High Pressure Liquid Chromatography

Separation of mixtures in microgram to gram quantities by passage of the sample through a column containing a stationary solid by means of a pressurized flow of a liquid mobile phase; components migrate through the column at different rates due to different relative affinities for the stationary and mobile phases base on adsorption, size or charge.

Apparatus and Instrumentation

1- Solvent delivery system

2- Stainless steel columns.

3- Injection port.

4- Flow through detector.

5- Recorder.

Applications

HPLC is used largely for the separation of non-volatile substances including 1onic and polymeric samples; complementary to gas chromatography.

Disadvantages

Column performance is very sensitive to settling of the packed bed or the accumulation of strongly adsorbed materials or particulate matter at the top; universal detection system not available.

High performance liquid chromatography (HPLC) has its origins in classical column chromatography) although in both theory and practice it is similar to gas chromatography. In column chromatography the sample is introduced into a liquid mobile phase which flows through a column of relatively coarse particles of the stationary phase, usually silica or alumina, under the influence of gravity. Flow rates are of the order of 0.1 cm^3 per min which results in extremely lengthy separation times and quite inadequate efficiencies and separations of multicomponent mixtures. The poor performance is largely due to very slow mass transfer between stationary and mobile phases and poor packing characteristics leading to multiple path effect. It was recognized that much higher efficiencies and hence better resolution could be achieved through the use of smaller particles of stationary phase, and that rapid separations would require higher flow rates necessitating the pumping of the mobile phase through the Column under pressure. The means of

meeting these two basic requirements were developed during the 1960s together with suitable pumps, injection systems and low dead-volume detectors and the technique, which is now at least as extensively used as GC, became known as "high performance liquid chromatography (HPLC) or simply liquid chromatography (LC). The mobile phase is typically pumped at pressures up to about 3000 psi (200 bar), and flow rates of 1-5 cm^3 min^{-1} can be achieved through 10-25 cm columns packed with particles as small as 3 micrometer in diameter. At its best HPLC is comparable to GC for speed, efficiency and resolution and it is inherently more versatile. It is not limited to volatile and thermally stable samples and the choice of stationary phase includes solid adsorbents, chemically modified adsorbents, ion-exchange and exclusion materials thus allowing all four sorption mechanisms to be exploited. A much wider choice of mobile phases than in GC facilitates a very considerable variation in the selectivity of the separation process. A schematic diagram of a high performance liquid chromatograph is shown in **Figure 1** and details of the components are discussed below.

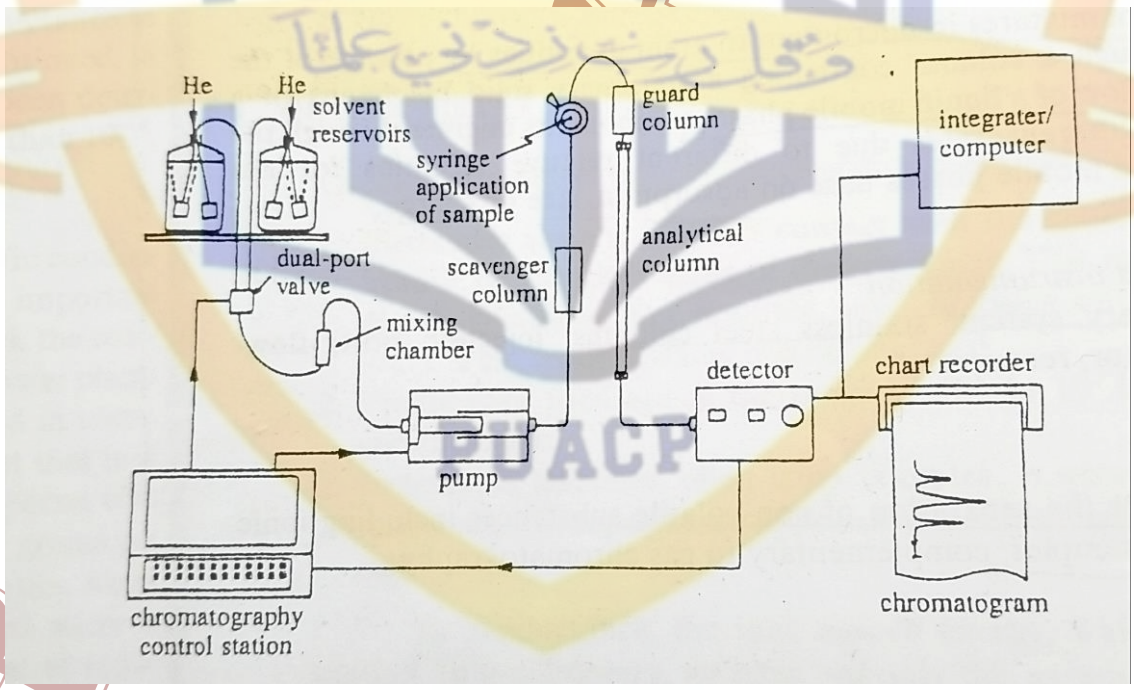


Figure 1

All materials which come into contact with the mobile phase are manufactured from stainless steel, PTFE, sapphire, ruby or glass for inertness.

Solvent Delivery System/HPLC Pump

These include solvent reservoirs and inlet filters, solvent degassing facilities and one or more pumps with associated pressure and flow controls. ~~(Most systems are microprocessor or computer controlled enabling parameters to be selected and monitored during operation).~~

A single solvent may be used as the mobile phase for **isocratic elution** or mixtures of two to four solvents (binary, ternary and quaternary) blended together under microprocessor or computer control for **gradient elution** i.e. where the composition of the mobile phase is altered during the chromatographic run so as to optimize the separation.

Pumps for HPLC should be capable of delivering a constant, reproducible and pulse-free supply of mobile phase to the column at flow-rates between 0.1 and at least 5 cm^3 min^{-1} and at operating pressures up to about 3000 psi (200 bar) They should be chemically inert to the various solvents that may be used and preferably have a very small hold-up volume to facilitate rapid changes of mobile phase and for gradient elution.

A number of types of pump have been developed and these can be classified according to whether they function at constant flow, which is desirable for reproducible retention data, or constant pressure. The later will deliver a constant flow only; if column back pressure, solvent viscosity and temperature also remain constant. Constant flow reciprocating pumps are now the most widely used type (Figure 2a) but because their mechanical action inherently produces a pulsating delivery of the mobile phase the flow must be smoothed so as to eliminate the pulsations. This can be achieved in several ways, the simplest being the incorporation of a pulse damper in the flow to the column. One such device is a flexible bellows or diaphragm enclosed in a small oil-filled chamber which absorbs the pulsations. Alternative designs of pump include a double-headed arrangement where two pistons operate in parallel but with delivery strokes 180° out-of-phase and sharing common solvent inlets and outlets. Another is an in-line double-headed pump with one piston (A) having twice the capacity of the other and delivering solvent both to the column and to the chamber of the second piston simultaneously (Figure 2 b). The smaller piston (B) then takes over the delivery whilst the larger one is refilling.

With both designs, residual pulsations can be virtually eliminated with a pulse damper or by suitable cam design that varies the speed of the pistons during the fill and delivery parts of the cycle so as to maintain a constant flow. The flow rates of reciprocating pumps are varied by altering the length of stroke of the piston(s) or through the use of a variable speed stepper motor.

Constant flow can be ensured by incorporating flow or pressure sensors into the design which automatically adjust pumping stroke or motor speed by means of a feed-back system. Automatic compensation for solvent compressibility can therefore be achieved).

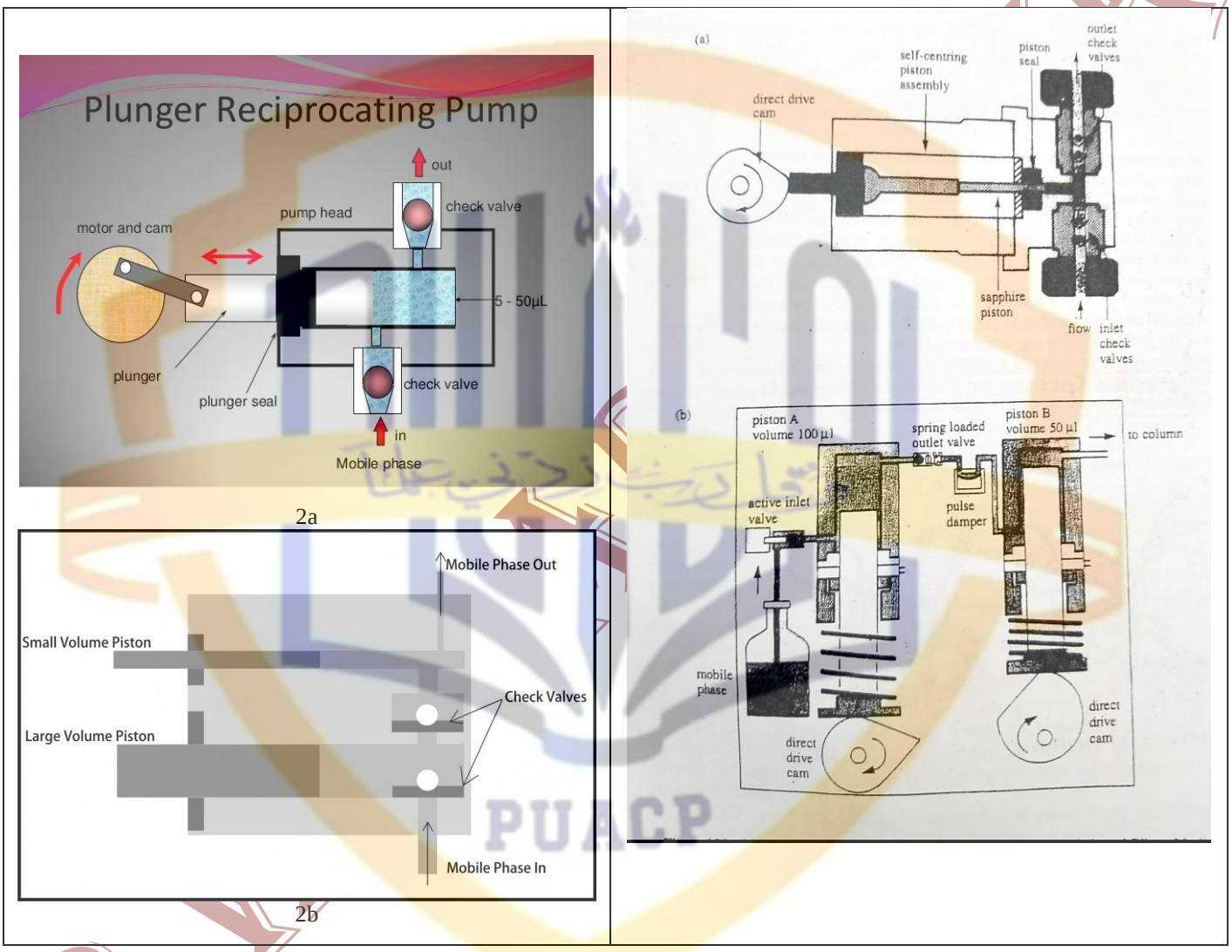


Figure 2: (a) Single head reciprocating pump. (b) Double head 'in series' reciprocating pump.

Two alternative but much less common types of constant flow pump are the screw-driven syringe and the hydraulic amplifier. The former consists of a variable-speed stepper motor which drives a plunger into a stainless-steel cylinder of large capacity (up to 500 cm^3) while in the latter a variable speed gear pump supplies oil under pressure to a pressure intensifier which in turn acts upon the mobile phase contained in a cylinder and delivers it to the column at a greatly increased

pressure. Disadvantages of these types of pump are cost and the need for frequent refilling especially when solvents need to be changed, although both achieve constant and pulseless flows without the need for pulse-dampers or feed-back controls.

Sample Injection System

Sample injection in HPLC is a more critical operation than in GC. Samples may be injected either by syringe or with a valve injector although the former is now rarely used. Valves, which can be used at pressures up to about 7000 psi (500 bar), give very reproducible results for replicate injections (<0.2% relative precision) and are therefore ideal for quantitative work. They consist of a stainless steel body and rotating central block into which are cut grooves to channel the mobile phase from the pump to the column (Figure 3). The sample is loaded into a stainless steel loop incorporated into the valve body or attached externally whilst the mobile phase is passed directly to the column. By rotating the central block, the flow can be diverted through the loop thereby flushing the sample onto the column. Returning the block to its original position enables the next sample to be loaded ready for injection. Although the sample injected is generally a fixed volume as determined by the size of the loop, these are interchangeable and range from 2 µl to over 100 µl. Multiport valves which can accommodate several loops of different sizes are available, and some loops can be used partially filed. ~~(Automated injection systems that allow a series of samples and standards to be injected over a period of time whilst the instrument is unattended and under variable chromatographic conditions are frequently used in industrial laboratories.)~~

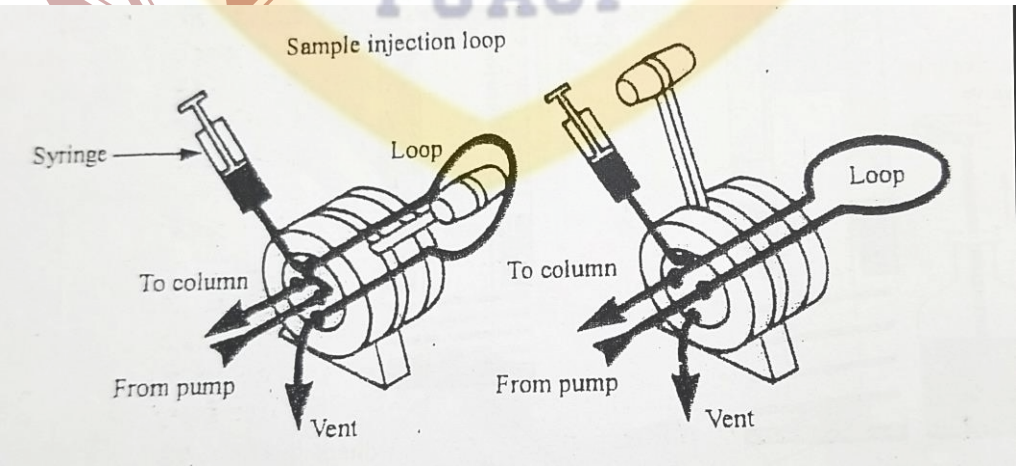


Figure 3: Six valve injector with external loop.

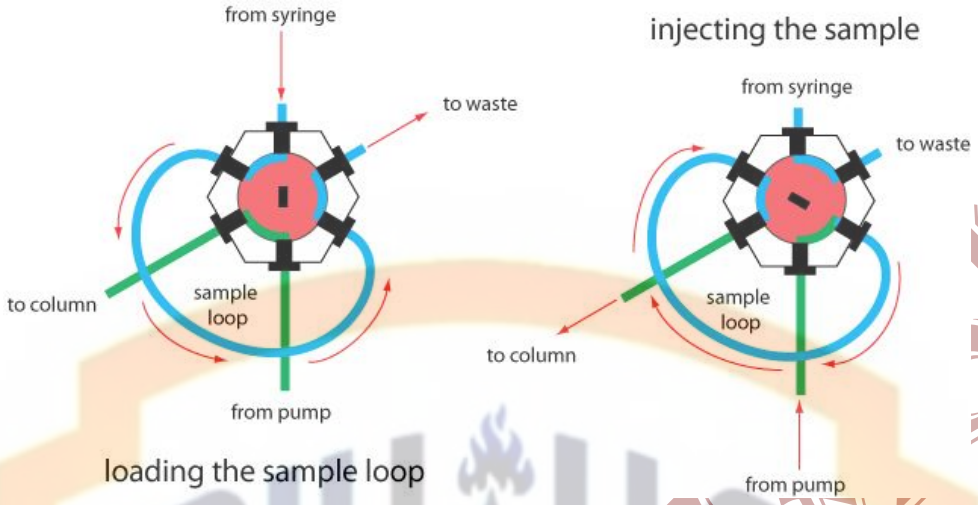


Figure 3: Six valve injector with external loop.

Column

Columns are made from straight lengths of precision-bore stainless-steel tubing with a smooth internal finish. Typically they are 10-25 cm long and 4-5 mm i.d.. Microbore columns, 20-50 cm long and with an i.d. of 1-2mm, are sometimes used where sample size is limited and to minimize solvent consumption because the volumetric flow rate through them is less than a quarter of that through conventional columns.

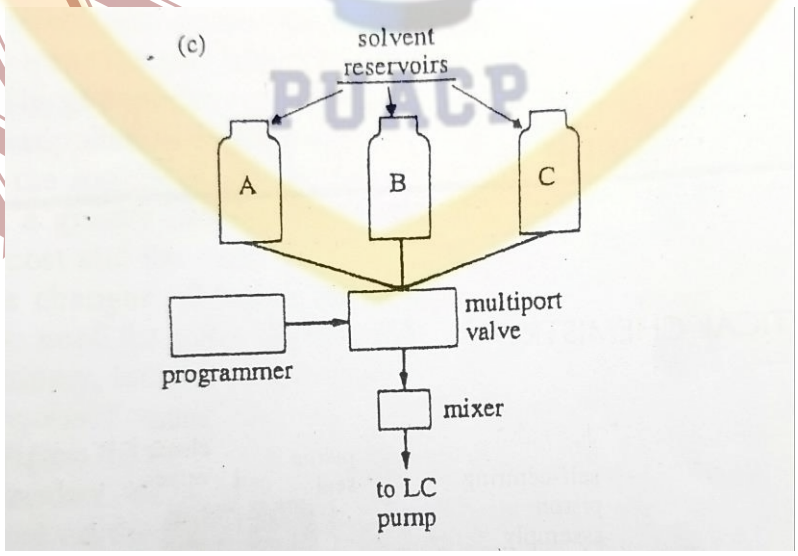


Figure 2c: A low-pressure gradient former with three solvents.

The stationary phase or packing is retained at each end by thin stainless-steel frits or mesh disks of 2 µm porosity or less. Columns are packed by a slurry method which involves suspending the particles of packing in à suitable solvent and 'slamming' it into the column rapidly and at pressures in excess of 3000 psi (200 bar). The choice of slurry solvent depends upon the nature of the packing and many solvents have been investigated. Balanced-density slurries, in which solvent density is matched with particle, density, are some- times used to minimize settling out of larger particles during the packing procedure or for packings which are larger than 10 µm. HPLC columns need more careful handling and storage than GC columns to avoid disturbance of the packed bed. They should be kept sealed at both ends when not in use and flushed with methanol prior to sealing.

Column life is generally six months or more and can be prolonged by use of a guard column and a scavenger column. The former (guard column) consists of a very short length of column placed **between the injection port and the analytical column** to trap strongly retained species or particulate matter originating from the mobile phase, the samples or from wearing of the injection valve. It is packed with relatively large particles (~30 um) of the same or a similar stationary phase to that used in the analytical column and require periodic renewal. Scavenger columns are short lengths of tube packed with large particle silica and **positioned between the pump and the injection valve** with the principal object of saturating an aqueous mobile phase with silica to reduce attack on the packing in the analytical column, especially by high or low pH buffers.

Stationary Phase (Column Packing)

Unmodified or chemically modified micro-particulate silicas (3, 5 or 10 um) are preferred for nearly all HPLC applications. The particles, which are totally porous, may be spherical or irregular in shape but it is essential that the size range is as narrow as possible to ensure high column efficiency and permeability. For separations based on adsorption, an unmodified silica, which has a polar surface due to the presence of silanol (Si-OH) groups is used. Appropriate chemical modification of the surface by treatment witn chloro- or alkoxy-silanes, e.g. $R(CH_3)_2SiCl$, produces bonded-phase packings which are resistant to hydrolysis by virtue of forming siloxane (Si-O-Si-C) bonds. The reactions are similar to those used to silanize GC

Supports. Materials with different polarities and chromatographic characteristics can be prepared. The most extensively used are those with a non-polar hydrocarbon-like surface, the modifying groups, R, being octa-decyl (C18 or ODS), octyl or aryl. More polar bonded-phases, e.g. amino-propyl, cyanopropyl (nitrile) and diol, and cation and anion exchange materials are also available. Mixed ODS/aminopropyl and ODS/nitrile phases having enhanced selectivity for certain classes of compound have also been produced. HPLC separations based on exclusion (GPC) are best achieved with microparticulate silicas which are sometimes chemically modified with such groups as trimethysilyil to eliminate or minimize adsorption effects.

Chiral stationary phases for the separation of enantiomers (Optically active isomers) are becoming increasingly important. Among the first Types to be synthesized were chiral amino acids ionically or covalently bound to amino-propyl silica and named Pirkle phases after their originator. The ionic form is susceptible to hydrolysis and can be used only in normal phase HPLC whereas the more stable covalent type can be used in reverse phase separations but is less stereoselective. Polymeric phases based on chiral peptides such as bovine serum albumin or α_1-acid glycoproteins bonded to silica, esterified cellulose and chiral cyclodextrins can all be used in reverse phase separations. The latter, α-, β- or γ-cyclodextrins form barrel shaped cavities into which enantiomers can fit. Chiral phases form complexes with analytes by binding them at specific sites through H-bonding, π–π and dipolar interaction. **In addition, steric repulsion, solvent, pH, ionic strength and temperature all affect chromatographic retention.** If the total adsorption energy of two enantiomers differs then a separation is possible.

The recently introduced graphitized carbon and a new generation or rigid porous polymeric micro-beads based on styrene/divinyl benzene as alternative of silica, can both be used over an extended pH range between 1 and 13. These materials have increased the choice of stationary phases and the scope of HPLC, particularly for highly polar and basic substances where peak tailing on silica-based columns is a frequent occurrence.

(Some examples of column packings used in HPLC and their applications are given in Table 4.13.)

Mobile Phase

Unlike GC, in HPLC appropriate selection of the mobile phase composition is crucial in optimizing chromatographic performance. The eluting power of the mobile phase is determined

by its overall polarity, the polarity of the stationary phase and the nature of the sample components. For normal phase separations (polar stationary phase/non-polar mobile phase) eluting power increases with increasing solvent polarity whereas in reverse phase separations (non-polar stationary phase/polar mobile phase) eluting power decreases with increasing solvent polarity). Some examples of solvents suitable for HPLC are given in Table 4.9 together with their polarities as measured by a solubility parameter, P' (applicable to partition-based separations) and an adsorption parameter, ε^{o} (applicable to adsorption-based separations). Such a list is often called an eluotropic series. Other properties of solvents which must be taken into account include boiling point and viscosity, detector compatibility, flammability and toxicity. Generally, lower boiling and hence less viscous solvents give higher chromatographic efficiencies and lower back pressures. The most commonly used detectors are based on absorbance of UV radiation and on refractive index. The UV cut-off and refractive indices of solvents therefore need to be known. These are included in Table 4.9.

Often, optimum retention and resolution are achieved by using a mixture of two solvents. The solubility-based parameter, P' varies linearly with the proportion of the two solvents, being given by the weighted arithmetic mean of the two individual values. The adsorption-based parameter, ε^{o}, however, increases rapidly with small additions of a more polar solvent to less polar one and levels off as the proportion increases. Elution with a single solvent or mixed solvent or fixed composition is called isocratic as opposed to gradient elution. Gradient elution is sometimes employed where sample components vary widely in polarity. (Table: 4.13). Gradient formers enable binary, ternary and quaternary mixtures of solvents to be blended reproducibly. In reverse phase separations the most widely used mobile phases are mixtures of aqueous buffers with methanol, or water with acetonitrile. In normal phase work pentane or hexane with dichloromethane, chloroform or an alcohol are frequently used.

Many soIvents for HPLC require purification before use as the impurities may either be strongly UV absorbing, e.g. aromatic or alkene impurities in n-alkanes, or they may be of much higher polarity than the solvent itself, e.g. traces of water or acids, or ethanol in chloroform, etc. All mobile phases should be filtered and degassed before pumping through the column, the former to prevent contamination and clogging of the top of the column and the latter to prevent noise in the

detector from the formation of air bubbles due to the pressure dropping to atmospheric at the column exit.

Table: 4.13

Packing	Mode of HPLC*	Applications
microparticulate silicas; spherical or irregular particles; mean particle size 3 µm, 5 µm, 10 µm	LSC (adsorption)	non-polar to moderately polar mixtures, e.g. polyaromatics, fats, oils, mixtures of isomers
chemically modified versions of the above (bonded-phase packings):		
octadecyl (ODS or C_{18})	BPC, IPC	wide range of moderately polar mixtures, e.g. pharmaceuticals and drugs, amino acids
octyl (C_8)	BPC, IPC	more polar mixtures, e.g. pesticides, herbicides, peptides, metabolites in body fluids
short chain (C_3 or less)	BPC, IPC	IPC applications of above three packings include bases, dyestuffs and other multiply charged species; can be used instead of IEC
diol	BPC	very polar and water-soluble compounds, e.g. food and drink additives
nitrile	normal phase and BPC	alternative to silica and can give better results
aminoakyl	BPC	carbohydrates including sugars
anion and cation exchangers (tertiary amine or sulphonic acid)	IEC	ionic and ionizable compounds, e.g. vitamins, water-soluble drugs, amino acids, food and drink additives
controlled porosity silicas (may be chemically modified to reduce adsorption effects) Trimethyl silyl	SEC	polymer mixtures, screening of unknown samples. Increasing use for separating mixtures of smaller molecules before other modes of HPLC
chiral amino acids bound to aminopropyl	CC	mixtures of enantiomers especially of drugs
chiral peptides	CC	
cyclodextrins	CC	

* LSC = liquid–solid chromatography
BPC = bonded-phase chromatography
IPC = ion-pair chromatography
ICE = ion-exchange chromatgraphy
SEC = size exclusion chromatography
CC = chiral chromatography

Table: 4.14

Detector basis	Type†	Maximum sensitivity‡	Flow rate sensitive?	Temperature sensitivity	Useful with gradient?
Ultraviolet absorption	*S*	5×10^{-10}	No	Low	Yes
Infrared absorption	*S*	10^{-6}	No	Low	Yes
Fluorimetry	*S*	10^{-10}	No	Low	Yes
Refractive index	*G*	5×10^{-7}	No	$\pm 10^{-4}$°C	No
Conductometric	*S*	10^{-8}	Yes	±1°C	No
Mass spectrometry	*S*	10^{-10}	No	None	—
Amperometric	*S*	10^{-10}	Yes	±1°C	—
Radioactivity	*S*	—	No	None	Yes

* Most of these data were taken from: L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*. New York: Wiley-Interscience, 1964, p. 165. With permission.
† *G* = general; *S* = selective.
‡ Sensitivity for a favourable sample in grams per millilitre.

What are guard columns and what benefits they offer in HPLC analysis?

HPLC Guard Column

A guard column is a protective column or cartridge installed between the injector and the analytical column. It serves to remove the impurities and suspended solids from reaching the analytical column. Typically it has a length of about 2 cm and internal diameter of 4.6 µm. Guard columns are packed with pelicullar particles of around 40 µm size to offer negligible pressure drop.

Desirable features of guard columns

- Guard column should have preferably the same packing as the analytical column to eliminate separation complications
- Internal ID of guard column should be comparable to analytical column to minimize back-pressure. Shorter guard column length is preferable but it should be long enough to prevent strongly retained compounds from reaching the main column
- Frit facing the injector should be removable for cleaning by removal of about 2 mm of material and filling with fresh material

- Disposable cartridge type guard columns are convenient and economical to use compared to refillable guard columns.

Guard columns need to be changed on regular basis but intermediate change becomes necessary through observation of changes in chromatographic behaviour such as increase in backpressure, peak broadening and, changes in retention time of peaks. However, the frequency of change can be decided on the basis of chemical composition of sample, presence of highly retained or irreversibly retained components, injection volume or number of injections.

Reverse and Normal Phase HPLC

A polar stationary phase and a non-polar mobile phase are used for normal phase HPLC. In normal phase, the most common R groups attached to the siloxane are: diol, amino, cyano, inorganic oxides, and dimethylamino. Normal phase is also a form of liquid-solid chromatography. The most non-polar compounds will elute first when doing normal phase HPLC.

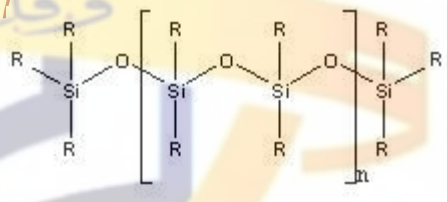


Figure: Basic structure of a siloxane.

The R groups can be varied depending on the type of column and analyte being analyzed.

Reverse phase HPLC uses a polar mobile phase and a non-polar stationary phase. Reverse phase HPLC is the most common liquid chromatography method used. The R groups usually attached to the siloxane for reverse phase HPLC are: C_8, C_{18}, or any hydrocarbon. Reverse phase can also use water as the mobile phase, which is advantageous because water is cheap, nontoxic, and invisible in the UV region. The most polar compounds will elute first when performing reverse phase HPLC. Check the animation on the principle of reversed-phase chromatography to understand its principle.

Ion Exchange Chromatographic Columns

Ion exchange columns are used to separate ions and molecules that can be easily ionized. Separation of the ions depends on the ion's affinity for the stationary phase, which creates an ion exchange system. The electrostatic interactions between the analytes, mobile phase, and the

stationary phase, contribute to the separation of ions in the sample. Only positively or negatively charged complexes can interact with their respective cation or anion exchangers. Common packing materials for ion exchange columns are amines, sulfonic acid, diatomaceous earth, styrene-divinylbenzene, and cross-linked polystyrene resins. Some of the first ion exchangers used were inorganic and made from aluminosilicates (zeolites). Although aluminosilicates are not widely used as ion exchange resins used.

Size Exclusion Chromatographic Columns

Size Exclusion Chromatographic columns separate molecules based upon their size, not molecular weight. A common packing material for these columns is molecular sieves. Zeolites are a common molecular sieve that is used. The molecular sieves have pores that small molecules can go into, but large molecules cannot. This allows the larger molecules to pass through the column faster than the smaller ones. Other packing materials for size exclusion chromatographic columns are polysaccharides and other polymers, and silica. The pore size for size exclusion separations varies between 4 and 200 nm.

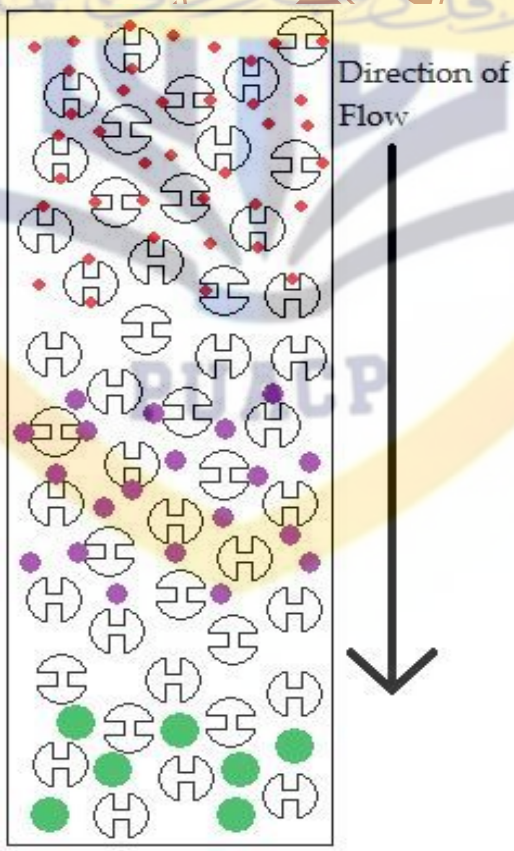


Figure: Schematic of a size exclusion column.

The larger particles will elute first because they are too big to fit inside the pores. The smallest particles will elute last because they fit very well inside the pores.

Column Efficiency

Peak or band broadening causes the column to be less efficient. The ideal situation would to have sharp peaks that are resolved. The longer a substance stays in the column it will cause the peaks to widen. Lengthening the column is a way to improve the separation of different species in the column. A column usually needs to remain at a constant temperature to remain efficient. Plate height and number of theoretical plates determines the efficiency of the column. Improving the efficiency would be to increase the number of plates and decrease the plate height.

The number of plates can be determined from the equation:

$$N = L/H \qquad (1)$$

Where, L is the length of the column and H is the height of each plate. N can also be determined from the equation:

$$N = 16(t_R W)^2 \qquad (2)$$

or

$$N = 5.54(t_R W_{1/2})^2 \qquad (3)$$

where t_R is the retention time, W is the width of the peak and $W_{1/2}$ is half the width of the peak. Height equivalent to a theoretical plate (HETP) is determined from the equation:

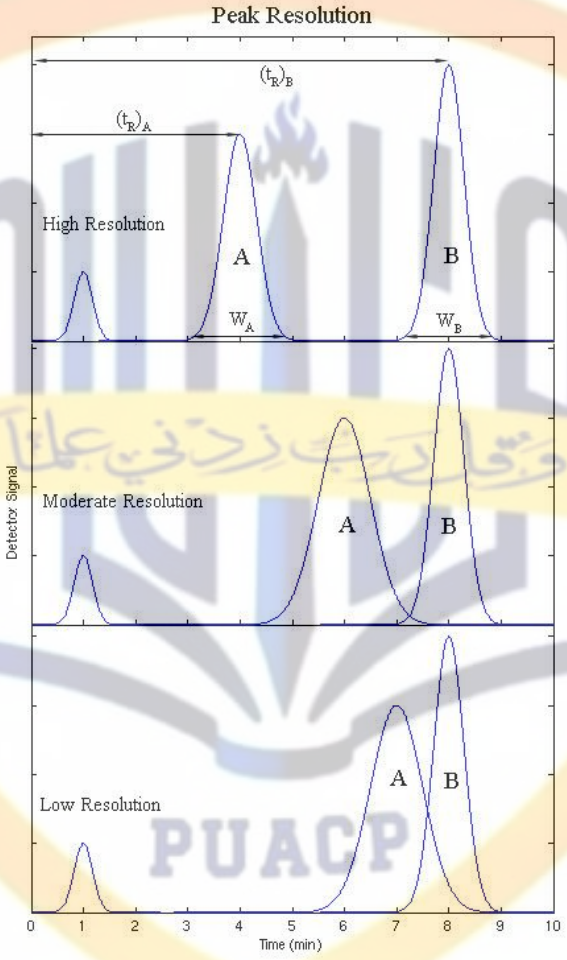
$$H = L/N \qquad (4)$$

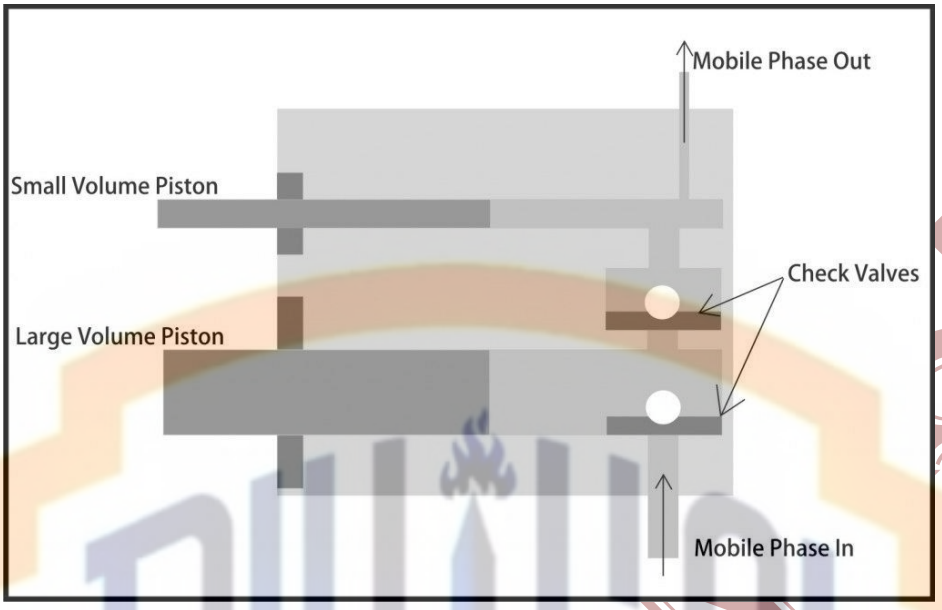
or HETP can also be determined by the *van Deemter equation*:

$$H = A + \frac{B}{\mu} + C\mu \qquad (5)$$

where H equals HETP, A is the term for eddy diffusion, B is the term for longitudinal diffusion, C is the coefficient for mass-transfer between the stationary and mobile phases, and μ is the linear velocity. The equation for HETP is often used to describe

the efficiency of the column. An efficient column would have a minimum HETP value. Gas chromatographic columns have plate heights that are at least one order of magnitude greater than liquid chromatographic column plates. However GC columns are longer, which causes them to be more efficient. LC columns have a maximum length of 25 cm whereas GC columns can be 100 meters long.





HPLC as compared with the classical technique is characterized by:

- small diameter (2-5 mm), reusable stainless steel columns;
- column packings with very small (3, 5 and 10 mm) particles and the continual development of new substances to be used as stationary phases;
- relatively high inlet pressures and controlled flow of the mobile phase;
- precise sample introduction without the need for large samples;
- special continuous flow detectors capable of handling small flow rates and detecting very small amounts;
- automated standardized instruments;
- rapid analysis; and
- high resolution.

The dead volume

The dead volume is the volume of an HPLC system between the points of injection to the point of detection, excluding the column.

Thus it includes the injection volume, the volume of the injector, the volume of the connecting tubing before and after the column, the volume of the end-fittings and frits, and the volume of the detector flow cell.

The dead volume can be measured by replacing the column with a zero dead-volume connector. By injecting a very small sample amount, the time can be measured between the moment of injection and the maximum peak height. This time multiplied by the flow rate gives you a very good estimation of the system dead volume.

Detectors

UV-Visible photometers and dispersive spectrophotometers:

These detectors respond to UV/visible absorbing species in the range l90 800 nm and their response are linear with concentration, obeying the Beer Lambert law. They are not appreciably flow or temperature sensitive, have a wide linear range and good but variable sensitivity. Photometers are designed to operate at one or more fixed wavelengths .i.e. 220, 254, 436 and 546 nm, whereas spectrophotometers facilitate monitoring at any wavelength within the operating range of the instrument. Both types of detector employ low-volume (10ul or less) flow-through cells fitted with quartz windows. Careful design of the cell, which should be of minimal volume to reduce band-spreading, and maximal path length for high sensitivity, is necessary to reduce undesirable refraction effects at the cell walls as solutes pass through. Although many substances absorb appreciably at 254 nm or one of the other fixed wavelengths available with a photometer, a much more versatile detection system is based on a spectrophotometer fitted with a grating monochromator and continuum source, e.g. a deuterium lamp for the UV region and a tungsten-halogen lamp for the visible region (Figure 1).

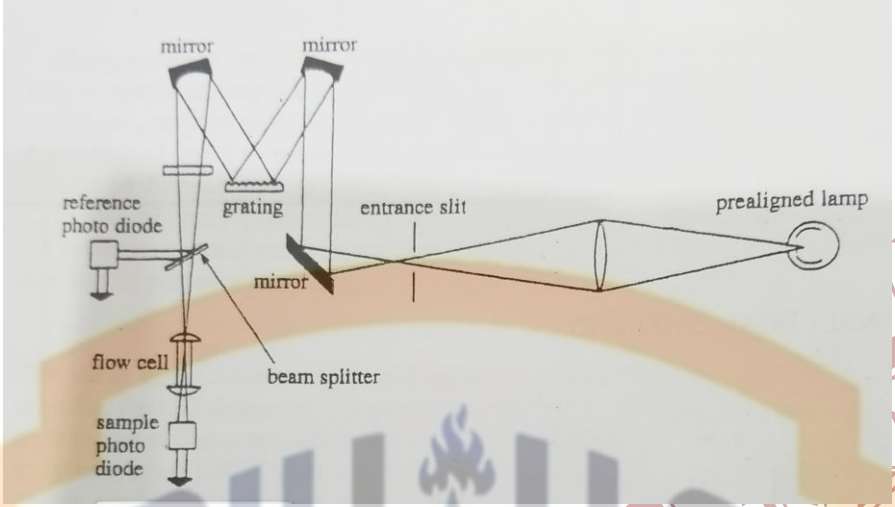


Figure: 1: Variable-wavelength detector, showing deuterium lamp, optical path, reference photodiode and monochromator.

They have double-beam optics, stable low-noise electronics and are often controlled by microprocessor. Some can be programmed to select a sequence of optimum monitoring wavelengths during or between chromatographic runs, and the recording of a complete UV spectrum after stopping the flow with a selected peak in the detector cell is a feature of other designs.

Another development is a rapid scanning capability that allows complete spectrum to be recorded in a fraction of a second without the need to stop the flow, therefore rivalling diode array detectors. Sensitivity and resolution are better than some diode array detectors. Photometers are more sensitive than spectrophotometers, are cheaper and more robust and are well suited to routine work where monitoring at 254 nm or some other fixed wavelength is acceptable. Spectrophotometers however, allow 'tuning' to the most favourable wavelength either to maximize sensitivity for a particular solute or to 'detune' the response to other solutes. By allowing monitoring down to 190 nm, weakly absorbing or saturated compounds can be detected.

Diode array spectrophotometers

These can provide more spectral information than photometers or conventional dispersive spectrophotometers but are much more expensive and generally less sensitive (Figure 2a). However, they enable sets of complete UV or UV and visible spectra of all the sample

components to be recorded as they elute from the column. The stored spectral information can be processed in several ways by the microcomputer and displayed using sophisticated colour graphics software packages. The most usual is a 3D chromatogram of time/absorbance/ wavelength ~~as shown in Figure 2(b).~~ This can be rotated on the screen to allow examination of otherwise hidden regions behind major peaks, (~~Figure 2 c~~) or viewed from directly above as an absorbance contour map to provide useful information on peak purity. (~~Figure 2(d)~~).

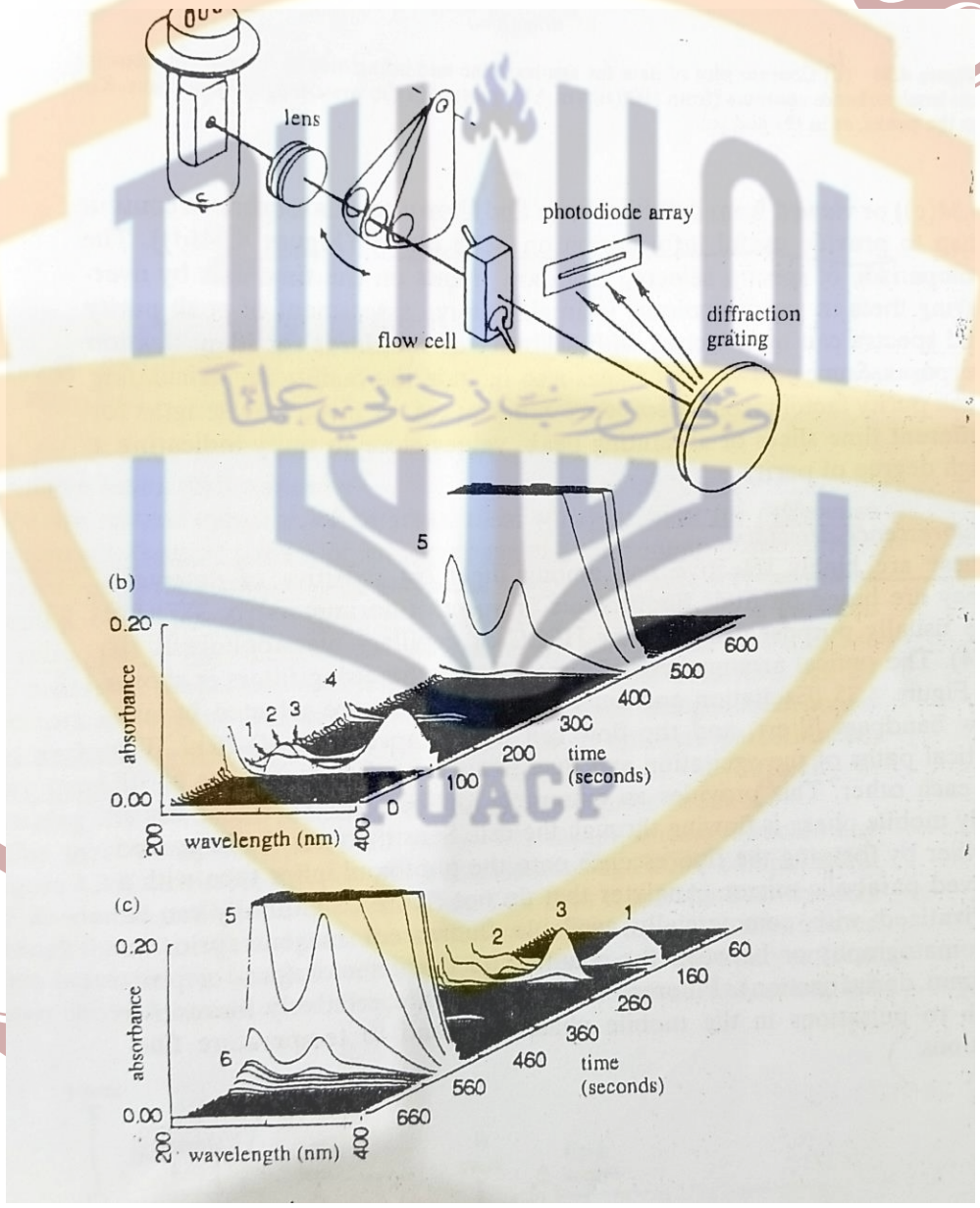


Figure: 2 a, b and c.

The comparison of spectra selected from any points on the time axis by over-1aying them in various colours is an alternative assessment of peak purity and spectra can be matched with a library of standards for identification purposes. Some software packages also include the facility for calculating peak purity factors from absorbance ratios at two or more wavelengths for different time slices of an eluting peak, values close to unity indicating a high degree of purity.

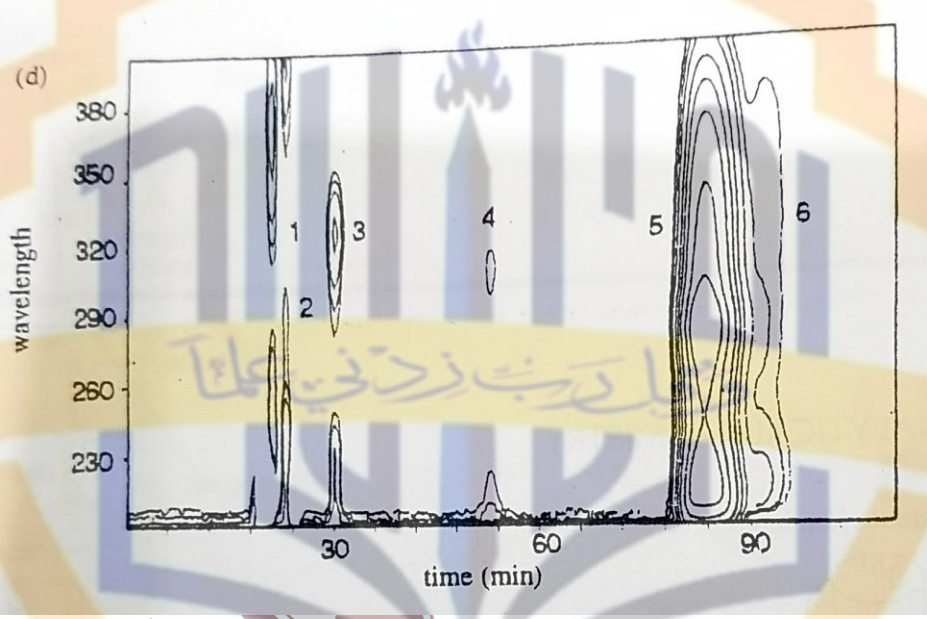


Figure 2 (d): Contour plot of data for azathioprine and impurities in (b) and (c), showing the iso-absorbance contours (from 1000 to 5 m AU) plotted in the wave-length-time plane. Key to the peaks, as in (b) and (c).

Fluorescence detectors

These are highly selective and among the most sensitive of detectors. They are based on filter fluorimeters or spectrofluorimeters, but are usually purpose-designed for HPLC or capillary electrophoresis. The optical arrangement of a typical detector using filters is shown in Figure 3. Excitation and emission wavelengths are selected by narrow bandpass filters, and the flow cell has a capacity of 10-25 ul. The optical paths of the excitation and fluorescent emission beams are at 90° to each other. This provides an extremely low background signal whilst only mobile phase is flowing through the cell. Sensitivity can be improved further by focusing the fluorescence onto

the photomultiplier tube with a curved parabolic mirror. Analytes that do not fluoresce naturally can be derivatized with commercially available fluorescent reagents prior to chromatography or between the column and the detector (pre- or post- column derivatization). Fluorescence detectors are relatively insensitive both to pulsations in the mobile phase flow and to temperature fluctuations.

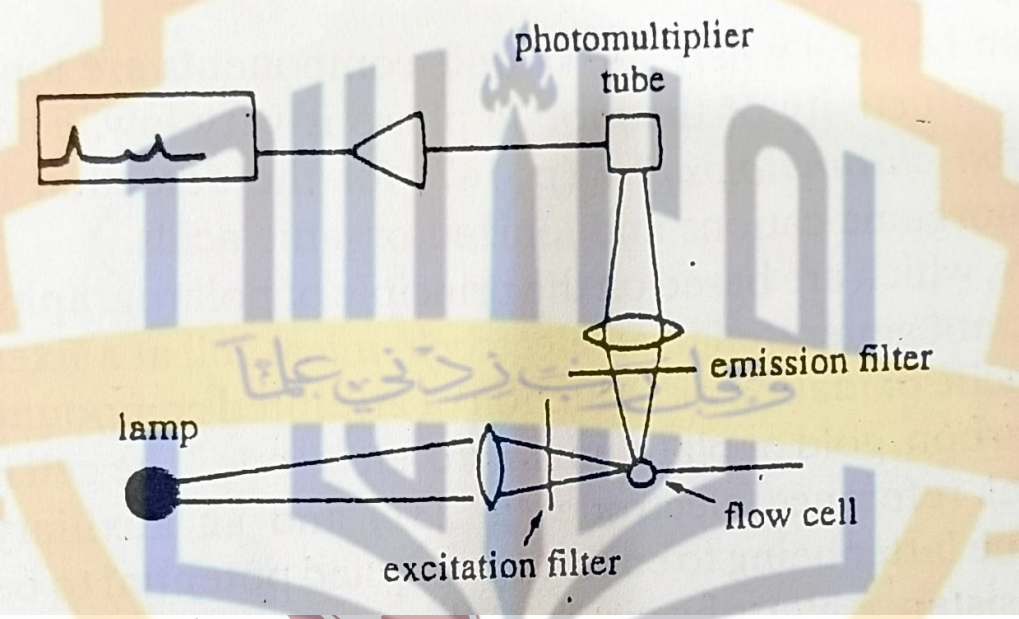


Figure 3: Schematic diagram of a simple dual-filter fluorimeter. Excitation using a Xenon lamp. Filters used to select the wavelength in both the excitation and emission beams.

Refractive index (RI) monitors

There are several types of RI detector, all of which monitor the difference between a reference stream of mobile phase and the column effluent. Any Solute whose presence alters the refractive index of the pure solvent will be detected, but sensitivity is directly proportional to the difference between the refractive index of the solute and that of the solvent. At best they are two orders of magnitude less sensitive than UV/visible detectors. All RI detectors are highly temperature-sensitive) and some designs incorporate heat exchangers between column and detector to optimize performance. They cannot be used for gradient elution because of the difficulty in matching the refractive indices of reference and sample streams. The most common type of RI

monitor is the deflection refractometer (Figure 4). A visible light source is directed through a two-compartment cell divided by a diagonal piece of glass. The light is refracted on the way through the cell, reflected back from a mirror behind the cell then refracted again before being focused on a phototube detector. Whilst only solvent passes through each half of the cell, the phototube signal is constant, but when a solute passes through the sample compartment, the light beam is deflected and the change in intensity of radiation falling on the phototube is registered by the recorder. This type of detector has a wide linear range of response but (like other Rl monitors) only moderate sensitivity.

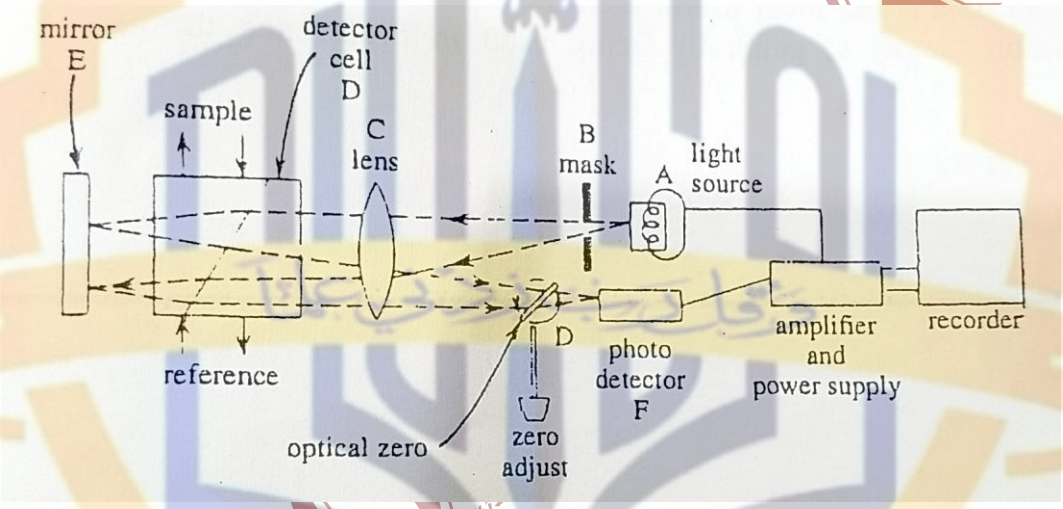


Figure 4: Refractive Index (RI) detector.

Two other types of RI monitor are based on Fresnel's laws of reflection and the principle of interferometry respectively. The former utilizes a very small volume (3 ul) sample cell and is therefore useful for highly efficient columns, but linearity is limited and the cell windows need to be kept scrupulously clean for optimum performance. The main advantages or the interferometric design are improved sensitivity and a wide linear range.

Electrochemical detectors

Conductance monitors can be used where the sample components are jo and providing that the conductivity of the mobile phase is very low. They are used extensively in ion chromatography for the detection of inorganic anions, some inorganic cations and ionized organic acids.

Amperometric detectors, which are based on the principle of polarography, rely on measuring the current generated in an electrochemical cell at a fixed applied potential by the facile oxidation or reduction of an eluted compound at the surface of a glassy carbon, gold or platinum micro-electrode. The cell is completed with a calomel reference electrode and an auxiliary electrode, the purpose of the latter being to enable the applied potential to be stabilized when the cell resistance alters by virtue of the currents generated. The mobile phase acts as a supporting electrolyte for the redox reactions and Composition is therefore restricted to predominantly aqueous solvent mixtures. Several designs have been produced, some with internal cell volumes as little as 1 ul. Amperometric detectors are amongst the most sensitive available but they are susceptible to noise, caused by any residual pulsations from the pump affecting the flow of mobile phase, and to surface contaminations of the micro-electrode due to the build-up-of electrode reaction products which impairs reproducibility. However, their high sensitivity and selectivity (through variation of the applied potential) enhances their value for the trace analysis of certain types of compound, e.g. phenols and aryl amines such as catechol-amines (by oxidation) and nitro or azo compounds (by reduction) although in the latter cases air must be purged from the mobile phase with nitrogen to eliminate interference by the reduction of dissolved oxygen of the remaining types of detector, those based on fluorimetry are both selective and particularly useful for trace analysis as they can be orders of magnitude more sensitive than UV/Visible photometers.