High Performance Liquid Chromatography

1. Introduction HPLC.

High Performance Liquid Chromatography (HPLC) is one mode of chromatography, one of the most used analytical techniques. Chromatographic process can be defined as separation technique involving mass-transfer between stationary and mobile phase. HPLC utilises a liquid mobile phase to separate the components of a mixture. The stationary phase can be a liquid or a solid phase. These components are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture separates into its components. The amount of resolution is important, and is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures. (fig.1)

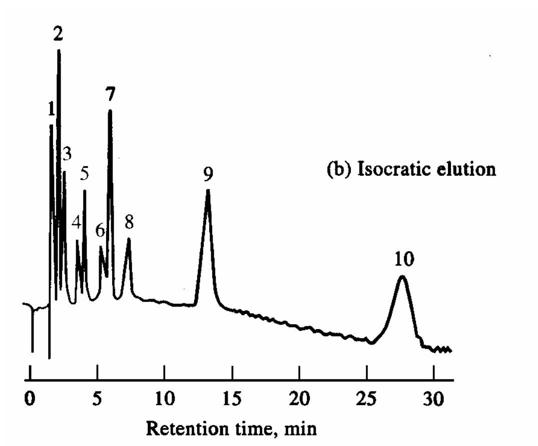

Fig. 1

2. Theory

HPLC is a dynamic adsorption process. Analyte molecules, while moving through the porous packing beads, tend to interact with the surface adsorption sites. Depending on the HPLC mode, the different types of the adsorption forces may be included in the retention process:
Hydrophobic (non-specific) interactions are the main ones in **reversed-phase (RP)** separations.
Dipole-dipole (polar) interactions are dominant in **normal phase (NP)** (mode.
Ionic interactions are responsible for the retention in **ion-exchange** chromatography.
All these interactions are competitive. Analyte molecules are competing with the eluent molecules for the adsorption sites. So, the stronger analyte molecules interact with the surface. The weaker the eluent interaction, the longer the analyte will be retained on the surface.
SEC (size-exclusion chromatography) is another case. It is the separation of the mixture by the molecular size of its components.
The basic principle of SEC separation is that the bigger the molecule, the less possibility there is for it to penetrate into the adsorbent pore space. So, the bigger the molecule the less it will be retained.

3. Types of HPLC

There are many ways to classify liquid column chromatography. If this classification is based on the nature of the stationary phase and the separation process, three modes can be specified.

a. Adsorption chromatography: the stationary phase is an adsorbent (like silica gel or any other silica based packing) and the separation is based on repeated adsorption-desorption steps.

b. Ion-exchange chromatography: the stationary bed has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time.

c. Size exclusion chromatography: the column is filled with material having precisely controlled pore sizes, and the sample is simply screened or filtered according to its solvated molecular size. Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the porous of the packing particles and elute later. This technique is also called gel filtration or gel permeation chromatography.
Concerning the first type, two modes are defined depending on the relative polarity of the two phases: normal and reversed-phase chromatography. In normal phase chromatography, the stationary bed is strongly polar in nature (e.g. silica gel), and the mobile phase is nonpolar (such as n-hexane). Polar samples are thus retained on the polar surface of the column packing for longer than less polar materials.
Reversed-phase chromatography is the inverse of this. The stationary bed is (nonpolar) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. Here the more nonpolar the material is, the longer it will be retained.
Reverse phase chromatography is used for almost 90% of all chromatographic applications.
Eluent polarity plays the major role in all types of HPLC. There are two elution types: isocratic and gradient. In the first type, constant eluent composition is pumped through the column during the whole analysis. In the second type, eluent composition (and strength) is steadily changed during the run.

HPLC as compared with the classical LC technique is characterised by:

- high resolution
- small diameter (4.6 mm), stainless steel, glass or titanium columns;
- column packing with very small (3, 5 and 10 μm) particles;
- relatively high inlet pressures and controlled flow of the mobile phase;
- continuous flow detectors capable of handling small flow rates and detecting very small amounts;
- rapid analysis;

Initially, pressure was selected as the principal criterion of modern liquid chromatography and thus the name was "high pressure liquid chromatography" or HPLC. This was, however, an unfortunate term because it seems to indicate that the improved performance is primarily due to the high pressure. This is, however, not true. In fact, high performance is the result of many factors: very small particles of narrow distribution range and uniform pore size and distribution, high pressure column slurry packing techniques, accurate low volume sample injectors, sensitive low volume detectors and, of course, good pumping systems. Naturally, pressure is needed to permit a given flow rate of the mobile phase.

4. Stationary Phases (Adsorbents)

HPLC separations are based on the surface interactions, and depend on the types of the adsorption sites. Modern HPLC adsorbents are the small rigid porous particles with high surface area.
Main adsorbent parameters are:

- Particle size: 3 to 10 µm
- Particle size distribution: as narrow as possible, usually within 10% of the mean;

The last parameter in the list represents an adsorbent surface chemistry.
Depending on the type of the ligand attached to the surface, the adsorbent could be normal phase (-OH, $-NH_2$), or reversed-phase (C_5, C_8, C_{18} CN, NH_2), and even anion ($CH_2NR_3^+OH^-$), or cation ($R\text{-}SO_3^-H^+$) exchangers.

5. Instrumentation HPLC system

HPLC instrumentation includes a pump, injector, column, detector and data system. The heart of the system is the column where separation occurs. Since the stationary phase is composed of micrometre size porous particles, a high pressure pump is required to move the mobile phase through the column. The chromatographic process begins by injecting the solute onto the top of the column. Separation of components occurs as the analytes and mobile phase are pumped through the column. Eventually, each component elutes from the column as a narrow band (or peak) on the recorder.
Detection of the eluting components is important, and this can be either selective or universal, depending upon the detector used. The response of the detector to each component is displayed on a chart recorder or computer screen and is known as a chromatogram. To collect, store and analyse the chromatographic data, computer, integrator, and other data processing equipment are frequently used.

6 Schematic of a HPLC instrument. (fig.3)

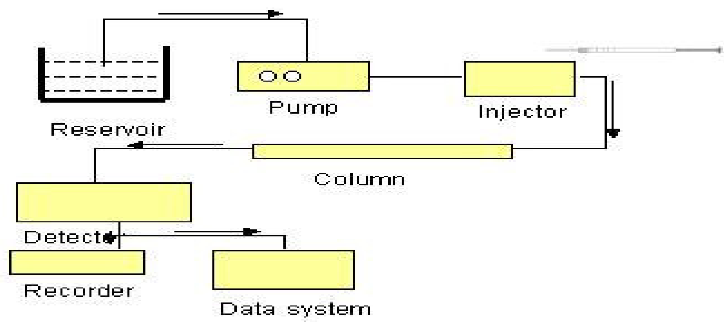

fig.3

7. Mobile phases

In HPLC, the type and composition of the eluent is one of the variables influencing the separation. Despite the large variety of solvents used in HPLC, there are several common properties:
Purity
Detector compatibility
Solubility of the sample
Low viscosity
Chemical inertness

For normal phase mode, solvents are mainly nonpolar; for reversed-phase, eluents are usually a mixture of water with some polar organic solvent such as acetonitrile or methanol.
Size-exclusion HPLC has special requirements. SEC eluents have to dissolve polymers, but the most important is that SEC eluent has to suppress possible interactions of the sample molecule with the surface of the packing material.

8. Functional description of the instrument

Mobile phase reservoir, filtering
Pump
Injector
Column
Detector
Data system

8.1 Mobile phase reservoir, filtering

The most common type of solvent reservoir is a glass bottle. Most of the manufacturers supply these bottles with special caps, Teflon tubing and filters to connect to the pump inlet and to the purge gas (helium) used to remove dissolved air. Helium purging and storage of the solvent under helium is not sufficient for degassing aqueous solvents. It is useful to apply a vacuum for 5-10 min. and then keep the solvent under a helium atmosphere.

8.2 Pump (fig.4)

High pressure pumps are needed to force solvents through packed stationary phase beds. Smaller bed particles require higher pressures. There are many advantages to using smaller particles, but they may not be essential for all separations.
The most important advantages are: higher resolution, faster analyses, and increased sample load capacity. However, only the most demanding separations require these advances in significant amounts. Many separation problems can be resolved with larger particle packings that require less pressure.
Flow rate stability is another important pump feature that distinguishes pumps.
Very stable flow rates are usually not essential for analytical chromatography.
However, if the user plans to use a system in size exclusion mode, then there must be a pump which provides an extremely stable flow rate.
An additional feature found on the more elaborate pumps is external electronic control. Although it adds to the expense of the pump, external electronic control is a very desirable feature when automation or electronically controlled gradients are to be run. Alternatively, this becomes an undesirable feature (since it is an unnecessary expense) when using isocratic methods. The degree of flow control also varies with pump expense. More expensive pumps include such state-of-the-art technology as electronic feedback and multiheaded configurations.
Modern pumps have the following parameters:

Flow rate range: 0.01 to 5 mL/min
Flow rate stability: not more than 1%
For SEC flow rate stability should be less than 0.2%
Maximum pressure: up to 300 hPa.
It is desirable to have an integrated degassing system, either helium purging,
or membrane filtering.

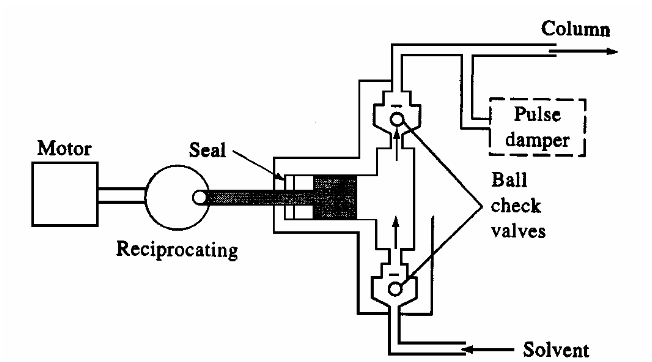

Fig.4 HPLC pump

8.3 Injector

Sample introduction can be accomplished in various ways. The simplest method is to use an injection valve. In more sophisticated LC systems, automatic sampling devices are incorporated where the sample is introduced with the help of autosamplers and microprocessors.
In liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. The solvent need not be the mobile phase, but frequently it is judiciously chosen to avoid detector interference, column/component interference, loss in efficiency or all of these.
It is always best to remove particles from the sample by filtering over a 5 µm filter, or centrifuging, since continuous injections of particulate material will eventually cause blockages in injection devices or columns.
Sample sizes may vary widely. The availability of highly sensitive detectors frequently allows use of the small samples which yield the highest column performance. Typical sample mass with 4.6 mm ID columns range from the nanogram level up to about 2 mg diluted in 20 ml of solvent. In general, it will be noted that much less sample preparation is required in LC than in GC since unwanted or interfering compounds, or both, may often be extracted, or eliminated, by selective detection. Examples of injectors see fig.5 and 6.

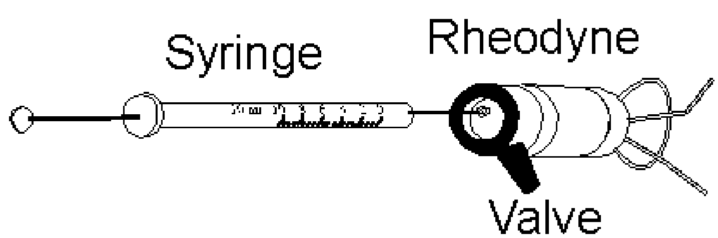

fig. 5

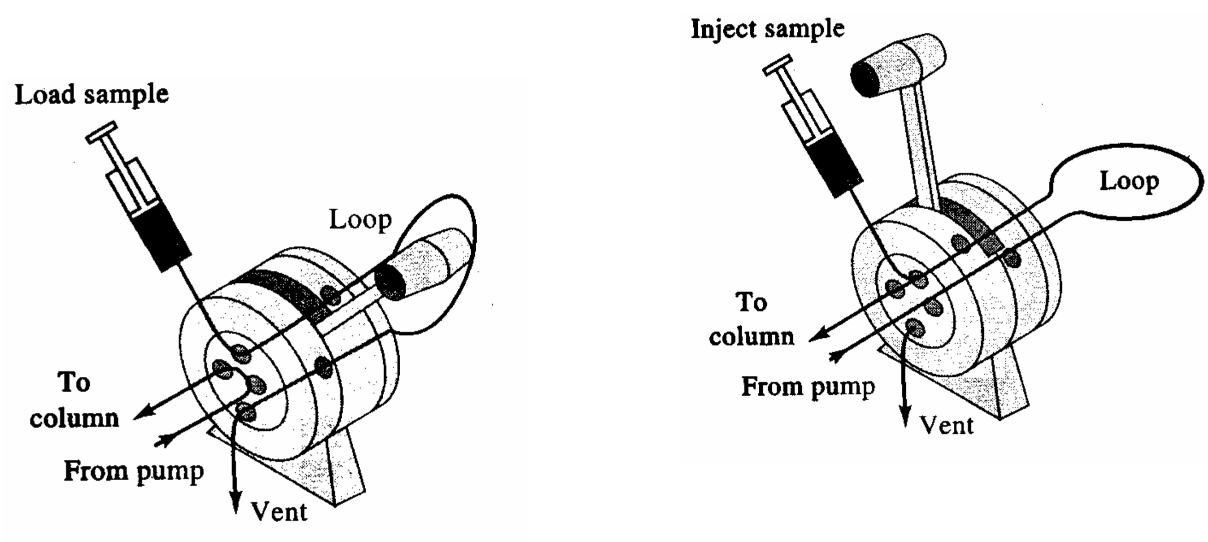

Fig 6.

8.4 Column

Typical HPLC columns are 5, 10, 15 and 25 cm in length and are filled with small diameter (3, 5 or 10 µm) particles. The internal diameter of the columns is usually 4.6 mm; this is considered the

best compromise for sample capacity, mobile phase consumption, speed and resolution. However, if pure substances are to be collected (preparative scale), then larger diameter columns may be needed. Packing the column tubing with small diameter particles requires high skill and specialized equipment. For this reason, it is generally recommended that all but the most experienced chromatographers purchase prepacked columns, since it is difficult to match the high performance of professionally packed LC columns without a large investment in time and equipment.
In general, LC columns are fairly durable and one can expect a long service life unless they are used in some manner which is intrinsically destructive, as for example, with highly acidic or basic eluents, or with continual injections of 'dirty' biological or crude samples. It is wise to inject some test mixture (under fixed conditions) into a column when new, and to retain the chromatogram.
If questionable results are obtained later, the test mixture can be injected again under specified conditions. The two chromatograms may be compared to establish whether or not the column is still useful.

8.5 Detector

Today, optical detectors are used most frequently in liquid chromatographic systems. These detectors pass a beam of light through the flowing column effluent as it passes through a low volume (~ 10 µl) flow cell. The variations in light intensity caused by UV absorption, fluorescence emission or change in refractive index, from the sample components passing through the cell, are monitored as changes in the output voltage. These voltage changes are recorded on a strip chart recorder and frequently are fed into a computer to provide retention time and peak area data.
The most commonly used detector in LC is the ultraviolet absorption detector (fig.8). A variable wavelength detector of this type, capable of monitoring from 190 to 400 nm, will be found suitable for the detection of the majority samples.
Other detectors in common use include: Photo Diode Array UV detector (PAD), refractive index (RI), fluorescence (FLU), electrochemical (EC). The RI detector is universal but also the less sensitive one. FLU and EC detectors are quite sensitive (up to 10-15 pmole) but also quite selective.

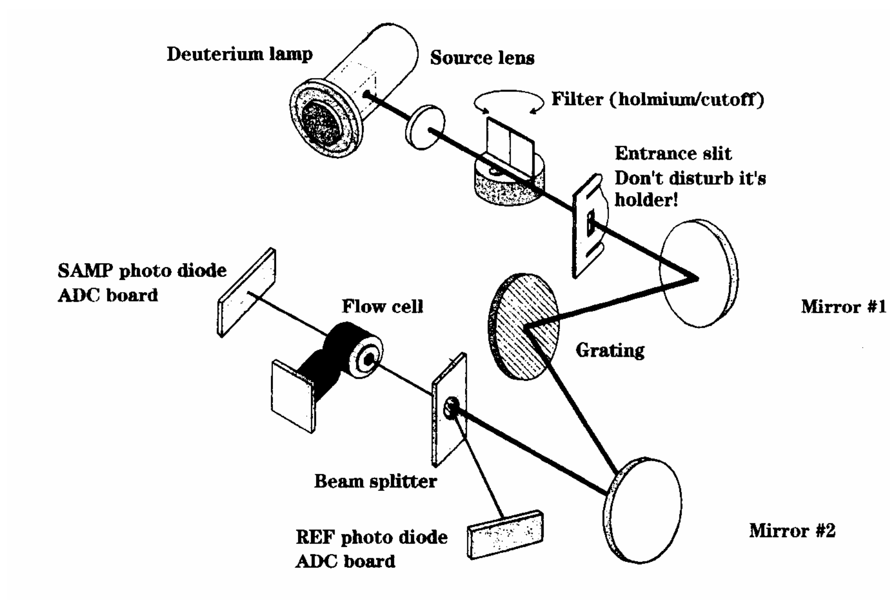

Fig.8

8.6 Data system

Since the detector signal is electronic, using modern data collection techniques can aid the signal analysis. In addition, some systems can store data in a retrievable form for highly sophisticated computer analysis at a later time.
The main goal in using electronic data systems is to increase analysis accuracy and precision, while reducing operator attention. There are several types of data systems, each differing in terms of available features. In routine analysis, where no automation (in terms of data management or process control) is needed, a pre-programmed computing integrator may be sufficient. If higher control levels are desired, a more intelligent device is necessary, such as a data station or minicomputer. The advantages of intelligent processors in chromatographs are found in several areas. First, additional automation options become easier to implement. Secondly, complex data analysis becomes more feasible. These analysis options include such features as run parameter optimisation and deconvolution (i.e. resolution) of overlapping peaks. Finally, software safeguards can be designed to reduce accidental misuse of the system.

Further information:

Reference textbooks

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A.Gratzfeld-Husgen, R.Schuster, HPLC for Food Analysis, Hewlett Packerd

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