25 FLAME SPECTROSCOPY

CONTAINS :

25.1. INTRODUCTION

Metallic salts (or metallic compounds) after dissolution in appropriate solvents when introduced into a flame (for instance : acetylene burning in oxygen at 3200°C), turns into its vapours that essentially contain mostly the atoms of the metal. Quite a few such gaseous metal atoms are usually raised to a particular high energy level that enables them to allow the emission of radiation characteristics features of the metal : for example-the characteristic flame colourations of metals frequently encountered in simple organic compounds such as : Na-yellow, Ca-brick-red ; Ba-apple-green. This forms the fundamental basis of initially called **Flame Photometry,** but more recently known as **Flame Emission Spectroscopy (FES).**

It is quite evident that a relatively large proportion of the gaseous metal atoms shall remain in the ground state *i.e.,* in an unexcited form. It has been observed that such ground-state atoms shall absorb radiant energy pertaining to their own particular resource wavelength. Therefore, when a light having the same resonance wavelength is made to pass through a flame consisting of such atoms, a portion of the light shall be absorbed accordingly. Furthermore, the extent or degree of absorption would be directly proportional to the total number of ground-state present in the flame. And this is the basis of **Atomic Absorption Spectroscopy** (AAS).

The emission spectrum thus obtained is made up of a number of lines that actually originate from the resulting excited atoms or ions ; and these steps may be shown diagrammatically as represented in Figure 25.1.

Figure 25.1 : Diagrammatic Representation of Emission Spectrum from excited Atoms or Ions.
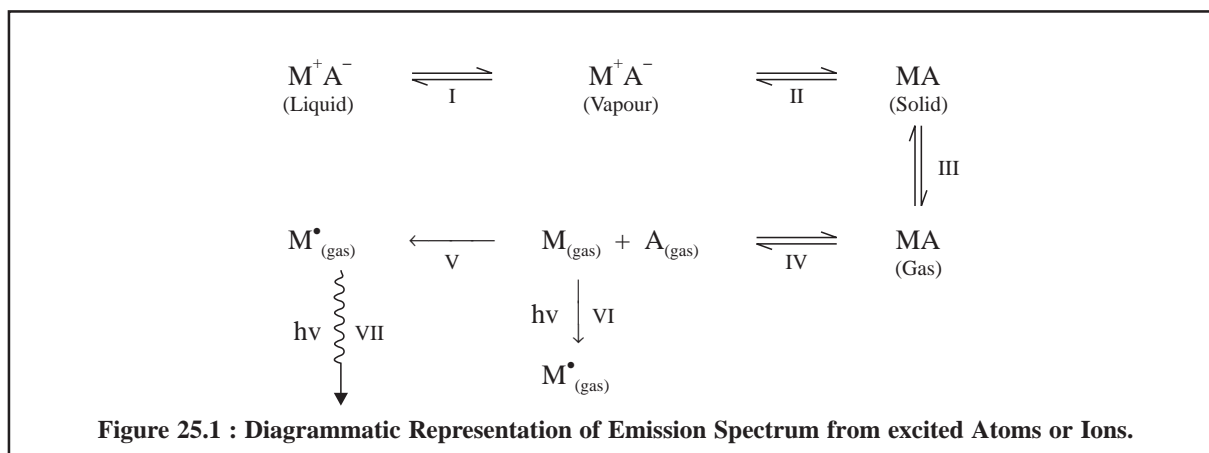
The various steps (I to VII) in Figure 25.1, above are explained as under :

Step-I   : The liquid sample containing a suitable compound of the metal ($M^+ A^-$) is aspirated into a flame, thereby converting it into its vapours or liquid droplets,

Step-II   : The evaporation of vapours (or droplets) give rise to the corresponding solid residue,

Step-III   : The vapourization of the solid residue into its gaseous state occurs,

Step-IV   : The dissociation of the gaseous state into its constituent atoms, namely : $M_{(gas)} + A_{(gas)}$ take place, that initially, is in ground state,

Step-V   : The thermal excitation of some atoms into their respective higher energy levels will lead ultimately to a condition whereby they radiate energy (flame emission) measured by Flame Emission Spectroscopy (FES), and

Step-VI   : The absorption of radiant energy by some atoms into their higher energy levels enable them to radiate energy (atomic absorption) measured by Atomic Absorption Spectroscopy (AAS).

25.2. THEORY

The underlying principle of **Flame Emission Spectroscopy (FES)** may be explained when a liquid sample containing a metallic salt solution under investigation is introduced into a flame, the following steps normally take place in quick succession, namely :

(*i*) the solvent gets evaporated leaving behind the corresponding solid salt,

(*ii*) the solid salt undergoes vaporization and gets converted into its respective gaseous state, and

(*iii*) the progressive dissociation of either a portion or all of the gaseous molecules gives rise to free neutral atoms or radicals.

The resulting neutral atoms are excited by the thermal energy of the flame which are fairly unstable, and hence instantly emit photons and eventually return to the ground state (*i.e.,* the lower energy state). The resulting emission spectrum caused by the emitted photons and its subsequent measurement forms the fundamental basis of FES.

Bohr's Equation : If we consider two quantized energy levels *e.g.,* higher as E_2 and lower as E_1, the radiation given out during the transition from E_2 to E_1 may be expressed by the following equation :

$$E_2 - E_1 = h\,v \qquad\qquad ...(a)$$

where, h = Planck's constant, and

v = Frequency of emitted light,

now, the frequency v may be defined as follows :

$$v = c/\lambda \qquad\qquad ...(b)$$

where, c = Velocity of light, and

λ = Wavelength of the absorbed radiation.

Combining equations (*a*) and (*b*) we have :

$$E_2 - E_1 = hc/\lambda$$
$$\lambda = hc/E_2 - E_1 \qquad\qquad ...(c)$$

The expression (*c*) is the **Bohr's equation** which enables us to calculate :

• Wavelength of the emitted radiation which is characteristic of the atoms of the particular element from which it was initially emitted,

- Wavelength of radiation given out from a flame is indicative of the element(s) that might be present in that flame, and

- Intensity of radiation may quantify the exact amount of the elements present.

Boltzmann Equation : The fraction of free atoms which are excited thermally, or in other words, the relationship between the ground-state and the excited-state quantum is exclusively represented by the Boltzmann equation given below :

$$N_1/N_0 = (g_1/g_0)\ e^{-\Delta E/kT} \qquad\qquad\qquad ....(d)$$

where, N_1 = Number of atoms in the excited state (high energy level),

   N_0 = Number of ground state atoms,

 g_1/g_0 = Ratio of statistical weights for ground and excited states,

    E = Energy of excitation (= $h\upsilon$),

    k = The Boltzmann's constant, and

    T = Temperature (in Kelvin).

Form equation (d) it may be observed that :

- Fraction of atoms excited (N_1) solely depends upon the temperature of the flame (T), and

- Ratio N_1/N_0 is dependent upon the excitation energy (ΔE).

Therefore, the fraction of atoms excited critically depends on the temperature of the flame thereby emphasizing the vital importance of controlling the temperature in **Flame Emission Spectroscopy (FES).**

25.3. INSTRUMENTATION

There are *two* types of **Flame Photometers** that are used invariably in *Flame Emission Spectroscopy* (FES), namely :

(*a*) Simple Flame Photometer, and

(*b*) Internal Standard Flame Photometer.

These *two* typical instruments shall be discussed briefly here highlighting their various components and procedural details.

25.3.1. SIMPLE FLAME PHOTOMETER

The line-sketch of a simple flame photometer is shown in Figure 25.2.

In general, Flame Photometers are designed and intended mainly for carrying out the assay of elements like : Sodium, Potassium, Calcium, and Lithium that possess the ability to give out an easily excited flame spectrum having sufficient intensity for rapid detection by a photocell.

Procedure : The compressed and filtered air (A) is first introduced into a Nebulizer (E) which creates a negative pressure (suction) enabling the liquid sample (C) to gain entry into the atomizer (E). Thus, it mixes with the stream of air as a fine droplet (mist) which goes into the burner (G). The fuel gas (D) introduced into the mixing chambers (F) at a given pressure gets in touch with the air and the mixture is ignited. Consequently, the radiation from the resulting flame (H) is made to pass through a convex lens (I) and ultimately through an optical filter (J) that allows specifically the radiation characteristic of the element under examination to pass through the photocell (K). Finally, the output from the photocell is adequately amplified (L) and subsequently measured on an appropriate sensitive digital-read-out device.
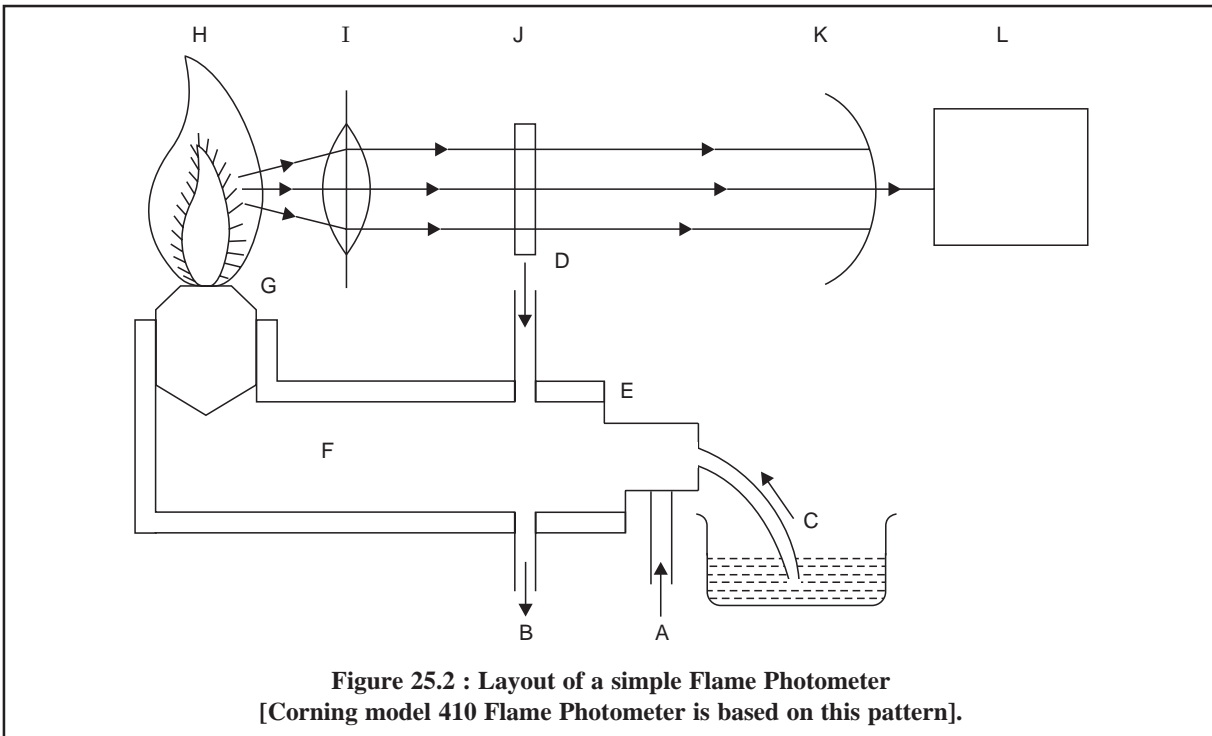
Figure 25.2 : Layout of a simple Flame Photometer
[Corning model 410 Flame Photometer is based on this pattern].

A = Inlet for compressed Air,

B = Drain outlet (to maintain constant pressure head in the mixing Chamber),

C = Liquid sample (sucked into the Nebulizer),

D = Inlet for Fuel-Gas to the Laminar-Flow-Burner,

E = Nebulizer to atomize the liquid sample,

F = Mixing Chamber for Fuel Gas, Compressed Air, and Atomized Liquid Sample,

G = Burner,

H = Flame,

I = Convex lens,

K = Optical filter to transmit only a strong-line of the element, and

L = Amplifier to amplify the feeble electrical impulse and a built-in direct read-out device.

25.3.2. INTERNAL STANDARD FLAME PHOTOMETER

The layout of an internal standard flame photometer is illustrated in Figure 25.3.

A = Inlet for compressed air,

B = Inlet for Acetylene (Fuel-Gas),

C = Liquid sample sucked in by an atomizer,

D = Flame,

E = Mirror,

F = An optical filter to allow the transmission of only one strong-line of the element,

G = Amplifier to amplify the weak electrical current,

H = A Null detector to record the intensity of the element under study and the internal-standard (Lithium),

Figure 25.3 : Layout of an Internal Standard Flame Photometer.
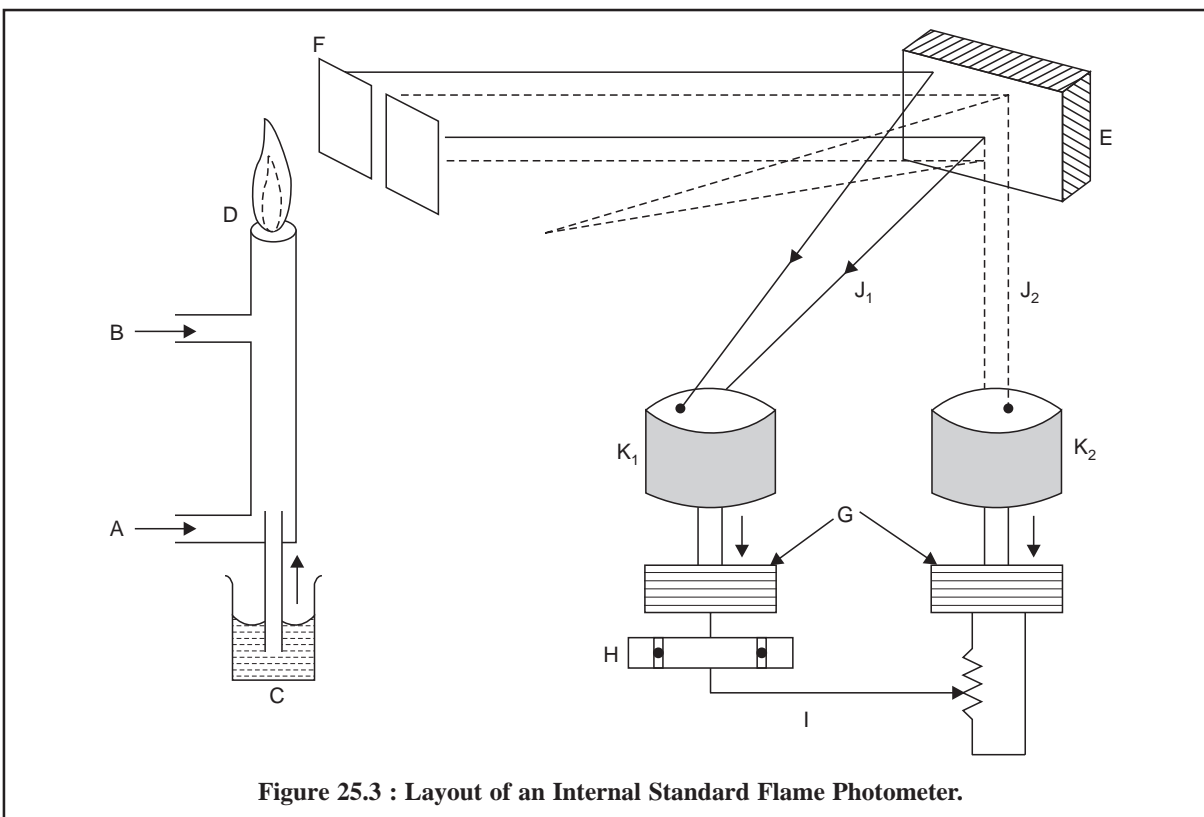
I = A calibrated potentiometer,

J_1 = Lines due to the 'sample'

J_2 = Lines due to the Internal Standard 'Lithium', and

K_1 & K_2 = Photocells to convert light-energy to electrical impulse.

The use of an internal standard flame photometer not only eliminates the visible effects of momentary fluctuations in the flame characteristics produced by variations in either the oxidant or under full pressures, but also the errors caused due to differences in surface tension and in viscosity are minimised to a great extent.

Procedure : In this particular instance 'Lithium' is employed as an internal standard and an equal concentration is added simultaneously to the sample and the standard solutions. The sample (C) solution having the internal standard (Lithium) is sucked in by an atomizer and a fine spray is thereby introduced into the flame (D). The radiation thus emitted is subsequently passed through a filter (F) and then collected by a mirror (E). The emitted radiation reflected from the mirror is split up into two parts : the first part is caused due to the internal standard (Lithium), whereas the second part arises due to the element under examination. Both these lines J_1 and J_2 are passed through the respective photocells K_1 and K_2 whereby the light energy is transformed into the electrical impulses. These electrical impulses are usually very weak and feeble and hence, they are duly amplified by a suitable amplifier (G) individually and are subsequently introduced into the common detecting device (H) *i.e.,* a **'Null detector'**-so as to enable it to record the intensity of the element under investigation and also the internal standard (Lithium) accurately using a calibrated potentiometer (I).

In short, an **internal-standard flame photometer** provides a direct and simultaneous result with respect to the ratio of intensities.

25.4. APPLICATIONS OF FLAME EMISSION SPECTROSCOPY IN PHARMACEUTICAL ANALYSIS

A few typical example of **'flame-emission spectroscopy'** are given below.

25.4.1. ASSAY OF SODIUM, POTASSIUM AND CALCIUM IN BLOOD SERUM AND WATER

(*i*) **Standard potassium and sodium solutions, approximately 500 ppm :** Weigh accurately 0.95 *g* of dried KCl and 1.25 *g* of dried NaCl into separate 1-litre volumetric flasks. Dissolve in water and dilute to the mark.

(*ii*) **Standard calcium solution, approximately 500 ppm :** Weigh accurately 1.25 g of $CaCO_3$, which has been dried at 110°C, into a 500-ml breaker. Add about 200 ml of DW and 10 ml of conc. HCl. Cover the breaker with a watch-glass during addition of acid to prevent loss of solution as CO_2 is evolved. After the solution is complete, transfer it quantitatively into a 1-litre volumetric flask and dilute to the mark with DW.

(*iii*) **Radiation buffer* for sodium determination :** Prepare a saturated solution with reagent-grade $CaCl_2$, KCl, $MgCl_2$, in that order.

(*iv*) **Radiation buffer for potassium determination :** Prepare a saturated solution with reagent-grade NaCl, $CaCl_2$ and $MgCl_2$, in that order.

(*v*) **Radiation buffer for calcium determination :** Prepare a saturated solution with reagent-grade NaCl, KCl, $MgCl_2$ in that order.

Procedure

(*a*) **Preparation of working curves :** Transfer 5 ml of the appropriate radiation buffer to each series of 100-ml volumetric flasks. Add a volume of the standard solution which will cover a concentration ranging between 0 to 100 ppm. Dilute to 100 ml with DW and mix well. Measure the emission intensity of these samples by taking at least three readings for each. Between each set of measurements, aspirate DW through the burner. Correct the average values for background luminosity, and prepare a working curve from these data.

(*b*) **Analysis of blood serum/water sample :** Prepare aliquot portions of the sample as described in the above paragraph (*a*). If necessary, use a standard to calibrate the response of the spectrometer to the working curve. Then measure the emission intensity for the unknown. After correcting the data for background, determine the concentration by comparison with the working curve.

25.4.2. ASSAY OF BARIUM, POTASSIUM AND SODIUM IN CALCIUM ACETATE

The technique of flame emission spectroscopy is used to determine the concentration of Ba, K, and Na ions by measuring the intensity of emission at a specific wavelength by the atomic vapour of the element generated from calcium acetate *i.e.,* by introducing its solution into a flame.

25.4.2.1. For Emission Measurements

Introduce water into the atomic vapour generator, adjust the instrument reading to zero, introduce the most concentrated solution into the generator and adjust the sensitivity to give a suitable reading ; again introduce water or the prescribed solution into the generator and when the reading is constant readjust, if necessary, to zero.

25.4.2.2. Method of Standard Addition

The various steps are as follows :

(1) Place in each of not fewer than three similar graduated flasks equal volumes of the solutions of the substance being examined, prepared as follows :

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* Radiation buffers are used to minimise the effect of each ion upon the emission intensity of the others.

(*a*) Prepare a 5.0% w/v solution and use barium solution ASp*, suitably diluted with water to prepare the standard solution.

(*b*) Prepare a 1.25% w/v solution and use potassium solution ASp**, suitably diluted with mater, to prepare the standard solution.

(*c*) Prepare a 1.0% w/v solution and use sodium solution ASp***, suitably diluted with water, to prepare the standard solution.

(2) Add to all but one these flasks a measured quantity of the specified standard solution (marked *; **; ***; above) to produce a series of solution containing increasing amounts of the element being determined.

(3) Dilute the contents of each flask to the required volume with water.

(4) After having calibrated the instrument as directed above, introduce each solution into the generator three times and record the steady reading. If the generator is a flame, wash the apparatus thoroughly with water ; if a furnace is used fire it after each introduction.

(5) Plot the mean of the readings against concentration on a graph the axes of which intersect at zero added element and zero reading.

(6) Extrapolate the straight line joining the points until it meets the extrapolated concentration axis. The distance between this point and the intersection of the axes represents the concentration of the element (*e.g.,* Mg, K, Na) being determined in the solution of the substance being examined.

25.4.2.3. Limits of Elements present in Calcium Acetate Sample

Mg : Not more than 500 ppm of Mg ;

K   : Not more than 0.1% of K, and

Na   : Not more than 0.5% of Na.

25.4.3. COGNATE ASSAYS

The following substance, namely : magnesium acetate ; potassium citrate ; potassium hydroxide ; potassium nitrate and sodium chloride can also be assayed for their respective elements as shown in Table 25.1 below. However, all the respective solutions of the said pharmaceutical substance and their standard solutions must be prepared as prescribed in BP (1993) strictly to obtain the best results.

Table 25.1 : Assay of Pharmaceutical Substances by Flame Emission Spectrophotometry (or FES):

S.No.	Name of Substance	Elements Present	Qty. Prescribed (% *w/v*)	Limits Prescribed***
1.	Magnesium Acetate	K ; Na ;	For K = 0.50 ; For Na = 1.00 ;	NMT 0.1% of K ; NMT 0.5% of Na ;
2.	Potassium Citrate	Na ;	For Na = 1 mg ; of Na per ml in DW ;	NMT 0.3% of Na ;

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* **Barium Solution ASp :** Dissolve 1.778 g of $BaCl_2$ in sufficient water to produce 1000 ml. Dilute it with water so that 1 ml contains 1 mg of Ba.

** **Potassium Solution ASp :** Dissolve 1.144 g of KCl, previously dried at 100° to 105° for 3 hours, in sufficient water to produce 1000 ml. (It contains 600 meg of K in 1 ml).

*** **Sodium Solution ASp :** Dissolve 0.5084 g of NaCl, previously dried at 100° to 105° for 3 hours, in sufficient water to produce 1000 ml. (It contains 200 mcg of Na in 1 ml).

3.	Potassium Hydroxide	Na ;	For Na = 1.0 g in 50 ml DW + 5 ml of 5M H_2SO_4. Dilute to 100 ml with DW. Dilute 1 ml to 10 ml in DW.	NMT 1.0% of Na ;
4.	Potassium Nitrate	Na ;	For Na = 1.0 and measuring at 589 nm	NMT 0.1% of Na ;
5.	Sodium Chloride	K ;	For K = 1.0 ;	NMT 500 ppm

*British Phrmacopeia, Vol. I and II, 1993.

THEORETICAL AND PRACTICAL EXERCISES

1. What is **'Flame Photometry'** ? Explain.

2. Discuss the following theoretical aspects of flame spectroscopy :

   (*a*) Bohr's Equation, and   (*b*) Boltzmann Equation.

3. What are the two types of Flame Photometers commonly used in **Flame Emission Spectroscopy** (FES) ? Describe them individually with a neat layout and explain their *modus operandi.*

4. How would you assay sodium, potassium and calcium in blood serum and water ? Explain.

5. Explain the assay of Ba, K and Na in calcium acetate using the **'method of standard addition'.**

6. Discuss the assay of the following pharmaceutical substances by **FES-method** :

   (*i*) Magnesium acetate,   (*ii*) Potassium nitrate,

   (*iii*) Potassium nitrate, and   (*iv*) Sodium chloride.

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26 ATOMIC ABSORPTION SPECTROSCOPY

26.1. INTRODUCTION

Allan Walsh, in 1955*, was the pioneer for the introduction of **atomic absorption spectroscopy** (AAS), which eventually proved to be one of the best-known-instrumental-techniques in the analytical armamentarium, that has since been exploited both intensively and extensively in carrying out the quantitative determination of trace metals in liquids of completely diversified nature, for instance : blood serum-for Ca^{2+}, Mg^{2+}, Na^+ and K^+ ; edible oils-Ni^{2+} ; beer samples-Cu^+ ; gasoline (petrol)-Pb^{2+} ; urine-Se^{4+} ; tap-water-Mg^{2+} ; Ca^{2+} ; lubricating oil-Vanadium (V).

The atomic absorption spectroscopy (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. It has been noticed that gaseous metal atoms in an unexcited form *i.e.,* ground state atoms, will absorb radiant energy related to their own specific resonance wavelength. Hence, when a light with the same resonance wavelength is passed through a flame comprising of such atoms, a part of the light will be absorbed accordingly. Besides, the degree of absorption would be directly proportional to the total number of ground-state atoms present in the flame, which ultimately forms the basis of **Atomic Absorption Spectroscopy (AAS)**.

AAS facilitates the estimation of a particular element in the presence of many other elements efficaciously. In other words, there is absolutely 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.

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*Walsh, A., **Spectrochim Acta., 7,** 108, 1955.

Because of the fact that AAS is free from any cumbersome-sample-preparation, it has proved to be an ideal, dependent and versatile analytical tool for the non-chemists specifically, for instance : biologists, clinicians and the engineers, whose interest lies only in the significance of the results.

26.2. THEORY

The underlying principle of atomic absorption spectroscopy (AAS) is the absorption of energy exclusively by ground state atoms while they are in the gaseous form.

It may be further expatiated as follows below :

A solution consisting of certain metallic species when aspirated into a flame, it will give rise to the corresponding vapours of metallic species. As it has already been discussed under flame emission spectroscopy (FES) [Chapter-25] : Some metal atoms would be raised directly to an energy level to such an extent as 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 shall 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, one logically determines the amount of light absorbed. In other words, the concentration of the metallic element may be determined directly from the value of absorption.

The total amount of light absorbed may be provided by the following mathematical expression :

$$\text{Total amount of light absorbed (at } \upsilon) = \frac{\pi e^2}{mc} \, Nf \qquad \qquad ...(a)$$

where υ = 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 υ, and

f = Ability for each atom to absorb at υ (oscillator strength).

The components in Eq. (*a*), namely : π, e, m and c are constants, therefore, it can be further written in a simplified form as below :

$$\text{Total amount of light absorbed} = K \times N \times f \qquad \qquad ...(b)$$

Hence, from Eq. (*b*) it may be inferred that :

(*a*) it is independent of the wavelength, and

(*b*) it is independent of temperature,

More explicitly, the absorption by atom is independent of both the wavelength of absorption and the temperature of the atoms. And these two specific characteristic features give AAS a clear distinct and positive edge over FES.

26.2.1. MERITS OF AAS OVER FES

The various points of merit of atomic absorption spectroscopy over flame spectroscopy are enumerated below :

S. No.	AAS		FES
1.	This technique is superior and specific because of the fact that only the atoms of a particular element can absorb radiation of their own characteristic wavelength.	1.	Spectral interferences usually take place in this technique.
2.	A relatively large number of metal atoms produce an atomic absorption signal whereby the effect of flame-temperature variation is negligible in AAS *i.e.,* independent of flame-temperature.	2.	A much smaller number of metal atoms do produce an emission signal in FES, showing that this technique is not independent of flame, temperature.
3.	The detection limits of sensitivity of the following elements are more by AAS technique, such as : Ag, As, Au, B, Bi, Cd, Co, and Fe.	3.	The detection limits (sensitivity) of the under mentioned elements are higher by FES technique, for instance : Al, Ba, Ca, Eu, Ho, In, K and La.

Note : The detection limits of Cr, Cu, Mn, Mo, Pd, Rh, Ni, and V are almost equal by AAS and FES techniques.

26.2.2. DEMERITS OF AAS

The various points of demerit of atomic absorption spectroscopy are as follows :

(*i*) It essentially requires a separate lamp for each element to be determined ; and this serious lacuna is usually overcome either by using a line-source with the introduction of flame or by using a continuous source with the introduction of a very high resolution monochromator,

(*ii*) AAS cannot be employed very effectively for such elements that produce their corresponding oxides when exposed in the flame, for example : Al, Mo, Si, Ti, W, V. Nevertheless, these estimations may be performed under suitably modified experimental parameters, and

(*iii*) When the solutions of metal salts are made in an aqueous medium the predominant anion present affects the resulting signal to a negotiable extent.

26.3. INSTRUMENTATION

The atomic absorption spectrophotometers are essentially of *two* types, namely :

(*a*) Single-beam Atomic Absorption Spectrophotometer, and

(*b*) Double-beam Atomic Absorption Spectrophotometer.

These two instruments shall be discussed briefly here along with their vital components.

26.3.1. SINGLE-BEAM ATOMIC ABSORPTION SPECTROPHOTOMETER

The schematic diagram of a single-beam atomic spectrophotometer in illustrated in Figure 26.1.

A = Anode (Tungsten),

B = Glass-shield,

C = Neon or Argon at 1-5 torr,

D = Quartz or Pyrex Window,

E = Inlet for Acetylene,

F = Inlet for air,

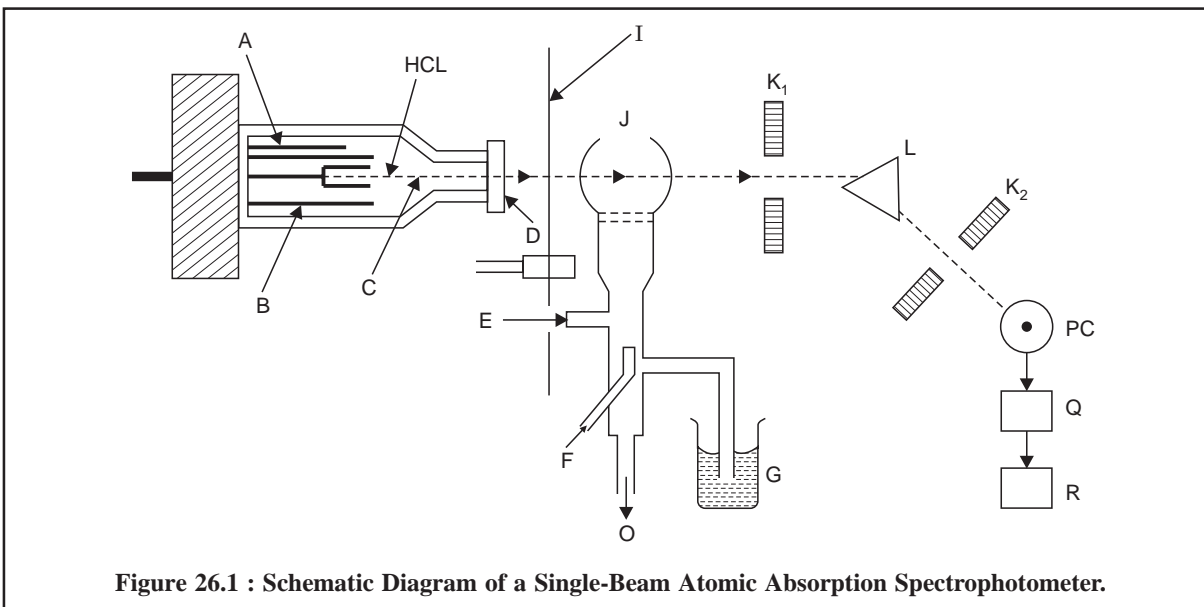G = Liquid sample sucked in by an atomizer,

HCL = Hollow cathode Lamp,

Figure 26.1 : Schematic Diagram of a Single-Beam Atomic Absorption Spectrophotometer.

I = Chopper (a rotating shutter),

J = Flame,

K_1 and K_2 = Slits,

L = Prism or Grating,

M = Photocell,

O = Drain outlet to maintain a constant pressure head in the mixing chamber,

PC = Photocell,

Q = Photodetector, and

R = Amplifier and Recorder.

The most common source for atomic absorption measurements in the 'hollow-cathode-lamp', which essentially consists of a Tungsten anode (A) and a cylindrical cathode (HCL) sealed in a glass tube (B) that is duly filled with neon or Argon (C) at a pressure of 1 to 5 torr. It is a practice to have the cathode constructed of the metal whose spectrum is desired or serves to support a layer of that particular metal. The chopper* (I) is interposed between the hollow-cathode-lamp (HCL) and the flame (J). Subsequently, the liquid sample (G) is sucked in by an atomizer into the flame (J). Just prior to its entry to the flame, the sample solution first gets dispersed into a mist of very small droplets that evaporates in the flame to yield initially the dry salt, and subsequently the vapour of the salt. At this particular stage a portion of this vapour will be dissociated into atoms of the element required to be measured. In this manner, the flame possesses free ground state (*i.e.,* unexcited) atoms that are worthy of absorbing radiations, from an external source when the radiation eventually matches exactly to the energy needed for a transition element from the lower ground-state-level to the upper excited-state-level. The resulting unabsorbed radiation from the flame (J), firstly passes through the slit (K_1) and then through the monochromator *i.e.,* the prism or grating (L) that exclusively isolates the exciting spectral lines of the light source ; secondly, through the slit (K_2) into the photocell (PC), thirdly, into a photodetector (Q) and fourthly, its output is adequately amplified and registered on a recorder (R). It is worthwhile to mention here that the final absorption is measured by the difference in the transmitted signal both in the absence and presence of the element under investigation.

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*Chopper is a rotating wheel whose function is to break the steady stream of light from the hollow-cathode-lamp into an intermittent light that gives rise to a **Pulsating current** into the photocell ; this current is duly amplified and recorded.

26.3.2. DOUBLE-BEAM ATOMIC ABSORPTION SPECTROPHOTOMETER

The major disadvantage of a single-beam atomic absorption spectrophotometer (Figure 26.1) lies in its very low stability. The introduction of a **double-beam atomic absorption spectrophotometer** (Figure 26.2) has completely eliminated the above main lacuna and provides much enhanced stability. In this particular instance the chopped beam of light from the hollow-cathode-lamp is split into two parts. The first portion, passes through the flame, while the second portion is made to bypass the flame completely. However, the two separate beams of light are recombined meticulously by an unique optically-designed assembly, passes through a monochromator to a strategically placed detector and ultimately to a sensitive read-out device.

It is pertinent to mention here that a double-beam atomic absorption spectrophotometer is absolutely independent of (*a*) lamp drift, (*b*) sensitivity of detector with time.

The optical path of a double-beam atomic absorption spectrophotometer is depicted in Figure 26.2. The various essential components comprising the optical arrangement in Figure 26.2 are enumerated after the figure.
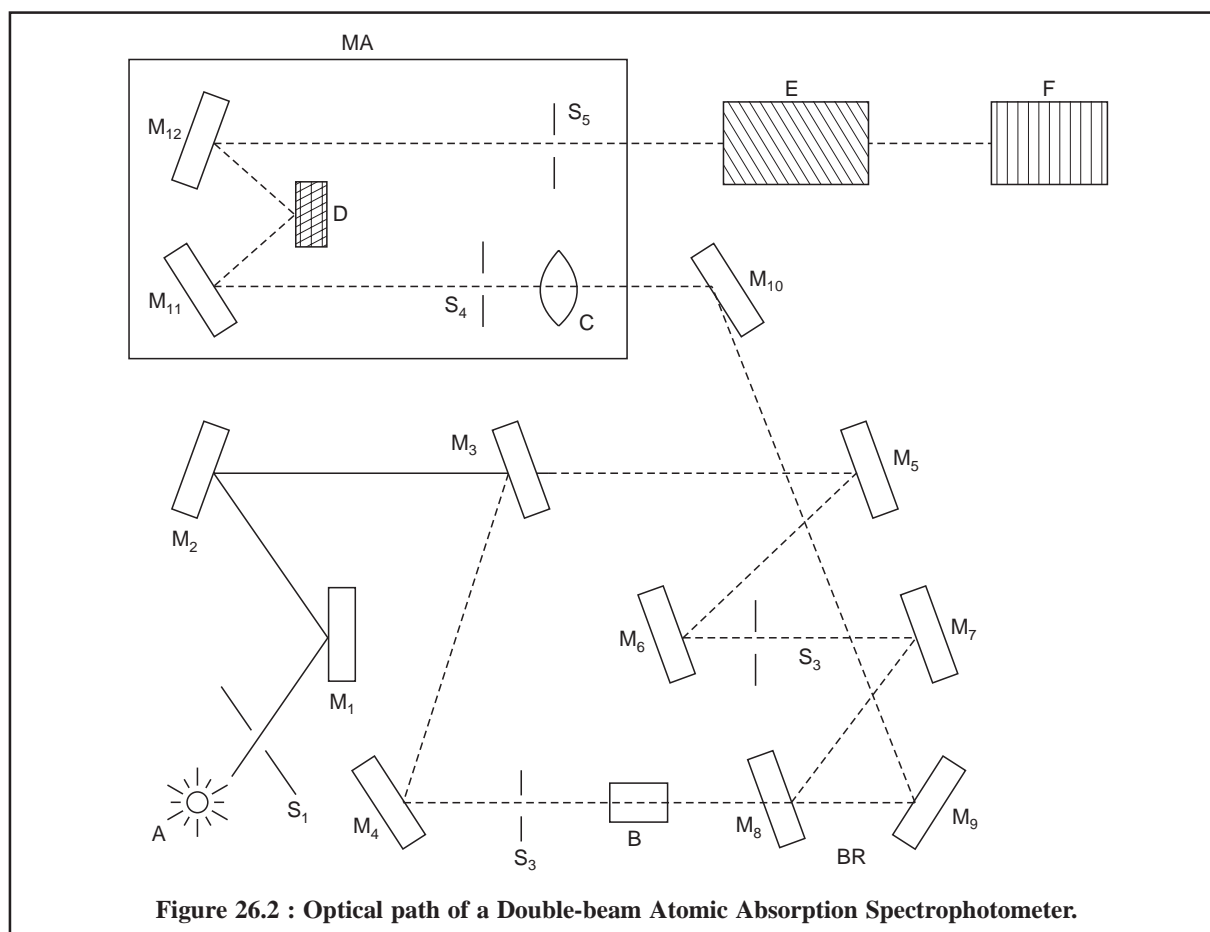

Figure 26.2 : Optical path of a Double-beam Atomic Absorption Spectrophotometer.

A = Source of light (Hollow-Cathode-Lamp),

B = Flame,

C = Field lens,

D = Grating,

E = Detector,

F = Read-out device,

BR = Beam recombination zone,

MA = Monochromator assembly,

S_1 to S_4 = Slits,

S_5 = Exit slit, and

M_1 to M_{12} = Mirrors

The light hollow-cathode-lamp source (A) passes through the slit S_1 and strikes at mirrors M_1 and M_2. The Mirrors M_3 splits chopped beam from the source into two parts ; one passes through the mirror M_4-slit S_2-flame (B)-mirror M_8 and strikes at mirror M_9 to reach mirror M_{10}, and the second strikes at mirror M_6-slit S_3-mirror M_7, M_8 and M_9 respectively to reach the mirror M_{10}. The mirror M_8 and M_9 serve as a **beam recombination zone** (BR). The recombined beam gets reflected by mirrors M_{10} passes through the field lens (C), slit S_4, strikes at M_{11}, passes through the grating (D), to the mirror M_{12} and ultimately passes out through the exit (S_5) and the monochromator assembly (MA) into the detector (E) and finally to the read-out device (F).

26.4. IMPORTANT ASPECTS OF ATOMIC ABSORPTION SPECTROSCOPY

The following *three* important aspects of atomic absorption spectroscopy shall be discussed here briefly, namely :

(*i*) Analytical Techniques,

(*ii*) Detection Limit and Sensitivity, and

(*iii*) Interferences.

26.4.1. ANALYTICAL TECHNIQUES

In atomic absorption spectroscopy (AAS) the technique using *calibration curves* and the *standard addition method* are both equally suitable for the quantitative determinations of elements.

26.4.1.1. Calibration Curves

Theoretically, the absorbance must be proportional to concentrations, however, deviations from linearity usually take place. Therefore, it is necessary to prepare an **empirical calibration curve** (ECC). For this, the standard solutions of the element(s) to be determined are employed to plot the ECC from which the contents in the '**test solutions'** may be measured conveniently.

Figure 26.3, represents the typical calibration curves of copper at 3428 Å, where :

X = Sample absorption reading,

Y = Sample concentration reading, and

Z = Calibration curve.

It is quite evident from the calibration curve (Z) in Figure 26.3, that the linearity between the concentration of Cu (in ppm) and absorbance prevails over the range 2.0 to 10.0 ppm specifically, whereas at higher concentrations the said relationship does not hold good anymore. Hence, it is pertinent to mention here that whenever the quantitative analysis of an element is to be carried out, the absorbance is preferably measured almost under the same experimental parameters whereby the calibration curve was initially constructed.
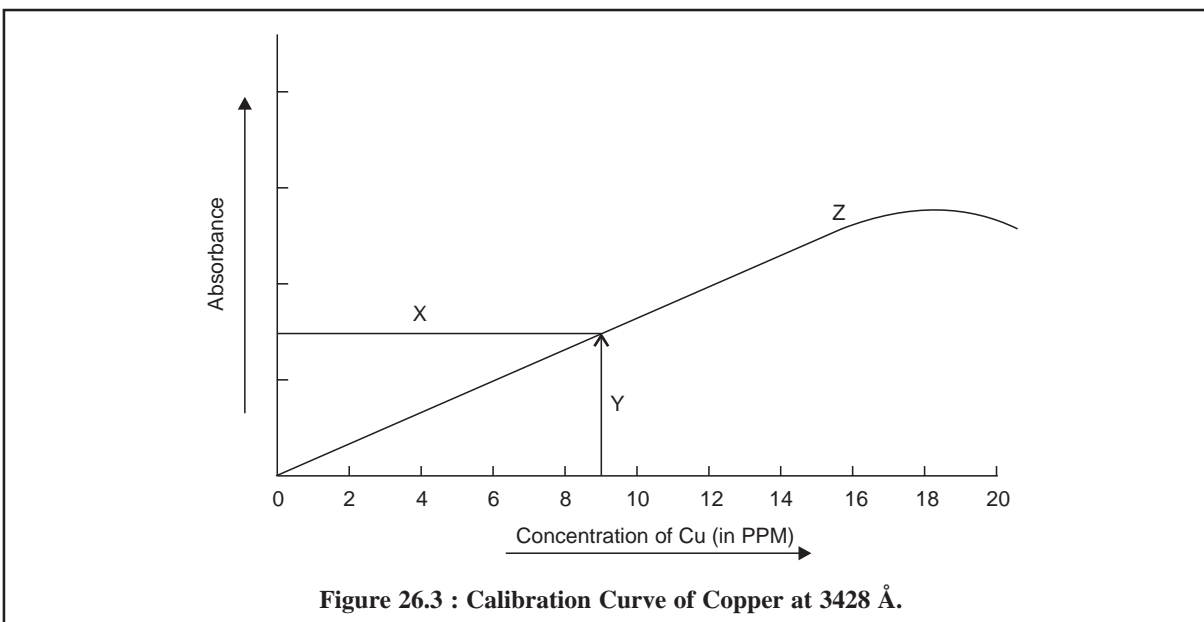
Figure 26.3 : Calibration Curve of Copper at 3428 Å.

26.4.1.2. Standard Addition Method

The **standard addition method** is widely employed in AAS. In this case, two more aliquots of the sample are transferred to volumetric flasks. The first, is diluted to volume, and the absorbance of the solution is measured. The second, receives a known quantity of analyte, whose absorbance is also measured after dilution to the same volume. Likewise, data for other standard additions may also be obtained.

If a plot between absorbance and concentration reveals a linear relationship, which may be accomplished by several stepwise standard additions, the following expressions hold good, namely :

$$A_X = k\, C_X \qquad\qquad\qquad ...(a)$$

$$A_T = k\,(C_S + C_X) \qquad\qquad\qquad ...(b)$$

where, C_X = Analyte concentration in the diluted sample,

C_S = Contribution of the added standard to the concentration, and

A_X and A_T = Measured absorbances of C_X and C_S.

Combining Eqs. (*a*) and (*b*) we have :

$$C_X = C_S\, \frac{A_X}{A_T - A_X} \qquad\qquad\qquad ...(c)$$

When a number of stepwise additions are performed, A_T can be plotted against C_X. Thus, the resulting straight line may be extrapolated to $A_T = 0$. By substituting this value in Eq. (*c*) we may have at the intercept :

$$C_X = -\,C_S \qquad\qquad\qquad ...(d)$$

Advantage : The major plus points of the standard addition method is that it tends to compensate for variations caused by physical and chemical interferences in the sample solution.

26.4.2. DETECTION LIMIT AND SENSITIVITY

Detection Limit : It may be defined as the concentration (meg ml^{-1}) 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 : It 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 the sensitivity takes no cognizance 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 a 1% absorption-is a pure theoretical number only that would undergo a change solely depending on the efficiency of the lamp (hollow-cathode-lamp), atomizer, flame-system employed, monochromator (prism, grating used), and finally the photomultiplier used.

The sensitivity for 1% absorbance is determined by the help of the expression given below :

$$C_1\% = \frac{C_{0.1} \times 0.0044}{0.1}$$

where, $C_1\%$ = Concentration that yields 1% absorption, and

$C_{0.1}$ = Concentration that yields an absorption of 0.1

Sensitivity is usually expressed in terms of mcg ml^{-1} for 1% absorbance.

It is an usual practice to perform an actual-test-run over a sufficiently large range by employing the necessary prevailing expansion facility so as to ascertain fully 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 is normally equals to twice the noise level.

26.4.3. INTERFERENCES

In general, atomic absorption methods are subject to *three* types of interferences, namely :

 (*i*) Spectral Interferences,

(*ii*) Chemical Interferences, and

(*iii*) Ionisation Interferences.

The different interferences shall be discussed briefly below :

26.4.3.1 Spectral Interferences

This type of interference normally takes place when the absorption of an interfering species either overlaps or lies very near to the analyte absorption, with the result that resolution by the monochromator almost becomes impossible, Hollow-cathode-source invariably give rise to extremely narrow emission-lines, hence interference caused due to overlap of atomic spectral lines is rather rare.

A few typical examples of spectral interferences are given below :

(*a*) Spectral interferences 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.

Remedy

(*a*) When the source of the combustion or particulate products is the full and oxidant mixture alone, then a blank is aspirated into the flame and the necessary corrections are effected from the observed absorbances.

(*b*) Spectral interferences may be produced due to an emission line of another element, radical or molecule and also by unresolved band spectra. Here, the lines are read together proportionately to the extent of overlap if the spectral band after passing through the monochromator allows the undersired radiation to reach the photoreceptor finally.

For instance : Manganese triplet (at 4031°, 4033° and 4035° A) : potassium doublet (at 4044° and 4047° A) and the gallium line (at 4033° A).

Remedy : The overlapping of this nature may be eliminated either by prior chemical separation or by selection other spectral lines.

(*c*) **Sample Matrix :** A relatively more complex and troublesome problem is usually faced when the source of scattering originates right in the sample matrix itself. In such a situation, it has been noticed that the power of the transmitted beam-designated as P, is reduced by the nonanalyte components, whereas the incident beam power-designated as Po, is not ; thereby resulting in a positive error in absorbance and hence in concentration.

Example : Determination of Barium in alkaline-earth mixtures affords a potential matrix interference due to absorption. It has been observed that an intense and useful absorption line for barium atoms, occurring at 553.6 nm, lies in the centre of a broad absorption band for Ca $(OH)_2$, that extends from 540 to 560 nm.

Remedy : (1) The effect due to sample matrix is quickly and effectively eliminated by replacing nitrous oxide for air as the oxidant for the acetylene, whereby the higher temperature completely decomposes the Ca $(OH)_2$ and eliminates the absorption band.

(2) If the source of interference is known, an excess of the interfering substance may be added to the sample as well as the standards ; provided the 'excess' is sufficient enough with respect to the concentration from the sample matrix, the concentration of the latter will thus become insignificant. Such an added substance is sometimes referred to as a radiation buffer.

26.4.3.2. CHEMICAL INTERFERENCES

In usual practice, the chemical interferences are found to be more common than the spectral interference. However, their effects may very often be minimized by appropriate choice of experimental parameters.

Examples : (*i*) **Chemical Interferences due to Anion (PO_4^{3-}) :** Phosphate ions have been found to interfere with determination of Mg and Ca by AAS. The absorption due to Mg and Ca are appreciably weaker in the presence of PO_4^{3-} ions than in their absence. This is evidently on account of the formation of fairly stable phosphates of Mg and Ca which do not readily split-up into the respective atoms in the mantle of a flame.

Remedy : The addition of an excess of strontium (Sr), or lanthanum (La), or thorium (Th) ion remarkably minimizes the interference of PO_4^{3-} ion in the determination of Mg, and Ca by replacing the analyte in the analyte in the compound formed with the respective interfering species. In short, these ions do combine preferentially with PO_4^{3-} ions.

(*ii*) **Chemical Interference due to Cations :** In certain specific cases cations also interfere in atomic absorption measurements, for instance : Boron interferes with Mg and Ca ; whereas aluminium interferes with alkaline earth elements.

Protective Agents : These agents are found to inhibit the interferences by virtue of their ability to form relatively stable but volatile species with the respective analyte. There are *three* reagents that are employed commonly for this purpose, namely :

(*a*) Ethylenediaminetetra-acetic acid (EDTA).

(*b*) 8-Hydroxyquinoline, and

(*c*) Ammonium salt of 1-pyrrolidinecarbodithioic acid (APDC). EDTA helps to eliminate the interferences of Al^3, Si^{4+}, PO_4^{3-} and SO_4^{2-} in the determination of Ca.

26.4.3.3. Ionization Interferences

It has been observed that the ionization of atoms or molecules is comparatively very small in magnitude in combustion mixtures which essentially involve air as the oxidant and, therefore, may be ignored and neglected. Consequently, the substitution of air with either oxygen or nitrous oxide, however, gives rise to

temperatures which are high enough to cause appreciable ionization., Hence, as a consequence of the at-tained equilibrium-a fairly significant concentration of electron exists as shown below :

$$M \rightleftharpoons M^+ + e^-$$
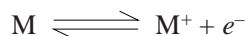
where,   M = Neutral atom or molecule,

M^+ = Its corresponding ion, and

e^- = An electron

Hence, if the medium has the species B in addition to species M, and if the former ionizes according to the following equation :

$$B \rightleftharpoons B^+ + e^-$$

then the extent of ionization of the latter will be minimized substantially be the *Law of Mass Action* of the electrons originated from the former species (*i.e.,* B).

Example : The intensity of atomic absorption lines for the alkali metals, such as : potassium (K) ; rubidium (Rb) ; and caesium (Cs), is found to be affected by temperature in a complex way. Under certain experimental parameters a noticeable decrease in absorption may be observed in hotter flames. Hence, lower excitation temperatures are invariably recommended for the analysis of alkali metals.

Remedy : The resulting effects of shifts in ionization equilibrium may be eliminated effectively by the addition of an ionization suppressor, that promptly gives a comparatively high concentration of electrons to the flame. This ultimately results in the suppression of ionization by the respective analyte.

26.5. APPLICATION OF ATOMIC ABSORPTION SPECTROSCOPY IN PHARMA-CEUTICAL ANALYSIS

The elements present in a host of pharmaceutical substances are determined quantitatively by atomic absorption spectroscopy, for example : Pd in carbenicillin sodium ; Cu, Pb and Zn in activated charcoal ; Fe in ascorbic acid ; Ag in cisplatin ; Ph and Zn in copper sulphate ; Zn in glucogen ; Zn in insulin ; Pb in oxprenolol hydrochloride ; Ni in prazosin hydrochloride ; Zn in sodium sulphite heptahydrate, and Cd and Pb in zinc oxide.

26.5.1. ASSAY OF TOTAL ZINC IN INSULIN ZINC SUSPENSION

Theory : Insulin zinc suspension is nothing but a neutral suspension of insulin in the form of water insoluble complex with $ZnCl_2$. Determination of both total zinc and zinc in solution is performed on a sample of the supernatant liquid obtained by centrifuging the suspension. The percentage of total zinc and of zinc in solution varies according to the strength of the preparation *viz*., 40, 80 or 100 units ml^{-1}.

Materials Required : Stock solution of Zn (5000 mcg ml^{-1}) : Dissolve Zn metal (Anala-R-Grade) 2.5 g in 5 M HCl (20 ml) and dilute to 500 ml with DW ; HCl (0.1 M) : 10 ml ;

Procedure : To the 2 ml of well-shaken suspension add HCl (0.1 M ; 1 ml) and dilute with water to 200 ml. Spray the solution by adopting the standard procedure and read off the concentration of zinc from a calibration curve prepared with solution containing 0.5, 1, 2, and 3 mcg ml^{-1} of Zn.

26.5.2. ASSAY OF PALLADIUM IN CARBENICILLIN SODIUM

Materials Required : Carbenicillin sodium : 1.0 g ; sulphuric acid (36 N or 18 M) : 2.0 ml ; mixture of nitric acid (70% w/v) and hydrochloric acid (35% w/w or 11.5 M) [3 : 4] : 5.0 ml ; hydrochloric acid (11.5 M) : 3.0 ml ; palladiun solution (standard) [Dissolve 1.670 g of Palladium (II) chloride in 200 ml of a 50% v/v solution of hydrochloric acid (11.5 M) with the aid of heat, cool and add sufficient water to produce 1 litre] : This standard palladium solution contains 1 mg of Pd in 1 ml ;

Procedure* : Moisten 1 g of carbenicillin sodium in a silica crucible with 2 ml of sulphuric acid. Heat, gently at first, then more strongly until all carbon is removed and a white ash is obtained. Allow to cool and add 5 ml of a mixture of nitric acid and hydrochloric acid and evaporate to dryness on a water-bath. Add 3 ml of hydrochloric acid, warm to dissolve and add sufficient water to produce 25 ml.

Place in each of three similar graduated flasks equal volumes of the solution of the substance prepared as above. Add to all but one of these flasks a measured quantity of the specified standard solution of palladium to produce a series of solutions containing increases amounts of Pd. Dilute the contents of each flask to the required volume with DW.

After calibrating the instrument as stated above, introduce each solution into the generator 3 times and record the steady reading at 248 nm. If the generator is a flame, wash the apparatus thoroughly with DW after each introduction ; if a furnace is used, fire it after each introduction. Plot the mean of the readings against concentration on a graph the axes of which intersect at zero added Pd and zero reading. Extrapolate the straight line joining the points until it meets the extrapolated concentration axis. The distance between this point and the intersection of the axes represents the concentration of Pd present in the prepared solution of carbenicillin sodium.

26.5.3. COGNATE ASSAYS

A number of pharmaceutical substances official in BP (1993) can be assayed by adopting the above procedures of AAS as detailed in the following Table 26.1.

Table 26.1 : Assay of Pharmaceutical Substances by Atomic Absorption Spectroscopy :

S. No.	Name of Substance	Elements Assayed	Qty. Prescribed	Comp. of Flame	Measured at(nm)	Limits Prescribed
1.	Activated Charcoal	Cu ; Pb ; Zn ;	2.0 g	Air-Acetylene	325.0	Cu = NMT** 25 ppm ; Pb = NMT 10 ppm ; Zn = NMT 25 ppm ;
2.	Ascorbic Acid	Fe ;	20% w/v soln. in 0.1 M HNO$_3$	—	248.3	Fe = NMT 2 ppm ;
3.	Cisplatin	Ag ;	0.1 g	Lean air-Acetylene	328 (Silver-hollow-cathode lamp)	Ag = NMT 250 ppm ;
4.	Copper Sulphate	Pb ; Zn ;	1.25 g in 10 ml HCl	—	Pb : 283.3 ; Zn : 213.9 ;	Pb = NT 75 ppm ; Zn = NMT 500 ppm ;
5.	Glucagon	Zn ;	0.2% w/v Air-in 0.01 M HCl	Acetylene (11.2) Vol	213.9 (Zn hollow-cathode-lamp)	Zn = NMT 0.15% of Zn ;
6.	Oxprenolol Hydrochloride	Pb ;	4% w/v	—	217.0 (Pb-hollow-cathode-lamp)	Pb = NMT 5 ppm ;

7.	Prazosin Hydrochloride	Ni ;	0.1 g	—	232	Ni = NMT 50 ppm ;
8.	Sodium Sulphite Heptahydrate	Zn ;	20.0 g	Air-Acetylene	213.9 (Zn-hollow-cathode-lamp)	Zn = NMT 12 ppm ;
9.	Zinc oxide	Cd ; Pb ;	2.0 g	Air-Acetylene	Cd : 228.8 ; Pb : 217. 0;	Cd = NMT 10 ppm ; Pb = NMT 50 ppm ;

*NMT = Not More Than ;

THEORETICAL AND PRACTICAL EXERCISES

1. Explain the following statements with respect to atomic absorption spectroscopy (AAS) :

   (*i*) One of the best known analytical methods for quantitative estimation of trace metals.

   (*ii*) AAS may be accomplished either by electrothermal device or by flame.

   (*iii*) AAS facilitates estimation of a specific element in the presence of other elements accurately and precisely.

   (*iv*) Merits of AAS over FES.

   (*v*) Demerits of AAS.

2. Discuss the underlying principle of AAS. How would you explain the mathematical expression derived from the total amount of light absorbed ?

3. Describe the various components that are essentially involved in any one of the following two types AAS :

   (*a*) Single-beam AAS,   (*b*) Double-beam AAS

   with the help of a schematic diagram.

4. Give a comprehensive account on the following *two* vital aspects of AAS, namely :

   (*a*) Analytical techniques,   (*b*) Detection limit and sensitivity.

5. How do the following *three* types of interferences affect the atomic absorption spectroscopic methods :

   (*a*) Spectral interferences,   (*b*) Chemical interferences, and

   (*c*) Ionisation interferences.

   Support your answer with appropriate examples.

6. Discuss the assay of the following medicinal compounds in an elaborated manner :

   (*i*) Total Zn in insulin Zn suspension,   (*ii*) Pd in carbenicillin sodium,

   (*iii*) Ag in cisplatin,   (*iv*) Fe in Ascorbic acid,

   (*v*) Pb in Oxprenolol hydrochloride, and   (*vi*) Ni in Prazosin hydrochloride.

RECOMMENDED READINGS

1. Kirkbright, G.F. and M. Sargent, '**Atomic Absorption and Fluorescence Spectroscopy,** New York, Academic Press, 1974.

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PART V

ASSAY METHODS BASED ON SEPARATION TECHNIQUES

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27 LIQUID-LIQUID EXTRACTION

27.1. INTRODUCTION

Liquid-Liquid extraction is a versatile and dependable separation technique wherein an aqueous solution is usually brought into contact with another organic solvent, exclusively immiscible with the former, so as to affect a legitimate and actual transfer of either one or more solutes into the latter. The normal-feasible separations which can thus be achieved are found to be rather easy, fast, convenient and effective resonably. Invariably such separations may be performed by shaking the two liquids in a separatory funnel for a few minutes ; and may be extended either to large quantities of pharmaceutical substances or trace levels.

In the case of pharmaceutical chemicals that are mostly **'organic solutes',** the liquid-liquid extraction system may very often make use of two immiscible organic solvents (*e.g.,* alcohol and ether) instead of the aqueous-organic type of extraction. On the contrary, the **'inorganic solutes'** normally encountered are invariably in aqueous solutions ; therefore, it has become absolutely necessary to produce such neutral substances out of them, for instance *ion-association complexes* and *metal-chelates* (using organic-ligands) that may be extracted into an appropriate organic solvent.

In short, **liquid-liquid extraction** has been employed predominantly and effectively not only for the pre-concentration and isolation of a 'single' chemical entity just before its actual estimation, but also for the extraction of classes of organic compounds or groups of metals, just prior to their usual estimation either by *chromatographic techniques* or by *atomic-absorption methods*.

27.2. THEORY

The behavioural pattern of two immiscible solvents, say 'a' and 'b', is essentially nonideal with respect to one another. Now, if a third substance is made to dissolve in a two-phase mixture of the solvents (*i.e.,* 'a' and 'b'), it may behave ideally in either phases provided its concentration in each individual phase is approximately small. Therefore, under these prevailing experimental parameters the ratio of the mole fractions of the solute in the two respective immiscible phases ('a' and 'b') is found to be a constant which is absolutely independent of the quantity of solute present. It is termed as the **Nernst Distribution Law** or the **Partition Law** and may be expressed as follows :

$$K_p = \frac{[A]_a}{[A]_b} = \frac{\text{Concentration of solute in solvent 'a'}}{\text{Concentration of solute in solvent 'b'}} \qquad ...(a)$$

where, $[A]_a$ = Mole fraction of solute A in Phase 'a'

$[B]_b$ = Mole fraction of solute B in Phase 'b', and

K_p = A constant.

The constant (K_p) is also known as the **distribution coefficient** or the **partition coefficient**. Interestingly, this particular relation [Eq. (*a*)] was originally derived for ideal solutions only, but it caters for a fairly good description of the behavioural pattern of a number of real-extraction-systems encountered in the analysis of pharmaceutical substances. However, the **Partition Law** offers the following *two* limitations, namely :

(*a*) It is not thermodynamically rigorous *i.e.,* it takes no cognizance of the activities of the different species. In other words, it is solely applicable to very dilute solutions in which case the ratio of the activities almost approaches unity, and

(*b*) It does not hold good when the distributing substances encounters association or distribution in either phases (*i.e.,* 'a' and 'b').

Consequently, a more rigorous treatment particularly specifies K_p as the ratio of the activities of the substance (A) in the two solvents instead of their concentrations. Hence, for dilute solutions, at a specified constant pressure and temperature, the mole fraction of a solute is directly proportional to its concentration in molarity or mass per unit volume ; which implies that these may be employed instead of mole-fraction in Eq. (*a*).

Thus, the **Partition Coefficient** K_p is also given by the following expression :

$$K_p = \frac{S_1}{S_2} \qquad ...(b)$$

where, S_1 = Solubility of substance A in solvent 'a', and

S_2 = Solubility of substance A in solvent 'b'.

Adequate precaution and care must be exercised in determining partition coefficients based on the solubility data as S_1 is not the solubility of substance 'A' in pure Solvent 'a', but rather the solubility in Solvent 'a' saturated with Solvent 'b'.

Example : In order to determine the exact partition coefficient of substance 'A' between water and ethyl acetate, the appropriate solubilities would be those of the substance 'A' in 3.3% ethyl acetate in water (composition of the 'aqueous' layer) and 8.7% in water in ethyl acetate (composition of the **'ester'** layer).

Likewise, the following Table 27.1, records the mutual solubilities of a few typical solvent pairs that are used frequently for **liquid-liquid extraction** procedures.

Table 27.1 : Physical Data on Binary Solvent Systems*

S.No.	Solvent Pair	Percentage Composition Phases		Composition of Azeotrope (%)	Boiling Pt. of Azeotrope (°C)	Boiling Pt. of Solvents (°C)
		Upper	Lower			
1.	1-Butanol-	79.9	7.7	55.5	93.0	117.7
	Water	20.1	92.3	44.5		100.0
2.	n-Butyl ether-	99.97	0.19	66.6	94.1	142.0
	Water	0.03	99.81	33.4		100.0
3.	Carbon tetrachloride-	0.03	99.97	95.9	66.8	76.8
	Water	99.97	0.03	4.1		100.0
4.	Chloroform-	0.8	99.8	97.0	56.3	61.2
	Water	99.2	0.2	3.0		100.0
5.	Dichloromethane-	2.0	99.9	99.0	38.8	40.0
	Water	98.0	0.1	1.0		100.0
6.	Ethyl ether-	98.53	6.04	98.2	34.2	34.6
	Water	1.47	93.96	1.2		100.0
7.	Hexane-	85.0	42.0	73.1	69.0	50.0
	Methanol	15.0	58.0	26.9	64.7	
8.	1-Octanol-	—	0.1	10.0	99.4	195.0
	Water	—	99.9	90.0		100.0
9.	Toluene-	99.95	0.06	79.8	85.0	110.6
	Water	0.05	99.94	20.2		100.0
10.	m-Xylene-	99.95	0.05	60.0	94.5	139.1
	Water	0.05	99.95	40.0		100.0

In liquid-liquid extractions the following *two* aspects are very crucial and important, namely :

(*a*) Error due to the Volume Change, and

(*b*) Effectiveness of an Extraction.

These *two* aspects shall be discussed briefly at this juncture.

27.2.1. ERROR DUE TO THE VOLUME CHANGE

In a situation wherein two immiscible solvents are employed in an extraction, the volumes of the two individual phases after attainment of equilibrium may be appreciably different in comparison to the initial volumes of the solvents used. Therefore, a number of procedures have been adopted to avoid **'error due to the volume change'** incurred thereby, namely :

(*i*) Measure the volume of the phase employed for the analysis and incorporate this volume in the calculations,

(*ii*) Separate the phase quantitatively and subsequently dilute to a known volume,

(*iii*) Separate the phase quantitatively and make use of the entire volume in the remaining steps of the ongoing analysis, and

(*iv*) Carry a marker substance through the extraction to automatically compensate for volume changes.

However, the latter procedure finds its abundant use in chromatographic methods of analysis.

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* Leo, A., Hansch, C. and Elkins, D., **Chem Rev., 71** 525, 1971.

27.2.2. EFFECTIVENESS OF AN EXTRACTION

Based on the appropriate partition coefficient of an immiscible solvent pair it is possible to calculate the 'effectiveness of an extraction'.

Let us assume that 'x' moles of solute present initially in a volume V_2 of Solvent 'b'. Now, this particular sample undergoes extraction with a volume V_1 of Solvent 'a' and subsequently 'y' moles of compound are left in V_2 at equilibrium.

Substituting these values in Eq. (a) and using molarity instead of mole fraction, we have :

$$K_p = \frac{M_1}{M_2} = \left(\frac{x-y}{V_1}\right)\bigg/\left(\frac{y}{V_2}\right) \qquad \qquad ...(c)$$

after simplifying and rearranging :

$$K_p = \left(\frac{x}{V_1} - \frac{y}{V_1}\right)\frac{V_2}{y}$$

or

$$= \frac{x}{y} \cdot \frac{V_2}{V_1} - \frac{V_2}{V_1}$$

or

$$= \frac{V_2}{V_1}\left(\frac{x}{y} - 1\right)$$

or

$$K_p \cdot \frac{V_1}{V_2} = \frac{x}{y} - 1$$

or

$$K_p \cdot \frac{V_1}{V_2} + 1 = x/y$$

or

$$y/x = \left(\frac{V_1}{V_2}K_p + 1\right)^{-1} = f \qquad \qquad ...(d)$$

where, f = Fraction not extracted.

Figure 27.1, represents the nomogram from which the unextracted fraction for various values of V_2/V_1 and K may be obtained.

From Eqs. (d) it is quite evident that the fraction extracted is absolutely independent of the initial solute concentration. Hence, the fraction left unextracted after 'n' extraction may be given by the following expression :

$$fn = \left(\frac{V_1}{V_2}K_p + 1\right)^{-n} \qquad \qquad ......(e)$$

assuming that the same volumes of solvents have been used for each extraction.

Form Eq. (e) it is distinctly obvious that a series of several extractions would definitely prove to be more efficacious than one single extraction using the same total volume of solvent.

From figure 27.1, the following steps may be adopted in order to determine the percentage of the analyte left in the sample after a single extraction :

(i) Hold a straight edge in such a manner that it is made to pass though the point on the 'left scale' which exactly corresponds to that ratio of extracting solvent to sample solvent volume and through the point representing the partition coefficient (K_p) on the **'middle scale'**, and

(*ii*) The percentage of the '**analyte**', left behind in the sample solvent after a single extraction is given by the intersection of the straight-edge with the **'right scale'**.

Figure 27.1 : A nomogram to calculate the 'fraction not extracted' after a single step liquid-liquid extraction process.
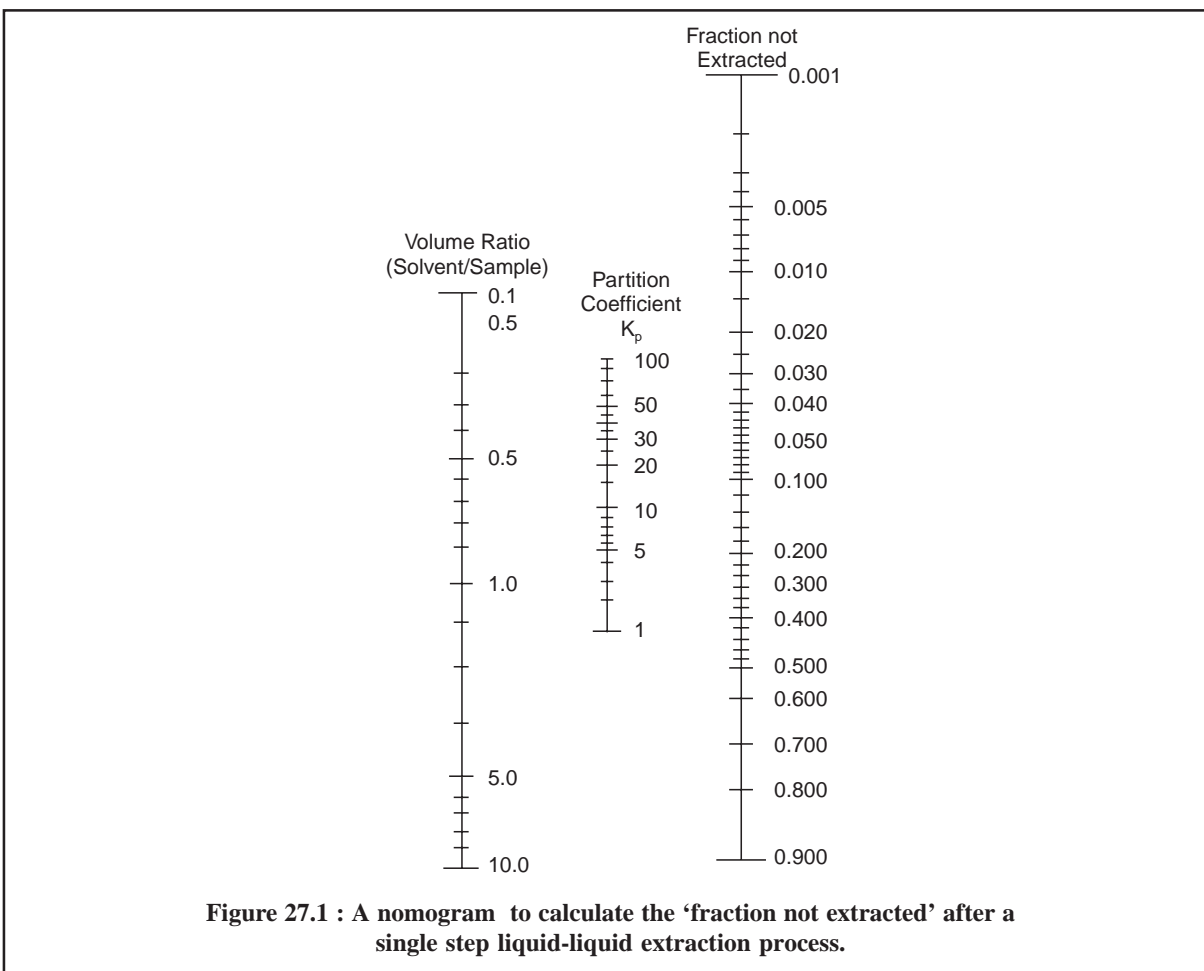
27.3. FACTORS INFLUENCING SOLVENT EXTRACTION

A number of cardinal factors exert a positive influence on the phenomenon of solvent extraction, namely :

(*a*) Effect of temperature and inert solutes,

(*b*) Effect of pH on extraction,

(*c*) Effect of ion-pair formation, and

(*d*) Effect of synergistic extraction.

These factors shall be discussed briefly below :

27.3.1. EFFECT OF TEMPERATURE AND INERT SOLUTES

The physical as well as chemical interactions of a solute is capable of changing its apparent partition coefficient between a pair of solvents. Therefore, it is absolutely necessary to take this into consideration while selecting an appropriate extraction-system. Craig and Craig* have advocated that the partition coefficients

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*   Craig, L.C. and Craig, D., Laboratory Extraction and Countercurrent Distribution, in, **Techniques of Organic Chemistry,** Vol III, I, Separation and Purification, 2nd ed., Weissberger, A., Ed., New York, Interscience 171, 1956.

are normally not sensitive to temperature when the two solvents in question are more or less immiscible and also the concentrations are fairly low in both the phases. Thus, the effect of temperature on the partition coefficient may be estimated conveniently from its effect on the solubilities of the substance in the two respective solvents. By substituting the solubilities (*e.g.,* S_1 and S_2) in Eq. (*b*) it is possible to estimate K.

The effect of inert solutes, such as : calcium chloride, magnesium chloride and sucrose, can also be employed judiciously and efficaciously in the development of solutions to difficult extraction problems by allowing efficient extractions from the water into such solvents as acetone, ethanol and methanol that are found to be completely miscible with water in the absence of salt. Matkovitch and Cristian* found the above three inert solutes to be the best agents for salting acetone out of water. It has been observed that the acetone layer that separated from a saturated aqueous solution of $CaCl_2$ exclusively contained 0.32 ± 0.01% water (v/v) and 212 ppm salt (w/w) at equilibrium.

27.3.2. EFFECT OF pH ON EXTRACTION

Generally, it has been found that the organic acids and bases do exist in aqueous solution as equilibrium mixtures of their respective neutral as well as ionic forms. Thus, these neutral and ionic forms may not have the same identical partition coefficients in a second solvent ; therefore, the quantity of a substance being extracted solely depends upon the position of the acid-base equilibrium and ultimately upon the pH of the resulting solution. Hence, extraction coefficient (E) may be defined as the ratio of the concentrations of the substance in all its forms in the two respective phases in the presence of equilibria ; and it can be expressed as follows :

$$E = \text{Extraction Coefficient} = \frac{\Sigma[Si]_2}{\Sigma[Si]_1} \qquad ...(i)$$

where, $\Sigma[Si]_2$ = The sum total of all forms of the compound in Phase-'2', and

$\Sigma[Si]_1$ = The sum total of all forms of the compound in Phase '1'.

In fact, the actual effect of the equilibrium on the extraction may be shown by determining the extraction coefficient for the system :

$$A + H \; \rightleftharpoons \; AH \quad \text{or} \quad K = \frac{[AH]_1}{[A]_1 \, [H]_1} \qquad ...(ii)$$
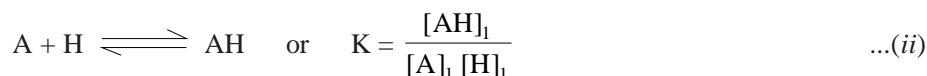
where, A = Extract with partition coefficient K_p, A and

AH = Extract with partition coefficient Kp, AH

Hence, $\qquad\qquad K_p, A = \dfrac{[A]_2}{[A]_1}$ and $K_p, AH = \dfrac{[AH]_2}{[AH]_1}$ $\qquad ...(iii)$

Therefore, for this particular system the efficiency coefficient E may be expressed as follows :

$$E = \frac{[A]_2 + [AH]_2}{[A]_1 + [AH]_1} \qquad ...(iv)$$

Now, substituting Eq. (*ii*) and Eq. (*iii*) into Eq. (*iv*) and subsequently simplifying, we shall get :

$$E = \frac{K_p, A[A]_1 \; K_p, AH[AH]_1}{[A]_1 + [AH]_1} \quad \text{From Eq. (}iii\text{)}$$

or $\qquad\qquad = \dfrac{[A]_1 \, \{K_p, A + K_p, AH[AH]_1 \, / [A]_1\}}{[A]_1 \, \{1 + [AH]_1 \, / [A]_1\}}$

or $\qquad\qquad E = \dfrac{K_p, A + K_p, AH. \, K[H]_1}{1 + K[H]_1} \quad \text{From Eq. (}ii\text{)} \qquad\qquad ...(v)$

* Matkovitch, C.E., and Cristian, G.D., **Anal. Chem., 45,** 1951, 1973

From Eq. (v) it is quite evident that E approaches K_p, A as $K[H]_1$ becomes small and K_p, AH as $K[H]_1$ becomes large.

Now, assuming that only A extracts (*i.e.,* A being a neutral organic base and AH the conjugate acid), Eq. (v) may be expressed as :

$$E = K_p, A \; \frac{1}{1 + K[H]_1} = K_p, A \; \frac{[A]}{[A] + [AH]} \qquad \qquad ...(vi)$$

The following inferences may be arrived at on the basis of Eq. (vi), namely :

(a) Extraction coefficient (E) is just the partition coefficient times the fraction of the analyte which is present in the extractable form,

(b) Under a given set of experimental parameters the ultimate effect of the 'equilibrium' shall be to reduce the amount extracted, and

(c) Forcibly shifting the 'equilibrium' toward the extractable species by adjusting the pH helps to minimise the effect of the equilibrium thereby rendering E almost equal to K_p, A.

In conclusion, it may be observed that the pH for an **'extraction system'** must be selected in such a fashion so that the maximum quantum of the analyte is present in the extractable form, that obviously suggests that the analyte should always be in the form of either a free base or a free acid. From the actual practical experience it has been noticed that a good-working range lies between 95 to 97% present in the extractable form.

27.3.3. EFFECT OF ION-PAIR FORMATION

Ion-pair formation needs its due recognition because it very often gives rise to unexpected extractions. In true sense, ion-pair may be regarded as a close association of an anion and cation, and therefore, it usually takes place either in a polar or a non-polar solvent. In reality, the ion-pairs are invariably formed by virtue of the union between comparatively large organic anions and (much smaller) cations. Interestingly, the resulting ion-pairs have been found to show their appreciable solubility in polar solvents ; and hence, these species may be extracted conveniently under such experimental parameters where neither individual component ion could.

A few vital criteria towards the formation of an improved aqueous extractable ionic species are, namely :

- Formation of a neutral metal-chelate complex or by ion association, and
- Creation of larger and more hydrophobic molecular species.

A few typical examples shall be discussed here to explain the chelate-formation :

Example 1 : **Cu^{2+} with 'acetylacetonate'** forms a fairly stable ring compound :

| Acetylacetonate (*keto*-form) | Acetylacetonate (*enol*-form) | | Copper(II)-acetylacetonate complex (Resonating ring compound) |
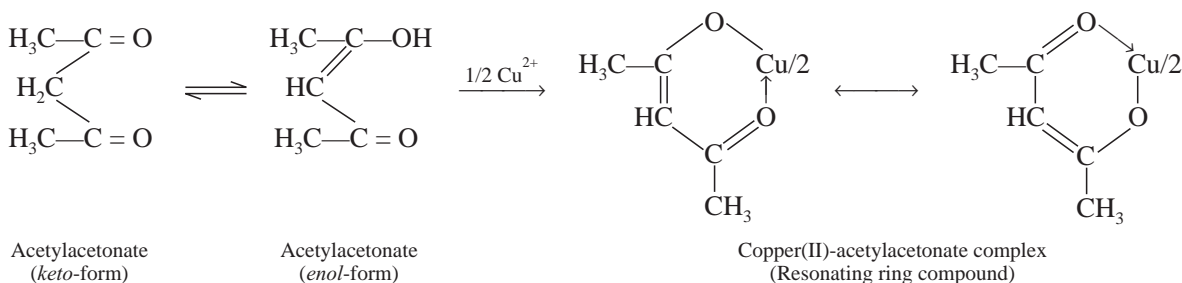
Example 2 **: Iron (III) 'cupferrate'** gives rise to a stable ring compounds as shown below :

$$\text{(ring structure)} \quad N—O^- \; \} \; NH_4^+ \xrightarrow{1/3 \; Fe^{3+}} \text{(ring structure)} \; Fe/3$$
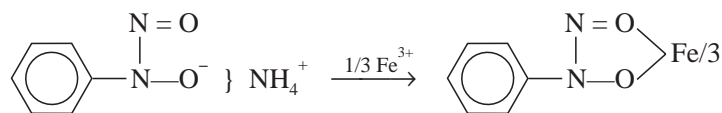
Example 3 **: Sulphonic acids** rapidly pair with a plethora of **'protonated amines'** to form an easily extractable complex

Primary amine (1°) :
$$R—\overset{\overset{\displaystyle H}{|}}{\underset{\underset{\displaystyle H^{\oplus}}{|}}{N}}—H. \; HSO_3^{\ominus} \qquad \text{A complex}$$

Secondary amine (2°) :
$$R_2—\overset{\overset{\displaystyle H}{|}}{\underset{\underset{\displaystyle H}{|}}{N^{\oplus}}}. \; HSO_3^{\ominus} \qquad \text{A complex}$$

Example 4 **: Cl⁻ ion** serves as an 'appropriate anion' that favourably combines with many aromatic amines and alkaloids which may ultimately be extracted from the corresponding aqueous solutions into chloroform as their respective chlorides*.
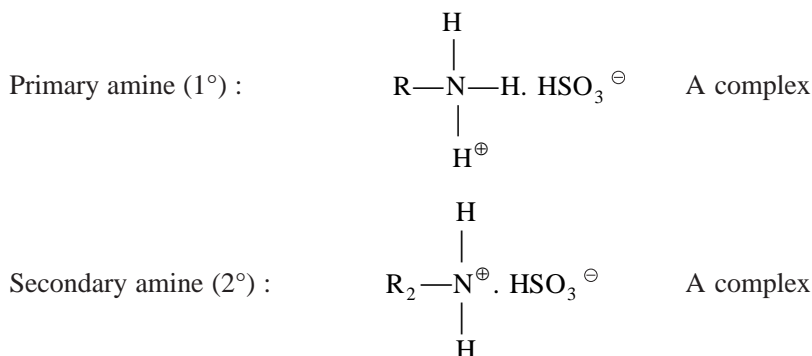
27.3.4. EFFECT OF SYNERGISTIC EXTRACTION

Synergism : It may be defined as **'the process whereby two different reagents when employed together are capable of extracting a metal ion with a distinct and marked efficiency, in comparison to a condition when the same two reagents are used individually'.**

Example **: (*i*) : Complexation of Mn²⁺ with dithizone and pyridine :**

It has been observed that the complex formed by Mn^{2+} with dithizone alone is of no practical analytical utility because of the fact that it undergoes decomposition very quickly. However, the addition of a base, such as : pyridine into the Mn^{2+} plus dithizone complex yields a red-complex, which is fairly stable to oxidation and light; and, therefore, forms the basis for a very sensitive **photometric method** employed in estimating trace amounts of Mn^{2+}.

Equation : Following is the chemical reaction of the above complex formation :

$$Mn^{2+} + 2S=C \quad \xrightarrow[\text{(Base)}]{\text{Pyridine}} \quad [\text{complex structure}] \; Mn + 2H^+$$

Dithizone
(or Diphenylthiocarbazone)

Manganese-dithizone
(red-complex)
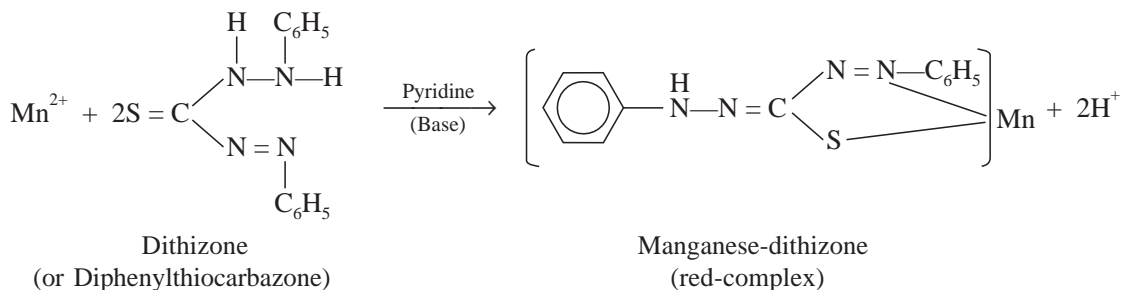
(*ii*) Complexation of Ni²⁺ with dithizone and 1, 10-phenanthrolone :

Noticeably, the reaction of Ni^{2+} with dithizone is quite slow and sluggish. Nevertheless, this slow reaction is significantly accelerated by the addition of nitrogen-containing bases like 1, 10-phenanthroline. The resulting complex may be represented by the following equation :

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* Manske, R.H.F., **Can. J. Chem., B14**, 347, 1936.

Equation :

Nickel-dithizone-phenanthroline complex

It is the basis of a very sensitive synergistic extraction photometric procedure for trace amounts of Ni^{2+}.

27.4. EMULSION PROBLEM ENCOUNTERED IN EXTRACTIONS

Emulsion : It may be defined as-**'a dispersed system containing at least two immiscible liquid phases'.**

The effective and meaningful extraction of an analyte is rendered almost impossible when one encounters an emulsion formation during an extraction process thereby the separation of the two phases becomes difficult. Actually, it offers a frequent and serious problem when dealing with the extraction of drugs from biological as well as pharmaceutical formulations.

Emulsion formation enhances the area of the interface between the two immiscible solvents and as a result also enhances the **'free energy'** of the system, which may be designated by the following expression :

$$\text{Free energy} = \gamma \times \Delta A$$

where γ = Interfacial tension, and

A = change in surface area resulting from emulsification.

Obviously the **'lowest free energy'** is given by the most stable state for a system at constant pressure and, therefore, in due course an emulsion shall **'break'** spontaneously to the two-layered system. However, the breaking of an emulsion could be relatively a rather slow phenomenon. There are a number of factors which may be responsible for the slow-coalescence of an emulsion, namely :

(*a*) Finely divided powders, albumin, gelatin and natural gums have a tendency to coat the droplets formed in an emulsion which ultimately prevent them from coalescing,

(*b*) Usually surfactants decrease the interfacial tension between the two immiscible liquids which help in stabilizing an emulsion, and

(*c*) Ionic species may get absorbed at the interface of two immiscible layers resulting in the formation of a net charge on the droplets. Because all droplets shall essentially bear the similar charge, naturally they will repel one another thereby preventing coalescence.

In fact, there are many natural and synthetic products that are profusely incorporated in the 'formulation of drugs' which are found to stabilize emulsions either by coating the droplets or by minimizing the interfacial tension, namely :

(*i*) **Coating the droplets :** *e.g.,* starch acacia, silica, gelatin finely divided talc, and

(*ii*) **Minimizing the interfacial tension :** *e.g.,* mono-and di-glycerides ; stearates and sorbitan monoleate.

It has been observed that once an emulsion is formed it is rather difficult to break it. Therefore, it is absolutely necessary to adhere to the following guidelines, as far as possible, in order to avoid forming emulsions in the course of an extraction process :

(1) Always affect very cautious and gentle agitation besides employing a sufficiently large liquid-liquid interface to obtain a reasonably good extraction. Especially when the two-liquid layers have a large contact surface in an extraction process, vigorous or thorough shaking of the two phases is not required at all,

(2) The removal of any finely divided insoluble material(s) in a liquid phase must be done by filtration before carrying out the extraction process,

(3) Always prefer and use such solvent pairs that have a large density difference and a high interfacial tension, for instance : water and hexane, as they are less prone to emulsion problems. In contrast, such solvent pairs as water and benzene should not be used in the extraction process,

(4) When performing extraction from water always ensure not to work at pH extremes and particularly at high pH ranges to avoid emulsification, and

(5) In cases, of acute emulsion-problems substances like-anion exchangers alumina or silicagel are used specifically to resolve the problem by adsorption of the emulsifying agents. In fact, it would be advisable to employ the technique of column chromatography for the effective separation of the analyte as compared to an extraction process.

Breaking of an Emulsion (*i.e.,* Coalescence) : Following are the various techniques invariably used so as to break an emulsion or to achieve coalescence, namely :

(1) **Mechanical Means :** Coalescence may be achieved by mechanically creating turbulence on the surfaces of the droplets either by passing the emulsion through a bed of glass-wool or by stirring with the help of a glass-rod simply,

(2) **Centrifugation :** In cases where the densities of the two liquids are appreciably different coalescence may be afforded by centrifugation-a physical means,

(3) **Addition of Monovalent and Divalent Ions :** Relatively simple emulsions are broken by adding monovalent salts like sodium chloride ; whereas charge-stabilized emulsions are specifically sensitive to the divalent ions, such as : $CaCl_2$; $MgCl_2$ etc.

(4) **Ethanol or Higher Alcohol :** Addition of small quantities of either ethanol or sometimes a higher homologous alcohol shall aid in coalescing an emulsion,

(5) **Sudden Cooling of Emulsion (Thermal Shock) :** Sudden temperature drop or freezing (*i.e.,* giving a thermal shock) of an emulsion mostly enhances the interfacial tension between the two immiscible phases thereby causing coalescence.

(6) **Altering the Ratio of Solvents :** Coalescence of an emulsion may also be achieved either by altering the ratio of the prevailing dispersed phase or even by partial evaporation of the solvent,

(7) **Silicone Defoaming Agent :** A few drops of the silicone-defoaming agent sometimes help in breaking an emulsion, and

(8) **Thin-Bed of an Adsorbent :** Sometimes simply passing an emulsion through a thin-bed of an adsorbent remarkably helps in achieving coalescence taking note of the fact that the analyte will not be absorbed from either solvent.

27.5. ASSAY METHODS BASED ON LIQUID-LIQUID EXTRACTION

A number of specific elements may be determined quantitatively based on liquid-liquid extraction method or **'solvent-extraction'** technique, namely :

(*a*) Determination of copper (I) as the neo-cuproin complex,

(*b*) Determination of Iron (III) as the 8-hydroxyquinoline complex or Iron (III) oxinate,

(*c*) Determination of lead (I) by the dithizone method,

(*d*) Determination of molybdenum (VI) by the thiocyanate method,

(*e*) Determination of Ni (II) :

   (*i*) as dimethylglyoxime complex, and

   (*ii*) by synergistic extraction.

All these assay methods shall be discussed in the following sections :

27.5.1. DETERMINATION OF COPPER (I) AS THE NEO-CUPROIN COMPLEX

Theory

'Neo-cuproin' (*i.e.,* 2, 9-dimethyl-1 : 10-phenathroline) under specific experimental parameters almost behaves as a critical reagent for copper (I). The resulting complex is freely soluble in chloroform and absorbs at 457 nm.
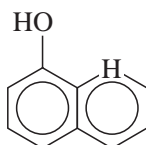
Materials Required : hydroxyammonium chloride solution (10% w/v) : 25 ml ; sodium citrate solution (30% w/v) : 50 ml ; ammonia solution ; **'neo-cuproin'** solution (0.1% w/v in absolute ethanol) : 50 ml ; chloroform ;

Procedure : The following steps may be adopted :

(1) Transfer 10.0 ml of the sample solution (containing upto 200 mcg of copper) in a separatory funnel, add 5 ml of hydroxyammonium chloride solution to affect the reduction of Cu (II) to Cu (I),

(2) To the resulting solution add 10 ml of solution citrate solution to enable complexation of any other metals that may be present,

(3) Add ammonia solution gradually until the pH is about 4.0 (use Congo Red) followed by 10 ml **'neo-cuproin'** solution,

(4) Shake for about 30 seconds with 10 ml of chloroform and allow the layers to separate,

(5) Repeat the extraction with a further 5 ml of chloroform, and

(6) Finally, measure the absorbance at 457 nm against a blank on the reagents that have been used identically to the sample.

27.5.2. DETERMINATION OF IRON (III) AS THE 8-HYDROXY QUINOLATE COMPLEX [IRON (III) OXINATE]

Theory : Iron (III) upto an extent of 50-200 mcg can be extracted effectively from an aqueous solution with a 1% solution of 8-hydroxyquinoline (symbolized as HQ) in chloroform by carrying out a double extraction when the pH of the resulting aqueous solution ranges between 2 and 10. Evidently, between pH 2.0 to 2.5 metals like Ni, Co, Ce (III) and Al do not interfere at all. However, iron (III) oxinate is dark-coloured in chloroform and absorbs at 470 nm.

8-Hydroxyquinoline (oxime)

The reaction may be expressed as follows :

$$3\,(HQ)_{org} + (Fe^{3+})_{aq} \rightleftharpoons (FeO_3)_{org} + (H^+)_{aq}$$
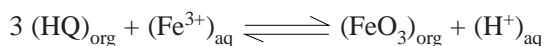
Materials Required : Hydrated ammonium iron (III) sulphate : 0.0266 g ; oxine solution ('AnalaR'-Grade, 1% w/v in chloroform) : 50 ml ; chloroform ; 100 ml ;

Procedure : The following steps may be followed :

(1) Weigh accurately 0.0226 g of hydrated ammonium iron (III) sulphate and dissolve it in 1 litre of DW in a volumetric flask ; 50 ml of this solution contains 100 mcg of iron,

(2) Place 50 ml of the solution ($\equiv$ 100 mcg of Fe) in a 100-ml separatory funnel, and add to it 10 ml of 1% oxine solution, and shake for 1 minute,

(3) Separate the chloroform layer,

(4) Transfer a portion of the chloroform layer to a 1 cm absorption cell and determine the absorbance at 470 nm in a UV-spectrophotometer, employing the solvent (chloroform) as a blank or reference, and

(5) Repeat the extraction with a further 10 ml quantity of 1% oxine solution, and measure the absorbance again so as to confirm whether all the iron was extracted or not. Usually three extractions suffice the complete extraction of Fe (III).

Note : From a glimpse of typical analytical results it may be seen that absorbance after first extraction 0.0592 ; after second extraction 0.0050 ; after third extraction 0.0010 ;

27.5.3. DETERMINATION OF LEAD (I) BY THE DITHIZONE METHOD

Theory : In solution, dithizone (diphenylthiocarbazone) exhibits tautomerism as shown below :

Dithizone
(*enol*-form) I

Dithizone
(*keto*-form) II
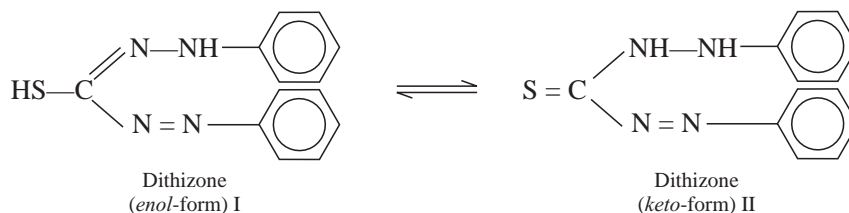
The enol-form of Dithizone (I) behaves as monoprotic acid having a dissociation constant *pKa* = 4.7 upto a pH range of about 12 : obviously, the acid proton is inherited due to the thiol moiety in (I). In reality, two kinds of **'metal dithizonates'** are invariably formed, namely :

(*a*) **'Primary' metal dithizonates :** These are produced as per the following reaction :

$$Mn^+ + n\,H_2\,Dz \rightleftharpoons M\,(HDz)_n + n\,H^+$$
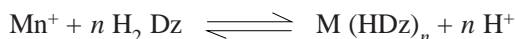
They are of greater analytical value because of their high stability and greater solubility in organic solvents.

(*b*) **'Secondary' metal dithizonates :** These are specifically formed by some metals, such as : Cu, Ag, Au, Hg, Bi and Pd. The second complex are produced under the following *two* conditions, namely :

(*i*) deficiency of the reagent, and

(*ii*) higher pH range,

and may be expressed as follows :

$$2\,M\,(HDz)_n \rightleftharpoons M_2\,Dz_n + n\,H_2\,Dz$$
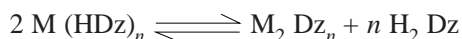
The poor stability and miserable solubility in organic solvents render these products of insignificant analytical importance.
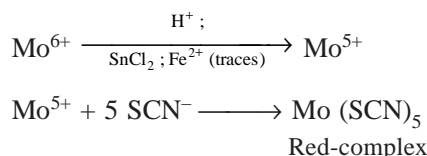
It is, however, pertinent to mention here that dithizone* is an extremely sensitive reagent and, therefore, helps in the determination of lead either from a neutral or faintly alkaline medium to the extent of a few micrograms.

Materials Required : Pure lead nitrate : 0.0079 g ; ammonia-cyanide-sulphite mixture (dilute 35 ml of conc. ammonia solution having sp. gr. 0.88 and 3 ml of 10% w/v solution potassium cyanide **(Caution : deadly poisonous, use protective gloves while handling)** to 100 ml, and then dissolving 0.15 g of sodium sulphite in this solution) : 75 ml ; dithizone (pure) solution (0.005% w/v in chloroform)** : 7.5 ml ; chloroform : 17.5 ml ;

Procedure : Dissolve 0.0079 g of pure lead nitrate in 1 litre of DW in a volumetric flask. To 10 ml of this solution (equivalent to about 50 mcg of Pb) contained in a 250-ml separatory funnel, add 775 ml of ammonia-cyanide-mixture, and adjust the pH of the resulting solution to pH 9.5 by the careful addition of HCl. Now, add 7.5 ml of dithizone solution and 17.5 ml of chloroform rapidly. Shake the contents of the separatory funnel thoroughly for 1 minute, and allow the phases to separate. Determine the absorbance at 510 nm *vis-a-vis* a blank solution in a 1.0 cm absorption cell. However, a further extraction of the same solution yields zero absorption thereby indicating that complete extraction of lead has taken place.

27.5.4. DETERMINATION OF MOLYBDENUM (VI) BY THE THIOCYANATE METHOD

Theory : Molybdenum (VI) is mostly converted to molybdenum (V) when an acidic solution of the former is treated with tin (II) chloride preferably in the presence a little Fe^{2+} ion. The resulting molybdenum (V) form a red complex with thiocyanate ion as follows :

$$Mo^{6+} \xrightarrow[\text{SnCl}_2 \text{ ; Fe}^{2+} \text{ (traces)}]{\text{H}^+ \text{ ;}} Mo^{5+}$$

$$Mo^{5+} + 5\ SCN^- \longrightarrow \underset{\text{Red-complex}}{Mo\ (SCN)_5}$$

Consequently, the red-complex is extracted with either solvents possessing donor oxygen atoms, such as : 3-methyl butanol. However, Mo (VI) may also be extracted with diethyl ether-an oxygenated solvent, because it yields the maximum percentage extractive with 7.0 M NH_4 SCN as could be seen from the following Table 27.2.

Table 27.2 : Diethyl Ether Extraction of Mo (VI) with Various Anions

Metal	HCl[a] (6.0 M)	HBr[b] (6.0 M)	HI[c] (6.9M)	NH₄SCN[b] (7.0M)	HNO₃[b] (8.0 M)
	PERCENTAGE EXTRACTIVES				
Mo (VI)	85	54	6.5	97	0.6

a-3 : 2 volume ratio of organic to aqueous phase

b-1 : 1 volume ratio of organic to aqueous phase

c-4 : 1 volume ratio of organic to aqueous phase

The molybdenum complex exhibits maximum absorption at 465 nm.

Materials Required :

(*i*) **Standard Molybdenum Solution :** Dissolve 0.184 g of ammonium molybdate $(NH_4)_6\ [Mo_7O_{24}]$ 4H₂O in 1 litre of distilled water in a volumetric flask : this yields a 0.01% solution which can be

---

* Dithizone of the **'purest quality'** must be used for the assay, since it readily oxidizes to form diphenylthiocarbadiazoles [S = C (N = N – C₆H₅)₂] which is non-reactive to metals.

** 1 ml of dithizone solution ≡ 20 mcg of Pb ;

diluted to 0.001% with 0.1 M HCl, thereby giving a Mo solution containing 100 mcg ml^{-1},

(*ii*) **Ammonium Iron (II) Sulphate Solution :** Dissolve 10 g of the salt in 100 ml of very dilute sulphuric acid,

(*iii*) **Tin (II) Chloride Solution :** Dissolve 10 g of Tin (II) chloride dihydrate in 100 ml of 1 M HCl, and

(*iv*) **Potassium Thiocyanate Solution :** Prepare a 10% w/v aqueous solution from the pure salt ('AnalaR'-Grade).

**Procedure :** The various steps involved are as follows :

(1) First of all construct a calibration curve by transferring accurately 1.0, 2.0, 3.0, 4.0 and 5.0 ml of the 0.001% Mo solution (*i.e.,* containing 10, 20, 30, 40, and 50 mcg Mo respectively) in individual 50-ml separatory funnels and diluting each of them with an equal volume of water.

(2) Add to each funnel 2 ml of conc. HCl, 1 ml of ammonia iron (II) sulphate solution, and 3 ml of the potassium thiocyanate solution,

(3) Shake gently and then induce 3ml of the tin (II) chloride solution,

(4) Add water to bring the total volume in each separatory funnel to 25 ml and mix thoroughly,

(5) Introduce exactly 10 ml of redistilled 3-methyl butanol into each funnel and shake them separately for 30 seconds,

(6) Allow the two phases to separate completely and carefully drain out the lower aqueous layer,

(7) Remove the glass-stopper and pour the alcoholic extract through a small plug of purified glass wool in a small funnel and transfer the organic extract to a 1 cm absorption cell,

(8) Measure the absorbance at 465 nm in a UV spectrophotometer against a 3-methyl butanol blank,

(9) Plot the graph by taking absorbance against concentration of Mo in Mcg, thereby obtaining a straight line spreading over a range 0-50 mcg of Mo (obeying **Beer's Law**), and

(10) Finally, determine the concentration of Mo in unknown samples provided and containing less than 50 mcg Mo per 10 ml ; make use of the calibration curve, and subject the unknown samples to the same treatment as the standard solutions.

27.5.5. DETERMINATIONS OF NICKEL (II)

A. As Dimethylglyoxime Complex

Theory : In ammoniacal solution, Ni (II) forms an insoluble red coordination compound with dimethylglyoxime ($C_4H_8O_2N_3$). Nickel dimethylglyoximate is only sparingly soluble in chloroform (35-50 mcg Ni ml^{-1}). It is, however, necessary to know the approximate amount of Ni present in the sample, so as to avoid adding a large excess of dimethylglyoxime, which is not very soluble in water and may precipitate easily along with the nickel-complex. The optimum pH range at which the extraction of this complex should be carried out ranges between 7-12 in the presence of citrate. It has been observed that the nickel-complex is quite bulky in nature when first precipitated and hence, shows a tendency to move up along the walls of the container. Therefore, care should be taken that the sample must not contain more than 50 mg of Ni. Lastly, the nickel complex absorbs at 366 nm and also at 465-470 nm.

The formation of nickel dimethylglyoximate complex may be expressed as follows :

$$Ni^{2+} + 2\ C_4H_8O_2N_2 \xrightarrow{\ NH_3\ } Ni\ (C_4H_7O_2N_2) + 2\ H^+$$
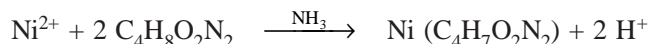
Materials Required : Ammonium nickel sulphate (pure) : 0.135 g ; citric acid : 5.0 g ; dilute ammonia solution ; dimethylglyoxime solution (dissolve 0.50 g of dimethyl-glyoxime in 250 ml of ammonia solution and diluting to 500 ml with water) : 20 ml ; chloroform : 50 ml ;

Procedure

(1) Weigh accurately 0.135 g of pure ammonium nickel sulphate (NiSO$_4$, (NH$_4$)$_2$ SO$_4$, 6H$_2$O) and dissolve in 1 litre of distilled water in a volumetric flask,

(2) Transfer 10 ml of the resulting solution (Ni $\simeq$ 100 mcg) into a breaker containing 90 ml of water,

(3) Add to it 5 g of citric acid, and then dilute ammonia solution carefully until the pH is 7.5,

(4) Cool and transfer to a separatory funnel, add 20 ml of dimethylglyoxime solution and, after standing for a minute 12 ml of chloroform,

(5) Shake the contents of the funnel for 1 minute, permit the two phases to separate out completely,

(6) Collect the lower red chloroform layer and determine the absorbance at 366 nm in a 1 cm absorption cell against a blank, and

(7) Extract once again with a 12 ml portion of chloroform and measure its absorbance at 366 nm ; usually very negligible Ni (II) may be found.

B. Synergistic Extraction

Theory : Dithizone and 1, 10-phenanthroline (see Section 27.3.3) help in the synergistic extraction of Ni (II) both quantitatively and rapidly over a wide range of pH between 5.5 to 11.0 to give rise to a dark coloured mixed-ligand complex that absorbs at 520 nm. The resulting complex bears the following vital characteristic features, namely :

 (*i*) It is fairly stable to allow the complete removal of excess dithizone by back-titration with 0.1 M NaOH, so as to make a 'monocolour' method feasible,

(*ii*) The molar absorptivity of the complex stands at 4.91×10^4 mol^{-1} L cm^{-1}, and

 (*iii*) The synergistic method is predominantly much more sensitive as compared to any other method for the determination of Ni (II).

Materials Required : Ammonium nickel sulphate* (pure) : 0.0135 g ; phthalate or acetate (ethanoate) buffer (pH 6.0) : 5 ml ; dilute ammonia solution ; chloroform : 15 ml ; sodium hydroxide (0.1 M) : 10.0 ml ;

Procedure

(1) To 5 ml of a solution containing from 1 to 10 mcg of Nickel (II) 5 ml of a phthalate or acetate buffer,

(2) In case, the sample is acidic, adjust the pH to 6.0 with dilute ammonia solution carefully,

(3) To the resulting solution add 15 ml of chloroform solution of dithizone and 1, 10-phenanthroline,

(4) Moderately shake the two phases for 5 minutes in a separatory funnel, allow them to separate distinctly into aqueous and chloroform (lower) layers,

(5) Excess dithizone may be removed from the chloroform layer by back-extraction with 10 ml of 0.1 M NaOH, (a through shaking for 60 seconds will suffice this extraction),

(6) Once again separate the chloroform layer (lower) and measure its absorbance in a 1 cm absorption cell at 520 nm *Vs* an identically treated blank, and

(7) Finally, draw a calibration curve using standard Ni (II) solution containing 2, 4, 6, 8, and 10 mcg in 10 ml (obeying **Beer's Law**).

Caution : All glassware must be rinsed with dilute acid and then thoroughly with distilled water.

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* Dissolve 0.0135g of ammonium nickel sulphate in 1 litre of DW ; dilute 10 ml of this solution to 100 ml with DW (1 ml of this solution $\equiv$ 2 mcg of Ni).

Note : The reagent must be prepared afresh using 'AnalaR-Grade' dithizone and 1, 10-phenanthroline, preferably taken from a new or recently opened reagent bottle.

THEORETICAL AND PRACTICAL EXERCISES

1. What is the importance of '*liquid-liquid extraction*' in the domain of actual estimation ? Explain.

2. Discuss the **Nernst Distribution Law** or **Partition Law** with reference to the theoretical aspects of liquid-liquid extraction support your answer with suitable examples.

3. Expatiate the *two* following vital aspects of liquid-liquid extraction :

    (*a*) Error due to the volume change,   (*b*) Effectiveness of an '*extraction*'.

4. Enumerate the following **four** cardinal factors which influence the solvent extraction mostly :

    (*i*) Effect of temperature and inert solutes,   (*ii*) Effect of pH on extraction,

    (*iii*) Effect of ion-pair formation, and   (*iv*) Effect of synergistic extraction

    Provide suitable examples wherever possible to make your explanation more plausible and understandable.

5. What do you understand by the term '*free energy*' of the system between two immiscible solvents ? How would you encounter the emulsion problem in liquid-liquid extractions ? Explain.

6. Discuss the assay of Ni(II) :

    (*a*) As dimethylglyoxime complex, and   (*b*) By synergistic extraction.

7. Describe the theory and methodology for the assay of Cu(I) as its neo-cuprin complex.

8. Discuss the theoretical and procedural aspects for the assay of Fe(III) as its Fe(III) oxinate complex.

9. How would you determine Pb(I) by the dithizone method ? Explain.

10. Give the theory and procedure for the determination of Mo(VI) by the thiocyanate method.

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6. Karger, B.L., L.R. Snyder and C. Horvath., **'An Introduction to Separation Science',** New York, John Wiley & Sons, Inc., 1975.

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28 THIN LAYER CHROMATOGRAPHY (TLC)

The following *four* aspects of chromatography shall be discussed in Part V of this book in addition to applications of these chromatographic techniques exclusively to quantitative analyses of pharmaceutical substances :

- Thin-Layer Chromatography (TLC),
- Gas-Liquid Chromatography (GLC),
- High Performance Liquid Chromatography (HPLC), and
- Size Exclusion Chromatography (SEC).

28.1. INTRODUCTION

Thin-layer chromatography (TLC) is an altogether new, versatile and specialized laboratory technique that was evolved in early Fifties, and since then has become an indispensable means of separation for analysts and researchers round the globe. It can be employed conveniently both for organic and inorganic substances,

either derived from natural sources or synthesized in the laboratories, on quantities ranging from the nanogram to microgram levels.

Kirchner in 1950 was the first who used adsorption chromatography on impregnated glass-plate coated with silicic acid or alumina. It may be emphasized, however, that **Egon Stahl's fundamental work** stands as a landmark in the world-wide acceptance of this new technique in the laboratory. Later on, Stahl in 1958, introduced a standard equipment for preparing uniform thin-layers of known thickness, which eventually led to the ultimate acceptance of this new technique as an additional modern tool for analytical chemistry.

This is invariably referred to in various literature as : **'open-column chromatography'; 'drop chromatography' ; 'strip-chromatography' ; 'spread-layer chromatography' ; 'surface chromatography'.**

TLC in addition to combining the meritorious plus points of column and paper chromatography, also considered to be extraordinarily superior to either of the two in certain aspects.

28.2. THEORY

The adsorbent used in TLC is a thin, uniform layer (normally 0.24 mm thick) of a dry, finely powered material applied to an appropriate support, such as a glass plate or an aluminium sheet or a plastic foil. Subsequently, the mobile phase is permitted to move across the surface of the plate (usually by capillary action) and the chromatographic phenomenon may solely depend upon adsorption, partition, or a combination of both, depending on the adsorbent, its treatment, and the nature of the solvents employed. During the chromatographic separation procedure the TLC-plate is placed in a chromatographic chamber, mostly made up of glass to enable clear observation of the movement of the mobile phase up the plate, that is pre-saturated with the solvent vapour. The inert solid supports invariably employed are, namely : alumina, silica gel, kieselguhr and cellulose, to these may be added appropriate substances, for instance : calcium sulphate (gypsum) so as to provide adequate adhesion to the solid support, example : silica gel-G (G-stands for gypsum).

The prepared layer may be impregnated with suitable materials to achieve specific purpose, namely :

(*i*) **Buffering materials :** To afford acidic, basic or neutral layers,

(*ii*) **Silver nitrate :** To modify its characteristics *e.g.*, for separation of methyl esters of fatty acids, and

(*iii*) **Ion-exchange materials :** To modify its properties, *e.g.*, admixture of cellulose with ion-exchange resins used for the separation of nucleic acids and their respective derivatives.

Therefore, the application of skill and wisdom may give rise to a fairly wide spectrum of possible layers, employed in conjunction with a vast combination of solvent systems permits and affords an almost infinite variation of separating power that really makes TLC such a versatile and useful technique in the domain of pharmaceutical analysis.

28.2.1. VERSATILITY OF TLC OVER PAPER AND COLUMN CHROMATOGRAPHY

In general, TLC essentially not only amalgamates the meritorious plus points of both paper and column chromatography but also it is distinctly superior and more versatile to either of the two methods. However, the versatility of TLC over paper and column chromatography are quite evident from the following points, namely :

(*i*) **Simple equipments :** TLC mostly requires very simple equipments, such as : micro-slides ; specimen jars with lid ; glass-sprayers ; strips of glass sheet ; small chromatank etc.

(*ii*) **Short development time :** In TLC, the separation is very rapid *i.e.*, the development time is of short duration (say 1 hour) for reasonably good separation on inorganic adsorbent layers. Hence, it has a positive edge over paper and column chromatography which normally takes several hours or days.

(*iii*) **Wide choice of stationary phase :** TLC may be used for adsorption, partition (including reversed phase) or ion-exchange chromatography,

(*iv*) **Quick recovery of separated constituents :** TLC permits the possibility of removal of the adsorbent coating on the plates by scraping with a spatula. In other words, a spot or a zone can be removed

quantitatively, and the separated constituent dissolved in an appropriate solvent is estimated either by suitable spectrophotometric or colorimetric analysis.

(*v*) **Separation effects :** The separation effects obtained by TLC are more distinctive and superior than those of paper chromatography,

(*vi*) **Easy visualization of separated components :** Detection of fluorescence components when exposed to UV light is much easier than on paper by virture of the fact that inorganic material (*i.e.*, adsorbent) has intrinsic fluorescence,

(*vii*) **Detection Limit :** TLC affords extremely sharp delineated spots and offer lower detection limit *i.e.*, one decimal power less than that in paper chromatography,

(*viii*) **Variable thickness of think layers :** The method employed in TLC may be further extended to preparative separations by using thicker layers and also to meet separations by column chromatography,

(*ix*) **Chemically inert stationary phase :** Use of inorganic adsorbents *e.g.*, alumina and silica, in TLC allows the application of corrosive sprays to detect fractionated substances, for instance : carbohydrates by 70% conc. H_2SO_4, and

(*x*) **Trace analysis :** TLC method is suitable as micromethod in trace analysis.

28.3. EXPERIMENTAL TECHNIQUES OF TLC

The various techniques with regard to thin layer chromatography (TLC) are as stated below, namely :

28.3.1. PREPARATION OF THIN LAYERS ON PLATES

The paramount importance with regard to the preparation of thin layer is that it must be uniform and consistent throughout. Various means have been put forward to apply thin layers of powdered or their suspensions or their slurries to the carrier plates with a view to achieve an uniform layer throughout the length of the plates. These are namely :

(*a*) **Pouring of Layers :** In order to obtain layers of equal thickness, a measured amount of the suspension or slurry is placed on a given-size plate that is rested on an absolutely labelled surface. The plate is subsequently tipped backward and forward to permit the slurry (or suspension) to spread uniformly on the surface of the plate.

(*b*) **Dipping :** Peifer* in 1962, was pioneer in introducing this technique, whereby two plates at a time back-to-back are dipped together in a slurry of the adsorbent in either chloroform or chloroform-methanol. However, this particular methods is not much in use now-a-days.

(*c*) **Spraying :** Reitsema** was first to develop this method by making use of a small paint-sprayer for the distribution of the suspension or slurry onto the surface of the glass-plate.

Disadvantages : There are mainly *two* major disadvantages of this technique, namely :

(*i*) Non-uniformity of layers on a single-plate, and

(*ii*) Variation observed from one plate to the other was significant.

Belgian Patent No : 625012 : It essentially consists of spraying either molten or partially molten absorbent onto a glass plate, for instance : an alumina film prepared by melting and aluminium rod with an oxyacetylene flame and subsequently spraying the molten adsorbent onto a glass plate.

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* Peifer, J.J., **Mikrochim Acta,** 529, 1962.

** Reitsema, R.H., **Anal. Chem., 26,** 960, 1954.

(*d*) **Spreading :** In this particular case, the suspension or slurry is put in an 'applicator', which is subsequently moved either over the stationary glass-plate or *vice-versa i.e.*, it is held stationary while the glass plate is pulled or pushed through. This technique termed as 'spreading' usually yields uniform thin layers on the glass plates.

Kirchner's* technique essentially consists of :

* selecting uniform surfaced glass plates,

* placing them between glass or metal guides which are thicker than glass plates by the amount that is desired for the adsorbent layer, and

* spreading the slurry on the glass plate with the help of a glass rod.

Egon Stahl's apparatus exclusively designed for the application of thin-adsorbent layers which broadly comprises of *two* major parts, namely :

 (*i*) **Aligning Tray :** It is a tray on which the glass plates are placed in a series or in-a-line, and

(*ii*) **Spreader :** It holds the spreading mixture (as a slurry or suspension) and applies it uniformly in a thin-layer.

Figure 28.1 (*a*) shows the operation of thin-layer spreader ; while Figure 28.1 (*b*) depicts the aligning tray with glass-slide partially coated.
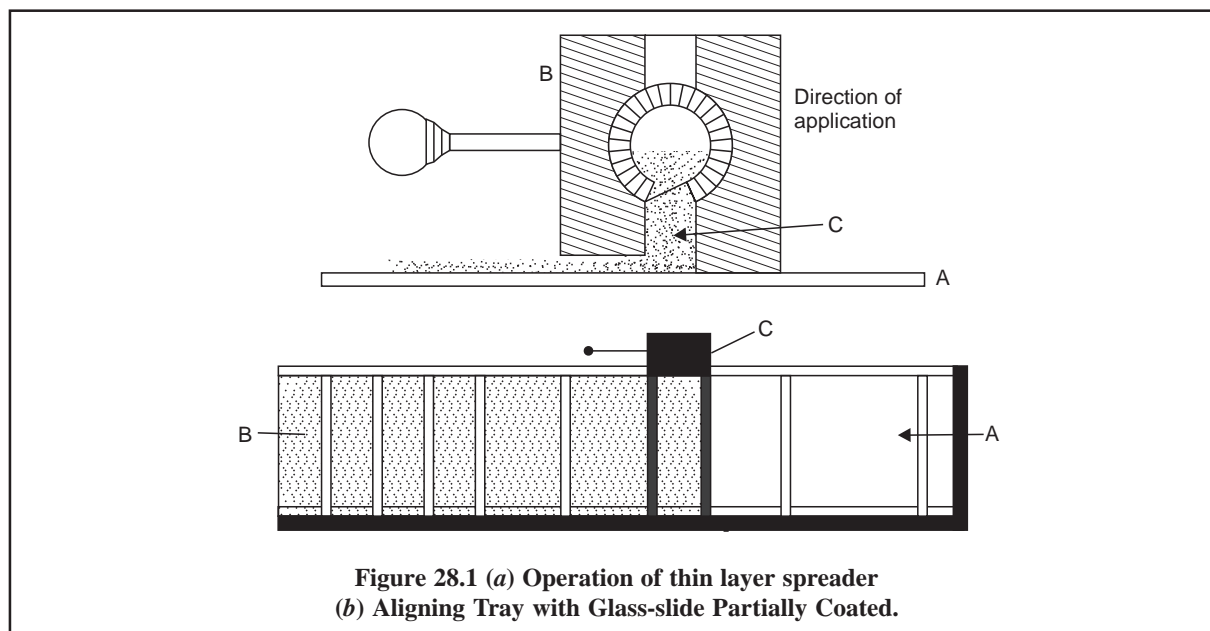
**Figure 28.1 (*a*) Operation of thin layer spreader
(*b*) Aligning Tray with Glass-slide Partially Coated.**

In Figure 28.1 (*a*)   A = Glass plate,

                                        B = Spreader, and

                                        C = Slurry of the adsorbent.

Here, the slurry (C) is put in the spreader (B) and then moved along the direction of application onto the surface of the glass plate (A) to obtain an uniform layer,

In Figure 28.1 (*b*)   A = Glass plate,

                                        B = Layer of adsorbent, and

                                        C = Aligning tray

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* Kirchner, J.G., Miller J.M. and Keller, G.J., **Anal, Chem., 23, 420, 1951.**

The slurry of the adsorbent is introduced into the aligning tray (C) which is then moved onto the glass plate (A) to obtain an uniform layer of adsorbent (B).

In 1962, a well known West German firm DESAGA introduced a much improved and simplified version of a TLC-spreader that could conveniently give uniform layer thickness raging from 0.00 to 2,0 mm.

(*e*) **'TLC-Plates ready-for Use' (or Pre-coated Plates)**

E. Merck AG (Darmstadt, West Germany) was pioneer in introducing *two* types of ready-for-use TLC plates either on glass or polysheets (ethyleneterephthalate), namely :

 (*i*) Ready-for-use TLC Plates with Cellulose-F, and

(*ii*) Ready-for-use TLC Plates with Silica Gel F-254.

Interestingly, these precoated TLC-plates essentially have : first, a special abrasive-resistant layer containing no gypsum ; and secondly, the layer contains a reliable fluorescent indicator that is excited to emit a fluorescence under either a short-wave or a long-wave UV light.

Advantages : These '*ready-for-use*' TLC-plates have the following advantages namely :

- It may be safely activated at 110-120° C, before, use,

- The properties of the layer minimise spot-diffusion that helps both more strong concentration of spots and more distinctive separations with higher sensitivity,

- It may accept more corrosive spray-reagents, for example : conc. sulphuric acid, phosphoric acid, phosphomolybdic acid, perchloric acid on antimony trichloride ; and the sprayed plates could be heated upto 110-120 °C without any darkening whatsoever,

- The migration rate is slightly enhanced when compared to hand coated plates, and

- The TLC plates may be cut into strips by the aid of a glass cutter applied on the reverse side.

28.3.2 CHOICE OF ADSORBENTS

The choice of proper adsorbent in TLC plays a vital role in the separation of components either belonging to natural origin or to purely synthetic origin. It is chiefly based on certain crucial informations like :

 (*i*) Solubility of the substance *e.g.*, hydrophilic and lipophilic,

 (*ii*) Nature of the compound *i.e.*, whether it is acidic/basic/neutral/amphoteric

 (*iii*) Reactivity of compound with either the solvent or the adsorbent, and

 (*iv*) Chemical reactivity of compounds with the binders.

In actual practice, the adsorbents are of two types : firstly the *inorganic*, and secondly, the *organic* adsorbents. A host of substances from each type are used in TLC and these shall be discussed briefly as below :

28.3.2.1. Inorganic adsorbents

These are namely :

(*i*) **Aluminium oxide- (Al_2O_3) :** The alkali (Na_2CO_3 ; $NaHCO_3$) present in alumina very often gives rise to secondary reactions that may be eliminated by washing with dilute mineral acid or with water, followed finally by methanol and ultimately by heating at 200 °C.

Note : Justisaz and Teichner* in 1947 suggested that 1 g of alumina for TLC has 90 sq. M surface area and the one having less than 6 sq. M is useless. Alumina is usually available in three grades :

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* Jutisaz, M. and S. Teichner, **Bull. Soc. Chim.** France, 389, 1947.

(*a*) acidic (pH $\simeq$ 4.0) ; (*b*) basic (pH $\simeq$ 9.0) ; and (*c*) neutral (pH $\simeq$ 7.5).

(*ii*) **Aluminium Silicate :** It permits the adsorption of sterols and sterol glycosides from oils without the use of solvent.

(*iii*) **Bauxite (aluminium oxide ore) :** Zechmeister used bauxite for the separation of enzymic hydrolysates of chitin (a nitrogen-containing polysaccharide found in certain fungi *e.g.*, ergot) ; whereas La Lande employed it for the refining of sugar.

(*iv*) **Bentonites :** It is used mostly for the separation of Vitamin D from vitamin A and sterols and 2,4-dinitrophenyl hydrazones of aldehydes and ketones.

(*v*) **Calcium Carbonate :** It is used as such for the separation of xanthophylls and napthaquinones or other pigments and elution is done with dilute acid to isolate the various components present.

Note : Vaterite-the unstable crystalline modification of calcium carbonate has much greater adsorbent capacity than aronite or calcite.

(*vi*) **Calcium Hydroxide :** It is used as an adsorbent for the separation of carotenoids.

(*vii*) **Calcium Oxalate :** It is used for the separation of anthraquinones and related hypericins (*i.e.*, a dianthrone pigment found in the leaves and petals of *Hypericum perforatum*, Family ; *Guttiferae*).

(*viii*) **Calcium Silicate :** It is employed frequently for the separation of carbohydrates and the corresponding phenylosazones.

(*ix*) **Calcium Sulphate :** It is found to be suitable for the separation of steroids and lipids.

(*x*) **Dicalcium Phosphate :** It is used for the purification of carotene-the natural red pigment.

(*xi*) **Fuller's Earth :** It is hydrous magnesium aluminosilicate which is employed extensively in the petroleum industry for the decolaration of oils. It is also employed for the separation of amino acids and pteridines.

(*xii*) **Hydoxyl-Apatite :** It is a complex calcium phosphate hydroxide which is used for the separation of proteins and glycerides. In may be used with/without binder.

(*xiii*) **Kieselguhr (Diatomaceous Earth) :** (pH 7.0) : It is available both with and without a binder. Its capacity of resolving constituents is less than either silica gel or alumina.

(*xiv*) **Magnesium Silicate (Magnesol : MgO 2.5 $SiO_2.H_2O$) :** It is usually employed for the separation of sugar acetates ; whereas, magnesium trisilicate is used for the separation of steroids, acetylate gycosides, esters, glycerides, lactones etc.

(*xv*) **Silica Gel :** (pH 6.0) : It is used extensively for the separation of sterols, fatty acids, glycerides, azoated carbohydrates, sugar acetates, amino acids.

(*xvi*) **Tri-calcium Phosphate :** It is mostly used for the separation of enzymes.

(*xvii*) **Water-soluble salts :** A number of water-soluble salts are used in TLC for affecting separation of constituents, namely :

$CuSO_4$ (anhydrous)　　:　for azobenzene derivatives,

$CuSO_4.5H_2O$　　　　　:　found to be better than alumina,

$ZnSO_4$; $MnSO_4$; $Al_2(SO_4)_3$ and $MgSO_4$: anhydrous salts good for azobenzene derivatives,

$Al_2(SO_4)_3$　　　　　　:　for hydroxyl anthraquinones, and

Na_2CO_3　　　　　　　:　for Vitamin A

(*xviii*) **Zinc Carbonate :** It is used for the separation of carotenoids and coloured derivatives of amino acids.

28.3.2.2. Organic Adsorbents

The organic adsorbents are known for their relatively milder action for the separation of good number of components, namely :

(*i*) **Cellulose and Acetylated Cellulose :** These adsorbents are commercially available in various forms *e.g.*, particle size, degree of acetylation, with or without binders like starch or Plaster of Paris.

(*ii*) **Charcoal and Activated Carbon :** Tiselius used charcoal for the frontal analysis of sugars, amino acids and other substances. Charcoal absorbs strongly aromatic substances, such as : amino acids, which may be explained by virtue of the fact that the carbon-carbon spacings in graphite are almost of the same order as those present in benzene. Charcoal is also employed for the adsorption of fatty acids.

Weiss* used impregnated activated carbon with fatty acid or non-electrolyte thereby modifying and attributing special and improved adsorption characteristics.

(*iii*) **Dextran Gels :** Proteins and nucleotides can be separated by using cross-linked dextran gels available in various types and particle sizes. The molecular weight of dextran-gels vary considerably depending upon the extent of cross-linked nature.

(*iv*) **Cellulose Ion-Exchange Powder :** Interestingly, the cellulose powder have been modified by state-of-the-art technique that they invariably mimic as real ion-exchangers, namely :

DEAE-Cellulose   = Diethaminoethyl cellulose,

ECTEOLA-Cellulose = Epichlorhydrin linked triethanolamine cellulose, and

PEI-Cellulose   = Polyethylenimine cellulose.

Note : These absorbents may be used both with or without binders, such as : colloidion.

(*v*) **Ion-Exchange Resins :** Nucleic acids and their respective derivatives may be separated either by using ion-exchange resins alone or in conjunction with cellulose powder.

(*vi*) **Polyamide :** Flavanoids-the phenolic substances may be separated effectively using polyamide as such or with a binder, for instance : plaster of Paris or starch.

(*vii*) **Polyethylene Powder :** Fatty acids and their corresponding esters are separated by using polyethylene powder.

(*viii*) **Sucrose :** Both xanthophylls and chlorophylls (*i.e.*, chlorophyll-*a* and -*b*) are separated by using sucrose powder effectively.

28.3.3. CHOICE OF SOLVENT SYSTEM IN TLC

The choice of solvent or a mixture of solvents used in TLC is solely guided by two important factors : (*a*) the nature of the constituent to be separated *i.e.*, whether it is polar or non-polar ; and (*b*) the nature of the process involved *i.e.*, whether it is a case of 'adsorption' or 'partition chromatography'. It has been observed that the rate of migration of a substance on a given adsorbent depends upon the solvent used ; therefore, the latter may be arranged in order of the elutive power, usually termed as the **elutropic series** as shown in the following Table 28.1.

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* Weiss, D.E., **Nature, 162,** 372, (1948).

Table 28.1 : Eluotropic Series of Solvents for Adsorbents Containing Oxygen

Series-'A'*		Series-'B'**
Petroleum Ether	↓ I N C R E A S I N G E L U T I V E P O W E R ↓	n-Pentane
Carbon Tetrachloride		Petroleum Ether (bp 30-50 °C)
Trichloro Ethylene		Petroleum Ether (bp 80-100 °C)
Benzene		n-Hexane
Dichloromethane		n-Heptane
Chloroform		Cyclohexane
Diethyl Ether		Carbon Tetrachloride
Dimethyl Formamide		Trichloroethylene
Ethyl Acetate		Benzene
Pyridine		Dichloromethane
Acetone		Chloroform (Alcohol-Free)
n-Propanol		Diethyl Ether (Absolute)
Ethyl Alcohol		Ethyl Acetate
Methyl Alcohol		Pyridine
Formamide		Acetone
Water		n-Propanol
Glycol		Ethyl Alcohol
Glycerine		Methyl Alcohol ; Water

Note :   (*i*) **These series are not always valid in precisely the same order for all substances,**

     (*ii*) **These series may be regarded as good guides for selecting a specific solvent only, and**

     (*iii*) **These series are good for hydrophyllic adsorbents and not for hydrophobic ones** *e.g.*, **charcoals.**

From actual experimental results it has been established beyond any reasonable doubt that the mixtures of two or three solvents of different polarity mostly offer distinct and much improved separation as compared to chemically homogeneous solvents. Table 28.2 records the elutropic series of one and two component solvents.

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*   Hesse, G.**J. Anal. Chem., 181,** 274, 1961.

**   Wohleben, G., Cited in **Handbuch der Lebensmitted Chemic,** Band II, Tell I, (Springechu Verlag, Berlin), 584, 1965.

Table 28.2 : Eluotropic Series of 1 and 2-Component Solvents

	↓	
Benzene	I	Benzene : Ether (1 : 9)
Benzene : Chloroform (1 : 1)	N	Ether : Methanol (9.9 : 0.1)
Chloroform	C	Ether
Cyclohexane : Ethyl Acetate (8 : 2)	R	Ether : DMF (9.9 : 0.1)
Chloroform : Acetone (95 : 5)	E	Ethyl Acetate
Benzene : Acetone (9 : 1)	A	Ethyl acetate : Methanol (9.9 : 0.1)
Benzene : Ethyl Acetate (8 : 2)	S	Benzene : Acetone (1 : 1)
Chloroform Ether (9 : 1)	I	Chloroform : Methanol (9 : 1)
Benzene : Methanol (95 : 5)	N	Dioxane
Benzene : Ether (6 : 4)	G	Acetone
Cyclohexane : Ethyl Acetate (1 : 1)		Methanol
Chloroform : Ether (8 : 2)	E	Dioxane : Water (9 : 1)
Benzen : Acetone (8 : 2)	L	
Chloroform : Methanol (99 : 1)	U	
Benzene : Methanol (9 : 1)	T	
Chloroform : Acetone (8.5 : 1.5)	I	
Benzene : Ether (4 : 6)	V	
Benzene : Ethyl Acetate (1 : 1)	E	
Chloroform : Ether (6 : 4)		
Cyclohexane : Ethyl Acetate (2 : 8)	P	
Butyl Acetate	O	
Chloroform : Acetone (7 : 3)	W	
Benzene : Ethyl Acetate (3 : 7)	E	
Butyl Acetate : Methanol (9.9 : 0.1)	R	
	↓	

Note : The numbers refer to proportionate volumes.

28.3.4. ACTIVATION OF ADSORBENT

In fact, it is extremely important to eliminate as completely as possible the solvent imbedded into the thin layer of coated adsorbent. It is achieved conveniently first by air-drying the TLC plates for a duration of 30 minutes and then in a hot-air oven maintained at 110 °C for another 30 minutes and subsequently cooling them in a dessicator. This drying process helps a great extent in rendering the adsorbent layer active. In order to achieve very active layers, silica gel and alumina coated plates may be heated upto 150 °C for a duration of 4 hours and colling them in a dessicator.

28.3.5. PURIFICATION OF SILICA GEL-G LAYERS

The iron present as an impurity in silica gel-G affords an appreciable distortion of the 'chromatogram'. Hence, it has become almost a necessary step to purify the adsorbent. The 'iron-free' layers may be achieved by providing the pre-coated and air-dried plates a preliminary development with a mixture of methanol and

concentrated hydrochloric acid* (9 : 1). By this process the entire iron gets migrated with the solvent front to the upper boundary of the TLC plate. Consequently, the purified plates are again dried and activated at 110°C.

The cleaning process usually washes out the $CaSO_4$ originally present as binder. Therefore, the silica gel thus obtained by purification may be reused to prepare TLC-plates with other appropriate binders like gypsum, starch etc.

28.3.6. SPOTTING OF THE COMPONENTS

The following points may be strictly adhered to while spotting the component or mixture of components on a TLC plate, namely :

   (*i*) The sample is normally applied as a solution in a '*non-polar solvent*' as far as possible, since the use of a polar solvent may cause:

   (*a*) spreading out of the starting spot, and

   (*b*) affect directly the Rf value of components,

   (*ii*) The solvent employed for dissolving the sample must be easily volatile-in-nature so that it should be removed from the TLC plate before development commences,

   (*iii*) The 'area of application' should be smallest as far as possible so as to achieve a sharper resolution,

   (*iv*) To maintain the size of the spot 'small' repeated applications is made by allowing the solvent to evaporate after each application. It can be easily achieved by :

   (*a*) Pre-warming the TLC-plate, and

   (*b*) Passing a stream of hot-air right below the sample spot (from a hair-drier).

   (*v*) For exclusively 'preparative work' the sample is applied in a narrow-band the width of which must be kept as narrow as possible, and

   (*vi*) Use of 'spotting templates', available commercially** may provide

   (*a*) Device for making the 'starting-line'

   (*b*) Device for making the 'finishing-line', and

   (*c*) Means for uniformly spacing the spots on the starting-line.

28.3.7. DEVELOPMENT OF THIN LAYERS

The spotted TLC plates, after evaporation of the sample solvent, is placed in a closed chamber saturated with vapours of the developing solvent(s). One end of the plate is then wetted with the developer by means of either 'ascending-technique or the 'descending-technique' as shown in Figure 28.2 (*a*), (*b*). After the developer has traversed one-half to two-thirds the total length of the TLC plate, the latter is removed from the chamber, air-dried and the positions of the components are located by any of several methods.

There are *three* major factors which essentially govern the 'development of thin-layers', namely :

   (*i*) Equilibration of the chamber (or chamber-saturation),

   (*ii*) Protection against oxidation (temperature and light), and

   (*iii*) Visualization.

---

*   Both Methanol and conc. HCl must be of 'AnalaR'-Grade Reagent.

** Manufactured by M/S DESAGA ; CAMAG ; CAMDEN ; & CHEMETRON ;

Figure 28.2 (*a*) : Ascending Flow A = Starting position of sample(s) ; B = Solvent System ;
C = Chromatographic Surface ;
Figure 28.2 (*b*) : Descending Flow A = Starting position of sample(s) ; B = Developer ;
C = Chromatographic surface ; D = Cotton wick ;
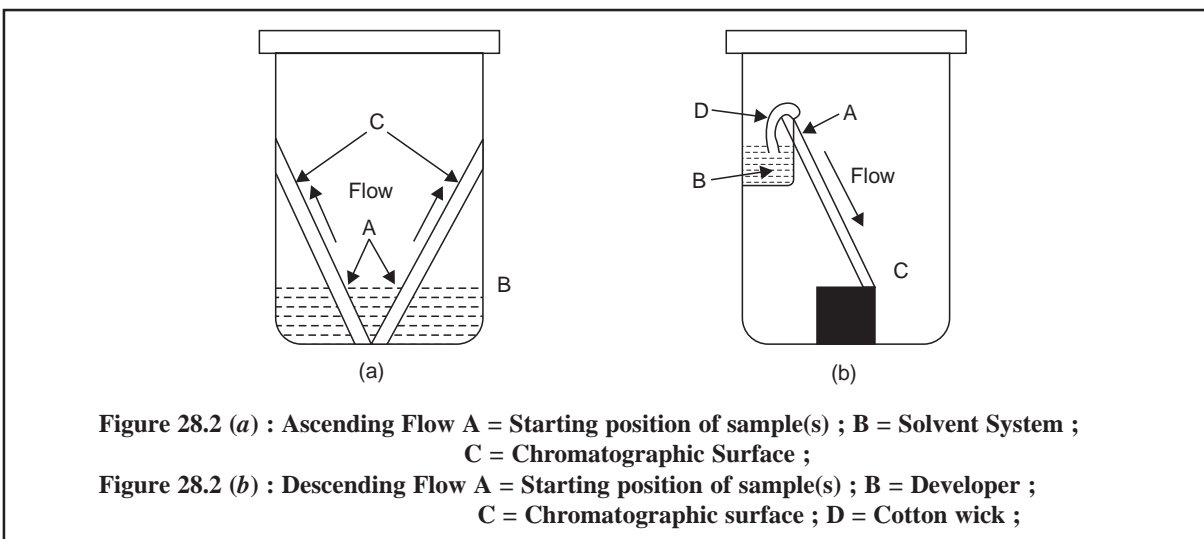
28.3.7.1. Equilibration of the Chamber

The equilibration of the chamber or chamber-saturation is a vital factor to obtain reproducible Rf values. It may be achieved by allowing the solvent system to remain in the chamber for at least 1 to 2 hours so that the vapours of the solvent(s) would pre-saturate the latter adequately. This is done to obtain distinct separation of constituents, uniform solvent from and prevent evaporation of the solvent on TLC-plates.

28.3.7.2. Protection against Oxidation

Both temperature and light augments oxidation and, therefore, ideally the following experimental parameters must be observed to obtain the best development of thin-layers, *viz.*,

Temperature : 18-23°C, and

Light   : Diffused daylight both natural and artificial,

However, direct sunlight (UV) or drought may give rise to 'oblique formation' of the solvent front.

28.3.7.3. Visualization

As a result of both intensive as well as extensive research a number of organic and inorganic substances have been identified that positively demonstrate an '*improved visualization*'. Such substances are termed collectively as '**fluorescent indicators**'.

Examples : Barium diphenylamine sulphonate ; 2,7-dichlorofluorescein ; Fluorescein (0.2% w/v in Ethanol) ; Morin (0.1% w/v in Ethanol) ; Sodium fluorescinate (0.4% w/v in water) ; Rhodamine B ; Zinc Silicate ; Calcium silicate ; Methylumbelliferone (or 7-hydroxy-4-methyl coumarin).

28.3.8. SPECIAL TECHNIQUES IN TLC

The various special techniques applicable in TLC are enumerated below briefly with specific examples wherever possible, namely :

(*i*) **Horizontal TLC :** Mistryukov in 1961 introduced this technique whereby the horizontal development of loose-layer TLC plates were made by using a shallow dish having a ground glass cover. The TLC plate was carefully rested on a T-shaped glass piece and the starting end was pressed duly against a filter paper held by another glass strip, which allowed the solvent to move to the thin-layer-film from the bottom of the dish by capillary action.

Example : Methyl esters of mixed fatty acid may be separated on loose-layer of alumina using suitable solvent-system.

(2) **Continuous TLC :** It is good for the separation of such components having small as well as very close Rf values and may be achieved by using :

(*a*) Rectangular horizontal plates where the solvent is allowed to move over them and subsequently evaporated after it has almost reached the end of the run, and

(*b*) Triangular glass-plates-where the mixture to be separated is spotted near the apex on a thin-layer and two different solvent mixtures are fed from two sides to the thin-layer and fractions subsequently collected at the base (Reisert,* 1963 ; Turins** ; 1965 ;).

(3) **Preparative TLC :** TLC may be skillfully extended to cater for extremely useful method for pre-parative separations. To maintain uniformity, as a rule, plates of 20 cm height and 20-100 cm length with layers between 0.5 and 0.2 mm thickness are normally employed. It essentially has three cardi-nal features, namely :

(*a*) Component mixtures is always obtained either in streaks or bands,

(*b*) Separation is invariably accomplished by multiple development, and

(*c*) Localization of separated components is only done under UV-light.
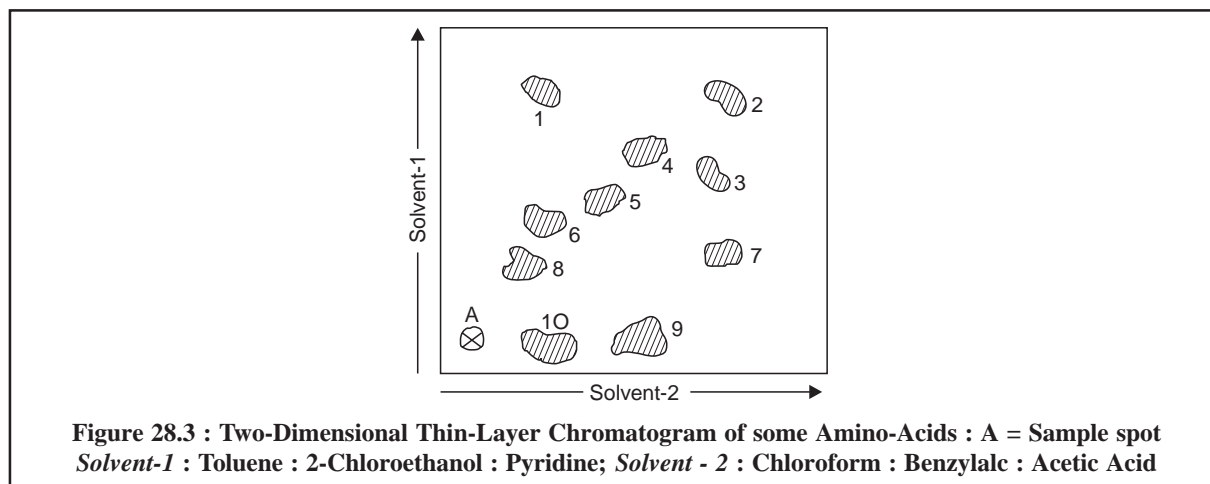
Note : Amount of constituents ranging between 0.1 and 100 g can be separated without any loss easily.

(4) **Multiple Dimensional TLC :** It can be regarded as a variant of multiple development chromatog-raphy. It could be expatiated with the help of the following typical example, namely :

Example : Separation of mixture of fatty acids, cholesterols and their esters ; lecithins and polar lipids*** :

S. No.	Solvent System	Position of Plate	Separation Caused
1.	Propanol : Ammonia (2 : 1)	Up-right	(*i*) Resolution of polar lipids and lecithins ; (*ii*) Carried fatty acids, cholesterol and its esters to the solvent front
2.	Chloroform : Benzene (3 : 1)	-do-	(*i*) Separated fatty acids and free cholesterols (*ii*) Carried esters to the solvent front.
3.	Carbontetrachloride	Plate turned to 180°	Separation of only cholesterol esters.

(5) **Two-Dimensional Chromatography :** It is also termed as **two-dimensional planar chromatography.** Here, the sample is spotted in one corner of a square TLC plate (size : 20 cm × 20 cm) as shown in Figure 28.3. The development is first carried out in the ascending direction using solvent-1 (see legend of Figure 28.3). The solvent is then eliminated by evaporation and the plate is rotated through 90°, following which ascending with the second solvent is accomplished. After

Figure 28.3 : Two-Dimensional Thin-Layer Chromatogram of some Amino-Acids : A = Sample spot
Solvent-1 **: Toluene : 2-Chloroethanol : Pyridine;** *Solvent - 2* **: Chloroform : Benzylalc : Acetic Acid**

*   Reisert, P.M. and D. Schumacher., **Experientia, 19** (84), 1963.

**   Turins, S. and V. Marianovio-Krojowan, V. Obradovic and M. Obradovic., **Anal. chem., 46,** 1905, 1965.

***   Weicker, H., **Klin Wache, 37,** 763, 1959.

removal of the solvent the spots of separated constituents are located by spraying with specific reagents.

Example : Mixture of amino acids obtained from protein hydrolysates are separated by this method* and spots located by using Ninhydrin Reagent that forms a pink to purple product with amino acids.

(6) **Centrifugal Chromatography :** It essentially makes use of the 'centrifugal force' so as to accelerate the flow of solvent through the thin-layer of the chromatogram. Korzum and Brody** in 1964, first applied this method to TLC, whereby the layers of plaster-of-paris bound alumina or silica gel were directly applied to either circular glass or aluminium plates with a hole in the centre to enable it to fit into the centrifuge. As usual, the sample mixture is applied 2.5 cm from the centre hole and the solvent system is set to allow a constant flow, with the centrifuge rotating at 500-700 RPM. In this manner, the usual developing time of 35 minutes is drastically reduced to mere 10 minutes by acceleration.

(7) **Wedged-Tip Chromatography :** Reindel *et al*** (1953) and Mathias**** (1954) introduced the wedged-tip technique in TLC which essentially exhibit the following *two* plus points, namely :

(*a*) Improved separation, and

(*b*) Constituents forced to assume an almost band-like path.

Figure 28.4, depicts the TLC-plate with wedged-tip divisions. The following steps are to be adopted sequentially, namely :

(*i*) Draw dividing lines 0.5 to 1.0 mm broad on the surface of the layer with a narrow-metal spatula,

(*ii*) Pentagons are facilitated by the help of a stencil made of transparent plastic material, and

(*iii*) Sample mixture are applied to the narrow portion of the wedge to get the best results.

Figure 28.5, illustrates TLC of an urine sample by the normal TLC-technique *vis-a-vis* the wedged-tip technique (Figure 28.5(*b*)). One may clearly visualize the beautiful separated bands in the latter as compared to the several odd and irregular-shaped spots in the former. Both the clarity of separation and the reproducibility of the results are predominant in the latter technique. Figure 28.5 (*a*) and (*b*) represent the typical analysis of a urine sample containing glucose, arabinose, lactose and fructose respectively.
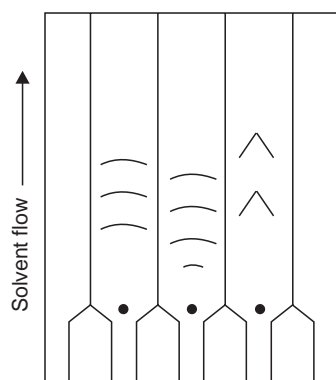
Figure 28.4 : TLC with Wedged-Tip Divisions
A = It occurs only with large quantities
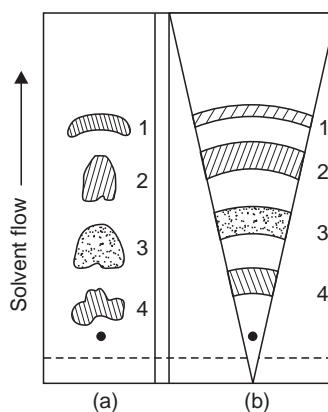
Figure 28.5 (a) TLC-Normal technique 28.5
(b) TLC-Wedged-Tip Technique
1 = Glucose (Blue-Violet) ; 2 = Arabinose (Blue)
3 = Lactose (violet) ; 4 = Fructose (Red) ;

* Von Arx. E., and R, Ann Nehr., **J. Chromatog, 12,** 329, 1963.

** Korzum, B.P., and Brody, S., **J. Pharm Sci., 53,** 454, 1964.

*** Reindel, F., and W. Hoppe., **Naturwissenschaften, 40,** 245, 1953.

**** Mathias, W., **Naturwissenschaften, 41,** 18, 1954.

28.3.9. CHEMICAL REACTIONS ON TLC PLATES

Glass being an inert material used for TLC-plates renders it ideal for utilization with strong corrosive reagents.

Miller and Kirchner* in 1953, were the pioneer in originating and developing the novel ideal of performing chemical unit-process reactions directly on TLC-plates. The *two* major steps involved in achieving this objective are, namely :

(*a*) Sample is spotted on a TLC plate in the usual manner and subsequently covered with a specific reagent, and

(*b*) Soonafter the reaction is completed, the TLC plate is developed using an appropriate solvent thereby separating the products of the reaction.

In actual practice, the resulting Rf value of the original compound together with the chromatographic results of the reaction are usually good enough to identify a compound accurately and precisely.

Example : (*i*) Citral reacts with 30% H_2O_2 in the presence of UV-light for a duration 10 minutes and undergoes catalytic oxidation to yield geranic acid as shown below :

Citral $CHO + H_2O_2$ (30%) $\xrightarrow[\substack{\text{10 minutes ;}\\\text{(Catalytic oxidation)}}]{\text{UV-Light}}$ COOH   Geranic acid
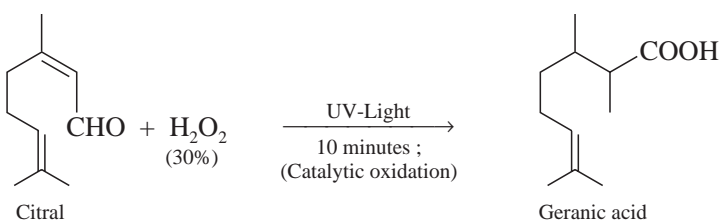
(*ii*) Citral undergoes reduction in the presence of 10% w/v solution of $LiAlH_4$ in ether to produce geraniol as represented in the following reaction :

Citral $CHO + LiAlH_4$ $\xrightarrow[\text{(10\% w/v soln. in Ether)}]{\text{Reduction}}$ CH_2OH Geraniol
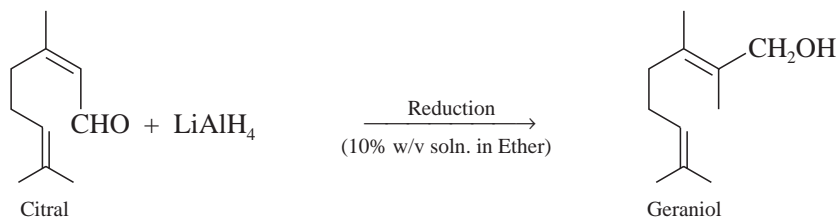
Exactly in the same manner, a number of other chemical unit-process reactions may be accomplished on TLC plates as stated here briefly :

(*a*) **Dehydration :** Sample spot of terpene alcohols *e.g.*, linalol, be converted to hydrocarbons by adding a drop of conc. H_2SO_4 as shown below :

Linalol $\xrightarrow{\text{Conc. } H_2SO_4}$ OH $\cdot H_2O$ OH   Terpin hydrate
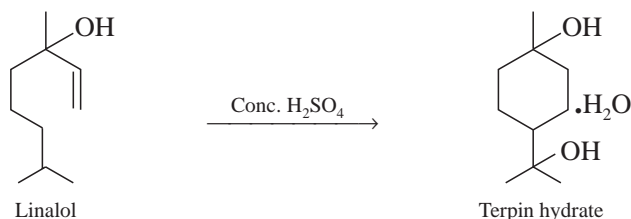
Consequently, the TLC plate is developed with hexane and since oxygenated compounds, do not move in hexane (*i.e.*, stay-back), only the hydrocarbons thus generated move away from the specific-reaction zone.

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* Miller, J.M., and J.G. Kirchner, **Anal. Chem., 25,** 1107, 1953.

(*b*) **Bromination :** Cargill* in 1962, separated cholestanol from cholesterol by TLC. The mixture is spotted on a TLC plate and reacted with a soln. of Br_2 (0.1% w/v in $CHCl_3$), taking care that its quantity must be 2 to 3 times the weight of the sample mixture. Development in a solvent system consisting of benzene and ethyl acetate (2 : 1) would result in a clear distinction of cholestanol and reaction products of cholesterol with Br_2.

(*c*) **Enzymatic Reaction :** Randerath and Randerath** in 1964, demonstrated an enzymatic reaction directly on an anion-exchange layer of cellulose impregnated with polyethylene imine. A buffered solution of phosphodiesterase is applied to the sample spot of cytidine dipohosphate glucose, which is subsequently covered with paraffin and allowed to stand for 45-60 minutes at 23°C. Chromatography of the resulting degradation products gives rise to cytidine 5-monophosphate and glucose 1-phosphate.

(*d*) **Esterification :** Benneth and Heftmann*** in 1962, showed that it was feasible to esterify the C-3, hydroxy steroids directly on TLC plates by means of tri-fluoroacetic anhydride. After treating the compounds with the anhydride, it is absolutely necessary to dry the pate in the hood for several minutes so as to get rid of the trifluoroacetic acid that is produced as a by-product.

28.3.10. COMBINATION OF TLC WITH OTHER TECHNIQUES

TLC may be combined with *column chromatography* and *vapour-phase chromatography* as discussed here briefly :

(*a*) **Column Chromatography :** TLC helps in selecting the best combination of solvent and adsorbent for a given column separation. Miller and Kirchner**** (1952) developed this combination thoroughly and employed it extensively for the separation of a large number of difficult types of compounds.

(*b*) **Vapour-phase Chromatography :** The various spots obtained from TLC may be eluted, concentrated and then subjected to vapour-phase chromatogrphy studies. Ikeda *et al*.***** (1961, 1962) exploited this combination for the analysis of a variety of naturally occurring constituents, namely :

(*i*) Citrus oils and other essential oils,

(*ii*) Oestrogens in urine sample,

(*iii*) Testosterone in urine sample, and

(*iv*) Progesterone in plasma.

28.3.11. DETECTION OF COMPONENTS

After development of TLC plates, the next important step is to detect the separated components so as to determine their respective Rf values.

Example : (*i*) **Coloured Substances :** *e.g.*, Xanthophylls, Chlorophylls, Carotenes, etc., may be located visually.

(*ii*) **Colourless Substances :** *e.g.*, alkaloids, steroids, amino acids and the like may be detected under short-wave UV-light or a long-wave UV-light. These substances may also be detected as brown/dark brown spots when exposed to I_2-vapours in a closed dessicator.

(*iii*) **Specific Detecting Reagents :** A few specific detecting reagents are normally used for a particular class of compounds *e.g.*,

*   Cargill, D.I., **analyst, 87,** 865, 1962.

**   Randerath, K., and E. Randerath., **Angew Chem Intern,** Ed, 3, 442, 1964.

***   Benneth, R.D., and E. Heftmann., **J. Chromatog., 9,** 353, 1962.

****   Miller, J.M. and J.G. Kirchner., **Anal Chem., 24,** 1480, 1952.

***** Ikeda *et al.,* **Food Technol, 15,** 379, 1961 ; Ikeda *et al,* **Food Science, 27**, 454, 1962.

Aniline-phthalate reagent :   for carbohydrates ;

Ninhydrin reagent   :   for amino-acids, and

Dragendorff's reagent   :   for alkaloids

(*iv*) **Chromic acid/conc. H_2SO_4 :** These corrosive reagents usually char the organic material on TLC plates and may be seen as dark brown spots.

28.3.12. EVALUATION OF THE CHROMATOGRAM

After completing the detection procedure the various separated solutes on the TLC plate are marked with the help of a sharp needle (*e.g.*, pithing needle) ; subsequently, their evaluation may be carried out either qualitatively or quantitatively, as stated below :

28.3.12.1. Qualitative Evaluation

The Rf value (Retention Factor) various separated solutes is determined accurately. The Rf value represents the differences in rate of movement of the components duly caused by their various partition coefficients *i.e.*, their different solubility in the mobile and stationary phases. In order words, the Rf value (relate to front) is-'the ratio between the distance starting point-centre of spot and distance starting point-solvent front', thus it may be expressed as :

$$Rf = \frac{\text{Distance of centre of spot from starting point}}{\text{Distance of solvent front from starting point}}.$$

Important Points : (*i*) Due to the always longer path of the solvent front, the Rf value is invariably lesser than 1.

(*ii*) Rf value is always constant for each component only under identical experimental parameters, and

(*iii*) Rf value depends upon a number of governing factors, such as : quality of the layer material ; activation grade of the layer ; thickness of layer ; quality of solvent ; equilibration of chamber ; chromatographic technique employed (*e.g.*, ascending, descending) ; presence of impurities ; and conc. of simple applied ; and

(*iv*) All possible anomalies in (*iii*) above may be eliminated by performing a co-chromatogram of a standard substance along with that of a sample. Thus, the distance traversed by a substance is compared with that of the standard (or reference). This 'new' relation is usually designated as Rst-value. Therefore, in short, it is expressed as follows :

$$Rst = \frac{\text{Rf of the substance}}{\text{Rf of the standard}}$$

Unlike the Rf value, the Rst value may be more than 1.00 because here the substance under investigation (*i.e.*, sample) usually travels further than the standard.

In TLC, the qualitative evaluation is solely based on the determination of Rf values of unknown spots *vis-a-vis* Rf values of standard substances preferably on the same TLC plate so as to avoid any possible error whatsoever.

28.3.12.2. Quantitative Analysis

The quantitative analysis of chromatographically separated constituents may be carried out with high degree of accuracy and precision in *two* manners, namely :

(*i*) **Direct Method :** *i.e.*, the quantitative determinations is performed directly on the adsorbent layer, and

(*ii*) **Indirect Method :** *i.e.,* the separated constituents are quantitatively removed from, the adsorbent and subsequently estimated after elution.

28.3.12.2.1. *Direct Methods*

The various methods under this category are, namely :

(*i*) **Measurement of Spot-areas :** This method is solely based on a mathematical relationship existing between the prevailing spot area and the amount of component present. It is not quite accurate due to high random errors.

(*ii*) **Densitometry :** The intensity of the colour of a component is measured on the chromatogram using a densitometer.

(*iii*) **Spectrophotometry :** Characterization of the separated spots by reading the absorption or fluorescence curves directly from TLC plates is carried out with the help of Chromatogram Spectrophotometer devised by Zeiss, Stahl and Jork.

Besides, IR-spectroscopy, reflectance spectroscopy, spark chamber method etc., may also be employed for the direct evaluation of chromatograms.

28.3.12.2.2. *Indirect Methods*

These methods are based on elution techniques, followed by micro-analysis of the resultant eluate by adopting one or more of the undermentioned known methods, namely :

Colorimetry ; Fluorimetry ; Radiometry ; Flame-photometry ; UV-Spectrophotometry ; Gravimetry ; Polarography ; Vapourphase Chromatography ;

28.4. APPLICATIONS OF TLC IN PHARMACEUTICAL ANALYSIS

The technique of thin-layer chromatography (TLC) has been used extensively in the domain of pharmaceutical analysis for a variety of specific and useful applications, for example :

(*i*) To identify the presence of undesirable specific organic compounds present as impurities in a number of pharmaceutical substances, namely : morphine in apomorphine hydrochloride ; hydrazine in carbidopa ; 3-aminopropanol in dexampanthenol ; etc.,

(*ii*) Related substances present in official drugs, namely : related substances present in a wide number of potent pharmaceutical substances *e.g.*, aminophylline ; baclofen ; chloramphenicol ; carbamazepine etc.,

(*iii*) Foreign alkaloids present in alkaloidal drugs, for instance : atropine sulphate ; codeine ;

(*iv*) Foreign steroids present in steroidal drugs, for example : betamethasone valerate ;

(*v*) Ninhydrin positive substances in official amino acids *e.g.*, glutamic acid ; leucine ;

The various applications of TLC as cited above would be discussed in the sections that follow :

28.4.1. PRESENCE OF SPECIFIC SUBSTANCES AS IMPURITIES IN DRUG SUBSTANCES

Examples : (1) **Morphine in Apomorphine Hydrochloride**

Materials Required : Silica gel-G ; Mixture of Acetonitrile : Dichloromethane : Ethyl acetate ; Anhydrous formic acid : Water (30 : 30 : 30 : 5 : 5) ; solution (1) : 0.20% w/v of apomorphine in methanol ; solution (2) : 0.004% w/v of apomorphine HCl in methanol ; (3) Morphine : 2% w/v in methanol ; sodium nitrite solution (3% w/v in DW) ;

Procedure : Prepare the chromatogrphic tank by lining the walls with sheets of filter paper ; pour the mobile-phase into the tank, saturating the filter paper in the process, to a depth of 5 to 10 mm, close the tank and allow it to stand at 20° to 25 °C for 1 hour for equilibration of the mobile-phase in the chromatank. Apply separately to the TLC plate 5 μ*l* of each of two solutions (1) and (2) of apomorphine hydrochloride and (3) of morphine in the form of circular spots about 2 to 6 mm in diameter, and 15 to 20 mm from one end of the plate and not nearer than 10 mm to the sides ; the two spots must be at least 10 mm apart. Mark the sides of the plate 15 cm from the line of application. Allow the solvent to evaporate and place in the chromatank,

ensuring that it is nearly vertical as possible and that the spots are above the level of the mobile-phase. Close the tank and allow to stand at 20° to 25°, unless the mobile-phase has ascended to the marked lines. Remove the plate and dry it in a current of cold air until all traces of solvent has disappeared and spray with a solution of sodium nitrite. Expose the plate to ammonia vapour for a few minutes and allow to stand in daylight for about 1 hour.

Observations : In the chromatogram obtained with solution (1), there is no reddish orange spot with an Rf value of 0.3 to .5 relative to the principal spot (about 2% of morphine). The test in not valid unless there is a clearly visible spot in the chromatogram obtained with solution (2).

28.4.1.1. Cognate Assays

A number of other typical examples of pharmaceutical substances containing specific organic compounds, that may be identified by adopting the similar TLC technique are stated in Table 28.3 :

Table 28.3 : Cognate Assays of Specific Organic Compounds Present in Pharmaceutical Substances

S. No.	Name of Substance	Name of Impurities	Adsorbent	Mobile Phase	Solutions	Detec-tion	Observations
1.	Amino benzoic acid	4-Nitro-benzoic acid	Cellulose-F254 (Merck)	2-Mthyl propan-1-ol : water : 5M ammonia (700 : 30 : 15)	(1) 10% w/v of amino-benzoic acid ; (2) 0.020% w/v of 4-nitro benzoic acid ; [in EtOH (96%)]	UV-Light (254 nm)	Spot obtained with soln. (1) for impu-rity should not be more than the spot with soln. (2)
2.	Benztropine Mesylate	Tropine	Silica gel-G	Ethanol (96%) : Ammonia (13.5 M) (75 : 15)	(1) 4.0% w/v of sample ; (2) 0.20% w/v of atropine ; [in acetone]	(1) Sod* iodobis-muthate soln. (2) 0.4% w/v soln. of H_2SO_4	Spot obtained with soln. (1) for impu-rity should not be more than the spot with soln (2).
3.	Cyprohepta-diene Hydro-chloride	Dibenzo-cyclohepta triene	Silica gel-60 ; F 254 (Merck)	Chlorofom : Methanol (90 : 10)	(1) 0 1.% w/v of sample ; (2) 0.0020% w/v dibenzo-cyclohepta-triene ; [in chloroform]	Spray with ethanolic H_2SO_4 (10%) heat at 110 °C and examine under UV-light 365 nm ;	

---

* **Sodium iodobismuthate solution :** Boil for a few minutes a mixture of 2.6 g of bismuth oxycarbonate, 7.0 g of sodium iodide and 25 ml of glacial acetic acid. Allow to stand for 12 hours and filter through sintered glass. To 20 mm of the filtrate add 8 ml of ethyl acetate (solution A). Immediately before use, mix 2 ml of solution A, 20 ml of glacial acetic acid and 40 ml of ethyl acetate.

Note :   (*i*) **For TLC, the sensitivity may be enhanced by spraying first with the above solution and then with sulphuric acid (0.2%), and**

(*ii*) **Solution A must be kept in a well-closed container.**

28.4.2. RELATED SUBSTANCES PRESENT IN OFFICIAL DRUGS

(1) **Aminophylline : Presence of Related Substances**

Materials Required : Silica gel-G F254 ; Mobile-phase (butan-1-ol : acetone : chloroform : 13.5 ammonia : : 40 : 30 : 30 : 10) : 100 ml ; Solution-1 : dissolve 0.2 g of sample in 2 ml of DW, warm and dilute to 10 ml with methanol ; Solution (2) : dilute 1 vol. of soln. 1 to 200 vols. with methanol ;

Procedure : Apply separately to the coated plate of silica get GF254 10 µl each of solution (1) and (2). Follow the procedure as detailed in Part II section 4.1, using the above mobile phase. After removal of the plate, allow it to dry in air and examine under UV-light (254 nm).

Observations : Any secondary spot in the chromatogram obtained with solution (1) is not more intense than the spot in the chromatogram obtained with solution (2).

28.4.2.1. Cognate Assays

A good number of pharmaceutical substances do contain 'related substances' which can be identified by TLC methods as summarized in Table 28.4 below :

Table 28.4 : Cognate Assays of Related Substances Present in Pharmacopoeial Drugs :

S. No.	Name of Substance	Adsorbent	Mobile Phase	Solutions	Detection	Observations
1.	Bisacodyl	Silica gel GF254 (Merck)	Butane-2-one : Xylene (1 : 1) ; Solvent front to run only 10 cm from line of application	(1), (2), (3) and (4) contain 2.0% w/v ; 0.20% w/v ; and 0.020% w/v ; and 0.010% w/v of sample in acetone ; (5) contains 0.20% w/v Bisacodyl EPCRS in acetone ;	Dried plate examined under UV-light (254 nm)	Any secondary spot obtained with soln. (1) is not more intense than spot with soln. (3) and not more than one spot is more intense than spot with sol. (4)
2.	Chloram-phenicol	Silica gel GF254 Apply separately to TLC plate 1 µl and 20 µl of sol (1) ; 1 µl of sol (2) and 200 µl of sol. (3)	Butane-2-one : Xylene (1 : 1) ; Solvent front to run only 10 cm from line of application	(1), (2), (3) and (4) contain 2.0% w/v ; 0.02% w/v ; and 0.01% w/v of sample in acetone ; (5) contains 0.2% w/v Bisacodyl EPCRS in acetone ;	Dried plate examined under UV-light (254 nm)	Any secondary spot obtained with 20 µl of soln. (1) is less intense than the spot in the chromatogram obtained with sol. (3)
3.	Chloram-bucil	Silica gel GF254 Apply separately to TLC plate 5 µl of sol. (1), (2) and (3)	Chloroform : Methanol : Water (90 : 10 : 1)	(1) 1% w/v of sample in acetone ; (2) 1% w/v of chloramphenicol EPCRS in acetone ; and (3) dilute 0.5 ml of (2) to 100 ml with acetone	Under UV-light (254 nm)	Any secondary spot obtained with soln. (1) is less intense than spot with soln. (2), and not more than one such spot is more intense than spot with soln. (3)

28.4.3. FOREIGN ALKALOIDS PRESENT IN ALKALOIDAL DRUGS

Examples :

(1) **Atropine Sulphate : Foreign Alkaloids and Development Products :**

Materials Required : Silica gel G ; mobile-phase (acetone : water : 13, 5 M ammonia : : 90 : 7 : 3) : 100 ml ; solution (1, 2% w/v of sample in methanol ; solution (2) : 0.02% w/v of sample in methanol ; solution (3 : 0.01% w/v of sample in methanol ; dilute potassium iodobismuthate solution (dissolve 100 g of (+) – tartaric acid in 500 ml of water and add 50 ml of potassium iodobismuthate solution RI*) : 100 ml ;

Procedure : Apply separately to the coated TLC plate 1 μl of each of three solutions (1), (2) and (3). Develop the plate in the above mobile-phase such that the solvent front is allowed to ascend only 10 cm above the line of application. After removal of the plate, dry it at 100 °C to 105 °C for 15 minutes, allow to cool and spray with dilute potassium iodobismuthate solution until spots appear.

Observations : Any secondary spot in the chromatogram obtained with solution (1) is not more intense than the spot obtained with solution (2), and not more than one such spot is more intense than the spot obtained with solution (3).

28.4.3.1. Cognate Assay

The presence of **'foreign alkaloids'** in Codeine (BP)** may be determined by more or less an identical method as already discussed in section 28.4.3 earlier.

28.4.4. FOREIGN STEROIDS PRESENT IN STEROIDAL DRUGS

Example :

(1) **Betamethasone Valerate : Related Foreign Steroids :**

Materials Required : Silica gel G ; mobile-phase : (1, 2-dichloroethane : methanol : water : : 95 : 5 : 0.2) : 100 ml ; mixture of chloroform and methanol (9 : 1) : 50 ml ; solution (1) : betamethasone valerate sample : 1. 5% w/v ; solution (2) : betamethasone valerate BPCRS/EPCRS*** : 1.5% w/v ; solution (3) : a solution containing 0.030% w/v each of betamethasone EPCRS and betamethasone 21-valerate BPCRS ; alkaline tetrazolium blue solution**** q.s. ;

Procedure : Apply separately to the coated TLC plate 1 μl of each of three solutions (1), (2) and (3) prepared in a mixture of chloroform/methanol stated above. After removal of the plate, allow it to cool dry in air until the solvents have evaporated, heat at 105 °C for 10 minutes, cool and spray with alkaline tetrazolium blue solution.

Observations : (1) The principal spot in the chromatogram obtained with soln. (1) corresponds in position, colour and intensity to that obtained with soln. (2),

(2) Any secondary spot in the chromatogram obtained with soln. (1) is not more intense than the proximate spot in the chromatogram, with soln. (3).

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*   Dissolve 100 g of (+)-tartaric acid in 400 ml of water and add 8.5 g of bismuth oxynitrate. Shake for 1 hour, add 200 ml of a 40% w/v solution of KI and shake well Allow to stand for 24 hours and filter. Store and protect from light.

**   BP (1993)

***   British Pharmacopoeia Chemical Reference Substance ; European Pharmacopoeia Chemical Reference Substance ;

****   Immediately before use mix 1 volume of a 0.2% w/v soln. of tetrazolium blue in methanol with 3 volumes of 12% w/v soln. of sodium hydroxide in methanol ;

28.4.5. NINHYDRIN POSITIVE SUBSTANCES PRESENT IN OFFICIAL AMINO ACIDS

Example :

1. **Glutamic Acid**

Materials Required : Silica gel-G ; mobile-phase (glacial acetic acid : water : butan-1-ol : : 20 : 20 ; 20 ; 60) : 100 ml ; solution (1) : dissolve 0.1 g of sample in 5 ml of 2 M ammonia* ; solution (2) : dilute 1 ml of soln. (1) to 50 ml with water ; solution (3) : dilute 5 ml of solution (2) to 20 ml with water ; Solution (4) : dissolve 10 mg of glutamic acid EPCRS in sufficient water to produce 50 ml ; solution (5) dissolve 10 mg of glutamic acid EPCRS and 10 mg of aspartic acid EPCRS in sufficient water to produce 25 ml ; ninhydrin solution (0.2% w/v solution of ninhydrin in a mixture of 95 vols. of butan-1-ol and 5 vols of 2 M acetic acid**) : 50 ml ;

Procedure : Apply separately to the silica gel G coated plates 5 µl of each of sols (1), (2), (3), (4) and (5) and dry the TLC plates in a current of air for 15 minutes before commencing development. Carry out the development using the above mentioned mobile-phase as usual. After removal of the plate, allow it to dry in air, spray with ninhydrin solution and heat at 100° to 105 °C for 15 minutes.

Observations

(1) Any secondary spot in the chromatogram obtained with soln. (1) is less intense than the spot obtained with soln. (3).

(2) The test is not valid unless the chromatogram obtained with soln. (5) show two distinctly separated spots.

28.4.5.1. Congnate Assays

The assay of leucine-an amino acid official in BP (1993) may also be carried out by adopting a similar procedure using the same adsorbent and mobile-phase but different solution from (1) to (5).

THEORETICAL AND PRACTICAL EXERCISES

1. Attempt the following aspect of '*Thin-layer Chromatography*' (TLC) :

    (*a*) Importance of TLC,

    (*b*) Theory of TLC, and

    (*c*) Versatility of TLC over paper and column chromatography.

2. Discuss comprehensively the various *experimental techniques* of TLC :

    (*i*) Preparation of TLC plates,   (*ii*) Choice of 'adsorbents',

    (*iii*) Choice of 'solvent system in TLC', and   (*iv*) Activation of 'adsorbent'.

3. How would you accomplish the following requirements in TLC ?

    (*a*) Purification of silica gel-G layers,   (*b*) Spolting of components (analytes),

    (*c*) Development of thin layers.

    Explain with appropriate examples.

4. Explain the following '*Special Techniques*' in TLC with suitable examples :

    (*i*) Multi dimensional TLC,   (*ii*) 2D-Chromatography,

    (*iii*) Centrifugal chromatography, and   (*iv*) Wedged-Tip Chromatography.

---

* Dissolve 15 ml of 13.5 M ammonia (25% w/w of NH_3) to 100 ml in water.

** Dilute 11.4 ml of 17.5 M glacial acetic acid to 100 ml with water.

5. Enumerate the various '*chemical reactions*' that can be carried out on TLC plates. Support your answer with typical examples, reactions and procedure involved.

6. Elaborate these aspects of TLC explicitly :

(*a*) Combination of TLC with other techniques *e.g*., column chromatography and vapour-phase chromatography,

(*b*) Detection of separated components,

(*c*) Qualitative evaluation of the '*chromatogram*', and

(*d*) Quantitative analysis of the '*chromatogram*'.

7. What are the various aspects of TLC in pharmaceutical analysis with regard to :

(*a*) Presence of specific substances as impurities in **'drugs'.**

(*b*) Presence of related substances n **'official drugs'.**

(*c*) Presence of foreign alkaloids in **'alkaloidal drugs'.**

(*d*) Presence of steroids present in **'steroidal drugs'.**

(*e*) Ninhydrin positive substances present in **'official amino acids'.**

Give at least **ONE** typical example to support your answer.

RECOMMENDED READINGS

1. Marini-Bettolo, G.B., **Thin Layer Chromatography,** Amsterdam, Elsevier, 1964.

2. Stahl, E., Ed., **Thin Layer Chromatography,** New York, Academic Press, 1965.

3. Stahl, E. and H. Jork, **Thin Layer Chromatography : A Laboratory Handbook',** New York, Springer Verlag, 1969.

4. Touchstone, J.C. Ed., **'Quantitative Thin Layer Chromatography',** New York, Wiley 1973.

5. Stock, R. and C.B.F. Rice, **Chromatographic Methods,** 3rd ed., London, Chapman and Hall, 1974.

6. Heftmann, E., **Chromatography,** 3rd ed., New York, Van Nostrand Reinhold Co., 1975.

7. Touchstone, J.C. and M. F. Dobbins, **Practice of Thin Layer Chromatography,** New York, John Wiley 1978.

8. Weissberger, A., Ed, **Thin Layer Chromatography,** New York, Wiley Interscience, 1978.

29 GAS LIQUID CHROMATOGRAPHY (GLC)

29.1. INTRODUCTION

One of the most difficult and frustrating problems ever encountered in the domain of pharmaceutical analysis is that of the simultaneous separation, identification and above all the quantitation of more than one compound from a complex mixture in a pharmaceutical product.

A good of sophisticated chromatographic techniques of separation have been put forward since early fifties that may be categorized into the following *four* groups, namely :

(*a*) Gas-Solid adsorption Chromatography (GSC),

(*b*) Gas-Liquid partition Chromatography, (GLC),

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(*c*) Liquid-Solid adsorption Chromatography (LSC), and

(*d*) Liquid-Liquid partition Chromatography (LLC).

The first two groups have been collectively termed as **'Gas Chromatography'.** Its phenomenal growth at almost logarithmic pace may be attributed to its unparalleled potential in resolving components of a complex mixture. **Gas chromatography** fundamentally is a separation technique that not only essentially provides *prima facie* indentification of a compound but also caters for quantitative estimation after due calibration.

Gas chromatography makes use, as the stationary phase, a glass or metal column filled either with a powdered adsorbent or a non-volatile liquid coated on a non-adsorbent powder. The mobile-phase consists of an inert-gas loaded with the vapourised mixture of solutes flowing through the stationary phase at a suitable temperature. In the course of the passage of the vapour of the sample through the column, separation of the components of the sample occurs in two ways, namely :

(*a*) **due to adsorption effects-***i.e.,* when the prepared column consists of particles of adsorbent only, and

(*b*) **due to partition effects-***i.e.,* when the particles of adsorbent are coated with a liquid that forms a stationary phase.

Martin and Synge in 1952, became the Nobel Laureates for their excellent, innovative research work on the development of **partition chromatography**.

It is, however, pertinent to mention here that GLC has a much greater application in the field of pharmaceutical analysis which extends over to most organic constituents that have a measurable vapour present at the temperature employed.

The principal advantages of GC are enumerated below, namely :

- It has high frequency of separation and even complex mixtures may be adequately resolved into constituents,
- It has a very high degree of sensitivity in detection of components *i.e.,* only a few mg of sample is enough for complete analysis,
- Speed of analysis is quite rapid,
- Gives reasonably good accuracy and precision,
- The technique is fairly suitable for routine analysis because its operation and related calculations do not require highly skilled personnel, and

The overall cost of equipment is comparatively low and its life is generally long.

29.2. THEORY

There are, in fact, *three* theories that have gained virtually wide recognition and acceptance in describing a gas chromatographic separation, namely :

(*a*) Plate theory,

(*b*) Rate theory, and

(*c*) Random walk and nonequilibrium theory.

These different theories will be discussed briefly in the sections that follows :

29.2.1. PLATE THEORY

Martin and Synge* first proposed the '**plate theory**' in 1941, whereby they merely compared the GC separation to fractional distillation. Thus, the 'theoretical' plate is the portion of the column wherein the solute is in complete equilibrium with the mobile and the stationary phase.

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* Martin, AP, and RLM Synge, **Biochem. J., 35,** 1358, 1941.

This equilibrium is represented by the following expression :

$$K_D = \frac{\text{Conc. of solute in Stationary Phase}}{\text{Conc. of solute in Mobile Phase}}$$

where, K_D = Distribution coefficient.

Thus, the distribution of a solute after 'n' equilibrium (plates) may be defined by the expansion of the binomial in Eq. (*a*) below :

$$(a + b)^{n-1} \qquad \qquad ...(a)$$

where, $(n - 1)$ = Number of transfers between the plates,

$a = 1/(K_D + 1)$, and

$b = K_D/(K_D + 1)$.

29.2.2. RATE THEORY

As the '*Plate Theory*' has two serious limitation, *viz.*, first : it does not speak of the separating power of a definite length of column, and second : it does not suggest means of improving the performance of the column ; the '**Rate Theory**' has been introduced which endeavours to include the vital fact that-'**the mobile-phase flows continuously, besides the solute molecules are constantly being transported and partitioned in a gas chromatographic column**'. It is usually expressed by the following expression :

$$h = 2\lambda d_p + \frac{2\gamma D_G}{u} + \frac{8k' d_f^2}{\pi^2 (1+k')^2 D_L} u \qquad \qquad ...(b)$$

where, u = Average linear gas velocity,

λ = Measure of the packing irregularities

d_p = Particle diameter,

γ = Tortuosity factor,

D_G = Coefficient of gaseous diffusion of the solute in the carrier gas,

k' = Ratio of the amount of solute in the stationary phase to that in the gas phase,

d_f = Film thickness (usually in μm), and

D_L = Diffusion constant of solute in liquid phase.

Eq. (*b*) was first advocated by Van Deemter* in 1956, and may be rewritten as given below [Eq. (*c*)] wherein all terms except '*u*' are constant :

$$h = A + \frac{B}{u} + Cu \qquad \qquad ...(c)$$

29.2.3 RANDOM WALK AND NONEQUILIBRIUM THEORY

Giddings** in 1958, first proposed the captioned theory wherein he suggested a chromatographic separation in terms of a random walk. Based on a statistical concept the virtual spreading of a '**solute band**' may be considered by virtue of **molecular diffusion, mass transfer**, and **Eddy diffusion** (*i.e., flow pattern effects*) were equated to standard deviation. In fact, this particular approach correlates the spreading of a chromatographic peak to various parameters, for instance : mass transfer, diffusion coefficient D_G, particle diameter, velocity of mobile-phase, and finally the length of column. Thus, coefficient D_G, particle diameter, velocity of mobile-phase, and finally the length of column. Thus, the plate height '*h*' employing the random walk approach may be expressed as in Eq. (*d*) in the next page :

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* Van Deemter, FJ Zuiderweg and A. Klinkengerg, **Chem., Eng., Sci., 5,** 271, 1956.

** Giddings JC, **J. Chem Ed., 35,** 588 1958.

$$h = \frac{B}{u} + Cu + \sum \frac{1}{1\,/\,A\ +\ (1/Cm)u} \qquad\qquad ...(d)$$

where, Cm = Resistance to mass transfer in gas phase should be treated independently,

　　　　A = Eddy diffusion,

　　　　B = Longitudinal molecular diffusion in both mobile and stationary phases, and

　　　　C = Kinetic or mass transfer term originating in the stationary phase.

In actual practice, there are *two* basic considerations that prevail upon in gas chromatography, namely :

(*a*) **Retention :** The phenomena affecting retention or hold up on the column, sometimes referred to as the thermodynamic effect, and

(*b*) **Column Efficiency :** The phenomena affecting column efficiency or the kinetic aspect that governs the tendency for a particular solute band to '*broaden*' as it traverses through the column.

However, the resolution or extent of separation of any two peaks from a column is solely dependent upon both *retention* and *column efficiency*.

Although separations may be caused by elution, frontal and displacement analyses, yet the elution technique is the most common. This method makes use of a stream of carrier-gas flowing through the column. Precisely, a sample is injected into the carrier-gas as a '**plug**' of vapour that is swept into the head of the packed chromatographic column. Separation of components that comprise the sample results from a difference in the multiple forces by which the column materials tend to retain each of the components.

Irrespective of the nature of the retention that is due to adsorption, solubility, chemical binding, polarity or molecular filtration, the column does retain some components longer than others. When in the gas phase the components are moved toward the column outlet, they are selectively retarded by the stationary phase. Consequently, all components pass through the column at **varying speeds** and **emerge in the inverse order of their retention by the column materials**. The aforesaid process may be outlined schematically as shown in Figure 29.1.

Figure 29.1 : Schematic Elution of Components A and B From A Column Vs Time
[X = 'Plug' of A and B ; Y = Carrier gas inlet]

Upon emerging from the column, the gaseous phase immediately enters a 'detector' attached to the column. Here, the individual components register a series of signals that appear as a succession of peaks above a base line on the chromatogram.

From Figure 29.1, it is evident that 'plug' of mixture A and B just enters the column 1 at time T_1, moves to the middle of the column I at time T_2, part of A has passed through the column III at time T_3 and finally A has passed completely and part of B passed through the column IV at time T_4.

29.3. INSTRUMENTATION

A gas chromatograph essentially comprises of *six* vital components, namely :

(*a*) Carrier Gas Regulator and Flow Meter,

(*b*) Sample Injection System,

(*c*) Separation Column,

(*d*) Thermal Compartment,

(*e*) Detectors,

(*f*) Recording of Signal Current, and

(*g*) Integrator.

These components shall be discussed briefly in the sections that follow : Figure 29.2, gives the schematic diagram of a standard GLC equipment showing the various parts :

Figure 29.2 : Schematic Diagram of A Standard GLC equipment.

The sample is introduced into the vaporizer and enters the column along with the carrier gas at a constant flow through the detector oven. The reference sample also passes through the detector oven into the column which is maintained by column-oven heat control device. The detector picks up the signals of the sample as well as the reference substance one after the other which is duly amplified and the signal current recorded on a strip-chart recording device or other suitable means. After passing through the detector oven the vapours of the sample plus the carrier gas leaves the equipment through an exhaust pipe.

Note : Ultrapure N_2 for use in flame-ionization devices may be generated by the Serfass Apparatus available commercially.

29.3.1. CARRIER GAS PRESSURE REGULATOR AND FLOW METER

The various carrier gas used in GC along with their characteristic features are stated below :

H_2 : It has a distinctly better thermal conductivity and lower density. Demerits are its reactivity with unsaturated compounds and hazardous explosive nature,

He : It has an excellent thermal conductivity, low density, inertness and it permits greater flow rates. It is highly expensive,

N_2 : It offers reduced sensitivity and is inexpensive, and

Air : It is employed only when the atmospheric O_2 is beneficial to the detector separation.

Importantly, the operating efficiency of a chromatograph is directly dependent on the maintenance of a highly constant carrier gas-flow-rate. Carrier gas passes from the tank through a toggle value, a flow meter, a few feet of metal capillary restrictors, and a 0-4 m pressure gauze. The flow rate could be adjusted by means of a needle value mounted on the base of the flow meter and is controlled by the capillary restrictors. On the downstream side of the pressure regulator, a tee (T) may split the flow and direct it to the sample and the reference side of the detector.

29.3.2. SAMPLE INJECTION SYSTEM

The sample injection system is very important and critical because GC makes use of very small amounts of the samples. A good and ideal sample injection system should be the one where the sample must not—

 (*i*) be decomposed at the point of injection,

 (*ii*) create pressure surges, and

 (*iii*) undergo fractionation, condensation or adsorption of components during the course of transfer to the column.

There are different modes of handling liquid, solid and gaseous samples in a GC which will be discussed briefly here :

(*a*) **Liquid Samples :** They are usually injected by hypodermic syringes through a self-sealing silicon-rubber septum into a preheated-metal-block flash evaporator. The sample is vapourized as a 'plug' and carried right into the column by the respective carrier gas. Sample size ranges between 1–10 μ*l*.

(*b*) **Solid Samples :** These are either dissolved in volatile liquids (solvents) or temporarily liquefied by exposure to infra-red heat.

(*c*) **Gas Samples :** They are best handled and injected by gas-light syrings or a gas-sampling valve, usually termed as a *stream-splitter*. In the simplest form this is merely a glass-system (Figure 29.2A) made up of three stop-cocks, between two of which there is a standard
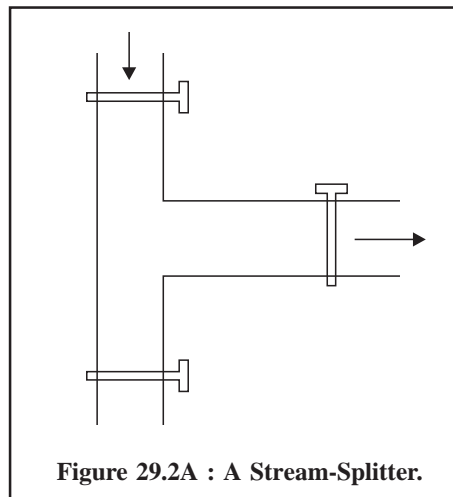

Figure 29.2A : A Stream-Splitter.

volume wherein the 'gas' is trapped. Gas from this bypass-capillary-loop is introduced right into the column by sliding or rotating a valve to connect the loop with the stream of carrier gas.

29.3.3. SEPARATION COLUMN

It is also known as the '**chromatographic column**'. In reality the heart of a GC is the column duly packed or capillary in which the separation of constituents is materialized. The packed-column is usually a tubing having an internal diameter of 4.0 mm and made up of stainless-steel, copper, cupronickel or glass either bent in U-shape or coiled. Its length varies from 120 cm to 150 M.

The general requirements of a liquid phas are :

*   Differential partitioning of sample components,
*   Reasonably good solvent properties for components,
*   High thermal stability, and
*   A lower vapour pressure at the column temperature.

Table 29.1, illustrates the characteristic features of some typical liquid-phases used in GC :

Table 29.1 : Typical Liquid Phases

S.No.	Solvent	Suitable for Solute Type	Upper Temp. Limit (°C)
1.	Paraffin Oil (Nujol)	Paraffin, Olefin, Halide,	150
2.	Silicone Oil	Paraffin, Olefin, Ester, Ether,	200
3.	Polyglycols (Carbowaxes)	Amine, Nitrile, Ether, Ketone, Ester, Alcohol, Aromatics,	100-200
4.	Apiezon L-Grease	General for polar types	300

29.3.4. THERMAL COMPARTMENT

A precise control of the column temperature is not only a must but also a requisite, whether it is intended to maintain an invariant-temperature or to provide a programmed-temperature. Importantly, the temperature of the column oven must be controlled by a system that is sensitive enough to changes of 0.01°C and that maintains an accurate control to 0.1°C. In normal practice, an air-bath chamber surrounds the column and air is circulated by a blower through the thermal compartment. However, separate temperature controls are very much desirable for the vaporizer block as well as the detector-oven.

More recently, programmes are also available that features both in linear and non-linear temperature programming as sample and reference columns. The compartment temperature can also be raised at various rates upto a maximum of 60 °C min^{-1} in the lower-temperature ranges and about 35 °C min^{-1} at higher temperatures.

29.3.5. DETECTORS

There are in all *six* different kinds of detectors used in 'Gas Chromatography', namely :

 (*i*) Thermal conductivity detector (TCD),

 (*ii*) Flame ionization detector (FID),

 (*iii*) Electron capture detector (ECD),

 (*iv*) Thermionic detector (NP–FID)

 (*v*) Flame photometric detector (FPD), and

 (*vi*) Photoionization detector (PID).

The *first three* detectors are invariably used in GLC and shall be discussed in details below ; whereas a passing reference shall be made with respect to the *second three* detectors.

29.3.5.1. Thermal Conductivity Detector (TCD)

The thermal conductivity detector, or **katharometer**, was the first ever detector employed for GLC; and is still being used today be virtue of its *versatility*, *stability*, *simplicity* and above all the *low-cost*.

Principle : The underlying principle of TCD is that the ability of a gas to dissipate heat, *i.e.,* its thermal conductivity, from a heated body shall change with the composition of the gas. It may be further explained by the fact that each specific carrier gas shall have a characteristic thermal conductivity that is picked up first-and-foremost by the equilibrium temperature of the detecting element to afford a baseline signal. Evidently, the thermal conductivity of the mixture of carrier gas plus sample must be altogether different from that of pure carrier gas ; and while the mixture takes its course through the detector, an obvious change in the temperature of the detecting element is duly recorded as a signal.

Figure 29.3 shows a simple diagram of a thermal conductivity detector. It essentially consists of two cells of small volumes, made within a metal block, termed as **reference cell** and **sample cell**. Each cell has a resistance wire or thermister or filament that possesses a high temperature coefficient or resistance *i.e.,* the resistance varies appreciably with slight variation in temperature. These two resistances, namely : reference cell (R) and sample cell (S) are included in two arms of a Wheatstone Bridge. Now, the carrier gas is passed into both the cells, but the column-effluents are allowed to enter only the sample cell. Thus, the temperature of the sample cell changes due to widely different thermal conductivity of the sample component than that of the carrier gas, thereby causing a change in resistance of (S) and the Wheatstone Bridge gets unbalanced. The off-balance current is transmitted to the recorder that finally draws the elution-curve for the sample(s) undergoing chromatographic separations.
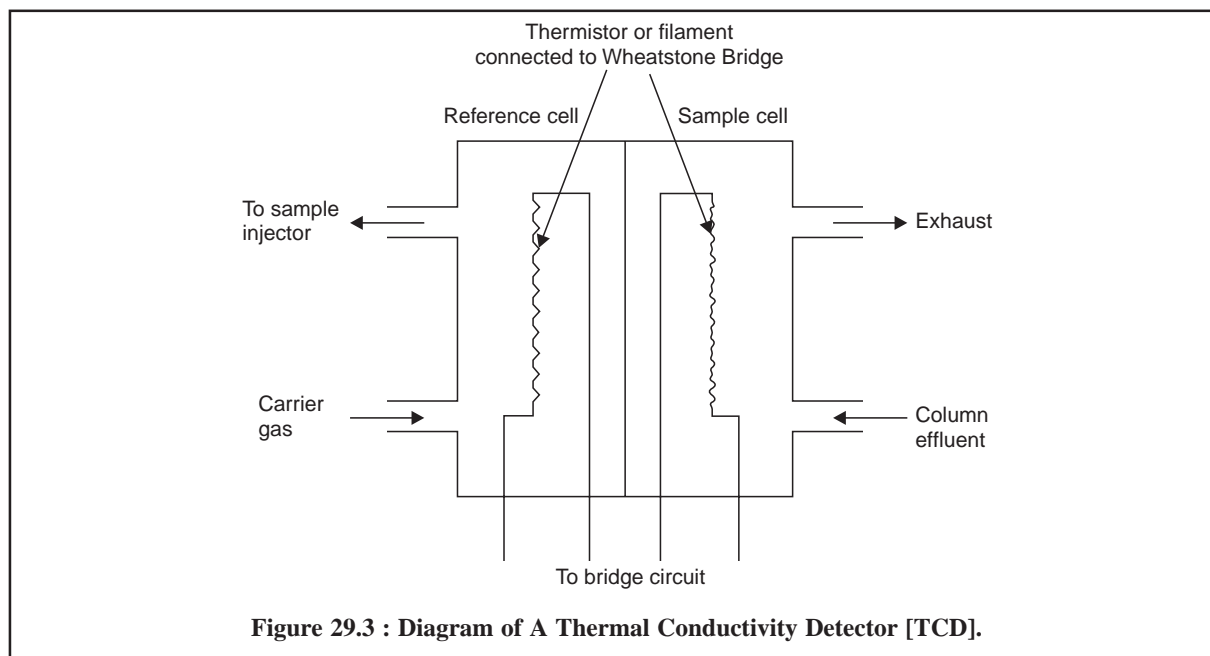
Figure 29.3 : Diagram of A Thermal Conductivity Detector [TCD].

Cautions

 (*i*) **First turn the carrier gas on and then switch on filament-current/detector block heater, and**

(*ii*) **Do not off the carrier gas before switching off the detector current or before the detector block has attained ambient temperature. This saves the filament from being damaged and enhances its life-span considerably.**

29.3.5.2. Flame Ionization Detector (FID)

The general class of '**ionization detectors**' comprise of the following important detectors, namely :

- Flame ionization detector,
- Electron capture detector,

- Thermionic detector, and
- Photoionization detector.

No other detector till date has surpassed the flame ionization detector (FID) as a universal gas chromatographic detector. It hardly meets, all the characteristic features of TCD in terms of simplicity, stability, and versatility besides having *two* distinctly positive plus points :

 (*i*) Its linearity over a wider concentration range, and

(*ii*) It being more sensitive with less flow and temperature dependency.

Principle : First, the principles of operation for all ionization detectors shall be discussed briefly and then the actual principles with specific details would be described under each particular detector.

Generally, the fundamental physical process underlying the operation of an ionization detector is the conduction of electricity by gases. At normal temperatures and pressures a gas essentially behaves as a perfect electrical insulator. However, if electrically charged particles (ions and electrons) are produced in a gas, it becomes a conductor. In other words, their free motion in the direction of the electrical field renders the gas conducting. Assuming a situation, when a vapour is held between two electrodes to which a voltage is applied, practically and absolutely no current shall flow at all in the electrical circuit until and unless charged particles are introduced. The quantum of electric current thus generated would become the signal of the ionization detector. On applying adequate voltage to the electrodes, all of the ions would be collected, and hence the ion-current shall be directly proportional to the number of ions between the electrodes. As the presence of only a few ions are capable of exhibiting the conductivity of the gas; therefore, ionization detectors are usually very sensitive.

Principle of FID : The underlying principle of FID is that invariably a mixture of hydrogen-oxygen or hydrogen-air flame burns with the generation of comparatively fewer ions, but when an organic compound *viz.*, most pharmaceutical substances is ignited in such a flame, ion production gets enhanced dramatically. Therefore, when such a flame is held between two electrodes to which a voltage ranging between 100-300 V is applied, it would instantly give rise to an ion current on burning an organic compound in the flame.

Figure 29.4, illustrates a schematic diagram of a **flame-ionization detector**. It comprises of a positively charged ring (also referred to as cylindrical collector electrode), whereas the flame jet serves as the negative electrode. The flame jet has two inlets ; from the bottom of the column effluent is introduced and from the side H_2 to form the fuel, whereas air is let in uniformly around the base of the jet.
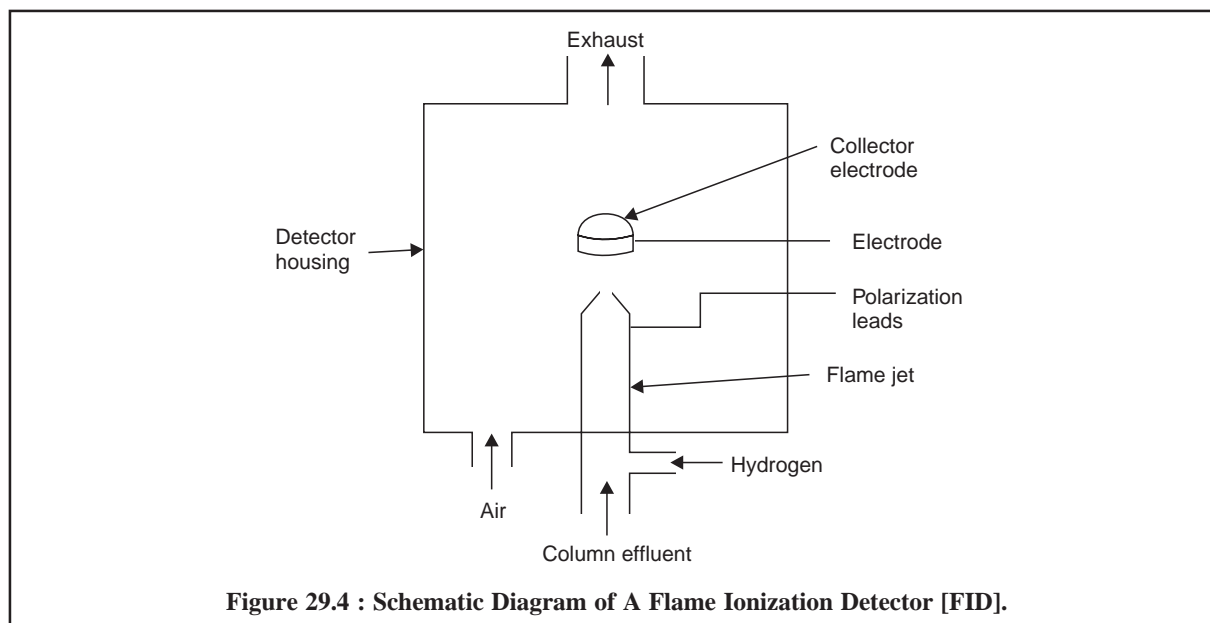

Figure 29.4 : Schematic Diagram of A Flame Ionization Detector [FID].

29.3.5.3. Electron Capture Detector (ECD)

In the domain of gas chromatography the **electron capture detector** (ECD) enjoys the reputation of being one of the *most sensitive* as well as *selective detectors*. However, this valuable detector needs to be handled with a lot of skill and expertise so as to achieve wonderful and dependable results.

Principle : ECD belongs to the general class of ionization detectors, the underlying principles of which have already been discussed. In ECD specifically a β-emitter serves as a source of radiation to generate the ions that helps in ionizing the carrier gas molecules to form positive ions and free electrons as expressed in the following Equation (*e*) :

$$C + \text{radiation} \rightarrow C^+ + e^- \qquad\qquad ...(e)$$

In a situation when the said phenomenon is conducted between a pair of charged electrodes, the mobility of the lighter negative ions *i.e.,* the electrons, would be much higher in comparison to the heavier positive ions *i.e.,* the charged carrier-gas-molecules, thereby ruling out the possibility of their 'recombination'. Thus mostly the cations and electrons will be collected, while generating a standing current that forms the baseline-signal of the ECD detector. At this stage, if an organic molecule, (*i.e.,* a pharmaceutical substance) possessing a comparatively high electron affinity is introduced, a portion of the electrons shall be captured to produce negatively charged ions. These heavy-negative-ions will have less mobility as compared to the electrons ; therefore, they will have no other coice than to unite with positive ions. Thus, the net result would be fewer ions and electrons available to migrate to the electrodes, thereby causing a marked and pronounced reduction in the standing current of the detector. Ultimately, this observed current decrease represent as the 'signal' of the electron capture detector.

Figure 29.5, depicts the diagram of an electron capture detector. The metal block of the detector housing itself serves as a cathode, whereas an electrode polarizing lead suitably positioned in the centre of the detector housing caters for a collector electrode (anode). The radioactive source from a beta-emitter is introduced from either sides of the detector housing below the electrode polarizing lead.

The column-effluent is passed into the detector from the bottom whereas its exhaust goes out from the top.
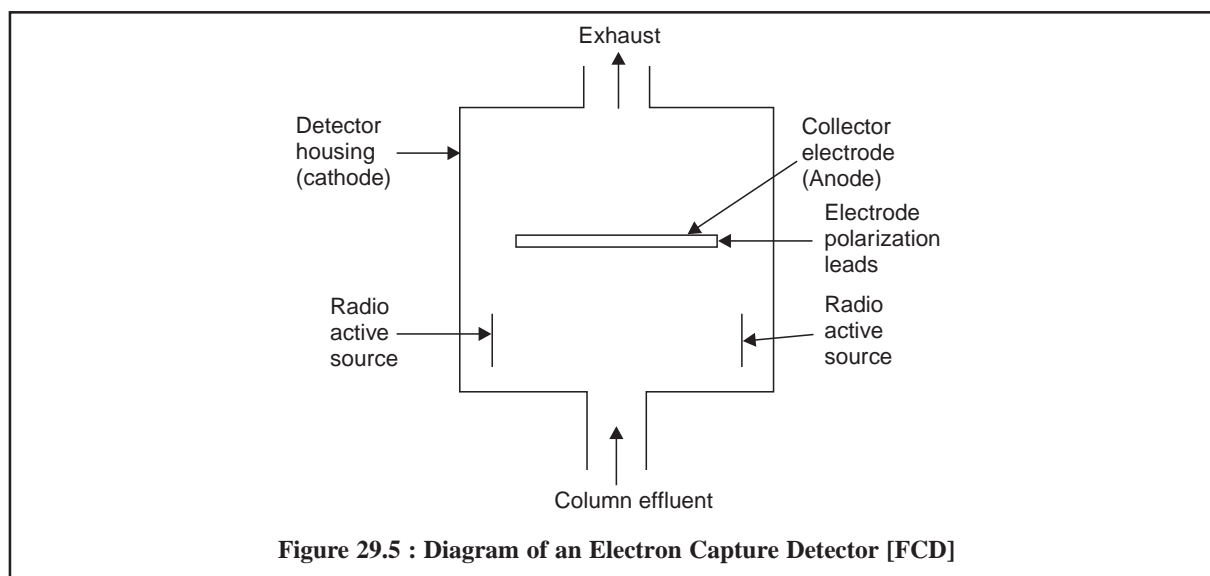
Figure 29.5 : Diagram of an Electron Capture Detector [FCD]

29.3.5.4. Thermionic Detector (NP-FID)

The very name suggests, the thermionic detector functions on the principle of ion-current generated by the thermal production of ions. It may also be invariably termed as a **nitrogen detector**, a **sulphur detector**, a **phophorus detector**, and a **halogen detector** by virtue of the fact that its specificity in detecting organic compounds essentially containing these elements. Furthermore, it is also widely known as NP-FID because it is invariably employed for carrying out the *analysis of N- or P-containing organic compounds*.

29.3.5.5. Flame Photometric Detector (FPD)

Brody and Chaney* in 1966, were the first and foremost to describe the **flame photometric detector** (FPD) which unfortunately could not get enough recognition in the field of gas chromatographic analysis due to the following reasons, namely :

(*i*) Its selectivity, and

(*ii*) Its poor commercial availability.

It solely operates on the principle of photon emission. If P- or S-containing hydrocarbons are ignited in a hydrogen-rich flame, it gives rise to *chemiluminescent species spontaneously* which may subsequently be detected by a suitably photomultiplier device. Hence, FPD is regarded as a specific detector for P- or S-containing compounds.

29.3.5.6. Photoionization Detector (PID)

Lovelock** in 1960, first introduced the photoionization detector but unfortunately its reported usages have been more or less scarce.

PID belongs to the generic class of ionization detectors whose principles have already been discussed earlier. As the very name signifies the PID induces ionization *via* photons emitted by an UV-lamp. A PID detector makes use of a photon energy of 10.2 electron volts (eV) emitted as a **Lyman alpha line***. Only such compounds having ionization potentials less than 10.2 eV shall absorb the UV-radiation and be subsequently converted to positive ions. Two-charged electrodes serve as an electric field in the detector, the cathode becoming the collector electrode for the ions. The ion-current thus generated, that will be directly proportional to the ion concentration, then becomes the signal of the detector.

29.3.6. RECORDING OF SIGNAL CURRENT

In general, the signal from a gas chromatograph is recorded continuously as a function of time by means of a potentiometric device. Most frequently, a recorder of 1-10 mV full-scale deflection ($\simeq$ 10 inches) and having a response time 1 second or less is quite adequate.

Variable chart speeds between the range of 5-50 mm. min^{-1} are most preferable in GC.

Essentially in a potentiometric recorder, the input signal is balanced continuously by a feedback signal making use of a servomechanism ; whereby a pen strategically connected to this system moves proportionally along the width of the chart paper, thus recording the signal, whereas simultaneously the chart paper keeps moving at a constant speed along its length.

The following important points should be noted before operating a recorder, namely :

(*i*) Its 'zero' must be adjusted (or synchronized) with the 'input zero' otherwise the baseline might shift with alterations in attenuation of the signal,

(*ii*) The amplifier gain must also be adjusted duly so as to avoid completely the dead-base and oscillation,

(*iii*) A recorder with inadequate shielding from AC circuits would display shifting of its zero point, and

(*iv*) A reasonably good recorder having quality performance must be employed so as to achieve correct recording of analog-signal, a topmost priority towards quantitative accuracy and precision.

29.3.7. INTEGRATOR

An '**intergrator**' may be regarded as a device that essentially facilitates simultaneous measurement of areas under the chromatographic peaks in the chromatogram either by *mechanical* or *electronic* means. It is, however, pertinent to mention here that '**manual techniques**' for determining peak areas are known, such as :

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* Brody, SS and JE Chaney., **J. Gas Chromatography, 4,** 42, 1966.

** Lovelock, JE, **Nature, 188,** 401, 1960.

***Driscoll, JN, **Amer. Lab., 8,** 71, 1976.

'triangulation', **cutting and weighting of peaks**, planimetry, but all these methods are quite time consuming, tedious and not accurate. Hence, based on the actual need, incorporation of an appropriate integrator in a reasonably good GC-set up is an absolute necessity.

There are *two* types of integrators generally employed in GC, namely :

(*a*) **Ball and Disk Integrator :** This is nothing but a purely mechanical device and installed at one end of the very strip-chart recorder itself. It carries a pen that writes along a span of about one inch, reserved for integrator on the recorder chart paper at the end. The zero line of the integrator moves almost parallel to the base line of the chromatogram and as soon as a peak appears on the recorder, the integrator-pen starts moving from right to left the *vice-versa* within its one-inch strip. Each one-inch traverse (counted along projection parallel to signal axis) is usually assigned a value of 100 counts ; the total number of counts corresponding to a peak are directly proportional to the area of the peak.

The type of mechanical integrator* affords fairly good accuracy and precision ; and above all it is quite cheap.

(*b*) **Electronic Integrator :** An '**electronic integrator**' is definitely a much superior, accurate and dependable device wherein the GC-signal is converted to a frequency pulse that are accumulated corresponding to a peak and later on digitally printed out as a measure of the peak area. The main advantages of an electronic integrator are, namely :

(*i*) Provides a much wider linear range,

(*ii*) Changing the 'attenuation' is not required, and

(*iii*) Offers highest precision in peak-area measurement.

Of course, the electronic integrators are quite expensive

Precision of the TWO methods : The '**electronic integrator**' is almost 3 times** more accurate and precise than the '**ball and disc integrator**' :

Method	Standard Deviation (%)
Ball and Disc Integrator	1.30
Electronic Integrator	0.40

GC-Computer System : Nowadays, a large number of data-processing-computer-aided instruments for the automatic calculation of various peak parameters, for instance : relative retention, composition, peak areas etc., can be conveniently coupled with GC-systems. A commercially available*** fairly sophisticated computer system of such type are available abundantly that may be capable of undertaking load upto 100 gas-chromatographs with ample data-storage facilities. In fact, the installation such **as 'multi GC-systems'** in the routine analysis in oil-refineries and bulk pharmaceutical industries, and chemical based industries have tremendously cut-down their operating cost of analysis to a bare minimum.

29.4. WORKING TECHNIQUES FOR QUANTITATIVE ANALYSIS

In actual practice, the following *three* working techniques are not only widely popular but also provide optimum accuracy and precision for the quantitative analysis of pharmaceutical substances, namely :

(*i*) Area Normalization,

(*ii*) Internal Standard Method, and

(*iii*) Comparison Method.

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* Manufactured by : Disc Instrument Company, USA.

** Source : M/s Varian Aerograph, USA.

*** Sumadzu, Japan ; Varian Aerograph, USA ; Perkin-Elmer, USA.

There three techniques will be discussed briefly in the sections that follow :

29.4.1. AREA NORMALIZATION

Assuming that the chromatogram is as represented in Figure 29.6, the formula employed is :

$$\% \ X = \frac{Ax \cdot fx}{Ax \cdot fx \ + \ Ay \cdot fy \ + \ Az \cdot fz} \times 100$$

$$= \frac{Ax \cdot fx}{\Sigma Af} \times 100 \qquad\qquad ...(f)$$

where,   A = Peak area, and

     f = Response factor.

Areas :   Az   Ay   Az

Response

factors :   fx   fy   fz

$$\% \ X = \frac{Ax. \ fx}{\Sigma Af} \times 100 \ \text{etc.}$$

Figure 29.6 : Area Normalization Method.
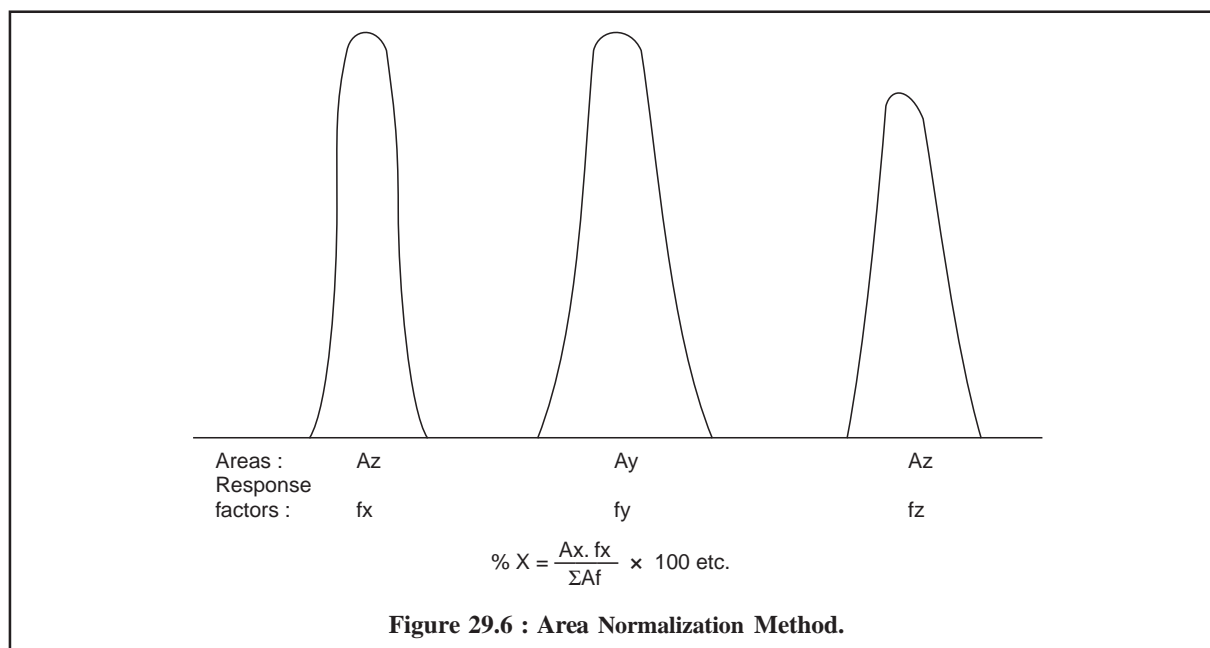
Generally, different components possess different response factors, application of which not only compensates for different detector response for different components but also take into consideration the other factors inherent with the procedure. However, these factors may be calculated by preparing a synthetic mixture absolutely identical to what is expected in the sample, and subsequently carrying out the gas-chromatography of this mixture exactly under idential experimental parameters as described in the method of analysis. Thus, we have :

$$fx = \frac{Wx / Ax}{Wr / Ar} \qquad\qquad ...(g)$$

where, W = Weight or conc. of component in the mixture, and

     r = A reference component present in the mixture which is assumed to have response factor of unity.

In certain instances, like petroleum fractions, where it may be possible to assume that most of the components possess almost equal response factors, the area normalization formula in Eq. (f) may be further simplified to :

$$\% \ X = \frac{Ax}{A} \times 100 \qquad\qquad ...(h)$$

Salient features of Area Normalization Method are as follows :

(*i*) Very suitable for routine type of samples where the variations in composition are only marginal *i.e.,* in such cases where the response factors need to be checked periodically only when necessary, and

(*ii*) An obligatory condition of this method being that all the components of the sample should elute and be recorded.

29.4.2. INTERNAL STANDARD METHOD

In this particular method it is necessary to select a reference compound (known as-**internal standard**) that should meet the following requirements rigidly :

(*i*) It is not a component of the sample but as far as possible, is chemically identical,

(*ii*) It is resolved from various components of the sample, and

(*iii*) It elutes near the components of interest.

The internal standard (IS) is usually added to the sample in such a concentration that matches favourably with that of components to be evaluated. Now, the respective chromatogram is obtained by the GC-method. The percentage of the sample is obtained by the following expression :

$$\%X = \frac{A x \cdot f x}{A_{IS} \cdot f_{IS}} \cdot \frac{w}{W} \times 100 \qquad \qquad ...(i)$$

where, W = Weight of the sample,

  w = Weight of the internal standard,

  A_{IS} = Peak area of internal standard, and

  f_{IS} = Response factor of the internal standard.

Graphical Approach : Many a times a '**graphical approach**' as illustrated in Figure 29.7 is also applied for the quantitative determination of components in the sample.
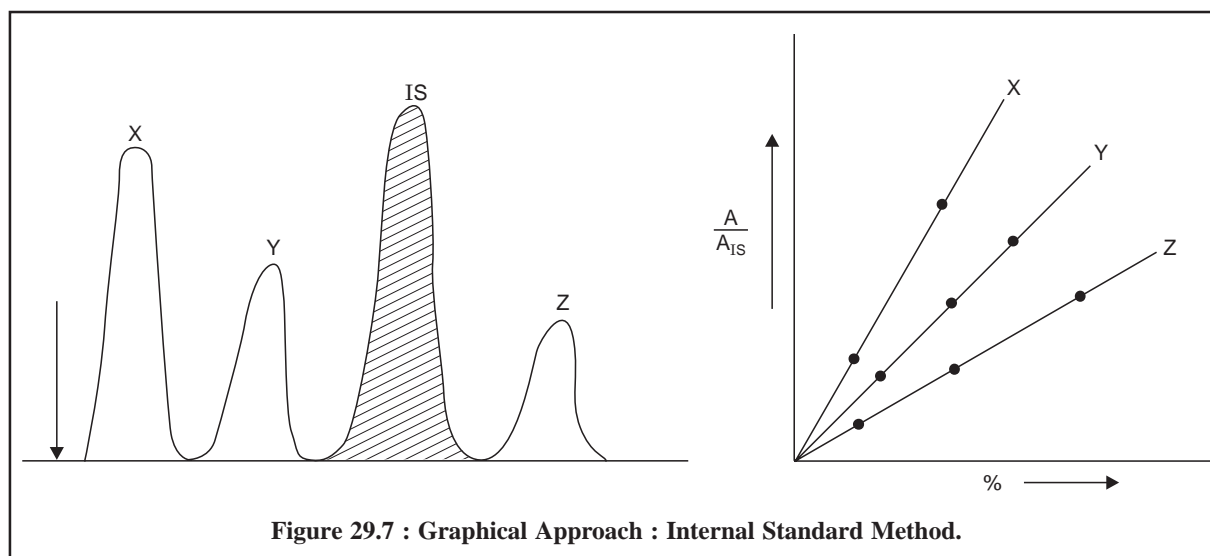
Figure 29.7 : Graphical Approach : Internal Standard Method.

First and foremost, the calibration curves are plotted for each component by GC-method using synthetic blends (containing varying concentrations of the component and fixed known concentration of IS) and also plotting A/A_{IS} Vs %-concentration. Then running separately the sample (plus IS) in a similar manner and determining A/A_{IS} value, %-concentration of the component may be observed from the calibration curve.

Salient features of **Internal Standard Method**-are as follows :

(*i*) It gives very accurate and precise results,

(*ii*) It completely eliminates possibility of error caused due to loss of some part of the sample (other than the determined components) during the initial preparation stage,

(*iii*) It eliminates error due to incomplete elution of all the sample components, and

(*iv*) It eliminates error caused due to inaccurate measurement of sample size before injection.

29.4.3. COMPARISON METHOD

The **'comparison method'** makes use of a purely synthetic blend containing the component to be determined in the same order of concentration as expected in the sample. In fact, the very purpose of this synthetic-blends is only to simulate a typical sample. Now, exactly equal (or known) amounts of both, the '**synthetic blend**' and the '**sample**'are separately injected and chromatograms obtained. Thus, by actually comparing the areas of the desired component in both the chromatograms, the 'unknown concentration' may be determined by the following expression :

$$\%X = \frac{A}{A'_X} \text{ (\% X in synthetic blend)}$$

where, A'_X = Peak area of component X in the chromatogram of 'synthetic blend'.

However, this method is less accurate in comparison to the first two methods described earlier for quantitative analysis. It should be used judiciously if only a few components present in small concentration (*i.e.,* < 5%) in the sample are required to be estimated *e.g.,* in trace-analysis.

Precautions : Following are certain precautions that must be observed in the quantitative analysis, namely :

(*i*) Detector response should always be linear in the concentration range covered in the analysis,

(*ii*) Distortion of the peak caused due to detector and recorder performance must be as negligible as possible,

(*iii*) Both sample decomposition and adsorption in any portion of the GC-assembly must be avoided, and

(*iv*) Adequate and precise sampling technique must be followed to permit injection of representative sample only. Obviously, this part of analyis is as vital and critical as the gas chromatorgraphic part of analysis.

29.5. APPLICATIONS OF GLC IN PHARMACEUTICAL ANALYSIS

Gas liquid chromatography (GLC) or **gas chromatography** (GC) finds its abundant applications in the accurate and precise analysis of plethora of official pharmaceutical substances covering a wide range as enumerated below :

(*i*) Assay of Drugs,

(*ii*) Determination of specific organic compounds as impurities in official pharmaceutical substance,

(*iii*) Determination of related substances in official drugs,

(*iv*) Determination of water in drug, and

(*v*) Determination of chloroform with head-space chromatography.

29.5.1. ASSAY OF DRUGS

Assay of Cetostearyl Alcohol

Materials Required : Solution (1) (1% w/v of cetostearyl alcohol sample in 96% ethanol) ; solution (2) (0.6% w/v of cetyl alcohol EPCRS in 96% ethanol) ; (3) (0.4% w/v of stearyl alcohol EPCRS in 96% ethanol ; solution (4) [mix 1 ml of solution (2) and 1 ml of solution (3) and add sufficient 96% ethanol to produce 10 ml] ;

Chromatographic Parameters-are as follows :

 (*i*) **Column :** Made of glass or stainless steel ; size : (3 M × 4 mm) ; adsorbent : diatomaceous support (125 to 180 mesh) impregnated with 10% w/w of polydimethylsiloxane and maintained at 200 °C,

 (*ii*) **Inlet-port and Detector :** are maintained at 250 °C,

 (*iii*) **Flow rate of Carrier Gas** (N_2) : 30 ml minute^{-1}, and

 (*iv*) **Resolution Factor :** between the two principle peaks in the chromatogram obtained with solution (1) must not be less than 1.25 (it may be achieved by adjusting the flow rate), and

 (*v*) **Detector :** Flame Ionization Detector (FID).

Procedure : After having maintained the above mentioned experimental conditions for gas chromatography inject 2μ*l* of solutions (1) through (4) sequentially.

Observations : The assay is not valid unless the chromatogram obtained with solution (4) shows two principal peaks with a signal-to-noise ratio of at least 5.

Calculations : Calculate the content of cetylalcohol and of stearyl alcohol from the chromatogram thus obtained with solution (1) by normalization. Identify the peaks by visual comparison with the chromatograms obtained with solutions (2) and (3) respectively.

29.5.1.1. Cognate Assays

A few other drugs can also be assayed by the same procedure and are stated below in Table 29.2 :

Table 29.2 : Cognate Assay of Drugs by GLC-Method

S.No.	Name of Substance	Column Parameters	Solutions	Calculations
1.	Ethyloestrenol	Glass column (1.0 M × 4 mm) packed with acid-washed silanised diatomaceous support (80 to 100 mesh) coated with 3% w/w of phenyl methyl silicone fluid (50% phenyl) maintained at 200 °C. OV-17 is also suitable.	(1) 0.2 w/v of ethylo-estrenol BPCRS and 0.1% w/v of arachidic alcohol (internal standard) ; (2) 0.2% w/v of sample ; (3) 0.2% of sample plus 0.1% w/v of the internal standard.	Calculate the content of $C_{20}H_{32}O$ using the declared content of $C_{20}H_{32}O$ ethylo-estrenol BPCRS.
2.	Lincomycin Hydrochloride	Glass column (1.5 M × 3 mm) packed with acid-washed silanised diatomaceous support impregnated with 3% w/w of phenyl methyl silicone fluid (50% of phenyl) (OV-17 is also suitable) and maintained at 260 °C ; Inlet-port and detector maintained at 260-290 °C ; Carrier gas : Helium-with a flow rate of about 45 ml minute^{-1}.	Solutions (1), (2) and (3)* ;	Calculate the content of $C_{18}H_{34}N_2O_6S$, HCl, H_2O in lincomycin hydro-chloride BPCRS

* BP (1993) Vol. I, p-384.

29.5.2. DETERMINATION OF SPECIFIC ORGANIC COMPOUNDS AS IMPURITIES IN OFFICIAL PHARMACEUTICAL SUBSTANCES

A number of organic compounds, such as : N, N-dimethylaniline-present in amoxycillin trihydrate ; cephalexin ; cloxacillin sodium ; dicloxacillin sodium ; 2-ethylhexanoic acid-in amoxycillin sodium ; 4-chlorophenol-in clofibrate ; acetone and butanol-in daunorubacin hydrochloride ; cineole : limonene ratio-in dementholised mint oil etc ;

A. Determination of N, N-dimethylaniline in Cephalexin

Materials Required : Cephalexin sample : 1.0 g ; Solution A [0.005% w/v soln. of naphthalene (internal standard) in cyclohexane] : 20 ml ; Solution B (mix 50 mg of N, N-dimethylaniline with 2 ml HCl and 20 ml of water, shake to dissolve, add enough DW to produce 50 ml ; and dilute 5 ml of the resulting solution to 250 ml with DW) : 20 ml ; solution (1) (add 5 ml of 1 M NaOH and 1 ml of solution A to 1 ml of solution B, shake vigorously for 1 minute, centrifuge and use the upper layer) ; solution (2) (dissolve 1 g of cephalexin sample in 5 ml of 1 M NaOH, add 1 ml of solution A, shake vigorously for 1 minute, centrifuge and use the upper layer) ;

Chromatographic Conditions : are as stated under :

Column* : Glass column ; size (2 M × 2 mm) ; adsorbent : packed with acid-washed, silanized diatomaceous support (80 to 100 mesh) impregnated with 3% w/w of phenyl methyl silicone fluid (50% phenyl, and maintained at 120 °C,

Detector : Flame Ionization Detector (FID),

Inlet-port and Detector : maintained at 150 °C, and

Flow Rate : 30 ml minute^{-1} for N_2 as the carrier gas.

Procedure : After having set the above experimental conditions for gas chromatography, inject 1 μl of the solutions (1) and (2) sequentially into the column. Repeat the determinations so as the ensure a consistent response. Determine the peak areas**.

Calculations : From the value obtained calculate the content of N, N-dimethylaniline present in the given sample of cephalexin. However, according to BP(1993) it should not be more than 20 ppm.

29.5.2.1. Cognate Assay

A few cognate determinations are listed in Table 29.3 :

Table 29.3 : Cognate Determination of N, N, Dimethylaniline in Official Pharmaceutical Substances

S.No.	Name of Substance	Chromatographic Conditions	Sample Injection only	Solns for GLC***	Pharmacopoeal Requirement
1.	Amoxicillin Sodium	Same as for Cephalexin	1 μl	Solution A ; Soln. (1) and (2) ;	NMT 20 ppm ;
2.	Cloxacillin Sodium	-do-	1 μl	Solution A ; Soln. (1) and (2) ;	NMT 20 ppm ;
3.	Dicloxacillin Sodium	-do-	1 μl	Solution A ; Solution B ; Solutions (1), (2) and (3) ;	NMT 20 ppm ;

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* OV-17 is also suitable ;

** BP (1993) Vol I ;

*** Applications where temperature programming is required in GLC, peak-areas determinations are to be used only.

29.5.3. DETERMINATION OF RELATED SUBSTANCES IN OFFICIAL DRUGS

Related substance present in good number of official drugs may be determined by GLC method, for instance : Bromopheneramine maleate ; Bronopol ; Cepahloridine ; Chlorocresol ; Chloroform ; Chloroxylenol., Cindamycin hydrochloride ; Griseofulvin ; Isometheptene mucate ; Levomenthol ;

A. Related Substances in Bromopheneramine Maleate

Materials Required : Solution (1) dilute 1 volume of solution (3) to 200 volumes with a 0.005% w/v solution of N-phenylcarbazole (internal standard) in toluence : 10 ml ; Solution (2) : (add 5 ml of DW to 0.1 g of bromopheneramine maleate sample, make the resulting solution alkaline with 13.5 M ammonia, add 2.5 ml of toluene, shake for 5 minutes, centrifuge and use the upper layer) ; solution (3) : (prepare it exactly in the same manner as solution-'2' but using the internal standard solution in place of toluene) ; Solution (4) : (dissolve 10 mg of bromopheneramine maleate BPCRS in 5 ml of DW, make alkaline with 13.5 M ammonia, add 2.5 ml of toluene, shake for 5 minutes, centrifuge and dilute 1 volume of the upper layer to 20 volumes with toluene) ;

Chromatographic Parameters : These are as mentioned below :

Column : Glass column ; size : (1.5 m × 4 mm) ; adsorbent : packed with acid-washed, silanized diatomaceous support (80 to 100 mesh) impregnated with 3% of phenyl methyl silicone fluid (50% phenyl) and maintained at 220 °C.

Flow rate of Carrier Gas : 30 l minute^{-1} ;

Carrier Gas : Nitrogen ;

Detector : Flame Ionization Detector (FID) ;

Procedure : After having maintained the aforesaid experimental parameter for gas chromatography, inject 1 μl each of solutions (1), (2), (3) and (4) in a sequential manner.

Calculations

(*i*) Calculate the ratio (γ) of the area of the peak due to bromopheneramine to that of the peak due to the internal standard in the chromatogram obtained with solution (1) ; and

(*ii*) In the chromatogram obtained with solution (3) the ratio of the sum of the areas of any secondary peaks to the area of the peak due to the internal standard is not greater than γ and the ratio of the area of any secondary peak to the area of the peak due to the internal standard is not greater than 0.4γ.

29.5.3.1. Cognate Determinations

A few cognate assays are summarized in Table 29.4 ; below :

Table 29.4 : Cognate Determinations of Related Substances in Official Drugs*

S.No.	Name Substance	Chromatographic Conditions	Sample Injection Qty.	Solutions for GLC*	Calculations/ Observations
1.	Bronopol	Same as under Bromopheneramine Maleate	1 μl	Solution A ; Solution B ; Solutions (1), (2) and (3)	Calculate ratio for sample and internal stand. from soln. (1) ; for soln. (3) ratio of area of any secondary peak and is not > γ.

* BP (1993) Vol I & II.

2.	Clindamycin Hydrochloride	Same as above except adsorbent (60 to 80 mesh) ; Column Temp. 170 °C. Inletport and Detector Temp. 200° to 210 °C. Record the chromatogram for soln. (1) twice the retention time of cindamycin.	1 µl	Solution (1) ; Solution (2) ;	(1) Test is not valid unless the height of the principal peak obted. with soln. (1) measured from base-line is at least 70% of fullscale deflection. (2) For soln. (1) the sum of the areas of any second ary peak is not > 3% and the area of any one such peak is not 2% by normalization.

29.5.4. Determination of Water in a Drug

Assay of Water Present in Mentrophin

Materials Required : Solution (1) [dilute 15 µl of anhydrous methanol (internal standard) with sufficient anhydrous propan-2-ol to produce 100 ml] ; Solution (2) (dissolve 4 mg of mentrophin sample in 0.5 ml of anhydrous propan-2-ol) ; Solution (3) [dissolve 4 mg of mentrophin sample in 0.5 ml of solution (1)] ; Solution (4) and 10 µl of water to 50 ml of solution (1) ;

Chromatographic Conditions

Column : Stainless steel ; size : (1 M × 2 mm) ; adsorbent : packed with porous polymer beads (60 to 80 mesh) and maintained at 120 °C.

[**Note :** Chromosorb 102 is also suitable.]

Carrier Gas : Helium ;

Detector : Thermal Conductivity Detector (TCD)-maintained at 150 °C.

Procedure : After having maintained the various experimental parameters stated above for gas chromatography and using throughout absolutely dry glassware which may be siliconized, inject 1 µl of solution (1) through solution 4 sequentially and obtain the chromatogram.

Calculations : From the chromatograms obtained and taking into account any water detectable in solution (1), calculate the percentage of water taking 0.9972 g as its weight per ml at 20 °C.

29.5.5. DETERMINATION OF CHLOROFORM IN COLCHICINE BY HEAD-SPACE GAS CHROMATOGRAPHY

Head-space gas chromatography is an analytical device specifically suitable for the separation and simultaneous determination of volatile constituents present in solid or in liquid samples.

Principle : The underlying principle of **head space gas chromatography** is the analysis of the vapour phase in equilibrium with the solid or liquid phase.

Apparatus : The introduction of sample(s) may be accomplished by using airtight syringes and a simple conventional gas chromatograph. Nevertheless, the equilibrium has got to be carried out in a separate chamber and the vapour phase is subsequently conveyed to the column taking necessary and every possible precautions so as to avoid any minute changes in the equilibrium.

Materials Required : Solution (1) dissolve 0.4 g of colchicine sample in sufficient water to produce 10 ml and place 1 ml of the solution in each of three identical stoppered vials ; solution (2) dilute 5 µl of chloroform to 10 ml with water and place 10 µl of the resulting solution and 1 ml of solution (1) in each of three identical stoppered vials ;

Chromatographic Conditions

Column : Fused-silica capillary column ; size : (50 M × 0.32 mm) coated with a 5-μm film of chemically-bonded polymethyl siloxane ;

Detector : Flame Ionization Detector (FID) maintained at 250 °C.

Carrier Gas : Nitrogen

Flow Rate : 4 ml minute^{-1} for the carrier gas ;

Procedure : First of all maintain the above experimental parameters of the gas chromatograph and then maintain the six solutions at 90 °C for 20 minutes, pressurise for a duration of 30 seconds only and transfer subsequently to the column at a temperature of 120 °C. Repeat the operation using a vial containing 1 ml of water. Perform each measurement at least three times.

Calculations : Calculate the percentage w/w of chloroform, taking into consideration 1.48 as the weight per ml at 20 °C.

THEORETICAL AND PRACTICAL EXERCISES

1. 'Nobel laureates Martin and Synge's innovative work on the development of '*partition chromatography*' introduced Gas Liquid Chromatography (GLC) as a versatile analytical tool'. Justify the above statement with plausible explanation.

2. Enumerate the following aspects of GLC :

   (*i*) separation occurs due to adsorption effects,

   (*ii*) separation occurs due to partition effects, and

   (*iii*) advantages of GLC over TLC, column chromatography.

3. Give a comprehensive account on the theoretical aspect of GLC with regard to :

   (*a*) Plate theory,   (*b*) Rate theory, and

   (*c*) Random walk and nonequilibrium theory.

4. Discuss the working of various components required for an efficient GLC equipment with the help of a neat diagramatic sketch.

5. What are the various **'detectors'** used in GLC equipment ? Describe the following two commonly used detectors in an elaborated manner :

   (*a*) Thermal Conductivity Detector (TCD)   (*b*) Flame Ionization Detector (FID).

6. **'Chromatographic Peaks'** in GLC may be measured accurately either by

   (*a*) Ball and Disc Integrator ;   (*b*) Electronic Integrator.

   Discuss the two aforesaid integrators and also affirm which one gives better results.

7. What are the *three* widely popular working techniques for **'quantitative analysis'** by GLC ? Expatiate each method along with graphic presentation wherever necessary.

8. How would you carry out the assay of the following drugs :

   (*i*) Cetostearyl alcohol,   (*ii*) Ethyloestrenol, and

   (*iii*) Lincomycin hydrochloride.

9. How GLC helps in the determination of specific organic compounds in **'official drug substances'** :

   (*i*) N, N-Dimethylaniline in CEPHALEXINE, and

   (*ii*) N, N-Dimenthylaniline in CLOXACILLIN SODIUM.

10. How would assay the *related substances* in **'Official Drugs'** ?

(*i*) Bromopheneramine moleate,   (*ii*) Bronopol, and

(*iii*) Clindamycin hydrochloride.

11. Give details for the assay of :

(*a*) Water present in mentrophin, and   (*b*) Chloroform present in colchicine.

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30 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

30.1. INTRODUCTION

The excellent and the most wonderful technique of **'high performance liquid chromatography'** (HPLC) is nothing but an outcome of the various theories and instrumentation that were originally advocated for liquid chromatography (LC) and gas chromatography (GC). By the late 1960's, analysts invariably used to have the bliss of excellent experience of achieving the goal of superb separations of complex mixtures in seconds rather than in minutes, with the aid of electronic integrators to get an exact access of areas under elution bands, and above all the **'computer-printouts'** of the complete analysis with the flick of a finger. In many favourable instances the smallest possible quantities ranging from nanogram to picogram* could be detected with utmost ease and convenience.

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* nanogram = 10^{-9} g ; 1 picogram = 100^{-12} g ;

In reality, some of the serious limitations too often encountered in GC ultimately brought about the development of HPLC, for instance :

(*i*) In GC the mixture of components are usually screened in the vapour phase. Hence, either a stable vapour from the mixture is obtained directly or indirectly converting the substance in it to such derivatives that are thermally stable. One 20% of chemical compounds usually come across in analysis are suitable for GC directly *i.e.*, without making their corresponding appropriate derivatives,

(*ii*) The remainder 80% of the chemical compounds are either thermally unstable or involatile in nature, and

(*iii*) Compounds essentially having highly polar or ionizable function groups are very prone to **'tailing'** by GC-analysis.

Therefore, HPLC has been evolved as a dire confluence of need, technological supremacy, the emergence of newer theoretical concepts and ideas towards development along rational lines, and above all-**'the human desire to minimise work'. HPLC offers numerous advantages as stated below :**

- Capable of handling **'macromolecules'**,
- Suitable for **pharmaceutical compounds,**
- Efficient analysis of **'labile natural products',**
- Reliable handling of **inorganic or other ionic species,** and
- Dependable analysis of **biochemicals**.

Interestingly, in HPLC the stationary phase and the mobile-phase is able to interact with the sample selectively. Besides, such interactions as hydrogen bonding or complexation which are absolutely not possible in the GC-mobile phase may be accomplished with much ease in the HPLC-mobile phase. Furthermore, the spectrum of these selective interactions may also be enhanced by an appropriate chemical modification of the silica surface *i.e.*, the stationary phase. Therefore, HPLC is regarded as a more versatile technique than GC and capable of achieving more difficult separations.

30.2. THEORY

The particle size of the stationary phase material plays a very vital and crucial role in HPLC. In fact, high-efficiency-stationary-phase materials have been researched and developed exclusively for HPLC having progressively smaller particle size termed as microparticulate column packings. These silica particles are mostly uniform, porous, with spherical or irregular shape, and having diameter ranging from 3.5 to 10 µm.

Bonded-Phase Supports : The bonded-phase supports usually overcome plethora of the nagging problems which is mostly encountered with adsorbed-liquid phases. Here the molecules, comprising the stationary phase, *i.e.*, the surfaces of the silica particles, are covalently bonded to a silica-based support particle.

However, the most popular bonded-phase, **siloxanes**, are formed by heating the silica particles-in dilute acid for a day or two so as to generate the reactive silonal group :

$$\begin{array}{ccccc} OH & & OH & & OH \\ | & & | & & | \\ -Si-O-Si-O-Si- \\ | & & | & & | \end{array}$$

which is subsequently treated with an organochlorosilane :

$$
\begin{array}{ccc}
& \overset{\displaystyle CH_3}{\underset{\displaystyle CH_3}{|}} & \overset{\displaystyle CH_3}{\underset{\displaystyle CH_3}{|}} \\
-Si-OH \ + \ Cl-Si-R \ \longrightarrow & -Si-O-Si-R \ + \ HCl
\end{array}
$$

These bonded phases are found to be fairly stable between the pH range 2 to 9 and upto temperatures of about 80 °C. The nature of the R group of the silane solely determines the surface polarity of the bonded phase. A fairly common bonded phase is made with a linear C_{18} hydrocarbon, also known as ODS (octadecyl silane) bonded phases, wherein the groups appear to be protruding out from the silica particle surface just as the bristles on a toothbrush. It takes care of almost 75% of the samples in HPLC.

Note : The exact mechanism by which the respective bonded phases actually alter the nature of the sorption mechanism is still not yet clear.

When such microparticulate-bonded-phases are packed compactly into a column by means of a suitable device, the small size of these particles offers a significant resistance to solvent flow ; therefore, the mobile phase has to be pumped through the column under a high positive pressure. For an analytical HPLC, the mobile-phase is pumped through the column at a flow rate of 1-5 cm^3. min^{-1}.

At this juncture usually two varying situations arise. These are, *firstly*, isocratic elution - *i.e.*, when the composition of the mobile-phase is constant, and

Secondly, gradient elution-*i.e.*, when the composition of the mobile phase can be made to change in a predetermined fashion during the course of separation.

Note : Here, the gradient elution may be simply compared to the temperature programming in GC.

In-line Detector : It broadly helps to sense the separated solutes, after they exit through the column. Invariably the detector is an electrical signal whose variation is displayed on a potentiometer recorder or a computing integrator or a video-screen. Modern HPLC units are provided with detectors having selective-devices thereby categorically restricting the response to all the solutes present in a mixture.

Note : However, no universal detector has so far been discovered for HPLC to cater for a wide-spectrum of components ; as the Flame-Ionization-Detector (FID) used for GC in Chapter 29.

Post-Column Derivatisation : There are certain stubborn and fairly difficult components that are not easily detectable in HPLC. Therefore, such component(s) have to be appropriately converted into their corresponding detectable form once they emerge from the column.

Table 30.1 records the comparison of HPLC and GLC specifically with respect to their advantages.

Table 30.1 : Comparison of HPLC and GLC

S. No.	HPLC	GLC
1.	Can accommodate non-volatile and thermally unstable samples	Not applicable.
2.	Applicable to inorganic ions	Not applicable.
3.	Complicated and expensive equipment	Simple and inexpensive equipment.
4.	Overall analysis is not that fast	Quite rapid
5.	No universal detector available	FID-as Universal Detector

Both HPLC and GLC are :

- Efficient, highly selective and widely applicable

- Only small quantum of sample required

- Ordinarily non-destructive of sample
- Readily adaptable to *'Quantitative Analyses'*
- Provide dependable, accurate and precise and reproducible results.

30.3. INSTRUMENTATION

Modern HPLC essentially comprises of the following main components namely :

 (*i*) Solvent reservoir and degassing system,

 (*ii*) Pressure, flow and temperature,

 (*iii*) Pumps and sample injection system,

 (*iv*) Columns,

 (*v*) Detectors,

 (*vi*) Strip-chart recorder, and

 (*vii*) Data handling device and microprocessor control.

All these vital components will be discussed with adequate details, wherever necessary, in the various sections that follow :
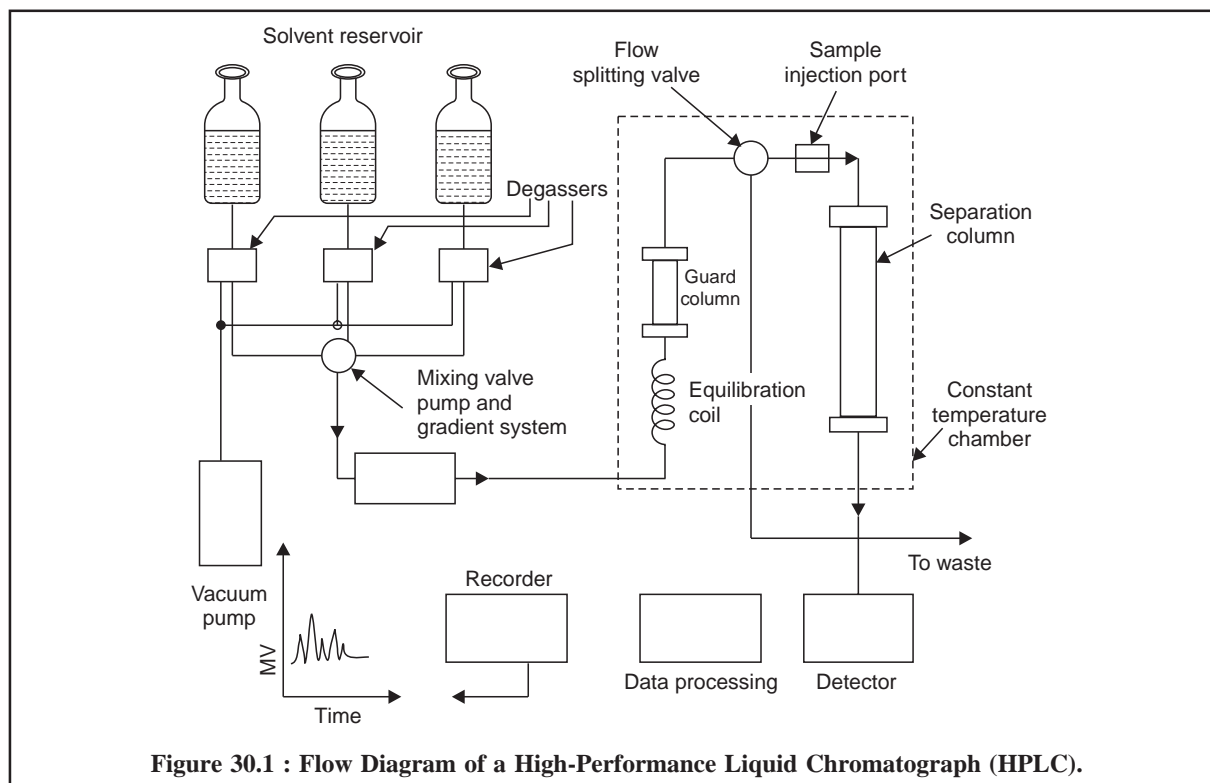
Figure 30.1 : Flow Diagram of a High-Performance Liquid Chromatograph (HPLC).

The Figure 30.1, illustrates the flow diagram of a high performance liquid chromatograph, wherein all the vital components have been duly represented.

The mobile phase, that may be either a single liquid or a mixture of two or more liquids, is pumped at high pressure into a temperature controlled oven, where it first, gains its entry into an equilibration coil to bring it to the operating temperature, and secondly, through a 'guarded column' specially designed and strategically positioned to protect the analytical column from impurities and ultimately extend its lifetime. In case a differential

type of detector is employed, the flow may be split at this juncture, with a portion going directly to the reference side of the detector and a portion to the analytical column housed in a constant temperature chamber. Just like GC, the sample is introduced to the mobile phase prior to the column. Finally, the column-effluent is made to pass through the sample side of all the detector, followed by a data-processing unit and then to the recorder.

30.3.1. SOLVENT RESERVOIR AND DEGASSING SYSTEM

Mobile-phase consisting of a mixture of organic solvents or an aqueous-organic mixture or a buffer solution may be employed depending upon the chromatographic method *vis-a-vis* the detector to be used. Special grades of solvents are commercially available for HPLC that have been adequately refined to eliminate completely the UV-absorbing impurities and any particulate matter., In case, other grades of solvents are employed, purification may have to be done at all cost because impurities present would, if strongly UV-absorbing, affect the detector or, if of higher polarity (*e.g.*, traces of H_2O or EtOH, commonly included as a stabilizer, in $CHCl_3$), influence the separation.

Solvent-reservoir comprises of a 1 dm^3 glass bottle having a lid and a 1/8 inch diameter ptfe-tube to convey the mobile phase from the reservoir to the degassers and then to the pump. As described above, any liquid entering the pump should be free from dust and particulate matter, otherwise these foreign substances may invariably give rise to irregular pumping action, damage seals and valves, irregular behaviour of column owing to its contamination, and ultimate blockade of column. Sometimes a stainless steel filter element (of filter size 2 μm) that could be conveniently positioned either in the ptfe-tube in the reservoir or an in-line-filter may be employed.

Degassing : Many liquids dissolve appreciable amounts of atmospheric gases *e.g.*, air or suspended air-bubbles that may be a major cause of practical problems in HPLC, specifically affecting the operation of the pump and the detector. However, all such problems may be avoided by degassing the mobile-phase by subjecting the mobile-phase under vacuum, distillation, spurging with a fine spray of an inert gas of low solubility such as Argon or Helium or by heating and ultrasonic stirring*.

30.3.2. PRESSURE, FLOW AND TEMPERATURE

Pressure : HPLC columns are packed usually upto 700 times atmospheric pressure and, therefore, the operating inlet-column-pressure in HPLC may be to a maximum of 200 times atmospheric pressure.

Hence, 1 N atmospheric pressure = 10^5 Pa (Pascal)**

or $$1 \text{ Pa} = 1 \text{ Nm}^{-2}$$

or $$1 \text{ Bar} = 10^5 \text{ Pa}$$

Pressures may also be expressed as psi (*i.e.*, pounds per square inch) or in kg cm^{-2}.

Conversion between bar and psi : It is known that—

1 pound = 0.4536 kg ; 1 inch = 2.54 cm ; and g = 9.81 ms^{-2} ; Therefore, 1 psi = a force of 0.4536 × 9.81 N acting over an area of 0.0254^2 m^2

or $$1 \text{ psi} = .4536 \times 9.81/(.0254)^2 = 6897 \text{ Pa}$$

or $$1 \text{ bar} = 10^5/6897 = 14.5 \text{ psi}$$

the conversion for kg cm^{-2} are :

$$\boxed{1 \text{ kg cm}^{-2} = 0.981 \text{ bar} = 14.2 \text{ psi}}$$

However, it is pertinent to mention here that most of the analytical HPLC is performed using pressures between 25 to 100 bar only.

---

*   Scott, RPW, **Contemporary Liquid Chromatography,** New York, Wiley, 1976.

** Standard International Unit of Pressure.

There are, in fact, several factors that are solely responsible for the '*pressure*' developed in a column, namely :

(*a*) the length of the column,

(*b*) particle size of the stationary phase,

(*c*) viscosity of the mobile-phase, and

(*d*) flow-rate of the mobile-phase.

The pressures mentioned above correspond to mobile-phase flow rates of approximately 1-5 cm^3 min^{-1} through the column.

Flow : The flow can be measured periodically at the column outlet by collecting the liquid for a known period, and thereafter, either measuring the volume or weighing it physically.

Temperature : In reality, the maintenance of strict 'temperature control' plays a vital role in measuring the retention-data correctly and precisely. It makes use of the refractometer detectors specifically. In HPLC, difficult separations may be achieved by increasing the temperature carefully, but this must be done initially on a hit and trial basis.

30.3.3 PUMPS AND SAMPLE INJECTION SYSTEM

Pumps : The *two* major functions of the pump in a modern HPLC are, namely :

(*i*) To pass the mobile-phase through the column at a high pressure, and

(*ii*) At a constant a controlled flow rate.

HPLC makes use of two types of pumps. They are :

(*a*) **Constant Pressure Pump :** A constant-pressure pump acts by applying a constant pressure to the mobile-phase. The flow rate through the column is determined by the flow resistance of the column.

(*b*) **Constant Flow Pump :** A constant-flow pump affords and maintains a given flow of liquid. The pressure developed entirely depends upon the flow resistance.

Importantly, in a constant-pressure pump the flow rate will change if the flow resistance changes. Whereas in the constant flow pumps the changes in flow resistance are compensated duly by a change of pressure. Therefore, it is always advisable to use constant flow pump in HPLC determinations.

Salient features of HPLC pump are as follows :

(*i*) Interior of the pump must not be corroded by any solvent to be used in the system,

(*ii*) Variable-flow-rate device must be available to monitor flow rate,

(*iii*) Solvent flow must be non-pulsing,

(*iv*) Changing from one mobile-phase to another must be convenient, and

(*v*) It should be easy to dismantle and repair.

The pump is a very delicate and sensitive part of HPLC unit ; therefore, all buffer solutions should be removed carefully after use either by pumping water (HPLC-grade) or an appropriate solvent (HPLC-grade) for several minutes.

Reciprocating Pump : Figure 30.2 represents the schematic diagram of a typical reciprocating pump along with its various essential components. The piston is moved in and out of a solvent chamber by an eccentric cam or gear. The forward-stroke closes the inlet-check value while the outlet valve opens and the respective mobile phase is duly pumped into the column. Consequently, the return-stroke-closes the outlet valve and it refills the chamber.

Advantages : It has the following advantages, namely :

(*i*) It has an unlimited capacity,

(*ii*) The internal-volume can be made very small from 10-100 µ*l*,

(*iii*) The flow-rate can be monitored either by changing the length of the piston or by varying the speed of the motor, and

(*iv*) It has an easy access to the valves and seals.

Figure 30.2 : Schematic Diagram of a Reciprocating Pump.
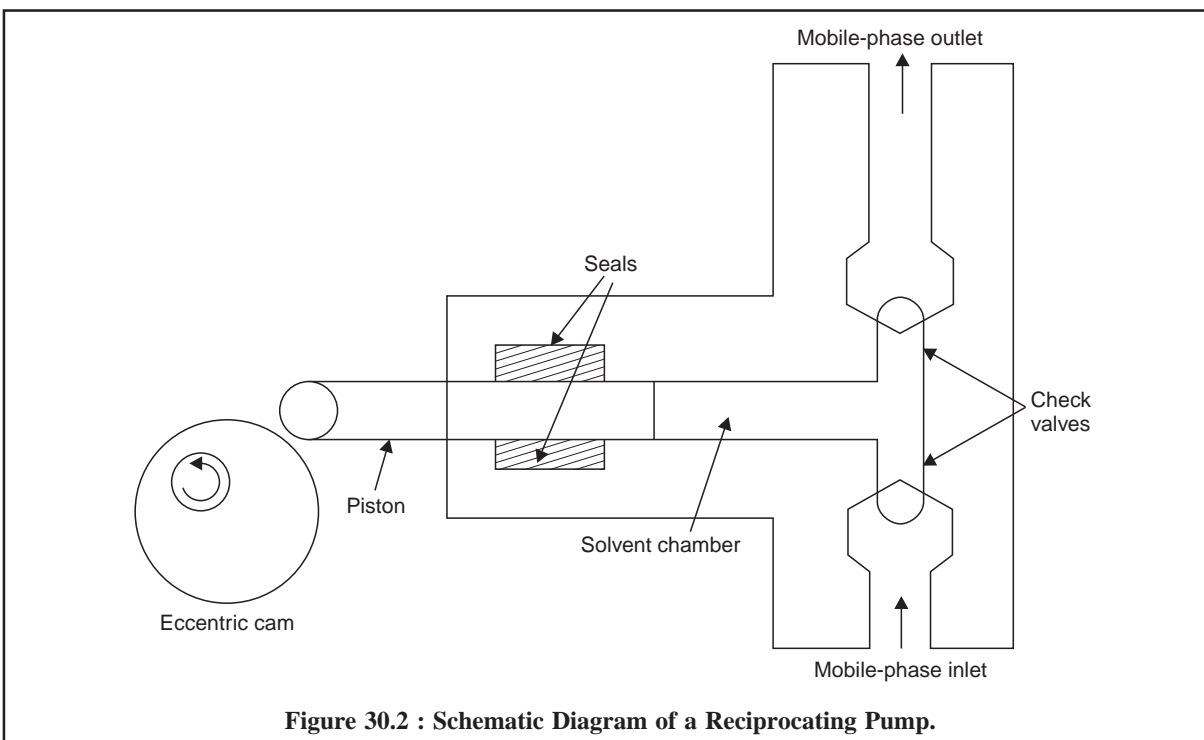
The use of twin-head reciprocating pump (*i.e.*, having the two heads operated 180° out of phase) functions in such a manner that while one head is pumping, the other is refilling as could be seen in Figure 30.3.

Figure 30.3 : Working of A Twin-Head Reciprocating Pump.
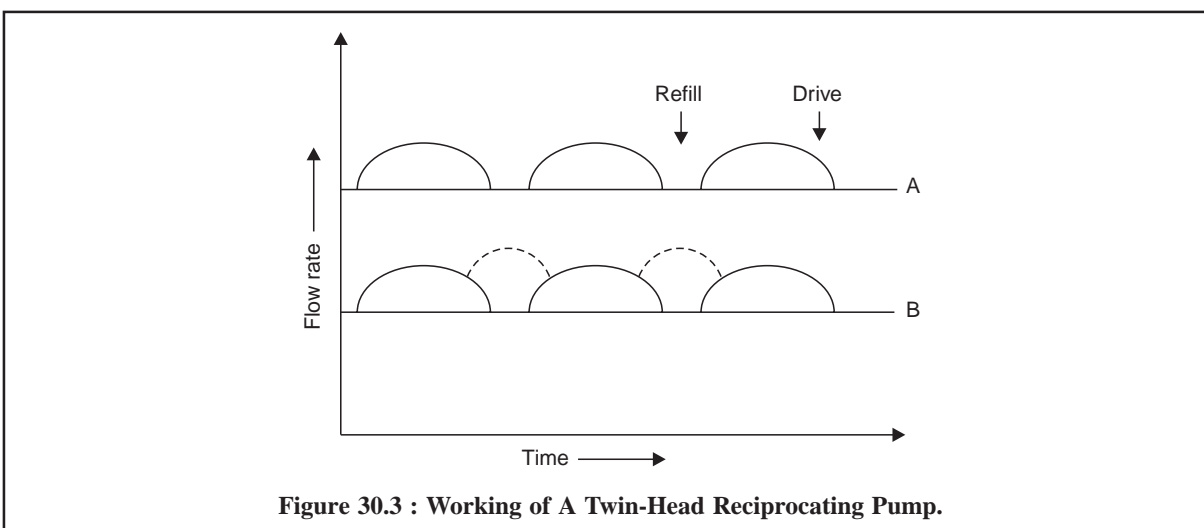
In Figure 30.3, the flow rate of a twin-head reciprocal pump has been plotted against time. The stage-A depicts the drive while the refill zone is vacant ; while the stage-B evidently shows the two-heads functioning simultaneously thereby the drive and the refill both zones could be visualized.

Sample Injection System : There are in all *three* different modes of sample injection system that are used in HPLC, namely :

(*a*) **Septum Injectors :** They usually permit the introduction of the sample by a high pressure syringe through a self-sealing elastometer septum. The major drawback associated with this type of injectors is **'leaching effect'** of the mobile-phase just in contact with the septum, thereby resulting in the formation of **'ghost peaks'** or **'pseudo peaks'.** In short, in HPLC the mode of syringe injection brings about more problems than in GC.

(*b*) **Stop-flow Septumless Injection :** Here, most of the problems associated with septum-injectors have been duly eliminated. The flow of the mobile-phase through the column is stopped for a while, and when the column reaches an ambient pressure the top of the column is opened and the sample introduced at the top of the packing.

The first two methods are relatively very cheap.

(*c*) **Microvolume Sampling Valves :** Highly sophisticated modern HPLC frequently make use of microvolume sampling valves for injection which not only give fairly good precision, but also are adaptable for automatic injection. These valves enable samples to be introduced reproducibly into pressurized columns without causing the least interruption of the mobile-phase flow.

Figure 30.4, displays the operation of a sample loop in two different modes *i.e.,* (*a*) sampling mode and (*b*) injection mode. Here, the sample is loaded at atmospheric pressure into an external loop in the microvolume-sampling valve, and subsequently injected into the mobile-phase by a suitable rotation to the valve. However, the volume of sample introduced usually ranges between 2 μl to over 100 μl ; but can be varied either by altering the volume of the sample loop or by employing specific variable-volume sample valves.

Figure 30.4 : Microvolume Sampling Valve : Operation of a Sample Loop.
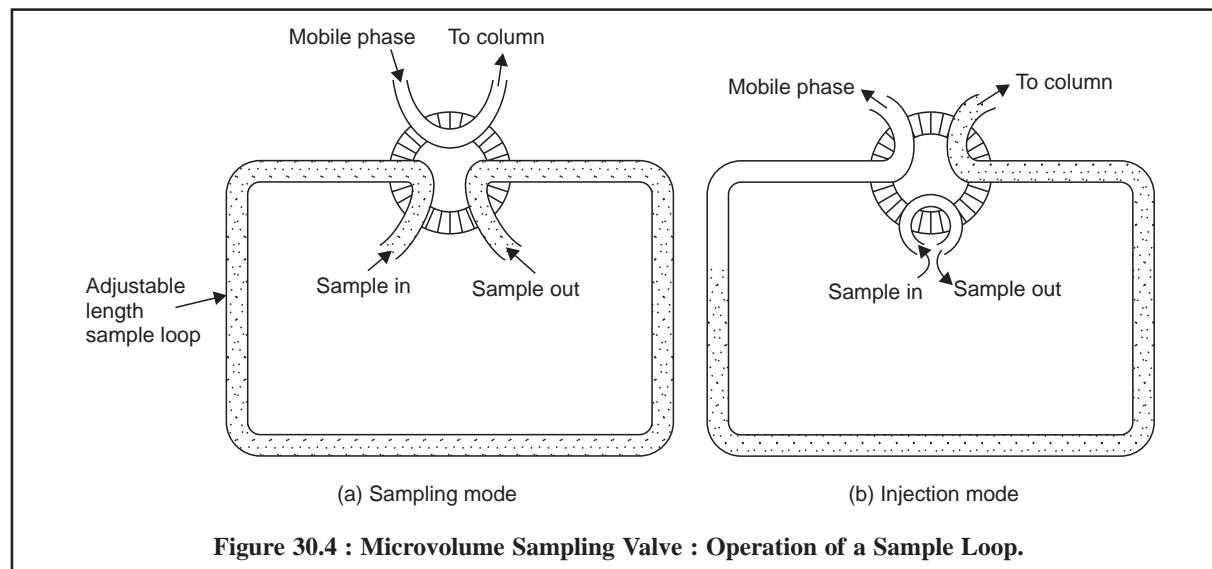
Therefore, it is always preferred for most quantitative work by virtue of its very high degree of precision and accuracy.

30.3.4. COLUMNS

(*a*) **Dimensions and Fillings :** Following are the various dimensions of HPLC columns :

|	
Material	: Stainless-steel (highly polished surface)
External Diameter	: 6.35 mm (or ≡ 0.25 inch),
Internal Diameter	: 4-5 mm (usual : 4.6 mm), and
Length	: 10-3 cm (usual : 25 cm).

(b) **Fittings :** Each end of the column is adequately fitted with a stainless-steel gauze or frit with a mesh of 2 μm or less so as to retain the packing material (usually having a particle diameter 10, 5 and 3 μm).

A stainless-steel-reducing union for a column of ID 4.6 mm type makes use of a 1/4-1/6 inch union with a short length of 0.25 mm (or 0.01 inch) ID ptfe tube so as to connect the column to the detector.

In actual practice, three conventional reducing unions available are employed, namely :

(i) Large Dead Volume (LDV)) Union   : Loss of efficiency,

(ii) Zero Dead Volume (ZDV) Union   : Loss of efficiency, and

(iii) Low Dead Volume (LDV) Union   : Most efficient, most expensive, and dead-volume 0.1 μl.

Figure 3.5 depicts the diagram of a typical LDV-Union having a SS-frit of 2 μm and a ptfe tubing of 1/6 inch.

Figure 30.5 : LDV-Union.
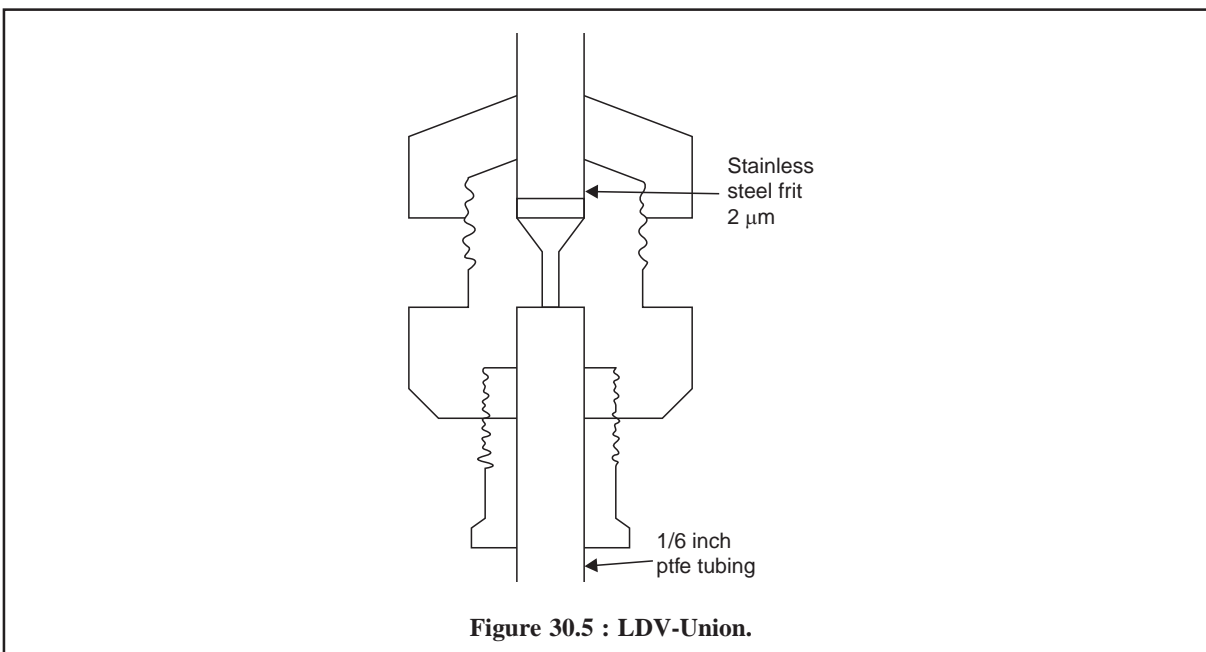
(ii) **Performance :** Inside a column the concentration of a band of solute decreases as it moves through the system. The column performance or the efficiency of a column entirely depends on the amount of spreading that takes place. The measurement is represented in Figure 30.6, below :

Figure 30.6 : Measurement of Column Performance.
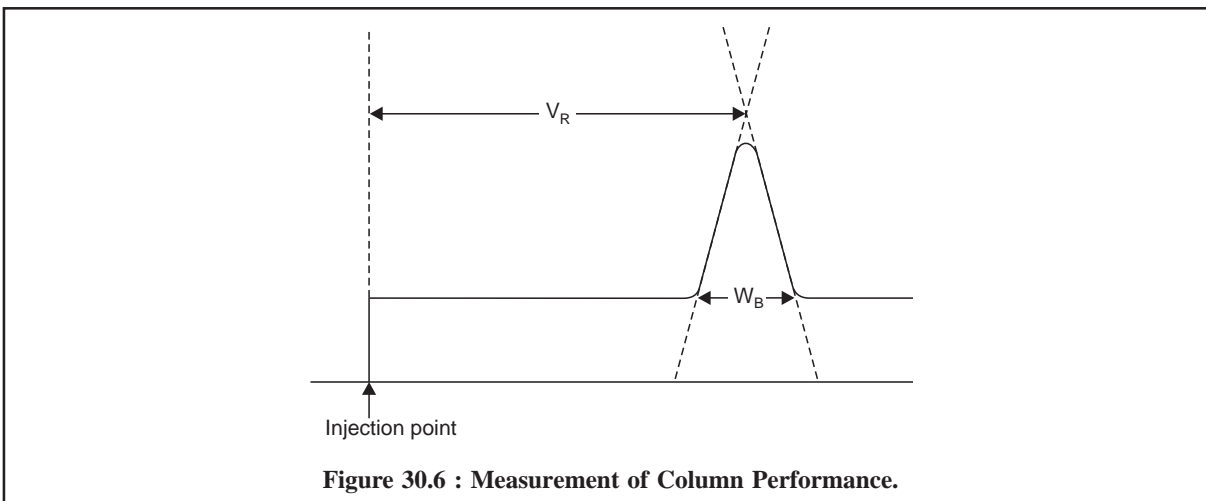
The efficiency or performance of a column may be measured by the following expression :

$$N = 16(V_R/W_B)^2 \qquad\qquad ...(a)$$

or $\qquad\qquad H = L/N \qquad\qquad ...(b)$

V_R = Retention volume of a solute,

W_R = Volume occupied by a solute (or **'Peak-Width'**). Evidently, for a more efficient column, W_B will be smaller at a given value of V_R,

N = Plate number of the column (dimensionless),

H = Plate height of the column (mm $\times$ μm), and

L = Length of the column (cm).

Based on Eq. (b) one may clearly observe that for a more efficient column 'N' gets larger and correspondingly 'H' gets smaller.

(iii) **Types of Packing :** Modern HPLC makes use of packing which essentially consist of small and rigid particles with a very narrow particle size distribution. Broadly speaking three types of packing are invariably used in HPLC column, namely :

(a) **Styrene-divinylbenzene copolymers** based porous polymeric beads have been employed exclusively for size-exclusion and ion-exchange chromatography, but now mostly been replaced by silica-based packings that proved to be more efficient and mechanically stable.

(b) **Porous-layer beads** with a diameter ranging between 30-35 μm comprising of a thin shell (1-3 μm) of silicon or modified silica, on an inert spherical core material, such as : glass beads are still being employed for certain ion-exchange procedures ; but of late their usage as such in HPLC have been superseded by 100% porous microparticulate packings, and

(c) **Porous-silica particles** (100%) with a diameter less than 1 μm and narrow-particle size range, nowadays, form the basis of most abundantly available important column packings used in analytical HPLC. In comparison to the porous-layer beads, as detailed in (b) above, the porous-silica particles yield significant improvements not only in column efficiency but also in sample capacity and speed of analysis.

30.3.5. DETECTORS

The main function of the detector in HPLC is to monitor the mobile-phase coming out of the column, which in turn emits electrical signals that are directly proportional to the characteristics either of the solute or the mobile-phase.

The various detectors often used in HPLC may be categorized into *three* major heads, namely :

(i) **Bulk-property detectors :** They specifically measure the difference in some physical property of the solute present in the mobile-phase in comparison to the individual mobile-phase, for instance :

(a) Refractive-index detectors, and

(b) Conductivity detectors.

(ii) **Solute-property detectors.** They critically respond to a particular physical or chemical characteristic of the solute (in question), which should be ideally and absolutely independent of the mobile-phase being used. But complete independence of the mobile-phase is hardly to be seen, however, signal discrimination is good enough to enable distinctly measurable experimental procedures with solvent changes, such as : gradient-elution.

The solute-property detectors include :

(a) UV-detectors, and

(b) Fluorescence Detectors.

(*iii*) **Multipurpose detectors :** Besides, providing a high degree of sensitivity* together with a broad-linear-response-attainable range, invariably a particular situation critically demands detectors of more selective nature in the domain of 'analytical chemistry' *vis-a-vis* 'Pharmaceutical Analysis' that could be accomplished by using 'multipurpose detectors', such as : **"Perkin-Elmer '3D' System"** that combines UV absorption, fluorescence and conductometric detection.

(*iv*) **Electrochemical detectors : 'Electrochemical detector'** in HPLC usually refers to either amperometric or coulometric detectors, that specifically measure the current associated with the reduction or oxidation of solutes. As only a narrow spectrum of compounds undergo electrochemical oxidation, such detectors are quite selective ; and this selectivity may be further enhanced by monitoring the potential applied to the detector so as to differentiate between various electroactive species. Naturally, electrochemical detection essentially makes use of conducting mobile phases, for instance : inorganic salts or mixtures of water with water-miscible organic solvents.

The **five important types of detectors** shall be discussed along with their simple diagrammatic sketches, in the sections that follow :

30.3.5.1. UV-Detectors

Principle : An UV-detector is based on the principle of absorption of UV visible light from the effluent emerging out of the column and passed though a photocell placed in the radiation beam.

Figure 30.7 represents the schematic diagram of a double-beam UV detector used in HPLC system. Initially, dual-wavelength instruments having 254 and/or 280 nm were introduced which is presently being replaced by more sophisticated and up-dated variable wavelength detectors spread over wide range between 210-800 nm capable of performing more selective detection possible.

Figure 30.7 : Schematic Diagram of a Double-Beam UV Detector.
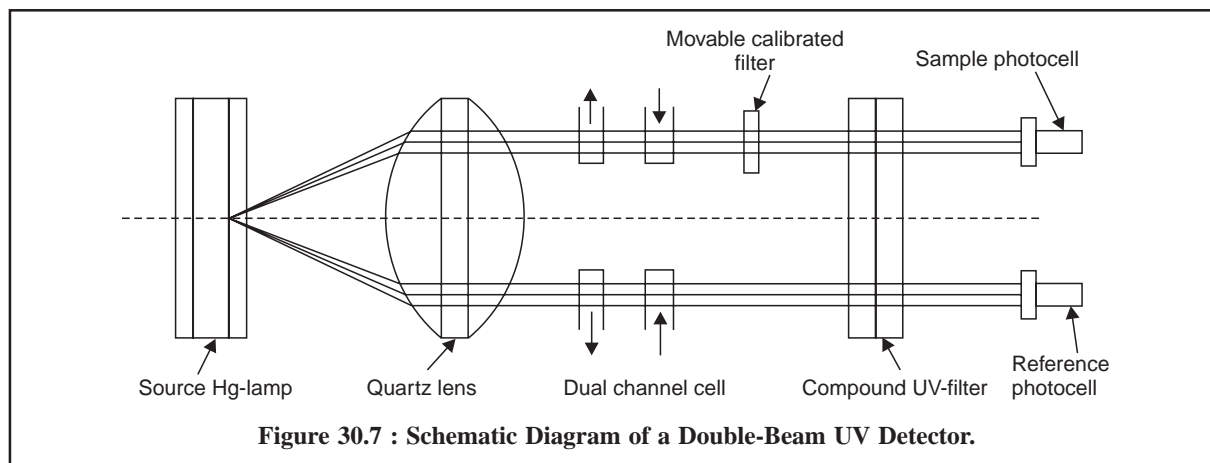
Diode Array Detector (or **Multichannel Detector**) is also a UV detector wherein a polychromatic light is made to pass through the flow cell. A strategically placed grating diffracts the outcoming radiation and subsequently meets an array of photodiodes whereby each photodiode receives a different narrow wavelength band. Here, a microprocessor scans the array of diodes several times in one second and the resulting spectrum is visualized on the screen of a VDU or subsequently stored in the instrument for a printout as and when required. Another extremely important and useful characteristic feature of a diode-array detector is that it may be 'programmed' so as to affect changes in the detection wavelength at particular points in the chromatogram. This versatile criterion is used to **'clean up'** a chromatogram *i.e.,* to discard all interfering peaks caused due to components irrelevantly present in the sample.

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*　High Sensitivity of about 1 in 10^9 being attainable using UV and fluorescence detectors.

Advantages : Various advantages are, namely :

(*a*) A very selective detector which will detect only such solutes that specifically absorb UV/visible radiation *e.g.*, alkenes, aromatics and compounds having multiple bonds between C, O, N and S.

(*b*) The mobile-phase* employed ideally must not absorb any radiation.

30.3.5.2. Fluorescence Detector

A plethora of compounds (solutes) present in the mobile-phase on being passed as column effluent through a cell irradiated with Xenon or Deuterium source first absorb UV radiation and subsequently emit-radiation of a longer wavelength in two different manners, namely :

(*a*) Instantly-termed as **'Fluorescence',** and

(*b*) After a time-gap-known as **'Phosphorescence'**.

Fluorescent compounds : A relatively small proportion of inorganic and organic compounds exhibit natural fluorescence, whereas a larger number of pharmaceutical substances and environmental contaminants [*e.g.*, polycyclic aromatic hydrocarbons (PAH)] having a conjugated-cyclic system are fluorescent. Such compounds having absorbed energy being re-emitted from 0.1-1.0 can be detected by a fluorescence detector. However, non-fluorescent compounds can be converted to fluorescent derivatives by treatment with appropriate solvents.

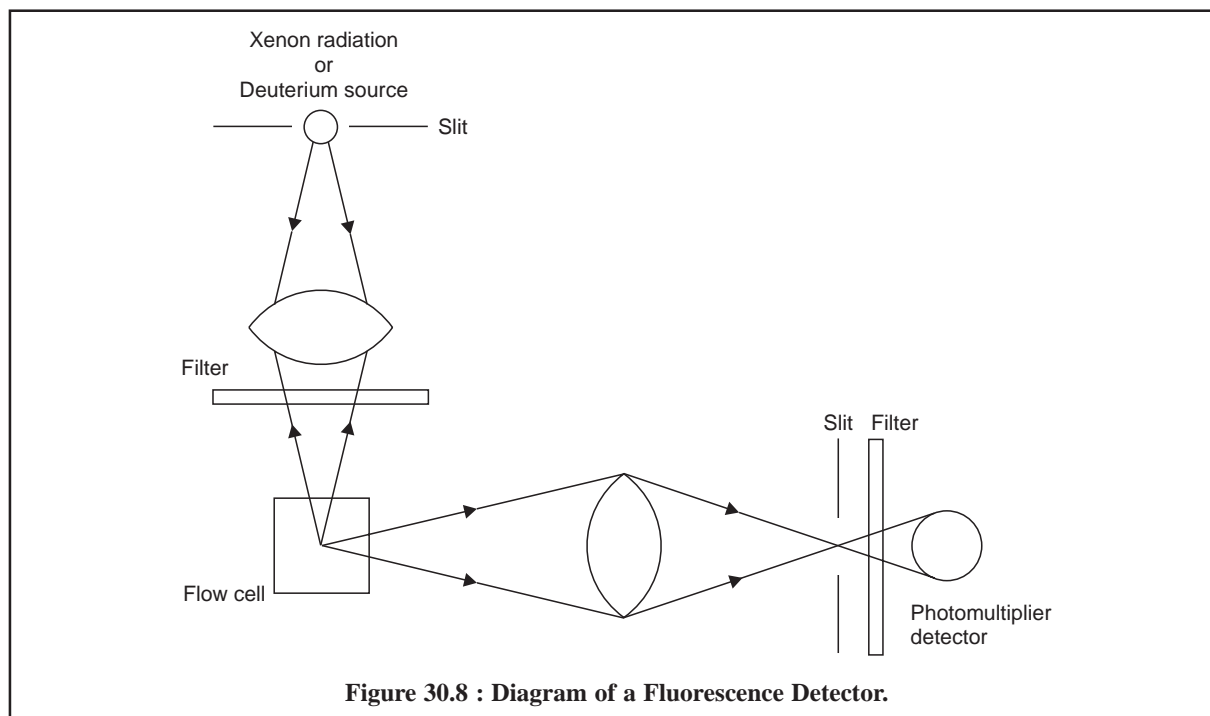Figure 30.8, illustrates the diagram of a fluorescence detector :

Figure 30.8 : Diagram of a Fluorescence Detector.

Radiation from a Xenon-radiation or a Deuterium-source is focussed on the flow cell through a filter. The fluorescent radiation emitted by the sample is usually measured at 90° to the incident beam. The second filter picks up a suitable wavelength and avoids all scattered light to reach ultimately the photomultiplier detector.

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*   Always use only HPLC-Grade solvents for better precision and reproducibility of results.

30.3.5.3. Refractive Index Detector

It is also known as **'RI-Detector'** and **'Refractmeter'.** Figure 30.9, represents the block-diagram of a refractive-index detector.
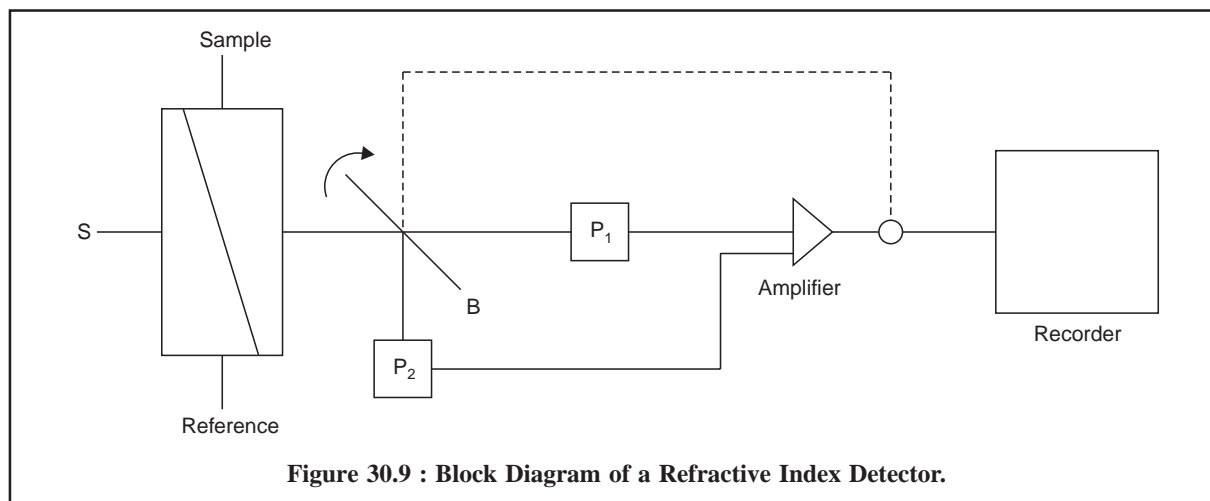

Figure 30.9 : Block Diagram of a Refractive Index Detector.

Light from the source(s) is focused into the cell, that consists of sample and reference sample ; and the two chambers are separated by a diagonal sheet of glass. After passing through the cell, the light is diverted by a beam-splitter (B) to two photocells (P_1 and P_2 respectively. A change in the observed refractive index (RI) of the sample stream causes a difference in their relative output, which is adequately amplified and recorded duly.

The RI of a few commonly used mobile-phase is stated below :

| Mobile-Phase	Refractive-Index
Benzene	1.501
Decane	1.410
Hexane	1.375
Octane	1.397
Tetrayhydrofuran	1.405

Any solute can be detected as long as there exists a measurable difference in refractive index between the solute and the mobile-phase.

30.3.5.4. Multipurpose Detector

A multipurpose detector essentially comprises of three detectors combined and housed together in a single unit. A typical example of such a detector is the one developed by Perkin-Elmer known as **"Perkin-Elmer '3D' System"** which is depicted in Figure 30.10.

The functions of the three different detectors used in Figure 30.10 are enumerated as under :

 (*i*) **Fluorescence Function :** It can monitor emission above 280 nm, based on excitation at 254 nm,

 (*ii*) **UV-Function :** It is fixed wavelength 254 nm detector, and

 (*iii*) **Conductance-Function :** The metal inlet and outlet tubes serve as electrodes to measure the conductance of the ions.

Figure 30.10 : Block Diagram of a Multipurpose Detector.
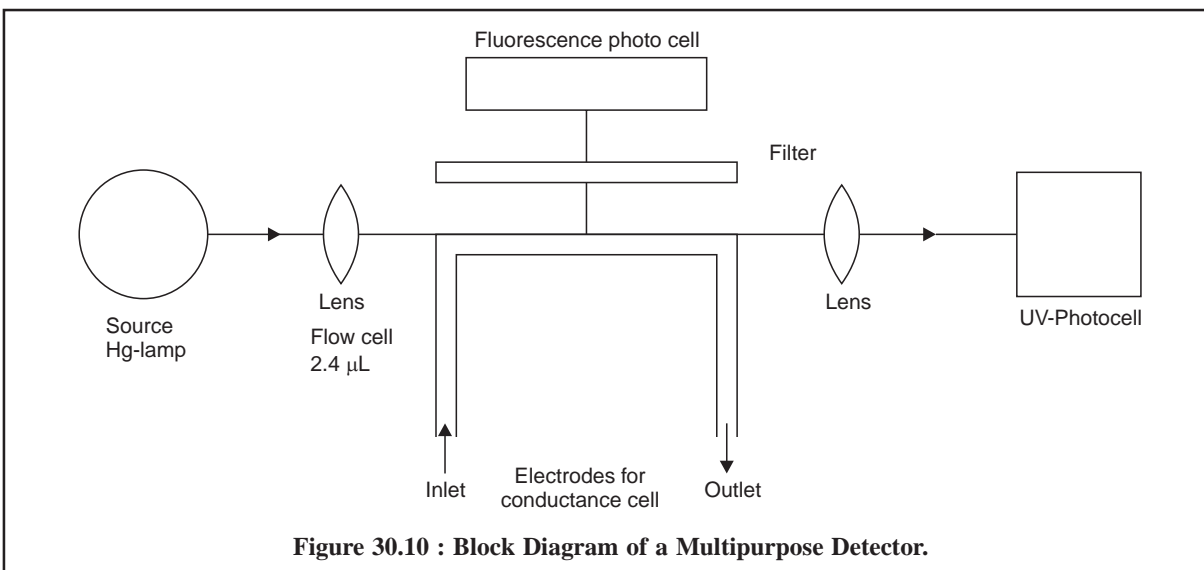
30.3.5.5. Electrochemical Detectors

In actual practice, however, it is rather difficult to utilize the functions of electrochemical reduction as a means of detection of HPLC by virtue of the fact that the serious interference (*i.e.*, large background current) generated by reduction of oxygen in the mobile phase. As complete removal of oxygen is almost difficult, therefore, electrochemical detection is normally based upon the oxidation of the solute.

Examples : The various compounds that may be detected conveniently are, namely : aromatic amines, phenols, ketones, and aldehydes and heterocyclic nitrogen compounds.

In short, the **amperometric detector** is presently considered to be the best electrochemical detector having the following distinct advantages, such as :

(*i*) very small internal cell-volume,

(*ii*) high degree of sensitivity,

(*iii*) more limited range of applications, and

(*iv*) excellent for trace analyses as UV-detector lacks adequate sensitivity.

Table 30.8, provides a comprehensive comparison of various typical detector characteristics invariably used in HPLC, such as : response, concentration expressed in g ml^{-1} and the linear range. However, the linear range usually refers to the range over which the response is essentially linear. It is mostly expressed as the factor by which the lowest factor (*i.e.*, Cn) should be multiplied in order to obtain the highest concentration.

Table 30.2 : Pattern of Typical Detector Characteristics in HPLC

S. No.	Type	Response	Cn (g ml^{-1})	Linear Range
1.	Amperometric	Selective	10^{-10}	10^4-10^5
2.	Conductometric	-do-	10^{-7}	10^3-10^4
3.	Fluorescence	-do-	10^{-12}	10^3-10^4
4.	UV/Visible Absorption	-do-	10^{-8}	10^4-10^5
5.	Refractive Index	Universal	10^{-6}	10^3-10^4

30.3.6. STRIP CHART RECORDER

The signal emerging from the detector of a HPLC is recorded continuously as function of time most commonly with the help of a potentiometric recorder. Invariably, a recorder of 1 to 10 mV full-scale deflec-

tion over a stretch of approximately ten inches and having a response-time of one second or even less is regarded as most appropriate. Strip-chart recorder with variable chart speeds ranging between 5 to 5 mm min^{-1} are usually preferred.

The input signal of a potentiometric-recorder is balanced continuously with the help of a feedback signal arrangement (device) using a *servomechanism*. A pen attached to this device moves proportionately, with preadjusted attenuation, along the width of the chart-paper thereby recording the signal accurately, while the chart-paper moves at a fixed speed along the length.

It is pertinent to mention here that before commencing the operation of a recorder, its zero point must be adjusted with the input zero, otherwise the baseline will also shift with slight changes in the attenuation of the signal.

Besides, it is also equally important to adjust properly the amplifier gain so as to eliminate completely the dead-band and the oscillations. A recorder having inadequate shielding from the AC circuits may display shifting of its zero point.

30.3.7. DATA HANDLING DEVICE AND MICROPROCESSOR CONTROL

Modern HPLC is adequately provided with complete data handling devices. Thousands of samples routinely analysed in Quality Assurance Laboratories in Pharmaceutical Industries/Bulk Drug Industries etc. are duly processed and the data stored in the computerised data-handling devices. Each stored data may be retrieved from the memory of the computerised device with the flick of a finger, as and when needed, in the form of print-out.

Microprocessor based analytical equipments is no longer an uncommon phenomenon towards the modernization, automation, and above all the ease of function and handling of sophisticated devices, for instance : a microprocessor scans the array of diodes many times a second in a **'diode array detector'** ; a microprocessor does the temperature programming of a constant temperature chamber of HPLC unit.

30.4. DERIVATIZATION

The main purpose of derivatization in HPLC is to improve detection specifically when determining traces of solutes in complex matrices, for example :

 (*i*) Pharmaceutical substances lacking an UV-chromophore in the 254 nm region but possessing a reactive functional group,

 (*ii*) Biological fluids *e.g.*, blood, serum, urine ; cerebrospinal fluid (CSF); and

 (*iii*) Environmental samples.

 Derivatization may be accomplished by *two* means, namely :

 (*a*) Pre-column off-line derivatization.

 (*b*) Post-column on-line derivatization.

 These two methods shall be discussed briefly at this juncture :

30.4.1. PRE-COLUMN OFF-LINE DERIVATIZATION

Merits : This technique has the following merits :

(*a*) Requires no modification to the instrument *i.e.*, a plus point when compared to the post-column methods, and

(*b*) Imposes fewer limitations with regard to reaction-time and conditions.

Demerits : The demerits include :

(*a*) Formation of a stable and well-defined product is an absolute necessity,

(*b*) Presence of excess reagent or by products may invariably interfere with separation, and

(*c*) Very often derivatization may altogether change the chromatographic properties of the sample which facilitated separation.

30.4.2. POST-COLUMN ON-LINE DERIVATIZATION

The following experimental parameters should be maintained, namely :

(*a*) Derivatization performed in a special-reactor strategically positioned between the column and the detector,

(*b*) Reaction must be completed rapidly at moderate temperatures,

(*c*) Derivatization reaction need not even go to completion provided it can be made reproducible,

(*d*) No detector-response should exist due to any excess reagent present, and

(*e*) Reaction must be carried out in a medium other than the mobile-phase.

Merit : The main merit of post-column-on-line derivatization is that ideally the separation and detection processes can be optimized individually.

30.4.3. REAGENTS FOR DERIVATIZATION

There are potentially viable reagents available that may be employed for the derivatization of compounds either for enhancing UV/visible radiation (called **chromatags**) or for reaction of non-fluorescent reagent molecules (called **fluorotags**) with solutes to yield fluorescent derivatives.

Examples : (*i*) **Derivatization for UV-Detectors :**

Ninhydrin (a **chromatag** is commonly employed to yield corresponding derivatives of amino acids that show absorption specifically at about 570 nm as shown in the following reaction :

Ninhydrin   'Blue-coloured complex'
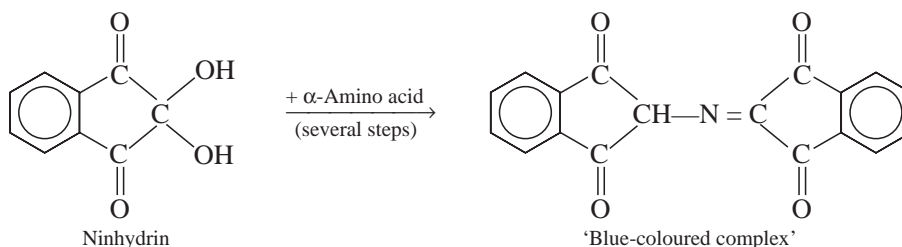
(*ii*) **Derivatization for Fluorescence Detectors :**

Dansyl Chloride (a **fluorotag**) is invariably used to obtain fluorescent derivatives of proteins, amines and phenolic compounds, the excitation and emission wavelengths being 335 to 365 nm and 520 nm respectively.

Densyl chloride
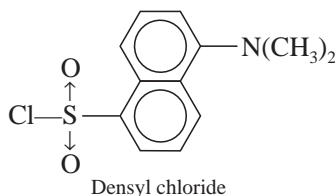
Some other important **'Fluorotags'** are stated below :

S. No.	Reagent	Reacting Functional Groups
1	Ce^4-salts + H_2SO_4	Dicarboxylic acids
2	Fluorescamine [Fluram[R]]	1°-Amines
3	*o*-Phthalaldehyde [Fluoropa[R]]	1°-Amines

30.5. APPLICATIONS OF HPLC IN PHARMACEUTICAL ANALYSIS

Modern HPLC finds its abundant applications not only confined to isolation of natural pharmaceutically active compounds, control of microbiological processes but also assay of pure drugs and their dosage forms. A few typical examples will be discussed below :

30.5.1. ISOLATION OF NATURAL PHARMACEUTICALLY ACTIVE COMPOUNDS

Some plant alkaloids and glycosides can be isolated as stated below :

Category of Natural Products	Constituents	Used as
Alkaloids	Morphine; Codeine	Analgesic, Antitussive
Glycoside	Digitalis glycosides Sennosides	Cardiovascular diseases, Laxatives

Chromatographic Conditions :

Column   : Size-25 cm × 4.6 mm ID ;

Adsorbent   : Lichrosorb RP-8 ;

Mobile-phase : Water/Acetonitrile-Gradient Elution ;

Detector   : UV 254 nm
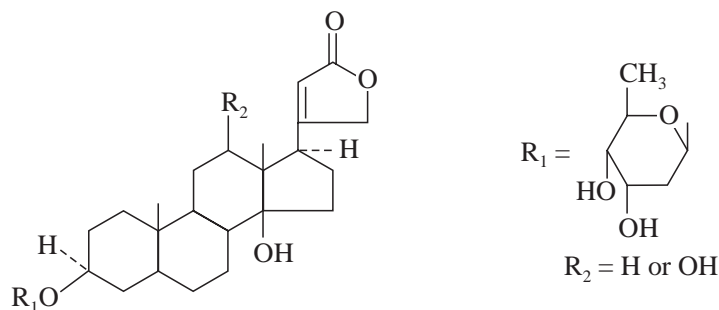
Digoxin : $R_2 = OH$; R_1 = as above ;

Digitoxin : $R_2 = H$; R_1 = as above ;

30.5.2. CONTROL OF MICROBIOLOGICAL PROCESSES

Various microbiological processes are used in the production of a number of antibiotics, for instance : **penicillins**, **tetracyclines**, **chloramphenicol** and **streptomycins**. The major areas of such operations being :

- kinetics of the microbiological process,
- monitoring of the on-going process,
- isolation and purification of active ingredients,
- purity control of active constituents, and
- monitoring derivatization reactions of these compounds.

HPLC-controlled analysis of a microbiological process during Penicillin Production : Chromatographic conditions are as follows :

Column   : Size-25 cm × 4.6 mm ID ;

Adsorbent   : Lichrosorb-NH$_2^{(R)}$ (10 μm) ;

Mobile-phase : 0.005 M H$_2$SO$_4$ buffer (pH 4.4))/acetonitrile (50 : 50) ; Flow rate : 3 ml min^{-1} ;

Detector   : UV-220 nm ;

Microbial cleavage of Penicillin-G into 6-AMP and phenylacetate is as shown below :
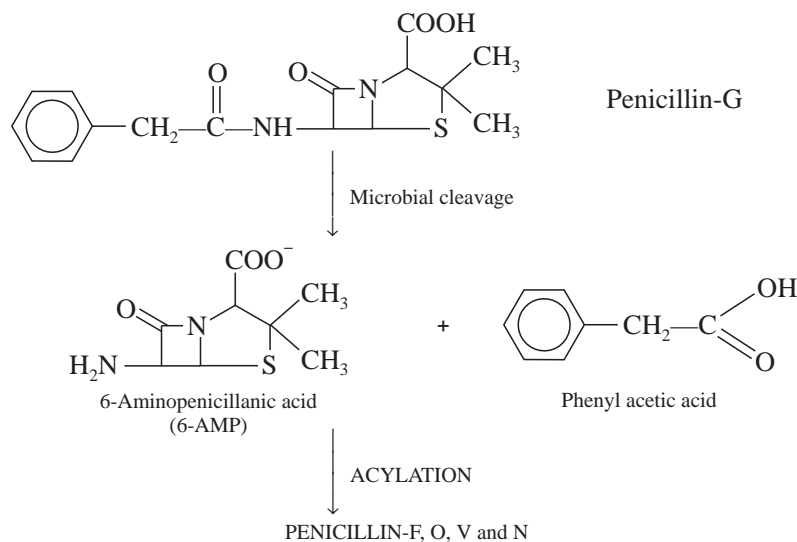
Figure : 11 (*a*) and (*b*) shows the HPLC chromatograms of penicillins in **'production sample'** and **'standard solution'** respectively :

Figure 30.11 (*a*) and (*b*) : HPLC Chromatograms of Penicillins In 'Production Sample' and 'Standard Solution'.
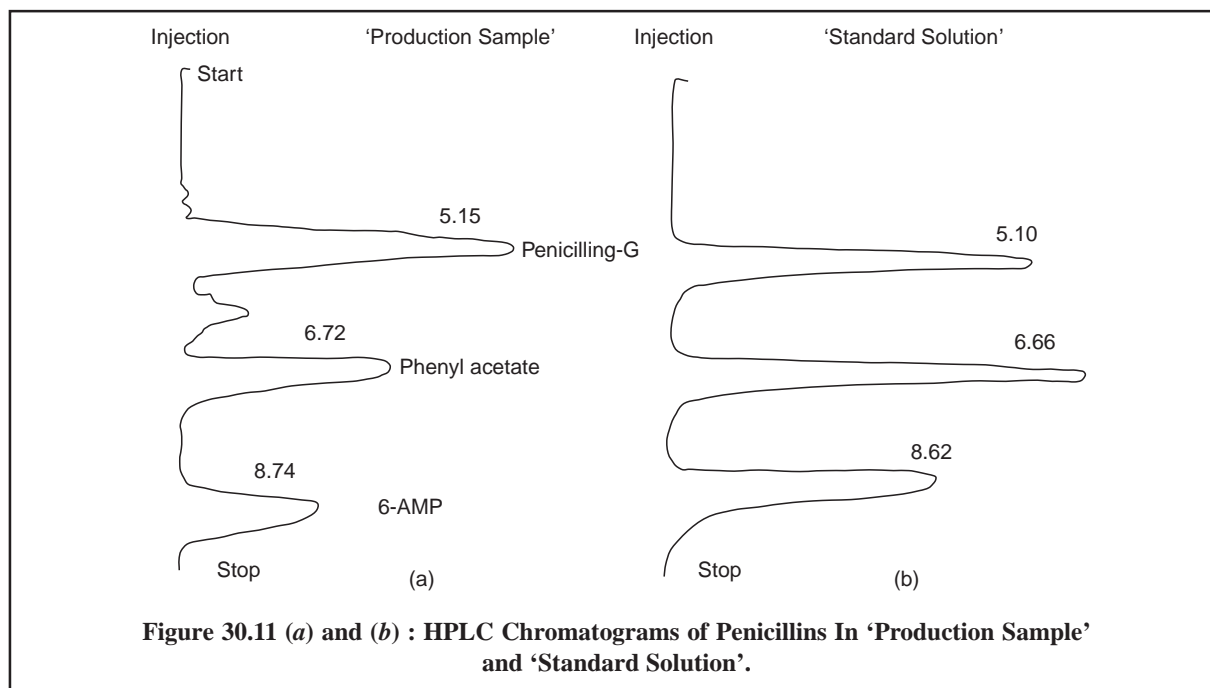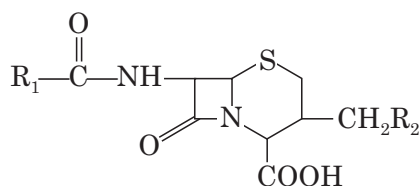
30.5.3. ASSAY OF CEPHALOSPORINS

Several commercially available cephalosporin antibiotics have been adequately separated by HPLC methods under the following experimental parameters
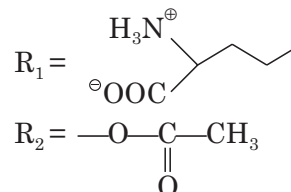
    Column   :   ODS-SIL-X-II,

    Mobile-phase :   0.95 M Ammonium Carbonate/Methanol (95 : 5) ;

Detector : UV-220 nm ;

Cephalosporin C :

The loss of absorption with cleavage of the β-lactam was used by Marrelli* to analyze the concentrations of cephalosporin C in the presence of other UV absorbing species.

30.5.4. ASSAY OF FRUSEMIDE**

Theory : HPLC analysis of frusemide and its decomposition products is carried out by using simultaneous fluorescence and UV detection.
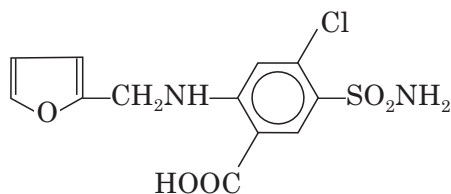
4-Chloro-N-furfuryl-5-sulphamoylanthranilic acid

It is noteworthy that fluorescence detection is a very specific technique, especially when excitation and emission wavelengths can be selected. In addition to this, sensitivity for compounds with photoluminescence properties can be higher by factors of 100 to 1000 when compared with that of other detectors.

The chromatographic conditions for Frusemide determination are as stated below :

Column : Size : 250 × 4.6 mm ID ;

Adsorbent : Lichrosorb$^{(R)}$ RP-8, 10 μm ;

Mobile-phase : Gradient elution-2 minutes, from 20% B to 37% B in 15 minutes, where, A = Water (pH 2.7) and B = Acetonitrile ;

Detector : (*i*) Fluorescence : Excitation : 275 nm ; Emission : above 405 nm ;

Figure : 12 (*a*) and (*b*) depict the HPLC chromatograms of frusemide by fluorescence and UV detectors respectively.

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* Flynn, EH, Ed., **'Cephalosporins and Penicillins',** Academic Press, New York, 1972.

** The name of this diuretic in the **Eur. Ph.** is **'furosemide'.**

Figure 30.12 : HPLC-Chromatogram of Frusemide (a) Fluorescence Detector ; (b) UV-Detector.
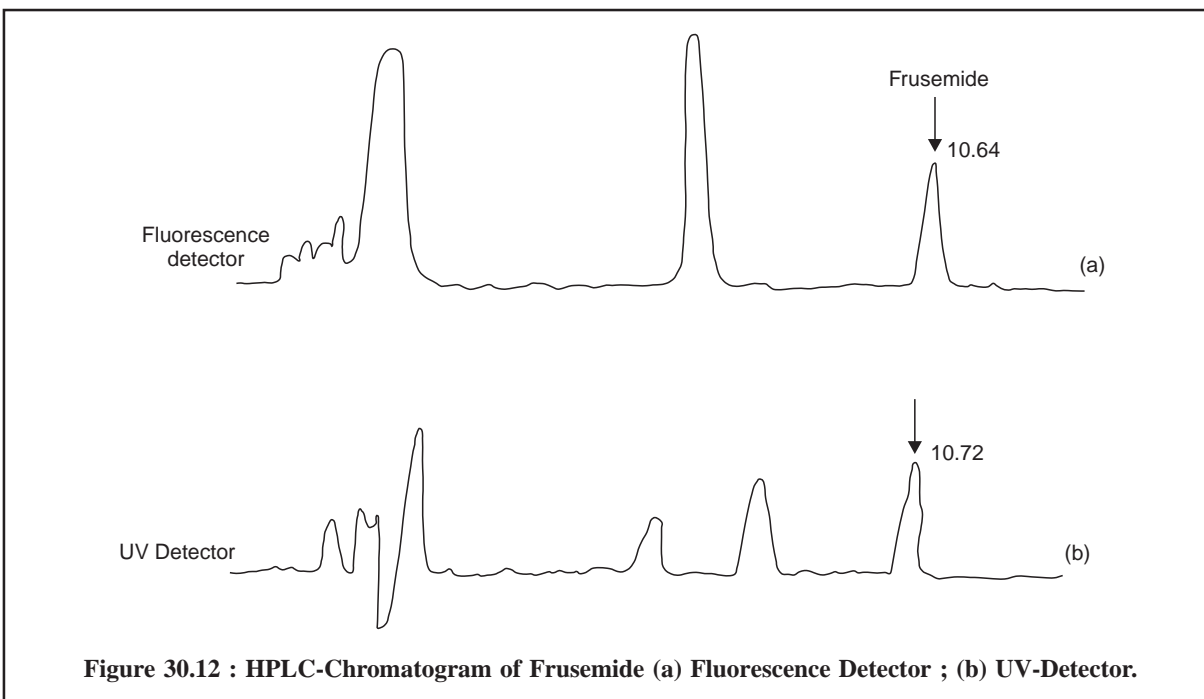
30.5.5. ASSAY OF THEOPHYLLINE

Theophylline invariably contains other related substances as impurities, namely : theobromine, caffeine and β-hydroxypropyltheophylline.
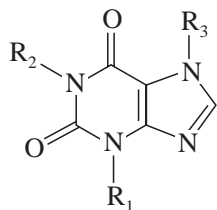
Caffeine : $R_1 = R_2 = R_3 = —CH_3$;

Theophylline : $R_1 = R_2 = —CH_3$; $R_3 = —H$;

Theobromine : $R_2 = —H$; $R_1 = R_2 = —CH_3$;

β-Hydroxypropyltheophylline : $R_1 = R_2 = —CH_3$; and

$$R_3 = —CH_2—CH—CH_3 ;$$
$$\quad\quad\quad | $$
$$\quad\quad\quad OH$$
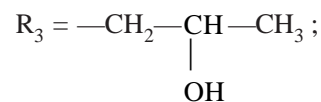
The chromatographic conditions for HPLC are as stated below :

Sample size : 10 μL ;

Column : size – 250 × 4.6 mm ID ;

Adsorbent : Lichrosorb (R) RP-8, 10 μm ;

Mobile-phase : 0.02 M KH_2PO_4 Buffer (pH 3.5)/Acetonitrile (95 : 5) ;

Detector : UV-254 nm ;

Figure 30.13, illustrates the HPLC chromatogram of **theophylline** assay with *four* distinct peaks.

Injection
Start
8.98 Theobromine
3.24 Theophyline
Caffeine
8.20 β-Hydroxypropyl theophyline
Finish

Figure 30.13 : HPLC Chromatogram of Theophylline.
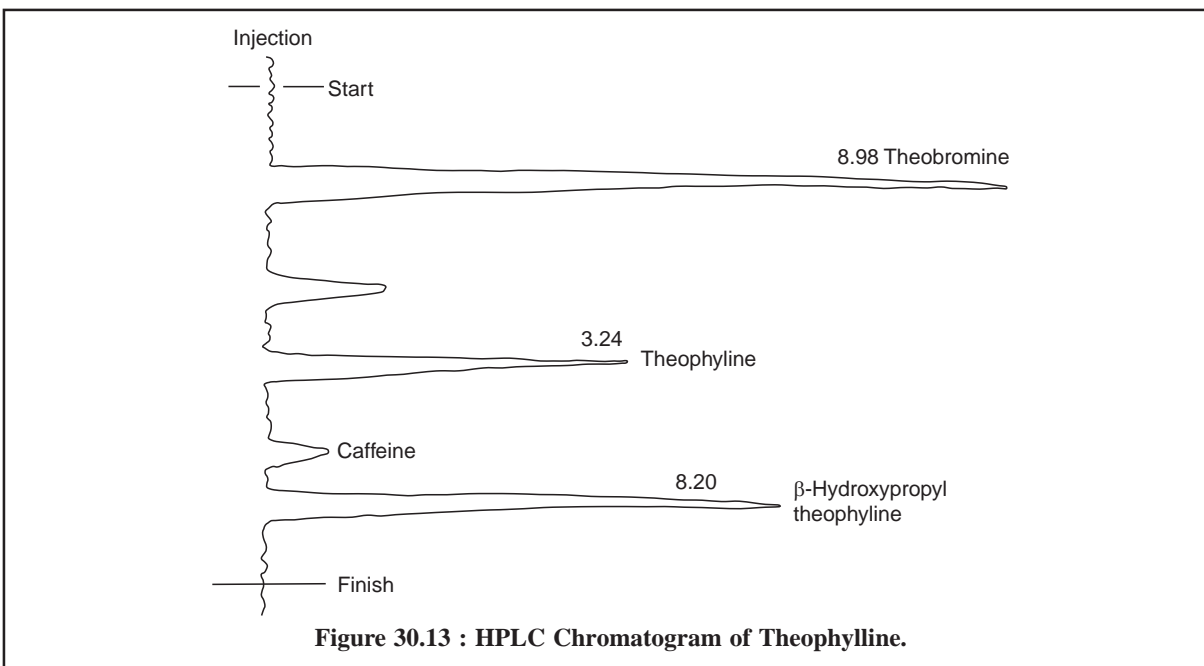
30.5.6. ASSAY OF CORTICOSTEROIDS

A mixture of **six corticosteroids**, namely : *deoxycortisone*, *hydrocortisone acetate*, *cortisone*, *prednisone*, *hydrocortisone* and *prednisolone* can be assayed by HPLC method. The chromatogrpahic parameters for the assay are as follows :

Sample size	:	10 μL
Column	:	size-250 × 4.6 mm ID ;
Adsorbent	:	Lichrosorb$^{(R)}$ DIOL : 10 μm ;
Mobile-phase	:	Gradient elution of A (*n*-Hexane) and B (Isopropanol) ;
Detector	:	UV-254 nm ;

Figure 30.14, clearly shows the six well-elaborated and distinct peaks of all the constituents stated earlier under the above HPLC parameters.

Injection
Start
4.52 Deoxycortisone
18.40 Hydrocortisone acetate
13.78 Cortisone
15.41 Prednisone
19.59 Hydrocortisone
20.04 Prednisolone
Finish

Figure 30.14 : HPLC Chromatogram of Corticosteroids.
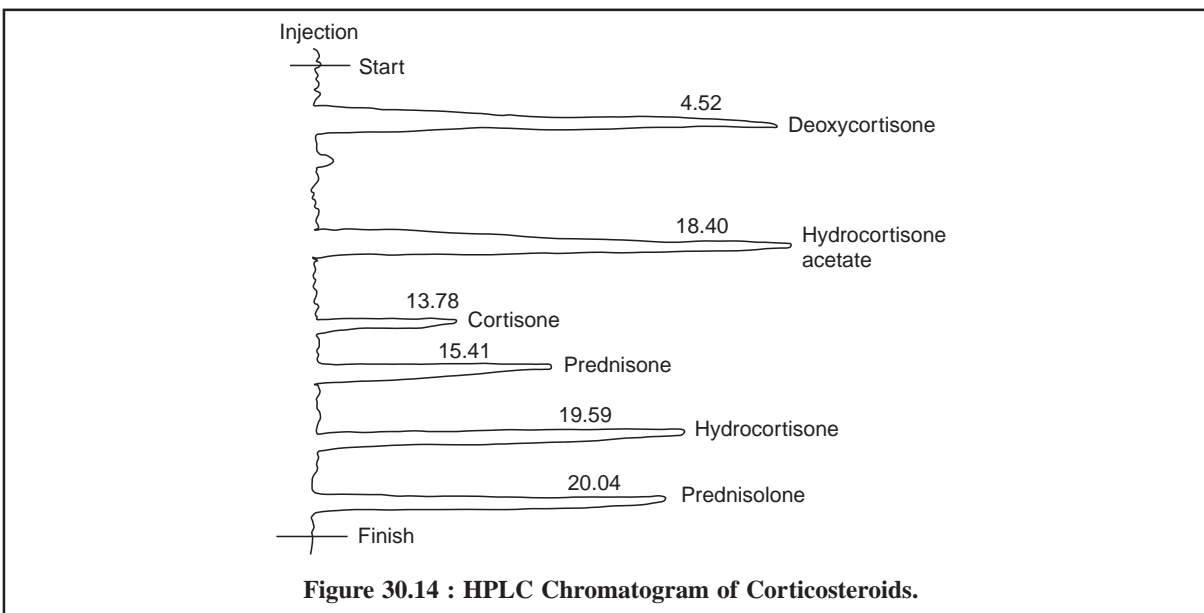
30.5.7. ASSAY OF DICHLORPHENAMIDE*

Materials Required : Dichlorphenamide sample : 100 mg ; dichlorphenamide RS : 100 mg ; Mobile-phase [solution containing 0.02 M NaH_2PO_4 and 0.2 M Na_2HPO_4 in a mixture of equal volumes of acetonitrile and water] : 50 ml ;

Procedure : The chromatographic procedure may be performed using μ **Bondapack C18 column** as the stationary phase and the above mentioned mobile-phase with a flow rate of 1.0 ml per minute and a detection wavelength of 280 nm. Carry out the HPLC analysis using solutions in the mobile-phase containing (1) 0.05% w/v of dichlorphenamide RS and (2) 0.05% w/v of dichlorphenamide sample.

Calculations : Calculate the content of $C_6H_6Cl_2N_2O_4S_2$ using the declared content of the same in dichlorphenamide RS.

30.5.8. ASSAY OF HUMAN INSULIN*

Materials Required : Solution (1) : dissolve 40 mg of Human Insulin in sufficient of 0.01 M HCl (0.3648 g of HCl in one litre DW) to produce 10 ml ; solution (2) : Mix thoroughly 900 μL of 0.01 M HCl to 100 μL of solution (1) ; Solution (3) : dissolve 40 mg of Human Insulin EPCRS in sufficient 0.01 M HCl to produce 10 ml ; Solution (4) : mix 1 ml of solution (3) with 1 ml of a solution containing 4 mg of porcine insulin RS : Mobile-phase 'A' : Dissolve 28.4 g of anhydrous Na_2SO_4 in sufficient water to produce 1000 ml, add 2.7 ml of orthophosphoric acid, adjust of pH 2.3, if necessary, with ethanolamine, filter and degas by passing He through the solution ; Mobile-phase 'B' : Mix 500 ml of mobile-phase 'A' with 500 ml of acetonitrile, filter and degas by passing He through the solution.

Procedure : The HPLC is carried out using (*a*) a **Vydac C18 column**, for proteins and peptides, maintained at 40 °C, (*b*) as the mobile phase at a flow rate of 1 ml per minute, a mixture of 48 volumes of mobile phase 'A' and 52 volumes of mobile phase 'B' prepared and maintained at a temperature of not less than 20 °C, and (*c*) a detection wavelength of 214 nm.

Adopt the following steps sequentially :

Step	Inject	Test is not valid unless
I	10 μL of soln. (4)	The resolution between the peaks corresponding to human and porcine insulin is at least 1.3. (If necessary, adjust the conc. of acetonitrile in the mobile-phase by slight decrease or increase until the required resolution is obtained.
II	Soln. (3) six times	The relative standard deviation of the area of the principal peak is at most 2%.
III	10 μL of each of solns. (1), (2) and (3)	The area of the principal peak in the chromatogram with soln. (1) is 10 ± 0.5 times the area of the principal peak in the chromatogram obtained with soln. (2).

Calculations : Calculate the content of human insulin, $C_{257}H_{383}N_{65}O_{77}S_6$, from the peak areas and using the declared content of $C_{257}H_{383}N_{65}O_{77}S_6$ in human insulin EPCRS.

30.5.9. COGNATE ASSAYS

A number of pharmaceutical substances can be assayed by HPLC method as detailed in Table 30.1 (see next page).

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* BP (1993)

Table 30.1 : Cognate Assays by HPLC Method

S. No.	Name of Substance	Stationary Phase	Mobile Phase/ Flow Rate	Solutions	Detection	Calculations
1.	Betamethasone Valerate	Spherisorb CDS 1	Abs. Ethanol and Water (42 : 58)/2 ml per minute	(1), (2) and (3)*	UV-Detector 238 nm	Based on the declared content of betamethasone valerate BPCRS ($C_{27}H_{37}FO_6$)
2.	Fluocinolone Acetonide	μ Bondapack C18	Methanol : Water (62 : 38)/2ml min^{-1} ;	(1), (2) and (3)	UV-Detector 254 nm	Based on the declared content of $C_{24}H_{29}Cl_2FO_5$ in BPCRS.
3.	Methotrexate	Nucleosil C18 in Acetonitrile	Phosphate Buffer (pH 6.0) and Acetonitrile (92 : 8)/1.4 ml min^{-1}.	(1), (2) and (3)	UV-Detector 303 nm	Based on the declared content of $C_{20}H_{22}N_8O_5$ in EPCRS
4.	Vinblastine Sulphate	Zorbax C8	Methanol : 1.5% w/v soln. of diethylamine adjusted to pH 7.5 with *o*-phosphoric acid : Acetonitrile (50 : 38 : 12)/1.0 ml min^{-1}	(1) to (5) kept in ice before use	UV-detector 262 nm	Based on the declared content of $C_{46}H_{58}N_4O_9.H_2SO_4$ in EPCRS.

* BP (1993) Vol. 1 ;

Along similar lines, the presence of related substances found in pharmaceutical drugs may be estimated by using HPLC method, for examples : atenolol, buclizine hydrochloride, ibuprofen, and the like.

THEORETICAL AND PRACTICAL EXERCISES

1. What are the major advantages of High Performance Liquid Chromatography (HPLC) over GLC ? Explain with typical examples.

2. Enumerate the vital theoretical aspects of HPLC giving suitable examples.

3. Describe the working of a HPLC-equipment highlighting the various important components with a labelled diagramatic presentation.

4. Elaborate on the following aspects of HPLC :

   (*a*) Reciprocatng pump,

   (*b*) Working of a twin-head reciprocating pump,

   (*c*) Microvolume sampling valve (*i*) Sampling mode ; and (*ii*) Injection mode, and

   (*d*) Measurement of column performance.

5. Discuss briefly the following detectors used in HPLC alongwith a diagramatic sketch :

   (*i*) Double-beam uv-detector,   (*ii*) Fluorescence detector,

   (*iii*) Refractive index detector, and   (*iv*) Multipurpose detector.

6. What do you understand by '*pre-column off-line derivatization*' and '*post-column on-line derivatization*' ? Explain.

7. Explain how one may use HPLC to accomplish the following :

(*a*) Isolation of alkaloid and glycoside,   (*b*) Control of microbiological processes.

Give suitable examples in support of your answer.

8. How would you assay the following **'medicinal compounds'** enlisted in *Official Compendia* :

(*i*) Cephalosporins,   (*ii*) Frusemide,

(*iii*) Theophylline,   (*iv*) Corticosteroids,

(*v*) Dichlorphenamide, and   (*vi*) Human Insulin.

RECOMMENDED READINGS

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