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CHROMATOGRAPHY: INTRODUCTION OF IMPORTANT SEPARATION METHODS AND MODERN TECHNIQUES FOR ADJUSTMENT OF COLUMN PARAMETERS AND FLOW RATE IN LIQUID CHROMATOGRAPHY TO REDUCE ANALYSIS TIME AND COST.

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Abstract

Chromatographic techniques play a major role in the pharmaceutical industries for separation, including clinical applications, environmental applications, forensics, food analysis, and the analysis of biological samples. Different chromatographic techniques are available for analysis, like TLC, HPLC, UPLC, GC, LCMS, ion exchange, affinity, and Size exclusion chromatography, etc. In each chromatographic technique, the movable (Mobile) phase and fixed (Stationary) phases play a major role in the separation of mixture compounds. In Liquid chromatography, the "small particle size" of the stationary phase increased the performance of chromatographic separation after adjustment in Column parameters (length & internal diameters), resulting in reduced analysis time, consumption of chemical/Solvent, and Cost of analysis which is support to Green Chemistry. Isolation of content depends on partitioning between mobile and stationary phases which is completed with the help of Vander Waals' forces through dipole-dipole, dipole-induce dipole, л-complex bonding, hydrogen bonding interaction, adsorption/desorption, Physico-chemical interaction, capillary ways holding-bonding pattern, size and shape pattern, and ligand-receptor phenomena. In all the above techniques the movable phase is liquid or gas whereas the stationary phase contains solid material and solid support coated with a liquid material.

Keywords: Separation Techniques, Chromatography, HPLC, TLC, UPLC, Column, Stationary phase, Green chemistry, Adjustment in Column Parameters.

INTRODUCTION OF CHROMATOGRAPHY:

Chromatography is more than 100 years old and is used for the simplification, detection, and determination of pharmaceutical substances in bulk and dosage forms, cosmetic products, amino acids, hormones, vitamins, proteins, carbohydrates, etc. Chromatography is used in clinical applications for the detection of endogenous

neuropeptides, the examination of blood and urine, structure elucidation, and the monitoring of unknown pollutant molecules in environmental applications. Chromatography is very useful in the forensic field for the analysis of drugs, steroid quantification, and analysis of cloth dyes in the field of textile, and it is very helpful for the analysis of food, flowers and recognition of polycyclic compounds in vegetables. On March 8, 1903, M.S. (Mikhail Semenovich) Tswett introduced the term "chromatography" during his lecture, which was presented in the Warsaw University Biological Section and titled “On a New Category of Adsorption Phenomena and Their Application to Biochemical Analysis"

Mikhail Semenovich performed an experimental study using leaf pigments with a particular adsorption method that is acceptable for the partition of the leaf pigments. Later that technique was refined, which finally became famous as ***chromatography*** in 1906. The meaning of chromatography is color-writing which was taken from Greek words. Colors mean “chroma” and write means “Graphein". Chromatography indicates a separation technique, whereas a chromatograph is a system for performing Chromatography, M.S. Tswett tested a plant extract with solvent (petroleum ether) using a glass tube which was filled with chemical ($CaCO_3$) fine particles and the plant extract separated with different colors, as shown in Fig. 1. The technique of M.S Tswett's was continued by scientist: Gottfried Kranzlin in Germany, Charles Dhere in Switzerland, and Leroy S. Palmer in the United States [1-4].

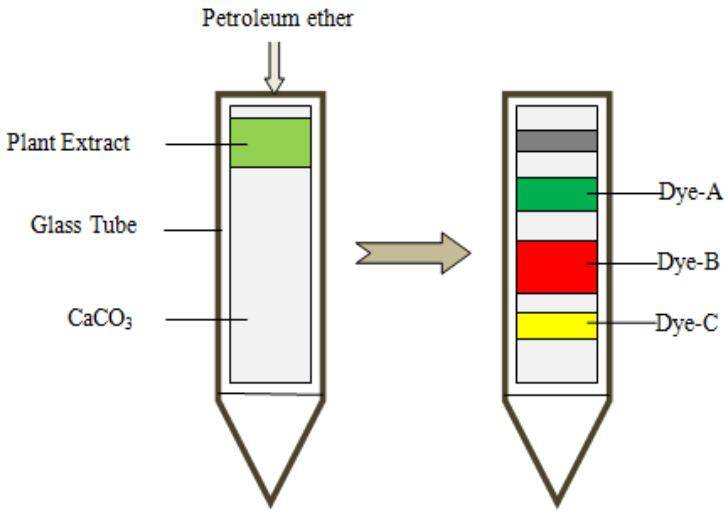


Fig. 1 Tswett's Experiment

CLASSIFICATION OF CHROMATOGRAPHY:

The term "chromatography" can be classified in three fundamental ways, as mentioned below [5-6].

Based on the nature of the Chromatographic Bed: In case shape of chromatographic bed look like a column then it is called; column chromatography. If the shape of the chromatographic bed is plain then it is called; planar chromatography example: Paper chromatography & TLC.

Based on Physical Nature of the Mobile Phase and stationary Phase: Chromatographic techniques are often classified in the below terms which specify the physical nature of MP (Mobile Phase) & SP (Stationary Phase): Once gas is employed as a mobile phase and liquid/solid is applied as a stationary phase then that term is called; *Gas-liquid* and *Gas-solid chromatography*. In case liquid is utilized as a mobile phase and liquid/solid is used as a stationary phase then that term is called; *Liquid-liquid and liquid-solid chromatography.*

***Based on the Mechanism of Separation*:** It is categorized as *Adsorption*, *Partition*, *Size Exclusion*, *ion exchange*, and *Affinity* chromatography.

ADSORPTION CHROMATOGRAPHY:

In Adsorption chromatography sample component is separated according to its adsorption/desorption affinities on an active solid surface [5]. The Liquid/Gas is used as the ***movable phase*** and the polar substance which has a broad Specific surface area utilized as the ***stationary phase*** (adsorbent), different types of adsorbents as mention in Table 1 are used for chromatographic separation [3].

Table 1

Solid	Used to separate
Silica gel	Sterols, Amino acids
Carbon	Peptides, carbohydrates, amino acids
Aluminum silicate	sterols
Alumina	Dyestuffs, vitamins, inorganic compounds
Magnesia carbonate	Porphyrins
Magnesia silicate	Esters, glycerides, alkaloids
Calcium Hydroxide	Carotenoids
Calcium carbonate	xanthophylls
Calcium phosphate	Enzymes, proteins, polynucleotides
Starch	Enzymes
Sugar	Chlorophyll, xanthophylls

A solute binds with an adsorbent and makes a frail life 'bond' via “Vander Waals forces/electrostatic interaction/hydrogen bonds/hydrophobic interactions/dipole-dipole/dipole-induce dipole/л-complex bonding ” on the outside of adsorbent as mention in Fig. 2 below [7-9].

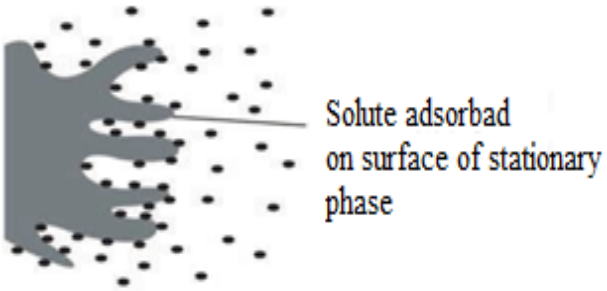


Fig. 2(Adsorption Chromatography)

During separation some components returned to the mobile phase, and adsorbent pores are free hence, these pores are occupied with other Components. Thus, there is a continual interchange process among both phases. Those molecules bind more tightly with adsorbent than others; the quantity of these molecules in holes of adsorbent will be more than its quantity in the nearby liquid. Hence, we can suppose that they can be perfectly separated, below is a separation factor:

k = (X / Y) S / (X/ Y) L

Where: k=separation factor for contents, X, Y= Two components, S= adsorbed phase, L= liquid phase [10-11].

An example of adsorption chromatography is given below [12].

- **Open Column Chromatography (LSC)**
- **TLC (LSC)**
- **HPLC (LSC)**
- **Gas- Chromatography (GSC)**

Open-Column Chromatography (LSC): Silica, Alumina, and cellulose are filled within a cylindrical glass tube; the size of the glass tube could be different in size depending on the number of components to be separated. Silica, Alumina, and cellulose are used as filling substances to prepare the column [3].It is a preparative application on a range from micrograms to kilograms of stationary phase. To prevent the loss of stationary phase different types of filters like glass wool plug/glass frit is used. A stopcock is attached to the column used to control the solvent flow. Filter & stopcock design in the bottom of the column, in most

conditions, two different types of techniques are applicable to arrange the column: one is the dehydrated method, and the second hydrated method.

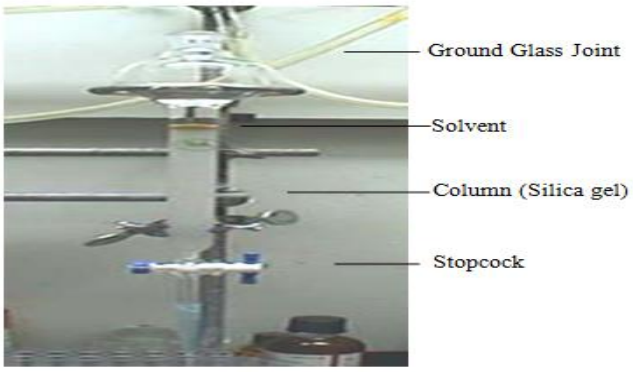


Fig.3 (Systematic Setup of adsorption column chromatography)

In this technique, each component is separately moved through the column at its dissimilar speeds based on its polarities, which means the component that has more holding or bonding with the stationary phase will be eluted later and the component that has less holding or bonding with the stationary phase will be eluted fast. When adding air pressure from the top, we can collect solvent fractions from the columns that contain isolated (purified) components; Fig. 3 shows a systematic setup of Open-column chromatography [13-15]. The Following are the benefits and drawbacks of open-column chromatography [16].

Benefits of open column chromatography:

- Different types of mixture components can be separated.
- Several quantities of the same mixture can be used for separated by open-column chromatography.
- Column chromatography has a broad range of mobile phases for the separation of mixture components.
- Components can be separated and used again and it is a robust method.
- It can be possible to run automation.

Disadvantages of open column chromatography:

- Time-taking chromatographic technique.
- Less capacity of separation comparison to other advanced separation techniques like HPLC, GC, and UPLC/UHPLC.
- More consumption of solvent in that technique which is very costly.
- The computerization of this technique makes it more difficult and pricey.

Thin-Layer Chromatography (LSC): The progress of TLC is divided into three periods: first 1938 - 1951, second 1956-1980, and third 1981 to the present, Progress of TLC mention in below Table 2 [17].

Table 2

1938	M. S. Shraiber and N. A. Izmailov use thin layer on microscope slides
1949	J. E. Meinhard and N. F. Hall used binders to advance TLC.
1956 to 1962	In 1956 Egon Stahl used silica gel as a sorbent film and introduced his job titled **Thin-Layer Chromatography**. 1958 Stahl worked with manufacturers to introduce commercial materials for TLC and In 1962 A varieties of progress chambers we re-established with linear reservoirs and flat compartments.
1975	Business manufacture of HPTLC plates leads to upgrading in practice and instrumentation.
1985	The technique for combining TLC with Mass Spectroscopy (MS) was first explained.

The TLC gives a rapid, low-cost, and moveable technique for qualitative examination, this technique is used to separate a mixture of components and identify unknown substances by matching their separation model to that of known reference substances (Like Pharmacopoeia Substance-USP/BP/CRS, Primary Reference Substance (PRS), Secondary Reference Substance (SRS), Related Compounds, etc.)[18]. It is *a liquid-solid adsorption chromatography***.** During separation, a solvent mixture moves the upper side of the plate by a capillary phenomenon with different rates. This process is called the growth of the plate [17]. The demonstration of the ascending growth for TLC is shown in Fig. 4, and the separation phenomenon is explained in Fig. 5. (A, B& C molecules are separate with polarity phenomena against the stationary phase). Component C of the sample mixture has more attraction with the stationary phase of TLC-Plate than components A& B. Component B has more attraction than Component A but it has less attraction than Component C, which means Components A, B &Care regularly separated from each other [19-20]. TLC technique was refined in different stages and in the 1970s a fine-particle coat (~5µm) and related instrumentation were linked for their correct use and finally, it was become known *as high-performance thin-layer chromatography (HPTLC)*. It provides high sensitivity, reproducibility, and separation commands. New HPTLC terminals are controlled by software and give superior results similar to those of HPLC and GC instruments [21-22].

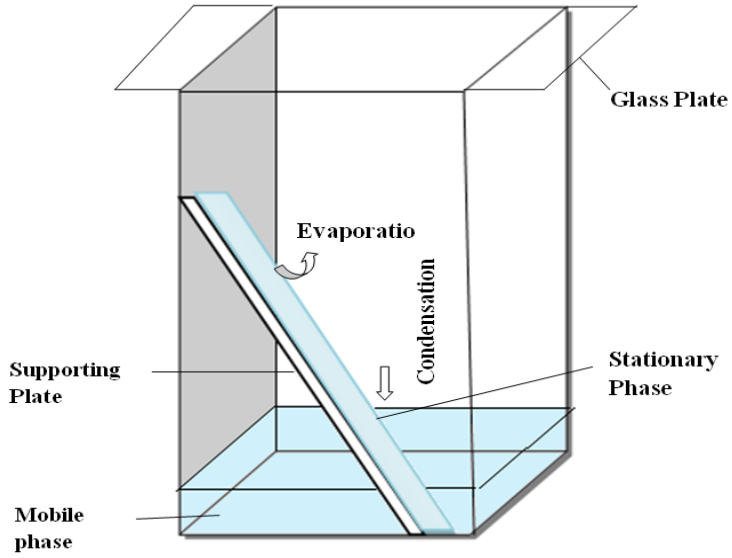


Fig. 4 (Demonstration of ascending growth of TLC)

The TLC technique has the following advantages and disadvantages [17, 23-24].
Advantages of TLC:

- It is a low-priced technique than the other modern techniques.
- Minimal material is required to perform that technique.
- TLC is easy to perform and interpret
- Less Solvent utilization.
- Minimal sample preparation.
- Useful for a wide range of applications.
- We can visualize the components in the presence of UV light.
- The non-volatile complex is separated by this technique.

Disadvantages Thin-Layer Chromatography (TLC):

- It needs a larger sample size
- It gives lower sensitivity, reproducibility, and Resolution capacity than other methods, such as HPLC, UHPLC/UPLC, or GC
- Limited selectivity for certain components.
- It may require several solvents during separation.
- It is an open system analysis so environmental factors (i.e. Temperature and humidity) can influence the results.
- Reproduce of results is very complex.

High-performance liquid chromatography (LSC): It is known as "Adsorption chromatography" In this technique movable phase is "liquid" and the fixed phase is "Solid". In analytical chemistry, HPLC has an important position for separation, identification, qualitative and quantitative analysis of drug material, drug products, cosmetic products, amino acids, hormones, vitamins, proteins, and carbohydrates in the fields of Pharmaceuticals, Nutraceuticals, Food industries, Cosmetics field, Environmental matrices, analysis of forensic samples, and industrial chemicals. According to the working principle in this technique, a mobile phase passes through packed columns (stationary phase) at high pressure, the value of pressure depends on the flow rate, column diameter, column length, and particle size used to prepare of Column. In HPLC back pressure increased when we used a column with has small particle size and increased flow rate of mobile phase. The functional pressure resistance range for regular HPLC systems (Make: Agilent, Waters & Shimadzu) is often 400–600 bars which is generally suitable for HPLC column packing material(fully porous particle)[25].According to the mode of operation, HPLC is categorized as *Normal phase* and *Reverse phase* in both conditions we can perform the analysis using Isocratic and Gradient Methods [26-29].

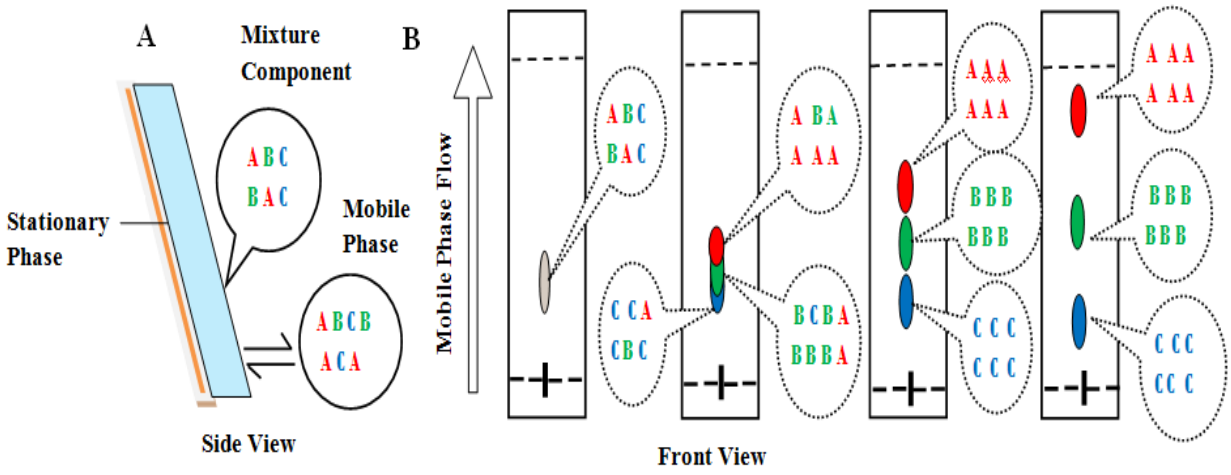


Fig. 5 Separation of three components (A, B and C)

Normal Phase Chromatography:

- Non-polar like: hexane, toluene, chloroform, octane, etc. used in reservoirs known as mobile phase.
- Polar adsorbent silica is mostly used to prepare the stationary phase but for normal phase conditions, it is chemically saturated with a **silanol group** at the end. These OH groups are statically spread outer walls of that stationary phase and build a frail bond with molecules through dipole-dipole/dipole-induce dipole/л-complex bonding, and hydrogen bonding interactions.
- The polar molecules have more affinity with the stationary phase than the Non-polar molecules hence non-polar molecules eluted first.
- HPLC instrument at Normal phase condition Use for analysis of Steroid hormones, phospholipids, saccharides, tocopherols, etc.

Reverse phase chromatography:
- The solvents that are soluble in water are used as mobile phases like Methanol, Acetonitrile, etc.
- Non-polar chemically modified silica with organic "silane groups" such as C8, C12, and C18 are used as stationary phases in Reverse phase conditions.
- The non-polar molecules will favor hold with the non-polar stationary phase instead of the polar molecules. As a result, it eluted later than the polar molecules.
- Used for Low molecular weight compounds.

Components of HPLC system: The HPLC system is assembled or connected with different compartments. In the market different brands/models of HPLC systems are available with advanced features; however working principles are the same for all brands of HPLC systems, the physical setup and working principle of HPLC are shown in Figs.6, 7 [30, 31].

- **Solvent Reservoir**
- **Solvent manager**
- **Sample manager**
- **Column management**
- **Detection**

Fig. 6(HPLC-Waters Alliance Systems)

Solvent Reservoir: A glass bottle or stainless steel container is used as a solvent reservoir. Generally, glass bottles are used for the solvent reservoir (mobile phase) glass bottles may be transparent or amber in color, and the mobile phase suck by the solvent manager (pump) through plastic tubing [32-33].
Solvent Manager: It is a separate compartment in the HPLC instrument, A solvent (Liquid Mobile Phase) Suck from the reservoir section by that compartment with the help of a pumping system, there is one or two pumps are fitted in this section (Single Pump for Low-pressure gradient system & Two Pumps for High-pressure gradient system)[34]. A degasser is fitted in this section to degas the mobile phase after that same mobile phase is forced through the pump at a fixed flow rate (milliliter /minutes) to

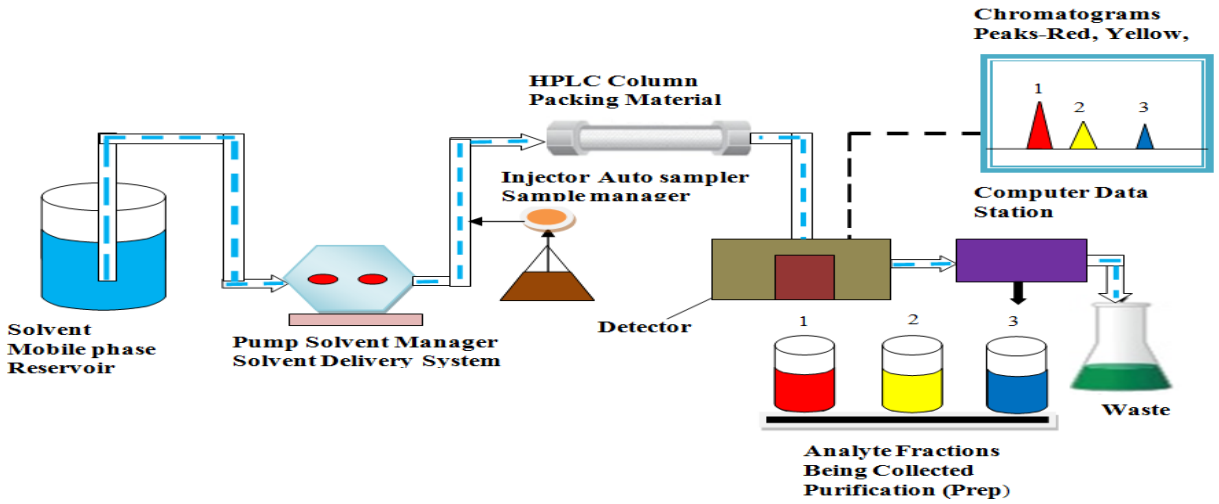


Fig. 7(Work Flow of HPLC-System)

The next section (Column management), A pump can supply the mobile phase in both conditions (isocratic gradient). During the analysis of the HPLC system, generally isocratic method are used due to its simplicity,

suitability, reproducibility, and essay to prepare on the system. However, it is not preferred when a sample containing components has a wide range of retention time [32-33].

Sample manager: The sampler manager is the section of HPLC where analyte solutions are kept in an HPLC tray by filling HPLC Vials with systematic order at controlled Temperature. The same volumes are automatically quantified (Like 10 µl, 50 µl, and 100 µl) and the same volumes of sample are injected in the path of the mobile phase hence, it passes away from the column resulting in the separation of mixture component [35].

Column management: It is the Thermostatic Column Compartment where the HPLC column joint with inlet and outlet tubing of the mobile phase. The temperature of that compartment is set as per requirement. The temperature plays a major role in separation as it can change the retention time & shape of peaks [36].

Column: In chromatographic techniques, the column plays a major role as a sample mixture separated into columns so that it is known as the "Heart of the chromatograph"[35]. Most columns are manufactured with stainless steel in a tubing shape, where an adsorbent material (Stationary phase) is filled, the stainless steel wall is capable of resisting the higher pressure that is generated during separation, as well is comparatively inert to chemical corrosion (chloride & lithium ions at low pH)[9]. Generally, columns are in straight shape, some time may be a "U" shape. The columns are packed using different methods/techniques such as dry packing with the "tap-fill" procedure, Slurry packing for hard gel columns, and Slurry sedimentation method for soft gels [10].Columns can be dissimilar in length and internal width as shown in Fig. 8[37].

Fig. 8(HPLC Columns)

Particle size of filled material and physical parameters (length and internal diameter) of the column played an important role in the separation of sample components, hence to understand the phenomena of separation following two theories are explained
[29, 38].

- **Plate theory**: It is an aged theory that was given by Martin and Synge in 1941.
- **Rate theory**: It was projected via way of means of Van Deemter in 1956 and these days carried out in use

Plate theory: According to this theory a chromatographic column contains imaginary theoretical plates (Fig. 9c); in each plate, the analyte equilibrates between the Stationary and mobile phase as mentioned in Fig. 9(a) there are two components (●▲) are applied in the column hence the component (▲) has more affinity with stationary phase eluted later, and the component (●) has less affinity with stationary phase eluted first. During that separation process, a new balance is made in addition to fresh eluent, sample molecules which are available in the mobile phase are partly adsorbed on the surface of the stationary phase, due to distribution coefficients.

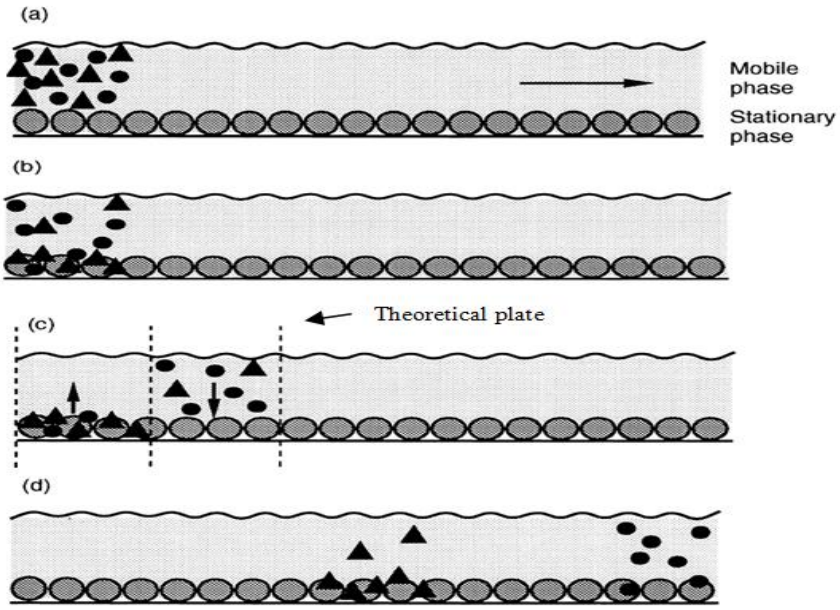


Fig. 9(Separation of two components)

Hence previously adsorbed molecules come out again in the flow of the mobile phase (Fig. 9.c) the movement of the solute continues in the column from one plate to the next plate [11, 9].

Theoretical plates: During the separation of both components a new balance was found [Fig. 9(c)] means approx 3.5 particle diameters of the stationary phase involved as a single section. Hence, that section of the stationary phase called as "Theoretical plate". The theoretical plate concept comes from the distillation theory [8].

A column with has higher number of theoretical plates will produce a narrow peak means superior separation, so for better separation high theoretical plates are required, and the following factors are used[29, 9].

- Use Longer column
- Columns have small plate height
- Short distribution paths in pores of solid phase

Theoretical plates, length, and plate height can be expressed as below;

$$H = \frac{L}{N} \qquad (1)$$

Where; H=Plate height, L= Length of column, N= Theoretical plates

The theoretical plates are determined from data obtained under isothermal, isocratic, or isodense conditions, depending on the technique, following the equation, used to calculate the theoretical plates [29, 38].

$$N = 5.54\left(\frac{t_R}{w_{1/2}}\right)^2 \qquad (2)$$

t_R: component retention time

$W_{1/2}$: width of peak at half-height

The width of the peak was measured at half height (1/2) instead of baseline because the broadening of the peak is fast near to Baseline hence accurate measurement of peak width is hard so that 5.54 is used.

When peak width is calculated at the base-line then theoretical plates are calculated as per below equitation:

$$N = 16\left(\frac{t_R}{w}\right)^2 \qquad (3)$$

t_R: Retention time of the compound
W: width of the peak at the baseline

Rate theory: when sample molecules are injected into a column those molecules are moved in an unintentional style and controlled by several factors:

- **Eddy Diffusion**
- **Flow Distribution**
- **Longitudinal diffusion**
- **Mass transfer**

The value of theoretical plate height (H) is influenced by the above factors, the minimum value of H shows the most efficient separation and the high value of H gives poor separation. The lower value of H turn maximizes N and gives way to sharp peaks. Any method that is responsible for band broadening of solute will increase HETP (Height equivalent Theoretical plates) and decrease column efficiency. A statistical term for the above factors, known as the ***van Deemter equation***, that was first resultant by a group of Dutch petroleum scientists by that equation we can find optimum velocity (Flow Rate) and minimum plate height [8, 29, 39, 40]. The Van Deemter equation and its plot are mentioned below:

$$H = A + B/u + Cu \qquad (4)$$

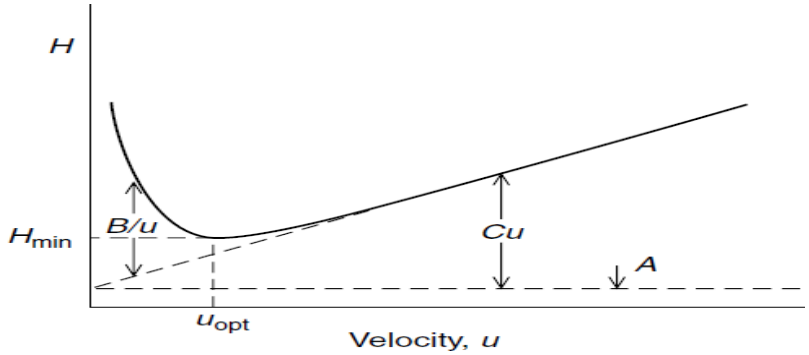
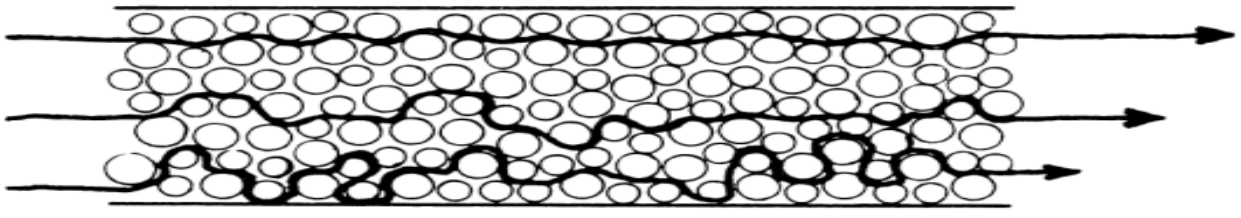


Fig. 10 (Van Deemter plot)

Where; A: Eddy diffusion, u: Flow Velocity, B: longitudinal diffusion C: Mass transfer, H: Theoretical Plates Height.

Eddy diffusion (A): The column filled using small particles of solid materials (stationary phase), during separation a mobile phase continuously runs through the column. The mixture components move with the help of the mobile phase in this column hence some molecules are very lucky to leave the column than others because they have a straight traveling path in the column, whereas other molecules leave the column later as they have several diversions traveling paths (Fig. 11a). All sample molecules take different traveling paths in columns, depending on mobile phase flow streams [9, 33]. A column packed with large particles has more eddy diffusion resulting broad peak shape whereas small particles of stationary phase reduced the term-A resulting in a well peak shape (Fig. 11 (b) & 11(c)) obtained as mentioned [41].



(a) Phenomena of Eddy diffusion

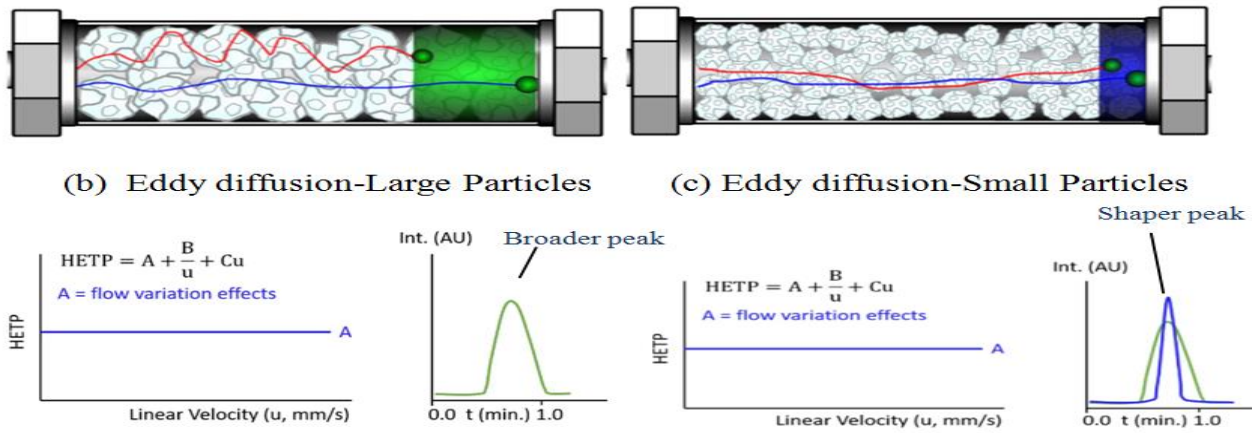


Fig. 11 (Eddy diffusion with Large and small particles)

Flow Distribution (u): When the mobile phase runs from the column then maximum flow shows at the center of particles rather than it's near of particles (Fig.. 12), which means particles are affecting the flow of the mobile phase, a high flow rate enhance the equilibration point (Cu) but reduce the longitudinal dispersion of the solute particles (B/u). However, at extremely high flow rates, the equilibrium time will be less resulting in enlargement of the band [8-9].

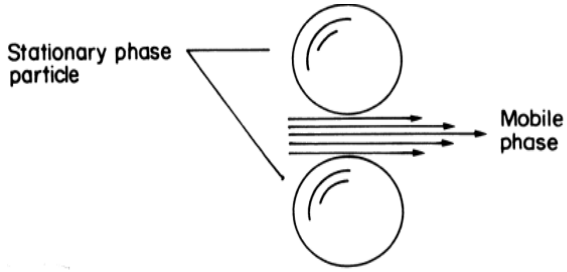
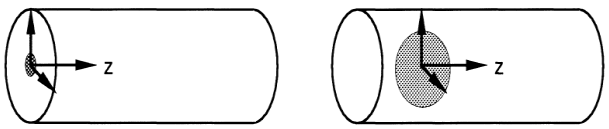


Fig. 12 (Flow distribution in a column)

Eddy diffusion and flow velocity affect the column performance, so to minimize the effects of these factors column should following features:

- The column should be packed with uniformly sized particles
- The packing of particles should be collected with a thin-size distribution.
- A tightly packed column with a small particle size gives a small effect of eddy diffusion means a well-packed column shows the value of eddy diffusion like zero (Fig.10) and can be negated [39-40].

Longitudinal diffusion (B): In longitudinal diffusion sample components are spread out in solvent just like a sugar piece melts in water slowly exclusive of external force as shown in below (Fig.13).



(a) (b)

Fig. 13 (a) Sample immediately after injection. (b)Sample a later moment.

Longitudinal diffusion is controlled inversely by the speed of the mobile phase, low velocity means longer staying of sample molecules in the column, resulting elution time of components will be longer (More time for diffusion) and Band broadening, whereas at high linear velocity, the sample molecules quickly out from column hence high flow gives less time for diffusion resulting "shaper peaks" In Simple word *"Faster flows = less time in the column = lower H = shaper Peaks"* see in Fig. 14. To minimize the impact of Term –B *The flow velocity of the mobile phase should be special so that longitudinal dispersion has no bad effect on separation* [9, 39, 33, 41].

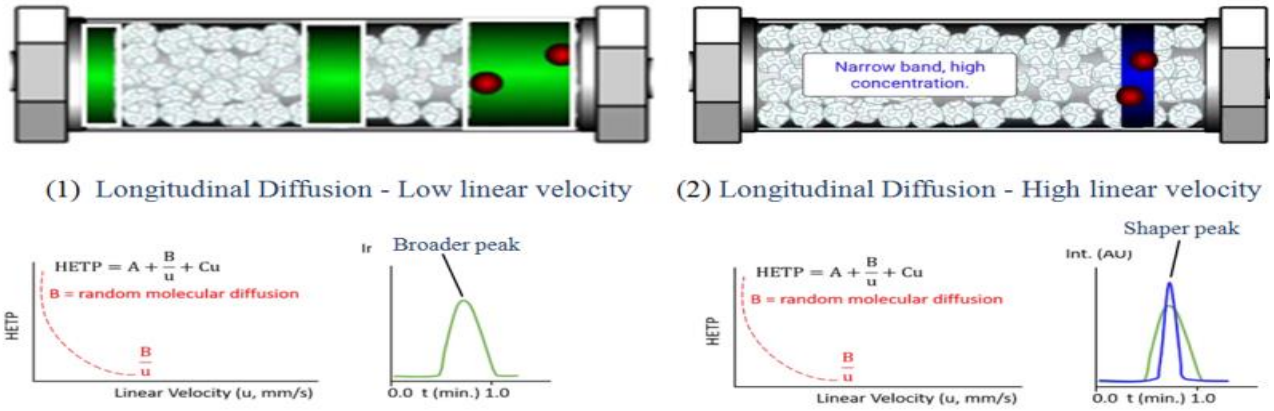


Fig. 14 (longitudinal diffusion at Low and High linear velocity)

Mass transfer (C): The mass transfer words arrive from the finite time required for the analyte to equilibrate within the stationary phase & mobile phase. The stationary phase particles have several pores, they are filled with a mobile phase that does not travel hence some molecules enter in pores of the stationary phase and take additional time to re-enter in mobile phase flow than the others that are not entered into the pores, now the gap of both molecules are small at low velocity resulting shaper peak observed (Fig.15a) but at higher flow rate this gap is more means there is less time available for equilibration resulting band broadening (Fig.15b) [39-41].

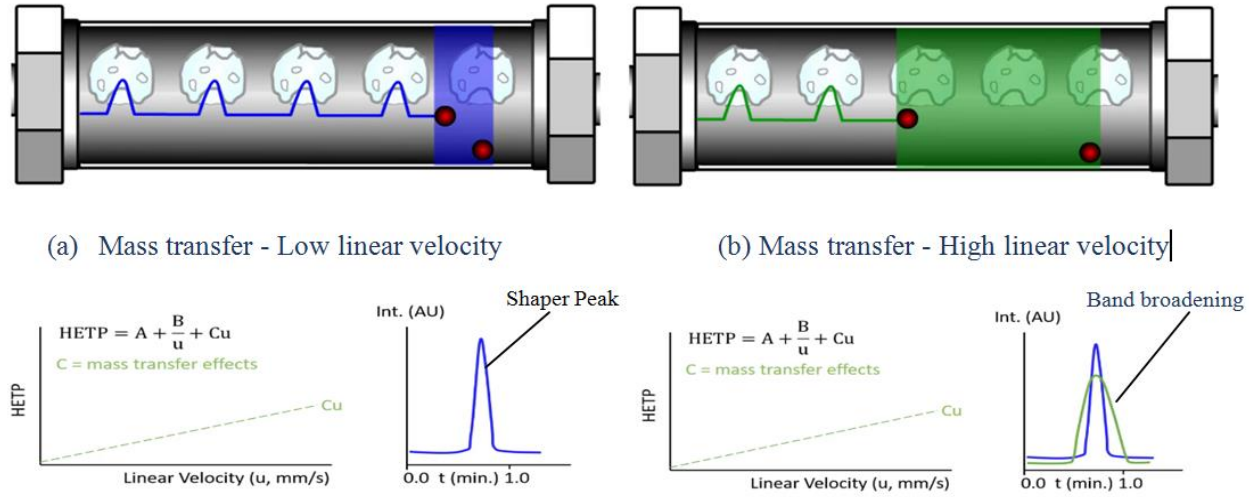


Fig. 15 (Mass Transfer at Low and high linear velocity)

In other words, sample molecules have small diffusion coefficients in "large particles" resulting in sample molecules traveling slowly. Other hand sample molecules have large diffusion coefficients in "smaller particles" resulting in sample molecules traveling fast.

To minimize the effect of the Term –C the column should be prepared with following features [8-9].
- Columns should be prepared using porous particles of small diameter.
- The column should be prepared using smaller particle sizes as they are reduced the mass transfer time means reduced Term-C.
- The separation with small particles can be sped up by raising the flow rates without drastically reducing efficiency (Fig.16) the slope of the line is reduced with a 1.8µm Particle size [40].

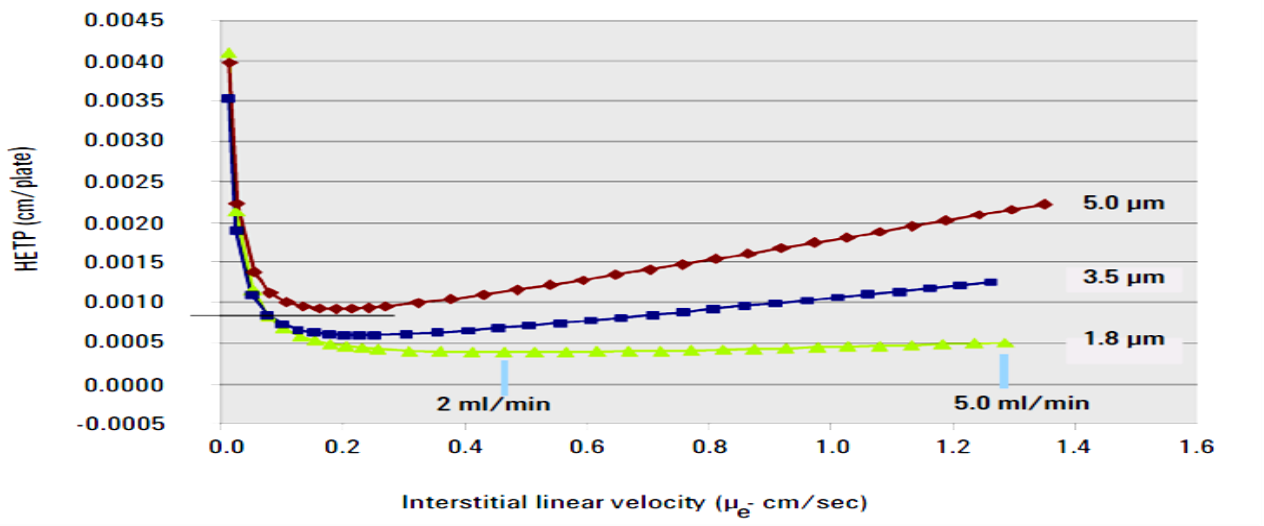


Fig. 16: (Van Deemter curve for different particle sizes)

As already discussed above particle size and column length play a major role in separation, small particle size produced higher flow resistance than large particles, a sharp peak was observed with small particles, and band broadening was observed with large particles. The particle size & length of the column can be customized; hence ratio of column length (L) and particle size (dp) should be stable or in the range between −25% to to+50% of the prescribed L/dp ratio. If particle size is reduced, then the flow rate needs to change, because the column with a smaller particle size requires high speed to produce equal performance. If we change the particle size and diameter of the column then the flow rate can be adjusted using the below equation [38].

$$\mathbf{F_2=F_1 \times [(dc_2^2 x\ dp_1)/ (dc_1^2 x\ dp_2)]}$$ where;

F_1 = Original flow rate (ml/min)
F_2 = Adjusted flow rate (ml/min)
dc_1 =Original internal diameter of the column in (mm)
dc_2 = Adjusted internal diameter of the column (mm)
dp_1 = Original particle size in (µm)
dp_2 = Adjusted particle size in (µm)

Example: Calculation of flow rate using the above formulas:
F_2=?, F_1=2.0 ml/min, dc_1=4.6 mm, dc_2=2.1 mm, dp_1=5µm, dp_2= 3µm.

$F_2=2.0 \times [(2.1^2 x\ 5)/ (4.6^2 x\ 3)]$
$F_2=2.0 \times [(4.41x\ 5)/ (21.16x\ 3)]$
$F_2=2.0 \times [(22.05)/ (63.48)]$
$F_2=2.0 \times [0.347]$
F_2=0.7 ml/min.
Hence Flow decrease 0.7ml/minute from 2.0ml/minute.
Details of adjusted variables and their original and adjusted Conditions are mention in Table 3.

Table 3

Variable	Original Conditions	Adjusted Conditions
Column length (*L*), in mm	150	100
Column diameter (*dc*), in mm	4.6	2.1
Particle size (*dp*), in µm	5	3
L/dp	30.0	33.3
Flow rate, in mL/min	2.0	**0.7**

The changes made to keep the constant Ratio of L/dp resulted in the performance of column (like; theoretical plates) obtained similar and separation time reduced almost 10 times after the adjustment of variables, Fig. 17 and Table 4; show the effect of adjusted variables [9].

Table 4

Variable	Original Conditions	Adjusted Conditions
Column length (L), in mm	200	60
Column diameter (dc), in mm	4.6	2.1
Particle size (dp), in µm	10	3
L/dp	20	20
Flow rate, in mL/min	2.0	0.7
Theoretical plates	7000	7000
Run time in minute	15	**1.5**

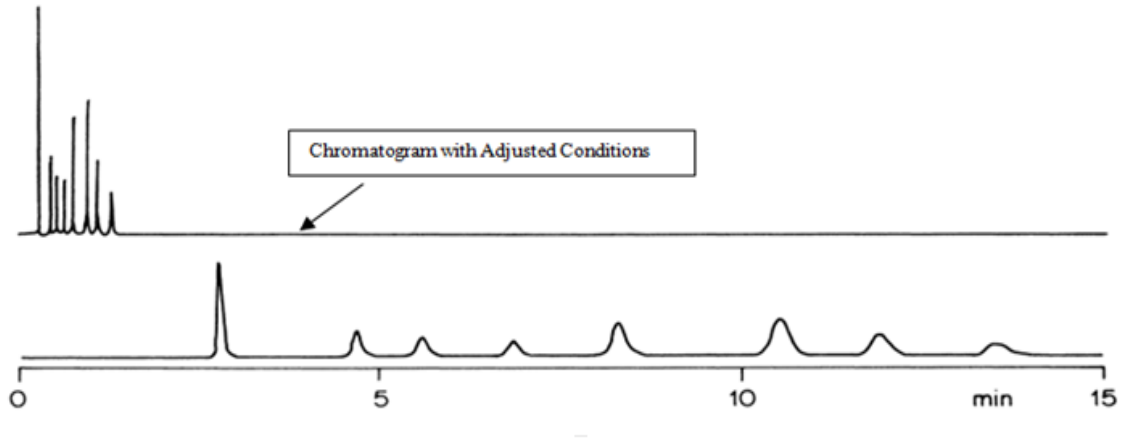


Fig. 17 (Comparison of performance between stationary phases of different particle diameters)

In case the gradient method is used for analysis, to reduce the time and Cost of analysis Column parameters have been adjusted, hence new Gradient time (t_{G2}) shall be calculated using the below equation [38]:

$tG2 = tG1 \times (F1/F2)\ [(L_2 \times dc_2^2)/(L_1 \times dc_1^2)]$ Where;

tG2= New gradient time
tG1=Original gradient time
F1= Original flow rate (ml/min)
F2= Adjusted flow rate (ml/min)
L_1= Original Column length in mm
L_2= Adjusted Column length in mm
dc_1=Original Column diameter in mm
dc_2= Adjusted Column diameter in mm

Example: Calculation of Gradient Time using the above formulas;
tG2=? tG1=@ (Table 5), F1=2.0 ml/min, F_2=0.7 ml/min, L_1=150mm, L_2=100mm, dc_1=4.6mm, dc_2=2.1mm

tG2= @ x (2.0/0.7) [(100x2.1^2)/ (150x4.6^2)]
tG2 =@x (2.857) [(441)/ (3174)]
tG2=@x (2.857) (0.138)
tG2=@x (2.857) (0.138)
tG2=@x0.4
Calculated new gradient time after adjustment of Column parameters and Flow rate shown in Table 5.

Table 5

Mobile phase Gradient Ratio		Original Time in Minute (@)	New Gradient Time in Minute (tG2)
Mobile Phase-A (%)	Mobile Phase-B (%)		
70	30	0	(0x0.4) =0
70	30	3	(3x0.4)=1.2
30	70	13	[1.2+(10x0.4)] =5.2
70	30	16	[5.2+(3x0.4)] =6.4

Detection: It is a very important part of liquid chromatography due to physical or chemical attributes turn into a measurable signal resulting in concentration or identity [42-43]. The detector is the ending section of the HPLC instrument; the detector analyzes the sample after separation into columns. The concentration of individual components is proportional to the electronic signal. In liquid chromatography, the Detectors are mostly categorized into two groups: Bulk Property Detectors and Solute Property Detectors [44-45].
Different types of HPLC detectors and their use are given below [46].

- UV, VIS, and PDA Detectors
- Evaporative Light Scattering Detector
- Mass Spectrometer
- Fluorescence Detector
- Optical Rotation Detector
- Refractive-Index Detector
- Multi-Angle Light Scattering Detector
- Conductivity Detector
- UV, VIS, and PDA Detectors

UV Detector: A ultra-violet detector is used to analyze those compounds that have unsaturated bonds, aromatic groups, or functional groups that have hetero-atoms, they have π* and σ* nonbonding orbital. A regular UV detector has a wavelength range between 195 to 370 nm.
VIS-Detector: The working principle is the same as a UV detector except for to range of wavelength i.e. 400 to 700 nanometers.
UV/Visible-Detector: the detector has a combined range of wavelength 195-700 nm so it is called a UV/VIS detector.
PDA-Detector: The detector that provides three dimension results (wavelength, Time, and Light Intensity) of the chromatogram is called a PDA Detectors whereas UV-Detector, Vis- Detectors and UV/VIS-Detector can provide only two dimension results (Time and Light Intensity).
Evaporative Light Scattering Detector (ELS): it is used for non-volatile Analytes like lipids, sugar, and high Molecular weight Analytes.
Mass Spectrometer: The Analytes are detected based on their molecular weight, due to that we can identify the structure of the analyte.
Fluorescence Detector (FLR): This detector mostly uses natural products, clinical samples, Pharmaceutical products, and petroleum products.
Optical Rotation Detector: It is used for the measurement of optical isomers. The column separates R- and L-type optical isomers, they move towards detectors hence an optical Rotational Detector is identified which is R or L.
Refractive-Index Detector (RI): Sugar, Alcohol, and Inorganic ions are analyzed with RI- Detector as they don't have UV- -absorbance.
Multi-Angle Light Scattering Detector: We can directly identify the molecular weight of the analyte by this detector without using the calibration curve.
Conductivity Detector: This detector is generally used for Ion- chromatography to measure electronic resistance as a sample solution containing ionic components and they conduct electricity.

Advantages of HPLC [47-48].

- Use qualitative and quantitative analysis with high accuracy, High precision, High sensitivity, Speed, and Cost-effective
- It can be used with mass spectroscopy

- It is a faster and more automated technique than other techniques like column chromatography, TLC, Paper chromatography, etc.
- In HPLC gradient elution is easily adjustable.

Disadvantages of HPLC [47-48].

- HPLC requires timely and costly maintenance.
- It required costly solvents, power supplies, expertise for operation, and Complexity.
- Precaution to avoid column contamination, column blockage, and Limited sample size.
- HPLC instrument is more costly than other chromatographic techniques like TLC, Paper chromatography, and Column chromatography.

Novel Improvements in HPLC Technique: As we already discussed the smaller particles give better separation, reduce the analysis time, and enhance *resolution, speed, and sensitivity*, the smaller particles require higher pressures during the separation of the analyte, hence carry the mobile phase at higher pressure HPLC instrument was refine and in 2004 "Waters" launched a new technique known as UPLC (Ultra performance liquid chromatography). Other vendor's refine the HPLC instrument similar to UPLC and it is known as UHPLC (ultra high performance liquid chromatography) since UPLC is a Waters Trade Mark. UPLC is capable of operating pressure up to 15000 psi whereas UHPLC is capable of up to 20000 psi however working principle of UPLC/UHPLC is the same as HPLC [49-51].

Advantages of UPLC/UHPLC:

- The analysis time is reduced by about 10 times than the HPLC analysis.
- The consumption of solvents, chemicals, water, and electricity is reduced.
- The productivity increased with the use of UPLC/UHPLC technology over the HPLC
- Present the selectivity, sensitivity, and dynamic range of LC analysis.
- For method development spent time can be reduced using the UPLC/UHPLC.
- In UPLC/UHPLC small particle size is used as a stationary phase which reduces the analysis time (Fig. 18).

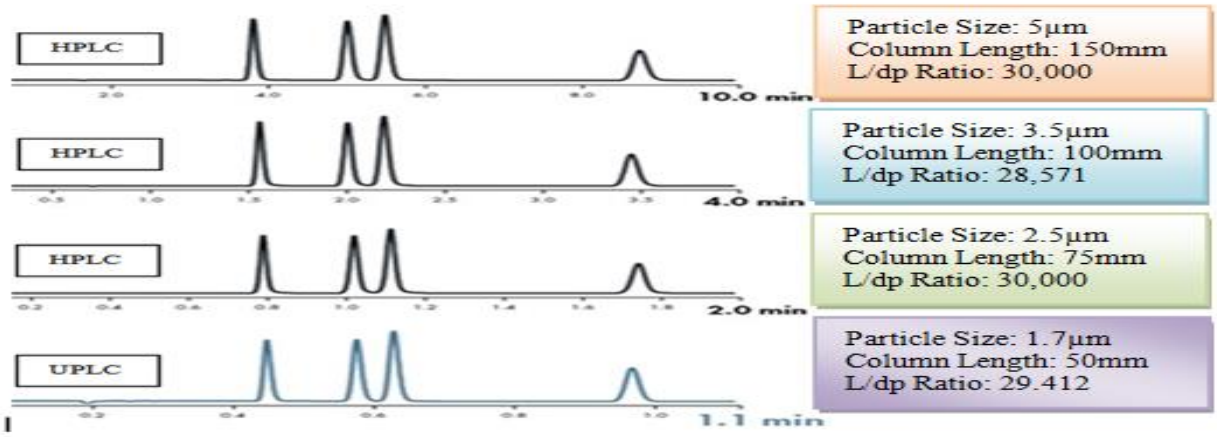


Fig. 18 HPLC and UPLC Chromatograms

The particle size of the column (stationary phase) plays a major role during the separation of Analytes, we can demonstrate the chromatographically in the above Fig. 18. A 50 mm long UPLC column packed with 1.7 µm particle size and another side HPLC column packed with 150 mm long 5 µm particle size, 100 mm long 3.5 µm particle size, and 75 mm long 2.5 µm particle size used for analysis. Hence L/dp (Length/Particle size) Ratio is about constant for each column (HPLC/UPLC), The analysis time is shorted 10 times on UPLC analysis while maintaining the resolution means after reducing the time on UPLC the Resolution is not affected, the peak are well separated. The following are the benefits of UPLC/UHPLC analysis [52].

Disadvantage of UPLC/UHPLC [53-56].

- UPLC or UHPLC works at higher back pressure than the usual HPLC which may reduce the life of the column.
- Costly instruments than the usual HPLC.
- Required Costly maintenance.

Gas Chromatography: During analysis of sample solution, sample solution changes into a volatile stage at a higher temperature then same transported with mobile phase (e.g. nitrogen, helium, or argon, etc.) and comes into contact with a stationary phase (Solid/Liquid) hence, that sample separated by adsorption/desorption and Physico-chemical interaction in a gas chromatographic column. The compounds having lower molecular weight and stable at high temperatures are mostly analyzed on gas chromatographic technique like; Residual solvents such as methanol, isopropyl alcohol, benzene, hexane, toluene, and others are analyzed in drug products and drug substances. In this technique, different types of detectors are used according to their suitability; the details of the detectors are mentioned below [57].

1. Electron Capture Detector (ECD)
2. Flame Ionization Detector (FID)
3. NPD stands for Nitrogen-Phosphorus Detector.
4. Flame photometric detector (FPD)

Use of detectors:

- **Electron Capture Detector (ECD**): Used to analyze halogenated compounds**.**
- **Flame ionization detector (FID):** Used to analyze carbon-containing compounds.
- **Nitrogen-phosphorous Detector (NPD):** Used to analyze compounds that contain nitrogen or phosphorous**.**
- **Flame photometric detector (FPD):** Used to analyze compounds containing sulphur or phosphorous**.**

GC-Column: In gas chromatography, capillary columns and packed columns are used for analysis**.** The capillary column provides improved resolution and analysis speed than the packed column. The analyte has been identified based on retention time (RT) which is similar to HPLC; Fig. 19 shows the basic hardware Components of Gas chromatography [58].

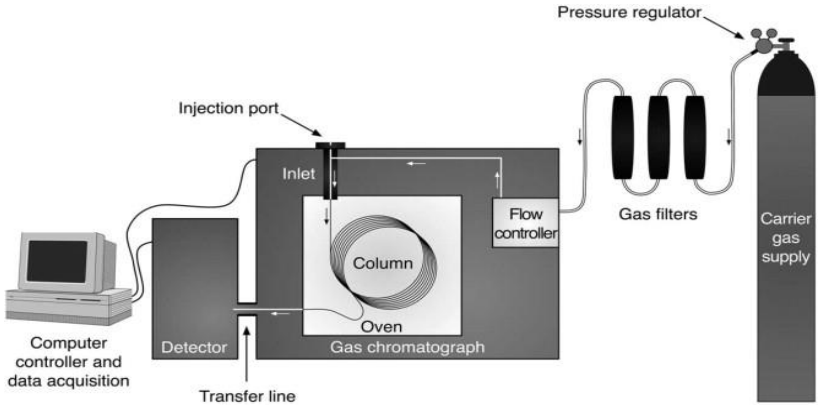


Fig. 19 (Set-up of Gas chromatography)

Gas chromatography is divided into two parts based on the mechanism of separation
(*A) Gas-Solid Chromatography which is part of adsorption chromatography*
*(B) Gas-Liquid Chromatography in comes under Partition chromatography***.**

Gas-Solid Chromatography:

The inert gas (nitrogen, helium, argon, etc.) is used as the mobile phase and solid substances are used as the stationary phase. The active solid adsorbent material used to prepare the stationary phase means the inner side of the column is coated with that material. During analysis on the Gas-Chromatographic instrument, sample molecules (converted in gas form due to higher temperature) move with the mobile phase which is also gas form; hence sample molecules are separated due to adsorption/desorption interaction**.** It is a very useful technique to analyze samples at higher temperatures [59-61].

PARTITION CHROMATOGRAPHY:

The Stationary phase is always liquid on a solid/gaseous surface and, the Mobile phase may be liquid and Gas in this technique [12]. During separation, the mixture compound is distributed among two segments

(Mobile & stationary phase), the solid stationary phase coated with liquid material according to its distribution coefficient shown in Fig. 20 [62-63].

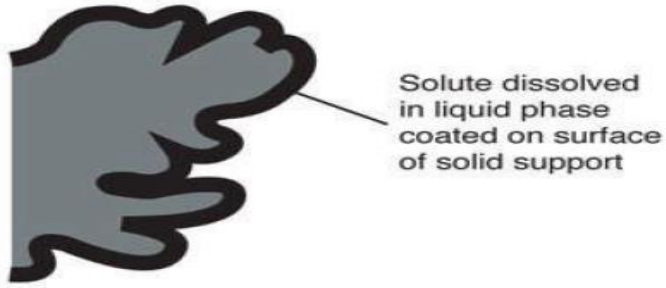


Fig. 20 (Partition Chromatography) [8]

- *Partition paper chromatography (liquid-liquid chromatography)***.**
- *Partition column chromatography (liquid-liquid chromatography)***.**
- *Gas chromatography (Gas-liquid chromatography).*

Partition paper chromatography: It is a very cheap method in chromatography. In this technique, a minute amount of sample is placed on paper sheets or strips, and that mixture is distributed in two liquid phases (stationary phase, which holds fiber) and
a moving liquid phase called the mobile phase. During this process, mixture components separate according to their different movement rates across the paper sheets, each compound of the mixture is separated according to its attraction with the stationary phase (paper sheet) and the mobile phase (solvent), and the entire separation is completed as per the capillary way of the pore in the stationary phase (paper sheet). This technique was invented in 1943 by Martin and Synge.
Based on the method of development of chromatograms, partition paper chromatography can be classified as mentioned below [64].

1. Ascending Paper Chromatography
2. Descending paper chromatography
3. Ascending-Descending Paper chromatography
4. Radial paper chromatography
5. Two-Dimensional Paper Chromatography

Ascending Paper Chromatography: It was first established by Consden, Gordan, and Martin and was later modified by Williams and Kirby, during separation the solvent moves upward direction on the paper/strip as it dips in a solvent which

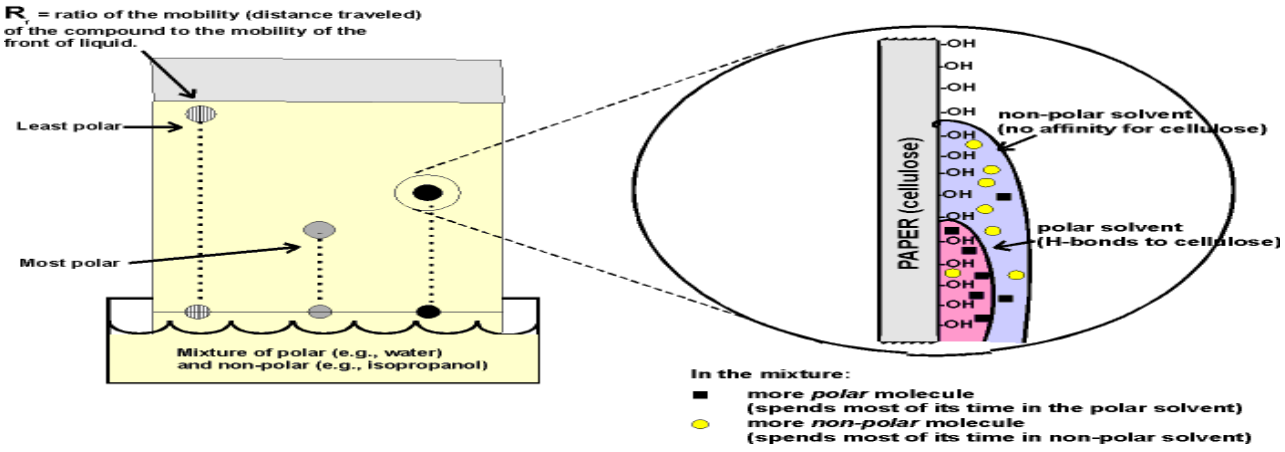


Fig. 21 (Separation of Mixture component in ascending paper Chromatography) [65]

Stores in the bottom of the beaker, that solvent is a mixture of polar and non-polar solvents, and, the stationary phase is cellulose paper having a polar nature with (-OH) group, during the separation of the mixture component, non-polar molecules spend maximum time with non-polar solvent resulting more mobility than the polar molecules. The polar molecules make an H-bond with a stationary phase that has less mobility resulting mixture compound separated. As a part of preventive action sample spot, should not be dipped in the solvent system; it should be up from the solvent level (Fig. 21) is an example of ascending paper chromatography [66].

Descending Paper Chromatography: The solvent reservoir is kept in the top position and the mobile phase runs from top to bottom on the paper sheet (stationary phase), the sample mixture is spotted on that paper sheet, and the mixture component is separated with polar and non-polar attraction phenomena. The chromatographic setup is depicted in Fig. 22. [67].

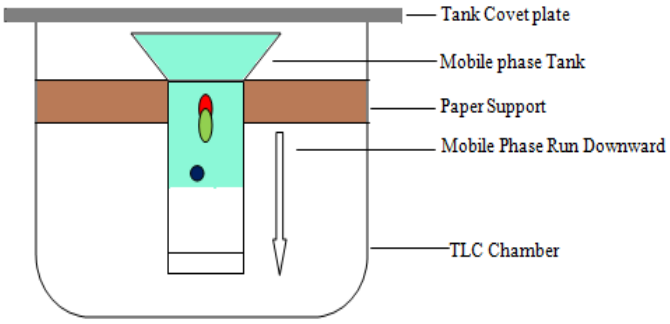


Fig. 22(Descending Paper chromatography)

Ascending-Descending Paper Chromatography: It is a combined (hybrid) technique of ascending and descending chromatography. The top section of ascending chromatography was turned down over a glass rod. The solvent moves upwards first like in ascending chromatography, and then the same solvent runs downwards in that technique. Fig. 23 shows a Setup of ascending and descending chromatography [68-70].

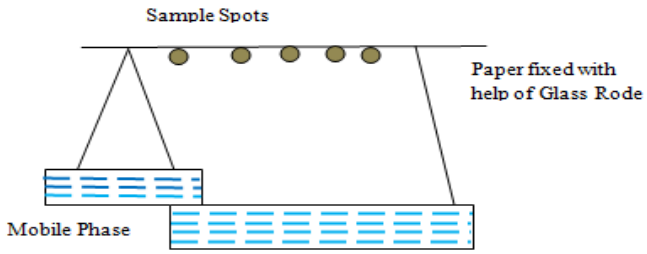
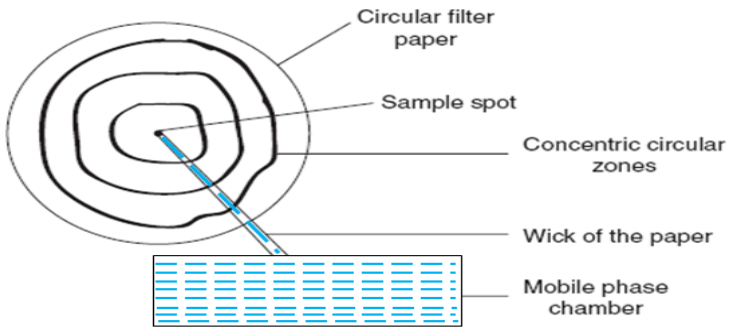
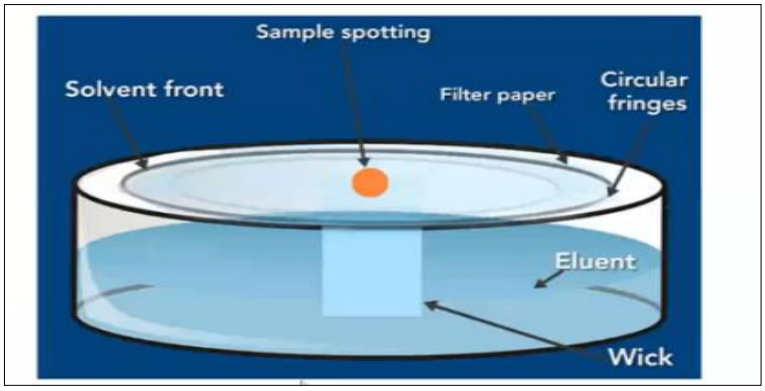


Fig. 23 (Ascending and descending Paper Chromatography)

Radial paper chromatography: First, it was introduced by Rutter; a circular filter paper was used in that technique. The mixture solvent was applied at the center of the circular filter paper, which allowed the wick of the paper to be dipped into the mobile phase. The sample gets separated in the form of concentric rings, whereas in other techniques the component is separated with a single spot. Radial paper chromatography gives fast results with sharpness and resolution; we can easily remove test samples during and after development. The setup of radial paper chromatography developments is shown in Fig. 24 (a, b) [66, 69, 71].



(a)



(b)

Fig. 24 (a & b) Radial paper chromatography developments

Two-Dimensional Paper Chromatography: The mixture components are speckled on the turn side of an angle paper sheet, hence sample spot is applied on one side of a paper sheet, and a single mobile phase is used for separation in one direction. After completing that step, the paper sheet chromatogram is dried, then turned 90° and developed again, using another mobile phase of different polarity for batter/clear separation. That technique is very useful in cases where a complex mixture of similar compounds is separated, like carbohydrates, amino acids, steroids, peptides, and many other organic compounds. Fig. 25: Setup of two-dimensional paper chromatography developments [72-73].

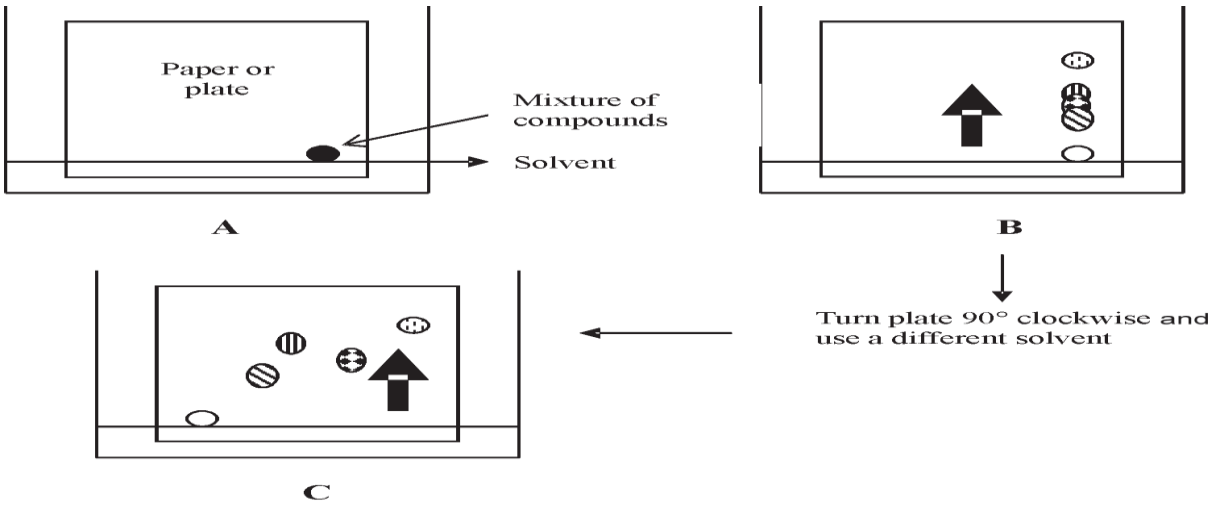


Fig. 25 Two- Dimensional paper Chromatography developments

Partition column chromatography: The mobile and stationary phases both are liquids (For the stationary phase; a film of solvent coated on solid support which is not miscible with the mobile phase). The separation depends on the relative solubility of the mixture sample in a stationary liquid layer because of different partition coefficients different components of the sample are separated [12, 74-76].

Gas-Liquid chromatography (GC:

In case of mobile phase is Gas and the stationary phase is liquid over a solid surface then it is called Gas-Liquid Chromatography, in that technique a thin layer coating of non-volatile liquid material like, dimethyl silicone, or polyethylene glycol, diethylene glycol succinate (DEGS), etc. applied over the inactive solid support (e.g. diatomaceous earth, crushed firebricks, glass powder, powdered Teflon, carbon black, etc.). In a capillary column, that thin layer makes a partition in the sample component between the mobile phase (carrier Gas) and stationary phase (Liquid-coated capillary column), that technique was not used at higher temperatures as the liquid-coated film is unstable at that temperature. The Advantages and Disadvantages of Gas chromatography are mentioned below [59-61].

Advantage:

- Using gas-liquid chromatography we can perform Qualitative and quantitative analysis.
- GC column separated the most complex mixture with higher resolution than the HPLC.

- It provided high sensitivity, precision, and resolving power.
- Thermal detectors or mass detectors can be used with gas-liquid chromatography.
- Analysis time can be completed in a short time Using gas-liquid chromatography.
- For analysis, low amounts of samples are required.

Disadvantage:

- The detectors used in GC are destructive.
- The sample cannot be recovered.
- Limited to analyze only volatile samples.
- We cannot change the ratio of the mobile phase.

SIZE-EXCLUSION CHROMATOGRAPHY (SEC):

It is also called gel-filtration chromatography (GFC) or gel-permeation chromatography (GPC). In this technique, sample molecules are separated based on size and shape, which means molecules are separated from largest to smallest; the molecules with a larger size than the pores of the stationary phase will be eluted first, and smaller size molecules will be able to penetrate the pores of the stationary phase for a longer time, resulting it will be eluted later. Generally, that technique is applied to large molecules and macromolecular complexes such as industrial polymers and proteins [77- 78].

- If an aqueous solution is used to transport the sample molecules through the column, then it is known as gel-filtration chromatography.
- In case an organic solvent is used to transport sample molecules, it is known as gel permeation chromatography.

AFFINITYCHROMATOGRAPHY:

This is another category of liquid chromatographic technique that makes use of a mixture of chromatography and bio affinity, which makes use of biological-like interactions for the separation and specific analysis of sample components it is also known as "affinity purification." specific ligand/substrate/nucleic acid is chemically immobilized or "fixed" to a solid support in a column. It's called a matrix, and the material for the matrix should be chemically inert, have good flow properties, and have functional groups like Agarose, polyacrylic amide, polystyrene, cellulose, silica, etc. when a complex mixture (Enzyme/Receptor/Protein) run through that column, then the sample molecules which is having a specific binding affinity with ligand/Substrate/Nucleic Acid, become bound and unbounded molecules (Enzyme/Receptor/Protein) are washed away from the column, the molecules those are bounded with ligand/Substrate/Nucleic Acid, stripped from the solid support with help of Buffer with different pH/Ionic Strength, Competitive inhibitor, resulting purified specific molecule from a complex mixture Fig. 26 shows the separation of affinity chromatography[79-84].

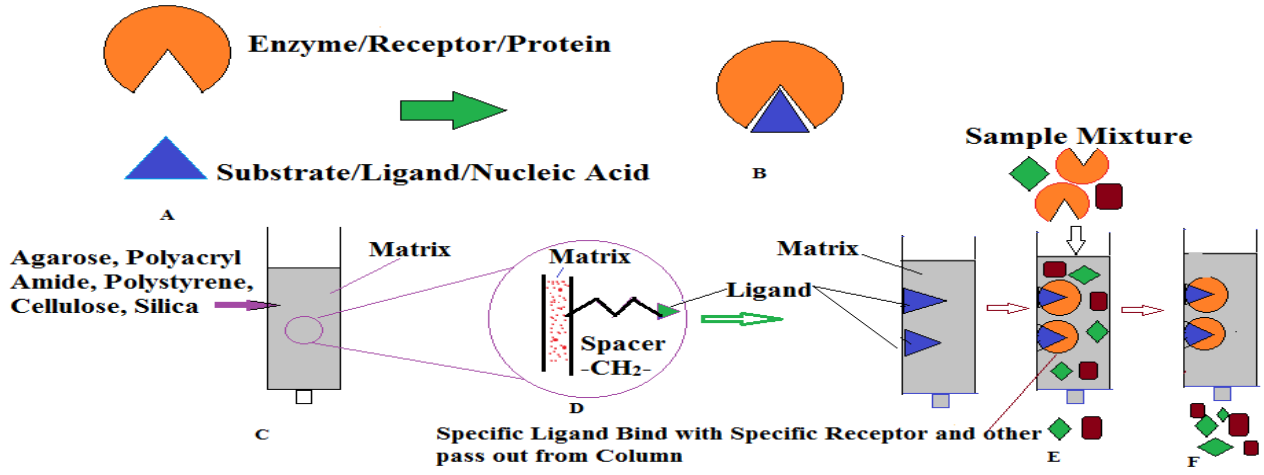


Fig. 26 (Affinity Chromatography flow of separation)

ION-EXCHANGE CHROMATOGRAPHY:

Ion-exchange chromatography is part of Ion-Chromatography, which is used for the separation and purification of ionic compounds like proteins, peptides, nucleic acids, polynucleotides, etc. Ions are made of a bond with attraction (positive and negative ions make a bond);
If two ions have similar charges, they will hate each other. The analyte ions; [anion (-) or cation (+)] are bound with a stationary phase, which is of two types: an anion exchanger stationary phase and a cation exchanger stationary phase. Fig. 27 shows the separation of ion exchanger chromatography [85-87].

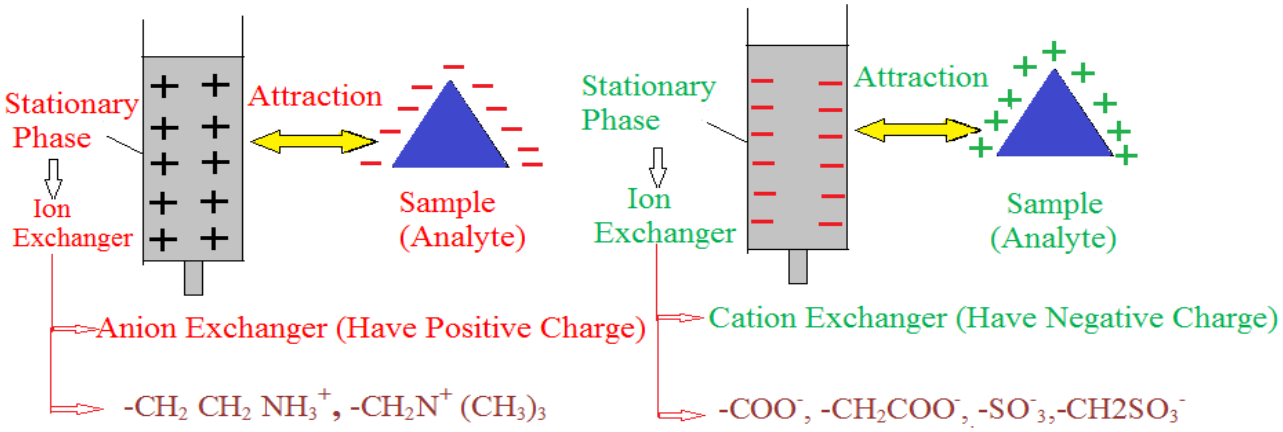


Fig. 27 (Ion exchange chromatography)

Anion exchanger stationary phase: The stationary phase contains a positive (+) charge function group, hence negative (-) ions attract with the positive charge stationary phase.
Cation Exchanger stationary phase: The stationary phase contains a positive (-) charge function group, hence positive (+) ions attract with the negative charge stationary phase [88-89].
Declarations Conflict of interest: For that Review Article no conflict of interest and there is no financial interest to report.
References:

1. Leslie S. (2003) Ettre LCGC (North America), M.S. Tswett and the invention of Chromatography, Milestones in chromatography, Vol.21, pp**.** 458
2. Mr.Gorhe SG. et al. (2018) A Review on High-performance Liquid Chromatography (HPLC), International Journal of Advance Scientific Research and Engineering Trends, Vol.3, Issue 1, ISSN: (Online)**.** 2456-0774
3. Braithwaite A. and Smith F.J. (1996) Chromatographic Method. 5thedn. Netherlands: Publisher by Kluwer Academic Publisher. pp-117-121
4. Hitachi. (2023) Technical Support, High-Performance Liquid Chromatograph （HPLC), Basic Course, Available athttps://www.hitachihightech.com/global/en/knowledge/analytical-systems/hplc/hplc-basics/course1.html#sec-1. Accessed in (2023).
5. L.S.ETTRE (1993) Nomenclature for Chromatography, Analytical Chemistry Division Commission on Chromatography And Other Analytical Separations Commission On Analytical Nomenclature, Department of Chemical Engineering, Yale University, New, Haven, CT 06520, USA. , Chem., Vol. 65, No. 4, pp. 825-827,
6. Ahuja Satinder(2003) Chromatography and Separation Science. Publisher by Academic Press. Elsevier Science (USA) Vol.4.pp-13
7. S. Harcum. (2008) Purification of protein solutions, Biologically Inspired Textiles.
8. Baraem Ismail (2010) Basic Principles of Chromatography, Department of Food Science and Nutrition, University of Minnesota, St. Paul, PP. N. 481-482. MN 55108-6099, USA
9. Veronika R. Meyer (2004) Practical High-Performance Liquid Chromatography.fourth edition, Adsorption Chromatography, British Library Cataloguing in Publication Data. ISBN 0-470-09377-3, ISBN 0-470-09378-1

10. eGyankosh, Ignou (2023) The people,s University, Unit-5 Liquid Column Chromatography, Method-1, Downloaded At https://egyankosh.ac.in/bitstream/123456789/43309/1/Unit-5.pdf. pp-36-37, Accessed in (2023).
11. Skoog, DA; Holler, FJ; Crouch, SR (2016) Principles of Instrumental Analysis, 7th ed. Belmont, CA. Thomson Higher Education. 28E , pp-766.
12. Jayesh M. Rajput, "et al" (2022) A Comprehensive Review on Advanced Chromatographic Techniques and Spectroscopic Techniques in Pharmaceutical Analysis, International Journal of Pharmaceutical Research and Applications, Volume 7, Issue 3, pp. 53-62, ISSN: 2456-4494, doi: 10.35629/7781-07035362
13. Ranjith Reddy Kondeti. "et al" (2014) an advantage in column chromatography: A Review, World Journal of Pharmaceutical Science, ISSN:2321-3310**.**
14. UCLA (2023) College chemistry & Biochemistry. Available at http://www.chem.ucla.edu/~bacher/General/30BL/tips/column1.html. Accessed in (2023).
15. OzlemCoskun, "et al"(2016) Separation techniques: Chromatography, North Clin Istanbul, Invited review, Department of Biophysics, CanakkaleOnsekiz Mart University, Canakkale, Turkey. Vol. 3(2) 156-60. doi: 10.14744/nci.2016.32757
16. Chrominfo (2023). About Science, Technology, Health, and Food. Available at https://chrominfo.blogspot.com/2018/10/advantages-and-disadvantages-of-column.html, Accessed in (2023).
17. Mary F. Striegel, Jo Hill (1996) Thin-Layer ChromatographyFor Binding Media Analysis, Scientific tools for conservation, pp-7-10
18. Elke Hahn-Deinstrop, E. (2006) Applied Thin-Layer Chromatography, 2nd ed. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany,ISBN: 978-3-527-31553-6, pp-1-8
19. Li Cai (2018) Thin Layer Chromatography. Division of Mathematics and Science, University of South Carolina. https://www.researchgate.net/publication/277703425_Thin_Layer_Chromatography,doi: 10.1002/9780470089941.et0603s08
20. Niamh Mac Fhionnlaoich, "et al" (2018). A Toolkit to Quantify Target Compounds in Thin-Layer Chromatography Experiments, Journal of Chemical Education, Department of Chemical Engineering, University College London, Torrington Place, London WC1E 7JE, U.K, ACS Publication: J. Chem. Educ. 95, 2191−2196, DOI: 10.1021/acs.jchemed.8b00144
21. E. Reich, A. Blatter (2005). Thin-Layer Chromatography Overview.Encyclopedia of Analytical Science (Second Edition)
22. C. F. Poole (2005), Thin-Layer Chromatography Principles.Encyclopedia of Analytical Science (Second Edition)
23. Pharmastuff4u(2023)Advantages and Disadvantages of Thin Layer Chromatography TLC, available at https://www.pharmastuff4u.com/2023/03/advantages-and-disadvantages-of-thin.html, Accessed in (2023)
24. What Is HPLC (2023) Advantages and Disadvantages of Thin Layer Chromatography, available at https://whatishplc.com/hplc-basics/advantages-and-disadvantages-of-thin-layer-chromatography/ Accessed in (2023)
25. Restek (2023) pure chromatography, Building up pressure on HPLC. Available at https://www.restek.com/row/chromablography/chromablography/building-up-pressure-on-hplc/#:~:text=Operating%20pressure%20limits%20for%20regular,fully%20porous%20particle)%20packing%20material. Accessed in (2023)
26. Abdu Hussein Ali (2022) High-Performance Liquid Chromatography (HPLC): A review, HPSI The open Access publisher, Chemistry Department, College of Natural and Computational Sciences, MekdelaAmba University, Ethiopia. doi: 10.29328/journal.aac.1001026
27. Sneha Lakshmi R.P (2015) A Review on Chromatography with High-Performance Liquid
Chromatography (HPLC) and its Functions: Research and Reviews: Journal of Pharmaceutical Analysis, Department of Pharmaceutical Analysis, Omega College of Pharmacy, Osmania University, Hyderabad, India. E-ISSN: 2320-0812
28. Shimadzu (2019) Excellence in science,Liquid Chromatography Mass Spectrometry (LCMS). First Edition: October, 2018. Accessed in (2023)
29. Mu. Naushad, "et al" (2014). Ultra Performance Liquid Chromatography Mass Spectrometry Evaluation and Applications in Food Analysis. History and induction of UPLC/MS, Boca Raton London New York, CRC Press (Taylor & Francis Group). PP.13, 9-11

30. Waters (2023), Chromatography system Available at https://www.waters.com/nextgen/in/en/products/chromatography/chromatography-systems/alliance-hplc-system.html. Accessed in (2023)
31. Waters (2023) Preparative Liquid Chromatography Primer Available at https://www.waters.com/nextgen/in/en/education/primers/preparative-liquid-chromatography-primer.html. Accessed in (2023)
32. Vaishnavi S. Kalamb (2022), HPLC Method Development, and Validation: A Review, International Journal of Advanced Research in Science, Communication and Technology Volume 2, Issue 2.doi: 10.48175/568
33. L. R. SNYDER (1979),Introduction to Modern Liquid Chromatography, Technicon Instruments Corporation Research & Development Division Tarrytown, New York, Second Edition, **A** Wiley-Interscience Publication
34. Klaus Unger (2007), Introduction to High-Performance Liquid Chromatography.
35. HPLC Basics (2023), Fundamentals of Liquid Chromatography (HPLC), Courtesy of Agilent Technologies, Inc. Available at https://polymer.ustc.edu.cn/_upload/article/files/23/46/8007190f4e29a2673dc0b5c292ba/P020110906263097048536.pdf. Accessed in (2023)
36. Zhimin Li, Paula Hong, Patricia R. McConville (2023). The Importance of Column Compartment Thermostatting and Preheating for Temperature Sensitive Separations in Liquid Chromatography
37. Orochem Molecular purification by design (2023) HPLC Column available at. https://orochem.com/product-category/chromatography/hplc-column/. Accessed in (2023)
38. USP48-NF43, General Chapters general Tests & Assays ⟨601⟩ To ⟨999⟩ Physical Tests & Determinations⟨621⟩ Chromatography. doi: https://doi.org/10.31003/USPNF_M99380_08_01.
39. Satinder Ahuja (2003), Chromatography and separation science, Volume -4, Amsterdam Boston London New York Oxford Paris San Diego San Francisco Singapore Sydney Tokyo, 2003 Elsevier Science (USA).
40. Agilent (2012) 1290 Infinity LC System Manual and Quick Reference, Edition-05/2012, Printed in Germany. Accessed in (2023)
41. Elements (2023) Crawford Scientific, Van Deemter equation https://www.crawfordscientific.com/chromatography-blog/post/van-deemter-equation-the-lockdown-guide. Accessed in (2023)
42. Scott, R.P.W (1996). Chromatographic Detectors. Design, Function and Operation, Marcel Dekker: New York.
43. Snyder, L.R.; Kirkland, J.J.; Dolan, J.W (2010). Introduction to Modern Liquid Chromatography, John Wiley and Sons: New York
44. Bhardwaj SK, Dwivedi K, Agarwal DD (2015), A Review: HPLC Method Development and Validation. International Journal of Analytical and Bioanalytical Chemistry 5(4): 76-81.
45. Sharma BK (2006) Instrumental methods of chemical analysis. (25th ed.); Goel Publishing House, pp. 286-385
46. Shodex TM (2023) Captures the science. Detectors for HPLC are available at https://www.shodexhplc.com/lessons/lesson-6-detectors-for-hplc/#01, Accessed in (2023)
47. Yury V. Timchenko (2021) Advantages and Disadvantages of High-Performance Liquid Chromatography (HPCL). Vol.8:10,Journal of Environmental Analytical Chemistry,ISSN: 2380-2391
48. HPLC (2023), Application available at https://whatishplc.com/hplc-basics/principle-and-procedure-of-hplc/ Accessed in (2023)
49. Chromatography (2023), Today, Difference between HPLC and UPLC, https://www.chromatographytoday.com/news/hplc-uhplc/31/breaking-news/what-is-the-difference-between-uhplc-and-uplc/30754. Accessed in (2023)
50. Ravsaheb H. Rathod, Suraj R. "et al"(2019) Ultra-high performance liquid chromatography-MS/MS (UHPLC-MS/MS) in practice: analysis of drugs and pharmaceutical formulations, Journal of Pharmaceutical Sciences.https://doi.org/10.1186/s43094-019-0007-8
51. Kharat Sachin Sadanand "et al"(2019). A Brief Review on Recent Advances in HPLC Techniques, International Journal of Pharmacy and Biological Sciences. doi: https://doi.org/10.21276/ijpbs.2019.9.3.164

52. Waters (2023). Beginners Guide to UPLC,The Promise of Small Particles Available at https://www.waters.com/nextgen/us/en/education/primers/beginner-s-guide-to-uplc/the-promise-of-small-particles.htmlAccessed in (2023)
53. S. Sridhar, "et al" (2023) UPLC - a dynamic and expeditious approach to Liquid chromatography, international journal of pharmaceutical, chemical and biological sciences
54. UKEssays.com (2023), Review of Ultra High-Performance Liquid Chromatography and High-Performance Liquid Chromatography, available at, https://www.ukessays.com/essays/sciences/review-of-ultra-high-performance-liquid-chromatography-and-high-performance-liquid-chromatography.php#:~:text=UHPLC%20uses%20high%20pressures%20for,UHPLC%20systems%20have%20concrete%20specifications. Accessed in (2023)
55. Swetha Sri, "et al" (2020) A Review on Comparative study of HPLC and UPLC, Research journal of pharmaceutical and technology. doi: 10.5958/0974-360X.2020.00284.X
56. RabiaBasharat, "et al" (2021) Ultra Performance Liquid Chromatography (Mini-Review), ORIENTAL JOURNAL OF CHEMISTRY, ISSN: 0970-020, CODEN: OJCHEG, 2021, Vol. 37, No.(4): Pg. 847-857. http://dx.doi.org/10.13005/ojc/370411
57. Bukirwa Irene Kamara (2010)Ethno Veterinary Botanical Medicine Herbal Medicines for Animal Health, Phytochemical Methods. London: CRC Press Taylor & Francis Group. New York. PP.57-58
58. Eric Stauffer, "et al" (2008) Fire Debris Analysis, Gas Chromatography, and Gas Chromatography-Mass Spectrometer, United States of America. Elsevier, Academic Press publications. 235-246
59. Dr. Deepak (2017) How is Gas Solid Chromatography different from Gas Liquid Chromatography?, Lab-Training.com, available at https://lab-training.com/gas-solid-chromatography-different-gas-liquid-chromatography
60. Easy Biology class (2023), Difference between Gas-Solid Chromatography and Gas-Liquid Chromatography: ComparisonsTable, Available at https://www.easybiologyclass.com/difference between-gas-solid-chromatography-and-gas-liquid-chromatography-comparison-table/ Accessed in (2023)
61. Madhu (2023) Difference Between Gas Solid Chromatography and Gas Liquid Chromatography available, at https://www.differencebetween.com /difference-between-gas-solid-chromatography-and-gas-liquid-chromatography/ Accessed in (2023)
62. Infinity Learn (2023), Partition Chromatography– Principle, Diagram, Types, and Applications, available at https://infinitylearn.com/surge/chemistry/partition-chromatography/ Accessed in (2023)
63. Britannica (2023), Partition chromatography, available at, https://www.britannica.com/science/partition-chromatography Accessed in (2023)
64. Mr.Shinde Ganesh S, "et al"(2021) A Review on Chromatography and Advancement in Paper Chromatography Technique, Asian Journal of Pharmaceutical Analysis, Vol.11, issue-01, ISSN:2231-5675(Online). doi: 10.5958/2231-5675.2021.00009.0
65. Topper (2023). Partition chromatography. Available at https://www.toppr.com/ask/content/concept/partition-chromatography-245013/ Accessed in (2023)
66. Mukta Gupta, "et al" (2018) Paper chromatography A: Review, Journal of Emerging Technologies and Innovative Research, Vol.5, Issue-10, ISSN: 2349-5162
67. MasarratMukadam, Saeed Chowdhry, Ayesha Karimji, Atafaraz Ansari (2021) Dimensional chromatography, International Journal of Pharmacy & Pharmaceutical Research, Vol.22, issue-2, 520-536.
68. Advanced biochemical instrumentation (BCH 582) the University of Nigeria, nsukka paper chromatography by ayolotumuyiwa Augustine paper chromatography, Available at, file:///C:/Users/omprakash.saini/Downloads/Paper_Chromatography.pdf. Accessed in (2023).
69. Pharmacy Study Material (2016), available at, http://rxpharmaworld.blogspot.com/2016/12/paper-chromatography.html, Accessed in (2023).
70. Daniel O. Omokpariola, "et al" (2019). Group Practical and Term -Paper on "Paper Chromatography" Research Gate, doi: 10.13140/RG.2.2.28961.33121
71. YouTube (2023), Radial Paper chromatography, Government Science College PardiDist, Valsad, Available at https://www.youtube.com/watch?v=Qz6Cr8kZLrw, Accessed in (2023).
72. Silvia Debenedetti (2009) TLC and PC in Isolation, Identification, and Characterization of Allelochemicals' Department of Biological Sciences, Faculty of Sciences, National University of La Plata, Calle 47 y 115, 1900 La Plata, Argentina.

73. Baraem Ismail (2010) Basic Principles of Chromatography, Department of Food Science and Nutrition, University of Minnesota, St. Paul, MN 55108-6099, USA. doi 10.1007/978-1-4419-1478-1_27
74. T.Yugandharudu "et al"(2012) A Review On Analytical Method Development And Method Validation, International Journal of Pharmaceutical Research & Analysis, Vol 2 / Issue 1, pp. 32-48, e-ISSN: 2249 – 7781
75. Slide share, Partition column Chromatography, available at https://www.slideshare.net/AsifPappu/partition-columnchromatography, Accessed in (2023).
76. Byju's, Column Chromatography, available at https://byjus.com/chemistry/column-chromatography/#:~:text=into%20discrete%20components,What%20is%20column%20chromatography%3F,phase%20and%20a%20mobile%20phase./ Accessed in (2023).
77. Agilent Technology Size Exclusion Chromatography for Biomolecule Analysis, Agilent Technologies, Inc. 2015 Printed in the USA, November 1, 2015 5991-3651EN Available at https://www.agilent.com/cs/library/primers/public/5991-3651EN_LR.pdf. Accessed in (2023).
78. MandawiTripathi (2022), Size exclusion chromatography is available at https://courseware.cutm.ac.in/wp-content/uploads/2020/06/size-exclusion-chromatography.pdf. Accessed in (2023).
79. Kenichi KASAI (2014) Proceedings of the Japan Academy, Series B, Physical and Biological Sciences Frontal affinity chromatography: A unique research tool for biospecific interaction that promotes glycobiology, University of Tokyo (Department of Biochemistry and Biophysics, Faculty of Sciences), Ser. B 90. doi: 10.2183/pjab.90.215
80. David S.Hage (1999) Clinical Chemistry, Affinity chromatography: A Review of clinical applications, 593-615
81. SamehMagdeldin and Annette Moser (2012) Affinity Chromatography: Principal And Application, Research Gate doi: 10.5772/39087 · Source: InTech
82. Pedro Cuatrecasas, Protein purification by Affinity chromatography, The Journal of Biological Chemistry, Volume 245, No.12, Issue of June 25, Thermo Fisher protein biology resource library handbook.
83. MajectaUrh, Dan Simpson, Kate zhao (2009) Methods in enzymology. volume 463, Pages 417-438, Chapter 26, Affinity chromatography: General methods. doi: 10.1016/S0076-6879(09)63026-3
84. Affinity Chromatography Available at https://www.youtube.com/watch?v=8_7cdfNO7OY, Accessed in (2023).
85. ÖzlemBahadirAcikara. Ion-Exchange Chromatography and Its Applications, Available at https://cdn.intechopen.com/pdfs/44033/InTech-Ion_exchange_chromatography_and_its_applications.pdf. Accessed in (2023). http://dx.doi.org/10.5772/55744
86. Bhattacharyya L, Rohrer JS (2012) Applications of Ion Chromatography for Pharmaceutical and Biological Products. New Jersey: John Wiley & Sons
87. Ion Exchange chromatography is available at https://www.youtube.com/watch?v=VOSkyj1dtbc, Accessed in (2023).
88. Dr. SK. Abdul Rahaman (2020) Ion exchange chromatography. Nirmala College of Pharmacyatmakur, Mangalagiri, Andhra Pradesh, India
89. Judith B. Ngere (2023) Ion-Exchange Chromatography Coupled to Mass Spectrometry in Life Science, Environmental, and Medical Research, and American Public Society-95-pp-152-166. https://doi.org/10.1021/acs.analchem.2c04298