Consortium for Educational Communication

Module on

CHROMATOGRAPHY

By

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INTRODUCTION

General description of chromatography*:* Chromatography was invented by the Russian botanist Mikhail Tswett. Chromatography is a technique in which the components of a mixture are separated based on the rates at which they are carried through a stationary phase by a gaseous or liquid mobile phase. Chromatography is usually introduced as a technique for separating and/or identifying the components in a mixture. The basic principle is that components in a mixture have different tendencies to adsorb onto a surface or dissolve in a solvent. It is a powerful method in industry, where it is used on a large scale to separate and purify the intermediates and products in various syntheses.

Theory of Chromatography:

There are several different types of chromatography currently in use, i.e., paper chromatography; thin layer chromatography (TLC); gas chromatography (GC); liquid chromatography (LC); high performance liquid chromatography (HPLC); ion exchange chromatography; and gel permeation or gel filtration chromatography.

Basic Principle of Chromatographic techniques:

All chromatographic methods require one static part (the stationary phase) and one moving part (the mobile phase). The various constituents of the mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus changing the separation.

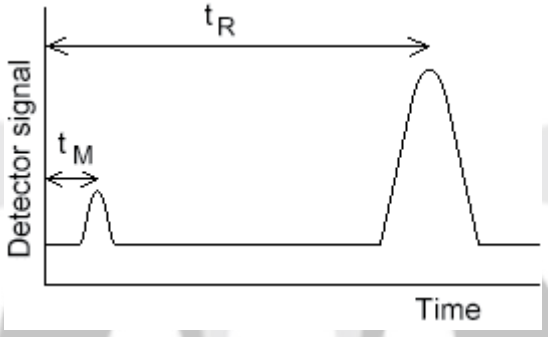
2.1 Partition-coefficient

The distribution of analytes between phases can often be described quite simply. An analyte is in equilibrium between the two phases;

$$A_{mobile} \rightleftharpoons A_{stationary}$$

The equilibrium constant, *K*, is termed the *partition coefficient*; defined as the molar concentration of analyte in the stationary phase divided by the molar concentration of the analyte in the mobile phase.

The time between sample injection and an analyte peak reaching a detector at the end of the column is termed the *retention time* (*t*R). Each analyte in a sample will have a different retention time. The time taken for the mobile phase to pass through the column is called *t*M.



A term called the *retention factor, k'*, is often used to describe the migration rate of an analyte on a column. You may also find it called the *capacity factor*. The retention factor for analyte *A* is defined as;

$$k'A = t\,R - tM \,/\, tM$$

t R and *t*M are easily obtained from a chromatogram. When an analytes retention factor is less than one, elution is so fast that accurate determination of the retention time is very difficult. High retention factors (greater than 20) mean that elution takes a very long time. Ideally, the retention factor for an analyte is between one and five.

2. Distribution coefficient

The distribution constant (or partition ratio) (K_D), is the equilibrium constant for the distribution of an analyte in two immiscible solvents. In chromatography, for a particular solvent, it is equal to the ratio of its molar concentration in the stationary phase to its molar concentration

in the mobile phase, also approximating the ratio of the solubility of the solvent in each phase.

$$(K_D)_A = \frac{(a_A)_{org}}{(a_A)_{aq}} \approx \frac{[A]_{org}}{[A]_{aq}}$$

Distribution constants are useful as they allow the calculation of the concentration of remaining analyte in the solution, even after a number of solvent extractions have occurred. They also provide guidance in choosing the most efficient way to conduct an extractive separation.

Chromatogram

A **chromatogram** is the visual output of the chromatograph. In the case of an optimal separation, different peaks or patterns on the chromatogram correspond to different components of the separated mixture.

The chromatographic techniques rely on one of the following phenomena: adsorption; partition; ion exchange; or molecular exclusion.

2.1 Adsorption

Adsorption chromatography was developed first. It has a solid stationary phase and a liquid or gaseous mobile phase. (Plant pigments were separated at the turn of the 20th century by using a calcium carbonate stationary phase and a liquid hydrocarbon mobile phase. The different solutes travelled different distances through the solid, carried along by the solvent.) Each solute has its own equilibrium between adsorption onto the surface of the solid and solubility in the solvent, the least soluble or best adsorbed ones travel more slowly. The result is a separation into bands containing different solutes. Liquid chromatography using a column containing silica gel or alumina is an example of adsorption chromatography. The solvent that is put into a column is called the eluent, and the liquid that flows out of the end of the column is called the eluate.

2.2 Partition

In partition chromatography the stationary phase is a non-volatile liquid

which is held as a thin layer (or film) on the surface of an inert solid. The mixture to be separated is carried by a gas or a liquid as the mobile phase. The solutes distribute themselves between the moving and the stationary phases, with the more soluble component in the mobile phase reaching the end of the chromatography column first. Paper chromatography is an example of partition chromatography.

Normal Phase chromatography

In normal-phase chromatography, the stationary phase is polar and the mobile phase is non-polar. In reversed phase we have just the opposite; the stationary phase is nonpolar and the mobile phase is polar. Typical stationary phases for normal-phase chromatography are silica or organic moieties with cyano and amino functional groups. For reversed phase, alkyl hydrocarbons are the preferred stationary phase; octadecyl (C18) is the most common stationary phase, but octyl (C8) and butyl (C4) are also used in some applications. The designations for the reversed phase materials refer to the length of the hydrocarbon chain.

Reverse Phase chromatography

Reversed-phase chromatography (RPC) is any liquid chromatography procedure in which the mobile phase is significantly more polar than the stationary phase. It is so named because in normal-phase liquid chromatography, the mobile phase is significantly less polar than the stationary phase. Hydrophobic molecules in the mobile phase tend to adsorb to the relatively hydrophobic stationary phase. Hydrophilic molecules in the mobile phase will tend to elute first. Separating columns typically comprise a C8 or C18 carbon-chain bonded to a silica particle substrate.

2.3 Ion exchange

Ion exchange chromatography is similar to partition chromatography in that it has a coated solid as the stationary phase. The coating is referred to as a resin, and has ions (either cations or anions, depending on the resin) covalently bonded to it and ions of the opposite charge are electrostatically bound to the surface. When the mobile phase (always a liquid) is eluted through the resin the electrostatically bound ions

are released as other ions are bonded preferentially. Domestic water softeners work on this principle.

2.4 Molecular exclusion/Size exclusion/Gel Permeation/Gel Filtration

Molecular exclusion differs from other types of chromatography in that no equilibrium state is established between the solute and the stationary phase. Instead, the mixture passes as a gas or a liquid through a porous gel. The pore size is designed to allow the large solute particles to pass through uninhibited. The small particles, however, permeate the gel and are slowed down so the smaller the particles, the longer it takes for them to get through the column. Thus separation is according to particle size.

3. Classification of Chromatographic techniques:

3.1 Paper chromatography

This is probably the first, and the simplest, type of chromatography that people meet. A drop of a solution of a mixture of dyes or inks is placed on a piece of chromatography paper and allowed to dry as represented in Figure 1. The mixture separates as the solvent front advances past the mixture. Filter paper and blotting paper are frequently substituted for paper chromatography if precision is not required. Separation is most efficient if the atmosphere is saturated in the solvent vapour

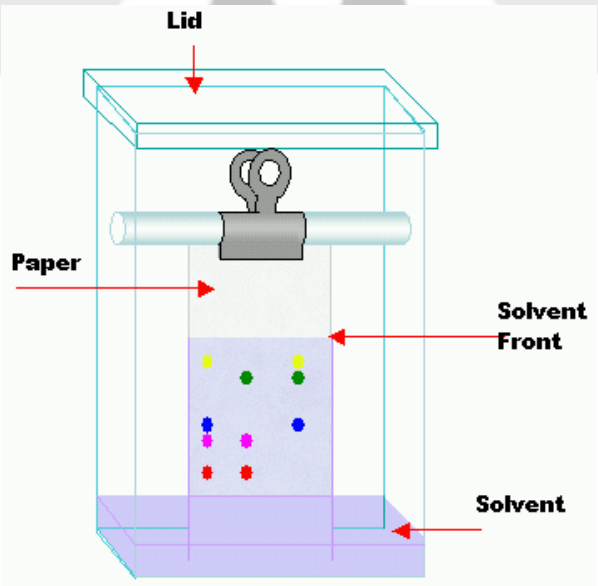


Figure 1.Schemmatic representation of Paper Chromatography.

Some simple materials that can be separated by using this method are inks from fountain and fibre-tipped pens, food colourings and dyes.

The components can be regenerated by dissolving them out of the cut up paper. The efficiency of the separation can be optimised by trying different solvents, and this remains the way that the best solvents for industrial separations are discovered (some experience and knowledge of different solvent systems is advantageous). Paper chromatography works by the partition of solutes between water in the paper fibres (stationary phase) and the solvent (mobile phase). Common solvents that are used include pentane, propanone and ethanol.

3.2 Thin layer chromatography (TLC)

Thin layer chromatography is similar to paper chromatography, but the stationary phase is a thin layer of a solid such as alumina or silica supported on an inert base such as glass, aluminium foil or insoluble plastic. The mixture is 'spotted' at the bottom of the TLC plate and allowed to dry. The plate is placed in a closed vessel containing solvent (the mobile phase) so that the liquid level is below the spot.

To thoroughly understand the process of TLC, as well as all types of chromatography, we must travel to the molecular level. All forms of chromatography involve a dynamic and rapid equilibrium of molecules between the two phases as represented in Figure2.

1. **Free** - completely dissolved in the liquid or gaseous mobile phase and
2. **Absorbed** - stuck on the surface of the solid stationary phase.

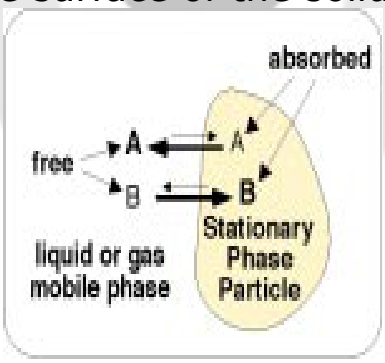


Figure 2. Mixture of A & B frees in mobile phase and absorbed on the stationary phase

Molecules are continuously moving back and forth between the free and absorbed states with millions of molecules absorbing and millions of other molecules desorbing each second. The equilibrium between the free and

absorbed states depends on three factors:

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

Thus, one has three different variables to change in chromatography. The polarity of the molecules is determined by their structures. By selecting different stationary and mobile phases, one can change the equilibrium between the free and absorbed states. It is important to understand chromatography at this molecular level because this allows one to choose mobile and stationary phases that will separate just about any mixture of molecules.

Partitioning: Different molecules partition differently between the free and absorbed state that is the equilibria between these two states is not the same. In Figure 3 below, molecule A is weakly absorbed, its equilibrium lies in the direction of the free state and there is a higher concentration in the mobile phase. Molecule B, on the other hand, is strongly absorbed, its equilibrium lies in the direction of the absorbed state, and has a higher concentration on the stationary phase.

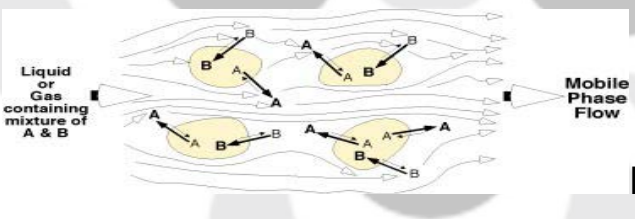


Figure 3: Dynamic equilibrium between A & B and the mobile and stationary phase.

Although alumina and silica are the most common stationary phases used for TLC, there are many different types. They range from paper to charcoal, non-polar to polar and reverse phase to normal phase. Several different types of stationary phases are listed according to polarity in Figure 4.

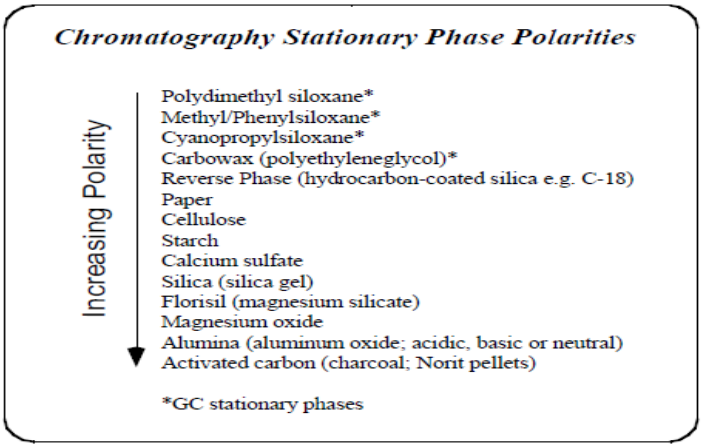


Figure 4. Common Stationary Phases listed by Increasing Polarity

The mobile phase polarities can also be varied to effect the chromatographic separation. Figure 5 lists some common mobile phases according to increasing polarity.

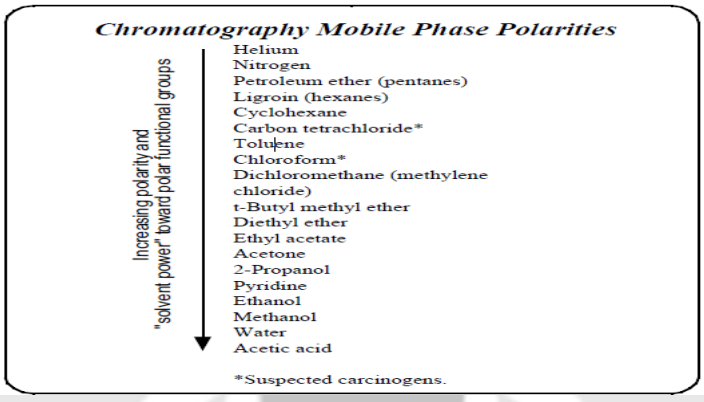


Figure 5. Common Mobile Phases listed by Increasing Polarity

Analysis of a Mixture by Thin Layer Chromatography

TLC is carried out on glass plates or strips of plastic or metal coated on one side with a thin layer of adsorbent. The adsorbent contains a small amount of gypsum ($CaSO_4$) which acts as a binder to give an adherent coating. For routine work, small TLC plates can be prepared by dipping microscope slides in slurry of the adsorbent in chloroform. More uniform plates are obtained by mixing the adsorbent with an equal weight of water, spreading the mixture on a glass plate and allowing it to set dry. Precoated TLC plates are commercially available with various adsorbents in very uniform

layers A series of photographs are provided at the end of this document to show the steps in running a TLC plate.

Since a TLC plate can run three as represented in Figure 6, if not four mixtures at one time, it is very important to properly label the plate. Notice that pencil is always used to mark a TLC plate since the graphite carbon is inert. If organic ink is used to mark the plate, it will chromatograph just as any other organic compound and give incorrect results.

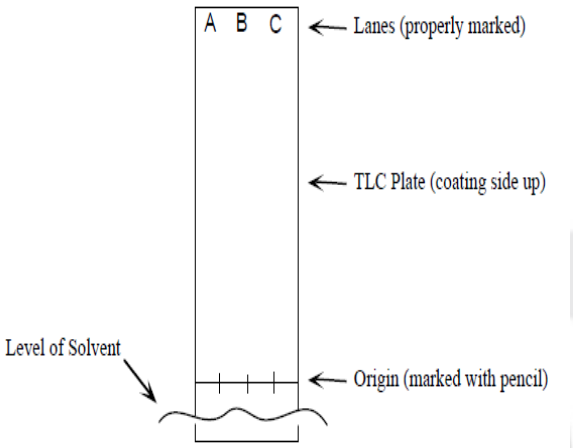


Figure 6. TLC Plate ready to be spotted

Once the dilute solution of the mixture has been spotted on the plate, the next step is the development. Just like paper chromatography, the solvent must be in contact with the stationary phase. shows a wide-mouth bottle commonly used to develop TLC plates. Figure 7 shows a wide-mouth bottle commonly used to develop TLC plates.

Figure 7. Development Chamber

The bottle is filled with a small amount of the mobile phase and capped

with a cork. In addition, a piece of filter paper is put in the bottle to help create an atmosphere saturated with solvent. Use your tweezers to place the plate in the development chamber; oils from your fingers can sometimes smear or ruin a TLC plate.

In situ and on-line detection or visualization of TLC spots:

In situ detection can be done visually or by a variety of spectroscopic methods and mass
spectrometric techniques. The MS techniques also include some on-line methods.

Visual detection

TLC plates normally contain a fluorescent indicator which makes the TLC plate glow green under UV light of wavelength 254 nm.

Spectroscopic methods

UV, diode-array (DAD), fluorescence spectroscopies, mass spectrometry (MS), and Fourier-transform infrared (FTIR) and Raman spectroscopies have all been applied for the in situ detection of analyte zones on a TLC plate.

R_f value

In addition to qualitative results, TLC can also provide a chromatographic measurement known as an R*f* value.The Rf value is used to quantify the movement of the materials along the plate. Rf is equal to the distance traveled by the substance divided by the distance traveled by the solvent as represented in Figure 8. Its value is always between zero and one.

$$R_f = \frac{\text{distance spot travels}}{\text{distance solvent travels}}$$

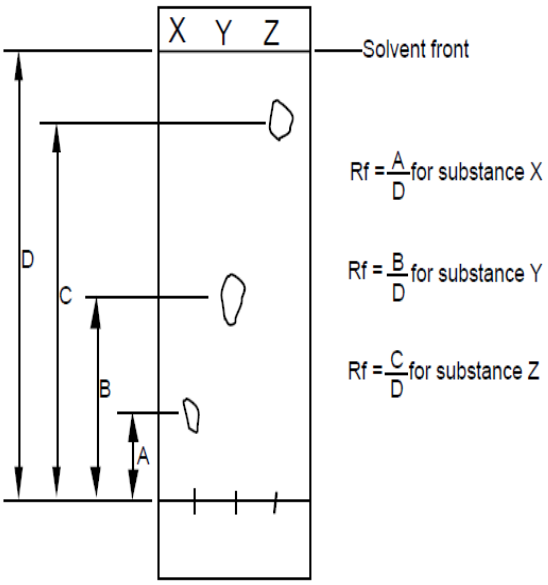


Figure 8. Calculation of R*f* value.

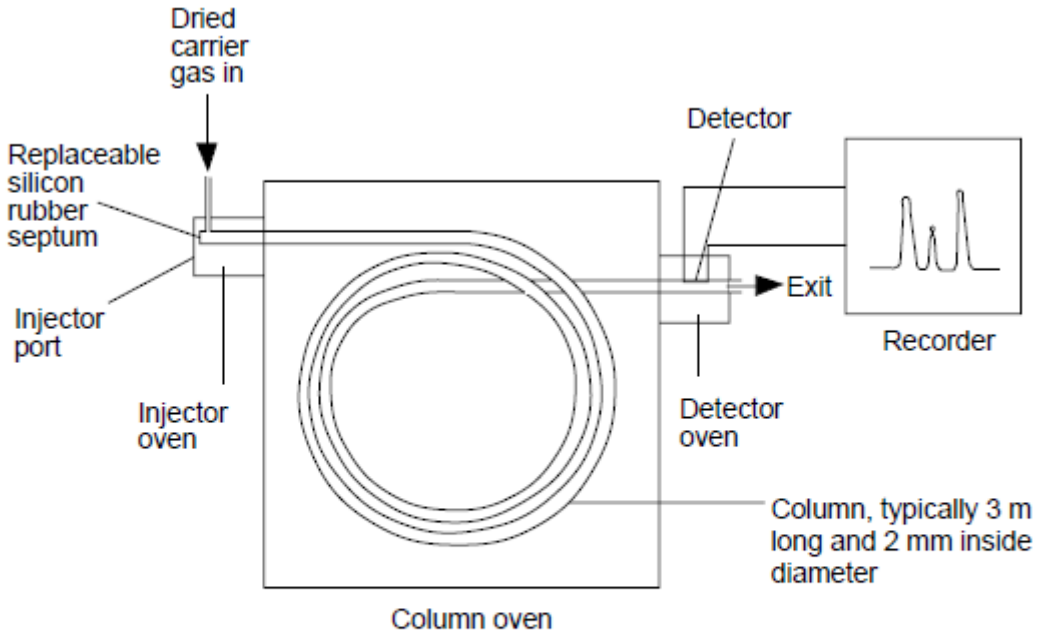
3.3 Gas chromatography (GC)

This technique uses a gas as the mobile phase, and the stationary phase can either be a solid or a non-volatile liquid (in which case small inert particles such as diatomaceous earth are coated with the liquid so that a large surface area exists for the solute to equilibrate with). If a solid stationary phase is used the technique is described as gas-solid adsorption chromatography, and if the stationary phase is liquid it is called gas-liquid partition chromatography. The latter is more commonly used, but in both cases the stationary phase is held in a narrow column in an oven and the stationary phase particles are coated onto the inside of the column.

Analysis of a Mixture by Gas Chromatography

For separation or identification the sample must be either a gas or have an appreciable vapour pressure at the temperature of the column – it does not have to be room temperature. The sample is injected through a self sealing disc (a rubber septum) into a small heated chamber where it is vaporised if necessary as represented in Figure 9. Although the sample must all go into the column as a gas, once it is there the temperature can be below the boiling point of the fractions as long as they have appreciable vapour pressures inside the column. This ensures that all the solutes pass

through the column over a reasonable time span. The injector oven is usually 50–100 °C hotter than the start of the column. The sample is then taken through the column by an inert gas (known as the carrier gas) such as helium or nitrogen which must be dry to avoid interference from water molecules. It can be dried by passing it through anhydrous copper (II) sulphate or self indicating silica (silica impregnated with cobalt(II) chloride). Unwanted organic solvent vapours can be removed by passing the gas through activated charcoal. The column is coiled so that it will fit into the thermostatically controlled oven.



Analytical columns tend to be narrow, and preparation columns tend to be wide to allow for the greater volumes passing through them.

The sensitivity of the technique is such that very small samples can be analysed, down to 10^{-7} dm^3 (0.1 μl).

Figure 9. Schematic representation of GC system

The temperature of the oven is kept constant for a straightforward separation, but if there are a large number of solutes, or they have similar affinities for the stationary phase relative to the mobile phase, then it is common for the temperature of the column to be increased gradually over a required range. This is done by using computer control, and gives a better separation if solute boiling points are close, and a faster separation if some components are relatively involatile. The solutes progress to the end of the column, to a detector.

Detectors Used:

After the components have been separated by the chromatograph columns, they are then passed over to the detector. Several types of detectors are available for gas chromatographs, including flame ionization detectors (for ppm-level hydrocarbons) and flame photometric detectors (for ppb- to ppm-level sulphur detection), but the most common detector used for most hydrocarbon gas measurements is the thermal conductivity detector (TCD).

3.4 Liquid chromatography (LC)

Liquid chromatography is similar to gas chromatography but uses a liquid instead of a gaseous mobile phase. The stationary phase is usually an inert solid such as silica gel ($SiO_2.xH_2O$), alumina ($Al_2O_3.xH_2O$) or cellulose supported in a glass column. The adsorbing properties of silica and alumina are reduced if they absorb water, but the reduction is reversed by heating to 200–400°C. Silica is slightly acidic, and readily adsorbs basic solutes. On the other hand, alumina is slightly basic and strongly adsorbs acidic solutes. Other stationary phases that can be used include magnesia, $MgO.xH_2O$ (good for separating unsaturated organic compounds); and dextran (a polymer of glucose) cross-linked with propan-1,2,3-triol (glycerol, $CH_2OHCHOHCH_2OH$), which is sold as Sephadex and can separate compounds such as purines. A wide range of solvents are used in this technique, including hydrocarbons, aromatic compounds, alcohols, ketones, halogenocompounds and esters. A mixture of solvents can also be used. The optimum solvent is chosen by running experiments on a small scale using TLC plates.

3.5 High performance liquid chromatography (HPLC)

The efficiency of a separation increases if the particles in the stationary phase are made smaller. This is because the solute can equilibrate more rapidly between the two phases. However, if the particles are made smaller, capillary action increases and it becomes more difficult to drain the column under gravity. Consequently, a high pressure has to be applied to the solvent to force it through the column. The stationary phase normally consists of uniform porous silica particles of diameter

10^{-6}m range, the surface pores having a diameter of 10^{-8}–10^{-9} m. (This gives the solid a very high surface area). The particles can be bonded with a non-volatile liquid that allows interactions of solutes with different polarities. These liquids are held on the silica particles by covalent bonds. Interaction is then possible between the lone pair of electrons on the nitrogen atom and the solute molecule. The stationary phase particles are packed into the HPLC column and are held in place by glass fibres coated with inert alkyl silane molecules. The separation in HPLC is normally so efficient that a long column is not necessary Figure 10. (If the column was too Reproducibility is essential, and this is only possible if a constant flow rate is maintained. This means that the pump used must be capable of generating a uniform pressure; twin cylinder reciprocating pumps are typical. This type of pump has two chambers with pistons 180°C out of phase, and can generate pressures up to 10 MNm^{-2} (10 MPa/100 atmospheres). The high pressures involved mean that the instrumentation has to be very strong, and the 'plumbing' is usually constructed from stainless steel. The pump and the piping must be inert to the solvent and solutes being passed through them. The flow rates of HPLC columns are slow – often in the range 0.5–5 cm^3 min^{-1}. The volumes of the columns are very small, and this means that the injection of the sample must be very precise and it must be quick without disturbing the solvent flow. Sample volumes are small 5–20 mm^3 is usually sufficient.

The passage of solutes through a GC can be speeded up by increasing the temperature of the column. The same effect in HPLC is achieved by changing the composition of the mobile phase i.e., there is a concentration gradient in which the proportion of methanol, say, in a methanol/water system is increased linearly from 10 per cent methanol to 60 per cent methanol, during the separation.

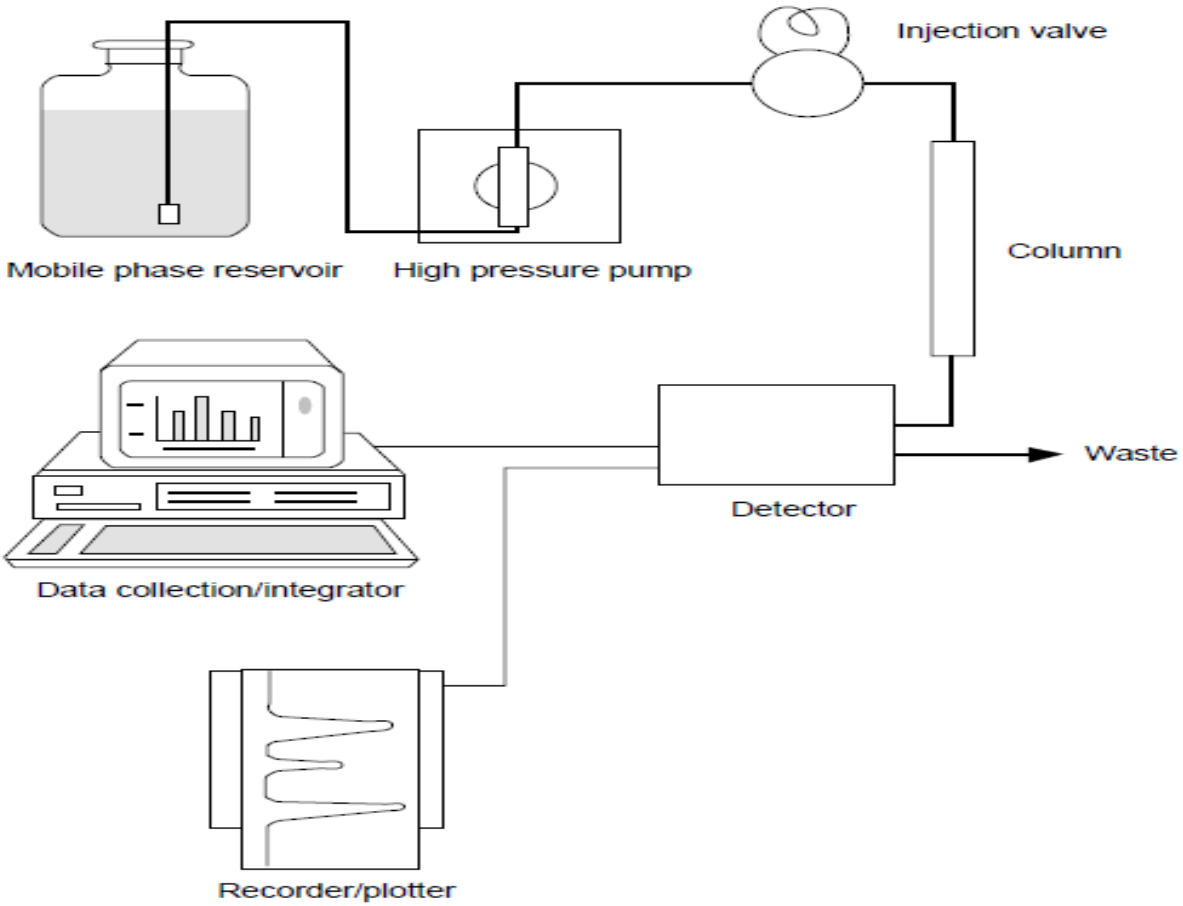


Figure 10. Schematic representation of an HPLC system

Detectors used:

1. Refractive Index Detector
2. Evaporative Light Scattering Detector
3. Conductivity Detector
4. Conventional UV-Vis Absorption Detector
5. Multi-wavelength UV-Vis Absorption Detector
6. Fluorescence Detector
7. Electrochemical Detector
8. ICP Detector Detector

Other methods used in conjunction with HPLC for determining the presence of solutes are based on:

1. Mass spectrometry;

2. Infrared spectroscopy;
3. Visible spectroscopy;
4. Ultraviolet spectroscopy;
5. Fluorescence spectroscopy;
6. Conductivity measurement; and
7. Refractive index measurement.

3.6 Ion exchange chromatography

Ion exchange chromatography is used to remove ions of one type from a mixture and replace them by ions of another type. The column is packed with porous beads of a resin that will exchange either cations or anions. There is one type of ion on the surface of the resin and these are released when other ions are bound in their place – e.g., a basic anion exchange resin might remove nitrate (V) ions (NO_3 –) from a solution and replace them with hydroxide ions (OH–). Many of the resins used are based on phenylethene (styrene) polymers with cross linking via 1,4-bis-ethenylbenzene (divinylbenzene). If the ion is a quaternary ammonium group the resin is strongly basic (eg $–CH_2N(CH_3)_3$ + OH–) then the resin will selectively remove the ions: $I^- > NO_3^- > Br^- > NO_2^- > Cl^- > OH^- (> F^-)$, thus liberating hydroxide ions while nitrate (V) ions, for example, are removed. The exchange site can be strongly or weakly acidic or basic depending on the group present.

3.7 Gel filtration or gel permeation chromatography

The separation of large molecules, often in biochemical situations, can be achieved in a column which works on the basis of molecular exclusion. The mixture of solutes is carried through the column by a solvent. The stationary phase (the gel) typically consists of particles of a cross-linked polyamide which contains pores. Separation occurs according to molecular size – the larger molecules passing through the column fastest . Different gels are available that allow the separation of proteins with relative masses ranging from a few hundred to in excess of 108. The greatest resolution is achieved by using very small gel particles, but the flow rate through the column then becomes much slower.

4. Factors that determine the efficiency of Chromatography:

a. Separation Efficiency: Plate Theory The plate theory supposes that the chromatographic column contains a large number of separate layers, called **theoretical plates**. Separate equilibrations of the sample between the stationary and mobile phase occur in these "**plates**". The analyte moves down the column by transfer of equilibrated mobile phase from one plate to the next

Greater theoretical plates Better separation resolution

Theoretical Plate Number-Resolution

A measure of separation efficiency: How many times the

Analyte mobile ☐ Analyte stationary equilibrium is achieved

b. In qualitative analysis by comparison with known components, retention time (Distance) is used for identification of a component of a mixture

Relative retention = Test Retention Time/ Reference Retention time

c. The mass distribution coefficient, *ki`*, is of central importance for the description of the chromatographic migration; nevertheless, it determines the extent of peak dispersion as well *ki`* is named retention factor, capacity factor or capacity ratio. It was already mentioned that *ki`* consists of an unspecific part, the phase ratio that is equal for all analytes for a given chromatographic system.

d. The retention time is the time in which halve of the quantity of a solute, *i*, is eluted from the chromatographic system. With other words, it is identical with the position of the peak maximum in case

of a Gaussian elution profile. It is determined by the length of the column, L, and the migration velocity of the solute.

1. Applications of chromatography:

a) Chromatography is widely used for recognizing the presence or absence of components in mixtures that contain a limited number of species whose identities are known. For example, 30 or more amino acids in a protein hydrolysate can be detected with a reasonable degree of certainty by means of a chromatogram.

b) It is important to note that while a chromatogram may not lead to positive identification of the species in a sample, it often provides sure evidence of the *absence* of species

c) Chromatography owes its enormous growth in part to its speed, simplicity, relatively low cost, and wide applicability as a tool for separations. However, it is doubtful that its use would have become so widespread had it not been for the fact that it can also provide quantitative information about separated species.

d) Quantitative chromatography is based on a comparison of either the height or the area of an analyte peak with that of one or more standards.

e) Chromatography is used to separate compounds in reaction mixtures both in the laboratory and on the industrial scale. However, technology has now advanced sufficiently to allow chromatographic techniques to be interfaced directly to other analytical methods. For example, gas chromatographs are routinely linked to mass spectrometers and HPLC columns are linked to UV-Vis spectrometers.

f) Chromatographic techniques have become very important in industry for the purification and separation of intermediates in multi-stage synthesis. Such separations have to be done in batches rather than in continuous flow. In terms of scientific advances, one of the major innovations in the past five years has been the development of efficient columns capable of separating specific chiral compounds

from a mixture. They work by the stereo-specific adsorption of one enantiomer onto the surface of the stationary phase. The resin contains only one enantiomer, hence its stereo-selectivity towards other chiral molecules. The cost of column for chiral separations can be roughly three times (or even higher) the cost of the standard analytical column. A similar type of chromatography, affinity chromatography works on similar principle- a substrate is covalently bound to a resin ans only vacant sites in the correct orientation can interact with these sites.

g) Research has also shown that graphite based HPLC packing materials can separate compounds with low or modest polarities very efficiently. Studies of particles and pore sizes have shown that the surface area of graphite based materials in HPLC columns can be as high as $1000 m^2 g^{-1}$. Some classes of compounds that have been separated by such columns include:
   1. Aromatic hydrocarbons
   2. Alkylnapthalenes
   3. Methylphenols
   4. Polychlorinated biphenyls
   5. Steroids
   6. Some amino-acids

By using different solvent systems are one area which has been found to have great potential is supercritical fluid chromatography (SFC). A supercritical fluid is one at a temperature and pressure above its critical point. Supercritical carbon dioxide (used to extract caffeine from coffee) in HPLC columns has the solvating properties of a liquid and transport properties of gas. Another advantage is that the extraction of the solutes from eluate is easy- the carbon dioxide is simply allowed to evaporate.

h) However, supercritical carbon dioxide would not be good solvent for a chromatography column if the eluate were to be analysed by infrared spectroscopy, because of the strong spectral absorption of carbon dioxide which would obscure the absorption of the eluted compounds. Supercritical xenon has been found to achieve good

separation of compounds such as polyaromatic hydrocarbons and if the eluate flows through a microcell no absorption due to xenon is observed. This is particular advantage if SFC is to be linked with infrared spectroscopy.