Unit 2.2 Thin Layer Chromatography

Thin-layer chromatography (TLC), is a solid-liquid form of chromatography where the stationary phase is normally a polar adsorbent and the mobile phase can be a single solvent or combination of solvents.

In thin-layer chromatography, the stationary phase is a polar absorbent, usually finely ground alumina or silica particles. The adsorbent is coated on a glass slide or aluminium sheet or plastic sheet creating a thin layer of the particular stationary phase. Almost all mixtures of solvents can be used as the mobile phase. By manipulating the mobile phase, organic compounds can be separated.

In TLC, molecules are continuously moving back and forth between the free and adsorbed states with millions of molecules adsorbing and millions of other molecules desorbing each second. The equilibrium between the free and adsorbed states depends on three factors:

- The polarity and size of the molecule
- The polarity of the stationary phase
- The polarity of the solvent

The polarity of the molecules is determined by their chemical structures. By selecting different stationary and mobile phases, we can change the equilibrium between the free and adsorbed states. Different molecules behaves partition differently between the free and adsorbed state, that is the equilibrium between these two states is not the same.

The stationary phase used in TLC is typically alumina $(Al_2O_3 .xH_2O)n$ or silica gel $(SiO_2 .xH_2O)n$. The covalent network of these adsorbents creates more polar nature.

The electropositive character of the aluminum or silicon and the electronegative oxygen create a more polar stationary phase. Therefore, the more polar the molecule to be separated, the stronger the attractive force to the stationary phase. The polar stationary phase will more strongly attract like or polar molecules. The equilibrium will be shifted as the molecules remain on the stationary phase. Nonpolar molecules will have a lower affinity for the stationary phase and will remain in the solvent longer. This is essentially how the partitioning separates the molecules. The equilibrium governs the separation, but the component's attraction to the stationary phase versus the mobile phase determines the equilibrium. In general, the more polar the functional group, the stronger the bond to the stationary phase and the more slowly the molecules will move. Sometimes, the molecules will not move at all. This problem can be ovecome by increasing the polarity of the mobile phase so that the equilibrium between the free and adsorbed state is shifted towards the free. Although alumina and silica are the most common stationary phases used in TLC.

Common stationary phases by Increasing polarities are polydimethyl siloxane, methylphenylsiloxane, cyanopropylsiloxane, calcium sulphate, silica, magnesium silicate, magnesium oxide, aluminium oxide and activated carbon.

In a mixture of compounds, the most polar compounds will adhere more strongly to the stationary phase.

Elution sequence of functional group based on increasing polarity of functional group of the separation viz., Alkane Hydrocarbons, Alkyl Halides (Halocarbons), Alkenes (Olefins), Dienes, Aromatic hydrocarbons, Aromatic halides, Ethers, Esters, Ketones, Aldehydes, Amines, Alcohols, Phenols, Carboxylic acids, Sulfonic acids.

In general, the more nonpolar the compound, the faster it will elute and the more polar the compound the slower it will elute. One should recognize the functional groups and easily determine which one is more polar than another.

Some of the common mobile phases listed by Increasing Polarity, viz., Helium, Nitrogen, Petroleum ether (pentanes), Ligroin (hexanes), Cyclohexane, Carbon tetrachloride, Toluene, Chloroform, Dichloromethane (methylene chloride) t-Butyl methyl ether, Diethyl ether, Ethyl acetate, Acetone, 2-Propanol, Pyridine, Ethanol, Methanol, Water, Acetic acid.

Finding a good solvent system is usually the most difficult part of TLC. If the mobile phase has not been identified or selected, it should be started with a nonpolar solvent such as ligroin and the separation should be observed. If the components in the mixture do not move very far, by adding a polar solvent such as ether or ethyl acetate to the ligroin, issue may be solved. We can compare with the trial basis and if there is no significant movement, then polar solvent should be increased to get a good result. By this methodology suitable mobile phase can be identified.

Advantages of TLC over paper and column chromatography.

The main advantages of TLC are

1. Requires simple equipment
2. Short development time: TLC takes one hour for development but paper and column chromatography take several hours to days.
3. Wide choice of stationary phase: Method may be employed for adsorption, partition (including reverse phase chromatography or ion exchange chromatography).
4. Easy recovery of separated compounds: While removing the Powderly coating of the plates by scraping with a knife or scrapper, the separated components can be recovered easily. The spot or zone may be removed quantitatively.
5. Preparation effect: superior than paper chromatography
6. Thickness of the layer: Variable thickness is available from preparative to analytical.

7. Easy visualization of separated compounds: detection of fluorescence compounds under UV light is easier than on paper because the inorganic background does not fluoresce
8. Sensitivity: 10 to 100 times more than paper chromatography
9. Chemically inert stationary phase.

Comparison between Thin Layer Chromatography and Paper chromatography

Thin Layer Chromatography	Paper chromatography
Principle of separation is adsorption	Principle of separation is partition
More quantity of sample is required	Minimum quantity of sample is required
Consuming less time (15-45 min.)	Consuming more time (1-3 h)
Plates may be heated in oven for long time	Paper cannot be heated for long time
Very sharp separation	Less sharp separation
Physical strength of the plate is high (Ascending/Descending)	Physical strength of the paper is low (Descending/ Small paper only ascending)
Plates are prepared as thin coat with adsorbents	Paper is used as such since coating can't be done on paper.
Corrosive reagent can be used	Corrosive agents can't be used since it destroys it.
Compound can be detected under UV	Can't be detected under UV
Sensitivity of detection is high	Less sensitivity of detection
Spots are less diffused	Spots are easily diffused.

Comparison between Thin Layer Chromatography and High Performance Thin Layer Chromatography

Parameters	Thin Layer Chromatography	High Performance Thin Layer Chromatography
Chromatographic Plate	Precoated/Hand made	Precoated
Adsorbent thickness	250µm	50-200 µm

Adsorbent size	5-10 µ	4-8 µ
Prewashing of plate	Not mandatory	Mandatory
Sample application	Manual/Semiautomatic	Semiautomatic/Automatic
Shape of application	Spot	Spot/Band
Spot size	2-4mm	0.1-1mm
Sample volume	1-10 µl	0.1-10 µl
Large volume application	Overloading on spot	Can be applied as bands
Number of sample spots	Upto 10	Upto 40
Development distance	10-15cm	5-7cm
Development time	Based on Mobile phase	Upto 40% less than TLC
Results reproduction	Moderate difficult	Reproducible

Requirements of TLC:

1. Stationary phase
2. Supporting material (Glass plates/ Aluminum /Rubber/Polymer sheet)
3. Preparation and activation of TLC plates
4. Application of sample
5. Development Tank
6. Mobile phase
7. Development techniques
8. Detecting agents

Adsorbents:

In the beginning of the development of TLC very few adsorbents were used like silica gel, alumina etc. nowadays a variety of adsorbents are used based on our need. Particle size and homogeneity are the main parameters considered for the adsorbents to decide its application. Adsorbents generally do not

adhere to the glass or aluminum plates. Hence binders like gypsum (calcium sulphate) in 10-15% or starch or hydrated silicon dioxide may be added as the binding agent.

Factors to be considered for selection of adsorbents:

1. Characteristics of compound to be separated
2. Solubility of compounds
3. Nature of substance to be separated (acidic/basic/amphoteric)
4. To see whether compound is liable to react chemically with adsorbent/mobile phase

   Adsorbents are classified into two types they are organic and inorganic adsorbents.

   Inorganic: Silica gel, alumina, magnesia, magnesium silicate, ferric oxide hydrate and calcium silicate.

   Organic: cellulose and its derivatives, charcoal and activated carbon.

Preparation of thin layers on plates: Using applicators

Types;

1. Pouring: Simple, but not used to the great extent
2. Dipping: Chloroform or chloroform-methanol mixture with the adsorbents as slurries were minimally used.
3. Spraying: Difficult to get uniform layer
4. Spreading: thickness can be adjusted from 0.01 to 2.0mm

Per-coated plates: 0.1-0.2mm

Activation of adsorbents: Developed plates are dried at 30° C. Then keep in 110^oC for another 30 min. If silica gel is used, then the layer will be kept at 150^oC for 4 hours.

Purification of silica gel as layer: Silica gel G contains iron as impurity, which causes a considerable distortion of the chromatographs. Iron free layers can be done by preliminary development with methanol –concentrated hydrochloric acid

(9:1). The iron gets migrated with the solvent front and again dried and activated at 110°C for 30 min.

Sample application: Agla microsyringe is generally used for quantitative work. Capillary tube for qualitative work. Sample volume and concentration possible is far greater than paper chromatography. Solvents used for sample solution should be volatile and as non-polar as possible.

Development tank: ascending and the angle of chamber θ = 45°

Solvent height in the chamber is 1mm, and the chamber should be saturated with solvent vapour.

Descending is available without any advantage

Horizontal and radial with little use.

Solvent system:

Development method: Solvent front should reach 15 -18 cm of normal 20cm tall plate (80%) will take 20-40 min.

Detection of compounds: UV light, Visualizing agents, fluorescence detector.

Chromic acid / Sulphuric acid can be used as visualizing agent, even though too corrosive which is used in TLC but not in paper chromatography.

Evaluation of chromatogram:

Quantitative: same as paper chromatography, except to say Rf value for TLC is inferior reproducibility.

Quantitative: 1. Direct and 2. Indirect

1. Direct:
    a. Visual assessment of chromatogram: first used method and very simple procedure.
    b. Determination by measurement of spot areas: spot dimension and materials relationship.

c. Quantitative TLC incorporating densitometry: concentration of compound measured directly.
d. Direct spectrophotometry on the TLC chromatogram: lambda max of the substance, reading the separated chromatogram by reading with UV/Fluorescence detection. In addition, IR, reflectance spectroscopy, spark chamber method can be used.

2. Indirect method (Elution technique):

   Chemical constituent can be recovery by quantitative elution and subsequent determination with suitable instrument. In this method the areas containing the adsorbed component after visualization is marked and scooped with the help of a vacuum cleaner. This is a preferable method where there is no loss of adsorbent will take place. By the simple agitation elution of solute is takes place and removal of adsorbent by centrifugation. Micro analysis of resultant eluate can be performed by any of the following method, gravimetric, colorimetry, UV spectrophotometry, fluorimetry, polarography, caulometry, radiometry, flame photometry or vapour phase chromatography.

   Advantages of Indirect method:

   a. Nature of the substance to be analyzed or assayed
   b. Sophisticated equipments are available for micro analysis and its sensitivity
   c. Time available
   d. Alternative method available, if any and their relative accuracies.

Spectrophotometry is a suitable method for all the substances. Citrus oils, essential oils can be done by vapour phase chromatography. Quinine sulphate and riboflavin can be measured by fluorimetry, paracetamol can be analyzed by UV spectroscopy or fluorescence spectroscopy.

Preparation of plates:

Glass plates or flexible plates are commonly used as support for adsorbent. Size of the plates used depends on type of chromatographic tank and spreading apparatus available.

The standard sizes are 20x5 cm, 20x20 cm.

The surface should be flat without irregularities.

The standard film thickness is 250µm.

Methods for application of adsorbent:

Pouring

Dipping

Spraying

Spreading

Pouring: The adsorbent of finely divided and homogeneous particle size is made into slurry using mortar and pestle and is poured on a plate and allowed to flow over it by tilting the plate, so that it is evenly covered.

Dipping: This technique is used for small plates, by dipping the two plates at a time, back to back in slurry of adsorbent in chloroform or other volatile solvents. Exact thickness of layer may not be good.

Spraying: Slurry is diluted further for the operation of sprayer. But this technique is not used now a days as it is difficult to get uniform layer.

Spreading: All the above methods fail to give thin and uniform layers. Modern methods utilize the spreading devices for the preparation of uniform thin layers on glass plates. Commercial spreaders are of two types a) Moving spreader, b) Moving plate type. It gives thickness from 0.2 to 2.0mm.

Activation of plates:

After spreading plates are allowed to dry in air and further dried and activated by heating at about 100°C for 30 min. By removing the liquids associated with layer completely, the adsorbent layer is activated.

Solvent system:

The choice of the mobile phase is depends upon the following factors.

1. Nature of the substance to be separated
2. Mode of chromatography (Normal phase or Reverse Phase)
3. Separation to be achieved – Analytical or Preparative

The organic solvent mixture of low polarity is used, and highly polar solvents are avoided to minimize adsorption of any components of the solvent mixture. Use of water as a solvent is avoided, because it may loosen the adhesion of a layer on a glass plate.

Solvents with an increasing degree of polarity are used in liquid-solid or adsorption chromatography. The solvents listed in eluotropic series are selected.

For example in an increasing order - n-Hexane, cyclohexane, toluene, benzene, diethyl ether, chloroform, dichloromethane, 1,2-dichloroethane, acetone, ethyl acetate, acetonitrile, propanol, methanol acetic acid, water.

Application of the sample:

Sample solution in a nonpolar solvent is applied.

The concentration of a sample or standard solution has to be prepared with concentration of 1% solution and spotted using a capillary tube or micropipette.

The area of application should be kept as small as possible for sharper and greater resolution.

Sample application: Two methodologies are performed for sample application. A) spotting (TLC) and b) spreading (HPTLC).

Spotting: Draw a guide lines lightly with a pencil. Dissolve the sample in chloroform. Use the capillary to transfer and spot the dissolved sample in the line of the plate.

TLC Plate-Development Methods

The plate-development is the main process of thin-layer chromatography in which the actual separation takes place. In this step, a solvent system is moving against the gravity through the sample on the TLC layer in order to separate the mixture into individual substances. Separation can be achieved through various methods. The most common TLC development techniques are explained below.

Vertical Development

In this common TLC development method, the plate is placed in a suitable TLC developing chamber such that the solvent wets the TLC layer below the starting line. Due to capillary forces, the solvent rises up the layer, transporting the sample mixture. Once the solvent front has reached the predetermined height (10-15 cm for TLC and 3-7 cm for HPTLC), the plate is removed from the chamber, the solvent front is marked with a pencil or spatula, and the plate is dried.

Horizontal Development

In this TLC developing method, the plate is positioned horizontally inside the chamber, and the solvent is applied using a wick or capillary slit. Development can be performed from one or both sides of the TLC plate.

Two-Dimensional Development

In two-dimensional TLC development, the sample is applied to a starting point in a corner of the TLC plate. The plate is placed in a normal chamber and developed once from bottom to top. After drying, the plate is turned 90° and placed in another chamber with a different solvent and developed again. The chromatogram track from the first development is used as the starting line for the second development. Two-dimensional TLC offers the advantage of running a standard with either development. However, the standard cannot be developed in two dimensions on the same chromatogram since it would mix with the sample.

Multiple Development

In this method, the TLC plate undergoes multiple developments with drying between each cycle. The solvent travels repeatedly through the layer, re-concentrating and deforming the spots, often producing elliptical shapes or narrow bands. This significantly improves resolution for substances with Rf values below 0.5. Multiple development can be performed over different separation distances, using the same solvent or different solvents of varying polarity.

AMD (Automated Multiple Development)

AMD is an automated version of multiple development, and is based on a gradient solvent system. In the case of silica gel TLC layers, solvents of decreasing polarity are used, and each consecutive development step is performed over a longer distance. Ideally, development is performed in special U-chambers, where the solvent is fed via a pump. After each run, the solvent is drawn off under vacuum. Since the process starts with polar solvents, all substances first migrate with the front and become concentrated to narrow bands. As solvent polarity decreases, first the most polar and later the non-polar substances remain in position. AMD offers excellent resolution and sensitivity, and allows each step to be preselected at will.

TLC Developing Chambers

Thin-layer chromatography is usually performed in developing chambers. As explained below, there are a variety of chambers are available to choose depending on the sample and application goals. Furthermore, TLC development can be performed in saturated or unsaturated chambers.

Normal Chambers

Normal development chambers are the most common type used in thin-layer chromatography. These glass chambers measure 21 x 21 x 9 cm, and are suitable for simultaneous development of at most two TLC plates.

Twin-Trough Chambers

The twin-trough chamber is divided into two sections by a ridge at the base. For standard linear development, solvent consumption (20 ml for a 20 x 20 cm plate and 4 ml for a 10 x 10 cm plate) is considerably lower in twin-trough chambers than in normal chambers. This reduces material and disposal costs.

Sandwich Chambers (S-Chambers)

In this method, a sandwich is created by covering the TLC layer with a glass plate. The TLC layer and cover plate should be slightly separated using spacers such that only the bottom-most zone (about 2 cm in width) remains uncovered. The cover plate should not be dipped into the solvent. The sandwich design can be used in any type of TLC development chamber.

Horizontal Chambers (Linear Chambers)

In this method, a sandwich chamber is used for horizontal development of TLC plates. TLC development can be performed from one or both sides of the plate.

Development of Chromatograms:

Generally ascending method is used to greater extent but various other methods are also used. They are

Ascending Development: The plates after spotting of the sample are placed in chamber containing solvent at the bottom. Flow of solvents is from bottom to top like capillary action against the gravity.

Descending: flow of the solvent from reservoir to the plate is by means of a filter paper strip. Solvent moves from top to bottom of the plate.

Two-dimensional development: It is used if the component of the mixtures is not completely separated by development in a single direction. In this sample spot is applied at corner of the plate. First development is carried out by ascending method in one solvent. The plate is taken out, solvent allowed to evaporate. Second development is carried in another solvent by changing the edge of the plate at 90°.

Reversed Phase TLC:

In this method the stationary phase used is non polar in nature and mobile phase is polar in nature. These chromatoplates are prepared by immersing the adsorbent layer very slowly in 5-10% of paraffin, silicone oil, undecane in petroleum ether or diethyl ether. After removing the plate and evaporating the solvent, the plate is ready for performing TLC experiment. Paraffin and silicone oil provides the permanent impregnation whereas undecane can be removed after development after heating the plate at $120^{o}C$.

High Performance Thin Layer Chromatography (HPTLC):

HPTLC is an analytical technique based on thin layer chromatography, but with enhancements intended to increase the resolution of the compounds to be separated and further allow quantitative analysis of the compounds.

Pre-conditioning (Chamber saturation):

Preconditioning has pronounced influence on the separation profile. If the sample applied in non saturated chamber, it requires larger quantity of solvent for a given distance. It also leads to increase the Rf values. The reasons for the chamber saturation are 1) if the chamber is saturated prior to development, solvent vapour gets uniformly distributed in chamber. 2) As plates are introduced in such chamber, it soon gets pre-loaded with solvent. 3) Less solvent required to travel particular distance. 4) Lower the Rf values.

Development and drying is done in HPTLC is similar to TLC. They are 1) Ascending, 2) Descending, 3) Two dimensional, 4) Horizontal (Continuous), 5) Gradient, 6) Radial and 7) Multidimensional.

Drying of the developed Plates: After the development the plates are removed from the chamber and mobile phase. Drying preferably performed in fume cupboard to avoid contamination of lab atmosphere. During drying plates should always laid horizontally. Usually hand dryer may be used either cold or hot. The following considerations are taken care of during drying process. 1) Essential oil component may evaporate. 2) Compound sensitive to oxygen may get destroyed. 3) Particle of dust from lab may deposit on chromatogram.

It is precisely for this reason the drying of chromatogram should preferably be done in desiccators with protection from heat and light.

Detection and visualization:

1) One of the most characteristic features of the HPTLC is possibly to utilize post chromatographic off line derivatization.
2) The zones can be located by various physical, chemical, biological, physiological methods.

3) There is no difficulty in detecting the coloured substance or colourless absorbing the short wave length in UV region (254nm) or with intrinsic fluorescence such as riboflavin or quinine sulphate.
4) The substance which do not have above properties have to be transferred into detectable form by means of chromogenic and fluorogenic reagent.
5) Iodine is the universal detecting agent.
6) Derivatization is essentially required for detection when individual compounds does not respond to UV or does not intrinsic fluorescence.
7) Other detection methods based on wetting and solubility phenomena.
8) As aluminum oxide, kieselguhr or silica gel are hydrophilic adsorbent, on dipping or spraying the chromatogram with water lipophilic such as steroid hydrocarbon appears as a white spot against semitransparent background.
9) Instead of water, one can employ hydrophilic or lipophilic dye solution for spraying or dipping.
10) Incase of hydrophilic dyes such as methylene blue the background is stained blue, whereas non-wetted zone appear pale.
11) While in case of lipophilic dyes non-wetted zone appears as a deeply coloured against as a pale background.
12) Fluorescent chemicals are used for the detection of lipophillic substances by wetting or non-wetting technique. Eg. Rhodomin B.
13) Several corrosive reagents are also used for detection of organic compounds.
14) These compounds produce colour or fluoresccnt zone on heating. eg. 10% v/v alcoholic sulphuric acid, 5% potassium dichromate.

Visualization of TLC results: Allow solvent to evaporate from surface of TLC plate. The view results under UV light (Look the grayish spots on the fluorescent green background). Mark spots with a pencil while viewing under UV.

Quantitative method in TLC and HPLC:

Techniques for quantitative analysis.

1) Analysis of fraction on plate-
    a. Visual comparison with standard.
    b. Precise measurement of spot area or spot size.
    c. Photo densitometry
    d. Spectrometry
2) Determination of fraction after elution from coating material-
    a. Scooped area containing adsorbed compound with vacuum cleaner and then analysed by various analytical methods like gravimetry, polography and chlorometry.
3) Radioactive substance can be assayed by-
    a. Photo densitometry of X-ray film.
    b. Direct radio-scanning of chromaplates.

Quantitative analysis:

Rf values are calculated by identifying the spots i.e., in quantitative analysis. Rf value ranges from 0 to 1. But the ideal values are from 0.3 to 0.8 when the Rf value of a sample and reference standard.

Applications of TLC:

1. Prior determination (Checking process): checking in small scale separation procedure and purification procedure. Also for distillation fractions and purification by molecular determination.
2. Separation chemistry: mainly for isolation and separation of individual compounds of a mixture. TLC is more advantageous because almost all chemical substances can be separated and high speed of separation.
3. Selectivity of TLC is high: very closely related chemical compounds can also be clearly resolved by adsorption or partition TLC
4. Purification of the sample can be done.
5. Identification of related compounds in the drugs.
6. Separation of mixtures of drugs of chemical or biological origin, plant extracts etc.
7. Separation of carbohydrates, vitamins, antibiotics, glycosides, etc.

8. Purification of organic compound: small amount of sample can be purified 30-40 mg of crude materials can be separated in two 20 cm plates.
9. Examination of reaction: By products identification or estimation can be done.
10. Characterization and isolation of organic compounds can be done. Eg. acids: Normal aliphatic acids with even number of carbon atom, carboxylic acid, phenol carboxylic acid, etc. Alcohols: Even numbered alcohols from decanol through hexanol. Glycols: some glycols. Alkaloids: Purine alkaloids, pyridine alkaloids. Amines: Alkylamines. Amino acids, proteins and peptides: cyclic pepetides. Antibiotics: tetracyclines, penicillins, neomycin, etc.
11. Separation of inorganic ions: used to separate cations, anions, purely covalent species and some organic derivatives of the metals. Zinc and Iron are separated by radial chromatography. Separation of cations are reported that their separation depends on the ion-exchange properties of the adsorbent and coordination tendencies of the solvent. Ag, Hg, Pb, Bi, Cd, As, Sb, Sn, Fe, Cr, Al, Zn, Co, Ni, Pt, V, Rb, Au, Se, Te, Mo, Ge, etc., can be separated by TLC.
12. Quantitative estimation
    a. Spectroscopic measurement: carboxy compounds, vitamins, lipids, epoxy’s , etc.
    b. Fluorometric measurement: Hormones
    c. Spectral reflectance: Dyes
13. Limitation: TLC is limited to small scale use only.
14. TLC is a quick, inexpensive microscale technique that can be used to:
    Determine the number of components in a mixture, verify a substance’s identity, monitor the progress of a reaction, determine appropriate conditions for column chromatography and analyze the fractions obtained from column chromatography.

THIRD YEAR PHARM.D-3.2-Pharmaceutical Analysis Theory

UNIT 4.3 ATOMIC ABSORPTION SPECTROSCOPY

Absorption of energy by ground state atoms in the gaseous state is the basis of atomic absorption spectroscopy (AAS). It is in contrast to emission spectroscopy, that the absorption of specific wavelength by atom is measured. AAS was introduced by Alan Walsh in the mid 1950's. It is the most powerful technique for the quantitative determination of trace metals in liquids. Using this technique most of the metallic elements can be determined with remarkable sensitivity and accuracy. By this technique, the determinations can be made in the presence of many other elements therefore no need of separation of other elements present in the samples. Thus, it saves a great deal of time. Atomic absorption spectroscopy is method of elemental analysis. It is particularly useful for determining trace metals in liquids and is almost independent of the molecular form of metal in the sample. It can be realized from the fact that 60-70 elements including most of the common rare earth metals, can be determined using this technique in the concentration range from trace to macro-quantities. This technique is not only restricted to aqueous solutions but also to non-aqueous solution.

Working principle of Atomic absorption spectrometer

The working principle of Atomic absorption spectrometer is based on the sample being aspirated into the flame and atomized, when the light beam is directed through the flame through the monochromator, and onto the detector that measures the amount of light absorbed by the atomized element in the flame. Since metals have their own characteristic absorption wavelength, a source lamp composed of that element is used, making the method relatively free from spectral or radiational interferences. The amount of energy of the characteristic wavelength absorbed in the flame is proportional to the concentration of the element in the sample. Absorbance is logarithm ratio of light intensities measured without the intensity of incident light (I_0) and with intensity of light absorbed (I) the analysed atoms present in light path (absorbance = log Io/I). Also based on Beer Lambert law, when monochromatic light passes through a transparent medium, the fraction of radiant energy absorbed is directly proportional to thickness and concentration of solution.

$I = Ioe^{-kct}$ where, k = absorption coefficient, c = concentration, t = thickness

Construction and working of Atomic absorption spectrometer

The Atomic Absorption Spectrophotometer used for the detection of trace metals have following components:

•Radiation Source: Hollow Cathode Lamp

•Atomiser or Burner: Graphite Burner

•Monochromator: Grating

There are eight basic components of an atomic absorption spectroscopy:-

•Radiation Source

•Chopper

•Atomiser

•Nebuliser

•Monochromator

•Detectors

•Amplifier

•Read Out Devices

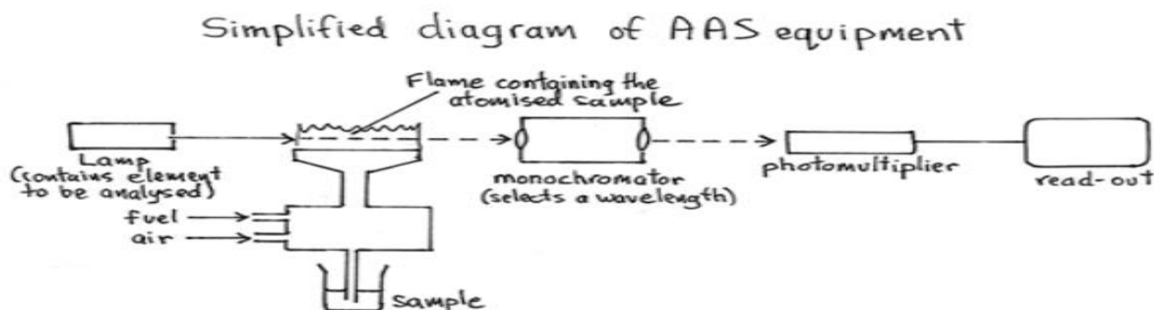


FIGURE 1

Radiation source

Main sources used for atomic absorption are hollow cathode lamps and electrode-less discharge lamps. The hollow cathode lamp is an excellent, bright, stable, line source for the estimation of most of the elements.

Hollow cathode lamp: The hollow cathode lamp consists of a glass cylinder filled with an inert gas usually Argon or Neon at low pressure and a metal or metal salts like Zn, Cd, Hg. Hollow cathode lamp consists of a tungsten anode and a cylindrical cathode sealed in a glass tube that is

filled with neon or argon at a pressure of 1 to 5 torr. As stated before, the light from this lamp is exactly the light required for the analysis, even in absence of monochromator. The reason for this is that atoms of the metal to be tested are present within the lamp, and when the lamp is glowing, these atoms are supplied with energy, which causes them to elevate to the excited states. Upon returning to the ground state, exactly the same wavelengths that are useful in the analysis are emitted, since it is the same wavelength for the test metal with exactly the same energy levels that undergoes excitation. The hollow cathode lamp therefore must contain the element being determined. A typical atomic absorption laboratory has a number of different lamps in stock which can be interchanged in the instrument, depending on what metal is being determined. Some lamps are "multi element" which means that several different specified kinds of atoms are present in the lamp and are excited when the lamp is glowing. The light emitted by such a lamp consists of the line spectra of all the kinds of atoms present. No interference will usually occur as long as the sufficient intense line for a test metal which is found can be efficiently separated from all other lines with the monochromator. The lamp itself is a sealed glass envelope filled with argon or neon gas (Fig…). When the lamp is switched on, argon atoms are ionized, as shown, with the electrons drawn to the anode (positive electrode), while the argon ions, Ar+, "bombard" the surface of the cathode (negative electrode). The metal atoms, M, in the cathode are elevated to the excited state and are ejected from the surface as a result of this bombardment. When the atoms return to the ground state, the characteristic line spectrum of that atom is emitted. The light, which is directed at the flame, where unexcited atoms of the same element absorb the radiation and are themselves raised to the excited state.

Spectral line width:

The natural width of an atomic absorption or an atomic emission line is on the order of 10^{-5} nm. Due to the several reasons two effects however, cause line broadening are Doppler and Pressure which always encountered when atomic absorption spectroscopy is used.

Doppler broadening:

The sources of radiation that are used for most measurements of atomic absorption spectroscopy contain energetically excited atoms that emit radiation upon returning to a lower energetic level. Doppler broadening results from the rapid motion of atoms as they emit or absorb radiation. Atoms moving towards the detector emit wavelengths that are slightly shorter than the wavelengths emitted by atoms moving at the right angles to the detector.

Pressure broadening:

Pressure broadening is also called as collisional broadening that occurs when the pressure of the atomic vapour is sufficient to cause a relatively large number of collisions that involve the emitting atoms. Similar to Doppler broadening, pressure broadening becomes greater with increase in temperature. Therefore, broader absorption and emission peaks are always encountered at elevated temperatures.

We have to distinguish between line source atomic absorption spectroscopy and continuous source atomic absorption spectroscopy. In classical line source, the high spectral resolution required for atomic absorption spectroscopy measurements is provided by the radiation source itself that emits the spectrum of the analyte in the form of lines that are narrower than the absorption lines. Continuum sources, such as deuterium lamps, are only used for background correction purposes. The advantage of this technique is that only a medium resolution monochromator is necessary for measuring atomic absorption spectroscopy, however, it has the disadvantage that usually a separate lamp is required for each element that has to be determined. In continuum source, in contrast, a single lamp, emitting a continuum spectrum over the entire spectral range of interest is used for all elements. Obviously, a high-resolution monochromator is required for this technique, as will be discussed later.

Hollow cathode lamps (HCL)

Hollow cathode lamps are the most common radiation source in line source atomic absorption spectroscopy. Inside the sealed lamp, filled with argon or neon gas at low pressure, is a cylindrical metal cathode containing the element of interest and an anode. A high voltage is applied across the anode and cathode, resulting in an ionization of the fill gas. The gas ions are accelerated towards the cathode and, upon impact on the cathode, sputter cathode material that is excited in the glow discharge to emit the radiation of the sputtered material, a typical machine will have two lamps and each lamp can handle 4-8 elements.

Electrodeless discharge lamps

Electrodeless discharge lamps contain a small quantity of the analyte as a metal or a salt in a quartz bulb together with an inert gas, typically argon gas, at low pressure. The bulb is inserted into a coil that is generating an electromagnetic radio frequency field, resulting in a low-pressure inductively coupled discharge in the lamp. The emission from electrodeless discharge lamps is

higher than that from an Hollow cathode lamps, and the line width is generally narrower, but electrodeless discharge lamps need a separate power supply and might need a longer time to stabilize.

Deuterium lamps

Deuterium hollow cathode lamps or even hydrogen hollow cathode lamps and deuterium discharge lamps are used in line source atomic absorption spectroscopy for background correction purposes. The radiation intensity emitted by these lamps decreases significantly with increasing wavelength, so that they can be only used in the wavelength range between 190 and about 320 nm.

Sample holder

Flame is used as sample holder, where in the burner, sample solution fuel & ordinary agents are passed through separate passages, to meet the opening of the base of flame. As the sample contains metallic element to be estimated by atomic absorption spectroscopy is a liquid, the flame breaks up the liquid sample into droplets which are then evaporated leaves the residue which is reduced to atoms.

Chopper

A rotating wheel is interposed between hollow cathode lamp and flame, to break the steady light from the lamp into an intermittent or pulsating light.

Atomizer

A flame atomizer consists of a pneumatic nebuliser, which converts the solution into a fine droplet like mist, or aerosol that is fed into a burner. Two types of atomisers or burners are available - Total consumption Burner and Premix Burner. In the total consumption burners, the aspirated sample and flame gases are separated but are mixed in the flame which is turbulent. In more commonly used premix burners, the sample and flame gases are mixed thoroughly before reaching the flame which is 'Laminar'. Flame used in AAS are given in the table… Fuels and oxidant employed in flame spectroscopy and the approximate range of temperature ranges with each of these mixtures also given in the table …. For heavy metal species, which are less readily excited, oxygen or nitrous oxide must be employed as the oxidant. These oxidants produce temperature of 2500ºC to 3100ºC with common fuels.

Flame Used in Atomic Absorption Spectroscopy

Fuels and oxidant	Temperature ^{0}C
Gas/Air	1700 – 1900
Gas/Oxygen	2700 - 2800
Hydrogen/Air	2000 - 2100
Hydrogen/oxygen	2550 - 2700
Acetylene/Nitrogen dioxide	2600 - 2800
Acetylene /Oxygen	3050 - 3100
Acetylene /Air	2100 – 2400

Specialized atomization techniques

While flame and electrothermal vaporizers are the most common atomization techniques, several other atomization methods are utilized for specialized use.

Glow-discharge atomization

A glow-discharge device (GD) serves as a versatile source, as it can simultaneously introduce and atomize the sample. The glow discharge occurs in a low-pressure argon gas atmosphere between 1 and 10 torr. In this atmosphere lies a pair of electrodes applying a DC voltage of 250 to 1000 V to break down the argon gas into positively charged ions and electrons. These ions, under the influence of the electric field, are accelerated into the cathode surface containing the sample, bombarding the sample and causing neutral sample atom ejection through the process known as sputtering. The atomic vapor produced by this discharge is composed of ions, ground state atoms, and fraction of excited atoms. When the excited atoms relax back into their ground state, a low-intensity glow is emitted, giving the technique its name.

Hydride atomization

Hydride generation techniques are specialized in solutions of specific elements. The technique provides a means of introducing samples containing arsenic, antimony, selenium, bismuth, and lead into an atomizer in the gas phase. With these elements, hydride atomization enhances detection limits by a factor of 10 to 100 compared to alternative methods. Hydride generation occurs by adding an acidified aqueous solution of the sample to a 1% aqueous solution of sodium

borohydride, all of which is contained in a glass vessel. The volatile hydride generated by the reaction that occurs is swept into the atomization chamber by an inert gas, where it undergoes decomposition. This process forms an atomized form of the analyte, which can then be measured by absorption spectrometry.

Cold-vapor atomization

The cold-vapor technique is an atomization method limited to only the determination of mercury, due to it being the only metallic element to have a large enough vapor pressure at ambient temperature. Because of this, it has an important use in determining organic mercury compounds in samples and their distribution in the environment. The method initiates by converting mercury into Hg^{2+} by oxidation from nitric and sulfuric acids, followed by a reduction of Hg^{2+} with tin(II) chloride. The mercury is then swept into a long-pass absorption tube by bubbling a stream of inert gas through the reaction mixture. The concentration is determined by measuring the absorbance of this gas at 253.7 nm. Detection limits for this technique are in the parts per billion.

Nebulizing of liquid sample

Before the liquid sample enters the burner, it is first of all converted into small droplets. This method of formation of small droplets from the liquid sample is called ‘Nebulisation’ and this is done by ‘Nebuliser’.

Monochromators

Monochromator is used to select a given absorbing line from spectral lines emitted from hollow cathode lamp. Hollow cathode lamp is used when high dispersion is essential. For resolving spectra such large dispersion and high resolving monochromator are advantageous. There are two types of monochromators that are used viz. Prism and Grating. Most common monochromators in atomic absorption spectroscopy are gratings and prism which is to select a given absorbing line from spectral lines emitted from hollow cathode lamps.

Detectors

PMT (Photomultiplier tube) is the most efficient detector. In the PMT, there is an evacuated envelope, which contains a photocathodes series of electrodes called dynodes and an anode. The photo cathode is fixed to the terminal of the power supply. As soon as the photon strikes the photocathode an electron is dialogued and photon is accelerated to Dynode (ten anodes), resulting in the liberation of more electrons. Thus, the current multiplied at each dynode

and the resultant electron current is received by the anode to produce an EMF across RL which goes to the external amplifier and read out system. Photomultiplier tube has good stability if used with a stable power supply.

Amplifier

Electric current from the photomultiplier detector is fed to the amplifier and amplifies the electric current in many times.

Read – out devices

In most of the atomic absorption measurement, chart recorders are used as read out devices. A chart recorder is a potentiometer using a servo meter to move the recording pen. In some Atomic absorption measurements digital read out devices are also used.

Operation

Electromagnetic radiation is provided by hollow cathode lamp which is coated with element to analyses and has anode and cathode. For analysis of Zn metal → Zn hollow cathode lamp and for Cd metal → Cd hollow cathode lamp. Hence difference cathodes are available for analysis of elements, which are made of same element. Vacuum is supplied inside the lamp. Radiation produced is the exact wavelength which is needed for promoting atom from ground to excited state.

First blank solution is sprayed, where there is no occurrence of absorption data, when the sample solution is sprayed, absorption occurs and radiation of absorbance is recorded.

Different methods of determination of elements:

I. **Calibration curve method**

A calibration curve is constructed using serial concentrations of standard solutions by plotting absorption in Y axis and concentration in X axis. From the calibration curve its evident that the relationship between absorbance and concentration. It's linear over the range of 2.0 to 10.0 ppm. But of higher concentration relationship is deviated from the linear line. **Disadvantage of this method is,** if interferences are present due to other elements, normal calibration curve cannot be constructed.

II. **Addition Method**

For the quantitative determination of elements. A known quantity of standard is added to sample solution. For example: For the analyses of lead, known concentration of standard solution of $PbNO_3$ is added to the assay sample.

Interferences in atomic absorption spectroscopy

Interference is a phenomena that leads to changes in intensity of the analyte signal in spectroscopy. Interferences in atomic absorption spectroscopy classified into two types. They are spectral and non-spectral interferences. Spectral interferences result in higher light absorption due to presence of light absorbing species other than the analyte and Non-spectral interferences that affect the formation of analyte items.

Spectral Interferences

Spectral interferences occur when two elements or an element and a poly atomic species in the cell absorb or emit radiation at wavelengths that are nearly identical. Spectral interference is caused by radiation overlap of absorption line due to emissions from another element or compound. Most common spectral interferences are due to molecular emissions from oxides of other elements in the sample. If an absorbing wavelength of an element, not being determined but present in the sample falls within the measuring line of the element of interest, the absorbance of the element will be measured together with the analyte of interest and give a higher absorbance value. Example: Vanadium line is 3082.11 A and Aluminium is at 3082.15 A. Choose a different Aluminium line at 3092.7 A. we should note that Interference due to overlapping lines is rare in AAS; Radiation interference in the determination of Na with Mg present, and in the determination of Iron with Cu or Ni present. In such case we have to use alternate wavelength or Use smaller slit width or Use blank.

Non-spectral interferences

Scattering

This is caused by scattering of the radiation source due to matrix impurities. Scattering as a spectral interference arise when particulate matter from flame atomization scatter the incident radiation from the source. Incomplete combustion of organic materials which give rise to molecular species that exhibits broad band spectra. The absorption and scattering of radiation by flame atomization can be corrected by analyzing a blank. Example: Refractory oxides formed by Ti, Zr, and W due to atomization of high concentration solutions. Using bank can solve this problem

Background Absorption

Background interferences is also known as nonspecific interferences usually caused by absorption of polyatomic species or scattering of radiation within the cell. Background absorption extends over a broad wavelength band. It is also referred to as Molecular absorption, Broadband absorption or Non specific absorption. Background absorption is caused by light absorption due to unvaporised solvent droplets in flame. It is also caused by absorption of unknown molecular species in flame. The absorbance and scattering of radiation due to matrix interference give rise to sample background which becomes a problem at wavelength below 350nm. Using Background correction methods will eliminate this interference. A deuterium source lamp is used for background correction which comes already fitted into the Atomic absorption instrument by Manufacturer. Background Absorption as an interferent cannot be corrected by Standard Addition method.

Matrix interference

Matrix interference is a physical interference, and can either suppress or enhance absorbance signal of analyte. It occurs when components of sample matrix other than the analyte react to form molecular species and sample background. When a sample is more viscous or has different surface tension than the standard it can result in differences in sample uptake rate due to changes in nebulization efficiency or when the sample and standards are prepared in different solvents or when the sample and standards are measured at different temperatures or when the sample contains a high concentration of dissolved salts or acid or when organics are present in sample matrix or when sample and standards differ in aspiration and atomization rate in flame. Such interferences are minimized by matching as closely as possible the matrix composition of standard and sample or by using a blank to zero instrument or using standard addition or using background correction.

Chemical interference

Chemical interferences are those that arise from chemical reactions that occur within the cell. If a sample contains a species which forms a thermally stable compound with the analyte that is not completely decomposed by the energy available in the flame then chemical interference exists. Refractory elements such as Ti, W, Zr, Mo and Al may combine with oxygen to form thermally stable oxides. Analysis of such elements can be carried out at higher flame temperatures using nitrous oxide – acetylene flame instead of air-acetylene to provide higher dissociation energy. Alternately an excess of another element or compound can be added e.g. Ca in presence of

phosphate produces stable calcium phosphate which reduces absorption due to Ca ion. If an excess of lanthanum is added it forms a thermally stable compound with phosphate and calcium absorption is not affected.

Ionization interference

Ionization interference occurs when a significant proportion of atoms in the cell become ionized. It happens only in hot flames. The dissociation process does not stop at formation of ground state atoms. Excess energy of the flame can lead to excitation of ground state atoms to ionic state by loss of electrons thereby resulting in depletion of ground state atoms. In low temperature flames such interference is encountered with easily ionized elements such as alkali metals and alkaline earths. Ionisation interference is eliminated by adding ionization buffer (which ionizes to give a mass of electrons that shift the ionization equilibrium of analyte to form atoms) an excess of an element which is easily ionized thereby creating a large number of free electrons in the flame and suppressing ionization of the analyte. Salts of such elements as K, Rb and Cs are commonly used as ionization suppressants.

THIRD YEAR PHARM.D-3.2-Pharmaceutical Analysis Theory

UNIT 4.1 Ultra Violet and Visible spectroscopy

Introduction

Electronic transitions

Chromophores and Auxochromes

Shifts in UV visible spectroscopy

Effect of solvents on absorption spectra

Beer Lambert's Law and their derivation. Deviation of the beer lamberts law

Instrumentation of UV Visible spectrophotometer

Applications

Ultra violet and visible spectroscopy

Introduction

In analytical chemistry, light and other forms of electromagnetic radiations are widely used for the measurements. Instruments ranges from the simple flame photometers to spectrophotometers and nuclear magnetic spectrometers which are widely used in the field of pharmaceutical analysis for structural elucidation and quantitative estimation of drugs in the bulk and pharmaceutical formulations.

The basis of the ultraviolet (190-380nm) and visible (380-800nm) spectroscopy is the absorption of light energy by the sample containing conjucated double bonds, or unsaturation or pi bonds. The basic requirement of the molecule is to absorb UV and visible light by unsaturation character present in the compound. After the absorption of electromagnetic radiation by the ground state molecules, elevated to the excited state. The measurement of amount of absorbed light radiation by the ground state sample is calculated by measuring the unabsorbed light, otherwise known as transmitted light, by the detector.

Electronic transitions

Electrons in the outer most molecular orbital are promoted to higher energy level. As a rule, energetically favoured electron promotion will be from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO), and the resulting species is called an excited state Organic compounds with π electron only will absorb UV visible radiation and excited.

There are several types of electronic transitions available to a molecule including:

σ to σ * (alkanes)

σ to π * (carbonyl compounds)

π to π * (alkenes, carbonyl compounds, alkynes, azo compounds)

n to σ * (oxygen, nitrogen, sulfur, and halogen compounds)

n to π * (carbonyl compounds)

σ to σ * **Transitions**

An electron in a bonding σ orbital is excited to the corresponding antibonding orbital. The energy required is large. For example, alkanes as methane (which has only C-H bonds and can only undergo σ to σ * transitions) shows an absorbance maximum at 125 nm. Absorption maxima due to σ to σ * transitions are not seen in typical UV-Visible spectra (200 - 700 nm). σ bonds are very strong and requires higher energy of vacuum UV.

n to σ * **Transitions**

Saturated compounds containing atoms with lone pairs (non-bonding electrons) are capable of n to σ * as transitions. These transitions usually need less energy than σ to σ * transitions. They can be initiated by light whose wavelength is in the range 150 - 250 nm. The number of organic functional groups with *n to* σ * peaks in the UV region is small. These transitions are involved in saturated compound with one hetero atom with unshared pair of electron i.e. saturated halides, ethers, aldehyde, ketones, amines etc.

These transitions are sensitive to hydrogen bonding, for example, alcohol and ethers which absorbs at wavelength shorter than 185 nm therefore used as a solvent in UV

π to π * **Transitions**

For molecules that possess π bonding as in alkenes, alkynes, aromatics, acyl compounds or nitriles, energy that is available can promote electrons from a π Bonding molecular orbital to a π Antibonding molecular orbital (π *). This is called a π to π * transition. The absorption peaks for these transitions fall in an experimentally convenient region of the spectrum (200 - 700 nm). These transitions need an unsaturated group in the molecule to provide the π electrons.

n to π * **Transitions**

Even lone pairs that exist on Oxygen atoms and Nitrogen atoms may be promoted from their non-bonding molecular orbital to a π antibonding molecular orbital within the molecule. This is called an n to π * transition and requires less energy (longer wavelength) compared to a n to π * transition within the same chromaphore. These are available in compounds with unsaturated centers, for example, Alkenes. They require lowest energy as compare to others

Chromophore

A covalently unsaturated group responsible for electronic absorption. Or Any group of atoms that absorbs light whether or not a color is thereby produced. Example c=c, c=o, No_2. A compound containing chromophore is called **chromogen.**

There are two types of chromophore

- Independent chromophore: single chromophore is sufficient to import color to the compound, for example, Azo group
- Dependent chromophore: When more than one chromophore is required to produce color. Example acetone having 1 ketone group is colorless whereas diacetyl having two ketone group is yellow

Auxochrome

A saturated group with non-bonding electron when attached to chromophore alters both wavelengths as well as intensity of absorption. Example: OH, NH_2, NHR etc.

OR A group which extends the conjugation of a chromophore by sharing of nonbonding electrons.

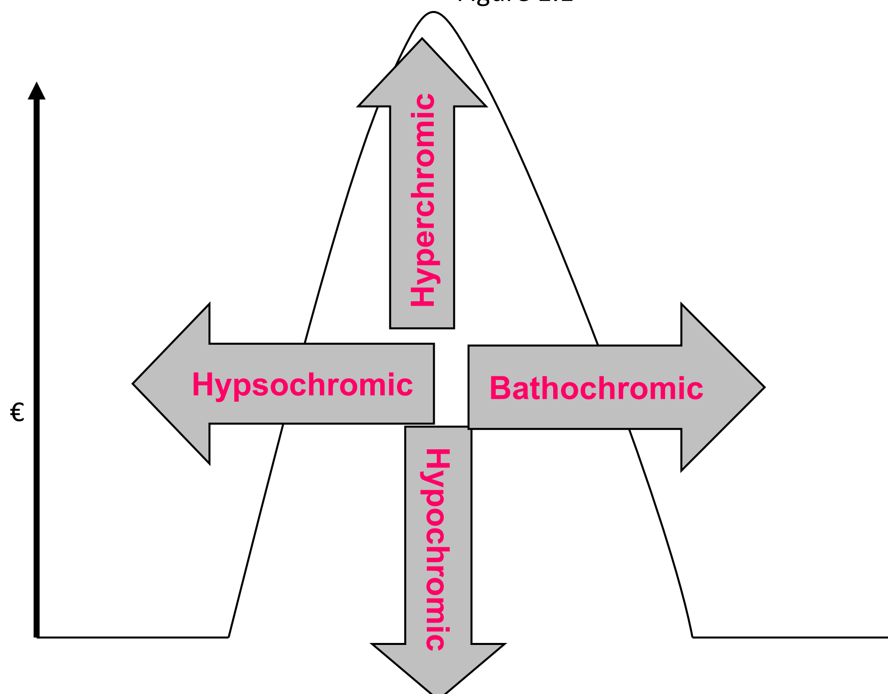
Bathochromic group: The group which deepen the colour of chromophore is called bathochromic group. Eg. Primary, secondary and tertiary amino groups

Shifts in UV Visible spectroscopy

The UV Visible spectrum, shows same pattern of graph, for a compound or drug, when it is dissolved in a same solvent and having same concentration. At the same time, if the concentration changes, the absorbance will also be changed at the …max. If concentration is more, then it is observed that there is more absorbance and if the concentration is less, then there is less absorbance at …max. When there is any change in pH, the spectrum may be shifted towards right side or left side according to the interaction of molecules with solvent. These phenomena are referred as shifts in UV visible spectroscopy, there are 4 shifts are possible, they are

- Bathochromic shift: (Red shift) shift of lambda max to longer side or less energy is called bathochromic shift or read shift. This is due to substitution or solvent effect.
- Hypsochromic shift: (Blue shift) shift of lambda max to shorter side and higher energy is called hypsochromic or blue shift. Eg solvent effect.
- Hyperchromic effect: an increase in absorption intensity
- Hypochromic effect: a decrease in absorption intensity

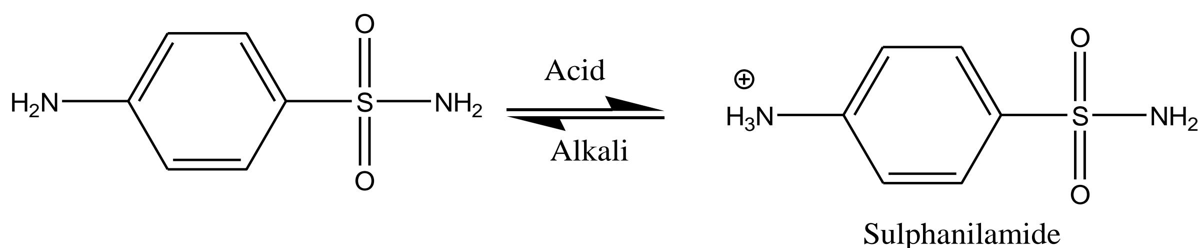
Figure 1.1



200 nm 700nm

Effect of solvents on absorption spectra

With the basic idea of the various shifts, the compound will also show the different pattern of spectrum, when the compound is placed in the different environment. Especially when the solvent is changed, one can observe the difference in shape of the UV spectrum from the normal spectrum due to the chemical interaction between the solvent and the compound. Foe example: Phenol in cyclohexane has slight interaction between solvent and solute arises and vibrational fine structure is observed. Phenol in water shows salvation of solute and hydrogen bonding are possible so that the fine structure is abolished, only the band envelope being attained. Similarly, if two solvents are aqueous and differ only in their ionic character, for example, Different strengths of the same buffer (salt) solution, it is possible for a 'salt effect' to cause slight differences in the absorption band of a compound. In the case of Sulphanilamide in alkaline solution, the primary amino group is retained as auxochrome. But the Sulphanilamide in acidic solution quaternisation occurs to a coordinately saturated auxochrome. This is much less effective in modifying absorption and characteristic benzenoid absorption is obtained at 265nm. 10mg of sulphanilamide in 1ml of Hcl (pH>1) shows λmax at 220nm whereas sulphanilamide in 1ml of NaOH (pH<13) shows λmax at longer wavelength at 240nm.



Sulphanilamide

Note that the solvent dimethyl formamide (DMF) absorbs radiation below about 240nm so that the readings are unreliable below 240nm, if DMF is chosen as choice of solvent. Similarly, the solution of sodium hydroxide also absorb radiation below 230 nm. The following table explains about the cut off wavelength for the solvents used in the UV visible spectroscopy.

Solvent	Minimum Wavelength (nm)
acetonitrile	190
water	191
cyclohexane	195
hexane	195
methanol	201
ethanol	204
ether	215
methylene chloride	220
chloroform	237
carbon tetrachloride	257

Beer Lambert's Law and their derivation

When a beam of monochromatic light is passed through a sample solution of an absorbing substance, reduction of the intensity of the incident light may occur. The reduction is due to reflections of outer and inner surfaces of the transparent cell, scatter by undissolved particles or dust in the solution and absorption of photons by molecules present in the solution. The amount of light absorbed by the molecule is known by measurement of transmitted light (I_T) using suitable detector. Lambert developed the relationship between incident light and transmitted light for different thickness of the substance and concluded that the rate of decrease in the intensity of light with thickness, b, of the medium is proportional to the intensity of incident light (I_0). Beer's law states that the intensity of beam of parallel monochromatic radiation declines exponentially with the number of absorbing molecules. In other words, the absorbance is directly proportional to the concentration of absorbing molecule present in the sample. The combination of both laws yields Beer-Lambert Law.

The Beer-Lambert law tells us quantitatively how the amount of attenuation depends on the concentration of the absorbing molecule and the path length over which absorption occurs. As the light traverses a medium containing an absorbing analyte, decreases in intensity occur as the analyte becomes excited. The transmittance T of the solution is the fraction of incident radiation transmitted by the solution, as shown in the Equation 1.1. Transmittance is often expressed as a percentage known as percent transmittance.

$$T = I_T/I_0 \qquad 1.1$$

The absorbance A of a solution related to the transmittance in a logarithmic manner, as shown in the Equation 1.2. One can find out that as the absorbance of a solution increases the transmittance decreases.

$$A = -\log T = \log I_0/I_T = abc \qquad 1.2$$

According to the above laws, the absorbance is directly proportional to concentration of the absorbing species *c* has the units of g/100ml or mg/ml and *b* has the units of cm, absorptivity *a* depends on the units of concentration. When *c* is the moles per litre, the constant is called molar absorptivity and has the symbol ϵ. The equation therefore takes the form

$$A = \epsilon bc \qquad 1.3$$

Another form of Beer Lambert proportionality constant is the specific absorbance, which is the absorbance of a specific concentration in a cell of 1 cm pathlength. Most common and widely used form in pharmaceutical analysis is the A (1%, 1 cm), which is equal to absorbance of a 1 gm/100ml (1% w/v) solution in 1 cm cell. The Beer-Lambert equation therefore takes the form. The units of A (1%, 1 cm) is dl g-1 cm-1

$$A = A_{1cm}^{1\%}\ bc \qquad 1.4$$

Deviation of the beer lamberts law

There are few exceptions to the linear relationship between absorbance and path length at a fixed concentration. Since path length *b* is constant, we frequently observe difference from the direct proportionality between absorbance and concentration. Some of the deviations are

- Real or True Deviation-It is the fundamental and represent the real limitation to the law

- Instrumental deviation -Result of the method that we used to measure the absorbance
- Chemical deviation- Result of chemical changes, that occur when concentration changes

The true deviation is due to the law tends to break down at very high concentrations, especially if the material is highly scattering. If the light is especially intense, nonlinear optical processes can also cause variances. Dilute solutions only follow the law. The reason is when concentration is high the molecule will react or interact with each other by collision and each molecule will not absorb radiation energy in same manner as it does in dilute solution. The index of refraction of absorbing species changes at high concentration. If the concentration is directly proportional to the absorbance, it shows the linearity (b). When increase the absorbance with solution of less concentration, shows the positive deviation (a) in other words, when a small change in concentration produces greater change in absorbance. When the absorbance is decreases or static with increasing concentration, shows negative deviation (c) in other words, when a large change in concentration produces small change in absorbance. The law strictly followed for the dilute solutions, as concentration increases causes deviation

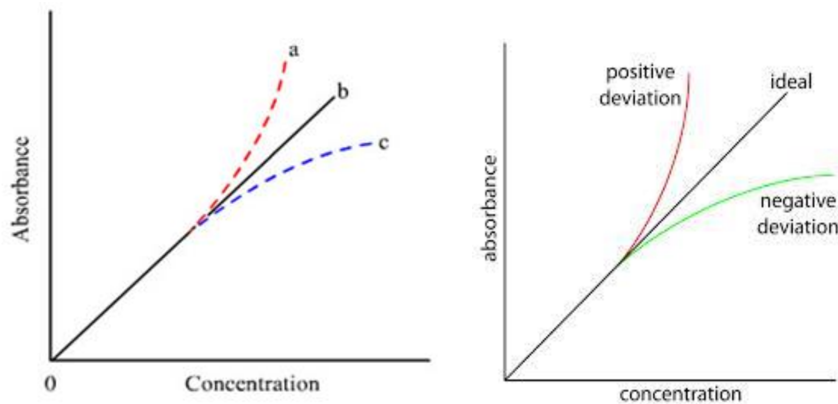


Figure 1.2 Real deviation of Beer lambert law.
a is the positive deviation, b is the linear and c is the negative deviation.

Instrumental deviation includes, stray radiations reaching the detector, when sensitivity changes of detector employed is occur, fluctuations of radiation source, defect in radiation amplifications system, working in broad band of wavelengths rather than monochromatic radiations. Stray radiation often is the result of scattering and reflection off the surfaces of gratings, lenses or mirrors, filters and windows. Usually the wavelength of the stray radiation is very different from the wavelength band selected. It is known that radiation exiting from a monochromator is often contaminated with minute quantities of scattered or stray radiation. If the analyte absorbs at the wavelength of the stray radiation, a deviation from Beer-Lambert law is observed similar to the deviation due to polychromatic radiation. When the measurements are made in the presence of stray light, the observed absorbance is given by

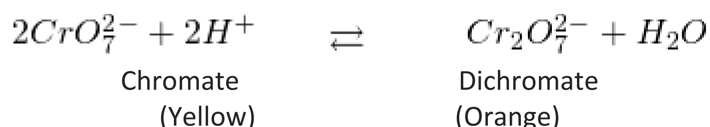
$$A' = \log I_0 - I_s / I_T - I_s \qquad 1.5$$

Where I_s is the radiant power of the stray light.

Deviation due to mismatched Cells or Cuvettes: If the cells holding the analyte and the blank solutions are having different path-lengths, or unequal optical characteristics, it is obvious that there would be a deviation observed in Beer-Lambert law.

Chemical deviations involve that, absorbing species may undergo ionization, dissociation or even may react with solvent or each other and these reactions may produce two or more species in solution with varying absorptivity values. These deviations also due to effect of pH, temperature and time factor for colored solutions (color may fade by due to deterioration of organic color by oxidation, reduction or hydrolysis) These deviations can be corrected by using buffers, selecting suitable solvent and appropriate band of wavelengths.

Some absorbing molecules undergo change in colour with change in pH of the solvent medium. An example is Phenol red which changes colour from yellow in acidic media to red in basic media due to internal migration of proton accompanied by a switch between a single and an adjacent double bond. Another example is aqueous solution of potassium dichromate which changes from yellow to orange on increasing the concentration of hydrogen ions due to chromate – dichromate conversion



Chromate (Yellow) Dichromate (Orange)

Instrumentation

Photometers or spectrometers in the ultraviolet and visible spectral region either are built only for the measurement of light absorption, fluorescence. reflection. or scattering. or have a modular con-struction so that they can be used for several meas-uring operations. Spectrometers always include a polychromatic light source, a monochromator for the spectral resolution of the beam. a sample hold-er, and a detector for measuring the radiation that has been modified by its passage through the sam-ple. Depending on the measuring problem. these components can be modified. and the light path changed. In the measurement of absorption, the radiation is measured against a reference for each monochromatic wave-length after attenuation by a sample. By this reference measurement, such effects as reflections at the cell windows or other optical surfaces can be corrected for, if sample and reference differ only with respect to the substance being examined and are otherwise optically identical. The equipment can be of the single- or double-beam type. In single-beam equipment, radiation of the selected wavelength is passed alternately through the reference cell and the sample containing cell before it strikes the detector. In older equipment, the position of the sample holder in the cell compartment is adjusted manually. Usually, only a small number of wavelengths are measured because a spectrum cannot be recorded automatically.

Photometers can be of the broadband type (broad spectral band light source and filter) or the narrow-band type (line spectrum light source and filter). In more expensive equipment, continuous radiation sources (broadband) are used, and the filter is replaced by a monochromator. so that a complete spectrum can be observed point by point. Only a very good monochromator can give resolution as good as that from a combination of a line source and filter since in the latter system, the very small width of the natural line determines the bandwidth of the radiation used.

Single-Beam Equipment: Modern single-beam equipment first records a spectrum of the standard in the reference optical path and then the spectrum of the sample. The internal microprocessor serves not only for control but also for calculating the intensity ratio according to the Beer -Lambert Law. The optical light path of such equipment is shown in Figure. This equipment has fewer optical components and is less mechanically complex (no beam splitter) than the double beam spectrometer, but the time required for each sample measurement is longer (successive

measurements of dark. reference, and test spectra). Good stability of the light source and of the electronics is necessary to avoid any difference between measurements.

Double-Beam Equipment: In double-beam equipment, the two light paths (through the sample and the reference) are automatically continuously interchanged during the wavelength scan. A rotating minor, oscillating mirror, or half-transparent mirror splits the beam after it emerges from the monochromator. However. for accurate measurement, at each wavelength, the "dark" signal must be observed as well as that for the reference cell and the sample.

Sample containers:

The cell or cuvettes that hold the samples must be made of material that is transparent to radiation in the region. Quartz or fused silica is required for UV region (blow 350 nm). Silicate glass can be employed in region between 350 and 2000 nm. Plastic containers are also available for visible region.

PROPER SELECTION, USE, AND CARE OF CUVETTES: Cuvettes are expensive, fragile laboratory items. It is important that one should use them properly and carefully. Use quartz cuvettes for UV work. Glass, plastic or quartz are acceptable for work in the visible range. There are inexpensive plastic cuvettes that are suitable for some UV work. Disposable cuvettes are often recommended for colorimetric protein assays, since dyes used for proteins tend to stain cuvettes and are difficult to remove. Matched cuvettes are manufactured to absorb light identically so that one of the pair can be used for the sample and the other for the blank. Do not touch the base of a cuvette or the sides through which light is directed. Do not scratch cuvettes, do not store them in wire racks or clean with brushes or abrasives. Do not allow samples to sit in a cuvette for a long period of time. Make sure the cuvette is properly aligned in the spectrophotometer. Wash cuvettes immediately after use

Light Sources

Line Sources

In filter photometers, the spectral bandwidth can be improved considerably if line sources are combined with filters. Mercury line sources emit various wavelengths. Such equipment is used mainly in clinical analysis, because in general, single wavelengths are only used for photometry or in simple kinetic experiments. Mercury vapor arcs are also employed in polarimeters.

Sources of Continuous Radiation

In spectrometers, a light source supplying continuous radiation must be available. Normally, a high-radiant power source that emits in the visible range (tungsten-halogen lamp) is combined with an ultraviolet emitter (hydrogen or deuterium lamp) to provide a wider spectral range.

Tungsten-Halogen Lamps: By addition of iodine to incandescent lamps a higher filament temperature can be reached, and consequently, a higher radiation density at the shortwave end of the spectrum. The emission maximum of a tungsten -halogen lamp is in the green part of the spectrum.

Hydrogen and Deuterium Lamps: Deuterium lamps. whose emission maximum is at200 nm, are used mainly in sequential spectrometers. The emission maximum of hydrogen lamps is at a longer wavelength, at more than 400 nm.

Selection of Wavelengths

In simple equipment, only relatively wide transmission bands are selected by means of coloured glass or interference filters. A working beam of narrow bandwidth is obtained by combining this with a mercury source. Interference filters give spectral bandwidths in the range of 3 to 10 nm.

Prism Monochromators: Prisms are relatively expensive and very difficult to manufacture in the highest optical quality. Dispersion by diffraction is not linear. Prism monochromators are mechanically very complex and therefore very expensive. They were at one time used widely in the UV region. Today they are often used as pre-monochromators (pre-dispersion devices) since the decomposition of white light into rainbow colours by a prism gives only one spectrum order.

Grating Monochromators

In the dispersion spectrum produced by gratings, several wavelengths of different orders appear at the same place. Incident while light is reflected at a number of grooves that are either ruled mechanically on a plane surface or produced holographically by superposition of two laser rays. The reflected beams interfere in different ways, depending on their wavelength, the distance between lines of the grating, and the angle of reflection. so that constructive or destructive interference can occur. Thus, reinforcement at a given wavelength takes place only in a certain direction. The greater the number of grooves, the more do the partial beams interfere. and the more significant is the destructive interference at certain wavelengths. The dispersion of the grating is almost linear. However, as already mentioned, individual orders overlap so that either an order filter must be fitted or a pre-dispersion device (prism) must be used. If the grooves are blazed, reflected intensities of mainly one order can be collected. This is at a maximum for a particular angle (blaze angle) and a particular wavelength (blaze wavelength). An inlet slit is usually located in front of the prism or grating symmetrical to the exit slit. If the inlet and exit slits are coupled and have the same width, the slit function forms a triangular intensity distribution. In high-quality equipment, the first monochromator is followed by a second. These double monochromators have con-siderably better resolving power.

Detectors

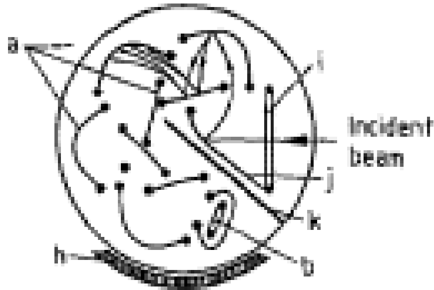
Properties of Ideal detectors are it should be high sensitivity, and high signal-to-noise ratio. It should give constant response over a considerable range of wavelengths. It is necessary to exhibit fast response time and zero output signal in the absence of illumination.

Detectors are of mainly two types. based on either the external or the internal photoelectric effect.

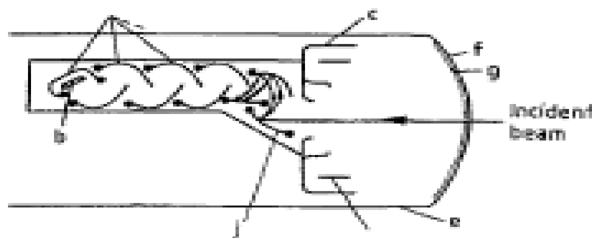
Photovoltaic cells (Barrier-Layer cells): In this the external photoelectric effect is used. It is widely used in visible region, has max sensitivity at about 550nm. It consists of flat copper or iron electrode upon which a layer of semiconductor material such as selenium is deposited. The outer surface is coated with a thin transparent metallic film of gold or silver, which serves as a collector and it is protected by a transparent envelope. When radiations of sufficient energy reach the semiconductor, covalent bonds are broken, with the result of electron and holes are formed. The migration of electrons produces an electrical current of magnitude which is proportional to the number of photons that strike the semiconductor surface. It is a rugged, low cost detector, does not required any external sources of energy. But he amplification of output is less convenient.

Phototubes (Vacuum photo tubes): It consists of semi cylindrical cathode and a wire anode sealed inside an evacuated transparent envelope. The concave surface of the electrode supports a layer of photo emissive material that tends to emit electron when it is irradiated. The emitted electrons flow to the wire anode generating a photocurrent that is equal to the amount of radiation fallowing on it. This results in increase of potential applied across the two electrodes. Phototube generally operates at 90V. They produce a small dark current that results from thermally inducted electron emission.

Photomultipliers: In photomultipliers, the external photoelectric effect is utilized. Photons incident on a photocathode, which have an energy greater than the work function of cathode material expel electrons, which are captured by an anode that is positively charged relative to the cathode. The number of electrons produced is proportional to light intensity. They can be measured by the current flowing between electrodes.



Dynodes are often placed between the photocathode and the anode as intermediate electrodes. High-quality photomultipliers have 10 to 14 dynodes. This internal amplification is critical because in addition to electrons from the light-induced process. other thermally activated electrons give rise to a dark current. Photomultipliers are therefore often cooled for the measurement of low light intensities. The head-on arrangement (Fig. 1…A) has a larger inlet angle and is extended lengthwise. with a correspondingly greater distance separating the dynodes (lower field strengths). It can accommodate more dynodes than the side-on arrangement, and therefore has a greater range of amplification. However, response times are longer. The side-on arrangement (Fig. 1…B) is considerably more compact, the distances between dynodes are shorter and the response times are relatively short. However, the "capture angle" is smaller. For detecting only a small number of photons, the more slowly reacting head-on photomultiplier is preferable.



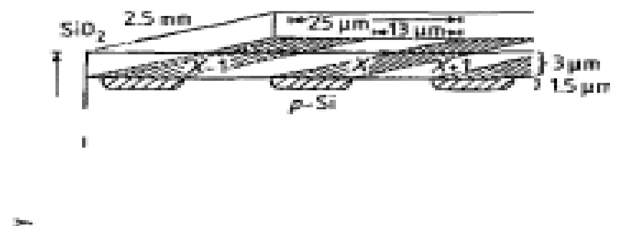


Figure 1.3 **A**

Figure 1.3 **B**

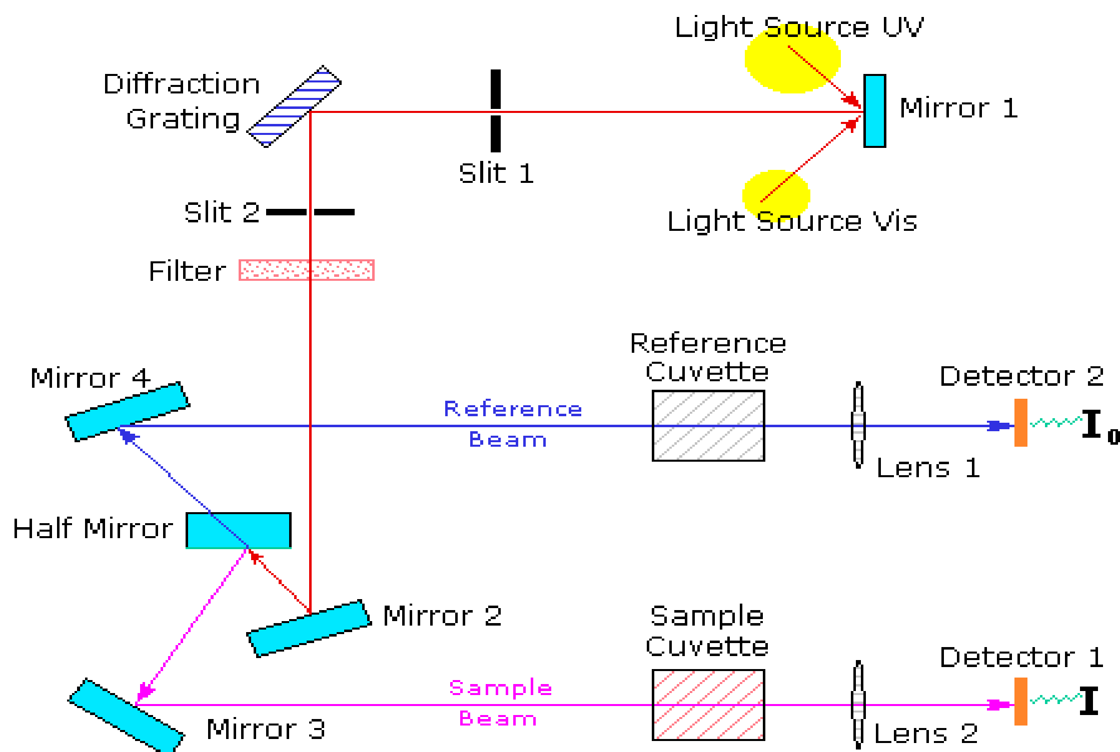
Figure 1.3 Types of photomultiplier

1.3 A) Head-on arrangement: 1.3 B) Side-on arrangement (on a larger scale)

a) Dynodes b) Anode c) Focusing electrode d) Focusing ring e) Conductive internal coating f) Front plate g) Semi-transparent photocathode h) Envelope i) Grill j) Opaque photocathode k) Shield

Photodiodes: on the other hand, use the internal photoelectric effect. When these diodes are arranged in arrays, they are known as diode arrays or diode matrices. Multiplex detectors are used in simultaneous spectrometers.

An example is the Reticon array, CCD arrays (charge coupled devices), which were developed for television cameras and fax machines, have also become important in spectroscopy. Apart from the advantage of being multiplex devices, these charge transfer components have characteristics more similar to those of photomultipliers than normal photodiodes.



Applications

UV spectra and Visible spectra can be used to identify an unknown compound by a comparative analysis. One can compare the UV or Visible spectra of the unknown with the spectra of known suspects. Those that match is evidence that they may be one and the same. However, using a match on UV or Visible is not conclusive.

UV and Visible spectra can also be used to determine the concentration of a chromophore compound in a mixture using the Beer Lambert Law. Usually 3-5 standard concentration of the chromophore are prepared, and the absorbance of each standard is measured. The Absorbance can be plotted against the concentration and a standard curve can be generated. By measuring the absorbance of the unknown mixture, one can locate the absorbance on the "y" axis of the Standard curve, draw a straight line over till it intersects the standard curve and then a perpendicular down until it intersects the "x" axis. At the point of intersection on the "x" axis, the concentration of the chromophore can be determined.

One can also use a proportional method where using the Absorbance of a known concentration of respective sample, and the Absorbance of the unknown sample can be measured.