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Learning Outcome of Module-IV

LO	Learning Outcome (LO)	Course Outcome Code
LO1	To understand the chromatographic separation and analysis of drugs.	BP701.1, BP701.4, BP701.6
LO2	To understand the chromatographic separation using HPLC	BP701.1, BP701.4, BP701.6
LO3	To understand the chromatographic separation using Gas chromatography	BP701.1, BP701.4, BP701.6

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

INTRODUCTION

The mobile phase in liquid chromatography refers to the solvent that is continuously applied to the column and stationary phase for solubilization and elution of analytes. When the liquid mobile phase passes through the column, analyte molecules interact with the mobile phase and solubilize in it at different time points. This results into separation of sample components by virtue of different physicochemical interactions with the stationary phase and mobile phase. These interactions depend upon the molecular size (as in size exclusion chromatography), charge (as in ion exchange chromatography), hydrophobicity (as in hydrophobic interaction chromatography), specific binding (as in affinity chromatography). The chromatographic process can also take advantages of several interactions simultaneously and employ a combination of these for higher separation efficiency. The mobile phase thus acts as a carrier phase for analytes. The component analytes elute from the column in a definite order depending upon the relative strengths of their interaction with the stationary and the mobile phase.

Throughout the chromatographic run, the mobile phase flows continuously through the column. The fractions consisting of different analyte components are collected and the concentration of each component in respective fraction is monitored by the detector over time and is read as a chromatogram.

Commonly used HPLC mobile phases are water, aqueous buffers, organic solvents or mixtures of organic solvents. While running the chromatography in an isocratic mode, the composition of the mobile phase is kept uniform throughout the analytical run. Whereas, while doing gradient elution, the mobile phase composition can be changed to obtain a pH gradient or a polarity gradient.

The mobile phases are selected on the basis of type of chromatographic modes-

Mobile Phases for Normal Phase Chromatography

In Normal Phase Chromatography non polar solvents are used such as hexane, heptane, iso-octane. The polarity is again adjusted by using slightly polar solvents like chloroform, isopropanol or ethyl-acetate in its combination.

Mobile Phases for Reverse Phase chromatography

In reverse phase chromatography water is commonly used as the solvent. The polarity of mobile phase can be adjusted by adding other polar solvents such as Methanol, Acetonitrile or Tetrahydrofuran, in fixed or varying ratio. For chromatographic separation and analysis of ionizable compounds, buffers and Ion-pairing reagents are added in the aqueous phase to increase retention.

Mobile Phases for Ion Exchange Chromatography

In Ion Exchange Chromatography, aqueous salt solutions or buffers are used as mobile phase. Desorption of analyte molecules takes place by increasing the salt concentration in a gradient manner or altering the pH. Since the solute molecules carry charge and reversibly bind to the stationary phase by displacing the counterions, they are eluted in the order of their strengths of binding; the most weakly bound substances elute out first and vice versa.

Mobile Phases for Size Exclusion Chromatography

The mobile phase is selected on the basis of its potential to dissolve the components of the sample and maintain its consistent viscosity at operating temperature. Ionic strength of the solvent is maintained by addition of salts. Highly polar solvents such as water, alcohols, acetone etc. are not used with polystyrene column packings.

Usage of Mobile Phases in HPLC

Only HPLC grade solvents are recommended to be used as mobile phases in HPLC. The solvents should be made homogenous by proper mixing and filters should be used in reservoirs. Degassing helps to prevent the bubble formation in solvent as it flows through the column. If buffer solutions are used as mobile phases, thorough washing should be done using water to prevent deposition of crystalline compounds. While performing gradient elution, the changeover of mobile phase polarity should be gradual and washing should be given using the solvent of intermediate polarity.

Solvent Delivery System

The solvent delivery system is an essential component of HPLC. It is meant to deliver the mobile phase to column with a constant and pulse-free flow. The efficiency of the system and reproducibility of retention time is directly affected by the flow rate. Therefore, the solvent

delivery system should function with high precision and accuracy. This requires the capability to maintain pressures high enough to force the solvent through compactly packed column. Also, if mixture of two or more solvents is to be used as mobile phase, then the solvent delivery system should be able to mix all solvents to give appropriate polarity.

The components of a Solvent Delivery System in a HPLC are:

1. Solvent Reservoir
2. Pump
3. Mixer
4. Dampner

Solvent Reservoir

The solvent delivery system in HPLC consists of a single or multiple solvent reservoirs usually made of glass. The mobile phase may be a liquid of single composition or a mixture of different solvents. The mixture may consist of polar and non-polar solvents mixed in variable proportions depending on the composition of the sample. Valves are provided to facilitate the solvent flow under high pressure and to allow rapid refilling of the solvent reservoir.

Degasser

Degassing is an important action performed prior to mobile phase delivery into HPLC system. Solvents remain in contact with the atmosphere and therefore essentially consist of an equilibrium level of dissolved air. When solvents are mixed, bubbles are formed due to dissolved air which can interfere with the pump operation and sensitivity of detectors. Also the solubility of analytes in air saturated solvents is lower as compared to pure solvents. Degassing of the solvent before its entry into pump can resolve all these issues.

The methods for degassing are-

1. Sonication-

Bubble formation often occurs when solvents are mixed in desired proportions to make appropriate mobile phase because the solubility of air is less in mixed solvents as compared to pure solvents. Bubbling can be prevented by keeping the mobile phase reservoir in an ultrasonic bath. The ultrasonic sound waves make the small bubbles to coalesce and allow them to escape easily. The excess gas is allowed to escape before it enters the pump,

1. Components of a Solvent Delivery System in HPLC

2. Vacuum Filtration-

Figure-2: Degassing of Mobile Phase by Sonication

Figure In this technique the solvent is filtered through a membrane under vacuum to eliminate bubbles and particulate matter if any. Although degassing of solvent occurs only once and soon the solvent starts equilibrating with air. This method is often combined with sonication for effective degassing. However, this technique is effective for premixed mobile phases and is not recommended for on-line mixing.

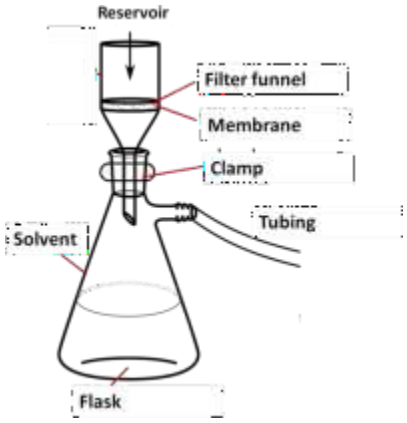


Figure-3: Degassing of Mobile Phase by Vacuum Filtration

3. Helium sparge-

In this technique, a stream of helium gas is passed through the solvent. Helium is insoluble in solvents and sweeps the dissolved air out of the solvent. This technique is very effective because it can lower down the dissolved air in common solvents below the saturation level of mixtures. However, if supplied under pressure, Helium sparging tends to evaporate the volatile components of the solvent mixture and slightly change the mobile phasecomposition.

4. On-line membrane degassing-

On-line membrane degassing is the most convenient approach for solvent degassing. It consists of a hollow fiber made of a semi-permeable membrane through which the mobile phase or solvent flows. A partial vacuum is maintained on the outside of the membrane. The principle is simple- since air can diffuse through the membrane but diffusion of solvent vapors is prevented, the dissolved air is removed from the solvent before it reaches the pump. The membrane degassing module is installed immediately upstream of the pump, into the inlet line,

so that re-dissolution of air into the solvent can be prevented.

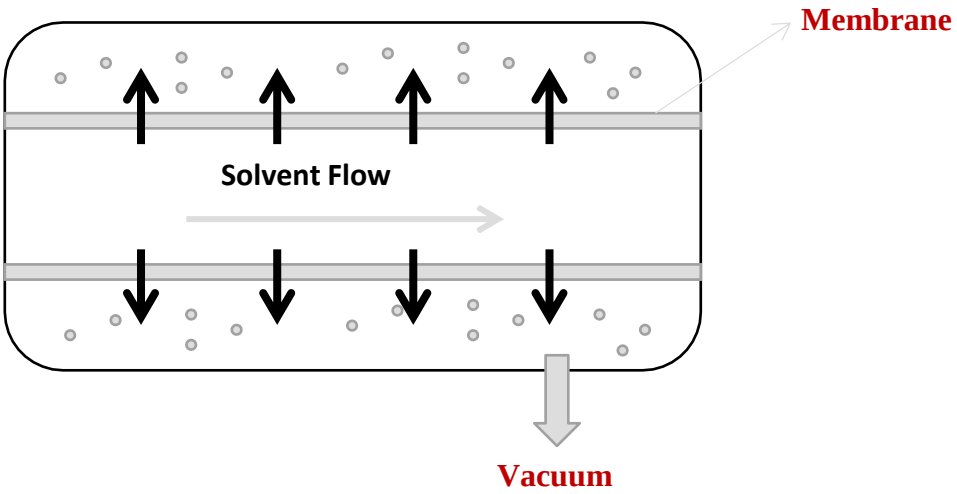


Figure-5: Degassing of Mobile Phase through Membrane

Pump

A pump is enabled to aspirate the solvent or mobile phase from the solvent reservoir and forces it with pressure through the injector, column and detector. The mobile phase has to pass through the stationary phase inside the column which may consist of a compact bed of adsorbant material or ion exchange resin, or a micro-porous packing material. This requires appropriate pressure to force the mobile phase through the compactly packed column and at the same time dissolve the analytes. The pump efficiently provides the operating pressures ranging upto 6000 psi, sufficient to overcome the resistance to solvent flow through the column.

As the solvent flows through the column, the components of the sample mixture equilibrate between mobile phase and the stationary phase and eventually separate on the basis of their solubility in the mobile phase. This equilibration requires sufficient time to occur that depends on the flow rate of the mobile phase. If the flow rate is too high, the analytes do not get enough time to equilibrate and may get eluted out from the column without separation. Conversely, if the flow rate is less than optimum, elution may take very long time. The separation of compounds is analysed through peaks appearing in a chromatogram at characteristic retention time. The retention time is strongly influenced by the flow rate, composition and volume of the mobile phase. The flow rate of mobile phase is a critical factor that directly affects the retention time and peak height in the chromatogram. The reproducibility of the chromatogram thus depends on the precision and accuracy of the flow rate. Therefore the pump system should

be capable of delivering a wide range of reproducible flow rates (commonly ranging from 0.1-10ml/min with reproducibility of 0.5%) and to cope up with the back pressures that arise due to fluid flow. Appropriate adjustment of flow rate enhances run-to-run reproducibility so that the chromatogram of sample can be analysed withrespect to the one of standards or calibrants.

There are three types of pumps that are commonly used in HPLC-

1. **Constant pressure pumps** – These pumps are connected to a low pressure gas cylinder and operate via gas pressure. The gas pressure is used to drive the pistons that further produce a consistent and continuous high pressure liquid flow. The solvent flow stops during the return stroke of pistons, therefore pulse damping is required to suppress the detector signal at this time.

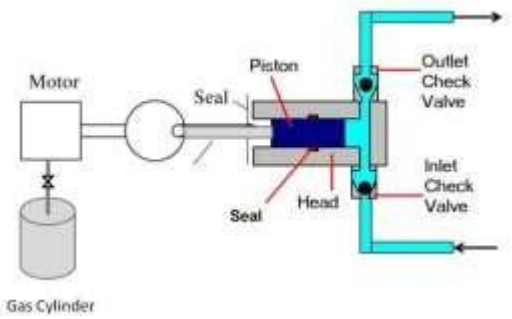


Figure-6: Constant Pressure Pump

2. ***Syringe Type Pumps-*** These are suitable for small bore columns and have limited solvent capacity. These are operated by an electrically driven lead-screw arrangement that moves a piston, which in turn generates the pressure enough to deliver a fixed volume of solvent to the column. The flow of solvent is pulseless and constant, and is not affected by column backpressure and viscosity changes, therefore these pumps are relatively reliable. Their drawback is that due to their limited solvent capacity (approx. 250 ml), solvent needs to be changed often and gradient operation cannot be accomplished.

3. **Reciprocating Piston pumps -** These pumps through reciprocating motion of a piston with in a hydraulic chamber. A motor electrically drives the piston back and forth; during the backward stroke the piston sucks in the solvent from the reservoir and during the

forward stroke it delivers the solvent into the column. The check valves are enabled that close the column outlet while aspirating the solvent in backward stroke, and close inlet from the reservoir during forward stroke. Flow rates can be fixed by adjusting piston displacement. These pumps are advantageous because their solvent delivery is smooth, constant flow rate can be achieved even at high pressure, supports gradient operation and no limitation of reservoir size or operating time. In case of minor pressure pulses, dampening may be required.

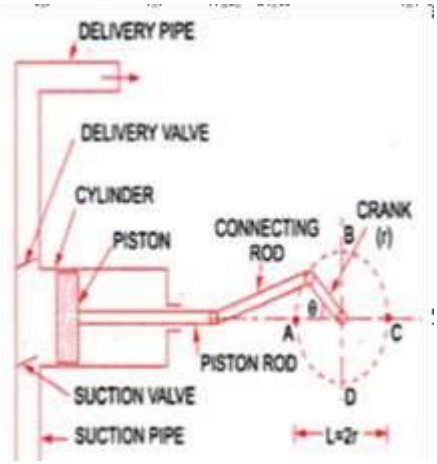


Figure-8: Reciprocating Piston Pump

Mixing of Solvents

The solvent delivery system must be capable of mixing solvents homogenously and vary polarity of mobile phase gradually. In an isocratic mode the solvent composition is kept constant throughout the run and the analytes elute out when a fixed volume of mobile phase volume has transited through the column. However, in a gradient elution, the mobile phase composition is required to change over the course of chromatographic run. Also the viscosity of the mobile phase may change due to change in its composition and the pressure may be required to adjust to maintain the required volumetric flow rate. The pump that delivers the mobile phase, can also be used to mix or blend the solvents. On the basis of automatic mixing of solvents, there are three types of pumps-

i) Binary pumps- Mix two solvents

ii) Ternary pumps- Mix three solvents

iii) Quaternary pumps- Mix four solvents

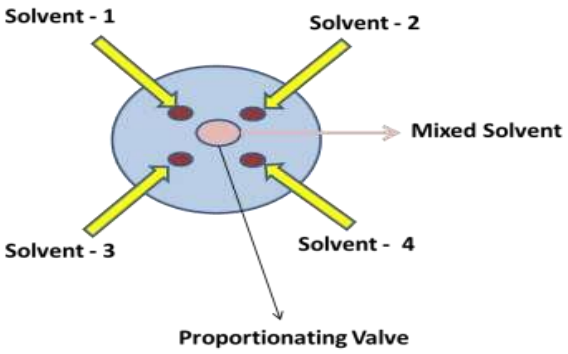


Figure-9: A Quaternary Pump

The mixing of solvents to prepare appropriate mobile phase can be done in any of the three ways:

- **Manual mixing-** it is the simplest way of mixing solvents in a precise manner, without using the pump.
- **Low-pressure mixing using a pump-** This is done by using a single pump with a proportioning valve to deliver the solvent.
- **High-pressure mixing using a pump-** In this system, two pumps are used to push
- thesolvents separately and the output liquids are blended by on line mixing

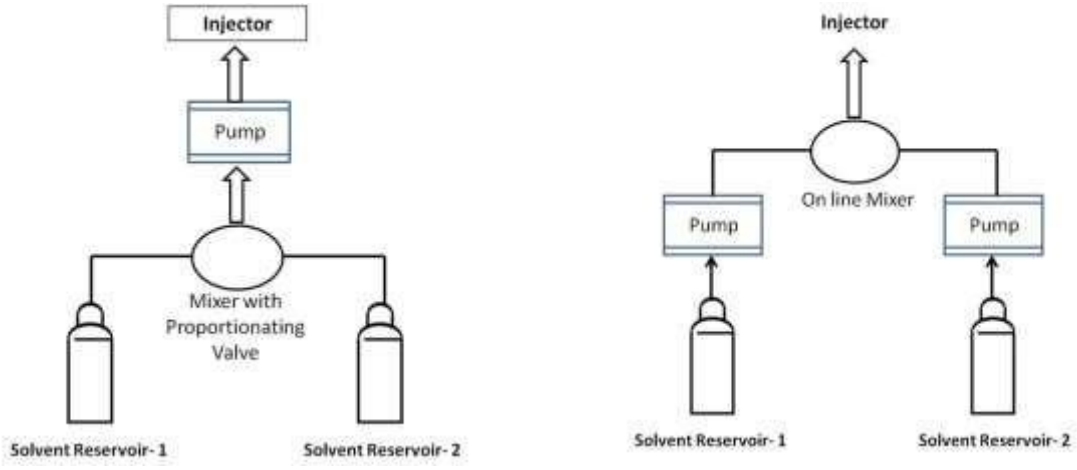


Figure-10: a) Low-pressure mixing pump; b) High-pressure mixing pump

The solvent delivery system is desired to have a low system volume so that the mobile phase changeover may be done rapidly with minimum volume. This is important in HPLC, firstly while using gradient mode, and secondly while optimizing a separation process.

In gradient elution mode, the volume or time required for the gradient to pass through the

HPLC system is known as system volume. In a chromatographic process that employs a mixture of two solvents as the mobile phase, initially both the solvents are discharged from the reservoirs separately and are mixed in a desired ratio before the mixture enters the column. The system volume refers to the actual fluid volume from the point the solvents are proportioned. In other words it is the volume between the point where the gradient is formed and the column inlet. It is also known as the delay volume or dead volume and affects reproducibility of gradients and throughput of a gradient separation. It also impacts the transfer of a gradient method from one system to another. The solvent delivery system in HPLC should be able to maintain the gradient volume at least equal to the gradient delay volume plus the column void volume.

Dampener

The solvent delivery system in HPLC is essentially required to deliver smooth, pulse-free flow of mobile phase through the column. Pumps often deliver a series of pulses in the flow of mobile phase. Pulsed flow of solvent influences the equilibrium distribution of analytes between stationary and mobile phase and affect the reproducibility of retention time of analytes. A stable baseline can be obtained in a chromatogram by controlling the pulses. Most of the detectors, particularly refractive index detector, are quite sensitive to pulses in the solvent flow and gather background noise, which is reflected in the chromatogram. Thus elimination of pulses also enhances precision and detection limits for trace compounds in a sample.

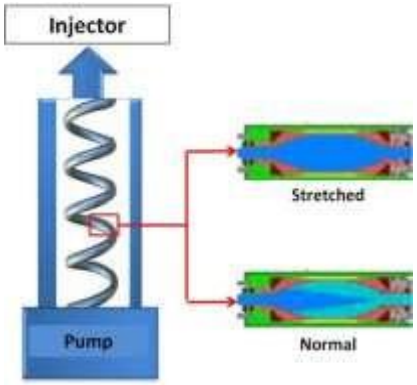


Figure-11: Pulse Dampener

Pulses in the flow of mobile phase can be eliminated by using dampeners. The simplest dampener consists of a large coil of narrow-bore tubing placed between the pump and the injector. When the pulsating flow is encountered by the dampener, the pressure rises in the

tube and the tube is stretched. Due to stretching, the volume of tube increases which adjusts the excess mobile phase, thus lowering down the pulsation. The tubular coil flexes on each stroke of the pump and absorbs the pulsation energy. As the pressure decreases, the volume of tube also decreases. Whole system works as a hydraulic accumulator and shock absorber, smoothening out the pulses in mobile phase flow..

Types of Sample Injectors

There are two basic types of sample injectors are –

1). Manual Injector Systems

Manual mode of sample injectors can be used in HPLC. These injectors transfer the sample from a syringe to a constant volume sample loop which is further connected to the high pressure stream of mobile phase through a valve, which allows the sample to enter inside the column. The valve is a six -port rotary injection valve, specially designed for this purpose. The sample is introduced at atmospheric pressure into the loop by a syringe. The loops are designed to take up a fixed volume of sample like 20µl, 50µl, 100µl and 200µl. When excess of sample is injected into the loop (for example 30 ul in a loop of 20µlcapacity), then only the stipulated volume (20µl) is passed into column and the excess is drained out.

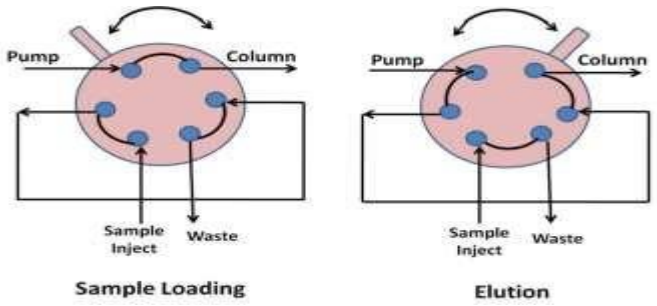


Figure-1: Manual Injector Systems

In manual sample injection, when the injector is in the LOAD position, the mobile phase flow is arrested, i.e. in the load position the sample loop is not aligned in the path of the mobile phase. At this stage thee sample is loaded into the loop. When the injector is turned into the INJECT position, the mobile phase passes through the sample loop and the sample in the loop is pushed by the mobile phase stream into the column. As a precaution, some amount of the sample is allowed to flow into waste from the loop, so that no air bubbles persist in the loop and there is no trace left of the previously used sample. The manual injection systems are generally used for the micro analytical methods.

2). Automatic injectors:

Most contemporary HPLC systems are now enabled with automatic injection systems consisting of auto samplers. The functioning of injection valve is similar to the manual valves, however, the sample is drawn by an automated syringe from sample vials placed in a mechanized tray.

These eliminate personal errors, provide high precision and accuracy and greatly enhance the productivity. Auto samplers also facilitate automatic injection of large number of samples in HPLC system. They also offer a high degree of injection volume precision and allow to introduce the required volume of sample accurately into the column.

The commercially available automatic sampling devices offer the advantage of analysis of large numbers of samples routinely. These are used for bulk analyses like quality control of pharmaceutical products, as they extremely beneficial for unattended operation with automatic data-handling.

Auto-samplers carry out the sample injection under pressure with the help of a motor which is required to push the sample against high back pressure in the column. Filling of the loop and delivery of the sample to the column with high precision is done through software programming. The sequence of samples for injection from vials is also controlled with the help of computer. For this purpose, the vials are numbered according to the sequence fed in the program. Mostly a 6- port loop injection valve is used to deliver the sample plug which consists of approximately 20 micro litres of sample.

While using an automatic sample injection system, certain precautions should be adopted to maintain the accuracy of the system and eliminate the errors. The vials should be number correctly on auto sampler rack and should be listed in the same sequence in the computer. The compatibility of the solvent should be checked and prime injection with the solvent should be done. In order to prevent carry over between injections, the needles and tubing should be thoroughly washed.

Components of an Auto- sampler

The components of an auto- sampler include:-

- Sample Vials & Needle
- Metering pump- to aspire the sample from the sample vial

- Sample loop
- Injection valve

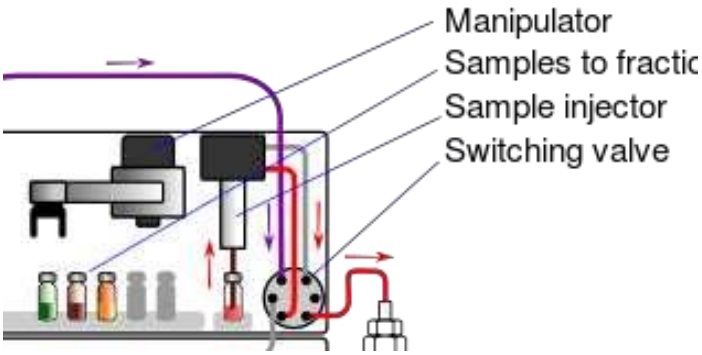


Figure-2: Components of an Auto- sampler

Sample vials & Needle

Autosamplers use a needle that penetrates the cap of a sealed sample vial. The needle is further attached to a flexible tube that helps to withdraw the sample from the vial by suction and passes it into the loop. After withdrawal of a sample and its injection into the column, the turn table rotates to position the next sample vial, which is now penetrated by the needle to draw next sample. After every use the needle, tubing and injector should be rinsed with wash fluid to remove traces of sample. All the operations like penetration, injection and rotation, are controlled through timers enabled in the auto-sampler. In order to avoid plugging of the needle and ensure reproducibility of sample volume for every injection, the samples should be filtered and made absolutely free from any particulate matter.

Metering pump

In most of the autosamplers, a metering pump is used to draw the sample through suction from the vials. It passes the sucked sample into an injection port, through which the sample is introduced through the loop. A piston metering syringe pump aspires the predetermined volume of sample from the vial and transfers it to a large loop in a six-port valve.

Sample Loop

The sample is transferred from the syringe to a sample loop which further transfers the sample onto the column under high-pressure mobile phase stream. The sample is injected into the loop when the loop is disconnected from the mobile phase flow path. After the sample is filled into the loop, it is connected back into the mobile phase flow path and the sample is

swept into the column for elution.

There are two modes of loading the sample in the loop:

1. **Complete filling/ Overfilling –**

In this technique the loop is overfilled with 3-5 times larger volume than the volume of the injection loop, for example, a 20 µL injection loop can be filled with 60 and 100 µL of the sample.

This is required because of two reasons:

i. The loop already contains the mobile phase. When the sample is loaded in the loop, it mixes with the resident mobile phase and gets diluted.

ii. While traveling through the loop, the velocity profile of sample fluid is parabolic i.e the velocity is higher at the center of the tube (about twice the average), while it is zero at the walls. In other words, the core of an injection plug travels faster than the peripheral fluid inside the loop.

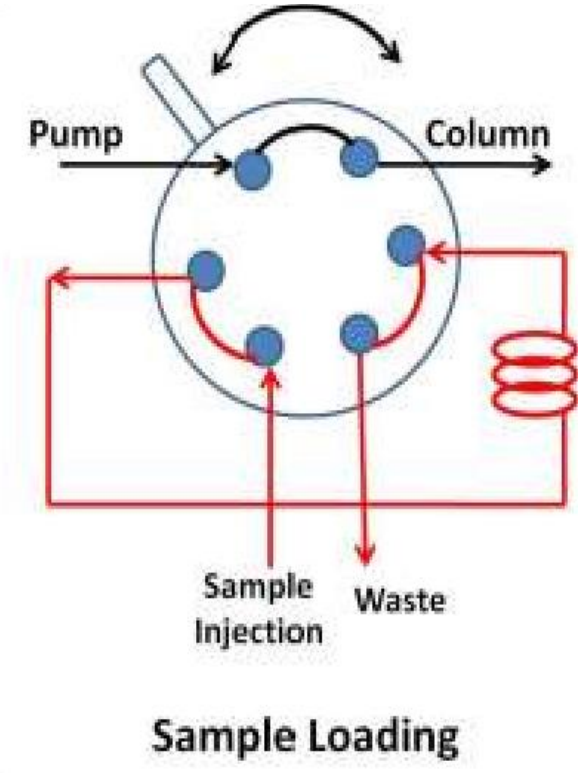


Figure-3: Complete-filling Mode

Therefore, in order to ensure that the sample remains homogeneous and dilution effect is minimized, the loop is overfilled with 3-5 times the sample volume. Although ample amount of sample is required for injection, this method maintains high degree of precision and linearity and gives reproducibility.

2. Partial-filling –

In this method the loop chosen is only partially filled with the sample and there is no wastage of sample. The same volume is injected onto the column to that dispensed from the syringe. The volume to be filled should not exceed 50% of the loop capacity, for example 50 µL of sample can be injected in a loop of 100 µL capacity. This method is useful when the amount of sample is limited, when the sample volume needs to be varied frequently, or when loop of absolute volume is not available for complete filling.

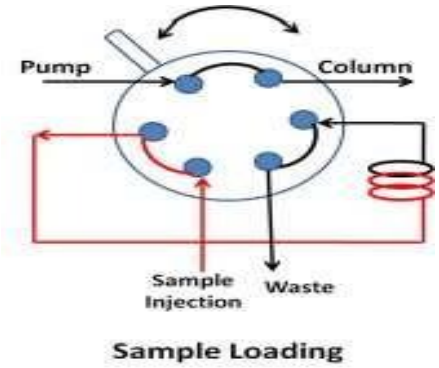


Figure-4: Partial-filling Mode

The sample is likely to get diluted with mobile phase during loading, and diluted sample occupies approximately double the volume of sample loaded from the syringe. In partial filling not more than 50% volume is occupied, due to which the diluted sample front is prevented from entering the column. This enhances the reproducibility and precision of the process. The sample volume that is to be injected onto the column is set by the syringe, therefore accuracy of dispensed volume is maintained.

Types of Injectors

On the basis of mode of sample loading in the loop, two types of injectors are available:

Dual mode injector: allow both partial- and complete-filling of the sample in loop. These are advantageous because as partial filling mode can be employed for varying the volumes.

Single mode injector: allow only complete-filling of the sample in loop and maximize the reproducibility of results.

Injection Valve

It plays the most critical role in delivery of the sample. It operates by switching between two configurations and controls the flow of solvent through the autosampler device. In one

configuration it allows loop filling and in the other configuration it allows to sweep the sample loaded in the loop to the column. The valve allows a definite sample volume to be pumped into the column.

The sample injection into the HPLC system is very important in an analytical process, and the quality and flexibility of the sample injection valve are therefore very important for reproducibility of data. Sample injection through valve arrangement allows unattended, fast and reproducible delivery of the sample. It also offers the advantage of injection a wider range of sample volumes (i.e. from 60 nl up to few ml), that too at pressures as high as 7000psi with negligible error (< 0.2%). If sample analysis is required at various temperatures, sampling valves can be kept within a temperature-controlled oven and another advantage can be derived for its usage.

Types of Autosampler Designs

There are three types of operational designs prevalent in an autosampler-

- Pull to fill autosampler
- Push to fill autosampler
- Integral-loop auto-samplers

Pull to Fill or Pulled Loop Auto Sampler:-

The sample is drawn or pulled into the injection loop through syringe suction with the help of a metering pump that is connected to the sample port of the injector. The sample is sucked through a needle that is penetrated into the sample vial and the plunger is drawn back to fill the loop with a definite volume of sample. The accuracy of volume is maintained by calibration of syringe to ensure the reproducibility of sample volume during its withdrawl from the sample vial. The needle is not included in the high pressure fluid path.

During 'inject' configuration, mobile phase flows from the pump into the column, through the sample loop. This type of orientation of the valve disconnects all other parts of the autosampler from the hydraulic path. The injection valve now has to load the sample. The syringe or metering pump pulls a definite volume of sample from the vial and passes it into the sample loop, in either the full or partial loop mode. The injection valve rotates again to direct the mobile phase through the loop effectively displacing the sample onto the analytical column. In this configuration, the other autosampler components are again disconnected from the

hydraulic pathway. After every delivery, the syringe and needle are rinsed with the solvent to eliminate any 'carry over' of the sample from one injection to the next.

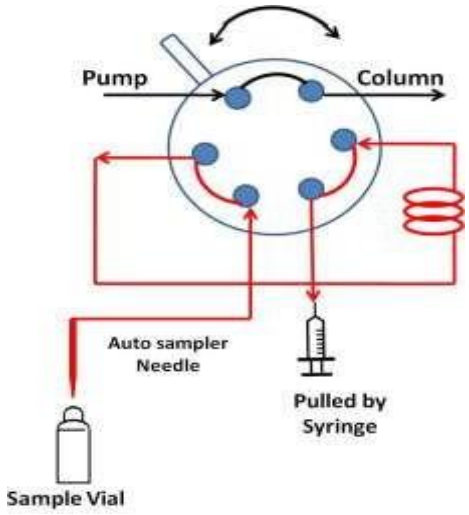


Figure-5: Pull to Fill or Pulled Loop Auto Sampler

Pull to fill autosamplers are advantageous due to their robust mechanical design that offer greater reliability. Being simple in design, they are comparatively inexpensive and have very low maintenance costs. Some drawbacks are however associated with these, i.e. considerable amount of sample (upto 100 microliters in some cases) is required for complete filling of needle and tubing before it reaches the sample loop. Excess of sample thus go wasted. Since the sample is drawn from the vial one after the other in mechanized way, the vials should be kept sequentially in the tray. Washing is required after every injection to eliminate sample carry over.

Push to Fill Auto Samplers:-

In 'push to fill' technique the syringe aligns on the sample vial and draws the desired volume of sample with the help of the metering pump. The injection valve switches the configuration and allows the mobile phase to flow directly from the pump to the analytical column. The sample content is then pushed by the metering pump into the sample loop. When the valve again changes the configuration, the mobile phase now flows through the sample loop into the column, and sample is also pushed into the column under the pressure of mobile phase flow.

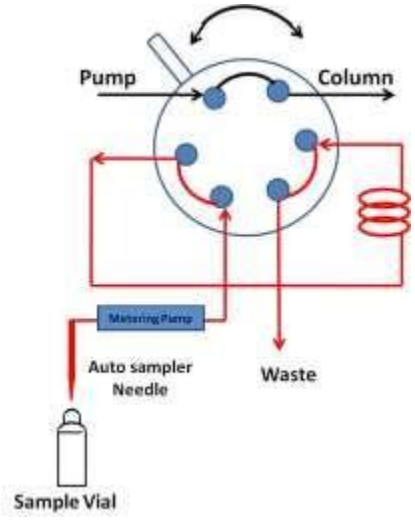


Figure-6: Push to Fill Auto Sampler

In push to fill design, sample loss is very less. A motor is enabled for filling and dispensing the sample from syringe, therefore error is also minimized to 0.5%. These autosamplers can be used for both the modes i.e. complete filling and partial filling modes. Adding further to the advantages, 'push to fill'autosamplers can access sample vials randomly, with variable number of injections.These can be used to inject a wide range of sample volumes, as less as 15 microlitresupto few mL.Thesecan be programmed for dispensing definite volume and can be washed off easily using the wash solvents.

Integral Needle Loop Auto-Samplers.:-

The integral needle loop autosamplers are used in most contemporary HPLC systems now days. The special feature of this system is that the sample loop and needle together form an integrated unit and operate as an in-line component of an autosampler, therefore it is also called direct injection system. The mechanism is similar to other autosamplers except that it includes a high pressure seal for introducing the sample into the column. The high pressure needle seal is connected to the rotor seal through a capillary tube. The eluent flow is directed from the pump to the analytical column, when the injection valve is in 'load' position and autosampler is disconnected from hydraulic path. The sample is drawn from the vial by the autosampler needle during this mode and filled into the integral loop with the help of metering pump. The needle again returns to the needle seal and the sample plug is dispensed from the loop into the analytical column, when the valve switches its mode and connects the autosampler components to the hydraulic path.

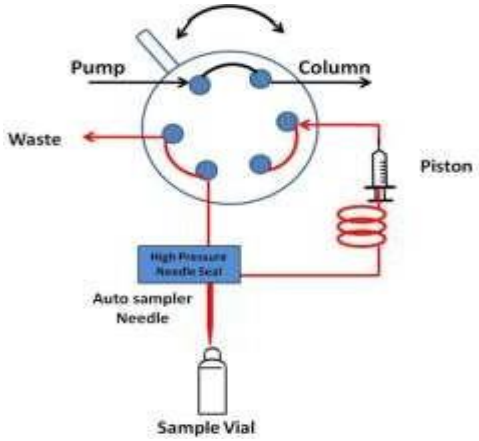


Figure-7: Integral Needle Loop Auto-Sampler

Several advantages are offered by integral loop auto samplers. Most importantly, in this mechanism sample is not wasted and is therefore very essential for trace analysis. The sample is contained completely within the portion of the loop that is swept and complete volume of loaded sample is injected into the column. The maximum sample volume that can be injected depends upon the integral loop size, usually it is 100 µL in most systems. Any amount of volume can be injected within this limit. For trace analyses or applications involving limitation of sample amount, it can be used for multi draw option that allows pooling of injection volumes of 100 µL, maximum upto 1,500 µL. This enables to increase the volume of sample for injection. The sample carryover can be reduced by washing of injection valve and loop with wash solvent after every delivery.

Another advantage is that it is flexible for selecting different sample volumes, different number of injections and can be programmed for accessing different sample vials. The flexibility of random access allows ease of analysis of variable number and types of sample as well as standards.

The disadvantage associated with integral loop system is that the high pressure seal require regular mechanical care and maintenance.

High performance liquid chromatography (HPLC) is a common and routinely used technique for the analysis of compounds in research laboratories and pharmaceutical industries. The main components of HPLC are columns and detectors which may be termed as the lifeline of this chromatographic separation procedure. The separation or purification of components from the

mixture occurs in the column. Detector is crucial for the analysis of any components that have been purified or separated. For efficient separation of analytes from a mixture of compound a suitable column and detector are needed. The choice for the column and detector is based on the type of method developed for the analysis and also on the type of analytes that are to be purified and detected.

1. Columns

In chromatographic separation, column is the component where the actual separation of analytes occurs. The capacity of columns and its nature determines the types of column that can be used for the separation or purification of analytes. Columns form an important part of high performance liquid chromatography for it is responsible for the separation of the mixture components. The sample that is to be separated or purified is introduced into the column along with the mobile phase and the separation of components takes place in the column. After their separation these components elute at different intervals depending on their binding interactions with the column matrix. The matrix in the column is the stationary phase which is usually made up of silica gel. Silica is a preferred choice due to its porosity and particle size. Also, silica being an inert material doesn't react with the mobile phase. HPLC columns have different lengths ranging from 30 mm to 250 mm and have porosity from 3-5 microns.

Efficiency of a column

The efficiency of a column in HPLC depends on some factors or parameters. These are:

- Length of the column
- Porosity of the column
- Mobile phase
- Life of Column

It is important to evaluate the column performance after every 1000 runs or as required. There are circumstances when the column may not be performing up to its prescribed standard of purification. This may be due to the clogging or contamination of the column or the column packing may have been compromised. In such cases, column needs to be replaced or their packing material has to be changed.

Principle of separation

The column in HPLC is filled with very small particles (also called gels) which interact differently with each sample component due to different binding capacity of each component.

This interaction leads to different retention times of each component inside the column. Thus, using these time differences, the components can be separated. However, in some situations the components are not properly resolved. In such a scenario, if we change some parameters such as temperature, composition of mobile phase, pH or the column, then it may lead to proper separation of components.

Types of columns

The columns used in HPLC can be classified on the basis of the purification protocol or the principle on which the separation of analytes occurs. These columns are classified as:

- Normal Phase Column
- Reverse Phase Column
- Ion exchange Column
- Size Exclusion Column

Normal Phase Column

The stationary phases of these columns are more polar than the mobile phase. This implies that the stationary phase is made up of a polar compound whereas the mobile phase has comparatively less polarity. The most commonly used polar compound for the stationary phase is the silica. Silica based columns are routinely used for HPLC in pharmaceutical industries. When normal phase columns are used, the mobile phase used is usually a mixture of methylene chloride, hexane and chloroform or a mixture of these with diethyl ether. The mobile phase compostions mentioned above are less polar than silica; hence the separation of analytes occurs on the basis of polarity. Compounds which are more polar remain in the polar stationary phase and are separated from less polar components which are eluted along with the less polar mobile phase.

Reverse Phase Column

The second type of column used in high performance chromatography is the reverse phase column. As the name implies, it is the reverse of normal phase column or the stationary phase is less polar compared to the mobile phase. The stationary phase is usually made up of bonded hydrocarbons such as C8 or C18 or non polar hydrocarbons. Here, the mobile phase used is aqueous organic solutions for e.g water-methanol or water-acetonitrile mixture. The separation of compounds is on the basis of polarity. The difference in using a normal phase column and a reverse phase column is that although the separation is on the basis of polarity in case of

reverse phase columns the polar compounds are eluted first whereas the less polar components are adsorbed on to the less polar column and elute later.

Ion Exchange Column

The third commonly used column in high performance liquid chromatography is the ion exchange column. The basis of separation or purification using these columns is the interaction between the charged column and the net surface charge of the ions to be separated. Therefore, those compounds which can be easily ionized are analyzed by ion exchange column. The stationary phase is a resin or matrix on which charged functional groups are present. If the column is positively charged then it is called anion exchanger and if negatively charged then it is known as cationic exchanger. The analytes on the basis of their charge are adsorbed on the stationary phase due to electrostatic intercations between the charged column and the sample ions. Since this type of HPLC involves exchange of ions during the purification process, it is known as ion exchange chromatography. The mobile phase for these columns comprises of buffers which may have additives such as salts, detergents, zwitterions, organic etc or organic solvents .

Size Exclusion Columns

In size exclusion column the separation of components is on the basis of their size. The stationary phase in these columns is a combination of polymers such as polysaccharides and silica. The small sample molecules penetrate deep into the stationary phase and are trapped while the large molecules remain loosely adsorbed or bound to the stationary phase. When the mobile phase is passed over this column, the loosely attached large molecules elute first. Since the elution is based on the size of molecules this is known as size exclusion chromatography. These columns are usually not preferred for the analysis of pharmaceutical compounds as the separation is on the basis of size only.

2. Detectors

Detector is an important component of a chromatograph which detects the analytes or the components of a mixture that have been separated or purified from the column. It senses the presence of the analyte when it elutes from the column and converts it into an electrical signal which is recorded by a data system or connected to a digital display board such as computer where the resolution of peaks can be observed.

An ideal detector for high performance liquid chromatography should have the following

properties:

- Highly sensitive and give fast response
- Good stability and reproducibility of the data
- Low dead volume: This minimizes the peak broadening
- Low drift and noise level: Low drift and noise levels helps in detection of analytes that are present in minute or trace quantities

Types of detectors in HPLC

- Absorbance Detectors
- Refractive Index Detectors
- Evaporative Light Scattering Detectors
- Multi Angle Light Scattering Detectors
- Mass Spectrometer Detectors
- Conductometric Detectors
- Fluorescence Detectors
- Chemiluminescence Detectors
- Electro Chemical Detectors
- Optical Rotation or Chiral Detectors

Absorbance detectors

The ultra violet, visible and photo diode array detectors are known as absorbance detectors as they absorb wavelength for the determination or detection of the analyte. These detectors are highly sensitive for light-absorbing compounds at very small quantities (nanogram and picogram levels). The main advantage of using these detectors are: they are easy to operate, handle and provide reproducible data.

Amongst the absorbance detectors, the most commonly used detector in HPLC is UV detector.

UV detector

In HPLC system having UV detector, during the chromatographic analysis, the sample goes to a clear and colorless flow cell. Now the UV light is irradiated on this sample solution which absorbs a part of UV light thereby causing a change in the UV intensity. Now, the intensity of mobile phase without the sample is also measured. There will always be a difference in observed UV intensity for the mobile phase without the sample and the sample solution. This

difference is measured to determine the amount of sample. UV absorbance by an analyte depends on the wavelength used hence it is important to choose an appropriate wavelength for analysis. The wavelength is selected on the basis of component or analyte that needs to be examined. The wavelength used in a standard UV detector usually ranges between 195 to 370 nm with 254 nm being commonly used.

Visible detector

A visible detector uses longer wavelength i.e. from 400 to 700 nm for detection as we know that in the spectrum range of visible light is from 390 to 700 nm. Some companies provide HPLC instruments having a detector whose detection range of wavelength is from 195-700 nm thereby coveing both the ultraviolet and visible ranges.

Photo diode array (PDA)

This detector detects an entire spectrum simultaneously. UV & VIS detectors use two dimensions to analyze the data. These dimensions are light intensity and time. However, PDA adds another dimension to light intensity and time which is the wavelength. This helps in determining the most suitable wavelength without repeating analyses. As a result, it saves time and is more efficient.

Refractive Index detector (RI) Detector

This detector measures the change in the refractive index of incident light when it passes through the sample and the mobile phase present in a flow cell. In this detector, the flow cell or the glass cell is divided into two chambers. These two chambers are known as reference cell and sample cell. The sample cell contains the eluent i.e sample present in mobile phase coming from the column whereas the reference cell contains only the mobile phase. Now, when there is no separation of analytes has occurred the sample cell does not contain any analyte hence the solvent inside both the sample and reference cell are the same, as shown in the figure A. Therefore when the light beam is incident on these cells there is no refraction or bending phenomena so the observed beam will be straight in this case. However, in case the eluent contains any components other than the mobile phase bending or refraction of the incident beam occurs at the surface of the two cells due to the difference in reflex index between the two solvents, as shown in figure B. By measuring this change in reflective index, the presence of analytes can be determined.

RI monly used as they have a lower sensitivity compared to the UV detector. Although RI detectors are less sensitive than UV detectors, they have certain advantages over UV detector. It can be used to detect all types of components. There are substances such as sugar, alcohol, or inorganic ions that do not have an UV absorption spectra and cannot be detected by UV detectors. However, all samples cause change in reflective index and therefore, RI detectors can be used to measure all such types of samples or analytes.

(i) During HPLC sometimes the mobile phase used may absorb UV light hence the detection of analytes in such mobile phases cannot be done using UV detector. In such cases RI detector can be used.

(ii) In RI detector the observed intensity is directly proportional to the concentration of the analytes. However, the amount of UV absorbed by an analyte which is in the form of peak absorbed will not give the concentration of analytes. Owing to these advantages, RI detector is preferrably used for the detection of sugars and for size exclusion chromatography analysis.

Evaporative Light Scattering Detector

This detector is highly specific and sensitive for non-volatile analytes at nanogram levels. In this, the eluent coming out from the column which contains analyte is nebulized and then evaporated to form fine particles. Then a laser beam is irradiated on these fine particles resulting in scattering of light. The intensity of the scattered light is measured to quantify the analytes. This detector provides a sensitive detection with stable base line. The samples that are used for detection by this detector include lipids, sugars, and analytes having high molecular weights.

Multi-angle Light Scattering Detector

Multi-angle light scattering detector is commonly used in size exclusion chromatography. This detector helps in determining an absolute molecular weight of the analyte even if the analyte is present in trace quantity. There is no need of using a calibration curve using a set of known standards for determining the molecular weight of analyte as is routinely done in size exclusion chromatography.

Mass Spectrometer Detector

This detector considers the mass to charge ratio (m/z) for an analyte to determine its identity. There is a low volume cell present in this detector where the eluent moves into from the column and the analytes are detected on the basis of m/z ratios. The obtained results also help in determining the analyte structure. This detector is highly sensitive and can detect the presence of components present in traces.

Conductometric Detector

Another type of detector used in HPLC is the conductometric detector. This is commonly used in ion exchange chromatography. Ions present in solutions conduct electricity due to the charge present on them. This detector measures the resistance developed due to the electrical charge and this resistance can be used to calculate the concentration of ions present in the sample solution. In this detector, two electrodes are arranged in a Wheatstones bridge present in a sensor cell. When the sample ions move into the sensor cell, the electrical resistance is calculated through an electric circuit which is detected by the detector. This output is linearly proportional to the sample ion concentration.

Fluorescence Detector

A commonly used detector in pharmaceutical industries is the fluorescence detector. This detector is highly sensitive for some selective groups of compounds even at femtogram levels. Here, a specified wavelength is used to excite the atoms of an analyte and this excitation results in a fluorescent light signal which is recorded and analyzed to quantify the samples. Most of the pharmaceuticals, natural products, clinical samples, and petroleum products are detected by this detector. There are some compounds which do not emit florescence light when excited so for carrying out the detection of these compounds they are tagged with fluorescence derivatives such as dansyl chloride and quantified. The main advantage of this detector is that it is easy to operate and gives repeatable results.

Chemiluminescence Detector

It is a type of detector which is similar to florescence detector, but instead of using a light source to excite the atoms, the excitation is carried out by a chemical reaction, hence the name chemiluminescence. Luminescence is a phenomenon where a compound or material emits light of a specific wavelength without emitting heat. The emission of the matter occurs due to absorbance of energy from heat, electrical field or chemical reaction. When the source of the external energy is a chemical reaction, then this phenomenon is known as chemiluminescence. As this detector is not relied on the external excitation source, the noise is small resulting in high signal to noise ratio thereby providing a higher sensitivity than the

florescence detector. When the eluent flow out of the column it is mixed with a luminescence reagent in a mixer. When the luminescence reaches its peak intensity it is measured by a photomultiplier tube and sent to the chemiluminescence detector.

Electrochemical Detector

Another type of detector used in HPLC is the Electro chemical detector. Electrochemical detector is based on the measurement of electrical current that results from an oxidation or reduction reaction of an analyte occuring at the electrode. The level of current is directly proportion to the concentration of analyte hence is used for the quantification of the analytes. Since the reaction occurring at electrodes involves oxidation/reduction reactions for accurate measurements of the the analyte concentrations pH adjustments are needed. Also, the eluent containing analytes must be pure as the presence of halides or metal contamination may result in background current. This background current can cause a drift in the base line of the chromatogram. They are highly sensitive and can detect analytes in the picomole range. They are simple and convenient to use and have a wide-spread applicability. This detector is usually beneficial for detection of phenol, catecholamines, nitrosamines, and organic acids. There are several different types of ECs.

Types of Analysis

Qualitative Analysis

HPLC is utilized for the recognition / identification (ID) of respective sample compounds and the most frequent parameter for compound ID is found to be its retention time (the time it proceeds for that precise compound to elute after injection from the column). The recognition of compounds depends on the chemical structure, detector used and molecular weight or few alternative molecular parameters.

Quantitative Analysis

HPLC helps in the estimation of the compound amount in a sample (concentration). For accomplishing a quantitative evaluation of the compound, a sample with a familiar amount of the compound of interest is infused and measurement of its peak height or peak area is taken. Hence, the two foremost aspects to enact a chromatogram i.e. perform quantification are specified:

1. To determine peak height of a chromatogram as deliberated from the baseline

2. To determine peak area of a chromatogram.

In innumerable cases, there is a linear (straight) relationship between the area or height and the amount of sample.

Preparation of Pure Compound(s)

A purified form of substance can be prepared for subsequent use by compiling the chromatographic peaks at the outlet of the detector and concentrating the compound (analyte) by eliminating or evaporating the solvent, - (e.g. clinical studies, toxicology and organic synthesis studies etc.). This methodology is termed as preparative chromatography.

Trace analysis

The estimation of trace compounds is vital in pharmaceuticals, biological, environmental and toxicology studies. A compound is termed as trace compound if its concentration is less than 1% by weight, generally ppm (parts per million) or lower but is of great interest to the analyst. Sensitive detectors and high resolution separations are used in HPLC as the trace elements are very difficult to separate or detect.

Applications of High Performance Liquid ChromatographyPharmaceuticals

High-performance liquid chromatography (abbreviated as HPLC or simply LC) is a method of choice for assessment of a huge variety of samples in the pharmaceutical industries. It is used throughout the process of designing a new drug from assessing new formulations, scrutinizing purity of new chemical subsistence, auditing changes in synthetic procedures or extent up to bearing out quality control of the eventual drug product.

Common applications in pharmaceutical analysis are:

- To regulate drug stability.
- Tablet dissolution application of pharmaceutical dosages form.
- To control quality of Pharmaceuticals: HPLC is used to see whether the prepared and manufactured drugs or pharmaceutics are according to the standards laid down by the regulating bodies of drugs like pharmacopoeia and others. When particularly specified HPLC solvents and mobile phases are utilized it will give an idea of how the peak of the drug will look in the formulation to be determined.
- To determine the shelf life of pharmaceutical products.

- To recognize the specific constituent or molecules in the mixture and for bio availability investigations etc. After the preparation of the specific formulation, the discharge of the drug during a particular period of time is analyzed for bio availability investigations.

- To evaluate the concentration of plasma, metabolic profile etc of chemical moieties or formulation during development or preclinical trials. The analysis is done to examine their blood concentration after certain periods of administration.

  - Quantification of drugs in biological samples.
  - Forensic analysis of textile dyes.
  - Analysis of dyes in lipstick smears
  - Determination of drugs like cocaine which abuse in blood, urine etc.
  - At parties, a mobile HPLC is used to identify and quantify drug ecstasy.
  - Steroids promoting growth can be determined by HPLC technique in sweat, hair, urine and serum.

- Analysis of purines and pyrimidines from urine, plasma etc.
- Assessment of corticoidsids from plasma in disorders of adrenal gland which secretes an endocrine hormone.
- Assessment of antibiotics.
- In the patients having liver cirrhosis, estimation of changes in the excretion of aquaporin 2 in the urine.
- Analysis of bilrubin, biliverdin in hepatic disorders.
- Disclosure of Neuropeptides which are endogenous in extracellular fluid of brain etc.

Petrochemicals

Bioactive natural products

- Purification of polyphenol and caffeine from tea (*Camellia sinensis*), maize, petunia etc by HPLC
- Purification of flavonoids
- Purification of alkanoids from tea (*Camellia sinensis*)
- Tea, Maize and Petunia are known to be rich in polyphenolic compounds. In this way, the point by point convention for the purification of compounds from these plant has been shown in procedure.

Advantages of HPLC

- High sensitivity : Evaluation of samples with minute concentrations (nano-gram and pictogram)
- High Precision and resolution: Detect similar molecules very firmly.
- Highest accuracy: Identification of components of complex mixtures.
- Severely fast and efficient: The procedure can be concluded in approximately up to 10 to 30 minutes.
- Highly reproducible
- Highly versatile
- Predominantly automated: Basic HPLC runs can be operated with minimal training.
- Provides management of data, safeguard features, reporting and instrument validation.
- Dynamic and compliant
- Expands production by administering all the areas of assessment - from sample to instrument, and from segregation to reporting results.
- Continuous monitoring of the column effluent
- Ion exchange, adsorption, exclusion and partition column separations are extremely outstanding.
- Minimum or no sample pre treatment required for evaluation of both aqueous and non aqueous samples.
- There is extreme property of selectivity for typical analysis due to specific solvents and column packing available easily.
- Multiple components can be determined in a single analysis.
- HPLC is not only restricted to volatile and thermally labile analytes due to wider choices of mobile and stationary phase. This advantage rendered HPLC more beneficial than GLC.

Disadvantages of HPLC

- HPLC can be high priced, demanding huge quantities of expensive organics.
- It is comparatively simple to use existing HPLC methods but due to design of various modules, columns and mobile phases can be complex to troubleshoot problems or to establish new methods.
- HPLC have lesser sensitivity for certain compounds. Some compounds cannot be determined by HPLC as they are irrevocably adsorbed. For example volatile substances are best segregated by gas chromatography.

GAS CHROMATOGRAPHY

Gas chromatography is an analytical technique which is helpful in separation of constituting components of a mixture thereby providing the information about their amount and molecular composition. The output is in the form of a chromatogram detailing the heights and the areas of resolved peaks.

1. THEORY AND PRINCIPLE

In GC, retention of sample analytes occurs due to higher interaction of analyte molecules with stationary phase than the mobile phase. Thus interaction of analyte molecules with stationary phase plays an important role in separation of sample components. This separation of sample into its constituent components occurs in the GC column. Depending upon the application type, columns differ in their length and internal diameter. Columns used for an application can be either packed (columns are packed with a solid support that is coated with immobilized liquid stationary phase) or capillary (these are hollow silica tubes in which inner wall is coated with immobilized liquid stationary phase of varying thickness). The rate and degree of partition of sample component depends on the chemical affinity of the analyte towards stationary phase and analyte vapor pressure, which in turn is governed by column temperature. During standardization of the process, the parameters such as chemical affinity of the analyte towards stationary phase and analyte vapor pressure are manipulated either by changing the chemical nature of the stationary phase and the column temperature.

In Gas Liquid Chromatography (GLC) the mobile phase is a carrier gas, which is inert in nature like helium, hydrogen or nitrogen. In most of the instruments Helium is used as carrier gas but for better separation hydrogen is the preferred gas. Stationary phase in GLC is a microscopic layer of liquid inside the column made up of metal or glass. The liquid material used for stationary phase have high boiling point like silicone grease or wax. To obtain better results and for maximum column efficiency, there should be optimum flow rate of mobile gas phase. Components of the volatilized sample interact with the stationary phase and elute out from the column at varying intervals depending upon their retention time. Every compound has a different retention time and thus some elute out faster while few elute slowly at later stage. Separation of components are achieved by a series of partitions between moving gas phase of sample and stationary liquid phase held in the column. The basic principle in Gas Liquid Chromatography (GLC) is that it exploits the differences in partition coefficients of volatilized sample between mobile gaseous phase and stationary liquid phase as the samples passes through the column.

Partition Coefficient (Distribution Coefficient) KC measures the affinity of an analyte to be attracted to the stationary phase.

$$KC = [CS]/[CM]$$

Where CS = concentration of analyte in the stationary phase CM = concentration of analyte in the mobile phase

Large KC values results in extended retention times of analyte. The value of KC can be controlled by column temperature or chemical nature of the stationary phase. The use of this technique is confined to analytes that are volatile and thermally stable. In this process of separation, highly volatile analytes elute out first thereby marking the inverse proportionality of partition coefficient and volatility of analyte.

Retention factor "k", sometimes called as capacity factor, is used to describe the migration rate of an analyte onto a column. Retention factor for analyte A is

$$kA = (tR - tM)/tM$$

Where tR is the retention time (time between sample injection and generation of analyte peak when sample reaches detector at theend of the column)

tM is the time taken for mobile gaseous phase to pass throughthe column

tR and tM can be obtained from a chromatogram. Ideally, retention factor for an analyte should be between one and five. Lower the retention factor, faster the analyte elutes out and high retention factors like greater than 15, mean that the elution will take very long time.

The liquid layer generally have low volatility and high decomposition temperatures like polyethylene glycol, dimethyl silicone, diethylene glycol succinate (DEGS) etc. The non-volatile liquid coated along the inner wall of capillary tubing or on the inert solid support as a thin layer helps in partitioning of the sample components between stationary phase (liquid layer) and mobile phase (carrier gas). The solid support used in GLC includes materials like glass powder, diatomaceous earths, powdered Teflon, crushed firebricks, carbon black etc.
The technique is highly sensitive and has high speed of resolutions. It not only determines what type of chemical is present in the mixture but also tells the quantity of each chemical in the mixture.

Basic Concepts involved in Gas Liquid Chromatography involves

1. Concept and structure of capillary column (Open Tubular Column)
2. Concept of Carrier gas as a mobile phase
3. Concept of Liquid stationary phase
4. Temperature and/or Pressure Programming

A thorough knowledge of above said points can give an idea about the operational parameters which can be tweaked for better performance of GLC. It helps in addressing the issues like general effect of the column dimensions, temperature programming, carrier gas type and flow rate etc. on separation, detection limits and peak retention times. Thus deep understanding can help the researcher to look for tradeoffs between these performance factors. The solute particles while migrating through the column might interact with the stationary phase. Thus, different solutes (components of a sample) will migrate differently due to their degree of distribution between gaseous mobile phase and stationary phase. Greater the interaction with stationary phase, solutes will migrate with low velocity and will take more time to elute out of the column. Due to difference in retention time of the solutes, components of the mixture elute out at different time point thereby separating the constituting components of the sample.

2. INSTRUMENTATION

The equipment generally consists of

1. Supply of carrier gas
2. An injector
3. Temperature regulated oven,
4. Column,
5. Detector (at a short distance from the column)
6. Recorder

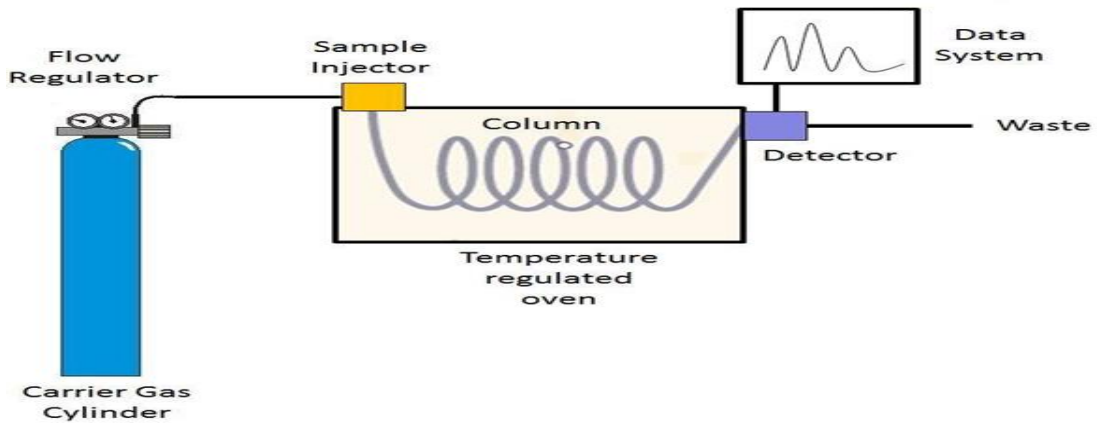


Figure: Basic instrumentation of Gas Liquid Chromatography

3. CARRIER GASES: SUPPLY AND CONTROL

Carrier gas or the mobile phase is an important component in Gas Liquid Chromatography. Carrier gas should be inert in nature such as helium, hydrogen or nitrogen. Pressurized cylinder or bottled gas supply are the most common gas source that readily supplied by vendors as steel tank with pressure regulator. The pressure regulator regulates the gas supply so that correct pressure is fed to the required part of the instrument. The control generally regulates the gas coming into the instrument and its further supply to various parts of the instrument. Modern GC instruments have Electronic Pneumatic pressure controller, while the older instruments had manual pressure control via regulators. In 90% of the GC systems, extremely pure Helium is used. For better separation, hydrogen is the preferred gas but is avoided due to its explosive nature. Helium is inert and non-reactive in nature. High grade helium, referred to as “five-nine gas” meaning it is 99.999% pure, is used in process. But this much purity is also not sufficient when analyzing trace elements (of the range of parts per million or parts per billion) in the sample. Helium gas entering the separating column is passed through at least a resin trap that removes oxygen, hydrocarbons, trace analytes, and/or water vapor which might interfere with the analysis part, or degrade the column or interfere with the detection system. Special attention should also be given to the purity of tubing which connects the source (cylinder) and the gas chromatograph (machine). Before entering GC system, pressure of the gas is reduced from 2500 psi (gas cylinder) to approximately 60 psi (system permissible lmits) with a two-stage regulator.

Difficulty faced in Gas chromatography is the compressibility of the carrier gas and its subsequent influence on separation. When the temperature of column is increased with constant inlet pressure, the average flow rate in the column decreases resulting in increased viscosity of the gaseous mobile phase, thereby decreasing the separation speed and efficiency. Flow may be kept constant through mass flow meters that have inlet and outlet openings which can be adjusted on the basis of pressure differences.

INJECTORS

After passing through the resin trap, purified helium enters the injector where it acts like the mobile phase by pushing the analytes through the separation column. Basically, the chromatographic process begins when sample is introduced into the column without disrupting the flow in column.

Here the sample is volatilized and the resulting gas is drawn along with the carrier stream entering the GC column. Numerous types of injectors are available like on column injector, split-splitless injector, cryogenic focusing injector etc., but split-splitless injector is the most commonly used. This type of injector operates in two modes, split and splitless-split modes. If the sample solution is containing highly concentrated levels of analytes (parts per thousands), the injector is operated in the split mode. In split mode, only a small fraction of sample injected truly enters the separation column and the rest is vented to the atmosphere. Due to high concentration of analytes in the sample solution, even the small fraction of analytes in the solvent are adequate for the identification and quantification. For sample solutions containing low levels of analytes (parts per million/parts per billion), the injector is operated in a dual or splitless-split mode. In this dual mode, when the sample is injected, the injector operates in splitless mode and whole injected volume is pushed onto the separating column. But if this splitless mode is continued throughout the run, the peaks will be non-symmetric and will interfere with peak integration. To overcome this problem, split mode is switched on approximately 30-60 sec after injection.

This dual splitless-split mode permits majority of the sample to load onto the column while clearing remaining sample to allow for a “clean”, well-shaped chromatographic peak.

The interface between the atmosphere and injector is separated by a septum which must be inert to leaching organic constituents. Samples are injected by septa to enter glass liner in the injection port, glass liner prevents exposure of analytes to reactive hot metal surfaces.

Samples are introduced into the GC system with a glass syringe having a metal needle. Samples can be injected either manually or with an automatic sampler. Earlier, when packed columns were used, samples were injected directly onto a section of the column. As the needle of the injection is withdrawn, the injector port is sealed and chromatographic run starts. Thus, on-column injection eludes exposure of analytes to any reactive surfaces.

Highly volatile analytes that cannot be analyzed in standard GC condition are examined using a cryogenic focusing injector. The bottom of the injector which encompasses the head of the capillary column contains liquid nitrogen. This cryogenic fluid cools the column and results in condensation of analytes at the head of the column. Once the analytes enters the column i.e. after injection, the liquid nitrogen is removed and column oven is heated to analyze the volatile analytes. Another consideration is with respect to volume of sample being analyzed which is in extremely small quantity and require precise handling to generate reproducible results. Auto-samplers helps in introduction of samples automatically into the inlets thereby

providing better reproducibility and time-optimization. Auto samplers can be used to inject upto 150 samples instantaneously and precisely the same amount of sample injected every time. Although GC systems have choice for manual insertion of sample but it is very rarely used because these automatic samplers can consistently and precisely reproduce small volume injections and save considerable labor costs.

With the introduction of robotic auto samplers and electronic pressure control for carrier and support gas, the gas chromatograph can now be operated for long hours without human intervention. This allows the researcher to return after hours or days to find the samples analyzed and data stored for further processing.

TEMPERATURE REGULATED OVEN

Samples of gas chromatography must be converted into and maintained in vapor state throughout the separation process. In general, temperature of the separating column in GC is controlled with the help of oven, it keeps the column at temperatures from 40 °C to 400 °C. Oven heats rapidly, however the temperature can be brought down using a fan that removesthe hot air from the back of oven, thereby providing thermal control for better separation of analyte. The column is provided a supported system to prevent it touching the oven walls that might damage the column. The connections of injector and detector are also within this oven. Depending on the operations, GC can be performed either isothermal or temperature controlled.

Early gas chromatographs performed isothermal operations and a steady temperature was maintained throughout the analysis. While in temperature programmed operations, oven temperature is regulated as per the requirement for GC separation and temperature program fed into the system. This allows separations of different chemical analytes which differ marginally in their vapor pressure in a single analysis. Temperature is an important factor while analyzing complex mixtures.

Conventional ovens consist of a resistive coiled wire that radiates the heat into the inner volume of the oven. With the help of fan, heat is spread in an even manner throughout the oven. Thermistor helps in regulating the oven temperature by feedback circuit that controls and programs the oven temperature. To generate reproducible chromatography results, it is necessary that no thermal gradient occurs inside the oven

COLUMNS FOR GC

Column is the important component of analytical gas chromatograph, the quality of separation can be only by this. Early GC used to have a typically 1–5 m long and 1–5 mm internal diameter, however, micro-packed columns used later on had <1 mm internal diameter. Limitation of packed column was improper resolution due to their length as pressure drop resisted the gas flow within the column. This restriction was overcome by the use of capillary column in which the stationary phase is coated on the inner wall as a thin film. Advantage of capillary column over packed columns is increased separation efficiency, ability to work at lower temperature using capillary columns thereby giving better separation in same time or same separation in shorter time. Results have been obtained upto 10 times faster than the packed column. Due to lesser amounts of stationary phase in a capillary column, its capacity is limited therefore it has special sample-introduction methods and sensitive detectors. The column is coiled to fit the temperature regulated oven (column oven) and is connected with the injector and detector inlets.

Glass columns have been the ideal choice in GC by most of the researchers, however, earlier various other column materials such as nickel, copper and stainless steel were also used.
Coiled glass capillary tubing was in culture for quite long time but due to fragile nature of glass, fused silica columns (based on fibre-optic technology) that are highly flexible, durable and chemically inert were later on employed in GC. In recent times, packed column GC is almost completely been replaced by flexible fused-silica tubing, wall coated open tubular (WCOT) capillary columns. Research and development have resulted in designing of columns that can be used for non-volatile substances and moderately thermally labile materials. For analyzing relatively non-volatile substances, columns have been designed for high temperature applications while cool on column injections have been introduced for moderately thermally labile samples. Quality monitoring of the fused-silica column is done by checking the point defects and general strength of the capillary tubing. During this step of quality check, internal surface chemistry of the fused-silica is also verified so as to meet the application for which it is intended to be used. Practically, the stationary phase in GC column should be unreactive, adequately wet column supports, and have reasonably solubility in commonly used volatile organic solvent. High- molecular-weight hydrocarbons such as hexadecane, squalane, and Apiezon greases are lately used as low selectivity stationary phases. Hydrocarbon are susceptible to oxidation and should be used with carrier gases having a low oxygen content. The most useful stationary phases contain either ether or ester

as “anchor” functional groups, like poly(perfluoroalkyl ether) oil.

An ideal support used in GLC should have sufficient surface energy so that the stationary liquid phase is able to wet the support by forming a thin, stable film over it. The support should be sufficiently inert to eliminate solute interactions with its surface. It should have a large surface area to weight ratio thereby facilitating the preparation of columns with a high phase loading, be mechanically stable and good conductor of heat to facilitate rapid thermal equilibrium. Preparation of packed column is a multistep process, which involves mixing of liquid phase with solid support in presence of suitable solvent followed by removal of solvent by evaporation and finally packing the dried material into empty column.

DETECTORS

Detectors play an important role in detection of analytes in gas chromatography because the whole idea of analyzing the sample will be wasted if the analyte is not detected precisely and accurately. Detector detects sample components/analytes on the basis of their physiochemical properties. And detectors of gas chromatography are one of the reason and key strength why the researchers prefer this technique over other forms of chromatography. Detectors generate an electronic signal thereby generating the chromatogram. The carrier gases used in gas chromatography are transparent to most of the GC detectors therefore their background levels and interference is quite low. As the sample gas stream comes out from the column, the detector monitors the separated components of the gaseous sample and signals are interpreted after data acquisition. Different type of detectors are available however choosing among them is mainly based on the application for which it is being used, chemistry of the analyte, sensitivity and on whether qualitative or quantitative data is required. Depending upon the signal they produce in response to an eluting solute, GC detectors can be of two types, i.e. detectors that generate a univariate (single) signal like Thermal conductivity detector (TCD), Flame ionization detector (FID), Alkali bead or nitrogen-phosphorus detector (NPD), Flame photometric detector (FPD) etc. and detectors that generate multivariate (multiple, multiplex) response, such as Atomic emission detector (AED), Infrared detector (IRD) and Mass spectrometer (MSD).

Depending upon the ability to detect various compounds, GC detectors can be of two types i.e. Universal and Selective detectors. Universal detector can detect almost all the types of compounds that elute out in the process e.g. Thermal conductivity detector (TCD),

Flame ionization detector (FID) etc. While Selective detectors detects only a specific class of compounds based on physical, molecular or elemental properties e.g. nitrogen-phosphorus detector (NPD), Flame photometric detector (FPD). Selectivity can also be looked as specificity, that is, the ability to detect a functional group or specific atom to the exclusion of others.

Detectors can also be classified on the basis whether detector responds to the flow of analyte mass passing through it or to the analyte concentration passing through it. In concentration- sensitive detector, a signal will be generated which will be proportional to the analyte concentration in the carrier gas as it passes through. e.g. Thermal conductivity detector (TCD) happens to be the most common concentration-sensitive detector that gives result in concentration terms like ng/mL. On contrary, mass sensitive detectors responds to the analyte mass passing through the detector in a given time. e.g. flame ionization detector (FID) that gives result in mass per time (pg/s). That is, detector response will not change even if make up gas is added at the end of column, because the same mass of analyte will pass through the detector in the same time.

Detection limit of a detector is the minimum quantity of analyte that it can distinguish from the background. It's the ability of detector to differentiate signal from eluting compound to that of signal from neighboring background noise. Numerically it can be measured as signal- to-noise ratio. Larger the Signal/Noise value for a given amount of solute in a given set of conditions, better is the detector as it would be helpful in detecting minute quantities of solute. Noise can originate from many sources like due to events in detection process, thermal and vibrations etc. Analog and/or digital detector electronics can be used to reduce high- frequency noise. It is discrete choice of the designer as to how much filtering is needed in analog and digital sections of the signal processing circuit. A middle path is looked upon to balance detector response time and noise suppression. Major source of noise in most analyses is because of chemical noise and considered as "low-frequency noise". Sensitivity of the detector is to measure the change in response to change in solute amount or concentration. Higher the sensitivity, better one can differentiate between small changes in analyte compounds. Specificity on the other hand is the ability of detector to detect a specific atom or functionality present in the analyte.

THERMAL CONDUCTIVITY DETECTOR (TCD)

Thermal Conductivity Detector is a non-destructive universal detector which is most commonly used for the analysis of light and permanent gases with packed columns or capillary PLOT columns. Non-destructive detectors can detect solutes without changing the sample analyte chemically and is beneficial in some instances. Although it is one of the least sensitive Gas Chromatography detectors, but due to advantages like, it has wide linear dynamic range, requires little power and no fuel gas, has no flame and inexpensive, Thermal Conductivity Detector is a popular detector and sometimes the only choice for few applications. Thermal Conductivity Detector can be used in portable micro gas chromatographs as it can be easily miniaturized and run on low power consumption.

Thermal Conductivity Detector integrates wheatstone bridge circuit that measures the change in resistance of heated filament as the sample passes through. In a simplest way, wheatstone

bridge circuit can be illustrated as shown in figure, column effluent, containing separated analyte, flows over one filament (termed sample column) and the clean carrier gas passes over the other (termed reference column). A constant voltage/current is applied to the circuit and the balanced current/voltage between opposite legs of the bridge is monitored.

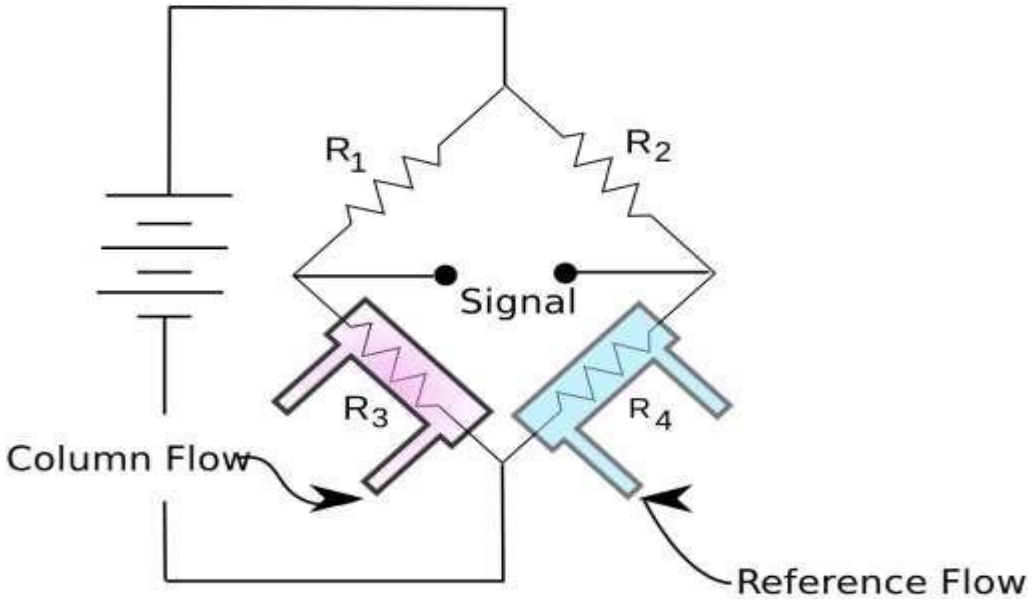


Figure: Wheatstone bridge circuit in Thermal Conductivity Detecto

The idea of introducing reference column in original Thermal Conductivity Detector designs was basically to cancel baseline rises that occurs due to stationary phase bleed through the run. For that, an identical column was attached to reference side with same column flow as that of analytical side assuming that both the column would bleed same and would cancel the signal rise due to stationary phase bleed on the analytical side. Due to advancements in column technology and stationary phase, the problem of stationary phase bleed is now not the major factor for using a column in reference side. As compared to other Gas Chromatography detectors, Thermal Conductivity Detector are more sensitive to temperature variation. Thus the function of reference side of the TCDs now a days is to minimize the drift due to temperature and flow change in the cell during the run.

The two columns present in the TCD apparatus can be employed to detect two sample, thus dual TCD analysis use one side of the TCD for channel 1 and the other for channel 2 detection. To avoid confusion, methods must be developed so that the peaks from each
channel elute at different times and data analysis tools to handle positive (from channel 1)and negative peaks (from channel 2) in the same chromatogram

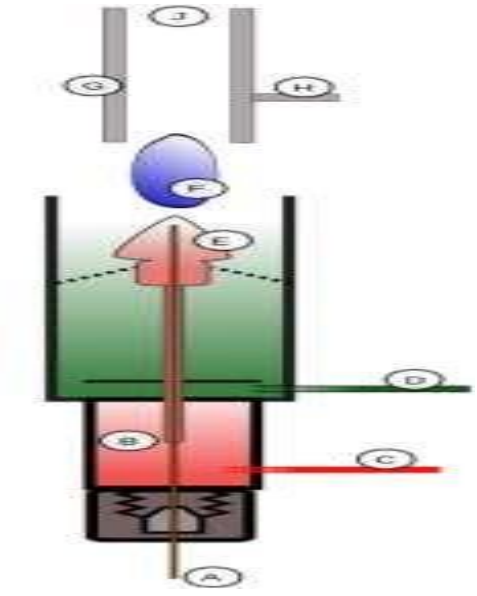
Universal GC detector, TCD is based on thermal conductivity, thermal conductivity is basically a physical property of molecule that signifies its ability to conduct heat. Carrier gases used in GC have high thermal conductivity thereby helping to drain heat away from heated filament. Carrier gases like H2, He, Ne, CH4 having thermal conductivity of 230.4, 190.6, 60.3, 49.1 respectively at 400K are examples of the carrier gases that are used for thermal conductivity detector. As the eluted solute passes over the detector's sample filament (channel 1), its temperature will rise relative to the reference filament (channel 2) because analytes have low thermal conductivity and are less efficient in draining the heat away. This in turn will increase the temperature in the sample filament. Therefore signal is a function of solute concentration and its thermal conductivity relative to that of carrier gas. Keeping this principle in mind, a researcher should use a carrier gas having a different thermal conductivity to that of lowest analyte concentration in the sample to obtain highest sensitivity from the Thermal Conductivity Detector.

To minimize problems associated with the TCD, like detector contamination, solute condensation and peak tailing, this universal detector should be operated at a temperature that equals isothermal column temperature or the highest column temperature in a temperature-

programmed method Other precautions taken into consideration while using TCD as a detector in gas chromatography are, high temperature should be avoided because sensitivity of TCD decreases at higher temperature, life of TCD filament do decrease with greater usage and eventually burn out with time. Thus, life time of TCD decreases with its usage at higher temperature. Lifetime also depends on corrosiveness of gases being analyzed and exposure to vibrations/shocks. As the solutes flow directly over the filament of detector, some polar or reactive solutes interact with filaments causing peak tailing. To minimize corrosiveness, filaments are coated to reduce interaction with the metal filament. To overcome repeated corrosion, most TCD designs allow replacement of filaments. To sum up all, TCD is a reliable, rugged, and useful GC detector, especially for the analysis of light and permanent gases.

FLAME IONIZATION DETECTOR (FID)

Flame ionization detector (FID) is the leading universal detector in gas chromatography and is a univariate detector. It has distinctive properties (unit carbon response and wide linear operating range) that puts it a step forward then rest of the general-use detectors in gas chromatography. Apart from above mentioned unique attributes, other positive points are low cost, ease of use, speed of response, and ruggedness.



It is a destructive mass-sensitive detector, meaning that its response is proportional to the carbon mass that passes through it in unit time. It is measured in terms of picograms carbon per second (pg/s) and detection limits are in the low pg C/s. The meaning of unit carbon response is that flame ionization detector responds linearly to the mass of carbon flowing

through it and is independent of compound structure. The accuracy of the detector is high as this detector gives unit response for most hydrocarbons with very less percent error. FID does not require standards for quantification as the attribute of unit carbon response in FID allows the researcher to quantify components in mixtures without having calibration standards for each and every component. That is, FID is useful in estimating concentration levels of components in a sample when the identities are unknown or when the standards are not available for calibration.

ELECTRON CAPTURE DETECTOR (ECD)

Electron Capture Detector is non-destructive, concentration sensitive. It is the detector of choice for many environmental GC methods due to its inherent sensitivity and selectivity for halogenated compounds. It is the most sensitive non-hyphenated GC detector. Invention of ECD in 1950s brought revolution in the environmental studies and subsequent improvements led to today's Electron Capture Detector. Due to its unique attributes, ECD is used to perform low-level analyses of chlorinated pesticides and PCBs on a routine and cost-effective manner. The principles behind electron-capture detection are a bit more complex and are highly dependent on specific detector design, experimental set points and solute characteristics. However the basic mechanism of detection and principle of Electron Capture Detector is that an electron plasma is established in the detector by the ionization of a continuously fed "reagent" gas of relatively low ionization potential such as Nitrogen, or a few percent of methane in helium. β-Particles emitted from a radioactive source ionizes the reagent gas thereby releasing electrons and sustaining the plasma. Typically, radioactive sources such as Nickel [^{63}Ni (65.9 keV max)] and Hydrogen [^{3}H (18.6 keV max)] are used. The resulting background abundance of electrons is measured, usually by using a pulsed circuit with 30-50 V bias and us pulses. During each pulse, the background of electrons is completely collected and the current is measured. The plasma quickly reestablishes after each pulse. One approach in electronics of Electron Capture Detector is to integrate the current measured during the pulses, and output the result as a continuous DC signal. Another approach is to use a feedback circuit wherein frequency of the pulses is adjusted to maintain a constant current, this provides a wider linear response range and more reliable measurement. It has been found that the response of the ECD is highly dependent on the molecular structure of the analytes.

NITROGEN/PHOSPHORUS DETECTOR (NPD)

Nitrogen/Phosphorus Detector (NPD) is a highly selective, destructive detector and finds its wide use in flavors, foods, forensics, and pharmaceutical and pesticide analysis applications. It is known as alkali bead detector or thermionic ionization detector. Most modern NPDs are based on a heated bead design model described by Kolb and Bischoff.

Response of the alkali bead detector rely on a selective moderated surface reaction between nitrogen- or phosphorus-containing compounds and certain alkali atoms in a cool plasma. Salts of cesium (Ce) and rubidium (Rb) have been used successfully for both N and P selective detection.

Salts of these alkali metals can be either integrated into or coated onto ceramic or glass beads that are in turn attached onto a wire that is used to heat them. Column effluent mixes with Hydrogen and air as it leaves the detector jet. The bead is kept cool for maximum sensitivity, especially for N-containing compounds. A flame would overheat the bead and cause similar problems as running the bead with too much current. Without a flame, Hydrogen is combusted just at the surface of the heated bead, thereby forming a localized plasma wherein alkali salts in the bead are reduced and evaporated so that the reduced alkali metals can then catalytically react with N- and P-containing compounds. These ions are collected by an electrode that is held at a few hundred volts relative to the jet. As the alkali metals are depleted from the surface, they are replenished from the bulk of the bead or coating material. There are many variables associated with response of NPD that combine to yield a different sensitivity, selectivity, and lifetime based on bead type and operating conditions. The various variables include, characteristics of the bead, operating variables, detector design and beadhistory.

FLAME PHOTOMETRIC DETECTOR (FPD)

Flame Photometric Detector is a selective and destructive detector. It uses a flame to measure the light emitted from phosphorus or sulfur combustion products. It is used for the specific detection of sulfur in petroleum and petrochemical samples and for the specific detection of phosphorus-containing pesticides in foods and the environment. Flame Photometric Detector

is based on the principle of light emission when the excited ions forms stable compounds. That is, during the combustion of organic molecules in a hydrogen flame, many different forms of excited fragments and recombination products are formed. When these excited ions recombine and relax into stable forms, they emit light. Spectral and temporal characteristics of the emission are species specific and can be exploited for selective detection. Typical excited species that emit light include those from oxygen (OH*), carbon (CH*, C2*), phosphorus (HPO*), and sulfur (S2*). FPD monitors emission using a photomultiplier tube (PMT) positioned above the flame (where recombination and emission occurs). An optical notch filter is placed between the emission zone and the photomultiplier tube to select a narrow wavelength region corresponding to where the sulfur or phosphorus emission is highest relative to background (usually carbon) emission. Filters of ~394 nm for S and ~525 for P are typically used in FPD. However to measure both sulfur and phosphorus simultaneously, dual detector designs are used with two filter/PMT combinations.

PHOTOIONIZATION DETECTOR (PID)

Photoionization detector (PID) is nondestructive, concentration sensitive, and partially selective. Since the PID is non-destructive, it can be used effectively in series with other detectors. It has no flame, is fairly simple, safe and can be effectively integrated into portable gas chromatographs for field use purpose. It is the detector of choice that can trace low levels of aromatic compounds of environmental or health concern. PID creates molecular ions using high-energy photons from a sealed light source that are strong enough to ionize molecules. However, the energy from the lamp usually does not cause molecules to fragment. The molecular ions are attracted and are neutralized at cathode, yielding the original intact molecule. This current generated due to neutralization is measured and represents the detector signal. The current is proportional to the number of ions neutralized, which is, in turn, proportional to the concentration of the compound in the cell.

ELECTROLYTIC CONDUCTIVITY DETECTOR (ELCD)

Electrolytic Conductivity Detector destructive, mass-sensitive selective detector, mainly used for selective detection of halogen-containing compounds. The detector consists of three main components: the reactor assembly, cell-solvent assembly, and detector controller. Although the prime mode of operation of the ELCD is the halogen mode, sulfur and nitrogen modes are also possible. However for that each detection mode requires a specific reactor, resin

cartridge, and solvent. This detector can be used as a stand-alone or in tandem following a photoionization detector or other non-destructive detectors.

Electrolytic Conductivity Detector converts eluting compounds with the target heteroatom like halogens, Sulfur or Nitrogen to an ionizable gas using reductive conditions at high temperatures (800 to 1,100°C) in a catalytic micro reactor. Products of the gaseous reaction proceeds to the detector cell where they quickly dissolve in a flowing deionized solvent stream and increases its electrolytic conductivity. Conductivity detector amplifies the change in conductivity and produces a signal that is proportional to the mass of target species. Deionizing resin bed filters the solvent stream which can be reused and continuously recycled. High levels of hydrocarbon solvents can cause elemental carbon buildup in the reaction tube, thereby decreasing the performance of the detector and requiring frequent maintenance. Thus to prevent detector overload by high amounts of solvent, solvent ventingis performed by time-programmed control of using a vent valve.

DATA SYSTEM

Since early days of gas chromatography, there has been a little change in recording of the chromatographic results. Due to digital revolution, strip chat recorder that were used in earlier gas chromatographs have been replaced by electronic recording-integrators or microcomputers.

Data system accepts analogue signal from detector and digitalizes to record it as separated peaks in the form of chromatogram due to chromatographic separation. It automatically reports peak retention times, peak areas, etc and the software displays the result in automated manner. Thus, gas chromatography can be used to perform various qualitative and quantitative operations thereby assisting in sample identification and its quantitation.

ypes of Analysis

Gas chromatography can be used for:

1) Qualitative analysis

The identification & separation is done by comparing the volume of the sample or retention time to the standard or by collection of the individual components as they emerge from the chromatograph and later on identifying them by other methods. The retention time is changed according to changes in operating conditions and this will also influence the

accuracy of identification. Thus GC is mostly used for operating a target compound analysis, where one has a better idea of the compounds commenced in a mixture so reference standards can be utilized for estimating retention times.

2) Quantitative analysis

In Quantitative analysis parameters of the chromatogram are determined by Gas Chromatography in relation to the amount of the corresponding component of the injected sample. It is necessary to measure the peak area or peak height of each component to analyze the components quantitatively. For the well resolved peaks, both peak height and area are proportional to the concentration of the component.

3) Checking the purity of a compound

In preparative chromatography purity of a compound can be checked by comparing the chromatogram of the standard & the sample.

Applications of Gas Chromatography

Pharmaceuticals

With the development of complex pharmaceuticals, high-tech delivery systems and advanced formulations, accurate analysis is needed to provide safe and effective drugs. In the pharmaceutical industry Gas Chromatography technique is used to analyze residual solvents in both raw materials (drug substance) and finished products (drug product). The samples like saliva, blood and other secretions containing large amounts of organic volatiles can be very expeditiously and conveniently analyzed with high sensitivity using GC. Recognizing the amount of which compound is in a given sample gives a huge advantage in estimating the effects of human health. The analysis with the help of GC takes place in a matter of minutes and routine analysis of few drugs can be done in few seconds.

A few examples are:

- Drugs quantification and their metabolites in urine and blood
- Related substances and volatile impurities
- Drug assays
- Biopharmaceutical applications include urine drug screens for ethylene oxide in uncontaminated products such as sutures and for barbiturates and underivatized drugs.

Long answer type Questions (10 Marks)

1. What is the function of detectors in HPLC? What are salient features required for detectors. Explain different detectors?
2. Describe in detail instrumentation and applications of HPLC.
3. Give the diagramatic representation of an HPLC instrument. What are the various detectors used in HPLC**.**
4. Explain the principle, instrumentation and applications of HPLC.
5. Discuss the theory, working and applications of GC.
6. Describe various columns used in Gas Chromatgraphy.
7. What is the function of detectors in GC? What are salient features required for detectors. Explain different detectors?
8. Describe Gas Chromatograph with a neat labelled diagram. Explain the type of GC columns, carrier gases and detectors used.

Short answer type questions (5Marks)

1. Write the construction and working of any two detectors used in HPLC.
2. Describe the pumps, sample injection techniques and applications of HPLC
3. Enlist the Detectors and sample injection techniques used in Gas Chromatography & explain in detail each of two.
4. Draw a neat schematic diagram of GC. Explain about columns used in GC.

Very Short answer type questions (2 Marks)

1. Define Gradient Pump.
2. Retention time and retention factor.
3. Comment on Stationery phase used in HPLC.

4. What is void volume of a HPLC column? How it is related to separation efficiency?
5. Types of pump used in HPLC.
6. Stationery phase of HPLC.
7. Define principle of HPLC
8. What is Normal phase & Reverse phase Chromatography?
9. What are the carriers used in GC.
10. What is derivatization in GC and write its significance
11. Explain any one derivatisation technique in GC.
12. Mention the importance of guard column in GC?
13. Define Guard column and its significance.
14. What is derivatization in Gas Chromatography?
15. What are two major limitations of GC