High Performance (Pressu
Liquid Chromatograp

35.1 Introduction

One of the early problems with liquid chromatography was the slow rate at which the analysis place. Early methods used gravity feed, and it was not uncommon for an analysis to take several to complete. This led to great delay, but also the excessive time on the column inevitably led to lo resolution by diffusion, and so on. Consequently, for a number of years liquid chromatography wa widely used as means of separating organic compounds. This problem was largely overcome by advent of high–performance liquid chromatography (HPLC). In this system pressure is applied to column, forcing the mobile phase through at much higher rate. The pressure is applied using a pu system. The action of the pump is critical, since it must not pulsate and mix up the sample by separated in the solvent, causing it to lose resolution. Development of pumps has proceeded quite qu over the last several years, and now it is possible to achieve good resolution under the conditions req for HPLC.

All of the factors affecting separation in liquid chromatography apply to HPLC. The factors affec plate height, the sample distribution between the stationary and mobile phases, and the selection stationary and mobile phases still pertain even under the conditions of HPLC. The principal advan of the system is the speed at which separations take place. Because of the decrease in time, diffusion the column is reduced and resolution improved.

Emphasis has been placed on the size of the particles making up the substrate. It has been fo that the smaller the size, the better the resolution. This is because of the decrease in the geometry fac A in the Van Deemter relationship.

Pressures used normally range from 30 to 200 atm, depending on the type of column used. T pressure is varied to provide the optimum linear flow rate of the mobile phase. It is that pressure wh gives the smallest theoretical plate height. It is derived experimentally as in gas chromatography wh the relationship H/V is developed.

35.2 Principle

It is known that the resolving power of a chromatographic column increases with column length the number of theoretical plates per unit length, although there are limits to the length of a column to the problem of peak broadening. As the number of theoretical plates is related to the surface area the stationary phase it follows that the smaller the particle size of the stationary phase, the better resolution. Unfortunately, the smaller the particle size, the greater the resistance to eluant flow. All of forms of column chromatography so far discussed rely on gravity or lower pressure pumping systems the supply of eluant to the column. The consequences of this is that the flow rates achieved are relati low and this gives greater time for band broadening by simple diffusion phenomena. The use of fas flow rates is not possible because it creates a back–pressure which is sufficient to damage the ma

structure of the stationary phase, thereby actually reducing eluant flow and impairing resolution. In the past decade there has been a dramatic development in column chromatography technology which has resulted in the availability of new 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 the chromatography and avoids creating the impression that high pressures are an inevitable pre–requisite for high performance. This is now known not to be the case and the term *medium pressure liquid chromatography* (MPLC) has been coined for some separations.

The new technology in stationary phases has been applied to thin–layer chromatography giving rise to high performance *thin–layer chromatography* (HPLTC). In general, however, the impact of this new technology has not been quite so great as it has been in column chromatography.

All factors affecting separations on liquid column chromatography apply to this technique also, *e.g.*, plate height, sample distribution between the stationary and liquid phases, and the selection of the stationary and liquid phases. Various methods of development of the chromatograms (elution, gradient elution *etc.*) can be used with this technique.

35.3 Instrumentation

A schematic diagram of a typical HPLC unit is shown in Figure 35.1. The system consists of :

1. a solvent reservoir and mixing system
2. a high pressure pump
3. a sample inlet pump
4. a column
5. a detector and recording unit.

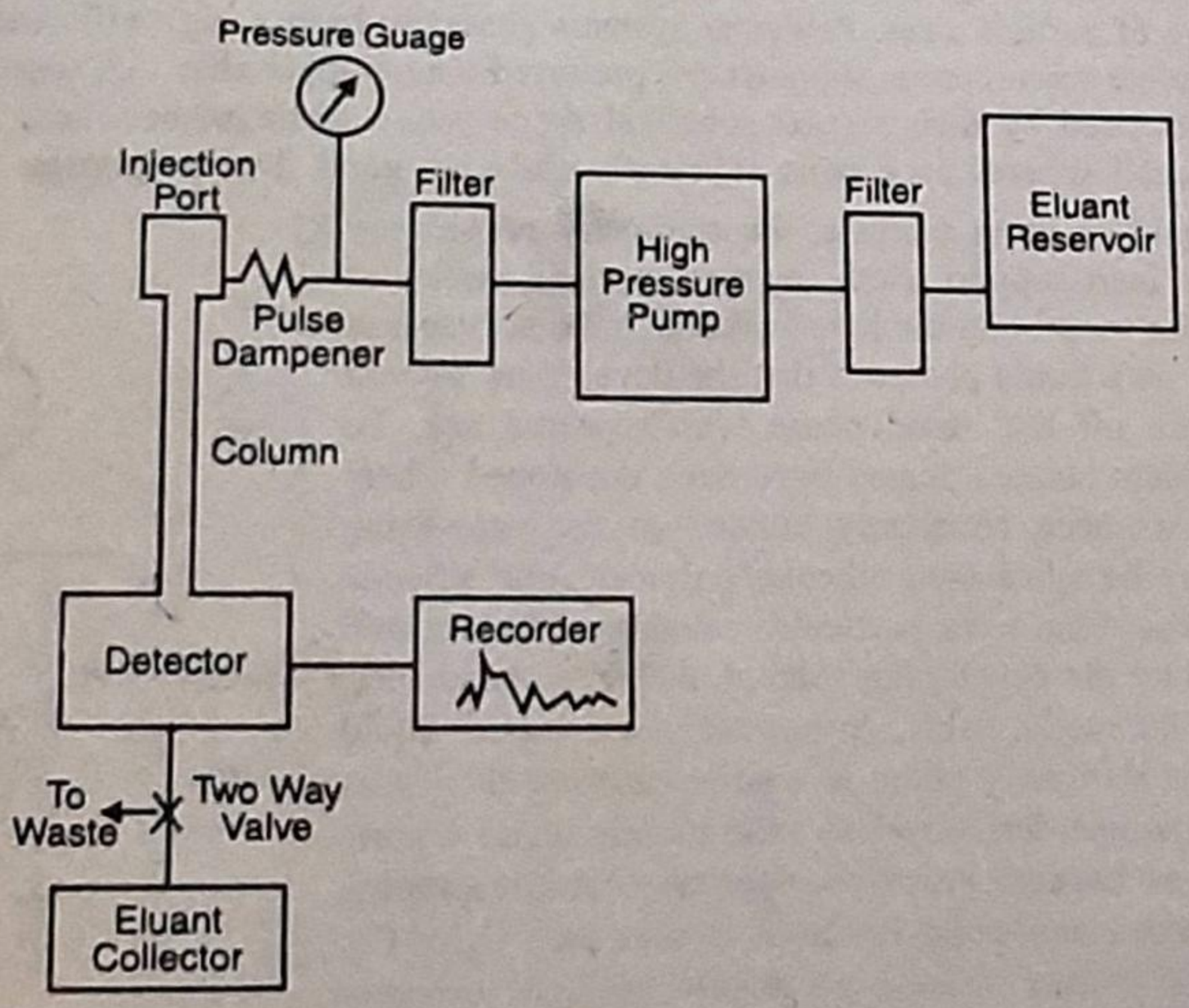


Fig. 35.1 : High Performance Liquid Chromatography (Schematic).

The appropriate solvents (mobile liquid phase) from the reservoirs are allowed to enter the chamber where a homogenous mixture is obtained. A pump capable of maintaining high pressure the solvent from the mixing chambers and pushes it through the column.

The sample is injected through a port into the high pressure liquid carrier stream between the and the column. The separation takes place on the columns which vary from 50–100 cm in length 2–3 mm in i.d. Typical flow rates are 1–2 ml/min with pressure up to several thousand psi. The effluent passes through a non–destructive detector where a property such as ultraviolet absorbance, index, or molecular fluorescence is monitored, amplified, and recorded as a typical detector response retention time chromatogram. The effluent may be either discarded, recycled, or saved for studies in a fraction collector which is synchronized with the detector.

35.4 Apparatus and Materials

1. **The Column.** The columns used for HPLC are generally made of stainless steel manufactured so that they can withstand pressures of up to 5.5×10^7 Pa (8000 p.s.i). Straight of 20 to 50 cm in length and 1 to 4 mm in diameter are generally used though smaller capillary are available. The best columns are precision bored with an internal mirror finish which allows packing of the column. Porous plugs of stainless steel or teflon are used in the ends of the column retain the packing material. The plugs must be homogenous to ensure uniform flow of solvents the column. It is important in some separations involving liquid partition and ion–exchange column temperature is thermostatically controlled during the analysis.

2. **Column Packing.** Three forms of column packing material are available based on a rigid (as opposed to gel) structure. These are :

(*i*) *microporous supports* where microporus ramify through the particles which are generally 5 µm in diameter;

(*ii*) *pellicular* (superficially porous) supports where porous particles are coated onto an inert solid such as a glass bead of about 40 µm in diameter.

(*iii*) *bonded phases* where the stationary phase is chemically bonded onto an inert support.

For adsorption chromatography, adsorbents such as silica or alumina are available as microporous forms with a range of particle sizes. Pellicular systems generally have a high efficiency but low sample capacity, and therefore microporous supports are preferred where applicable. All form of HPLC column packing are characterised 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 coated with liquid phases is that the 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 silicone polymer. The silicone polymer bonded phases have 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 nitrile or alkylamine derivatives and the mobile phase a non–polar solvent such as hexane. For reverse–phase chromatography, the stationary phase is a non–polar compounds such as a C_8 or C_{18} hydrocarbon and the mobile phase a polar solvent such as water/acetonitrile or water/methanol mixtures.

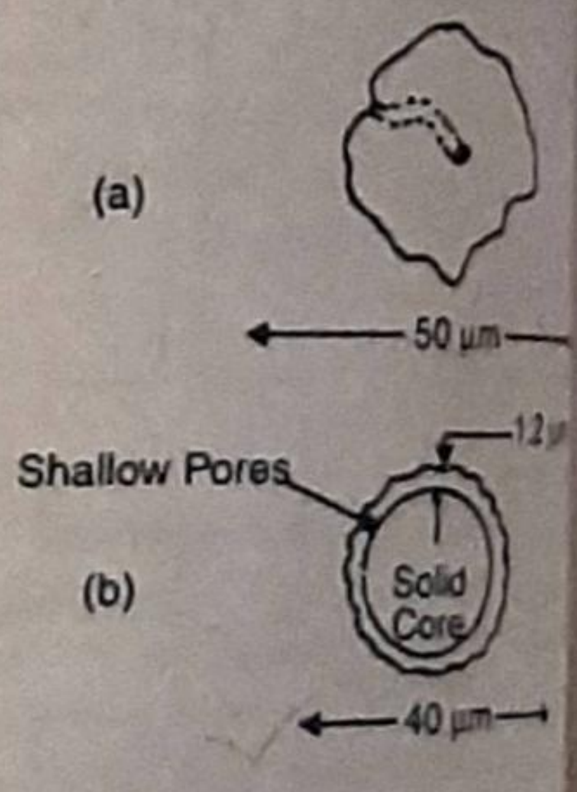


Fig. 35.2 : HPLC Packing Bead

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 a cross-linked silicon network. These resins are classed as hard gels and readily withstand the pressure required during analysis.

The stationary phases for exclusion separations are generally porous silica, glass, polystyrene or polyvinylacetate beads. These are generally used where the eluting solvent is an organic system, and the beads are available in a range of pore sizes. Semi-rigid gel such as Sepadex or Bio-Gel P and non-rigid gels such as Separose and Bio-Gel A are only of limited use in HPLC since they can withstand only low pressures. 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.

The porous material like silica gel and alumina (Fig. 35.2a) have large surface area (50–500 m²/g) and high pore volume (0.2–2 ml/g) but with particle size less than 50 μm. The pellicular consist of a rigid core of about 40 μ diameter. The outer 1–2μm is a porous shell which is prepared from alumina or silica or other adsorbent (Fig. 35.2b). The relatively shallow pores give rise to much greater efficiencies when compared with the porous particles. But the thin layer of the adsorbent on the rigid core severely limits the sample capacity.

Another type of column packing material consists of pellicular support. By bonding organic compounds to the surface hydroxyl groups of silica, the chromatographic behaviour of the surface can be changed drastically. All these processes involve reactions with the reactive silanol (=Si–OH) groups on the silica gel. Many functional groups can be bonded to the silanol. The most commonly used groups include octyl, octadecyl, phenyl, alkylamine, and alkyl nitrile. These packings introduce a wide range of polarities for the stationary phase enabling a variety of separations to be carried out. When non-polar

Table 35.1 : Porous Materials for HPLC

Type and Name	*Description*	*Surface Area* (m^2/g)	*Use*		
			LLC	*LSA*	*LSP*
Silica					
Porasil	37–75 μm, spherical types with different surfaces areas	up to 500	×	×	—
Lichrosorb-Si	5, 10, 20 & 30 μm irregular	300	×	×	—
Zorbax	5 μm, spherical	300	×	×	—
MicroPak-Si	5 and 10 μm irregular	500	×	×	—
Aluminia					
Lichrosorb Alox	5, 10, 20μm irregular	70–90	×	×	—
Chemically bonded					
Micro Pak-NH_2	Alkaylmine bonded to Silica 10μm		—	—	×
DurPak Carbowax 400	Carbowax 400 chemically bonded to Porsil	50–100	—	—	×
MicroPak					
SP-ODS	Octadecyl silane bonded to silica 5μm	125–250	—	—	×
BondaPak/C18					
Porasil	Octadecylsilane chemically bonded to Porasil 10μm		—	—	×

LLC = liquid–liquid chromatography, LSA = Liquid–solid adsorption,
LSP = Liquid–solid partition (reversed phase chromatography)
(Porasil, Pora Pak, Durapak and Bondapak supplied by Water Associates, Lichrosorb and microPak supplied by Varian and E. Merck, Zorbax supplied by Du Pont)

compounds are being separated, one normally uses a stationary phase less polar than the mobile phase (e.g. $Si-(CH_2)_6\ CH_3$). This becomes reversed phase chromatography. For separation of polar compounds stationary phase that is more polar (e.g. $Si(-)\ (CH_2)_n\ NH_2$), is used. Some reaction pathways for the preparation of chemically bonded phases, also called *"brushes" have been depicted* in Fig. 35.3.

Polyamides precipitated onto nonporous as well as porous supports also find use as column materials. Details of some commercially available packing materials and their characteristics are given in Table 35.1 and 35.2.

Table 35.2 : Pellicular Column Materials for HPLC

Type and Name	Description	Surface Area (m^2/g)	Use		
			LLC	*LSA*	*LSP*
Inactive silica					
Zipax	25–37 µm, spherical	1	×	—	—
Active Silica					
Corasil	37–50 µm, spherical	7 & 14	×	×	—
Perisorb	30–40 µm, spherical	10	×	×	—
Active alumina					
Pellumina	37–44 µm, spherical	4 & 8	×	×	—
Polymer coated					
Zipax ANH	Cynoethylsilicone coated on Zipax	1	—	—	×
Zipax PAM	Polyamide coated on Zipax	1	—	—	×
Chemically bonded					
Permaphase ODS	octadecylsilyl bonded to Zipax	1	—	—	×
BondedPak C_{18}	octadecylsilyl bonded to Corasil	7	—	—	×
DuraPak	Carbowax 400 chemically bonded to Corasil	7	—	×	—

(Zipax products supplied by Du Pont, Perisorb supplied by Varian and E. Merck, Corasil products supplied by Water Associates).

3. **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 particle. 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. The most widely used technique for column packing is the high pressure slurring technique. A suspension of packing is made in a solvent of equal density to the packing material. The slurry is then rapidly pumped at high pressure onto a column with a porous plug at its outlet. The resulting bed of packed material within the column can then be prepared for use by running the developing solvent through the column, hence equilibrating the packing with the developing solvent. When hard gels are packed, it is necessary for them to be allowed to swell first in the solvent to be used in the chromatographic process before packing under pressure. Soft gels cannot be packed under pressure and have to be allowed to pack from a slurry in the column under gravitational sedimentation only, in a similar way to the packing of columns for conventional column chromatography.

4. **Chromatography 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 proportion. 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 the 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 co
interfere with the detection system. This is particularly the case if the detection system is
absorbance at below 200 nm. Purified solvents for use in HPLC systems are available comm
even with these solvents a 1 to 5 μm microfilter is generally introduced into the system prior to the
It is also essential that all solvents are degassed before use otherwise gassing tends to occur
pumps. It tends to be particularly bad for aqueous methanol and ethanol solvents. Gassing (the
of air bubbles in the solvent) can alter column resolution and interfere with the continuous mon
of the column effluent. Degassing may be carried out in several way; by warming the solvent, by
it vigorously with a magnetic stirrer, subjecting it to a vacuum, ultrasonic vibrations or by
helium gas through the solvent reservoir.

5. **Pumping Systems.** The pumping systems is one of the most important features of
system. There is a high resistance to solvent flow due to the narrow columns packed with small
and high pressures are therefore required to achieve satisfactory flow rates. The main feature of a
pumping system is that it is capable of output of at least 3.4×10^7 Pa (5000 p.s.i.) and ideally
be no pulses of flow through the system. There must be a flow delivery of at least 10 cm^3 min^{-1}
normal analysis, and up to 30 cm^3 min^{-1} for preparative analysis. All materials in the pump
chemically resistant to all solvents. Various pumping systems are available which operate on the
of constant pressure or constant displacement.

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

Constant displacement pumps maintain a constant flow rate through the column irrespective
changing conditions within the column. One form of constant displacement pump is a motor-
syringe type pump where a fixed volume of solvent is forced from the pump to the column by a
driven by a motor. Such pumps, as well as providing uniform solvent flow rates, also yields a
solvent flow which is important as certain detectors are sensitive to changes in solvent flow rate.
reciprocating pump is the most commonly used form of constant displacement pump. The
moved by a motorised crank, and entry of solvent from the reservoir to the pump chamber and
solvent to the column is regulated by check valves. On the compression stroke solvent is forced
pump chamber into the column. During the return stroke the exit check valve closes and solvent is
in via the entry valve to the pump chamber, ready to be pumped onto the column on the next
stroke. Such pumps produce pulses of flow and pulse dampeners are usually incorporated into the
to minimise this pulsing effect. All constant displacement pumps have in–built safety cut–out
so that if the pressure within the chromatographic systems changes from pre–set limits the
inactivated.

6. **Detector Systems.** Since the quantity of material applied to the column is frequently very
it is imperative that the sensitivity of the detector system is sufficiently high and stable. Most
the detector is a variable wavelength ultraviolet–visible spectrophotometer, a fluorimeter, a
index monitor or an electrochemical detector. A recent development has been the interfacing of
to a mass spectrometer.

For the detection of anions in ion chromatography, a conductivity detected by eluant
is used.

7. **Practical Procedure.** The correct application of a sample onto a HPLC column is
particularly important factor in achieving successful separations. Ideally the sample ought to be
as an infinitely narrow band into the column. There are two methods which are generally used.
method makes use of microsyringe designed to withstand high pressure. The sample is injected
a septum in an injection port, either directly onto the column packing or onto a small plug of

material immediately above the column packing. 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 atmospheric, the injection is made and the pump switched on again. This is termed a *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 eluant from the pump is then passed through the loop, the outlet of which leads directly onto the column. The sample is thus flushed onto the column by the eluant, without interruption of solvent flow to 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 not preferably been deproteinated, may eventually cause the column to lose its resolving power. To prevent this occurrence a *guard column* is installed between the injection and the analytical column. This guard column is a short (2 to 10 cm) column of the same internal diameter, and packed with similar material to that present in the analytical column. The packing of the guard column can be replaced at regular intervals.

Column Efficiency and Selectivity

The column selectivity depends upon the partition coefficient, K, where K is given by the relation

$$K = \frac{\text{concentration of solute in the stationary phase}}{\text{concentration of solute in the mobile phase}}$$

The van Deemter equation

$$\text{HETP} = A + \frac{B}{\mu} + C\mu$$

where A represents an eddy diffusion term, B/μ represents the longitudinal term, and $C\mu$ represents the non-equilibrium in the mass transfer terms, was discussed in the section on gas chromatography. In HPLC the plot of HETP against the mobile phase velocity yields a curve such as is shown in Fig. 35.4 not the inverted parabola of gas chromatography.

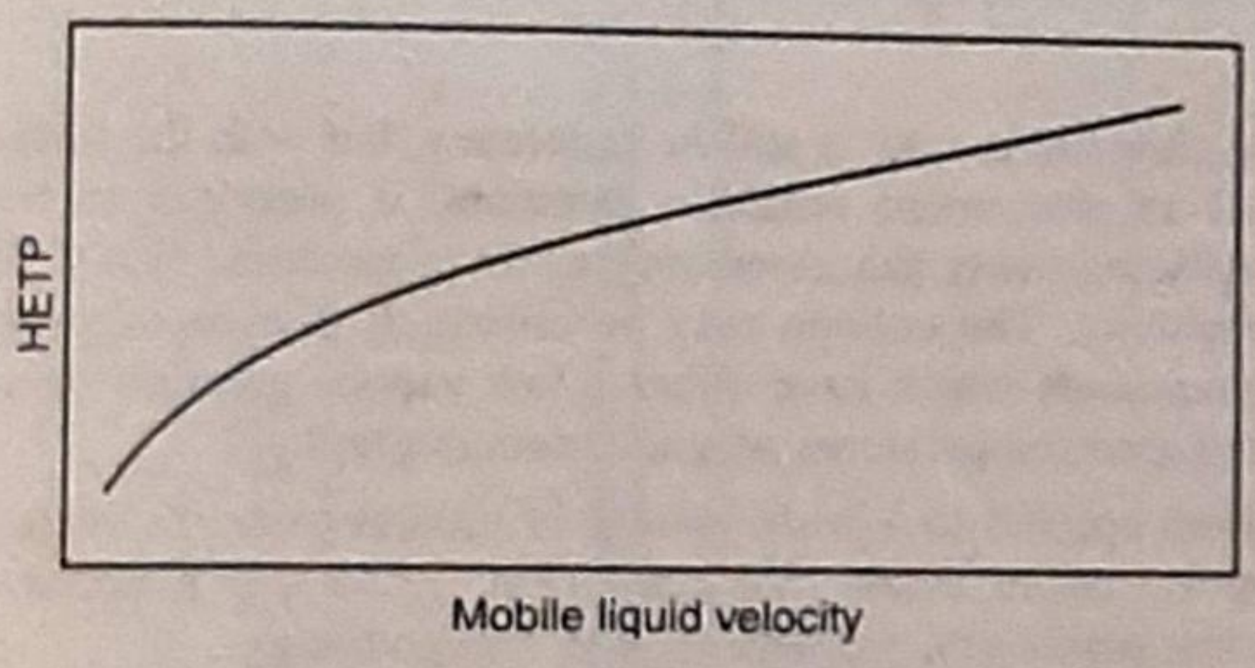


Fig. 35.4 : A van Deemter Plot for HPLC.

A rapid mass transfer between the solute in the mobile phase and the solute in the stationary liquid phase must be obtained in HPLC. As can be seen from the van Deemter plot, increased pressure alone will not accomplish this. In fact, rapid flow rates resulting from increased pressures are incompatible with good separations. The increase in transfer rate may be accomplished to a limited extent by a raise in temperature, but the most important factor is the proper choice of the packing material. The original packing materials were porous particles of relatively large diameter. The large pores filled with immobile pools of the mobile phase liquids and effectively removed some of the solute from the more efficient separation process and increased the separation time. Recently new support packing, known as "porous layer beads" or specifically porous "supports" have been marketed under the trade names of Zipax® and

Corasil®. Zipax® contains a series of impervious spheres each of which is coated with a thin layer of porous material. The beads are then partially bonded by fusion. Enough space is present in the material to allow the passage of solute molecules to the surface of the individual beads. Corasil® has a surface of a thin layer of silica gel bonded to the outer surface of the bead.

The column selectivity may be increased by changing the liquid used in the stationary phase as by changing the pore size or modifying surface of the stationary phase. The mobile phase may be altered by changing the polarity, pH and/or ionic strength.

35.6 Comparison of High Performance Liquid Chromatography and Gas Liquid Chromatography

Table 35.3 provide a comparison between high-performance liquid chromatography (HPLC) and gas–liquid chromatography (GLC), which is discusses in the next chapter. Where either is applicable gas–liquid chromatography offers the advantages of speed and simplicity to equipment. On the other hand, HPLC is applicable to non–volatile substances (including inorganic ions) and thermally unstable materials whereas gas–liquid chromatography is not. The two methods tend to be complementary.

Table 35.3 : Comparison of High–Performance Liquid and Gas–Liquid Chromatography

Characteristics possessed by both methods :
Efficient, highly selective, and widely applicable
Only small sample required
Ordinarily nondestructive of sample
Readily adapted to quantitative analysis.
Particular advantages of higher–performance liquid chromatography :
Can accommodate non–volatile and the thermally unstable samples
Generally applicable to inorganic ions.
Particular advantages of gas–liquid chromatography :
Simple and inexpensive equipment
Rapid.

35.7 Applications

High pressure liquid chromatography is still in its infancy, but with the further development of support materials, as well as new, more sensitive detectors, it promises to become more and more important. It is already replacing many gas chromatographic procedures. HPLC offers the advantages of speed, resolution, and sensitivity. The column may be reused. It is especially useful for separating high molecular weight compounds which have either a low vapour pressure or undergo pyrolysis when subjected to the higher required temperatures of gas chromatography.

The process have been applied to a wide variety of natural products such as nucleic acids, urine, serum, carbohydrates, lipids, amino acids, bile acids (Fig. 35.5) and manufactured products such as pharma-ceuticals, pesticides, herbicides, surfactants, and antioxidants.

Chromatography of separation of barbiturates by HPLC is shown in Fig. 35.6.

The wide applicability, speed and sensitivity of HPLC have resulted in it becoming the most popular form of chromatography and virtually all types of biological molecules have been purified using this approach. *Reverse phase partition HPLC* is particularly useful for the separation of polar compounds such as drugs and their metabolities, peptides, vitamins, polyphenols and steroids. Prior to the advent of this form of chromatography, the separation of such polar compounds was not easily accomplished and often required pre–derivatisation to less polar compounds. The technique is particularly widely used in clinical and pharmaceutical work as it is possible to apply biological fluids such as serum and urine directly to the column, preferably using a guard column. The separation of some highly polar compounds such as amino acids, organic acids and the catercholamines which are difficult to resolve, adequately, by normal phase chromatography, can often be improved by one of two possible approaches. The

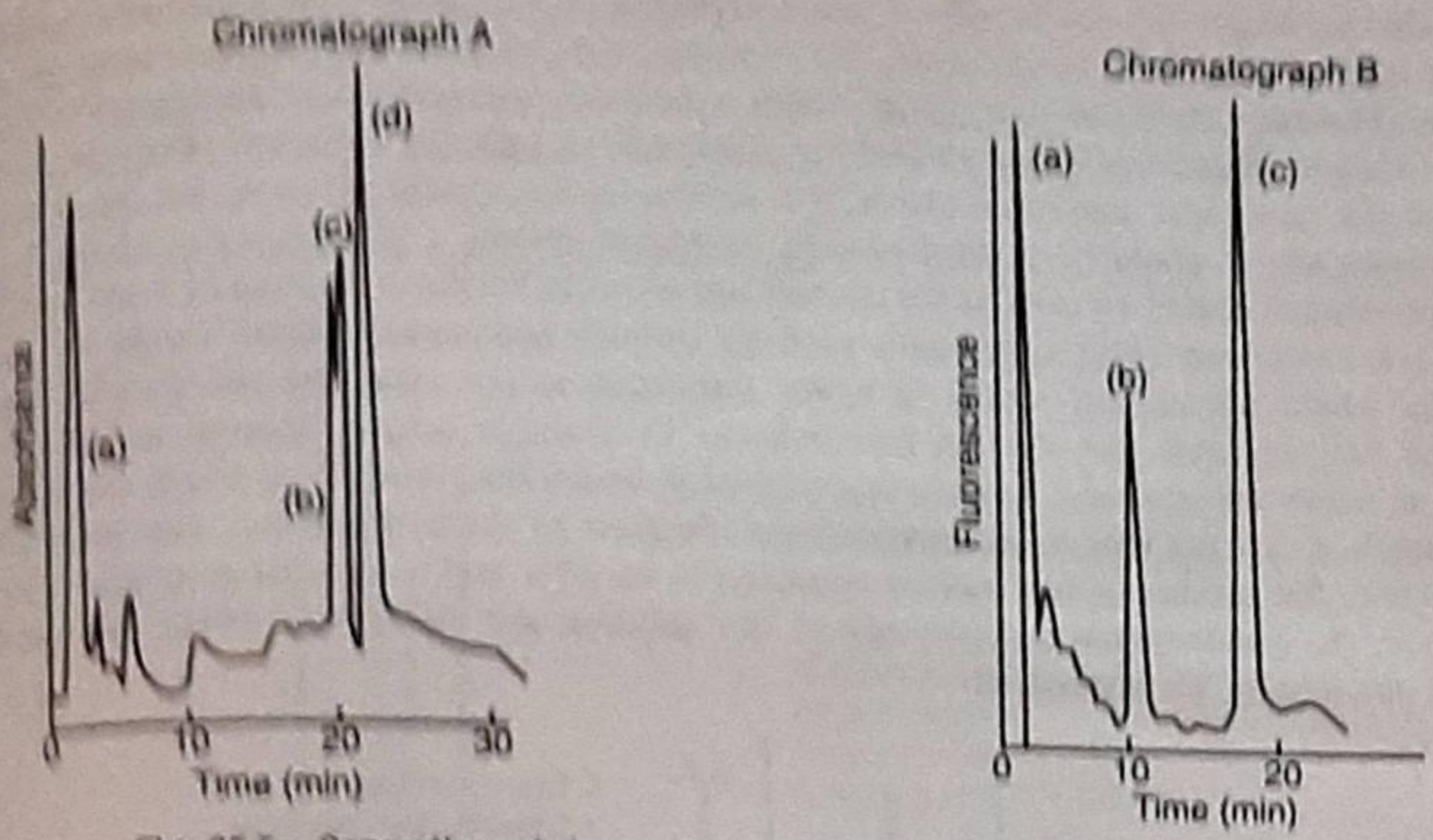


Fig. 35.5 : Separation of the methyl anthracyl esters of the bile acids.
Chromatograph A : separation on Sil–X–I® (0.5 m) with 80% isooctane/20% isopropanol with a flow rate of 0.75 ml/min gave peaks (*a*) chloromethylanthracene, (*b*) chenodeoxycholic acid, (*c*) desoxycholic acid, (*d*) cholic acid. *Chromatograph B* : separation on μBondapak® C^{18} (0.30 m) with 85% methanol/15% water with a flow rate of 1.0 ml/min gave peaks (*a*) chloromethylanthracene, (*b*) cholic acid, (*c*) chenodeoxycholic acid and desoxycholic acid.

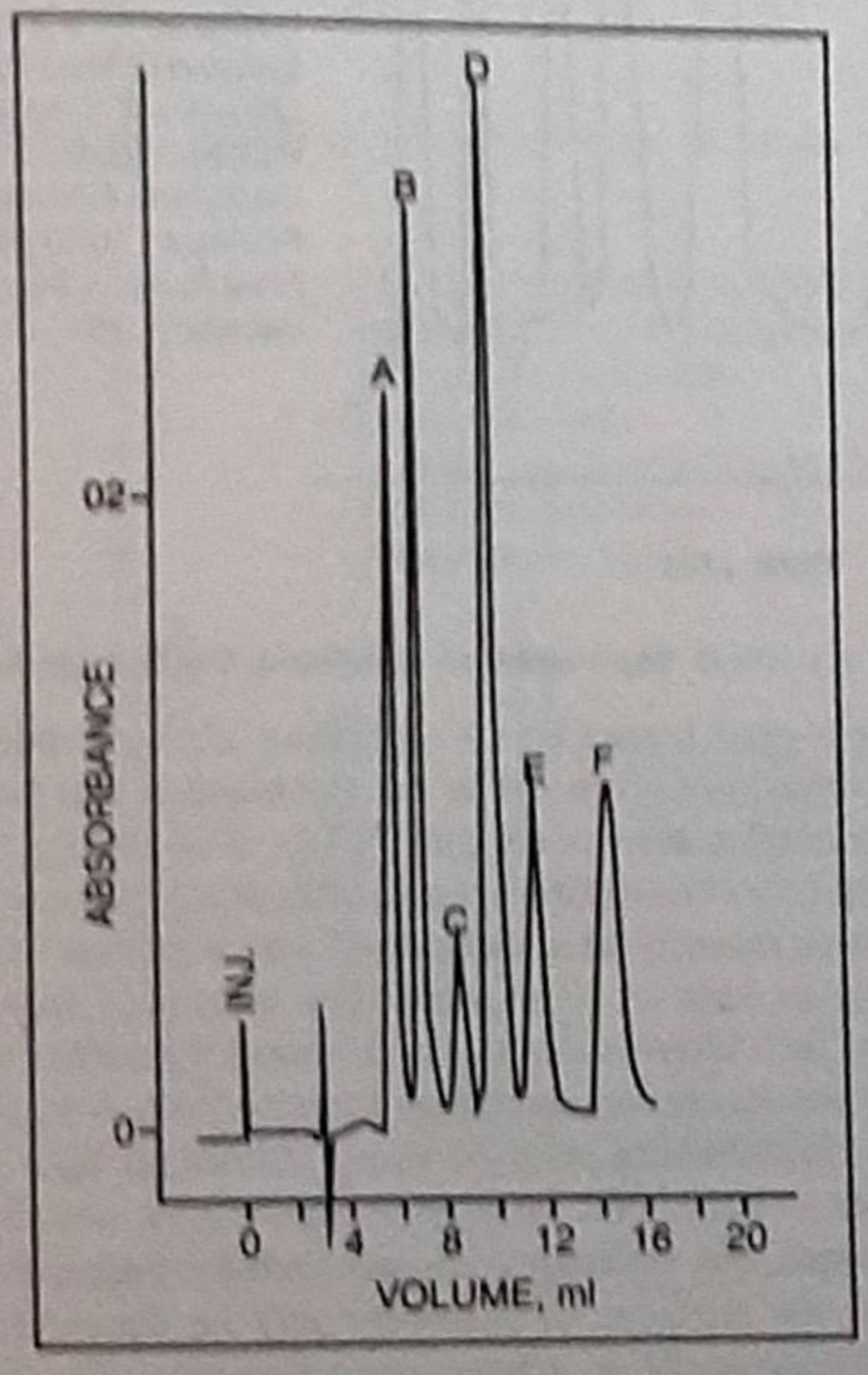


Fig. 35.6 : Separation of barbiturates by HPLC; A. phenobarbital; B. butabarbital; C. mephobarbital; D. pentabarbital; E. secobarbital; F, methohexital.

ion–suppression in which the ionisation of the compound is suppressed by chromatographing at an appropriately high or low pH. Weak acids, for example, can be chromatographed using an acidified mobile phase. The second is *ion–pairing* in which a *counter ion* with opposite charge to that to be separated is added to the mobile phase so that the resulting ion–pair has sufficient lipophile character to be retained by the non–polar stationary phase of a reverse phase system. Thus to aid the separation of acidic compounds which would be present as their conjugate anions, a quarternary alkylamine ion such as tetrabutylammonium would be used as the counter ion whereas for the separation of bases which would be present as a cation, an alkyl sulphonate such as sodium heptanesulphonate would be used. The mechanism by which ion–pairing results in better separation is not cleat but two theories have been proposed. The first suggests that the ion–pair behaves as a single neutral species, whilst the second suggests that an active ion–exchange surface is produced in which the counter ion, which has considerable lipophilic properties, and the ions to be separated are adsorbed by the hydrophobic, non–polar stationary phase. In practice, the success of ion–pairing approach is variable and somewhat empirical. The size of the counter ion, its concentration and the pH of the solution are all factors which may profoundly influence the outcome of the separation.

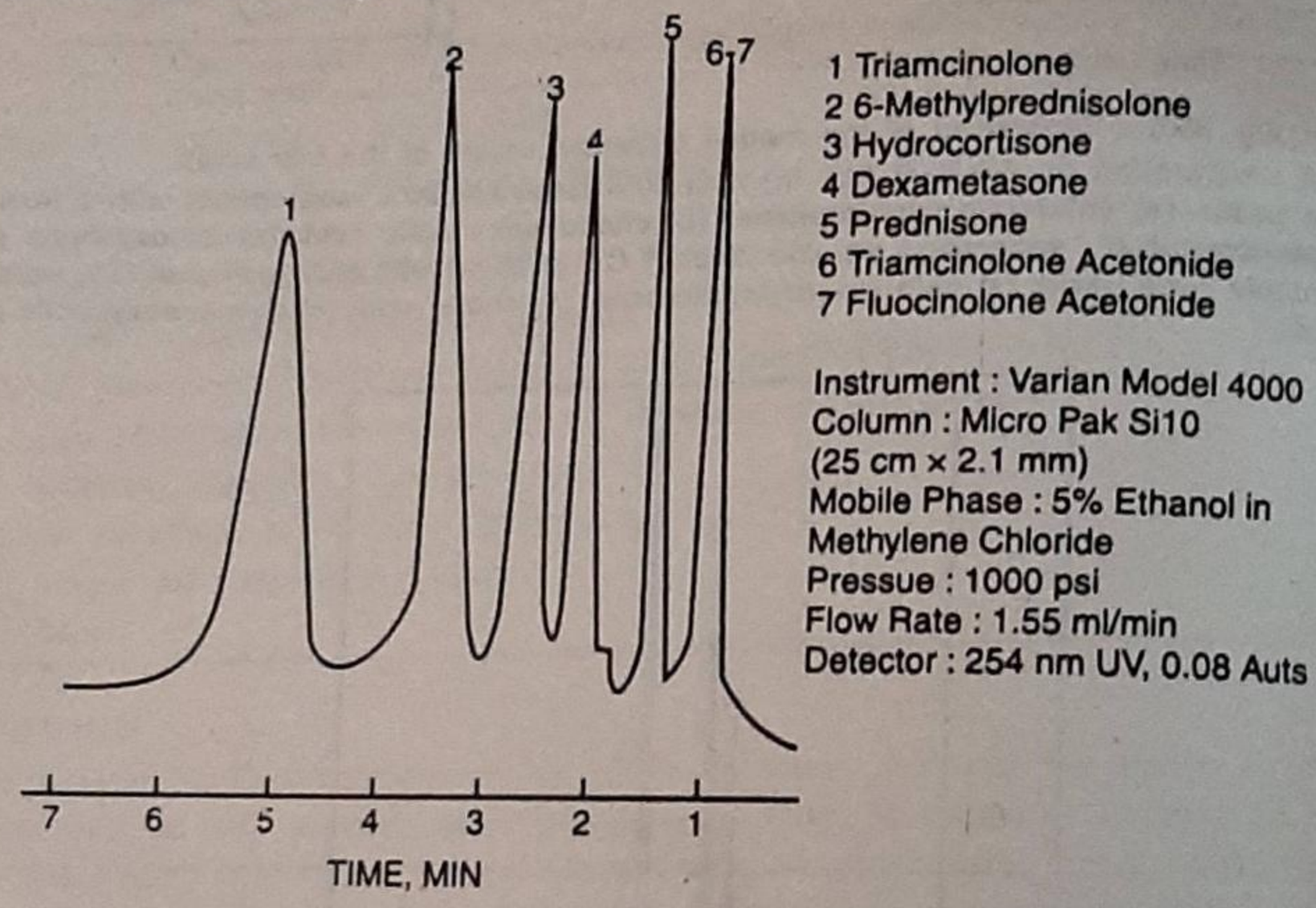
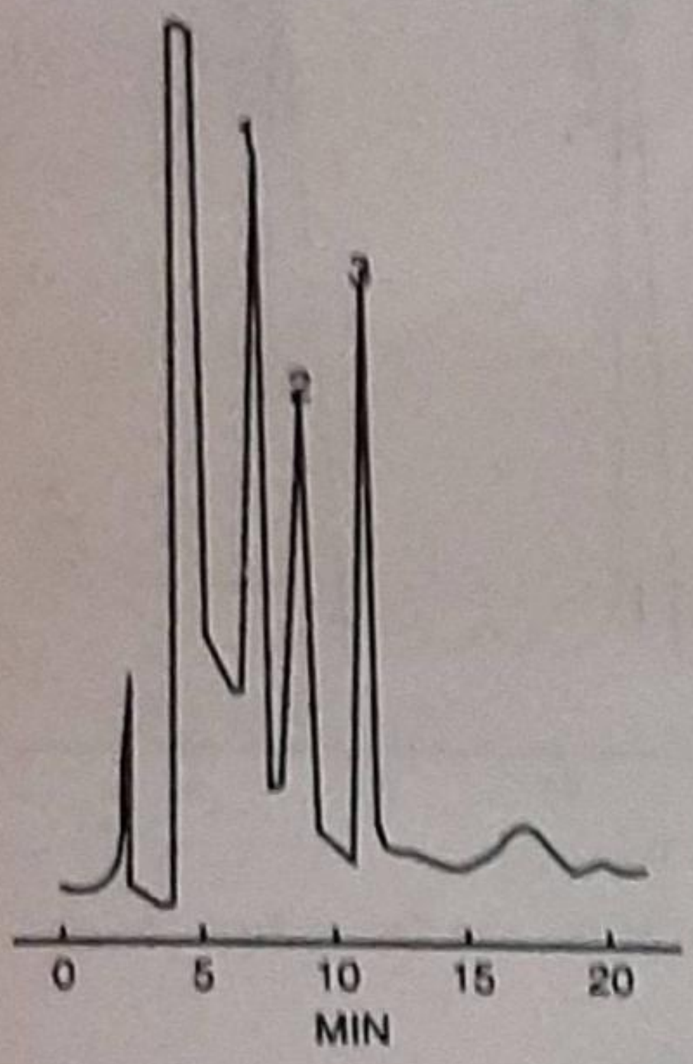


Fig. 35.7 : HPLC Separation of Synthetic Corticosteroids.

HPLC has probably had the biggest impact on the separation of oligopeptides and proteins. Instruments dedicated to the separation of proteins have given rise to the technique of *fast protein liquid chromatography* (FPLC). There are no unique principles associated with FPLC, it is simply based on reverse phase and ion–exchange chromatography and on chromatofocussing. Microbore glass–lined stainless steel columns 1 mm diameter and 2.5 cm long have recently been developed which enable very small amounts of sample to be used with separation taking as little as 10 minutes. The technique enables such complex mixtures as tryptic digests of proteins and the culture supernatant of microorganisms to be applied directly to the column which most commonly contains an ion–exchange system. Protein mixtures from cell extracts still need some form of preliminary fractionation prior to study. Although high performance exclusion and ion–exchange chromatography are so successful for protein separations, not all proteins can be completely purified using them. In these cases, the technique of *hydrophobic interaction chromatography* which exploits hydrophobic regions on the surfaces of proteins, may be successful. The stationary phase is strongly hydrophobic and most commonly is octylor phenylagarose. The hydrophobic regions of the protein surface interacts with this phase by π–π bond interaction. This minimises interaction of the protein

the aqueous environment. Binding is accomplished in dilute (0.01 M) buffer and elution carried out r with aqueous ethylene glycol or ethanol or by the addition of so-called *chaotropic compounds* chlorate, trifluoroacetate or thicyanate ions or urea) which disrupt water structure and thus discourage ophobic interactions. Proteins purified by this technique include aldolase, transferrin, cytochrome *c* thyroglobulin.

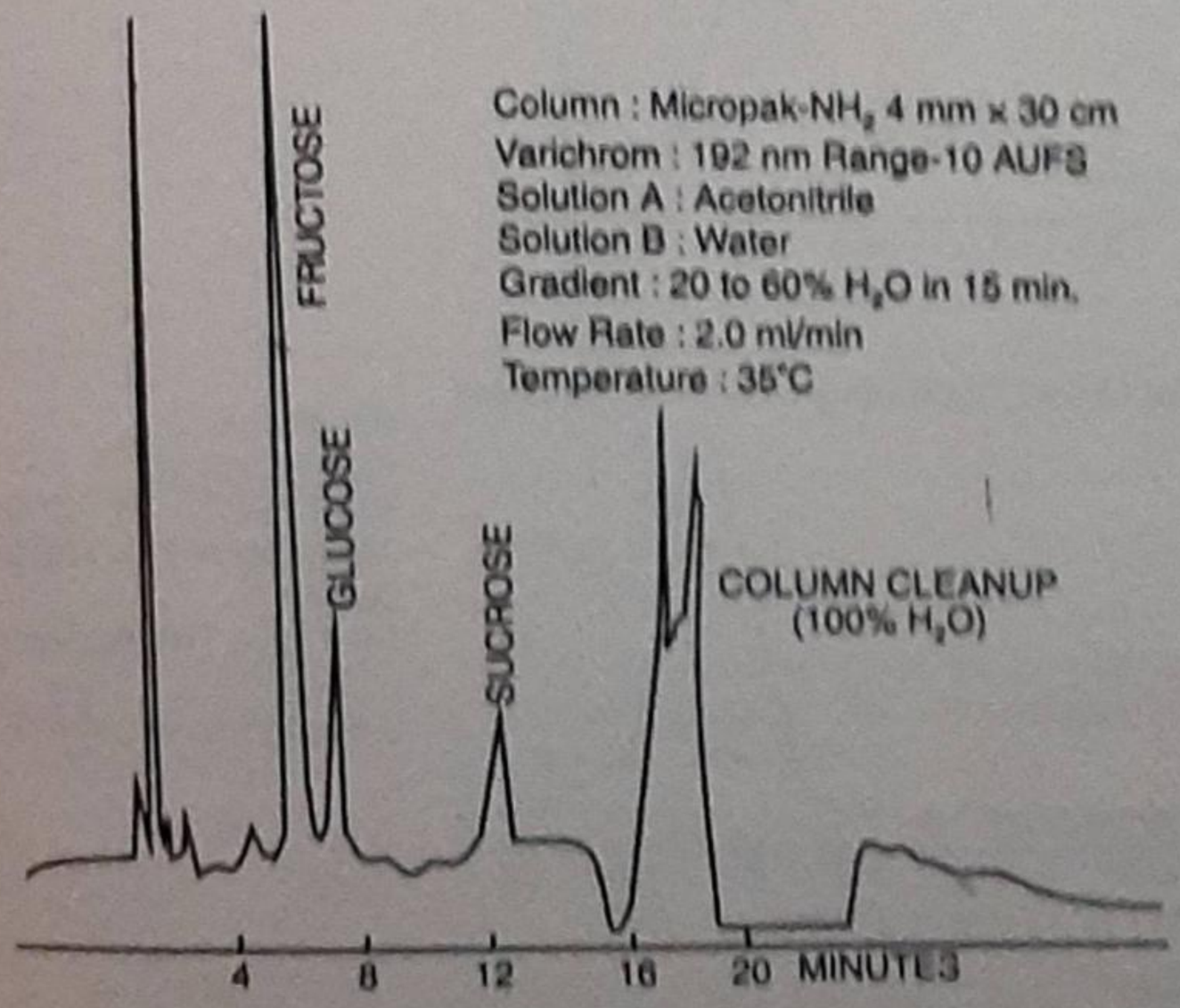
Some typical separations employing the HPLC technique are given in Fig. 35.7 to 35.12.



1. Oxphenbutazone (20 µg/ml)
2. Phenylbutazone (20 µg/ml)
3. Furosemide (20 µg/ml)

Note : Sample filtered through 0.2 um Membrane prior to direct injection
Column : 15 cm × 4.6 mm ID
Packing : ISRP, 5 micron
Flowrate : 1 ml/min
Mobile Phase : 84% 0.1 MKH_2PO_4 (pH 6.8), 10% Isopropanol, 6% Tetrahydrofuran
Detector : 254 nm, 0.08 AUFS

Fig. 35.8 : HPLC (reversed phase) separation of drugs of abuse in horse plasma.



Column : Micropak-NH_2 4 mm × 30 cm
Varichrom : 192 nm Range-10 AUFS
Solution A : Acetonitrile
Solution B : Water
Gradient : 20 to 60% H_2O in 15 min.
Flow Rate : 2.0 ml/min
Temperature : 35°C

Fig. 35.9 : HPLC separation of sugars in Pepsi.

35.8 HPLC Adsorption Chromatography

All of the pioneering work in chromatography was based upon liquid–solid adsorption in which the

stationary phase was the surface of a finely divided solid. In such a packing, the solute
the eluting solvent for sites on the surface of the solid, retention is the result of adso
Currently, liquid–solid HPLC is used extensively for the separation of neutral organic

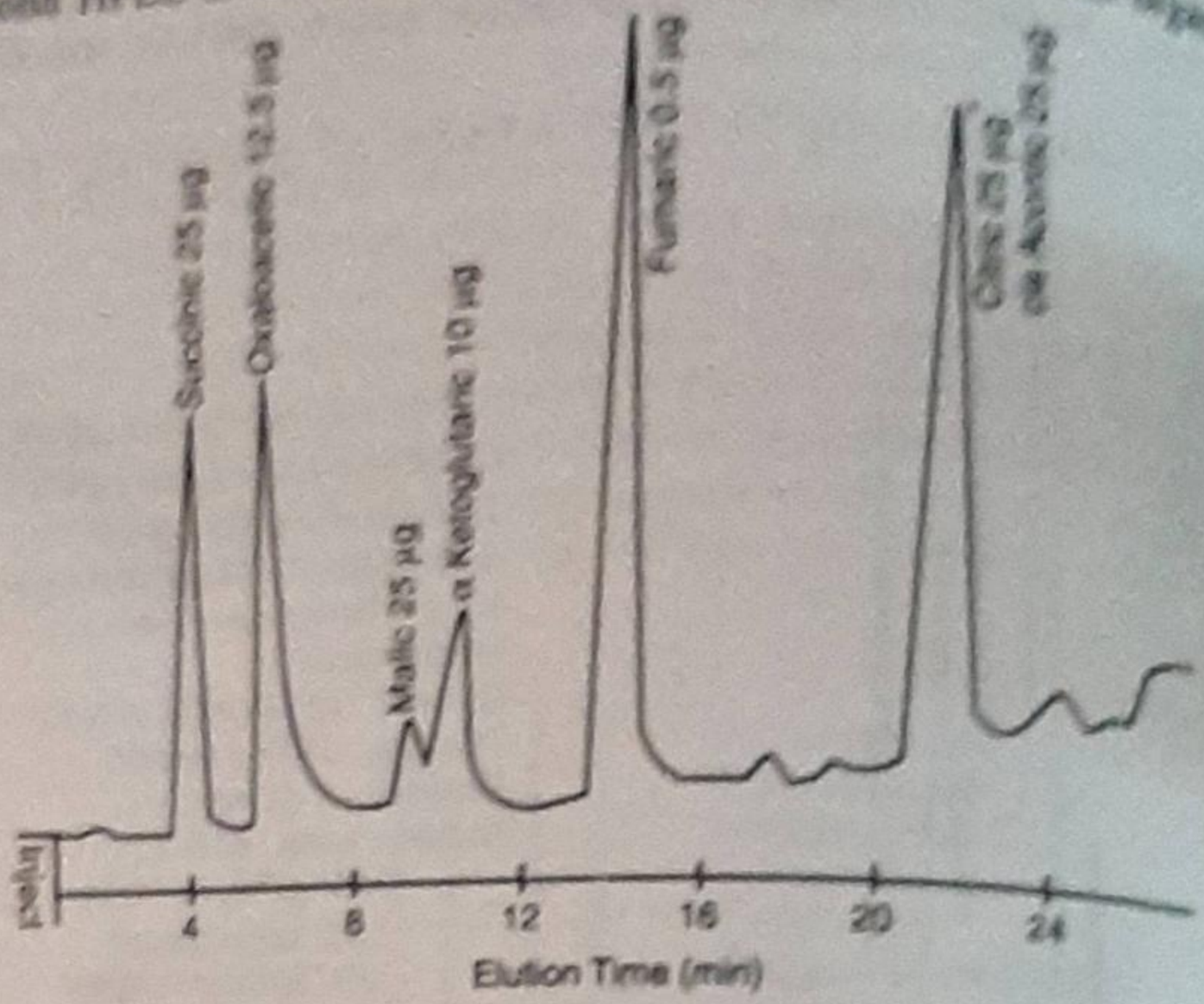
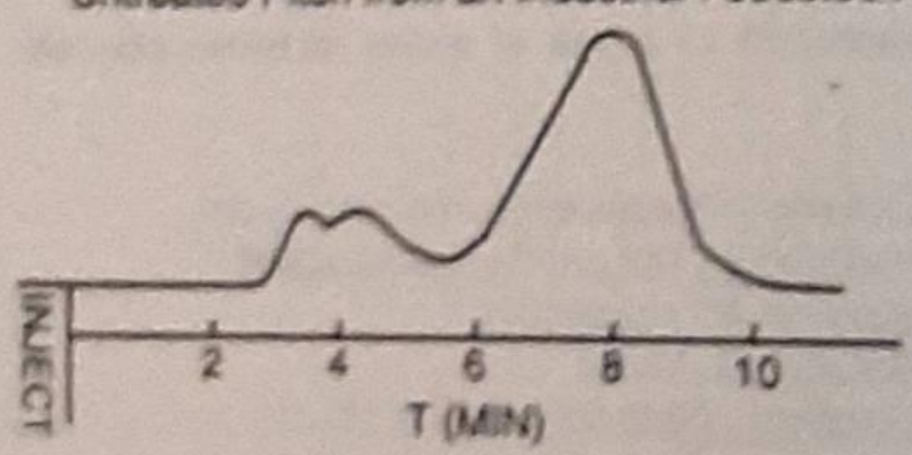
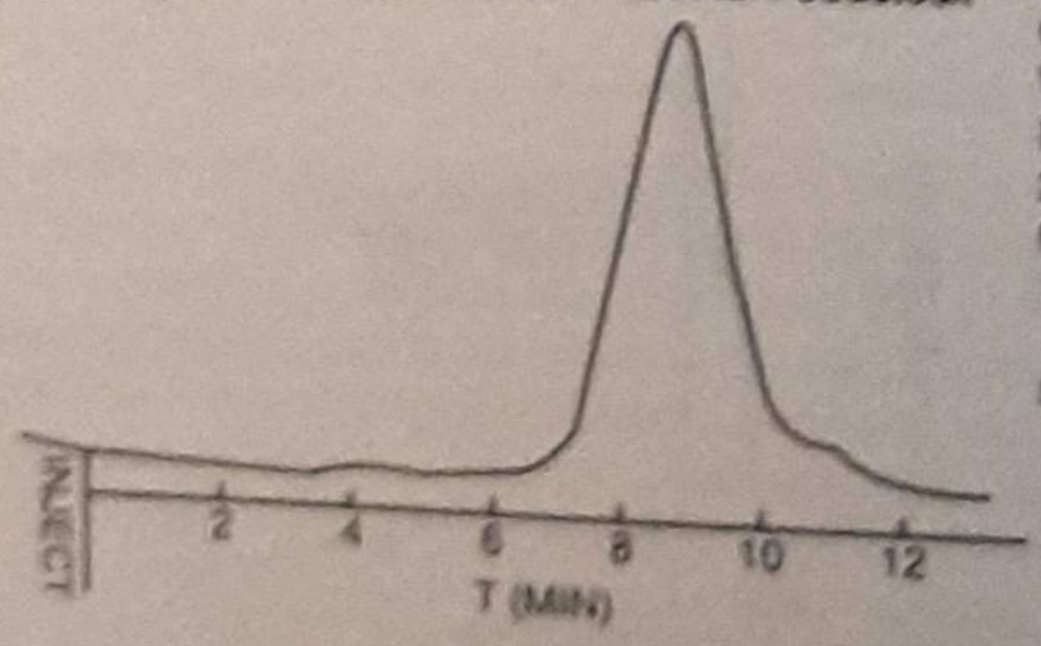


Fig. 25.10 : Gradient separation of Di– and Tricarboxylic Acids.

Untreated Pitch from an Industrial Feedstock



Thermally Altered Pitch from an Industrial Feedstock



Conditions :
Varian 5010 LC
Fixed 254 nm .64 AUFS
3000H Micro Pak TSK 30 cm Column
1.0 ml/min THF Ambient

Saturated Solution $\xrightarrow{25\ \mu l}$ 5 ml THF

Fig. 25.11 : HPLC (Exclusion) Separation of untreated and thermally treated pitch

Stationary and Mobile Phases. Silica gel is by far the most common adsorbent for liq chromatography. Alumina also finds considerable use. Packing materials of specified particle ranges are available from several commercial sources, typical for HPLC is a 10–µm silica g

in which 80% of the particles range between 8 and 12 μm. One company offers columns packed with specially prepared spherical silical gel particles, with 95% of these particles having diameters within ±1 μm of the nominal particle size of 3 μm.

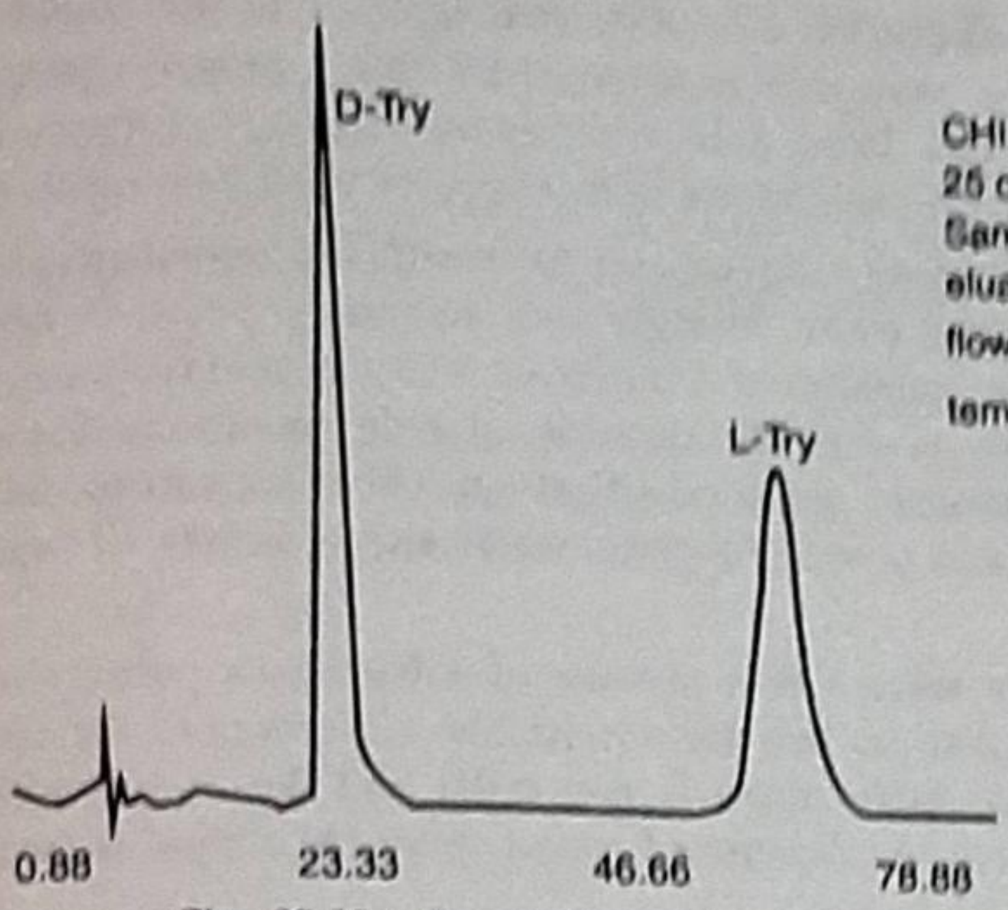


Fig. 35.12 : Separation of D, L– isomers of Tryptophan.

The choice of mobile phase is all–important for successful in liquid–solid chromatography; by varying the solvent, the capacity factor (k') for solute can be varied until they fail in the ideal range of 1 to 10.

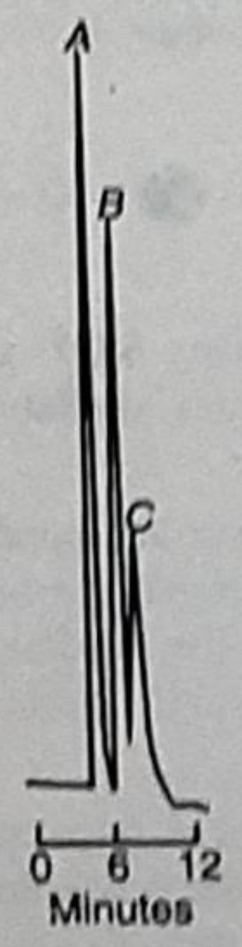


Fig. 35.13 : A liquid–solid chromatogram for adernal steroid–cortisones. Peaks : (A) cortisol; (B) cortisone; (C) corticsterone. Column: 25 cm × 4.6 μm packed with 10 mm silica gel. Pressure: 225 psi. Flow rate: 59.8 mL/hr. Mobile Phase: 75% heptane/25% ethanol.

Applications. Large differences exist in the tendencies of compounds to be adsorbed, and their differences serve as the basis for adsorption chromatography. For example, a positive correlation can be discerned between adsorption properties and the number of hydroxyl groups in an organic molecule. A similar correlation exists with double bonds. Compounds containing certain functional groups are more strongly held than others. The tendency to be adsorbed decreases in the order : acid > alcohol > carbonyl > ester > hydrocarbon. The nature of the adsorption is also influential in determining the order of adsorption. Much of the available knowledge in this field is empirical; the choice of adsorbent and solvent for a given separation frequently must be made on a trial–and–error basis.

Fig. 35.13 shows a typical liquid–solid separation by HPLC.

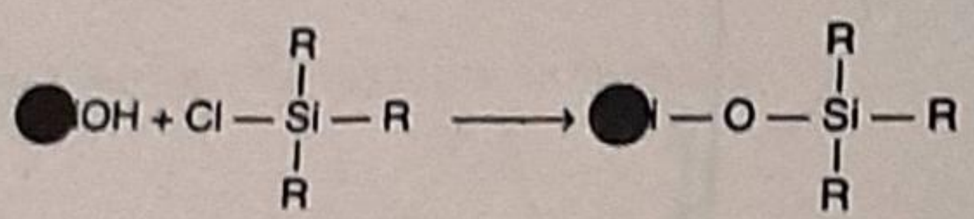
35.9 HPLC Partition Chromatography

Partition of liquid–liquid chromatography was originated in 1941 in the Nobel prize–winning Matin and Synge. In their studies, they were able to demonstrate that a properly prepared columns have a plate height as small as 0.002 cm. Thus, a 10 cm column of this type may contain as many 5000 theoretical plates. High separation efficiencies are to be expected even with relatively short colu

Solid Supports. The most widely used solid support for partition chromatography has been si acid or silica gel. This material adsorbs water strongly; the stationary phase is often aqueous a consequence. For some separations, the inclusion of a buffer or a strong acid (or base) in the water f has proved helpful. Polar solvents such as aliphatic alcohols, glycols, or nitromethane, alone or mix with water, have also served as the stationary phase on silical gel. Other support media include alum diatomaceous earth, starch, cellulose, and powdered glass, water and a variety of organic liquids ha been used to coat these solids.

The mobile phase may be a pure solvent or a mixture of solvents; its polarity must be marke different from the stationary liquid so that the two are immiscible and serves as the stationary phase. *reverse–phase* chromatography, the stationary phase is non–polar and the mobile phase is polar. Th choice of liquid pairs is largely empirical. As mentioned earlier, gradient elution is frequently employe to enhance separation efficiency.

Bonded Phase Packings. A type of packing is becoming increasingly popular for reverse–phase HPLC consists of pure silica gel particles onto which an organic group has been chemically attached. As an example, a hydrocarbon surface can be formed by the reaction of chlorooctadecyl silane with the OH groups on the surface of silica gel. That is, where R is the octadecyl group and the Si in the circle



attached to an OH group represents one of many SiOH groups on the surface of the gel particle. Other groups that have been bonded to silica gel include aliphatic amines ethers, and nitrates as well as aromatic hydrocarbons.

The behaviour of the chemically silica surface appears to be intermediate between a solid surface at which adsorption occurs and an immobilized liquid at which a liquid–liquid equilibrium exists. Chemically bonded surface offer a considerable advantage over ordinary solid–supported liquids in that the stationary phases cannot be stripped of its liquid by the mobile phase. On the other hand chemically bonded surfaces suffer from limited loading capacities.

Applications. Partition chromatography has become a powerful tool for the separation of closely related substances. Typical examples include the resolution of the numerous amino acids formed in the hydrolysis of a protein, the separation and analysis of closely related aliphatic alcohols, and the separation of sugar derivatives.

TEST YOUR KNOWLEDGE

Q.1. What do you understand by HPLC?
Q.2. What is the principle of HPLC?
Q.3. Name the components of a typical HPLC unit.
Q.4. Write a short note on column packing in HPLC.
Q.5. Discuss some commercially available packing material and their characteristics.
Q.6. Write briefly procedure for column packing.
Q.7. Write a short note on pumping systems used in HPLC.