Chapter 10

High performance liquid chromatography

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General Principles and Techniques of Chromatography

Chromatography is a technique by which two closely related compounds can be separated. It can be used for the separation of large amounts or small amounts of materials. The selection of a particular form of chromatography to achieve a separation is dependent on the material to be isolated and often several chromatographic methods may be used sequentially to achieve the complete purification of a compound.

The basis of all forms of chromatography is the partition or distribution coefficient (Kd) which describes the way in which a compound distributes itself between two immiscible phases. For a compound distributing itself between equal volumes of two immiscible solvents A and B, the value for this coefficient is a constant at a given temperature and is given by the expression:

Kd = Concentration in solvent A / Concentration in solvent B.

The term effective distribution coefficient is defined as the total amount as distinct from the concentration of substance present in one phase divided by the total amount present in the other phase. It is infact the distribution coefficient multiplied by the ratio of the volumes of the two phases present. Basically all chromatographic systems consist of two phases. One is the stationary phase which may be solid, liquid or a solid /liquid mixture which is immobilized. The second mobile phase may be liquid or gaseous and flows over or through the stationary phase. The choice of stationary or mobile phases is made so that the compounds to be separated have different distribution coefficients. This may be achieved

by setting up:

1. An adsorption equilibrium between a stationary solid and a mobile liquid phase (adsorption chromatography).
2. A partition equilibrium between a stationary liquid and a mobile liquid phase (partition chromatography).
3. A partition equilibrium between a stationary liquid and a mobile gaseous phase (gas–liquid chromatography).
4. An ion exchange equilibrium between an ion exchange resin stationary phase and a mobile electrolyte phase (ion–exchange chromatography).
5. An equilibrium between a liquid phase inside and outside a porous structure or molecular sieve (exclusion chromatography).
6. An equilibrium between a macromolecule and a small molecule which has a high biological specificity and hence affinity (affinity chromatography).

The principle of separation may be depicted by considering a column packed with a solid granular stationary phase to a height of 5 cm surrounded by the mobile liquid phase of 1 cm^3 per cm of column. If 32 μg of a compound is added to the column in 1 cm^3 of solvent, then as this 1cm^3 moves on to the column to occupy position, 1cm^3 of solvent will leave the base of the column. If the compound added has an effective distribution coefficient of 1, it will distribute equally between the solid and liquid phases. If a further 1cm^3 of solvent is introduced onto the column 32 μg of substance will be distributed to a larger area making 16 μg in each area. If a further 1cm^3 of solvent is introduced, further distribution takes placing making 8 μg in each area and 16 μg in the centre. It is apparent that after each equilibration, the compound is getting distributed with maximum concentration at the centre part. The two factors which affect the pattern of separation (resolution) of a mixture of compounds ie. effective distribution coefficient and the number of equilibrations that take place. In a real situation, the equilibrations take place continuously on a column since the solvent is being poured continuously. The number of equilibrations is called number of theoretical plates and the each zone is called a theoretical plate. And its length in the column is called the height equivalent to one theoretical plate. The more efficient the column, the greater the number of theoretical plates that are involved.

In practice, chromatographic separations may take one of the three modes: column chromatography, thin layer chromatography and paper chromatography, based on how the stationary phase is supported.

In column chromatography, many times the peak can be asymmetrical displaying either fronting or tailing. Peak asymmetry has many reasons including application of too much solute to the column, poor packing of the column, poor application of sample to the column or solute support interactions.

The success of any chromatographic procedure is measured by its ability to separate completely (resolve) one compound from a mixture of similar compounds. Peak resolution Rs = 2(tRa –tRB)/wA

+wB where tRA and tRB = the retention times of compounds A B respectively.

wA and WB = the base widths of the peaks for A and B respectively.

It is shown that where Rs = 1.5, the separation of the two peaks is 99.7% complete. In most practical cases Rs values of 1.0, corresponding to 98% separation, is satisfactory.

The number of theoretical plates (plate number)(N) involved in the elution of a particular compound is given by:

N = 16(tR/w)2

High Performance Liquid Chromatography

The resolving power of a chromatographic column increases with column length and the number of theoretical plates per unit length, although there are limits to the length of a column due to the problem of peak broadening. As the number of theoretical plates is related to surface area of the stationary phase, it follows that the smaller the particle size of the stationary phase, the better the resolution. Unfortunately the smaller the particle size, the greater the resistance to eluent flow. In the last decade there has been a dramatic development in column chromatography technology which has resulted in the availability of new smaller particle size stationary phases which can withstand these pressures and of pumping systems which can give reliable flow rates. These developments which have occurred in adsorption, partition, ion–exchange, exclusion and affinity chromatography have resulted in faster and better resolution and explain why HPLC has emerged as the most popular, powerful and versatile form of chromatography.

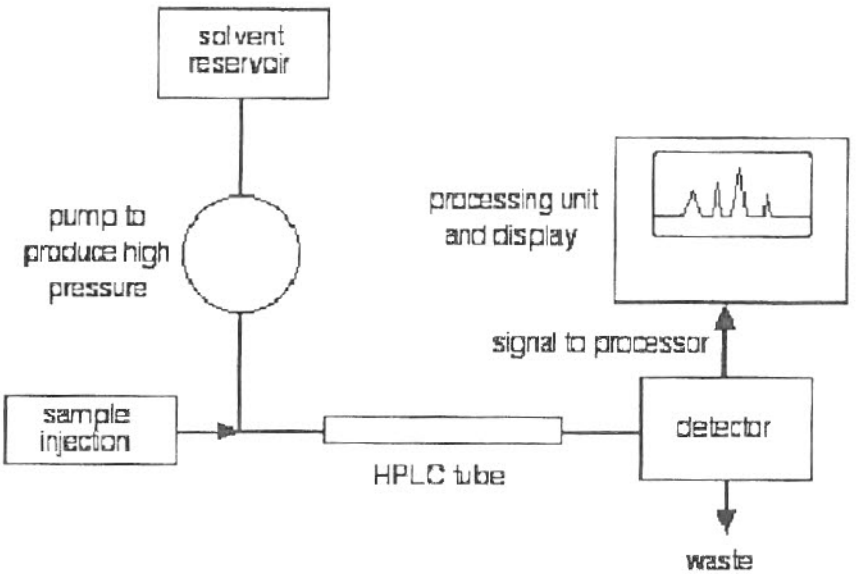
Originally HPLC was referred to as high pressure liquid chromatography, but nowadays the term high performance liquid chromatography is preferred since it better describes the characteristics of chromatography.

Apparatus and materials

The principal components of a HPLC system are explained below.

The column used for HPLC are generally made of stainless steel and are manufactured that they can withstand pressures of upto 5.5 x 10^7 (8000 p.s.i). Straight columns of 20 to 50 cm in length and 1 to 4 mm in diameter are generally used though smaller capillary columns are available. The best columns are precision bored with an internal mirror finish which allows efficient packing of the column. Porous plugs of stainless steel or teflon are used in the end of the column to retain the packing material.

The plugs must be homogeneous to ensure uniform flow of solvent through the column. It is important in some separations involving liquid partition and ion exchange that the



column temperature is thermostatically controlled during the analysis

Column Packing

Three forms of column packing material are available based on a rigid solid structure. These are:

1. Microporous supports where micropores ramify through the particles which are 5 to 10 μm in diameter.
2. Pellicular (Superficially porous) supports where porous particles are coated onto an inert solid core such as glass bead of about 40 μm in diameter.
3. Bonded phases where the stationary phase is chemically bonded to an inert support.

For adsorption chromatography, adsorbants silica or alumina are available as microporous or pellicular forms with a range of particle sizes. Pellicular systems generally have a high efficiency but low sample capacity. All forms of HPLC column packing are characterized by their regular spherical shape which distinguishes them from conventional materials. These small spheres pack most efficiently and give good flow properties.

In liquid–liquid partition systems, the stationary phase may be coated onto the inert support. Both microporous and pellicular supports are used for supporting the liquid phase. One disadvantage of supports quoted with liquid phases is that developing solvent may gradually wash off the liquid phase with repeated use. To overcome this problem, bonded phases have been developed where the liquid phase has been covalently bonded to the supporting material which may be silica or a silicon polymer. The silicon polymer bonded phases the particular advantage that as well as not being eluted by the developing solvent,

they are chemically, hydrolytically and thermally stable. In normal phase liquid-liquid chromatography, the stationary phase is a polar compound such as alkyl or alkyl amine derivatives and the mobile phase a non–polar solvent such as hexane. For reverse phase chromatography, the stationary phase is a non-polar compound such as C_8 or C_{18} hydrocarbon and the mobile phase a polar solvent such as water/acetonitrile or water/ methanol mixtures.

Many different types of ion exchangers are available of which the cross linked microporous polystyrene resins are widely used. Pellicular resin forms are also available, as are bonded phase exchangers covalently bonded to across – linked silicon network. The resins are classed as hard gels and readily withstand the pressures required during analysis.

The stationary phases for exclusion separations are generaly porous silica, glass, polystyrene or polyvinyl acetate beads. These are generally used where the eluting solvent is an organic system and the beads are available in a range of pore sizes.

The supports for affinity separations are similar to those for exclusion separations. The spacer arm and ligand are attached to these supports by similar chemical means to those used in conventional low pressure affinity chromatography.

Column Packing Procedure

Columns may be purchased already packed from commercial companies with specified packing material structure and dimensions. Many workers however prefer to pack their own columns since this is cheaper than purchasing pre–packed columns. Several methods are available for packing columns and the method used will depend on the nature of the packing material and the dimensions of the particles. The major priority in the packing of a column is to obtain a uniform bed of material with no cracks or channels. Rigid solids and hard gels should be packed as densely as possible, but without fracturing the particles during the packing process.

Chromatographic solvent (mobile phase)

The choice of mobile phase to be used in any separation will depend on the type of separation to be achieved. Isocratic separations may be made with a single solvent, or two or more solvents mixed in fixed proportions. Alternatively a gradient elution system may be used where the composition of the developing solvent is continuously changed by use of a suitable gradient programmer. In majority of cases this involves the use of two pumps. All solvents for use in HPLC systems must be specially purified since traces of impurities can affect the column and interfere with the detection system. Purified solvents for use in HPLC systems are available commercially, but even with these solvents a 1 to 5 μm microfilter is generally introduced into the system prior to the pump. It is also essential that all solvents are degassed before use, otherwise gassing tends to occur in most pumps. Gassing (the presence of air bubbles in the solvent) can alter column resolution and can interfere with the continuous monitoring of the column effluent. Degassing can be carried out in several ways; by warming the solvent, by stirring it vigorously with a magnetic stirrer, subjecting

it to a vacuum, ultrasonic vibration, or by bubbling helium gas through the solvent reservoir.

Pumping Systems

The pumping system is one of the most important features of an HPLC system. There is a high resistance to solvent flow due to the narrow columns packed with small particles, and high pressures are therefore required to achieve satisfactory flow rates. The main feature of a good pumping system is that it is capable of outputs of atleast 3.4×10^7 Pa (5000 p.s.i) and ideally there must be no pulses of flow through the system. The materials in the pump should be chemically resistant to all solvents.Various pumping systems are available which operate on the principle of constant pressure or constant displacement.

Such pumps as well as providing uniform solvent flow rates, also yield a pulse-less solvent flow which is important as certain detectors are sensitive to changes in solvent flow rate.

Constant Pressure Pumps

Constant pressure pumps produce a pulse-less flow through the column; but any decrease in the permeability of column will result in lower flow rates for which the pump will not compensate. These pumps operate by the introduction of high pressure gas into the pump, and the gas in turn forces the solvent from the pump chamber into the column. The use of an intermediate solvent between the gas and the eluting solvent reduces the chances of dissolved gas directly entering the eluting solvent and causing problems during the analysis.

Constant Displacement Pumps

These pumps maintain a constant flow rate through the column irrespective of changing conditions in the column. One form of constant displacement pump is a motor–driven syringe type pump where a fixed volume of solvent is forced from the pump to the column by a piston driven by a motor. The reciprocating pump is the most commonly used form of constant displacement pump. The piston is moved by a motorized crank and entry of solvent from the reservoir to the pump chamber and exit of solvent to the column is regulated by check valves. On the compression stroke solvent is forced from the pump chamber into the column. During the return stroke the exit check valve closes and solvent is drawn in *via.* the entry valve to the pump chamber, ready to be pumped on to the column on the next compression stroke.

Detector Systems

Since the quantity of material applied to the column is frequently very small, it is imperative that the sensitivity of the detector system is sufficiently high and stable. Most commonly the detector is a variable wavelength ultraviolet visible spectrophotometer, flourimeter, a refractive index monitor or an electrochemical detector. A recent development has been the interfacing of HPLC to a mass spectrometer.

Practical Procedure

The correct application of a sample onto a HPLC column is another particularly important factor in achieving successful separations. Ideally sample should be introduced as an infinitely narrow band on to column. There are two methods which are generally used. The first method makes use of a microsyringe designed to withstand high pressures. The sample is injected through a septum in an injection port, either directly onto the column packing or onto a small plug of inert material immediately above the column material. This can be done while the system is under pressure, or the pump may be turned off before injection, and when the pressure has dropped to near atmosphere, the injection is made and the pump switched on again. This is termed stop flow injection. The second method of sample introduction is by use of a loop injector. This consists of a metal loop of small volume which can be filled with the sample. By means of an appropriate valve, the eluent from the pump is channeled through the loop, the outlet of which leads directly onto the column. Automatic versions of loop injectors are commercially available.

Repeated application of highly impure samples such as sera, urine, plasma or whole blood, which have preferably been deproteinated, may eventually cause the column to loose its resolving power. To prevent this a guard column is installed between the injector and the analytical column. This column is a short (2 to 10cm) column of the same internal diameter, and packed with similar material to that present in the analytical column. Packing of the guard column can be replaced at regular intervals.