

$a_x = F a_y$ .....(4

here, F = Absorptivity factor.
a_x and a_y = Absorptivities of x and y, respectively.

r mixture of x and y, the total absorbance of x and y at absorptivity factor point (k_F) n be expressed as:

$A_m = A_x + A_y$

$A_m = a_x b_x C_x + a_y b_y C_y$ (where $b_x = b_y = 1cm$)

$A_m = a_x C_x + a_y C_y$

here,
A_x, A_y and A_m = Absorbance of x, y and their mixture at k_F.
C_x and C_y = Concentrations of x and y, respectively.
a_x and ay = Absorptivities of x and y at k_F.

substituting a_x with Fa_y:

$A_m = a_y F C_x a_y C_y$

$A_m = a_y (F C_x + C_y)$

the total concentration of the mixture ($FC_x + C_y$) can be calculated by using a ression equation representing the linear relationship between the absorbance of y and corresponding concentration at the absorptivity factor point.

The concentration of x can be determined after subtraction of concentration of y an multiplication by the inverse of F.

$$C_x = [(FC_x + C_y) - C_y] \cdot 1/F$$

1.5.4.11. Dual Wavelength Method

Dual wavelength method (or **two wavelengths method**) facilitates analysing component in presence of an interfering component by measuring the absorban difference (ΔA) between two points in the mixture spectrum. In this method (**figu 1.28**), one of the drugs is considered a component of interest and the other drug considered an interfering component and *vice versa*. The basis for such method is th selection of two wavelengths where the interfering component shows the sam absorbance (ΔA equals zero), whereas the component of interest shows significa difference in absorbance with concentration. The ΔA between two points on the mixtu spectra is directly proportional to the concentration of the component of intere independent of interfering component.

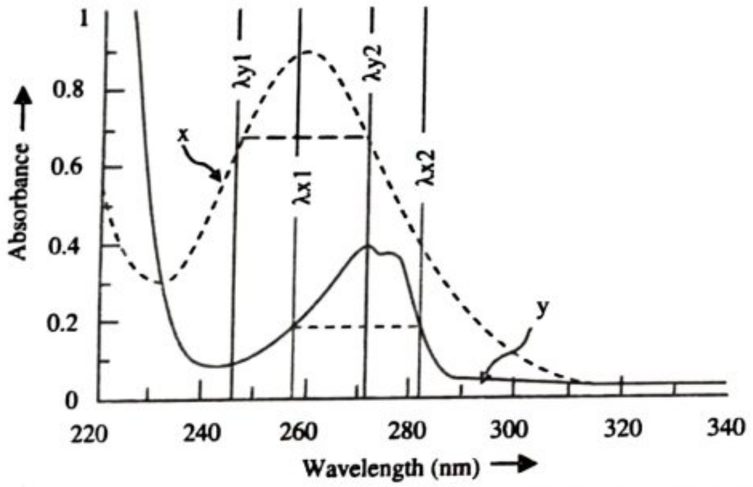


Figure 1.28: Selection of Wavelengths for Dual Wavelength Method

This method is used for simultaneous determination of different drugs, **e.g.,** atenolol an indapamide, drotaverine and aceclofenac, atorvastatin and ezetimibe, chlorpheniramin and phenylpropanolamine, and dexketoprofen and tramadol.

1.5.4.12. Ratio Subtraction Method (RSM)

If a mixture of two drugs (**x** and **y**) have overlapping spectra, and the spectrum of y extended more than x, the determination of x can be done by dividing the spectrum of t mixture by a certain concentration of y as divisor (y^0).

The division will give a new curve that represents $\frac{x}{y^0}$ + constant. If this constant subtracted and the new curve obtained after subtraction is multiplied with y^0, the origi zero-order D^0 spectrum of x is obtained. This can be summarised in the follow equations:

$$\frac{x+y}{y^0} = \frac{x}{y^0} + \frac{y}{y^0} = \frac{x}{y^0} + \text{constant}$$

$$\frac{x}{y^0} + \text{constant} - \text{constant} = \frac{x}{y^0} +$$

$$\frac{x}{y^0} * y^0 = x$$

The constant can be determined directly from the curve $\frac{x+y}{y^0}$ by the straight line that is parallel to the wavelength axis in the region where y is extended. RSM was successfully applied for determination of multicomponent pharmaceutical products containing, metronidazole and diloxanide, and amlodipine and atorvastatin.

To determine the second component (y), an extension of the already developed method has been established as a new approach, known as **Extended Ratio Subtraction Method** (ERSM), in which y could be determined by dividing the obtained D^0 spectrum of x by a known concentration of x as a divisor (x^0) to get the value of the constant $\frac{x}{x^0}$. On dividing the spectrum of the mixture (x + y) by the same divisor (x^0), a new curve is obtained that represents $\frac{x}{x^0} + \frac{y}{x^0}$, where $\frac{x}{x^0}$ is the previously obtained constant. If this constant is subtracted and the obtained curve after subtraction is multiplied with x^0 (the divisor), the zero-order absorption spectrum (D^0) of y (original spectrum of y) is obtained.

$$\frac{x+y}{x^0} = \frac{x}{x^0} + \frac{y}{x^0} - \frac{x}{x^0} = \frac{y}{x^0} \times x^0 = y$$

Concentration of y is calculated by using the regression equation representing the linear relationship between the absorbance at its λ_{max} *versus* the corresponding concentration of y.

1.5.4.13. Mean Centring of the Ratio Spectra

This method is applied for further improvement of the selectivity to resolve the overlapping between drugs in binary and ternary mixtures. This eliminates the derivative step, and therefore the signal-to-noise ratio is enhanced. To explain the mean centring expression, a three-dimensional vector is considered:

$$y = \begin{bmatrix} 5 \\ 1 \\ 3 \end{bmatrix}$$

This column is centered or Mean Centered (MC) by subtracting the mean of three numbers:

$$\bar{y} = \begin{bmatrix} 3 \\ 3 \\ 3 \end{bmatrix} \qquad MC(y) = y - \bar{y} = \begin{bmatrix} 5 \\ 1 \\ 3 \end{bmatrix} - \begin{bmatrix} 3 \\ 3 \\ 3 \end{bmatrix} = \begin{bmatrix} +2 \\ -2 \\ 0 \end{bmatrix}$$

It could be proved that if the vector y is multiplied with **n** (a constant number), the mean centred vector is also multiplied with **n** and also if a constant number is added to the vector **y**, the mean centre of this vector is not changed. If there is no interaction among the two components of a mixture (i.e., **x** and **y**), and if Beer's law is obeyed for each compound, it can be expressed as:

$$A_m = a_x C_x + a_y C_y \qquad \text{.....(48)}$$

Where, A_M = Absorbance of the mixture.
a_x and a_y = Molar absorptivities of x and y, respectively.
C_x and C_y = Concentration of x and y, respectively.

On dividing **equation (48)** with a_y, the ratio spectrum is obtained as:

$$B = \frac{A_m}{a_y} = \frac{a_x C_x}{a_y} + C_y \qquad \text{..... (49)}$$

Since the mean centering of a constant C_y is zero, mean centering (MC) of **equation (49)** would be obtained as:

$$MC(B) = MC\ \frac{a_x C_x}{a_y} \qquad \text{..... (50)}$$

Equation (50) illustrates the **mathematical explanation for analysis of binary components** that permits the determination of concentration of one compound without interference from the other compound of the binary system, and *vice versa*.

1.5.4.14. Absorption Factor Method (AFM)

This method describes the analysis of a binary mixture where the two components (**x** and **y**) have overlapped spectra. '**y**' shows interference at λ_{max} of **x**, while **x** shows no interference with **y** at another wavelength (λ_2).

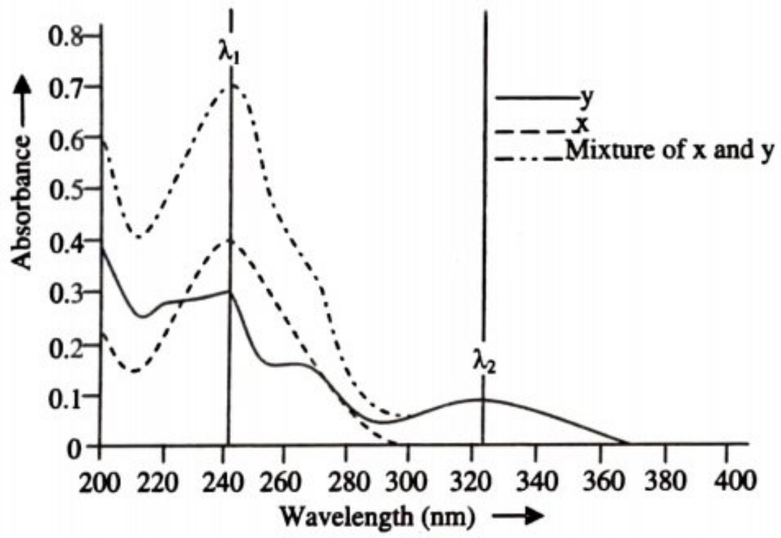


Figure 1.29: Zero-Order Spectra of x and y and their Mixture

In figure 1.29, the absorption spectra of x and y show severe overlapping in the wavelength region of 200-300nm. So, the absorption spectra of the standard solutions of **y** with different concentrations were recorded in the wavelength range of 200-400nm and the average value of absorption factor $\left(\frac{A_y, \lambda_1}{A_y, \lambda_2}\right)$ was calculated. Since the absorbance of the mixture (x + y) at λ_2 is equal to that of pure y due to lack of contribution of x at this wavelength, the absorption of x at λ_1 could be calculated as:

$$A_x, \lambda_1 = A\lambda_1(x+y) - \frac{A_y, \lambda_1}{A_y, \lambda_2} * A\lambda_2(x+y)$$

Where, $A\lambda_1(x+y)$ and $A\lambda_2(x+y)$ = Absorbance values of mixture at λ_1 and λ_2.

$\frac{A_y, \lambda_1}{A_y, \lambda_2}$ = Absorption factor of pure y.

The concentrations of x and y were calculated from the corresponding regression equation obtained by plotting the absorption values of the zero-order spectra at λ_1 and λ_2 against the corresponding concentrations, respectively.

1.5.4.15. Multivariate Chemometric Methods

Chemometrics is the art of processing data with various numerical techniques in order to extract useful information. Drug separation, identification, determination, and validation have been studied using chemometrics. It recognises that it is often better to measure many

non-selective signals and then combine them in multivariate model (multivariate analysis), whereby multiple variables are considered simultaneously. A multivariate measurement is defined as one in which multiple measurements are made on a sample of interest. So, more than one variable or response are measured for each sample. Multivariate methods include **Multiple Linear Regression** (MLR) and **factor-based methods**.

In spectroscopy, if the absorbance spectra of a number of samples of known composition are measured, all these spectra are assembled into one matrix called **absorbance matrix**. All of the concentration values for the components of the sample are also assembled into a separate matrix called **concentration matrix**. The data of matrices are organised into pairs; each absorbance matrix is paired with its corresponding concentration matrix. The pair of matrices comprises a data set. Data sets have different names depending on their origin and purpose.

Training set is a data set containing measurements on a set of known samples. It is used to develop the calibration which is applied to predict the concentrations of unknown samples. Training set should contain all expected components, span the concentration ranges of interest and contain mutually independent samples. **Validation set** is an additional data set containing independent measurements on samples that are independent from the samples used to create the training set. Validation set is used to test the validity of the calibration developed with the training set. The developed calibration is used to predict the concentrations of the components in the validation samples. Then these predicted concentrations are compared to the actual concentrations. The absorbance matrix containing the unknown(s) spectra together with the corresponding result matrix containing the predicted concentrations comprise an **unknown set**.

1.6. SUMMARY

The details given in the chapter can be summarised as follows:

1) The **UV region** extends from **10-400nm**.
2) UV region is sub-divided into the **near UV (quartz) region** (200-400nm) and the **far or vacuum UV region** (10-200nm).
3) The **visible** region extends from **400-800nm**.
4) Since **UV** and **visible** spectroscopy involves electronic transitions, it is often called **electronic spectroscopy**.
5) **Ultraviolet absorption spectra** arise from transition of electron(s) within a molecule or of ions from lower to higher electronic energy levels.
6) The **ultraviolet emission spectra** arise from the reverse type of transition.
7) **σ-Electrons** are involved in saturated σ-bonds, such as those between carbon and hydrogen in paraffin.
8) **π-Electrons** are involved in unsaturated hydrocarbons.
9) **n-Electrons** are not involved in the bonding between atoms in molecules.
10) **Chromophoric group** is defined as any isolated covalently bonded group that shows a characteristic absorption in the UV or visible region.
11) An **auxochromic group** can be defined as any group which does not itself acts as a chromophore but whose presence brings about a shift of the absorption band towards the red end of the spectrum.
12) **Bathochromic shift** is an effect in which the absorption maximum is shifted towards longer wavelength due to the presence of an auxochrome or by the change of solvent. It is also called as red shift.

13) **Hypsochromic shift** is an effect in which the absorption maximum is shifted towards shorter wavelength. It is also called as blue shift.
14) **Hyperchromic shift** is an effect in which the intensity of absorption maximum increases.
15) **Hypochromic shift** is an effect in which the intensity of absorption maximum decreases.
16) **Woodward-Fieser Rules** describes the effect of alkyl substituents or ring residues.
17) In **hydrogen discharge lamps**, hydrogen gas is stored under relatively high pressure.
18) **Absorption filters** derive their effects from bulk interactions of radiation within the material.
19) **Interference filters** are based on the phenomenon of optical interference.
20) The **monochromator** is used to disperse the radiation according to the wavelength.
21) Detectors used in UV-visible spectrophotometers can be called as **photometric detectors**.
22) **Photomultiplier tubes** is the most sensitive of all the detectors, expensive and used in sophisticated instruments.
23) **Photovoltaic cell** or **barrier-layer cell** is also known as **photronic cell** and operates without the use of a battery.
24) One **problem** with the single beam system is that it **measures the total amount of light reaching the detector**, rather than the percentage absorbed.
25) **Chemometrics** is the art of processing data with various numerical techniques in order to extract useful information.
26) **Training set** is a data set containing measurements on a set of known samples.
27) **Validation set** is an additional data set containing independent measurements on samples that are independent from the samples used to create the training set.

1.7. EXERCISE

1.7.1. True or False

1) The UV region extends from 100-400nm.
2) The visible region extends from 400-800nm.
3) Ultraviolet absorption spectra arise from transition of electron(s) within a molecule or of ions from higher to lower electronic energy levels.
4) The visible emission spectra arise from the reverse type of transition.
5) σ-Electrons are involved in saturated σ-bonds, such as those between carbon and hydrogen in paraffin.
6) n-Electrons are involved in the bonding between atoms in molecules.
7) Chromophoric group is defined as any isolated ionic bonded group that shows a characteristic absorption in the UV or visible region.
8) Bathochromic shift is also called as red shift.
9) Hypsochromic shift is also called as yellow shift.
10) In hydrogen discharge lamps, hydrogen gas is stored under relatively low pressure.
11) Barrier-layer cell is also known as photronic cell and operates without the use of a battery.

1.7.2. Fill in the Blanks

12) The UV region extends from _____.
13) The visible region extends from _____.
14) _____ are involved in unsaturated hydrocarbons.
15) _____ are not involved in the bonding between atoms in molecules.

16) _____is an effect in which the intensity of absorption maximum increases.
17) _____ is an effect in which the intensity of absorption maximum decreases.
18) _____ describes the effect of alkyl substituents or ring residues.
19) _____ derive their effects from bulk interactions of radiation within the material.
20) _____ are based on the phenomenon of optical interference.
21) The _____ is used to disperse the radiation according to the wavelength.
22) _____ is the most sensitive of all the detectors, expensive and used in sophisticated instruments.
23) In _____, hydrogen gas is stored under relatively high pressure.
24) _____ is the art of processing data with various numerical techniques in order to extract useful information.
25) _____ is a data set containing measurements on a set of known samples.

Answers:

1) False	2) True	3) False
4) False	5) True	6) False
7) False	8) True	9) False
10) False	11) True	12) 10-400nm
13) 400-800nm	14) π-Electrons	15) n-Electrons
16) Hyperchromic shift	17) Hypochromic shift	18) Woodward-Fieser rules
19) Absorption filters	20) Interference filters	21) Monochromator
22) Photomultiplier tubes	23) Hydrogen discharge lamps	24) Chemometrics
25) Training set		

1.7.3. Very Short Answer Type Questions

1) Define and classify chromatophores.
2) What do you mean by auxochromes? Give few examples.
3) Differentiate between bathochromic and hypsochromic shift.
4) What is the effect of conjugation on absorption maxima?
5) Enlist the parts of a spectrophotometer.
6) Give the differences between single-beam and double-beam spectrophotometers.
7) Give the advantages of UV-Vis spectroscopy.
8) Draw a well-labelled diagram of single-beam UV spectrophotometer.

1.7.4. Short Answer Type Questions

1) Write a short note on electronic transitions of organic species.
2) What optimum conditions are required for spectrophotometric measurements?
3) What are the different radiation sources used in UV-Vis spectroscopy?
4) Write a short note on grating monochromator.
5) Write about double-beam UV spectrophotometer.
6) Demonstrate the working of double beam UV spectrophotometer.
7) Name the different detectors used in UV-Vis spectroscopy. Explain any one.
8) Write a note on derivative spectrophotometry.

1.7.5. Long Answer Type Questions

1) Mention the theory and principle involved in UV-Vis spectroscopy.
2) Give the Beer-Lambert's law, along with its deviations and limitations.
3) Explain briefly any three multicomponent analysis techniques.
4) Write a detailed note on spectrophotometric titration.
5) Give the instrumentation of spectrophotometer.

CHAPTER 2

Fluorimetry

2.1. FLUORIMETRY

2.1.1. Introduction

The method of measuring the intensity of fluorescent light emitted by the substance being examined with respect to that emitted by the given standard substance is termed as **fluorimetry**.

Fluorescence (a spectrochemical analytical method) involves exciting the analyte molecules by irradiation at a certain wavelength and emitting radiation of a different wavelength. The emitted light produces a spectrum that is useful for qualitative and quantitative analysis. When a molecule undergoes excitation by absorbing light of appropriate wavelength, its ground electronic state converts into one of the many vibrational levels in one of the excited electronic states.

This excited electronic state is the **first excited singlet state** (S_1). If a molecule reaches this excited state, it undergoes relaxation through several processes; fluorescence being one among them which emits light.

2.1.2. Molecular Luminescence

In **molecular luminescence**, analyte molecules get excited and results in emission spectrum useful for qualitative or quantitative analysis.

Molecular fluorescence is the optical emission resulting from molecules that absorb electromagnetic radiation and get excited to higher energy levels. Detection of fluorescence is preferred over absorption measurements due to its greater sensitivity because the fluorescence signal has a zero background. Quantitative measurements of molecules in solution and fluorescence detection in liquid chromatography are the analytical applications of molecular fluorescence.

Phosphorescence is the emitted light (by an atom or molecule) that continues to emit even after the removal of exciting source. It is almost similar to fluorescence with the only difference that the molecules excite to a metastable state from where getting back to the initial state is not possible. Emission results when thermal energy excites the electron which reaches a state from where it can de-excite, thus, concluding that phosphorescence is influenced by temperature.

2.1.3. Theory

Wavelength distribution of the emitted light measured with a single constant excitation wavelength is termed as an **emission spectrum**. The dependence of emission intensity at a single wavelength on the excitation wavelength is termed as an **excitation spectrum**. These spectrums can be presented either on a wavelength scale or a wave number scale. Wavelength (λ), frequency (ν), or wave number ($\bar{\nu}$) are the terms used for describing light of a given energy. The wavelengths (expressed in nm) and wave numbers (expressed in cm^{-1}) are

inter-convertible by reciprocating each value. **For example,** 400nm means $400 \times 10^{-7} cm^{-1}$ = 25,000cm^{-1}. Whether the fluorescence spectra should be presented in terms of wavelength or wave number has always been a matter of debate. The fact is that wave number scale is linear to energy, but most of the commercial instruments produce spectra in wavelength domain, which can be easily interpreted visually. Accurately corrected spectra cannot be easily obtained and are also not used routinely; therefore, the directly recorded technical spectra are used preferably on the wavelength scale.

The emission spectrum recorded by an ideal instrument represents the photon flux emitted at each wavelength, over a wavelength interval determined by the slit widths and dispersion of the emission monochromator. The excitation spectrum in the same way represents the relative quantum yield at each excitation wavelength. The quantum yields and emission spectra for most of the fluorophores do not depend on excitation wavelength. This is the reason why excitation spectrum of a fluorophore superimposes its absorption spectrum. Even if ideal conditions prevail, such correspondence demands the presence of only a single type of fluorophore and absence of other complicating factors (**e.g.,** a sample having high optical density produces a non-linear response).

2.1.4. Concepts of Singlet, Doublet and Triplet Electronic States

The theory of phosphorescence can be understood with the knowledge of singlet and triplet states. These are related to multiplicity considerations of atomic spectroscopy. The terms **singlet** and **triplet states** denote the number of unpaired electrons when there is no magnetic field. If 'n' number of unpaired electrons are present, (n+1) fold degeneracy (equal energy states) is linked to the electron spin, no matter which molecular orbital is occupied. If zero unpaired electrons are available (n = 0), that means n+1 or 0+1 or 1 spin state is present. This is a **singlet state**. Similarly, systems with 1, 2, 3, 4 and so on number of unpaired electrons are termed as doublet, triplet, quartet, quintet, etc., states respectively.

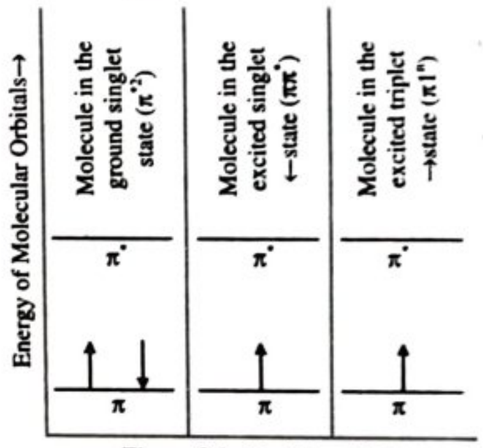


Figure 2.1: (a), (b) and (c)

Molecules in ground state bear no unpaired electrons, thus are in singlet state. Upon absorption of UV or visible radiation of particular frequency, one or more paired electrons (generally a π-electron) of these molecules jumps to an excited singlet state. Here, the electron spin does not change, and thus the net spin remains zero. It may also happen that one set of electron spins can unpair, forming two unpaired electrons building an excited triplet state. **Figure 2.1 (a)** shows a molecule in the ground singlet state, **figure 2.1 (b)** depicts a molecule present in the excited singlet state, and **figure 2.1 (c)** illustrates a molecule present in an excited triplet state.

Excited-State Processes in Molecules

When light of appropriate frequency falls on the molecules, it gets absorbed within 10^{-15} second. During absorption, shift in electronic state occurs as the molecule move from the ground to the first excited singlet electronic state (**figure 2.2**). At room temperature, the molecules remain in their ground vibration level. After absorption, they become excited and jump to any one of the vibrational levels of the first excited electronic state. From the excited singlet state, any of the three phenomena may occur as per the molecule involved and the conditions:

1) The **first possibility** is that the excited singlet state is unstable. In this situation, the excited molecules do not emit any radiation and revert back to the ground state through the phenomena of **collisional deactivation**.

2) The **second possibility** is that the molecule in the excited singlet state may emit an UV or visible light photon by the phenomena of **fluorescence**.

3) The **third possibility** is that the molecule in stable excited singlet state transit to a metastable triplet state and then return back to the ground state by emitting an UV or visible light photon by the phenomena of **phosphorescence emission**. The crossing from a singlet state (no unpaired electron) to a triplet state (two unpaired electrons) is known as **intersystem crossing**.

The decay from the triplet state to the ground state singlet has no spin symmetry, hence is slow, i.e., life-time of phosphorescence is longer than fluorescence.

The phosphorescence mechanism where singlet-triplet decay occurs has been confirmed through magnetic susceptibility and ESR measurements.

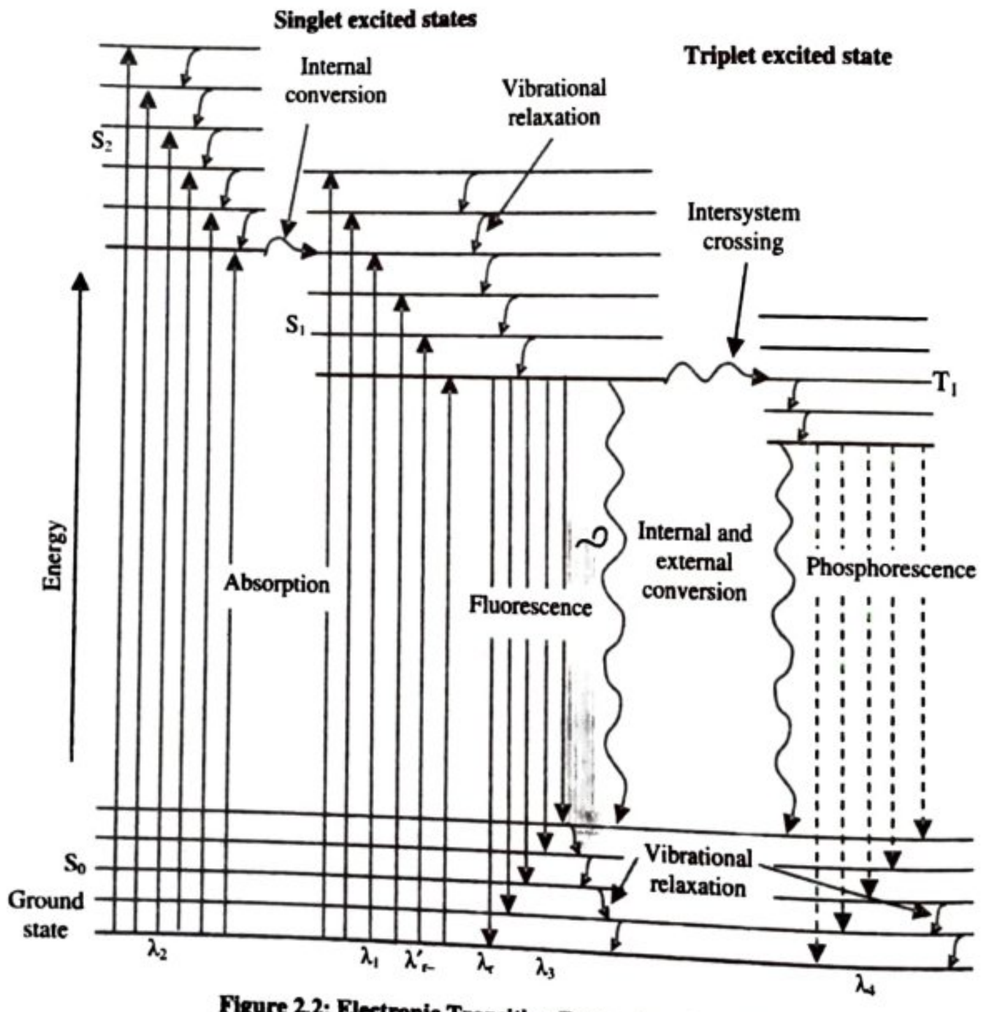


Figure 2.2: Electronic Transition Energy Level Diagram

Process of Excitation and Emission

After absorption, a number of vibrational levels of the excited state occur. The molecules in higher vibrational levels of the excited state undergo **vibrational relaxation** and reach the lowest vibrational levels of the excited state. From here the molecules further relax down to their ground state via several processes or pathways (discussed below).

2.1.4.1. External Conversion or Collisional Deactivation

This pathway leads to a non-radiative relaxation. An excited molecule and solvent or other solutes interact and transfer energy among each other to deactivate an excited electronic state. This process is termed as **external conversion** or **collisional quenching**. This process is characterised by the solvent effect on fluorescence intensity. The conditions under which the particle collisions reduce lead to enhanced fluorescence.

2.1.4.2. Internal Conversion

This pathway involves **direct vibrational coupling** between the ground and excited electronic states (vibronic level overlap) and **quantum mechanical tunnelling** (no direct vibronic overlap but small energy gap). This process is rapid (i.e., 10^{-12} seconds) in comparison to average lifetime of the lowest excited singlet state (i.e., 10^{-8} seconds), therefore, competes with fluorescence.

Internal conversion is an intermolecular process which brings down a molecule to a lower energy electronic state without emitting light. This process is not clear yet; however, it is known to be highly efficient, because only a few compounds exhibit fluorescence. Internal conversion may also lead to **pre-dissociation**, which is different from dissociation in which the electron of a chromophore gets excited by absorbing radiation and reaches the highest vibrational level resulting in the breakdown of the chromophoric bond. Internal conversion is not involved in the process of dissociation; and this process also competes with the fluorescence process.

2.1.4.3. Intersystem Crossing

This pathway involves overlapping of the triplet energy states by the singlet energy states. This overlapping results in vibrational coupling between the two states. Molecules in the singlet excited state can cross over to the triplet excited state. The average lifetime of this process is 10^{-9} second. In intersystem crossing process, the spin of an excited electron is reserved, resulting in a charge multiplicity of the molecule. This process commonly occurs in molecules containing heavy atoms (iodine or bromine), in the presence of which large number of spin/orbital interactions occur and a charge in spin becomes more favourable. If paramagnetic species (**e.g.,** molecular oxygen) are present in solution, the intersystem crossing enhances, decreasing the fluorescence.

2.1.4.4. Phosphorescence

This pathway involves emission of light by the molecules which relax from the triplet excited state to the singlet ground state. This transition is restricted, therefore, the lifetime of triplet state is long (i.e., 10^{-2} to 100 seconds) and the phosphorescence rate is slow.

2.1.4.5. Fluorescence

This pathway involves emission of light by the molecules which relax from the singlet excited state to the singlet ground state. The energy gap between the two states influences the wavelength (and thus, the energy) of the light emitted. The lifetime of fluorescence is short (i.e., approximately 10^{-8} second) so that it can compete with the above mentioned processes (i.e., collisional deactivation, intersystem crossing, and phosphorescence). The **net energy balance for the process of fluorescence** is given by:

$$E_{fluor} = E_{abs} - E_{vib} - E_{solv.relax.}$$

Where,

E_{fluor} = Energy of the emitted light.

E_{abs} = Energy of the light absorbed by the molecule during excitation.

E_{vib} = Energy lost by the molecule from vibrational relaxation.
$E_{solv.relax}$ = Need for the solvent cage of the molecule to undergo re-orientation when in the excited state and when the molecule relaxes to the ground state.

The above equation states that the fluorescence energy for a given molecule is always less than the absorption energy. Thus, the light emitted is observed at longer wavelengths than the excitation.

2.1.4.6. Vibrational Relaxation

The average lifetime of this process is 10^{-12} second or less (i.e., shorter than the average lifetime of an electronically excited state). Consequently, when the fluorescence process occurs from solution, a transition from the lowest vibrational level of an excited state to an excitation level occurs. As a result of vibrational relaxation, **Stokes shift** occurs in which the fluorescence band for a given electronic transition gets displaced towards the lower frequencies or longer wavelengths from the absorption bands.

2.2. FACTORS AFFECTING FLUORESCENCE

2.2.1. Introduction

The fluorescence intensity is affected by the following two categories of factors:

1) Structural factors, and
2) Non-structural factors.

2.2.2. Structural Factors

The structural factors affecting fluorescence intensity of compounds are:

1) **Conjugation:** A molecule should be unsaturated to absorb UV-visible radiation; because if radiation is not absorbed, fluorescence does not occur.

2) **Nature of Substituent Groups:** These groups significantly affect the fluorescence of molecules. There are no rigid rules but the following generalities may be useful:
   i) **Electron Donating Groups:** Amino (NH_2) and hydroxyl (OH) groups enhance the fluorescence intensity.
   ii) **Electron Withdrawing Groups:** Nitro (NO_2) and carboxylic (COOH) groups reduce the fluorescence intensity.

   SO_3H or NH_4 groups do not produce any effect on fluorescence intensity. The table below describes the effect produced by several substituents on the emissive wavelength and fluorescence intensity:

Table 2.1: Substituents and their Effect on Wavelength and Intensity

Substituents	Effect on Wavelength	Effect on Intensity
Alkyl	No effect	Slight ↑ (increase) or ↓ (decrease)
COOH, CHO, COOR, and COR	↑ (increase)	↓ (decrease)
OH, OME, and OEt	↑ (increase)	↑ (increase)
CN	No effect	↑ (increase)
NH_2, NHR, and NR_2	↑ (increase)	↑ (increase)
NO_2 and NO	Large ↑ (increase)	Large ↑ (increase) or complete quenching
SH	↑ (increase)	↓ (decrease)
SO_3H	No effect	No effect
F, Cl, Br, and I	↑ (increase)	↓ (decrease)

3) **Rigidity of Structures:** Rigid structures (**e.g.,** fluorene) have more fluorescence intensity, while the flexible structures (**e.g.,** biphenyl) have less fluorescence intensity.

4) **Intermolecular Hydrogen Bonding:** Fluorescence becomes stronger by intermolecular bonds if dioxane and acetic acid esters (other than ethyl chloroacetate) are used as proton acceptors.

   If β-napthol is used as a proton acceptor, the intermolecular hydrogen bonding reduces the inner quenching rate. If ethyl chloroacetate is used as a proton acceptor, the hydrogen bonds mask the fluorescence quenching.

5) **Intramolecular Hydrogen Bonding:** By using steady state and time-resolved spectroscopic techniques, the effect of intramolecular hydrogen bonding on 6-dodecanoyl-2-dimethylaminonapthalene (laurdan) in neat and binary solvent mixtures has been investigated. Therefore, it stabilises and increases the fluorescence intensity of intramolecular hydrogen bonding.

2.2.3. Non-Structural Factors

The non-structural factors affecting fluorescence intensity of compounds are:

1) **Temperature:** If **temperature is increased**, the molecular collisions increase. This in turn results in deviation, which **decreases the fluorescence intensity**. Contrarily, if the **temperature is decreased**, the molecular collisions decrease, which **increases the fluorescence intensity**.

2) **Viscosity:** If **viscosity is increased**, the molecular collisions decrease, which in turn **enhances the fluorescence intensity**. Contrarily, if the **viscosity is decreased**, the molecular collisions increase, thus, **decreasing the fluorescence intensity**.

3) **Oxygen:** It mainly **reduces the fluorescence intensity** by acting in any of the following two ways:
   i) It oxidises the fluorescent substance into a non-fluorescent substance, or
   ii) It reduces fluorescence due to its paramagnetic properties of molecular energy (as it has triplet ground state).

4) **Effect of pH:** This effect depends on the chemical structure of the molecule. **For example,**
   i) Aniline gives visible fluorescence in neutral or alkaline medium, and gives fluorescence in UV region only in acidic medium.
   ii) Phenols do not give fluorescence in acidic medium as they are undissociated, and gives good fluorescence in alkaline medium as they are dissociated (ionic).

5) **Photochemical Decomposition:** In some cases where UV-visible absorption leads to photochemical reaction, fluorescence is not observed. Hence, a wavelength that cannot be absorbed strongly should be preferred to avoid such a reaction. Otherwise, up to 20% errors may occur.

6) **Effect of Concentration:** Fluorescence and concentration are **directly proportional** but the equation applies only to small values of fluorescence and thus, low sample concentration. Thus, it is true to about 5% when the extinction of a solution of the sample is about 0.05.

   When such conditions prevail, the test solution absorbs a very small amount of light, and the extinction should not be greater than about 0.02 for a linear calibration curve. When stronger solutions are used, fluorescence concentrate at the irradiated face of the cell in large amount, and this effect is clearly visible in the simple experiment with quinine. **Figure 2.3** represents the cells.

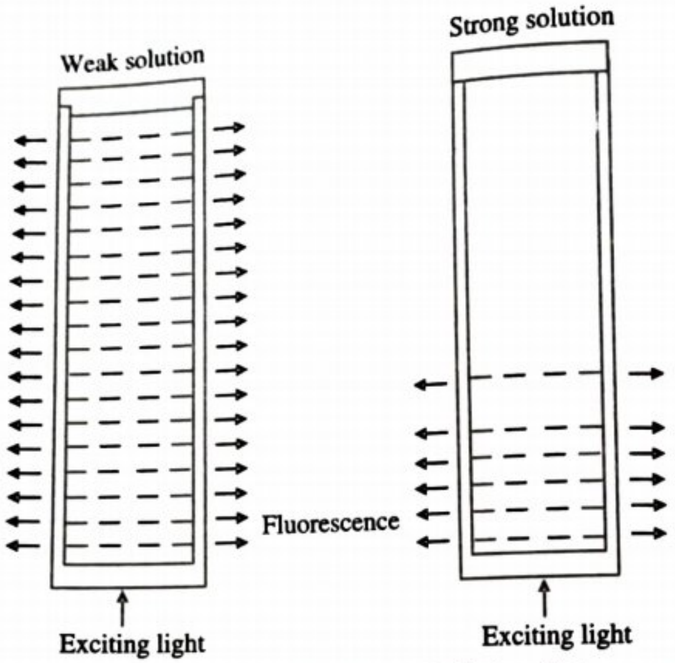


Figure 2.3: Effect of Solute Concentration on Emission of Fluorescence from Cell

When the concentration of fluorescent substance is very high, all the incident radiation gets absorbed, and the following equation is obtained:

$$F = I_0Q$$

This equation indicates that **fluorescence does not depend on concentration**, and is proportional to the intensity of incident radiation. This property is helpful in determining the approximate emission curve of a light source.

7) **Adsorption:** Extreme sensitiveness of the method requires very dilute solutions (10-100 times weaker than those employed in spectrophotometry). Therefore, if the fluorescent substance gets absorbed on the container walls (**e.g.,** quinine gets absorbed on the cell walls), a serious problem may occur. Thus, strong stock solutions should be kept and diluted whenever required.

8) **Light:** Monochromatic light is required for the excitation of fluorescence during a quantitative analysis because the intensity and wavelength vary linearly. Purity of the irradiating beam obtained from filters should be checked by examining the light scattered by a slightly turbid solution. The trace obtained should show one peak only, corresponding in wavelength to that expected.

Even after a monochromatic light is obtained, there is a possibility of another source of error, i.e., the Raman emission from the solvent. This emission in case of fluorescence occurs at a wavelength longer than that of the exciting beam. The effect of this emission increases with decreasing solute concentration because in order to produce an adequate response to the fluorescence, the detector sensitivity should be increased. The interference from Raman scatter can be avoided by using radiation of a wavelength removed from that of the fluorescence peak.

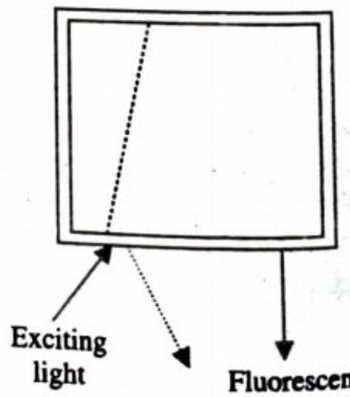


Figure 2.4: Frontal Illumination

Presence of Raman emission can be confirmed by observing a small change in its frequency that may occur as a result of a change in the exciting radiation. Difference in the frequency of excitation and Raman emission is around $3.4 \times 10^3 cm^{-1}$ for aqueous solutions. In terms of wavelength, some Raman peaks of common solvents with excitation by the mercury line at 336nm are:

i) **Water** - 416nm,
ii) **Ethanol** - 409nm, and
iii) **Chloroform** - 410nm.

9) **Methods of Illumination:** The right-angle method is the illumination method utilising the **Spekker fluorimeter**. This method gives a smaller blank value for scattered light and fluorescence from container walls compared to that given by the alternative method of **frontal illumination** (**figure 2.4**).

Frontal illumination method is used for opaque solutions and solids. It is favourable for both dilute and concentrated solutions. However, the problem associated with this method is that the inner-filter effects occur, which alters the spectra.

2.2.4. Quantitative Aspects of Fluorescence

A molecule absorbs radiation and then undergoes fluorescence. When the absorbing substance is present in very high concentration, the first layers of solution absorbs the entire incident light and very little amount of light reaches the distant portions of the sample. Such a sample will fluoresce non-uniformly and the fluorescence will be independent of the concentration of the absorbing substance. Analytically, such a condition is not acceptable; therefore, very low solution concentrations of fluorescing substances are used so that the entire incident light is not absorbed.

The entire light absorbed by a molecule may emit as fluorescence; however, there are more chances that some parts of the absorbed light or energy are lost in other ways. A value of Φ can be obtained from **equation (1)**:

$$\Phi = \frac{F}{I_{ab}} \qquad \text{.....(1)}$$

Where, Φ = Quantum yield of fluorescence with value ranging from 0 to 1.
F = Fluorescence intensity (i.e., intensity of the emitted radiation).
I_{ab} = Intensity of the absorbed radiation.

Difference between the intensity of incident radiation and the intensity of transmitted radiation gives the value for intensity of absorbed radiation:

$$I_{ab} = I_0 - I_0e^{-2.3abc} \qquad \text{.....(2)}$$

Where, a = Molar absorptivity.
b = Path length.
c = Molar concentration.

If 'c' has a very small value, **equation (2)** can be written as:

$$I_{ab} \cong \frac{2.3abcI_0}{1+2.3abc} \cong 2.3abcI_0 \qquad \text{.....(3)}$$

Where, the approximation $e^x \cong 1 + x$ has been used. On combining **equations (1) and (3)**:

$$F = 2.3I_0 \Phi abc \qquad \text{.....(4)}$$

Equation (4) shows that at very low concentrations, the **fluorescence intensity and concentration are directly proportional**. As per **equation (4)**, the value of F is proportional to I_0; so by increasing the intensity of the incident excitation radiation, the sensitivity can be increased for a given concentration. Based on this feature, fluorimetry is distinguished from spectrophotometry, as in the latter absorbance is independent of the incident intensity.

Equation (4) also states that **fluorescence intensity and molar absorptivity are proportional to each other**. Thus, the light corresponding to the absorption band maximum is used as the excitation radiation. A beam of radiation with many wavelengths in width is employed for excitation, and the wavelength range should be selected such that it includes the maximum in the excitation spectrum.

A standard curve is prepared with known solutions of a pure sample having fluorescence intensity against fluorescing substance concentration. The same procedure is followed using an unknown sample and its concentration is noted from the graph. Same experimental conditions (i.e., the excitation source, solvent, pH, and temperature) should be maintained for both known and unknown measurements. There are several foreign substances that can reduce the Φ value, and thus the sensitivity of a fluorescent compound. This suppression in fluorescence is termed as **quenching**.

2.2.5. Quenching

Due to the specific effects produced by the constituents of the solution being used, the fluorescence (or phosphorescence) intensity is reduced. Such a reduction in fluorescence intensity is termed as **quenching**.

There are many ways by which quenching may occur. **For example,** when the solution absorbs excess amount of either primary or fluorescent radiation, **concentration quenching** occurs (also known as **inner filter effect**); when the fluorescent substance absorbs the same radiation, **self-quenching** occurs; when the chemical nature of the fluorescent substance changes and reduces its emission, **chemical quenching** occurs. An **example** of chemical quenching is when aniline is excited at 290nm, a blue fluorescence appears between pH 5-13. Aniline at low pH exists as the anilinium cation and in higher pH it exists as the anion, and neither of them produces fluorescence.

Sometimes the non-radioactive energy loss from the excited molecules also results in quenching. This occurs because the quenching agent (**e.g.,** quenching of many organic compounds by dissolved oxygen) enables the molecules to convert from the excited singlet state to an excited triplet state, from where emission is restricted.

Some structural features also are capable of initiating quenching by inhibiting fluorescence. **For example**, iodide, bromide, nitro, and carboxylate functional groups decrease the fluorescence of molecule.

Formation of complexes between the molecule and heavy metal ions (**e.g.,** mercury) also results in quenching.

Generally, quenching is applied to all those processes which decrease the quantum yield. **For example,**

1) Collision deactivation by solvent,
2) Energy consumed by bond breakage, and
3) Fluorescence absorption by another component of the solution.

2.3. INSTRUMENTATION

2.3.1. Introduction

Fluorimeter is the instrument measuring fluorescence intensity (F). It includes the following components:

1) Light sources,
2) Monochromators/filters,
3) Sample cells/cuvettes,
4) Detectors, and
5) Polarisers.

2.3.2. Light Sources

The radiant energy required for exciting the fluorescent molecule or fluorophore is supplied by the light source. **Intensity, stability,** and **wavelength variability** are some of the desired features that the light source should possess. A high intensity radiant energy should be supplied from the source since fluorescence is directly proportional to source intensity, i.e., the **more intense is the source**, the **greater fluorescence** is produced. If the **source does not remain stable** and flickers, the **fluorescence measurements will not be reproducible**. The fluorescent molecules become excited only at certain wavelength range of the source, therefore, the source should supply energy only at these wavelengths.

Some of the commonly used light sources are:

1) **Lasers:** A source producing high intensity radiation and having high stability is the lasers. These light sources undergo molecular excitation, but are monochromatic, i.e., **wavelength variability is their major drawback**. Due to this reason, the fluorophore should excite at the wavelength to which the laser has been tuned, otherwise fluorescence will not occur. Another limitation of lasers is their **high cost**. Therefore, some other light sources, **e.g.,** deuterium lamp, mercury vapour lamp, and xenon arc are utilised in fluorimetry.

2) **Deuterium Lamp:** It provides radiation of high intensity but its wavelength range is limited to 200-350nm.

3) **Mercury Vapour Lamp:** It also provides high intensity radiation and is a stable source, but it does not provide a continuous spectrum. Mercury vapour lamp is also termed as a **line source**, since it emits radiation at 257.7nm, 313.0nm, 365.0nm, 404.7nm, 407.8nm, 435.8nm, 546.1nm, 577.0nm, and 579.1nm wavelengths. The mercury lines also provide a monochromatic beam at each wavelength. If other lines are filtered efficiently, stray light is minimised. Therefore, mercury lamps are sometimes used in filter fluorimeters. This source is discontinuous, but most of the fluorescing molecules absorb radiation at one of the mercury lines. Fluorescence can be observed by setting the excitation source at the mercury line nearest to the absorption maximum.

4) **Xenon Arc:** It is also a commonly used light source in fluorescence measurement. It produces a continuous spectrum at 250-600nm wavelength (major advantage), but produces radiation of different intensity at different wavelengths. Peak intensity of xenon arc is observed at 470nm. Desired fluorescence can be observed by setting the source at such wavelength that maximum absorption occurs. The **disadvantages** are that a cooling apparatus may be required for proper operation. Ozone produced by the xenon arc needs to be removed and this may lead to instrumental limitations. Xenon arc is preferred over deuterium lamp as the former extends the wavelength range at which excitation occurs and also enhances the selectivity.

5) **Tungsten Halogen Lamp:** It is used as the fluorescence polarisation source, and provides a continuous spectrum in the visible range. This source is less intense, still accurate fluorescence measurements can be obtained with the use of highly efficient fluorophores (high quantum yield). One wavelength in the range is chosen to match the excitation maximum of the fluorescent tag chosen. The **advantages** of tungsten halogen lamp in fluorescence polarisation are its instrumental simplicity required for operation and its low cost.

2.3.3. Monochromators/Filters

Filters are categorised into:

1) A **short-pass filter** that permits light at wavelengths below to that given for the filter to pass.
2) A **long-pass filter** that permits light at wavelengths above to that given for the filter to pass.
3) A **band-pass filter** that permits light between two given wavelengths to pass. These filters efficiently eliminate the use of different sources of interfering light.

Mercury vapour lamp is commonly used in fluorimeters because it supplies narrow lines of radiation. The wavelength suitable for excitation is selected by a band-pass filter, while excluding the other undesired wavelengths. On the emission side of the fluorimeter, a long-pass filter is used. The excitation band-pass filter and the emission long-pass filter do not have any common wavelengths, thus, the stray light is eliminated.

Filters are made up of glass or are dye-containing Wratten filters. The glass or dye is selected properly for regulating optical transmission as they absorb only the undesired wavelengths. Thus, indicating that the desired wavelengths are not absorbed and they pass through the filters.

Spectrofluorimeters employ the same monochromators used in spectrophotometers. In the earlier instruments, prisms were used, but at present **gratings** are more common. Gratings are either ruled (more commonly used) or holographic. The latter can reduce the stray light, but is less efficient than the former, which permits light of greater intensity to pass for a given wavelength. Monochromator adjusts the angle which the incident radiation makes with the grating surface after striking. It separates the incident light into its component wavelengths by rotating the gratings. The component wavelengths are focused on the sample by passing through slits. In case of emission, the fluorescent sample serves as the light source and the gratings are used for selecting the emission wavelength.

2.3.4. Sample Cells/Cuvettes

For excitation wavelength greater than 320nm (i.e., visible region), **glass cuvettes** are used because they themselves absorb large amount of radiation at wavelengths below this value. For excitation wavelength at UV region or for very sensitive fluorescence measurements, **quartz cuvettes** are used. **Matched quartz cuvettes** are also available so that the reference and sample are contained in identical cuvettes. The problem arising due to cuvette variability is eliminated, thereby, allowing for sensitive measurements.

Fluorescence measurements with the use of matched quartz cuvettes is generally avoided in clinical laboratories; and a filter fluorimeter, round glass test tubes are preferred for this purpose. However, measurements using round tubes may be interfered with stray light; but, the optical and electronic null of the instruments compensates this. Apart from this, baffles are used to block the stray radiation, enabling only the radiation emitted from the sample to be focused on the detector.

2.3.5. Detectors

Fluorimetry employs **photomultiplier tube** as the detector. For fluorescence measurements in the visible range, a **glass-encased tube** is used. While, in the UV range, glass itself absorbs the radiation; therefore, special **UV-responsive tubes** are used.

The **principle involved** in the working of photomultiplier tube is **amplification**. When the incident light strikes the light-sensitive screen surface in the detector, a potential is generated which is proportional to the light intensity. This **potential** is magnified using several electron emitter or collector cells; and when an **electronic** signal passes from one cell to the other, it gets amplified till it reaches the end-stage **collector**. Here, the potential is measured and recorded on a meter, digital display, or other **recording** device.

The instruments available now are equipped with **microprocessors** for making concentration calculations and direct printouts. Such instruments **are** calibrated using a series of standards, and the fluorescence concentration curve is stored in the instrument memory. After calibration, samples are analysed and the fluorescence obtained is compared to the stored curve for determining the analyte concentration.

2.3.6. Polarisers

An electromagnetic radiation is made up of electronic and magnetic components. During absorption, only the electronic component interacts with the electrons. In this electronic component, radiations vibrating in two perpendicular planes are present. Thus, the electronic component for any given radiation beam is defined in terms of two planes perpendicular to each other. Fluorimetry employs certain **non-cubic (anisotropic) crystals** as polarisers because they can filter one of the two perpendicular planes. When two planes of incident light falls on the crystal, one plane of light passes through it while the other gets absorbed. This depends on the orientation of crystal to the incident beam. The plane of light that passes through the crystal is obtained as the plane-polarised light.

2.3.7. Single Beam Filter Fluorimeter

Figure 2.5 illustrates the basic arrangement of a single beam 90°, filter fluorimeter, consisting of the following **components**:

1) A mercury vapour lamp, 2) A condensing lens, 3) A primary filter,
4) A sample container, 5) A secondary filter, and 6) A receiving photocell.

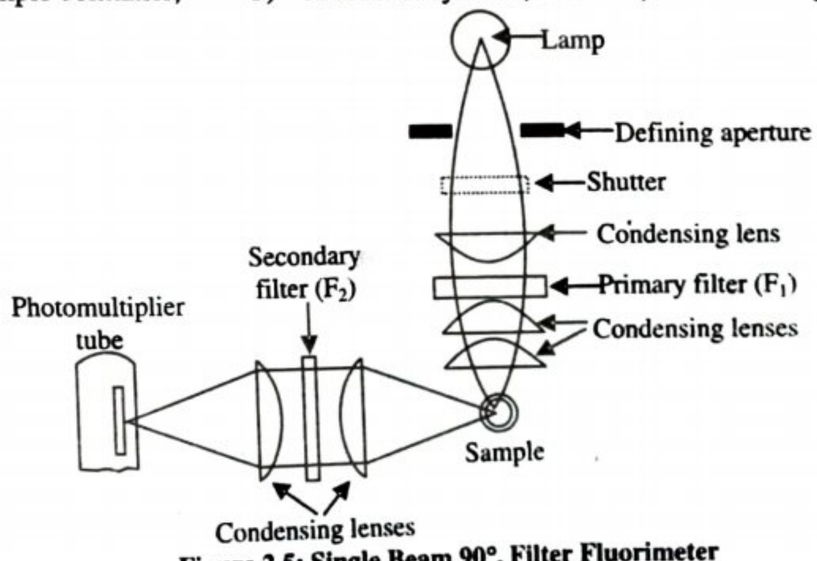


Figure 2.5: Single Beam 90°, Filter Fluorimeter
Source: Adapted from *Instrumental Methods of Chemical Analysis* (pp 2.406), by Chatwal G.R. (2006) (Himalaya Publishing House)

The single beam fluorimeter works as follows:

1) A light is passed from the mercury vapour lamp, through a condensing lens, and then through a primary filter.
2) The primary filter selects only UV radiation but absorbs the visible radiation.
3) The UV radiation from the primary filter reaches the sample container.
4) UV and fluorescent radiations obtained in the sample container passes through a secondary filter.
5) This filter absorbs the primary radiant energy and transmits the fluorescent radiation.
6) This transmitted radiation is received by a photocell placed at right angles to the incident beam.
7) The output of the photocell is measured by a sensitive galvanometer or other device.

The light source used should be of adequate stability because the fluorescence intensity is proportional to the irradiation intensity. The fluctuations in irradiation intensity in most of the fluorimeters are not compensated for. In such fluorimeters, two photocells are used and the readings are recorded on a potentiometer in balancing the photocells against each other.

The primary filter used selects the UV radiation (and not the visible radiation), while the secondary filter transmits visible fluorescent radiation and absorbs incident UV radiation.

2.3.8. Double Beam Filter Fluorimeter

Figure 2.6 illustrates a double-beam filter fluorimeter, consisting of a specially designed mercury vapour lamp, and two anodes equipped on the opposite sides of a centre structure. This lamp works by alternating current, thus, the two anodes receive the discharge and generate radiation on alternate half-cycles of the exciting voltage. Consequently, the sample and the standard receive identical radiations.

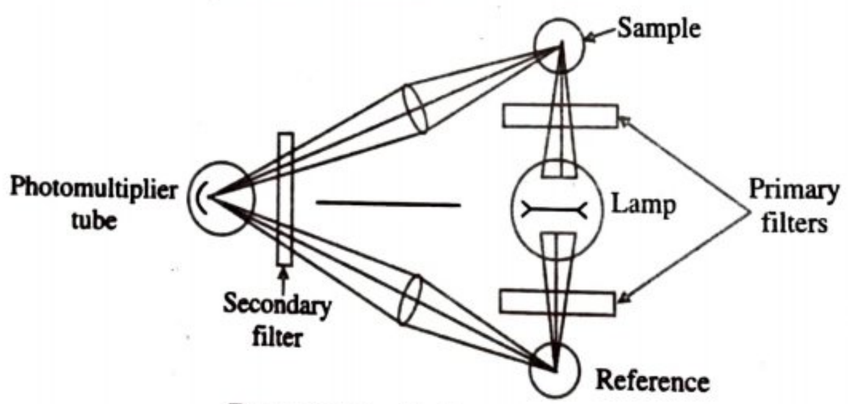


Figure 2.6: Double-Beam Filter Fluorimeter
Source: Adapted from *Instrumental Methods of Chemical Analysis* (pp 2.406), by Chatwal G.R. (2006) (Himalaya Publishing House)

The double beam fluorimeter works as follows:

1) The two beams from the lamp initially pass through two primary filters, and then through the sample and reference.
2) The fluorescent radiations obtained from the sample and reference converges through a common secondary filter into a single photomultiplier tube.
3) This tube records the ratio of two fluorescence signals (one from the sample and the other from reference).

In this system, the effect of temperature changes and variation in source and detector can be minimised.

2.4. APPLICATIONS

2.4.1. Introduction

Fluorimetric titrations are used for the following purposes:

1) Determination of ruthenium,
2) Determination of vanadium,
3) Determination of boron in steel,
4) Determination of aluminium in alloys,
5) Determination of chromium and manganese,
6) Determination of uranium salts,
7) Estimation of rare earth terbium,
8) Estimation of bismuth,
9) Determination of beryllium in silicates,
10) Estimation of 3,4-benzpyrene,
11) Determination of vitamin B_1, and
12) Determination of vitamin B_2 (riboflavin).

2.4.2. Determination of Ruthenium

In inorganic chemistry, fluorimetry technique is used for determining ruthenium in the presence of other platinum metals. At pH 6, the complex between ruthenium (II) ion and 5-methyl-1,10-phenanthroline undergoes fluorescence. Palladium and the reagent together form a precipitate that is removed by centrifugation. Iron (if present) forms a complex and quenches the fluorescence. Any other platinum elements can be present in up to 30µg/ml range without causing any interference in the determination of ruthenium in 0.3-2.0µg/ml range.

2.4.3. Determination of Vanadium

Fluorimetry is also used for determining vanadium by reacting with benzoic acid in an acetate buffer at pH 2. When the sample is excited at 300nm, fluorescence occurs linearly at 410nm over the range 0.5-400ppb. Vanadium determination is interfered with the presence of iron and large amounts of titanium.

2.4.4. Determination of Boron in Steel

Fluorimetry also aids in determining the traces of boron present in steel by complexing with benzoin. In this process, the boron in an acid solution of the sample is converted into boric acid which is co-distilled with methanol to separate from other components. The distillate obtained contains boric acid, thus, is neutralised with NaOH and evaporated to remove the methanol. The residue left behind is taken in an alcohol, followed by the addition of an alcoholic benzoin solution to it. After 2 minutes, fluorescent intensity of the mixture is measured which is linear with the boron concentration up to 100µm in 50ml volume, but declines with increasing concentrations.

2.4.5. Determination of Aluminium in Alloys

Fluorimetry proves to be an accurate, sensitive, and rapid method for determining small amount of aluminium found in alloys using dye pontachrome blue black F reagent at pH 4.8 in a buffered solution. This technique can determine 0.01-1.00% of acid soluble aluminium present in steel. This method involves a complex formation between aluminium and azo dye 2,2′-dihydroxy-1,1′-azo naphthalene-4-sulphonic acid, sodium salt (pontachrome blue black R). Iron and other interferences are removed by mercury cathode electrolysis, followed by fluorescence measurement of the complex at pH 4.9 in a buffered solution.

2.4.6. Determination of Chromium and Manganese

Fluorimetry can also determine chromium and manganese in steel by dissolving the steel in acid and oxidising the solution with persulphate. Chromium and manganese are present as $Cr_2O_7^{2-}$ and MnO_4^- ions absorbing in the violet and yellow green regions, respectively. The absorption bands are slightly overlapped; however, on treating a portion with $NaNO_2$, the MnO_4^- ion (and not $Cr_2O_7^{2-}$ ion) gets reduced. Now, manganese is determined by the difference measurement and a measurement at $\lambda = 4100$Å on the reduced portion gives chromium.

2.4.7. Determination of Uranium Salts

For determining uranium salts with fluorimetry, the sample solution is boiled with nitric acid, followed by fusion with sodium fluoride. This yields a melt, containing sodium fluoride and uranium fluoride, which solidifies into a glass on cooling. The solidified product can be examined using a fluorimeter. Palladium and the reagent together form a precipitate that is removed by centrifugation. Iron (if present) forms a complex and quenches the fluorescence.

2.4.8. Estimation of Rare Earth Terbium

This element forms a fluorescent complex with EDTA and SSA (Sulpho-Salicylic Acid). Its excitation spectrum can be related to the absorption spectrum of SSA ($\lambda_{max} = 3200$Å). The fluorescent spectrum obtained presents characteristic peaks of the Tb ion at 4850, 5450, 5750 and 6300Å. This method is specifically used for determining terbium and it has been observed that the excitation energy of SSA gets intra-molecularly transferred to Tb. This energy transfer, however, does not occur when SSA and other rare earth ions form complexes. The reason is incompatibility of the rare earth excited energy levels with the SSA excited state triplet level.

2.4.9. Estimation of Bismuth

For determining bismuth spectrofluorimetrically, the sample solutions are evaporated in an argon hydrogen flame, followed by irradiation with iodine emission line at λ = 2061.63Å (which is very close to bismuth line at $\lambda = 2061.70$Å) to absorb the radiation. Bismuth is estimated by measuring fluorescence emission line at 3025Å and the detection limit of Bi^{3+} ion is 2.5×10^{-7} molar concentration of bismuth.

2.4.10. Determination of Beryllium in Silicates

Fluorimetry can detect 0.001µg of beryllium in 25ml and 0.25µg can be detected with a precision of better than 1%. A linear calibration curve is obtained up to 0.50µg in 25ml volume. This determination method involves formation of a fluorescent complex between beryllium and morin (2′,4′,3,5,7-pentahydroxyflavone). Iron and rare earths may cause interference, and thus are removed by mercury cathode electrolysis.

2.4.11. Estimation of 3,4-Benzpyrene

This is a cancer-causing substance that can be extracted either with tobacco solution or from tobacco smoke deposits and separated out by chromatography (Al_2O_3) and elution. The fluorescent solution obtained is placed in a glass cell and irradiated with a mercury lamp and glass filter ($\lambda = 3650$Å). The resultant fluorescence radiation (λ = 4030, 4089, 4270Å) after being collected from the face of the cell absorbing the radiation is focused on the entrance slit of a photographic spectrograph.

2.4.12. Determination of Vitamin B_1 (Thiamine)

This vitamin itself is non-fluorescent, however, its oxidation product (thiochrome) produces a blue coloured fluorescence. This property helps in its determination in food samples like meat, cereal, etc.

Phosphatase and the food sample are reacted, thereby, hydrolysing the phosphate esters of thiamine present in the sample. Phosphatase and other insoluble matter are removed by filtering the solution. The filtrate obtained is diluted to a known volume, from which one aliquot for analysis and the other for a blank are withdrawn. Sodium hydroxide and isobutyl alcohol are added in equal quantities to both the aliquots. Thereafter, an oxidising agent (**e.g.,** potassium ferricyanide) is added to the first aliquot and after shaking, the alcoholic and the aqueous solution are separated. The separated alcoholic solution is examined using a fluorimeter. Now the complete procedure, including a blank, is repeated using a standard thiamine solution.

2.4.13. Determination of Vitamin B_2 (Riboflavin)

This vitamin is determined by fluorescence method because the fluorescent power depends on the exact conditions and the nature and amount of impurities present. The **standard increment method** is used to confirm that the effect of impurities on the standard and unknown is the same. In this method, the fluorescence of a portion of the standard in the same solution is measured with the unknown. This method also believes that riboflavin oxidises to yield a non-fluorescent substance.

This method involves treating the acid solution of the food sample with different reagents. As a result, various interfering ions precipitate out. Thereafter, this solution is oxidised using dilute permanganate. The residual fluorescence is measured as a blank and solid sodium dithionite ($Na_2S_2O_4$) is added in slight excess amount, followed by fluorescence determination. After the fluorescence determination of blank, a known volume of standard is added and fluorescence is re-measured. Finally, the results are computed by the following scheme:

Fluorescence of Solution	Designation
10ml oxidised sample + 1ml water	F_A
Same + dithionite	F_B
Same + 1ml standard	F_C

Final results are established by calculating the **fluorescing material concentration** using the following formula:

$$\frac{F_B - F_A}{F_C - F_A} = \frac{m_x}{m_x + m_s}$$

Where,

m_x and m_s = Masses of riboflavin from sample and standard in the cuvette, respectively.

2.5. SUMMARY

Details given in the chapter can be summarised as follows:

1) The method of measuring the intensity of fluorescent light emitted by the substance being examined with respect to that emitted by the given standard substance is termed as **fluorimetry**.

2) In **molecular luminescence**, analyte molecules get excited and results in emission spectrum useful for qualitative or quantitative analysis.
3) **Phosphorescence** is the emitted light (by an atom or molecule) which continues to emit even after the removal of exciting source.
4) The wavelength distribution of the emitted light measured with a single constant excitation wavelength is termed as an **emission spectrum**.
5) The dependence of emission intensity at a single wavelength on the excitation wavelength is termed as an **excitation spectrum**.
6) The terms **singlet** and **triplet states** denote the number of unpaired electrons when there is no magnetic field.
7) An excited molecule and solvent or other solutes interact and transfer energy among each other to deactivate an excited electronic state and it is termed as **external conversion** or **collisional quenching**.
8) **Internal conversion** is an intermolecular process which brings down a molecule to a lower energy electronic state without emitting light.
9) If **temperature is increased**, the molecular collisions increase. This in turn results in deviation which **decreases the fluorescence intensity**.
10) If **viscosity is increased**, the molecular collisions decrease, which in turn **enhances the fluorescence intensity**.
11) Due to the specific effects produced by the constituents of the solution being used, the fluorescence (or phosphorescence) intensity is reduced. Such a reduction in fluorescence intensity is termed as **quenching**.
12) When the chemical nature of the fluorescent substance changes and reduces its emission, **chemical quenching** occurs.
13) **Deuterium lamp** provides radiation of high intensity but its wavelength range is limited to 200-350nm.
14) Fluorimetry employs **photomultiplier tube** as the detector.

2.6. EXERCISE

2.6.1. True or False

1) In molecular luminescence, analyte molecules get excited and results in emission spectrum useful for qualitative or quantitative analysis.
2) Phosphorescence is the emitted light which stops to emit after the removal of exciting source.
3) The wavelength distribution of the emitted light measured with a single constant excitation wavelength is termed as an emission spectrum.
4) The wavelength distribution of the emitted light measured with a single constant excitation wavelength is termed as an emission spectrum.
5) The terms singlet and triplet states denote the number of paired electrons when there is no magnetic field.
6) External conversion is an intermolecular process which brings down a molecule to a lower energy electronic state without emitting light.
7) If temperature is increased, the molecular collisions increase. This in turn results in deviation which decreases the fluorescence intensity.

8) Deuterium Lamp provides radiation of high intensity but its wavelength range is limited to 250-370nm.
9) Fluorimetry employs photomultiplier tube as the detector.

2.6.2. Fill in the Blanks

10) The method of measuring the intensity of fluorescent light emitted by the substance being examined with respect to that emitted by the given standard substance is termed as _____.
11) _____ is the emitted light (by an atom or molecule) which continues to emit even after the removal of exciting source.
12) The dependence of emission intensity at a single wavelength on the excitation wavelength is termed as an _____.
13) An excited molecule and solvent or other solutes interact and transfer energy among each other to deactivate an excited electronic state and it is termed as _____.
14) If _____ is increased, the molecular collisions increase. This in turn results in deviation which decreases the fluorescence intensity.
15) If _____ is increased, the molecular collisions decrease, which in turn enhances the fluorescence intensity.
16) When the chemical nature of the fluorescent substance changes and reduces its emission, _____ occurs.
17) _____ provides radiation of high intensity but its wavelength range is limited to 200-350nm.
18) Fluorimetry employs _____ as the detector.

Answers

1) True	2) False	3) True
4) True	5) False	6) False
7) True	8) False	9) True
10) Fluorimetry	11) Phosphorescence	12) Excitation spectrum
13) External conversion	14) Temperature	15) Viscosity
16) Chemical quenching	17) Deuterium Lamp	18) Photomultiplier tube

2.6.3. Very Short Answer Type Questions

1) Define fluorimetry and molecular luminescence.
2) What is the principle involved in the working of photomultiplier tube?
3) What is intersystem crossing?
4) Name the factors affecting fluorescence.

2.6.4. Short Answer Type Questions

1) Write a short on excitation and emission spectra.
2) Mention the advantages and disadvantages of fluorimetry.
3) Explain the working of single beam fluorimeter along with a well-labelled diagram.
4) What are the different light sources employed by fluorimeter?
5) Mention the quantitative aspects of fluorimeter.

2.6.5. Long Answer Type Questions

1) Briefly explain the effect of different factors on fluorescence.
2) Explain the basic instrumentation of fluorimeter.

CHAPTER 3

IR Spectroscopy

3.1. IR SPECTROSCOPY

3.1.1. Introduction

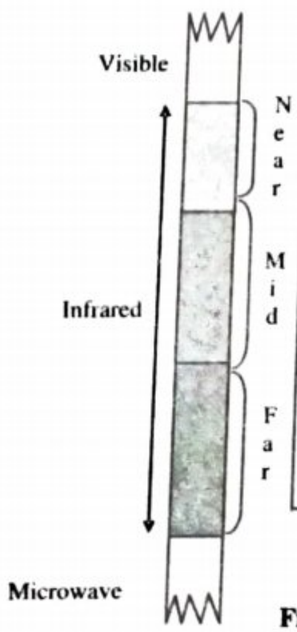
Infrared (IR) spectroscopy **deals with the infrared region** (12800-10cm^{-1}) **of electromagnetic spectrum**. The term **infra** means **beyond**, thus **infrared** means **beyond red**. It covers a range of techniques, the most common being a form of absorption spectroscopy. This spectroscopic technique is used for identifying compounds and determining the sample composition. Infrared spectrophotometer is a common laboratory instrument used for this technique.

The infrared region in electromagnetic spectrum is divided into the following **three regions** named for their relation with the visible spectrum (**figure 3.1**):

1) The **far-infrared**, approximately 400-10cm^{-1} (1000-30μm), lies adjacent to the microwave region, has **low energy** and is used for rotational spectroscopy.

2) The **mid-infrared**, approximately 4000-400cm^{-1} (30-2.5μm) is used to study the fundamental vibrations and associated rotational-vibrational structure. This region has wavelengths between 3×10^{-4} and 3×10^{-3}cm.

3) The **near- infrared**, approximately 14000-4000cm^{-1} (2.5-0.8μm) has **higher energy** and can excite overtone or harmonic vibrations.



λ (cm)	λ (μm)	λ (cm^{-1}) (Wavenumber)	Energy (E)
7.8×10^{-5} to 3×10^{-4} (0.000078-0.0003)	2.5 to 0.8	14,000 to 4,000	10-37Kcal/mole
3×10^{-4} to 3×10^{-3} (0.0003-0.003)	30 to 2.5	4,000 to 400	1-10Kcal/mole
3×10^{-3} to 3×10^{-2} (0.003-0.03)	1000 to 30	400 to 10	0.1-1Kcal/mole

cm = 10^{-2}m
mm = 10^{-3}m
μm = 10^{-6}m

Figure 3.1: IR Regions of the Electromagnetic Spectrum

IR spectra are mostly reported in μm; however, $\bar{\nu}$ (nu bar or wavenumber) is another currently preferred unit. Organic molecules absorb the IR radiation and convert them into energy of molecular vibrations. In IR spectroscopy, an organic molecule is exposed to IR radiation, and absorption occurs when the radiant energy matches a specific molecular vibration energy.

3.1.2. Principle

Infrared spectroscopy works on the principle that all molecules vibrate and absorb energy in the infrared region. Most of the vibrational absorption states correspond to 2.5-25μm (4000-400cm^{-1}) wavelength. The **vibrational frequency** (ν) in a two atomic system containing two masses (m_1 and m_2) (**figure 3.2**) is related to the force constant (k) and reduced mass (μ) by the following equation:

$$\nu = \frac{1}{2\pi}\sqrt{\frac{k}{\mu}} \text{ with } \mu = \frac{m_1 m_2}{m_1 + m_2}$$

An IR spectrum represents transmission *versus* wave number (cm^{-1}), i.e., frequency (ν) divided by the speed of light. Absorption occurs only at those frequencies at which higher vibrational states can be reached. If a molecule absorbs energy, the signal at this frequency decreases and forms a peak in the spectrum.

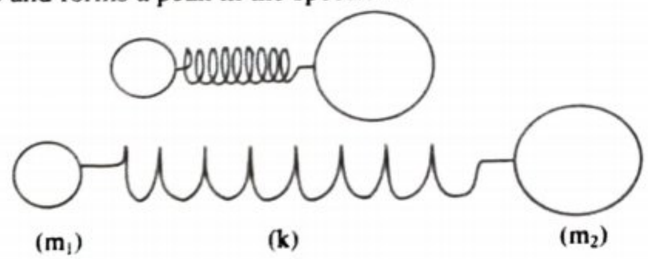


Figure 3.2: Atomic System Consisting of Two Masses (m_1 and m_2)

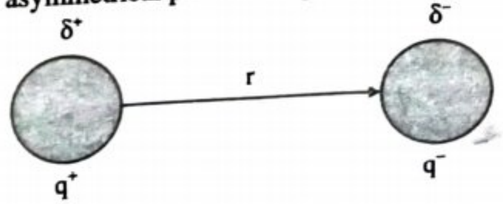
As per the formula, larger the force constant (k) of the bond between two atoms, higher is the vibrational frequency. Thus, indicating that a C≡C bond will absorb at a higher frequency than a C—C bond, forming a peak at 1600cm^{-1} and 1000cm^{-1} respectively. If lighter atoms will be involved in the vibration, the frequency will be higher. The stretching vibrations of C—H bond can be found in the 3000cm^{-1} region of IR spectrum.

3.1.2.1. Selection Rule

Given below are the selection rules for IR spectroscopy:

1) **Absorption of Correct Wavelength of Radiation (Matching of Frequency):** When a molecule's natural vibration frequency matches with the frequency of incident radiation, it will absorb the radiation (i.e., a net transfer of energy occurs, and changes the amplitude of molecular vibration). Natural frequency of HCl molecule is 8.7×10^{13}Hz (vib/sec) or 2890cm^{-1}. When HCl sample is exposed to IR radiation, the transmitted radiation is analysed and it is observed that part of this radiation having the same frequency (8.7×10^{13}Hz) is absorbed and the remaining is transmitted to give the characteristic value of HCl.

2) **Dipole Moment:** When the change in the vibrational state of a molecule is related to the change in its dipole moment, it absorbs IR radiation. A heteronuclear diatomic molecule consists of two different atoms having different electron withdrawing capacity. The electron density shifts towards the more electronegative atom, and such molecule possesses an electric dipole moment and is said to be polar. Dipole moment arises due to asymmetrical partial charge distribution.



$$\mu = q \times r$$

Where, μ = Dipole moment.
q = Magnitude of charge.
r = Distance between charges.
$e = 1.602 \times 10^{-19}C$

But atomic charge is $q \times e$

$$\mu = q \times e \times r$$

Here charge is measured in Coulomb and distance in meters; so, **SI unit of μ** is Coulomb meter (i.e., Cm). But for convenience μ is often given in unit **Debye** ($1D = 3.336 \times 10^{-30}$cm).

If HCl has dipole moment of 1.83D bond length and r is 92pm:

$$\mu = q \times e \times r$$
$$q = 0.41$$

This value indicates that charge in HCl molecule is asymmetrically distributed in a manner that Cl atom effectively gains 0.41 of an electron and H atom loses 0.41 of an electron. The total dipole moment in a polyatomic molecule is the vector sum of the dipole moment of the individual bond. No dipole moment exists in a symmetrical molecule (like CCl_4), however the C—Cl bond is polar.

3.1.2.2. Overtones

Overtone bands (harmonics) appear at integer multiples of fundamental vibrations, thus the strong absorptions at $800cm^{-1}$ and $1750cm^{-1}$ give rise to weaker absorptions at $1600cm^{-1}$ and $3500cm^{-1}$, respectively. Two frequencies interact and give beats (combination or difference frequencies); thus, absorptions at xcm^{-1} and ycm^{-1} interact and produce two weaker beat frequencies at $x \pm ycm^{-1}$.

On absorbing IR radiation, transitions from the ground state (V = 0) to the second excited state (V = 2) give rise to weak bands, termed **overtones**. If all the vibrational bands are equally spaced (which actually are not), **the energy of first overtone** is given by:

$$\Delta E_{vib} = E_{vib(V=2)} - E_{vib(V=0)} = \left(2+\frac{1}{2}\right)hv - \left(0+\frac{1}{2}\right)hv = 2\,hv$$

On adding reagent, the chemical shift of each proton or proton group of the sample molecule changes and the extent of this induced shift is measured. The resultant induced shift is plotted against the ratio of shift reagent to substrate to give a straight line at low ratio values. Structural information for each proton group in a molecule can be obtained from these plots.

They may be used to resolve overlapping signals from different proton groups in a molecule, or can be used in more quantitative studies to provide information on molecular configuration. **For example**, chiral lanthanide shift reagents are used for quantitative estimation of enantiomer mixtures.

The actual IR spectrum becomes quite complicated due to the presence of additional bands at various wavelengths. **For example**, additional bands can appear at 1/2, 1/3, ... of the wavelength (or two times, three times,..... the frequency) of the fundamental absorption bands; such bands are termed **overtones**. These are present in the spectra of carbonyl compounds, in the range $3200\text{-}3500cm^{-1}$. This value is two times the characteristic absorption frequency due to C=O stretching vibrations.

3.2. THEORY

3.2.1. Introduction

Infrared spectroscopy relies on the theory that **molecules absorb specific frequencies** that are characteristic of their structure. These absorptions are **resonant frequencies**, as the frequency of absorbed radiation matches the frequency of the vibrating bond or group. The energies are determined by the shape of molecular potential energy surfaces, the masses of atoms, and the associated vibronic coupling.

In the IR spectroscopy (region 2.5-15μ), the absorbed energy causes major changes in the vibrational energy that depends on the following factors:

1) Masses of the atoms in the molecule,
2) Bond strengths, and
3) Arrangement of atoms in the molecule.

No two compounds, except the enantiomers, can have similar IR spectra.

3.2.2. Fundamental Modes of Vibrations in Polyatomic Molecules

The molecular atoms are not tightly held. A molecule appears to be consisting of different sized balls (atoms) tied with springs (chemical bonds) of variable strengths. On passing IR light through the sample, the vibrational and rotational energies of the molecules increase.

Fundamental vibrations are of the following **two types**:

1) **Stretching Vibrations:** In this type, the distance between the two atoms increases or decreases, however, keeping the atoms in the same bond axis. Stretching vibrations are of the following **two types**:
   i) **Symmetric Stretching Vibrations:** In this type, the movement of atoms with respect to a particular atom in a molecule is in the same direction.
   ii) **Asymmetric Stretching Vibrations:** In this type, one atom approaches and the other atom moves apart from the central atom.

2) **Bending or Deformation Vibrations:** In this type, the positions of atoms change with respect to the original bond axis. The **stretching absorptions of a bond appear at higher frequencies** (i.e., higher energy) than the bending absorptions of the same bond. Bending vibrations are of the following **four types**:
   i) **Scissoring:** In this type, two atoms approach each other.
   ii) **Rocking:** In this type, the movement of atoms is in the same direction.
   iii) **Wagging:** In this type, two atoms move up and down the plane with respect to the central atom.
   iv) **Twisting:** In this type, one atom moves up the plane and the other moves down with respect to the central atom.

In bending vibrations, the bond lengths change if required to do so by the centre of gravity resisting displacement. Bending vibrations describe two dimensional motions, thus there is **2n–5 bending vibration** for non-cyclic and linear molecules. These vibrations appear at lower frequencies than the stretching vibrations (appear at higher frequencies). Since the force constants of bending vibrations are also less than those of the stretching vibrations, the former are more sensitive to environmental conditions.

In a polyatomic molecule, the same bond can perform stretching and bending vibrations simultaneously.

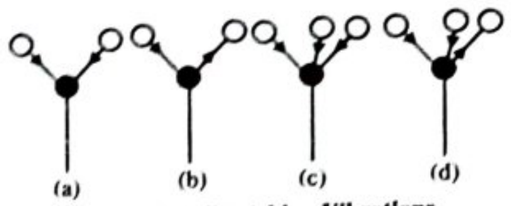


Figure 3.3: Stretching Vibrations
(a) = Symmetric stretching vibration of AB_2 molecule
(b) = Asymmetric stretching vibration of AB_2 molecule
(c) = Symmetric stretching vibration of AB_3 molecule
(d) = Asymmetric stretching vibration of AB_3 molecule

The two types of stretching vibrations, i.e., symmetric and asymmetric, are denoted by 'ν'. The symmetric and asymmetric stretching vibrations of AB_2 molecule are illustrated by the **figure 3.3 (a)** and **(b)**; while the symmetric and asymmetric stretching vibrations of AB_3 molecule are represented in **figure 3.3 (c)** and **(d)**. The most important bending vibrations are illustrated in **figure 3.4**.

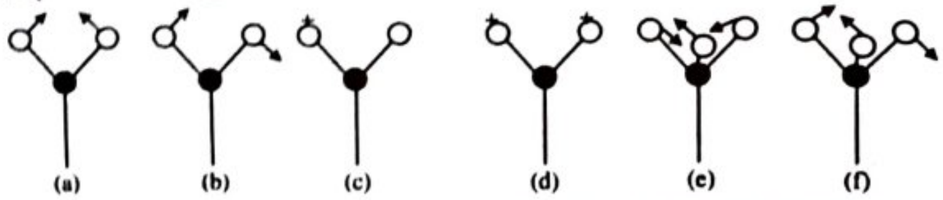


Figure 3.4: Bending Vibrations of AB_2 and AB_3 Molecules. The in-plane deformation vibrations - (a) Scissoring and (b) Rocking Vibrations of AB_2 Molecule; The out-of-plane deformation vibrations - (c) Twisting and (d) Wagging Vibrations of AB_2 Molecule; (e) Symmetric and (f) Asymmetric Vibrations of AB_3 Molecule.

Bending vibrations are of the following **two types**:
i) In-plane deformation vibrations, and
ii) Out-of-plane deformation vibrations.

The ***in-plane deformation vibrations*** include scissoring **[figure 3.4 (a)]** and rocking *vibrations* **[figure 3.4 (b)]**; and the **out-of-plane deformation vibrations** include *twisting* **[figure 3.4 (c)]** and wagging vibration **[figure 3.4 (d)]**. This may also comprise symmetric **[figure 3.4 (e)]** and asymmetric **[figure 3.4 (f)]** vibrations. **Water** is a triatomic non-linear molecule with 3×3–6 or **3(3n–6)** normal modes of vibrations, which can be calculated by applying the following two primary forces:
i) The force acting against stretching or shortening of O–H bond, and
ii) The force acting against the bending of H–O–H molecule.

These forces are applied to the water molecule and the nature of three normal modes of vibration of water is determined with the help of **Herzberg method**. The normal modes of vibrations of water determined by these calculations are shown in **figure 3.5**.

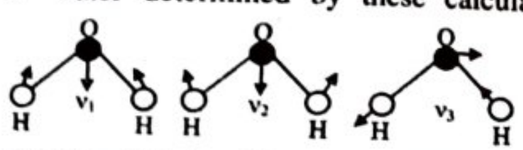


Figure 3.5: Normal Modes of Vibration of Water Molecule

In **figure 3.5**, ν_1 is the symmetric stretching mode, ν_2 is the in-plane deformation (scissoring) vibration, and ν_3 is the asymmetric stretching mode. The three vibrations are infrared active. The stretching vibrations (ν_1 and ν_3) appear at 3652 and 3756cm^{-1}, whereas the in-plane deformation vibration (ν_2) appears at 1515cm^{-1} in the IR spectrum of water.

Carbon dioxide is a linear triatomic molecule with 3×3–5 or **4(3n–5)** modes of vibrations, which are shown in **figure 3.6** as calculated with Herzberg method.

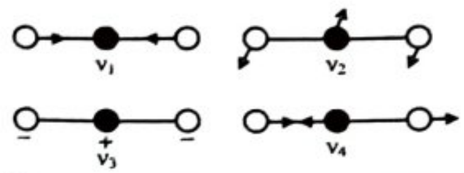


Figure 3.6: Normal Modes of Vibration of Carbon Dioxide Molecule

In **figure 3.6**, ν_1 is the symmetrical stretching vibration, which does not appear in the infrared spectrum (i.e., it is inactive) as it produces no change in the dipole moment of the molecule. The ν_2 and ν_3 are in-plane deformation vibrations and appear perpendicularly to the surface of page. These two kinds of vibrations occur independently of each other, but have similar frequencies because the frequency cannot be altered on turning the plane of vibrations. Thus, the deformation vibrations (ν_2 and ν_3) of CO_2 are degenerate and appear at 666cm^{-1} region in the infrared spectrum. The ν_4 is asymmetrical stretching vibration and appears in the infrared spectrum in 2350cm^{-1} region.

The number of modes of vibrations in polyatomic molecules is different from those calculated theoretically due to the following reasons:
1) The overtones (multiples of a given frequency) and combination of tones (sum of two other vibrations) increase the number of modes of vibrations.
2) Some other phenomenon may reduce the number of bands.

While determining the number of modes of vibrations in the IR spectrum, the following experimental **limitations** are observed:
1) Vibrations not falling in the IR region do not appear in the IR spectrum.
2) Weak vibrational bands are not observed in the IR spectrum.
3) The vibrational bands with same or slightly different frequencies overlap each other and are observed as a single band in the IR spectrum.
4) Some vibrational bands may degenerate and appear at the same place in IR spectrum.
5) No band appears in the IR spectrum of a molecule if no change occurs in its dipole moment.

3.2.3. Translational Energy

Translational energy and the uniform velocity of a molecule are associated. This motion is described with respect to the molecule's centre of mass. The classical energy due to translational motion is given by:

$$E_t = \frac{1}{2}mv^2 \qquad \text{.....(1)}$$

Where, E_t = translational energy of molecular mass (m) moving with velocity (v) with respect to the centre of mass (m).

A molecule moves freely in three perpendicular directions (i.e., x, y and z) due to translational motion, thus indicating that it has **three degrees of freedom**. As per the quantum mechanical equations of translational motion of an isolated molecule of mass M in a rectangular box of dimensions a× b× c, the **translational energy value** is given by:

$$E_t = \frac{h^2}{8M} = \left[\left(\frac{n_x}{a}\right)^2 + \left(\frac{n_y}{b}\right)^2 + \left(\frac{n_z}{c}\right)^2\right] \qquad \text{.....(2)}$$

Where, n = integer values. Continuous ranges of translational energies are available as the number of translational energy levels is large and the difference between them is small.

3.2.4. Rotational Frequencies

Absorption occurs in the IR region due to changes in the vibrational and rotational levels. When radiations of less than $100cm^{-1}$ frequency are absorbed, molecular rotation occurs in the substance. As this absorption is quantized, discrete lines appear in the spectrum due to molecular rotation. Along with a single change in vibrational energy, a large number of changes occur in the rotational energy; thus, vibrational spectra appear as vibrational rotational changes. On passing IR light through a sample, the molecule's vibrational rotational energies increase. The three different ways in which molecules can store energy as a result of motion of atoms in a non-linear triatomic molecule are shown in the figure 3.7.

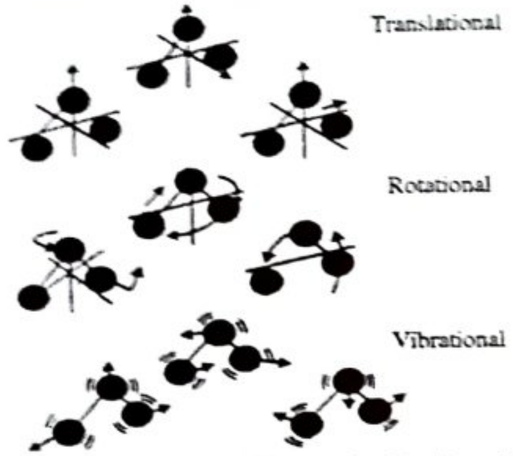


Figure 3.7: **Three Different Ways in Which Molecules Can Store Energy as a Result of Motion of Atoms in a Non-Liner Triatomic Molecule.**

Rotational frequencies are the result of **rotation of a molecule** about an axis through the centre of gravity. This energy is associated with the overall rotation of the molecule with the atoms considered as fixed point masses. As per the classical theory, the **rotational energy** value is given by:

$$E_{rot} = \frac{1}{2} I\omega^2 \quad \text{..... (3)}$$

Where, I = Moment of inertia.
ω = Angular velocity of the rotating molecule.

However, the **quantum mechanical formula for rotational energy** of a simple linear molecule is given by:

$$E_{rot} = J(J+1)\frac{h^2}{8\pi^2 I} \quad \text{..... (4)}$$

Where, I = Moment of inertia of a simple linear molecule.
J = 0 or a positive integer (the rotational quantum number).

Each rotational level has a (2J + 1) fold degeneracy.

In a **monoatomic molecule**, only **one rotational degree of freedom** exists. But a non-linear tri-or poly-atomic molecule rotates about the three perpendicular axes passing through the centre of gravity, thus, indicating that it has three rotational degrees of freedom.

3.2.5. Vibrational Energy

Vibrational energy and the **oscillation of atoms** of a molecule (considered as point masses) about equilibrium positions are associated. This energy can be treated on a quantum mechanical basis.

The **vibrational energy** of a molecule is given by:

$$E_{vib} = h\nu\left(v + \frac{1}{2}\right)$$

Where, ν = Vibrational frequency
v = 0 or a positive integer (the vibrational quantum number).

Non-linear molecules (**e.g.,** H_2O, NO_2, and CH_4) have **3n–6** vibrational degrees of freedom; while the linear molecules (**e.g.,** CO_2, C_2H_2, and I_3^-) have **3n–5** vibrational degrees of freedom.

Vibrational frequency or wave number is influenced by the bond strength and reduced mass. With increase in bond strength or decrease in the reduced mass, the value of vibrational frequency increases.

The C═C stretching gets absorbed at a frequency higher than the C—C stretching, since the bond strength (value of k) of double bond is higher than that of the single bond.

3.2.6. Factors Affecting Vibrations

Discussed below are the factors under the effect of which the vibrational frequencies of some groups shift from their normal values:

1) **Coupled Interactions:** Two bond oscillators sharing a common atom never behave as individual oscillators, except when they exhibit different oscillation frequencies. This behaviour is the result of mechanical coupling interaction between the oscillators. **For example,** CO_2 consists of two C═O bonds (O═C═O) with a common carbon atom. Thus, CO_2 has two fundamental stretching vibrations, of which one is asymmetrical and the other is symmetrical stretching vibration.

The symmetrical stretching vibration mode consists of an in-phase stretching or contracting of the C to O bond. This absorption occurs at a wavelength longer than the wavelength for C═O group in aliphatic ketones. This mode also does not alter the molecule's dipole moment; hence is infrared inactive, but can be observed in the Raman spectrum near $1340cm^{-1}$ region. In asymmetrical stretching vibration mode, the two C to O bonds stretch out of plane; and one C═O bond stretches as the other contracts. This mode alters the molecule's dipole moment, and hence is infrared active. This absorption occurs at a wavelength shorter or a frequency higher ($2350cm^{-1}$) than that for a C═O (carbonyl) group in aliphatic ketones. This difference in C═O absorption frequencies in CO_2 molecule is due to strong mechanical coupling or interaction.

The two ketonic carbonyl groups separated by one or more carbon atoms show normal carbonyl absorptions near $1715cm^{-1}$ region because appreciable coupling has been prevented by the intervening carbon atom(s).

Labelling of symmetric or asymmetric vibrations is done with reference to the axis of symmetry about which if a molecule is rotated, an identical view is presented more than once in a complete rotation. The vibrational mode is labelled as symmetric if rotation about this axis does not alter the nature of vibration, while if it is altered the vibrational mode is labelled as asymmetric. The modes are either parallel or perpendicular depending on whether the change in dipole moment is along the axis of symmetry or perpendicular to it. Modes are numbered as ν_1, ν_2, ν_3, ... in order of decreasing frequency within each symmetry group, starting from the symmetric mode of vibration.

Coupling also accounts for two N–H stretching bands in 3497-3077cm^{-1} region in primary amine and primary amide spectra, for two C=O stretching bands in 1818-1720cm^{-1} region in carboxylic anhydride and imide spectra, and for two C–H stretching bands in 3000-2760cm^{-1} region for methylene and methyl groups. Characteristic group frequency bands involve coupled vibrations.

The important **requirements for effective coupling interactions** are:

i) The vibrations should be of the same symmetry species for the interactions to occur.
ii) There should be a common atom between the groups for strong coupling between stretching vibrations.
iii) Maximum interaction occurs when the coupled groups individually absorb near the same frequency.
iv) Coupling between bending and stretching vibrations occurs when stretching bond forms one side of the changing angle.
v) A common bond is required for the coupling of bending vibrations.
vi) Separation of groups by one or more carbon atoms and/or mutually perpendicular vibrations causes no coupling.

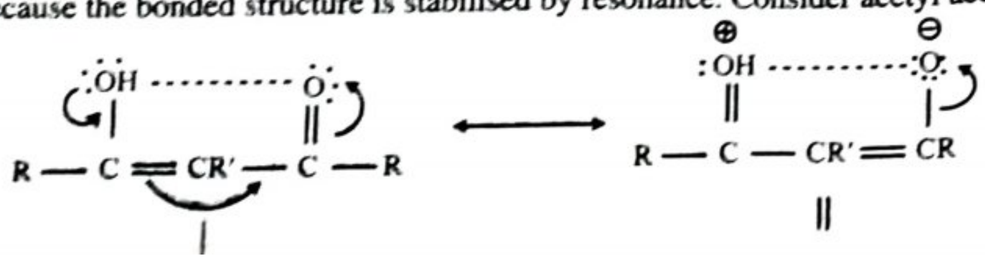
2) **Hydrogen Bonding:** This gives rise to downward frequency shifts. **Stronger the hydrogen bonding, greater is the absorption shift** from the normal value towards the lower wave number. With the help of infrared technique, the two types of hydrogen bonds can be distinguished. The **inter-molecular hydrogen bonds** give rise to **broad bands**, while the **intra-molecular hydrogen bonds** give rise to **sharp and well-defined bands**.

Inter-molecular hydrogen bonds depend upon concentration. The intensities of such bands decrease and ultimately disappear on dilution. Intra-molecular hydrogen bonds do not depend on concentration. The frequency difference between free and associated molecules in intra-molecular hydrogen bonding is smaller than that in inter-molecular hydrogen bonding.

***Non-associating solvents** (e.g., CS_2, $CHCl_3$, and CCl_4) are most commonly used because solvents, like benzene, acetone, etc., highly influence the O–H and N–H compounds. The electronegativity of nitrogen atom is less than that of oxygen atom, thus the hydrogen bonding in amines is weaker than that in alcohols and the frequency shifts in amines are less drastic.*

***For example,** amines show N–H stretching at 3500cm^{-1} region in dilute solutions, while absorption in condensed phase spectra occurs at 3300cm^{-1} region. Alcohols with intermolecular hydrogen bonding are absorbed between 3400-3200cm^{-1} region, while O–H_{str} in free alcohol absorbs near 3600cm^{-1} region.*

The hydrogen bonding in enols and chelates is very strong and absorption due to O–H stretching occurs at very low values. As these bonds are not broken on dilution with an inert solvent, free O–H stretching is not observed at low concentrations. This is because the bonded structure is stabilised by resonance. Consider acetyl acetone.



The O–H group involved in chelation gives rise to broad absorptions between 3000 and 2500cm^{-1} region. The νCO absorption in enolic and ketonic forms occurs at 1630cm^{-1} and 1725cm^{-1} region, respectively. The quantities of enolic and ketonic forms can be determined from the intensities of two peaks.

3) **Fermi Resonance:** Coupling of two fundamental vibrational modes give rise to two new modes of vibration having higher and lower frequencies than that observed in the absence of interaction. Interactions that may occur between fundamental vibrations and overtones or combination tone vibrations are known as Fermi resonance.

For an isolated C–H bond only one C–H stretching vibration takes place. The C–H stretching vibrations in CH_3 groups combine together to exhibit two coupled vibrations (asymmetric and symmetric) of different frequencies. The vibrational frequencies of C–H coupled vibrations of CH_3 groups will be different than CH_2 groups, and thus the detection of all the four C–H stretching vibrations in the high resolution IR spectra of the compounds having CH_2 and CH_3 groups becomes easy.

A C–H stretching absorption can be detected from its position (around 2925 cm^{-1}) in the IR spectra. However, atleast three of these C–H stretching bands (in the same spectra) due to CH_2 and CH_3 groups, **e.g.,** in the spectrum of propionic anhydride, should be visible. **Vibrational coupling** occurs between two bonds (close in the molecule) vibrating individually at the same frequency. The coupling vibrations may be fundamentals or there may be a coupling of a fundamental vibration with the overtone of some other vibration (this is called Fermi resonance). This type of coupling also accounts for two N–H stretching bands in the spectra of primary amines and amides.

For strong vibrational coupling between stretching vibrations, presence of a common atom between the two groups is necessary. This type of coupling is seen in acid anhydrides (R–CO–O–CO–R), in which two C–O stretching absorptions takes place with a separation of 60cm^{-1}. Coupling occurs between two C=O groups indirectly linked via O. This interaction is effective due to a slight double bond character in the (C=O)–O (carbonyl–oxygen) bonds arising due to resonance. The system becomes coplanar due to resonance.

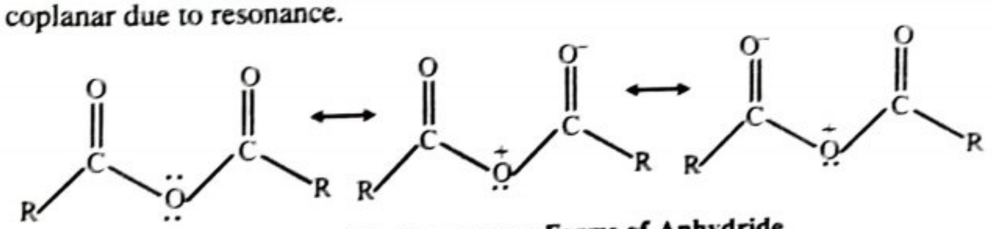


Figure 3.8: Reasonance Forms of Anhydride

C=O stretching absorption bands for acyclic saturated acid anhydrides occur at 1850cm^{-1} region due to asymmetric and symmetric stretching. The high frequency band is more intense in acyclic anhydrides, while the lower frequency band is more intense in cyclic anhydrides. Hence, the acyclic and cyclic anhydrides can be easily differentiated. In conjugated anhydrides, a decrease in C=O stretching is observed due to resonance. The acid anhydrides exhibit C–O stretching vibration as one or two bands near 1300-1050cm^{-1} region.

Fermi resonance is an apparent splitting of a fundamental, such as C=O stretching into two bands. It has its origin in the sharing of intensity between the fundamental and an overtone or combination tone of almost similar frequency.

Fermi resonance can also be explained with the help of the absorption pattern of CO_2. The symmetrical stretching band of CO_2 appears in the Raman spectrum at $1340cm^{-1}$ region. Two bands are observed at $1286cm^{-1}$ and $1388cm^{-1}$ regions. Splitting is caused by the coupling between the fundamental C=O stretching vibration near $1340cm^{-1}$ region and first overtone of the bending vibration. The fundamental bending vibration occurs in $666cm^{-1}$ region and the first overtone occurs near $1334cm^{-1}$ region Fermi resonance commonly occurs in the IR and Raman spectra. For **Fermi resonance to occur**:

i) The vibrational levels should be of same symmetry species, and
ii) The interacting groups should be in the molecule so that mechanical coupling can take place.

4) **Electronic Effects:** Let us assume a saturated aliphatic ketone, **e.g.,** acetone (CH_3COCH_3), in which C=O stretching absorption occurs at $1715cm^{-1}$. This absorption frequency changes due to the changes in the environment of carbonyl group (C=O).

The vibrational frequency of absorption is increased by a halogen on a carbon atom α- to the ketone functional group. **For example,** α-chloroacetone (CH_3COCH_2Cl) gives C=O absorption in $1725cm^{-1}$ region . This halogen effect can be seen when C–X (X = halogen) bond becomes coplanar with the C=O bond. **For example,** α,α-dichloroacetone ($ClCH_2COCH_2Cl$) shows C=O absorption in $1725cm^{-1}$ region (just as in CH_3COCH_2Cl); but the α,α' dichloroacetone ($ClCH_2COCH_2Cl$) shows twice the effect and absorbs at $1740cm^{-1}$ region.

Adding a halogen atom and an electronegative atom causes inductive effect, which either shortens or strengthens the band. As a result, the force constant increases along with the increase in frequency or wave number of absorption. On attaching an alkyl group at α-position of C=O group, a +I effect is produced and the wave number of absorption is decreased (as force constant decreases as a result of lengthening or weakening of bond). **For example,** the C=O stretching absorption of HCHO and CH_3CHO occurs at $1735cm^{-1}$ and $1730cm^{-1}$ region, respectively.

The C=O group of acetone (CH_3COCH_3) is lined by two $-CH_3$ groups. Thus two +I methyl groups weaken the C=O bond strength than that in acetaldehyde (CH_3CHO) in which only one $-CH_3$ group is present. Hence, acetone C=O absorption occurs at lower frequency ($1715cm^{-1}$) and acetaldehyde C=O absorption occurs at higher frequency ($1730cm^{-1}$).

The aldehydes in comparison to ketones absorb at higher wave number because of C=O stretching vibrations.

3.3. INSTRUMENTATION

3.3.1. Introduction

The usual optical materials (glass or quartz) absorb strongly in the IR region, and thus the apparatus for measuring IR spectra is different from that for the visible and UV regions. The **main components** of an IR spectrometer are:

) Sources of radiation,
) Monochromators,
) Sample cells, and
) Detectors.

IR spectrophotometers are of **two types**:

1) Dispersive spectrophotometer with monochromator (single beam IR spectrophotometer),
2) Fourier Transform Infrared Spectrometer (FTIR).

3.3.2. Sources of Radiation

The IR spectrometer requires a source of radiant energy for isolating narrow frequency bands. The IR radiation emitted by the source should be steady, of intensity sufficient for detection, and should extend over the desired wavelengths.

Infrared sources are hot bodies that emit continuously throughout the IR region, and which approximate a black body radiator in their emission properties. An **incandescent solid** is chosen as a source of IR radiation so that its emission closely approaches that of a **black body radiator**. This solid is electrically heated at 1200-1500°C temperature.

The following sources can be used as a source of IR radiation:

1) **Nernst Glower:** It consists of a rod or hollow tube (2cm long and 1mm in diameter) made by sintering a mixture of cerium, zirconium, thorium and yttrium oxides. It is heated between 1000-1800°C temperature. It provides maximum radiation at 7100 cm^{-1} region.
2) **Globar:** It is a silicon carbide rod (5cm long and 0.5cm in diameter) which is also electrically heated between 1300-1700°C temperature and has a positive coefficient of resistance.
3) **Nichrome Wire:** A coil of this wire is heated by passing current and is used as a source when the required wavelength range and intensity are not sufficient.
4) **Rhodium Wire:** This wire is sealed in a cylinder.
5) **Tungsten Filament Lamp:** It is used for near infrared region.

3.3.3. Monochromators (Wavelength Selectors)

Radiations of various frequencies are emitted by the radiation source. Since the sample in IR spectroscopy absorbs at certain frequencies, desired frequencies from the radiation source should be selected and the radiations of other frequencies should be rejected.

This selection is achieved using monochromators of the following **two types**:

1) Prism monochromator, and
2) Grating monochromator.

3.3.3.1. Prism Monochromator

A prism to be used as a dispersive element should be made up of materials that transmit in the infrared region (**e.g.,** various metal halide salts). Glass and quartz are utilised in the visible and UV region, but they are unsatisfactorily absorbed in the IR region.

Sodium chloride is the **most common prism salt** due to its high dispersion in the 4-15μm region (a region significantly important in the study of functional groups). Many of these salt materials are inappropriately subjected to mechanical and thermal instability and/or water solubility; thus, protection against damage should be continuously implemented.

A single- or mono-pass monochromator is shown in the **figure 3.9**:

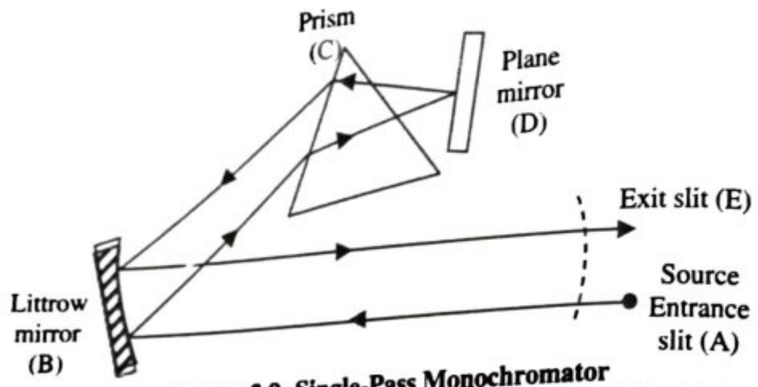


Figure 3.9: Single-Pass Monochromator
Source: Adapted from *Instrumental Methods of Chemical Analysis* (pp 2.42), by Chatwal G.R. (2006) (Himalaya Publishing House)

The sample is placed at or near the beam focus, just before the **entrance slit** (A) to the monochromator. The radiation from the source pass through the sample and the entrance slit, and then strikes the off-axis parabolic **Littrow mirror** (B) which makes the radiation parallel and sends it to **prism** (C).

The dispersed radiation reflects from the **plane mirror** (D) and returns through the prism a second time and focuses into the **exit slit** (E) of the monochromator. Through this slit, it finally passes into the detector section.

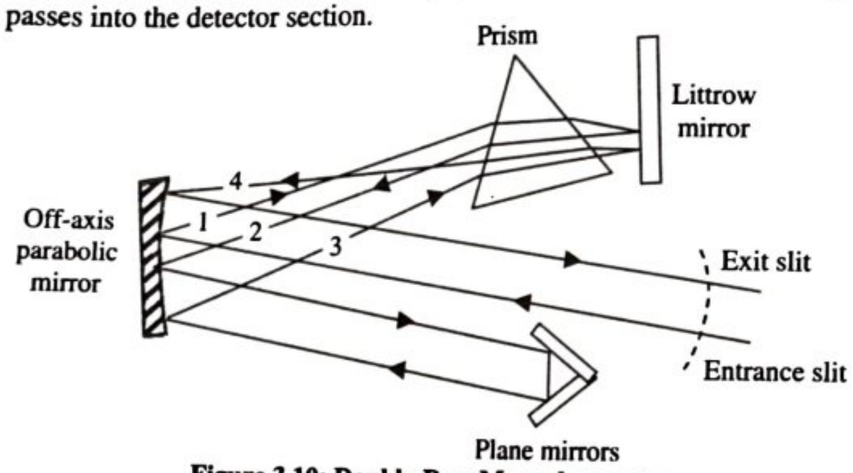


Figure 3.10: Double-Pass Monochromator
Source: Adapted from *Instrumental Methods of Chemical Analysis* (pp 2.42), by Chatwal G.R. (2006) (Himalaya Publishing House)

The double-pass monochromator is shown in the **figure 3.10**. In this monochromator, the radiation passes four times through the prism as shown (1), (2), (3) and (4) in **figure 3.10**. The double pass monochromator produces more resolution than the monochromator in the radiation, before reaching to the detector. In both mono- and double-pass monochromators, **sodium chloride** (rock-salt) prism is used for 4000-650cm^{-2} (2.5015.4μ) region. Prisms of **lithium fluoride** or **calcium fluoride** give more resolution in the region of significant stretching vibrations.

3.3.3.2. Grating Monochromator

Gratings are made up of various materials and cause linear dispersion. On replacing the prism in a prism monochromator with a grating, higher dispersion is achieved. **Reflection gratings** are preferred over transmittance gratings.

Some gratings with different rulings (lines/cm) are used for covering the wide wavelength (energy) range associated with IR radiation. Different combinations of transmission or interference filters with or without gratings are used.

Grating is a series of parallel straight lines cut into a plane surface. Dispersion by a grating follows the law of diffraction (**figure 3.11**), and also the following mathematical relation:

$$n\lambda = d(\sin i \pm \sin \theta)$$

Where, n = The order (a whole number).
λ = Wavelength of the radiation.
d = Distance between grooves.
i = Angle of incidence of IR radiation beam.
θ = Angle of dispersion of light of a particular wavelength.

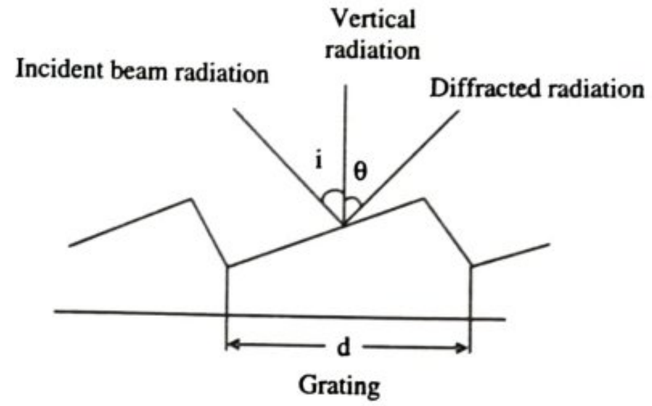


Figure 3.11: Path of IR Radiation Diffracted by a Grating Monochromator

The angle of dispersion is different for radiations of different wavelengths. Separation of light occurs at a grating because light of different wavelengths disperses at different angles.

Grating monochromator has the following **advantages over prism monochromator:**
1) They can be used over considerable wavelength ranges.
2) Grating can be made with aluminium which remains unaffected by moisture; while, the prisms of metal salt are subjected to etching due to atmosphere moisture.

A grating is used along with a small prism that acts as an order sorter. Sometimes it is used with filters transparent over limited wavelength ranges.

3.3.4. Sample Cells and Sample Handling

IR spectroscopy is used for the characterisation of solid, liquid or gas samples, thus the samples of different phases have to be handled and differently treated. But, the only common point to the sampling of different phases is that the material containing the sample should be transparent to IR radiation.

This condition restricts only to certain salts like potassium bromide (KBr) or sodium chloride (NaCl). However, the salt is selected depending on the wavelength range to be studied.

The sampling processes of different samples are discussed below:
1) **Sampling of Solids:** Solid whose IR spectrum is to be recorded is sampled as follows:
   i) **Solid Dissolved in Solvent:** The solid sample is dissolved in a solvent (**e.g.,** carbon tetrachloride, chloroform, alcohol, acetone, cyclohexane, and carbon disulphide), and the resulting solution is used in one of the cell.

As Solid Film: The sample solution is placed on KBr or NaCl surface and the solvent is evaporated. As a result, the solid sample leaves behind a thin film on the cell surface. This technique is used for rapid qualitative analysis.

Mull Technique: The solid sample is mixed with heavy mineral oil (Nujol) to form a paste, which is sandwiched between two salt plates and then used for spectral measurement. Nujol is transparent in most part of the IR region, but has absorption maxima at 2915, 1462, 1376 and 719 cm^{-1} regions. This is the **limitation** in the use of Nujol for compounds having absorption in the region similar to Nujol. The mull technique is used for qualitative analysis.

Pressed Pellet Technique (Disk Method): A small amount of finely ground solid sample is mixed with powdered potassium bromide (of 100 times its weight). The obtained finely ground mixture is passed under very high pressure in a press (at least 25,000 psig) to form small 1-2mm thick pellets (of 1cm diameter). These pellets are transparent to IR radiation and are run as such.

Figure 3.12 shows a device used for preparing small pellets by pressing the mixture of KBr and solid sample. The powdered mixture of KBr and sample is introduced and the upper screw (A) is tightened till the powder is compressed into a thin disc. When the sample is sufficiently compressed, the bolts (A and A′) are removed and the steel cylinder with the sample disc is put inside it in the path of the beam of IR spectrometer. A blank potassium bromide pellet of same thickness is also put in the path of the reference beam. This method has some **advantages over Nujol Mull method**:

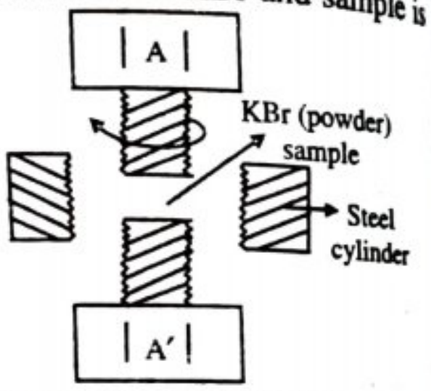


Figure 3.12: Device for Preparing Potassium Bromide Discs

a) Due to the use of KBr, no bands appear in the IR spectrum that used to appear due to the mulling agent.
b) KBr pellets can be stored for prolong time periods.
c) The pellets can be used for quantitative analysis as the sample concentration can be adjusted.
d) The spectrum resolution in the KBr is superior to that obtained with mulls.

However, the pellet technique has a few **disadvantages** also:

a) It shows a band at 3400cm^{-1} region due to the OH group of moisture present in the sample. Thus, care should be taken during investigations related to the OH band region in the sample.
b) The high pressure involved in the formation of pellets causes polymorphic changes in the crystallinity of samples (especially, inorganic complexes), and this may further complicate the IR spectrum.
c) This method is not suitable for polymers that are difficult to grind with KBr.

Thus, it can be concluded that the Nujol method is suitable for running crystalline compounds (including complexes) in the solid state and the KBr pellet method is used for the remaining solid samples.

ampling of Liquids: The simplest way of sampling a liquid is in a thin film (0.1-3mm) squeezed between two sodium chloride plates (sodium chloride is transparent IR light). Calcium fluoride plates are used for water-containing samples. A drop of quid sample is placed on the top of sodium chloride plate and another sodium

chloride plate is placed over it. This pair of sodium chloride plates enclosing the liquid film is placed in the path of sample beam. In the same way, a drop of the low melting substance is placed between the two plates for spectral analysis.

3) **Sampling of Gases:** The gaseous sample is introduced into a gas cell (10cm long) having sodium chloride walls. Sodium chloride windows allow the cell to be placed directly in the path of the sample beam. The low frequency rotational changes in the gaseous phase split the high frequency vibrational bands. Very few organic compounds are examined as gases.

3.3.5. Detectors

Except in the near IR region, where a photoconductivity cell is basically used, no better choice than thermal detectors is available. These give responses for all frequencies. If the radiant power for the IR region is low, the detector signal will also be low. The various types of detectors are as follows:

1) Golay cell,
2) Bolometer,
3) Thermocouple,
4) Thermistor, and
5) Pyroelectric detector.

3.3.5.1. Golay Cell

The golay cell is used in some commercial spectrophotometers. It has a small metal cylinder, whose one end is closed by a blackened metal plate and the other end is closed by a flexible metalised diaphragm. The cylinder is filled with xenon and sealed. Then IR radiation is allowed to fall on the blackened metal plate. As a result, the gas heats up and expands. The resulting pressure increases and deforms the metalised diaphragm that separates into two chambers. A light from a lamp is allowed to fall on the diaphragm that reflects the light on to a photocell. The diaphragm motion changes the output of cell. The signal seen by the phototube is modulated with respect to the power of the radiant beam incident on the gas cell.

3.3.5.2. Bolometer

A bolometer (**figure 3.13**) works on the principle that the electrical resistance of a metal increases by 0.4% for every Celsius degree increase of temperature.

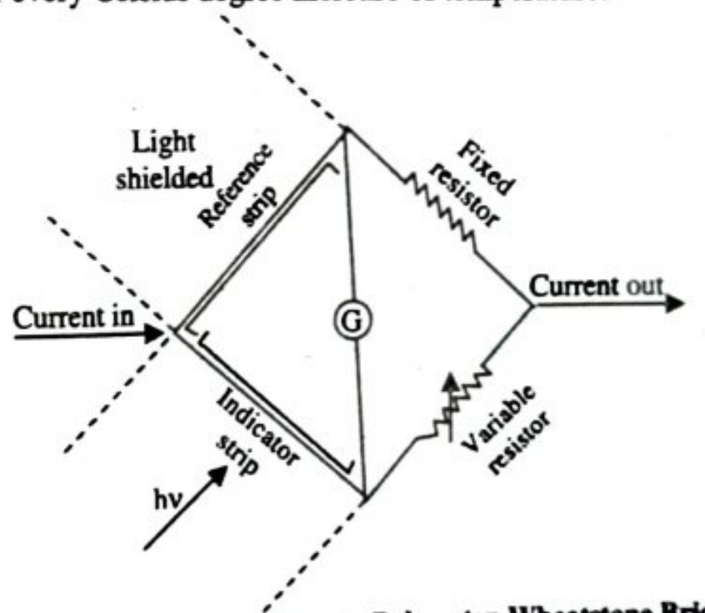


Figure 3.13: Schematic Presentation of a Bolometer-Wheatstone Bridge for Infrared Detection

A bolometer consists of a thin metal conductor whose temperature changes when IR radiation falls on it. The resistance of this conductor also changes with temperature, and the degree of change in resistance is the measure of the amount of radiation that has fallen on the bolometer.

3.3.5.3. Thermocouple

Thermocouple detector (**figure 3.14**) works on the principle that when two dissimilar metal wires are connected together at both ends, a temperature differential exists and an electric current flow between the two ends. The end exposed to the IR radiation is generally a black body so as to increase the energy gathering efficiency, and is called the **hot junction**. The other end is thermally insulated and carefully screened from stray light and is called the **cold junction**. The flowing electricity and the energy differential between the two connections are directly proportional.

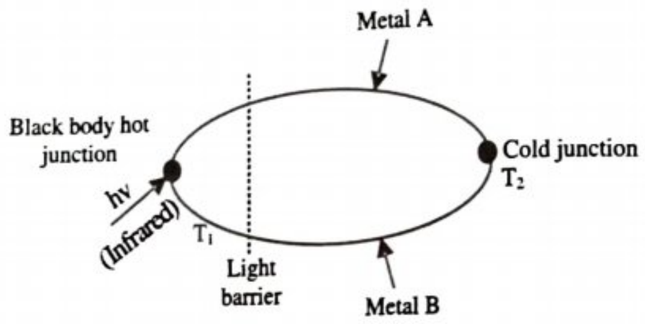


Figure 3.14: Schematic Presentation of a Thermocouple.

3.3.5.4. Thermistor

Thermistor is made up of a fused mixture of metal oxides. The electrical resistance of the mixture decreases with increase in temperature. This relationship between electrical resistance and temperature allows the thermistors to be used as IR detectors in the same way as bolometers. Thermistor changes resistance by 5% per degree Celsius change in temperature. Its response time is also slow.

3.3.5.5. Pyroelectric Detector

Pyroelectric detector is made up of a non-centrosymmetrical crystal, which has an internal electrical field along its polar axis. On applying IR radiation, a change in polarisation is observed due to an alteration of the crystal lattice. The pyroelectric detector acts as a capacitator if two electrodes are connected to the crystal. The effects of this detector depend on the rate of temperature change and not on the temperature change itself. Pyroelectric detector also ignores the effects of background radiation. They are usually used in FTIR spectrometers.

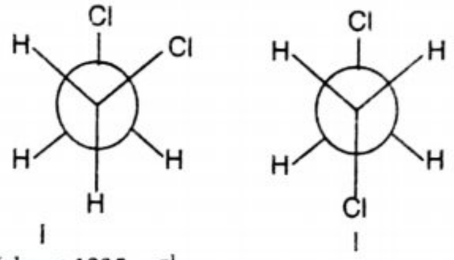
3.4. APPLICATIONS

3.4.1. Introduction

Infrared spectroscopy is widely used in industries and in research work. It is a simple technique that is effectively used for the measurement, quality control, and dynamic measurement. It is also used in forensic analysis in civil and criminal analysis. Quantitatively infrared spectroscopy is used to determine the quantity of a substance either in pure form or as a mixture of two or more compounds.

3.4.2. Qualitative Analysis

Qualitative analysis by infrared spectroscopy is used for determining the structure of complex molecules. IR spectroscopy helps in detecting Gauche (skew) and staggered conformations. The two conformations of 1,2-dichloroethane are shown below.



Gauche (skew) 1235cm^{-1} (CH_2 rocking)

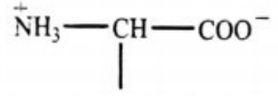
Staggered (*trans*) 1291cm^{-1} (CH_2 rocking)

Infrared spectral studies are used for identifying the presence of various functional groups, like olefinic protons, N-methyl, O-methyl and C-methyl groups.

The qualitative applications of IR spectroscopy are discussed below:

1) **Identification of an Organic Compound:** An organic compound's identity can be established from its fingerprint region (1400-900cm^{-1}) that should exactly match with the known spectrum of that compound.

2) **Structure Determination:** This technique helps in determining an unknown compound's structure. All the major functional groups absorb at their characteristic wave numbers. The shifts due to environmental effects are also observed. The data available due to absorption frequencies can be used for predicting the possible structure. The available chemical data can help in the confirmation of the structure. It is known that the IR spectra of amino acids exhibit bands for only ionised carboxylic acids and amine salts ($-\overset{+}{N}H_3$), and not for free $-NH_2$ and $-COOH$ groups. Amino acids exist as Zwitter ions as below:



Similarly, from the IR bands of sulphanilic acid it can be seen that the compound contains $\overset{+}{N}H_3$ and SO_3^- salts and not $-NH_2$ and SO_3H free groups.

3) **Distinction between Two Types of Hydrogen Bonding:** This technique is used for differentiating between the types of hydrogen bonding. In hydrogen bonding, the electron cloud in a hydrogen atom transfers to the neighbouring electronegative atom. The s-orbital of proton should overlap the p-orbital of the acceptor group.

On dissolving such a substance in non-polar solvent (**e.g.,** CCl_4), the aggregates or polymers break in dimers and monomers. Due to this, the $O-H_{str}$ (stretching vibration) absorption shifts to higher frequencies and sharp peaks are obtained. Intramolecular and intermolecular hydrogen bonding can also be distinguished using this technique. **For example,** *o*-nitrophenol shows intramolecular hydrogen bonding and *p*-nitrophenol shows intermolecular hydrogen bonding.

On dilution, the compounds showing intramolecular hydrogen bonding do not undergo any absorption shifts, while the compounds showing intermolecular hydrogen bonding do so.

3.4.3. Quantitative Analysis

Quantitative analysis by infrared spectroscopy helps in evaluating an organic mixture quantitatively. Estimation of mixture components can be done by:

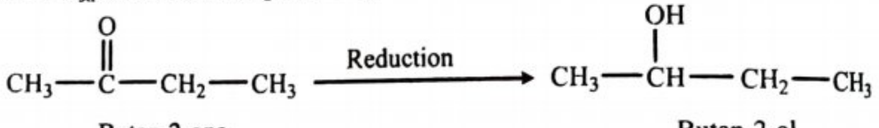
1) Measuring the intensities of absorption bands characteristic of each component, and
2) Knowing the optical density of the absorption band for a pure component.

The commercial xylene exists as a mixture of three isomers (i.e., *ortho*-, *meta*- and *para*-xylenes), and this mixture cannot be separated easily. However, the percentage composition of mixture can be determined by taking its IR spectrum. The bands in the spectrum are formed at:

1) $740cm^{-1}$ for *ortho* isomer,
2) $880cm^{-1}$ for *meta* isomer, and
3) $830cm^{-1}$ for *para* isomer.

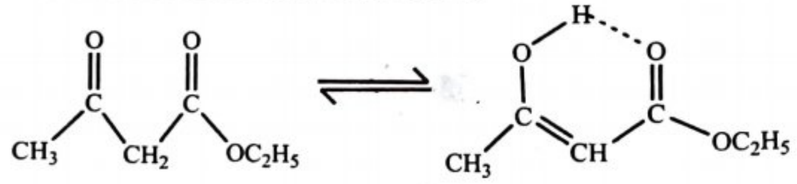
Mixtures of known composition are recorded and the working curves are drawn for the above bands for *ortho*-, *meta*- and *para*-isomers. The quantitative applications of IR spectroscopy are discussed below:

1) **Study of a Chemical Reaction:** This technique is used to study the chemical reactions. **For example**, a saturated aliphatic ketone is reduced to form a secondary alcohol. Ketone forms a strong band at $1710cm^{-1}$ region. On reduction, it forms butan-2-ol, which absorbs at $3300cm^{-1}$ region due to $O—H_{str}$ bond. The reaction can be studied at definite intervals and complete reduction occurs when a strong band due to $C=O_{str}$ will be missing and only a band due to $O—H_{str}$ is present.



Butan-2-one Butan-2-ol

2) **Study of Keto-Enol Tautomerism:** Diketones and ketoesters show keto-enol tautomerism only if they have α-H atom. The IR spectrum of such a compound contains bands due to C=O, O—H and C=C bonds. **For example**, ethyl acetoacetic ester exists in keto-enol isomers in equilibrium.



$\nu C=O_{str}$ 1733, $1710cm^{-1}$(s) $\nu O–H_{str} \sim 3300cm^{-1}$(b)
$\nu C=O_{str}$ $1645cm^{-1}$(s)

3.5. SUMMARY

The details given in the chapter can be summarised as follows:

1) Infrared (IR) spectroscopy **deals with the infrared region ($12800-10cm^{-1}$) of electromagnetic spectrum.**
2) The term **infra** means **beyond**, thus **infrared** means **beyond red**.
3) **Infrared spectroscopy** works on the principle that all molecules vibrate and absorb energy in the infrared region.
4) **Infrared spectroscopy** relies on the theory that **molecules absorb specific frequencies** that are characteristic of their structure.

5) **In stretching vibrations** the distance between the two atoms increases or decreases, however, keeping the atoms in the same bond axis.
6) **Bending or deformation vibrations** the positions of atoms change with respect to the original bond axis.
7) **Absorption** occurs in the IR region due to changes in the vibrational and rotational levels.
8) **Rotational frequencies** are the result of **rotation of a molecule** about an axis through the centre of gravity.
9) Vibrational energy and the **oscillation of atoms** of a molecule (considered as point masses) about equilibrium positions are associated.
10) **Stronger the hydrogen bonding, greater is the absorption shift** from the normal value towards the lower wave number.
11) **Vibrational coupling** occurs between two bonds (close in the molecule) vibrating individually at the same frequency.
12) **Nernst glower** consists of a rod or hollow tube (2cm long and 1mm in diameter) made by sintering a mixture of cerium, zirconium, thorium and yttrium oxides.
13) **Globar** is a silicon carbide rod (5cm long and 0.5cm in diameter) which is also electrically heated between 1300-1700°C temperature and has a positive coefficient of resistance.
14) **Sodium chloride** is the **most common prism salt** due to its high dispersion in the 4-15μm region.
15) In both mono- and double-pass monochromators, **sodium chloride** (rock-salt) prism is used for $4000-650cm^{-2}$ (2.5015.4μ) region.
16) **Reflection gratings** are preferred over transmittance gratings.
17) **The golay cell** is used in some commercial spectrophotometers.
18) **Thermistor** is made up of a fused mixture of metal oxides.
19) **Pyroelectric detector** is made up of a non-centrosymmetrical crystal, which has an internal electrical field along its polar axis.
20) **Infrared spectroscopy** is a simple technique that is effectively used for the measurement, quality control, and dynamic measurement.

3.6. EXERCISE

3.6.1. True or False

1) Infrared spectroscopy deals with the infrared region ($12600-20cm^{-1}$) of electromagnetic spectrum.
2) The term infra means beyond, thus infrared means beyond red.
3) Infrared spectroscopy works on the principle that all molecules vibrate and absorb energy in the infrared region.
4) In Bending Vibrations the positions of atoms do not change with respect to the original bond axis.
5) Absorption occurs in the IR region due to changes in the vibrational and rotational levels.
6) Vibrational energy and the oscillation of atoms of a molecule (considered as point masses) about equilibrium positions are not associated.
7) Rotational frequencies are the result of rotation of a molecule about an axis through the centre of gravity.
8) Vibrational coupling occurs between two bonds (close in the molecule) vibrating individually at the different frequency.

9) The golay cell is used in some commercial spectrophotometers.
10) Sodium chloride is the most common prism salt due to its high dispersion in the 4-15µm region.

3.6.2. Fill in the Blanks

11) _____ works on the principle that all molecules vibrate and absorb energy in the infrared region.
12) In _____ Vibrations the distance between the two atoms increases or decreases, however, keeping the atoms in the same bond axis.
13) In _____ Vibrations the positions of atoms change with respect to the original bond axis.
14) _____ are the result of rotation of a molecule about an axis through the centre of gravity.
15) _____ occurs between two bonds (close in the molecule) vibrating individually at the same frequency.
16) _____ consists of a rod or hollow tube (2cm long and 1mm in diameter) made by sintering a mixture of cerium, zirconium, thorium and yttrium oxides.
17) _____ is a silicon carbide rod.
18) _____ is the most common prism salt due to its high dispersion in the 4-15µm region.
19) _____ is made up of a fused mixture of metal oxides.
20) _____ is made up of a non-centrosymmetrical crystal, which has an internal electrical field along its polar axis.

Answers

1) False 2) True 3) True
4) False 5) True 6) False
7) True 8) False 9) True
10) True 11) Infrared spectroscopy 12) Stretching
13) Bending 14) Rotational frequencies 15) Vibrational coupling
16) Nernst Glower 17) Globar 18) Sodium chloride
19) Thermistor 20) Pyroelectric detector

3.6.3. Very Short Answer Type Questions

1) Define IR spectroscopy.
2) Give the principle of Infra-red spectroscopy.
3) What is transitional energy?
4) Give examples of some detectors used in IR spectroscopy.
5) Discuss vibrational energy.

3.6.4. Short Answer Type Questions

1) Define translational and rotational energy.
2) Give the applications of IR spectroscopy.
3) Write a short note on sources of radiation.
4) Write a short note on monochromator.

3.6.5. Long Answer Type Questions

1) Explain the theory of IR spectroscopy and the factors influencing vibrational frequencies.
2) Demonstrate the detailed instrumentation of IR spectroscopy.

CHAPTER 4

Flame Photometry

4.1. FLAME PHOTOMETRY

4.1.1. Introduction

When a small amount of sodium is introduced into a Bunsen burner flame, a characteristic yellow light is produced. The brightness of the flame may vary with the quantity of sodium or other metal introduced. Relation between the emission intensity and the concentration of the element producing the emission of such characteristic radiation forms the basis of flame photometry (a form of emission spectroscopy).

In the spectrum of flame photometry, often the emission lines are overlapped by the bands emitted by oxides or other molecular species present in the sample. This problem can be prevented by either scanning for a few nanometres on both sides of the analyte peak or by taking single measurements on both sides of the peak. The average obtained from these two measurements is subtracted from the total peak height. Often lithium is used as an internal standard to control the effect of variables like flame temperature, fuel flow rate, and background radiation. Normally, a definite quantity of lithium is required for each standard and sample.

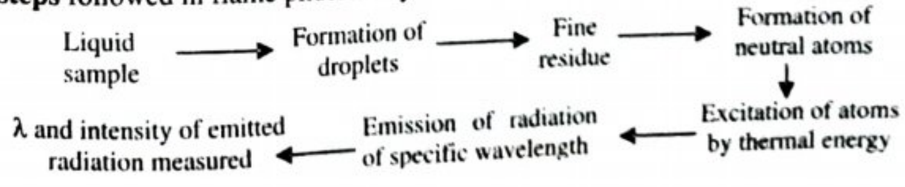
Flow Injection Analysis (FIA) technique can be employed for simultaneously carrying out the flame photometric analysis of elements like sodium (Na), potassium (K), lithium (Li), and calcium (Ca). This technique utilises a fast scanning monochromator for scanning the sample emission spectra from the flame source. Taking Li as an internal standard (by scanning two different levels of the dispersed sample zone for every single injection), the soil extracts and tap water are determined for elements like Na, K, and Ca, which may be present in wide concentration ranges.

4.1.2. Theory

Flame photometry is also known as **flame emission spectroscopy** because it involves the emission of radiation by the neutral atoms introduced into the flames.

Fine droplets are produced on spraying a solution containing metallic salt on to a flame. Thermal energy of the flame results in the evaporation of solvent droplets, leaving behind a solid residue in the form of neutral atoms. These atoms are then exposed to thermal energy of the flame which converts them into excited state atoms. These excited atoms are unstable, thus, they return to their ground state and emit radiation of definite wavelength. This wavelength is characteristic of the element and is used for its identification (i.e., qualitative analysis). The emitted radiation intensity depends on the concentration of the element analysed (i.e., quantitative analysis).

The **steps** followed in flame photometry are:



Theoretically it is possible to analyse all the elements by flame photometry. The availability of burner, fuel and oxidant combinations, and some technical reasons allow the analysis of Group IIA (Li, Na, and K) and Group IA (Ca and Mg) elements by this technique. The flame temperature defines the fraction of thermally excited atoms, which in turn affects the emitted radiation intensity.

4.1.3. Principle

When a solution of metallic salt is sprayed on a flame (acetylene burning in air), vapours containing metallic atoms may be produced. Few of these metallic atoms may be raised to a higher energy level, thus, imparting characteristic colour to the flame followed by the emission of radiation. **For example**, introduction of sodium salts to the flame imparts a yellow colour. This is the principle of flame photometry. Though, larger number of gaseous metallic atoms will remain in their unexcited or ground state.

These ground state atoms absorb radiation of their own characteristic resonance wavelength (the radiation wavelength emitted by the atoms excited from the ground state). Therefore, if light of the resonance wavelength is passed through a flame containing atoms (specific to that wavelength), some part of the light will be absorbed and the absorption range will be proportionate to the number of ground state atoms present in the flame. This is the basis of Atomic Absorption Spectroscopy (AAS).

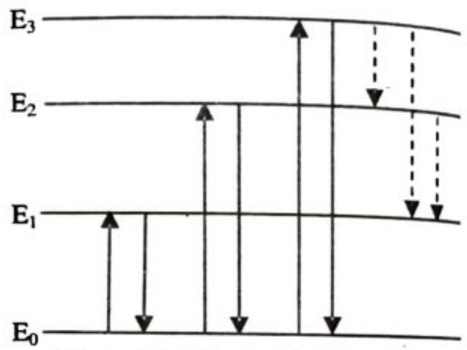


Figure 4.1: Electronic Transition

Figure 4.1 presents an energy level diagram in which the ground state having electrons of a given atom at their lowest energy level is presented by E_0, and E_1, E_2, E_3, etc., represent the higher or excited energy levels.

The transition of electrons from one quantised energy level to another ($E_0 \rightarrow E_1$) depends on the absorption of radiation energy. **Bohr's equation** expresses the amount of energy absorbed (ΔE) in the process:

$$\Delta E = E_1 - E_0 = h\nu = h\frac{c}{\lambda}$$

Where, c = Velocity of light; h = Planck's constant;
ν = Frequency; and λ = Wavelength of radiation absorbed.

Transition of electrons from E_1 to E_0 results in the emission of radiation of ν frequency. Since each atom of an element gives rise to a definite, characteristic line spectrum, different excitation states for different elements are produced. The resulting emission spectra involve transitions from excited to ground state, i.e.,
$E_3 \rightarrow E_0$, $E_2 \rightarrow E_0$ and also $E_3 \rightarrow E_2$, $E_3 \rightarrow E_1$ (dotted lines) (**figure 4.1**).

The resulting emission spectrum of a given element is quite difficult to interpret. The absorption of radiation by previously excited atoms (**e.g.,** $E_1 \rightarrow E_2$, $E_2 \rightarrow E_3$, etc.) is a generally accepted hypothetical phenomenon. In reality, the ratio of excited state atoms to the ground state atoms is extremely small. Therefore, the absorption spectrum of a given element is commonly associated with transitions from the lower energy states to higher energy states and possesses much simpler features than the emission spectrum.

Boltzmann equation signifies the relationship between ground state and excited state atoms:

$$\frac{N_1}{N_0} = \left(\frac{g_i}{g_0}\right) e^{\frac{-\Delta E}{kT}}$$

Where,

N_1 = Number of atoms in the excited state.
N_0 = Number of atoms in the ground state.
g_i/g_0 = Ratio of statistical weights for excited and ground states.
ΔE = Energy of excitation (hν).
k = Boltzmann constant.
T = Absolute temperature (K).

The Boltzmann equation states that the ratio $\left(\frac{N_1}{N_0}\right)$ depends on the excitation energy (ΔE) and temperature (T). If the temperature is increased and the excitation energy is decreased while dealing with transitions at longer wavelengths, a greater value for $\frac{N_1}{N_0}$ ratio will be achieved.

Flame emission spectroscopy involves more inter-element interferences in comparison to AAS. However, because of comparatively higher concentration of ground state atoms, the AAS appears more sensitive to inter-element interferences. In this regard, wavelength of the resonance line is an important factor. The elements having comparatively low energy values for the resonance lines are more sensitive in comparison to flame emission spectroscopy, whose resonance lines are associated with higher energy values. **For example**, sodium (emission line of wavelength = 589.0nm) shows great sensitivity in flame emission spectroscopy, whereas zinc (emission line wavelength = 213.9nm) is relatively insensitive. In atomic absorption spectroscopy, as with molecular absorption, the absorbance (A) is given by the logarithmic ratio of the intensity of the incident light signal (I_0) to that of the transmitted light (I_t):

$$A = \log\frac{I_0}{I_t} = KLN_0$$

Where,

N_0 = Concentration of the atoms in the flame (number of atoms per cm^3).
L = Path length through the flame (cm).
K = Absorption coefficient constant.

The **detector response** (E) in flame emission spectroscopy is given by the expression:

$$E = K\alpha C$$

Where,

K = Related to a variety of factors including the efficiency of atomisation and of self-absorption.
α = Efficiency of atomic excitation.
C = Concentration of the test solution.

Atomisation

Flame Emission Spectroscopy (FES) utilises the free analyte atoms in their ground state, obtained through the atomisation of analyte present within the aerosol. These flameless electrothermal methods can be employed in the handling of very small sample volumes (5-100 ILL) or solid samples.

Ionisation

Atoms having low ionisation potentials are generally ionised at elevated flame and furnace temperatures. The ionisation process decreases the quantity of neutral free atoms in both the ground state and excited state, therefore lowers the acuity of the analysis. This defect can be avoided through addition of extra (100 fold) quantity of easily ionised elements (like K, Cs, or Sr) to subdue ionisation in both sample and calibration solutions. The vapour containing higher concentration of electrons can be produced by utilising easily ionised atoms. These electrons utilise the concept of mass action for subduing the ionisation of analyte atoms. Therefore, suppressants are usually added to samples containing variable quantities of alkali metals determined by acetylene/air flames for stabilising free-electron concentrations. The suppressants are added as they are essential for analyses requiring hotter acetylene/nitrous oxide flames.

4.1.4. Origin of Spectra

The theory of thermal equilibrium fails to explain the phenomenon behind the emissions from the primary combustion zone or partly from the flame region above it. Therefore, a thorough quantitative analysis of flame emissions requires information regarding the concentrations of the emitting molecules, the flame temperature, and various formation and excitation mechanisms. Even though accomplishing a number of thorough researches, these mechanisms are not yet clear. Furthermore, the possibility of decreasing collisions between the super-thermally excited species and the gas molecules, and the probability of self-absorption must also be considered. Very prominent three-dimensional variants of the gas constituents may create additional problems associated with the primary combustion zone.

The combustion reactions or the radiating molecule structure can be fully understood through the studies undertaken in this field. However, this field does not provide any specific information regarding the background emissions of flame. These studies were performed under definite experimental surroundings, like low flame temperature burning at low pressure, discharge tubes, explosions, and shock waves. Thus, the results obtained from this study cannot be always applied to flames.

Besides these problems, there is a common agreement regarding the following **excitation mechanisms**:

1) **Formation of Excited OH:** The chemiluminescent reaction (given below) takes place in the primary combustion zone, resulting in the formation of an excited OH radical (OH*):

$$CH + O_2 \rightarrow CO + OH^*$$

The other important reactions for hydrogen flames are:

$$O + H \rightarrow OH^*$$
$$H + O_2 \rightarrow OH^* + O$$

These reactions can also produce elements in their intermediate excited states or complexes. Moreover, during the transition of an OH radical from its lower energy state to higher energy, it may produce an intermediate as a result of combination of the two other particles:

$$OH + (O)H + H \rightarrow OH^* + H_2O$$

The reason behind the super-thermal excitation of the OH band above the primary combustion zone in the presence of H and OH radical in hydrogen and acetylene flames in excess of their equilibrium concentration can be described through this reaction.

2) **Formation of Excited CH and C_2:** The fact that the formation of excited CH and C_2 (CH* and C_2*) takes place in flame is debated for a long time. The comparative examination of CH* emission from various flames shows that CH* is generally

produced through a side reaction rather than direct breakdown of acetylene. Since CH* ($A^2\Delta$ or $B^2\Sigma$") occurs in a higher reaction zone than C_2, there is a possibility that it may be formed from C_2 by the reactions.

$$C_2 + OH \rightarrow CO + CH^*$$
$$C_2H + O \rightarrow CO + CH^*$$

These reactions are exothermic in nature and are adequate enough to bring CH up to A and B states. Regarding the creation of C_2* most of the studies favour the following reactions:

$$CH_2 + C \rightarrow H_2 + C_2^*$$
$$CH + C \rightarrow H + C_2^*$$

3) **Formation of Excited CO_2:** There is a dispute regarding the origin and structure of the "blue continuum" radiation of carbon monoxide and hydrocarbon flames. Recent studies show that this blue continuum does not involve simple recombination reactions. The flame radiation is generally not a continuum, but has rotational fine structure which is smeared out at only moderate spectral resolution. The origin of emission is given by the following three-body collision:

$$^1CO + {}^3O + X \rightarrow {}^3CO_2^* + X$$

Where, X = Flame molecule.

^1CO = A CO molecule in the singlet ground state.

^3O = An O atom in the triplet ground state.

$^3CO_2$* = A CO_2 molecule in an excited triplet state.

The recently formed CO_2 molecule utilises the third partner (X) as a stabiliser. This molecule without radiating goes over into a neighbouring excited singlet state ($^1CO_2$*) from which it can undergo a radiative transition to the electronic singlet ground state:

$$^1CO_2^* \rightarrow {}^1CO_2 + h\nu$$

The frequencies (ν) of the emitted photons can be represented by their distinct values if the initial and ground states in the electronic transition remains steady; consequently, producing a spectrum having a distinct structure. The usual complexity of the spectrum may explain the reason behind the appearance of a quasi-continuum spectrum, usually at low resolution and high temperature. Consequently, after colliding with a flame molecule, $^1CO_2$* can also pass radiation-less into the ground state:

$$^1CO_2^* + X \rightarrow {}^1CO_2 + X$$

The X in the equation absorbs the released excitation energy. Since all the reactions are reversible in nature, reverse transition is also possible which is explained by the arrow pointing in the opposite direction in the above reaction. There are **two ways involved in the formation of excited $^1CO_2$* molecules**, i.e., either by recombining a CO molecule with an O atom or by collisional excitation of a CO_2 molecule in the ground state by an X molecule.

4.2. INTERFERENCES

4.2.1. Introduction

The existence of other materials in the sample may interfere with the analytical procedure involved in measuring the radiation intensity of the sample concentration. This interference needs to be controlled to attain good results of analysis. Some of the commonly encountered interference processes in flame photometry are:

1) Spectral interferences, 2) Ionisation interferences, 3) Cation-anion interferences, 4) Cation-cation interferences, and 5) Oxide formation interferences.

4.2.2. Spectral Interferences

Spectral interference can be categorised into **three types:**

1) The **first type** of spectral interference may arise due to the partial overlapping between the spectra of two elements (or compounds) emitting radiations at particular wavelengths. In such a case, the detector fails to identify individual radiation sources and thus reads the entire signal, giving inaccurate results. This interference type is most commonly experienced at elevated flame temperatures, since a large number of spectral lines are formed at such temperatures.
2) The **second type** of spectral interference does not involve any spectral overlapping but occur due to the production of much closer spectral lines of two or more elements. This interference produces more inconvenience while using a filter as the spectral isolation device. Utilisation of filters produce incorrect readout signals, since spectral lines separated by 50-100Å are passed through these filters to the detector limit. These interferences can be minimised significantly by increasing the resolution of the spectral isolation system.
3) The **third type** of spectral interference may arise due to the presence of higher salt concentrations in the sample, thereby producing spectral interference between a spectral line and a continuous background. This interference type is more commonly concerned with salts of alkali and alkaline earth metals. Utilisation of scanning technique may correct this interference. The alternations in viscosity and surface tension of the organic solvent (present in a sample) may influence the emission intensity of spectral line. This in turn alters the deliverance rate of the sample to the flame. Moreover, the organic compounds also affect the flame temperature by contributing to the heat of combustion, thus, enhancing the line intensities.

4.2.3. Ionisation Interferences

Few metallic atoms (like Na) can only be ionised through an elevated temperature flame.

$$Na \rightleftharpoons Na^{+} + e^{-}$$

Sodium ion produces its own characteristic emission spectrum, whose frequencies are dissimilar to those of the atomic spectrum of sodium. Therefore, the radiant power of atomic emission decreases with ionisation. This interference type can be corrected by the addition of higher amount of potassium salts in unknown and standard solutions. The potassium added undergoes ionisation but prevents the sodium from getting ionised. Consequently, the emission spectrum of the sodium atom is enhanced. This interference is limited to the first group elements of the periodic table.

4.2.4. Cation-Anion Interferences

Generally, anions are not competent to emit radiations. However, some polyvalent anions are capable of forming less volatile salts in the flame, thereby minimising the emission from definite cations. **For example,** less intensive radiations are emitted by the calcium solution (10µg/ml) containing phosphate or sulphate (10µg/ml) in comparison to the chloride solution. This may occur because of the formation of lesser volatile calcium phosphate or calcium sulphate. These compounds provide lesser quantity of free excited calcium atoms than an equimolar solution of calcium chloride.

This interference can be corrected by the addition of higher quantity of calcium precipitating agent (like lanthanum chloride), which forms a complex with the phosphate and thus precipitates calcium. A chelating agent (like EDTA) can also be utilised to form complexes with calcium to remove interference. Hydrochloric acid in concentration not higher than 1mol/l can be utilised for dissolving the sample, since chloride is known to show no such type of interference.

4.2.5. Cation-Cation Interferences

Filter instruments provide a suitable resolution for the principal lines of sodium (589nm), potassium (767nm), and lithium (671nm) to facilitate an interference free analysis of different elements. Yet, interference may occur in the sample containing sodium and calcium having comparatively closer emission wavelengths. Combination of calcium with the products of combustion of the flame gases forms calcium hydroxide, which produces a broad band molecular emission at 554nm.

A broad band-pass sodium filter may transmit a few emissions, and thus result in false interpretation for sodium. In contrast, the emission attained at 589nm from high sodium concentrations usually produces interference in the analysis of calcium, with its principal wavelength measured at 626nm; whereas sodium does not create any interference when measured at the less intense line of calcium at 423nm. The factors, like comparative concentrations of alkali and alkaline earth metals present in the sample and the filter bandwidths, affect the range of existing interference. The use of an effective monochromator other than filters plays an important role in eliminating cationic interference.

4.2.6. Oxide Formation Interferences

In this type of interferences, a large percentage of the free metal atoms usually combine with oxygen (present in the flame) to form stable oxides, and thus depresses the emission intensity of free metals. Most of the alkaline earth elements are known to form oxides and suffer this type of interference. This interference type can be corrected either by applying very high temperature flames, which break the oxide-producing free atoms for their excitation or by utilising oxygen-free environment to produce excited atoms.

4.3. INSTRUMENTATION

4.3.1. Introduction

In flame photometry, the sample is introduced into a flame where it undergoes a number of processes leading to the formation of excited atomic species that emit the radiation, which is then measured and suitably analysed. The instrument used for this purpose is called **flame photometer (figure 4.2).**

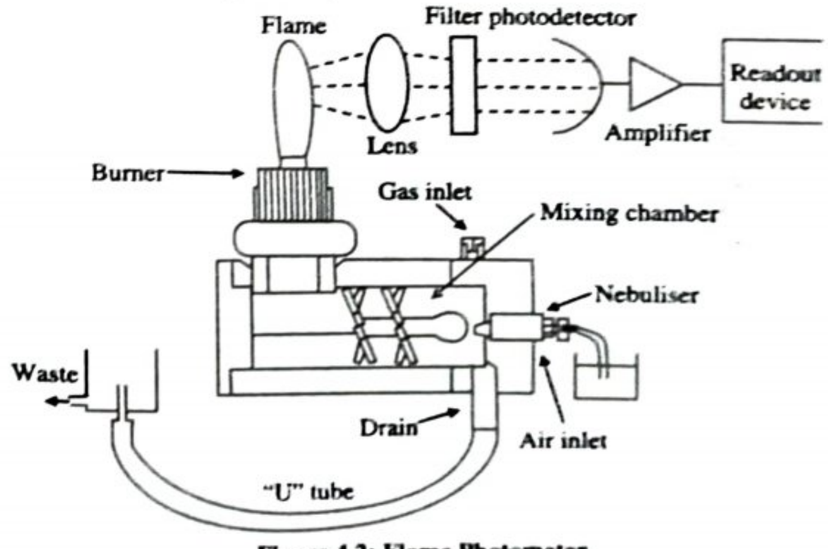


Figure 4.2: Flame Photometer

4.3.2. Atomisers

The role of atomiser is to generate the vapours of analyte which gets excited by the thermal energy of the flame and then emits characteristic radiation that is measured. The flame atomiser assembly consists of two components. The prior is a nebuliser where the sample in the form of a solution is drawn in and converted to a fine mist or an aerosol. It is then passed onto the second component, i.e., the burner along with air or oxygen and a fuel gas. In the flame, a number of processes occur that convert the analyte to excited species.

Different types of atomisers used in the atomisation process are:

1) **Flame Atomisers:** These atomisers utilise a pneumatic nebuliser for converting the sample solution into mist or aerosol. **Figure 4.3** presents three electrode DC plasma jets. A single cathode is provided for two separate DC plasma jets. The burning of plasma takes place in the form of an inverted Y. The samples are commonly injected as aerosol between the two graphite anodes. The plasma background emission is prohibited since the emissions are observed from the region beneath the strongly emitting plasma core.

2) **Non-Flame Atomisers:** Certain atomic spectrometric techniques utilise non-flame atom reservoirs. Electrothermal atomisers, like carbon rods, carbon furnaces, or tantalum ribbons, are utilised in Atomic Absorption Spectroscopy (AAS) or Atomic Fluorescence Spectrometry (AFS) techniques because they involve the generation of ground state atoms, whereas Auger Electron Spectroscopy (AES) involves the application of atmospheric pressure inductively coupled argon plasmas to produce excited atoms.

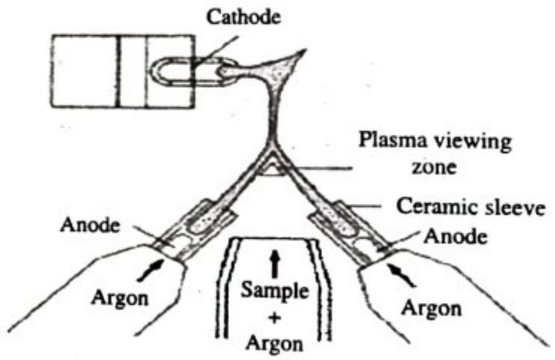


Figure 4.3: Flame Atomiser

3) **Electrothermal Atomisers:** These atomisers (**figure 4.4**) utilise a syringe or an auto sampler for placing a small quantity (up to a few microliters) of sample in the furnace. This step is subsequently succeeded by dry ashing and atomisation steps performed through instrument programming.

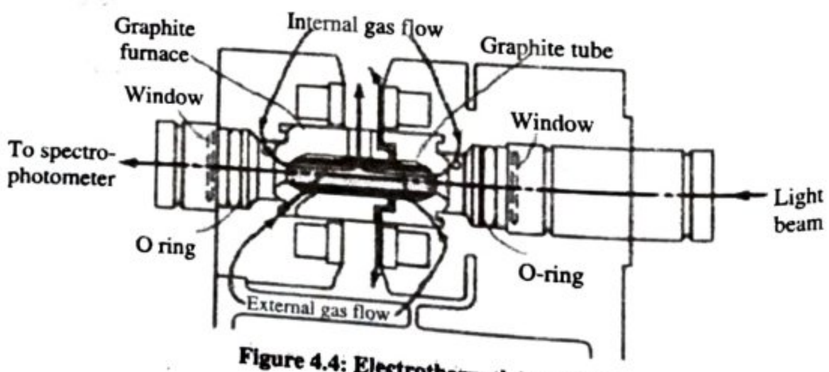


Figure 4.4: Electrothermal Atomiser

4.3.3. Nebulisers

It is a device used for introduction of sample into the flame. This process is called **nebulisation**, which involves thermal vaporisation and dissociation of aerosol particles at high temperatures producing small particle size with high residence time. Some of the **nebulisation methods** are:

1) Pneumatic nebulisation (most commonly used),
2) Ultrasonic nebulisation,
3) Electrothermal vaporisation, and
4) Hydride generation (used for certain elements only).

4.3.4. Burners

Flame photometry utilises a number of burners and combinations of fuel and oxidants for producing an analytical flame. Some commonly employed burners are:

1) **Mecker Burner:** This burner was employed in the past. It utilises natural gas and oxygen to produce a flame of low temperature and excitation energies. Thus, it is commonly employed for analysing alkali metals. Chemically, the flame produced by Mecker burner is not homogeneous, indicating its different regions, i.e., **oxidising** and **reducing regions**. Different quantities of excited atoms are estimated to depend on the regions in which atomic excitation process is taking place.

2) **Total Consumption Burner: Figure 4.5** presents a total consumption burner utilising hydrogen and oxygen gases as the fuel and oxidant, respectively. In this burner, the liquid sample is drawn directly into the flame. Hydrogen and oxygen gases are entered through the side tubing and are burned at the upper end of the burner to produce a flame. When the liquid sample is exposed to the base of flame, oxygen evaporates the sample solution and leaves behind a solid residue. Later, atomisation and excitation processes are performed for the sample. This apparatus is named as total consumption burner because the sample flowing through the capillary tube will also be driven into the flame irrespective of droplet size.

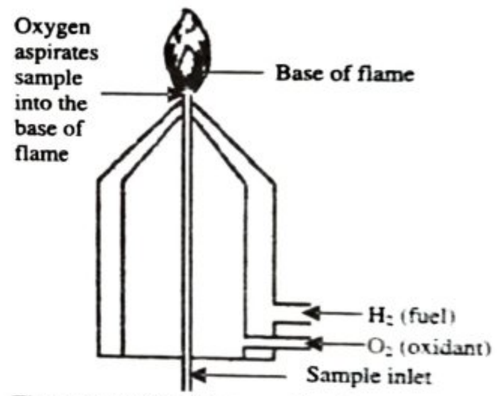


Figure 4.5: Total Consumption Burner

Total consumption burner has the following **advantages**:

i) No loss in the fuel and oxidant,
ii) Eliminates the errors, and
iii) Combustible sample, **e.g.,** petroleum, can be directly aspirated into the flame without any danger of explosion.

However, total consumption burner suffers from the following **disadvantages**:

i) Droplets of widely varying sizes are formed during aspiration.
ii) The flame is more strongly cooled by heavier load of the liquid.
iii) The burner tip can become encrusted with salts left after evaporation of solvent leading to a change in the aspiration rate of the solvent.
iv) It is very noisy (both physically and electronically), thus leads to poor reproducibility of analytical results.

For the above reasons, **total consumption burner** is not much used except for explosive flames, **e.g., hydrogen with other oxidants**.

3) **Premix or Laminar Flow Burner: Figure 4.6** presents a premix or laminar flow burner which involves thorough mixing of aspirated sample with the fuel and oxidant before it is driven to the opening of burner and then entered into the flame. The gases involved flow in non-turbulent manner (laminar flow).

In laminar flow burner, only about 5% of sample is exposed in the form of small droplets to the flame, followed by its easy decomposition. Thus, indicating that sample atomisation in the flame will occur efficiently. Larger droplets of the sample from the aspirator strike the side of the spray chamber and are drained off. Therefore, this burner only utilises 5% of the sample and the remaining 95% is wasted; hence resulting in loss of sensitivity. Nevertheless, this loss needs to be adjusted against the loss of larger droplets.

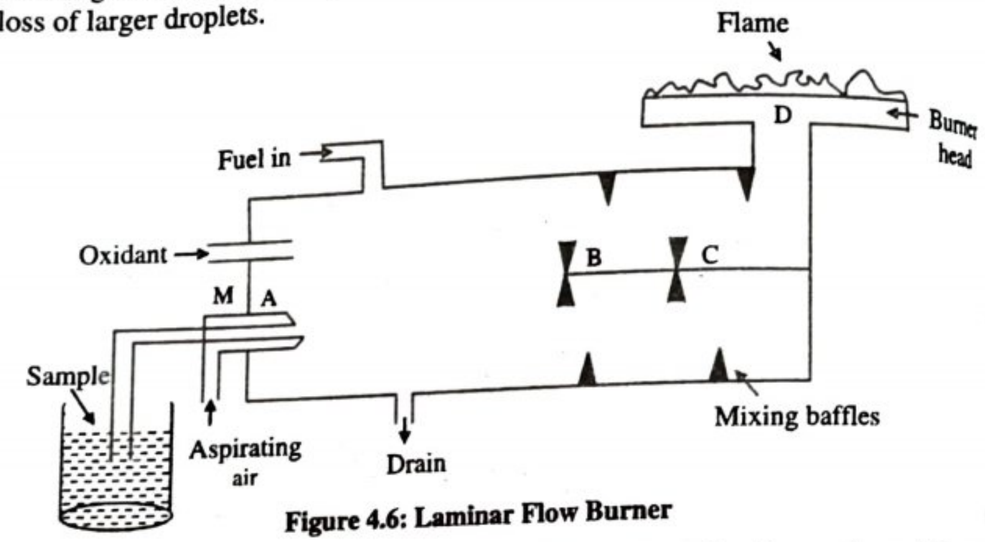


Figure 4.6: Laminar Flow Burner

4) **Lundergarph Burner:** This burner (**figure 4.7**) utilises liquid samples which are aspirated into the spray chamber. The bigger droplets condense on the chamber sides and drain off, while the minor droplets and the evaporated sample flows into the base of the flame in the form of a mist. The nebulisation stage in this type of burner can be improved by utilising a number of strategies, like using the impact bead (Perkin-Elmer), ultrasonic vibrators, and thermospray heaters.

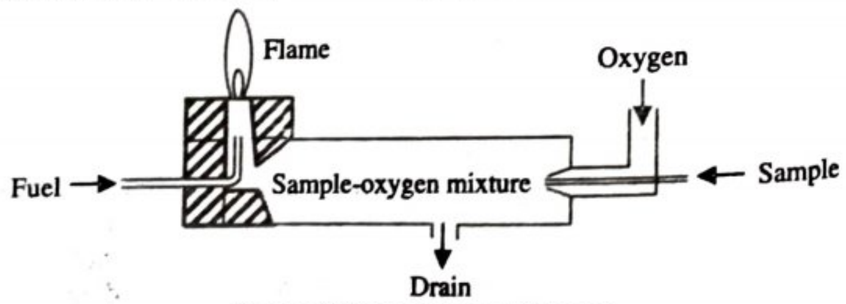


Figure 4.7: Lundergarph Burner

4.3.4.1. Structure of Flame

As seen in **figure 4.8**, the flame may be divided into the following regions or zones:

1) **Pre-Heating Zone:** In this zone, the combustion mixture is heated to the ignition temperature by thermal conduction from the primary reaction zone.

2) **Primary Reaction or Inner Zone:** This zone is about 0.1mm thick at atmospheric pressure and is visible by virtue of its blue green light ascribed to C_2 and CH radicals. There is no thermodynamic equilibrium in this zone and the concentration of ions and free radicals is very high. This region is not used for flame photometry.

3) **Interconal or Reaction-Free Zone:** This zone can extend up to considerable height. The maximum temperature is achieved just above the tip of the inner zone. The higher temperature favours both production of free atoms and maximum excitation for atomic emission spectroscopy. Therefore, this zone is used for flame photometry.

4) **Secondary Reaction Zone:** Within this zone, the products of the combustion processes are burnt to stable molecular species by the surrounding air. The shape of an unmixed flame is generally different. The inner zone can still be recognised, but it is very vague and is thickened. A laminar flame makes a strong hissing noise which gets louder when a liquid is atomised into it.

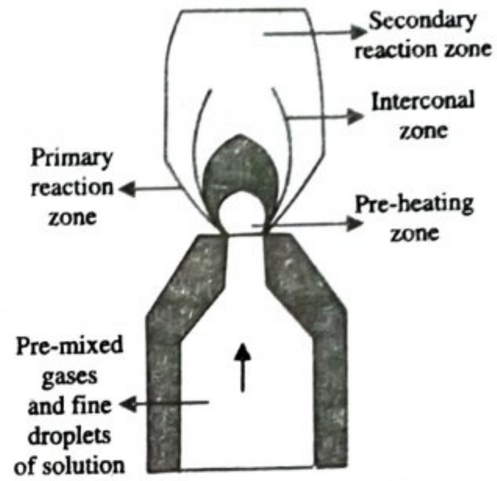


Figure 4.8: Different Zones of a Flame

4.3.4.2. Events Occurring in the Flame

Flame photometry employs a variety of fuels (mainly air) and oxidants (oxygen or nitrous oxide). The fuel-oxidant ratio defines the intensity of temperature produced. The different events occurring in the flame are:

1) **Desolvation:** In this process, the flame dehydrates the metal particles, thus, facilitating solvent evaporation.
2) **Vapourisation:** In this process, the metal particles in the sample are dehydrated, thus, evaporating the solvent.
3) **Atomisation:** In this process, the flame heat reduces the metal ions in the solvent to metal atoms.
4) **Excitation:** In this process, a specific amount of energy is absorbed by the electrons and nucleus of the atom due to the existing force of attraction between them, thus, resulting in the transition of atom to a higher energy level.
5) **Emission Process:** In this process, the atom in the unstable higher energy state returns to the stable lower energy state by emitting energy in the form of radiation of characteristic wavelength, which can be measured using a photodetector.

4.3.4.3. Fuel and Oxidants

The flame produced by the fuel and oxidant used is required in the conversion of samples to neutral atoms and then subsequently exciting them by heat energy. The flame produced should have an ideal and constant temperature. The sample elements get converted into ions (and not neutral atoms) at high temperature; and the atoms may not reach the excited state at lower temperature. Therefore, fuel and oxidants are used in combined form (**table 4.1**) to produce the preferred temperature.

Table 4.1: Fuel-Oxidant Combinations

Fuels	Oxidants	Temperature (°C)
Propane	Air	2100
Propane	Oxygen	2800
Hydrogen	Air	1900
Hydrogen	Oxygen	2800
Acetylene	Air	2200
Acetylene	Oxygen	3000

4.3.5. Mirrors

Radiation emitted by the flame is released in all directions in space. Large amount of radiation is lost during this process, thus, resulting in the loss of corresponding signals. A mirror is placed behind the burner for reflecting the radiation back to the entrance slit of the monochromator. This increases the amount of radiation used in the analysis. Since the mirror used is concave, it is capable of covering a wide range of angle from the flame. Best results can be obtained by reflecting the hottest and steadiest part of the flame onto the entrance slit of the monochromator, which reduces the flickering of the upper part of flame (where light intensity is reduced and noise is increased).

The reflecting surface of the mirror is front-faced rather than rear-faced (as in the normal household mirror). In the latter case, the flame radiation needs to pass through the support material (glass or quartz) twice before getting reflected to the entrance slit. However, the supporting material is also capable of absorbing radiations of shorter wavelengths, thus resulting in the loss of corresponding signal. Front-faced mirrors are efficient but not physically protected, i.e., they are more prone to chemical attack (especially from acid vapours in a hood) and scratches. Such mirrors can be protected by placing the instruments in corrosion-free environment.

4.3.6. Slits

The entrance and exit slits are commonly placed prior to and after the dispersion elements of a monochromator system. The **entrance slit** prevents the access of most of the radiations, allowing only the radiations emitted by the flame and the mirrored reflection of the flame to enter the optical system. The **exit slit** on the other hand, allows only a selected wavelength range to pass through the detector. A narrow range of wavelength, i.e., of the order of a few nanometres is usually required for this purpose. This is necessary if the wavelength of emission lines from other components in the flame is similar to those of the emission lines of the elements being determined. The entrance of these interfering lines into the detector is restricted by the slit. **Figure 4.9** diagrammatically explains the utility of slits in monochromator system.

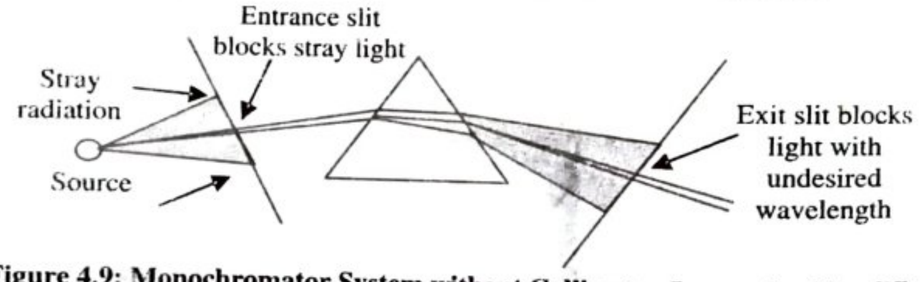


Figure 4.9: Monochromator System without Collimator Lenses for Simplification

4.3.7. Monochromators

A monochromator is required for measuring a single wavelength but it can also be applied for scanning a wide range of wavelength. If a polychromatic light is passed through an entrance slit it gets dispersed by diffraction gratings. These instruments are used in a systematic manner to facilitate continuous determination of elements.

Two most commonly used monochromator designs are:

1) **Czerny-Turner Monochromator:** In this monochromator (**figure 4.10**), two mirrors are required for reflecting and focusing the polychromatic and diffracted beams. It mostly operates on a computer-driven rotation of gratings in diverse ways to select a required wavelength. As the grating rotates, a different wavelength is focused onto the exit slit.

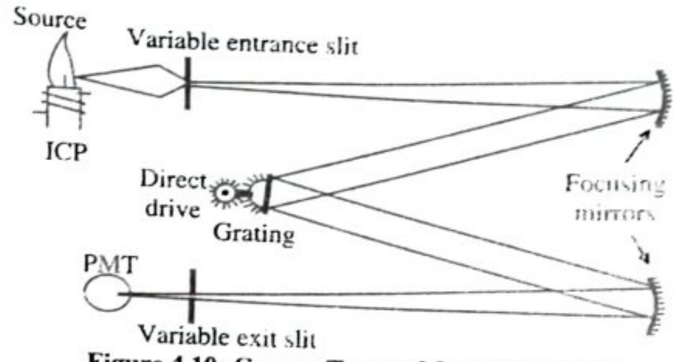


Figure 4.10: Czerny-Turner Monochromator

2) **Echelle Monochromator:** This monochromator is a high-resolution instrument which attains resolutions of 5ppm in comparison to 10-20ppm of most traditionally used instruments. Since the spectra produced by this system may overlap each other, these systems are very compact.

4.3.8. Detector

The radiation coming from the optical system reaches the detector to measure its intensity. A detector should be capable of perceiving wide range of emitted radiations having different wavelengths. Either **vacuum phototubes** or **photomultiplier tubes** are utilised as detectors in flame photometers. The photomultiplier detectors utilised in good flame photometers are capable of generating electrical signals from the radiations falling on them.

4.4. APPLICATIONS

4.4.1. Introduction

The applications of flame photometry are:

1) It is extensively employed in industries associated with chemicals, pharmaceuticals, soils, agriculture, ceramics, glass, plant materials, water, oceanography, and in various biological or microbiological laboratories.
2) It is employed for detecting the presence of sodium, potassium, calcium, and magnesium in biological fluids like serum, plasma, urine, etc.
3) It is also employed as a standard procedure for analysing industrial and natural water for estimating the elements responsible for water hardness (**e.g.,** calcium, magnesium, barium, etc.).
4) It is commonly used for detecting the presence of sodium, potassium, calcium, and magnesium (after removing other interfering elements) in soil samples.
5) It is used for determining few vital elements, **e.g.,** aluminium, barium, calcium, cesium, chromium, copper, iron, lead, magnesium, manganese, potassium, sodium, strontium, and zinc.
6) It is commonly exploited by glass industries for determining elements, **e.g.,** sodium, potassium, boron, lithium, etc.
7) It is employed by cement industries also for determining sodium (Na_2O), potassium (K_2O), calcium (CaO), magnesium (MgO), manganese (MnO_2), and lithium (Li_2O).
8) It is employed in the analysis of alkali-alkaline earth metals and other metals existing in metallurgical products, catalysts, alloys, etc.
9) It is a commonly used method for determining metals (like lead and manganese) in petroleum products (like gasoline), lubricating oils, and organic solvents.
10) It is employed in industries for determining ash and estimating the quantity of alkali and alkaline earth metal oxides.

4.4.2. Qualitative Analysis

Flame photometry enables detection of group I and II elements in the periodic table, e.g., sodium, potassium, lithium, magnesium, calcium, strontium, and barium. Visual detection of few elements is possible just by looking at the colour in the flame, for **example,** sodium gives yellow flame. However, it is not a very reliable method. Hence, it is better to use flame photometer with a filter or monochromator that divides radiation as per the wavelengths of different metals from other radiations present. Detection of characteristic wavelength radiation indicates that the sample carries a metal.

Flame photometry method of metal detection is fast, simple, and reliable. However, this method has certain **limitations**:

1) It is less reliable as compared to atomic absorption spectroscopy.
2) It gives no information on the molecular structure of compound in the sample solution.
3) It cannot detect the non-radiating elements (like carbon, hydrogen, and halides) except under special circumstances. **For example,** for chlorine detection in a liquid sample, it is to be precipitated as silver chloride and then aspirated into flame of a flame photometer to determine silver. From the obtained data, chloride can be quantified.

4.4.3. Quantitative Analysis

Flame photometry is a rapid method for quantitative determination of groups I and II elements of the periodic table. Metallic elements other than of groups I and II can also be determined using high optical resolution equipment.

Experimental Procedure for Quantitative Analysis

For quantitative analysis, the initial step is to introduce the sample solution into the flame and then measure the radiation intensity at the pertinent wavelength.

Metal concentration in the sample is determined from the radiation intensity using one of the following methods:

1) **Standard Addition Method:** This method utilises various solutions containing the unknown and known amounts of standard substance.

   Firstly the signal intensity for the unknown (X) is deduced by aspirating its solution into the flame of a flame photometer. Then, a series of solutions with the unknown (X) along with different amounts of the standard (A) are prepared. The signals obtained by aspirating them into the flame are recorded.

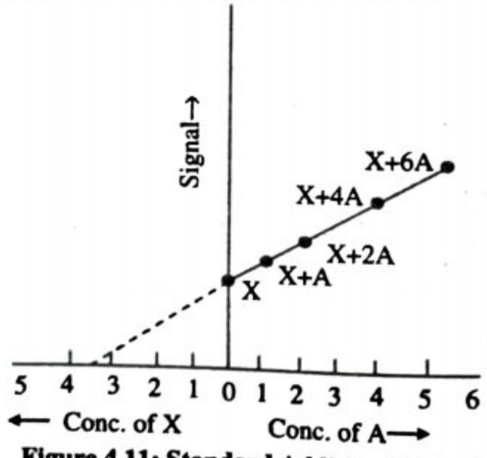


Figure 4.11: Standard Addition Method for Determining the Concentration of Unknown (X).

A plot for signal intensity (y-axis) *versus* concentration of unknown (X) plus concentration of standard (A) (x-axis) is plotted (**figure 4.11**). The concentration of unknown is determined from the intersection of the curve with the concentration axis.

Advantages

i) This method is capable of measuring very low concentrations of the elements present in the sample.
ii) This method counterbalances for interfering material that may be present in the sample solution.
iii) This method can also estimate the rarely analysed elements.

Precaution

While using the standard addition method, a correction for background emission from the flame should be done by injecting the solvent into the flame and then measuring its signal intensity, called **background emission**. For calculations, the background emission intensity should be subtracted from the emission due to sample.

2) **Internal Standard Method:** For applying this method to a simple flame photometer, the following steps are taken:
   i) Standard sample solutions with known amounts of internal standard element (usually lithium) are prepared and aspirated into the flame to record the signal intensity for each sample.
   ii) Then the samples carrying unknown concentrations are aspirated into the flame and the signal intensity is recorded.
   iii) The ratio of two intensities is determined and plotted against the concentration of unknown element (the internal standard element's concentration remains constant). From the calibration curve obtained, the concentration of element present in the sample can be deduced if its signal intensity is known by aspirating it into the flame.

   When using with simple flame photometer, this method shows some momentary fluctuations in flame characteristics. These fluctuations can be avoided by using a direct-reading instrument that provides direct and simultaneous reading for the ratio of the two intensities. The elements possessing the following **characteristics** can be chosen as the **internal standard**:
   i) It should be useful in very small amount in the original sample.
   ii) It should provide an emission line that reacts to interferences same as the line of the unknown element does.

4.5. SUMMARY

The details given in the chapter can be summarised as follows:

1) **Flow Injection Analysis (FIA) technique** can be employed for simultaneously carrying out the flame photometric analysis of elements like sodium (Na), potassium (K), lithium (Li), and calcium (Ca).
2) Flame photometry is also known as **flame emission spectroscopy** because it involves the emission of radiation by the neutral atoms introduced into the flames.
3) Theoretically it is possible to analyse all the elements by **flame photometry**.
4) **Boltzmann equation** signifies the relationship between ground state and excited state atoms.
5) **Flame emission** spectroscopy involves more inter-element interferences in comparison to atomic absorption spectroscopy.

6) The **first type** of spectral interference may arise due to the partial overlapping between the spectra of two elements (or compounds) emitting radiations at particular wavelengths.
7) The **second type** of spectral interference does not involve any spectral overlapping but occur due to the production of much closer spectral lines of two or more elements.
8) The **third type** of spectral interference may arise due to the presence of higher salt concentrations in the sample, thereby producing spectral interference between a spectral line and a continuous background.
9) Generally, **anions** are not competent to emit radiations.
10) The role of **atomiser** is to generate the vapours of analyte which gets excited by the thermal energy of the flame and then emits characteristic radiation that is measured.
11) **Flame atomiser** utilises a pneumatic nebuliser for converting the sample solution into mists or aerosol.
12) **Nebuliser** is a device used for introduction of sample into the flame.
13) A **monochromator** is required for measuring a single wavelength but it can also be applied for scanning a wide range of wavelength.
14) Either **vacuum phototubes** or **photomultiplier tubes** are utilised as detectors in flame photometers.
15) **Flame photometry** enables detection of group I and II elements in the periodic table like sodium, potassium, lithium, magnesium, calcium, strontium, and barium.

4.6. EXERCISES

4.6.1. True or False

1) Flame photometry is also known as flame emission spectroscopy because it involves the emission of radiation by the negative atoms introduced into the flames.
2) Theoretically it is possible to analyse all the elements by flame photometry.
3) Boltzmann equation signifies the relationship between ground state and excited state atoms.
4) Flame emission spectroscopy involves less inter-element interferences in comparison to atomic absorption spectroscopy.
5) Generally, anions are not competent to emit radiations.
6) Nebuliser is a device used for introduction of air into the flame.
7) A monochromator is required for measuring a single wavelength but it can also be applied for scanning a wide range of wavelength.
8) Either vacuum phototubes or photomultiplier tubes are utilised as detectors in flame photometers.
9) Flame photometry enables detection of group II and III elements in the periodic table.

4.6.2. Fill in the Blanks

10) Flame photometry is also known as _____.
11) _____ signifies the relationship between ground state and excited state atoms.
12) _____ spectroscopy involves more inter-element interferences in comparison to atomic absorption spectroscopy.
13) The _____ of spectral interference may arise due to the presence of higher salt concentrations in the sample
14) Generally, _____ are not competent to emit radiations.

15) The role of _____ is to generate the vapours of analyte which gets excited by the thermal energy of the flame and then emits characteristic radiation that is measured.
16) _____ utilise a pneumatic nebuliser for converting the sample solution into mists or aerosol.
17) _____ is a device used for introduction of sample into the flame.
18) Either vacuum phototubes or _____ are utilised as detectors in flame photometers.

Answers

1) False	2) True	3) True
4) False	5) True	6) False
7) True	8) True	9) True
10) Flame emission spectroscopy	11) Boltzmann equation	12) Flame emission
13) Third type	14) Anions	15) Atomiser
16) Flame atomisers	17) Nebuliser	18) Photomultiplier tubes

4.6.3. Very Short Answer Type Questions

1) Define flame photometry.
2) Give the theory of flame photometry.
3) What is the Boltzmann equation?
4) Define atomisers.
5) Define nebulisers
6) Enlist few detectors used in flame photometry.

4.6.4. Short Answer Type Questions

1) Give the detailed principle of flame photometry.
2) Write in detail about the origin of spectra in flame photometry.
3) Give the application of flame photometry.
4) Write short notes on:
   i) Burners
   ii) Mirrors
   iii) Slits
   iv) Fuel and oxidants

4.6.5. Long Answer Type Questions

1) Give the interferences of flame photometry.
2) Give the instrumentation of flame photometry.

CHAPTER 5

Atomic Absorption Spectroscopy

5.1. ATOMIC ABSORPTION SPECTROSCOPY

5.1.1. Introduction

Allan Walsh, in **1955**, was the pioneer for the introduction of Atomic Absorption Spectroscopy (AAS). This technique eventually proved to be one of the best known instrumental techniques in the analytical industry. Since then the technique is being exploited both intensively and extensively for quantitative determination of trace metals in liquids of completely diversified nature, **for example,** Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} ions in blood serum; Ni^{2+} ions in edible oils; Cu^{+} ions in beer samples; Pb^{2+} ions in gasoline (petrol); Se^{4+} ions in urine; and Mg^{2+} ions in tap water.

AAS may be accomplished either by using a flame, whereby the sample solution is aspirated directly into a flame or by using an electrothermal device, whereby the sample solution is first evaporated and then ignited on a hot surface. AAS facilitates the estimation of a particular element in the presence of many other elements efficaciously. In other words, there is no necessity to separate the test element from the rest, thereby not only saving a great deal of time but also eliminating the possibility of various sources of error incurred by these processes. In addition, AAS may be used for the estimation of both aqueous and non-aqueous solutions.

5.1.2. Principle

The underlying principle of AAS is the absorption of energy by ground state atoms while they are in the gaseous form.

It may be further expatiated as follows: When a solution consisting of certain metallic species is aspirated into a flame, the corresponding vapours of metallic species are developed. Some metal atoms would be raised directly to an energy level to an extent to emit the particular radiation of the metal. At this critical point, a sufficiently large quantum of the metal atoms of a particular element would still remain in the non-emitting ground-state, which in turn should be receptive of light radiation having their own specific wavelength.

Consequently, when a light of this wavelength is passed through a flame; along the atoms of the metallic species, a portion of the same would be absorbed; and the resulting absorption has been found to be directly proportional to the density of the atoms present in the flame at that material time. In AAS, the amount of light absorbed is determined. In other words, the concentration of metallic element may be determined directly from the value of absorption.

The **total amount of light absorbed** can be determined as:

$$\text{Total amount of light absorbed (at } \nu) = \frac{\pi e^2}{mc} Nf \qquad \text{.....(1)}$$

Where, ν = Frequency of the light path.
e = Charge on the electron.
m = Mass of the electron.
c = Speed of light.
N = Total number of atoms which can absorb at ν.
f = Ability for each atom to absorb at ν (oscillator strength).

The components in **equation (1)**, namely π, e, m and c are constants, therefore, it can be further written in a simplified form as below:

$$\text{Total amount of light absorbed} = K \times N \times f \qquad \text{.....(2)}$$

Hence, from equation (2), it may be inferred that the amount of light absorbed is independent of the wavelength and temperature.

More explicitly, the absorption by atom is independent of both the wavelength of absorption and the temperature of atoms.

5.1.3. Theory

The process of atomic absorption is illustrated in **figure 5.1**:

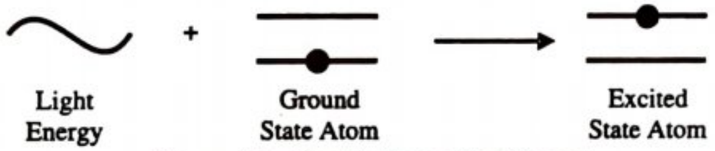


Figure 5.1: Atomic Absorption Process

The **ground state** atom absorbs light energy of a specific wavelength as it enters the **excited state**. As the number of atoms in the light path increases, the amount of light absorbed also increases. By measuring the amount of light absorbed, a quantitative determination of the amount of analyte can be made. Use of special light sources and careful selection of wavelengths allow the specific determination of individual elements.

When a solution of metallic salt is sprayed on to a flame, fine droplets are formed. Due to the thermal energy of flame, the solvent in the droplets evaporate, leaving a fine residue, which are converted to neutral atoms.

These neutral atoms absorb radiation of specific wavelength, emitted by Hollow Cathode Lamp (HCL). This lamp is filled with the vapour of element, which gives specific wavelength of radiation. For the determination of every element, hollow cathode lamp is selected, which contains vapour of the element to be analysed. Although this appears to be the demerit of AAS, specificity can be achieved only by the use of HCL.

The intensity of light absorbed by the neutral atoms is directly proportional to the concentration of the element and obeys Beer's law over a wide concentration range. The intensity of radiation absorbed by neutral atoms is measured using photometric detectors (photo tube or photo multiplier tube).

In AAS, the flame temperature is not critical, since the thermal energy of flame is used just to atomise the sample solution into fine droplets, to form a fine residue and later to neutral atoms. Excitation of neutral atoms is brought about only by radiation from hollow cathode lamp and not by the thermal energy of the flame.

5.1.3.1. Atomic Structure

The electrons travel in **orbits** around the nucleus; in the classical quantum theory, these orbits take the form of **orbitals**: while in the quantum mechanical theory, they represent the probability-density distributions. Since the potential energy of the electrons in these orbitals increases with increasing distance from the nucleus, they can also be represented as energy levels. Each of these energy levels can be characterised by **three quantum numbers**, namely the **principal quantum number** (n), the **orbital angular momentum** (or azimuthal) **quantum number** (l), and the **inner quantum number** (j). These quantum numbers arise from the fact that the energy levels can only absorb well-defined amounts of energy, i.e., they are quantised, since the wave functions must fulfil determined conditions of symmetry.

Principal quantum number (n) defines the shell in which the electron is located and is thus a measure for the relative distance of the electron shell from the nucleus. Electrons can be taken up in a shell in increasing order according to the selection rule that $2n^2$ is the maximum number of electrons that can exist in a shell.

Within the shells the electrons reside in orbitals (wave functions) of differing symmetry that are described by the **angular momentum quantum number** (l), which can take values of 0, 1, 2,....., n-1.

Each characterised orbital can take up to two electrons which must have opposite spins. Each of these electrons is characterised by the **inner quantum number** (j), which can take values of $j = l \pm 1/2$. Sodium atom with 11 electrons indicates that the first two shells with $2 + 8 = 10$ electrons are completely filled. The 11th electron is located on its own in the 3rd shell and is termed the **valence electron**. Since its transitions are responsible for the occurrence of spectral lines, it is also termed the **photoelectron**. In the ground state, the valence electron is at the lowest possible energy level, which in the sodium atom is the 3s state.

5.1.3.2. Atomic Spectra

The interaction of radiation and matter is the basis of spectroscopy. Radiation can be either absorbed by matter or emitted by matter. The measurement of these phenomena provides the basis of both absorption and emission spectroscopy.

The absorption spectra in atomic spectroscopy are relatively simple compared to those in molecular spectroscopy. The energy levels permitted in atoms are the energies of the electronic orbitals. The energy differences are the difference between these energies. If an electron goes from a ground state (E_0) to an excited state (E_1), there is a difference of energy ($E_0 - E_1$). In order to undergo transition it must absorb a photon with the same energy, i.e., $E_0 - E_1 = E = h\nu$ ($h\nu$ = energy of the absorbed photon). This absorption of energy is the basis of atomic absorption spectroscopy.

Similarly, an excited atom will have an electron in an upper excited orbital. The electron descends to a lower orbital of lower energy. The change in energy is equal to $E_4 - E_3$, where E_4 and E_3 are the energies of the upper orbitals concerned. In the process, a photon of energy is emitted. The energy of this photon must be E′, which is the difference in energy between E_3 and E_4, i.e., $E' = h\nu = E_4 - E_3$ and it is specific to the transition E_4 to E_3. This is the basis for emission spectroscopy, plasma emission, and flame photometry.

The emission spectra of atoms have been observed and studied for many years. By a slow, methodical, mathematical process of trial and error, the emission lines have been assigned to transitions between particular energy levels and the energy of those levels has been calculated. Diagrams have been developed for each element assigning the energies of each orbital and the wavelength of the transition between those orbitals. These are called **Grotrian diagrams**. They are used extensively in atomic absorption and atomic emission.

5.1.3.3. Natural Line Broadening

The **minimum possible half-width** is termed the **natural line width**. An excited atom remains only for a very short period (τ) in the excited state (typically 10^{-9} to 10^{-8}s) before it releases the energy of excitation, **e.g.,** as a photon. According to the **Heisenberg uncertainty principle**, the energy levels of the transition can only be determined with an uncertainty of ΔE over the observation time Δt.

$$\Delta E \Delta t = \frac{h}{2\pi} \qquad \text{.....(3)}$$

From this uncertainty of the energy levels of a radiative transition:

$$\Delta E = h\nu \qquad \text{.....(4)}$$

An uncertainty of the frequency ν of the respective spectral line:

$$\delta\nu = \frac{\Delta E}{h} = \frac{1}{2\pi\tau} \qquad \text{.....(5)}$$

If we observe the transition between two energy levels (E_k and E_j), the times of relaxation of each level (which represent the inverse values of all Einstein transition probabilities for the levels) enter the uncertainty relationship, as depicted schematically in **figure 5.2**, and an **expression for the natural half-width** (ΔV_N) can be obtained:

$$\Delta V_N = \frac{\nu_0^2}{2\pi}\left(\frac{1}{\tau_k} - \frac{1}{\tau_j}\right) \qquad \text{.....(6)}$$

Where, ν_0 = Frequency of the line.
τ_k = Time of relaxation of the upper (excited) state.
τ_j = Time of relaxation of the lower (ground) state.

If a transition to a stable ground state (resonance line) occurs, only the time of relaxation of the excited state enters the expression, since the ground state is stable ($\tau_j = \infty$). For alkaline-earth metals, **e.g.,** the half-widths are in the order of 0.01pm (strontium) to 0.14pm (beryllium), which is negligible compared to other mechanisms of broadening in AAS. The same applies to other elements determinable by AAS.

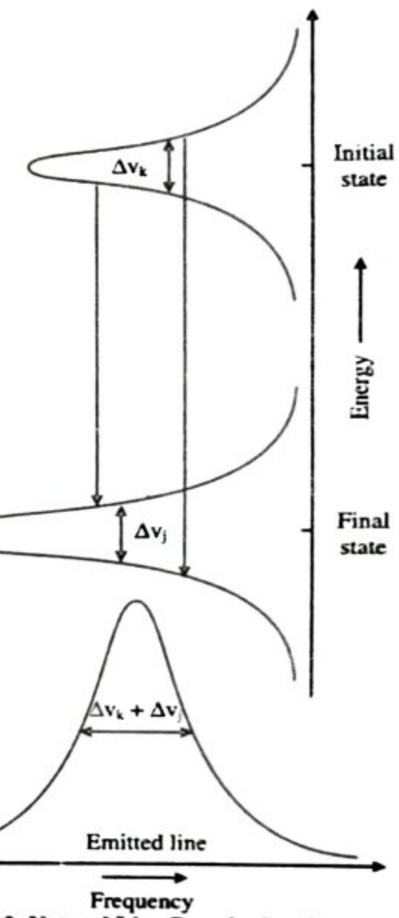


Figure 5.2: Natural Line Broadening due to Broadened Energy Levels of the Transition

5.1.3.4. Doppler Effect

Spectral lines also undergo broadening due to the random thermal movement of the atoms. This movement can be described by **Maxwell distribution**. Although the velocity of atoms is in the order of 1000m/s, and thus far below the velocity of light (3,00,000,000m/s), it still has an influence that cannot be neglected. As a result of the positive and negative velocity vectors along the line of observation relative to the observer, the emitted or absorbed photons receive an additive velocity component which brings about a corresponding positive or negative shift in the frequency. A **Doppler**

profile, thus results from the Maxwell distribution and this can be described by a **Gaussian function**. The **half-width ($\Delta\lambda_D$) of a spectral line** influenced by the Doppler effect is given by:

$$\Delta\lambda_D = \frac{\nu}{c}\sqrt{\frac{2RT}{M}} = 716.10\lambda_0\sqrt{\frac{T}{M}}$$

This equation states that the Doppler width ($\Delta\lambda_D$) is directly proportional to the wavelength (λ_0) and the root of absolute temperature (T), and inversely proportional to the root of relative atomic mass (M) of the emitting species.

5.1.3.5. Detection Limit and Sensitivity

Detection limit may be defined as the concentration (mcg/ml) of an element that gives rise in the shifting of absorbance signal to an amount which equals to the peak-to-peak noise of the base-line.

Sensitivity may be defined as the concentration of element present in the sample solution that produces 1% absorption.

From the above definition it is quite evident that sensitivity takes no cognisance of the noise-level of the base-line, therefore, it is more or less of no use as a definite guide to the least quantity of an element which may be estimated. However, the sensitivity of 1% absorbance is a pure theoretical number that would undergo a change depending on the efficiency of lamp (hollow cathode lamp), atomiser, flame system, monochromator (prism, grating used), and the photomultiplier used.

The sensitivity for 1% absorbance is determined as:

$$C_1\% = \frac{C_{0.1} \times 0.0044}{0.1}$$

Where,

$C_1\%$ = Concentration that yields 1% absorbance.
$C_{0.1}$ = Concentration that yields an absorbance of 0.1.

Sensitivity is usually expressed in terms of **mcg/ml for 1% absorbance.**

It is a usual practice to perform an actual test run over a sufficiently large range by employing the necessary prevailing expansion facility so as to ascertain whether or not the atomic absorption technique is reasonably applicable to a specific low-level estimation. Such a data may ultimately reveal the exact and true detection limit which normally equals to twice the noise level.

5.2. INTERFERENCES

5.2.1. Introduction

There are the following types of interferences in AAS:

1) Spectral interferences,
2) Chemical interferences,
3) Ionisation interferences,
4) Matrix or bulk interferences,
5) Solvent interferences, and
6) Dissociation of metal compounds.

5.2.2. Spectral Interferences

These interferences may be **caused by overlapping of any radiation with that of characteristic radiation of the test element to be estimated**. The interfering radiation may be an emission line of another element, radical or molecule, unresolved band spectra or

general background radiation from the flame, solvent, etc. In this case, the lines will be read together in proportion to the degree of overlap if the resolving power and spectral band pass of the monochromator permit the undesired radiation to reach the photoreceptor.

If the spectral interference is due to sample matrices or flame components, one can **overcome** this by **working with AC amplifiers tuned to the frequency at which the source is chopped or modulated**. However, the signals from the flame, which are not being modulated, are rejected.

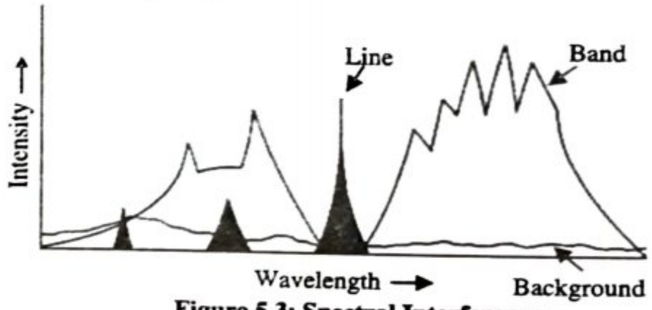


Figure 5.3: Spectral Interferences

Most of the quantitative work in atomic absorption is accomplished with **line** spectra, but the analyst must be aware of band and continuous spectra to guard against and correct for interferences at relatively low temperatures (**figure 5.3**). The band spectra are due to the rotational and vibrational characteristics of undissociated molecules or complex ions remaining in the flame. A flame background spectrum is also present. This receives contribution from all the elements present in the sample as well as from the fuel and the oxidant, and may be corrected by instrumental or graphical procedures.

Spectral interferences are caused either by the combustion products which show broad-band absorption or the particulate products which scatter radiation. In fact, both these products distinctly lower the power of the transmitted beam of light and ultimately give rise to positive analytical errors.

For example, manganese triplet (at 4031°, 4033° and 4035°A), potassium doublet (at 4044° and 4047°A), and the gallium line (at 4033°A). The overlapping of this nature may be **eliminated** either by **prior chemical separation** or by **selection of other spectral lines**.

5.2.3. Chemical Interferences

These interferences must be considered in atomic absorption procedures. If compounds or complex ions of the element being determined incompletely dissociate into their atoms, low results will occur. The more concentrated the solution, greater will be the deviation from the correct value. This incomplete dissociation is a chemical interference and might be **removed with the use of a higher flame temperature.**

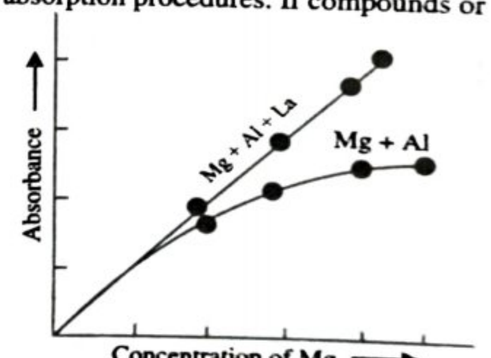


Figure 5.4: Chemical Interferences of Al on Mg

In situations where a hotter flame cannot be utilised, chemical means are suggested, **for example,** aluminium (Al) and magnesium (Mg) form a thermally stable mixed oxide. Because of their thermal stability, low results for magnesium are obtained in the presence of

aluminium. The **interference may be removed by the addition of lanthanum (La) to the system**. Since the mixed oxide of lanthanum and aluminium is more stable than that of magnesium and aluminium, lanthanum-aluminium oxide is formed and magnesium oxide is released. Magnesium is then determined after this latter compound is reduced and vaporised in the flame (**figure 5.4**).

5.2.4. Ionisation Interferences

These interferences arise in atomic absorption **if the flame temperature is too high**. When this occurs, a number of vaporised atoms become ionised by the flame. The resulting ions absorb at a different wavelength than the vaporised atoms; the new wavelength will not be selected by the monochromator, and low values result. Any attempt to alleviate the ionisation interference by lowering the flame temperature might result in incomplete dissociation and more chemical interferences. The interference is usually **minimised by the addition of a more easily ionisable element**.

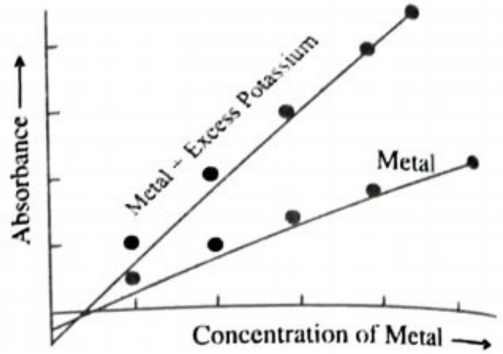


Figure 5.5: Ionisation Interferences

For example, ionisation interference of calcium may be corrected by the addition of large quantities of sodium or potassium salts to the solution. Since each of these elements possesses a lower ionisation potential than does calcium, their electrons will be more easily removed and calcium will remain as the vaporised metal (**figure 5.5**).

The processes may be represented by a series of equations for the determination of the metal (M). The desired reaction is:

$$My \xrightarrow{\Delta} M^0 + Y^0 \qquad \text{.....(7)}$$

And the interfering reaction is:

$$My \xrightarrow{\Delta} M^+ + e^- \qquad \text{.....(8)}$$

Where, My = Molecular compound.
M^0 and Y^0 = Vaporised elements.
M^+ = Metal ion.

When the potassium compound (Ky) is added to the sample, it is vaporised and the potassium is ionised according to the equations:

$$Ky \xrightarrow{\Delta} K^0 + Y^0 \qquad \text{.....(9)}$$

And,
$$K^0 \xrightarrow{\Delta} K^+ + e^- \qquad \text{.....(10)}$$

The excess of electrons from **equation (10)** reverses the direction of **equation (8)** as follows:

$$e^- + M^+ \rightarrow M^0 \qquad \text{.....(11)}$$

Ionisation interferences may also arise in atomic emission. In this case, calibration curves which are concave upward are produced.

5.2.5. Matrix or Bulk Interferences

A **change in the viscosity of the solution** caused by either the change in a solvent or a change in concentration may result in a matrix or bulk interference. The addition of an organic solvent usually results in a decreased viscosity and a two to fourfold increase in absorbance.

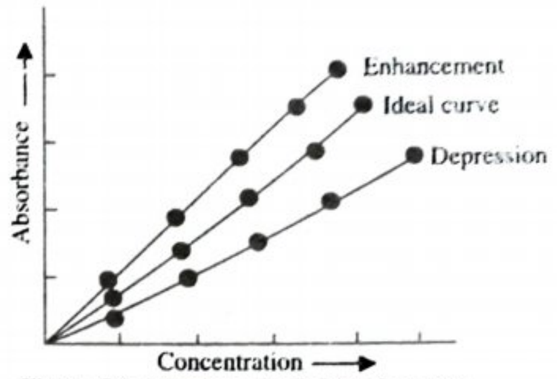


Figure 5.6: Matrix or Bulk Interferences

On the other hand, an increase in concentration results in an increase in viscosity, a slower flow through the burner, and a corresponding decrease in absorbance (**figure 5.6**). The viscosity changes would also cause corresponding intensity changes in emission measurements.

Both ionisation and bulk interferences are greatly affected by burner design.

5.2.6. Solvent Interferences

Another potential source of interference is the solvent. In general, metals in aqueous solutions give lower absorbance readings than the same concentration of such metals in an organic solvent.

In order to produce free atoms from a solution containing molecular compounds, as opposed to ions, it is first necessary to drive-off the solvent, leaving a residue containing the metals of interest. The residue is then decomposed, generating free metal atoms. The rate at which a solvent is driven-off depends on the temperature of the atomiser and the volatility of solvent. If a flame is used, hotter the flame, more rapidly the solvent is driven-off. If the solvent is water, evaporation is slow and tends to decrease the flame temperature.

This in turn retards the entire atomisation process. If the solvent is organic in nature, such as acetone, alcohol, ether, or a hydrocarbon, the solvent not only evaporates rapidly, but may also burn, thus increasing the flame temperature. The atomisation process is more efficient. More free atoms are produced from this system. A higher absorption signal is registered from organic solvents than from aqueous solutions, even though the metal concentration in the two solutions is equal. This process is shown in **table 5.1**:

Table 5.1: Formation of Atoms is Flames

Combustion of an Aqueous Solvent
Ions in water $\xrightarrow{\text{Step 1}}$ Evaporation leaving a hydrated residue $\xrightarrow{\text{Step 2}}$ Residue (dehydrated atoms formed) $\xrightarrow{\text{Step 3}}$ Excited atoms (emission) and neutral atoms (absorption)
Combustion of an Organic Solvent
Metals in organic solvent $\xrightarrow{\text{Step 1}}$ Solvent burns $\xrightarrow{\text{Step 2}}$ Organic addend burns $\xrightarrow{\text{Step 3}}$ Excited atoms (emission) and neutral atoms (absorption)

5.2.7. Dissociation of Metal Compounds

When metals like La, Al, and Ti are aspirated into the flame, metal atoms are not obtained but extremely stable refractory oxides are obtained. Thus, the atomic absorption studies with these elements become complicated. For such elements, **nitrous oxide-acetylene flames are used** which could dissociate these metal oxides to enable analysis of these elements by atomic absorption spectrometry.

5.3. INSTRUMENTATION

5.3.1. Introduction

There are five basic components of an atomic absorption spectrophotometer:

1) The **light/radiation source** that emits the spectrum of the element of interest,
2) An **absorption cell** in which atoms of the sample are produced (flame, graphite furnace, MHS cell, FIAS cell, FIMS cell),
3) A **monochromator** for light dispersion,
4) A **detector** which measures the light intensity and amplifies the signal, and
5) A **read-out device** that shows the reading after it has been processed by the instrument electronics.

There are two basic **types of atomic absorption spectrophotometer**:

1) Single beam atomic absorption spectrophotometer, and
2) Double beam atomic absorption spectrophotometer.

5.3.2. Radiation Source

An atom absorbs light at discrete wavelengths. In order to measure this narrow light absorption with maximum sensitivity, it is necessary to use a line source, which emits the specific wavelengths that can be absorbed by the atom. Narrow line sources not only provide high sensitivity, but also make atomic absorption a very specific analytical technique with a few spectral interferences.

The two most **common line sources** used in atomic absorption are:

1) **Hollow Cathode Lamp:** This lamp (**figure 5.7**) consists of a hollow cup containing the element to be determined (in this case the element is sodium). The anode is a tungsten wire. The two electrodes are housed in a tube containing an inert gas. The lamp window is of quartz, silica, or glass. The exact material used depends on the wavelength to be transmitted.

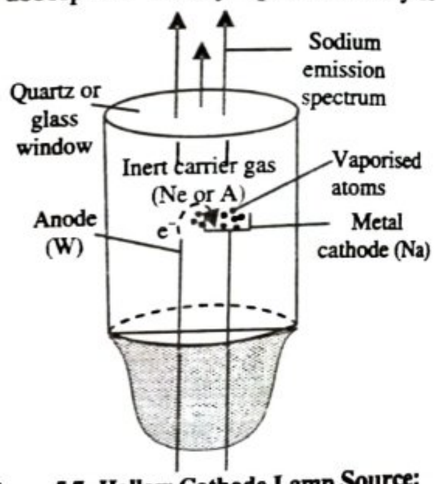


Figure 5.7: Hollow Cathode Lamp Source: Adapted from *Instrumental Methods of Chemical Analysis* (pp 2.345), by Chatwal G.R. (2006) (Himalaya Publishing House)

When a potential is applied between the two electrodes, a current in the milliampere range arises, the inert gas is charged at the anode, and the charged gas is attracted at high velocity to the cathode. The impact with the cathode vaporises some of the sodium atoms. These are excited and on returning to the ground state give rise to the **sodium emission spectrum**. The manufacturer's recommended current for the lamp should not be exceeded or the lifetime of the lamp may be considerably shortened. Excess current may also give rise to a self-absorption process wherein the sputtered ground-state atoms absorb some of the emitted energy, resulting in a lessening of intensity of the emitted. On the other hand, if the source lamp is run below the recommended current, a loss of intensity and a corresponding loss of sensitivity will result.

2) **Electrode-less Discharge Lamp:** For most elements, the hollow cathode lamp is a satisfactory source for atomic absorption. In a few cases, however, the quality of analysis is impaired by limitations of the hollow cathode lamp. The primary cases involve the more volatile elements where low intensity and short lamp life are a problem. The atomic

absorption determination of these elements can often be dramatically improved with the use of electrode-less discharge lamp which is a brighter and more stable source.

Figure 5.8 shows the design of Perkin-Elmer System 2 Electrode-less Discharge Lamp (EDL). A small amount of the metal or salt of the element for which the source is to be used is sealed inside a quartz bulb, which is placed inside a small, self-contained RF generator or "driver". When power is applied to the driver, an RF field is created. The coupled energy will vaporise and excite the atoms inside the bulb, causing them to emit their characteristic spectrum. An accessory power supply is required to operate an EDL.

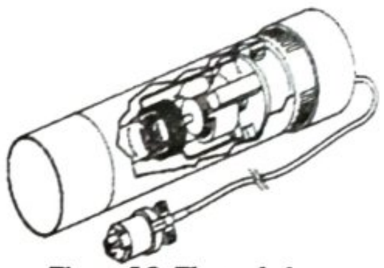


Figure 5.8: Electrode-less Discharge Lamp

5.3.3. Chopper

A rotating wheel, known as chopper, is interposed between the hollow cathode lamp and the flame. This rotating wheel is interposed to break the steady light from the lamp into an intermittent or pulsating light. This gives a pulsating current in the photocell. There is also a steady current caused by light which is emitted by a flame. But only the pulsating (or alternating) current is amplified and recorded and thus, the absorption of light will be measured without interference from the light emitted by the flame itself.

5.3.4. Flame Atomisers

Flame is used for converting the liquid sample into the gaseous state and also for converting the molecular entities into an atomic vapour. There are **two types of burners** in common use:

1) **Total Consumption Burner:** In this burner (**figure 5.9**), the sample solution, fuel, and oxidising gases are passed through separate passages to meet at the opening of the flame base. As the sample containing metallic element to be estimated by atomic spectroscopy is a liquid, the flame breaks up the liquid sample into droplets which are then evaporated or burnt, leaving the residue which is reduced to atoms. Total consumption burners use oxygen with hydrogen or acetylene, and give very hot flames. This burner is however noisy, hard to use, and its efficiency is not very good.

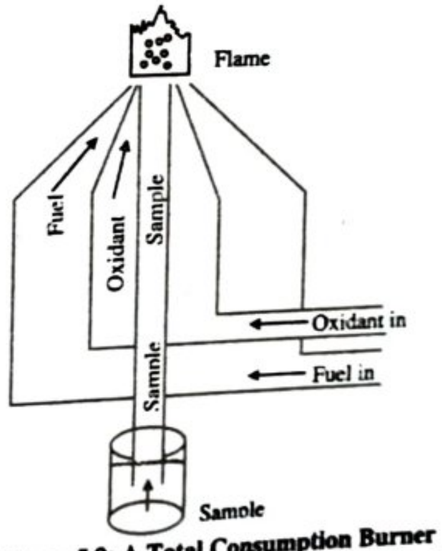


Figure 5.9: A Total Consumption Burner
Source: Adapted from *Instrumental Methods of Chemical Analysis* (pp 2.347), by Chatwal G.R. (2006) (Himalaya Publishing House)

2) **Premixed Burner:** In this burner (**figure 5.10**), a mixture of the sample (liquid) and premixed gases ($C_2H_2 + O_2$) is allowed to enter the base. From the base, the gases enter a region, from where the unburnt hydrocarbon gaseous mixture and liquid droplets are allowed to enter a region which is of free heating and about 1mm in thickness. In this region, the liquid is evaporated leaving a residue. Heating in this region is done by the heat obtained by conduction and convection and by diffusion of radicals into it which initiate the combustion. After this, the sample residue is burnt to produce atoms. Production of atoms is initiated in one region and is completed in another region.

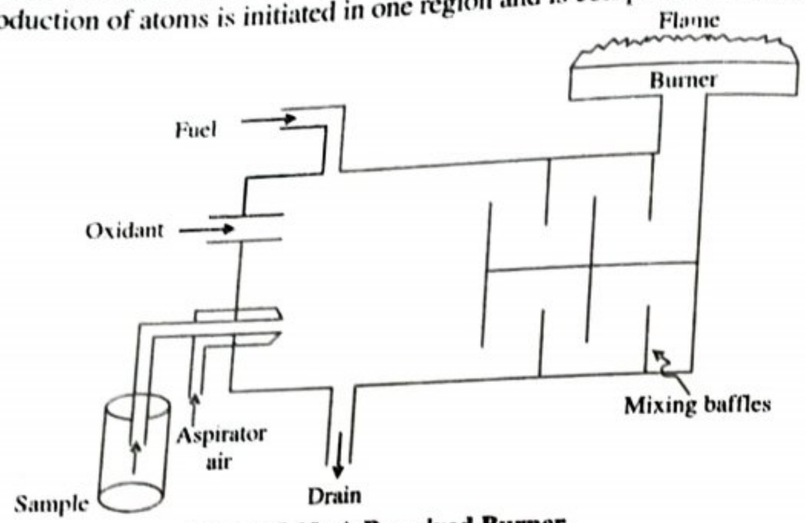


Figure 5.10: A Premixed Burner

Source: Adapted from *Instrumental Methods of Chemical Analysis* (pp 2.348), by Chatwal G.R. (2006) (Himalaya Publishing House)

Premixed burner is very suitable for the atomic absorption studies of metals of IA, IB and IIB groups, together with Ga, In, Ti, Pb, Te, Mn, Ni, and Pd.

5.3.4.1. Properties of Flames

When a nebulised sample is carried into a flame, de-solvation of the droplets occurs in the primary combustion zone, located just above the burner tip (**figure 5.11**). The resulting finely divided solid particles are carried to the **inner cone** (a region in the centre of the flame). In this hottest part of the flame, the particles are vaporised and converted to gaseous atoms, elementary ions, and molecular species. Excitation of atomic emission spectra also takes place in this region.

Finally, the atoms, molecules, and ions are carried to the **outer edge** or **outer cone**, where oxidation may occur before the atomisation products disperse into the atmosphere. Because the velocity of fuel/oxidant mixture through the flame is high, only a fraction of the sample undergoes all these processes; indeed, a flame is not a very efficient atomiser.

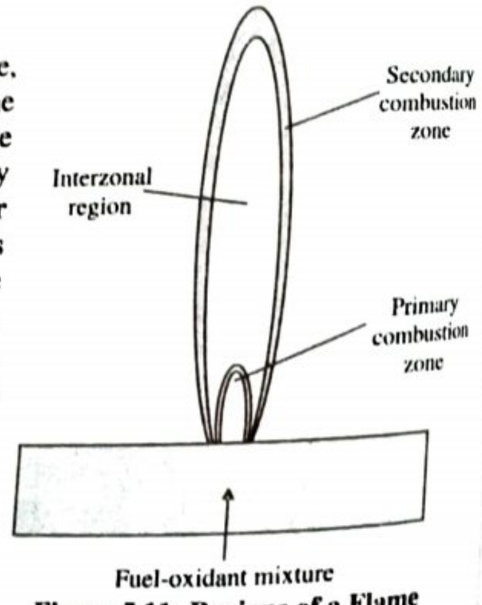


Figure 5.11: Regions of a Flame

5.3.4.2. Types of Flames/Fuels and Oxidants

Natural gas, **e.g.,** propane, butane, hydrogen, and acetylene, are the common **fuels** used for flame production; acetylene being most widely used. Air and air enriched with oxygen (O_2) and nitrous oxide (N_2O) are the common **oxidants**.

For a hot flame nitrous oxide-acetylene mixture is often used because of its low explosion hazard. Low-temperature flame is advantageously used for elements, such as copper, lead, zinc, and cadmium, which are easily converted to the atomic state. Alkaline earth metals on account of their forming refractory oxides require higher temperatures for decomposition. Aluminium, beryllium and rare earths form very stable oxides and require still higher temperatures which are provided by oxygen-acetylene or nitrous oxide-acetylene flame. The ratio of fuel to oxidising agent also affects the extent of atom formation in a flame.

The **table 5.2** depicts the commonly used fuels and oxidants in AAS:

Table 5.2: Fuels and Oxidants used in Atomic Absorption Spectroscopy

Fuels	Oxidants	Temperature (°C)	Maximum Burning Velocity (cm/s)
Natural gas	Air	1700-1900	39-43
Natural gas	Oxygen	2700-2800	370-390
Hydrogen	Air	2000-2100	300-440
Hydrogen	Oxygen	2550-2700	900-1400
Acetylene	Air	2100-2400	158-266
Acetylene	Oxygen	3050-3150	1100-2480
Acetylene	Nitrous oxide	2600-2800	285

5.3.4.3. Factors Affecting Flame Profile

The factors affecting flame profiles are given in **table 5.3**:

Table 5.3: Factors Affecting Flame Profiles

Physical Form of Sample in Flame	Reactions	Factors Controlling Reaction	Part of Flame
Oxide	No reaction or reduction	Stability of metal oxide, and flame composition	Outer mantle
Atoms	Accumulation or oxidation	Flame composition and stability of atoms	Reaction zone
Solid particles	Disintegration	Stability of compound, anions, flame temperature, and ultraviolet light emitted from the flame	Inner cone
Droplets	Evaporation	Droplet size, solvent, flame temperature feed rate, and combustibility	Base

5.3.5. Monochromators

In atomic absorption measurements, the most common monochromators are **prisms** and **gratings**. Commercially packaged atomic absorption instrumentation commonly includes a monochromator of about ½m focal length with a linear reciprocal dispersion in the range of 16-35Å.

Function of a monochromator is to select a given absorbing line from spectral lines emitted from the hollow cathode. For many elements, high dispersion is not necessary. In such cases, a resolution in the order of 0.5Å is desirable. When the cathode in hollow cathode lamp is made up of transition metals, the emission spectrum from the hollow cathode is so complicated that high dispersion is essential. For such cases, large dispersion and high resolving monochromators are advantageous for resolving spectra.

5.3.6. Detectors

For AAS, the **photomultiplier tube** is a most suitable detector. It has good stability if used with a stable power supply. It works satisfactorily and enables to compare intensity in a satisfactory manner. In photomultiplier tubes, there is an evacuated envelope which contains a photocathode, a series of electrodes called dynodes, and an anode. The photocathode is fixed to the power supply terminal (**figure 5.12**).

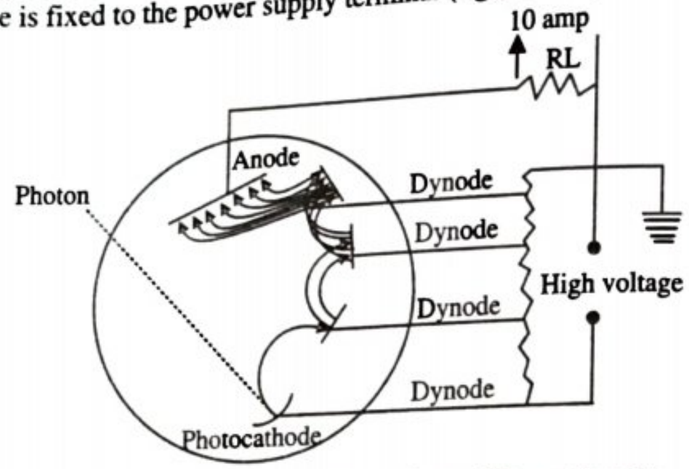


Figure 5.12: Photomultiplier Tube and Power Supply

As soon as a photon strikes the photocathode, an electron is dislodged and the photon is accelerated to the first dynode, resulting in the liberation of two or more electrons from it. Similarly, the electrons from this dynode are accelerated to the next dynode, resulting in the liberation of more electrons. Thus, the current multiplied at each dynode and the resultant current is received by the anode to produce an EMF across RL, which goes to the external amplifier and read-out system.

5.3.7. Amplifier

The elcctric current from the photomultiplier detector is fed to the amplifier that amplifies the electric current many times. Generally, "**Lock-in**" amplifiers are preferred which provide a very narrow frequency band pass and help to achieve an excellent signal-to-noise ratio.

A block diagram of a lock-in amplifier is shown in **figure 5.13**:

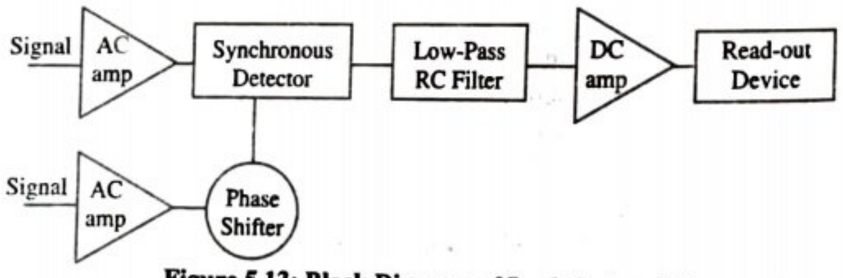


Figure 5.13: Block Diagram of Lock-in Amplifier

From **figure** 5.13, it clearly follows that the signal from the PMT is led to an AC amplifier to modulate the energy source. The modulated signal finally enters the synchronous detector. At the same time, the signal from a reference source (usually a flashlight bulb) is modulated at the same frequency as the signal from the PMT by passing through an AC amplifier.

This modulated reference signal also enters the synchronous detector through a phase shifter. The two signals are combined in a synchronous detector to produce sum and difference frequencies, the **sum frequency** will be twice the **chopping frequency** and the **difference frequency** will be zero. Thus, the difference frequency will be a DC signal.

When the signals from synchronous detector are allowed to pass through a low-pass RC filter, it removes the sum frequencies and a DC difference signal is amplified by a DC amplifier and then goes out to the read-out device.

5.3.8. Read-Out Device

In most of the atomic absorption measurements, **chart recorders** are used as read-out devices. A chart recorder is a potentiometer using a servomotor to move the recording pen. The displacement is directly proportional to the input voltage. In some atomic absorption measurements, **digital read-out devices** are used.

5.3.9. Single Beam Atomic Absorption Spectrophotometer

A single beam atomic absorption spectrophotometer and its components are illustrated in **figure 5.14**:

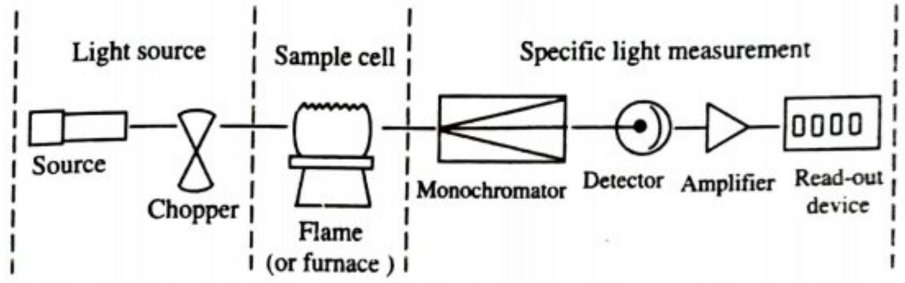


Figure 5.14: Single Beam Atomic Absorption Spectrophotometer

A **light source** which emits the sharp atomic lines of the element to be determined is required. The most widely used source is the **hollow cathode lamp**. These lamps are designed to emit the atomic spectrum of a particular element, and specific lamps are selected for use depending on the element to be determined.

It is also required that the source radiation be modulated (switched on and off rapidly) to provide a means of selectively amplifying light emitted from the source lamp and ignoring emission from the sample cell. Source modulation can be accomplished with a **rotating chopper** located between the source and the sample cell, or by giving the power to the source.

Special considerations are also required for a **sample cell** for atomic absorption. An atomic vapour must be generated in the light beam from the source. This is generally accomplished by introducing the sample into a **burner system** or electrically heated furnace aligned in the optical path of the spectrophotometer. Several components are required for specific light measurement.

A **monochromator** is used to disperse the various wavelengths of light which are emitted from the source and to isolate the particular line of interest. The selection of a specific source and a particular wavelength in that source is what allows the determination of a selected element to be made in the presence of others.

The wavelength of light which is isolated by the monochromator is directed onto the detector, which serves as the "eye" of the instrument. This is normally a **photomultiplier tube**, which produces an electrical current dependent on the light intensity. The electrical current from the photomultiplier is then amplified and processed to produce a signal which is a measure of the light attenuation occurring in the sample cell. This signal can be further processed to produce instrument read-out directly in concentration units.

5.3.10. Double Beam Atomic Absorption Spectrophotomet

A double beam atomic absorption spectrophotometer and its components are illustrate **figure 5.15**:

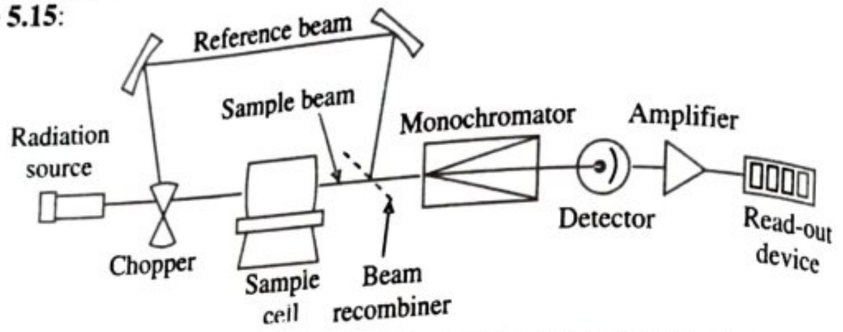


Figure 5.15: Double Beam Atomic Absorption Spectrophotometer

The light from the **source lamp** is divided into a **sample beam**, which is focused throu the **sample cell**, and a **reference beam**, which is directed around the sample cell. In double-beam system, the read-out represents the ratio of the sample and reference bea Therefore, fluctuations in source intensity do not become fluctuations in instrument rea out, and stability is enhanced. Generally, analyses can be performed immediately with lamp warm-up required.

The major **disadvantage of a single beam atomic absorption spectrophotometer** lie in its very **low stability**. Introduction of a double beam atomic absorption spectrophotometer has completely eliminated this problem and provides much enhance stability. In this particular instance, the chopped beam of light from the hollow cathod lamp is split into two parts. The first portion passes through the flame, while the secon portion is made to bypass the flame completely. However, the two separate beams light are recombined by a unique optically-designed assembly, passes through **monochromator** to a strategically placed **detector** and ultimately to a sensitiv **read-out device**.

It is pertinent to mention here that a double beam atomic absorption spectrophotometer absolutely independent of:

1) Lamp drift, and
2) Sensitivity of detector with time.

5.4. APPLICATIONS

5.4.1. Introduction

Atomic absorption spectroscopy is one of the most important methods of chemical analysis which is used in almost all the branches of chemical analysis. This technique is used in analytical chemistry, ceramics, mineralogy, biochemistry, water supplies metallurgy, and soil analysis. Some of its applications are described below:

1) Qualitative analysis,
2) Quantitative analysis,
3) Simultaneous multicomponent analysis,
4) Determination of metallic elements in biological materials,
5) Determination of metallic elements in food industry,
6) Determination of calcium, magnesium, sodium, and potassium in blood serum, and
7) Determination of lead in petrol.

5.4.2. Qualitative Analysis

It is performed by using a hollow cathode lamp. The cathode of this lamp is made up of the element to be detected, therefore for every new element to be tested a different hollow cathode lamp is used. For qualitative analysis, AAS is used rarely because the process is very tedious and involves detection of only one element at a time.

5.4.3. Quantitative Analysis

In this technique, the amount of radiation absorbed by the sample is detected. On substituting the value of radiation in **equation (1)**, the number of absorbing atoms in the light path (N) can be obtained. But N cannot be used to calculate the concentration of the element in the sample because the efficiency of producing atoms from a sample cannot be calculated. The calculations involved in quantitative analysis are generally based on calibration curves.

Calibration Curves

In quantitative analysis, the first step is to prepare the calibration curve by adjusting the read-out device to 100% transmittance with a blank and 0% transmittance when no radiant energy is entering the monochromator slit. Similar adjustment is made if the read-out device is a chart recorder.

The standard samples of the elements to be quantitatively determined are introduced into the burner and percentage absorption is determined. After this, the absorbance values are plotted against concentrations (**figure 5.16**). If the calibration curve obtained is linear, the slope is calculated and then the concentrations of unknown solutions are determined using the equation given below:

$$A = mc$$

Or, Absorbance = Slope × Concentration

It is important to remember that the sample whose concentration is to be determined is atomised and absorbance is measured under the same conditions that were used while preparing the calibration curve.

Once the recording is completed on a strip chart, the atomic absorption signals of standard samples of zinc and an unknown sample (X) are obtained as shown in **figure 5.16**:

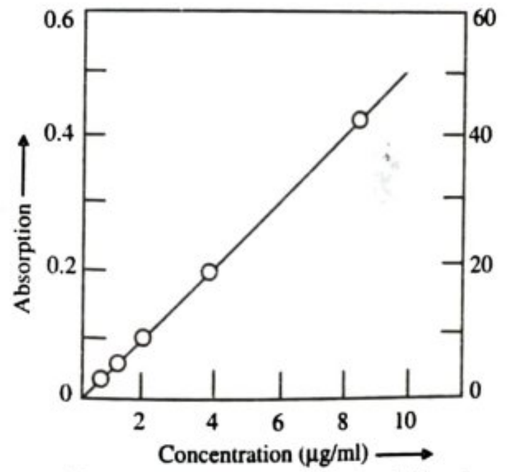


Figure 5.16: Calibration Curve of Atomic Absorption Signals Using Absorption

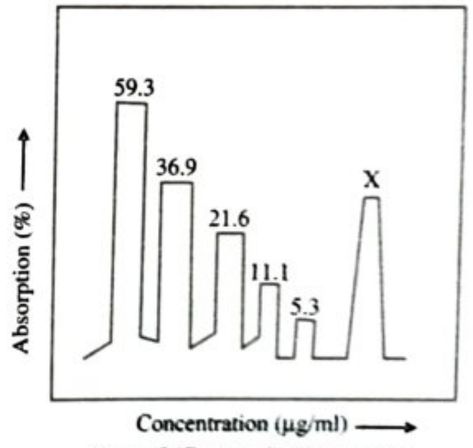


Figure 5.17: Atomic Absorption Signals of Zinc Standards (2138.6A°) and Unknown (X)

In **figure 5.17**, the baseline shows 100% transmission. The 0% transmission is obtained vertically above the figure (not given in the figure). Baseline is obtained with a blank sample solution having 0.00µg/ml of zinc. The numbers above each absorption signal and below each absorption signal represent the percentage absorption and the concentration

of standard samples, respectively. These results are used to plot a calibration curve (similar to the graph in **figure 5.16**). In **figure 5.17**, X denotes the zinc sample of unknown concentration. From the chart, the absorption value can be obtained. Then the unknown concentration of the zinc sample (X) can be determined using the equation:

$$A = mc$$

5.4.4. Simultaneous Multicomponent Analysis

This method of analysis can be carried out if a multi-element emission source is available. Earlier, multicomponent determinations were not possible since multi-element emission source were not available; but in **1973**, **Mitchell** developed a multi-element atomic absorption system, in which he used a multi-element hollow cathode source and a Videocon detection system. He detected eight elements (Zn, Cd, Ni, Co, Fe, Mn, Cu, and Ag) at a time using a spectral region from 2320-3281Å. After this, the method further developed for quantitative evaluation of various compounds.

5.4.5. Determination of Metallic Elements in Biological Materials

AAS is used frequently to determine trace metals in biological materials. The recently developed procedure offers the advantages of the speed of attack and decomposition of the carbonaceous material using 50% of H_2O_2. In this method, a combination of concentrated sulphuric acid and nitric acid can also be used.

For the analysis of biological materials, a standard method cannot be given because the quantities of hydrogen peroxide and acid required depend on the material to be analysed. Use of nitric acid or sulphuric acid also depends on the nature of trace metal. Therefore, sulphuric acid is not suitable for lead extraction because it forms insoluble lead sulphate.

5.4.6. Determination of Metallic Elements in Food Industry

The commonly found toxic elements during the food analysis are copper, zinc, and nickel. For the analysis of solid foodstuffs, the trace metals are extracted from the sample by the process of digestion using dilute sulphuric acid or nitric acid or 50% hydrogen peroxide. In **figure 5.18**, the results obtained after determining nickel in vegetable oils are shown; and in **figure 5.19**, the results of copper determination in beers are shown.

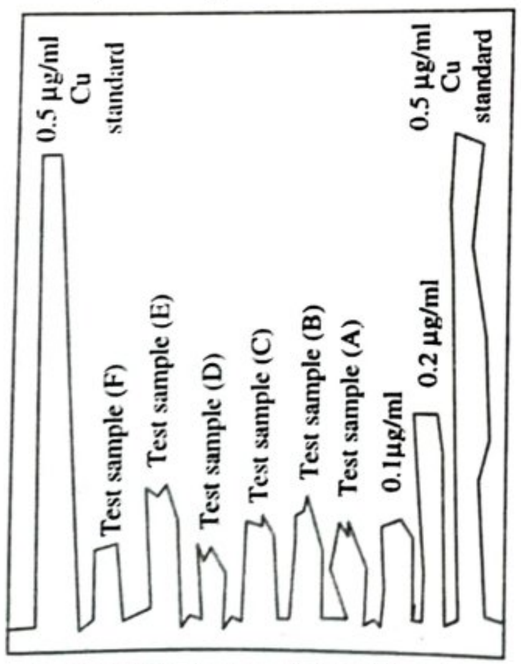


Figure 5.18: Determination of Nickel in Vegetable Oils

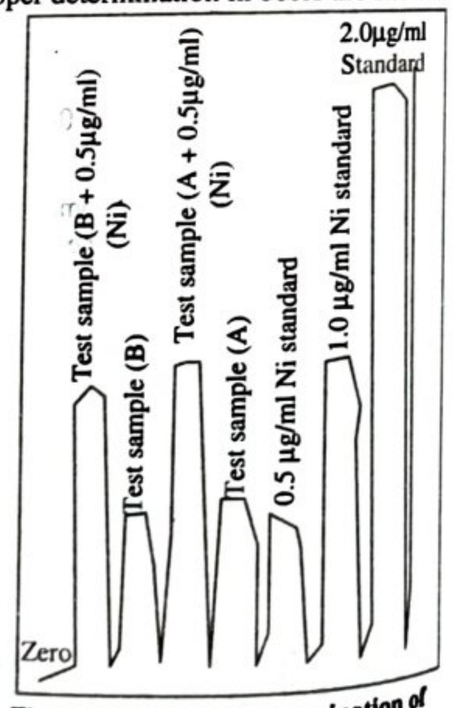


Figure 5.19: Direct Determination of Copper in Beers

5.4.7. Determination of Calcium, Magnesium, Sodium, and Potassium in Blood Serum

AAS is used to diagnose many pathological conditions, like diabetes and primary aldosteronism, by detecting the presence of various inorganic elements (Ca, Ma, Na, and K) in blood serum.

The sample solution of the serum is prepared by diluting the serum 10, 20 or 50 times in the presence of lanthanum chloride. This element helps in reducing the possibility of under-estimation of calcium due to phosphate suppression. Then, the test solutions are aspirated to atomic absorption spectrophotometers to measure their absorbance. The absorbance values obtained from the sample serum solution are then compared with those of aqueous standard solutions. Solution of lanthanum chloride is used for calcium and magnesium estimation.

By operating the atomic absorption spectrophotometer in the emission mode, presence of sodium and potassium in blood serum can also be measured. By emission method, sodium and potassium can be easily determined in sera at dilutions of 50 or 100:1.

5.4.8. Determination of Lead in Petrol

Two anti-knocking additives, tetraethyl and tetramethyl lead are found in petrol. The presence of lead in petrol can be determined by AAS using the **direct** and **indirect methods**. The direct method is preferred if the analytical chemist has knowledge about the components (tetraethyl or tetramethyl lead) to be analysed or what mixture of the two has been included in the sample to be analysed. If the analytical chemist has no information about the nature of lead additive, the indirect method is preferred. In this case, tetraethyl and tetramethyl lead are destroyed and extracted into aqueous phase. Both the direct as well as indirect methods are discussed below:

1) **Direct Method:** The presence of lead in petrol can be determined by using this method if the nature of the anti-knock additive is known. Following **steps** are included in this method:
   i) The standard solutions, having tetraethyl and tetramethyl lead (known concentrations), are prepared in cyclohexanone. If the tetramethyl lead is being estimated, the standard solutions should possess 0-5µg/ml lead whereas for the estimation of tetraethyl lead, the standard solutions should possess 0-50µg/ml lead.
   ii) Then, the standards are aspirated to atomic absorption spectrophotometer at wavelength 2833Å using air-acetylene. The response curves are prepared between the absorbance and concentration (µg/ml) for the solutions of tetraethyl lead, tetramethyl lead, and mixture of these two (**figure 5.20**).
   iii) The sample of petrol is diluted with cyclohexanone and aspirated then in the absorption spectrophotometer at 2833Å. The absorption value is noted and compared with the values on the standard curves. In this way, the content of lead in the petrol can be measured.

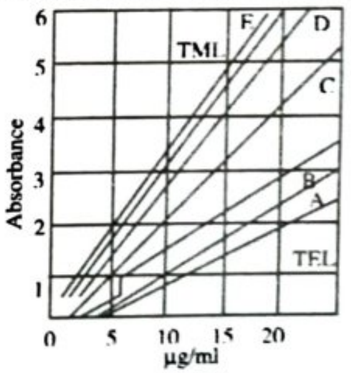


Figure 5.20: Typical Response Curves for TEL/TML

2) **Indirect Method:** This method is used when the analytical chemist has no information about the nature of the lead additive. This method involves the following **steps**:

i) Standard lead samples having 50, 25, 10 and 5µg/ml are prepared using deionised water. Then, the standard samples are aspirated into the atomic absorption spectrophotometer at 2833Å and the absorbance of each standard sample is recorded.

ii) Further, the petrol sample is treated with bromine to convert lead into lead bromide, which is extracted with dilute nitric acid. The obtained extract is introduced to the atomic absorption spectrophotometer and the absorbance is recorded.

iii) In the last step, the lead content in the petrol sample is determined by using the following expression:

$$\text{Pb as } \mu g/ml \text{ (wt/wt) present in petrol} = \frac{S_2 - S_1}{S_0 - S_2} \times C \times \frac{1}{10 \times \text{s.g. of original sample}}$$

Where, S_0 = Scale reading for blank, i.e., 1% nitric acid.
S_1 = Scale reading for sample extract.
S_2 = Scale reading for standard.
C = Concentration of standard lead (µg/ml) (wt/wt)
s.g. = Specific gravity.

5.5. SUMMARY

The details given the chapter can be summarised as follows:

1) **Allan Walsh**, in **1955**, was the pioneer for the introduction of Atomic Absorption Spectroscopy (AAS).
2) The underlying **principle of AAS** is the absorption of energy by ground state atoms while they are in the gaseous form.
3) The **ground state** atom absorbs light energy of a specific wavelength as it enters the **excited state**.
4) In AAS, as the number of atoms in the **light path increases**, the amount of light absorbed also increases.
5) **Principal quantum number** (n) defines the shell in which the electron is located and is thus a measure for the relative distance of the electron shell from the nucleus.
6) Within the shells the electrons reside in orbitals (wave functions) of differing symmetry that are described by the **angular momentum quantum number** (l) which can take values of 0. l, 2,....., n l.
7) The **interaction of radiation** and matter is the basis of spectroscopy.
8) **Radiation** can be either absorbed by matter or emitted by matter.
9) The **absorption spectra** in atomic spectroscopy are relatively simple compared to those in molecular spectroscopy.
10) **Grotrian diagrams** are used extensively in atomic absorption and atomic emission.
11) The **minimum possible half-width** is termed the **natural line width**.
12) Spectral lines also undergo broadening due to the random thermal movement of the atoms and this movement can be described by the **Maxwell distribution**.
13) **Detection limit** may be defined as the concentration (mcg/ml) of an element that gives rise in the shifting of absorbance signal to an amount which equals to the peak-to-peak noise of the base-line.
14) **Sensitivity** may be defined as the concentration of element present in the sample solution that produces 1% absorption.

15) A rotating wheel, known as **chopper**, is interposed between the hollow cathode lamp and the flame.
16) In atomic absorption measurements, the most common monochromators are **prisms** and **gratings**.
17) For AAS, the **photomultiplier tube** is a most suitable detector.
18) The most widely used light source used in AAS is the **hollow cathode lamp**.
19) The light from the **source lamp** is divided into a **sample beam**, which is focused through the sample cell, and a **reference beam**, which is directed around the sample cell.
20) The major **disadvantage of a single beam atomic absorption spectrophotometer** lies in its very **low stability**.

5.6. EXERCISES

5.6.1. True or False

1) Allan Walsh, in 1955, was the pioneer for the introduction of atomic absorption spectroscopy.
2) The underlying principle of AAS is the absorption of energy by ground state atoms while they are in the solid form.
3) The ground state atom absorbs light energy of a specific wavelength as it enters the excited state.
4) In AAS, as the number of atoms in the light path increases, the amount of light absorbed decreases.
5) Principal quantum number (n) defines the shell in which the electron is located.
6) Within the shells the electrons reside in orbitals (wave functions) of differing symmetry that are described by the angular momentum quantum number (l), which can take values of 0. l. 2,....., n l.
7) Radiation cannot be either absorbed by matter or emitted by matter.
8) The absorption spectra in atomic spectroscopy are relatively simple compared to those in molecular spectroscopy.
9) Grotrian diagrams are used extensively in atomic absorption and atomic emission.
10) Sensitivity may be defined as the concentration of element present in the sample solution that produces 7% absorption.
11) For AAS, the photodiode array is a most suitable detector.
12) The most widely used light source used in AAS is the hollow cathode lamp.

5.6.2. Fill in the Blanks

13) The _____ atom absorbs light energy of a specific wavelength as it enters the excited state.
14) The interaction of _____ and matter is the basis of spectroscopy.
15) _____ can be either absorbed by matter or emitted by matter.
16) The _____ in atomic spectroscopy are relatively simple compared to those in molecular spectroscopy.
17) _____ are used extensively in atomic absorption and atomic emission.
18) The minimum possible half-width is termed the _____.

19) _____ may be defined as the concentration of element present in the sample solution that produces 1% absorption.

20) A rotating wheel, known as _____, is interposed between the hollow cathode lamp and the flame.

21) For AAS, the _____ is a most suitable detector.

22) The most widely used light source used in AAS is the _____.

23) The major disadvantage of a single beam atomic absorption spectrophotometer lies in its very _____.

Answers

1) True	2) False	3) True
4) False	5) True	6) True
7) False	8) True	9) True
10) False	11) False	12) True
13) Ground state	14) Radiation	15) Radiation
16) Absorption spectra	17) Grotrian diagrams	18) Natural line width
19) Sensitivity	20) Chopper	21) Photomultiplier tube
22) Hollow cathode lamp	23) Low stability	

5.6.3. Very Short Answer Type Questions

1) Define atomic absorption spectroscopy
2) Give the principle of AAS.
3) Define atomic spectra.
4) What is the common radiation sources used in AAS?
5) Define quantum numbers.
6) What are grotrian diagrams?

5.6.4. Short Answer Type Questions

1) Write a short note on doppler effect.
2) Define detection limit and sensitivity.
3) Explain flame atomisers
4) Write a note on single beam atomic absorption spectrophotometer.
5) Write a note on double beam atomic absorption spectrophotometer.

5.6.5. Long Answer Type Questions

1) Give the interference of AAS.
2) Demonstrate the instrumentation of AAS.
3) Briefly write about AAS.

CHAPTER 6

Nepheloturbidometry

6.1. NEPHELOTURBIDOMETRY

6.1.1. Introduction

Passing light through moderately stable suspensions shows that some portion of the incident radiant energy dissipates due to absorption, refraction, and reflection, while the remaining portion gets transmitted. The concentration of dispersed phase alters the optical characteristic of each suspension. Intensity of the light transmitted through such suspensions is measured where the dispersed phase concentration forms the base of turbidimetric analysis.

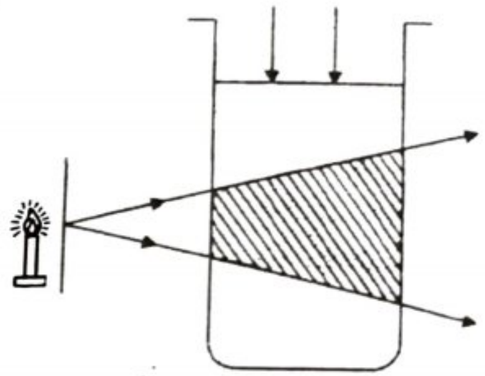


Figure 6.1: Tyndall Effect

On viewing the above mentioned suspension at 90° (i.e., right angles) to the direction of the incident light (**figure 6.1**), it appears opalescent due to reflection of light by the suspension particles. This phenomenon of light scattering is termed as the **Tyndall effect**. The opalescence or cloudiness occurs because of the irregularly and diffusely reflected light from the suspension. Thus, the intensity of the scattered light is measured that forms a true representation for the dispersed phase's actual concentration. This serves the basis of nephelometric analysis (derived from the Greek word ***nephele*** meaning **cloud**). For very dilute suspensions with concentration less than 100mg/l, nephelometric analysis is proved to be sensitive and effective. Observation reveals that turbidimetric analysis resembles flame photometry technique and nephelometric analysis technique is similar to fluorimetry.

6.1.2. Theory

Turbidimetry measures the degree of attenuation of an incident radiant beam falling on suspended particles in a medium. This measurement is carried out under the directly transmitted beam. Thus, turbidity (T) may be expressed as:

$$T = \frac{1}{\ell} \cdot \ell n \cdot \frac{I_o}{I_t}$$

Where, T = Turbidity.
ℓ = Length of dispersion through which the light passes.
I_o = Intensity of incident light.
I_t = Intensity of transmitted light.
n = Refractive index of the dispersion medium.

The I.P. states **Turbidance** (S) as a **measure of the light scattered by the suspended particles**; and **Turbidity** (r) as a **measure of the decrease in intensity of incident beam per unit length of a given suspension.**

Nephelometry is the measure of the scattered light from suspended particles at right angles (perpendicular) to the incident beam. Turbidimetry or nephelometry could aid to measurement of the precipitate produced as a result of the interaction of very dilute reagent solutions, or other particular matter; **for example,** measuring the concentration of colloidal dispersion of organic and inorganic compounds and suspensions of bacterial cells by microbial assays.

The **experimental parameters** observed during the formation of suspensions having uniform characteristic features are:

1) Extremely dilute suspensions of bacterial cells are used to avoid the problems due to birefringence,
2) Concentrations of the two ions combining to form a precipitate, besides the concentration ratio in the solutions mixed,
3) Details of procedure including the order and rate of mixing,
4) Concentration of other salts and substances present, like protective colloids, e.g. dextrin, gelatin, gum Arabica, etc., and
5) Temperature.

6.1.3. Principle

Nephelometry and turbidimetry are techniques resembling colorimetry, since its theory and equipment are applied to these techniques with little modifications. Nephelometry and turbidimetry rely on light scattering by non-transparent particles suspended in a solution. Difference between the two techniques lies in the measurement of the scattered radiation. When light passes through a suspension, some part of the radiant energy is dissipated through absorption, reflection, and refraction, while the other part is transmitted. **Turbidimetric analysis** utilises the concept where intensity of the transmitted light is measured as a function of the suspended particles concentration **(figure 6.2)**.

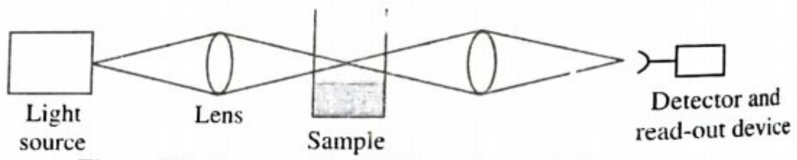


Figure 6.2: Concept of Turbidimetric Analysis

In **nephelometry**, the light directly passes through the sample solution carrying suspended particles. The radiation scattered by the particles at right angles to the incident beam is measured. So the basic principle of nephelometry is measuring the intensity of the scattered light which provides concentration of the dispersed phase **(figure 6.3)**.

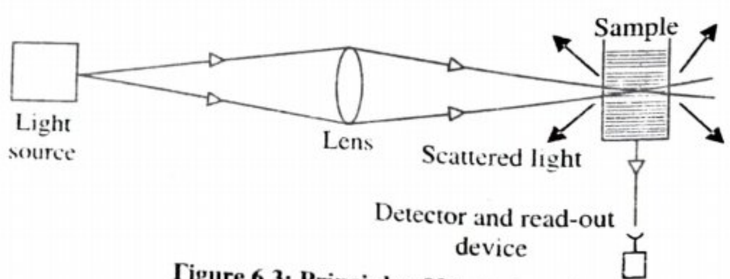


Figure 6.3: Principle of Nephelometry

6.1.4. Instrumentation

Much of the instrumentation used in nephelometry and turbidimetry is very similar to spectrophotometric devices. The components of a nephelometer and turbidimeter are:

1) Sources,
2) Detectors, and
3) Cells.

6.1.4.1. Sources

White light could be used in nephelometers but preference is given to **monochromatic radiation** which is also used in turbidimeters to minimise absorption. Sources that give high intensity monochromatic radiation is a better choice and also employment of short wavelengths may increase the Rayleigh scattering efficiency. The most convenient source is a **mercury arc** or a **laser** along with suitable filter combinations that isolates one of its emission lines.

For determining the concentration of a specific entity, a **polychromatic source** (e.g., a tungsten lamp) could be a better option. Employing the blue spectral region along with filters to block other wavelengths, provides better results.

6.1.4.2. Detectors

Nephelometers use **photomultiplier tubes** since scattered radiation intensity is very small. The detector is fixed at 90° to the primary beam in nephelometers. In order to obtain maximum versatility and sensitivity, the detector angle is changed and positioned close to the primary beam. Also certain nephelometers contain detector mounted on a circular disc (the outer edge of this disc has graduated marks which is readable from the outside), allowing measurement at different angles like at 0° and from 30-135°.

In turbidimeters, ordinary detectors such as **phototubes** may be used.

6.1.4.3. Cells

Cylindrical cells with flat faces are used for facilitating the entry and exit of beams. This also reduces the reflections and multiple scatterings from the cell walls. Preference is given to a cell having rectangular cross-section. **Semi-octagonal cells (figure 6.4)** are employed for measurements at angles other than 90°. The octagonal face **(figure 6.5)** aids in measurements at 0°, 45°, 90°, or 135° to the primary beam. To restrict the passage of light beam through a wall, it is painted dull black so that it absorbs unwanted radiation and minimises stray radiation.

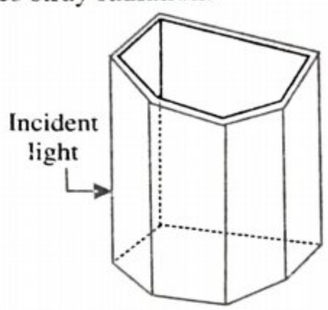


Figure 6.4: Semi-Octagonal Cells

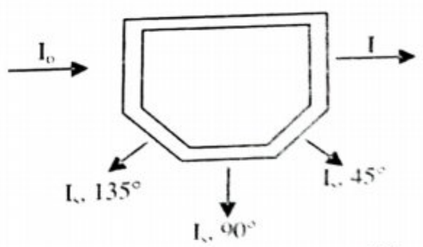


Figure 6.5: The Octagonal Face

In **experimental cells**, a black curved horn is fixed to the wall opposite to the beam entering so that the entire unscattered beam is trapped. A light trap can also be used for fitted beam trapping purpose in the cell of the chamber in which the cell is located.

6.1.4.4. Nephelometers

Nephelometric measurements are made by ordinary fluorimeters. For certain instance spectrophotometers can also be used as nephelometers.

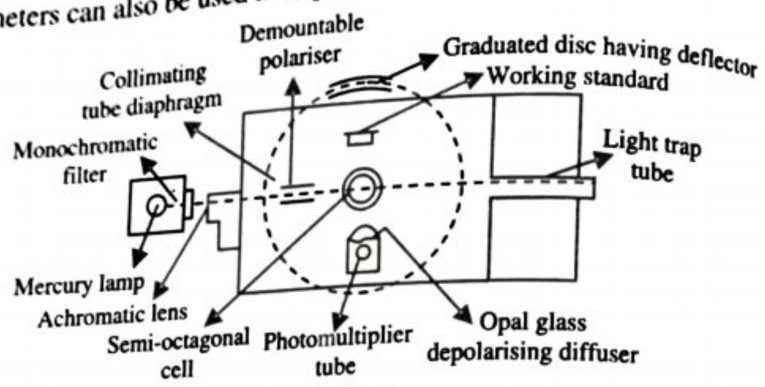


Figure 6.6: Nephelometer
Source: Adapted from *Instrumental Methods of Chemical Analysis* (pp 2.395), by Chatwal G.R. (2006) (Himalaya Publishing House)

A precision nephelometer (**figure 6.6**) functions at low intensities. The photomultiplier tube acting as a receiver is fitted on a turn-table positioned at desired angles from 0-180° relative to the exit beam. However, most nephelometric measurements are carried out at 45° or 90° to the primary beam. The black tube is used for trapping the undeviated beam, hence called a **light trap**. **Figure 6.6** depicts a nephelometer that determines the particle size, shape, and molecular weight along with nephelometric measurements.

Figure 6.6 shows another modification to the nephelometer suggested by **Debye** in which the detector and turn-table are situated in closed compartment below the cell. Radiation scattered by the cell gets intercepted through a small right-angle prism, which is also linked to the turn-table, and reflects downward to the photomultiplier tube upon opening of floor shutter.

6.1.4.5. Turbidimeters

For turbidity measurements, ordinary colorimeters or spectrophotometers can be used. Simple visual instruments like the **Parr turbidimeter** or the **Duboscq colorimeter** can also be used. But the turbidimeter of interest is **DuPont model 430 (figure 6.7)**. This is more sensitive to low concentrations of suspended particles than an ordinary turbidimeter. This is a double beam instrument which depends for its operation on the relative degree of polarisation of transmitted and scattered light.

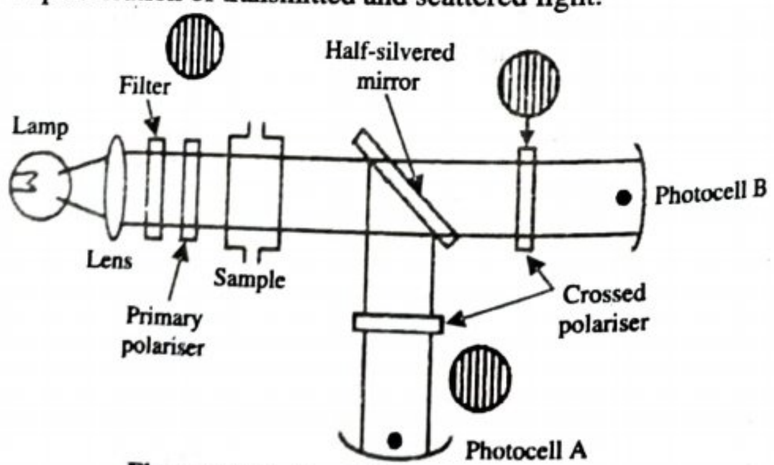


Figure 6.7: DuPont Model 430 Turbidimeter
Source: Adapted from *Instrumental Methods of Chemical Analysis* (pp 2.394), by Chatwal G.R. (2006) (Himalaya Publishing House)

The principle of DuPont model 430 turbidimeter states that the particles suspended in a solution cause scattering and changes the plane of polarisation of light. The lamp provides a light beam that passes through the primary polariser, making the incident beam to be plane-polarised. This plane-polarised light passes through the sample where the half-silvered mirror divides the beam into two parts. These beams are then detected with two separate photocells. Sample solution with no suspended particles shows maximum response by the photocell A; while for the sample solutions with suspended particles, the photocell B gives minimum or zero response. The measure of the concentration of suspended particles is taken to be the ratio of signal B to signal A. Any increase in the sample concentration of suspended particles causes the photocell B response to increase and photocell A to decrease. Hence, the ratio of two signals gives a sensitive measure of turbidity.

The double beam arrangement of DuPont model 430 turbidimeter is a **beneficial property** since it minimises the absorption by the solution particles. Individual samples can be analysed by this instrument along with online monitoring of flowing streams. Additional features like insensitivity to colour of solvent, particles, and to lamp fluctuations may provide accuracy. **Limitation** of DuPont model 430 turbidimeter is that it cannot be used with solutions of optically active substances.

6.1.5. Applications

Turbidimetry and nephelometry are useful for analysis of gaseous, liquid or solid samples (transparent in nature) in varying proportions. Applications of both these techniques are stated below:

1) **Inorganic Analysis:** If precipitates cannot be filtered due to small sized particles or gelatinous nature, gravimetric operations cannot be performed. In such cases, nephelometry and turbidimetry are employed in which the precipitates are converted into ideal suspension under controlled conditions. This is done due to the fact that light scattering depends on particle size, number and concentration.

In nephelometry or turbidimetry analysis for quantitative determination, calibration curves are prepared using samples of known metal concentrations. These calibration curves give results for suspensions of unknown concentrations.

Nephelometry and turbidimetry determine sulphate (as $BaSO_4$), carbonate (as $BaCO_3$), chloride (as AgCl), fluoride (as CaF_2), cyanide (as AgCN), calcium (as oxalate or oleate), and zinc (as ferrocyanide). Sulphate determination is most significant and provides regular estimation of total sulphur content in coke, coal, oils, rubbers, plastics, and other organic substances. Sulphur needs to be converted into sulphate for its determination. Then sodium chloride solution is added and shaken with the sulphate. Excess of solid barium chloride is added to yield barium sulphate suspension, which is then analysed by nephelometry or turbidimetry method. At last, calibration curve is prepared to determine the concentration of suspension.

Nephelometry and turbidimetry are also used for carbon dioxide determination by bubbling the gas through the alkaline solution of barium salt, and analysing the barium carbonate suspension.

Nephelometry and turbidimetry methods are advantageous than colourimetry methods due to their precision and sensitivity. **For example**, phosphorus is determined at a concentration of 1 part in more than 300 million parts of water in precipitate form with strychnine-molybdate reagent. Also, ammonia at a

concentration of 1 part in 160 million parts of ammonia can be estimated
Nessler's reagent.

2) **Organic Analysis:** Turbidimeter is used to determine the turbidity in sugar pro
(like food and beverages) and to determine the clarity of citrus juices. It is also
for detection of benzene in alcohol by dilution with water to produce an immis
suspension.

3) **Biochemical Analysis:** Turbidimetry measures the growth of a test bacterium
broth medium. This method aids in estimation of amount of amino acids, vitam
and antibiotics. Nephelometry detects protein, yeast, glycogen, and β- and γ-glob
in blood serum and plasma.

4) **Turbidimetric Titrations:** These titrations are performed in a manner simila
photometric titrations. Here absorbance is plotted against the titrant volume. W
rise in titrant volume, the concentration of precipitate rises and thereby absorba
also increases. Upon complete precipitation of substance, the absorbance becom
constant. This results in an abrupt change in the slope denoting the end po
(**figure 6.8**).

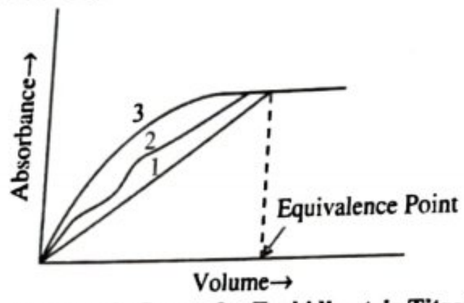


Figure 6.8: Curves for Turbidimetric Titrations

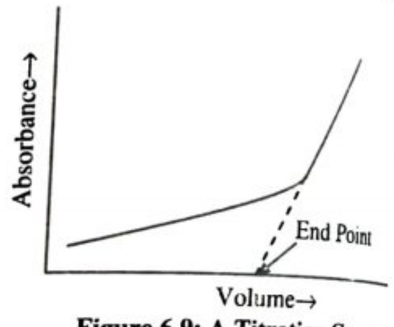


Figure 6.9: A Titration Curve

Figure 6.8 shows curve 1 which is ideal, and curves 2 and 3 are obtained from
precipitates resulting from mixed particle size, poor stirring, etc. In the latter
curves, detection of end point is difficult to obtain. Turbidimetric titration has
formal range of 10^{-5} to 10^{-6} with an average relative error of ±5% or more. T
apparatus used for turbidimetric titrations is very simple. It bears a light source and
photocell on the opposite side of the titration vessel.

Examples of turbidimetric titrations are titration of fluoride with calcium, bromi
with silver, and sulphate with barium. Through turbidimetric titration, silica
estimated in the approximate concentrate range of 0.1-150ppm SiO_2.

5) **Phase Titrations:** Turbidimetry is employed for titrating a mixture of two liquid
with a third liquid that is miscible with one of the two liquids. Upon introducing
adequate quantity of the third liquid, separation of phase occurs, causing turbidity
For result interpretation, either one should have knowledge about the three
component phase diagram or should titrate the unknown with known mixtures
Figure 6.9 shows a titration curve of water-pyridine mixture with chloroform.

6) **Determination of Molecular Weights of High Polymers:** The molecular weights
of macromolecules can be determined by measuring the intensity of light scattered
by polymer solutions. The turbidity of a macromolecule sol is related to its molecular
weight through the following relation:

$$\lim_{C \to 0} \frac{HC}{\text{Turbidity}} = \frac{1}{M}$$

Where,

H = A polymer constant for a dis

Turbidity = Fraction of incident light scattered per cm length of the solution through which it passes.

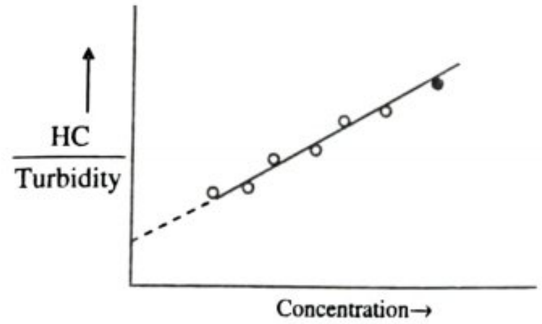


Figure 6.10: A Plot of HC/Turbidity *versus* Concentration

To estimate the molecular weight of a polymer, turbidity is measured at varied concentrations of polymer solution in a solvent. The plot of HC/turbidity *versus* concentration is extrapolated to zero concentration (**figure 6.10**), and the intercept gives the value for 1/M.

6.2. SUMMARY

The details given in the chapter can be summarised as follows:

1) The phenomenon of light scattering is termed as the **Tyndall effect**.
2) **Turbidimetry** measures the degree of attenuation of an incident radiant beam falling on suspended particles in a medium.
3) The I.P. states **Turbidance** (S) as a **measure of the light scattered by the suspended particles**; and **Turbidity** (r) as a **measure of the decrease in intensity of incident beam per unit length of a given suspension**.
4) **Nephelometry** and **turbidimetry** are techniques resembling to colorimetry, since its theory and equipment are applied to these techniques with little modifications.
5) **Nephelometry** and **turbidimetry** rely on light scattering by non-transparent particles suspended in a solution.
6) In **nephelometry**, the light directly passes through the sample solution carrying suspended particles.
7) In nephelometry the most convenient source is a **mercury arc** or a **laser** along with suitable filter combinations.
8) Nephelometers use **photomultiplier tubes** since scattered radiation intensity is very small.
9) **Cylindrical cells** with flat faces are used for facilitating the entry and exit of beams.
10) **Nephelometric** measurements are made by ordinary fluorimeters
11) Simple visual instruments like the **Parr turbidimeter** or the **Duboscq colorimeter** can also be used for measurement of turbidity.

6.3. EXERCISE

6.3.1. True or False

1) The phenomenon of light scattering is termed as the Tyndall effect.
2) Turbidimetry measures the degree of attenuation of a reflected radiant beam falling on suspended particles in a medium.
3) Nephelometry and turbidimetry are techniques resembling to colorimetry.

4) Nephelometry and turbidimetry do not rely on light scattering by non-transparent particles suspended in a solution.
5) In nephelometry, the light directly passes through the sample solution carrying suspended particles.

6.3.2. Fill in The Blanks

6) The phenomenon of light scattering is termed as the _____.
7) _____ measures the degree of attenuation of an incident radiant beam falling on suspended particles in a medium.
8) In _____, the light directly passes through the sample solution carrying suspended particles.
9) In nephelometry the most convenient source is a _____ or a laser along with suitable filter combinations.
10) Nephelometers use _____ since scattered radiation intensity is very small.
11) _____ with flat faces are used for facilitating the entry and exit of beams.
12) _____ measurements are made by ordinary fluorimeters.

Answers

1) True 2) False 3) True
4) False 5) True 6) Tyndall effect
7) Turbidimetry 8) Nephelometry 9) Mercury arc
10) Photomultiplier tubes 11) Cylindrical cells 12) Nephelometric

6.3.3. Very Short Answer Type Questions

1) Define tyndall effect.
2) What is turbidance?
3) Define nephelometry.
4) What are the detectors used in nephelometry?
5) Give the theory of turbidimetry.

6.3.4. Short Answer Type Questions

1) Give the principle of Nephelometry.
2) Write a short note on nephelometers and turbidimeters.
3) Write about the cells used in nepheloturbidimetry.

6.3.5. Long Answer Type Questions

1) Give the instrumentation of nephelometry and turbidimetry.
2) Give the applications of nephelometry and turbidimetry.

CHAPTER 7

Introduction to Chromatography

7.1. CHROMATOGRAPHY

7.1.1. Introduction

Chromatography is a combination of laboratory techniques that are used for **separating the mixture components**. Chromatography deals with a sample (or sample extract) dissolved in a mobile phase (a gas, liquid, or a supercritical fluid), which is allowed to move through an immobile and immiscible stationary phase. Selection of phases is done on the basis of solubility of components of the sample in each phase.

A component moves slowly through the stationary phase if it is soluble in it compared to the component which is less soluble in the stationary phase but highly soluble in the mobile phase. These differences in mobilities aid in the separation of sample components from each other while passing through the stationary phase.

Chromatography could be either preparative or analytical. **Preparative chromatography** separates the mixture components for further use (thus is a type of purification). **Analytical chromatography** requires small amounts of material and determines the relative proportions of mixture analytes. Both these techniques lack mutual exclusivity.

7.1.2. Related Terms

The various technical terminologies related to chromatography are:

1) **Analyte:** It is a component that gets separated during chromatography.
2) **Analytical Chromatography:** This technique determines the concentration of analyte(s) in a sample.
3) **Chromatogram:** It is a visual interpretation of chromatograph. In optimal separation, different peaks related to different components of a mixture are obtained on the chromatogram.
4) **Chromatograph:** It is an equipment used for sophisticated separation, **e.g.,** separation via gas or liquid chromatography.
5) **Effluent:** It is the mobile phase coming out of the column.
6) **Immobilised or Stationary Phase:** It is a phase covalently bonded on supporting particles or on the inside wall of column tubing. It involves a solid or liquid fixed in a position to achieve chromatographic separation, **e.g.,** silica layer is used in thin layer chromatography as the stationary phase.
7) **Mobile Phase:** It moves in a specific direction. It could be a liquid (used in liquid chromatography and cation exchange chromatography), gas (used in gas chromatography), or a supercritical fluid (used in supercritical-fluid chromatography).
8) **Preparative Chromatography:** It is a non-destructive technique used for purifying sufficient quantities of a substance that can be further used.
9) **Retention Time:** It is the time taken by an analyte to pass through the system (from the column inlet to the detector) under definite conditions.

10) **Sample:** It is the single component or mixture of components analysed in chromatography. When the sample is being analysed, the phase or the phases holding the desired components (or analytes) are referred to as the sample, whereas the non-desirable separated components from the sample (prior to or during the analysis) is called waste.

11) **Solute:** It is the sample components in partition chromatography.

12) **Solvent:** It is the medium solubilising other substances, like liquid mobile phase in LC.

7.1.3. Classification

Chromatography consists of a stationary phase (solid or liquid) and a mobile phase (liquid or gas). Separation of components takes place as a result of combined effect of two or more factors, i.e., migration rate, capillary action, extent of adsorption, etc. These factors rely on stationary and the mobile phase employed. Chromatography methods are categorised as per these two phases.

Table 7.1: Modern Classification of Chromatography Techniques

Classes	Examples	Abbreviations
Adsorption chromatography	Columnar method	LC
	Gas solid chromatography	GSC
Partition chromatography	Liquid-liquid partition chromatography	LLPC
	Paper chromatography	PC
	Thin layer chromatography	TLC
	Reverse phase partition or extraction chromatography	RPPC
Ion exchange chromatography	Cation exchange chromatography	CEC
	Anion exchange chromatography	AEC
	Inorganic exchange chromatography	IE
	Liquid exchange chromatography	LIE
	Ion chromatography	LAE/LCE
Exclusion chromatography	Gel-permeation chromatography	GP
	Ion exclusion chromatography	IExd.
	Molecular sieve chromatography	MS
Electro chromatography	Zone electrophoresis	ZE
	Boundary layer method	BLE
	Curtain chromatography	CC
	Capillary electrophoresis	CZE

7.1.4. General Principles

Principle of chromatography involves separation of components into varied bands (or colour graphs) and their identification. Preferential separation occurs based on the differential affinities of compounds for stationary and mobile phase. Upon separation, the compounds are identified by different methods of detection. The variation in affinities among components occurs due to **relative adsorption** or **partition coefficient** in both the phases.

7.1.4.1. Adsorption

Adsorption is based on the polarity of components with respect to the stationary phase. If the stationary phase and the sample component are polar, the polar component will migrate slowly, separate from the sample, and will be the last one to pass out of the column. In the same manner, if the stationary phase and sample component are non-polar.

the non-polar component will migrate slowly in the column under the influence of mobile phase and will be the last one to leave the column. Hence, the stationary and mobile phases are opposite, i.e., polar stationary phase, non-polar mobile phase and *vice-versa*. The solvent used in the column as mobile phase is called **eluent**. The liquid reaching the column end with the separated components is called the **eluate**.

7.1.4.2. Partition

Distribution of compounds among immiscible phases is termed **partition** or **distribution coefficient** (K_d). It is the basis of separation by chromatographic methods. For a compound that distributes itself between two immiscible solvents (A and B) of equal volumes, the partition coefficient at a given temperature is constant. It is stated by the following equation:

$$\frac{\text{Concentration in Solvent A}}{\text{Concentration in Solvent B}} = K_d$$

The test compound separates between the two immiscible solvents and two phases (solid/liquid or gas/liquid phases). **For example,** a substance distributed between silicic acid and benzene has a partition coefficient of 0.5, meaning the concentration of substance in benzene is twice than that in silicic acid.

Effective partition coefficient is the net amount (different from the concentration) of substance in one phase divided by the net amount in the other phase. It may also be defined as the partition coefficient multiplied by the ratio of the volumes of both the phases; **for example,** if a compound having partition coefficient 1 and distributed among solvents A and B is equilibrated between $10cm^3$ of A and $1cm^3$ of B, the compound in solvent A will be 10 times greater than that in solvent B.

Partition depends on the components solubility in different liquids; hence, mobile and stationary phases should be liquid. The liquid stationary phase is a thin film on a solid support in the column. Thus, if the components of a sample mixture have differences in their solubility in different liquids and are of same nature (i.e., either polar or non-polar), they split into two liquid phases (i.e., mobile and stationary phases). This is based on their partition coefficient between two liquids.

7.1.5. Theories of Chromatography

There are two theories of chromatography depending on the migration rate of solute and the development of peaks in the chromatogram:

1) Plate theory, and
2) Rate or kinetic theory.

7.1.5.1. Plate Theory

Martin and **Synge** proposed the plate theory as per which a column used in chromatography is made up of **theoretical plates** (**figure 7.1**) arranged in a series of different but continuous horizontal layers. Solute equilibrium between the stationary and mobile phases occurs at each plate. Solute migration occurs from one plate to another, present just below, by a sequential step by step transfers.

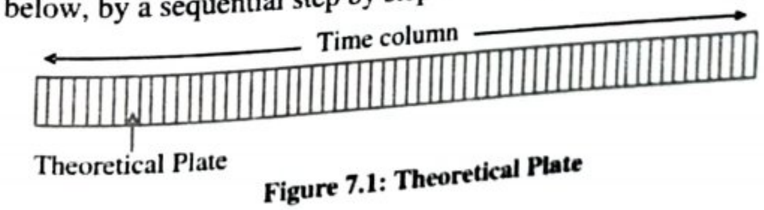


Figure 7.1: Theoretical Plate

By increasing the number of theoretical plates (n) in the column, the establishment of equilibrium also increases, which further enhances separation efficiency. Thus, the column efficiency can be indicated by the number of theoretical plates. If the length of column is '**l**' and the height equivalent of a theoretical plate is '**h**', then '**n**' is given by:

$$n = \frac{l}{h} \qquad \ldots(1)$$

Height Equivalent of a Theoretical Plate (HETP) is the height of a column layer, such that the solution leaving the layer is in equilibrium with the average concentration of the solute in the stationary phase throughout the layer.

The **number of theoretical plates** in a real column can be determined by examining a chromatographic peak after elution.

$$N = 5.55\ t_R^2/w_{1/2}^2$$

$$N = \frac{5.55 t_R^2}{w_{1/2}^2}$$

Where, $w_{1/2}$ = Peak width at half-height.

This equation denotes that columns behave as if they have different numbers of plates for different solutes in a mixture.

7.1.5.2. Rate or Kinetic Theory

Optimisation of chromatography is required for facilitating complete separation of all the sample components in minimum time. This can be done by modifying the mobile phase composition, by choosing a different stationary phase, or by altering the flow rate. A typical chromatogram is shown in **figure 7.2**. The sample is fed into the chromatographic column at t = 0s. Substances not retarded by the stationary phase pass out of the column at zero retention time (t_0) corresponding to the flow rate of mobile phase. Compounds A and B are retarded by the stationary phase and pass out of the column at their retention times $t_R(A)$ and $t_R(B)$, respectively. Peak width (w) is the intersection of the tangents on each side of the peak with the baseline. Retention time and peak width are the basic parameters used to derive other parameters that define the quality of chromatographic separation.

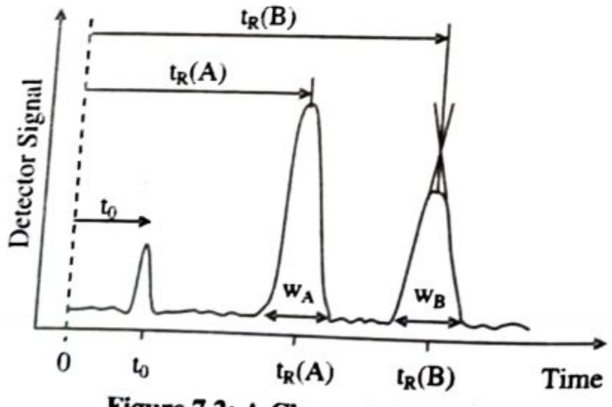


Figure 7.2: A Chromatogram

Capacity factor (k′) (equation 2) **is the velocity of the analyte relative to the velocity of the mobile phase**. Each compound interacts with the mobile and stationary phases for a different amount of time. The average velocity of a sample compound depends on the time for which it interacts with the mobile phase. If $k' >> 1$, the analyte moves quickly

and the elution time is so short that t_R cannot be determined precisely. If the sample moves slowly, the separation time is very high. Capacity factor is considered to be good if its value is between 1 to 5.

$$\text{Capacity factor, } k' = \frac{t_R - t_0}{t_0} \qquad \ldots..(2)$$

Selectivity factor (α) (equation 3) is the relative velocities of the analytes with respect to each other. Selectivity indicates how a chromatographic method can distinguish between two analytes.

$$\text{Selectivity factor, } \alpha = \frac{k'_B}{k'_A} = \frac{t_R(B) - t_0}{t_R(A) - t_0} \qquad \ldots..(3)$$

Parameters influencing band broadening can be estimated by the **Van Deemter equation** (equation 4). This equation is valid for gas and liquid chromatography, and also capillary electrophoresis.

$$\text{Van Deemter equation } H = A + \frac{B}{u} + C \cdot u \qquad \ldots..(4)$$

In **equation (4)**, the height of theoretical plates (H) is given as the summation of three terms. The first term (A) describes the influence of column packing on band broadening. This is called **Eddy diffusion**, which is constant for a given column and is independent of the flow rate. The second term (B/u) describes diffusion either towards or opposite to the direction of flow. This longitudinal diffusion and the flow rate (u) are inversely proportional. The third term (C·u) describes resistance to mass transfer between the stationary and mobile phases, which is directly proportional to the flow rate. From the plot of H against u, the optimum flow rate for a chromatographic separation can be determined **(figure 7.3)**.

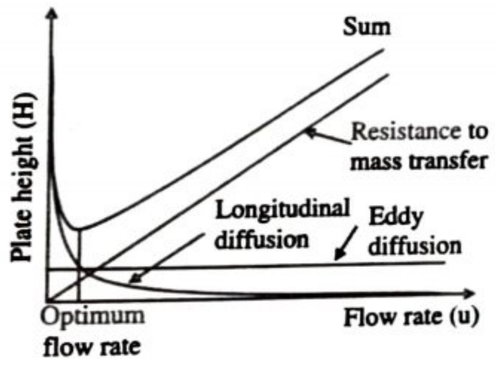


Figure 7.3: Van Deemter Plot for Determining the Optimum Flow Rate

Separation mainly aims to achieve a high resolution (R_s) (equations 5 and 6). If $R_s = 1.5$, peaks of identical area overlap by 0.3%, and if $R_s = 1$, peaks of identical area overlap by 4%. Peak resolution can be optimised by increasing the selectivity and reducing the band broadening.

$$\text{Resolution, } R_s = \frac{2.[t_R(A) - t_R(B)]}{w_A + w_B} \qquad \ldots..(5)$$

$$R_s = \frac{\sqrt{N}}{4}(\alpha - 1)\left[\frac{k'}{1 + k'}\right] \qquad \ldots..(6)$$

(Valid for $\alpha < 1.2$)

The equation (6) denotes that capacity factor (k′) greatly influences the resolution. The k′ values vary widely in the sample components. On optimising the conditions in such a way that the compounds to be eluting first have k′ values between 1 to 5, the other compounds with higher k′ values will elute later and show excessive band broadening. On the other hand, if conditions are optimised for the compounds eluting later, the resolution for the compounds eluting first will be poor. This elution problem can be solved by minimising k′ during the separation.

In liquid chromatography, the mobile phase composition can be changed during the separation. This is called **gradient elution**, which is just the opposite of **isocratic elution**, in which the mobile phase composition remains constant during the separation. In gas chromatography, a temperature gradient can be applied during separation rather than operating under isothermic conditions.

The first step to achieve a good separation of the sample mixture involves selecting a stationary phase for the analyte to interact. The next step involves selecting the composition and gradient of mobile phase to optimise the capacity factor and resolution.

7.1.6. Retardation Factor

The chromatographic activity of the solute can be described in terms of retardation factor or retention ratio (R). The **ratio of migration velocity of a solute to the migration velocity of the mobile phase** is termed the retardation factor (**R value**). It may be regarded as the fraction of the total time ($t_m + t_s$) spent by the solute in the mobile phase (where, t_m and t_s are the times spent by the solute in the mobile and stationary phases, respectively).

$$R = \frac{t_m}{t_m + t_s} \quad \ldots\ldots (7)$$

The ratio t_m/t_s is similar to Q_m/Q_s; where, Q_m and Q_s = quantities of solute in the mobile and stationary phases, respectively:

$$\frac{t_m}{t_s} = \frac{Q_m}{Q_s} \quad \ldots\ldots (8)$$

If C_s and C_m = solute concentrations in the stationary and mobile phases, respectively:

$$\frac{t_m}{t_s} = \frac{C_m \times V_m}{C_s \times V_s} \quad \ldots\ldots (9)$$

Where, V_m and V_s = volumes of mobile and stationary phases, respectively. On introducing the value of partition coefficient (K) in equation (9):

$$\frac{t_m}{t_s} = \frac{V_m}{KV_s} \quad \ldots\ldots (10)$$

On re-arranging equation (10):

$$R = \frac{t_m}{t_m + t_s} = \frac{V_m}{V_m + KV_s} \quad \ldots\ldots (11)$$

Therefore, with an increase in partition coefficient, the retardation factor (R) for a given V_s and V_m decreases. To achieve an efficient separation, the retardation factor can be changed by adjusting the adsorbent-solvent combination and other operating factors.

7.1.7. Retention Volume

Retention volume for a solute is the **volume of mobile phase required to carry the solute peak value through the column to elution**. It is also the fraction of time the solute spends in the mobile phase. When a solute peak at the column exit is maximum, one half of the total solute has eluted in the retention volume (V_R) and the other half remains in the mobile and stationary phases, i.e.,

$$V_R C_m = V_R C_m + V_s C_s \quad \ldots\ldots (12)$$

Or $V_R = V_m + KV_s$

$$\therefore \frac{V_m}{V_R} = \frac{V_m}{V_m + KV_s} = R \quad \ldots\ldots (13)$$

If the time taken (t) by a solute peak to appear at the column exit is termed the column length (L), the migration velocity of the solute is L/t.

7.1.8. Column Capacity

Column capacity (k) is the **ratio of the amount of solute in the mobile and stationary phases**, i.e.,

$$k = \frac{C_s V_s}{C_m V_m} \quad \ldots\ldots (14)$$

The ratio of the volumes of two phases is termed the **volumetric ratio (β)**.

$$\beta = \frac{V_m}{V_s} \quad \ldots\ldots (15)$$

$$\therefore k \times \beta = \frac{C_s}{C_m} = K \quad \ldots\ldots (16)$$

$$\text{And, } k = K/\beta \quad \ldots\ldots (17)$$

Relationship between column capacity (k) and R value is given by:

$$R = \frac{1}{(1-k)} \quad \ldots\ldots (18)$$

Relationship between column capacity (k) and retention volume (V_R) is given by:

$$k = \frac{V_R - Vm}{Vm} \quad \ldots\ldots (19)$$

7.1.9. Development of Chromatogram

The following three methods are used for developing chromatograms:

1) **Frontal Analysis:** This method of elution analysis was developed by **Teselius**. In this method (preparative technique), the sample solution is passed through the adsorbent packed in a column. While travelling through the column, the solutes having high affinity for the adsorbent occupy the active sites of adsorbent column; and the solutes having low affinity are weakly adsorbed or are accumulated in the migrating solvent front. Consequently, the minimum adsorbed solute first elutes out of the column, followed by the other solutes based on their degree of adsorption.

   For better understanding of frontal analysis, a mixture of two samples (A and B) is continuously introduced in the adsorbent packed column. This results in complete adsorption of the sample and saturation of the adsorbent. On further adding the

sample solution, A (the least adsorbed component) elutes out first in pure form, accompanied by the mixture of A+B (the strongly adsorbed component) that elutes out slowly. Retention volume (V_R) is used to determine the concentration of A in mobile phase and the concentration of other components can be determined from the volume-concentration ratio (**figure 7.4**).

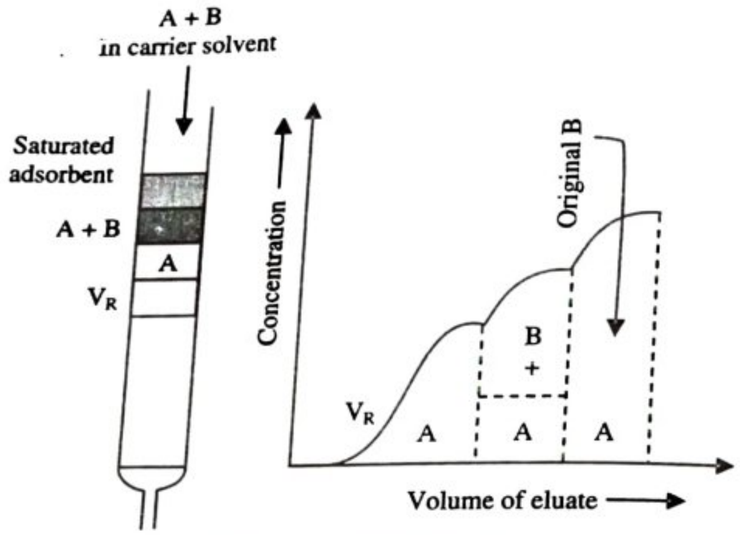


Figure 7.4: Frontal Analysis

In frontal analysis, the component which elutes out fast has low affinity for the adsorbent, and thus is separated from the component having high affinity. This method is used for eliminating traces of strongly adsorbed unwanted substances, while retaining the least adsorbed desirable components.

2) **Elution Analysis:** This method is widely used for developing chromatograms. In this method, small amount of sample solution is added on the column top. The pure solvent (eluent) is made to run down the column. This leads to differences in the migration of solutes in mobile phase. Different solutes elute out of the column at different rates depending on their partition coefficient. Differences in partition coefficient of the solutes in a sample lead to separation of mixtures that appear as bands or zone.

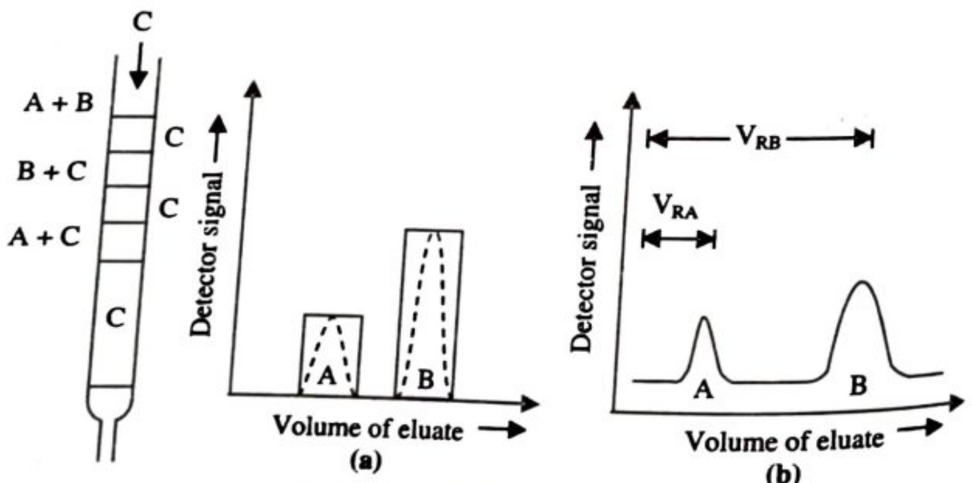


Figure 7.5: Elution Analysis

For better understanding of elution analysis, a small amount of mixture (of A and B components) is added at the column top. A mobile phase (having lower affinity than the components for the stationary phase) is made to run through the column.

A small fraction of column capacity is represented by the loading in column. The migration rate of components is slower than that of the eluent, depending on their affinity for the stationary phase. The components elute out on the basis of their degree of affinity but their migration depends on the mobile phase. The components completely separate from each other by eluting out with another intermediate zone or band of mobile phase (eluent C). This elution method can be improved by changing the eluent after a specific time period. **For example,** in stepwise elution, a combination of eluents (with increased eluting powers) is used to elute out the components having greater affinity for the stationary phase and also to aid their movement throughout the system.

Gradient elution is a technique of separating the components (having variable affinity for the stationary phase) by changing the eluent composition. The eluting power of mobile phase to be used in this technique can be increased by changing the ratio of two or more eluting components. The linear gradient of solvent composition increases or decreases depending on the concentration, pH, polarity, or ionic strength.

3) **Displacement Analysis:** In this method, a small amount of sample solution is introduced at the column top. The mixture components are separated by the downward flow of **displacing agent**, which is a solution of a substance having stronger affinity than the other components for the stationary phase.

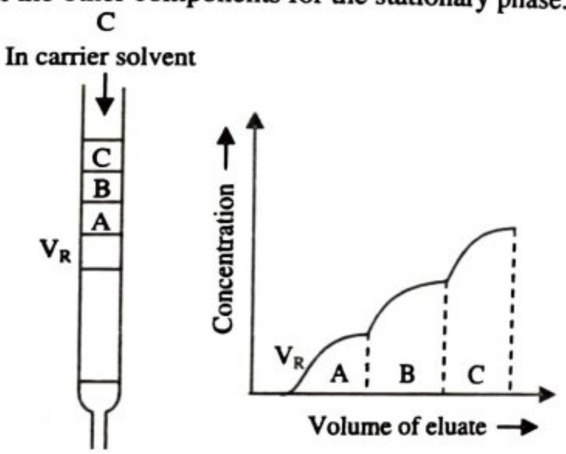


Figure 7.6: Displacement Analysis

Column adsorbs the displacing agent and forms a zone or band at the top. This band moves downwards and displaces out the sample components from the adsorption sites. Thus, the component bands are formed farther from the band of displacing agent. The components continuously displace each other from the column. The component having minimum affinity will first elute out of the column, followed by the elution of other components depending on their adsorption degree. Displacement analysis has an **advantage** that the column can be heavily loaded. The amount of sample solution added in the column is termed **loading**. However, the **disadvantage** of displacement analysis is that after one flow the displacing agent saturates the column.

For better understanding of displacement analysis, an eluent (C) that adsorbs on the stationary phase more strongly than the sample components (A and B) is used. A sample of mixture (of A and B components) is introduced at the column top, where it gets adsorbed on the stationary phase. Then the displacing agent (C) is made to run through the column. This agent while moving down the column displaces the components (A and B) from the stationary phase and elutes them out. This leads to

separation of components depending on the differences in their partition coefficient and adsorption. Displacement analysis does not give complete separation components as the zones of pure components have regions of mixtures between the that can be collected for further treatment.

7.1.10. Chromatogram Visualisation

The chromatogram formed by elution can be visualised by:

1) Retention factor (R_F), and
2) Prediction of column volumes (CV).

7.1.10.1. Retention Factor (R_F)

Retention factor is analysed for evaluation purpose if a system carries sufficient solvent. Retention factor (R_F) is the **distance travelled by the compound divided by the distance travelled by the solvent front**. This indicates that the distance travelled by the compound and the R_F value of the same compound are directly proportional to each other. This also indicates that when R_F values of different compounds are compared under the same chromatographic conditions, the compound with larger R_F value is less polar due to its weak interaction with the polar adsorbent on the plate.

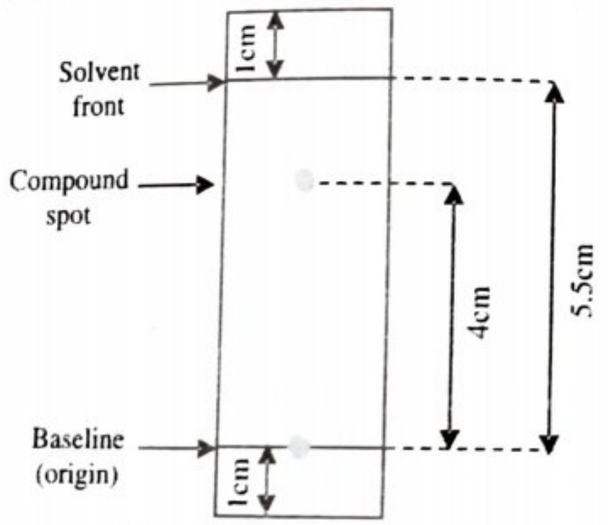


Figure 7.7: Detection of R_F Value

$$\text{Retention Factor } (R_F) = \frac{\text{Distance Travelled by the Compound}}{\text{Distance Travelled by the Solvent Front}}$$

Calculation of R_F value based on an **example**:

$R_F = 4.0\text{cm}/5.5\text{cm} = 0.73$

The **ideal value for R_F** is considered to be **0.2-0.4**. If all the components move such a distance from the baseline that the R_F values lay between 0.15 and 0.85, the solvent system used is considered adequate. If not so, another solvent system is used.

7.1.10.2. Prediction of Column Volumes (CV)

Elution from the column can be determined by TLC depending on the relationship between the retention factor (R_F) and Column Volume (CV). The **number of column volumes required to elute the component from the column**, regardless of its dimensions is termed as CV, i.e., [(Bed Volume) – (Volume of Packing)].

$$CV = 1/R_F$$

And, $$\Delta CV = 1/R_{F1} - 1/R_{F2}$$

Greater the value of ΔCV, easier will be the separation and resolution between the spots. This also allows loading the column with more sample.

7.2. SUMMARY

The details given in the chapter can be summarised as follows:

1) Chromatography is a combination of laboratory techniques that are used for **separating the mixture components**.
2) **Preparative chromatography** separates the mixture components for further use.
3) **Analytical chromatography** requires small amounts of material and determines the relative proportions of mixture analytes.
4) **Analyte** is a component that gets separated during chromatography.
5) **Chromatogram** is a visual interpretation of chromatograph.
6) **Chromatograph** is equipment used for sophisticated separation.
7) **Stationary phase** is a phase bonded covalently on supporting particles or on the inside wall of column tubing.
8) **Mobile phase** moves in a specific direction.
9) **Retention Time** is the time taken by an analyte to pass through the system (from the column inlet to the detector) under definite conditions.
10) **Principle of chromatography** involves separation of components into varied bands (or colour graphs) and their identification.
11) **Adsorption** is based on the polarity of components with respect to the stationary phase.
12) The solvent used in the column as mobile phase is called **eluent**.
13) The distribution of compounds among immiscible phases is termed **partition** or **distribution coefficient (K_d)**.
14) **Capacity factor (k′)** is the velocity of the analyte relative to the velocity of the mobile phase.
15) **Selectivity factor (α)** is the relative velocities of the analytes with respect to each other.
16) In liquid chromatography, the mobile phase composition can be changed during the separation which is called **gradient elution.**
17) The ratio of migration velocity of a solute to the migration velocity of the mobile phase is termed the **retardation factor (R_F value)**.
18) **Column capacity (k)** is the ratio of the amount of solute in the mobile and stationary phases.
19) **Elution analysis** is the method which is widely used for developing chromatograms.
20) **Gradient elution** is a technique of separating the components by changing the eluent composition.

7.3. EXERCISE

7.3.1. True or False

1) Chromatography is a combination of laboratory techniques that are used for separating the mixture components.
2) Analytical chromatography separates the mixture components for further use.
3) Analyte is a component that gets separated during chromatography.
4) Chromatogram is equipment used for sophisticated separation.
5) Stationary phase is a phase bonded covalently on supporting particles or on the inside wall of column tubing.
6) Mobile phase moves in a specific direction.
7) Retention time is the time taken by a chromatograph to pass through the system under definite conditions.

8) Adsorption is based on the polarity of components with respect to the stationary phase.
9) The distribution of compounds among miscible phases is termed partition or distribution coefficient.
10) Column capacity is the ratio of the amount of solute in the mobile and stationary phases.
11) Gradient elution is a technique of separating the components by changing the eluent composition.

7.3.2. Fill in the Blanks

12) _____ requires small amounts of material and determines the relative proportions of mixture analytes.
13) _____ is a component that gets separated during chromatography.
14) _____ is equipment used for sophisticated separation.
15) _____ is a phase bonded covalently on supporting particles or on the inside wall of column tubing.
16) _____ moves in a specific direction.
17) _____ is the time taken by an analyte to pass through the system (from the column inlet to the detector) under definite conditions.
18) _____ is based on the polarity of components with respect to the stationary phase.
19) The solvent used in the column as mobile phase is called _____.
20) _____ is the velocity of the analyte relative to the velocity of the mobile phase.
21) _____ is the ratio of the amount of solute in the mobile and stationary phases.
22) _____ is a technique of separating the components by changing the eluent composition.

Answers

1) True 2) False 3) True
4) False 5) True 6) True
7) False 8) True 9) False
10) True 11) True 12) Analytical chromatography
13) Analyte 14) Chromatograph 15) Stationary phase
16) Mobile phase 17) Retention time 18) Adsorption
19) Eluent 20) Capacity factor 21) Column capacity
22) Gradient elution

7.3.3. Very Short Answer Type Questions

1) Define chromatography.
2) What is a chromatogram?
3) Define stationary phase.
4) What to do you understand by mobile phase?
5) Define retention time.

7.3.4. Short Answer Type Questions

1) Give the principle of chromatography.
2) Classify chromatography.
3) Write a note on retardation factor.
4) Write a note on column chromatography.

7.3.5. Long Answer Type Questions

1) Give the theories of chromatography.
2) Briefly explain development of chromatogram and chromatogram visualisation.

CHAPTER 8

Column Chromatography

8.1. COLUMN CHROMATOGRAPHY

8.1.1. Introduction

Column chromatography is a separation technique in which the **stationary bed** (phase) is placed in a tube. The solid stationary phase particles or the solid support coated with liquid stationary phase cover the inner entire volume of the packed column. The stationary phase may also remain concentrated along the inside wall of the tube. This creates an unobstructed open path in the mid portion of the tube, thus forming an open tubular column for the mobile phase.

In column chromatography, the **mobile phase** is a gaseous or liquid mixture solution, which is allowed to flow via column for separating the components of a mixture. The different flow rates of the mixture components through the tube facilitate their separation. However, this can be determined by the degree up to which each of them is delayed due to their interaction with the stationary phase.

The modified version of column chromatography, termed **flash column chromatography**, was developed in **1978** by **W.C. Still**. This technique is somewhat similar to the conventional chromatography with the only difference that the solvent flows through the column under the influence of a positive pressure, thus separating the components in less than 20 minutes. A flash column chromatography system consists of pre-packed plastic cartridges to help in pumping the solvent. This system is also connected to detectors and fraction collectors to provide automatic processing. Gradient pumps are also connected to facilitate rapid separation and minimum usage of solvent.

8.1.2. Principle

Column chromatography works on the principle of adsorption of the solutes of a solution through a stationary phase, thus leading to the separation of mixture into its individual components (**figure 8.1**). This relies on the affinity towards the mobile phase and stationary phase. The molecules having high affinity towards the stationary phase elute later, while the ones having less affinity elutes first.

In column chromatography, the desired components are isolated from a mixture, allowed to flow through the top of the column that is made up of glass or plastic with sinter frits to hold the packing. The liquid solvent flows down through the column packed with adsorbent, under the effect of gravitational force or air pressure. The liquid obtained at the bottom of column is termed **eluent**. Equilibrium is established between the adsorbed solute and the solvent flowing down through the column.

In column chromatography, **adsorbents** (substances that cause the adherence of passing molecules or ions to the surface of its particles) are most commonly used as **stationary phases**; and a **solvent** that flows through the stationary phase and dissolves the molecules of the compounds to be separated is the **mobile phase**. Different mixture components have different interactions with the stationary and mobile phases, thus the mobile phase carries them to varying degrees to achieve separation. The individual components obtained are termed **elutants**, and are collected as solvent drips from the bottom of the column.

Chromatogram is the banded column of the adsorbent. When a substance occupies a particular area of the column, that area is termed the **zone**. Narrower the zones, more is the number of substances that can be separated in a column of particular length, thus, more concentrated elutes are obtained.

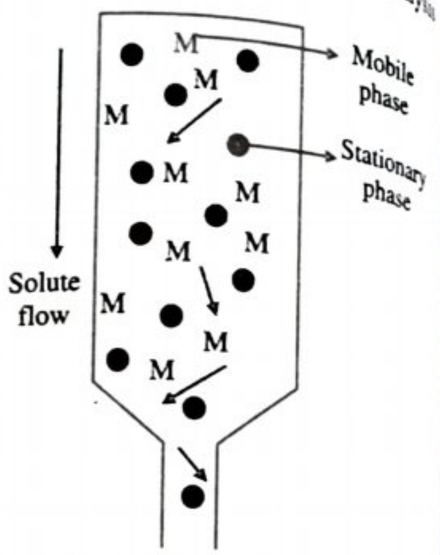


Figure 8.1: Separation by Column Chromatography

The following **two procedures** can be applied for determining the separated components:

1) After development, the adsorbent column is pushed out of the tube. The different zones are cut with a knife at the boundaries and the components present in them are extracted using a suitable solvent. This process of obtaining the components from the chromatogram is termed **elution**.
2) After development, the column is washed with a suitable solvent. When each component reaches the column end and is released out, it is collected separately.

8.1.3. Theory

The sample solution of concentration (C) is made to pass through the adsorbent column and 'n' gram of the sample is adsorbed per gram of the adsorbent.

$$n = f(C)$$

The solvent volume taken for the adsorption of 'n' grams of the sample is the **retention volume** (R):

$$R = f(C)/(C)$$

Separation of sample is achieved by dissolving the sample in a suitable solvent and passing through the column packed with adsorbent. To achieve effective separation, elution is carried out using the same solvent. The molecules with high affinity adsorb on the first layer of the stationary phase and those with less affinity adsorb on the last layer of the stationary phase. The separation process of a mixture of two substances (A and B) is represented in **figure 8.2**.

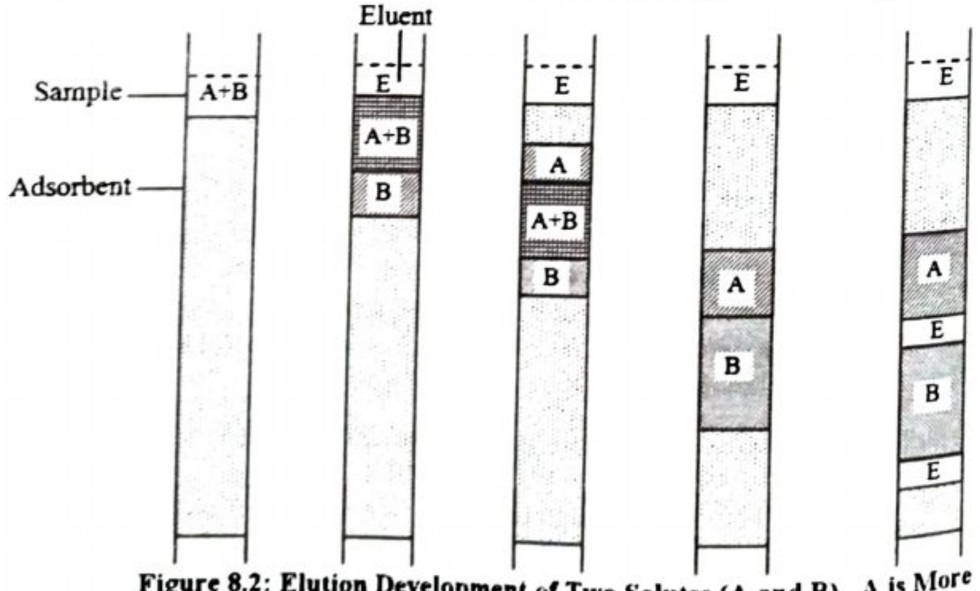


Figure 8.2: Elution Development of Two Solutes (A and B). A is More Strongly Adsorbed than B; E is the Eluent

8.1.4. Types of Column Chromatography

Column chromatography is of the following four types:

1) Adsorption column chromatography,
2) Partition column chromatography,
3) Ion exchange chromatography, and
4) Gel chromatography.

8.1.4.1. Adsorption Column Chromatography

In adsorption column chromatography, the mixture components get selectively adsorbed on the surface of the material packed in column (i.e., the adsorbent).

In this technique, the mixture components travel towards the stationary phase at different rates due to differences in their affinity. Adsorption indicates a physical attachment between a compound and the stationary phase particles. The polar compounds being separated adsorb to the polar stationary phase with more intensity, while the non-polar compounds adsorb to the non-polar stationary phase. Therefore, when a polar stationary phase is used, the polar components due to greater adsorption elute out later while the non-polar one elute out first. On the contrary, when a non-polar stationary phase is used, the reverse happens.

8.1.4.2. Partition Column Chromatography

In partition column chromatography, the mixture components distribute between the liquid stationary and mobile phases held on an inert solid support.

In this technique, the mixture components distribute between the two liquid phases due to the differences in their partition coefficients during the flow of mobile phase in the chromatography column. Since both the stationary and mobile phases are liquid in nature, the molecules disperse into either of the phases. The polar molecules get partitioned into the polar phase, while the non-polar ones get partitioned into the non-polar phase.

In partition chromatography, the commonly used stationary phases are diatomaceous earth (kieselguhr, celite, etc.), cellulose, and silica gel. However, glass beads, brick powder, starch, or purified sand can also be used limitedly.

8.1.4.3. Ion Exchange Chromatography

In ion-exchange chromatography, the ions and polar molecules get separated based on their charge. This technique can be used for any type of charged molecules like large proteins, small nucleotides, and amino acids. The solution to be injected is termed **sample**, and each separated component is termed an **analyte**. Ion-exchange chromatographic technique is used in protein purification, water analysis, and quality control.

8.1.4.4. Gel Chromatography

In gel chromatography, the column is packed with a permeable gel, which helps in separating the substances by molecular filtration or sieving action.

8.1.5. Methodology

The column chromatography following steps procedure includes:

1) Selection of column,
2) Selection of adsorbents and solvents,
3) Preparation of column,
4) Application of sample,
5) Elution procedures, and
6) Detectors.

8.1.5.1. Selection of Column

The chromatographic columns having stopcocks, loaded with a spring, and fitted with a sintered glass disc at the bottom can be commercially obtained. They can also be made of less costly glass tubes and old burettes. Good separation can be achieved by keeping the column's length to breadth ratio at 8:1 or 5:1. The stopcock attached to the column is used to control the solvent flow rate. A porous plate or small plug of glass wool is used to provide support to the adsorbent packed in the column. During the separation process, the flow of neutral gas (**e.g.,** nitrogen) should be maintained to prevent the substance from getting oxidised in the presence of air. Large sized columns are available in larger diameter (cm) and a greater length (m) than the small sized columns, which have a smaller diameter (mm) and length (cm).

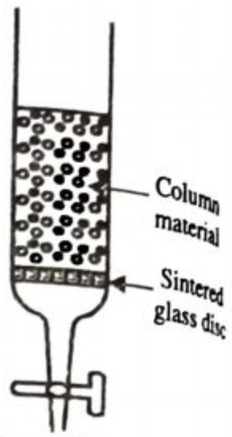


Figure 8.3: Adsorbent Used in Column

The chromatographic columns are divided into the following **types** based on the principle of mobile phase movement:

1) **Gravity Columns:** In these columns, the mobile phase moves through the stationary phase under the influence of gravitational force.
2) **Flash Columns:** In these columns, the mobile phase is pushed by a stream of air or inert gas using the adaptors.
3) **Pumped Columns:** In these columns, the mobile phase is moved by using the pumps generated at low or medium pressures.
4) **Vacuum Columns:** In these columns, the mobile phase is moved under the influence of vacuum.
5) **HPLC Columns:** In these columns, the mobile phase is pushed through the stainless steel columns.

8.1.5.2. Adsorbents and Solvents

Adsorbents

Silica gel and **alumina** are commonly used as adsorbents (stationary phase). Sometimes **florisil** is also used as an adsorbent. An **ideal adsorbent** should possess the following **characteristics**:

1) It should have an appropriate size and shape.
2) It should be mechanically stable.
3) It should be chemically inert.
4) It should have neutral surfaces.

Table 8.1 enlists the adsorbents commonly employed in column chromatography:

Table 8.1: Adsorbents Used in Column Chromatography

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

The amount of sample that gets adsorbed on an adsorbent is determined by mixing the known amount of adsorbent with the known volume of solvent at a constant temperature till equilibrium is achieved. Thereafter, the above solution is filtered and the amount of sample adsorbed per gram of adsorbent is plotted against the concentration.

The following curves can be seen in the plot (**figures 8.4, 8.5, and 8.6**):

1) **Linear Adsorption Isotherm:** This curve appears when the adsorbed substance and the solution concentration are directly proportional.
2) **Convex Adsorption Isotherm:** This curve appears when the weak concentration solution gets adsorbed.
3) **Concave Adsorption Isotherm:** This curve appears when the strong concentration solution gets adsorbed.

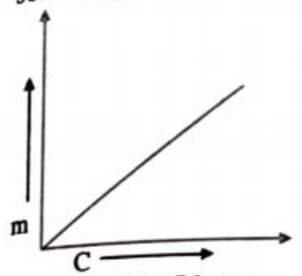


Figure 8.4: Linear Adsorption Isotherm Curve

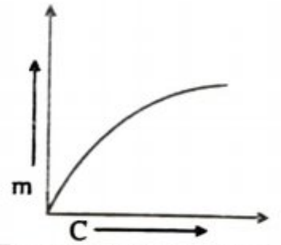


Figure 8.5: Convex Adsorption Isotherm Curve

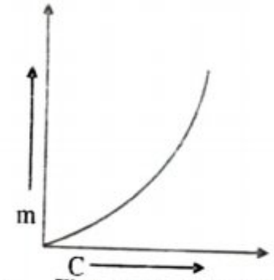


Figure 8.6: Concave Adsorption Isotherm Curve

Solvents

The solvents are used to elute the sample by mixing with it. The commonly used solvents are petroleum ether, cyclohexane, carbon tetrachloride, benzene, chloroform, pyridine, acetone, ethanol, methanol, water, and formamide. They are based on solvent polarity.

An ideal solvent should possess the following **functions**:

1) It should be able to introduce the sample into the column.
2) It should be used as developers.
3) It should be removed after each step.

8.1.5.3. Preparation of Column

For appropriate chromatographic separation and to achieve highest efficiency, the column should be packed uniformly with much care and attention.

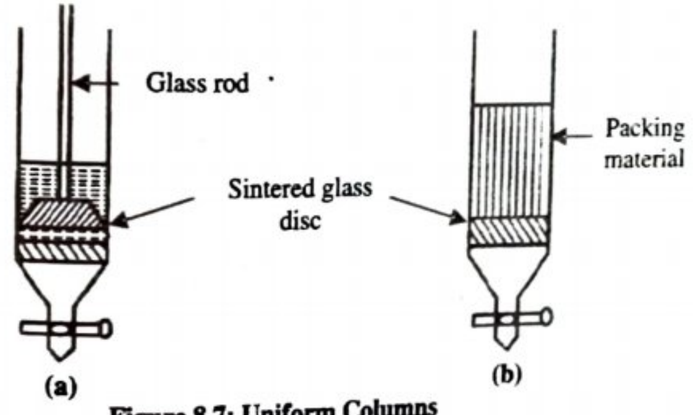


Figure 8.7: Uniform Columns

In **open tubular chromatography** utilising gravity of solvent, the packing particles should be more than 150μm in size to obtain acceptable flow rates. The column should be packed uniformly to reduce the distortion of chromatographic boundaries. Packing of columns should be done carefully to avoid formation of channels due to the entrapment of air bubbles in the column.

The chromatographic **columns are packed** by the following **two methods**.

1) **Wet Packing or Slurry Method:** In this method, the adsorbent and solvent are mixed to obtain a slurry of different nature depending on whether silica gel or alumina is used. This slurry is poured into the prepared column. A piece of glass wool is placed in the bottom of column and is tapped down with a glass rod. The column is attached to a ring stand and secured in a vertical position. After adding a pinch clamp to the bottom of the column, the clamp is closed. Half-length of the column is filled with a non-polar solvent (**e.g.,** hexanes).

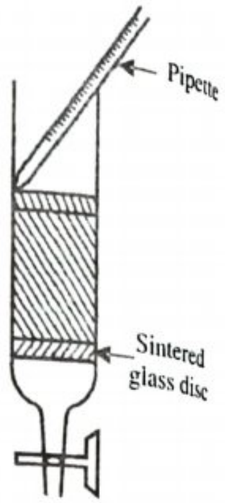


Figure 8.8: Tapping Technique

Alumina is weighed in a beaker. Hexane is placed in a flask and slowly added with alumina powder with swirling. Using a Pasteur pipette, slurry is mixed and quickly pipetted out into the column. The flask is placed under the column, the pinch clamp is opened, and the liquid is allowed to drain into it. This transfer of slurry to the column is continued till all the alumina is added. More hexanes are added (if required). The hexanes collected in the flask can be re-used to add more alumina to the column. After packing, the excess solvent is drained out till it reaches the top level of alumina. The pinch clamp is closed and the column is packed and ready to use. Sometimes, a small amount of sand is added to the top of the column so that it does not get disturbed on the addition of fresh solvent.

2) **Dry Packing:** This method is suitable for a micro-scale column. In this method, the column is filled with a non-polar solvent, followed by the gradual addition of powdered alumina or silica while gently tapping the side of the column using a pencil. The solid should float to the bottom of the column. The column should be packed uniformly so that no cracks, air bubbles, and channels are formed or else proper separation would not be achieved. There is **another dry pack method** in which the stationary phase is deposited in the column before the solvent. In this method, the column is filled with the stationary phase up to the intended height and then gradually added with the non-polar solvent to avoid irregular channelling. This dry method is used with alumina only as silica gel expands and does not pack well.

8.1.5.4. Application of Sample

The sample should be applied uniformly from the top of the column using a small pipette. The tip of this pipette is placed just above the adsorbent surface and opposite to the column wall. The sample can also be applied by a method in which one or two small filter paper discs (present at the column top), saturated with the sample material already dissolved in volatile solvent, are used.

8.1.5.5. Elution Procedures

The following three elution techniques are employed in column chromatography:

1) **Isocratic Elution:** In this elution technique, the solvent mixture of same composition is made to run through the column to achieve separation. The development is stopped when the separated mixture components appear as different coloured bands on the column. Now the column contents are extruded and the components are extracted with a suitable solvent. The bands are isolated using a transparent nylon tube as the column container. After complete development, the tube is cut in small portions to separate the column contents.

A common alternative method is to continue the column to run till the separated components are detected in the eluate (effluent) of the column.

2) **Stepwise or Fractional Elution:** On using a single solvent, some mixture components are only eluted; while the firmly held components can be isolated when a stronger eluting solvent is used. In this elution technique, different solvents of slowly increasing polarity are used for successive replacement of different components. This elution procedure provides sharper separations. But it has a **disadvantage** that a given compound can produce multiple peaks in the elutes of subsequent steps.

3) **Gradient Elution:** This elution technique was first described by **Williams** and **Tiselius**. In this technique, a constantly changing eluting medium is used, and the adsorbed substances (more strong) are sequentially eluted so that tailing is reduced. The eluent composition is continuously changed as per the conditions favouring separation from the chromatography medium. Elution position differs between substances depending on their binding strength. Microprocessor controlled solvent modules are currently available, and can generate the required gradient profile for isocratic, linear or stepwise development.

8.1.5.6. Detectors

Detectors are used for determining and monitoring the dissolved substances from the column. The different types of detectors employed in column chromatography are discussed below:

1) **Optical Detectors:** These detectors are small cells of glass or quartz. They are used as flow analysers for stable photometric analysis with visible or UV light of proper wavelength. If adequate absorption of samples in the required region is not achieved, suitable reagents are added to produce colour reactions that can be photometrically measured.

2) **Differential Refractometers: Claesson** applied the refraction of emerging power for detecting the components. This method however has been improvised to form a sensitive differential refractometer having a limit of detection of 10^6gm.

3) **Detectors Based on Heat of Adsorption (Micro-Adsorption Detectors):** In these detectors, a separating column releases out a liquid that is passed via two cells situated above each other, of which the cell present below contains an adsorbent. The mid packing of each cell has a glass-covering measuring point of the small thermistor. However, the entire deflection (i.e., +ve and –ve peaks) and concentration (up to 10^2 range) are directly proportional.

4) **Flame Ionisation Detectors:** In these detectors, a continuous metal wire passes through the column exit. The substances containing decomposition products (transported via wire) lead to argon or flame ionisation detectors.

5) **Conductivity Detectors:** These detectors are considered suitable for ionised materials in aqueous solution. The effluent is passed through the detector's measuring cell, containing two or three platinum electrodes inside the Wheatstone bridge circuit. Conductivity detectors function by the alternating current.

Some other types of commonly used detectors are microwave detector, ultrasonic detector, mass detector, vapour pressure detector, and solvent front detector.

8.1.6. Factors Affecting Column Efficiency

The column efficiency is affected by the following factors:

1) **Nature of Solvents:** Low viscosity solvents are used for high efficiency separations because the flow rate and viscosity are inversely proportional. Therefore, the selected solvent should be of **lowest viscosity** and **good elution strength**.

2) **Dimensions of Columns:** The column efficiency can be improved by increasing its width to length ratio. **Column to sample packing ratio** ranging from **1:20 to 1:100** is selected for preparative separations.

3) **Particle Size of Column Packing:** The column efficiency can be improved by decreasing the size of adsorbent particles. **Adsorbent particles of size** ranging between **100-200 mesh** are generally selected.

4) **Pore Diameter of Column Packing:** The column efficiency is not affected by decreasing the average pore diameter from **170Å -20Å**. The **pore diameter** of polar adsorbent **≤ 20 Å** is considered appropriate.

5) **Temperature of the Column:** Samples that are difficult to solubilise are separated at higher temperatures, while the other samples are separated at room temperature.

8.1.7. Advantages

Column chromatography has the following advantages:

1) It can be used to separate any type of mixture.
2) It can be used to separate any quantity of mixture.
3) A wide range of mobile phases can be used.
4) The sample can be separated and reused.
5) Automation is also possible.

8.1.8. Disadvantages

Column chromatography has the following disadvantages:

1) It is a time-consuming method.
2) It is an expensive method as it utilises a large amount of solvent.
3) Automation is possible and this makes the technique complicated and more expensive.

8.1.9. Applications

Column chromatography has limited applications in analytical field. Some of its uses are:

1) **Analytical Uses:** Glass or copper capillaries having an internal diameter of 0.05-2mm and length of 1-20m are used for analytical purposes. The narrow tubing's internal surface provides support or serves as an adsorbent for the liquid phase. The glass capillaries having internal surface treated with concentrated ammonia at 300°C temperature help in amino acids separation using pyridine:butanone:dilute acetic acid (5:5:1).

2) **Separation of Geometrical Isomers:** Silica gel and charcoal is used for separating *cis/trans* isomers of carboxylic acids. Their separation depends on steric factors. Those functional groups of isomers that can reach the adsorbent surface more easily get strongly adsorbed on the stationary phase.

3) **Separation of Diastereomers:** Diastereomeric 7-chloro-azibicycl-heptane can be separated by silica gel using pentane/diethyl ether as a solvent.

4) **Separation of Tautomeric Mixtures:** Separation of tautomeric mixtures is carried out at high temperature by column chromatography only; **for example,** the keto- and enol-forms of indolyl pyruvic acid and *p*-hydroxyl-phenyl pyruvic acid are separated in the liquid phase (a weak acidic medium), and the elute contains the enol form prior to the keto form.

5) **Separation of Racemates:** The racemates are isolated using organic solvents on lactose, **e.g.,** separation of Troger's base enantiomers.

8.2. SUMMARY

The details given in the chapter can be summarised as follows:

1) **Column chromatography** is a separation technique in which the stationary bed (phase) is placed in a tube.
2) The **stationary phase** may also remain concentrated along the inside wall of the tube.
3) The **mobile phase** is a gaseous or liquid mixture solution, which is allowed to flow via column for separating the components of a mixture.
4) The **modified version of column chromatography**, termed flash column chromatography, was developed in 1978 by W.C. Still.
5) The **flash column chromatography** systems consist of pre-packed plastic cartridges to help in pumping the solvent.
6) The liquid obtained at the bottom of column is termed **eluent**.
7) In column chromatography, **adsorbents** are most commonly used as **stationary phases**; and a **solvent** that flows through the stationary phase and dissolves the molecules of the compounds to be separated is the **mobile phase**.
8) The individual components obtained are termed **elutants**.
9) **Chromatogram** is the banded column of the adsorbent.
10) When a substance occupies a particular area of the column, that area is termed the **zone**.
11) Narrower the zones, more is the number of substances that can be separated in a column of particular length, thus, more concentrated elutes are obtained.
12) In **adsorption column chromatography**, the mixture components get selectively adsorbed on the surface of the material packed in column (i.e., the adsorbent).
13) In this technique, the mixture components travel towards the stationary phase at different rates due to differences in their affinity.
14) In **partition column chromatography**, the mixture components distribute between the liquid stationary and mobile phases held on an inert solid support.
15) In this technique, the mixture components distribute between the two liquid phases due to the differences in their partition coefficients during the flow of mobile phase in the chromatography column.
16) In **ion-exchange chromatography**, the ions and polar molecules get separated based on their charge.
17) In **gel chromatography**, the column is packed with a permeable gel, which helps in separating the substances by molecular filtration or sieving action.
18) In **gravity columns**, the mobile phase moves through the stationary phase under the influence of gravitational force.
19) In **flash columns**, the mobile phase is pushed by a stream of air or inert gas using the adaptors.
20) In **pumped columns**, the mobile phase is moved by using the pumps generated at low or medium pressures.
21) In **vacuum columns**, the mobile phase is moved under the influence of vacuum.
22) In **HPLC columns**, the mobile phase is pushed through the stainless steel columns.
23) **Silica gel** and **alumina** are commonly used as adsorbents (stationary phase).
24) **Linear adsorption isotherm** curve appears when the adsorbed substance and the solution concentration are directly proportional.
25) **Convex adsorption isotherm** curve appears when the weak concentration solution gets adsorbed.

26) **Concave adsorption isotherm** curve appears when the strong concentration solution gets adsorbed.
27) In **wet packing or slurry method**, the adsorbent and solvent are mixed to obtain slurry of different nature depending on whether silica gel or alumina is used.
28) In **dry packing** method, the column is filled with a non-polar solvent, followed by the gradual addition of powdered alumina or silica while gently tapping the sides of the column using a pencil.
29) In **isocratic elution**, the solvent mixture of same composition is made to run through the column to achieve separation.
30) In **stepwise or fractional elution**, different solvents of slowly increasing polarity are used for successive replacement of different components.
31) In **gradient elution**, a constantly changing eluting medium is used, and the adsorbed substances (more strong) are sequentially eluted so that tailing is reduced.
32) **Optical detectors** are small cells of glass or quartz.
33) In **micro-adsorption detectors**, a separating column releases out a liquid that is passed via two cells situated above each other, of which the cell present below contains an adsorbent.
34) In **flame ionisation detectors**, a continuous metal wire passes through the column exit.
35) **Conductivity detectors** are considered suitable for ionised materials in aqueous solution.
36) The selected solvent should be of lowest viscosity and good elution strength.
37) Adsorbent particles of size ranging between 100-200 mesh are generally selected.
38) The pore diameter of polar adsorbent ≤ 20 Å is considered appropriate.

8.3. EXERCISE

8.3.1. True or False

1) Column chromatography is a separation technique in which the stationary bed is placed in a cell.
2) The individual components obtained are termed elutants.
3) Narrower the zones, more is the number of substances that can be separated in a column of particular length.
4) When a substance occupies a particular area of the column, that area is termed as chromatogram.
5) In adsorption column chromatography, the mixture components distribute between the liquid stationary and mobile phases.
6) In gravity columns, the mobile phase is pushed by a stream of air or inert gas via the adaptors.
7) Convex adsorption isotherm curve appears when the weak concentration solution gets adsorbed.
8) In fractional elution, the solvent mixture of same composition is made to run through the column to achieve separation.
9) In optical detectors, a continuous metal wire passes through the column exit.

8.3.2. Fill in the Blanks

10) The modified version of column chromatography, termed flash column chromatography, was developed in 1978 by _____.
11) The liquid obtained at the bottom of column is termed _____.

12) _____ is the banded column of the adsorbent.
13) In _____ columns, the mobile phase is moved by using the pumps generated at low or medium pressures.
14) In HPLC columns, the mobile phase is pushed through the _____.
15) _____ curve appears when the adsorbed substance and the solution concentration are directly proportional.
16) _____ curve appears when the strong concentration solution gets adsorbed.
17) In _____ elution, different solvents of slowly increasing polarity are used for successive replacement of different components.
18) In _____ elution, a constantly changing eluting medium is used, and the adsorbed substances are sequentially eluted so that tailing is reduced.
19) _____ detectors are considered suitable for ionised materials in aqueous solution.

Answers

1) False 2) True 3) True
4) False 5) False 6) False
7) True 8) False 9) False
10) W.C. Still 11) Eluent 12) Chromatogram
13) Pumped 14) Stainless steel columns 15) Linear adsorption isotherm
16) Concave adsorption isotherm 17) Fractional 18) Gradient
19) Conductivity

8.3.3. Very Short Answer Type Questions

1) What is column chromatography?
2) What is ion exchange chromatography?
3) Define isocratic elution.
4) Give any two factors affecting column efficiency in column chromatography.
5) Give the advantages and disadvantages of column chromatography.
6) Name the detectors used in column chromatography. Write about any one of them.

8.3.4. Short Answer Type Questions

1) Give the theory of column chromatography.
2) Write a note on adsorption and partition column chromatography.
3) Write a note on adsorbents and solvents used in column chromatography.
4) Give the applications of column chromatography.
5) Discuss the methods of column preparation in column chromatography.

8.3.5. Long Answer Type Questions

1) Give the principle and theory of column chromatography.
2) Briefly explain the methodology of column chromatography.
3) Discuss about the types of column chromatography.

CHAPTER 9

Thin Layer Chromatography

9.1. THIN LAYER CHROMATOGRAPHY

9.1.1. Introduction

Thin Layer Chromatography (TLC) is a sub-division of liquid chromatography, in which the mobile phase is a liquid and the stationary phase is applied as a thin layer on a flat plate. TLC and paper chromatography combines to form **planar liquid chromatography** due to the layered stationary phases or the flat surface of the paper.

Mein Hard and **Hall** first planned TLC method in **1949** by using starch as a binder to separate the inorganic ions. **Kirchner** proposed the conventional ascending TLC method. **Izmailov** and **Shraiber** first introduced the working principle of TLC. **Stahl** first designed the standard equipment for TLC, which is similar to that of paper chromatography.

TLC is a simple technique which is used for achieving rapid separation of mixture components. It utilises a sheet of glass, plastic, or aluminium foil on which a thin and uniform layer of adsorbent (silica gel, aluminium oxide, or celiulose) is applied; this adsorbent layer is the stationary phase. The sample to be separated is applied over the stationary phase and a solvent or mixture of solvents (mobile phase) is made to travel upward along the plate through capillary mechanism. Separation occurs when different analytes rise up the plate at different rates.

9.1.2. Principle

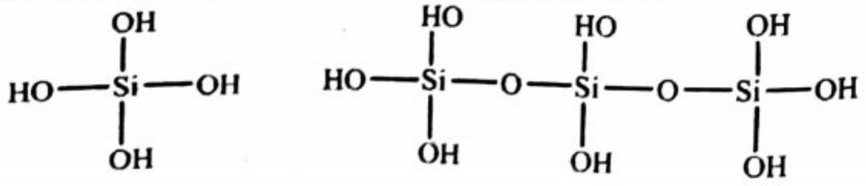
TLC works on the principle that the mobile phase flows through the stationary phase, on which a solid or liquid is supported and the mixture components are separated into individual analytes. This separation occurs depending on the migration of analytes between the stationary and mobile phases. The components having more affinity towards the stationary phase elute later, while those having low affinity elutes first.

The two major types of TLC methods are:

1) **Normal Phase TLC:** In this type, the stationary phase is polar and the mobile phase is non-polar.
2) **Reverse Phase TLC:** In this type, the stationary phase is non-polar and the mobile phase is polar.

9.1.3. Theory

The plastic, glass or aluminium sheet used in TLC method is coated with a layer of silica gel (in the form of silicon dioxide) or alumina powder. The silicon atoms join through the oxygen atoms and form a giant covalent structure (shown below).



The sample solution of the substance to be analysed is taken in a very small amount using a capillary tube, and then applied as a small spot on TLC plate 1cm from the bottom. This plate is developed in a chamber containing the mobile phase, which travels up through the plate by capillary action, dissolves the sample, and moves up based on the intermolecular forces existing between the stationary and mobile phases with the sample components. The free particles completely dissolve in the liquid or gaseous mobile phase. The adsorbed particles bind to the stationary phase when equilibrium is attained between the mobile and stationary phases. This equilibrium depends on:

1) The polarity and size of sample,
2) The polarity of stationary phase molecules, and
3) The polarity of mobile phase.

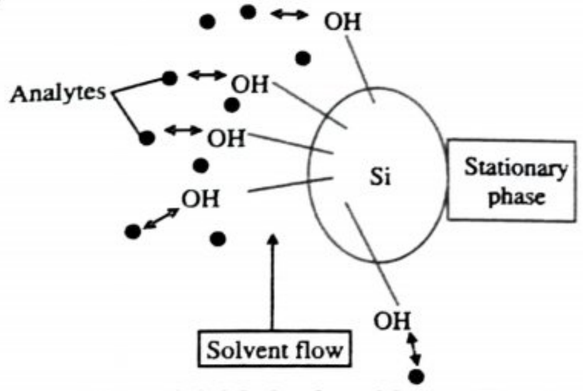


Figure 9.1: Mechanism of Solvent Flow through the Stationary Phase

9.1.4. Advantages

TLC combines the advantages of paper and column chromatography, and in some cases is also superior to them. The main advantages of TLC are:

1) **Simple Equipment:** It requires simple equipment.
2) **Short Development Time:** A good separation on inorganic adsorbent layers can be achieved rapidly as the development time is only an hour; while paper and column chromatographic techniques require several hours or days for development.
3) **Wide Choice of Stationary Phase:** It can be employed for adsorption, partition (including reversed phase) or ion exchange chromatography.
4) **Early Recovery of Separated Components:** The powdery coating of the plates can be removed by scraping with a knife. This means that a spot can be removed quantitatively, and the required component can be determined by calorimetric or spectrophotometric analysis by dissolving in a suitable solvent.
5) **Separation Effects:** The separation effects in TLC are superior to those obtained by paper chromatography.
6) **Easy Visualisation of Separated Compounds:** The fluorescence compounds can be easily detected under UV light because the inorganic background does not fluoresce.
7) **Sensitivity:** Extremely sharp delineated spots are obtained, thus spray reagents can easily detect even small quantities in comparison to paper chromatography in which more diffused spots can only be detected. This increased sensitivity is of the order of 10 to 100 folds than that of paper chromatography.
8) **Variable Thickness of Thin Layers:** The method used in TLC analysis can be applied for the preparative separation using thicker layers as well as to most separations by column chromatography.

9) **Chemically Inert Stationary Phase:** The biggest advantage of using an inert stationary phase in TLC is that it affords a vigorous means of detection including the application of strong heat or corrosive reagent, like concentrated H_2SO_4.

9.1.5. Disadvantages

The main disadvantages of TLC are:

1) There is a no longer stationary available in TLC plates, therefore, its separation length is insufficient in comparison to other chromatographic techniques.
2) The results obtained are difficult to reproduce.
3) Only soluble components of the mixtures can be separated.
4) It is not automatic.
5) It works in the open system, thus can get affected by temperature and humidity.

9.2. METHODOLOGY

9.2.1. Introduction

The various steps involved in TLC are:

1) Selection of coating materials or adsorbents,
2) Preparation of plates,
3) Activation of adsorbent,
4) Purification of silica gel G layers,
5) Marking the plate and spotting of the sample,
6) Preparation of the development tank,
7) Selection of solvent system,
8) Plate development,
9) Detection of components - R_f value,
10) Evaluation of the chromatogram, and
11) Recovery of components.

9.2.2. Selection of Coating Materials or Adsorbents

The adsorbent adheres on the TLC plate depending on particle size (1-25µm) and homogeneity. The adsorbents and binders (**e.g.,** gypsum, starch, etc.) are mixed as they do not adhere sufficiently to the glass plates. The **most commonly used binder** along with the adsorbents is **10-15% calcium sulphate** (gypsum).

The adsorbents should be selected based on the following **factors**:

1) Properties of the compounds to be separated,
2) Solubility of the compounds to be separated,
3) Nature of the compounds to be separated (i.e., acidic, basic, or amphoteric), and
4) Compounds' chemical reactivity with the adsorbent (solvent).

The following adsorbents can be employed in TLC:

1) **Inorganic Adsorbents:** Some of the inorganic adsorbents used in TLC are:
   i) **Silica Gel:** Sodium silicate hydrolyses into polysilicic acid, which condenses and polymerises into silica gel. Although, silica gel is available with or without binder, still 10% w/w binder is added to it for enhancing the adhesion of adsorbent layer to the plate and also for providing mechanical strength to the layer. Silica gel added with gypsum binder (calcium sulphate hemi-hydrate) is termed **silica gel G**. TLC utilises silica gel having mean particle size of 15µm and a particle size range of 5-40µm.

Amino acids, alkaloids, fatty acids, lipids, steroids, essential oils, sugars, terpenoids, etc. are separated using silica gel. The binders and other additives used along with silica gel are:

Types of Silica	Binders and Other Additives Added
Silica gel G	10% gypsum (calcium sulphate) as the binder
Silica gel H	Silicon dioxide or aluminium oxide binder
Silica gel F	Fluorescent indicator added, a subscript indicates the wavelength
Silica gel P	Preparative TLC
Silica gel R	Specially purified adsorbent
Silica gel R.P.	A silanized gel for reverse-phase work

   ii) **Alumina (Al_2O_3):** It is another commonly used adsorbent. Presence of sodium carbonate and bicarbonate affect its adsorption properties. Aluminium hydroxide undergoes many modifications to form aluminium oxide (alumina), which can be acidic, basic, or neutral in nature.

   Neutral alumina is used with organic eluents, and is suitable for substances that either bind or are already bound to strong alkalis. Acidic alumina is used for separating neutral or acidic materials. Basic alumina is used for separating steroids, alkaloids, and aromatic and unsaturated hydrocarbons.

   iii) **Kieselguhr or Diatomaceous Earth:** It is neutral in nature, may have or not have binder, and has less resolution capacity than the alumina and silica gel.

   iv) **Magnesia or Magnesium Oxide (MgO):** It is used as a substitute for alumina. Magnesium hydroxide undergoes dehydration to form the active magnesium oxide (magnesia), which is finely divided, thus allows filtration and can be used with the filter aid.

   v) **Magnesium Silicate and Calcium Silicate:** These adsorbents are used for separating sugars and its acetates, and phenylosazones. They have increased power of adsorption due to less water content.

   vi) **Others:** Aluminium silicate, bauxite, bentonites, barium sulphate, calcium carbonate, calcium hydroxide, calcium sulphate, dicalcium phosphate, fuller's earth, zinc carbonate, etc., are various inorganic materials that can be used as adsorbents in TLC.

2) **Organic Adsorbents:** The organic materials that can be used as adsorbents in TLC are:
   i) **Cellulose and its Acetylates:** Cellulose is a fibrous adsorbent and is preferred over paper as it is fast, allows uniform flow of the mobile phase, and less diffusion of the dissolved substances. Modified cellulose with or without binders are used for ion-exchange separations. Cellulose has water adsorbed within it, thus is used for separating hydrophilic substances (**e.g.,** amino acids, sugars, etc.) by the mechanism of partition.

   ii) **Charcoal and Activated Carbon:** Charcoal is a strong adsorbent and can adsorb aromatic substances. Its adsorption capacity can be enhanced by depositing a non-electrolyte or a fatty acid film over activated carbon.

3) Some **other organic adsorbents** that can be used for separation processes are dextran gels, ion-exchange resins, polyamides, polyethylene powder, sucrose, etc. When a mixture of two adsorbents is used, either one of them causes adsorption while the other acts as a diluent, or both causes adsorption linear with the percentage composition of the mixture.

9.2.3. Preparation of Plates

TLC plates can be prepared using the slurry of adsorbent, **e.g.,** silica gel, cellulose powder, etc., which is evenly spread on the plate with the help of spreaders. The adsorbent layer should be 150-250μm thick. After spreading, the plates are either dried at 80-90°C temperature for 30 minutes in hot air oven or are air-dried overnight. The adsorbent slurry can be applied on the plate by any of the following methods:

1) **Pouring:** In this method **(figure 9.2)**, a measured quantity of slurry is poured over the plate kept on a level. The plate is then inclined back and forth so that the slurry spreads homogenously over its surface.

Figure 9.2: Pouring Technique

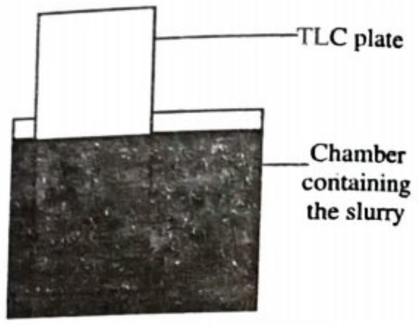


Figure 9.3: Dipping Technique

2) **Dipping:** In this method **(figure 9.3)**, the plates are dipped back to back either in chloroform or in chloroform-methanol adsorbent slurry.

3) **Spraying:** In this method, the adsorbent slurry is distributed over the glass plates using a small point sprayer. This method, however, has a few **drawbacks**; like homogenous layers of slurry cannot be obtained over the glass plates and the plates also vary from each other. Thus, this method of preparing plates is not in use these days.

4) **Spreading:** In this method **(figure 9.4)**, an applicator is used for the formation of layers over the glass plates. The plates can be prepared by either keeping the applicator stationary and pushing or pulling the plate over it, or by keeping the plate stationary and moving the applicator over it.

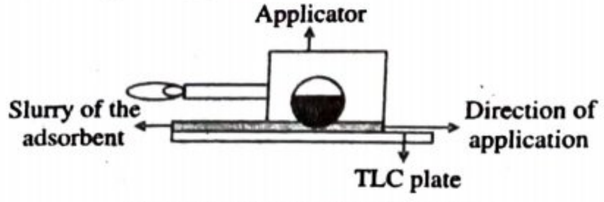


Figure 9.4: Spreading Technique

5) **Pre-Coated Plates:** These are commercially available pre-coated thin layers of adsorbents on glass or plastic plates.

9.2.4. Activation of Adsorbent

After the preparation of plates, the next step is activation of adsorbent layers over the plates. In this step, the liquid is quickly removed from the thin layer of adsorbent on plates by air drying the plates for 30 minutes and then drying in oven at 110°C temperature for another 30 minutes.

9.2.5. Purification of Silica Gel G Layers

Silica gel G contains iron as an impurity and it causes distortion of the chromatographs. Hence, purification of the adsorbent is essential. Iron free layers are obtained by giving the coated and air-dried plates a preliminary development with methanol-conc. HCl (9:1, v/v). The iron migrates with the solvent front to the upper edge of the plate, and then the plates are again dried and activated at 110°C temperature.

Calcium sulphate, initially present as a binder, dissolves out during the cleaning process. The purified silica gel G is reused to obtain plates with any other suitable binder such as gypsum, starch or agar.

9.2.6. Marking the Plate and Spotting of the Sample

The plate is marked with a pencil at 2-4cm from the bottom. However, to obtain accurate results marking should be done using a marker pen.

The sample is applied as a minute spot using a microsyringe or a microcapillary. The capillary is filled with the sample solution where the sample is dissolved in the solvent and then the solvent is completely evaporated from the spot.

9.2.7. Preparation of the Development Tank

The development tank used in TLC method is same as used in paper chromatography and the most common technique used is the ascending chromatography.

In the development chamber, the TLC plate (**figure 9.5**) is kept at 45° angle. Then the chamber is filled with the mobile phase (solvent) up to a height of 1mm. The three sides of the chamber are covered with papers soaked in solvent and the top of the chamber is closed with a lid. In the procedure of TLC, the solvent vapours should fill up the development chamber.

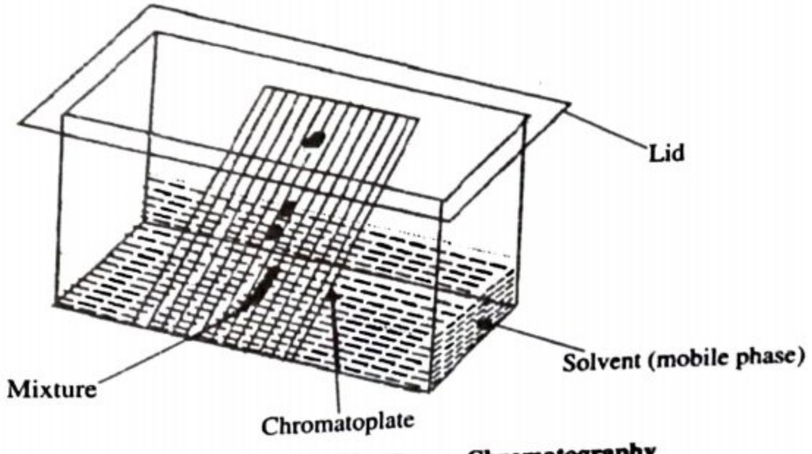


Figure 9.5: Apparatus for Thin Layer Chromatography

9.2.8. Types of Development in Thin Layer Chromatography

The commonly used development techniques of thin layer chromatography are:

1) **Ascending Development:** In this development method **[figure 9.6 (a)]**, after the plates are applied with samples, they are put in the development chamber containing mobile phase in the bottom. Consequently, the mobile phase runs from bottom to top of the plate, i.e., in ascending pathway.

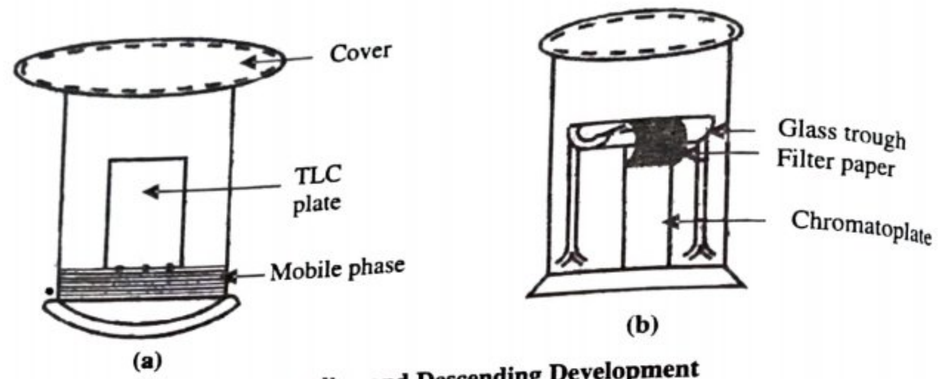


Figure 9.6: Ascending and Descending Development

2) **Descending Development:** In this development method [**figure 9.6 (b)**], the mobile phase is made to flow from the reservoir to the plate with the help of a filter paper strip. Consequently, the mobile phase runs from top to bottom of the plate, i.e., in descending pathway.

3) **Horizontal Development:** In this development method, plates with loose and non-sticky layers are utilised by using a shallow dish covered with a ground glass. The plate is held over a T-shaped glass piece and its end is pressed over a filter paper strip (of same width as the plate). Consequently, the solvent flows from the dish bottom to the thin layer plate.

4) **Stepwise and Multiple/Repeated Development:** In this development method, after developing the chromatogram with the mobile phase, it is removed from the chamber and the solvent is allowed to evaporate. A chromatogram is re-developed using the same solvent. This step is repeated a number of times to achieve the desired separation. If the same solvent is used in each run, this method is termed **multiple** or **repeated development**; and if different solvents are used each time, this method is termed **stepwise development**.

5) **Two-Dimensional Development:** In this development method (**figure 9.7**), a second chromatogram is developed with another solvent in perpendicular direction of the first chromatogram. This is done when the components are not completely separated by chromatogram development in one direction.

In two-dimensional development method, the sample is applied at the corner of the plate and the first development is run in ascending manner using a single solvent. Then the plate is removed and the solvent is evaporated. Thereafter, another solvent is used for the second development and also the edge of the plate is rotated by 90°.

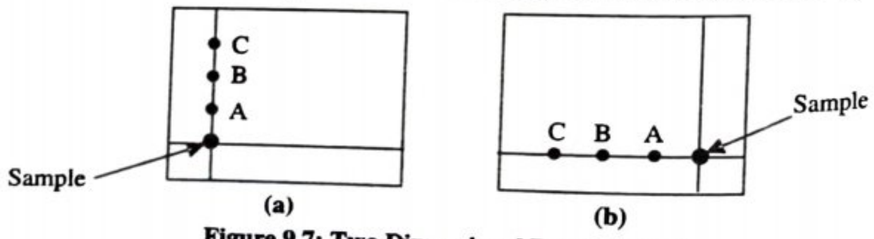


Figure 9.7: Two Dimensional Developments

6) **Preparative TLC:** In this development method, the sample is applied as streaks (bands) and multiple developments are carried out to achieve the desired separation. UV light is used for observing the spots, and then the bands are scrapped off and the sample is recovered by extracting with a suitable solvent.

7) **Gradient Elution:** In this development method, a specific type of specially designed tank is used since the solvent composition is continually changed during the development.

8) **Reverse Phase TLC:** In this development method, chromatoplates are used. These plates are prepared by slowly dipping the adsorbed layer in 5-10% of paraffin, silicone oil, undecane in petroleum ether, or diethyl ether. Then the plate is removed, solvent is evaporated, and chromatography is carried out. Permanent impregnation is achieved with paraffin or silicone oil; while, undecane can be removed by heating the plate at 120°C temperature.

9.2.9. Selection of Solvent System

The most suitable developing solvent (mobile phase) should be selected in TLC. In case the nature of components to be separated is unknown, **trial and error technique** is applied, in which small and rapid running TLC plates are used for selecting the best eluent.

Selection of the Mobile Phase: A mobile phase of ideal composition is selected in TLC, and this is a difficult and time-consuming step. The mobile phase is selected wisely; still the solvent composition is determined at the end of TLC technique by carrying out experiments.

The interactions controlling the retention of components in the stationary phase and the interactions (dispersive, polar, or ionic) governing the mobile phase are co-dependent. This is followed as a rule during the development of TLC. Reversed phase is always preferably selected.

Strong polar interactions govern the mobile phase (either methanol/water mixture or acetonitrile/water mixture) if dispersive interactions control the retention of components in stationary phase. Similarly, if the retention of components in stationary phase (silica gel) is controlled by strong polar interactions, a mixture of strong dispersive solvent is selected as the mobile phase (either an alkane or alkane/methylene dichloride mixture).

The elution rate of the solutes can be enhanced:
1) By decreasing the complementary interactions of the stationary phase, and
2) By increasing the dominative interactions of the mobile phase.

9.2.10. Plate Development

Plates having 5 × 20 or 20 × 20cm dimensions are selected for the development process in TLC. The following steps are followed thereafter:

1) Initially, a drop of sample is applied at one end of the plate and the spot is marked with a pencil.
2) Then the spot is air-dried for evaporating the solvent.
3) The plate is introduced in a closed chamber containing the developing solvent (mobile phase) and also solvent vapours.
4) One end of the plate should be dipped in the mobile phase to allow the solvent to travel up the plate.
5) When the developing solvent has travelled a sufficient distance (one-half to two-third of the plate length) on the plate, the plate is removed.
6) Finally, the plates are air-dried and the different positions of the components are determined.

9.2.11. Detection of Components - R_f Value

The dried spots are detected either by using UV light or by treating them with chemical reagents. Firstly, the spots are lined by marking their boundary with pencil. Then the spots are detected with any of the following methods:

1) **Iodination:** In this method, the plates are kept in a chamber containing iodine crystals.
2) **Ninhydrin Technique:** In this method, the spots are sprayed with ninhydrin reagent. This method is used for determining the compounds containing amine as a functional group.
3) The spots can be determined using fluorescent radiation.
4) The spots can be determined using UV radiation.
5) **R_f Measurement:** The R_f value is the ratio of the distance covered by the solute front to that covered by the solvent front (**figure 9.8**).

$$R_f = \frac{\text{Distance of the solute front}}{\text{Distance of the solvent front}}$$

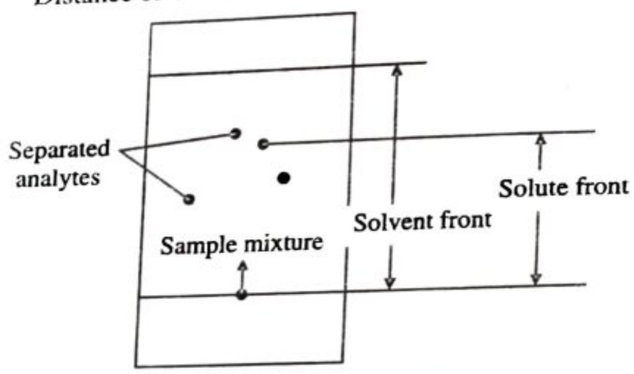


Figure 9.8: Measurement of R_f Value

Resolution (R_s) is the separation of the two analytes on TLC chromatogram (**figure 9.9**)

$$R_f = \frac{\text{Distance between the centre of spots}}{\text{Average diameter of spots}}$$

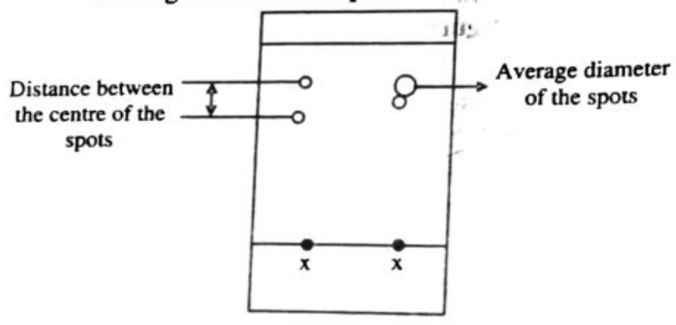


Figure 9.9: Resolution of the Spots

Flow constant (K) can be determined by taking the migration rate of the solvent front.

$$K = \frac{Zf^2}{t}$$

Where,

K = Flow constant
Z = Distance between the solute front and solvent front
t = Development time.

9.2.12. Evaluation of the Chromatogram

After detecting the solutes and their positions on the plate, they are evaluated by qualitative or quantitative analysis. In **qualitative evaluation**, the R_f value of test sample (unknown) is determined and compared with that of the standard sample (known).

Quantitative evaluation can be done either by the following methods:

1) **Direct Methods:** These methods involve:
   i) **Visual Comparison:** The intensity and size of the spots are visually observed and compared with the standard spots. This helps in evaluating the quantity of components semi-quantitatively.
   ii) **Spot Areas and Weight Relationship:** The area covered by the spot is determined to establish the quantity of components, which is represented by a linear relationship.
   iii) **Spot Densitometry:** This is used for detecting all types of components. The plate after development is sprayed with certain reagents to develop colour, which is measured using either a densitometer or spots on photographs or negatives.
   iv) **Direct Spectrometry:** A chromatogram spectrometer at wavelength of maximum absorption of the component is used to read the absorption or fluorescence of the zones on TLC plates.
   v) **Spectral Reflectance:** The spectral reflectance of the dyes adsorbed over the adsorbent layer is determined.
2) **Indirect Method:** This method involves elution, i.e., separation by TLC, recovery by quantitative elution, and estimation with a suitable method. In this method, the localised spots of the components are marked and scrapped out using a vacuum cleaner (preventing any loss). Then the solutes are eluted from the adsorbent by simple agitation with a solvent. Finally, the eluate is examined using the techniques of colorimetry, spectrophotometry, fluorimetry, radiometry, flame photometry, etc.

9.2.13. Recovery of Components

The sample is recovered using the Craig tube for extracting the solvent from the powder and for removing the adsorbent (**figure 9.10**). Another method used is removing the zones with spatula and then extracting with solvent.

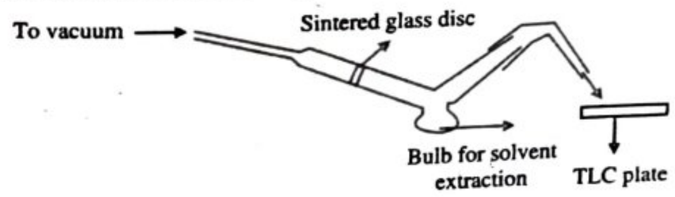


Figure 9.10: Recovery of the Samples

9.3. APPLICATIONS

9.3.1. Introduction

Thin layer chromatography has the following applications:

1) Qualitative estimation, and
2) Quantitative estimation.

9.3.2. Qualitative Estimation

The qualitative applications of TLC are as follows:

1) **As a Check on Processes:** TLC can control some other separation and purification processes (**e.g.,** distillation fractions and purification by molecular distillation).
2) **In Organic Chemistry:** It can be used for the isolation and separation of individual components of a mixture.
   i) **For Checking the Purity of Sample:** It is used for measuring the sample purity obtained by other methods.
   ii) **As a Purification Process:** A pure form of an organic compound is used in small quantity for characterisation of the same compound by elemental analysis and other physical methods.
   iii) **Examination of Reaction:** It is used for measuring the reaction progress and its completion.
   iv) **For Identifying Organic Compounds:** It can be used for rapid isolation, separation and purification of organic substances.
3) **Applications of TLC for Separation of Inorganic Ions:** It can be used for the separation of cations, anions, pure covalent species, and some organic derivatives of the metals.
4) **Separation of Amino Acids:** It can be used for the separation of amino acids in a mixture.
5) **Separation of Vitamins by Thin-layer Chromatography:** F_{254} pre-coated silica gel TLC plates (20 × 2cm dimension) are used for the separation of vitamins E, D_3 and A.

9.3.3. Quantitative Estimation

The spot area is measured based on pre-calibration, and this helps in quantitative analysis of the components in the spot. The samples separated by TLC can be quantitatively analysed by the following techniques:

1) **Photodensitometry or Visual Comparison:** Photodensitometry is used in case of transmission, reflectance or fluorescence modes. Visual comparison of the spot intensity of the test sample with that of the standard (with known intensity) is done.
2) The sample spots (eluted with a proper solvent) are determined with a suitable technique (visual spectrophotometry or spectrofluorimetry). Incorrect results are obtained if the eluate is directly measured in the UV region. This is because the substances extracted from the adsorbent undergo inappropriate absorption. This incorrectness can be overcome by measuring the extracts at longer wavelength.
3) **Measurement of Spot Areas:** The spot area on the plate and the component quantity in that spot can be correlated by many available techniques. **Purdy** and **Truter** in **1962** stated that a linear relationship exists between the logarithm of the weight of the substance and the square root of the spot area.

9.4. SUMMARY

The details given in the chapter can be summarised as follows:

1) **Thin layer chromatography** is a sub-division of liquid chromatography, in which the mobile phase is a liquid and the stationary phase is applied as a thin layer on a flat plate.
2) TLC and paper chromatography combines to form **planar liquid chromatography** due to the layered stationary phases or the flat surface of the paper.

3) **Mein Hard** and **Hall** first planned TLC method in **1949** by using starch as a binder to separate the inorganic ions.
4) **Stahl** first designed the standard equipment for TLC, which is similar to that of paper chromatography.
5) TLC works on the **principle** that the mobile phase flows through the stationary phase, on which a solid or liquid is supported and the mixture components are separated into individual analytes.
6) In **normal phase TLC**, the stationary phase is polar and the mobile phase is non-polar.
7) In **reverse phase TLC**, the stationary phase is non-polar and the mobile phase is polar.
8) The **most commonly used binder** along with the adsorbents is **10-15% calcium sulphate** (gypsum).
9) Silica gel added with gypsum binder (calcium sulphate hemi-hydrate) is termed **silica gel G**.
10) In **pouring** method, a measured quantity of slurry is poured over the plate kept on a level.
11) In **dipping** method, the plates are dipped back to back either in chloroform or in chloroform-methanol adsorbent slurry.
12) In **spraying** method, the adsorbent slurry is distributed over the glass plates using a small point sprayer.
13) In **spreading** method, an applicator is used for the formation of layers over the glass plates.
14) Silica gel G contains iron as an impurity and it causes distortion of the chromatographs.
15) In **descending development** method, the mobile phase is made to flow from the reservoir to the plate with the help of a filter paper strip.
16) In **ascending development** method, after the plates are applied with samples, they are put in the development chamber containing mobile phase in the bottom.
17) If the same solvent is used in each run, this method is termed **multiple** or **repeated development**; and if different solvents are used each time, this method is termed **stepwise development**.
18) In **two-dimensional development** method, a second chromatogram is developed with another solvent in perpendicular direction of the first chromatogram.
19) In **reverse phase TLC** development method, chromatoplates are used.
20) The **R_f value** is the ratio of the distance covered by the solute front to that covered by the solvent front.
21) **Resolution** is the separation of the two analytes on TLC chromatogram.
22) **Flow constant** can be determined by taking the migration rate of the solvent front.
23) In **qualitative evaluation**, the R_f value of test sample (unknown) is determined and compared with that of the standard sample (known).
24) The sample is recovered using the Craig tube for extracting the solvent from the powder and for removing the adsorbent.

9.5. EXERCISE

9.5.1. True or False

1) TLC and paper chromatography combines to form planar liquid chromatography due to the layered stationary phases or the flat surface of the paper.
2) In reverse phase TLC, the stationary phase is polar and the mobile phase is non-polar.
3) The most commonly used binder along with the adsorbents is 10-15% silica.

4) In spreading method, an applicator is used for the formation of layers over the glass pl
5) In ascending development method, the mobile phase is made to flow from
reservoir to the plate with the help of a filter paper strip.
6) The R_f value is the ratio of the distance covered by the solute front to that covere
the solvent front.

9.5.2. Fill in the Blanks

7) Mein Hard and _____ first planned TLC method in 1949 by using starch
binder to separate the inorganic ions.
8) _____ first designed the standard equipment for TLC, which is similar to
of paper chromatography.
9) In _____, the stationary phase is non-polar and the mobile phase is polar.
10) Silica gel G contains _____ as an impurity and it causes distortion of
chromatographs.
11) In _____ method, a second chromatogram is developed with another solve
perpendicular direction of the first chromatogram.
12) _____ can be determined by taking the migration rate of the solvent front.
13) The sample is recovered using the _____ for extracting the solvent from
powder and for removing the adsorbent.

Answers

1) True 2) False 3) False 4) True 5) Fals
6) True 7) Hall 8) Stahl 9) Reverse phase TLC 10) Im
11) Two-dimensional development 12) Flow constant 13) Craig tube

9.5.3. Very Short Answer Type Questions

1) What is TLC?
2) Name the organic adsorbents used in TLC.
3) How silica gel layers are purified in TLC?
4) What is two-dimensional development of tank?
5) Give any two methods of evaluation of chromatogram in TLC.
6) How components are recovered in TLC?
7) Give any two applications of TLC.

9.5.4. Short Answer Type Questions

1) Give the theory of TLC.
2) Write a note on inorganic adsorbents used in TLC.
3) Write a note on preparation of plates in TLC.
4) Discuss the solvent system used in TLC.
5) How components are detected in TLC?
6) Give the advantages and disadvantages of TLC.

9.5.5. Long Answer Type Questions

1) Briefly explain the methodology of TLC.
2) Discuss about the types of TLC.
3) Write about the applications of TLC.

CHAPTER 10

Paper Chromatography

10.1. PAPER CHROMATOGRAPHY

10.1.1. Introduction

Technique of paper chromatography was introduced by **A.J.P. Martin**, **R. Consden**, **A.H. Gordon**, and **R.L.M. Synge** (workers of Cambridge school).

Paper chromatography is a type of partition chromatography in which two liquids are used and the components are distributed between them. One liquid is the **stationary phase** (mostly water) that remains in the fibres of paper, and the other liquid is the **mobile phase** (development solvent). The mixture components get separated according to their rates of migration, and can be seen in the form of spots on different points on the paper. Previously, paper chromatography was only used to separate organic compounds, such as dyes and amino acids; however, now it is used to separate anions and cations of inorganic compounds.

10.1.2. Principle

This technique is generally used in the analysis, identification, purification and quantification of the components of mixtures into individual compounds. Paper chromatography is based on the principle of partition and the substances are distributed according to their migration rates between two liquids, of which one is the stationary phase (stationary liquid held in the paper) and the other is the mobile phase (moving liquid).

The mobility of mixture components depends on the nature of stationary phase and partition coefficient. On the basis of separation principle, paper chromatography is of **two types**:

1) **Paper Partition Chromatography:** In this type, paper is used along with the mobile phase as an inert support.
2) **Paper Adsorption Chromatography:** In this type, modified papers, such as paper saturated with silica or alumina, are used to separate the mixture components.

10.1.3. Theory

The test solution (mixture of two or more substances) is applied on the filter paper in the form of a small spot and is air-dried. In a closed chamber, the filter paper is placed with its edge dipped in a developing solvent. The developing solvent runs through the capillary axis and reaches the spot of test solution. Then, the different components of the test mixture start moving with different speeds under the influence of the developing liquid.

When an adequate distance (15-18cm) is covered by the substances (cations) along with the solvent, the paper is kept for drying. Thereafter, the paper is observed for different spots using visualising reagents. The movement of substances corresponding to the solvent is known as **migration parameter** or **R_F value**.

Migration Parameters

Different terms like R_F, R_x, and R_M are used to denote the position of migrated spots (chromatograms). These parameters show both qualitative and quantitative properties of a substance.

R_F
This value denotes the migration of solute front with respect to the solvent (**figure 10.1**):

$$R_F = \frac{\text{Distance Travelled by the Solute from the Origin Line (A)}}{\text{Distance Travelled by the Solvent from the Origin Line (B)}}$$

R_F value (a function of partition coefficient) remains constant for a given substance provided that the following chromatographic conditions remain constant

1) Temperature.
2) Paper type.
3) Direction and duration of development.
4) Nature, shape and size of the wick (in radial chromatography).
5) Amount of liquid in the reservoir, etc.

R_F is defined as the movement of the substance related to solvent front in a particular chromatographic system.

The R_F value of a substance depends on the following **factors**.

1) Solvent used.
2) Separation medium (paper quality in paper chromatography).
3) Nature of the mixture.
4) Temperature, and
5) Size of the vessel for carrying out the operation.

By keeping the above mentioned factors constant, the R_F values of different substances can be compared.

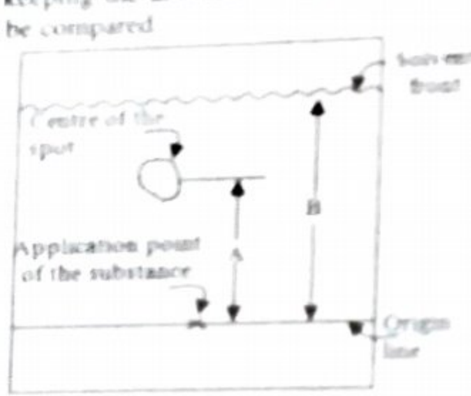


Figure 10.1: Diagrammatic Representation of R_f Value

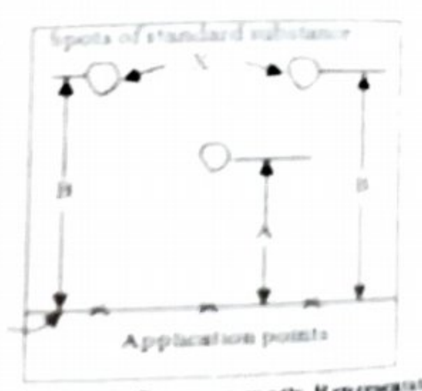


Figure 10.2: Diagrammatic Representation of R_x Value

R_x
This value denotes the movement of a substance if the solvent front runs off the strip of filter paper (**figure 10.2**)

$$R_x = \frac{\text{Distance Travelled by the Substance from the Origin Line (A)}}{\text{Distance Travelled by the Standard Substance from the Origin Line (B)}}$$

R_M
This value represents the R_f values of closely related compounds. It gives a rough approximation of the effect of individual functional groups. **Bate-Smith** and **Westall** defined R_M as:

$$R_M = \log\left(\frac{1}{R_F} - 1\right)$$

The R_M value is additive and comprises of the individual functional groups or other groups of atoms in a molecule.

By using suitable methods, the solute bands are cut from the paper, extracted and resolved. This technique is also used in quantitative determinations. The paper may also be scanned using densitometer after colour development with a ligand.

10.1.4. Development Techniques

There are five development techniques of paper chromatography.

1) Descending chromatography.
2) Ascending chromatography.
3) Ascending-descending chromatography.
4) Radial paper chromatography, and
5) Two-dimensional chromatography.

10.1.4.1. Descending Chromatography

In descending chromatography, the paper is developed by allowing the solvent to move in downward direction along the paper (**figure 10.3**). The apparatus used in this technique is a sealed glass tank of sufficient shape and size, and a trough for the mobile phase is placed in the upper part of the tank.

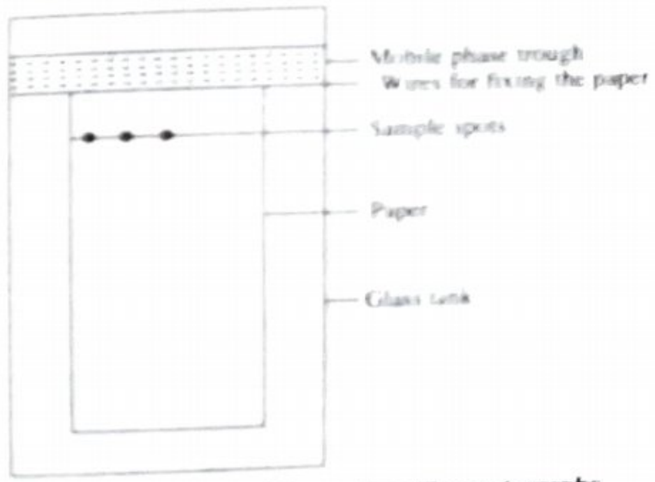


Figure 10.3: Technique of Descending Chromatography

Before performing elution, the glass tank is equilibrated with the mobile phase and then the paper having the sample spot is kept with its upper end in the trough containing the mobile phase.

This technique is **advantageous** because the paper development can be carried out for an indefinite period, even when the sample runs off at the other side of the paper.

10.1.4.2. Ascending Chromatography

In ascending chromatography, the paper is developed by allowing the solvent to move in upward direction along the paper. In this technique, the mobile phase is kept either in the chamber or in a container placed at the bottom of the chamber. The sample is applied at a few centimetres above the bottom edge of the paper. Then the paper is hanged from a hook within the chamber [**figure 10.4 (a)**]. The paper is rolled and its ends are fixed with strings, staples or plastic clips and kept within the chamber [**figure 10.4 (b)**].

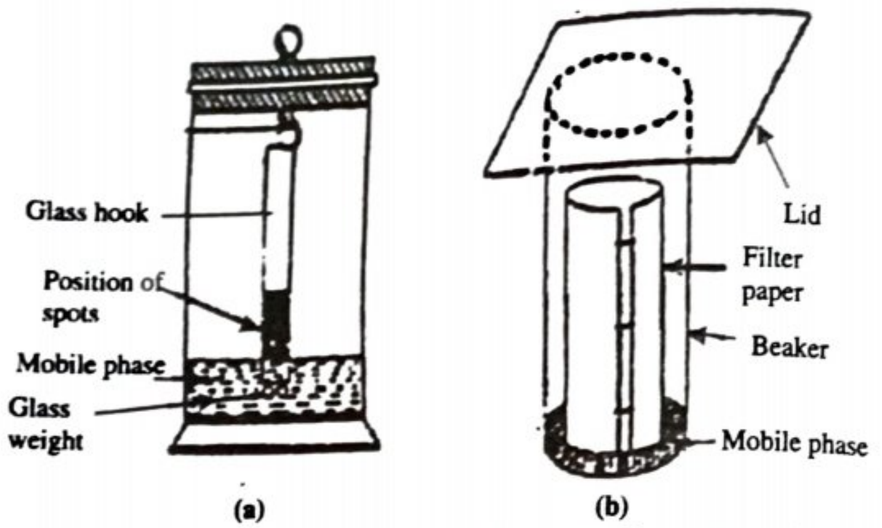


Figure 10.4: Technique of Ascending Chromatography

Both ascending and descending techniques are used for the separation of organic and inorganic components; however, if the R_F values are relatively close the descending technique is preferred.

10.1.4.3. Ascending-Descending Chromatography

The ascending-descending chromatography is a fusion of ascending and descending techniques. In this technique, firstly the paper is kept in an ascending manner and then the upper end of the paper is folded across the glass rod in a descending manner.

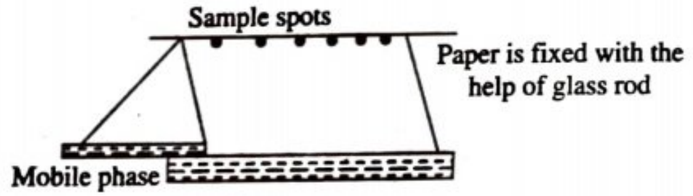


Figure 10.5: Technique of Ascending-Descending Chromatography

10.1.4.4. Radial Paper Chromatography

The radial paper chromatography is also known as **circular paper chromatography**. As the name indicates it is based on radial development of chromatogram. In this technique, the sample solutions are applied at the centre of filter paper and air dried. Then, the paper is placed horizontally over a petri dish having the mobile phase, in such a way that the wick gets dipped in the mobile phase and the paper is covered with another petri dish. Now the solvent travels upward through the wick and the components get separated in the form of circular zones/bands.

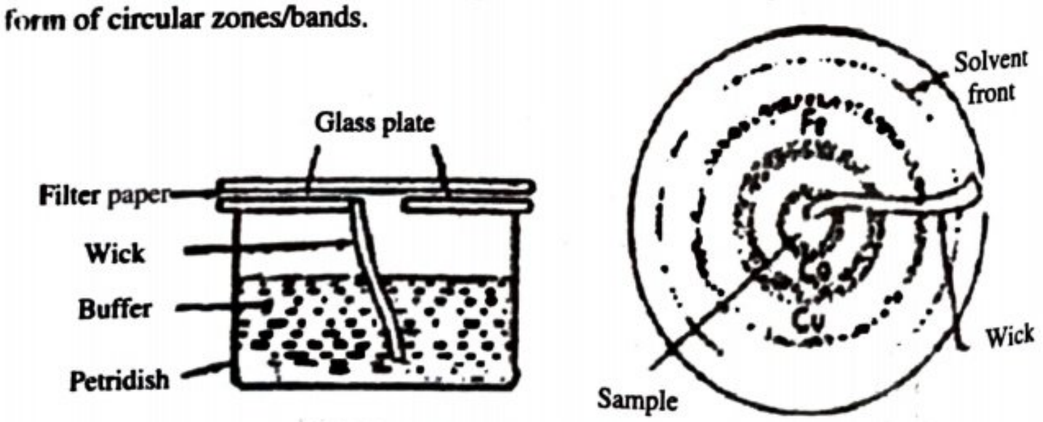


Figure 10.6: Technique of Radial Chromatography

10.1.4.5. Two-Dimensional Chromatography

In two-dimensional chromatography technique, a square or rectangular shaped paper is used, at one corner of which sample solution is applied. After the spot is dried, the solvent is allowed to travel a sufficient distance for first chromatogram development. The paper is rotated at right angle to the direction of the first run and the solvent movement is again allowed to develop the second chromatogram. In this technique, either similar or two different solvent systems can be used to develop chromatograms in both the directions.

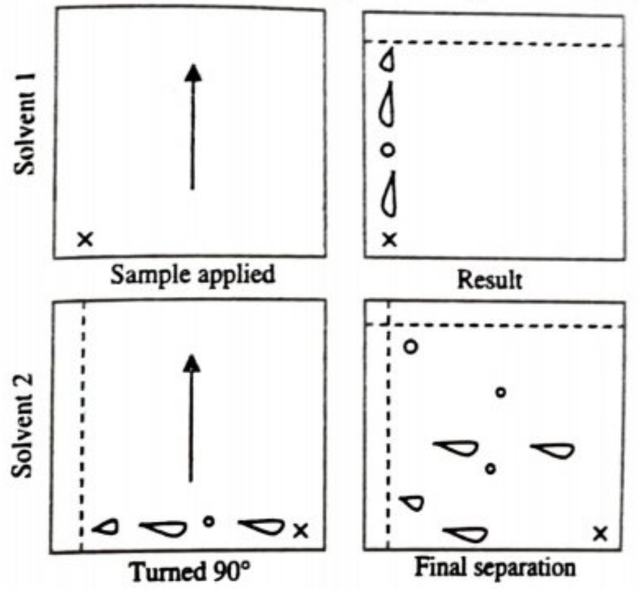


Figure 10.7: Technique of Two-Dimensional Chromatography

At one lower corner of a rectangular piece of filter paper, the sample is spotted to get more clear and precise results. The sample is applied in such a way that the spot remains above the surface of the solvent in the trough. After this, the paper is kept for drying and placed in the trough in such a way that its edge remains dipped in the solvent. The solvent front is allowed to run by either ascending or descending method. Then, the paper is taken out from the trough and air dried when the solvent reaches the opposite side of the paper.

10.1.5. Methodology

The various steps involved in paper chromatography are:

1) Choice of the proper chromatographic technique,
2) Choice of the filter paper,
3) Choice of solvent,
4) Preparation of sample,
5) Spotting,
6) Drying the chromatogram,
7) Detection of spots, and
8) Calculation of R_F values.

10.1.5.1. Choice of the Proper Chromatographic Technique

Before carrying the chromatography process, the method of paper chromatographic technique, i.e., ascending, descending, ascending-descending, radial, or two-dimensional, is selected according to the nature of the substances to be separated.

10.1.5.2. Choice of the Filter Paper

The success of paper chromatography depends on the filter paper used, therefore it is important to choose a correct one. The selection of paper depends on the type of sample

to be investigated. Following are the primary factors which determine the selection of filter paper:

1) Whether the filter paper is to be used for quantitative or qualitative analysis,
2) Whether the filter paper is to be used for analytical or preparative chromatography. or
3) Whether the substances to be separated are hydrophilic or lipophilic, neutral or charged species.

A wide range of Whatman filter papers are available and their selection depends on the type of separation. Whatman filter papers used in chromatography have up to 99% of α-cellulose and the rest is mineral content.

If the substances to be separated have sufficiently wide-apart R_F values, a coarser and faster paper (Whatman 31 ET paper) is used. Use of slow papers (**e.g.,** Whatman 20 *Schleicher* and *Schuell* 2045 a, Macherey Nagel 261 and Edrol 208) is very rare. These **slow Whatman papers** facilitate a better resolution of substances with close R_F values. For preparative purposes, generally **heavy Whatman papers** are used, **e.g.,** Whatman3MM. Even the gram quantities of substances can be separated using a single sheet of *Schleicher* and *Schuell* 2071 papers.

Table 10.1: Characteristics of Whatman Chromatographic Papers

Whatman Chromatographic Papers	Rate of Flow		
	Fast	Medium	Slow
Thin papers	No. 4	No. 7	No. 2
	No.54	No. 1	No. 20
	No.540		
Thick papers	No. 31	No. 3	
	No. 17	No. 3MM	

Cellulose papers are used to support various adsorbents (alumina, silica, zirconium oxide, etc.). These adsorbents get precipitated in the pores of filter paper to produce a thin sheet with the flexibility of the paper along with the adsorbent qualities of the precipitate. Various chelating agents (**e.g.,** 8-hydroxquinoline, dimethylglyoxime, etc.) are used to impart specific qualities to the papers. The **ion exchange papers** are produced by saturating the papers with powdered or liquid ion exchangers. The applications of these papers are described in the **table 10.2**:

Table 10.2: Modified Papers for Paper Chromatography

Types of Papers	Typical Uses
Carboxyl papers	Cationic separation of protonated amines and amino acids.
Acetylated papers	Reversed phase chromatography of lipophilic substances, like steroids, insecticides, pigments, and also metal cations.
Kieselguhr papers, alumina papers, zirconia papers, and silica papers	Separation of low polarity substances, like amines, fatty acids, steroids, triglycerides, vitamins, and pesticides.
Ion exchange papers	Ion exchange paper chromatography.

10.1.5.3. Choice of Solvent

For the separation of substances under examination, the best possible developing solvent should be selected based on the R_F values, which should be different for different mixture constituents. Various combinations of the solvent systems are available, but the condition

is that they should not be completely miscible with each other. A good solvent system should possess the following qualities:

1) The range of R_F values of the sample should be between 0.05 and 0.85. The minimum difference between the R_F values of any two components should be 0.05. It is the minimum required value for the separation of any two components.
2) To get circular spots, the distribution ratios of the components present in the solvent system should not depend on the concentration.
3) The solvents should not be chemically reactive with the components present in the sample mixture.
4) The solvents should not obstruct the detection of spots on the developed chromatogram.
5) The solvent system composition should remain constant with time.

Solvents used in paper chromatography are enlisted in **table 10.3** according to their order of increasing polarity. The polarity of a solvent can be measured from the dielectric constant.

Table 10.3: Solvents Listed in Order of their Increasing Polarity

Solvents	t_{20}	t_{25}
n-Hexane	1.89	
Cyclohexane	2.02	
Carbon tetrachloride	2.24	
Benzene	2.29	
Toluene	2.44	
Trichloroethylene	3.40	
Diethyl ether	4.34	
Chloroform	4.91	
Ethylacetate		6.02
n-Butanol	17.80	17.10
n-Propanol		20.10
Acetone		20.10
Ethanol		24.30
Methanol		32.60
Water	80.40	78.50

If a pure solvent is not giving satisfactory results, solvent of suitable polarity may be formed by trying out mixtures in different proportions of solvents as listed in **table 10.3**. Eleven solvent systems have been developed that can be used to separate almost all the mixtures (**table 10.4**). These solvents are enlisted in the decreasing order of the polarity of stationary phase. The solvent systems at the top are best suited for mixtures of polar compounds, while those at the bottom are suitable for non-polar compounds.

Table 10.4: Suitable Solvents Systems for Paper Chromatography

Stationary Phases	Mobile Phases
Water	Isopropanol-ammonia (9:1:2)
Water	n-Butanol-acetic acid-water (4:1:5)
Water	Phenol saturated with water
Formamide	Chloroform
Formamide	Benzene
Formamide	Benzene-cyclohexane (from 1:9 to 9:1)
Dimethyl formamide	Cyclohexane
Phenoxy ethanol	Heptane
Kerosene	70% iso-propanol
Liquid paraffin	Dimethyl formamide-methanol-water (10:10:1)

10.1.5.4. Preparation of Sample

There is no standard procedure for preparation of samples and this problem is due to various factors of the given samples, such as the presence of other systems, like fats, salts, proteins, etc. However, the ideal quantity of a substance required to be spotted is 10-20µ volume of sample with as many µg of the substance.

10.1.5.5. Spotting

If separation is done by ascending technique, a strip of Whatman filter paper of suitable size (25cm × 7cm) is used. A horizontal line, known as **origin line** is drawn on the filter paper by a lead pencil. Cross marks (×) are made on the origin line with a pencil in such a way that each cross (×) is at least 2cm away from other one (**figure 10.8**).

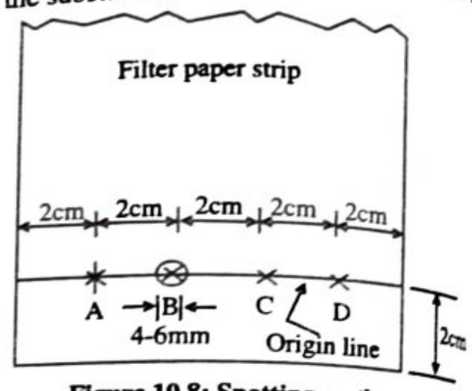


Figure 10.8: Spotting on the Chromatographic Paper

The test solutions are applied on cross (×) marks using a graduated micropipette. The spots are dried carefully by a stream of hot or cold air. Four cross (×) marks (A, B, C and D) can be seen in **figure 10.8**. Solutions of pure substances are applied on the first three marks, while the mixture of A, B and C is applied on the last (or fourth) point.

10.1.5.6. Drying the Chromatogram

After development, the wet chromatogram is kept in special drying cabinets that are electrically being heated with temperature controls.

10.1.5.7. Detection of Spots

The spots can be detected by any of the two methods.

1) **Physical Methods:** UV or fluorescence radiation is used for spot detection. **For example,** UV radiation at 254nm wavelength in propanol:water:acetic acid:triethyl amine (75:33:8:8) mobile phase is used for the detection of antibiotics. Fluorescence radiation is used for the detection of quinine.

2) **Chemical Methods:** In this method, the following chemical agents are used for detection based on the nature of functional group present on the sample:
   i) Ninhydrin reagent is used to detect amino acids (gives purple spots).
   ii) Bromocresol green is used to detect acidic compounds (gives yellow spots on green background).
   iii) Dragendorff's reagent is used to detect alkaloids (gives brown or orange spots).
   iv) Sodium nitrite is used to detect sulphonamides (gives purple or red spots).
   v) Potassium permanganate is used as a general reagent.
   vi) Iodine vapour is used to detect organic bases (gives brown spots).

10.1.5.8. Calculation of R_F values

R_F value is the distance travelled by the chromatographed species (or chemical compound) from its centre to the origin line. It can be determined from the distance of solvent front from the origin line.

10.1.6. Advantages

Paper chromatography has the following advantages:

1) It needs fewer quantitative materials to carry a separation.
2) It requires less time for the separation of compounds.

3) It requires a small amount of sample for analysis.
4) It is economic as compared to other chromatography methods.
5) It is suitable for the separation of both organic and inorganic compounds.
6) Its setup can be installed even in a small space.
7) It is very convenient to use.

10.1.7. Disadvantages

Paper chromatography has the following disadvantages:

1) It cannot be used to separate volatile substances.
2) It is not compatible with large amounts of sample.
3) It is not useful for quantitative analysis of the compounds.
4) It cannot be used to separate complex mixture.
5) It has less accuracy as compared to HPLC or HPTLC methods.
6) In this technique, the data cannot be saved for long periods.

10.1.8. Applications

Paper chromatography is used to:

1) Separate plant pigments, **e.g.,** chlorophyll a and b,
2) Determine sequence in the DNA and RNA molecules,
3) Determine sequence of the amino acid in proteins,
4) Detect forensic samples,
5) Separate sugar molecules,
6) Separate vitamins,
7) Separate antibiotics,
8) Analyse drug metabolites in blood and urine samples,
9) Detect unknown compounds, and
10) Determine the insecticides in food components.

Table 10.5 enlists the detailed applications of paper chromatography:

Table 10.5: Separation of Amine Antioxidants by Paper Chromatography (Acetylated Paper, Mobile Phase – Benzene: Methanol (1:1). Spray Reagent – 4% Benzoyl Peroxide in Benzene)

Systematic Names	Trade Names	Colour of the Reaction Product	Identification Limits before/after Chromatographic Separation (µg)		R_F Values
Phenyl-α-naphthylamine	Neozone A, Nonox A, and PAN	Light yellow	5	10	0.64
Phenyl-β-naphthylamine	Neozone D, Nonox D, and PBN	Blue-grey	5	20	0.64
Diphenyl-*p*-phenylenediamine	JZF	Yellow-orange	< 1	2	0.56
Phenyl-cyclohexyl-*p*-phenylenediamine	Alterungsschutzmittel 4010	Yellow	< 1	10	0.73
Di-β-naphthyl-*p*-phenylenediamine	Agerite White, Santowhite, Cl Nonox CI, DNP, and Antioxidant 123	Pink	1	5	0.55 (tailing)
p-isopropoxydiphenylamine	**For example,** in Agerite Hipar, a mixture of 2, 3 and 6	Yellow-brown	–	–	0.73
p,p'-dimethoxy-diphenylamine	**For example,** in Thermoflex A, a mixture of 2, 3 and 7.	Brown-pink	–	–	0.68

p (*p*-tolyl-sulphonylamino)-diphenylamine	Arabox	Brown-red	< 1	5	0.60
p-(*p*-tolyl-sulphonylamino)-phenyl-*p*-tolylamine	MUF	Red	1	5	0.65
Mono- and diheptyldiphenylamine	Agerite Stalite	Green			0.81 and 0.91
2,4-diamino-diheptyldiphenylamine	Oxynone	Brown	< 1	10	0.78
p,p'-diamino-phenylmethane	Tonox	Red-brown	2	40	0.50
Diphenyl-ethylenediamine	Stabilite	Red-brown	10	80	0.65

10.2. SUMMARY

The details given in the chapter can be summarysed as follows

1) Technique of **paper chromatography** was introduced by **A.J.P. Martin**, **R. Consden**, **A.H. Gordon**, and **R.L.M. Synge**
2) Paper chromatography is a type of partition chromatography in which two liquids are used and the components are distributed between them.
3) In **paper partition chromatography**, paper is used along with the mobile phase as an inert support.
4) In **paper adsorption chromatography**, modified papers such as paper saturated with silica or alumina are used to separate the mixture components.
5) The movement of substances corresponding to the solvent is known as **migration parameter** or **R_f value**.
6) **R_x** value denotes the movement of a substance if the solvent front runs off the end of filter paper.
7) **R_M** value gives a rough approximation of the effect of individual functional groups.
8) In **descending chromatography**, the paper is developed by allowing the solvent to move in downward direction along the paper.
9) In **ascending chromatography**, the paper is developed by allowing the solvent to move in upward direction along the paper.
10) The **radial paper chromatography** is also known as **circular paper chromatography**.
11) In **two-dimensional chromatography**, a square or rectangular shaped paper is used at one corner of which sample solution is applied. After the
12) If the substances to be separated have sufficiently wide apart R_f values, a **coarse and faster paper** (Whatman 31 ET paper) is used.
13) The **slow Whatman papers** facilitate a better resolution of substances with close R_f values.
14) For preparative purposes, generally **heavy Whatman papers** are used, e.g. Whatman3MM.
15) The **ion exchange papers** are produced by saturating the papers with powdered or liquid ion exchangers.
16) **Ninhydrin reagent** is used to detect amino acids (gives purple spots).
17) **Bromocresol green** is used to detect acidic compounds (gives yellow spots on green background).
18) **Sodium nitrite** is used to detect sulphonamides (gives purple or red spots).
19) **Iodine vapour** is used to detect organic bases (gives brown spots).
20) **Dragendorff's reagent** is used to detect alkaloids (gives brown or orange spots).
21) **Potassium permanganate** is used as a general reagent.

10.3. EXERCISE

10.3.1. True or False

1) In paper adsorption chromatography, paper is used along with the mobile phase as an inert support.
2) The radial paper chromatography is also known as circular paper chromatography.
3) The fast Whatman papers facilitate a better resolution of substances with close R_f values.
4) For preparative purposes, generally heavy Whatman papers are used.
5) Ninhydrin reagent is used to detect amino acids.
6) Iodine vapour is used to detect carbohydrates.

10.3.2. Fill in the Blanks

7) Technique of paper chromatography was introduced by A.J.P. Martin, R. Consden, A.H. Gordon, and _____.
8) In _____, modified papers such as paper saturated with silica or alumina are used to separate the mixture components.
9) The movement of substances corresponding to the solvent is known as _____.
10) In _____, the paper is developed by allowing the solvent to move in upward direction along the paper.
11) The _____ are produced by saturating the papers with powdered or liquid ion exchangers.
12) Sodium nitrite is used to detect _____.

Answers

1) False 2) True 3) False 4) True 5) True 6) False
7) R.L.M. Synge 8) Paper adsorption chromatography 9) R_f value
10) Ascending chromatography 11) Ion exchange papers 12) Sulphonamides

10.3.3. Very Short Answer Type Questions

1) What is paper chromatography?
2) What is R_f and R_M value?
3) Name the solvents used in paper chromatography.
4) Give any two chemical methods of detection of spots in paper chromatography.
5) Give any two applications of paper chromatography.
6) Give the advantages of paper chromatography.

10.3.4. Short Answer Type Questions

1) Give the theory of paper chromatography.
2) Write a note on two-dimensional paper chromatography.
3) Write a note on choice of filter paper in paper chromatography.
4) Discuss the solvents used in paper chromatography.
5) Give the applications of paper chromatography.

10.3.5. Long Answer Type Questions

1) Briefly explain the methodology of paper chromatography.
2) Discuss about the development techniques of paper chromatography.

CHAPTER 11 Electrophoresis

11.1. ELECTROPHORESIS

11.1.1. Introduction

Electrophoresis is a phenomenon describing the motion of particles in a gel or flu within a relatively uniform electric field. Electrophoresis is used for separating sh molecules based on their charge, size, and binding affinity. It is used for separating an analysing biomolecules, such as DNA, RNA, proteins, nucleic acids, plasmids, and the fragments. Electrophoresis is also used for identifying the source DNA (as in paterni testing and forensic science).

Electrophoresis of anions (negatively charged particles) is termed **anaphoresis**, while th electrophoresis of cations (positively charged particles) is termed **cataphoresis**.

Ferdinand Frederic Reuss (of Moscow State University) first discovered the technique of electrophoresis in **1807** when he noticed that clay particles migrated in water subjected to a continuous electric field.

11.1.2. Principle

The method of electrophoresis is used for separating the charged particles from one another based on the differences in their migration speed. During electrophoresis, tw platinum electrodes are immersed in two discrete buffer chambers, which are not flui isolated from each other. The charged particles migrate from one chamber to the other By applying an electric power, **electric potential** (E) is generated between the tw electrodes, under the influence of which the electrons move between the two electrode (i.e., from anode to cathode) through a wire. Hence, anode will be positively charged an cathode will be negatively charged.

Electrons that migrate to the cathode will leave the electrode and undergo reduction wit water to produce hydrogen gas and hydroxide ions. Simultaneously, electrons that migrate to the anode will undergo oxidation. Electrons that are released from the water molecules will enter the electrode and produce oxygen gas and free protons. These protons will immediately form hydroxonium ions with water molecules. The amount of electrons that leave the cathode and the amount of electrons that enter the cathode becomes equal. Since the buffer chambers are distinct yet interconnected, the charged particles migrate between them driven by the electric potential between the two electrodes. The anions (negatively charged ions) move towards the positively charged anode, while the cations (positively charged ions) move towards the negatively charged cathode.

The ions migrate at different speeds based on their sizes and the number of charges they carry. Thus, electrophoresis can separate different ions from each other. The basic physics governing the dependence of ion speed as a function of the number of charges the ion carries, the ion size, the magnitude of the applied electric field, and the nature of the medium in which the ions migrate should be known. Once these basic relationships are known, the principles of different specific electrophoresis methods become understandable. The central principle of electrophoresis is illustrated in **figure 11.1**.

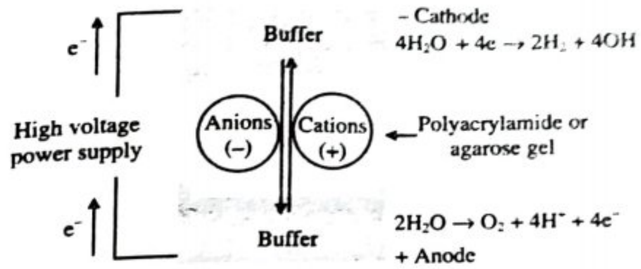


Figure 11.1: Principle of Electrophoresis

The force during electrophoresis can be easily described mathematically. An electric force (F_e) is exerted on the charged particle. The magnitude of this force and the product of the charge (q) of the particle and the electric field (E) generated between the two electrodes are equal. Thus,

$$F_e = q \times E$$

Dimensions of the electric field (E) are denoted by either Newton/Coulomb or volt/cm units. During electrophoresis, the magnitude of electric field (E) is denoted by volt/cm units. This value can be calculated from the voltage (volt) set by the electric power supply and the distance between two electrodes (cm).

When the electric field is applied and the electric force accelerates the charged particles, the medium exerts a drag force (F_d) called **friction** on the particles. The direction of this force is opposite to the direction of particle movement. This force is also proportional to the particle velocity (v). At a very low speed of particle migration during electrophoresis, F_d is a linear function of v.

$$F_d = f \times v$$

The ratio of the force and velocity is the **frictional coefficient** (f), whose value is a function of the particle size and shape, and medium viscosity. Larger the particles and more the obstruction in the medium, higher is the value of frictional coefficient.

When the electrophoresis process begins, the particles instantly accelerate to a velocity (v) at which the magnitude of the drag force and the magnitude of the (opposite) accelerating electric force becomes equal:

$$q \times E = f \times v$$

When the magnitude of the two opposing forces becomes equal, the resultant force nullifies. Consequently, each particle moves at a constant velocity characteristic of the given particle at the given accelerating potential and medium. **Electrophoretic mobility** (μ) is the particle velocity in a given medium when one unit of electric field is applied. This parameter is a linear function of the particle charge and it is a reciprocal function of the frictional coefficient (depends on the particle size and the medium nature).

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

Particles with different electrophoretic mobility migrate at different speeds in the same medium and electric field. Such particles can be separated by electrophoresis. In biochemical and molecular biological studies, proteins and nucleic acids are the most typical charged molecules that are analysed and separated by electrophoresis. This technique is performed using a special medium, which is most often a gel. The corresponding method is therefore denoted as gel electrophoresis.

11.1.3. Factors Affecting Electrophoretic Mobility

Electrophoretic mobility is the solute's response to the applied electrical field. T... following factors affect electrophoretic mobility:

1) **Sample:** The charge/mass ratio of the sample indicates its electrophoretic mobil... The mass depends on molecular size and shape.
   i) **Charge:** Higher the charge, greater is the electrophoretic mobility. However, ... charge is dependent on medium pH.
   ii) **Size:** Bigger the molecule, greater are the frictional and electrostatic forces exer... on it by the medium. Thus, **larger particles have smaller electrophoretic mobili...**
   iii) **Shape:** Rounded contours produce lesser friction and electrostatic retardat... than the sharper contours. **For example,** among globular and fibrous protei... the former will migrate faster.
2) **Electric Field:** The migration rate increases with an increase in potential gradien... Buffer ions carry the current in a solution placed between two electrodes. A... increase in potential difference increases the current. Resistance to current flow an... the migration rate are inversely proportional; therefore, a power pack has to be ma... available to control the current to a constant level without producing heat.
3) **Medium:** An **inert supporting medium** is generally selected for electrophores... This medium can exert adsorption or molecular sieving effect on the particles, thu... influencing the migration rate.
4) **Buffer:** The buffer maintains the pH of supporting medium and also affects th... electrophoretic mobility of the sample:
   i) **Composition:** The commonly used buffers are formate, phosphate, citra... acetate, barbitone, etc. The choice of buffer depends on the type of sample to b... separated by electrophoresis. Buffer affects the electrophoretic mobility if i... binds to a sample component being separated.
   ii) **Ionic Strength: Buffer having ionic strength between 0.05 and 0.1M** ... usually selected. If the ionic strength of buffer increases it indicates that the buffe... ions will carry a larger share of the current, while the sample ions will carry a... smaller part. This slows down the migration of sample components. If the ionic... strength of buffer decreases it indicates that the sample ions will carry a larger shar... of the current, thus resulting in faster separation. However, this causes diffusion of... the smaller components to be high with the associated loss of resolution.
   iii) **pH:** The direction and extent of migration of ampholytes depend on the buffer... pH. **Buffers having pH between 1 to 11** are used for achieving the desired... separation.
5) **Medium:** The inert medium can exert adsorption and molecular sieving effects on... the particle, thus influencing its migration rate.
   i) **Adsorption:** It indicates a component's retention on the supporting medium surface... Adsorption can reduce the rate and resolution of electrophoretic separation.
   ii) **Molecular Sieving:** Polyacrylamide, agar, starch, and sephadex media have... cross-linked structures that give rise to pores within the gel beads.
      a) **Sephadex:** Molecules larger than the pores do not enter the gel beads and... migrate faster.
      b) **Polyacrylamide, Starch, and Agarose:** Larger molecules squeeze through... the pores. The smaller molecules easily pass through the pores, but the... larger ones are retarded.

11.1.4. Applications of Electrophoresis

Electrophoresis has the following applications:

1) It is used for determining the high molecular weight compounds (**e.g.,** amino acids, proteins, and nucleic acids).
2) It is used for DNA sequencing.
3) It is used for DNA hybridisation.
4) It is used for determining biological fluid constituents.
5) It is used in the analysis of antibiotics.
6) It is used in the purification of vaccines.
7) It is used in chiral analysis.
8) It is used for determining impurities.
9) It is used in the assay of atropine phosphate.
10) It is used in the assay of codeine.
11) It is used in the diagnosis of genetic toxicology.
12) It is used in the diagnosis of hereditary diseases.
13) It is used in the analysis of carbohydrates.
14) It is used in the field of forensic science.
15) It is used in the field of molecular biology.

11.1.5. Types of Electrophoresis

Electrophoresis relies on the principle of migration of charged particles under the influence of electric field. The technique of electrophoresis is of the following two types:

1) **Zone Electrophoresis**
   i) Paper electrophoresis,
   ii) Gel electrophoresis,
   iii) Thin layer electrophoresis, and
   iv) Cellulose acetate electrophoresis
2) **Moving Boundary Electrophoresis**
   i) Capillary electrophoresis,
   ii) Isotachophoresis,
   iii) Isoelectric focusing, and
   iv) Immunoelectrophoresis.

11.2. TECHNIQUE OF PAPER ELECTROPHORESIS

11.2.1. Introduction

Paper electrophoresis is a type of zone electrophoresis, and is a very important laboratory method.

11.2.2. Principle

The charge a molecule carries depends on the medium pH. Electrophoresis at low voltage cannot separate low molecular weight compounds because of diffusion; however, it is easy to illustrate the relationship between charge and pH using amino acids, instead of proteins or other macromolecules.

11.2.3. Filter Paper

Paper of good quality and containing approximately 95% of α-cellulose should be used. The adsorption capacity of the selected paper should be very low.

11.2.4. Apparatus

The equipment of electrophoresis consists of a **power pack** and an **electrophoretic cell** (**figure 11.2**). The power pack provides a stabilised direct current and can be programmed to produce a constant voltage or a constant current. The electrophoretic cell comprises of platinum electrodes, two buffer reservoirs, a supporting medium for paper, and a transparent insulating cover.

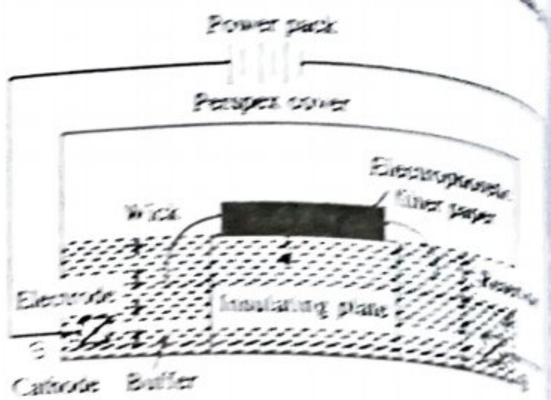


Figure 11.2: Horizontal Paper Electrophoresis

Each buffer reservoir is divided into two sections that are interconnected. One section contains the electrode and the other is in contact with the supporting medium. To prevent electrodes undergo any change in pH, the buffer in contact with the supporting medium should remain unaffected; thus, separate compartments are necessary. Wicks made up of several layers of filter paper or gauze are used to keep the buffer reservoir and the supporting medium in contact. Paper is placed on an insulating material (usually Perspex sheet). Then the entire electrophoretic unit is covered with an insulating material for reducing evaporation during run and for assuring electrical insulation.

The filter paper strips are commonly used in horizontal and vertical arrangements.

11.2.5. Sample Application

The sample is applied as a spot (of 0.5cm diameter) or as a narrow line streak. Special devices are commercially available for sample application. The sample can be applied before or after equilibrating the paper with buffer.

11.2.6. Electrophoretic Run

After applying the sample to the paper and equilibrating the paper with buffer, the current is switched on. The electrophoretic equipment is equipped with devices that provide stable voltage or current, still the entire unit is placed in a cold room to prevent overheating. The separation process completes within two hours, after which the power is switched off, the paper is removed, and vacuum dried in oven at 110°C temperature.

11.2.7. Detection and Quantitative Assay

The unknown electropherogram can be identified by comparing the unknown electrogram with the standard one under standard conditions. Individual compounds are identified as per their physical properties by the following methods:

1) **Fluorescence**
   i) The electrophoretogram is stained with **ethidium bromide** and visualised under UV light. The DNA and RNA fluoresce, thus facilitating their detection.
   ii) The electrophoretogram is stained with **fluorescamine** to detect amino acids, amino acid derivatives, peptides, and proteins.
   iii) **Dansyl chloride** can be used instead of fluorescamine.
2) **UV Absorption:** Proteins, peptides, and nucleic acids absorb in the range of 260-280nm, thus enabling their detection.
3) **Staining:** The compounds given in **table 11.1** are stained with the given dyes:

Table 11.1: Compounds Stained with Dyes for Quantitative Detection

Compounds	Dyes	Comments
Proteins	Bromophenol blue in acetic acid	Visual, quantitative
	Dansyl chloride	Fluorescent, quantitative
	Fluorescamine	Fluorescent, very sensitive
Nucleic acids	Methyl green-pyronin	DNA-Blue, RNA-Red, sensitive
	Ethidium bromide	Fluorescent, very sensitive
Polysaccharides	Iodine	Visual, sensitive
Lipoproteins	Sudan black in 60% ethanol	Visual, sensitive
Glycoproteins	Alcian blue	Visual, sensitive

4) **Detection of Enzymes in situ:** When the component to be separated is an enzyme, special techniques are used for its detection. The paper strip having the enzyme to be separated is saturated with the substrate to facilitate enzyme separation. Thereafter, the paper strip is placed in a suitable buffer along with electrophoretogram. Colour bands appear indicating the enzyme position.
5) **Quantitative Estimation:** On multiplying the colour density of the zone with the area of zone, the resulting value gives an estimate of the component concentration.

11.2.8. Applications

Paper electrophoresis has the following uses:

1) It is used for serum analysis for diagnostic purpose.
2) It is used for the analysis of muscle proteins, egg white proteins, milk proteins, and snake and insect venom.

11.3. TECHNIQUES OF GEL ELECTROPHORESIS

11.3.1. Introduction

Gel electrophoresis is a method utilised for the separation and analysis of macromolecules (DNA, RNA, and proteins) and their fragments, based on their size and charge. The technique is used in the field of clinical chemistry for separating proteins. It is also used in biochemistry and molecular biology fields for separating a mixed population of DNA and RNA fragments based on their length; thus, the size of DNA and RNA fragments can be estimated.

In gel electrophoresis, a gel is used as an anti-convective medium for suppressing the thermal convection produced by the application of electric field. The gel also serves as a sieving medium and retards the passage of molecules. Gels also maintain the finished separation, so that stain can be applied post electrophoresis.

DNA gel electrophoresis is performed for analytical purposes, often after amplification of DNA via PCR; however, it may be used as a preparative technique before using other methods such as mass spectrometry, RFLP, PCR, cloning, DNA sequencing, or Southern blotting for further characterisation. **Figure 11.3** represents the schematic diagram of gel electrophoresis.

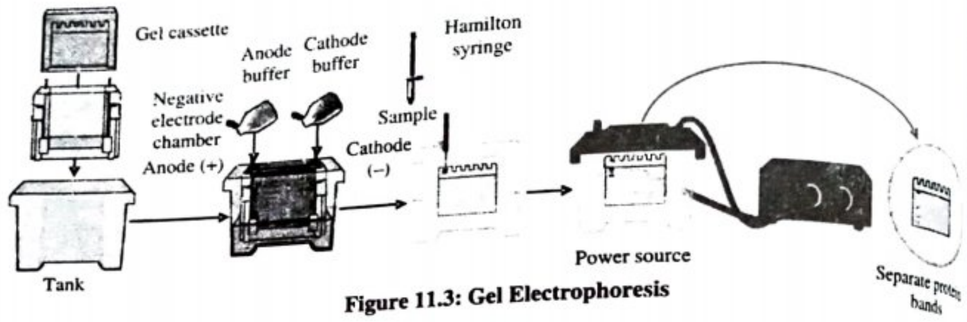


Figure 11.3: Gel Electrophoresis

11.3.2. Theory

A molecule with a net charge migrates in an electric field. This phenomenon is termed electrophoresis, which effectively separates proteins, DNA, and RNA. A molecule's migration velocity (v) in an electric field depends on the electric field strength (E) and electrophoretic mobility (μ) of the molecule. Thus,

$$v = \mu E \quad \text{.....(i)}$$

Electrophoretic mobility is unique for each molecule and medium. Electrophoretic separation is usually carried out in gels as they act as a molecular sieve, thus enhance the separation by modifying electrophoretic mobility. Molecules that are smaller than the pores in the gel easily pass through the gel, the larger molecules are more or less immobile, and the intermediate sized molecules pass through the gel with various degrees of facility. In gel electrophoresis, the following relation is found:

$$\log\mu = \log\mu_o - K_r\tau \quad \text{.....(ii)}$$

Where, μ_o = Free electrophoretic mobility of the molecule (mobility in a non-sieving medium).
K_r = Retardation coefficient.
τ = Concentration of the gel.

The value of μ_o depends on the mass-to-charge ratio of the molecule, and the value of K_r is related to the propriety of gel, and the size and shape of the migrating molecule.

11.3.3. Principle

On placing a mixture of electrically charged biomolecules in an electric field of field strength (E), the particles freely move towards the oppositely charged electrode. Depending on the physical conditions, different molecules move at different rates. A charged molecule's velocity (v) in an electric field depends on the variables:

$$v = E.q / f$$

Where, f = Frictional coefficient.
q = Net charge on the molecule.

Frictional coefficient describes frictional resistance to mobility and depends on the mass and degree of compactness of molecule, viscosity of buffer, and porosity of the matrix in which the experiment is performed. The number of positive and negative charges carried by the molecule determines the net charge on the molecule. Proteins gain charges by amino acid side chains and by the groups resulting from post-translational modifications (like deamination, acylation, or phosphorylation). DNA carries a uniform charge distribution since nucleotide receives a single negative charge by a phosphate group.

The equation above shows that the molecules move faster with increase in their net charge and with the strengthening of electric field. With a decrease in 'f' (a function of molecular mass/shape), the molecules having similar net charge separate due to differences in frictional coefficient while the molecules having similar mass/shape carry different net charge. Therefore, very high resolution separation can be achieved by electrophoresis.

11.3.4. Types of Gel

Different types of gels used in gel electrophoresis are:

1) **Starch Gel:** Potato starch is hydrolysed with acidified acetone at 37°C temperature. The resultant suspension is neutralised with sodium acetate, washed with excessive distilled water, and dried with acetone. On heating and cooling in an appropriate buffer, the obtained hydrolysed starch sets as a gel. The major **drawback** of starch gel is that its pore size cannot be controlled.

2) **Agar:** It is made up of agarose and agaropectin (galactose-based polymers). Agar solution is mixed with an equal volume of 40% polyethylene glycol at 80°C temperature. This precipitates agarose, which is collected, washed with distilled water, and dried with acetone.

   In aqueous buffers, agar solubilises at above 40°C temperature and sets as a gel at 38°C temperature. High molecular weight macromolecules, like proteins and nucleic acids, are separated by using agar.

3) **Polyacrylamide:** The components used for forming polyacrylamide include acrylamide monomer, N,N′-methylene-bis-acrylamide ammonium persulfate, and tetramethylenediamine (TEMED). These components are neurotoxic in nature, and thus should be used carefully.

Other gels that can be used are pectin, gypsum, sephadex, polyvinyl chloride, and polyvinyl acetate.

11.3.5. Agarose Gel Electrophoresis

An arrangement of a horizontal slab gel is commonly used. The gel is poured on a glass or plastic tray, which is then installed on a platform in the electrophoresis tank. The gel is immersed beneath the buffer surface and electrophoresis is performed. The resistance of gel to electric current is similar to that of the buffer, and thus a significant portion of the applied current passes along the length of the gel (**figure 11.4**).

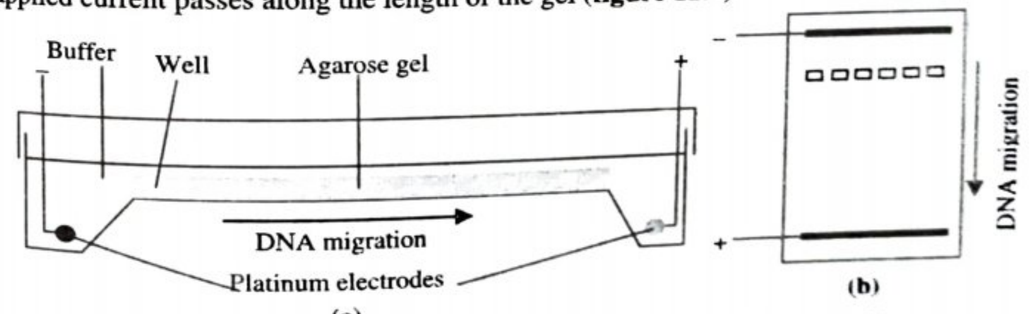


Figure 11.4: Agarose Gel Electrophoresis Apparatus a) Side view b) Top Down View

Samples are inserted in the wells placed at one end of the gel. Since the DNA in the buffer (having pH between 7.5 to 7.8) is negatively charged due to deprotonation of the phosphate linkage, it moves to the cathode.

11.3.5.1. Preparation and Staining of Gel

Agarose powder is boiled and melted with a buffered solution. The resultant solution is poured in a gel tray and allowed to cool at room temperature, as a result of which polymerisation occurs.

By staining with ethidium bromide (a fluorescent dye), DNA in agarose gels can be conveniently visualised.

11.3.5.2. Factors Affecting the Rate of DNA Migration

There are many factors that affect the velocity of DNA molecules in the gel (v). Some of these factors are:

1) **Voltage Applied:** Voltage directly influences the electric field strength (E) of the. At low voltages, the migration rate of linear DNA fragments is proportional to the voltage applied. At high voltages also, μ is considerably affected, thus modifying the migration speed. Maximum resolution of DNA fragments can be obtained by running the gel at not more than 5V/cm (distance between the electrodes).

2) **Agarose Concentration:** Equation (2) suggests a linear relationship between the logarithm of electrophoretic mobility of DNA and agarose concentration in the gel. Thus, if gels of different concentrations are used, a wide range of DNA molecules can be resolved (**table 11.2**).

Table 11.2: Range of Separation in Gels Containing Different Amounts of Agarose (1kb = 1 kilobase = 1000 Nucleotides)

Amount of Agarose in Gel (%[w/v])	Efficient Range of Separation of Linear DNA Molecules (kb)
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

3) **Molecular Size of the DNA:** Electrophoretic mobility of linear double-stranded DNA and the $\log_{10}$ of the number of base pairs (size component of K_r) are inversely proportional.

4) **Conformation of the DNA:** Migration rate of circular and linear DNA having same molecular weight through agarose gels is different (shape component of K_r).

5) **Electrophoresis Buffer:** Composition and ionic strength of electrophoresis buffer affects the electrophoretic mobility of DNA. The mass-to-charge ratio is constant for all DNA molecules in a given condition, and depends on the buffer pH. The buffering capacity is also significant, as the buffer becomes exhausted during extended electrophoresis. The buffers used usually contain EDTA and Tris-Acetate (TAE), Tris-Borate (TBE), Tris-Phosphate (TPE) at 50mM concentration (pH 7.5-7.8). The added EDTA inhibits the DNA degrading enzymes (nucleases), thus the Mg^{2+} ions required by these enzymes are trapped.

6) **Intercalating Dyes:** Ethidium bromide affects the DNA shape and K_r, thus reduces the electrophoretic mobility of linear DNA by about 15%.

11.3.5.3. Application

Agarose gel electrophoresis is the standard method for separating, identifying, and purifying DNA and RNA. The resolving power of agarose gels is lower than that of polyacrylamide gels, but the separation range is greater and they can be easily prepared.

Agarose gels are used for separating DNA fragments ranging from 200bp-50kb. They are also used for isolating a defined DNA fragment or as a preparative step in Southern blotting (DNA specific detection) or Northern blotting (RNA specific detection).

11.3.6. Polyacrylamide Gel Electrophoresis (PAGE)

The technique of PAGE is run vertically (**figure 11.5**). SDS-PAGE is carried out by fusing together two different types of polyacrylamide gels; thus, the gel has two parts, i.e., the **stacking gel** and the **resolving gel**. The stacking gel concentrates the protein mix loaded in the wells into a sharp band prior to its entry in the resolving gel.

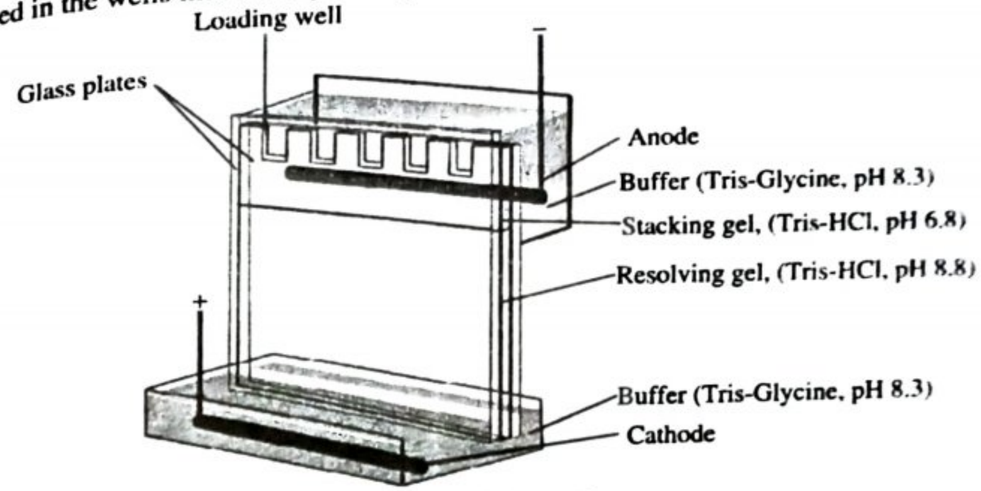


Figure 11.5: PAGE Apparatus

11.3.6.1. Preparation and Staining of Gel

Acrylamide and bisacrylamide mixture is prepared in a buffer, and added with ammonium persulphate to provide free radicals that initiate polymerisation. The obtained solution is poured between two glass plates to attain the shape of a gel and to hold it in place.

Different **staining techniques** are used for revealing the protein in the polyacrylamide gels. Two of such techniques are:

1) **Staining Resulting from a Chemical Reaction:** In silver staining, Ag^+ ions react with glutamate, aspartate and cysteine residues of the proteins and form complexes. The Ag^+ ions bound to the complexes undergo reduction with alkaline formaldehyde and form Ag. This precipitated Ag reveals the protein in the gel.

2) **Staining with Protein Binding Dyes:** Different dyes that bind to the proteins are used, but the most common one is Coomassie brilliant blue. This dye binds to proteins via physisorption to arginine, histidine, and aromatic amino acids. Coomassie staining is quantitative.

11.3.6.2. Factors Influencing the Migration

The migration of SDS-protein complexes in the resolving gel is affected by the **molecular weight**, the **acrylamide-bisacrylamide ratio** (affecting the gel proprieties component of K_r), and the **total polymer concentration** (τ). Cross-links formed from bisacrylamide provide rigidity and tensile strength to the gel and form pores to allow the passage of SDS-polypeptide complexes. These pore sizes decrease with the increase in bisacrylamide-acrylamide ratio, and becomes minimum when the ratio is approximately 1:20. Most SDS-polyacrylamide gels are cast with a molar ratio of 1:29, which can resolve the polypeptides of different sizes by as little as 3%.

The linear range of separation obtained with gels containing 5-15% of polymer is shown in the **table 11.3**:

Table 11.3: Effective Range of Separation of SDS-Polyacrylamide Gels, with a Bisacrylamide – Acrylamide Ratio of 1:29

Acrylamide Polymer Concentration (%)	Linear Range of Separation (kDa)
15	12-43
10	16-68
7.5	36-94
5.0	57-212

11.3.7. Applications

Given below are the applications of gel electrophoresis:

1) It is used for estimating the size of DNA molecules after restriction enzyme digestion, **e.g.,** in restriction mapping of cloned DNA.
2) It is used for analysing PCR products, **e.g.,** in molecular genetic diagnosis or genetic fingerprinting
3) It is used for separating restricted genomic DNA before Southern transfer, or for separating RNA before Northern transfer.
4) It is used in the field of forensic science, molecular biology, genetics, microbiology and biochemistry.
5) Western blot technique that is slightly different but related to gel electrophoresis is used for separating proteins and probing with labelled antibodies for specific proteins.
6) It also helps in comparing similarities and differences between species.
7) It is used for determining genetic relationship among species.

11.4. TECHNIQUES OF CAPILLARY ELECTROPHORESIS

11.4.1. Introduction

The technique of capillary electrophoresis is used for separating ions on the basis of their electrophoretic mobility by applying voltage. Electrophoretic mobility depends on the molecular charge, viscosity, and atomic radius. The migration rate of the particle and the applied electric field are directly proportional, i.e., greater the field strength, faster will be the mobility of particles. Only the ions migrate with the electric field, while the neutral species remain unaffected. In case two ions have the same size, the one carrying greater charge will move faster. In ions carrying same charge, the smaller particle exhibits less friction and faster migration rate. Capillary electrophoresis is widely in use since it provides high resolution separation in much less time.

11.4.2. Instrumentation

A quite simple in design instrumentation is required for capillary electrophoresis (**figure 11.6**). The basic instrument of capillary electrophoresis includes an auto-sampler, a detection module, a high-voltage power supply, a capillary, and a computer.

The capillary ends are placed in different buffer reservoirs, each carrying an electrode connected to a high-voltage power supply (can supply voltage of 30kV). Sample is injected into the capillary by temporarily replacing one of the buffer reservoirs (at the

anode) with a sample reservoir. Then either an electric potential or external pressure is applied across the capillary for a few seconds, and the separation is performed.

The separated analytes are optically detected (UV-visible or fluorometric) through the capillary wall near the opposite end (near the cathode). Capillary electrophoresis is suited to automation, and the arrangement of its commercial instruments is much familiar to that of modern HPLC.

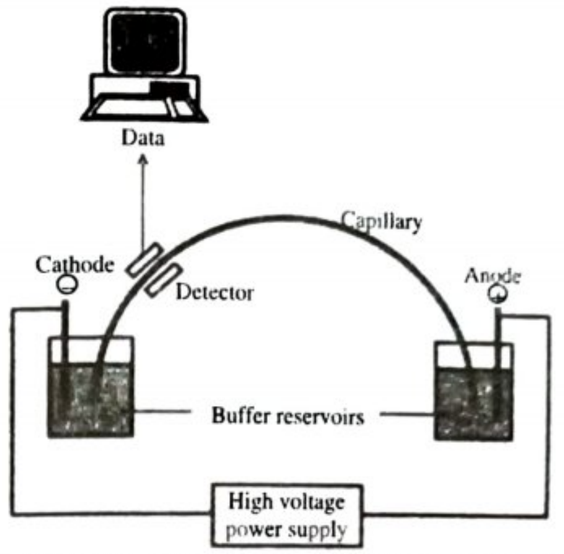


Figure 11.6: Schematic Representation of the Main Components of a Capillary Electrophoresis Instrument

11.4.3. Electrophoretic Mobility

In the process of electrophoresis, the sample ions move under the influence of an applied voltage. The ions undergo a force equal to the product of the net charge and the electric field strength. The sample ions are also affected by a drag force equal to the product of the translational friction coefficient and the velocity. This results in the expression for electrophoretic mobility:

$$\mu_{EP} = \frac{q}{f} = \frac{q}{6\pi\eta r} \quad \ldots(3)$$

Where, f = Translational friction coefficient for spherical particle (given by the Stokes' law).

η = Viscosity of the solvent.

r = Radius of the atom.

Migration rate of sample ions is represented by the charge-to-mass ratio. The actual velocity of the ions is proportional to the magnitude of electrical field (E), and can be determined by the following equation: ...(4)

$$v = \mu_{EP} E$$

This relationship indicates that in the presence of greater voltage, the migration of ionic species will hasten.

11.4.4. Electro-Osmotic Flow (EOF)

When a high voltage is applied to an electrolyte-filled capillary, the electro-osmotic flow occurs. This flow results when the pH of the buffer running through the silica capillary is more than 3 and the SiOH groups lose a proton to become SiO^- ions. Consequently, the capillary wall has a negative charge that develops a double layer of cations attracted to it. The inner cation layer is stationary and the outer one freely moves along the capillary

Under the influence of the applied electric field, the free cations move towards the cathode and create a powerful bulk flow. The electro-osmotic flow rate is governed by the following equation:

$$\mu_{EOF} = \frac{\varepsilon}{4\pi\eta} E\zeta \quad \ldots(1)$$

Where, ε = Dielectric constant of the solution.
η = Viscosity of the solution.
E = Field strength.
ζ = Zeta potential.

The electrophoretic mobility is greater than the electro-osmotic flow, thus the negatively charged particles (naturally attracted to the positively charged anode) separate out. The EOF works best when the zeta potential between the cation layers is large, a large diffuse layer of cations drag more molecules towards the cathode, the resistance from the surrounding solution is low, and the buffer has pH of 9 so that all SiOH groups are ionised.

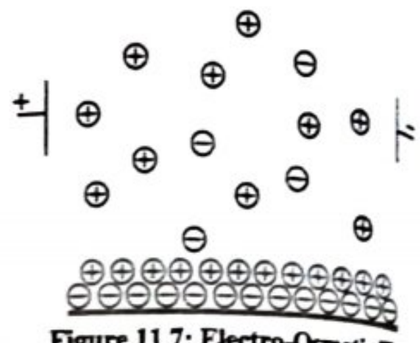


Figure 11.7: Electro-Osmotic Flow Due to Applied Voltage

11.4.5. Zeta Potential

Zeta potential and the pH dependent charge density on the capillary wall are proportional to each other. Therefore, mobility of EOF varies with the buffer pH. i.e., the EOF mobility will be comparatively greater at high pH. **Figure 11.8** represents the variation in EOF mobility with pH for a fused-silica capillary.

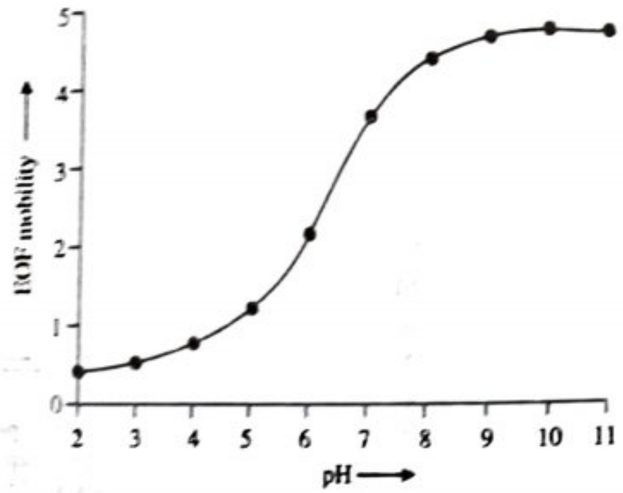


Figure 11.8: Variation of EOF Mobility with Changing pH for a Typical Uncoated Fused-Silica Capillary

Silanols completely ionises and its EOF mobility is highest above pH 9. Ionisation of silanols is low and its EOF mobility is insignificant below pH 4. Zeta potential also depends on the buffer's ionic strength. This is because with increase in ionic strength, the double layer compresses, the zeta potential decreases, and EOF mobility reduces.

The EOF mobility is sufficient above pH 7 to make sure that most of the ions migrate towards the cathode, irrespective of their charge. Due to this reason, the observed migration velocity of a solute is not related to its electrophoretic mobility alone, but to the combination of its electrophoretic mobility and EOF mobility. Therefore, a solute's apparent electrophoretic

mobility (μ_a), calculated from its observed migration velocity, is the vector sum of its real (or effective) electrophoretic mobility (μ_e) and EOF mobility (μ_{EOF}), i.e.,

$$M_a = M_e + \mu_{EOF}$$

The samples are introduced at the anode and EOF moves from the anode to the cathode. The cations have positive, neutrals have zero, and anions have negative real electrophoretic mobility. This indicates that the cations migrate faster than the EOF, neutrals migrate with the same velocity as the EOF, and anions migrate slowly than the EOF.

11.4.6. Flow Profile in CE

Another key feature of EOF is that along with the parabolic flow profile (generated by an external pump), it has a **flat flow profile (figure 11.9)** since its driving force (i.e., charge on the capillary wall) is uniformly distributed along the capillary. This indicates that no pressure drops are encountered and the flow velocity is uniform across the capillary. This is different than the pressure-driven flow, in which a pressure drop is exerted across the column by the frictional forces at the column walls. This yields a **parabolic** or **laminar flow profile**. The flat flow profile of EOF minimises zone broadening and enhances the high separation efficiencies, thus allowing separations based on mobility differences as small as 0.05%.

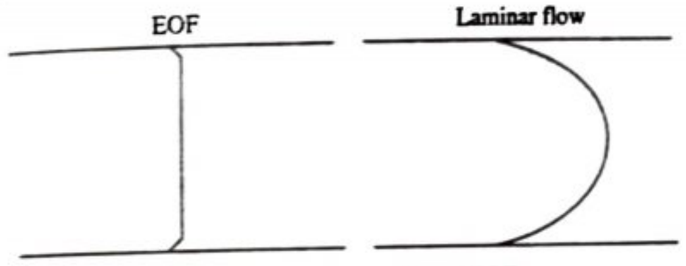


Figure 11.9: Flow Profiles of EOF

11.4.7. The Electropherogram

The data output from capillary electrophoresis is presented as an electropherogram (similar to a chromatogram), which is a **plot of migration time *versus* detector response**. The detector response is concentration dependent, such as UV-visible absorbance or fluorescence. A typical electropherogram obtained for the separation of a three component mixture of cationic, neutral and anionic solutes is shown in **figure 11.10**.

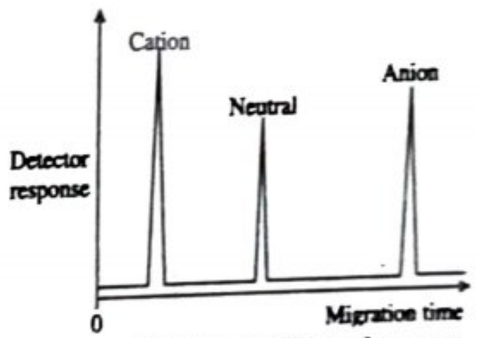


Figure 11.10: Typical Electropherogram Showing the Separation of a Cation, a Neutral Ion and an Anion

11.4.8. Applications of CE

Capillary electrophoresis is used in pharmaceutical industries for quality testing to ensure the absence of side products or interferents. Since the capillary walls can be neutralised with an acidic pH, the drugs having a basic amino group will not stick to the capillary, and thus capillary electrophoresis can be used for their separation.

Capillary gel electrophoresis is used for sequencing the human genome and separating DNA. For such separations, the capillary is injected with a polymer, which provides an additional mode of separation based on the size as the smaller fragments travel faster through the gel. This method is termed **sieving**.

11.5. SUMMARY

The details given in the chapter can be summarised as follows:

1) **Electrophoresis** is a phenomenon describing the motion of particles in a gel or fluid within a relatively uniform electric field.
2) Electrophoresis of anions is termed **anaphoresis**, while the electrophoresis of cations is termed **cataphoresis**.
3) **Ferdinand Frederic Reuss** first discovered the technique of electrophoresis in **1807**.
4) By applying an electric power, **electric potential** is generated between the two electrodes, under the influence of which the electrons move between the two electrodes (i.e., from anode to cathode) through a wire.
5) The method of electrophoresis is used for separating the charged particles from one another based on the differences in their migration speed.
6) The ratio of the force and velocity is the **frictional coefficient**, whose value is a function of the particle size and shape and the medium viscosity.
7) **Electrophoretic mobility** is the particle velocity in a given medium when one unit of electric field is applied.
8) Higher the charge, greater is the electrophoretic mobility.
9) Bigger the molecule, greater are the frictional and electrostatic forces exerted on it by the medium. Thus, larger particles have smaller electrophoretic mobility.
10) The migration rate increases with an increase in potential gradient.
11) An inert supporting medium is generally selected for electrophoresis.
12) Buffers having pH between 1 to 11 are used for achieving the desired separation.
13) **Paper electrophoresis** is a type of zone electrophoresis
14) The equipment of electrophoresis consists of a **power pack** and an **electrophoretic cell**.
15) The electrophoretogram is stained with **ethidium bromide** and visualised under UV light.
16) **Gel electrophoresis** is a method utilised for the separation and analysis of macromolecules (DNA, RNA, and proteins) and their fragments, based on their size and charge.
17) **DNA gel electrophoresis** is performed for analytical purposes, often after amplification of DNA via PCR
18) **SDS-PAGE** is carried out by fusing together two different types of polyacrylamide gels; thus, the gel has two parts, i.e., the **stacking gel** and the **resolving gel**.
19) The migration of SDS-protein complexes in the resolving gel is affected by the **molecular weight**, the **acrylamide-bisacrylamide ratio**, and the **total polymer concentration**.
20) The technique of **capillary electrophoresis** is used for separating ions on the basis of their electrophoretic mobility by applying voltage.
21) The electrophoretic mobility is greater than the electro-osmotic flow, thus the negatively charged particles separate out.
22) The data output from capillary electrophoresis is presented as an electropherogram (similar to a chromatogram), which is a **plot of migration time *versus* detector response**.

11.6. EXERCISE

11.6.1. True or False

1) Ferdinand Frederic Reuss first discovered the technique of electrophoresis in 1807.
2) The method of electrophoresis is used for separating the charged particles from one another based on the differences in their partition coefficient.

3) Higher the charge, greater is the electro-osmotic flow.
4) Larger particles have larger electrophoretic mobility.
5) Buffers having pH between 1 to 11 are used for achieving the desired separation.
6) Gel electrophoresis is a method utilised for the separation and analysis of micromolecules, based on their size.
7) The electrophoretogram is stained with ethidium bromide and visualised under UV light.
8) The technique of iontophoresis is used for separating ions on the basis of their electrophoretic mobility by applying voltage.

11.6.2. Fill in the Blanks

9) Electrophoresis of anions is termed _____, while the electrophoresis of cations is termed _____.
10) The ratio of the force and velocity is the _____.
11) _____ is the particle velocity in a given medium when one unit of electric field is applied.
12) The migration rate increases with an increase in _____.
13) Paper electrophoresis is a type of _____ electrophoresis
14) The equipment of electrophoresis consists of a power pack and an _____.
15) SDS-PAGE is carried out by fusing together the _____ and the resolving gel.
16) The data output from capillary electrophoresis is presented as an electropherogram, which is a plot of _____ *versus* _____.

Answers

1) True 2) False 3) False 4) False
5) True 6) False 7) True 8) False
9) Anaphoresis and cataphoresis 10) Frictional coefficient
11) Electrophoretic mobility 12) Potential gradient 13) Zone
14) Electrophoretic cell 15) Stacking gel 16) Migration time and detector response

11.6.3. Very Short Answer Type Questions

1) What is electrophoresis?
2) Give the types of electrophoresis.
3) Give the applications of paper electrophoresis.
4) Draw a well-labelled diagram of the apparatus used in gel electrophoresis.
5) Give the applications of gel electrophoresis.
6) What is electrophoretic mobility?

11.6.4. Short Answer Type Questions

1) Discuss the factors affecting electrophoretic mobility.
2) Write a note on PAGE.
3) Give the applications of electrophoresis.
4) Write a note on capillary electrophoresis.
5) Discuss agarose gel electrophoresis.

11.6.5. Long Answer Type Questions

1) Briefly explain the principle of electrophoresis.
2) Discuss about the techniques of paper electrophoresis.
3) Write an illustrative note on gel electrophoresis.

CHAPTER 12 Gas Chromatography

12.1. GAS CHROMATOGRAPHY

12.1.1. Introduction

A.T. James and **P. Martin** first time used the gas chromatography technique in **1952** for separating long chain fatty acids. The gases and vaporisable substances can also be separated by gas chromatography based on differential adsorption.

In gas chromatography, gas is used as the mobile phase and solid or liquid is used as the stationary phase. When the stationary phase is solid, it is known as **Gas Solid Chromatography (GSC)** and when the stationary phase is liquid, it is known as **Gas Liquid Chromatography (GLC)**.

In gas chromatography, a moving gas phase is passed over a stationary sorbent to separate the mixture components. This technique is similar to that of liquid-liquid chromatography, with the only exception that in the former a moving gas is used as the mobile phase while in the latter it is a liquid. The stationary phase remains the same, i.e., a solid or a liquid.

12.1.2. Principle

In gas chromatography, the substance to be analysed is partitioned between the mobile and stationary phases. During the separation, the sample is vaporised and carried through the column by the mobile gas phase (i.e., the carrier gas). The different components get separated based on their vapour pressure and affinities for the stationary phase. The affinity of a component towards the stationary phase is termed as **distribution constant** (K_c), which is also known as the **partition coefficient.**

$$K_c = [A]_s / [A]_m$$

Where, $[A]_s$ = concentration of component A in the stationary phase and $[A]_m$ = concentration of component A in the mobile phase.

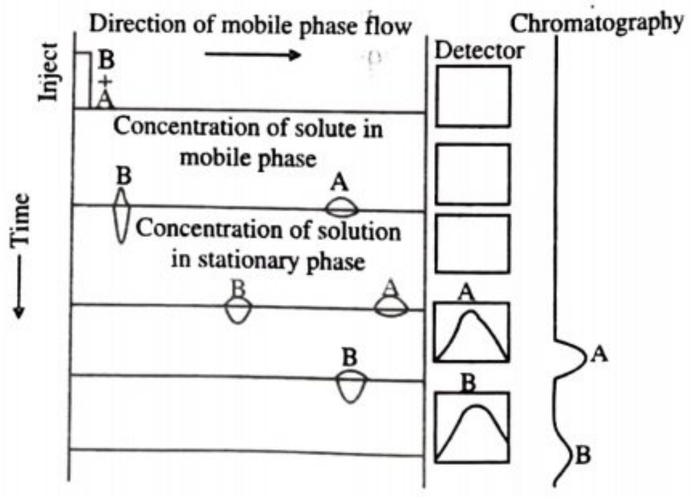


Figure 12.1: Schematic Representation of the Chromatographic Process

Movement of different components through the column is controlled by the distribution constant (K_c), thus the chromatographic separation occurs based on the differences in distribution constant. **Figure 12.1** shows a schematic representation of gas chromatography. The distribution constant depends on the temperature and the chemical nature of stationary phase. Thus, temperature can be used for enhancing the separation of different components through the column or a different stationary phase.

12.1.3. Theory

Gas chromatography is carried out in a column containing the mobile phase. **Gas-Liquid Chromatography** (GLC) employs liquid as the stationary phase and is a form of **partition chromatography**; while **Gas-Solid Chromatography** (GSC) employs a solid surface as the stationary phase and is a form of **adsorption chromatography**.

The migration rate of solutes through the chromatographic system relies on their affinities for the stationary phase. **Resolution** is a function of differential zone migration rates that separate the zone centres, and of zone broadening processes that merge the zones. The mobile phase (the carrier gas) in gas chromatography is an inert gas, thus the solute's affinity for the stationary phase determines the distribution coefficient. The solute in gas chromatography moves through the column only if it is a gas. Therefore, the relationship between the solute's vapour pressure and its concentration in a liquid solvent (the stationary phase) is determined. If an ideal solution is used, the relationship is **Raoult's law** [equation (1)].

$$p = xp^0 \qquad \text{..... (1)}$$

Where, p = vapour pressure of solute over the solution, x = mole fraction of solute in the solution, and p^0 = vapour pressure of the pure solute].

This equation is not obeyed by the real solutions, but a similar relationship, termed **Henry's law** can be written as:

$$p = \gamma x p^0 \qquad \text{..... (2)}$$

Where, γ = Activity coefficient, which is a function of x. The activity coefficient remains constant at very low concentrations:

$$\frac{x}{p} = \frac{1}{\gamma p^0} = \text{constant} \qquad \text{..... (3)}$$

The quantity x/p has the nature of a partition coefficient, and it controls selectivity in gas chromatography. Rather than work with x/p itself, the **partition coefficient** is defined by:

$$K = \frac{\text{gm of solute per gm of liquid phase}}{\text{gm of solute per cm}^3\text{ of gas phase}} \qquad \text{..... (4)}$$

Equation (3) suggests that the affinity of solute for stationary phase depends on its vapour pressure and activity coefficient. This dependence indicates that the solutes having same vapour pressures can be separated even if their activity coefficients are different in the stationary phase. A gas or a vapour on coming in contact with an adsorbent gets adsorbed on the solid surface in a specific amount. This phenomenon takes place as per the laws of **Freundlich** (i.e., $x/m = Kc^{1/n}$) or **Langmuir** (i.e., $x/m = K_1c + K_2c$, where, x = Mass of the gas or vapour adsorbed in mass m of the sorbent; c = Vapour concentration in the gas phase; and K, K_1, and K_2 = Constants). A gas or a vapour on coming in contact with a liquid gets dissolved in the liquid in a fixed amount. This phenomenon takes place as per the **Henry's law of partition** (i.e., $x/m = K_c$). Both the phenomena are selective and different vapour-sorbent pairs have different K-values.

12.1.4. Types

The two major types of gas chromatography are:

1) **Gas-Solid Chromatography:** In this type, the stationary phase is solid (adsorbent like alumina, silica, active carbon, etc. are used). This method provides a long column life time; however, catalytic changes are observed in this technique.
2) **Gas-Liquid Chromatography:** In this type, the stationary phase is an immobilised liquid coated on the solid support (like polymers). In this method, the liquid gradually bleeds off, and this is the disadvantage of this method.

12.1.5. Temperature Programming

Temperature programming combines the best results of runs at different temperatures. The sample is introduced into the chromatographic system, and the column is maintained at a temperature below that of the lowest-boiling component of the sample (preferably below 90°C). The column temperature is increased at some pre-selected heating rate. Earlier peaks that represent the low-boiling components appear as they would form an isothermal column maintained at a low temperature. With the increase in column temperature, the high-boiling components are forced through the column at an ever-increasing rate. In this way, the approximate proper temperature programme can be estimated.

Partition coefficient rapidly increases the solute amount in the vapour phase. Solute concentration in the liquid phase is always more than in the gas phase, i.e., the fraction $1/(1 + k')$ is less than unity; therefore, it is a good approximation to ignore $C_M V_M$ in the denominator. Remembering that $k' = K/\beta$, on ignoring the temperature dependency of the first term on the right-hand side from the equation, one gets:

$$\ln\left(\frac{1}{k'}\right)=\frac{\Delta\overline{H}_v}{RT}+C' \qquad \ldots\ldots(5)$$

The average increase in temperature should be determined to halve the value of k' (to double the total solute in the vapour phase). If k′ is halved by increasing the temperature from T_1 to T_2, the ratio of k' values can be determined from **equation (5)** as follows:

$$\ln 2=\frac{-\Delta\overline{H}_v/RT_2}{-\Delta\overline{H}_v/RT_1}=\frac{\Delta\overline{H}_v}{RT}\left(\frac{\Delta T}{T}\right) \qquad \ldots\ldots(6)$$

Which gives:

$$\Delta T=\frac{0.693R(\overline{T})^2}{\Delta\overline{H}_v} \qquad \ldots\ldots(7)$$

Where, $\overline{T}$ = Geometric mean of the two temperatures

$$\Delta T = T_2 - T_1 \qquad \ldots\ldots(8)$$

Trouton's rule is approximately valid, so $\Delta\overline{H}_v/T_b \simeq 23$.

The chromatographic process is operated near the solute boiling point, thus $\overline{T} = T_b$. The temperature increase (ΔT) halves the k′ value to be 21°C at an operating temperature of 75°C, 24°C at 125°C, and 30°C at 225°C. The k′ value is important because more the solute in the vapour state, faster the peak migrates. Thus, if the value of k′ is 5, one-fifth of the solute molecules exist as vapour.

The final temperature should be near the final solute's boiling point (if the maximum temperature limit of the stationary phase is not exceeded). With the increase in temperature range, individual compounds automatically select their own ideal temperature to migrate and separate in the column.

On increasing the column temperature linearly, the members of any homologous series are eluted at equally spaced intervals (**figure 12.10**), rather than proportional to $\ln t'_R$ as in isothermal elution.

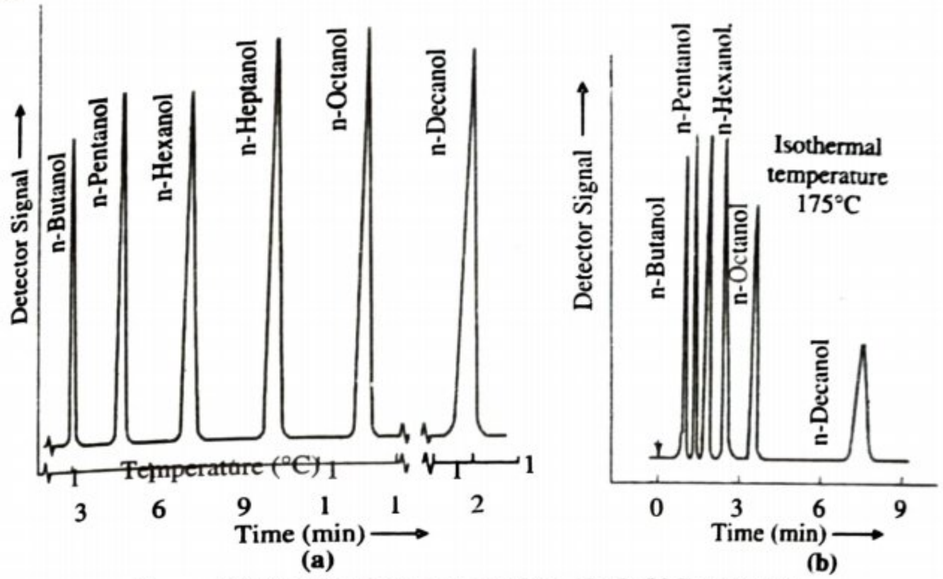


Figure 12.10: Chromatograms of an Alcohol Mixture: (a) Programmed Temperature from 100 to 175°C and (b) Isothermal Operation at 175°C.

12.1.6. Advantages

Gas chromatography has the following advantages:

1) It is a reliable technique and provides rapid analysis.
2) It is highly efficient and leads to high resolution.
3) It utilises sensitive detectors.
4) It requires small samples (<1 ml).
5) It is non-destructive as it enables the coupling to mass spectrometers, which measures the masses of individual molecules converted into ions, i.e. molecules that have been electrically charged.
6) It provides high quantitative accuracy.
7) It is a well-established technique with extensive literature and applications.

12.1.7. Disadvantages

Gas chromatography has the following disadvantages:

1) It is limited to volatile samples.
2) It is not suitable for thermolabile samples (that degrade at elevated temperatures).
3) It is not suited to preparative chromatography.
4) It requires MS detector for structural elucidation of the analyte, since most of the non-MS detectors are destructive.

12.2. INSTRUMENTATION

12.2.1. Introduction

The basic instrumentation of gas chromatographic technique has six components; however, many commercial variations are available.

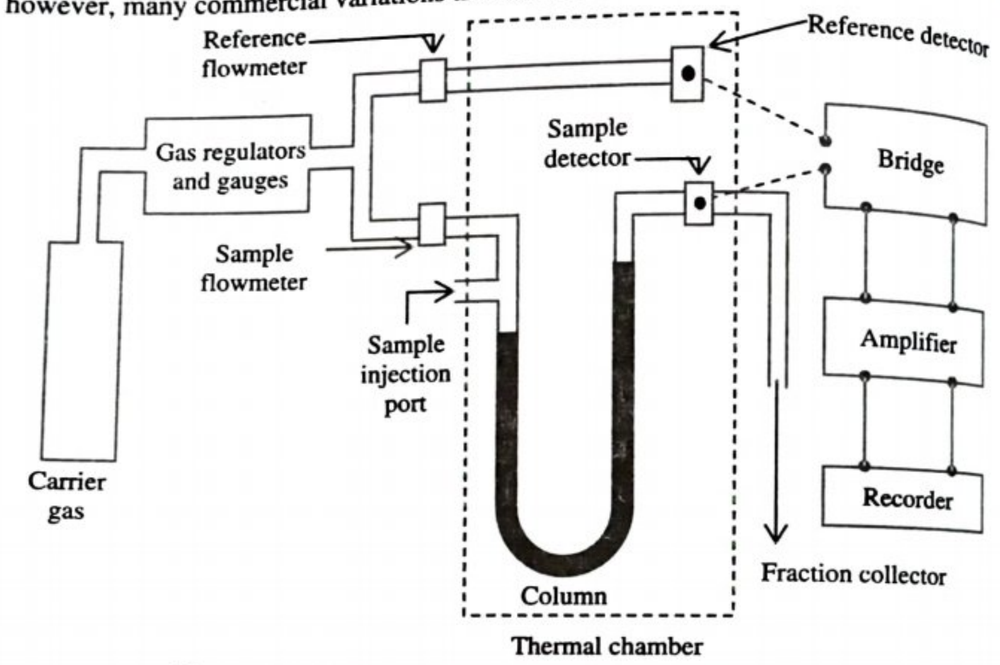


Figure 12.2: Schematic Representation of a Gas Chromatograph
Source: Adapted from *Instrumental Methods of Chemical Analysis* (pp 2.677), by Chatwal G.R. (2006) (Himalaya Publishing House)

The following components make up the instrumentation of gas chromatography (**figure 12.2**):

1) Carrier gas maintained at a high pressure and delivered at a rapid and reproducible rate,
2) Sample injector,
3) Separation columns,
4) Detectors,
5) Thermostated chambers for regulating the temperature of column and detectors, and
6) Amplifier and recorder system.

Separation in gas chromatography is carried out in a tubular column (of glass, metal, or Teflon) filled with an adsorbent (the stationary phase). The adsorbent is packed as fine size graded powder, while the liquids before being packed in the column are either coated as a fine film on the column wall or are coated over an inert size graded porous support (such as firebrick powder). A carrier gas (the mobile phase) is continuously made to flow through the column so that the sample components are distributed in the column.

The sample vapour is introduced in the column through the carrier gas entrance end. The different sample components adsorb on the stationary phase to different extents, and this depends on their distribution coefficients. The carrier gas immediately sweeps further the portion of each component in the gas phase. Thus, a fraction of the adsorbed amount desorbs out to maintain the value of distribution coefficient. Simultaneously, out of the amount swept away, some amount again goes into the adsorbent at the next point in the column to maintain the distribution coefficient value. This process continues and the band for each component moves further in the column attaining the shape of Gaussian distribution.

12.2.2. Carrier Gas

Hydrogen, **helium**, **nitrogen**, and **air** are the most widely used carrier gases. Hydrogen in comparison to other gases is more advantageous and also dangerous to use. Helium is the next best gas, and is used because of its exceptional thermal conductivity, inertness, low density, and greater flow rates; but it is expensive. Nitrogen is inexpensive but reduces sensitivity. Air is used only when the atmospheric oxygen is useful to the detector or separation. The following considerations should be kept in mind while **selecting a carrier gas**:

1) It should be inert, i.e., it should not react with the sample, stationary phase, or contacted hardware.
2) It should be suitable for the detector used and the type of sample being analysed.
3) It should be available in high purity.
4) It should give best column performance reliable with required speed of analysis.
5) It should not be expensive.
6) It should not cause any fire or explosion hazard.

12.2.3. Sample Injector

The system of sample injector is used for introducing the sample in a reproducible manner and should vaporise it rapidly so that the sample enters the column as a single slug.

Figure 12.3: Hypodermic Syringe

Liquid samples are introduced into a small inlet chamber using hypodermic syringes (**figure 12.3**) through a self-sealing rubber septum. The chamber is heated to cause flash evaporation, and the temperature should not be very high to avoid sample decomposition. Solid samples are either dissolved in volatile liquids prior to their introduction or are directly introduced if they are liquefiable. Gas samples are introduced into the carrier gas stream using a special gas sampling valves.

12.2.4. Separation Columns

The columns used are made of glass or metal tubing, and have a diameter of 4.8mm. They may be of any length ranging from a few centimetres to a hundred meters. They may be coiled, bent, or straight. The following **six types** of analytical columns are used in gas chromatography:

1) **Packed Columns:** These columns are prepared by packing metal or glass tubing with granular stationary phase. In gas liquid chromatography, the packing is prepared by coating a size graded inert solid support with the liquid phase.
2) **Open Tubular or Capillary or Golay Columns:** These columns are made of long capillary tubing (30-90m) and have uniform and narrow internal diameter (0.025-0.075cm). They are of stainless steel (most popular), copper, nylon, glass, etc. The liquid phase is coated over the inner wall of capillary tubing as a thin (0.5-1μ) and uniform film. Since there is no packing in these columns, the flow of carrier gas experiences least resistance.
3) **Support Coated Open Tubular Columns:** These columns are prepared by coating the inner wall of a capillary column with a micron size porous layer of support material, followed by coating with the liquid phase as a thin film.
4) **Wall Coated Open Tubular Columns:** These columns are prepared by coating the unmodified smooth inner wall of the tube with the liquid stationary phase.

5) **Porous-Layer Open-Tubular (PLOT) Columns:** These columns are prepared by coating the inner wall with a porous layer. Porosity can be achieved either by chemical methods (**e.g.,** etching) or by depositing porous particles on the wall from a suspension. The porous layer either provides support to the liquid stationary phase or acts as the stationary phase itself.

6) **Support-Coated Open-Tubular (SCOT) Columns:** In these columns, the porous layer consists of support particles and was deposited from a suspension.

12.2.5. Detectors

Gas chromatography employs a wide range of detectors, of which **Flame Ionisation Detector (FID)** and the **Thermal Conductivity Detector (TCD)** are the most common ones. Both these detectors are sensitive to various components, and both work over a wide range of concentrations. The **TCDs are universal** and can be **used for detecting any component**; however, they **cannot detect carrier gas till their thermal conductivities are different from those of the carrier gas at detector temperature.**

The **FIDs are sensitive to hydrocarbons**, and their sensitivity is more than that of TCDs; however, they **cannot detect water**. Both TCDs and FIDs are quite robust. The TCDs being non-destructive can be operated in-series before FIDs (destructive), thus providing complementary detection of the same eluents.

Some gas chromatographs utilise a **mass spectrometer** as the detector; this combination is known as **GC-MS**. Some GC-MS utilise an **NMR spectrometer** as a backup detector; this combination is known as **GC-MS-NMR**. Some GC-MS-NMR utilise an **IR spectrophotometer** as a backup detector; this combination is known as **GC-MS-NMR-IR**.

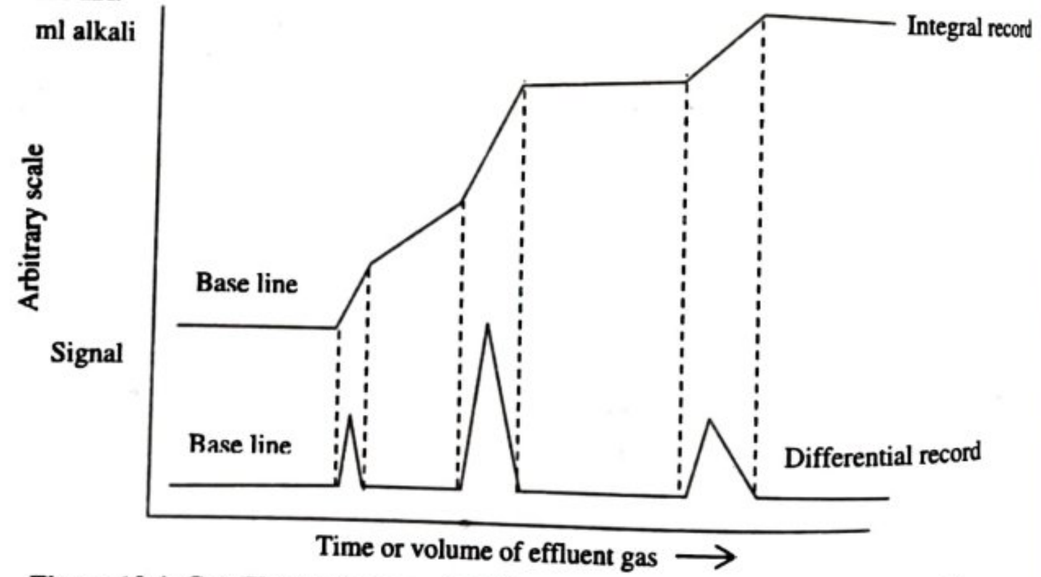


Figure 12.4: Gas Chromatogram of a Mixture of Three Acids using Differential and Integral Detectors

In **Gas-Liquid Chromatography (GLC)**, the gas from the column is constantly monitored and any difference between it and the normal gas is recorded, i.e., detectors in most apparatus are differential. The **chromatogram** is a record of **detector response against time**, or is a record of the known **gas flow against carrier gas volume**.

Figure 12.4 shows the trace obtained with a detector and also shows the appearance of a corresponding curve for an integral detector.

Some desirable **properties of a detector** are:

1) Its sensitivity should be high and should not show instability at high sensitivities.
2) Its volume should be low so that the compound eluted from the column in a small plug of carrier gas does not undergo further dilution within the detector.
3) Its response should be rapid and linear with the concentration of compound. It should be calibrated to determine the optimum range.
4) Its response should not be affected by the flow rate of carrier gas and temperature.

Some commonly used detectors in gas chromatography are discussed below:

1) **Katharometer:** This detector relies on the variation in thermal conductivity of the carrier gas in the presence of an organic compound. The principle of this detector is illustrated in **figure 12.5**. The platinum wires are heated by electric means and equilibrium conditions of temperature and resistance are attained when the carrier gas passes over them. They are mounted in a Wheatstone bridge arrangement, and when a compound emerges, the thermal conductivity of the gas surrounding wire changes; also changing the temperature and resistance of the wire along with the associated out-of-balance signal, which is amplified and recorded.

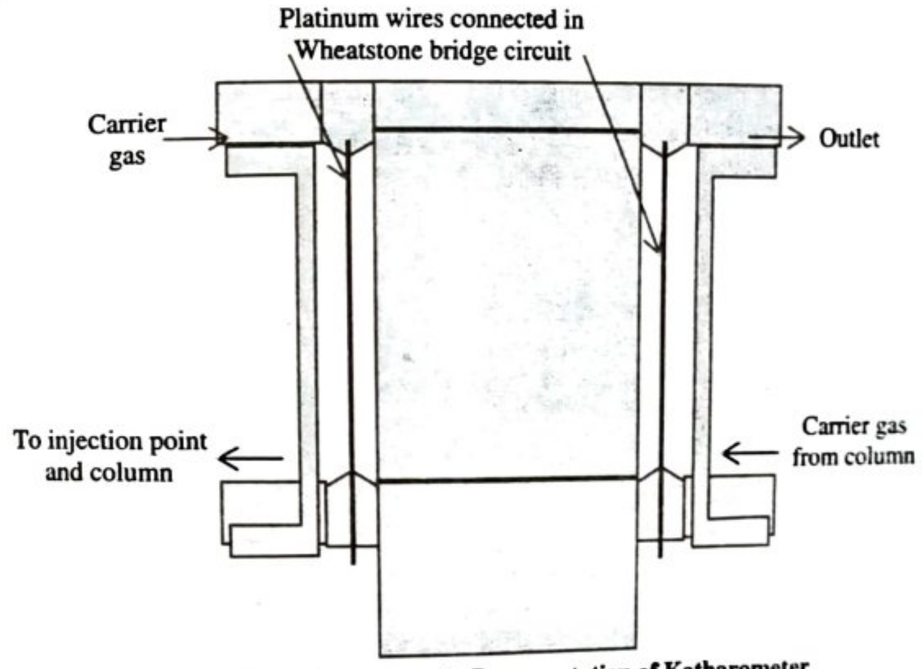


Figure 12.5: Diagrammatic Representation of Katharometer

The sensitivity of katharometer is lower than the sensitivities of other detectors. Its sensitivity is affected by temperature and flow rate fluctuations.

2) **Flame Ionisation Detecto (FID):** This detector is simpler in design (**figure 12.6**) and relies on the change in conductivity of the flame as the compound is burnt. The change in flame conductivity is not because of simple ionisation of the compounds emerging from the detector.

The molecule undergoes partial or complete stripping and gives charged hydrogen-deficient polymers or aggregates of carbon with low ionisation potential.

The carrier gas used is nitrogen or argon mixed with hydrogen before passing to the burner tip (made of a platinum capillary). This forms one electrode, and the other one is silver gauze or brass collector electrode about 1cm above the flame.

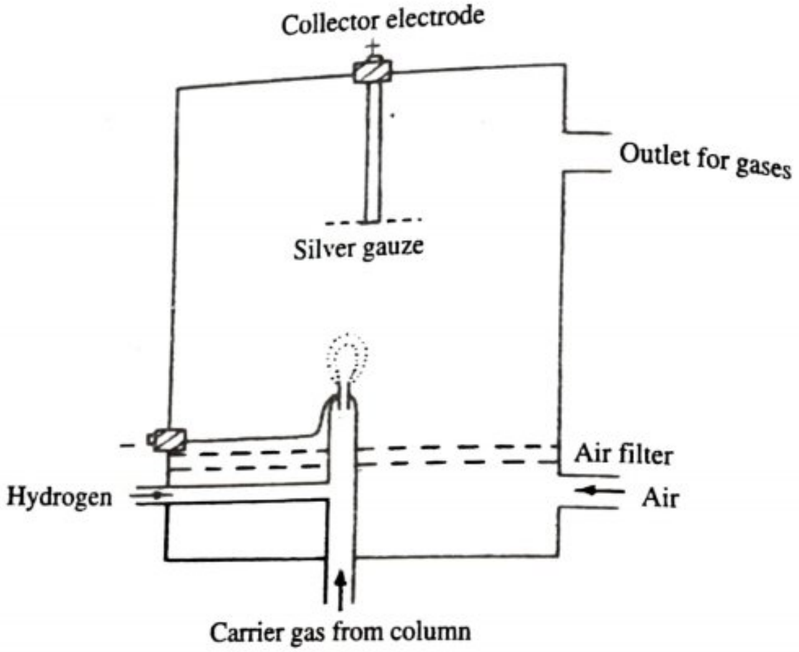


Figure 12.6: Diagrammatic Representation of Flame Ionisation Detector

3) **Thermal Conductivity Detector (TCD):** This detector (**figure 12.7**) utilises a heated filament placed in the emerging gas stream. Thermal conductivity of the gas phase governs the amount of heat the filament loses by conduction to the detector walls.

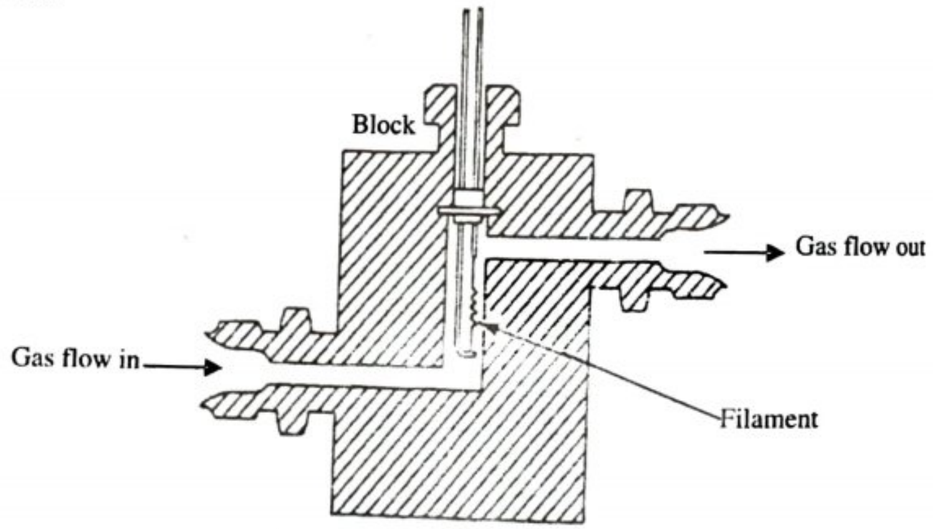


Figure 12.7: Cross-Sectional View of a Thermal Conductivity Detector

The cavity in the metal block has a tightly coiled filament of tungsten metal, tungsten-rhenium alloy, or tungsten sheathed with gold. A regulated DC current is supplied to heat the filament up to a constant temperature till it reaches less than a dull-red condition. If only carrier gas is flowing through the detector, the heat lost by the filament to the metal block is constant.

Hydrogen and helium have 6-10 times greater thermal conductivities than other organic compounds. Due to this reason, even trace amounts of organic materials adversely decrease the thermal conductivity of the column effluent. The filament retains more heat, and its temperature and electrical resistance increases. A standard detector comprises of four identical filaments (mounted in a brass block), which form the arms of a Wheatstone bridge.

A TCD can be constructed with thermistor, which is a metal oxide bead having attached electrical leads. The bead possesses 8000Ω resistance at 25°C temperature and a negative temperature coefficient of resistance. One bead is mounted in the pure carrier gas stream, and the other in the column effluent.

The Wheatstone bridge circuit is completed by a matched pair of 500Ω, 3W resistors. Thermistors are neither mechanically nor electrically exchangeable with filaments. They can operate at ambient or sub-ambient column temperatures.

4) **Thermionic Emission Detector (TED):** This detector utilises fuel-poor hydrogen plasma and a low temperature flame. This flame suppresses the normal flame ionisation response of compounds not containing nitrogen or phosphorus (**figure 12.8**).

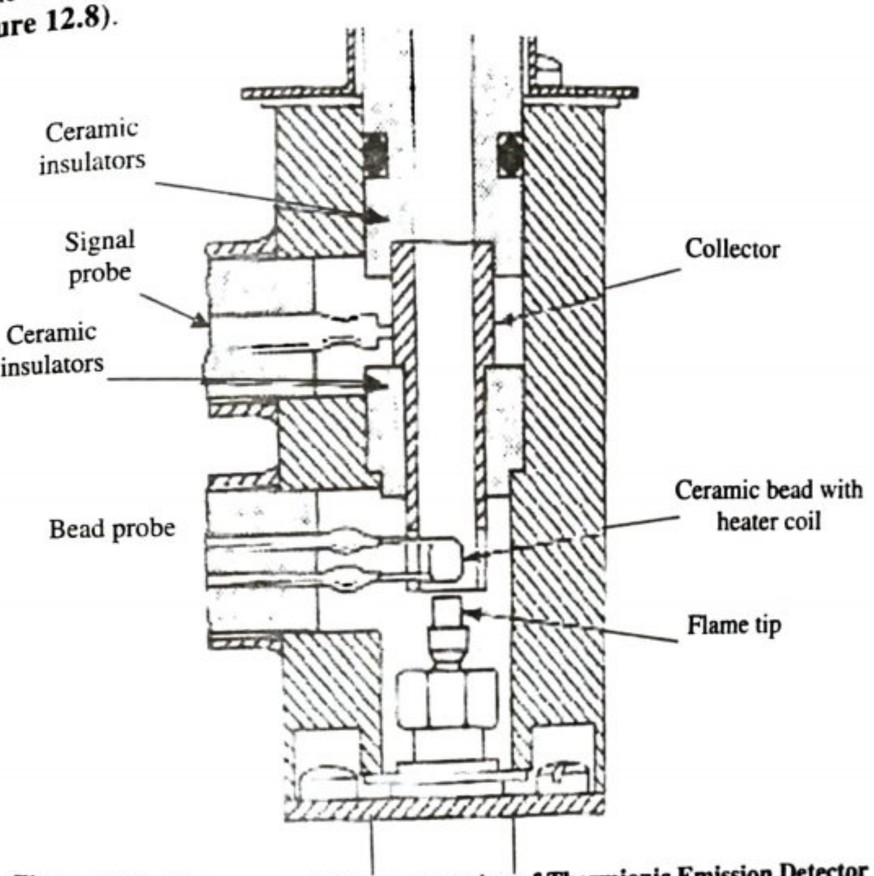


Figure 12.8: Diagrammatic Representation of Thermionic Emission Detector

TED has a very small hydrogen flow, and responds well to nitrogen and phosphorus compounds. It can be made to respond to only phosphorus compounds by increasing the plasma size and altering the polarity between the plasma tip and collector.

5) **Electron Capture Detector (ECD):** This detector relies on the electron affinity of different substances. It responds to compounds whose molecules have electron affinity, **e.g.,** chlorinated compounds, alkyl lead, etc. It responds less to hydrocarbons. A diagrammatic representation of an ECD using a metal foil coated with a tritium-containing compound as a steady source of slow electrons is shown in the **figure 12.9**.

ECD shows high sensitivity to some compounds, **e.g.,** chlorinated pesticides can be determined down to sub-picogram levels. ECD has a **drawback** of having a very narrow linear range; however, this can be solved by proper calibration and adjustment of the sample size.

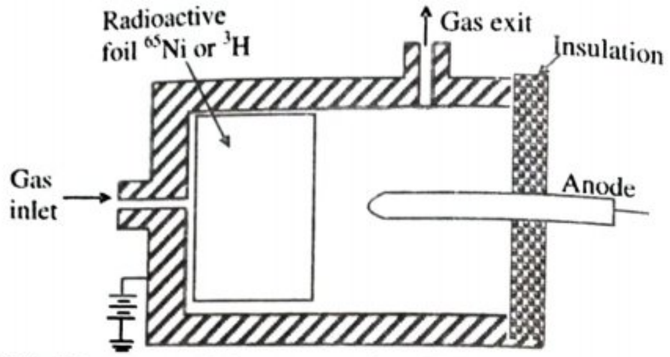


Figure 12.9: Diagrammatic Representation of Electron Capture Detector
Source: Adapted from *Instrumental Methods of Chemical Analysis* (pp 2.684), by Chatwal G.R. (2006) (Himalaya Publishing House)

ECD is used for detecting trace environmental pollutants. It is highly sensitive to halogenated compounds and is used for detecting herbicides, pesticides, SF_6 traces in fuel gases, organometallics (**e.g.,** lead tetraethyl), polynuclear aromatic carcinogens, and NO_2 and SO_2 in chimney stack gases.

6) **Alkali Flame Ionisation (Thermionic) Detector (AFID):** This is a flame ionisation detector using a source of alkali metal salt placed between the flame jet and the collector electrode. The alkali source is generally a glass bead having rubidium silicate (non-volatile salt) fused on platinum wire and maintained at a negative potential. AFID can be made to respond to compounds containing both nitrogen and phosphorus or only phosphorus by changing the polarity of jet.

7) **Flame Photometric Detector (FPD):** This is a special type of flame emission filter photometer. It is used for determining the volatile sulphur or phosphorus compounds. The column effluent passes into a dual hydrogen-enriched, low temperature flame within a shield. The carrier gas is supplied with air and hydrogen as make-up gases. Only the upper flame is viewed.

   Phosphorus forms an HPO species that emit band emissions at 510 and 526nm around the sides and base of the flame. Sulphur compounds form an S_2 entity that emits a series of bands centred on 394nm but also overlaps the phosphorus spectrum. FPD responds linearly to phosphorus, whereas its response to sulphur depends on the square of concentration. The sensitivity of FPD to phosphorus is about 100 times less than the TED.

8) **Photo-Ionisation Detector (PID):** This detector produces ionisation of solute molecules by using UV radiation from lamps with energies ranging between 9.5-11.7eV. The ions are collected at a positively charged electrode and the current is measured. Compounds having ionisation potentials lower than the lamp ionising energy produce a response.

9) **Electrolytic Conductivity Detector (ECD):** This detector relies on electrolytic conductivity. Organic compounds eluting from the gas chromatography column are burned in a miniature furnace to form simple molecular species. These species ionise and contribute to the conductivity of deionised water. The changes in electrolytic conductivity are monitored.

   The analyte ions are removed from the liquid by an ion-exchange column, part of a continuous circulating system that regenerates conductivity-grade water. On mixing the combustion products with hydrogen gas and hydrogenating over a nickel catalyst in a quartz tube furnace at 850°C temperature, ammonia from organic nitrogen, HCl from organic chlorides, and H_2S from sulphur compounds are formed.

12.2.6. Substrates

The solid support is coated with a substrate, which is a high boiling liquid that acts as the **immobile phase** in gas-liquid chromatography. The **general requirements** for the liquid phase are:

1) Good solvent property of the component,
2) Differential partitioning of sample components,
3) Low vapour pressure at the column temperature, and
4) High thermal stability.

Some typical substrates are given in **table 12.1**:

Table 12.1: Some Typical Substrates

Substrates	Solute Types	Temperature (°C)
1) Polyglycols	Amines, ethers, alcohols, ketones, esters, and aromatics	100-200
2) Paraffin oil (Nujol)	Paraffins, olefins, and halides	150
3) Silicone oils	Paraffins, olefins, esters, and ethers	200
4) Didecyl phthalate	Polar compounds	170

12.2.7. Temperature Control

A temperature programming enables controlled increase of temperature during an analysis. As a result, the latter peaks also become sharp and emerge quickly. Thus in temperature programming, the components of a wide boiling range mixture can be efficiently determined. The temperature programming can be carried out in the following three modes:

1) Natural or ballistic,
2) Linear,
3) Matrix or multi-linear.

The operation with linear temperature programme is more common. The **requirements for good temperature programming** are:

1) A dual column system to compensate for bleeding of liquid phase from columns when temperature is increased,
2) Separate heaters for injector, column, oven, and detector system,
3) Differential flow controllers,
4) Low mass column oven to enable rapid heat transfer,
5) Thin walled columns,
6) Low liquid phase loading,
7) Pure dry carrier gas, and
8) Stable and non-bleeding injection septums.

12.2.8. Data Acquisition System

A data acquisition system for gas chromatography comprises of two basic components. The analog signal coming from the detector is amplified and converted into digital data by the **first component**. The **second component** is a computer with a suitable software program that receives the digitalised signal. Some field portable gas chromatography systems can be connected to a laptop and the chromatography program is run from a compact disc. The chromatography software set up the run conditions for gas chromatography, acquires data, integrates peaks, display peaks on the computer screen, prints chromatograms, and run reports.

12.2.9. Gas-Solid Chromatography (GSC)

The apparatus and technique for GSC are similar to that for GLC, with the only difference in the nature of stationary phase and column length. Information on the columns used in GSC and their applications is illustrated in **table 12.2**.

Table 12.2: GSC Columns and their Applications

Columns	Separation Uses
1) Carbon (Carbosieve B)	Light hydrocarbons, H_2, and O_2
2) Silica (Spherosil, Porasil)	Light hydrocarbons, H_2, and O_2
3) Alumina	Hydrocarbons
4) Molecular sieves	H_2, O_2 and N_2, and branched chain hydrocarbons
5) Porus polymers (Pora Pak, Chromosorb)	Aqueous solutions, acids, and other polar materials.

12.3. DERIVATIZATION

12.3.1. Introduction

Derivatization reactions transform an analyte for detectability in gas chromatography or other instrumental analytical methods. Derivatization in gas chromatography analysis is a **technique that modifies the functionality of an analyte to allow chromatographic separations**. The resultant modified analyte is the product, and is known as the **derivative**, whose structure may be similar or closely related to but not the same as the original non-modified chemical compound.

Apart from particular analytes such as pharmaceuticals, biomolecules such as organic acids, amides, poly-hydroxy compounds, amino acids, pesticides, and other persistent organic compounds, some new classes of desirable compounds, like fluorinated alkylated substances and polycyclic aromatic hydrocarbons, are also being developed. Thus, new chemical analytical methods need to be developed and the existing ones need to be improvised. For this, one should be familiar with the derivatization methods applicable to GC analysis.

12.3.2. Derivatization Reagent

Derivatization reagent is a substance **used for** chemically modifying a compound so that a new compound, with properties suitable for analysis in gas and liquid chromatography, can be produced. A suitable derivatization reagent for gas chromatography should be selected based on the following **criteria**:

1) It should produce more than 95% complete derivatives.
2) It should not cause any rearrangements or structural alterations in the compounds during the formation of a derivative.
3) It should not cause any sample loss during the reaction.
4) It should produce a derivative that will not interact with the column used in gas chromatography.
5) It should produce a derivative that remains stable with time.

12.3.3. Objectives for Derivatization

Derivatization technique has the following objectives:

1) It improves resolution and reduces tailing of polar compounds containing –OH, –COOH, –NH, $–NH_2$, –SH, and other functional groups.
2) It enables the analysis of relatively non-volatile compounds.
3) It reduces the volatility of compounds prior to gas chromatography analysis.
4) It improves the analytical efficiency and increases detectability.
5) It causes stabilisation of compounds for gas chromatography analysis.

12.3.4. Types of Derivatization

Derivatization reactions for gas chromatography are of three types, i.e., **alkylation** (the general process is esterification), **acylation**, and **silylation**. These processes make the highly polar materials (**e.g.,** organic acids, amides, poly-hydroxy compounds, and amino acids) sufficiently volatile, thus suitable for gas chromatography analysis. The three derivatization reactions are discussed below.

12.3.4.1. Alkylation

Alkylation is the first step for further derivatization. It is most widely used as a protection method for some active hydrogen in a sample molecule. It represents esterification in which the active hydrogen is replaced with an aliphatic or aliphatic-aromatic group (**e.g.,** benzyl). The equation below shows the general reaction of esterification [X = halogen or alkyl group (R) and H = another alkyl group (R)]:

$$RCOOH + PhCH_2X \rightarrow RCOOCH_2Ph + HX$$

The **major chromatographic use** of alkylation is the **transformation of organic acids into esters** (particularly methyl esters) **that produce better chromatograms than the free acids**. Alkylation reactions are also used for preparing ethers, thioethers and thioesters, N-alkylamines, amides, and sulphonamides. The polarity of alkylation products is generally less than that of the starting materials. This is because active hydrogen has been replaced with an alkyl group. The obtained alkyl esters provide exceptional stability, and can be isolated and stored for longer periods. In esterification, an acid and an alcohol react to form an ester.

Dialkylacetals, diazoalkales, Pentafluorobenzyl Bromide (PFBBr), benzylbromide, Boron trifluoride (BF_3) in methanol or butanol, and Tetrabutylammonium Hydroxide (TBH) are the commonly used **derivatization reagents in alkylation** reactions. These alkylation reagents can be used either alone to form esters, ethers. and amides or in combination with acylation or silylation reagents. The reaction conditions for alkylation range from strongly acidic to strongly basic; however, stable derivatives are produced under both the conditions. The alkylation reagents are more limited to amines and acidic hydroxyls. A **drawback** is that sometimes the **reaction conditions become severe and the reagents become toxic**.

12.3.4.2. Acylation

Acylation reaction involves introduction of an acyl group to an organic compound. Acylation in carboxylic acid involves addition of an acyl group and loss of a hydroxyl group. Acylation can convert the compounds with active hydrogen, **e.g.,** –OH, –SH, and –NH, into esters, thioesters, and amides, respectively. This reaction is popularly used for the **production of volatile derivatives of highly polar and volatile organic materials**. The reaction also improves the stability of thermolabile compounds by adding protecting groups into the molecule. Acylation can make the extremely polar materials (such as sugars) amenable to separation by gas chromatography, and thus, are a valuable alternative to silylation. An **example** of acylation reaction between acetic anhydride and an alcohol to produce acetate ester and acetic acid is shown below.

$$CH_3OCOCOCH_3 + HOR \xrightarrow{H^- \text{(Catalyst)}} CH_3OCOR + HOCOCH_3$$

Acylation reaction has the following **advantages** in gas chromatography analysis:

1) It improves the stability of analyte by protecting the unstable groups.
2) It provides volatility to compounds having many polar groups (carbohydrates or amino acids), as they are non-volatile and decompose on heating.

3) It aids in chromatographic separations which is not possible with compounds inappropriate for gas chromatography analysis.
4) It can detect compounds at very low levels using an electron capture detector.

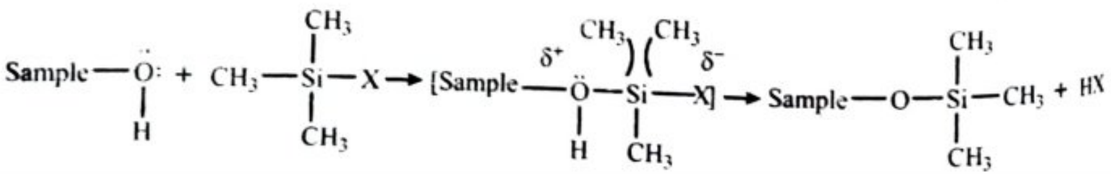
Fluoracylimidazoles, fluorinated anhydrides, N-Methyl-bis(Trifluoroacetamide) (MBTFA), Pentafluorobenzoyl Chloride (PFBCl), and Pentafluoropropanol (PFPOH) are the **reagents** commonly used for acylation. These reagents target the highly polar, multi-functional compounds, like carbohydrates and amino acids. They also allow the introduction of electron-capturing groups, and therefore enhance the detectability during analysis. The acylating reagents are available as acid anhydrides, acyl derivatives, or acyl halides, of which the latter two are highly reactive and can be suitably used where issues of steric hindrance may be a factor.

Acid anhydrides are available in a number of fluorinated configurations, which can improve detection. These fluorinated anhydride derivatives are used for detection using electron capture and flame ionisation detectors. Fluorinated anhydrides are used in derivatizing samples to confirm drugs of abuse. Still due to their acidic nature, it is necessary to remove any excess of by-products before gas chromatography analysis. This is done to prevent column deterioration. Because of the acidic by-products, the derivatization process has been carried out in pyridine, tetrahydrofuran, or another solvent that can accept the acidic by-product.

12.3.4.3. Silylation

Silylation is the most predominant derivatization method. It readily turns the sample volatile, and is very suitable for gas chromatography analysis of non-volatile samples. In silylation reaction, a silyl group, such as dimethylsilyl [$SiH(CH_3)_2$], t-butyldimethylsilyl [$Si(CH_3)_2C(CH_3)_3$], and chloromethyldimethylsilyl [$SiCH_2Cl(CH_3)_2$] is added into a molecule to replace active hydrogen. As a result of this replacement, the polarity of the compound as well as hydrogen bonding reduces. Silylation reaction has been successfully used in the gas chromatography analysis of hydroxyl and amino compounds that are considered non-volatile or unstable at 200-300°C temperature. The silylated derivatives are more volatile and stable, thus yield narrow and symmetrical peaks.

General Reaction Mechanism: Silylation reaction is driven by a good leaving group having a low basicity, the ability to stabilise a negative charge in the transitional state, and little or no π back bonding between the leaving group and silicon atom. In this reaction, the active hydrogen (in –OH, –COOH, –NH, $–NH_2$, and –SH groups) is replaced with a trimethylsilyl group. Silylation occurs through nucleophilic attack (SN_2), where the degree of silylation is better if a good leaving group is used. As a result, a bimolecular transition state occurs in the intermediate step of reaction mechanism. The reaction for the formation of trialkylsilyl derivatives is shown below, where X = Cl. The leaving group in case of trimethylchlorosilane (TMCS) is the Cl atom.



In silylation derivatization, the sample as well as the solvents should be dry. Silyl reagents should be stored in tightly closed containers as they are moisture-sensitive, and thus should be used in pure form and as little as possible. This will eliminate excessive peaks and prevent a large solvent peak.

The most commonly used **solvent** in silylation is **pyridine**. It may produce peak tailing, still it is an acid scavenger and drives the reaction forward. In many cases of silylation, a solvent is not required with silylating reagents. The completion of silylation derivatization is observed when a sample readily dissolves in the reagent.
According to **Regis**, the ease of reactivity of the functional group towards silylation follows the order:

Alcohol > Phenol > Carboxyl > Amine > Amide/Hydroxyl

For alcohols, the order is as follows:

Primary > Secondary > Tertiary

Many reagents need to be heated at temperature not more than 60°C for 10-15 minutes. This is necessary to prevent the breakdown of derivative. However, the hindered products may require to be heated for a long-term.

Hexamethyldisilzane (HMDS), Trimethylchlorosilane (TMCS), Trimethylsilylimidazole (TMSI), Bis(trimethylsilyl)acetamide (BSA), Bis trimethylsilyl Trifluoroacetamide (BSTFA), N-Methyl trimethylsilyl Trifluoroacetamide (MSTFA), Trimethylsilyl Diethylamine (TMS-DEA), N-Methyl-N-t-Butyldimethylsilyl Trifluoroacetamide (MTBSTFA), and Halo-methylsilyl are the **reagents** used for silylation derivatization.

Halo-methylsilyl derivatization reagents can produce silylated and halogenated derivatives for Electron Capture Detectors (ECDs). Silyl reagents form trimethylsilyl ether and trimethylsilyl ester derivatives by reacting with alcohols and acids, respectively. These derivatives are volatile and can be easily separated. Silyl reagents show compatibility with most detection systems, but they cause difficulties with Flame Ionisation Detectors (FIDs) when used in excess. Silyl reagents can be influenced by the solvent system and by adding a catalyst (**e.g.,** trimethylchlorosilane or pyridine), which increases the reagent reactivity. Reagents that introduce a t-butyldimethylsilyl group instead of trimethylsilyl group impart greater hydrolytic stability to the t-butyldimethylsilyl derivatives.

These derivatives have better stability against hydrolysis, and also have distinctive fragmentation patterns, thus are useful in various GC/MS applications. Trimethylsilyl and t-butyldimethylsilyl derivatives mostly have exceptional thermal stability and are amenable to a variety of injection and column conditions. Silylation provides the ability to derivatize various compounds for gas chromatography analysis.

12.4. APPLICATIONS

12.4.1. Introduction

The major applications of gas chromatography are qualitative and quantitative analysis of liquids, gases, and vapours (mainly of organic compounds). This technique can be used for determining a stable compound that can be vaporised below 300°C temperature.

However, if the compound is not stable to isomerisation and decomposition at these temperatures, the method will give rise to inaccurate results. The non-volatile compounds or the ones unstable at this temperature can be analysed by liquid chromatography.

12.4.2. Qualitative Analysis

The individual components of a mixture can be qualitatively analysed by either:

1) Comparing the retention times or volumes of the unknown with that of the series of standards, or
2) Collecting the individual components emerging from the chromatograph and identifying them using other methods.

All the experimental parameters of the standards and unknowns, mainly the flow rate and temperature, should be carefully controlled and duplicated if the analysis is to be done using retention times or volumes. There are many compounds with the same retention time.

Due to this reason, the data from a single set of conditions are not sufficient for positive identification; and hence, confirmation should be made using different substrate-solvent combinations.

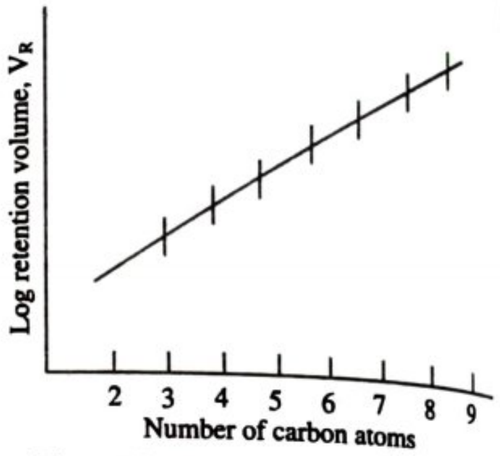


Figure 12.11: Plot of Log of Retention Volume against the Number of Carbon Atoms for a Homologous Series
Source: Adapted from *Instrumental Methods of Chemical Analysis* (pp 2.692), by Chatwal G.R. (2006) (Himalaya Publishing House)

Sometimes, the sample mixture should be spiked with a known compound. If the area of a previously present band increases and no additional elution band is obtained under several sets of conditions, positive identification can be practically confirmed.

Under a given set of experimental conditions, the logarithm of retention volume and the number of carbon atoms in each homologous series are linear. Each different homologous series yields a different line (**figure 12.11**). If the series is known, the number of carbon atoms can be determined using these graphs.

The series to which a compound belongs can be determined by plotting the logarithm of retention volume on a polar column against the logarithm of retention volume on non-polar column (**figure 12.12**).

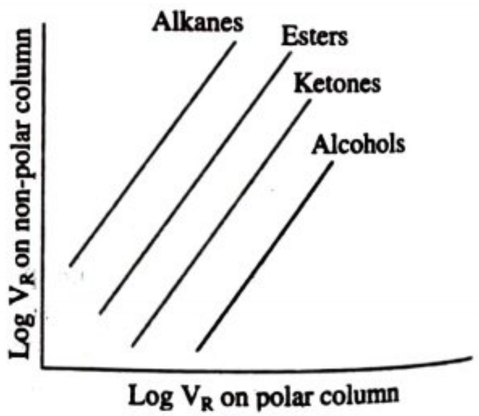


Figure 12.12: Plot of Log Retention Volume on a Polar Column against the Log of Retention Volume on a Non-Polar Column
Source: Adapted from *Instrumental Methods of Chemical Analysis* (pp 2.692), by Chatwal G.R. (2006) (Himalaya Publishing House)

After collecting the samples, the final identification can be made through mass spectroscopy, nuclear magnetic resonance, or infrared absorption techniques.

Interfacing devices are mostly required for compensating the sample size, sample composition, and/or the pressure requirements of different instruments.

12.4.3. Quantitative Analysis

The quantitative analysis of a chromatogram relies on the basis that the area of a single component elution peak and the quantity of the detected component are proportional. Accurate measurements of the peak area can be obtained if the output at the recorder and the concentration are linear, and also if the time response of the recorder matches the time response of the detector (if not so an automatic integrator should be coupled directly to the detector as an alternative). The flow rate of the carrier gas should also be reproducibly constant to allow a conversion between the flow rate and time.

The automatic integrators are preferably used because of their speed, accuracy, and ease of result interpretation. If an integrator is unavailable, **cut and weigh procedure** is used for area determination. In this procedure, sample of known weight is injected, the paper of constant thickness and moisture content is cut, and a weight component per weight of paper factor is determined.

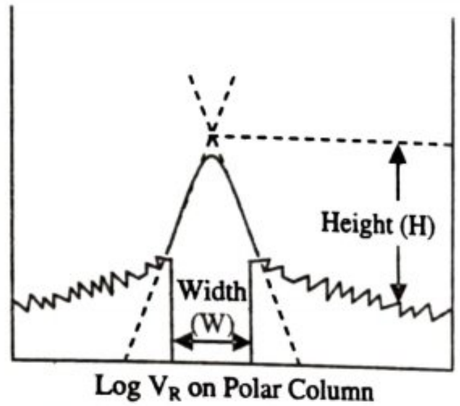


Figure 12.13: Triangulation Method of Determining Peak Area
Source: Adapted from *Instrumental Methods of Chemical Analysis* (pp 2.693), by Chatwal G.R. (2006) (Himalaya Publishing House)

The peak area can also be determined by **triangulation procedure**, in which tangents are drawn on both sides of the curve (**figure 12.13**). The intercept of the two tangents at the top is the height (H), the distance between the baseline and the intercepts of the two tangents is the width (W), and the area (A) can be calculated as:

$$A = \frac{1}{2} WH$$

12.4.4. Specific Applications

Gas liquid chromatography has a wide range of applications. In the last few decades, rapid changing complexities of the product (**e.g.,** drugs, foodstuffs, and consumer products) and the technology with associated environmental problems have been seen. This has forced the development of systems that can be used for the separation and identification of a large number of known and unknown species having increased sensitivity. GLC has an essential role in this task.

Some applications of GLC are:

1) **Detection of Steroidal Drugs:** GLC is used for detecting steroids in athletes in international sports competitions and the steroids administered to animals in traces. It is also used for detecting hazardous pollutants (**e.g.,** formaldehyde, carbon monoxide, trichloroethylene, benzene, and acrylonitrile).

   It can be used for analysing the volatile fatty acids produced by anaerobic bacteria. It also enables fingerprinting of particular microorganisms, thus facilitating identification of the bacteria.

2) **Analysis of Foods:** GLC is used for analysing foods. It is also used for separating and identifying lipids, proteins, carbohydrates, preservatives, flavours, colourants, texture modifiers, vitamins, steroids, drug and pesticide residues, and trace elements.

   Most of the compounds are non-volatile, thus HPLC is now used for food analysis; however, GLC is also used for the same purpose by converting the compounds into a volatile form to make them suitable for separation. This is termed derivatization, **e.g.,** lipids are converted to fatty acid methyl esters, proteins are converted by acid hydrolysis and esterification, and carbohydrates are converted by silylation.

3) **Analysis of Dairy Products:** GLC is used for analysing the dairy products, aldehydes and ketones (for rancidity), fatty acids (by derivatization), and milk sugars. It is also used for analysing butter (for determining the butter fat content), and added colours and flavours.

4) **Drug Analysis:** GLC is used in drug analysis, **e.g.,** analysis of commercial drug preparations, illicit drug samples, blood, urine samples, and stomach contents.

5) **Separation of Metal Chelates:** GLC is used for separating various metal chelates, **e.g.,** separation of β-diketonates of chromium (III) and other thermolabile di-, tri-, and tetravalent metals that are soluble in organic solvents and volatile.

   Acetyl acetone, trifluoroacetyl acetone, and hexafluoroacetone are the most commonly used ligand for derivatization of metal ions. Increasing fluorination of the ligand yields a more volatile metal chelate.

12.5. SUMMARY

The details given in the chapter can be summarised as follows:

1) **A.T. James** and **P. Martin** first time used the gas chromatography technique in 1952 for separating long chain fatty acids.

2) When the stationary phase is solid, it is known as **Gas Solid Chromatography (GSC)** and when the stationary phase is liquid, it is known as **Gas Liquid Chromatography (GLC).**

3) The affinity of a component towards the stationary phase is termed as **distribution constant** (K_c).

4) **Gas Liquid Chromatography** (GLC) employs liquid as the stationary phase and is a form of **partition chromatography.**

5) **Gas-Solid Chromatography** (GSC) employs a solid surface as the stationary phase and is a form of **adsorption chromatography.**

6) **Resolution** is a function of differential zone migration rates that separate the zone centres, and of zone broadening processes that merge the zones.

7) **Hydrogen, helium, nitrogen,** and **air** are the most widely used carrier gases.

8) **Packed columns** are prepared by packing metal or glass tubing with granular stationary phase.

9) **Open tubular or capillary or golay columns** are made of long capillary tubing (30-90m) and have uniform and narrow internal diameter (0.025-0.075cm).

10) **Support coated open tubular columns** are prepared by coating the inner wall of a capillary column with a micron size porous layer of support material, followed by coating with the liquid phase as a thin film.

11) **Wall coated open tubular columns** columns are prepared by coating the unmodified smooth inner wall of the tube with the liquid stationary phase.

12) **Porous-Layer Open-Tubular (PLOT) columns** are prepared by coating the inner wall with a porous layer.

13) In **Support-Coated Open-Tubular (SCOT) columns,** the porous layer consists of support particles and was deposited from a suspension.

14) Some gas chromatographs utilise a **mass spectrometer** as the detector; this combination is known as **GC-MS.**

15) Some GC-MS-NMR utilise an **IR spectrophotometer** as a backup detector; this combination is known as **GC-MS-NMR-IR.**

16) Some GC-MS utilise an **NMR spectrometer** as a backup detector; this combination is known as **GC-MS-NMR.**

17) The **chromatogram** is a record of **detector response against time,** or is a record of the known **gas flow against carrier gas volume.**

18) **Katharometer** detector relies on the variation in thermal conductivity of the carrier gas in the presence of an organic compound.

19) **Flame Ionisation Detecto (FID)** detector relies on the change in conductivity of the flame as the compound is burnt.

20) **Thermal Conductivity Detector (TCD)** utilises a heated filament placed in the emerging gas stream.

21) **Thermionic Emission Detector (TED)** utilises fuel-poor hydrogen plasma and a low temperature flame.

22) **Electron Capture Detector (ECD)** relies on the electron affinity of different substances.

23) **Alkali Flame Ionisation (Thermionic) Detector (AFID)** is a flame ionisation detector using a source of alkali metal salt placed between the flame jet and the collector electrode.

24) **Flame Photometric Detector (FPD)** is used for determining the volatile sulphur or phosphorus compounds.

25) **Photo-Ionisation Detector (PID)** produces ionisation of solute molecules by using UV radiation from lamps with energies between 9.5-11.7eV.

26) **Electrolytic conductivity detector** relies on electrolytic conductivity.

27) **Derivatization** in gas chromatography analysis is a technique that modifies the functionality of an analyte to allow chromatographic separations.

28) **Derivatization reagent** is a substance used for chemically modifying a compound so that a new compound, with properties suitable for analysis in gas and liquid chromatography, can be produced.

29) **Alkylation** is most widely used as a protection method for some active hydrogen in a sample molecule.

30) Dialkylacetals, diazoalkales, Pentafluorobenzyl Bromide (PFBBr), benzylbromide, Boron trifluoride (BF_3) in methanol or butanol and

Tetrabutylammonium Hydroxide (TBH) are the commonly used **derivatization reagents in alkylation** reactions.

31) **Acylation** reaction involves introduction of an acyl group to an organic compound.

32) Fluoracylimidazoles, fluorinated anhydrides, N-Methyl-bis(Trifluoroacetamide) (MBTFA), Pentafluorobenzoyl Chloride (PFBCl), and Pentafluoropropanol (PFPOH) are the **reagents** commonly used for acylation.

33) **Silylation** is the most predominant derivatization method. It readily turns the sample volatile, and is very suitable for gas chromatography analysis of non-volatile samples.

34) The most commonly used **solvent** in silylation is pyridine.

12.6. EXERCISE

12.6.1. True or False

1) Gas liquid chromatography employs liquid as the stationary phase and is a form of adsorption chromatography.
2) Hydrogen, helium, nitrogen, and air are the most widely used carrier gases.
3) Golay columns are made of long capillary tubing and have uniform and narrow internal diameter.
4) Wall coated open tubular columns are prepared by coating the inner wall of a capillary column with a micron size porous layer of support material, followed by coating with the liquid phase as a thin film.
5) In support-coated open-tubular columns, the porous layer consists of support particles and was deposited from a suspension.
6) Katharometer detector relies on the variation in thermal conductivity of the carrier gas in the presence of an organic compound.
7) Flame ionisation detector utilises fuel-poor hydrogen plasma and a low temperature flame.
8) Derivatization in gas chromatography analysis is a technique that modifies the functionality of an analyte to allow chromatographic separations.
9) Silylation is most widely used as a protection method for some active hydrogen in a sample molecule.
10) The most commonly used solvent in alkylation is pyridine.

12.6.2. Fill in the Blanks

11) A.T. James and _____ first time used the gas chromatography technique in 1952 for separating long chain fatty acids.
12) The affinity of a component towards the stationary phase is termed as _____.
13) Gas-solid chromatography employs a solid surface as the stationary phase and is a form of _____.
14) _____ is a function of differential zone migration rates that separate the zone centres, and of zone broadening processes that merge the zones.
15) _____ columns are prepared by coating the inner wall with a porous layer.
16) Some GC-MS utilise an NMR spectrometer as a backup detector; this combination is known as _____.
17) The chromatogram is a record of detector response against time, or is a record of the known gas flow against _____.

18) _____ detector utilises a heated filament placed in the emerging gas stream.
19) _____ detector is used for determining the volatile sulphur or phosphorus compounds.
20) _____ reaction involves introduction of an acyl group to an organic compound.

Answers

1) False 2) True 3) True 4) False 5) True
6) True 7) False 8) True 9) False 10) False
11) P. Martin 12) Distribution constant 13) Adsorption chromatography
14) Resolution 15) Porous-layer open-tubular 16) GC-MS-NMR 17) Carrier gas volume
18) Thermal conductivity 19) Flame photometric 20) Acylation

12.6.3. Very Short Answer Type Questions

1) What is gas chromatography?
2) Give the types of gas chromatography.
3) Give the advantages of gas chromatography.
4) Draw a well-labelled diagram of the apparatus used in gas chromatography.
5) Give the objectives of derivatization of gas chromatography.
6) What substrates are used in gas chromatography?

12.6.4. Short Answer Type Questions

1) Discuss the theory of gas chromatography.
2) Give the quantitative applications of gas chromatography.
3) Write a note on temperature programming.
4) Write a short note on derivatization of gas chromatography.
5) Discuss the columns used in gas chromatography.

12.6.5. Long Answer Type Questions

1) Briefly explain the detectors used in gas chromatography.
2) Discuss about the applications of gas chromatography.
3) Write an illustrative note on the instrumentation of gas chromatography.

CHAPTER 13

High Performance Liquid Chromatography (HPLC)

13.1. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

13.1.1. Introduction

High Performance Liquid Chromatography (HPLC) is used to separate complex chemical mixtures. Previously, it was known as High Pressure Liquid Chromatography, but now the term **performance** is used instead of **pressure** which indicates that pressure is not essential for high performance, and also defines the technique in a better way. HPLC is a highly rapid process as it involves high resolution and high speed columns.

Following are the points showing the **advantages** of HPLC **over gravity-fed (classical) column chromatography**:

1) The separated substances show better resolution,
2) Less time is required for separation,
3) Substances are separated with more accuracy, precision, and sensitivity, and
4) The technique is useful for qualitative as well as quantitative analysis.

The HPLC technique was developed in the early 1970s using the principles of traditional chromatographic techniques (mainly the column chromatography). The system involves pumping of mobile phase through the packed column under the influence of high-pressure, therefore the technique is also named as **high-pressure liquid chromatography**. The technique was first time presented by **Kirtland** and **Haber**, who proposed that high-pressure systems can operate at pressures up to 3000 psi.

The high-pressure liquid chromatography separation method involves a stationary phase contained in one end of the column and mobile phase (a source of pressurised liquid eluent) connected to the other end of the column.

13.1.2. Principle

In HPLC, the following four methods are used for separating chemical mixtures:

1) Adsorption,
2) Partition,
3) Ion exchange, and
4) Exclusion.

Selection of method for the separation process depends on the stationary phase nature. Some **examples** of HPLC stationary phases are given in **table 13.1**.

HPLC involves separation of mixture compounds on an analytical column that is packed with small particles of stationary phase (**e.g.,** silica) by elution with a liquid mobile phase. A high pressure is applied to pump the mobile or liquid phase through the packed columns. The working of HPLC is based on the principle that separation of molecular forms involves elution of a sample from a solid inorganic support by using a mixture of organic solvents. In

HPLC, capillary columns packed with cross-linked dextran or silica is used for solid support.

Table 13.1: Some Examples of HPLC Stationary Phases

Chromatographic Separation Principles	Commercial Names	Nature of Stationary Phases	Types of Support
Adsorption	Partisil C_2	Octylsilane	Porous
	Corasil	Silica	Pellicular
	Pellumina	Alumina	Pellicular
	Partisil	Silica	Microporous
	MicroPark A1	Alumina	Microporous
Partition	Bondapak-C_{18}/Corasil	Octadecylsilane (ODS)	Pellicular
	µBondapak-C_{18}	Octadecylsilane	Porous
	ULTRApak TSK ODS	Octadecylsilane	Porous
	µBondapak-NH_2	Alkylamine	Porous
	ULTRApak TSK-NH_2	Alkylamine	Porous
Ion-exchange	Partisil SAX	Strong base	Porous
	MicroPak-NH_2	Weak base	Porous
	Partisil-SCX	Strong base	Porous
	AS Pellionex-SAX	Strong base	Pellicular
	Zipak-WAX	Weak base	Pellicular
	Perisorb-KAT	Strong acid	Pellicular
Exclusion	BioGlas	Glass	Rigid solid
	Styragel	Polystyrene-divinylbenzene	Semi-rigid gel
	Superose	Agarose	Soft gel
	Fractogel TSK	Polyvinylchloride	Semi-rigid gel

In HPLC, the separation efficiency depends on the effective size of the interface of stationary and mobile phase in which the sample is dissolved. Therefore, it can be concluded that the separation efficiency depends on the physical and chemical interactions between the packed material of the solid phase and the compound to be separated.

This can be done using an inert column, which acts as a sieve, and sorts the components retained on the column based on the size and distribution of the components along the support. This may also be performed by **non-specific absorption** of one or more components on a solid phase.

High pressure applied on the organic solvents flowing through the column enables the use of solid support phases at high pressure, which otherwise would be impermeable to the organic phase.

The application of high pressure also reduces the run time, and the methods can be automated due to more reproducibility of the running conditions.

Retention Time (t_R)

The time required for emergence of the maximum peak of a component after sample injection is known as **retention time** (t_R). It is the sum of the time the component spends in the stationary and mobile phase (t_M).

The time duration that a component spends in the stationary phase is the **adjusted retention time** (t'_R), which is given by:

$$t'_R = t_R - t_M$$

The value of t_M is obtained by measuring the time required to elute an un-retained substance, **e.g.,** air or methane.

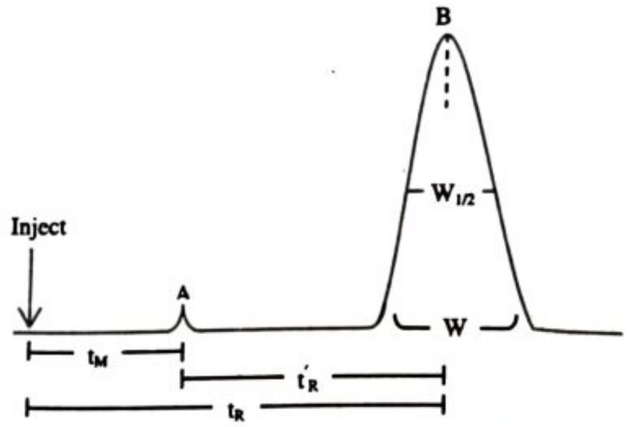


Figure 13.1: Diagrammatic Chromatogram of a Pure Substance (B) Injected along with an Unretained Substance (A)

13.1.3. Theory

HPLC overcomes the limitations found in standard liquid chromatography. In classic liquid chromatography, the separation process is very slow as the movement of solvent occurs under gravity. The limiting factor is the size of column packing in liquid chromatography. In the HPLC setup, the apparatus should operate efficiently under high pressure and should be specialised at low tolerances. Thus, HPLC is highly expensive than other chromatography techniques.

A mixture of polar and non-polar liquid components forms the mobile phase of HPLC. The concentration of these liquids depends on the sample composition. The solvent should not have dissolved gases, which might hinder solution mid-separation and particulates. In HPLC column, the components are separated according to their differing interactions with the column packing.

If the interaction between species and stationary phase is weak, it spends a comparatively less time in the column and reduces the retention time. For homogeneous columns, silica or alumina can be used as stationary phase, while a liquid stationary phase is considered as a bonded column.

HPLC pump is used to introduce solvent and sample in the column. Use of HPLC pump also helps to maintain a constant, pulse free flow rate. The HPLC pump can be a multi-piston pump or syringe pump. At the column end, HPLC detector is present that registers the presence of components in the sample but not the solvent. In the HPLC system, a UV absorption detector or an NMR detector is preferred.

Following terms are used to study a chromatogram:

1) **Injection Point:** It is the position time/point in time from where/when the sample is introduced in the column.
2) **Baseline:** It is a part of the chromatogram where only mobile phase emerges from the column.
3) **Peak Maximum:** It is the highest point of the peak.
4) **Dead Point:** It is the position of the peak-maximum of an unretained solute.
5) **Dead Volume (V_o):** It is the volume of mobile phase passed through the column between the injection point and the dead point.
6) **Dead Time (t_o):** It is the time elapsed between the **injection point** and the **dead point**.

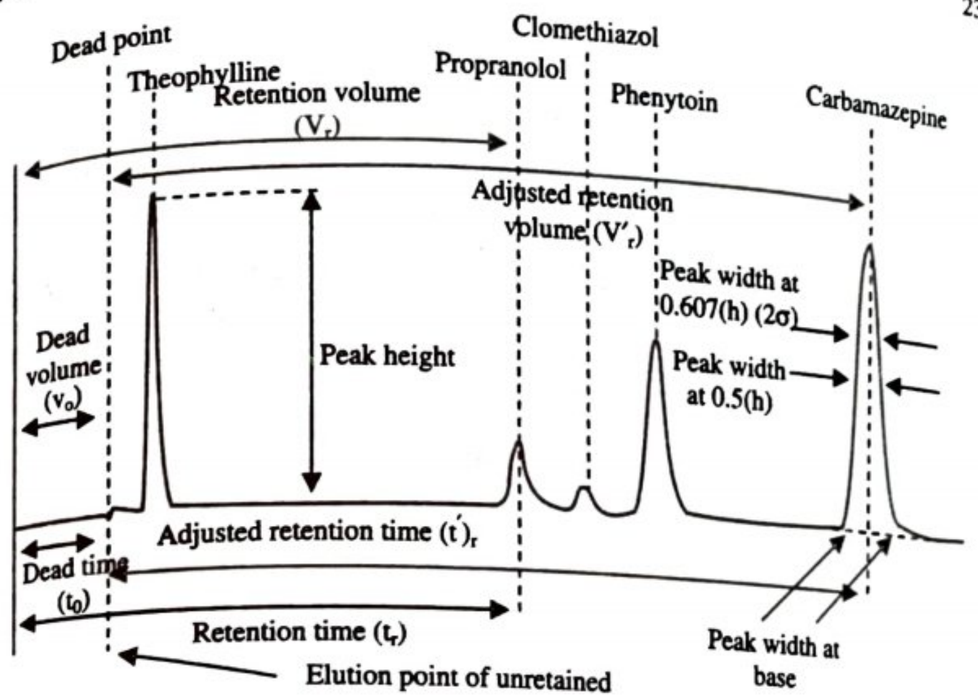


Figure 13.2: Nomenclature of HPLC

13.1.4. Types

Depending on the stationary phase system, the HPLC technique is of the following types:

1) **Normal Phase HPLC (NP-HPLC):** This method utilises **a polar stationary phase** and a **non-polar mobile phase** for the separation of mixture components on the basis of polarity. Silica is generally used as the stationary phase and hexane, methylene chloride, chloroform, diethyl ether, and mixtures of these are the selected mobile phases.

   The polar components interact with the polar stationary phase and are retained. Adsorption strengths can be increased by increasing the polarity of component, while the elution time can be increased by increasing the interaction between the polar component and the polar stationary phase.

2) **Reverse Phase HPLC (RP-HPLC or RPC):** This method utilises a **non-polar stationary phase** and **an aqueous, moderately polar mobile phase**. RPC is based on the principle of hydrophobic interactions, resulting from repulsive forces between a polar eluent, the relatively non-polar component, and the non-polar stationary phase.

   The binding of component to the stationary phase occurs according to the contact surface area around the non-polar segment of the component molecule upon association with the ligand in the aqueous eluent.

3) **Size-Exclusion HPLC (SEC) or Gel Permeation or Filtration Chromatography:** This method involves separation of particles based on their size. It is also used to determine the tertiary and quaternary structures of proteins and amino acids. This method is highly useful for the determination of molecular weight of polysaccharides.

4) **Ion-Exchange HPLC:** In this method, retention occurs according to the attraction between the solute ions and charged sites bound to the stationary phase, excluding the similarly charged ions. This chromatography is widely used in purifying water, ligand-exchange chromatography, ion-exchange chromatography of proteins, high-pH anion-exchange chromatography of carbohydrates and oligosaccharides, etc.

5) **Bio-Affinity Chromatography:** This method involves separation according to the specific reversible interaction of proteins with ligands that are attached to the solid support on a bio-affinity matrix through covalent bonds. Proteins interact with these column-bound ligands, are retained, and can be eluted by the following two ways:
   i) **Biospecific Elution:** Inclusion of free ligand in elution buffer which competes with column bound ligand.
   ii) **Aspecific Elution:** Change in pH, salt, etc. weakens the interaction of protein with the column-bound substrate.

   Due to the specificity of interaction, the bio-affinity chromatography gives highly efficient purification results even in a single step (10-1000 folds).

13.1.5. Advantages

HPLC has the following advantages:

1) It is a simple, rapid, and reproducible technique.
2) It is highly sensitive.
3) It shows a better performance.
4) It is a rapid process and is less time consuming.
5) Its resolution and separation capacity is high.
6) It is accurate and precise.
7) It utilises a chemically inert mobile and stationary phases.
8) It needs a small amount of mobile phase for developing chamber.
9) It involves early recovery of separated components.
10) It enables easy visualisation of separated components.
11) It shows a good reproducibility and repeatability.
12) It is useful in qualitative and quantitative analysis.
13) It is used for analytical and preparative purposes.
14) It is used for validation and quality control studies of product.

13.1.6. Disadvantages

The disadvantages of HPLC focus on the detection systems available, and include:

1) The most commonly used detectors in HPLC are UV spectrometers; however, the compound to be analysed should have a UV absorbing chromophore.
2) Variable wavelength UV spectrometers offer versatility but some steroids and other drugs must be derivatized before UV detection.
3) Another slight disadvantage is that the chemically bonded stationary phases applicable in drug analysis should be used within 3-7 pH range to ensure long term stability.

Inspite of these limitations, HPLC technique is widely used in the field of drug analysis.

13.2. INSTRUMENTATION

13.2.1. Introduction

Modern HPLC Instrument includes the following components (**figure 13.3**):

1) Solvent reservoir and degassing system,
2) Pumping system,
3) Sample injection system,
4) Columns,
5) Detectors,
6) Stripchart recorder, and
7) Data handling system.

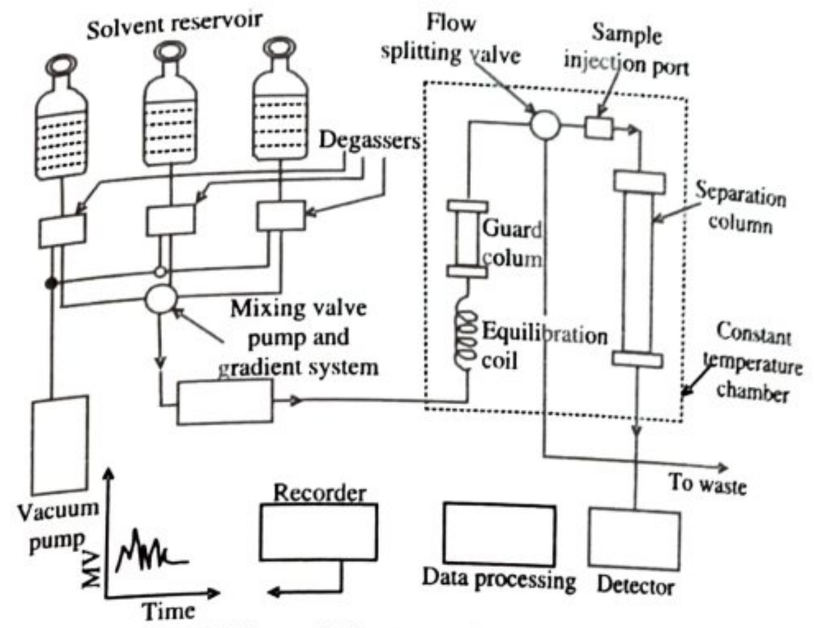


Figure 13.3: Schematic Representation of HPLC Instrument
Source: Adapted from *Pharmaceutical Drug Analysis* (pp 455), by Kar Ashutosh (2008) (New Age International Publishers)

The mobile phase, which may include either a single liquid or a mixture of two or more liquids, is pumped into a temperature controlled oven under high pressure. From the oven, the mobile phase enters an equilibration coil to attain the operating temperature, and then it passes through a guarded column designed and positioned such to protect the analytical column from impurities and to increase its lifetime. If a differential type of detector is used, the flow is splitted at this juncture; of which one portion directly goes to the reference side of the detector and the other portion reaches the analytical column, present within a constant temperature chamber.

The sample is introduced to the mobile phase before injecting into the column. The column-effluent is allowed to pass through the sample side of all the detector, then through the data-processing unit and the recorder.

13.2.2. Solvent Reservoir and Degassing System

Mobile phase used in the HPLC may be **a mixture of organic solvents or an aqueous-organic mixture or a buffer solution**. Selection of mobile phase depends on the chromatographic method and the detector to be used. Commercially available special grades of solvents that have been refined to completely remove the UV-absorbing impurities and any particulate matter are also used in HPLC. Prior to using other grades of solvents, purification should be performed. This is because the separation may get influenced if the impurities (that may be present) are strongly UV-absorbing, affect the detector, or are of high polarity (**e.g.,** traces of H_2O or EtOH, commonly included as a stabiliser, in $CHCl_3$).

Solvent-reservoir consists of a $1dm^3$ glass bottle with a lid and a 1/8 inch diameter PTFE-tube to transfer the mobile phase from the reservoir to the degassers and pump. Liquid entering the pump should be free from any impurity (dust and particulate matter), or else these impurities will result in irregular pumping action, irregular behaviour of column owing to its contamination, damage the seals and valves, and ultimately block the column. Sometimes, a stainless steel filter element (of filter size 2μm) is used that can be installed either in the PTFE-tube in the reservoir or an in-line filter can be used.

Degassing

Generally, liquids dissolve some amounts of atmospheric gases (**e.g.,** air or suspended air-bubbles) that cause some major practical problems in HPLC, specifically affecting the working of pump and the detector. These problems can be avoided by degassing the mobile phase. Degassing is performed by:

1) Subjecting the mobile-phase under vacuum,
2) Distillation,
3) Spurging with a fine spray of an inert gas of low solubility (argon or helium), or
4) Heating and ultrasonic stirring.

13.2.3. Pumping System

The liquid chromatographic pumps require:

1) The capacity to offer pressures of up to 6000 psi (lb/in^2),
2) Pulse-free output,
3) Flow rates within 0.1-10 ml/min,
4) Flow reproducibilities of 0.5% or more, and
5) Resistance to corrosion from various solvents.

The high pressures of liquid chromatographic pumps exhibit no explosion hazard since the liquids are not very compressible. However in case of some solvents, if rupture in a component occurs, it causes solvent leakage that may result in a fire or environmental hazard.

The types of pumps that are used in HPLC are:

1) Screw-driven syringe pump,
2) Reciprocating pump, and
3) Pneumatic or constant-pressure pump.

13.2.3.1. Screw-Driven Syringe Pump

A variable speed stepper motor turns a screw that drives a piston, which displaces the mobile phase from a chamber (of 200-500cm^3 volume). The flow is pulse free and varies with the motor speed. The mobile phase capacity depends on the solvent chamber volume. This volume is quite large for running numerous chromatograms before the chamber is required to be refilled. Much solvent is wasted in flushing out the pump when a change is required (**figure 13.4**).

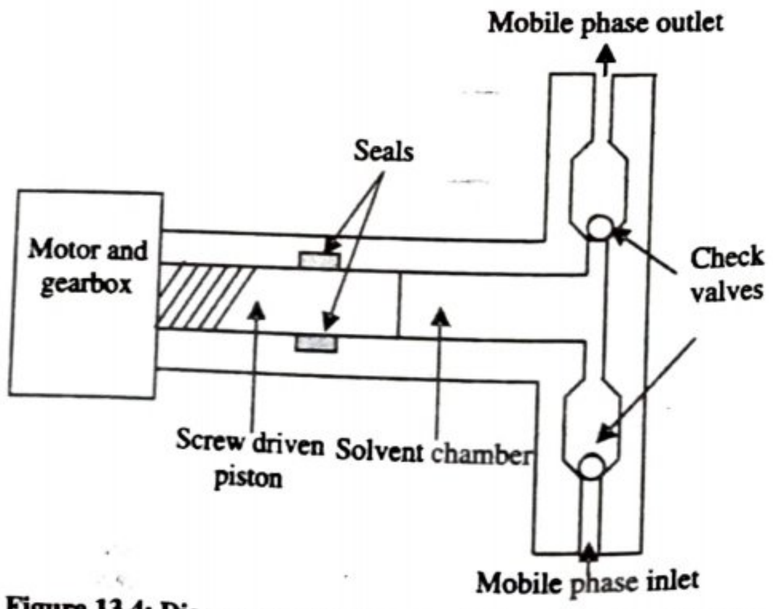


Figure 13.4: Diagrammatic Representation of Syringe Pump

13.2.3.2. Reciprocating Pump

In this pump (**figure 13.5**), an eccentric cam or gear drives the piston in and out of a solvent chamber. Upon moving forward, the inlet check valve closes, the outlet check valve opens, thus pumping the mobile phase into the column. Upon moving back, the outlet valve closes and the chamber is refilled. In comparison to syringe pumps, the reciprocating pumps have unlimited capacity, and their internal volume can be adjusted to very low, ranging from 10-100µl. The flow rate can be altered by varying the length of piston stroke or the motor speed. Access to the valves and seals is direct.

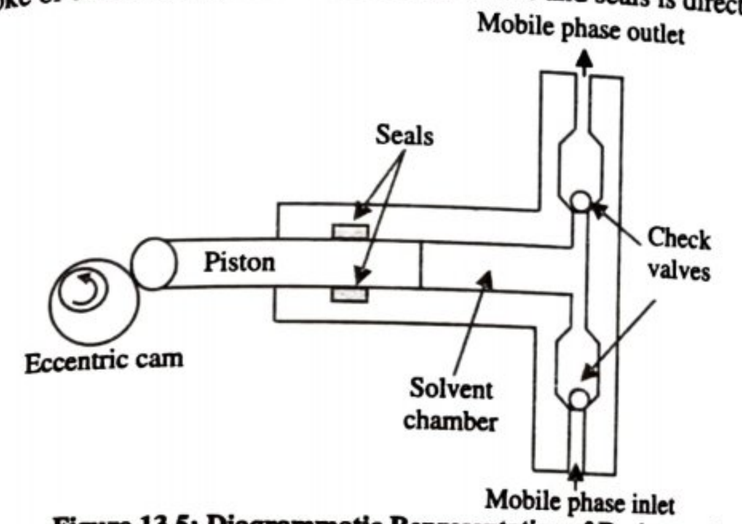


Figure 13.5: Diagrammatic Representation of Reciprocating Pump

A single headed reciprocating pump (**figure 13.6**) delivers the mobile phase to the column for half the time it operates; and during the piston drive stroke, the flow rate does not remains constant (since the piston speed varies with time). **Figure 13.6(i)** depicts the output of the pump. The output can be produced by using twin headed pump having two heads functioning at 180° out of phase (where one head pumps and the other gets refilled) [**figure 13.6(ii)**].

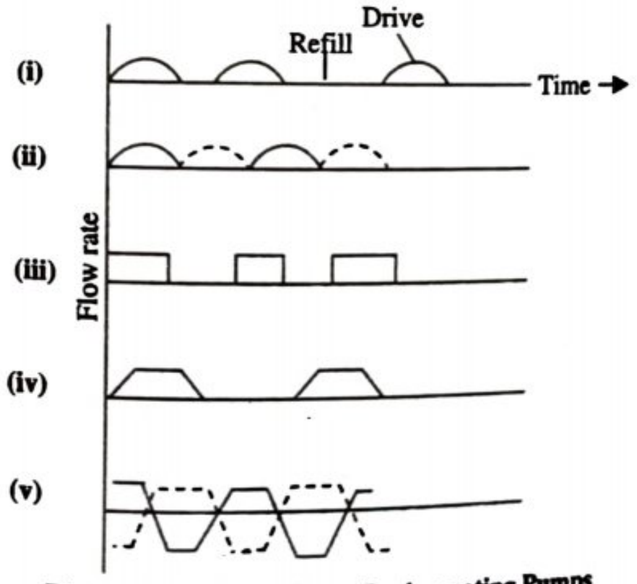


Figure 13.6: Output from Reciprocating Pumps

i) **Single Headed Pump**
ii) **Twin Headed Pump, Heads 180° Out of Phase**
iii) **Single Headed Pump with Constant Speed (Ideal)**
iv) **Single Headed Pump with Constant Piston Speed (in Practice)**
v) **Twin Headed Pump, Heads 180° Out of Phase with Different Constant Speeds on the Drive and Refill Strokes**

Modern twin headed pumps bear two pistons driven through a shaped cam or gear producing constant piston speed. The output of one head should be as in **figure 13.6 (iii)** and operation of two heads at 180° out of phase give pulseless flow.

Practically, every drive stroke presents a change in flow rate (which is not instantaneous), and produces an output as demonstrated in **figure 13.6 (iv)**. In order to avoid this, the driving cam is arranged to allow the piston travel faster upon refill than on the drive stroke, yielding an output as in **figure 13.6 (v)**.

The flow rate is the sum of the output of both the heads and remains constant. The flow noise in single headed pumps can be reduced by using a rapid stroke rate (one model employs 23 strokes per second so that the detector may not respond instantly to sense the flow changes). Some pumps employ feedback flow control, in which the measurement of flow rate is done downstream of the pump. Difference between the measured and the set flow activates motor speed modulation that reduces the flow difference to zero.

13.2.3.3. Pneumatic or Constant-Pressure Pump

This is the simplest form of pump, consisting of collapsible solvent container inside a vessel pressurised by a compressed gas. These pumps are inexpensive and pulseless. However, they suffer from limited capacity, pressure output, and their pumping rates rely on solvent viscosity. They also cannot be operated in gradient elution mode.

13.2.4. Sample Injection System

The following three modes of sample injection system are used in HPLC:

1) **Septum Injectors:** In this system, the sample is introduced through a high pressure syringe via self-sealing septum of elastometer. The major **shortcoming** of this system is that the mobile phase in immediate contact with the septum, gives rise to a leaching effect that results in ghost or pseudo peaks.

2) **Stop-Flow Septum-Less Injection:** In this system of sample introduction, most of the problems associated with septum injectors have been overcome. In this system, the flow of mobile phase through the column is stopped for a few moments, and when the column attains ambient pressure, the column top is opened and the sample is introduced at the top of the packing.

   The first two methods are inexpensive.

3) **Micro-Volume Sampling Valves:** In highly sophisticated modern HPLC apparatus, micro-volume sampling valves, having good precision and adaptable for automatic injection, are used. These valves allow sampling to be done reproducibly into pressurised columns with minimum interruption of the mobile phase flow.

   Figure 13.7 describes the operation of a sample loop in two varied modes:
   i) Sampling mode, and
   ii) Injection mode.

   The sample is introduced into an external loop in the micro-volume sampling valve at atmospheric pressure, and then injected into the mobile phase through rotation to the valve. The sample volume ranges between 2-100µl; but can be varied by changing the sample loop volume or by using specific variable-volume sample valves.

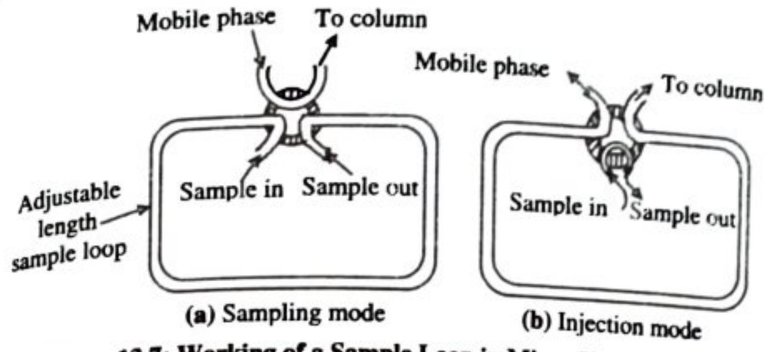


Figure 13.7: Working of a Sample Loop in Micro-Volume Sampling Valve
Source: Adapted from *Pharmaceutical Drug Analysis* (pp 459), by Kar Ashutosh (2008) (New Age International Publishers)

Therefore, the micro-volume sampling valve is mostly employed for quantitative work due to its high degree of precision and accuracy.

13.2.5. Columns

Construction of liquid chromatographic columns is done with stainless steel tubing. Glass or tyfon tubing is used for lower pressure (<600psi). The inside diameter and length of column ranges between 2-5mm and 10-30cm, respectively. Column packings have particle size ranging from 3-10µm. These types of columns carry 40,000-60,000 plates/m. At the present time, micro-columns with 1-4.6mm diameter and 3-7.5cm length are available. These columns require less solvent and have greater speed. Their packing is done with 3-5µm sized particles and they have around 1,00,000 plates/m. **Advantage** of micro-columns over normal columns is that they need less solvent, while high purity solvents are required in liquid chromatography, making it expensive and prone to decomposition.

Sub-micron sized silica particles agglomerate under favourable conditions and form large silica particles having uniform diameter. These particles are employed in liquid chromatography as a packing material. Thin layers of organic solvents are coated on these silica particles, and the solvents bound to the particle surface physically and chemically. Alumina particles, ion-exchange resins, and porous polymer particles can also be used as column packing material.

The following two types of columns are used in HPLC:
1) Guard columns, and
2) Column thermostats.

13.2.5.1. Guard Columns

It is a short column present between the injector and analytical column. Although the packing composition of guard columns and analytical columns is similar, but particle size is larger in guard columns to aid in the reduction of pressure drop.

The **benefits** of guard columns are:
1) They eliminate foreign particles and contaminants from the solvents, thereby improving the life of analytical columns.
2) In liquid-liquid chromatography, they minimise the loss of stationary phase from the analytical columns since the mobile phase is saturated with the stationary phase.

A guard column and retention gap are the same but function differently. They are 1-10m deactivated fused silica tubing linked to the front of the column (**figure 4.8**). This tubing does not contain stationary phase, and its interactions with the solute is reduced by deactivating its surface. A union attaches the tubing and the column. Usually, the

diameter of retention gap or guard column is same as that of the column. If the tubing sizes vary, a larger diameter guard column or retention gap is used than a smaller one.

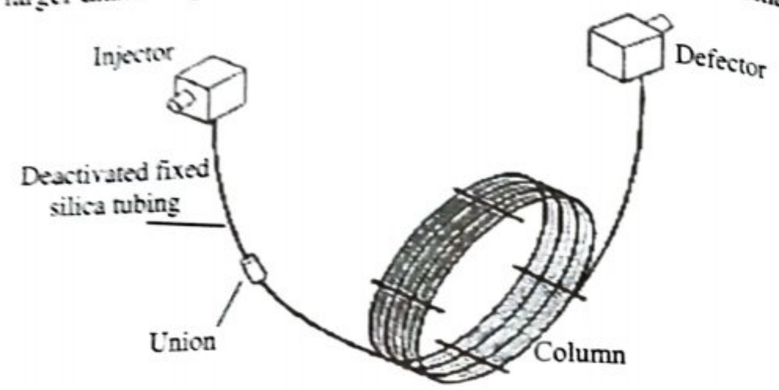


Figure 13.8: Retention Gap or Guard Column

Guard columns are suitably used with samples containing non-volatile residues that could contaminate a column. These residues get deposited in the guard column, and thus the interaction between the residues and the sample is reduced since the solute may not adhere in the guard column (as it lacks the stationary phase). The residues also do not coat the stationary phase, thus resulting in poor peak shapes. Guard column requires periodic cutting or trimming upon a build-up of residues. Guard columns are usually of 5-10m long to favour trimming before replacement. If peak shape problems initiate this means the guard column needs trimming or changing.

Retention gaps improve the peak shapes for special samples, columns, and GC conditions. Generally, around 3-5m of tubing provides the benefits of a retention gap. Benefit of retention gaps are seen in case of large volume injections (>2µl) and solvent-stationary phase polarity mismatches for split-less, Megabore direct and on-column injections. Combinations of these conditions may give rise to distorted peak shapes. Polarity mismatches result due to differences in polarity of sample solvent and column stationary phase. The betterment is observed in case of peaks eluting closest to the solvent front or solutes having similar polarity as the solvent. The benefits of a retention gap are best obtained on using a guard column.

13.2.5.2. Column Thermostats

Chromatographic operations are done at room temperature without the requirement of sharp control of column temperature. Improved chromatograms are obtained if the column temperature is maintained constant to the few tenths of a degree Celsius. To achieve a constant and precise temperature control, water jackets are fitted in the columns. The modern commercial instruments contain heaters for controlling the column temperature to a few tenths of a degree from near ambient to 150°C.

13.2.6. Detectors

In HPLC, the detector monitors the mobile phase passing out of the column, which further releases electrical signals directly proportional to the characteristics of the solute or the mobile phase.

The commonly used detectors in HPLC are:

1) **Bulk-Property Detectors:** These detectors measure the dissimilarity in certain physical properties of the solute in the mobile phase compared to the individual mobile phase. Refractive-index detectors and conductivity detectors are the **examples** of bulk-property detectors.

2) **Solute-Property Detectors:** These detectors respond to a specific physical or chemical characteristic of the solute (under investigation), and this property should be independent of the mobile phase being used. However, totally independent of mobile phase is not possible, yet signal discrimination helps in differentiating measurable experimental procedures with solvent changes, like gradient-elution. UV-detectors and fluorescence detectors are the **examples** of solute-property detectors.

3) **Multipurpose Detectors:** These detectors provide a high degree of sensitivity and broad-linear-response-attainable range. Detectors with greater selectivity is required in analytical chemistry, which is fulfilled by employing multipurpose detectors, **e.g.,** Perkin-Elmer 3D System that combines UV absorption, fluorescence and conductometric detection altogether.

4) **Electrochemical Detectors:** These detectors include amperometric or coulometric detectors that involve current measurement associated with the reduction or oxidation of solutes. As fewer compounds undergo electrochemical oxidation, these detectors show selectivity that is enhanced by monitoring the applied potential to the detector for differentiating various electro-active species. Electrochemical detection employs conducting mobile phases (**e.g.,** inorganic salts or mixtures of water) with water-miscible organic solvents.

13.2.6.1. UV-Detector

Principle

An UV-detector works on the principle of absorption of UV visible light from the effluent emerging out of the column and passing through a photocell positioned in the radiation beam **(figure 13.9).**

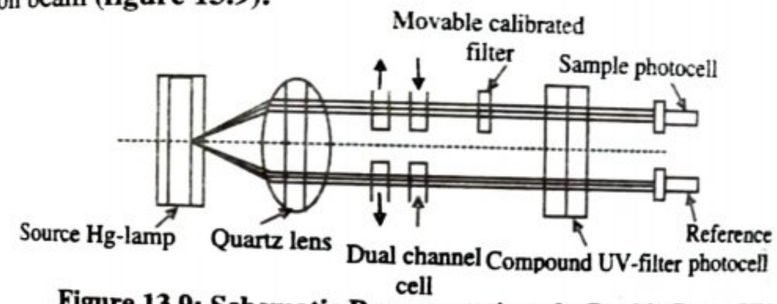


Figure 13.9: Schematic Representation of a Double-Beam UV Detector
Source: Adapted from Pharmaceutical Drug Analysis (pp 462), by Kar Ashutosh (2008) (New Age International Publishers)

Originally, dual-wavelength instruments with 254 and/or 280nm were used, but now more sophisticated and updated variable wavelength detectors with wavelengths ranging between 210-800nm are used for performing more selective detection.

13.2.6.2. Fluorescence Detector

Many compounds (solutes) are present in the mobile phase. When they are allowed to pass as column effluent through a cell irradiated with xenon or deuterium source, first UV radiation is absorbed and subsequently radiation of a longer wavelength is emitted in the following two ways:

1) If instantly, named as 'Fluorescence', and
2) If after a time-gap, named as 'Phosphorescence'.

Fluorescent **Compounds:** The number of inorganic and organic compounds exhibiting natural fluorescence property is very less, while most of the pharmaceutical substances and environmental contaminants [(**e.g.,** Polycyclic Aromatic Hydrocarbons (PAH)] with

a conjugated-cyclic system are fluorescent in nature. Energy absorbed by these substances is re-emitted from 0.1-1.0 and can be detected by a fluorescence detector. For the detection of non-fluorescent compounds, they are firstly converted to fluorescent derivatives by treating with appropriate solvents.

Figure 13.10 shows the diagram of a fluorescence detector. Radiation emitted from a xenon or deuterium source is concentrated on the flow cell using a filter. Usually at 90° to the incident beam, the fluorescent radiation emitted by the sample is measured. A second filter is used to filter only a suitable wavelength and reject all scattered light to reach the photomultiplier detector.

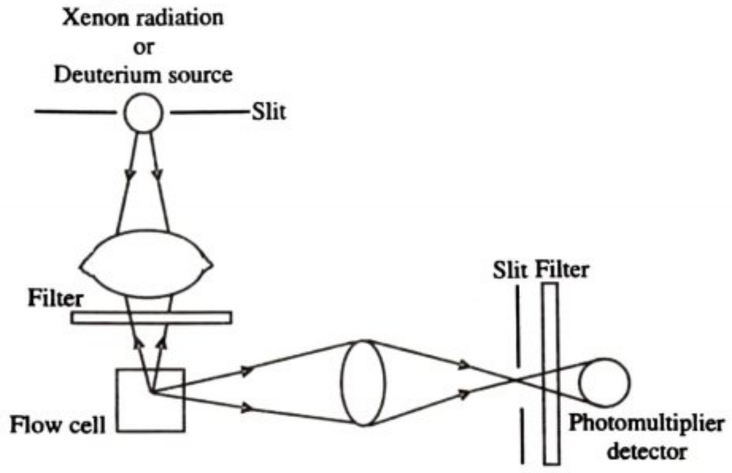


Figure 13.10: Schematic Representation of a Fluorescence Detector

13.2.6.3. Refractive Index Detector or RI-Detector or Refractometer

In refractive index detector (**figure 13.11**), light emitted from the source(s) is concentrated into the cell containing the sample and reference sample. Both the chambers of cells are separated by a diagonal glass sheet. The light passes through the cell and reaches the beam splitter (B) that diverts the light towards two photocells (P_1 and P_2).

A change in the observed refractive index of the sample results in a difference in their relative output, and this difference is amplified and recorded.

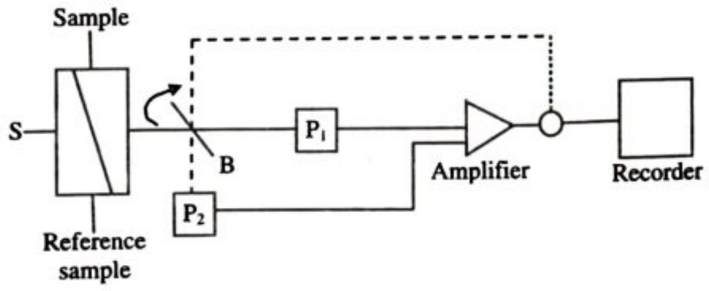


Figure 13.11: Block Diagram of a Refractive Index Detector

13.2.6.4. Multipurpose Detector

A multipurpose detector includes three detectors that are combined and kept together in a single unit. An **example** of this type of detector is Perkin-Elmer 3D System, developed by **Perkin-Elmer (figure 13.12)**.

The three different detectors perform the following functions:

1) **Fluorescence Function:** They monitor emission above 280nm, based on excitation at 254nm.
2) **UV-Function:** It is a fixed wavelength (254nm) detector.

3) ***Conductance-Function:*** The metal inlet and outlet tubes function like electrodes that measure the conductance of ions.

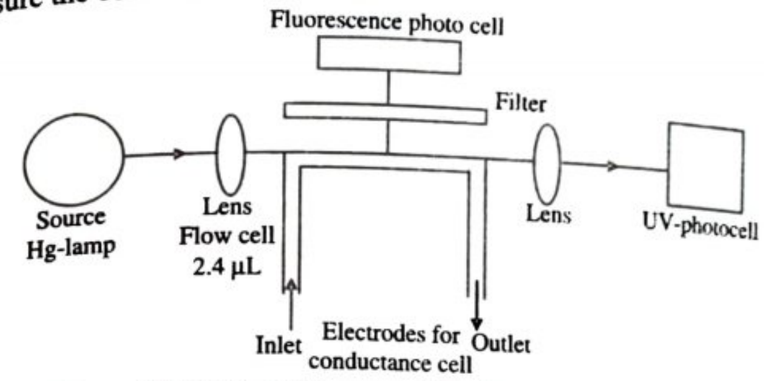


Figure 13.12: Block Diagram of Perkin-Elmer Detector
Source: Adapted from Pharmaceutical Drug Analysis (pp 465), by Kar Ashutosh (2008) (New Age International Publishers)

13.2.6.5. Electrochemical Detectors

Practically, it is difficult to utilise the functions of electrochemical reduction as a mean of detection of HPLC. This is because reduction of oxygen in the mobile phase causes serious interference (i.e., large background current). It is almost impossible to remove oxygen completely; therefore electrochemical detection generally depends on the oxidation of solute. Compounds like aromatic amines, phenols, ketones, aldehydes, and heterocyclic nitrogen compounds can be detected conveniently.

At the present time, amperometric detector is considered the best electrochemical detector and it possesses the following distinguished features:

1) Small internal cell-volume,
2) High sensitivity,
3) Limited range of applications, and
4) Best for trace analyses as UV-detector lacks adequate sensitivity.

13.2.7. Solvent Selection

Solvents for HPLC are selected on the basis of the following factors:

1) **Selection of Starting Solvent:** The starting solvent is selected based on the relative polarity of the solvent and the sample to be separated. For this, the solvent that matches to the most polar functional group of the sample molecule (**e.g.,** alcohols for OH, amines for NH_2, etc.) is selected. Separation can be done by the following methods:
   i) The solvent is too polar to allow the adsorbent to retain the sample, if the sample appears at the solvent front. In such a case, a solvent higher up (lower polarity) on the scale is selected.
   ii) If the sample does not appear at the solvent front in a reasonable time, a solvent or solvent blend lower down (higher polarity) on the scale is selected.
2) **Solvent Miscibility:** Solvents on the bottom half are soluble with each other, those present on the upper half are completely soluble, and those on the centre half are mutually soluble with each other. So from the given statement it is clear that solvents present on the same half of the scale (from 0 to 1) are completely soluble with each other. Solvents which get solubilise with almost all other solvents (except pentane and hexane) are called **universal solvents** (**e.g.,** tetrahydrofuran and acetonitrile). Among all, iso-propanol is the most useful **rinsing** or **cleanout solvent**, which is soluble at all levels of concentrations with all the other solvents.

3) **Solvent Blends:** They are more useful if present in pairs. The absorption energy of the solvent blends should not deviate by more than 100%; **for example,** chloroform at energy 0.40 and propanol at energy 0.82.

4) **Solvent Gradients:** Range of solvent gradient is not sufficient for all but for most of the separation problems. When solvents are changed or generated after gradient, minimum 5-10 column volumes of the new starting solvent should flow through the system before making another sample injection.

13.2.8. Strip Chart Recorder

The signals emerging from the HPLC detector are continuously recorded as function of time. For these purposes generally, a potentiometric recorder is used. The most efficient recorder is that which records 1-10mV full-scale deflection over a stretch of approximately ten inches and having a response-time of one second or less. Therefore, the most preferred recorder is a strip-chart recorder with variable chart speeds ranging between 5-5mm/min. A feedback signal arrangement (device) using a servomechanism is used to balance the input signal of a potentiometric-recorder continuously. With pre-adjusted attenuation a pen is attached with this device, which moves proportionately along the width of the chart paper so that signals can be accurately recorded. The paper attached with the device moves at a fixed speed along the length.

Before operating the recorder, its zero point should be adjusted with the input zero, or else the baseline will also shift with little changes in signal attenuation. Along with this, it is also important to appropriately adjust the amplifier gain so that the dead-band and the oscillations can be eliminated completely. A recorder with inadequate shielding from the AC circuits may show shifting of its zero point.

13.2.9. Data Handling System

In HPLC, there is a tremendous development in the data handling devices that ranges from a strip chart recorder, an electric integrator, and a PC-based workstation to a client-server network system (the latest one) (**figure 13.13**). The automation and sophistication has also advanced with the time

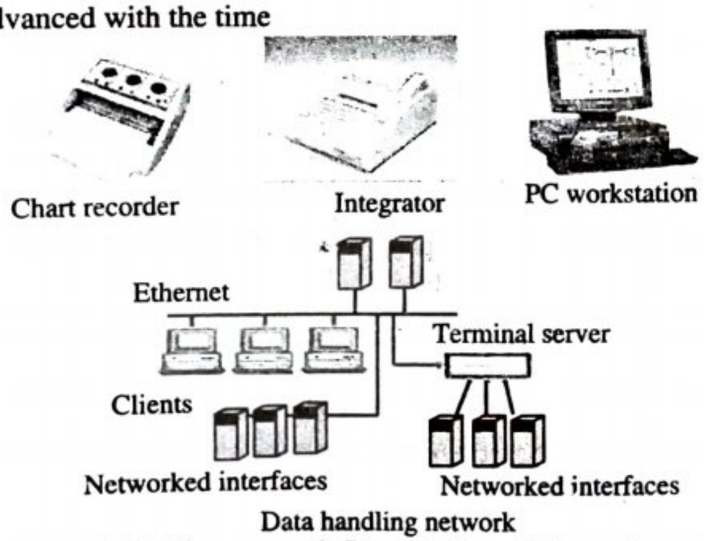


Figure 13.13: Diagrammatic Presentation of Chromatography Data Handling System

In a **chart recorder**, the peak heights are measured manually. The **integrators** include built-in algorithms for peak integration, calculation, and report generation. At the present time, **PC based workstations** are in use. These devices perform some additional functions like archiving the data and have a full HPLC system control over a user-friendly graphic interface.

13.3. APPLICATIONS

13.3.1. Introduction

HPLC is widely used in analytical chemistry. Its applications range from the isolation of naturally active pharmaceutical compounds, assay of pure drugs and their dosage form, to control of microbiological processes.

Following are some common applications of HPLC:

1) Stability studies,
2) Bioassays and its complementation,
3) Design of dosage form,
4) In cosmetic industry,
5) Isolation of natural pharmaceutically active compounds,
6) Control of microbiological processes,
7) Assay of cephalosporins,
8) Assay of furosemide,
9) Assay of theophylline,
10) Assay of corticosteroids, and
11) Assay of dichlorphenamide.

13.3.2. Stability Studies

Stability of various pharmaceutical compounds, degradation products (**e.g.,** stability studies of atropine), and other chemical substances can be studied using the technique of HPLC.

13.3.3. Bioassays and its Complementation

HPLC is used in the bioassay test of many complex molecules (**e.g.,** peptide hormones and antibiotic molecules). Bioassay of a compound includes complete estimation of the potency (but it does not give detailed chemical composition). It is a very costly, time taking, repeatable, and a poor precision test. Therefore, HPLC is preferred for complement bioassay and for complete study of the given chemical compound. HPLC is commonly used for the bioassay and analysis of peptide hormones, and some antibiotics (**e.g.,** cotrimoxazole, penicillins, sulphates, and chloramphenicol).

13.3.4. Design of Dosage Form

Biopharmaceutics of the dosage form and the pharmacokinetic properties of the drugs are studied with the help of HPLC. These properties are involved in dosage form designing.

13.3.5. In Cosmetic Industry

In this industry, HPLC is used for analysing the quality of various cosmetic products such as lipsticks, gels, creams, etc.

13.3.6. Isolation of Natural Pharmaceutically Active Compounds

HPLC is the most specific and sensitive method used for the separation of different therapeutically active components present in plant extracts. HPLC method is used in the isolation of different types of alkaloids and glycosides. **For example**, analysis of cinchona, liquorice, ergot extracts, and digitalis. Examples of some plant alkaloids and glycosides isolated using HPLC are given below:

Categories	Constituents	Uses
Alkaloids	Morphine and codeine	Analgesic and antitussive
Glycoside	Digitalis glycosides and sennosides	In cardiovascular diseases and as laxatives

13.3.7. Control of Microbiological Processes

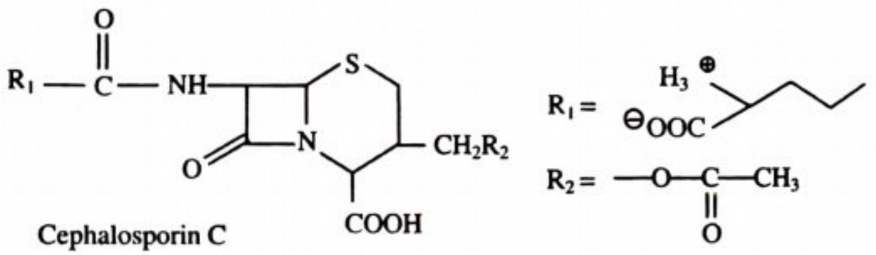
HPLC is used to analyse antibiotics (**e.g.,** tetracyclines, chloramphenicol, streptomycin and penicillins) produced by various microbiological processes. The main areas of the operations are:

1) Studying the kinetics and monitoring the microbiological processes,
2) Isolating and purifying the active ingredients,
3) Controlling the purity of active constituents, and
4) Monitoring the derivatization reactions of these compounds.

13.3.8. Assay of Cephalosporins

Many derivatives of cephalosporin class of antibiotics can be precisely separated by HPLC. The assay of this class of antibiotics can also be performed using HPLC under the given conditions:

1) **Column:** ODS-SIL-X-II,
2) **Mobile-Phase:** 0.95M ammonium carbonate/methanol (95: 5), and
3) **Detector:** UV-220nm.

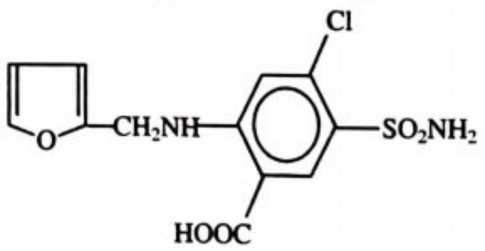


Cephalosporin C

Marrelli analysed the concentrations of cephalosporin C in the presence of other UV absorbing species using the loss of absorption with cleavage of the β-lactam.

13.3.9. Assay of Furosemide

The study of furosemide and its decomposition products is performed using fluorescence and UV detection methods during the HPLC analysis.

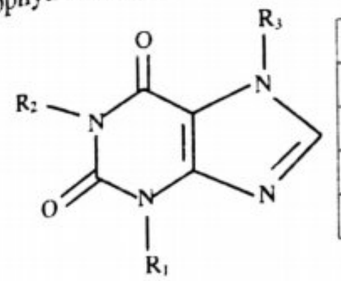


4-Chloro-N-furfuryl-5-sulphamoylanthranilic acid

Fluorescence detection is a very specific technique because excitation and emission wavelengths are selected. If compared with other detectors, sensitivity for compounds with photoluminescence properties should be higher by factors of 100 to 1000.

13.3.10. Assay of Theophylline

Impurities like theobromine, β-hydroxypropyltheophylline and caffeine found in theophylline can be easily detected and removed by HPLC analysis.



Caffeine	$R_1 = R_2 = R_3 = —CH_3$;
Theophylline	$R_1 = R_2 = —CH_3$; $R_3 = —H$;
Theobromine	$R_2 = —H$; $R_1 = R_3 = —CH_3$;
β-Hydroxypropyltheophylline	$R_1 = R_2 = —CH_3$; and
	$R_3 = —CH_2—CH_2—CH_3$

13.3.11. Assay of Corticosteroids

The mixture of six corticosteroids (hydrocortisone acetate, cortisone, deoxycortisone, hydrocortisone, prednisolone, and prednisone) can be assayed by HPLC. The chromatographic parameters for the assay of these corticosteroids are:

1) **Sample Size:** 10µl,
2) **Column Size:** 250 × 4.6mm ID,
3) **Adsorbent:** Lichrosorb DIOL: 10µm,
4) **Mobile-Phase:** Gradient elution of A (n-hexane) and B (isopropanol), and
5) **Detector:** UV-254nm.

13.3.12. Assay of Dichlorphenamide

Materials Required: Dichlorphenamide sample (100mg), dichlorphenamide RS (100mg), mobile phase (solution with 0.02M NaH_2PO_4 and 0.2M Na_2HPO_4 in a mixture of equal volumes of acetonitrile and water) (50ml).

Procedure: The above mentioned mobile phase with µBondapack C18 column having 1.0ml/minute flow rate is used in the chromatographic operation and detection is done at 280nm wavelength. In the mobile phase, following solutions are used to run HPLC analysis:

1) 0.05% w/v of dichlorphenamide RS, and
2) 0.05% w/v of dichlorphenamide sample.

Calculations

The content of $C_6H_6Cl_2N_2O_4S_2$ is calculated using the declared content of the same in dichlorphenamide RS.

13.4. SUMMARY

The details given in the chapter can be summarised as follows:

1) **High Performance Liquid Chromatography** (HPLC) is used to separate complex chemical mixtures.
2) The time required for emergence of the maximum peak of a component after sample injection is known as **retention time** (t_R).
3) The time duration that a component spends in the stationary phase is the **adjusted retention time** (t'_R).
4) **Injection point** is the position time/point in time from where/when the sample is introduced in the column.
5) **Baseline** is a part of the chromatogram where only mobile phase emerges from the column.
6) **Peak maximum** is the highest point of the peak.
7) **Dead point** is the position of the peak-maximum of an unretained solute.

8) **Dead volume (V_o)** is the volume of mobile phase passed through the column between the injection point and the dead point.
9) **Dead time (t_o)** is the time elapsed between the injection point and the dead point.
10) **Normal Phase HPLC (NP-HPLC)** utilises a polar stationary phase and a non-polar mobile phase for the separation of mixture components on the basis of polarity.
11) **Reverse Phase HPLC (RP-HPLC or RPC)** utilises a non-polar stationary phase and an aqueous, moderately polar mobile phase.
12) **Size-Exclusion HPLC (SEC) or gel permeation or filtration chromatography** involves separation of particles based on their size.
13) In **ion-exchange HPLC**, retention occurs according to the attraction between the solute ions and charged sites bound to the stationary phase, excluding the similarly charged ions.
14) **Bio-affinity chromatography** involves separation according to the specific reversible interaction of proteins with ligands that are attached to the solid support on a bio-affinity matrix through covalent bonds.
15) In **reciprocating pump**, an eccentric cam or gear drives the piston in and out of a solvent chamber.
16) In **screw-driven syringe pump**, a variable speed stepper motor turns a screw that drives a piston, which displaces the mobile phase from a chamber.
17) **Pneumatic or constant-pressure pump** consists of collapsible solvent container inside a vessel pressurised by a compressed gas.
18) In **septum injectors**, the sample is introduced through a high pressure syringe via self-sealing septum of elastometer.
19) In **stop-flow septum-less injection** system, most of the problems associated with septum injectors have been overcome.
20) **Micro-volume sampling valves** allow sampling to be done reproducibly into pressurised columns with minimum interruption of the mobile phase flow.
21) Construction of liquid chromatographic columns is done with stainless steel tubing.
22) **Guard column** is a short column present between the injector and analytical column.
23) **Bulk-property detectors** measure the dissimilarity in certain physical properties of the solute in the mobile phase compared to the individual mobile phase.
24) **Solute-property detectors** respond to a specific physical or chemical characteristic of the solute, and this property should be independent of the mobile phase being used.
25) **Multipurpose detectors** provide a high degree of sensitivity and broad-linear-response-attainable range.
26) **Electrochemical detectors** include amperometric or coulometric detectors that involve current measurement associated with the reduction or oxidation of solutes.
27) The **starting solvent** is selected based on the relative polarity of the solvent and the sample to be separated.
28) Solvents which get solubilise with almost all other solvents (except pentane and hexane) are called **universal solvents (e.g.,** tetrahydrofuran and acetonitrile).

13.5. EXERCISE

13.5.1. True or False

1) The time required for emergence of the maximum peak of a component after sample injection is known as retention time.
2) Baseline is the position of the peak-maximum of an unretained solute.
3) Gel permeation or filtration chromatography involves separation of particles based on their migration rate.

4) In reciprocating pump, an eccentric cam or gear drives the piston in and out of a solvent chamber.
5) In stop flow septum-less injectors, the sample is introduced through a high pressure syringe via self-sealing septum of elastometer.
6) Construction of liquid chromatographic columns is done with glass tubing.
7) Guard column is a short column present between the injector and analytical column.

13.5.2. Fill in the Blanks

8) The time duration that a component spends in the stationary phase is the _____.
9) _____ is a part of the chromatogram where only mobile phase emerges from the column.
10) Normal phase HPLC utilises a polar stationary phase and a non-polar mobile phase for the separation of mixture components on the basis of _____.
11) _____ consists of collapsible solvent container inside a vessel pressurised by a compressed gas.
12) _____ allow sampling to be done reproducibly into pressurised columns with minimum interruption of the mobile phase flow.
13) _____ detectors provide a high degree of sensitivity and broad-linear-response-attainable range.
14) Solvents which get solubilise with almost all other solvents (except pentane and hexane) are called _____.

Answers

1) True 2) False 3) False 4) True 5) False
6) False 7) True 8) Adjusted retention time 9) Baseline
10) Polarity 11) Pneumatic pump 12) Micro-volume sampling valves
13) Multipurpose 14) Universal solvents

13.5.3. Very Short Answer Type Questions

1) What is HPLC?
2) Define baseline, dead point, and dead volume.
3) Draw a well-labelled diagram of the apparatus used in HPLC.
4) Give the advantages of HPLC.
5) Write about any one sample injection system in HPLC.
6) What is retention time?

13.5.4. Short Answer Type Questions

1) Discuss the principle of HPLC.
2) Give the types of HPLC.
3) Write a note on the pumps used in HPLC.
4) Discuss the columns used in HPLC.
5) Write a short note on solvent selection in HPLC.

13.5.5. Long Answer Type Questions

1) Briefly explain the detectors used in HPLC.
2) Discuss about the applications of HPLC.
3) Write an illustrative note on the instrumentation of HPLC.

CHAPTER 14 Ion Exchange Chromatography

14.1. ION EXCHANGE CHROMATOGRAPHY

14.1.1. Introduction

Ion exchange chromatography (or **ion chromatography**) is an economical and versatile technique for rapid and effective separation of ions, amino acids, peptides, nucleotides, nucleic acids, etc. It is used for pre-fractionation or purification of a target protein from crude biological samples. Ion exchange chromatography is defined as **a process that allows the separation of ions and polar molecules based on their affinity to the ion exchanger**. This technique can be used for any kind of charged molecule including large proteins, small nucleotides, and amino acids.

14.1.2. Classification

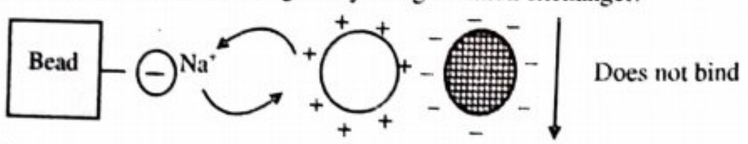
Ion exchange chromatography is carried out in columns packed with ion exchangers, and is divided into the following based on the type of exchanger used for separation:

1) Cation exchange chromatography, and
2) Anion exchange chromatography.

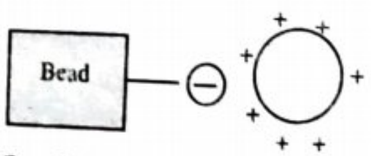
14.1.2.1. Cation Exchange Chromatography

Solid support with desired functional groups is prepared with various beads differing in flow rate, stability, binding capacity (linked with porosity), etc. Cation exchangers based on dextran (Sephadex), agarose (Sepharose), and cross-linked cellulose (Sephacel), are the ion exchange matrices having high porosity. This improves the flow properties and high capacities for macromolecules. The cation exchange chromatography is carried out with buffers having pH between 4 to 7, and a gradient is run from a solution containing only this buffer to a solution containing this buffer and 1M NaCl.

Step 1: Na^+ is attached to negatively charged cation exchanger:



Step 2: Positively charged protein exchanges with Na^+ and binds to cation exchanger. Negatively charged protein does not bind.



Step 3: A competing Na^+ (NaCl gradient) elutes the protein. Protein with less positive charge at given pH elute at lower NaCl concentration while protein with higher positive charge get eluted at higher concentration at NaCl.

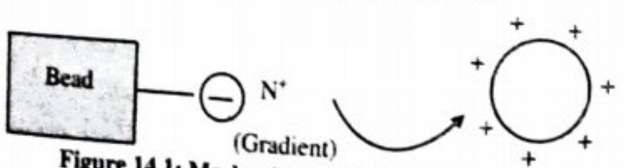


Figure 14.1: Mechanism of Cation Exchange Chromatography

A positive surface charge should be present on the molecules (proteins, nucleic acids, etc.) that bind to cation exchangers. Thus, for specific protein binding, the pH should be below the pI (isoelectric point) of that protein. With increasing salt concentration, the concentration of Na^+ (cation) and Cl^- (anion) ions also increases. After a point, the positively charged protein exchanges with the cation, and thus this type of chromatography is termed cation exchange chromatography (**figure 14.2**).

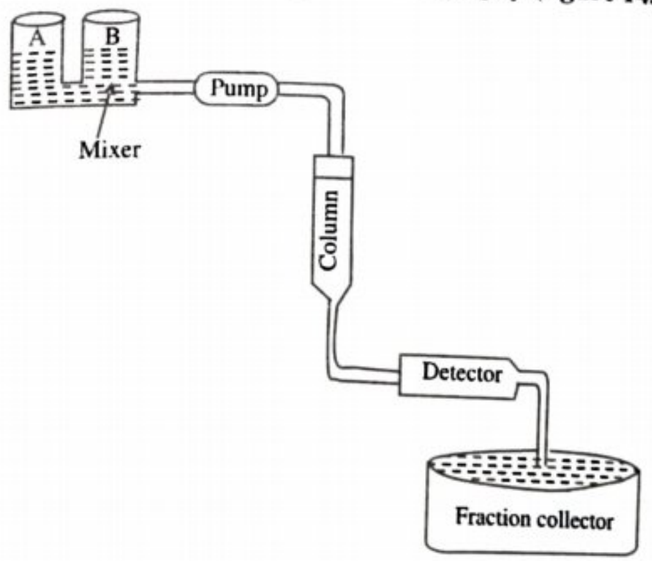


Figure 14.2: Experimental Set-Up of Cation Exchange Chromatography

14.1.2.2. Anion Exchange Chromatography

Solid support with desired functional groups is prepared with various beads differing in flow rate, etc. Anion exchangers based on dextran (Sephadex), agarose (Sepharose), or cross-linked cellulose (Sephacel), are commonly used.

The anion exchange chromatography is carried out with buffers having pH between 7 to 10, and a gradient is run from a solution containing only this buffer to a solution containing this buffer and 1M NaCl.

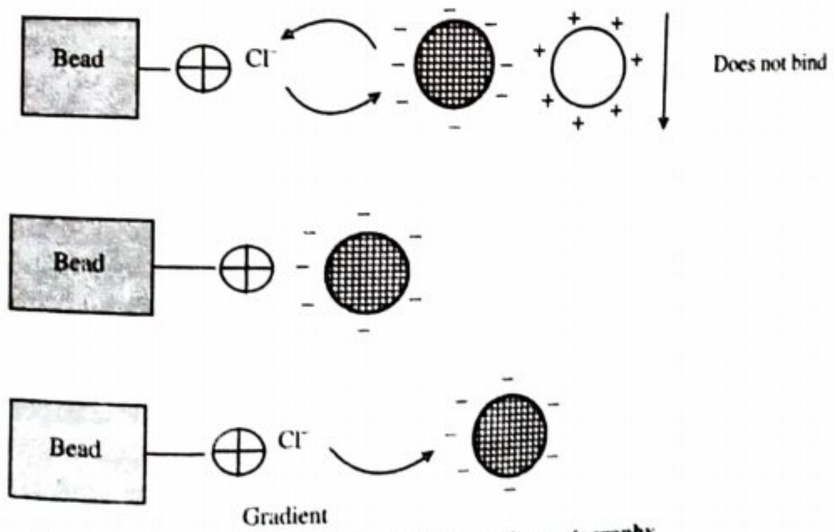


Figure 14.3: Mechanism of Anion Exchange Chromatography

A negative surface charge should be present on the molecules (proteins, nucleic acids, etc.) that bind to the anion exchanger. Thus, for specific protein binding, purification should be done above the pI of that protein. The salt in the solution binds competitively to the column matrix at a given concentration and releases the protein from its bound state.

The proteins separate because the amount of salt required for the release of protein differs with the protein's external charge.

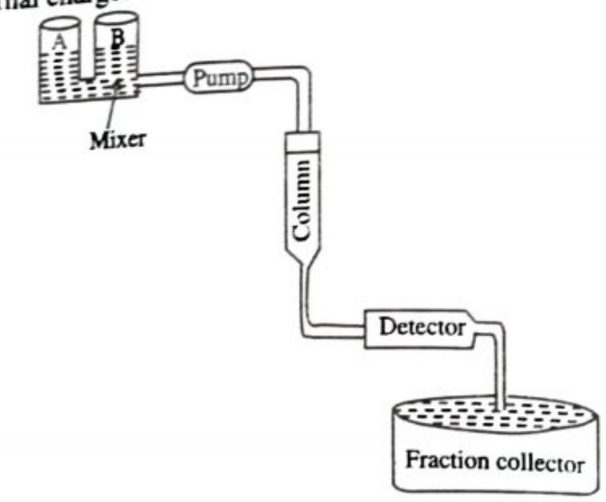


Figure 14.4: Experimental Set-Up of Anion Exchange Chromatography

Method: Depending on the column dimensions and capacity of exchanger, the amount of sample to be applied on the column is determined. After packing, the column is washed with distilled water and equilibrated with the starting buffer. When the molecules bind, the column is washed to equilibrate it in the starting buffer of low ionic strength. Thereafter, the bound molecules are eluted off using a gradient of a second buffer that gradually increases the ionic strength of eluent solution. Alternatively, the pH of eluent buffer is modified by adding a charge to the protein or matrix; and at this charge, they will not interact and the desired molecule elutes from the resin.

Gradient elution is more common than isocratic elution. Continuous or stepwise pH and ionic strength gradient may be used; however, continuous gradient yields better results. The pH gradient as well as ionic gradient increases for cation exchanger, while the pH decreases and ionic strength increases for anion exchanger. Anion (Cl^-) is attached to a positively charged matrix. The negatively charged protein binds to resin by competing with anion. A competing anion concentration (NaCl gradient) replaces the protein, and results in elution. A less negatively charged protein elutes first, and the more negatively charged one elutes later (**figure 14.4**).

14.1.3. Mechanism of Ion Exchange Process

Ion-exchange chromatography is involved in the separation of ionisable compounds (having different charges) and comprises of mobile and stationary phases like other column-based liquid chromatography techniques.

The mobile phase in this method is an aqueous buffer system, and the stationary phase is an inert organic matrix, chemically derivatized with ionisable functional groups (fixed ions) carrying displaceable oppositely charged ions. **Counter ions** exist in a state of equilibrium between the mobile and stationary phases, resulting in anion and cation exchanges (**figure 14.5**). Protons (H^+), hydroxide groups (OH^-), single charged mono-atomic ions (Na^+, K^+, and Cl^-), double charged mono-atomic ions (Ca^{2+} and Mg^{2+}), polyatomic inorganic ions (SO_4^{2-} and PO_4^{3-}), and organic bases (NR_2H^+) and acids (COO^-) are the exchangeable matrix counter ions.

Cation and anion exchange resin columns are used for the separation of cations and anions, respectively. Separation may also take place based on the binding of analytes to positively- or negatively-charged groups fixed on a stationary phase and in equilibrium with free counter ions in the mobile phase as per the differences in their net surface charge (**figure 14.5**).

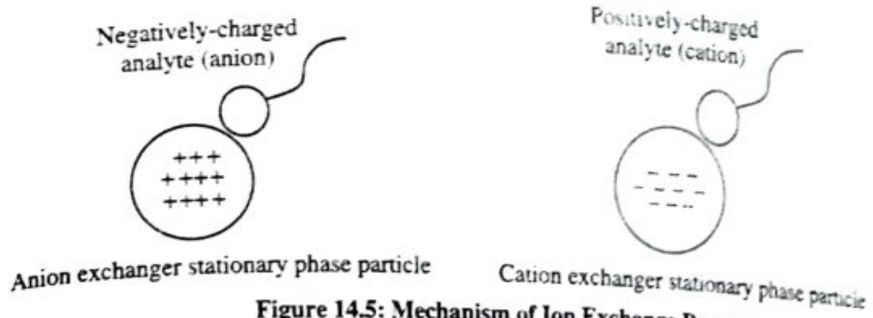


Figure 14.5: Mechanism of Ion Exchange Process

In ion exchange chromatography, ionic and polar analytes are separated using chromatographic supports derivatized with ionic functional groups carrying charges opposite to that of the analyte ions.

These ions compete with the similarly charged ions of the eluent to bind to the oppositely charged ionic functional group attached on the stationary phase. This competition can be explained using the equation given below by considering the exchanging ions (analytes and ions in the mobile phase) as cations.

$$S\text{–}X^-C^+ + M^+ \leftrightarrow S\text{–}X^-M^+ + C^+$$

In this process, the eluent cation (M^+) is replaced with the analyte cation (C^+) that is bound to the anion (X^-) fixed on the surface of chromatographic support (S).

In anion exchange chromatography, the exchanging ions are anions and this is represented by the equation given below:

$$S\text{–}X^+A^- + B^- \leftrightarrow S\text{–}X^+B^- + A^-$$

The eluent anion (B^-) is replaced with the analyte anion (A^-) that is bound to the positively charged ion (X^+) fixed on the surface of stationary phase. Adsorption of the analyte to the stationary phase and desorption by the eluent ions occurs repetitively while travelling in the column, and this results in the separation due to ion exchange.

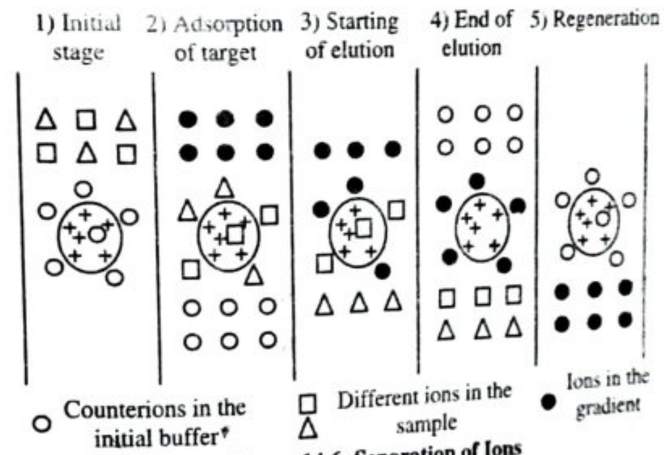


Figure 14.6: Separation of Ions

The charges present on the molecules keep on varying, and thus they undergo different degrees of interaction with the charged chromatography support. This interaction depends on the differences in the net charge, charge density, and surface charge distribution of the molecules.

Since the net surface charge of all the molecules having ionisable groups depends on pH, the mobile phase pH should be selected such that the net charge on a desirable protein in a mixture is opposite to that of the matrix functional group; and this is required so that the ionic protein will displace the functional group counter ion and bind to the matrix.

In contrast, the oppositely charged proteins will not be retained. If the pH of the mobile phase is altered, the net charge and the matrix binding capacity of the adsorbed protein analytes are affected, thus they are eluted. Likewise, if the concentration of a similarly charged species within the mobile phase is increased, the bound proteins can be eluted.

Ion exchange chromatography can separate the complex mixtures of anions or cations, and can measure the quantitative amounts of each ion in a short time. Earlier, separations by ion-exchange chromatography were used to be carried out in open-column mode. The column was loosely packed with the stationary phase comprising of small glass particles (1-2cm in diameter).

The mobile phase or eluent comprised of the competing ion and was continuously passed into the column and allowed to percolate through it under gravity. Sample mixture was applied to the column top and allowed to pass into the bed of ion-exchange material.

Eluent flow was resumed and the eluent fractions were collected from the column outlet at definite intervals. **Open column ion exchange chromatography** is a slow process due to low eluent flow rates; and if the flow rate is increased, the separation efficiency deteriorates.

These problems were overcome in **modern ion exchange chromatography** by using high efficiency ion exchange materials along with flow-through detection. Separations in modern ion chromatography are performed using columns filled with uniformly-sized ion exchange particles that are much smaller than those used in the open column ion chromatography. But, the ion exchange resins used in modern chromatography are of lower capacity than those used in the older method. In the modern method, the eluent should be pumped through the column since the stationary phase particles are quite small. The sample mixture is applied to the eluent through the injection port. The separated ions are detected using a flow-through detection instrument.

Even if ion exchange chromatography is widely in use, its separation mechanism is still not completely clarified. Many efforts have been made to theoretically describe the separation process of ion exchange. The ion exchange equilibrium is determined by the balance between the solute and the eluent interactions with the active sites of resin; and **ion exchange chromatography does not provide any information about the reactions taking place on the stationary phase surface**; this is a major **drawback** of this technique.

Ion exchange is alike sorption, as in both the methods the dissolved sample is taken up by a solid. An important difference between them is the stoichiometric nature of ion exchange. The ions removed from the solution are replaced with equivalent amount of other ions carrying the same charge, while the solute is taken up non-stoichiometrically without getting replaced in sorption. Stoichiometric displacement based on mass action law describes the retention of solute ion as an exchange process with the counter ion bound to the surface.

This model explains that the retention of a protein under isocratic and linear conditions is related to counter ion concentration, and this can be represented by equilibrium as follows:

$$\log k = -(Z_p / Z_s) \log C_m + \log(\ell Q)$$

Where, k = Retention factor.
C_m = Concentration of the counter ion in the mobile phase.
Z_p/Z_s = Ratio of the characteristic charge of protein to the counter ion value.

This ratio presents a statistical average of the electrostatic interactions between the protein and the stationary phase while travelling through the column. The behaviour of ion exchange chromatography can be explained by stoichiometric models. But, the mechanism is complex and stoichiometric consideration is inapt to long-range mechanisms, like electrostatic interactions due to the distribution of ions in solution are also affected by the electrostatic potential.

Other solute-solute, solute-solvent and solvent-solvent interactions also involve in retention and selectivity in ion exchange separation process. Entropic contribution originating from solvent (such as water) and structures around ion exchange sites are also important. In ion exchange chromatography, the primary separation mechanism is the electrostatic interaction between ion exchange sites and counter ions.

A distinguishing feature between the ion exchange resins and other types of gels is the presence of functional groups on the matrix. The ion exchange process between the ions in solution occurs on these functional groups. Exchange reactions occur on the basis of equivalency as per the electro-neutrality principle. The number of millimoles of an ion sorbed by an exchange and the number of millimoles of an equally charged ion released from the ion exchange should relate to each other.

On introducing a sample in the ion exchange chromatography system, equilibrium for each sample component is attained between the eluent and stationary phases. Distribution of component A between the two phases is expressed by the **distribution coefficient** (D_A):

$$D_A = \frac{[A]_r}{[A]_m}$$

The value of distribution coefficient depends on the population size of component A molecules in the stationary and eluent phases. Since the equilibrium is dynamic, a continuous and rapid interchange of component A molecules occurs between the two phases. The fraction of time (f_m) for which an average component A molecule remains in the mobile phase is given by:

f_m = Amount of A in the mobile phase/Total amount of A
$f_m = [A]_m V_m / [A]_m V_m + [A]_r w$
$= 1 + D_A (w/V_m)$
$k' = D_A (W/V_m)$
$f_m = 1/1 + k'$

Where, w = Weight of the stationary phase.
V_m = Volume of the mobile phase.

The anion and cation exchange mechanisms are quite similar. The analytes on entering the ion exchange column bind to the oppositely charged ionic sites on the stationary phase through Coulombic attraction. As per the **Coulomb's law**, electrostatic forces are responsible for the interactions between the ions in solute and oppositely charged ligands on the matrix in ion-exchange chromatography. Coulomb's law is given by:

$$f = q_1 q_2 / \varepsilon r^2$$

Where, f = Interaction electrostatic force.
$q_1 q_2$ = Charge on ions.
ε = Dielectric constant of the medium.
r = Distance between charges.

When both the ions carry similar charges (i.e., both are either positive or negative), the force is repulsive in nature; and when both are oppositely charged (i.e., one is positive and the other is negative), the force is attractive in nature. The interactions are increased

when the ion charge of the species increase (divalent ion should interact more strongly than a monovalent ion) and the dielectric constant decrease (two oppositely charged molecules increased more strongly in an organic solvent than in water). Conversely, the interactions decrease when the distance between the charges increases. Other interactions, especially Van der Waals forces, also involve in the Coulombic forces.

14.1.4. Factors Affecting Ion Exchange

Some general rules that contribute in predicting the affinity order are based on the following properties of solute and the ion exchanger:

1) The charge on solute ion,
2) The solvated size of the solute ion,
3) The degree of cross-linking of the ion exchange resin,
4) The polarisability of the solute ion,
5) The ion exchange capacity of the ion exchanger,
6) The functional group on the ion exchanger, and
7) The interaction degree of the solute ion with the ion exchange matrix.

On increasing the **charge on solute ion**, its affinity for an ion exchanger also increases through increased coulombic interactions; this is known as **electro-selectivity**, which becomes more distinct with the further dilution of the external solution in contact with the ion-exchanger. Electro-selectivity can be explained in terms of the Donnan potential, which is the difference in potential resulting due to imbalance in the ionic concentrations in the resin bead and in the external solution. An exchange process, in which two bound monovalent ions are replaced with a single divalent ion, moderates this imbalance, and thus is favourable. Electro-selectivity is reflected in the following order of selectivity coefficients:

$Pu^{4+} >> La^{3+} >> Ba^{2+} >> Tl^{+}$.

The **solvated solute ion's size** also exerts a substantial effect; the smaller solvated sized ions exhibit a greater binding affinity than the larger ions. Thus, **the selectivity sequence**, i.e., $Cs^+ > Rb^+ > K^+ > Na^+ > H^+ > Li^+$, is the opposite of the ionic radii sequence for hydrated ions, and follows the lyotropic series in which the most strongly hydrated ion (Li^+) is held most weakly. This behaviour is related to the swelling of resin, since a smaller ion gets easily retained in the resin pores.

Thus, **higher the degree of cross-linking, greater is the preference of the resin for smaller solute ions**. The combination of above mentioned (1) and (2) factors suggests that binding affinity increases with increasing polarising power, i.e., for ions having a high charge and small hydrated radius.

Ion exchange selectivity coefficients increase with the **polarisability degree of the solute ion**. Thus, the fixed ions of sulphonic acid exhibit a greater affinity for the more polarisable Ag^+ and Tl^+ ions than for the alkali metal ions. Similarly, I^- ion, in comparison to Br^- or Cl^- ions, is more strongly retained on an anion-exchanger. However, the reason for ClO_4^- ion having a higher anion exchange affinity than the I^- ion cannot be explained by polarisation.

The strong retention of large anions (ClO_4^-) having low charge and weakly basic in nature, can be attributed to their interaction with the water structure at the resin surface. Large, polarisable ions carrying a diffuse charge form a well-orientated layer of water molecules at their surface with much difficulty, and thus disrupt the surrounding water structure. This increases the **free energy**, which is the driving force for these ions to bind (i.e., to form an ion pair) to the fixed ion of an ion exchanger, thus reducing the disruption of water structure and weakening the free energy; this binding process is termed **water-structure induced ion-pairing**.

The **ion exchanger's capacity of ion exchange** affects the selectivity coefficients for some anions and cations; however, the selectivity coefficients remain constant for most ions as the ion exchange capacity is reduced. Similarly, the **nature of functional group** affects most of the ions, but the selectivity coefficients for other ions are mainly affected. Large, polarisable anions (BF_4^-, I^-, ClO_4^-, and ClO_3^-) show altered selectivity with variation in the alkyl-substituents in trialkyl-ammonium strong-base anion-exchangers.

This is related to the mechanism of water-structure induced ion-pairing, with larger functional groups (i.e., those with the largest alkyl substituents) disrupting the water structure, and causing them to bind large, polarisable ions stronger than the smaller functional groups. **Interactions between the solute ion and the ion-exchange matrix** cannot be easily predicted, and are specific to individual ions.

14.2. ION EXCHANGE RESINS

14.2.1. Introduction

Ion exchangers are either strong or weak. The terms **strong** and **weak** indicate the acid/base properties of the functional group. A ligand derived from a strong acid or a strong base is a **strong ion exchange resin**, and a ligand derived from a weak acid or a weak base is a **weak ion exchange resin**.

Variations in the ionisation state of the functional groups at different pH values decide the type of ion exchange resin suitable for a particular process. A strong ion exchange resin has the same charge density on its surface over a broad pH range, while a weak ion exchange resin has a charge density that varies with pH. At different pH, a weak ion exchanger has different selectivity and capacity.

While developing a purification process, the chromatographer should begin with a strong ion exchanger (**e.g.,** quaternary amine, sulfonic acid, or sulfopropyl), and use a weak ion exchanger (**e.g.,** DEAE or carboxymethyl) in case of unsatisfactory selectivity. Ion exchange resins are classified into the following **classes**:

1) Cation exchangers:
   i) Strong acid cation exchanger, and
   ii) Weak acid cation exchanger
2) Anion exchangers:
   i) Strong base anion exchangers, and
   ii) Weak base anion exchangers
3) Chelating ion exchangers,
4) Liquid ion exchangers,
5) Organic ion exchangers, and
6) Inorganic ion exchangers.

14.2.2. Cation Exchangers

In **strong acid cation exchangers**, the insoluble matrix has sulfonic acid groups ($-SO_3^-H^+$) attached to it. These exchangers split the neutral salts and convert them to corresponding acids.

$R\text{-}SO_3^- : H^+ + NaCl \rightarrow HCl + R\text{-}SO_3^- : Na^+$ .....(1)

They can be regenerated using strong acids like H_2SO_4 and HCl.

$2R\text{-}SO_3^- : Na^+ + H_2SO_4 \rightarrow R\text{-}SO_3^- : H^+ + Na_2SO_4$ .....(2)

They have a regeneration efficiency of 30-50%.

In **weak acid cation exchangers**, the insoluble matrix has carboxylic acid groups (–COOH) attached to it. These exchangers split the neutral salts, but remove the cations associated with water's alkalinity, thus forming carbonic acid.

$$R\text{-}COO^- : H^+ + NaHCO_3 \rightarrow R\text{-}COO^- : Na^+ + H_2CO_3 \quad \text{.....(3)}$$

They can be regenerated using an acid that is stronger than the functional group.

$$R\text{-}COO^- : Na^+ + HCl \rightarrow R\text{-}COO^- : H^+ + NaCl \quad \text{.....(4)}$$

They have a regeneration efficiency of 100%.

14.2.3. Anion Exchangers

In **strong base anion exchangers**, the insoluble matrix has quaternary ammonium group ($R\text{–}NR_3^+ : OH^-$, where R is alkyl/aryl group) attached to it.

$$R\text{-}NR_3^+ : OH^- + NaCl \rightarrow R\text{-}NR_3^+ : Cl^- + NaOH \quad \text{.....(5)}$$

$$R\text{-}NR_3^+ : OH^- + HCl \rightarrow R\text{-}NR_3^+ : Cl^- + H_2O \quad \text{.....(6)}$$

They can be regenerated using NaOH.

$$R\text{-}NR_3^+ : Cl^- + NaOH \rightarrow R\text{-}NR_3^+ : OH^- + NaCl \quad \text{.....(7)}$$

They have a regeneration efficiency of 30-50%.

In **weak base anion exchangers**, the insoluble matrix has primary, secondary, or tertiary amines attached to it. These exchangers do not split the neutral salts, but remove strong acids by adsorption.

$$R\text{-}NH_2 + HCl \rightarrow R\text{-}NH_2.HCl \quad \text{.....(8)}$$

They can be regenerated by treatment with excess of 1M NaOH solution or NH_3 solution.

$$7R\text{-}NH_2.HCl + NaOH \rightarrow R\text{-}NH_2 + H_2O + NaCl \quad \text{.....(9)}$$

They have a regeneration efficiency of 100%.

Table 14.1: Some Common Ion Exchange Resins

Resin Types	Chemical Constitution	Usual Form As Purchased	Selectivity	Thermal Stability
Strongly acidic cation exchanger	Sulphonic acid groups attached to styrene and divinylbenzene copolymer	Aryl $SO_3^-H^+$	$Ag^+ > Rb^+ > Cs^+ > K^+ > NH_4^+ > Na^+ > H^+ > Li^+ > Zn^{2+} > Cu^{2+} > Ni^{2+} > Co^{2+}$	Good, up to 150°C
Weakly acidic cation exchanger	Carboxylic acid groups attached to acrylic and divinylbenzene copolymer	R–COO^-Na^+	$H^+ >> Ag^+ > K^+ > Na^+ > Li^+ > H^+ >> Fe^{2+} > Ba^{2+}$ $Sr^{2+} > Ca^{2+} > Mg^{2+}$	Good, up to 100°C
Strongly basic anion exchanger	Quaternary ammonium groups attached to styrene and divinylbenzene copolymer	Aryl $CH_2N(CH_3)_3^+Cl^-$	$I^- > C_6H_5O^- > HSO_3^- > ClO_3^- > NO_3^- > Br^- > CN^- > HSO_3^- > NO_2^- > Cl^- > HCO_3^- > IO_3^- > HCOO^- > > CH_2COO^- > OH^- > F^-$	Fair for OH^- form, up to 50°C; good for Cl^- and other forms, up to 150°

Weakly basic anion exchanger	Polyalkylamine groups attached to styrene and divinylbenzene copolymer	Aryl $NH(R)_2^+Cl^-$	Aryl-SO_3H > citric > CrO_3 > H_2SO_4 > tartaric > oxalic > H_3PO_4 > H_3AsO_4 > HNO_3 > HI > HBr > HCl > HF > HCO_2H > CH_3CO_2H > H_2CO_3	Extensive information not available; tentatively limited to 65°C

14.2.4. Chelating Ion Exchangers

The chelating ion exchangers have various chelating or functional groups attached to their matrix. However, functional groups, like oxygen, nitrogen, sulphur, phosphorous, or arsenic, only act as electron donors. A metal ion's affinity for a chelating resin depends on the nature of chelating group and the stability of metal complexes formed on the exchanger under various pH conditions. The chelating resins combine with the metals to form tetra- or hexa-coordinated complexes.

Chelating ion exchangers are also termed as **specific ion exchangers** because of its specificity. **For example**, D-picrylamine was synthesised as a specific resin for potassium ion. There are many chelate-forming compounds (**e.g.,** anthranilic acid and iminodiacetic acid groups) that are introduced into resins by polycondensation (phenol and aldehydes) and styrene-type resin. A chelating resin, chelex 100, prefers copper, iron, and other heavy metals instead of sodium, potassium and calcium cations. The chelating ion exchangers being widely used are mostly synthetic chelating exchangers.

14.2.5. Liquid Ion Exchangers

Liquid ion exchangers are organic in nature, similar to solid resin, but exist in liquid form. High Molecular Weight Amines (HMWA) is a class of liquid anion exchangers. Depending upon the type of amine, there are primary, secondary, tertiary and quaternary amines with 18 to 27 carbon atoms. Aliphatic amines, **e.g.,** Amberlite LA-1and LA-2, are also used. The first is dodecenyl (tri akyl methyl) amine and the latter is lauryl (tri alkyl methyl) amine. Salt formed from liquid anion exchangers are as follows:

$$RNH_3\text{-}X + [MCl_4] \Leftrightarrow [RNH_3\text{-}MCl_4]_R + X^- \quad \text{..... (10)}$$

[Anionic complex]

Liquid anion exchangers involve transfer of ionic species across the liquid-liquid interface. This system is similar to solvent extraction by ion pair formation. They have a low solubility in water, large specific exchange capacity, large solubility in organic solvents, high selectivity and stability, and small surface activity. They have a faster exchange rate, possess no interstitial water, have no problem with suspended matter, provide true counter current process, and have greater permselectivity (permeation of certain ionic species through ion-exchange membranes).

Liquid cation exchangers are similar to solid cation exchange resins as H_2R. Their replaceable group is H^+. These resins are named so as they exist in liquid form. The common **examples** are HDEHP [di-(2-ethylhexyl) phosphoric acid] and DNN (dinonylnaphthalene sulfonic acid).

They serve as solid cation exchangers during separation of ions. ..... (11)

$$(H\text{-}DEHP)_o + CaCl_2.(Ca\text{-}DEHP)_o + 2HCl$$

This represents exchange of calcium on cation exchange resin in the liquid form. Inert diluents (**e.g.,** xylene, toluene, or benzene) should be used for separation. Unlike solid cation exchange resins, they can be converted back to H^+ as follows:

$$(Ca\text{-}DEHP)_o + HCl.CaCl_2 + (H\text{-}DEHP)_o \quad \ldots\ldots (12)$$

In the above equations, **o** represents the **organic phase**.

14.2.6. Organic Ion Exchangers

The matrix of organic ion exchangers comprises of an irregular, macromolecular, and 3-D network of hydrocarbon chains. Depending on the ionic groups present in the matrix, these exchangers are of two types.

When ionic groups, like $-SO_3^-$, $-COO^-$, $-PO_3^{2-}$, and $-AsO_3^{2-}$, are present in the matrix of organic ion exchangers, they are termed as **cation exchange resins**. When ionic groups, like $-NH_3^+$, $=NH_2^+$, $=N^+$, and $=S^+$, are present in the matrix of organic ion exchangers, they are termed as **anion exchange resins**.

The cation exchange resin obtained by the co-polymerisation of styrene and a small amount of divinyl benzene are commonly used. **For example**, cation exchange resin prepared by the co-polymerisation of methacrylic acid with glycol bis-methacrylate as the cross-linking agent. This contains free –COOH groups and has weak acidic properties.

The anion exchange resin prepared by co-polymerisation of styrene and a small amount of divinyl benzene by chloromethylation and interaction with trimethylamine base are commonly used.

14.2.7. Inorganic Ion Exchangers

In the last few years, a great increase have been observed in the researches on synthetic inorganic ion exchangers due to some important characteristics of inorganic ion exchangers. These characteristics involve resistance to temperatures, oxidising radiation, strongly oxidising solutions, and the major one was development of new materials with thermal stability, chemical stability, reproducibility in ion exchange behaviour, and selectivity for metal ions that are essential for analytical and environmental purposes.

Clay minerals, zeolites, heteropolyacid salts, oxides/hydrous oxides, hexacyanoferrates, and tetravalent metal acid salts are the significant classes of inorganic ion exchangers. **Table 14.2** enlists some natural and synthetic inorganic ion exchangers:

Table 14.2: Principle Classes of Inorganic Ion Exchangers

Types	Examples	Exchange Capacity (meq/g)
Smectite clays	Montmorillonite	0.5 – 1.5
Zeolites	$Na_x(AlO_2)_x\,(SiO_2)_y\,ZH_2O$	3 – 7
Substituted aluminium phosphates	Silico alumino phosphates $(M_x^{n+}\,Al_{1-x}O_2)\,(PO_2)\,(OH)_{2x/n}$	Depends upon the value of x
Hydrous oxides	$SiO_2 \times H_2O$, $Z_rO_2 \times H_2O$	1 – 2
Group IV phosphates	$Zr\,(HPO_4)_2\,H_2O$	4 – 8
Other phosphates	Uranium phosphate	
Condensed phosphates	$NaPO_3$	8
Heteropolyacids	$M_n\,X\,Y_{12}O_{40}\,nH_2O$ ($M = H^+, Na^+, NH_4^+$; X = P, As.) Y = Mo, W	0.2 – 1.5

Ferrocyanides	$M_{4/n}^{n+}\,Fe\,(CN)_6$ ($M = Ag^+, Zn^{2+}$ …)	1.1 – 1.6
Titanates	$Na_2\,Ti_nO_{2n+1}$ (n = 2 – 10)	2 – 9
Apatites	$Ca_{10-x}H_x\,(PO_4)_6\,(OH)_{2-x}$	
Anion exchangers	Hydrotalcite	2 – 4
Miscellaneous types	Alkaline earth sulphates	1.5 – 3
Fast ion conductors	β-alumina, NASICON	2 – 7

14.3. PROPERTIES

14.3.1. Introduction

Following are the properties of ion exchange resins:

1) Ion-exchange capacity,
2) Swelling characteristics, and
3) Ion-exchange selectivity.

14.3.2. Ion-Exchange Capacity

This property of an ion-exchanger is determined by the number of functional groups per unit weight of the resin. It is measured in various units, of which milliequivalents (of charge) per gram of dry resin or milliequivalents per millilitre of wet resin are the most common. In the latter unit, the type of counter-ion in the resin should be noted as it affects the degree of swelling of the resin and its volume.

Ion-exchange capacity is measured by saturating resin (of known weight) with a particular ion, then washing the resin followed by quantitative displacement of the ion: this helps in determining the number of moles of the displaced ion. The capacity measured in this way is higher than that applicable when the resin is packed as the stationary phase in a chromatographic column.

The ion-exchange capacity of a resin is useful in the concentration estimation of the competing ion used in an eluent to be used along with the resin. Resins with higher capacity use more concentrated eluents. The concentration of eluent is important in ion exchange chromatography. **Classical ion-exchange resins** have **capacities** in the range of **3-5mequiv/g**.

14.3.3. Swelling Characteristics

Organic resin exchangers have cross-linked polymer chains containing ionic functionalities. When they come in contact with water, the outer functional groups get solvated and the randomly arranged polymer chains unfold to house the larger solvated ions. Therefore, a concentrated internal solution of fixed and counter ions exists. The counter ions of this solution are mobile and diffuse out of the exchanger to reach the external aqueous solution.

However, the fixed ions fail to diffuse, and the external water molecules are forced into the resin to reduce its internal ionic concentration. Cross-linking of the resin provides mechanical stability to prevent resin dissolution; although, swelling remains due to the equilibrium pressure as a result of concentration differences between the external and internal ionic solutions. A polymeric resin with high ion exchange capacity has a very high swelling pressure (approximately 300 atmospheres).

Thus, the degree of swelling of the resin depends on the composition of solution with which it is equilibrated. Therefore, the eluent changes result in the changes in the level of swelling. This exerts important effects on the types of resins that can be appropriately used as stationary phases in chromatographic columns of fixed volume. Resins of low cross-linking (<2%) are present as soft gels in aqueous solution, and experience large volume changes with alterations in the eluent.

Thus, they are inappropriately used as stationary phases for high performance applications where the eluent is delivered under pressure. The high cross-linking effect of **macroporous resins** makes them highly rigid; their resistance to swelling effects also makes them suitable as chromatographic stationary phases for column packing purposes.

14.3.4. Ion-Exchange Selectivity

Selectivity coefficients can be used for determining the relative affinities of an ion-exchanger for different ions. A well-defined affinity series for anions and cations can be obtained by simple experiments; however in actual, the relative affinities vary with the type of ion exchanger and its usage conditions. Sometimes, simple ion-exchange is not the only operating retention mechanism, **e.g.**, partitioning of solute ions between the eluent and the pores of stationary phase may occur, or the solute ion can get adsorbed on the ion exchange matrix surface.

With respect to these factors, only approximate guidelines can be provided for the relative affinities of ion-exchangers for different ions. Selectivity coefficients for the uptake of cations by a strong acid cation exchange resin are in the following order:

$Pu^{4+} > > La^{3+} > Ce^{3+} > Pr^{3+} > Eu^{3+} > Y^{3+} > Sc^{3+} > Al^{3+} >> Ba^{2+} > Pb^{2+} > Sr^{2+} > Ca^{2+} > Ni^{2+} > Cd^{2+} > Cu^{2+} > Co^{2+} > Zn^{2+} > Mg^{2+} > UO_2^{2+} >> Tl^{+} > Ag^{+} > Cs^{+} > Rb^{+} > K^{+} > NH_4^{+} > Na^{+} > H^{+} > Li^{+}$

This series suggests that a cation exchange eluent of 0.1M KCl will be stronger than the eluent containing 0.1M NaCl, only if other factors are equal. Selectivity coefficients for anions on strong base anion exchangers are in the following order:

$Citrate > salicylate > ClO_4^{-} > SCN^{-} > I^{-} > S_2CO_3^{2-} > WO_4^{2-} > MoO_4^{2-} > CrO_4^{2-} > C_2O_4^{2-} > SO_4^{2-} > SO_3^{2-} > HPO_4^{2-} > NO_3^{-} > Br^{-} > NO_2^{-} > CN^{-} > Cl^{-} > HCO_3^{-} > H_2PO_4^{-} > CH_3COO^{-} > IO_3^{-} > HCOO^{-} > BrQ_3^{-} > ClO_3^{-} > F^{-} > OH^{-}$

14.4. METHODOLOGY

14.4.1. Introduction

The following two techniques are used to bring the solutions and ion exchange resins in contact:

1) Batch method, and
2) Column method - ion exchange chromatography.

14.4.2. Batch Method

In this method, which involves single step equilibrium, the resin and the solution are mixed in a vessel to attain equilibrium, and the resultant solution is filtered. The extent to which the ions in the solution exchange with the ions on resin, depends on selectivity coefficient. In single step equilibrium of this type, the exchange capacity of the resin is employed in a small portion. The batch method is used for water softening and production of deionised or demineralised water. In **softening of water**, the hardness causing calcium and magnesium ions are exchanged with the sodium ions. The sodium form of sulphonic acid resin is used in this method.

$$2RSO_3^{-}\,Na^{+} + Ca^{2+} \rightleftharpoons (RSO_3^{-})_2\,Ca^{2+} + 2Na^{+}$$

The resin on getting exhausted is added with 15% sodium chloride solution to remove the calcium or magnesium ions from it by exchange with sodium ions.

From **deionised or demineralised water**, all cations and anions of electrolytes have been removed. It is prepared by the treatment of water with a cation exchanger in the acid or the hydrogen form. Subsequently, all the cations of electrolyte are replaced with hydrogen ions. **For example,**

$$RSO_3^{-}\,H^{+} + M^{+} \rightleftharpoons RSO_3^{-}M^{+} + H^{+}$$

Then, water is treated with an anion exchanger in the basic or the hydroxide form.

$$R^{+}OH^{-} + Cl^{-} \rightleftharpoons R^{+}Cl^{-} + OH^{-}$$

The H^{+} and OH^{-} ions combine to form water, which has no ions of any dissolved electrolytes. Deionised water has multiple uses in the laboratory. The conductivity of deionised water and the conductivity of water prepared by distillation (time-consuming process) is the same. It can be used in conductance experiments and for preparing silver nitrate solutions. It is also used in biological studies. Biologists need to prepare culture media of accurate composition very often. If the solutions are prepared in distilled water, the ions present can give improper experimental results. This inaccuracy and errors can be eliminated with the use of deionised water.

14.4.3. Column Method - Ion Exchange Chromatography

This chromatography technique involves separation of mixture components on the basis of differences in the selectivity coefficients for the resin. These differences result in different migration rates on an ion exchange column.

Figure 14.7: Ion Exchange Column

The apparatus used in column method comprises of a glass column fitted with a glass wool plug or a sintered glass disc at the lower end (**figure 14.7**). A small particle sized resin should be used to provide a large surface area for contact between the resin and solution. The diameter of resin beads should be less than $1/10^{th}$ of the column diameter. The column should not be packed with dry resin. A slurry of the resin is prepared using distilled water and fine particles are removed by decantation. This slurry is slowly introduced into the column containing some water. No air bubbles and fine particles of the resin should be trapped in the column and the resin should be uniformly distributed. To ensure this, the column is backwashed by running distilled or deionised water from the bottom. Once the air bubbles are removed, the flow of water is stopped, the resin is allowed to settle, and excess water is drained off.

The water level should never fall below the resin surface, or else the resin may become dry and channels may form in the resin bed. If this happens, the solution and the resin will not be completely in contact.

Ion Exchange Column Used in Chromatographic Separations

Ion exchange chromatography relies on the principle that different ions (cations or anions) exhibit different capacity to undertake exchange reactions on the surface of a given exchanger.

An ion's capacity to undergo exchange reaction depends on the charge and size of the hydrated ion in solution. The ion exchange capacity increases the ionic charge (i.e., the valency of ion), but decreases with the increase in the size of hydrated ion under similar conditions.

On comparing other same sized hydrated ions, the charge on the ions is found to play a significant role in determining their capacity to undergo exchange reactions. The capacity of same sized cations decreases in the following order:

$$Th^{4+} > Al^{3+} > Ca^{2+} > Na^{+}$$

And the capacity of same sized anions decreases in the following order:

$$PO_4^{3-} > SO_4^{2-}$$

On comparing the similarly charged ions, the size of hydrated ions plays a significant role in determining their capacity to undergo exchange reactions. The capacity of univalent cations decreases in the following order:

$$H^{+} > Cs^{+} > Rb^{+} > K^{+} > NH_4^{+} > Li^{+}$$

The capacity of doubly charged cations decreases in the following order:

$$Ba^{2+} > Pb^{2+} > Sr^{2+} > Ca^{2+} > Ni^{2+} > Cu^{2+} > Co^{2+} > Zn^{2+} > Mg^{2+}$$

The capacity of univalent anions decreases in the following order:

$$I^{-} > NO_3^{-} > Br^{-} > CN^{-} > Cl^{-} > OH^{-} > F^{-}$$

The exchange between the active polyvalent ion in a resin and an ion of lower valency is found to be favourable if higher concentration of the solution is used.

Quality of an ion exchange resin depends on its capacity, which further depends on the total number of ion active groups per unit weight of the material. Thus, **greater the number of ions, greater is the capacity of the ion exchange resin to undergo exchange reactions.** Efficiency of an ion exchange resin depends on the degree of cross-linking. Thus, **greater the cross linking, higher is the efficiency of the ion exchange resin.**

Selection of Suitable Systems

The ion exchangers can be classified as per the pKa values of their ion groups. Table 14.3 represents a simplified survey of their working ranges:

Table 14.3: Operating Range of Columns Used Ion Exchangers

Specification of Ion Exchanger	Ionic Groups	Suitable Chromatographic Medium
Strong Acid	$-SO_3^-$	Acidic and alkaline
Weak acid	$-COO^-$	Only alkaline
Strong base	$-N^+R_3$	Acidic and alkaline
Weak base	$-NHR_2$	Only acidic

For practical purpose, the dissolved material should be in dissociated form so that it can be retained by the resin. Conveniently, the entire process should be carried out at a pH wherein one of the components is retained and the other does not interact with the ion exchange. Concentration of the involved oppositely charged ions is also of utmost importance. The retained ions can be eluted by using a high concentration of cations or anions without changing the pH, and hence, the dissociation relationships.

14.5. APPLICATIONS

14.5.1. Introduction

Due to the particular selectivity forces of material, separation with ion exchange is mostly used in inorganic chemistry. However, organic ions forming salts with the oppositely charged ions in another phase can also be separated. Most of the ion exchanger reactions require aqueous solvents. Some applications of ion exchange chromatography and the ion exchangers are given below.

14.5.2. Separations of Similar Ions from One Another:

This chromatography technique is used for separating similarly charged ions from one another as different ions undergo exchange reactions to different extents. **For example,** for separating a mixture of Li^+, Na^+ and K^+ ions, their solution is passed through a cation exchanger and 0.1N HCl is used as an eluent; for separating a mixture of Cl^-, Br^- and I^- ions, their solution is passed through a basic anion exchanger and $NaNO_3$ (sodium nitrate) solution is used as an eluent. When 0.5N $NaNO_3$ is used, Cl^- ion elutes out first. On increasing the concentration of $NaNO_3$, Br^- ion elutes next. On further increasing the concentration of $NaNO_3$, I^- ion elutes out.

A typical chromatographic separation of alkali metal ions with an acid eluent is given in the **figure 14.8:**

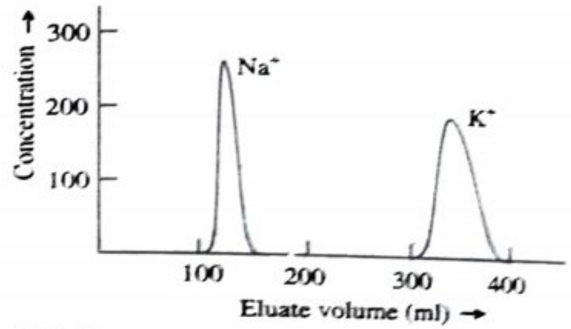


Figure 14.8: Separation of Potassium from Sodium on Dowex 50 × 12 (in the Hydrogen Form) by Elution with 0.6M HCl

14.5.3. Removal of Interfering Radicals

While estimating the Ca^{2+} or Ba^{2+} ions by oxalate or sulphate method, the PO_4^{3-} phosphate ion is found to be interfering; and it is removed by passing a solution of Ca^{2+} or Ba^{2+} ions having PO_4^{3-} ions through a sulphonic acid cation exchanger. The Ca^{2+} or Ba^{2+} ions exchange with the H^+ ions, while the PO_4^{3-} ions do not undergo any exchange process and pass through the column.

This process is repeated to remove the PO_4^{3-} ions completely. The Ca^{2+} and Ba^{2+} ions held by the resin are removed by using a suitable eluent, after which the ions are estimated by standard methods.

14.5.4. Softening of Hard Water

Presence of Ca^{2+}, Mg^{2+}, and other divalent ions in water makes it hard. These ions can be removed by passing the hard water through cation exchangers carrying Na^+ ions.

While passing, the following exchange reaction occurs:

$$\underset{\text{(Resin)}}{2NaR} + \underset{\text{(Hard water)}}{n\,Ca^{2+}} \rightleftharpoons \underset{\text{(Resin)}}{CaR^{2+}} + \underset{\text{(Solution)}}{2nNa^{+}}$$

The Ca^{2+} and Mg^{2+} ions are retained in the column, while the Na^+ ions pass into the solution. The Na^+ ions are harmless for washing purposes. An ion exchanger loses its activity after being used for a long time, and its activity can be rejuvenated by permeating a concentrated NaCl solution through it.

During this passage, the following reverse reaction occurs:

$$\underset{\text{(Resin)}}{CaR_2^{2+}} + \underset{\text{(Solution)}}{2nNa^{+}} \rightleftharpoons \underset{\text{(Resin)}}{nCa^{2+}} + \underset{\text{(Regenerated)}}{2Na_nR}$$

14.5.5. Demineralisation of Water

For demineralising the water, both cations and anions should be removed. First, the water is passed through an acidic cation exchanger; during the passage, the metallic cations (Na^+, Ca^{2+}, Mg^{2+}, etc.) exchange with the H^+ ions. Then the water obtained from cation exchanger is passed through a basic anion exchanger; during the passage, the anions present in water (Cl^-, NO_2^-, SO_4^{2-}, etc.) exchange with the OH^- ions of the exchanger.

The H^+ and OH^- ions that pass into the solution by getting exchanged with the cations and anions, respectively combine to form unionised water. Sulphonic acid resin is mostly used as the cation exchanger and a strong basic resin is used as the anion exchanger.

The mixed bed has been regenerated by making a resin of lower specific gravity in comparison to the other. Flotation can be used for separating them and a specific regeneration treatment is given to each resin.

14.5.6. Separation of Lanthanides

During the World War II, analytical chemistry faced much difficulty during the separation of fission products, especially the mixture of La, Sm, Eu, Y, Ce, Pr, Nd, and Pm and the mixtures of Cs and Rb, Sr and Ba, and Zr and Nb in trace amounts. Synthetic resins along with chromatographic elution technique were used for the separation of these mixtures.

On passing a solution having a mixture of lanthanides, through a column packed with a suitable ion exchange resin particles, the cations in solution get exchanged with the hydrogen or any other cation present in the ion exchanger.

The cations are held in the column in the order of their decreasing capacities to undergo the exchange reaction. Thus, the cation exhibiting maximum capacity to undergo exchange reaction is retained near the top, and the other cations are retained down in the column. If a citrate buffer solution is used as an eluent, the La^{3+}-citrate complex ion with maximum preference for eluent elutes out first and the La^{3+}-citrate complex ion with minimum preference elutes out later.

The separations can be improved by using other complexing agents like H_4EDTA and other amino acids. This has simplified the separation of kilogram quantities of lanthanides in the highest degree of purity.

14.5.7. Separation of Actinides

Ion exchange chromatography has an important role in the discovery of transplutonium elements in the actinide series. In this series also, elution occurs in the reverse order of the atomic number due to actinide contraction. This is the only way for identifying these elements, some of which have been produced in atom quantities.

14.5.8. Purification of Organic Compounds Extracted in Water

There are many natural products extracted in water that are contaminated with ions already present in water. These ions can be removed by an ion exchange process.

14.5.9. Separation of Sugars

Khym and **Zill** developed the method for separation of sugars by ion exchange.

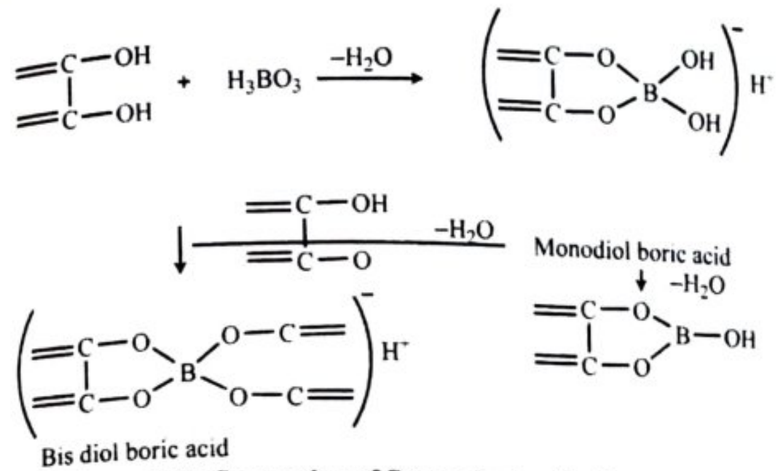


Figure 14.9: Separation of Sugars by Ion Exchange Process

The sugars are first converted into borate complexes in 11 × 0.9cm columns of 200-400 mesh Dowex 1 resin, using a loading of 5-10mg of borate complex and flow rates of 0.5-1ml/min. The sugars can be quantitatively recovered after the separation of borate complexes. In the same way, disaccharides can be separated from monosaccharides and the individual compounds of hexose and pentose mixtures are resolved.

14.5.10. Separation of Amino Acids

Ion exchange chromatography is used for separating the complex mixture of 18 amino acids obtained by the acid hydrolysis of proteins. The mixture of amino acids to be separated is introduced in a very short column at pH 2 and eluted using 0.35N sodium citrate buffer (pH 5.25). The column has been previously equilibrated with the eluent.

Acidic and neutral amino acids leave the column without getting separated. Then lysine, histidine, NH_3, and arginine amino acids elute out. A second sample is chromatographed in a longer column with 0.2N sodium citrate buffer (pH 3.28) and eluted using 0.88N citrate buffer (pH 4.25). A diagrammatic representation of such an analyser is shown in **figure 14.10**:

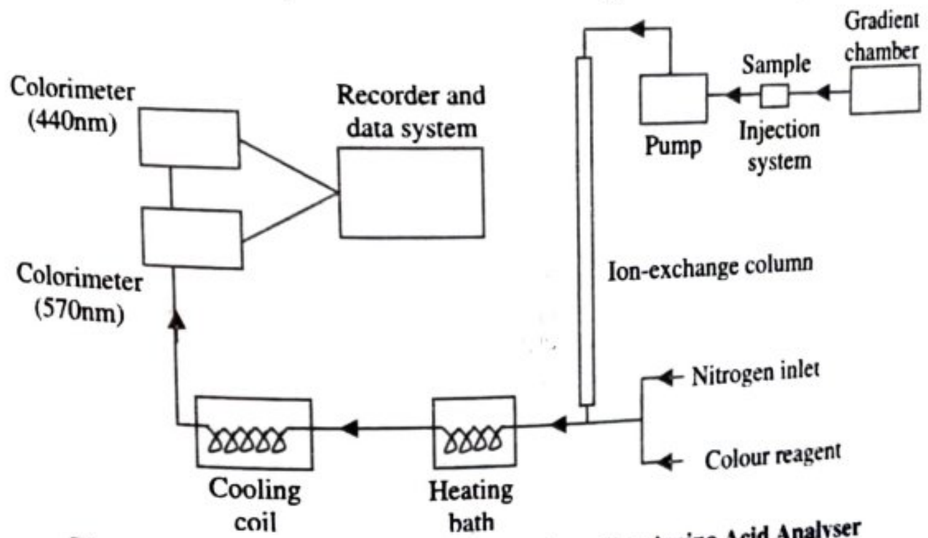


Figure 14.10: Diagrammatic Representation of an Amino Acid Analyser

The column effluent is mixed with ninhydrin colour reagent and nitrogen is fed to break the effluent stream into discrete bubbles. The mixture is heated at 105°C to develop a colour. Two colorimeters, set at 570nm to monitor the majority of amino acids and at 440nm to monitor the colour produced by proline and hydroxyproline, are used for determining the intensity of colour produced. On the other hand, amino acids can be

detected by converting to fluorescing derivatives. Although the method utilises two detectors, it is still more tiresome and less reproducible than the ninhydrin method. Many amino acid analysers employ two separate columns, of which the second one contains an anion exchanger for faster and efficient separation of basic amino acids and ammonia.

14.5.11. Preparation of Pure Reagents

Sodium hydroxide solutions used for volumetric determinations carry carbonates in them, thus resulting in acid-base titration errors. These errors can be eliminated if the carbonate is removed by passing the solutions through a column of strongly basic anion exchange resin in hydroxide form. During the passage, the carbonate is absorbed and an equivalent concentration of hydroxide is released.

Tetramethyl ammonium hydroxide is not available in the required degree of purity, thus it can be prepared by passing tetraethyl ammonium chloride solution through a cation exchanger bed that will be converted to tetraethyl ammonium form. Thereafter, the bed is washed with water followed by passing sodium hydroxide. The ready-to-use tetramethyl ammonium hydroxide is obtained in the effluent.

One more commercially important **example** involves silicic acid preparation from sodium silicate. When silicate passes through the hydrogen form of a cation exchanger, the sodium ions (of the silicate) is replaced with the hydrogen ions from the hydrogen form of the cation exchanger to yield silicic acid.

14.5.12. Hydrometallurgy

Ion exchange chromatography is used for recovering and purifying variety of metals, **e.g.,** uranium, thorium, lanthanides, actinides, gold, silver, and platinum. In some cases, the operation scale is small, **e.g.,** as in lanthanides, but their intrinsic value is quite high. Separation and recovery of trace amounts of toxic metals from effluent and waste streams is an important application. Some **examples** include recovery of chromium from spent metal-plating solutions; and recovery of copper and zinc from effluents in rayon and synthetic fibre industry.

Ion exchange is used for the treatment of low-grade uranium deposits. The ore in Rossing uranium mine has about 0.035% triuranium octoxide (U_3O_8). The process of continuous cation exchange is used for upgrading the low concentration feed (0.19gm/litre U_3O_8) and producing an eluate feed (3.49gm/litre), which is introduced into a liquid-liquid extraction plant that effectively separates uranium from trace impurities. The final strip solution contains l0gm/litre of U_3O_8, which precipitates and produces a product containing 97% U_3O_8 by weight. Ion exchange chromatography is used for uranium extraction from sea water in low concentrations (10^{-8}%). Resins incorporating the chelating amido oxime group are specific for uranium.

14.6. SUMMARY

The details given in the chapter can be summarised as follows:

1) **Ion exchange chromatography** (or **ion chromatography**) is a technique for rapid and effective separation of ions, amino acids, peptides, nucleotides, nucleic acids, etc.
2) Ion exchange chromatography is defined as **a process that allows the separation of ions and polar molecules based on their affinity to the ion exchanger**.
3) A ligand derived from a strong acid or a strong base is a **strong ion exchange resin**, and a ligand derived from a weak acid or a weak base is a **weak ion exchange resin**.

4) In **strong acid cation exchangers**, the insoluble matrix has sulfonic acid groups attached to it.
5) In **weak acid cation exchangers**, the insoluble matrix has carboxylic acid groups attached to it.
6) In **strong base anion exchangers**, the insoluble matrix has quaternary ammonium group attached to it.
7) In **weak base anion exchangers**, the insoluble matrix has primary, secondary, or tertiary amines attached to it.
8) The **chelating ion exchangers** have various chelating or functional groups attached to their matrix.
9) Chelating ion exchangers are also termed as **specific ion exchangers** because of its specificity.
10) **Liquid ion exchangers** are organic in nature, similar to solid resin, but exist in liquid form.
11) The matrix of **organic ion exchangers** comprises of an irregular, macromolecular, and 3-D network of hydrocarbon chains.
12) Clay minerals, zeolites, heteropolyacid salts, oxides/hydrous oxides, hexacyanoferrates, and tetravalent metal acid salts are the significant classes of **inorganic ion exchangers**.
13) **Ion-exchange capacity** of an ion-exchanger is determined by the number of functional groups per unit weight of the resin.
14) The **degree of swelling** of the resin depends on the composition of solution with which it is equilibrated.
15) **Selectivity coefficients** can be used for determining the relative affinities of an ion-exchanger for different ions.
16) **Open column ion-exchange chromatography** is a slow process due to low eluent flow-rates; and if the flow rate is increased, the separation efficiency deteriorates.
17) As per the **Coulomb's law**, electrostatic forces are responsible for the interactions between the ions in solute and oppositely charged ligands on the matrix in ion-exchange chromatography.
18) On increasing the **charge on solute ion**, its affinity for an ion-exchanger also increases through increased coulombic interactions
19) The smaller solvated sized ions exhibit a greater binding affinity than the larger ions.
20) Higher the degree of cross-linking, greater is the preference of the resin for smaller solute ions.
21) The ion-exchange selectivity coefficients increase with the polarisability degree of the solute ion.
22) In **batch method**, the resin and the solution are mixed in a vessel to attain equilibrium, and the resultant solution is filtered.
23) **Column method-ion exchange chromatography** involves separation of mixture components on the basis of differences in the selectivity coefficients for the resin.
24) Greater the number of ions, greater is the capacity of the ion exchange resin to undergo exchange reactions.
25) Greater the cross linking, higher is the efficiency of the ion exchange resin.

14.7. EXERCISE

14.7.1. True or False

1) In weak acid cation exchangers, the insoluble matrix has sulfonic acid groups attached to it.
2) The matrix of inorganic ion exchangers comprises of an irregular, macromolecular, and 3-D network of hydrocarbon chains.
3) Ion-exchange capacity of an ion-exchanger is determined by the number of functional groups per unit weight of the resin.
4) Higher the degree of cross-linking, greater is the preference of the resin for smaller solute ions.
5) The ion-exchange selectivity coefficients decrease with the polarisability degree of the solute ion.
6) Greater the number of ions, lesser is the capacity of the ion exchange resin to undergo exchange reactions.

14.7.2. Fill in the Blanks

7) Chelating ion exchangers are also termed as specific ion exchangers because of its _____.
8) In _____ exchangers, the insoluble matrix has primary, secondary, or tertiary amines attached to it.
9) _____ can be used for determining the relative affinities of an ion-exchanger for different ions.
10) On increasing the charge on solute ion, its _____ for an ion-exchanger also increases through increased coulombic interactions
11) In _____ method, the resin and the solution are mixed in a vessel to attain equilibrium, and the resultant solution is filtered.
12) Greater the _____, higher is the efficiency of the ion exchange resin.

Answers

1) False	2) False	3) True	4) True
5) False	6) False	7) Specificity	8) Weak base anion
9) Selectivity coefficients	10) Affinity	11) Batch	12) Cross-linking

14.7.3. Very Short Answer Type Questions

1) What is ion exchange chromatography?
2) Classify ion exchange resins.
3) What are cation exchangers?
4) Name the factors affecting ion exchange.
5) What are inorganic ion exchangers?

14.7.4. Short Answer Type Questions

1) Discuss the cation exchange chromatography.
2) Write a note on the cation and anion exchangers.
3) Discuss the properties of ion exchange chromatography.
4) Discuss the factors affecting ion exchange.

14.7.5. Long Answer Type Questions

1) Briefly explain the mechanism of ion exchange process.
2) Discuss about the applications of ion exchange chromatography.
3) Write an illustrative note on the methodology of ion exchange chromatography.

CHAPTER 15

Gel Chromatography

15.1. GEL CHROMATOGRAPHY

15.1.1. Introduction

Gel chromatography is a type of partition chromatography used for separating different sized molecules. This technique is also termed as **gel filtration**, **gel permeation**, **gel exclusion**, **size exclusion**, and **molecular-sieve chromatography**.

The gel structure being used contains pores of different diameters up to a maximum size. The test molecules are washed through a gel column and the molecules larger than the largest pores in the gel are excluded from the gel structure. Smaller molecules penetrate the gel and the extent of penetration depends on the molecular size. This delays their movement through the column.

15.1.2. Principle

In gel chromatography, the molecules are separated based on their size, therefore is also called **molecular sieve chromatography**.

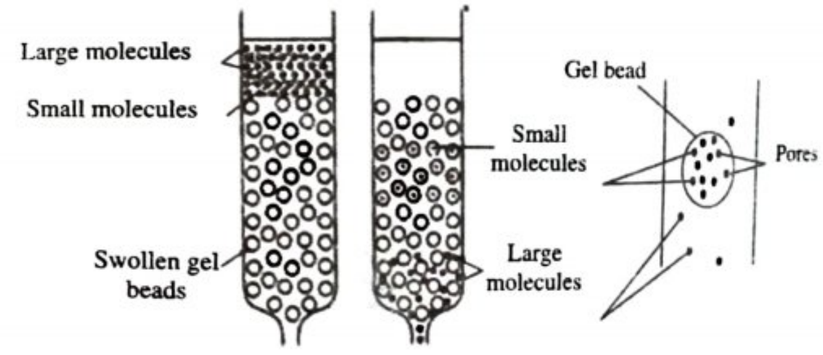


Figure 15.1: Principle of Gel Chromatography

A column is filled with swollen gel beads or porous glass beads, and this column serves as a molecular sieve. A mixture with molecules of different sizes is poured over the column. The large molecules are collected first as they pass through the spaces between the gel or glass beads. The pores of beads are smaller, and the large molecules cannot pass through them.

The small molecules enter the beads through the pores and get separated from the solvent. They slowly travel down the beads and are collected in a separate stream. Large molecules cannot pass through the pores, and are excluded from the beads; hence, this technique is also called **exclusion chromatography**.

15.1.3. Theory

In gel chromatography, the molecules are partitioned between a mobile phase and a stationary phase containing a porous matrix (of defined porosity) as a function of their relative sizes. A column of such a beaded matrix will have two measurable liquid

volumes, namely the **external volume** comprising of the liquid between the beads, and the **internal volume** comprising of the liquid inside the beads. The external volume is termed as the **void volume** (V_0), and the sum of external and internal volumes is termed as the **total volume** (V_t).

After the sample is applied in the column, molecules larger than the pores of the stationary phase matrix are excluded from the internal volume in the beads, and then rapidly migrate through the column, emerging at V_0. Molecules smaller than the matrix pores equilibrate with the external and internal liquid volumes, and migrate slowly through the column, emerging at a volume greater than V_0. Thus, the molecules are eluted in decreasing order of their size. The **elution volume** (V_R) of a particular molecule depends on the fraction of stationary phase available for diffusion. This can also be termed as the **partition coefficient** (represented by K_d or K_{av} constant). Therefore:

$$V_e = V_0 + K_{av}(V_t - V_0)$$

On rearranging this equation:

$$K_{av} = \frac{(V_e - V_0)}{(V_t - V_0)}$$

The size or mass of molecules and their flow behaviour through a gel filtration column is a function of their molecular shape or **hydrodynamic diameter**, which is the diameter of spherical volume (hydrodynamic volume), created by a molecule as it rapidly tumbles in solution. While performing gel filtration chromatography, it is assumed that all the molecules in a mixture are of the same symmetrical shape, so that the elution will occur in decreasing order of molecular weight. This assumption is acceptable in most of the cases; however, it should also be considered that the operative molecule dimension during gel filtration is the hydrodynamic volume, and an asymmetrical molecule will elute with high molecular weight compared to a symmetrical molecule of similar molecular weight. For separating proteins, it is assumed that all the proteins in the mixture are globular proteins. However, the asymmetrical proteins (fibrous proteins and some glycoproteins) elute with high molecular weight compared to globular proteins of similar molecular weight.

Performance

The column performance is determined from the number of theoretical plates per meter (n), which is calculated as follows:

$$n = \frac{5.54V_R^2}{LW_h^2}$$

Where, V_R = Retention volume for the desired component.
W_h = Width of the desired peak at half peak height, (Measured in the same units as V_R).

Retention volume is the distance along the baseline between the point of injection and a perpendicular dropped from the maximum of the desired peak.

15.2. INSTRUMENTATION

15.2.1. Introduction

A gel chromatography system (**figure 15.2**) is a specialised isocratic high performance liquid chromatography. A solvent reservoir (1-4L in size) is filled with the mobile phase. It is sparged with helium or ultrasonically treated to remove the gas and prevent the air bubbles from entering the detector downstream. A high pressure pump that can

operate at pressures up to 6000psi, forces the mobile phase to pass through the line filters and pulse dampeners to the sample injector where an aliquot of dilute polymer solution is injected.

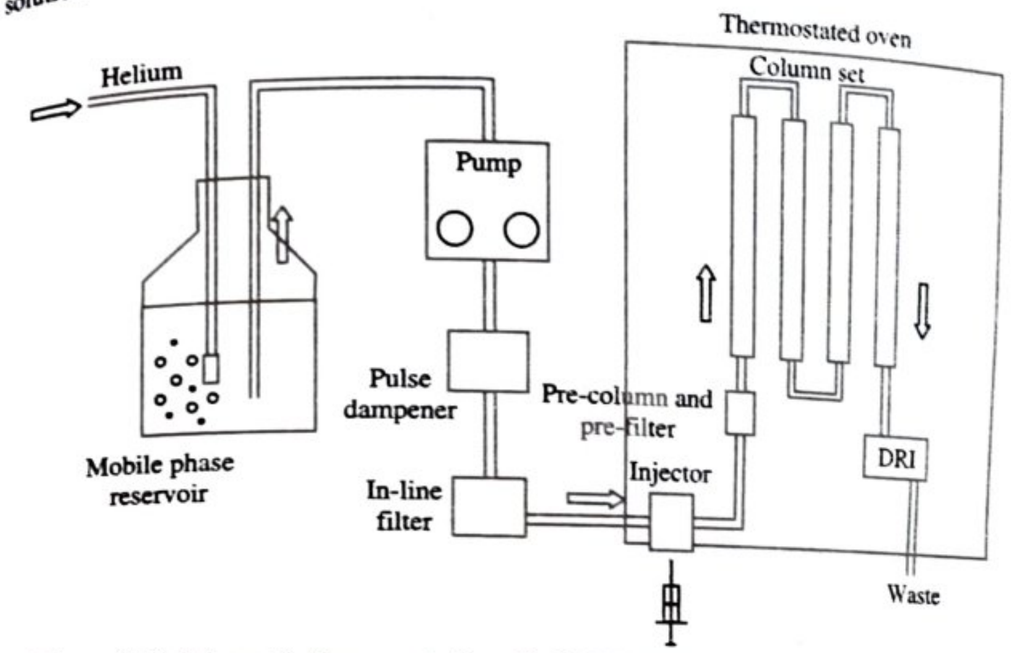


Figure 15.2: Schematic Representation of a Gel Filtration Chromatography Instrument

The sample initially existing as a narrow band in the system is carried through the pre-column and the analytical column set for molecular size discrimination to occur. The resultant discriminated sample elutes from the column set and passes through a universal detector, which generates an electrical (mV) signal proportional to the sample concentration. The sample and mobile phase exit the detector and move to a waste container. The electrical signal is transmitted to an integrator, recorder, or computer to be displayed and/or further processed.

15.2.2. Matrix Choice

The gel filtration matrices comprise of porous beads containing cross-linked polyacrylamide, agarose, dextran (**table 15.1**), or combination of these. These matrices are supplied in suspended form or as dry powders. They should be compatible with the molecules to be separated and should be stable to organic solvents, pH, and temperature. They should be inert when molecules are undergoing separation so that the molecules do not get partially adsorbed to the matrix; if this happens the migration of molecules through the column will be retarded and also tailed peaks will arise.

Table 15.1: Some Media used for Gel Filtration Chromatography

Materials	Media and Fractionation Range (Globular Proteins)	Advantages	Disadvantages
Dextran	Sephadex G-10 0 – 700 Da Sephadex G-25 1–5 kDa	Smaller fractionation range (G-10, G-25) is good for desalting.	Expanded forms require low pressures/hydrostatic heads.

	Sephadex G-50 1.5–30 kDa Sephadex G-100 4–150 kDa Sephadex G-200 5–600 kDa		
Agarose	Sepharose 6B 10–4,000 kDa Sepharose 4B 60–20,000 kDa Sepharose CL–4B 60–20,000 kDa Sepharose CL–2B 70–40,000 kDa	Good for larger molecules; cross-linked (CL) forms are more robust.	Must be kept wet and not allowed to dry out.
Allyl dextran-bis-acrylamide	Sephacryl S–200 HR 5–250 kDa Sephacryl S-300 HR 10–1,500 kDa Sephacryl S–400 HR 20–8,000 kDa	Non-biodegradable and mechanically robust.	Must be kept wet and not allowed to dry out.

The selected matrix should have such a molecular mass fractionation range that the desired molecule is eluted after V_0 and before V_t. The most appropriate fractionation range depends on the molecular mass of the target molecule and on the composition of the sample applied to the column. Hence, to achieve the best separation of sample molecules with similar molecular masses, a matrix with a narrow fractionation range should be used.

Various products are available for gel chromatography, and these products are classified according to the relative molecular mass above which all the molecules are excluded from the gel structure, i.e., their exclusion limit.

Sephadex is the original medium based on dextran (a linear glucose polymer) that has been modified to offer varying degrees of cross-linking to determine the material pore size. It is a strongly hydrophilic polymer and swells in water, thus fulfilling the condition that the gel should be fully hydrated before a column is prepared.

Polyacrylamide beads are available in a wide range of pore sizes. They are also hydrophilic and swell in aqueous solutions, like dextran. However, their chemical stability is more than that of the dextran gels.

Agarose gels are used when gels with very large pore sizes are needed. They are also hydrophilic but are sold in swollen form. Their **limitation** is that they become soft at above 30°C temperatures.

Mixed gels of polyacrylamide and **agarose** are available (Ultra-gel and LKB) in which the polyacrylamide contributes to the 3-D structure that provides support to the interstitial agarose gel.

Polystyrene gels are hydrophobic, and thus used with non-aqueous solvents for organic chemical applications.

15.2.3. Sample Size and Concentration

Maximum resolution in gel chromatography depends on the application of sample in a small volume (usually 1-5% of the total bed volume). Thus, the sample-handling capacity of this chromatography method is low, and should be performed late in a purification procedure when the numbers of different molecules in a sample are relatively low. The sample concentration to be applied in the column will be limited by the sample viscosity (increases with the sample concentration) relative to that of the eluent. A high viscosity results in irregular sample migration through the column along with resultant loss of resolution. In some cases, a high viscosity will reduce the column flow rate. When proteins are being separated by gel chromatography, the protein concentration in the sample should not be more than 20mg/ml.

15.2.4. Column Parameters

Maximum resolution in gel chromatography can be obtained by the use of long columns. The ratio of column diameter to column length ranges from 1:20 to 1:100.

The methods used for gel preparation are:

1) A weighed amount of the dry powder of gel is mixed with a solvent (to be used as an eluent) and made to swell. The resultant mixture is kept aside till equilibrium is attained.
2) Then gel slurry is warmed at 100°C in a water bath. The gel swells in a few days.
3) The slurry is cooled and packed in the column.

Two types of **column packings** are generally used:

1) Porous glasses or silica, and
2) Porous cross-linked organic gels, **e.g.,** dextrans, methacrylate-based gels, polyvinyl alcohol-based gels, and hydroxyethyl cellulose gels.

The detectors used in gel chromatography are based on UV fluorescence, UV absorption, or changes in refractive index.

15.2.5. Choice of Eluent

In gel chromatography, the molecules are separated based on their relative sizes. Due to this reason, the technique is independent of the type of eluent used. The elution conditions (pH, essential ions, cofactors, protease inhibitors, etc.) that will fulfill the requirements of desired molecule should be selected. However, the ionic strength of eluent should be sufficiently high to reduce protein-matrix and protein-protein associations by electrostatic or Van der Waals interactions. These interactions can be prevented by adding 0.1M NaCl or KCl to the eluent.

15.2.6. Effect of Flow Rate

Maximum resolution in gel chromatography can be achieved by low flow rates, as flow rate and resolution are inversely proportional. Optimum flow rate for resolution of proteins is 2ml/cm^2/h; however, much higher flow rates can be used with rigid matrices such as the Sephacryl HR range (30ml/cm^2/h). Low flow rates indicate longer separation times, therefore, a compromise between desired resolution and speed should be decided upon.

15.2.7. Separation Mechanism

In gel chromatography, the polymer molecules are separated based on their molecular size or hydrodynamic volume in solution. Separation is achieved when the polymer molecules elute through the column(s) packed with a porous material. Smaller molecules are retained in the pores while the larger ones are excluded. Thus, first the largest molecule (having the greatest hydrodynamic volume) elutes from the column and then the smaller molecules.

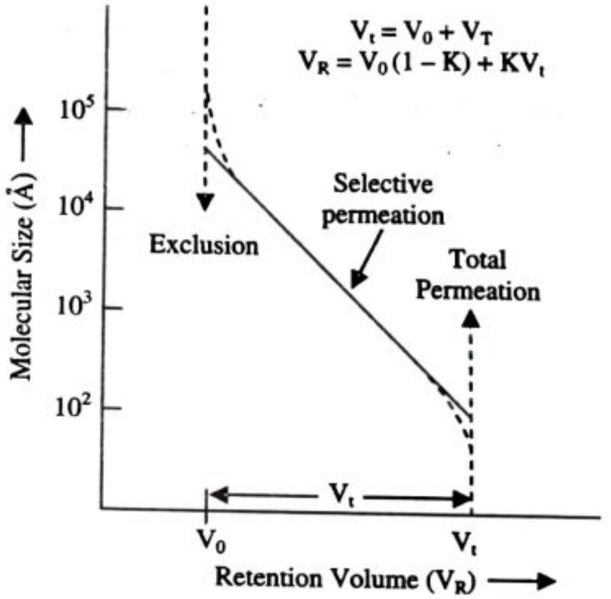


Figure 15.3: Calibration Curve for Gel Filtration Chromatography

The volume of liquid at which a solute elutes from a column or the volume of liquid corresponding to the retention of a solute on a column is the **retention volume** (V_R), which is related to the interstitial (void) volume and internal pore volume of the column.

The dependence of molecular size in solution on retention volume is illustrated in **figure 15.3**. The void volume (V_0) corresponds to the total exclusion of solute molecules from the pores. The excluded solute molecules are larger than the largest available size pores. The solute molecules are selectively separated between V_0 and V_t based on their molecular size in solution. Separation by a liquid exclusion chromatography mechanism does not occur beyond the total column volume (V_T). If the molecules separate beyond this volume, they are retained on the column support by an affinity mechanism.

15.2.8. Column Cleaning and Storage

The matrices used in gel chromatography are mostly cleaned by using 0.2M sodium hydroxide or non-ionic detergents. When not in use for long time periods, they should be stored at 4°C temperature in the dark and in the presence of an antimicrobial agent [0.02-0.05% (w/v) sodium azide or 20% (v/v) ethanol].

15.3. APPLICATIONS

15.3.1. Introduction

The gel chromatography is used for the following two specific purposes in the analysis of pharmaceutical substances:

1) Determination of relative component composition,
2) Determination of molecular weight, and
3) Determination of purity.

15.3.2. Determination of Relative Component Composition

The assay method employed for this purpose along with specific experimental parameters is mentioned in the official monograph. Two situations may arise:

1) **Equivalent Responses:** The relative quantity of each sample component can be determined by dividing each peak area by the sum of peak areas of the desired components. However, this is possible only if all the components show equivalent responses to the detector.
2) **Non-Equivalent Responses:** The relative component composition is determined either from the calibration curve (obtained with the calibration standards in the official monograph) or by any other standard method stated in the official monograph when the responses achieved are non-equivalent.

15.3.3. Determination of Molecular Weight

The following steps are followed for molecular weight determination of a pharmaceutical substance:

1) The sample is treated by the method given in the official monograph.
2) A graph of the retention volume of the calibration standards is plotted against the logarithm of molecular weight.
3) The curve obtained is a straight line within the exclusion and total permeation limits.
4) The molecular weight of desired component is determined from the calibration curve.
5) This calibration is valid only for the system employed under specified experimental parameters.

15.3.4. Determination of Purity

The gel chromatography techniques have been used for determining the purity of the following pharmaceutical substances:

1) **Corticotrophin – For Impurities of Higher Molecular Weights**

Materials Required

Corticotrophin: 1mg; Acetic acid (1M) (prepared by dissolving 57ml of glacial acetic acid in 1000ml of distilled water) : 100ml; Sodium dodecyl sulphate (1% w/v): 10ml.

Procedure

Accurately weighed 1mg of corticotrophin is dissolved in 1ml of 1M acetic acid containing 1% (w/v) of sodium dodecyl sulphate. The resultant solution is heated at 100°C for 10 minutes and cooled.

The chromatography is performed as follows:

i) A column (85cm × 10mm) is packed with polyacrylamide or cross-linked dextran having a fractionation range for peptides with relative molecular weights of 1000-10,000.
ii) 1M acetic acid (mobile phase) is allowed to flow through the column at a flow rate of 7ml/hour.
iii) The detection wavelength is set at 276nm.

The detector fitted with a flow-cell suitable for liquid chromatography having a volume of 1ml is connected to a strip-chart recorder. Both are set at a full-scale sensitivity of 0.5 absorbance unit.

The column is equilibrated with 1M acetic acid. Cold solution is applied to the column top using 0.4ml/cm^2 of column cross-sectional area. The sum of the peak areas eluted before the principal peak is not greater than 5.0% of the sum of the areas of all the peaks in the chromatogram.

2) **Insulin – For Proteins of Higher Molecular Weight**

Materials Required

Solution (1): Prepared by dissolving 10mg of insulin in 1ml of the mobile phase.
Solution (2): Prepared by diluting 100µl of solution (1) to 10ml with the mobile phase.
Solution (3): Prepared by dissolving 10mg of bovine insulin in 1ml of mobile phase.

Procedure

The chromatography is performed as follows:

i) A column (60cm × 7.5mm) is packed with silica gel (pore size about 13nm).
ii) A filtered and degassed solution is used as the mobile phase, which has been prepared by mixing 20 volumes of glacial acetic acid with 50 volumes of water, and pH is adjusted to 3 by adding a 25% (v/v) ammonia solution; this mobile phase has a flow rate of 0.5ml/minute.
iii) The detection wavelength is set at 276nm.

50µl of each solution is injected, and the detector sensitivity is adjusted so that the height of principal peak in the chromatogram obtained with solution (2) is 50-70% of full-scale deflection. In the chromatogram obtained with solution (1), the sum of the peak area eluting before the principal peak is not greater than the area of the principal peak in the chromatogram obtained with solution (2).

3) **Human Insulin – For Proteins of Higher Molecular Weight**

Materials Required

Solution (1): Prepared by dissolving 10mg of human insulin in 1ml of the mobile phase.
Solution (2): Prepared by diluting 100µl of solution (1) to 10ml with the mobile phase.
Solution (3): Prepared by dissolving 10mg of human insulin in 1ml of the mobile phase.

Procedure

The chromatography is performed as follows:

i) A column (60cm × 7.5mm) is packed with silica gel (pore size about 13nm).
ii) A filtered and degassed solution is used as the mobile phase, which has been prepared by mixing 20 volumes of glacial acetic acid with 50 volumes of water, and pH is adjusted to 3 by adding a 25% (v/v) ammonia solution; this mobile phase has a flow rate of 0.5ml/minute.
iii) The detection wavelength is **set at 276nm.**

50µl of each solution is injected, and the detector sensitivity is adjusted so that the height of principal peak in the chromatogram obtained with solution (2) is 50-70% of full-scale deflection. In the chromatogram obtained with solution (1), the sum of the peak area eluting before the principal peak is not greater than the area of the principal peak in the chromatogram obtained with solution (2).

4) **Plasma Protein Solution – For Polymers and Aggregates**

Materials Required

Plasma protein solution: 2.0ml; Phosphate buffer of pH 7 mixed with azide [to 1000ml of a solution containing 1.8% w/v of disodium hydrogen orthophosphate and 2.3% w/v of sodium chloride and sufficient of a solution containing 0.78% w/v of sodium dihydrogen orthophosphate and 2.3% w/v of sodium chloride (about 280ml) to produce pH 7. Sufficient sodium azide is added in the resulting solution to give a 0.02% w/v solution]: 1000ml.

Procedure

The chromatography is performed as follows:

i) A column (1M × 25mm) is packed with a cross-linked dextran suitable for fractionation of globular proteins in the range of molecular weights from 5,000 to 3,50,000.
ii) Phosphate buffer of pH 7 mixed with azide is used as the mobile phase having a flow rate of 20ml/hour
iii) The detection wavelength is set at 280nm.

The eluate is collected in fractions of 4ml, and the fractions corresponding to each peak are combined. For each combined fraction, nitrogen is determined as per the B.P. (1993). Not more than 10% of the total nitrogen is present in the combined fraction associated with non-retained proteins.

15.4. SUMMARY

The details given in the chapter can be summarised as follows:

1) **Gel chromatography** is a type of partition chromatography used for separating different sized molecules.
2) In gel chromatography, the molecules are separated based on their size, therefore is also called **molecular sieve chromatography**.
3) Large molecules cannot pass through the pores, and are excluded from the beads; hence, this technique is also called **exclusion chromatography**.
4) The external volume is termed as the **void volume** (V_0), and the sum of external and internal volumes is termed as the **total volume** (V_t).
5) The **elution volume** (V_R) of a particular molecule depends on the fraction of stationary phase available for diffusion.
6) **Retention volume** is the distance along the baseline between the point of injection and a perpendicular dropped from the maximum of the desired peak.
7) **Agarose gels** are used when gels with very large pore sizes are needed.
8) **Polystyrene gels** are hydrophobic, and thus used with non-aqueous solvents for organic chemical applications.
9) Maximum resolution in gel chromatography can be obtained by the use of long columns.
10) In gel chromatography, the polymer molecules are separated based on their molecular size or hydrodynamic volume in solution.
11) The matrices used in gel chromatography are mostly cleaned by using 0.2M sodium hydroxide or non-ionic detergents.

15.5. EXERCISE

15.5.1. True or False

1) In gel chromatography, the molecules are separated based on their size, therefore is also called exclusion chromatography.
2) The retention volume of a particular molecule depends on the fraction of stationary phase available for diffusion.
3) Agarose gels are used when gels with very small pore sizes are needed.
4) Maximum resolution in gel chromatography can be obtained by the use of long columns.
5) The matrices used in gel chromatography are mostly cleaned by using 0.2M hydrogen peroxide.

15.5.2. Fill in the Blanks

6) Large molecules cannot pass through the pores, and are excluded from the beads; hence, this technique is also called _____.
7) The external volume is termed as the _____, and the sum of external and internal volumes is termed as the _____.
8) _____ is the distance along the baseline between the point of injection and a perpendicular dropped from the maximum of the desired peak.
9) _____ are used with non-aqueous solvents for organic chemical applications.
10) In gel chromatography, the polymer molecules are separated based on their molecular size or _____ in solution.

Answers

1) False 2) False 3) False 4) True 5) False
6) Exclusion chromatography 7) Void volume and total volume
8) Retention volume 9) Polystyrene gels 10) Hydrodynamic volume

15.5.3. Very Short Answer Type Questions

1) What is gel chromatography?
2) Draw a well-labelled diagram of the apparatus used in gel chromatography.
3) What is retention volume?
4) Name the different compounds used as matrix in gel chromatography.
5) What are the column parameters in gel chromatography?

15.5.4. Short Answer Type Questions

1) Discuss the theory of gel chromatography.
2) Write a note on the matrix choice in gel chromatography.
3) Discuss the separation mechanism of gel chromatography.

15.5.5. Long Answer Type Questions

1) Discuss about the applications of gel chromatography.
2) Write an illustrative note on the instrumentation of gel chromatography.

CHAPTER 16

AFFINITY CHROMATOGRAPHY

16.1. AFFINITY CHROMATOGRAPHY

16.1.1. Introduction

Affinity chromatography technique is used to separate biochemical mixtures based on a highly specific biological interaction between an antigen and antibody, enzyme and substrate, or receptor and ligand. In affinity chromatography, the size fractionation ability of gel permeation chromatography and the ability to design a stationary phase that reversibly binds to known molecules are involved.

Affinity chromatography is used for:

1) Purifying and concentrating a molecule from a mixture into a buffering solution,
2) Reducing the amount of a molecule in a mixture, and
3) Determining the biological compounds that bind to a particular molecule, such as drugs.

16.1.2. Principle

Affinity chromatography used for separating the protein of interest works on the principle of reversible interactions that occur between the protein to be purified and the affinity ligand coupled to chromatographic matrix.

The proteins mostly have a characteristic recognition site, which is used for selecting the appropriate affinity ligand. The desired protein and the selected ligand should bind specifically and reversibly.

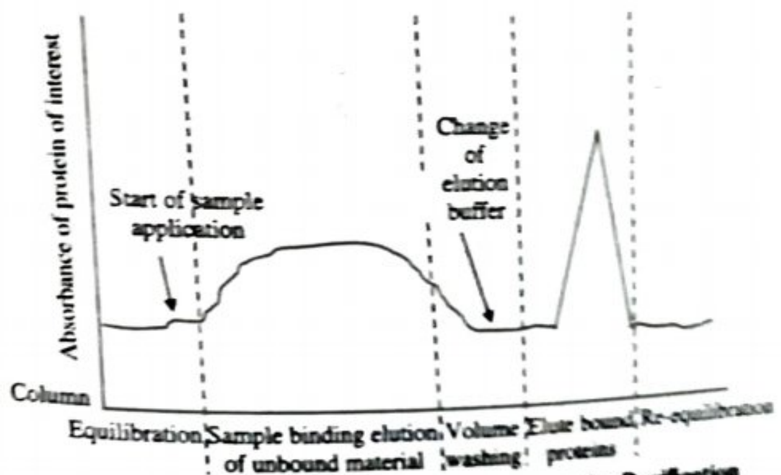


Figure 16.1: Typical Affinity Chromatography Purification

A typical process of **purification by affinity chromatography** involves the following steps (**figure 16.1**):

1) The samples are applied under favourable conditions for maximum binding with the affinity ligand.
2) Then the unbound substances are removed by washing, so that only the desired bound molecules remain attached to the affinity support.

3) The bound molecules are released and eluted by desorption, which is performed either specifically using a competitive ligand or non-specifically by altering the media atmosphere (**e.g.,** altering the ionic strength, pH, or polarity).
4) During the elution process, the purified protein is obtained in a concentrated form.

16.1.3. Theory

Affinity chromatography is a type of liquid chromatography, used for separating and specifically analysing the sample components. It utilises a reversible biological interaction (or molecular recognition) so that the specific analytes of a sample can be separated and analysed, **e.g.,** enzyme with an inhibitor and antigen with an antibody. The ligand (one of the components) is immobilised on a solid matrix, and then used for selective purification of the desired protein. This protein can also be eluted out by adding a competing ligand in the mobile phase or by altering the pH. **For example**, Ni-affinity chromatography is used for purifying 6xHis-tagged proteins, in which Ni is the chelating metal attached on NTA matrix.

Affinity chromatography leads to absolute purification in a single step. It was mainly designed for the purification of enzymes, however, now it is used for other purposes also, like purification of nucleotides, nucleic acids, immunoglobulins, membrane receptors, etc.

The biological interactions that occur in affinity chromatography are mostly non-covalent interactions between the reactive groups of targeted molecule for purification and ligand with a dissociation constant (K_d). The value of K_d varies between 10^{-3} to 10^{-7}M for affinity binding.

$$K_d = \frac{[A][B]}{[AB]}$$

Where, A = Targeted molecule.
B = Ligand.
AB = Complex formed between A and B.

16.1.4. Advantages

Following are the advantages of affinity chromatography:
1) It is a highly specific method.
2) It involves a single step purification.
3) The solid matrix can be easily washed, dried, and reused rapidly.
4) It yields the target molecules in a highly pure state.
5) It gives purified product with high yield.
6) It is also used for removing specific contaminants, such as proteases.

16.1.5. Disadvantages

Following are the disadvantages of affinity chromatography:
1) It is a time consuming method.
2) It requires large amounts of solvents, thus making it expensive.
3) It demands intense labour.
4) It involves non-specific adsorption, which can only be reduced and not completely eliminated.
5) The immobilised ligands are limitedly available and are expensive.
6) If the pH is not adjusted to required value, the proteins get denatured.

16.2. INSTRUMENTATION

16.2.1. Introduction

While using affinity chromatography for purification and separation of large biomolecules from complex mixtures, the support matrix, spacer arms, and ligand should be considered.

16.2.2. Support Matrix

The support materials used earlier in affinity chromatography included porous support materials, like **agarose**, **polymethacrylate**, **polyacrylamide**, **cellulose**, and **silica**. These materials are commercially available in a wide range of particle and pore sizes. Some available supports are already immobilised with common affinity ligands (**e.g.,** protein A, Cibacron Blue, heparin). Other support materials include non-porous supports, membranes, flow-through beads (perfusion media), monolithic supports, and expanded-bed adsorbents.

Non-porous support materials contain non-porous beads of 1-3μm diameter. These materials facilitate rapid purifications, but their surface areas are lesser than those of the traditional porous supports. Diffusion pores are also absent in the membranes used in affinity chromatography, thus restricting the surface area; however, they still facilitate faster separations just like the non-porous beads.

Small as well as large pores are present on the flow-through beads or perfusion media (formerly developed for ion-exchange chromatography). **Large flow-through pores** facilitate direct transport of the substances to the interior of the particle; this indicates that diffusion occurs through short distances.

Monolithic supports work on the same principle as perfusion media, and contain large flow-through pores and small diffusion pores. **Expanded-bed adsorbents** prevent column clogging and utilise a reversed flow to allow the column bed to expand so that the particulates freely flow through the column without clogging it.

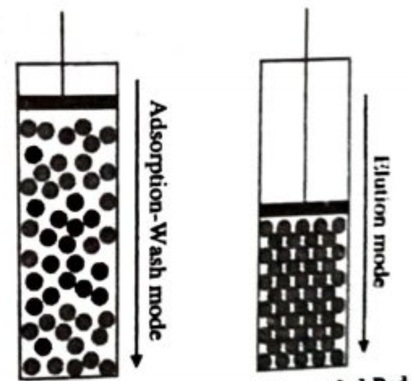


Figure 16.2: Mechanism of Expanded-Bed Chromatography

Elution is performed in a normal packed-bed, but during the adsorption-wash step, the flow is reversed and the column bed expands. This allows the particulate contaminates to pass freely through the column and prevent column clogging.

16.2.3. Spacer Arm

Sometimes the binding sites are located deep in the target molecule, and thus are difficult to access due to steric hindrance.

In such cases, efficient binding can be achieved by introducing a spacer arm between the matrix and ligand. This also provides a more effective and better environment for binding. The length of spacer arms should be carefully considered. If they are too short or too long, either non-specific binding will occur or binding will not occur. The spacer arms are generally used when coupling molecules are less than 1000Da.

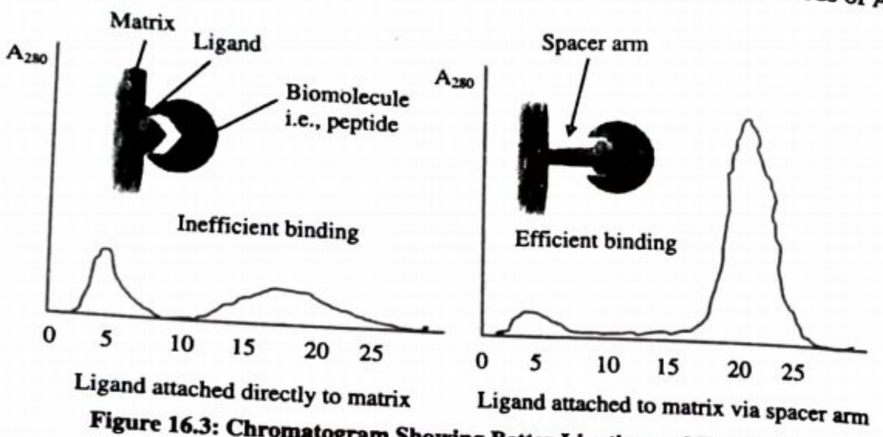


Figure 16.3: Chromatogram Showing Better Ligation and Elution when Spacer Arms are Introduced between the Ligand and Matrix

16.2.4. Ligand

Ligands (or **antibodies** or **immunoglobulins**) have high specificity and large binding constants. They are a type of glycoprotein, and are produced when the body's immune system responds to a foreign antigen. The amino acids in the Fc region have the same sequence, whereas the amino acids in the Fab region have variable amino acid sequences which allows for the specificity of the binding interaction against a wide range of antigens.

Antibodies produced by separate cell lines are called **polyclonal antibodies**. Antibodies produced when a single antibody-producing cell and a carcinoma cell are combined to create a hybridoma that can be grown in a cell culture, are called **monoclonal antibodies**. Affinity chromatography prefers the monoclonal antibodies over the polyclonal ones due to their lack of variability, which creates a more uniform affinity support.

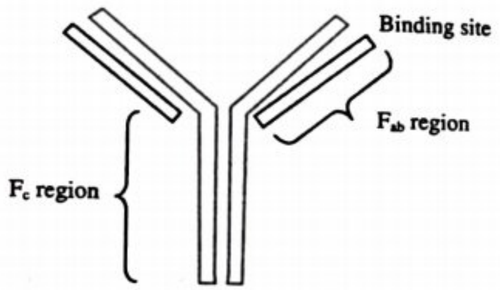


Figure 16.4: Typical Structure of an Antibody

Dye-ligand is a type of affinity ligand, used for purifying biomolecules from complex mixtures. **Dye-ligand chromatography** was devised in **1968** by **Haeckel *et al.***, when they were purifying pyruvate kinase by gel filtration chromatography and observed that blue dextran (a small dye molecule) eluted along with the desired protein. On further investigation, they determined that this co-elution was the result of dye-enzyme binding. This binding was also used in **1971** for purifying pyruvate kinase using a blue dextran column.

Biomimetic dye-ligand chromatography is the modified version of dye-ligand chromatography and employs modified dyes that mimic the natural receptor of the desired protein. These dyes offer better binding affinities, and were primarily developed due to the purity, leakage, and toxicity problems of the original commercial dyes. **Cibacron blue 3GA** is a commonly used modified triazine dye for protein purification.

Covalent attachment of the dye occurs when the dye's chlorine atom is displaced (under nucleophilic effect) by hydroxyl groups on the support's surface. **Chlorotriazine polysulfonated aromatic molecules** (triazine dyes) have been used for purifying albumin, oxidoreductases, decarboxylases, glycolytic enzymes, nucleases, hydrolases, lyases, synthetases, and transferases.

Dye-ligands and biomimetic dye-ligands have the **advantages** of being economic and resistant to chemical and biological degradation. However, their **disadvantage** is that the selection process for a particular biomolecule is empirical and requires screening processes during method development.

DNA is also used as an affinity ligand. It can purify DNA-binding proteins, DNA-repair proteins, primases, helicases, polymerases, and restriction enzymes.

Peptide affinity chromatography utilises **peptide affinity ligands** for purifying biomolecules. These ligands are identified using biological combinatorial peptide libraries (**e.g.,** phage-displayed libraries) or solid-phase combinatorial libraries (**e.g.,** one-bead-one-peptide libraries). This affinity ligand is used for isolating peptide sequences for many target molecules and for purifying *Staphylococcal* enterotoxin B, β-tryptase, and α-cobratoxin. Peptide affinity ligands have the **advantages** of being economic and stable.

16.3. METHODOLOGY

16.3.1. Introduction

The various steps involved in affinity chromatography are:

1) Column preparation,
2) Sample preparation,
3) Affinity purification,
4) Elution of the desired molecules by changing the mobile phase composition, and
5) Regeneration.

16.3.2. Column Preparation

The solid support (i.e., the bead matrix) is a gel, which is loaded in the elution column. The most commonly used solid supports are **sepharose**, **agarose**, and **cellulose**. In these materials, the hydroxyl groups present on the sugar residues can be employed to accept a ligand, which is then selected according to the preferred isolate. **Figure 16.5** shows the chemical structure of blue sepharose dye ligand, which is commonly used for Purification of Albumin and Enzymes (NAD+ and NaADP+)

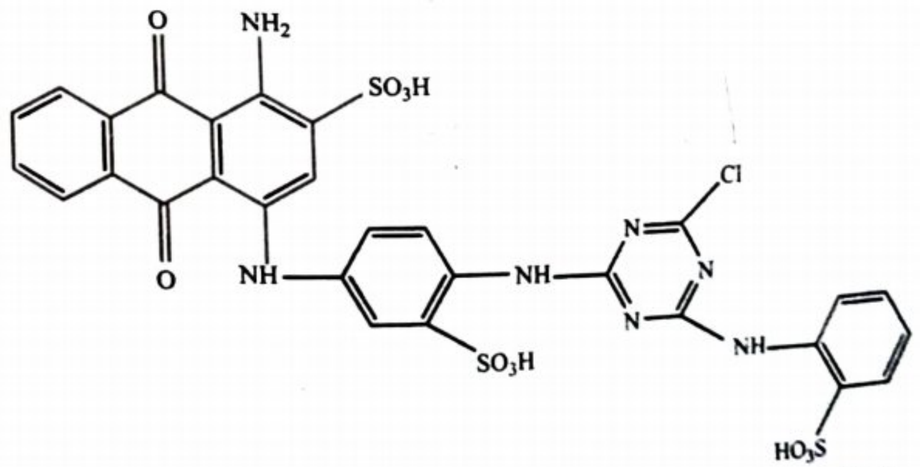


Figure 16.5: Chemical Structure of Blue Sepharose Dye-Ligand (Cibacron Blue 3GA)

For isolating antigen A-specific antibodies from an antiserum, antigen A should be utilised as a ligand. **Examples** of some **methods** used for **immobilising a protein ligand on the solid support** are given below (**figure 16.6**).

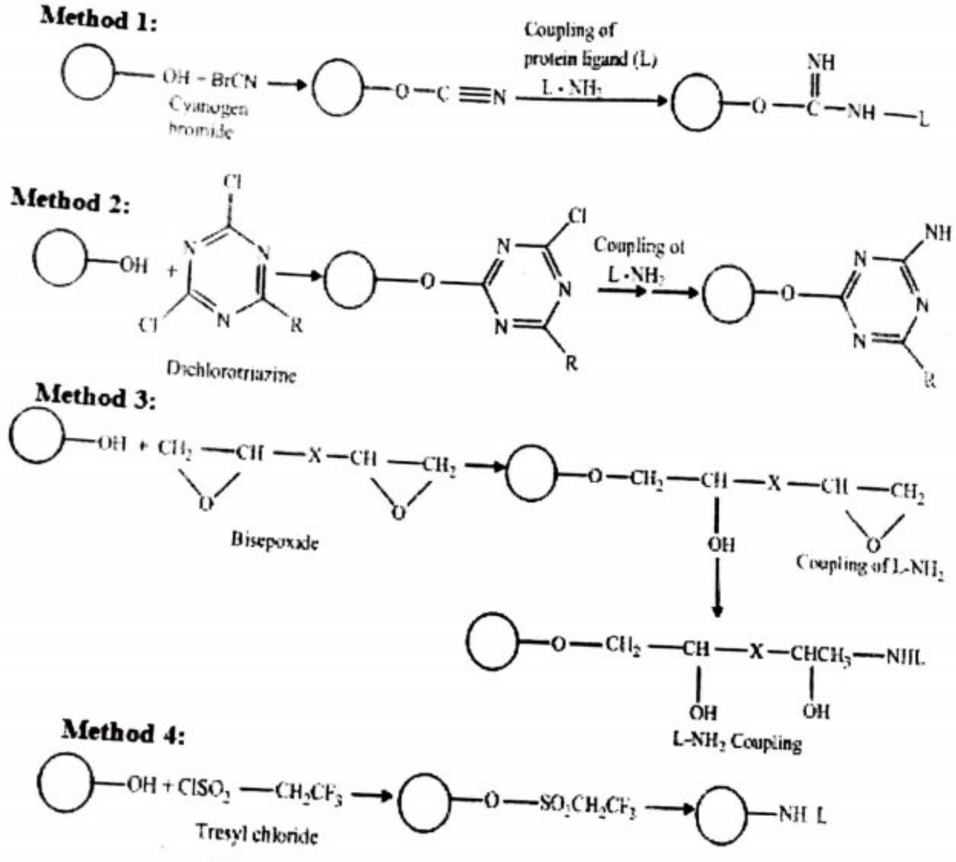


Figure 16.6: Immobilising Protein Ligand

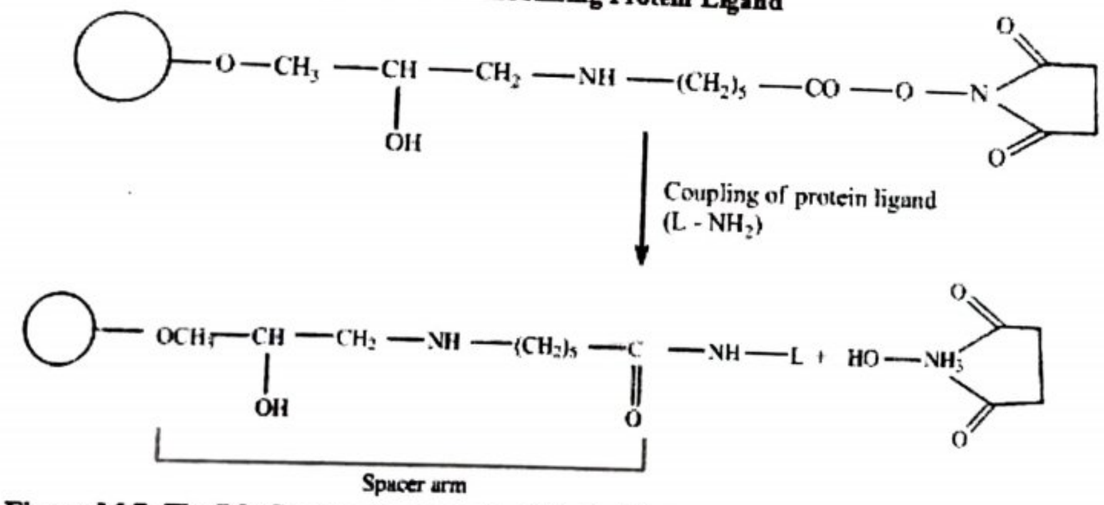


Figure 16.7: Flexible Spacer-Arm may be Attached between Ligand and Solid Support

For providing better flexibility to ligand, a flexible **spacer arm** can be attached between the ligand and solid support. **For example**, for protein ligand immobilisation, NHS-activated Sepharose [agarose beads with 10-atom spacer arms (6-aminohexanoic acid) attached by epichlorohydrin and activated by N-hydroxysuccinimide] is used (**figure 16.7**).

Similarly, for separating a particular enzyme, its substrate, an inhibitor, or a cofactor should be immobilised on the solid support.

Two factors should be considered for the ligand:

1) It should specifically and reversibly bind to the desired protein.
2) It should bind covalently to the matrix without disturbing its binding activity. This covalent binding is enabled by attaching spacer arms between the ligand and the matrix, so that even if the active site is deep within the ligand, it is not physically hidden from its binding substrate.

16.3.3. Sample Preparation

The sample should be centrifuged or filtered to obtain a clear solution with no solid particles. Protein solutions and cell lysates should be centrifuged. For filtration, a 0.45µm pore size filter is used. The effect of pH, salt concentration, or presence of any organic solvent on the solubility and stability of the sample or the desired protein should be considered. The effect of pH, salt concentration, and temperature on the interactions between the desired protein and the matrix-bound ligand should also be monitored. The composition of the initial binding buffer should be adjusted consequently. Prior to loading in the elution column, the sample components which might interfere with the target protein and/or the ligand **(e.g. metabolites in cell lysates) should be removed.**

16.3.4. Affinity Purification

The solution is rich in protein (such as antiserum). It is fed in the elution column and made to run through the gel at a controlled rate. The flow-rate is high for high-affinity samples. The rate of sample loading is reduced in case of a weak interaction and/or a slow equilibration process. Proteins having specific affinity for the immobilised ligand bind to it, while the other proteins go in flow-through. After this, the column is washed with buffer so that all the unbound proteins are removed **(figure 16.8)**.

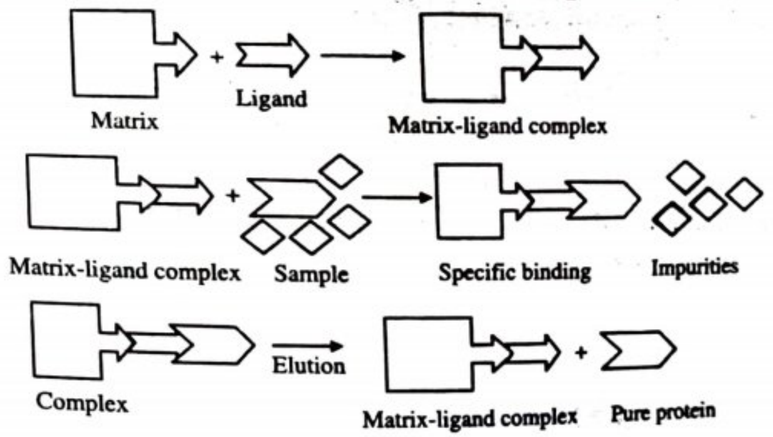


Figure 16.8: Outline of Affinity Purification Scheme

16.3.5. Elution of the Desired Molecules by Changing the Mobile Phase Composition

The desired molecules can be eluted as follows:

1) **Elution via pH and/or Ionic Strength Changes:** Elution can be simply carried out by decreasing the intensity of interaction between the ligand and the desired protein.

   This is brought about changing the pH, which will change the ionisation state of charged groups of the ligand and/or the desired protein, thus changing the intensity of interaction. In the same manner, the ionic strength is increased (by raising the NaCl concentration) to reduce the intensity of interaction. In both the cases, the solubility and stability of the desired protein should be measured.

2) **Competitive Elution:** In this type of elution, those materials are used which react with the desired protein or ligand and compete for the pre-existing interaction. **For example**, imidazole buffer is used for displacing the His-tag fusion proteins from the metal chelate matrix; glutathione is added in excess to the elution buffer for detaching GST-tagged target proteins from their column-conjugated glutathione ligand.

The cell extract containing the His-tag fusion protein is purified with a Ni^{2+} chelate column. The immobilised Ni^{2+}chelate ligand structure is in the upper panel. The sample is loaded in the column in a neutral buffer.

The His-tag fusion protein binds with the Ni^{2+} chelate ligand. When the other proteins are washed off, competitive elution with imidazole buffer is performed for isolating the pure fusion protein (in the lower right panel). The purity of fractions assessed by SDS gel electrophoresis is in the lower left panel.

In all the cases, the buffer flow rate should be reduced during the elution, so that the target protein is not excessively diluted. The desired proteins cannot be eluted by the above mentioned methods if the target-ligand interaction is very strong. Thus in such cases, the target protein is washed off from the column by using chaotropic agents (**e.g.,** urea and guanidine). This involves protein denaturation, which can then be re-natured in some cases provided the conditions are favourable; this might occur if urea or guanidine is used.

16.3.6. Regeneration

When the process of elution is complete, the column is washed with binding buffer (taken in several column volumes), and can be reused. The column can be stored for a long-term if it is prevented from bacterial or fungal infection by using sodium azide, which is a toxic compound.

16.4. APPLICATIONS

16.4.1. Introduction

Affinity chromatography has the following applications:

1) Immunoglobulin purification (antibody immobilisation),
2) Recombinant tagged proteins,
3) Protein a, g and l purification,
4) Biotin and biotinylated molecules purification,
5) Affinity purification of albumin and macroglobulin contamination,
6) Lectin affinity chromatography, and
7) Reversed phase chromatography.

16.4.2. Immunoglobulin Purification (Antibody Immobilisation)

Antibodies can be immobilised by covalent and adsorption methods. Random covalent immobilisation methods are used for linking the antibodies to the solid support via their free amine groups using cyanogen bromide, N-hydroxysuccinimide, N,N'-carbonyldiimidazole, tresyl chloride, or tosyl chloride. On the other hand, the free amine groups react with aldehyde or free epoxy groups on an activated support. These are random immobilisation methods, so the antibody binding sites may get blocked due to improper orientation, multi-site attachment, or steric hindrance.

Site-specific covalent immobilisation of antibodies is done by converting the carbohydrate residues (in the F_c region of the antibody) into aldehyde residues, which react with amine or hydrazide supports. Another site-specific immobilisation of antibodies is done by using the free sulfhydryl groups of F_{ab} fragments to link the

antibody fragments to an affinity support using epoxy, divinylsulfone, iodoacetyl, bromoacetyl, thiol, maleimide, TNB-thiol, tresyl chloride, or tosyl chloride.

Immobilisation of antibodies can also be done by adsorbing them on secondary ligands. **For example**, on reacting an antibody with hydrazide biotin, the latter reacts with oxidised carbohydrate residues on the F_c region of the antibody; the biotinylated antibody formed is then adsorbed on an avidin or streptavidin affinity support. This type of biotin immobilisation facilitates site-specific immobilisation of the antibody and can be performed using the commercially available biotinylation kits.

16.4.3. Recombinant Tagged Proteins

Purification of proteins becomes easier and simpler if the desired protein is tagged with a known sequence (termed as a **tag**). This tag ranges from a short sequence of amino acids to entire domains or whole proteins. It serves as a marker for protein expression and also assists in protein purification.

The properties of fusion tags allow the tagged proteins to be easily employed in the laboratory. The well-characterised tag-ligand chemistry allows single step affinity purification of tagged molecules using immobilised versions of their corresponding affinity ligands. Antibodies to fusion tags are also available and can be used for universal purification and detection of tagged proteins (i.e., without the need of obtaining or developing a probe for each specific recombinant protein). The most commonly used tags are Glutathione-S-Transferase (GST), Histidine fusion (His or polyHis tag), and protein A fusion tags. Other types of fusion tags are maltose-binding protein, thioredoxin, NusA, GB1 domain for protein G, etc.

16.4.4. Protein A, G and L Purification

These are native or recombinant proteins of microbial origin. They bind to specific immunoglobulins, including immunoglobulin G (IgG, representing 80% of serum immunoglobulins). Native and recombinant protein A is cloned in *Staphylococcus aureus*, recombinant protein G (cell surface protein) is cloned in *Streptococcus*, and recombinant protein L is cloned in *Peptostreptococcus magnus*. The proteins A and G bind to the F_c region of IgG, while the protein L binds to the kappa light chains of IgG.

Beaded agarose (**e.g.,** Sepharose CL-4B; agarose cross-linked with 2,3- dibromopropanol and desulphated by alkaline hydrolysis under reductive conditions), polyacrylamide, and magnetic beads are the most common matrices or supports that make use of proteins A, G, or L.

All the three proteins bind to the IgG sub-class. Protein A is suitable for cat, dog, rabbit and pig IgG; while, protein G is preferred when purifying mouse or human IgG. The proteins A and G are combined and used for purifying mammalian IgG samples. The protein L binds to the kappa light chain of immunoglobulins and these chains exist in other immunoglobulins also (i.e., IgG, IgM, IgA, and IgE), thus protein L is used for purifying antibodies of different classes. The IgGs from most species bind to protein A and G near the physiological pH and ionic strength. For the elution of purified immunoglobulins from protein G sepharose, pH should be maintained at less than 2.7.

16.4.5. Biotin and Biotinylated Molecules Purification

By incorporating a biotin tag into a biomolecule, it is used for purifying the biomolecule using a streptavidin or avidin affinity support. This can be done by inserting a biotinylation sequence into a recombinant protein. Then biotin protein ligase is used to add biotin (vitamin H or vitamin B_7) in a post-translational modification step. In affinity

chromatography, biotin is used as an affinity tag due to its strong interactions with avidin and streptavidin. Biotin has an **advantage** as an affinity tag that due to its small size (244 Da) it produces minimum effect on the activity of a large biomolecule.

Streptavidin is a large protein (60 kDa) obtained from *Streptomyces avidinii* and binds biotin with an affinity constant of $1013M^{-1}$. **Avidin** is a slightly larger glycoprotein (66 kDa) having stronger binding affinity to biotin ($1015M^{-1}$). Avidin and streptavidin have four sub-units, each of which binds to one biotin molecule. Biotinylated biomolecules can be purified by immobilising streptavidin on a support material, and using streptavidin for extracting the biotinylated molecules out of solution. Avidin and streptavidin can be immobilised using amine reactive coupling chemistries. Avidin can also be immobilised by its carbohydrate residues.

Biotin is used in Isotopically Coded Affinity Tags (ICATs), used for comparing the protein content in two different samples. The ICAT comprises of two labels, one containing deuterium (heavy) and the other containing hydrogen (light). These light and heavy labels are independently added to the cell lysates under comparison.

A thiol-specific reactive group is present in the reagent, which covalently binds to free cysteine on proteins. The labelled lysates are combined, digested with trypsin, and isolated on a streptavidin column. After a second separation step, the labelled proteins are analysed with mass spectrometry. The change in protein expression between the two cell lysates can be quantitatively analysed and related to the different conditions applied to the two sets of cell lysates.

16.4.6. Affinity Purification of Albumin and Macroglobulin Contamination

Affinity purification is used for cleaning up and removing excess of albumin and α_2-macroglobulin contamination from samples. It is necessary to remove them as they can interrupt with the analysis (**e.g.,** mass spectrometry and immunoprecipitation). **Blue sepharose affinity chromatography** is a purification method that can be used for removing these contaminants either before or after other purification steps.

In this method, the dye ligand is covalently bound to sepharose via chlorotriazine ring. Albumin binds non-specifically by electrostatic and/or hydrophobic interactions with the aromatic anionic ligand. **Cibacron blue F-3-GA** is the most commonly used dye and can be immobilised on sepharose to create an affinity column and remove around 90% of albumin from the sample.

Lectin Affinity Chromatography: This technique is used for studying glycosylation as a protein post-translational modification. Lectins are carbohydrate-binding proteins containing two or more carbohydrate binding sites. They are classified into five groups based on their specificity to the monosaccharides.

They have highest affinity for mannose, galactose/N-acetylgalactosamine, N-acetylglucosamine, fucose, and N-acetylneuraminic acid. In lectin affinity chromatography, protein is bound to an immobilised lectin via its sugar moieties (N-linked or O-linked). After the glycosylated protein binds to the affinity support, the unbound contaminants are washed off, and the purified protein is eluted. A wide range of lectins are commercially available in immobilised form; Concanavalin A (Con A) Sepharose and Wheat Germ Agglutinin (WGA) are most commonly used for glycoprotein purification.

16.4.7. Reversed Phase Chromatography

This technique involves affinity interaction between a biomolecule (**e.g.,** proteins, peptides, and nucleic acids) dissolved in a solvent (mobile phase) having some hydrophobicity and an immobilised hydrophobic ligand (stationary phase). Reversed phase chromatography is suitably used for separating non-volatile molecules.

The macromolecules (**e.g.,** protein or peptides) are adsorbed on the hydrophobic column surface. Elution is carried out using a mobile phase, which is a combination of water and organic solvents (**e.g.,** acetonitrile or methanol), applied to the column as a gradient (**e.g.,** starting with 95:5 aqueous:organic and gradually increasing the organic phase until the elution buffer is 5:95 aqueous:organic). Macromolecules remain bound to the hydrophobic column surface till the organic phase concentration is sufficiently high to elute the macromolecules from the hydrophobic surface. In reversed phase chromatography, the highly polar macromolecules are eluted first and the non-polar ones are eluted later, i.e., **more the polarity (hydrophilic) of a solute, faster is the elution** and *vice versa*.

16.5. SUMMARY

The details given in the chapter can be summarised as follows:

1) **Affinity chromatography** technique is used to separate biochemical mixtures based on a highly specific biological interaction between an antigen and antibody, enzyme and substrate, or receptor and ligand.
2) The **support materials** used earlier in affinity chromatography included porous support materials, like **agarose, polymethacrylate, polyacrylamide, cellulose,** and **silica**.
3) **Non-porous support materials** contain non-porous beads of 1-3μm diameter.
4) **Monolithic supports** work on the same principle as perfusion media, and contain large flow-through pores and small diffusion pores.
5) **Expanded-bed adsorbents** prevent column clogging and utilise a reversed flow to allow the column bed to expand so that the particulates freely flow through the column without clogging it.
6) The **spacer arms** are generally used when coupling molecules are less than 1000Da.
7) **Ligands** (or **antibodies** or **immunoglobulins**) have high specificity and large binding constants.
8) Antibodies produced by separate cell lines are called **polyclonal antibodies**.
9) Antibodies produced when a single antibody-producing cell and a carcinoma cell are combined to create a hybridoma that can be grown in a cell culture, are called **monoclonal antibodies**.
10) Affinity chromatography prefers the monoclonal antibodies over the polyclonal ones due to their lack of variability which creates a more uniform affinity support.
11) **Dye-ligand** is a type of affinity ligand, used for purifying biomolecules from complex mixtures.
12) **Biomimetic dye-ligand chromatography** is the modified version of dye-ligand chromatography and employs modified dyes that mimic the natural receptor of the desired protein.
13) **DNA** is also used as an affinity ligand. It can purify DNA-binding proteins, DNA-repair proteins, primases, helicases, polymerases, and restriction enzymes.
14) Peptide affinity chromatography utilises **peptide affinity ligands** for purifying biomolecules.
15) The solid support is a gel, which is loaded in the elution column. The most commonly used solid supports are **sepharose, agarose, and cellulose.**

16) For providing better flexibility to ligand, a flexible **spacer-arm** can be attached between the ligand and solid support.
17) **Elution** can be simply carried out by decreasing the intensity of interaction between the ligand and the desired protein.
18) In **competitive elution**, those materials are used which react with the desired protein or ligand and compete for the pre-existing interaction.

16.6. EXERCISE

16.6.1. True or False

1) Monolithic supports contain large flow-through pores and small diffusion pores.
2) The spacer arms are used when coupling molecules are less than 100Da.
3) Affinity chromatography prefers polyclonal antibodies over the monoclonal ones due to their lack of variability which creates a more uniform affinity support.
4) Dye-ligand is a type of affinity ligand, used for purifying biomolecules from complex mixtures.
5) In competitive elution, those materials are used which react with the desired protein or ligand and compete for the pre-existing interaction.

16.6.2. Fill in the Blanks

6) _____ utilise a reversed flow to allow the column bed to expand so that the particulates freely flow through the column without clogging it.
7) Antibodies produced by separate cell lines are called _____ antibodies.
8) _____ chromatography employs modified dyes that mimic the natural receptor of the desired protein.
9) For providing better flexibility to ligand, a flexible _____ can be attached between the ligand and solid support.
10) Elution can be carried out by decreasing the intensity of interaction between the _____ and the desired protein.

Answers

1) True 2) False 3) False 4) True 5) True
6) Expanded-bed adsorbents 7) Polyclonal 8) Biomimetic dye-ligand
9) Spacer-arm 10) Ligand

16.6.3. Very Short Answer Type Questions

1) What is affinity chromatography?
2) Give the advantages of affinity chromatography.
3) How sample is prepared in affinity chromatography?
4) Give the outline of affinity purification.

16.6.4. Short Answer Type Questions

1) Discuss the principle and theory of affinity chromatography.
2) Write a note on the ligand used in affinity chromatography.
3) Discuss about spacer arm of affinity chromatography.

16.6.5. Long Answer Type Questions

1) Discuss about the applications of affinity chromatography.
2) Write an illustrative note on the methodology of affinity chromatography.

Index