SEPARATION CHEMISTRY

R.P. Budhiraja

SEPARATION
CHEMISTRY

This page intentionally left blank

SEPARATION CHEMISTRY

R.P. Budhiraja

Ex-Reader in Chemistry,
Deshbandhu College (Delhi University)

To the Memory of my Parents with affection and reverence

This page intentionally left blank

Preface

The author's fascination for the field dealing with Separation and Purification techniques in Chemistry and Biochemistry arose out of a realisation that this Science is one more manifestation of the ingenuity of human mind for solving problems as they arise.

Though the role played by the field of Separation and Purification techniques in the advances made by Pure and Applied Chemistry and Biochemistry has been vital all along, somewhat surprisingly the wide-spread awareness of the importance of this field is only of recent origin. The recognition of the importance of this field is now increasingly forthcoming in the form of the emphasis being laid on its inclusion in the latest and updated versions of Chemistry and Biochemistry syllabi by institutions of higher learning all over.

In this book, the author has aimed at gathering in one place the numerous techniques of separation and purification evolved by chemists and biochemists over a long period of time, and introducing the reader interested in this field to the theory, practice and applications of each of these methods. Presently, these numerous techniques are mostly to be found in places scattered all through the literature relating to "Science of Separation"—the distinctive name of its own now acquired by this field of Science. These scattered accounts deal with the various methods at varying levels of technical detail—elementary to advanced—depending on the needs of their respective target readership. In the present book, the needs of its target readership, namely, students pursuing courses in Pure and Applied Chemistry and Biochemistry at the under-graduate and post-graduate levels, have been kept in view and topics have been dealt with in the requisite detail so as to meet the requirements of this readership adequately.

Specific courses of study in Chemistry and Boichemistry for which the present script should serve as a Textbook on Science of Separation are : M.Sc. Chemistry; B.Sc. (Hons.) Chemistry; B.Sc. (Hons.) Biochemistry.

In the fields of Applied Chemistry and Biochemistry, students pursuing University Degree courses in Pharmacy (B. Pharm.), Biotechnology, Environmental Science, Analytical Chemistry, Industrial Chemistry, Agricultural Chemistry, Food Technology and Medical Lab Science will also find this book as Textbook' on Separation and Purification techniques.

Teachers dealing with the aforesaid courses of study will also find a glance through the book worthwhile.

The author will welcome suggestions for improvement of the book.

R.P. BUDHIRAJA

Contents

1

Introduction

Nowadays, discovery of most of the new compounds results from synthesis in the laboratory. Laboratory preparation of a compound often yields a crude mixture containing some quantities of the unreacted starting materials as also impurities that are by-products from side reactions. A substance that occurs in a natural source is also rarely present as a single entity in that source. The first demand on the chemist or the biochemist is the isolation of the pure compound from the crude mixture that has resulted from either the laboratory synthesis of the compound or its extraction from one of its natural sources. The chemist or the biochemist proceeds to study the properties or details of the structure of a compound only after it has become available in the pure state.

In the realm of Applied Chemistry or Biochemistry also, separation and purification of compounds is as necessary a step as it is in the field of Pure Chemistry and Biochemistry. Here the necessity arises from reasons that are best elucidated by the following examples.

In the manufacture of synthetic drugs, usually a mixture results that contains the desired drug along with variable proportions of several other compounds of which some may even be dangerous to life. The necessity of freeing the desired compound from these by-products to get the drug of the required quality and uniform potency is too obvious.

In the analysis of air for pollutants, for example, some of the compounds may be present in too low concentrations to be analysed straightaway. In such a case, it becomes necessary to raise concentrations of those compounds to the level where the required analysis becomes practical. Situation of a different type may arise in certain other analyses. Thus in the analysis of river water for metals that are present in trace concentrations, erroneous results are obtained because of the interference caused in the analysis by organic compounds contained in the sample. In a situation like this interfering substances have to be removed before the analysis can be performed. Also, many analyses now carried out daily in the clinical and forensic laboratories all over the world involve separation as an integral part of the procedure.

Little progress could have been possible in the fields of either Pure or Applied Chemistry and Biochemistry in the absence of the various separation and purification techniques that have been developed and improved upon with the

passage of time. Due recognition of the importance of these separation techniques to the development of chemical and biochemical sciences has increasingly dawned on scientists in these fields only in recent times. This awakening has resulted in attention of these workers being drawn to the comprehension of the underlying theory of separation processes, this being especially so after the advent of gas chromatography. The theory of chromatography has developed rapidly during the past four decades providing fundamentals that stimulated further advances in details of technique and equipment used so as to get high column efficiency, good resolution and quick analysis. Much of this theory is applicable to non-chromatographic separation methods as well. With the advances already made in the development of theory, the realm of separations is no longer just an art practised by the chemist or the biochemist, but it is increasingly assuming the status of a science. This field is becoming known as Separation Science and its knowledge is now being disseminated through new monographs, treatises and journals.

1.1 Basic Principle of Separation Techniques

The basis of most of the techniques which achieve separations and purifications is that they bring about distribution of components of the starting material between two phases. This partitioning is brought about by taking advantage of difference of components in any of their properties like volatility, solubility, adsorption on a suitable solid material, molecular size etc. In the process of separation, one of the two phases gets considerably enriched in respect of one component, and the other phase in respect of the second component of the starting mixture. The respective distributed components are subsequently recovered from the two phases by using appropriate procedures.

The separation of two substances consists in carrying out (batch-wise or continuously) a successive series of operations, each of which is aimed at establishing an equilibrium between the two phases, say, A and B, which contain the two substances in different proportions. This scheme of separations can be explained with the help of the following mathematical derivation.

Suppose that as a consequence of the equilibrium established between the two phases at the end of the first operation, the two phases A and B contain molar fraction, C_1 of the first component, and molar fraction, C_2 of the second

component, then the expression $\left(\dfrac{C_1}{C_2}\right)_{A(1)}$ gives the composition of phase A, and

similarly $\left(\dfrac{C_1}{C_2}\right)_{B(1)}$ represents the composition of phase B, the subscript (1) in each

case signifying that these quantities refer to a single, once through operation. The relative values of $\left(\dfrac{C_1}{C_2}\right)_{A(1)}$ and $\left(\dfrac{C_1}{C_2}\right)_{B(1)}$ are determined by the *separation factor* of the particular system. The separation factor, α, is defined by the relationship:

$$\alpha = \frac{\left(\dfrac{C_1}{C_2}\right)_{A(1)}}{\left(\dfrac{C_1}{C_2}\right)_{B(1)}} \qquad \qquad ...(1.1)$$

The value of α is found experimentally by determining the values of $\left(\dfrac{C_1}{C_2}\right)_{A(1)}$ and $\left(\dfrac{C_1}{C_2}\right)_{B(1)}$ at the end of the aforesaid first equilibration. It follows from Equation (1.1) that the composition of phase A may be expressed in terms of the composition of phase B by the equation:

$$\left(\frac{C_1}{C_2}\right)_{A(1)} = \alpha \left(\frac{C_1}{C_2}\right)_{B(1)} \qquad \qquad ...(1.2)$$

In the process of separation by fractional distillation, the liquid phase B_1 is in equilibrium with the vapour A_1 being condensed to the liquid phase B_2, whose composition is evidently the same as that of the vapour A_1. The liquid phase B_2 is in equilibrium with its vapour A_2 which is condensed to the liquid B_3 having the same composition as the vapour A_2, and so on.

In the case of solvent extraction, the operating processes are alike to those given above. The liquid phase B_1 (which has dissolved the mixture of substances to be separated) is in equilibrium with the liquid phase A_1 which extracts the mixture at a separation factor of α. When the solvent is removed, the substance absorbed by the extractant phase is again dissolved in phase B (step 2 in Figure 1.1) and extracted by the liquid A_2, and so on.
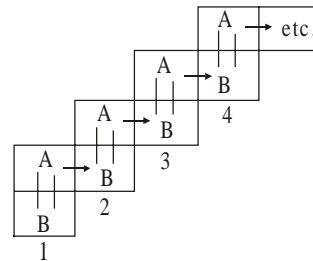

Figure 1.1 The stepping-off process for fractional separation of substances

Fractional crystallisation of a mixture of two or more substances proceeds in a similar manner.

The sequence of such equilibria (with subscripts 1, 2, 3...) may be represented in the manner shown in Figure 1.1. Since in all cases $\left(\dfrac{C_1}{C_2}\right)_{A(n)} = \left(\dfrac{C_1}{C_2}\right)_{B(n+1)}$, by taking into account relation 1.2, we may write for the scheme of separation under discussion the equation:

$$\left(\frac{C_1}{C_2}\right)_{A(n)} = \alpha^{n-1}\left(\frac{C_1}{C_2}\right)_{A(1)} \hspace{2cm} ...(1.3)$$

From Equation (1.3), it is evident that calculation of the composition of say, phase A at the end of the nth operation can be made from the value of $\left(\dfrac{C_1}{C_2}\right)_{A(1)}$ and α, which had already been found experimentally at the end of the very first operation. Taking the value of α as 2 in a particular experiment of, say, solvent extraction, the molar fraction of the first component in the extractant phase A will be sixteen times that in $A_{(1)}$, at the end of the 5th extraction.

In some separatory methods chemical reactions are required to convert or modify a component of the mixture to a form which either constitutes a new phase or enables the substance to be distributed to a second phase.

1.2 Distinction between 'Separation' and 'Purification'

Before giving an enumeration of the various techniques used for carrying out separation and purification in Chemistry and Biochemistry, the essential difference between these two very similar processes needs to be pointed out. In the case of purification the starting material consists mainly of one compound, and the small amounts of other compounds (termed contaminants or impurities) must be removed to get the main component as a pure entity. On the other hand in separation one would need to isolate several, or all, of the compounds in the pure state or an essentially pure state, from the starting material in which each of these compounds was present in significant amount. As the aim of either process basically is separation, the techniques employed to carry out separations and purifications are very similar.

1.3 Enumeration of Separation and Purification Techniques

An enumeration of several purification and separation methods is given in Table 1.1. Along with the commonly used descriptive titles of the various methods, Table 1.1 gives the underlying basis of each method. The list given in the table is

not exhaustive and discussion of several other separation and purification methods, not included in the table, is given along with the discussion of the methods listed in Table 1.1 in the chapters that follow.

Methods of separation of, say, metal ores from the gangue e.g. froth floatation and gravity-flow processes, which deal exclusively with suspensions of 'matter in bulk', are excluded from the book, since separations of concern to chemists and biochemists are the ones that deal with the smallest subdivisions of matter, such as atoms, molecules or small particles like those of colloids. For the same reason, the book also excludes separation of the grain from the chaff!

TABLE 1.1

Method	*Underlying Basis*
1. Crystallisation	Difference in solubility at higher and lower temperatures.
2. Fractional Crystallisation	Separation of the mixture into a number of fractions by successive crystallisations which can be induced through successful exploitation of small differences in solubility of the mixture components.
3. Fractional Precipitation	Separation of the individual components from a solution of the given mixture through successive precipitations which can be made to materialize by employing techniques like 'salting out' etc.
4. Sublimation	Difference in vapour pressure.
5. Solvent Extraction	Difference in solubility in two phases.
6. Distillation	Difference in volatility.
7. Chemical Methods	Conversion of one (or more) of the components of the given mixture into a derivative (or derivatives) whose physical properties are further removed from those of the other, chemically unchanged, components of the mixture there by making separation easier.
8. Counter-current Distribution	Using difference in solubility in two phases, in a series.
9. Chromatography	Difference in adsorption, partition, ion-exchange, molecular size etc. (depending on the concerned chromatographic method).
10. Dialysis	Differential migration across a semipermeable membrane which allows certain types of molecules to pass through it but excludes others.
11. Ultra-filtration	Flow of solvent, usually water, and diffusible colutes out of a solution through a membrane under applied pressure.

Method	*Underlying Basis*
12. Electrodialysis	Transportation of electrolytes from a dilute solution to a more concentrated solution across a membrane by applying an electric field.
13. Electrophoresis	Separation on a sheet in the presence of an electrical field.
14. Zone Refining	Crystallisation at elevated temperatures.
15. Fractional Melting	The phenomenon of a contaminant whose presence depresses the freezing point getting concentrated in the liquid phase at any temperature.
16. Inclusion-compound Formation	Separation of constituents of a mixture is achieved by removing that component which happens to consist of molecules of the requisite size and shape. Molecules of this component alone get accomodated as 'guest' species within the molecular structure of a 'host' species while the latter is crystallising out from a solution of itself and the mixture.
17. Centrifugation	Difference in rates of sedimentation of particles of the various constituents present in suspension when subjected to centrifugal forces.
18. Foam Separation	Tendency of surface-active solutes to collect at the gas-liquid interface.
19. Thermal Diffusion	Relative motion of the various molecular species of the given mixture under temperature gradient.
20. Electrorefining	Difference in values of the voltage required to convert different ionic species to the respective neutral substance.

1.4 Choice of the Appropriate Technique

The choice of the appropriate method, or sometimes two or more alternative methods for the separation of a particular mixture into its constituents, will most often depend upon the physical state (gaseous, liquid or solid) in which those constituents are present at room temperature. Crystallisation, for example, will not normally be contemplated for effecting the separation of the components of a mixture if they are gaseous or liquid, nor would purification of a solid by distillation be attempted normally.

The basic requirement in most of the separation and purification methods is the formation of second phase, whatever be the method of choice. Obviously, the choice of the nature of the second phase that needs to be formed during the process of separation depends upon whether the components of the mixture are originally present in the gaseous, liquid or solid phase, as the feasibility of the separation of this first phase from the newly formed second phase has to be taken care of. This choice of the second phase will, in turn, determine the required choice with regard

to the appropriate technique (or, two or more alternative techniques) that should succeed in carrying out the desired separation. Thus, separation of the constituents of a mixture of two miscible liquids is carried out by distillation. Distillation involves formation of vapour as the second phase, which is different in composition from the boiling liquid phase. In this method the separation of the two phases is being effected mechanically by the process of the vapours distilling over into the receiver and the liquid phase being left in the distillation flask. Gas chromatography could be an alternative method of separation if the two liquids constituting the mixture were sufficiently volatile.

Based on the nature of the second phase, the methods that are commonly used for separation can be classified as follows:

(i) Methods involving a solid second phase: These methods involve a second solid phase either through the formation of a sparingly soluble product or by adsorption on a suitable solid material.

Crystallisation, precipitation, chromatography, and electrophoresis belong to this category of separatory methods.

(ii) Methods involving a liquid second phase : The outstanding example of methods falling in this class is provided by solvent extraction. In this method extraction of the desired component into the second added liquid phase may be carried out from either a solid starting material or from a solution that has been prepared in a liquid solvent which is immiscible with the added liquid extractant. Another method that belongs to this category of separatory methods is dialysis in which the second liquid phase is kept separated from the first liquid phase by a suitable membrane. Zone refining and fractional melting are also examples of separation techniques that involve a liquid second phase.

(iii) Methods involving a gaseous second phase : Distillation, sublimation and gas chromatography belong to this class.

It may be added here that a combination of more than one separation or purification method may have to be used at times. Solvent extraction, for example, is very often only the first step in a multiple-method separation. Similarly, zone refining is the final step in getting an extremely pure sample of a substance which has already been obtained in more than 99per cent purity through the use of other purification techniques.

Although the basic requirement of most of the separation and purification methods is the formation of second phase, there are some methods e.g. the electromagnetic method of separation of isotopes, fractional diffusion and thermal diffusion for the separation of gases, that do not need to meet that requirement.

The book deals with the various separation techniques in the following four parts:

   I.   Traditional Methods of separation and purification.
  II.   Chemical Methods of separation and purification.
 III.   Chromatographic Techniques.
 IV.   Other Diverse Techniques of separation and purification.

■

This page intentionally left blank

PART I

Traditional Methods of Separation and Purification

This page intentionally left blank

<div align="center">

2

Methods of Everyday Use

</div>

The present chapter deals with separation and purification methods which may be termed as 'traditional' in view of the discovery of the newer techniques. Nevertheless, the mastery of these traditional methods (with the latest improvements in their practice) is a basic prerequisite even today for every chemist and biochemist.

2.1 Crystallisation

Crystallisation is the simplest and, perhaps, the oldest method of purifying solids. Because of the effectiveness of the method, crystallisation finds extensive use for the purification of solids. Appreciable difference of solubility of the given solid in a suitable solvent at the boiling point of the solvent and at room temperature forms the basis of this purification technique. The amount of impurities present in the solid is, of course, much less than that of the principal substance and it is unlikely that they will saturate the solution at room temperature. Therefore, these impurities remain in the mother liquor in the end. The steps involved in crystallisation are: (i) Preparation of a clear saturated, or nearly saturated, solution of the solid, (ii) Cooling the solution, (iii) Collecting the solid by filtration and removing the residual solvent from the solid.

(i) The solution usually is made in the boiling solvent. The solvent chosen should possess certain characteristics, so that, in general, purification can be effected conveniently and with minimum loss: (1) The solvent should not boil at too low a temperature; (2) it should not boil at too high a temperature; (3) it should possess slight, or at best, moderate tendency to dissolve the solid at the temperature of crystallisation; (4) it should dissolve the solid much better at elevated temperature i.e. the temperature co-efficient of solubility of the solid in the liquid should be reasonably high.

If the boiling point of the solvent is low, or if the solution is prepared at a temperature below 50ºC, generally the change in solubility on cooling to room temperature even in winter is not sufficiently great. If the boiling point of the solvent is too high, the solute may decompose. Moreover, complete removal of the solvent after filtration is difficult. Sometimes mixed solvents are used e.g. aqueous alcohol.
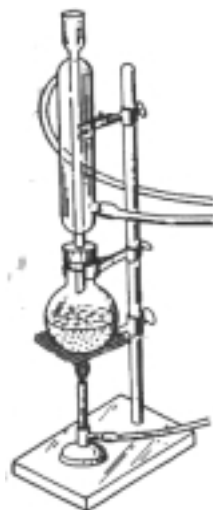
Figure 2.1
Reflux assembly

Figure 2.2
Jacketed funnel for filtration

Figure 2.3
Fluted paper,
for hot liquids

The solute, preferably taken in a fine state of subdivision to aid dissolving, along with just sufficient quantity of the appropriate solvent, is placed in a flask of suitable size. A reflux (upright) condenser is attached in case the solvent is not of high boiling point (Figure 2.1). The mixture is agitated while being heated, till the solute has dissolved completely. The complete dissolution should take place only after the solvent has boiled for a few minutes showing thereby that the proper quantity of the solvent had been taken. The hot solution is next to be filtered unless it is clear.

Filtration of the hot solution is to be carried out so that the drop in temperature is kept to a minimum to prevent the solid from separating out in the filter and in the stem of the funnel. Prevention of excessive cooling during filtration is achieved either (a) by heating the funnel fitted with the filter paper by surrounding it with a copper jacket containing hot water (Figure 2.2), or (b) by rapid filtration carried out by one of the following procedures: (1) Gravity flow through a fluted paper fitted in a glass funnel, preferably a stemless funnel set in a beaker (Figure 2.3), (2) Suction through a flat piece of filter paper properly fitting a Buchner funnel (Figure 2.4).

(ii) The filtrate is a hot clear solution, saturated or almost saturated with the desired solid. Crystals of the solid separate out as the solution cools. It may be mentioned here that transition temperatures are often observed in Inorganic Chemistry between different hydrates of a given salt or between double and single salts. For example, a solution of sodium sulphate and magnesium sulphate in water deposits crystals of $Na_2SO_4 \cdot 10H_2O$ and
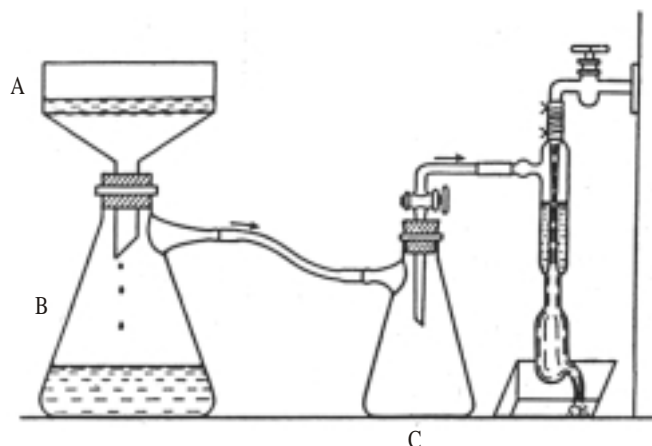
Figure 2.4 Filtration with suction (A) Buchner funnel, (B) Filter flask, (C) Trap

$Na_2SO_4 \cdot 7H_2O$ at temperatures below 22ºC, but yields the double salt, $Na_2SO_4 \cdot MgSO_4 \cdot 4H_2O$ at higher temperatures. The mixture of the liquid and crystals is allowed to stand with occasional shaking until crystallisation is complete.

(iii) In the next step the mother liquor is removed from the crystals by suction filtration. Since the mother liquor contains the impurities, it is the aim of the suction treatment to remove as much of the liquor as possible from the solid without evaporating the solvent. The solid is then washed with the cold solvent under suction. The object of washing the solid is the removal of the residual mother liquor still sticking to the crystallised compound.

In case the crystals obtained are discoloured due to the presence of traces of impurities, they are redissolved in a small quantity of the solvent and the solution in boiled with a small amount of animal charcoal. The hot solution is filtered, cooled and crystals separated and washed as before.

The washed solid may contain as much as 50% of the solvent by weight. As much of the residual solvent as possible is removed by adsorption either by pressing the wet solid firmly between pieces of filter paper, which is a good absorbing medium, or by pressing out the wet mass on a porous tile. After this treatment, a considerable amount of solvent still remains. This can be removed by evaporation. This can be accomplished by either (1) exposure to air at room temperature, or (2) heating in an open space. Heating is a good method of drying and may be done in a hot oven. If the wet compound liquefies or undergoes decomposition or oxidation or evaporation on heating, drying is carried out at room temperature which is accomplished (more

Figure 2.5
Vaccum desiccator

slowly, of course) by leaving the substance in a vacuum desiccator (Figure 2.5) charged with a suitable solid or liquid desiccating agent. Amongst the several desiccants commonly employed, the most efficient one is phosphorus pentoxide. Potassium hydroxide pellets are very efficient. Concentrated sulphuric acid is another efficient desiccant which is used quite commonly. Anhydrous calcium chloride granules can be used and these are as efficient as sulphuric acid and safer. For drying of organic solvents silica gel or fresh paraffin wax shavings can be used.

In the Abderhalden drying pistol (Figure 2.6), the solid is dried *in vacuo* while being heated by the vapour of a boiling liquid. The pistol consists of a tube that holds the sample for drying and a flask that holds a suitable desiccating agent. Tube T is inserted in a vapour jacket. The temperature of the sample compartment depends on the vapour which in turn depends on the boiling point of the liquid chosen for boiling in flask B.

The sample is spread out in a porcelain or platinum boat or on a filter paper which is then inserted in T. The desiccating agent, usually phosphorus pentoxide or fresh paraffin wax shavings, is placed in F and the apparatus assembled. The drying chamber is connected to a filter pump and evacuated. The liquid in B which should be perfectly non-inflammable, is then heated and maintained in a state of gentle boiling. Enough time is allowed to the wet sample to remain inside the heated apparatus so that it becomes completely dry.

Figure 2.6 represents an example of a ground-glass-joint assembly. Such an assembly dispenses with the use of corks. Ground-glass-joint assemblies can be set up on command as no time-consuming jobs like boring of corks etc. are involved and, also, these assemblies are leak-proof straight away. Further, the probability of the undesirable situation arising from reaction between the substance

Figure 2.6 Abderhalden drying piston

being processed and the material of the cork is completely eliminated by using ground-glass-joint assemblies.
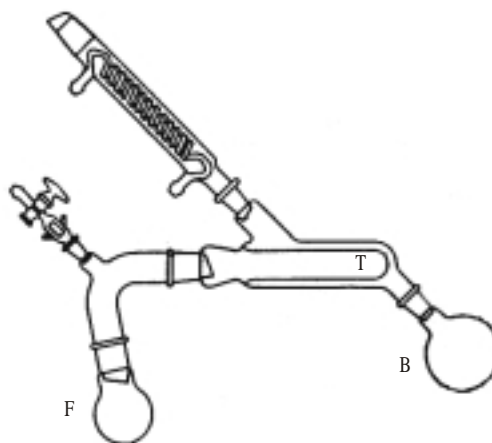
2.2 Fractional Crystallisation

If a mixture which contains two or more substances in comparable quantities, or a mixture which has substances of closely similar solubility, is to be separated into its constituents by crystallisation, a laborious process of repeated fractional

crystallisation has to be adopted. In this, the more soluble a particular component of the given mixture is, the greater will be its accumulation in the mother liquors. Thus, by working systematically, the least soluble constituent is increasingly freed from the more soluble ones on each successive crystallisation till finally it is obtained pure as established by the criterion of constancy of melting point of the product of recrystallisations.

The practice of the technique can be illustrated as follows:

Consider a 1 : 1 mixture of two components A and B of more or less the same solubility. Isolation of the two constituents from the mixture as individual compounds belongs to the realm of separation rather than purification. Separation of A from B would not be accomplished by the technique of crystallisation outlined above even if it were carried out on the mixture repeatedly. In theory the desired separation of A from B could be carried out by using a solvent in which A dissolves out completely and B is completely insoulble. But the attempts to find such a solvent are not very often successful. A more practical proposition, therefore, could be to use a solvent that produces two samples, one of those being mainly A and the other mainly B, and the operation being completed by subjecting each of these two samples to crystallisation, repeatedly if needed, in order to get pure A and pure B. In actual practice, however, a more elaborate process has to be gone through in order to obtain the components of the mixture as pure substances. The given mixture is dissolved in an appropriate quantity of a suitable hot solvent or mixture of solvents, and from the resulting solution only a small quantity of solid is allowed to separate out by crystallisation. This solid is collected, and the mother liquor is made to yield a little more of the solid by altering one factor selected from amongst the following three controlling factors whose variation results in crystallising out further quantities of solid :-

  (i) concentration,
 (ii) temperature,
(iii) solvent composition.

Collection is made of this fresh quantity of solid too, and the resulting mother liquor is made to yield again a small quantity of solid by making a further small alteration to the controlling factor selected earlier. In this manner, that is, by making continued small alterations to the controlling factor, small differences in solubility of the components of the given mixture can be exploited to divide the mixture into a number of fractions of continually varying composition. The name 'fractional crystallisation' for this separatory technique thus arises from the basis that separation in this method is effected through the formation of a number of fractions by successive crystallisations.

If the controlling factor selected is concentration, the initial solution is prepared by using a quantity of the boiling solvent which is much in excess of the minimum quantity required for complete dissolution of the mixture, so that only a small

quantity of the solid would crystallise out from the solution on cooling. After collection of the separated solid, the mother liquor is concentrated a little, by boiling or evaporating off some solvent, so that it yields a second crop of crystals on cooling. On repeating the above series of steps several times, the starting mixture can be got separated into a number of fractions.
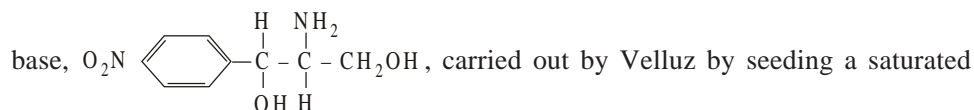
Instead of concentration, temperature, which is listed at (ii) above as another controlling factor, can sometimes be successfully made use of in separating a given mixture into a number of fractions by collecting, at intervals, the solid crystallising out from the prepared hot solution at successively lower temperature.

With the solvent composition selected as the controlling factor, the given mixture is dissolved in a rather excessive quantity of an appropriate hot solvent, which may be one pure liquid, or a mixture consisting of two or more liquids mixed together in a definite proportion. After collecting the first crop of crystals yielded by the hot solution as a result of its cooling, small quantities of a second liquid are added to the mother liquor whereby the altered solvent composition would yield a second crop of crystals which also is collected. Further crops, or fractions, will be obtained by repeating the aforesaid series of steps several times.

Now, suppose ten fractions have been obtained for the particular case under consideration, namely, a 1:1 mixture of the two compounds A and B, using one particular controlling factor selected out of the three factors referred to above. Of the two compounds, A may be assumed to be the one which has the lower solubility in the starting solvent. Out of the separated ten fractions, perhaps the first two fractions may contain exclusively A, and the last two fractions may be of exclusively B, with the 'middle' six fractions being mixtures containing A and B in varying proportions. Of these intervening six fractions, those which are rich either in A or B can respectively be made to yield further quantities of pure A or pure B by simple recrystallisations. The remaining ones of the middle fractions may be combined and refractionated.

An example of the application of the technique of fractional crystallisation in Organic Chemistry that merits special mention is the separation of a mixture of optical isomers. Separation of a mixture of d- and 1-isomers (optical antipodes or enantiomers) into its constituents is known as optical resolution. Since enantiomers are a pair of substances which have identical physical properties e.g., melting point, solubility etc. and also the same chemical properties, modified procedures of fractional crystallisation are required for their separation from a mixture with each other.

A method introduced by Velluz (1957) involves seeding of a supersaturated solution of a racemate (an equimolar mixture of d- and 1-isomers obtained in the last step of synthesis of a compound capable of showing optical isomerism) with crystals of the optically active isomer sought. Resolution of dl-chloramphenicol

base, O_2N ⟨benzene ring⟩ $-\overset{\displaystyle H}{\underset{\displaystyle OH}{C}} - \overset{\displaystyle NH_2}{\underset{\displaystyle H}{C}} - CH_2OH$, carried out by Velluz by seeding a saturated

solution is particularly interesting because it is necessary to seed only once with one optically pure enantiomer. Thereafter, the two enantiomers crystallise alternatively in successive crops, because only the isomer of the greater degree of supersaturation will crystallise. The procedure was carried out in the presence of hydrochloric acid which permitted substantial reduction of the volume of solvent required.

A noteworthy example of the application of the technique of fractional crystallisation in Inorganic Chemistry is the accomplishment of the challenging job of separation of lanth-anides. Simple salts like nitrates, oxalates, bromates and sulphates as well as double salts like 2 La $(NO_3)_3 \cdot 3$ Mg $(NO_3)_2 \cdot 24H_2O$ of these elements are easily crystallisable. Taking advantage of small differences of solubilities of these salts in water, separation of lanthanides has been achieved by repeated fractional crystallisation of these salts.

2.3 Fractional Precipitation

Fractionation by precipitation can be brought about by methods which are either chemical or physical in nature. Separations carried out by chemical precipitation are dealt with in the next chapter, and the present Section deals with separations by precipitation carried out by physical methods.

Fractional precipitation may be employed to bring about separations in cases where it is difficult to induce the dissolved solutes to separate out as solid by crystallisation. Thus, for example, whereas fractional crystallisation cannot bring about separation of proteins from complex mixtures in which they are found in biological preparations, fractional precipitation succeeds in effecting isolation of individual proteins from such complex mixtures. For effecting separations, fractional precipitation, like fractional crystallisation, makes use of controlling factors which influence the respective solubility of the compounds of the given mixture in the chosen solvent. These factors are: (i) Variation of the dielectric constant of the solvent, (ii) Salting-out.

(i) Variation of the Dielectric Constant of the solvent: The reduction of the effective dielectric constant of the solution of a polar solute in a polar solvent by the addition of a weakly polar solvent will increase the attraction between charged protein molecules and decrease their interaction with water, so that the result of the addition of the weakly polar solvent frequently is the precipitation of the solute. The method is widely used for the recovery of solutes from solution. The method must, however, be used with caution, since lowering of dielectric constant of the medium besides bringing about an increase in electrostatic interactions between ionic groups also reduces dispersion interactions between non-polar groups. This

may produce changes in the tertiary protein structure which are usually described as denaturation, so that the precipitated protein is irreversibly changed. The most effective use of media of reduced dielectric constant for the fractionation of mixtures of proteins is by the use of moderate concentrations (upto 25%) of solvents such as methanol or ethanol together with variations of ionic strength. The fractionation of the serum proteins providws many examples of this technique.

(ii) Salting-out: All solid solutes are precipitated from solution by the addition of neutral salts to the solution. The present discussion of salting-out will be confined to protein solutes, as the effect is most important for the purification of these substances.

The salting-out of proteins is only evident at high salt concentrations. Ammonium and sodium sulphates and potassium phosphate have been most widely used for the salting-out of proteins. Althouth the salting-out effects of phosphates are greater than those of ammonium sulphate, the considerably greater solubility of the latter, and its relatively small dependence on temperature has made it the first choice for the majority of salting-out methods. Sodium citrate solutions have also been used in some cases. A disadvantage of ammonium sulphate in comparison with phosphate and citrate solutions, is its weak buffering capacity. Concentrated ammonium sulphate solutions are acidic (pH approximately 5.5) owing to hydrolysis of the salt, and the pH should be adjusted by addition of NH_4OH or H_2SO_4 before use.

Salting-out methods can be classified into two groups:

(i) Methods that involve variation of ionic strength (salt concentration) at constant pH and temperature; and

(ii) Methods that involve variation of pH and temperature at constant ionic strength.

The first group are considered methods of choice for the fractionation of crude extracts and the second group of methods are more valuable for carrying out finer sub-fractionation within a previously precipitated fraction. Fractionation by the second group of methods can be carried out by variation of pH or temperature or both. pH fractionation has been more widely used, but in special cases temperature variation at constant pH and ionic strength can be a valuable method for inducing crystallisation of proteins (e.g. carboxyhaemoglobin and myosin).

Fractional precipitation as exemplified gives an initial separation of the constituents of a mixture but the product of each successive precipitation is far from being a pure compound, because precipitates carry a lot of other solutes along with them from the solution, even though the solubility of such salts may not have been exceeded. Thus, whereas fractional crystallisation is an adequate separatory technique in itself and can be used independently to obtain individual constituents of a given mixture in the pure state, fractional precipitation has to be combined with other purification processes e.g. electrophoresis to get pure compounds from the fractions obtained by successive precipitations.

2.4 Sublimation

Substances like camphor, naphthalene and anthraquinone having appreciable vapour pressure in the solid state can be freed from less volatile impurities by sublimation, which is the phenomenon of a solid passing directly to the vapour phase on heating and the change in phases getting reversed on cooling.

The theory of sublimation will become clear by reference to Figure 2.7, which is the vapour pressure-temperature diagram for camphor. The vapour pressure of a solid increases with temperature in much the same way that the vapour pressure of a liquid does. This is clear from the slope of the line AB which lies between the regions marked solid and vapour and is the vapour pressure curve of the solid, that is, it represents the vapour pressure of the solid at the corresponding temperature in Figure 2.7. The line CB separating the regions marked liquid and

Figure 2.7 Vapour pressure—Temperature diagram of camphor

vapour is the vapour pressure curve of the liquid whereas the line DB, which lies between the regions marked solid and liquid, represents the melting point at various pressures. The point B (179°C at a pressure of 370 mm) is the triple point where all three phases solid, liquid and vapour, coexist. This is the melting point of camphor at a pressure of 370 mm.

The melting point of a substance is practically independent of pressure; therefore, the temperature at the triple point will be practically the same as at the normal melting point. Thus if camphor is heated under normal conditions of 1 atmosphere, it melts at 179°C. If pure camphor vapour at atmospheric pressure is cooled, liquid is first formed and on further cooling this solidifies, as shown by passing from right to left along the line EF at 760 mm. Liquid is first formed at all pressures above the triple-point pressure of 370 mm. However, at all pressures lower than 370 mm camphor may pass from solid to vapour, and *vice-versa*, without going through the liquid state, as shown by passing along the line GH.

Thus sublimation may be carried out with camphor at pressures below 370 mm. Also, it may be done at total pressures above 370 mm in the presence of some gas, for example, air, provided the partial pressure of the camphor vapour does not exceed 370 mm. Thus sublimation is possible at atmospheric pressure. Suppose for example that the temperature of solid camphor is raised to 170°C. The pressure
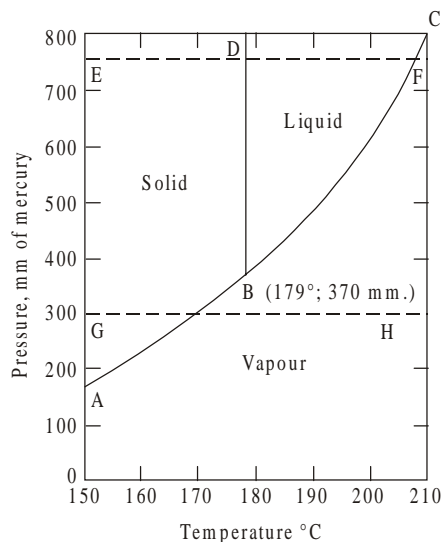
exerted by the camphor vapour then is 300 mm irrespective of whether or not air is present. The solid and vapour are in equilibrium. If part of the surface of the containing vessel is cooled, say to 25°, solid camphor will be deposited because the pressure of camphor vapour in equilibrium with the solid at 25° is 0.7 mm. Thus camphor is carried, partly by diffusion and partly by convection, from the region of higher vapour pressure to the one of lower vapour pressure, from the hot region to the cold region, without passing through the liquid state.

Various types of sublimation apparatus are in use. Purification by sublimation is relatively a slow process and depends upon the rate of diffusion of the vapour to the cold condensing surface. In order to make the method sufficiently efficient, as large a

Figure 2.8 Sublimation set-up

cooled condensing surface as possible should be used, and condensing and evaporating surfaces should be close together. A simple laboratory sublimation set-up which incorporates these features is shown in Figure 2.8.

In this set-up, impure substance is taken in an evaporating dish which is covered with a perforated asbestos plate over which is placed an inverted funnel. The dish is heated on a sand bath to avoid overheating which would be caused by the use

of a direct flame. Heating yields vapours of the pure compound which is recovered as a solid deposited on the inside walls of the funnel provided with cooling arrangement as shown in Figure 2.8. The perforated asbestos sheet prevents the sublimate from falling back into the impure material.

Since the temperature of the solid at the triple-point and at the melting point is practically the same, the vapour pressure of the substance at the triple point may be taken to be the same as vapour pressure at its melting point. It is very rare for a substance to have a vapour pressure of 760 mm or greater at the temperature of the triple point or, in other words, at normal melting point. Substances like camphor which have large enough vapour pressure near their melting point so as to yield to sublimation at atmospheric
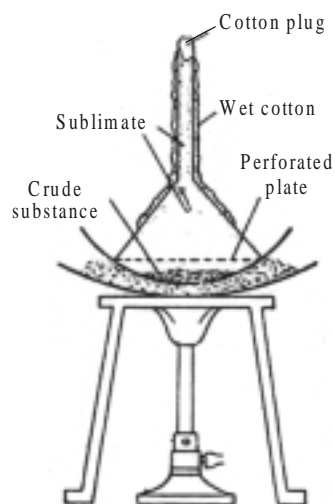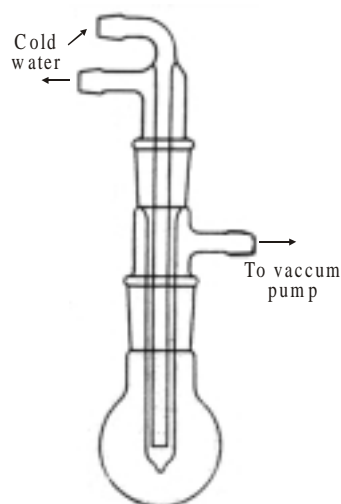

Figure 2.9 Set-up of sublimation under reduced pressure

pressure at some worthwhile rate are also not many. Generally reduced pressure (vacuum) is needed to increase the rate of evaporation of the solid to make purification by this technique a practical proposition. Also, only sublimation under reduced pressure can be employed in case the compound in question decomposes on heating under atmospheric pressure. In this modified procedure called vacuum sublimation, decomposition of the compound does not occur as sublimation takes place at a lower temperature because of the reduced pressure inside the apparatus. Vacuum sublimation is most easily carried out in apparatus shown in Figure 2.9. This is simply a cold finger condenser fitted into a flask via a straight receiver adapter.

Sublimation is restricted to relatively non-polar substances of fairly symmetrical structure. Should this criterion not be met, the time necessary to pass any significant quantity of material through the vaporisation-solidification sequence would usually be prohibitively long. In this case either recrystallisation or chromatographic techniques must be used.

2.5 Solvent Extraction

Extraction is the removal of a substance from a solid or from a liquid by means of a solvent. The extracting solvent should preferably extract the required substance without extracting other materials. Extraction is frequently employed in the separation of substances from reaction mixtures or from natural sources and is also useful in the purification of substances. Extraction is carried out either as a *batch* process or as a continuous process, details of these processes being given in the following sections.

2.5.1 Extraction from solids

Fats and other naturally occurring organic non-volatile compounds may often be extracted from plant seeds, animal tissues etc. with a readily volatile solvent, such as ether, or ether mixed with alcohol. The Soxhlet apparatus (Figure 2.10) is a very effective apparatus for this purpose. The macerated material is contained in a porous 'thimble' T, usually made of stout filter paper but also of porcelain or stoneware clay, and maintained in a glass cylinder provided with a syphon S. The volatile solvent, boiling gently in the flask F beneath, rises through the side-tube A and is condensed in the reflux condenser C forming the upper portion of the fit-up of the apparatus, so that a constant stream of pure solvent drops into the thimble, and a solution of the extracted matter returns

Figure 2.10
Soxhlet extraction apparatus

periodically into the flask via the syphon S. The process is thus automatic and *continuous* and has the virtue of always being carried out with pure solvent. The extracted matter is then recovered from the solution collected in the flask by removal of the solvent by distillation. (Section 2.6)

Application of solid-liquid extraction in the field of Inorganic Chemistry can be illustrated by taking the examples of separation of (i) lithium chloride from the chlorides of other members of the alkali metal group and (ii) calcium nitrate from the nitrates of other members of the alkaline earth group. The solubilities of sodium chloride and potassium chloride are very small in *n*-hexanol and 2-ethylhexanol, whereas the solubility of lithium chloride is large enough so that it can be separated from a mixture of the three chlorides by extraction with these solvents. Similarly, using a 50-50 per cent mixture of absolute ethanol and ether calcium nitrate can be removed from a mixture of the anhydrous nitrates of calcium, barium and strontium.

2.5.2 Extraction from liquids

2.5.2.1 Batch and continuous extractions

Although no two liquids are absolutely immiscible, yet many examples of pairs of liquids, such as water and benzene are known which may be regarded as quite immiscible for all ordinary practical purposes. This property may sometimes be used to separate one component from a mixture of liquids. Thus in the *Batch* process, benzene is isolated from a solution of benzene and alcohol by shaking this mixture of the two liquids with an excess of water in a separating funnel. Two representative types of the separating funnel used in the Batch process are shown in Figure 2.11(a)

(a)   (b)

Figure 2.11
Separating funnels

and Figure 2.11(b). When the mixture is left for a while the liquid separates into two layers. Benzene, which forms the upper layer [(1) in Figure 2.11(a) or (3) in Figure 2.11(b)] gets separated by running off the bottom aqueous layer [(2) in Figure 2.11(a) or (4) in Figure 2.11(b)]. By repeated
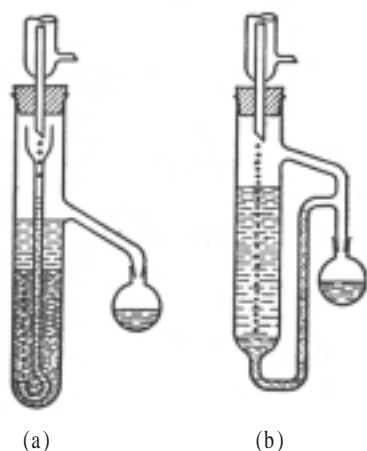

(a)   (b)
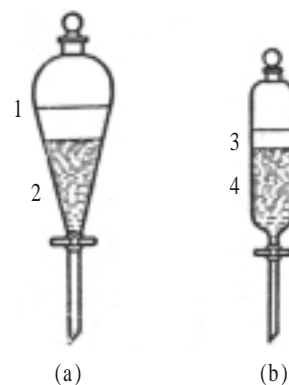
Figure 2.12 Apparatus for continuous extraction of a liquid

extraction of the traces of alcohol remaining in the benzene layer by shaking the latter every time with quantities of fresh water, alcohol-free benzene is obtained.

Continuous extraction from a liquid by an appropriate solvent is carried out by boiling the solvent into a reflux condenser, allowing the condensate to flow into and through the liquid containing the solute being extracted and returning the solvent to the boiler, from which it repeats the extraction cycle. In this way, the solute is extracted by a much smaller volume of the solvent than is required by repeated extraction involved in the Batch process discussed in the preceding paragraph. Types of apparatus for continuous extraction are shown in Figure 2.12(a) and (b), where the extracting solvent is lighter and heavier respectively than the solution.

2.5.2.2 *Extraction by transfer to a more volatile solvent*

It is sometimes of advantage to transfer a dissolved solid from one solvent (usually water) to another which is more volatile (e.g. ether, benzene, chloroform, light petroleum etc.). Thus salicylic acid dissolved in water cannot easily be recovered by evaporating the solution to dryness, owing to its volatility in steam. When the aqueous solution is shaken with a relatively small amount of ether in a separating funnel and the mixture allowed to settle, a good deal of the salicylic acid is transferred to the ether; when the upper ether layer is separated and deprived of the ether by distillation, the extracted salicylic acid remains in the distillation flask.

The same principle of extraction by a solvent is involved in the laboratory preparation of aniline by reducing nitrobenzene in which method the product is steam distilled from the reaction mixture, giving a little aniline with a relatively large volume of water. These are best separated by shaking the mixture with a volatile solvent in which the aniline dissolves (e.g. ether); the lower aqueous layer is separated from the ether layer. The ethereal extract is then dried by allowing it to stand over anhydrous calcium chloride to remove the water carried by ether. Finally, distillation of this dried extract yields aniline by removal of the volatile solvent, ether.

2.5.2.3 *Distribution coefficient (K_D)*

In all the cases of extraction from liquids given above, the solute is distributed between the two solvents in such a manner that a definite ratio (the Distribution coefficient or the Partition coefficient) is maintained between the two concentrations i.e. in the instance under consideration.

$$\text{Distribution coefficient } (K_D) = \frac{\text{Concentration of solute in ether layer}}{\text{Concentration of solute in water layer}}$$

The ratio depends largely upon the respective solubility of the substance in each of the pure solvents, and for the process to be effective in the above example the

solubility in ether must be considerably greater than the solubility in water. Urea, for instance, which is much more soluble in water than in ether, would not lend itself to extraction in this way. It follows from a consideration of the nature of the partition coefficient that a given amount of ether will afford a more efficient extraction when applied in successive quantities than when used all at once.

2.5.2.4 *Manipulations used for enhancing solubility*

Solute solubility in the solvent chosen for extraction can be enhanced by various manipulations of which the more commonly employed are: Variation of the dielectric constant of the solvent, salting-in, hydrogen bonding and pH adjustment. These manipulations are illustrated below with special reference to biochemically important extractions wherein their role is crucial.

(i) Variation of the dielectric constant of the solvent: This is perhaps the most powerful method for increasing solubilities (and, also, for decreasing solubilities, as mentioned earlier under 'Fractionation by Precipitation'), owing to the fundamental importance of the dielectric constant of the solvent in determining the solubility mechanism. In general, polar compounds are best extracted with polar solvents, and non-polar compounds with non-polar solvents, but selective extraction of polar compounds can often be carried out in media of reduced dielectric constant by making use of the enhanced salting-in effects under these conditions. Thus, although the total solubility may be markedly reduced by the reduction in the dielectric constant, the solubility of a particular component may be enhanced by the salting-in mechanism. Mixtures of methanol, ethanol, and dioxan with water are particularly useful for this purpose, although with labile solutes, it may be necessary to work at reduced temperatures.

The great disadvantages of fractionation of dipolar ions such as proteins using media of reduced dielectric constant are the increased lability of the solutes and the tendency to form intersolute complexes due to the increased electrostatic interactions between the different solute species.

(ii) Salting-in: The method of salting-in is particularly effective for increasing the solubilities of ions and dipolar ions to which categories the majority of biologically and metabolically active substances belong. Thus dilute aqueous salt solutions are among the most important extraction media for large dipolar ions such as proteins and peptides. In most cases salt solutions with concentrations in the range 0.05–0.2 M have been used, although higher salt concentrations up to 0.5N–1M may be useful in certain instances, and considerably lower salt concentrations may be employed in solvents of lower dielectric constant.

Sodium chloride solutions have been very widely used for extraction purposes, although the nature of the salting-in salt does not appear to be of primary importance. Salts such as potassium iodide and potassium thiocyanate have been employed with success in certain instances, although specific interactions may be involved in these cases.

Salting-in effects are produced by dipolar ions as well as by ions and glycine may be used to increase the solubility of certain proteins.

(iii) Hydrogen bonding: Hydrogen bonding is one of the most important mechanisms of solute-solvent interactions and these interactions increase solute solubilities. Thus the presence of a proton donor or acceptor function in the solvent may be expected to increase the solubility of solutes having an appropriate hydrogen-bonding function. This is perhaps the most important method for enhancing the solubilities of non-electrolytes, where the methods discussed earlier are less useful. Monofunctional donor or acceptor solvents may be more selective for extraction than polyfunctional solvents, and the reduced solvent-solvent interactions in such cases may also afford a reduced energy barrier to solution.

In those cases where other considerations preclude the use of solvents with intrinsic hydrogen-bonding functions, addition of proton donors, such as phenols, or proton acceptors, such as amines, to the solvent may greatly enhance the solubility of an acceptor or donor solute.

(iv) pH Adjustment: The pH of the extraction medium is often of particular importance and the use of acidic or alkaline solvents to extract basic or acidic solutes may be seen in this context. Dipolar ions such as proteins and peptides usually show their minimum solubilities at their isoelectric points where they have zero net charge, and they show greatly enhanced solubilities at even 0.5 pH unit on either side of the isoelectric point.

At 1–2pH units away from the isoelectric point many proteins behave as normal ions, and may be salted-in by dilute salt solutions. The effect of pH on the solubility is in fact most evident at low ionic strengths.

The three variables of pH ionic strength and dielectric constant form an interrelated system for the fractionation of proteins by variation of solubilities, the classic example of separation achieved in this manner being the fractionation of plasma proteins carried out by Cohn and Edsall.

(v) Salt formation: The formation of certain salts by a particular component of a mixture of solutes may be used to enhance the solubility of that component. Some striking examples of the application of salt-formation include the enhanced solubilities of amine hydrochlorides compared with the free amines in non-polar solvents such as chloroform, and the highly specific solubilities of the trichloroacetates of serum albumin and insulin in organic solvents such as methanol, ethanol and acetone.

(vi) Solubilization by surface-active materials: Many surface-active materials, both natural and synthetic, have a solubilizing action on proteins and other polar solutes. For example, tetra-alkylammonium and pyridinium chlorides have been used for the extraction of visual proteins from frog retinae. Another striking example of the application of surface-active materials for effecting separations in the biochemical field is the resolution of the subcellular microsome fraction into

ribosomes and a membrane fraction by using sodium deoxycholate and polyethylene glycols (Lubrols).

The use of detergents for extraction is, however, subject to many reservations. They may combine irreversibly with the solutes or may be otherwise difficult to remove. They may, however, be of value when other methods are unpromising, and when suitable means (such as ultracentrifugation) are available for the isolation of the required component from the detergent solution.

(vii) Temperature changes: Since the majority of solutes dissolve with the absorption of heat, their solubilities commonly increase with increasing temperature. A number of proteins are, however, known to have negative temperature coefficients of solubility, and no general theory for the prediction of this effect for protein solutes exists.

Although in principle increased solubilities might be obtained by working at an elevated temperature, in practice the thermolability of most biochemically important solutes and the increased rates of enzymatic or bacterial destruction severely limit the temperature range which can be used.

2.5.2.5 Solvent extraction of metal ions

Solvent extraction of metal ions in Inorganic Chemistry is based on the formation of an uncharged complex followed by distribution of this complex between the two concerned solvents which are immiscible in each other. The complex may be produced either by creation of coordinate covalent bonds or by solvation or by ion-pair formation.

The formation of a coordinate complex of a metal ion arises from the tendency of the ion to acquire a stable electronic configuration by filling up its vacant atomic orbitals by electrons which the ligand makes available. The coordination complex obtained may be an uncharged or a charged species.

A complex with a ring structure results when a metal ion conbines with a polyfunctional ligand which is capable of occupying two or more positions in the coordination sphere of the metal ion. Such complexes containing ring structures are called chelates. An example of a chelate is shown by the structure :
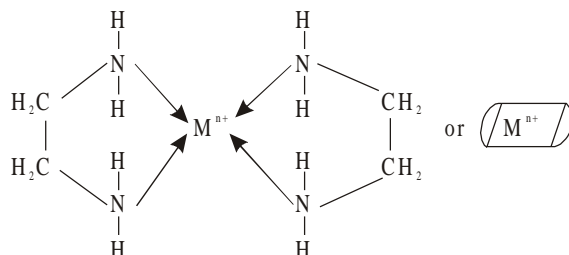
wherein the ligand ethylene diamine is shown complexed with a metal ion M^{n+}. An ordinary coordination covalent complex not possessing any ring structure

results when the ligand employed uses only one atom at a time as a donor towards the metal ion. On account of their stability chelates constitute the most important class of complexes in liquid-liquid extraction.

Form amongst the numerous ligands used for metal ion complexation, a few are given below as examples:

H_2O NH_3

Aquo   Ammino

8-Hydroxyquinoline

Neocuproin
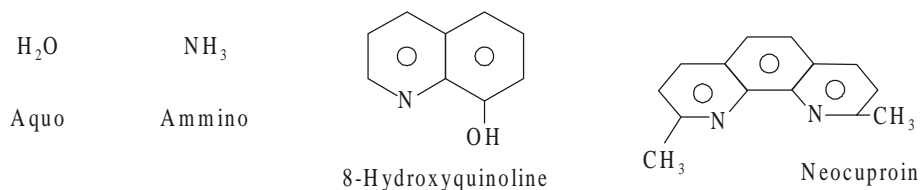
Neutral chelates are easily extracted in organic solvents. A charged chelate combines with an oppositely charged ion to produce a neutral entity which is extractable by an organic solvent. For example, the cationic chelate formed between Cu^+ and neocuproin combines with the anion ClO_4^-, to give an uncharged entity which is extractable with an organic solvent like chloroform.

An alternative to the above procedure of separation of metal ions from a solution is masking or sequestering. The given solution may be supposed to contain two metal ions, A^{m+} and B^{n+}, of which the separation is to be carried out by liquid-liquid extraction through complexation. A complexing ligand is added to the solution so that the metal ion A^{m+} is changed into a complex that is not extractable by the added organic extractant. By this masking or sequestering of the ion A^{m+}, it is only the metal ion B^{n+} which will distribute itself between the original liquid phase and the added liquid extractant. By repeated extraction using fresh quantities of the organic extractant, almost complete separation of A^{m+} and B^{n+} can be achieved.

In liquid-liquid extraction by solvation, the solvent itself takes part in complexation of the metal ion. This is brought about by donation of electrons by the donor atom present in the solvent molecule to the coordination sphere of the metal ion. Such an association of the solvent molecules with the metal ion results in the formation of a solvated complex that is extractable in the solvent. Thus, for example, octanol, C_8H_{17}–OH, extracts Co^{2+} in the presence of perchlorate ion as $[Co(C_8H_{17}$–OH$)](ClO_4)_2$. Extraction by solvation generally employs oxygenated solvents such as alcohols, ethers or ketones or neutral organophosphorus compounds. These solvents stand the competition from water for solvation of the metal ion successfully because of the higher availability of electrons from their donor atoms.

Liquid-liquid extraction by ion-pair formation involves formation of an uncharged species as exemplified below for a case in which a tertiary amine like trioctylamine (TOA) or triisooctylamine (TIOA) is used as the solvent to extract the anionic metal complex represented by B^-.

$$R_3N_{org} + H^+_{aq} + A^-_{aq} \rightleftharpoons R_3NH^+A^-_{org} \text{ (extraction)}$$

$$R_3NH^+A^-_{org} + B^-_{aq} \rightleftharpoons R_3NH^+B^-_{org} + A^-_{aq} \text{ (anion extraction)}$$
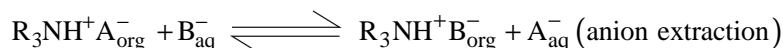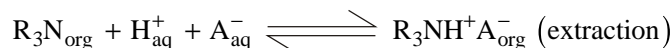
The net result is that the liquid tertiary amine has acted as anion exchanger between the anions A^- and B^-. Therefore, the extracting amine can also be designated as a liquid anion-exchanger.

Anionic metal complexes are best extracted by tertiary amines in the presence of mineral acids, whereas good extractions are obtained with primary and secondary amines using carboxylic acids. A typical example of the application of this separatory technique is the extraction of Fe^{3+}, and it is represented by the following sequence of chemical equilibria:

$$Fe^{3+}_{aq} + 4Cl^-_{aq} \rightleftharpoons FeCl^-_{4\,aq}$$

$$R_3N_{org} + H^+_{aq} + Cl^-_{aq} \rightleftharpoons R_3NH^+Cl^-_{org}$$

$$R_3NH^+Cl^-_{org} + FeCl^-_{4\,aq} \rightleftharpoons R_3NH^+FeCl^-_{4\,org} + Cl^-_{aq}$$
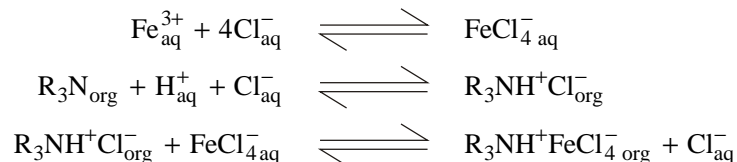
Extraction of Sc and that of U are two important examples of liquid-liquid extraction by ion-pair formation brought about in the presence of a mineral acid using a tertiary amine of high molecular weight, nemely, trioctylamine.

Selectivity of separation in liquid-liquid extraction by ion-pair formation is achieved by making the required variations in parameters like pH used for complexation, the complexing ligand, and concentration of the extractant effected by mixing with differing amounts of a suitable diluent which aids extraction. Most difficult separations, for example, those of acids like malonic, ascorbic, tartaric and citric in Organic Chemistry have been achieved by this separatory technique.

In the examples considered above, use has been made of liquid anion-exchangers for extraction by ion-pair formation. Use can also be made of liquid cation-exchangers for extraction by ion-pair formation and the mechanism of such an extraction is represented below.

$$M^{n+} + 2n(HX)_{org} \rightleftharpoons M(HX_2)_{n\,org} + nH^+$$

$$M(HX_2)_{n\,org} + nCaCl_{2aq} \rightleftharpoons [M(Ca\,X_2)_n]Cl_{n\,org} + nH^+_{aq} + nCl^-_{aq}$$
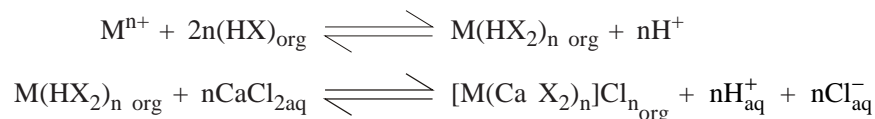
Synergic extraction is still another class of liquid-liquid extractions. Efficiency of extraction is enhanced in this technique by using two solvents for extraction, either solvent aiding the extraction by the other and hence the technique being named as synergic extraction. One of the extractants may be a chelating agent and the other one a solvating solvent or both may be chelating agents, or bothe may be solvating agents. An example of synergic extraction is the separation of U using tributyl phosphate along with 2-thenoyl trifluoroacetone. Although either of these

two solvents can extract U if used alone, a mixture of these two solvents acts as a more efficient extractant.

2.5.3 Applications of Solvent Extraction

The technique possesses a great advantage in that it involves a simple procedure and employs simple apparatus.

Solvent extraction is widely used for the isolation of single chemical species prior to a determination and the most important application of the technique is in the determination of metals present as minor and trace constituents in a variety of inorganic and organic materials. Thus the analysis of metallurgical and geological samples as well as petroleum products, foodstuffs, plant and animal tissue, and body fluids, in regard to their respective content of metals is carried out by selective solvent extraction and spectrometric determination of the metals as coloured complexes.

Solvent extraction is also used as a method of separating or concentrating of purely organic species. This separation method, however, does not possess the same degree of selectivity for organic species as for systems involving metals because of the general lack of suitable complexing and masking reactions. Consequently, large quantities of organic solvents are sometimes required for the isolation of a sufficiently pure organic compound from the starting material. The later-day technique of *counter-current distribution* is an improvement on the batch process of solvent extraction in that it is an automated one. Counter-current distribution has played an enormous role in the isolation and characterization of organic substances from complex natural mixtures of plant or animal origin, and this technique is discussed in a subsequent chapter.

2.6 Distillation

The process of converting a liquid into its vapour and subsequently collecting it as liquid by condensation of the vapour is known as distillation.

This operation is employed to separate a liquid from non-volatile impurities or to separate the various constituents of a mixture of liquids boiling at different temperatures. Amongst the numerous industrial applications of distillation, an interesting example is that of purification of metals like zinc, cadmium, mercury and bismuth which have comparatively low melting and boiling points and leave behind impurities in the rotorts when subjected to distillation.

The practical technique of distillation to be employed in a particular case depends upon the nature of the constituents of the mixture. Since boiling is the key step involved in any mode of distillation, it is essential to understand the 'Phenomenon of Boiling' for a proper appreciation of the details concerning the various laboratory techniques of distillation, and hence follows discussion of this phenomenon.

Constant evaporation takes place from a liquid surface except when the space above it is saturated with vapour. When heat is supplied to the liquid, part of it is used to evaporate the liquid. When the amount of heat supplied exceeds the amount utilized by the evaporation process, the temperature of the liquid rises. When the temperature of a liquid reaches the point where its vapour pressure is slightly above the pressure that is applied to the surface of the liquid, vapour formation can take place in the body of the liquid. The resulting disturbance is termed boiling. Even though the space above the boiling liquid is saturated by vapour, vaporization continues since vapour actually is forced out of the liquid into the space above, because the vapour pressure of the liquid exceeds the applied pressure. The temperature at which the vapour pressure is just equal to the applied pressure is the boiling temperature. The boiling temperature at 1 atmosphere pressure is called boiling point.

Superheating is necessary before boiling can take place at atmospheric pressure, as for example in an open vessel, because the vapour pressure of the liquid must exceed atmospheric pressure by a pressure equal to the weight of the liquid above the point of bubble formation. In addition another and much more important pressure which must be overcome is the surface tension of the liquid. The tendency of a bubble to collapse, resulting form the surface tension of the liquid, must be balanced by the pressure within the bubble. The smaller the bubble, the greater must be the pressure within the bubble to prevent collapse. At zero radius, the pressure becomes infinite. Conversely, the vapour pressure would have to be infinite in order for a bubble to start from zero radius. Actually, however, there usually are flaws in the glass and particles of dust or bubbles of air in the liquid. These serve as nuclei of finite radius for bubbles of vapour to form. Nevertheless, under ordinary conditions of boiling, the temperature of the liquid is usually a few degrees above the boiling point. If a liquid is carefully freed of air by boiling and then cooling, it is possible to raise the temperature considerably above the boiling point. Water has been raised to 137ºC, before it boiled. Of course, once the bubble forms it rapidly increases in size because of the large vapour pressure of the liquid. Often in the case of superheating, the bubble expansion takes place with explosive violence. This is called bumping. Bumping may lead to a loss of product, the contamination of the pure distillate or sometimes shattering of the container and injury to the experimenter. Bumping is avoided by employing remedies exemplified below.

In no case should a liquid be boiled without the addition of a boiling aid. Bumping is stopped by adding to the liquid a source of bubble nuclei, such as a boiling chip, a platinum wire, or an ebullition tube. Boiling chips are most commonly used. They are small chips of clean hard porous plate or tile about 2 to 5 mm in diameter. The small pores induce smooth boiling. Platinum wire acts as a porous material supposedly because of dissolved gas.

An ebullition tube is one of the best devices to aid boiling. It can be made easily, it does not contaminate the liquid and it can be used repeatedly. This is a glass tube about 3 mm in diameter, sealed about 5mm from the bottom, so that there is an open cup about 5 mm long. The tube should be long enough to reach well into the neck of the vessel so that it remains vertical. When placed in the liquid the cup on the bottom serves as a chamber into which the liquid may vaporize.

Bumping may be minimized by supplying heat through a small temperature gradient and over a large surface, as for example by the use of a liquid bath in which a flask is immersed.

Detailed below are the various distillation techniques employed in the chemistry laboratory.

2.6.1 Simple Distillation

The given liquid may be purified by simple distillation if it does not undergo decomposition before its boiling point is reached at atmospheric pressure. The non-volatile impurities as well as higher boiling impurities will be left behind in the distillation flask; impurities which are more volatile than the compound being purified will distil over earlier and can be removed before the desired compound begins to distil over.

Distillation at atmospheric pressure is carried out using the set-up of apparatus shown in Figure 2.13(a), which consists of distilla-tion flask (F), a water condenser
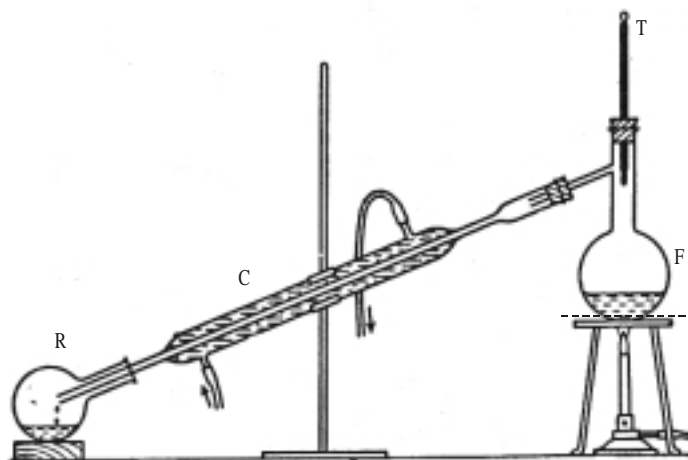
Figure 2.13 (a) Distillation assembly

(C) and a receiver (R). All distilla-tions should include the addition of a few chips of a broken porous plate to prevent bumping. The vapour of the substance, which is caused to boil at a regular rate, is condensed during its passage through a
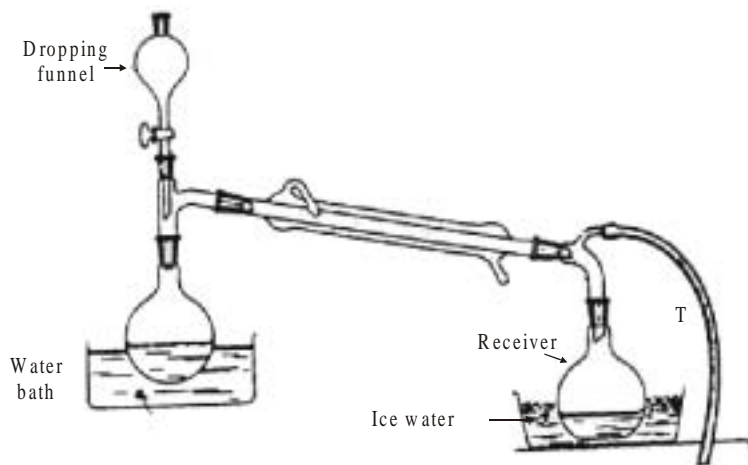
Figure 2.13 (b) Ground-glass-joint distillation assembly

condenser tube, surrounded by a jacket through which cold water circulates. The thermometer (T) is held with its bulb above the liquid at a level slightly lower than that at which the side tube is fused in the neck of the distillation flask. The thermometer with the bulb being surrounded by the vapours of the boiling

(a)   (b)   (c)   (d)   (e)   (f)
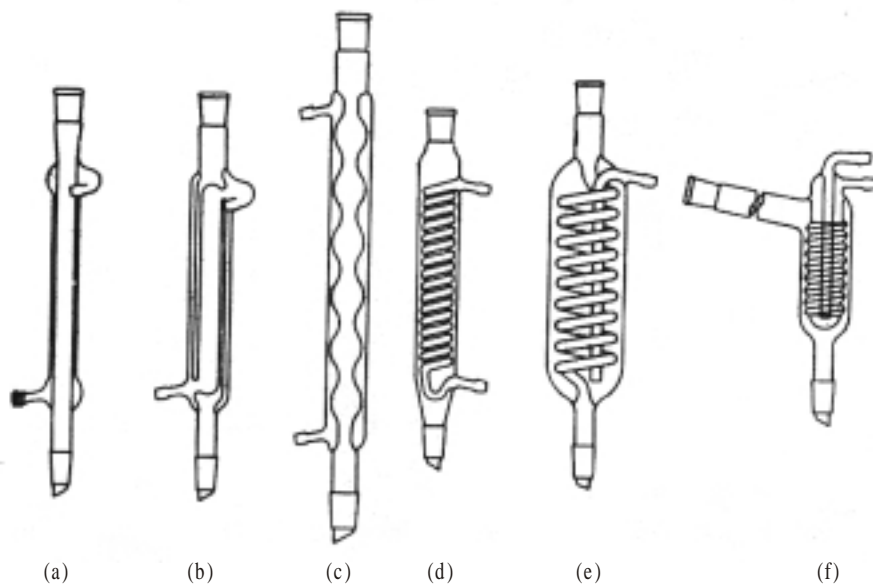
Figure 2.14 Water-cooled glass condensers

(a) Liebig   (d) Graham
(b) Davies double surface   (e) Thrope inland revenue pattern
(c) Allihn   (f) Friedrich

substance immediately before the vapours are carried over into the condenser indicates the boiling range of the substance.

If the hot vapours entering the condenser are above 140ºC an air condenser should be used. Above this temperature, a water condenser is liable to crack due to the temperature gradient prevailing across the glass (cold water on one side, hot vapours on the other). A water-bath or steam-bath is usually employed as the source of heat in distilling inflammable liquids boiling appreciably below 100ºC; for less volatile liquids an oil-bath or electrically heated isomantle may be used. Very volatile liquids such as ether should be collected in a receiver surrounded by an ice-bath to ensure maximum possible condensation; the fumes should also be directed to some safe extraction system. Care must be taken not to fit the apparatus without an outlet system for the uncondensed vapours—the air in the apparatus together with the vapour will expand during the distillation and may blow the equipment apart as the pressure builds up. Figure 2.13(b) shows a ground-glass-joint assembly used for distilling very volatile liquids like ether. The rubber tube T extends below bench.

Various devices have been adopted with the object of increasing the cooling surface of the condenser, and thus reducing its length. The commonly used condensers are shown in Figure 2.14. Figure 14(a) is a jacketed tube or Liebig type condenser. Figure 14(b) is the double surface condenser and has an obvious advantage that it may be half the length of the Liebig condenser for the same effective condensing surface area. Figure 14(c) is a bulbed or Allihn condenser; 14(d) is a coiled condenser (Graham), the coil taking the cooling fluid; there are many designs of this type, some with two or more integral coils for increased efficiencyl. In 14(e) the coil takes the distillate. 14(f) is the Friedrich condenser which has a side entry for the vapours.

2.6.2 Fractional Distillation (Rectification)

If a binary mixture of volatile substances contains a relatively large amount of each constituent, or if the two compounds have very similar vapour pressure characteristics, then a simple distillation is not a very efficient means of separating the two substances. It then becomes necessary to resort to the technique of fractional distillation, which is the process of collecting the distillate in aeveral fractions and subjecting the fractions to systematic redistillation.

The discussion which follows will bring out clearly how this technique operates to effect the separation of a mixture into its constituents. Separation of a mixture of benzene and toluene into its constituents has been chosen as the example for this discussion.

2.6.2.1 Theory underlying Fractional Distillation

At its boiling point, any mixture of benzene and toluene is in equilibrium with a vapour phase of a definite composition. From data of a number of mixtures, two

temperature-composition curves have been drawn, one for the liquid and the other one for the vapour phase, as shown in Figure 2.15. Points a and b are the points that have been calculated for 90ºC. When the composition of the liquid is 58 mole per cent benzene and 42 mole per cent toluene, the total vapour pressure at 90ºC is 760 mm, that is, the boiling point of a solution of this composition is 90ºC. This is the point a in Figure 2.15.

According to Raoult's law partial vapour pressures of benzene and toluene are proportional to their respective mole fractions in the vapour mixture, that is,

$$\frac{P_{C_6H_6}}{P_{C_7H_8}} = \frac{Y_{C_6H_6}}{Y_{C_7H_8}},$$

where $P_{C_6H_6}$ and $P_{C_7H_8}$ are respectively the partial pressures of benzene and toluene and $Y_{C_6H_6}$ and $Y_{C_7H_8}$ are respectively the mole fractions of benzene and toluene in the vapour phase at the given temperature.

At 90ºC the partial vapour pressures of benzene and toluene are 580 mm and 180 mm respectively.

Thus $\dfrac{Y_{C_6H_6}}{Y_{C_7H_8}} = \dfrac{580}{180} \approx 3.22$,

so that the vapour mixture consists of $\approx \left(\dfrac{3.22}{4.22} \times 100 \right)$ or 77 mole per cent benzene and $\approx \left(\dfrac{1}{4.22} \times 100 \right)$ or 23 mole per cent of toluene. This is the composition of the

vapour phase that is in equilibrium with the liquid containing 58 mole per cent of benzene and 42 mole per cent of toluene. From this it is evident that the vapour is richer in the more volatile component, in this case benzene, than is the liquid. If a mixture of benzene and toluene is distilled from a simple distilling flask, the distillate will have the composition of the vapour in equilibrium with the liquid in the distillation flask. Thus, for the mixture containing 58 mole per cent benzene and boiling at 90ºC, the very first portion of the distillate will contain 77 mole per cent benzene. The composition of the liquid in the distillation flask becomes progressively richer in toluene
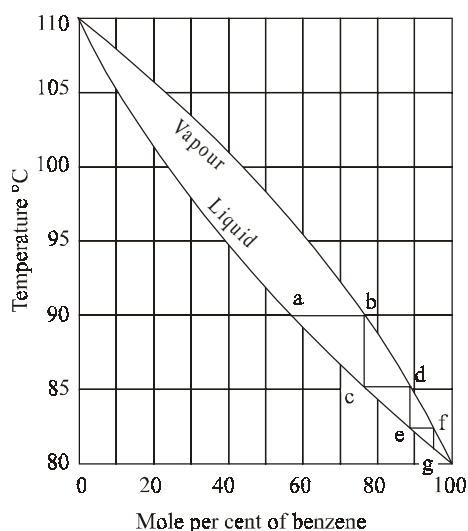

Figure 2.15 Boiling point-Composition curves of mixtures of benzene and toluene at 760 mm

as the distillation proceeds owing to the fact that the distillate is richer in benzene at all times relative to the liquid. Thus the composition of the very last portion of the distillate approaches that of pure toluene.

A very small amount of liquid approaching the composition of pure benzene can be obtained by a succession of distillations in the following way. When a small amount of the mixture containing 58 mole per cent of benzene is distilled, the vapour contains 77 mole per cent (point b of Figure 2.15). This can be condensed to a liquid and when it is heated, it begins to boil at 85ºC as shown by point c. The first vapour that comes over has the composition indicated by d, namely, 88 mole per cent benzene. This first small amount can in turn be condensed to a liquid, and when this is heated, boiling begins at e, about 83ºC, and the vapour has the composition corresponding to f, about 95 mole per cent benzene. The operation can be continued until essentially pure benzene is obtained.

2.6.2.2 Fractionating Columns

Separation of a mixture which contains two or more volatile liquids into its constituents can be effected in one step by using fractionating columns, or still-heads.

A fractionating column or still-head is an apparatus that increases the degree of separation when a mixture of volatile components is distilled. The following discussion will enable one to understand how a fractionating column achieves this increased degree of separation.

There are various types of fractionating columns. The 'bubble-plate' column shown in Figure 2.16 is chosen to elucidate the principle underlying the functioning of fractionating columns. To simplify the discussion, it may be assumed that the column is empty, that it is cold and that it is completely insulated. At the start of the distillation, liquid condenses on the plates, since the apparatus is cold. If the original composition is 58 mole per cent benzene, the composition at the first plate is 77% (c, Figure 2.15); that at the second plate is indicated by e (Figure 2.15); and so on. There is a drop in temperature between one plate and the next higher one. Thus the column acts like a serial bank of distilling flasks giving a theoretical separation equivalent to that achieved by as many distillations from distillation flasks as there are plates in the column. If there are enough plates the composition of the first vapour that issues from the top of the column approaches that of pure benzene.

Figure 2.16
Section of 'bubble plate' column.

As the distillation proceeds, vapour ascending from the boiler bubbles through cooler liquid on the bottom-plate. Establishment of equilibrium between the vapour and the liquid at the plate results in the condensation of a part of the

higher-boiling component of the vapour, and the vaporization of a part of the lower-boiling component of the liquid. This causes the liquid to become richer in the higher boiling component and the vapour to become richer in the lower boiling component. At the same time the temperature of the liquid on the plate rises, for the boiling point rises as the proportion of the high-boiling component increases. This heating effect leads to a gradual decrease in the efficiency of the column, for in time all the plates reach the temperature corresponding to a (Figure 2.15). Then the composition of the vapour leaving the column is that indicated by b (Figure 2.15).

The efficiency of the column is greatly enhanced by returning some of the final product in the form of liquid to the top of the column, and permitting all but a small proportion of liquid at the plates to flow down to the plate below. The easiest way to return liquid to the top of the column is to use a reflux condenser at the top of the column. This causes a general flow of liquid down the column, and an enrichment at the plates in the lower-boiling component. It is thus possible to maintain the composition of the liquid on the lowest plate at the point c (Figure 2.15) so that the vapour, if complete equilibrium is assumed, issues with the composition of d (Figure 2.15). The other plates perform similarly. Then there is a temperature gradient up the column, which coninues to function as a succession of distillations from the distilling flasks.

The ordinary laboratory fractionating columns do not hold liquid on plates, as does the bubble plate column, but provide extra surface on which vapour may come in an intimate and prolonged contact with the liquid. The net effect is the occurrence of successive distillations on these large exposed surfaces on account of absorption of heat by the condensed liquid from the rising vapours, a greater amount of the more volatile substance being vaporised at each successive stage at the expense of the component with the higher boiling point.

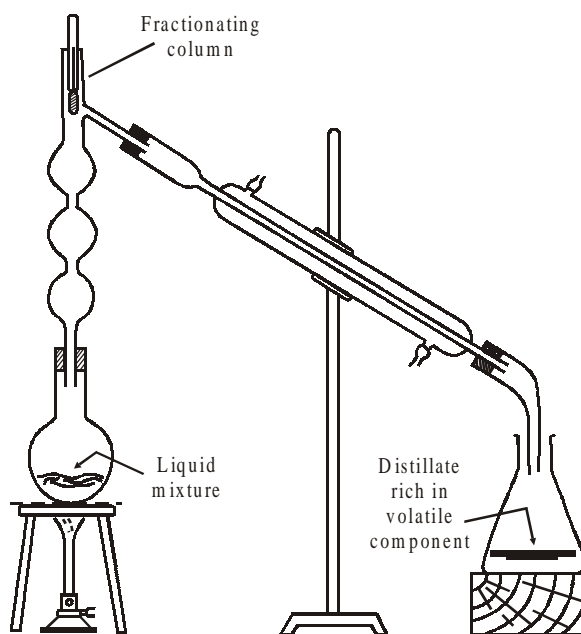In theory, the vapours emerging from the top of the column should consist eventually of the compo-

Figure 2.17 Fractional distillation

Labels in figure: Fractionating column; Liquid mixture; Distillate rich in volatile component

nent with the lower boiling point, namely, benzene in the example cited. As the concentration of benzene in the mixture being distilled decreases, however, a point is reached at which the vapour arriving at the top of the column does contain both benzene and toluene. This point is easily detected, because while pure benzene is distilling out of the column the boiling point of benzene will register on the thermometer. As soon as a mixture begins to emerge from the column, the temperature will rise. Very shortly thereafter, the residual benzene will be completely exhausted and pure toluene will distil. A separation can thus be effected by changing receivers at the right moments. Figure 2.17 shows the set-up of the apparatus for fractional distillation.

2.6.2.3 Column Efficiency

The heat exchange process within the fractionating column should, ideally, bring about complete separation of the substances in the mixture, but the degree of success that actually is achieved depends on a number of factors. Before discussing the factors that contribute to the effective functioning of a column, the terms "number of theoretical plates" and "height equivalent to a theoretical plate" (HETP) which are conventionally used to convey information on the efficiency of a column, need to be defined.

The word 'plate' is derived from 'bubble plate' column which had been chosen above to illustrate the principle underlying the functioning of fractionating columns. A *theoretical plate* is defined as one on which a perfect simple distillation takes place, resulting in an enrichment of the vapour phase identical to that which theoretically should occur according to the appropriate phase diagram like the one shown in Figure 2.15 for the benzene-toluene system. A column with 12 theoretical plates is thus capable of achieving the degree of separation that would be realised if 12 simple distillations were carried out. The HEPT is calculated by dividing the effective length of the column by the number of theoretical plates, though such a calculation need rarely be done unless special requirement for the same exists.

The factors on which the efficiency of a fractionating column in achieving separations depends are: (i) Amount of contact between vapour and liquid; (ii) amount of reflux, and (iii) rate of distillation.

(i) The amount of contact between vapour and liquid is determined by the length of the column, the diameter of the column, and the type of packing. In general the longer and narrower the tube, the more effective it will be. But compromises must be made in the interests of practicality in the ultimate design. In order to achieve a more intimate and prolonged contact between vapour and liquid during their passage through the column, the available exposed surface area within the column of a given length is increased in one of several ways shown in Figure 2.18 (a) e.g. by providing glass bulbs (A), glass balls (B) or glass plates

<center>(A)   (B)   (C)   (D)   (E)</center>
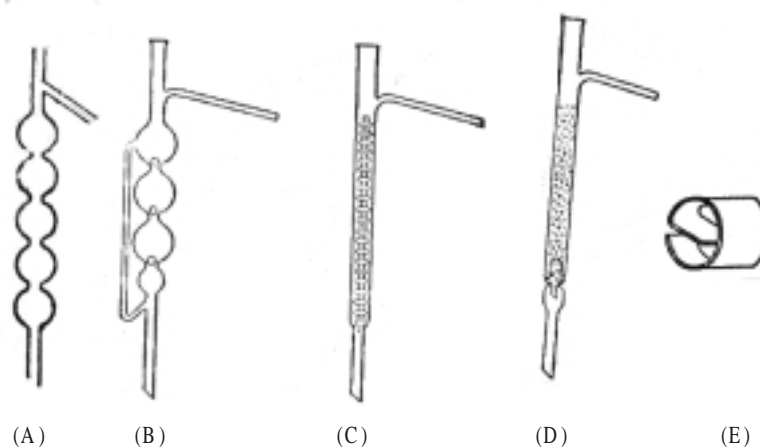
Figure 2.18 (a)

(C), or more satisfactorily by various types of packing such as glass beads (D) or Lessing's contact rings (E). The 'fractional distillation curves' obtained by plotting percentage distilled against temperature summarise the results obtained by distilling a 1:1 mixture of benzene (b.p. 80°) and toluene (b.p. 111°) from: (I) An ordinary distilling flask with side tube, (II) a Hampel still-head (D) [Figure 2.18 (a)] with glass beads, and (III) still-head packed with Lessing's contact rings (E) [Figure 2.18 (a)]. A particularly sharp separation into two fractions is noticeable in the last instance.
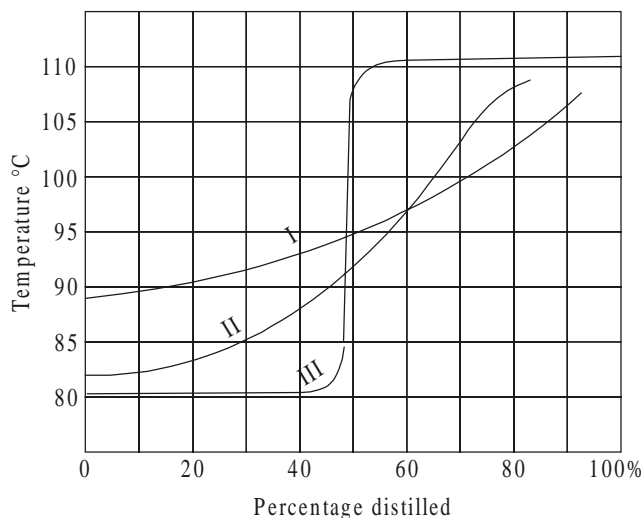
Figure 2.18 (b)
Fractional distillation curves

It should be apparent that those packings that present the greatest amount of surface area (and thus are most efficient) accumulate and hold more liquid than do less-efficient packings. At the conclusion of the distillation, therefore, a certain amount of the original sample is lost because of this hold up. A remedy applied to overcome this drawback is the delibrate addition to the original mixture of another liquid with a very high boiling point (considerably above that of the highest boiling component) as a consequence of which complete

distillation of all the components of the given mixture will be forced through the column. In the end the 'chaser' i.e. the outside liquid that had been deliberately added, will be left in the column as the holdup.

(ii) As already discussed, one requirement of the fractionation process is that there be counter-flow of condensate against the rising vapour. The term 'reflux ratio' is used to designate the relative quantities of liquid returned and taken off. Appropriate reflux ratio is required to be maintained for conducting fractionation process efficiently. Usually, some means need to be provided for controlling the amount of liquid that is taken off and the amount returned to the column to attain the needed reflux ratio for the particular fractionation being conducted. Installation of a reflux condenser at the top of the column is one such means. This aid increases the quantity of the liquid flowing downward and reference to it has already been made in connection with the working of the 'bubble-plate' column. Also, some means of insulating the fractionating column is usually provided to prevent excessive condensation of the rising vapour by loss of heat to the outside air, in order to maintain the proper reflux ratio for the efficient working of the column. A relatively simple way to minimise this loss of heat is to insulate the column with layers of aluminium foil. Also columns with evacuated jackets (much like Dewar flask), or columns with electrical heating jackets which compensate for heat lost by radiation or conduction from the column, are available.

Fractional distillations in the chemistry laboratory are often carried out using ordinary fractionating columns without incorporation of the reflux condenser as they generally attain the proper reflux ratio on account of condensation along the column resulting from heat losses to the outside through the sides of the column.

(iii) The rate of distillation should be slow enough to allow sufficient time for equilibrium to be established between vapour and liquid. The rate of heating must be such that a temperature gradient (higher temperature at the bottom of the column, lower temperature at the top) is maintained. However, heat input must not be restricted so much that the liquid boils in the distillation flask but no distillate emerges from the top of the column. The amount of heat supplied must, in fact, be increased gradually as the distillation progresses, because the boiling temperature of the residual liquid in the distillation flask increases as the more volatile components are removed.

When mixture comprises of members which individually are all gases ordinarily, separation of its constituents can be brought about by liquefying the gaseous mixture and subjecting the resulting liquid mixture to *fractional evaporation*. This process involves raising the temperature of the liquefied mixture in a controlled manner so that the individual components of the mixture are recovered as gases at their respective boiling points. Application of the method can be exemplified by the recovery of the individual gases from the liquid mixture consisting of oxygen, kryton and xenon, which is one of the fractions obtained in the first instance in the

Claude process for the fractionation of liquefied air. From this fraction, oxygen (B.P.–182.9), kryton (B.P.–151.7) and xenon (B.P.–106.9) can be easily separated from one another by fractional evaporation and collected as gases, because their boiling points are sufficiently far apart.

2.6.2.4 Constant Boiling Mixtures

The case of benzene-toluene mixture discussed above is an example of a solution which follows Raoult's law sufficiently closely. Solutions which obey Raoult's law are called ideal or perfect solutions. According to this law, the total vapour pressure, P, of an ideal solution gets expressed by the relation:

$$P = X_1 . P_1 + X_2 . P_2$$

where P_1 and P_2 are the vapour pressures of components 1 and 2, and X_1 and X_2 are the mole fractions of the respective components in the solution. Relatively few solutions are ideal or perfect solutions. Solutions which do not obey Raoult's law are called imperfect or non-ideal solutions. A non-ideal solution can belong to one or the other of the two following categories:

(1) When a solution displays total vapour pressure greater than that predicted by Raoult's law, it is said to show a *positive deviation*. A water-ethanol solution shows such a deviation. The molecules of each substance in such mixture seem to prefer their own kind and have an abnormally large tendency to escape from a mixture. The solution thus has an abnormally low boiling point.

(2) If the vapour pressure of a mixture is lower than that predicted by Raoult's law, the solution has *negative deviation*. Acetone-chloroform system shows such deviation. This type of mixture exhibits an abnormally high boiling point. This behaviour is to be expected whenever the two components react in some way with each other (formation of loose molecular complexes).

The process of fractional distillation is complicated in the case of solutions that show deviations from Raoult's law. A complete separation into components by fractional distillation of such a solution is not possible because of the formation of *a constant boiling mixture or an azeotrope*. For example, solutions of alcohol in water yield a constant boiling mixture (95.6% of alcohol and 4.4% of water) having the maximum vapour pressure and the minimum boiling point of all the possible alcohol-water mixtures; this mixture consequently distils first (78.13ºC/ 760 mm). Certain other mixed liquids yield constant boiling mixtures having the minimum vapour pressure, and hence the maximum boiling point; here the constant boiling mixture forms the last fraction to pass over. Thus mixtures of acetone and chloroform containing less than 36 mole per cent acetone yield pure chloroform and the constant boiling mixture on fractionation, and mixtures containing more than 36 mole per cent yield pure acetone and the constant boiling mixture.

2.6.3 Vacuum Distillation

If a liquid boils at such a high temperature at atmospheric pressure that it undergoes partial decomposition, its distillation is done at a reduced pressure. Use
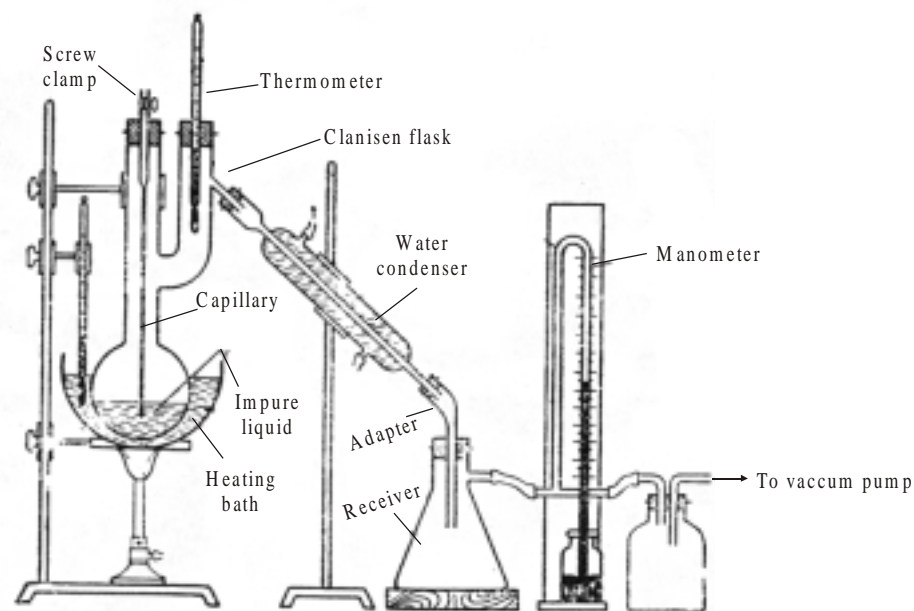
(a)
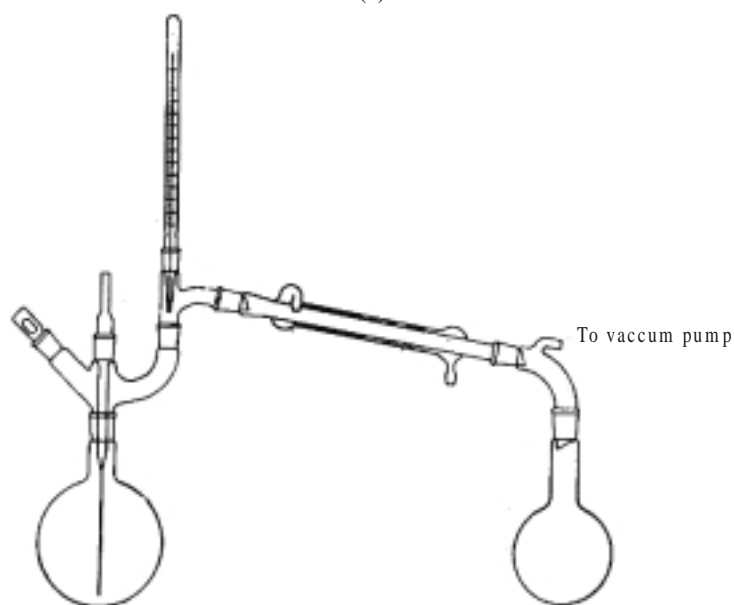
(b)

Figure 2.19 Distillation under reduced pressure

is made of a water pump (which gives a residual pressure of 10-20 mm of mercury) or of an oil pump (which may give a residual pressure as low as tenths or hundredths of a fraction of a millimeter of a mercury column) to get the necessary reduced pressure in the system in which the distillation is carried out. Distillation in vacuum is applied to substances that boil above 200ºC at atmospheric pressure. At a residual pressure of 20 mm of mercury the boiling point decreases by about 100ºC. High-vacuum distillation is used to separate even higher-boiling compounds (300ºC and upwards). At a residual pressure of 0.02 – 0.05 mm of mercury the boiling temperature of such compounds is reduced by about 200ºC.

The fit-up of the laboratory apparatus used in vacuum distillation is shown in Figure 2.19(a). The impure liquid is taken in a Claisen flask. Into one neck of the flask is inserted a capillary so that it nearly reaches the bottom, the other end of the capillary being fitted with a length of a thick-walled rubber tube gripped by a screw clamp. A current or air is carefully supplied through the capillary to attain a uniform boiling of the liquid during the distillation. Into the second neck of the flask is fitted a thermometer in such a way that its bulb is just below the point where the vapour enters the side tube and is led to the water condenser on heating the liquid. At the lower end of the condenser is attached an adapter connected to the receiver. The air is sucked off from the system by a water pump or an oil pump.

Figure 2.19(b) shows a ground-glass-joint assembly used for vacuum distillation.

2.6.4 Fractional Distillation at Reduced Pressure

Usually better separation of two substances by fractional distillation may be achieved at a lower pressure. This is exemplified in the separation of *n*-octane and bromobenzene. At 126ºC which is the boiling temperature of *n*-octane at 760 mm, the vapour pressure of *n*-octane is 2.3 times the vapour pressure of bromobenzene, whereas at 32ºC the vapour pressure of *n*-octane is 3.3 times that of bromobenzene. This greater spread of the vapour pressure at lower pressure (which is implied in the lowering of B.P. from 126ºC to 32ºC) results in an improved separation of the given mixture of *n*-octane and bromobenzene into its constituents.

Figure 2.20
Receivers for making cuts in vaccum distillation

Vacuum fractionation may be useful also in the separation of components of azeotropic mixtures, for the composition of the azeotropic mixture will change with pressure, and a better separation may be possible at reduced pressure. Thus, absolute alcohol may be obtained from 96% alcohol by first fractionating at pressures below atmospheric, followed by fractionating at atmospheric pressure, because the azeotropic mixtures at lower pressures are richer in alcohol.

It is convenient to use a modification in the apparatus that permits fractions to be taken without the necessity of breaking the vacuum. The method is to evacuate a system of more than one receiver fitted to a special adapter which provides a means of directing the distillate into the receivers as shown in Figure 2.20. These receivers are successively employed by rotating them into the receiving position.

2.6.5 Molecular Distillation (Ideal Distillation)

This type of distillation is employed to separate substances that decompose at the boiling temperature even in high vacuum. The principle involved in molecular distillation is: Under high vacuum (10^{-5} to 10^{-8} mm Hg) at temperatures ranging from 50ºC to 300ºC the molecules break away from the heated surface of the molten substance being subjected to distillation and pass, without occurrence of boiling, to the gas phase.

In a conventional distillation carried out at atmospheric pressure or even under reduced pressure, molecules that leave a liquid surface as vapour travel in straight paths during their movement towards a condensing surface until they collide with a molecule of the residual gas (usually air), with another vapour molecule, or with the surface of the distilling vessel. These collisions either return the vapour to the liquid or greatly slow down its rate of travel from the liquid to the condenser. In molecular distillation the residual gas is virtually removed from the distillation vessel (for example, evacuated to pressures approximating 0.001 mm Hg), and in this situation the vapour molecules can travel a path without collision, that is the mean free path characteristic of the molecules concerned, which is so great that if a cooled condensing surface is placed at a distance smaller than the mean free path of the molecules the gas will be condensed on this surface. Since evaporation is slow at lower temperatures, the liquid temperature is usually

(a)

A simple pot molecular still

(b)
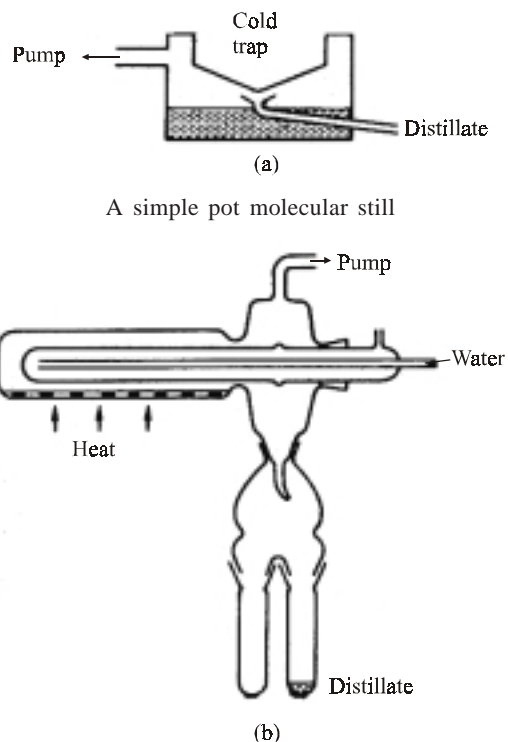
Figure 2.21 An improved molecular still with fraction collector

raised until the rate of evaporation meets practical requirement. In this way, substances with relatively high molecular weight (400 to 1200) and having structures which are unstable at higher temperatures, can be purified effectively, since molecular distillation can distil these compounds at temperatures 50–150ºC lower than those needed by other means. Also an extra advantage is the elimination of any tendency toward oxidation during distillation, since there is no atmospheric oxygen present.

Two of the several molecular stills in use are shown in Figure 2.21. Figure 2.21(a) shows a simple pot still. An improved design shown in Figure 2.21(b), can successfully carry out fractionation of a mixture into its components. In this still, the horizontal boiler is heated by resistance coil wound on a glass tube. The cold finger type condenser slopes so that the condensate runs back and drips off a rim into a fractionating adaptor to the receiver. The boiler holds approximately 100g of material which is inserted by removing the condenser and turning the boiler to the vertical. It is placed under vacuum in this position and gently heated to remove trapped gases. This is followed by release of vacuum, the condenser being replaced and the whole returned to the horizontal position for the distillation. A high vacuum is obtained by using a vapour diffusion pump.

Molecular distillation is widely used in the laboratory for analytical work, as well as in the chemical industry for improvement of flavours and fragrance, pharmaceuticals and edible products e.g. distilling vitamin A esters from dog-fish liver and shark liver oils.

2.6.6 Steam Distillation

If a mixture of two immiscible liquids is distilled, the composition of the vapour does not depend at all on the composition of the mixture. Each component exerts the same vapour pressure that it has when alone, and the boiling temperature is below that of the lower-boiling substance.

In the distillate the two components are obtained in the ratio:

$$\frac{\text{No. of moles of component A}}{\text{No. of moles of component B}} = \frac{\text{Vapour pressure of A at the distilling temp.}}{\text{Vapour pressure of B at the distilling temp.}}$$

Steam distillation is a particular case of this type of distillation in which one of the two components of the mixture of immiscible liquids is always water.

When a volatile substance is distilled with water, the substance is said to be steam-distilled if water comes over in the distillate. Steam distillation is not feasible if the substance reacts rapidly with water. It is especially useful under one or more of the following conditions: (1) When the presence of solid matter makes ordinary distillation, filtration and extraction impractical as, for example, in the preparation of aniline; (2) when the presence of tarry matter makes extraction with an organic solvent messy and inconvenient, as in the case of preparation of phenol;
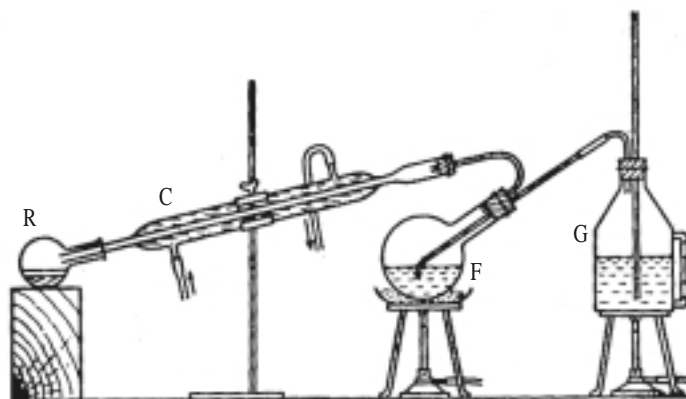
Figure 2.22 Steam distillation apparatus

(3) when the volatile product is a solid, for then the water in the inner tube of the condenser serves to keep the condenser free from clogging; (4) when the desired substance boils much above 100°C and decomposes at the boiling point; (5) when separation from a volatile impurity can be accom-plished better at reduced pressure, as the separation of bromobenzene from *p*-dibromo-benzene.

Steam disti-llation may in some cases take the place of vacuum distilla-tion, and has the advantage of greater convenience.

The fit-up of the laboratory apparatus used in steam distillation is shown in Figure 2.22. This consists of the steam generator, a tilted flask F, a long condenser C, a receiver R, and two burners. The generator must be provided with a safety tube leading to the bottom of the vessel. The exit tube should reach almost the bottom of F, so that the incoming steam will keep the contents well agitated, thus helping to maintain equilibrium between the phases. Tilting of flask F serves to decrease the splashing of liquid into the exit tube connected to the condenser and to keep that which has splashed from being carried over into the condenser. The flame from the burner below the flask need only be large enough to compensate for radiation losses. The condensate is collected in the receiver R. The distillate forms two layers and the organic substance is thus separated readily from the distinct aqueous layer.

The distillate is composed of the two components in molar proportions relative to their vapour pressures at the temperature of distillation in accordance with the mathematical expression given above. Though the vapour pressure of the substance being steam-distilled may be much smaller than that of water, it still may constitute an appreciable fraction of the distillate, since the molecular weight of the substance would be much greater than that of water. Thus, although the vapour pressure of water at 100°C is 42 times that of *p*-dibromobenzene which boils at 218°C, the weight of water in the distillate is only 3 times the weight of dibromobenzene because the molecular weight of the latter is 235.

2.7 Criteria of Purity

When a pure sample of a substance is available, its physical properties besides its chemical characteristics, can be employed as the standards by which another sample of that substance can be identified. Amongst the physical properties most often used to establish purity and identity of substances are the melting point, the boiling point and the refractive index. When a given sample is not pure, the progress of its purification can be followed by comparison of the value of one of its these properties with the value of the corresponding property of the pure compound before and after the application of purification procedures to the given sample.

2.7.1 Melting Point

The melting point of a substance is the temperature at which the liquid and solid phases are in equilibrium. A good way of experimental determination of the melting point of a solid is to place a small amount of the perfectly dry substance in a thin-walled capillary melting point tube attached to the bulb of a thermometer suspended in some suitable liquid and to raise the temperature of the liquid bath until the solid melts.

A few powdered crystals of the compound are placed in the capillary tube 10-15 cm long and about 1 mm in internal diameter, which has been sealed at one end. Obviously, to determine the temperature at which the substance is melting, the thermometer and the sample must be at the same temperature while the sample melts. This requires that the rate of heating of the bath be very low as the melting point is approached (about 1ºC per minute). Otherwise, the temperature of the mercury in the thermometer bulb and the temperature of the compound in the capillary will not be the same as the temperature of the bath liquid, and probably not equal to each other. This will be so because the transfer of heat energy by conduction takes place slowly.

The solid should be present tightly packed in the capillary to a depth of 2 – 3 mm. If the sample sublimes rapidly at the melting point it is necessary to seal the capillary before attempting to determine the melting point; in an unsealed tube the sample would sublime to the upper cooler part of the capillary which is not immersed in the bath. The capillary can be melted shut about 2 cm above the sample by briefly holding that part of the capillary in a small burner flame. The entire sealed portion should be immersed in the bath during heating. When an oil bath is used, the capillary can be fastened to the thermometer by means of a thin rubber band; if the capillary is straight, it may stick to the thermometer by the capillary action of the bath oil, without the help of a rubber band. Adjustment is made so that the sample of the substance present in the capillary at its bottom is opposite to the middle of the thermometer bulb. The thermometer is placed in the heating bath and heat applied. If a compound begins to decompose near the melting point, the capillary with the sample should be placed in the bath after the

temperature has been raised to within 5 or 10 degrees of the expected melting point so as to minimize the length of time for which the sample is heated.

If the approximate temperature at which the sample will melt is not known, a preliminary melting point determination should be made in which the temperature of the bath is raised quickly. Then the more accurate determination using a second capillary tube containing the sample should be carried out with a rate of heating of not more than 1°C per minute near the melting point.

The temperatures to be noted are: The point of first observable shrinkage, the point at which liquid begins to form, and the point at which the solid is completely

(a)   (b)
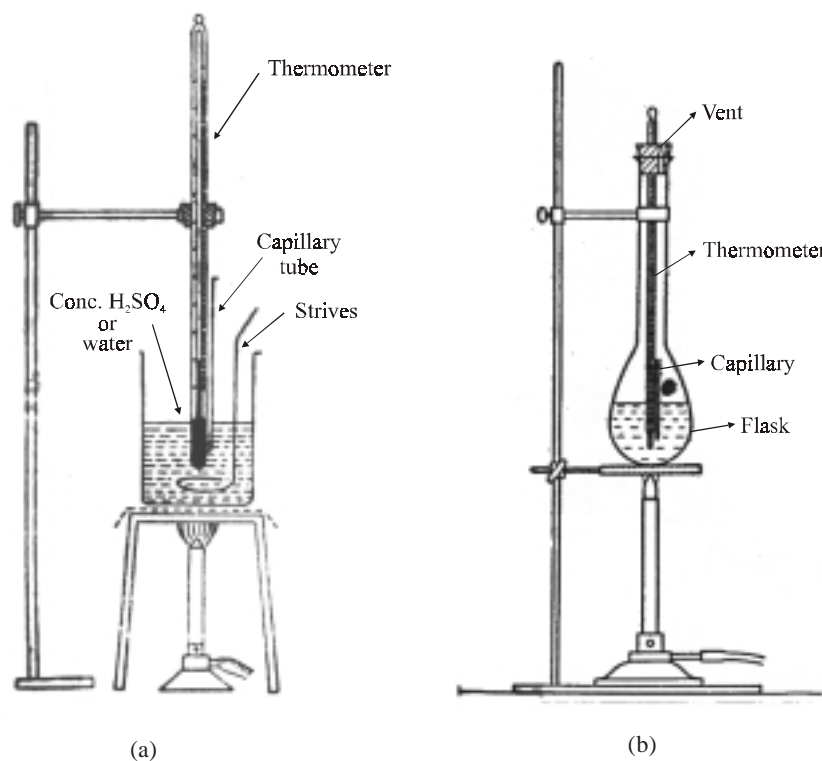
Figure 2.23 Hot baths for melting-point determination

liquefied. The first one is the softening point. The second and third points constitute the range of the melting point.

Several of the heating baths used for melting point determination are illustrated in Figure 2.23.

The simplest liquid bath [Figure 2.23(a)] consists of a beaker of liquid set on wire gauze above burner, with a thermometer suspended in the liquid. The beaker is filled one-half to two-thirds full with some suitable liquid which is stirred by an up-and-down motion of a circular stirrer.

The apparatus shown in Figure 2.23(b) consists of a long necked hard-glass flask to which a thermometer is fitted by means of a cork having a shallow vertical vent cut (as shown in the figure) to allow expansion of contents of the flask without pressure developing inside it.

In the Thiele apparatus illustrated in Figure 2.23(c), the burner is so positioned that hot liquid rises in the outer arm.

Liquids that may be used are high boiling and stable, such as cotton seed oil, paraffin oil, sulphuric acid and butyl phthalate.

In another method of determining the melting point, the solid sample is placed between thin glass plates on top of a copper block heated from below.

The success of any of these methods depends on having the thermometer record the temperature of the melting solid.

Melting points which are taken by visual methods give a range, rather than a point, because the values determined are based upon judgment of the observer who makes the decision as to which point within the observed range is the melting point.

The exact melting point can be deter-

Figure 2.23 (c)

mined automatically with instruments which use a beam of light to survey the process and a photocell to signal the instant at which the sample melts. Readout indicators give the measured value of the melting point. As the substance melts, the light transmission increases and resistance changes in the photocells are produced by the variations in the transmission. Some instruments permit the simultaneous determination of the melting point of three samples. *Mixed melting point* (the melting point of the mixture of the sample with the known compound) used in the identification of organic compounds can be measured simultaneously so that the melting point of the known and that of the mixture can be readily determined and compared.
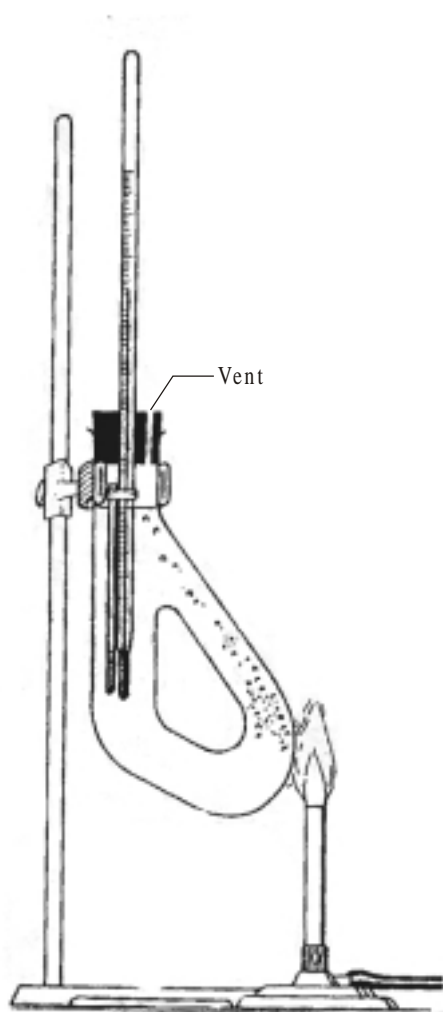
2.7.1.1 Deductions to be drawn from the Melting Point

The melting point is an important physical constant because it can be easily determined and serves as a criterion of purity of the given compound and is also a lot useful in establishing the identity of an unknown compound.

The best melting points ordinarily obtainable rarely have range of less than 0.5ºC, and a substance with a range of one or two degrees is usually regarded as pure enough for most purposes.

If a substance is impure, not only is the melting range broad, but the top of the range is below that of the true melting point. Thus the purification of a substance may be followed by the determination of the melting point, and, when this becomes sharp and reaches the correct temperature, the substance may be considered pure.

Also, the identity of a compound may be established by the use of the melting point, even if one is confronted with two or more substances having nearly identical melting points. Suppose a substance melting at 120ºC is isolated from a reaction. A study of its chemical properties and a search in the literature reveals several compounds melting at or very near 120ºC that might be identical with the unknown compound. To prove or disprove the identity, a sample of the unknown compound is mixed with a sample of the known compound and the melting point of the mixture is determined. If the compounds are the same, the mixture will melt at the same temperature as that at which each compound melts separately. If the compounds are different, the melting point of the mixture will be lower and have a broad range. This comparison of melting-point behaviour is most effectively accomplished by placing three melting-point tubes in the same heated bath. One tube contains the compound of known identity, another contains the compound of unknown identity, and the third contains the mixture of the two.

There can be a possibility that the substance is a eutectic mixture of two or more compounds. A eutectic mixture has a definite composition and melts 'sharply', that is, at a constant temperature, just as a pure compound does. The chance of a given mixture containing two or more compounds in just the proportion to give a sharp melting eutectic mixture is remote. All the same, to remove any doubt in this regard, mixed melting points should be determined for mixtures of more than one composition.

Identification by 'mixed melting points' is a valuable technique and it is used frequently by the chemist.
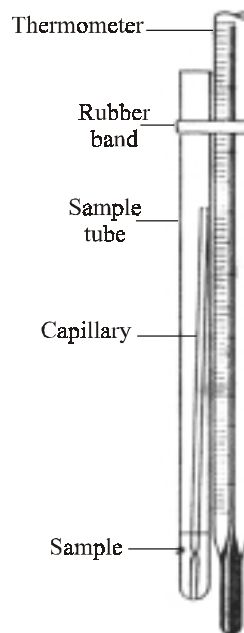
Figure 2.24
Apparatus for small-scale determination of boiling point

2.7.2 Boiling Point

The term 'boiling point' of a liquid has already been defined earlier and, if enough of pure liquid is available, its experimental determination is most easily made by carrying out a simple distillation [Figure 2.13(a)] during which the vapours of the boiling liquid come into thermal equilibrium with the thermometer bulb. The temperature of the vapour should be observed during the course of the distillation, and the temperature range over which most of the material distills should be taken as the boiling point.

Siwoloboff's Method

This technique that is quite well adapted to boiling point determination on a very small scale uses a capillary inverted in a thin-walled boiling tube. A few drops of the given liquid are placed into the boiling tube which is a 5-7 cm long glass tube having a diameter of about 5 mm and is sealed at the lower end. A length of melting point capillary, sealed about 5 mm above its lower end, is dropped inside the boiling tube. The boiling tube is thereafter fastened, to a thermometer employing a rubber band (Figure 2.24). Next, the thermometer is supported in a melting point bath (Figure 2.23), which is heated until a very rapid, steady stream of bubbles issues from the sealed capillary. The bath is then allowed to cool slowly, and the temperature at which a bubble just fails to come out of the capillary and the liquid starts to enter it is taken as the boiling point of the liquid.

If the barometric pressure is not 760 mm, the observed boiling point may be corrected to the temperature that would be expected at 760 mm. This correction amounts to about 0.5ºC for each 10 mm deviation of atmospheric pressure from 760 mm—the observed boiling point will be low if the atmospheric pressure is low.

The boiling points of very small amounts of liquid can be determined accurately and speedily with electric sensing equipment using photocells. The substance is put into a special boiling-point sample tube, which is designed to prevent superheating and achieve smooth and continuous boiling. The tube is illuminated from the bottom by dark field illumination, and as long as no bubbles are present (the compound is not boiling), no light passes through the liquid to reach the photocell sensor. When the boiling point is reached and bubbles begin to rise, the bubbles reflect light to the photocell. Light is reflected to the photocell with sufficient intensity and frequency to trigger the readout indicator when the true boiling point is reached. The initial outgassing bubbles (if any) do not actuate the sensor. Boiling points are determined with extreme accuracy to ± 0.3ºC.

2.7.2.1 Deductions to be drawn from the Boiling Point

A pure compound boils at a single fixed temperature—the boiling point. In case the identity of the liquid compound is known, its purity is established if the boiling

point of the liquid as determined by the Siwoloboff's method is in agreement with the literature value of the boiling point of the compound. In case the experimental determination of the boiling point is carried out by simple distillation, the compound can be inferred to be pure if the temperature recorded by the thermometer had remained constant throughout the distillation. However, while making such an inference, consideration has to given to the possibility of the impurities and the liquid having formed a 'constant boiling mixture', examples of such mixtures having been cited earlier in Section 2.6.2.4. Uncertainty arising from such a possibility can be eliminated by combining the experimental determination of the boiling point with the experimental determination of another physical property viz. the refractive index, which is dealt with in the next Section.

2.7.3 Refractive Index

The refractive index, n, of a liquid is defined as the ratio of the velocity of light in a vacuum to the velocity of light in that liquid. The refractive index is theoretically referred to vacuum as the first medium, but the index referred to air differs from this by only 0.03 per cent and, for convenience, is more commonly used, so that, $n = \dfrac{\text{velocity of light in air}}{\text{velocity of light in the liquid}}$.
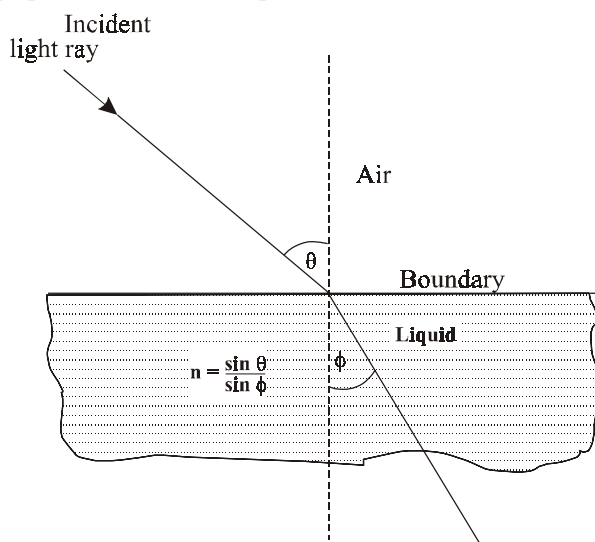
Figure 2.25 Refraction of light

The ray of light under- goes changes in wave velocity and in direction at the boundary surface and the refractive index can be experimentally determined as the ratio, $\sin \theta / \sin \phi$, in which θ is the angle of incidence for a light wave striking the surface of the liquid and ϕ is the angle of refraction within the liquid (Figure 2.25). Direct measurements of the angles of incidence and refraction are not feasible; hence specialised optical systems have been devised whose working makes use of the critical angle of reflection at the boundary of the liquid with a glass prism of known refractive index.

The index of refraction varies with the wave length of the incident light used. The refractive index also varies with the temperature at which its experimental determination is carried out—the refractive index decreases with increase in

temperature, because the density decreases with rise in temperature and this brings about decrease in the number of molecules per unit volume. If these factors are kept constant, the refractive index, n, is a characteristic constant for the particular liquid. The symbol n_D^{20}, thus, means the refractive index of the given liquid for the D lines of sodium (the yellow doublet at 5890/5896 Å) measured at 20ºC. Commercial refractometers have built-in compensators which allow for the use of ordinary white light instead of yellow sodium light for illumination. The compensator comprises of two direct-vision prisms, called Amici prisms, placed one above the other in front of the objective of the telescope of the refractometer. These two prisms are constructed of different varieties of glass and are so designed that they deviate all rays of light but not the ray corresponding to the sodium D line. The index of refraction that is obtained from the instrument, therefore, is that which would have been obtained if the yellow sodium light had been used. Incorporating such a feature is the Abbe refractometer which is described below. The Abbe refractometer has also provision for temperature control so that the determination of refractive index of the given sample can be carried out at the desired temperature. Another important advantage of this instrument is that only a few drops of the liquid are needed for measurement of the refractive index.

A schematic diagram of the optical system of the Abbe refractometer is shown

(a)

(b)
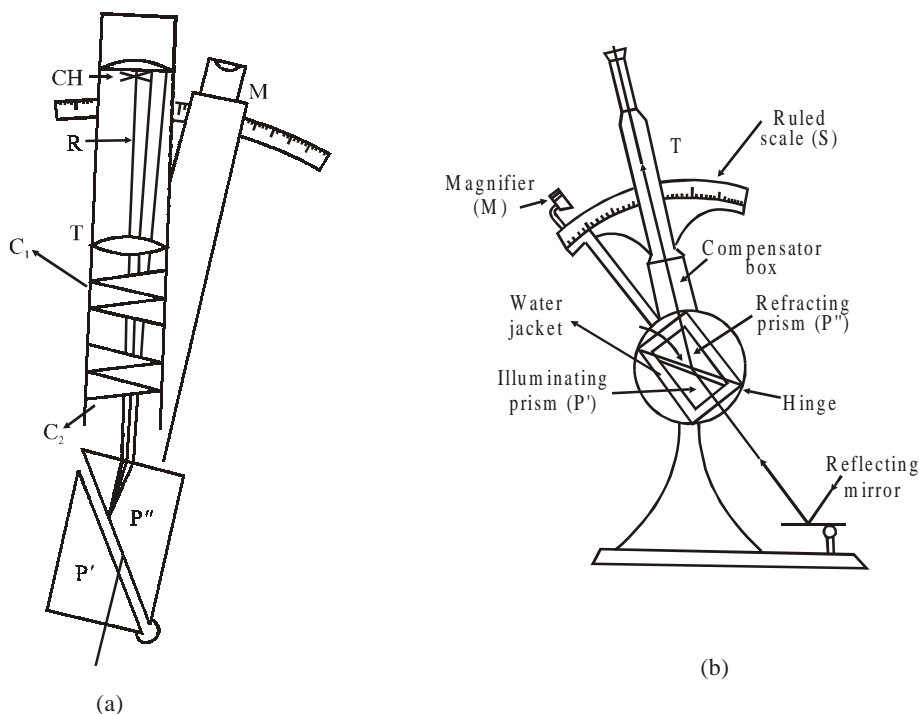
Figure 2.26 The Abbe refractometer.

in Figure 2.26(a) and the instrument in Figure 2.26(b). Water at 20ºC flows through the water jacket surrounding the prisms (P′, P″). The upper prism P″ (which can be lifted and lowered around a hinge common to both the prisms) is lifted and a few drops of the given liquid are spread on prism P′ with a wooden applicator. The prisms are then cooled slowly, any excess liquid being squeezed out. The reflecting mirror is adjusted so that the light source is reflected into the illuminating prism P′, the upper surface of which is rough ground. The rough surface acts as the source of an infinite number of rays which pass through the liquid layer (0.1 mm) in all directions. These rays then strike the polished surface

Figure 2.27 View through the eyepiece of the Abbe Refractometer (when the refractometer is set to be read, the intersection of the cross-hairsfalls on the border between the light and dark fields; the border should be as sharp and free from colour as possible).

of the refracting prism P″ and are refracted. The Abbe refractometer in effect measures the critical angle for refraction of light passing from the liquid to the glass prism P″ of known refractive index. The critical ray, R [Figure 2.26(a)], forms the border between the light and dark portions of the field when viewed with the telescope, T, which moves with the scale, S. The scale is provided with a scale magnifier, M. The field seen through the eye-piece appears as shown in Figure 2.27. The intersection of the cross-hairs, C.H. [Figure 2.26(a)], should be on the border between the light and dark sections of the field. Since white light is being used for illumination, coloured indistinct boundary between the light and dark portions of the field is only to be expected because of the differences in the refractive indices for light of different wave langths which constitute the white light. As already mentioned, provision of a compensator is made in the instrument to counter this problem arising from dispersion of white light at the liquid interface. The Amici prisms, C_1 and C_2 [Figure 2.26(a)] comprising the compensator, are then set as the next step of operating the refractometer so as to sharpen and achromatise the border between the light and dark sections until the difference is as sharp and as near black and white as possible. Finally, the refractive index is read from the scale when the cross-hairs and the border are lined up as shown in Figure 2.27.

■

This page intentionally left blank

PART II

Chemical Methods of
Separation and Purification

This page intentionally left blank

3

Chemical Methods of Separation and Purification

These methods involve carrying out appropriate chemical reactions for preparing selected derivatives of all or particular constituents of the given mixture of which all the components are, of course, present originally in one phase. Each such selected derivative is produced in a form which may either (i) constitute a new phase by itself, (ii) pass into a second phase that is immiscible with, and hence easily separable from, the first phase that orginally contained all the components of the given mixture, or (iii) remain in the original phase but is separable from it by such a physical process as fractional distillation, fractional crystallisation or dissolution. Physical separation of the phases is carried out as the next step, followed by suitable processing of these isolated phases to obtain the original components as separated individuals. The following examples illustrate the versatile applicability of chemical methods for separation of mixtures into their respective components in the fields of Organic Chemistry, Biochemistry and Inorganic Chemistry.

3.1 Separations in Organic Chemistry

3.1.1 Separation of a Mixture of an Alkane, an Alkene and an Alkyne into its constituents

A mixture of ethane, ethylene and acetylene can be resolved into its constituents by first passing the gaseous mixture through an ammoniacal solution of cuprous chloride which retains acetylene as a precipitate of cuprous acetylide.

$$\begin{matrix} CH \\ \| \\ CH \end{matrix} + 2\left[Cu(NH_3)_2\right]^+ \rightarrow \begin{matrix} C.Cu \\ \| \\ C.Cu \end{matrix} + 2NH_4^+ + 2NH_3$$
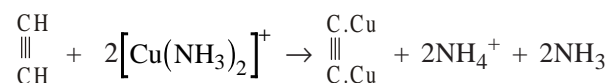
The escaping gaseous mixture consisting of ethane and ethylene is next bubbled through cold concentrated sulphuric acid which dissolves out ethylene by converting the latter into ethl hydrogen sulphate; ethane remains unaffected and can be collected separately as gas.

$$\begin{matrix} CH_2 \\ \| \\ CH_2 \end{matrix} + H_2SO_4 \longrightarrow \begin{matrix} CH_3 \\ | \\ CH_2 \cdot HSO_4 \end{matrix}$$
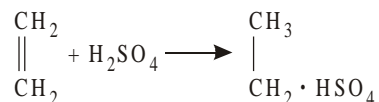
The precipitated cuprous acetylide is collected by filtration and acetylene recovered from it by treatment with dilute acid.

$$
\begin{array}{c}
\text{C . Cu} \\
\parallel\!\parallel\!\parallel \quad + 2H^+ \\
\text{C . Cu}
\end{array}
\longrightarrow
\begin{array}{c}
\text{CH} \\
\parallel\!\parallel\!\parallel \quad + 2Cu^+ \\
\text{CH}
\end{array}
$$
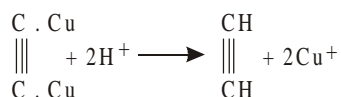
Ethylene is recovered by heating the solution of ethyl hydrogen sulphate in concentrated sulphuric acid to 160º–170ºC.

$$
\begin{array}{c}
\text{CH}_3 \\
\mid \\
\text{CH}_2 . \text{HSO}_4
\end{array}
\xrightarrow{\;160° - 170° \text{ C}\;}
\begin{array}{c}
\text{CH}_2 \\
\parallel \quad + \text{H}_2\text{SO}_4 \\
\text{CH}_2
\end{array}
$$
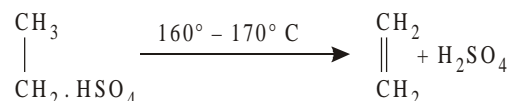
3.1.2 Separation of Carbonyl from Non-Carbonyl Compounds

A carbonyl compound (an aldehyde or a ketone) can be separated from its admixture with non-carbonyl compounds by shaking the mixture with a saturated solution of sodium bisulphite. This treatment yields a crystalline addition bisulphite derivative of the carbonyl compound:

$$
{>}C = O + \text{NaHSO}_3 \longrightarrow {>}C{\Large\langle}_{\text{SO}_3\text{Na}}^{\text{OH}}
$$
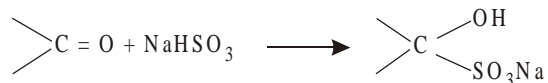
This solid derivative is separated out by filtration and the original carbonyl compound regenerated from it by heating with dilute acid or sodium carbonate solution.

$$
{>}C{\Large\langle}_{\text{SO}_3\text{Na}}^{\text{OH}} \quad + \ \text{H}^+ \longrightarrow {>}C = O + \text{Na}^+ + \text{H}_2\text{O} + \text{SO}_2
$$

$$
2 \ {>}C{\Large\langle}_{\text{SO}_3\text{Na}}^{\text{OH}} \quad + \ \text{Na}_2\text{CO}_3 \longrightarrow {>}C = O + 2\text{Na}_2\text{SO}_4 + \text{CO}_2 + \text{H}_2\text{O}
$$
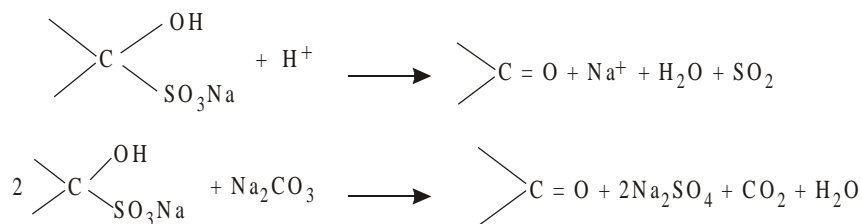
3.1.3 Separation of Neutral and Basic Components of Mixture

The constituents of a homogeneous mixture of aniline and toluene can be separated from each other by shaking the mixture with dilute hydrochloric acid in a separating funnel. The reaction mixture is allowed to stand for some time, and the aqueous layer of aniline hydrochloride separating at the bottom of the funnel is withdrawn, leaving the toluene layer in the funnel. This aqueous solution is transferred to another separating funnel and rendered alkaline with sodium hydroxide solution whereby aniline is renererated as an oily layer.

3.1.4 In the Hofmann's method of separating a mixture of primary, secondary and tertiary amines into its components, the mixture is treated with diethyl oxalate

whereby the primary amine yields a solid oxamide, the secondary amine gives a liquid oxamic ester and the tertiary amine is left unaffected.

$$2\,RNH_2 \;+\; \begin{matrix} H_5C_2OOC \\ | \\ H_5C_2OOC \end{matrix} \longrightarrow \begin{matrix} RHN.OC \\ | \\ RHN.OC \end{matrix} + 2\,C_2H_5OH$$

<div align="center">oxamide
(solid)</div>

$$R_2NH + \begin{matrix} H_5C_2OOC \\ | \\ H_5C_2OOC \end{matrix} \longrightarrow \begin{matrix} R_2N.OC \\ | \\ H_5C_2O.OC \end{matrix} + C_2H_5OH$$

<div align="center">oxamic ester
(liquid)</div>
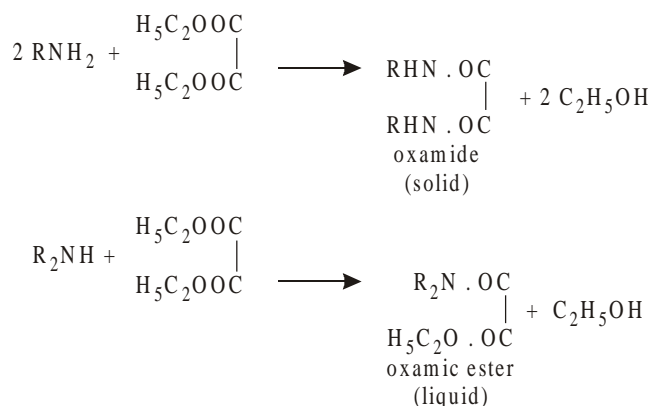
The solid oxamide is separated out by filtration and the solid yields the original primary amine on its being heated with caustic potash:

$$\begin{matrix} CO.NHR \\ | \\ CO.NHR \end{matrix} + 2KOH \longrightarrow \begin{matrix} COOK \\ | \\ COOK \end{matrix} + 2\,RNH_2$$

<div align="center">primary amine</div>
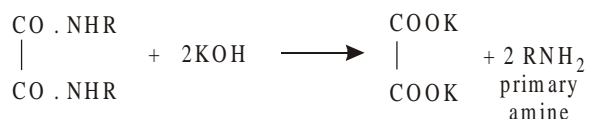
The liquid oxamic ester is separated from its admixture with the unchanged tertiary amine by fractional distillation. On decomposition by heating with caustic potash, the oxamic ester regenerates the original secondary amine:

$$\begin{matrix} CO.NR_2 \\ | \\ CO.OC_2H_5 \end{matrix} + 2KOH \longrightarrow R_2NH + \begin{matrix} COOK \\ | \\ COOK \end{matrix} + C_2H_5OH$$

<div align="center">secondary amine</div>
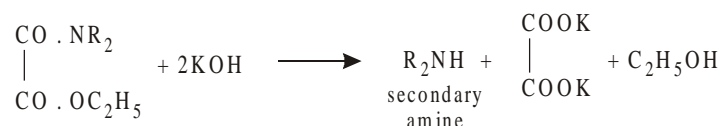
3.1.5 Separation of a Racemic Mixture into its Constituents

(a) The most commonly used method of separation of the racemic mixture into its constituent enantiomers is as follows:

A chemical reaction is carried out between the racemate and an *optically active* form (either laevo-or dextro-) of a substance capable of reacting with the racemate. This other optically active compound is usually derived from a natural source. To resolve the racemates of amines (or other bases) and alcohols, for example, use may be made of the naturally occurring d-tartaric acid (from wine tartar). The reaction with amines gives salts and esters are formed with alcohols. For the resolution of racemates of acids, use is frequently made of alkaloids such as quinine or strychnine extracted from plants in which each of these alkaloids is present in an optically active form. The racemate mixture forms two diastereoisomers (compounds that are stereoisomers of each other, but are not enantiomers) of a derivative, with the optically active reagent used. If the

enantiomers constituting the racemic mixture are denoted by letters L and R, and the optically active reagent by L′, then the two compounds (salts, esters etc.) obtained will be represented as:

$$\begin{array}{ccc} L & & R \\ L' & \text{and} & L' \end{array}$$

These two diastereoisomers posses different properties, say, different solubilities, so that the two can be separated by one of the ordinary methods, say, by fractional crystallisation. After separation, the optically active reagent is removed from the molecule (if the diastereoisomer is a salt, it is treated with acid or alkali and if it is an ester, hydrolysis is carried out) and pure forms of enantiomers are isolated. Resolution of dl-tartaric acid is the classical example of the application of this method.

With modification, the aforesaid method can sometimes be applied to the resolution of a neutral compound. Such a resolution is accomplished by first converting the neutral compound into a derivative which can form a salt. Resolution of dl-octanol-2 is an example of the application of this modification. The racemic mixture of the alcohol is converted by treatment with phthalic anhydride into the acid phthalate. In the next step the acid phthalate is reacted with the naturally occurring laevorotatory base, brucine. Fractional crystallisation of the resulting mixture of the brucine salts yields the separated salts. Decomposition of the separated salts with hydrochloric acid removes brucine, and the two resulting acid phthalates are then hydrolysed with alkali to get the d- and l-forms of octanol-2.
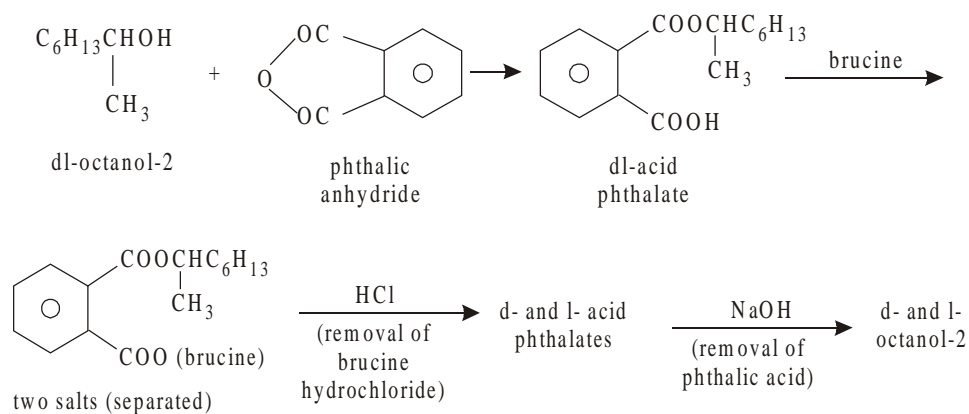
(b) Enzymes are highly specific biological catalysts capable of discriminating between molecules having similar structures. One compound may be chemically changed in the presence of an enzyme, while another, otherwise inseparable from the first, is not affected. This selectivity has been used as a basis of separation. The method is not frequently used but may be resorted to when two very closely related

molecules cannot be separated by other means. Separation of dl-alanine, for example, has been accomplished by such an enzymic method. For this separation, dl-alanine, obtained by synthesis, is acetylated, and the acetyl dl-alanine is treated with a solution containing an enzyme which can bring about deacetylation. The solution containing the required enzyme is prepared from fresh pork kidneys. The action on the acetyl dl-alanins is complete in about four hours. The enzyme acts preferentially on the acetyl derivative of the natural amino acid viz. l-alanine and the result is a mixture of l-alanine and acetyl d-alanine.

$$CH_3CHCOOH \atop NH_2$$

dl-alanine

$$\longrightarrow$$

$$CH_3CHCOOH \atop NHCOCH_3$$

dl-acetyl
alanine

$$\longrightarrow$$

$$COOH \atop H_2N-\underset{CH_3}{\overset{|}{C}}-H$$

l-alanine
(insoluble in ethanol)

$$+$$

$$COOH \atop HC\underset{CH_3}{\overset{|}{N}}HCOCH_3$$

acetyld-alanine
(soluble in ethnol)
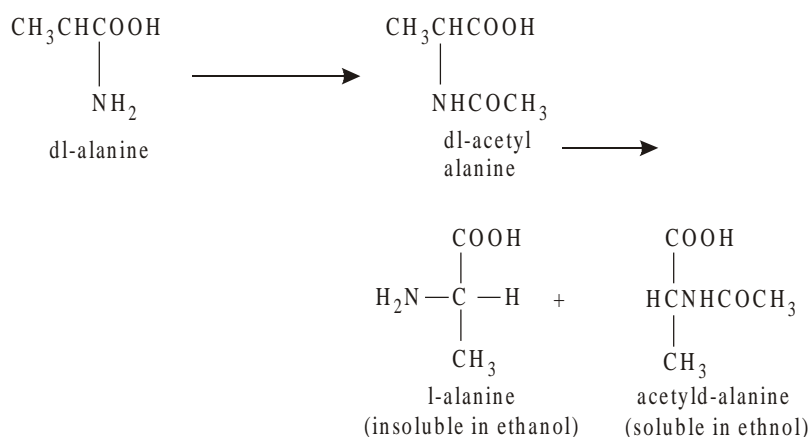
The resulting mixture is easily separated into its constituents because free alanine is insoluble in ethanol and the acetyl derivative is soluble.

3.2 Separations in Biochemistry

3.2.1 Isolation of Proteins from their naturally occurring Mixtures

Many polyvalant metals form salts with organic acids which are markedly less soluble than either the free acids or their sodium and potassium salts. Organic bases, similarly, react with these salts to form complexes which are difficultly soluble. Although these effects are of general occurrence, they find an important application in the field of Biochemistry in the purification of proteins, and for this purpose the polyvalent metals may be classified into the following groups on the basis of their interactions with the different functional groups present in proteins.

The first group which includes Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+} is bound strongly both to carboxylic acids and to nitrogenous substances such as amines and heterocylic compounds. The second group which includes Ca^{2+}, Ba^{2+}, Mg^{2+} and Pb^{2+} is bound strongly to carboxylic acids, but shows negligible affinity to nitrogenous ligends. Zn^{2+} and Ba^{2+} may be taken as representatives of the two classes, and their interactions with proteins have been studied extensively. A third

group of metals have a particularly strong affinity for sulphydryl groups. These include Hg^{2+}, Ag^+ and Pb^{2+}.

These metals can react with the carboxyl, amino, imidazole, or sulphydrl groups of proteins, and the particular type of complex formed will depend on the nature of the metal and of the protein and on the extent of ionization of the ligand groups.

An important property of the protein metal complexes is that their solubilities are particularly influenced by the dielectric constant of the medium. Thus, a moderate reduction of the dielectric constant of an aqueous solution will precipitate the Ba^{2+} or Zn^{2+} salts of many proteins which are readily soluble in the aqueous solutions. This property has been used for the fractionation of the plasma proteins. The use of barium and zinc salts not only gave superior fractionation, but also allowed the use of lower ethanol concentrations with diminished risk of denaturation.

The recovery of proteins from their complexes with metals has been greatly facilitated by the introduction of ion-exchange desalting, and of the complexones such as ethylene diamine-tetra-acetic acid (EDTA), which remove the metals as non-ionised complexes. The use of specific precipitants for effecting separations covers a large field in Biochemistry, and some examples from protein chemistry are given below to illustrate the possibilities of this method.

Electrophoretically well characterized α_1-and γ-globulins may be prepared directly from serum by precipitation with 0.5 per cent uranyl acetate solution. The γ-globulins were precipitated at pH 4.3 essentially free from other serum proteins, while the α_1-globulins could be precipitated at pH 3.2.

Another method for the isolation of a serum protein fraction uses amylopectin sulphate which is particularly effective for the precipitation of β_1-lipoproteins and this observation forms the basis of its potential use for the estimation of these proteins in plasma.

The acridine dye, Rivanol (2-ethoxy-6:9-diaminoacridine lactate), has been shown to be useful for the preparation of γ-globulins. A 0.4 per cent solution of the dye added to plasma at pH 7.6–7.8 will precipitate all the plasma proteins except the γ-globulins. The excess dye can then be removed by adsorption on Norit charcoal, and the γ-globulins recovered by precipitation with 25 per cent ethanol at –5ºC.

3.3 Separations in Inorganic Chemistry

3.3.1 Refining of Metals

One method of refining of metals, called the van Arkel process, involves the formation of a volatile halide by the direct action between the metal and a halogen followed by purification of the volatile halide (by fractional distillation, for example) and its subsequent decomposition to the metal and halogen at a higher temperature. The sequence of reactions involved in the van Arkel process is

exemplified below for the refining of Zr:

$$Zr + 2I_2 \longrightarrow ZrI_4$$

$$ZrI_4 \xrightarrow[800°C]{filament} Zr + 2I_2$$
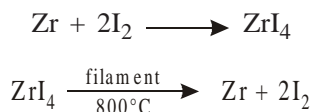
Besides Zr, the elements Ti, Hf, V, W, Si and Be have been purified by this method.

Purification via the volatile carbonyl compounds is similar to the van Arkel process, though used widely only for nickel:

$$Ni + 4CO \xrightarrow{50°\ C} Ni\,(CO)_4$$

$$Ni(CO)_4 \xrightarrow{180°\ C} Ni + 4CO$$
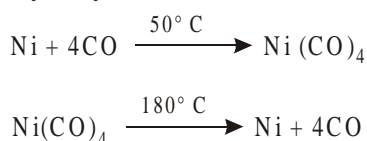
3.3.2 Separation of Lanthanides

(a) Complex formation constitutes an important technique of separation in Inorganic Chemistry. An important example of such an application is provided by the separation of lanthanides by means of complex formation. Lanthanide oxalates are insoluble in water but they become soluble on complexation with EDTA. However, towards acids the stability of each complex formed between EDTA and a particular member of the lanthanide series is different. Advantage is taken of this fact for effecting the aforesaid separation. On adding an acid the least stable complex is destroyed first to yield the precipitate of the corresponding exalate, whereas the lanthanide which forms the most stable complex is the last to precipitate out as its oxalate.

(b) Fractional precipitation makes use of the concept of solubility product and its application can be elaborated by taking the example of separation of lanthanides. This separation is effected by taking advantage of differences in solubility products of the hydroxides of the various members of this group of elements. On addition of NaOH to a solution of lanthanide nitrates, $Li\,(OH)_3$ precipitates out first because of its lowest solubility product, whereas $La\,(OH)_3$ precipitates out last of all because of its highest solubility product. By repeating dissolution and subsequent fractional precipitation of the hydroxides several times, it becomes possible to achieve complete separation of lanthanides.

(c) Change in oxidation state of an ion brought about by oxidation or reduction under conditions which do not affect the oxidation state of the other ions present in the mixture is yet another chemical method of separating lanthanides. The usual oxidation state of members of the lanthanide series is 3^+ and in this oxidation state they are very close to one another in properties. But some of these elements exhibit oxidation states of 2^+ and 4^+ also, and these changed values of oxidation state cause a marked change in the properties of such lanthanides.

Ce^{3+} alone is oxidised to the tetrapositive state by alkaline $KMnO_4$ when it is present in solution along with other tripositive lanthanide cations. Separation of cerium from other cations is then effected by carrying out this oxidation followed by the addition of a small quantity of an alkali. Carium alone is precipitated out as $Ce(OH)_4$ leaving all other tripositive lanthanides in solution. Thus 99% pure cerium can be obtained starting with a mixture having a cerium content of only 40%.

Eu^{3+} present in a solution along with other tripositive lanthanide ions can be isolated almost quantitatively through its reduction to Eu^{2+}. Zn-amalgam reduces only Eu^{3+} to the divalent ion leaving the tripositive lons of the other lanthanides unaffected. Eu^{2+} is then precipitated out from the aqueous solution as $EuSO_4$, which is insoluble, leaving behind other lanthanides in solution because the corresponding sulphates are all soluble in water.

3.3.3 Separations of Isotopes by Exchange Reactions

In 'exchange reactions' chemical reaction gives appreciable exchange of isotopes between the reactants. These reactions can be used for the separation of isotopes. Obviously, only partial separations can be achieved through exchange reactions, because these are equilibrium reactions. Some examples of application of exchange reactions are given below.

(i)   When carbon dioxide is kept in contact with water, the liquid gets richer in ^{16}O and the gas in ^{18}O:

$$C^{16}O_2 + 2H_2{}^{18}O \rightleftharpoons C^{18}O_2 + 2H_2{}^{16}O$$

(ii) Isotopic exchange reaction between hydrogen gas and water establishes the following equilibrium:

$$HD + H_2O \rightleftharpoons H_2 + HDO$$

As a result of this equilibrium, concentration of deuterium in water gets three to four times as great as in hydrogen.

(iii) Passage of lithuim chloride solution down a column containing sodium zeolite yields an outgoing solution which is richer in ^7Li in comparison with the starting solution:

$$Na[AlSiO_4] + {}^6Li^+ \rightleftharpoons {}^6Li\,[AlSiO_4] + Na^+$$

$$Na[AlSiO_4] + {}^7Li^+ \rightleftharpoons {}^7Li\,[AlSiO_4] + Na^+$$
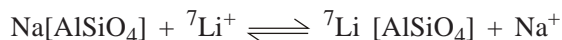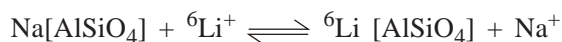
This happens because $^6Li^+$ reacts faster than $^7Li^+$.

3.4 Conclusion

The examples cited in this chapter are only illustrative of the versatile applicability of chemical methods for carrying out separation and purification in Chemistry and

Biochemistry and are far from being meant as a comprehensive list of the numerous chemical reactions that have been used by chemists and biochemists to obtain substances in the pure state. More examples of chemical reactions used as aids in separation processes are dispersed throughout the book. Two such noteworthy examples are: (i) Ion-exchange chromatography, and (ii) electro-refining which is purification by chemical reactions carried out with the help of an electric current. In the same context one is also reminded of the use of some chemical reactions for separation of inorganic ions mentioned in the previous chapter during discussion of Solvent Extraction.

■

This page intentionally left blank

PART III

Chromatographic Techniques

This page intentionally left blank

<div style="text-align: center">

┌─────┐
│ 4 │
└─────┘

Methods Involving Two Immiscible Phases of which One Phase is Stationary and the other One Mobile

</div>

Although the conventional techniques of purification like distillation, sublimation and crystallisation etc. with the latest improvements in them in terms of technique and equipment are as important even today as ever before, they have been increasingly supplanted by new separation and purification techniques amongst which chromatography, as practised in its numerous forms, is the most outstanding example. The importance of these newer techniques can be easily appreciated when it comes to dealing with compounds of high molecular weights such as proteins whose purification is not easily amenable to conventional methods dealt with in the previous two chapters. The present chapter discusses two of these newer techniques viz. counter-current distribution and chromatography. The technique of counter-current distribution (counter-current chromatography) is *atypical* of the usual chromatographic techniques in that neither of the two phases in it is supported on an inert support, though the separation of compounds by it is also based upon the different distribution coefficients between two immiscible phases of which one phase is mobile and the other one stationary, as are the separations in conventional chromatography. Because of its bulk (which arises from the numerous techniques by which chromatography is usually practised, the topic of typical chromatography has been discussed in detail, technique-wise, in several subsequent chapters, this topic having been dealt with in just an introductory manner in this chapter.

4.1 Counter-current Distribution [Counter-current Chromatography (CCC)]

Liquid-liquid partition methods referred to in the previous chapter can be extended even to the separation of solutes possessing only small differences in their partition coefficients by a method called counter-current distribution. The technique of counter-current distribution, developed mainly by Craig, is a multiple partition process with a large number of stages, entirely discontinuous and stepwise in nature.

4.1.1 Theory of Counter-current Distribution

The following discussion explains the theory underlying the separation of constituents of a mixture effected by the counter-current distribution procedure.

For the purpose of this discussion, the distribution or partition coefficient will stand defined by the equation:

$$K_D = \frac{\text{Amount/ml of the given solute in the upper phase}}{\text{Amount/ml of the solute in the lower phase}}$$

In this method, of the two immiscible liquid phases which may be mixtures of solvents, buffers, salts and various complexing agents, the heavier one constitutes the stationary phase. This heavier phase is placed in a series of tubes or cells. The lighter phase in each cell is moved after each equilibration by a simple movement to the adjoining cell while the first cell is supplied with a fresh quantity of the lighter phase. The whole operation of agitating the cell train for the extraction and demixing of the phases and moving the upper phase one cell forward is called 'a transfer'. The different components of the mixture put into the first cell at the start of the distribution advance through the train at different rates. By adding fresh quantity of the lighter phase to the first cell after each transfer, it is possible to carry out as many transfers as needed.

DISTRIBUTION OF A SINGLE SOLUTE

Distribution of a single solute through the cell train over several transfers may be illustrated by analysing its advance through a series of tubes numbered 0, 1, 2,......, r, each containing (for the sake of simplicity) equal volumes of two immiscible liquids (for which the distribution coefficient of the solute is K_D), the solute being placed in the lower phase of tube 0 to begin with. After equilibrium is reached the top layer of tube 0 is transferred to tube 1. The process is repeated n times; each time, all top layers are transferred to the next higher tube in sequence.

After the first transfer ($n = 1$), the fraction of solute remaining in tube 0 is $\dfrac{1}{K_D + 1}$, since the two immiscible liquids forming the two phases have equal volumes. Upon equilibration, the second tube (tube 1; $r = 1$) contains $\left(\dfrac{1}{K_D + 1}\right)\left(\dfrac{K_D}{K_D + 1}\right) = \dfrac{K_D}{(K_D + 1)^2}$ in the bottom phase and $\left(\dfrac{K_D}{K_D + 1}\right)\left(\dfrac{K_D}{K_D + 1}\right)$ $= \left(\dfrac{K_D^2}{(K_D + 1)^2}\right)$ in the top phase. In the second transfer, the top phase is moved to tube 2. Tube 1 then contains $\dfrac{K_D}{(K_D + 1)^2}$ transferred from tube 0 and an equal

amount remaining after the second transfer, to give a total of $\dfrac{2K_D}{(K_D+1)^2}$. The distribution of solute during four extractions is shown in Table 4.1.

TABLE 4.1

Fraction of Solute in Succeeding Tubes in Counter-current Distribution

Tube No. r/ No. of extractions n	0	1	2	3	4
0	1	0	0	0	0
1	$\dfrac{1}{K_D+1}$	$\dfrac{K_D}{K_D+1}$	0	0	0
2	$\dfrac{1}{(K_D+1)^2}$	$\dfrac{2K_D}{(K_D+1)^2}$	$\dfrac{K_D^2}{(K_D+1)^2}$	0	0
3	$\dfrac{1}{(K_D+1)^3}$	$\dfrac{3K_D}{(K_D+1)^3}$	$\dfrac{3K_D^2}{(K_D+1)^3}$	$\dfrac{K_D^3}{(K_D+1)^3}$	0
4	$\dfrac{1}{(K_D+1)^4}$	$\dfrac{4K_D}{(K_D+1)^4}$	$\dfrac{6K_D^2}{(K_D+1)^4}$	$\dfrac{4K_D^3}{(K_D+1)^4}$	$\dfrac{K_D^4}{(K_D+1)^4}$

Separation of Two Solutes

In general, two or more solutes act independently of each other, so that each will move according to its value of $\dfrac{K_D}{K_D+1}$.

Table 4.2 (derived from Table 4.1 by substituting proper values of K_D) is a schematic representation of extraction for four transfers following the counter-current distribution procedure for the case of separation of a mixture of two solutes, A and B, the values of distribution coefficients for A and B respectively being $K_{D(A)} = 0.5$ and $K_{D(B)} = 2.0$, and it being given that each of the two solutes is taken in unit quantity and the volumes of the two immiscible liquid phases are equal.

TABLE 4.2

Transfer number (n)\Tube   number (r)

		0				
0	B	1				
	A	1				
		0				

		1	0			
1	B	0.666	0.333			
	A	0.666	0.333			
		0	1			

		2	1	0		
2	B	0.444	0.444	0.111		
	A	0.444	0.444	0.111		
		0	1	2		

		3	2	1	0	
3	B	0.296	0.444	0.222	0.037	
	A	0.296	0.444	0.222	0.037	
		0	1	2	3	

		4	3	2	1	0
4	B	0.198	0.395	0.296	0.099	0.0123
	A	0.198	0.395	0.296	0.099	0.0123
		0	1	2	3	4

Schematic representation of extraction effected by four transfers carried out in the counter-current distribution procedure employed for the separation of constituents of a mixture of two solutes, A and B [$K_{D(A)} = 0.5$ and $K_{D(B)} = 2.0$; each solute has been taken in unit quantity and the two liquid phases have equal volumes].

The expression:

Total fraction of the solute in a particular tube $\times \dfrac{K_D}{K_D+1}$, will give the fraction

of that solute in the upper layer of that particular tube by substituting the K_D value of the solute in the expression; substitution of the K_D value of a solute in the expression:

Total fraction of the solute in a particular tube $\times \dfrac{1}{K_D+1}$, will give the fraction

of that solute in the lower layer of that tube.

Thus, the fractions of solute B in the upper layer and the lower layer of tube 3 after the third transfer (r = 3 and n = 3 in Table 4.2) are:

$$0.296 \times \frac{2}{2+1} = 0.296 \times \frac{2}{3} = 0.1973, \text{ and}$$

$$0.296 \times \frac{1}{2+1} = 0.296 \times \frac{1}{3} = 0.0987, \text{ respectively, since } K_{D(B)} = 2.$$

An examination of the different tubes at the end of each of these transfers yields the analysis given Table 4.3 with regard to the ratio:

$$\frac{B \left(\text{i.e. total amount of B in both the phases of any particular tube}\right)}{A \left(\text{i.e. total amount of A in both the phases of that particular tube}\right)}.$$

TABLE 4.3

Ratio B/A at the end of Transfer Number	Ratio B/A in Tube Number				
	0	*1*	*2*	*3*	*4*
0	$\dfrac{1}{1}$				
1	$\dfrac{0.333}{0.666}$	$\dfrac{0.666}{0.333}$			
2	$\dfrac{0.111}{0.444}$	$\dfrac{0.444}{0.444}$	$\dfrac{0.444}{0.111}$		
3	$\dfrac{0.037}{0.296}$	$\dfrac{0.222}{0.444}$	$\dfrac{0.444}{0.222}$	$\dfrac{0.296}{0.037}$	
4	$\dfrac{0.0123}{0.198}$	$\dfrac{0.099}{0.395}$	$\dfrac{0.296}{0.296}$	$\dfrac{0.395}{0.099}$	$\dfrac{0.198}{0.0123}$

Whereas at the beginning of the experiment (the state of affairs in tube number 0 at transfer number 0 the proportion of B to A was 1:1, at the completion of the

extraction after 4 transfers, the ratio $\dfrac{B}{A}$ is $\dfrac{0.198}{0.0123}$ (say 16:1) in tube number 4, and

$\dfrac{0.0123}{0.198}$ or 1:16 in tube number 0. Each successive transfer has brought about enrichment of B relative to A in one direction of the series of the five tubes and enrichment of A relative to B in the opposite direction. On this conclusion is based the name 'Counter-current Distribution' for this particular separatory technique. It is obvious from the above analysis that such a multi-stage scheme of extraction can afford an excellent separation of a mixture of solutes into its constituents each one of which has its own particular value of partition coefficient for a given pair of the liquid phases. Of course, one way to improve the separation is to increase the number of stages in which extraction is completed, but it is sometimes more effective to attempt to increase the difference in distribution coefficients by, for example, varying the composition of the solvent phases. As mentioned earlier, these phases may be mixtures of solvents, buffers, salts and various complexing agents.

4.1.2 Experimental Technique

Although excellent results may frequently be obtained by fractional extraction with such simple equipment as a set of separating funnels, still more efficient separations may be effected when more elaborate apparatus is used. There is commercially available a fully automatic equipment, named after Craig, which is so designed that several 'transfers' occur simultaneously in a train of contacting tubes. There are counter-current instruments containing from a few dozen tubes up to 1000 or more.

Figure 4.1 illustrates a Craig counter-current distribution tube. Chamber C [Figure 4.1(a)] in each such tube is filled with solvent Z that is heavier than the extracting solvent S. The volume of solvent Z is small enough so that when the
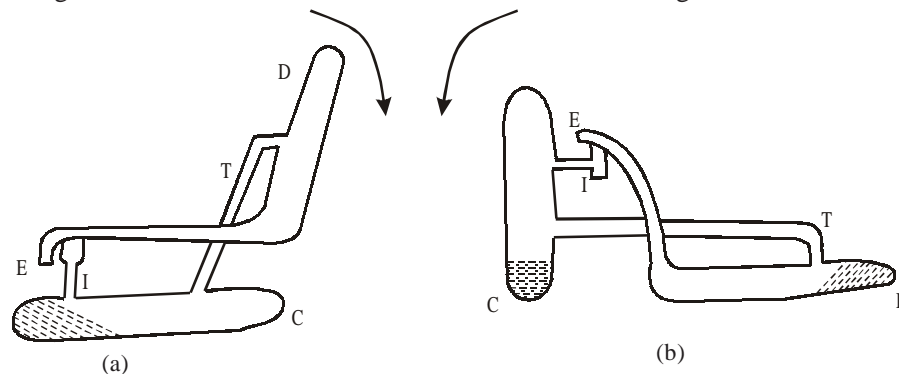

Figure 4.1 A Craige counter-current distribution tube

tube is tilted 90º as in Figure 4.1(b), it will not flow through the tube T. The given sample of mixture which is to be separated into its constituents, A and B, is present in solution in the quantity of solvent Z contained in the number 0 tube. Extracting solvent S is introduced into the first tube (i.e. the number 0 tube) through inlet I. After shaking by rocking back and forth, and after allowing the phases to separate, the tube is tilted 90º as in Figure 4.1(b). The lighter solvent S flows through tube T into chamber D. Now the assembly is rotated back to its original position, and the solvent S flows out through exit tube E into the next tube, namely the number 1 tube (via its tube I), leaving the original solvent Z in chamber C of the number 0 tube. The entire equilibration and transfer cycle is carried out by an adjustable, automatic mechanism in about 1–2 minutes. Fresh solvent is added to the number 0 tube and the process is repeated, this time the solutes being distributed between the two phases in two tubes; the amounts of constituents A and B of the mixture originally extracted into solvent S have been transferred to the second tube (i.e. the number 1 tube) containing solvent Z (no sample), where these are redistributed. The procedure is repeated several times, depending on the number of tubes.

4.1.3 Applications of Counter-current Distribution

The counter-current distribution method is predominantly used for the separation of complex mixtures of organic (often natural) substances. Separations are achieved under very mild conditions; thus the method is ideally suited for handling materials whose stability is sensitive to experimental conditions employed during their separation. No material is wasted, and all material put into a distribution machine can be recovered. It is not successful with compounds of limited solubility, with salts or other strongly polar compounds, or with saturated hydrocarbons.

A special feature of counter-current distribution is that it can be scaled up to permit the isolation of small quantities of components from large amounts of starting material. For example, the technique gave isolation of protogen from 4 tons of mixed beef and pork liver using a 40-tube apparatus consisting of stages with 1 litre in each phase.

The counter-current distribution technique is of particular value whenever it is desired to follow in detail the progress of a separation. Thus, in the resolution of a complex mixture, an initial extraction may be performed using relatively few tubes of large size. Any desired fraction from the initial separation may then be subjected to further fractionation, using if necessary an apparatus of a large number of tubes, and performing a large number of transfers. A good example of such a separation is the isolation of large peptide fragments from the degradation of the andrenocorticotropic harmone (ACTH) using up to 10,000 transfers in a 200-tube apparatus.

4.2 Chromatography

Chromatography is the most important of all purification methods now. Though the technique finds the widest applicability, chromatography is a comparatively simple method of separating a desired compound from its impurities, or of isolating individual components of a mixture. Apart from the high selectivity in separation shown by the technique, one other important advantage of chromatography is that its use involves mild experimental conditions as, for example, it can be generally carried out at room temperature.

Mikhail Tswett, a Russian botanist, used the word 'Chromatography' to describe his separation of plant pigments, which was carried out by passing an extract of the pigments through a column packed with calcium carbonate. The result was a series of coloured zones on the column and, thus, the name chromatography from the Greek *chromatus* and *graphein*, meaning 'colour' and 'to write'. Since Tswett, a wide variety of independent techniques that have little or nothing to do with colour have come to be called chromatography. At least one, paper chromatography, was recognized centuries before Tswett, whilst most evolved from his work.

'Chromatography' now refers to any of a diverse group of techniques that effect a separation through a distribution of sample between two immiscible phases. The requirement to distinguish chromatography from other separation techniques is that one phase be stationary while the second phase be mobile and percolating through the first phase resulting in selective retention of the components of a mixture by the stationary phase. The stationary phase, which may be solid or liquid or may consist of a mixture of a solid and a liquid, is finely divided and is fixed in place. The mobile phase, which may be liquid or gaseous, fills the interstices of the stationary phase and is able to flow through the stationary phase.

4.2.1 Classification of Chromatographic Systems

The physical states of the mobile and stationary phases give rise to four basic types of chromatography: (i) Liquid-solid chromatography, LSC, (ii) liquid-liquid chromatography, LLC, (iii) gas-liquid chromatography, GLC, and (iv) gas-solid chromatography, GSC.

Separation of the components, or solutes, of a sample results from differences in their rates of adsorption, solution, or reaction with the mobile and stationary phases. In the light of these observations distinguishing the numerous chromatographic techniques only on the basis of specification of the physical states of the stationary and mobile phases is inadequate, and a more adequate classification of these techniques must additionally also take into account (i) the nature of the separation e.g. adsorption, and (ii) the configuration of the system e.g. columnar. Table 4.4 gives a system of classification which incorporates these considerations.

TABLE 4.4

Chromatographic Systems

System	Mobile phase	Stationary phase	Configuration	Separation
Liquid solid Chromatography	Liquid	Solid	Column	Adsorption
Liquid-liquid Chromatography	Liquid	Liquid	Column	Partition
Ion-exchange Chromatography	Liquid	Solid	Column	Ionic replacement reactions
Gel Chromatography	Liquid	Liquid	Column	Sieving dependent upon molecular size
Complexing Chromatography	Liquid	Solid	Column	Rapid and reversible formation of complexes
Ion-pair Chromatography	Liquid	Solid	Column	Ion replacement reactions
Ion Chromatography	Liquid	Solid	Column	Ion replacement reactions
Paper Chromatography	Liquid	Paper	Sheet or strip	Partition or adsorption
Thin-layer Chromatography	Liquid	Solid	Thin film	Adsorption or partition
Gas Chromatography: (i) Gas-solid Chromatography	Gas	Solid	Column	Adsorption
(ii) Gas-liquid Chromatography	Gas	Liquid	Column	Partition
Capillary Supercritical Fluid Chromatography	Supercritical fluid	Liquid	Column	Partition

Any particular technique is designated as *preparative* when it is applied to the purification of a relatively large amount of a material. The purpose of such an experiment is to obtain a constituent in the pure state for further characterization and study or for some practical application. The same technique is designated as an *analytical* one when it is used for the determination of composition of a sample; however, it may be used to evaluate any physical or chemical characteristic of individual constituents isolated by application of the technique to the given sample.

4.2.2 Choice of suitable Chromatographic Technique

For each chromatographic separation, certain governing factors have to be taken into consideration while making choice of a particular method from amongst the methods listed in Table 4.4. Provided the system is sufficiently volatile, gas-liquid column chromatography will normally be the most convenient method. Use of heated columns and vaporization chambers allows separation by gas-liquid chromatography of compounds that boil at temperatures as high as 400ºC. Gas chromatography has the great advantage that no problems of removing the separated components from the column or the solvent arise. Substances which are non-volatile, constitute an overwhelming majority and their separation can most often be carried out successfully by liquid-solid adsorption or liquid-liquid partition column chromatography. A very convenient method of separation, which does not depend on volatility, is afforded by liquid chromatography with paper (cellulose) strips or cellulose layered on a plane surface (thin-layer chromatography). The limitation here is that, since the separation depends on distribution between an organic solvent and water adsorbed on to the cellulose, appreciable water solubility is required. Although the method is thus singularly well suited to the separation of many natural products, which normally occur in aqueous media, many classes of organic compound are ruled out. Water-soluble compounds which are strongly ionic are best chromatographed by an ion-exchange system, using either an anionic or a cationic resin. Ion-pair chromatography can bring about simultaneous separation of ionised and ionisable molecules present together in a mixture. Ion chromatography finds wide application for the separation of inorganic and organic ions. Complexation chromatography may be used for the successful separation of the constituents of mixtures of constitutional, configurational and isotopic isomers whose separation is otherwise difficult. Compounds differing in molecular size are best separated by gel chromatography. Rapid and efficient separation of compounds having limited volatility and thermal stability can be achieved using capillary columns in conjunction with supercritical fluids.

Detailed accounts of the various chromatographic systems enumerated in Table 4.4, along with the applications of these systems, follow in Chapters 5–14.

Besides the chromatographic techniques listed in Table 4.4, there are some chromatographic methods which primarily are of interest to biochemists and Chapter 15 deals with examples of such separation techniques. Such methods dealt with in Chapter 15 are: Affinity chromatography, dye-ligand chromatography, covalent chromatography, hydroxylapatite chromatography and hydrophobic interaction chromatography. Of course, several of those methods that constitute Table 4.4 are also capable of effecting separation of biomolecules, and such separations find their mention at appropriate places in the book.

■

5

Adsorption Column Chromatography
[Liquid-Solid Chromatography (LSC)]

Adsorption chromatography, the oldest of the chromatographic methods, originated from the classical investigations of Tswett (see page 67), who first described these in 1906. It was not before a lapse of many years that Kuhn Winterstein and Lederer rediscovered this method of separation of complex natural mixtures in the early thirties of the twentieth century. In a chromatographic system in which the stationary phase is a solid as it is in liquid-solid chromatography carried out in a vertical tube (column), the separation that results is brought through the process of adsorption and desorption.

5.1 Working of the Technique

5.1.1 Theory underlying Adsorption Column Chromatography

The solid material or *adsorbent* provides a very large surface area and has the ability to absorb chemical substances on its surface through such physical and chemical interactions as (i) Van der Waals forces, (ii) Inductive force, (iii) Hydrogen bonding, (iv) Charge transfer and (v) Covalent bonding.

(i) Vander Waals forces hold neutral molecules together in the liquid or solid state. Adsorption based on this is purely physical in nature characterized by low adsorption energies and rapid equilibrium being set up, and results in giving good separation. Adsorption of non-polar solutes on non-polar adsorbents occurs by play of van der Waals forces as, for example, in the case of hydrocarbons on graphite.

(ii) Inductive forces or dipole-dipole attractions arise when a chemical bond has a permanent electrical field with it (e.g. $C–NO_2$, $C–Cl$ etc.). The electrons of an adjacent atom or group or molecule get polarised under the influence of this field. This in turn gives rise to an induced dipole-dipole attraction between the adsorbent and the solute. Many adsorptions on alumina illustrate operation of these inductive forces.

(iii) Hydrogen bonding becomes important when the solutes have a proton-donor group which can undergo hydrogen bonding with the polar groups present at the surface of the adsorbent (e.g. the surface hydroxyl groups possessed by silica or alumina). These surface hydroxyl groups will

themselves act as proton-donor groups, thus giving rise to hydrogen bonding on coming in contact with, for example, ethers, nitriles or aromatic hydrocarbons.

(iv) The contribution of charge transfer to adsorption energy is reported to be very little in the case of most compounds. An adsorbed complex of the type, (Solute)$^+$ (Adsorbent site)$^-$ results by the transfer of an electron from the solute to a surface site.

(v) Covalent bonding (chemisorption) results owing to operation of relatively strong chemical forces between the solute and the adsorbent. Components of a mixture obtained by chromatographic separation may not possess any high degree of purity in cases where these strong chemical forces are operating.

If a small amount of a concentrated solution of a mixture of two substances, A and B, is applied to the top of the column, both become adsorbed to the packing material. If a solvent in which both A and B are soluble to some degree is now passed through the column the substances may be removed, or desorbed, from the adsorbent. However, the rates at which A and B are desorbed are usually not the same because one of the substances is likely to be adsorbed more strongly than the other. The flowing solvent which is in competition for the solutes with the adsorbent, will remove the less strongly held substance (say, compound A) more readily and carry it further down the column, where it is again adsorbed. Compound B is desorbed more slowly, so it trails behind A. As the flow of solvent down the column continues, the process is repeated many times. This repetition magnifies the difference of distances travelled through the column by the two solutes so that components A and B become finally separated from one another. The process of moving a solute mixture through a chromatographic system is called *development*. [see Figure 5.1(b)].

A close analysis of the earlier paragraphs will reveal that adsorption column chromatography bears the same relationship to simple methods of separation by adsorption as distillation with a fractionating column bears to simple distillation. The analogy with the fractionating column is indeed close. Just as distillation with a fractionating column is equivalent to thousands of separate distillations, chromatography with its ordered movement of the mobile phase relative to the adsorbent is equivalent to a repeated stirring up with adsorbent and filtering.

5.1.2 Criteria to be met by Adsorbents

Adsorbents should satisfy several general requirements: (i) They are the ones chosen by keeping in view the relevance to the nature of the substances to be separated so that success in separation is attained (a knowledge of the nature of the adsorbent and the forces responsible for adsorption will be a pre-requisite for obtaining the best conditions for achieving a satisfactory separation); (ii) they

should not dissolve in or react with the solvents; (iii) they should be chemically inert to the components of the mixture; (iv) they should have high adsorption capacity; (v) they should exhibit very good reproducibility and reversibility in adsorption characteristics.

A few materials in order of decreasing polarity and adsorption strength are: Alumina > magnesium oxide > charcoal > silica gel > calcium oxide > magnesium carbonate > calcium carbonate > potassium carbonate > sodium carbonate > sucrose > starch > cellulose. Increasing the polarity of the stationary phase increases the retention time i.e. the time for which components of the mixture are retained on the column while these are being washed down the column.

Amongst the adsorbents listed above, alumina and silica gels are used most often.

Based on their capacity and activity, adsorbents have been classified as weak (e.g. sucrose, starch), medium (e.g. calcium carbonate, magnesium oxide) and strong (e.g. activated alumina, activated carbon, activated magnesia). Changes in adsorption activity of adsorbents can be brought about via one of the following pathways:

(i) Removal or addition of substances such as water, which block the active sites. The application of this procedure can be illustrated for the case of preparation of an alumina of the desired activity. The adsorptive activity of alumina is dependent upon the amount of water present in it and, in fact the Brockman scale of activity is based on the amount of water that the alumina contains:

I, 0% H_2O; II, 3% H_2O; III, 6% H_2O; IV, 10% H_2O and V, 15% H_2O. The alumina of desired activity can be prepared by dehydrating the adsorbent at 360ºC for 5 hours and then allowing the dehydrated material to adsorb suitable amount of water.

(ii) Improving the surface properties by a change in the functional groups or size of the pores. Standardized adsorbents constituted of particles of definite activity and mesh size are available commercially.

Mesh size is a conventional term that is used to define the size of particles constituting an adsorbent, the alternative to the use of this term being, of course,

Table 5.1

Mesh Sizes of Adsorbents and Typical Applications

Mesh size	Applications
20—50	Crude preparative work, very high flow rate
50—100	Preparative applications, high flow rate
100—200	Analytical separations, medium flow rate
200—400	Highly efficient separations, slow flow rates

the usual defining of particle size in terms of μ[micron(10^{-6} m). The use of this expression refers to a standard sieve through which the particles can pass. A 100 mesh sieve has 100 small openings per square inch. Adsorbing material with high mesh size (400 and greater) is extremely fine and gives highly efficient chromatographic separations. Table 5.1 lists standard mesh sizes with corresponding most appropriate application.

5.1.3 Criteria to be met by Solvents

The role of solvent in maximizing performance (i.e. achieving adequate resolution within a reasonable time) is significant and, in practice, it is found more convenient to change the solvent to effect a separation rather than to use different adsorbents. Amongst the requirements which have to be met by the selected solvent the obvious one is that the sample must dissolve in the solvent and the solvent should not interfere with the recovery of separated fractions. Also, the selected solvent has to be one which does not react irreversibly with the stationary phase (e.g. acids are not to be used along with basic alumina). In view of the large volumes of solvent employed, even trace impurities present in it can gradually alter the stationary phase in liquid chromatography. Consequently, stringent purity requirements are laid down for solvents employed in liquid chromatographic (LC) techniques.

The mobile phase competes with the sample components for adsorption sites on the stationary phase and thereby decreases the number of adsorption sites which are available for the solutes (i.e. sample components). Consequently, use of increasingly polar mobile phases decreases the retention times of solutes. Several solvents used in LSC in order of increasing polarity are: Fluoroalkanes, petroleum ether, carbon tetrachloride, cyclohexane, toluene, benzene, esters, chloroform, ethyl ether, dichloroethane, methyl ethyl ketone, acetonitrile, alcohols, water, pyridine, organic acids.

In practice, the selection of the solvent of the right strength can be made either by tedious trial and error method or by using the rapid open bed separation (thin layer chromatography). For strongly adsorbed compounds, it is usually best to use a polar solvent such as an alcohol, pyridine, or an ester, whereas with weakly adsorbed solutes the solvent is normally petroleum ether, carbon tetrachloride or cyclohexane. Mixtures of two or three solvents of different polarity often give better separation than unmixed solvents.

5.1.4 Order of Elution of Different Classes of Compound

Successful separations depend on proper selection of both the stationary phase as well as the solvent (mobile phase). This is partly a matter of trial and error, but there are certain guiding principles. Some classes of compound are adsorbed much

more strongly than others. Roughly speaking acids and strong bases are most strongly adsorbed, followed by alcohols and amines, then by aldehydes, ketones esters and halides. Lower in the list come unsaturated and aromatic hydrocarbons, and finally saturated hydrocarbons. Thus the following compounds, each containing 3 carbon atoms, stand arranged in the order in which they will be eluted from the column:

$CH_3CH_2CH_3$; $CH_3CH = CH_2$; $CH_3CH_2CH_2Cl$; CH_3COOCH_3; $CH_3CH(NH_2)$ CH_3; $CH_3CH_2CH_2OH$; CH_3CH_2COOH.

5.1.5 *Procedure for Separation by LSC and Detection and Quantification of Separated Constituents*

A packed column for LSC ordinarily consists of a glass tube packed with the adsorbent which is finely divided solid. Packed columns are usually prepared by pouring a slurry of the stationary phase in the mobile phase into a glass tube closed at the bottom by a cottonwool pad or perforated plate having openings too small to permit passage of the solid particles used. The column is then allowed to form by use of one or more of the following methods:

1. Simple gravity filtration.
2. Application of air pressure to the top of the column to hasten filtration and promote packing of the stationary phase.
3. Use of a ramrod (which often consists of a cork at the end of a glassrod) of diameter slightly less than that of the glass tube, to aid even packing of the stationary phase.

When wet packing as outlined above is carried out, care is taken to avoid air bubbles within the column and to produce as homogeneous a column as possible. After packing, the mobile phase is allowed to flow out of the column only until its upper level is at the level of the packing, in order to avoid air bubbles in the packing.

The sample, dissolved in the mobile phase is then added to the top of the column in the minimum possible volume. It is important to avoid disturbing the column packing by addition of the sample. Addition should be made in such a way that the sample forms a thin layer at the top of the column and penetrates the packing to an equal depth at all points. The sample is generally rinsed into the column with one or two small aliquots of mobile phase, each time allowing just enough effluent to issue from the column to bring the level of mobile phase just down to the surface of the packing. A large volume of the mobile phase is now added to the section of the chromatographic column above the packing.

The basic experiment of adsorption column chromatography or liquid-solid chromatography (LSC) is illustrated in Figure 5.1(a). The progressive separation of the components by the flowing solvent (eluent) is depicted in Figure 5.1(b). As is seen from the figure, very simple apparatus may be used; minor modifications

Solution

Column

Adsorbent

Direction
of solvent
flow

Cottonwool
pad

Increasing time

(b)

Receiver

(a)
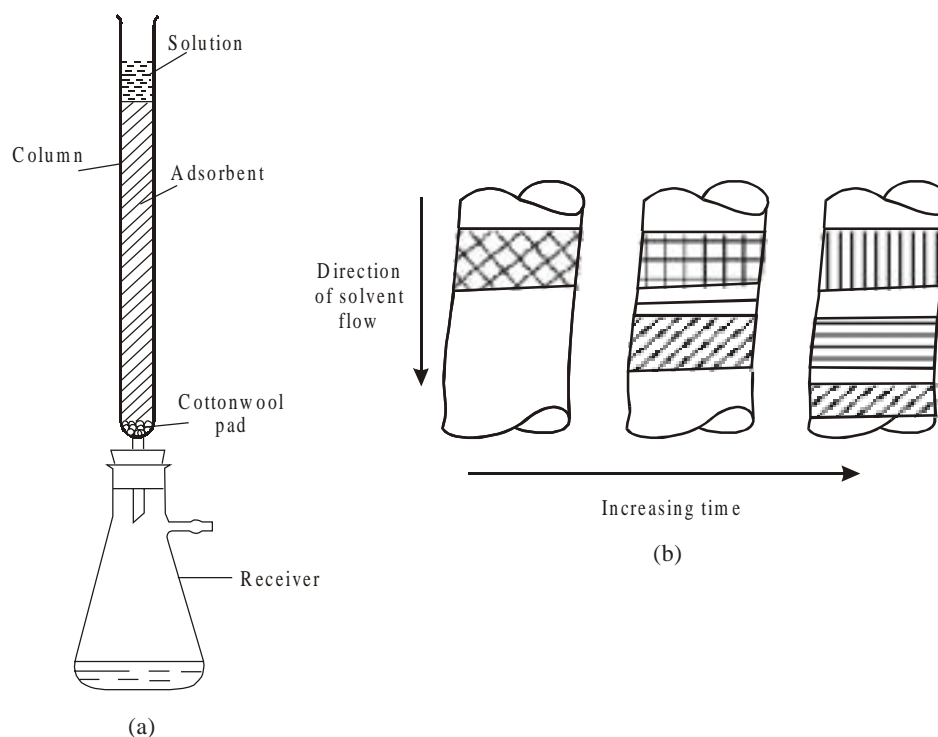
Figure 5.1 Adsorption column chromatography

may be made if, for example, one wishes to speed the process by suction or to work in an inert atmosphere. Columns from a few millimeters to several centimeters in diameter may be used, according to the scale of the experiment. Generally speaking a long thin column gives the best separation in difficult cases, but large quantities of readily separable substances can be treated more rapidly in a wide column.

It is much simpler to carry out the separation of coloured substances, because in such cases the separation can be followed visually and stopped at the right point [Figure 5.1(b)]. The contents are then pushed out bodily and cut into the various coloured zones, from which the components may be dissolved and recovered.

In the case of colourless materials, the separated substances may sometimes be rendered visible on the column by fluorescence, excited by an ultra-violet lamp. However, with colourless materials it is usually best to continue passing solvent down the column till all the sample has been washed through the column by the mobile phase. The mobile phase which leaves the column and now contains the solute molecules is called the effluent or eluate and it is collected as a number of fractions or 'cuts' at different times. The collection of liquid fractions (especially if the number of fractions is large) is both tedious and time-consuming so that, in practice, some form of mechanical device is employed. An automatic fraction

collector consists of a turntable in which a series of collection tubes are placed. The turntable is operated by an electrical device, and the receiver changes automatically after a given time or after a certain volume of the eluate has been delivered; at the same time provision is made for continuous feeding of the eluent (solvent) from a reservoir. If the chromatographic process has been effective, different collected fractions will each contain only one of the different components of the original sample.

The procedure of removing the components of a mixture sequentially from the column by sweeping them through with the mobile phase, as described above, is known as elution.

In the simplest case the eluent strength is kept constant during elution. This elution using throughout the same eluent (i.e. single solvent or mixed solvent of fixed composition) is called isocratic elution.

'Gradient elution' is the procedure wherein the composition of the mobile phase is progressively varied throughout the elution process. For example, if the solvent used is a mixture of ethyl alcohol and water, its composition is varied continuously with time from pure water to pure alcohol.

In the similar procedure of 'stepwise elution' the conditions under which elution is carried out are changed at predetermined intervals rather than progressively.

Besides 'elution analysis' there are two other procedures of separating and removing the various components of the given mixture from a column; these are 'frontal analysis' and 'displacement analysis'.

In 'frontal analysis' the sample solution is continuously added to the stationary phase at the top of the column. During such addition a stage reaches when components of the given sample start emerging from the column, the beginning being made by the component which has the smallest ratio of distribution between the stationary phase and the mobile phase. Other components follow and exit from the column in order of increasing distribution ratio but not separated as well as in the elution method.

In 'displacement analysis' the sample solution is added in one lot to the column packed with the stationary phase and this is followed by continuous addition of a solution of a substance which is more strongly attracted to the stationary phase than any of the sample components. Continuous addition of this solution moves the sample components down the column and these components leave the column in the order of their distribution ratios, but with much less overlap than in frontal analysis.

Frontal analysis and displacement analysis are of secondary importance only in comparison with elution analysis and neither of these two methods has any advantage over elution, except in preparative-scale separations where quantities to be dealt with are large.

The analytical part of the chromatographic experiment can be performed on the individual eluate fractions which have been collected at various time intervals of

the separation process; this process, though tedious in practice is usually accurate.

Alternatively, as is the usual practice now, analysis of the column effluent with regard to identification and quantitative estimation of the different sample components exiting from the column at various intervals can be carried out during the experiment by use of proper instrumentation, alongside of making collection of the eluate fractions. The changes indicated by the detector employed in the instrumentation system used are recorded on a chart recorder by a pen recorder coupled to the detector. The recorded plot of the detector response as a function of time or mobile phase volume is called a chromatogram. Detectors used in liquid chromatography are detailed in Chapter 6.

5.2 Applications of Adsorption Column Chromatography

Although developments in the field of chromatography since Tswett's work with a simple column have brought about a revolution in experimental techniques, conventional adsorption column chromatography still keeps its importance in its gravitational arrangement, owing to its simple working technique and large capacity.

Since the strength of adsorption is generally characteristic of functional groups in organic compounds, LSC is particularly useful in separating classes of compound. Adsorption chromatography has been widely used in the separations of phenols and amines. For amines, alumina is usually preferred since amines are basic and tend to be very strongly absorbed on silica which is acidic. On the other hand, acidic compounds such as phenols are chromatographed better on silica rather than on alumina.

From amongst the numerous applications of adsorption column chromatography, an interesting example is provided by the separation of a mixture of two enantiomers into its constituents whose separation cannot be carried out by the other usual physical methods like fractional crystallization or fractional distillation. A well-known illustration of the separation of enantiomers without their having to be converted into diastereoisomers by chemical reaction with optically active acids is the resolution of Troger's base, effected by V. Prelog and P. Wieland in 1944,
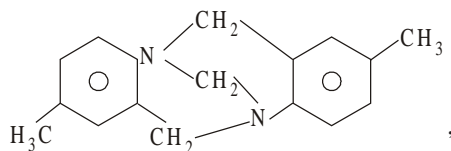
using adsorption column chromatography. The method is based on selective adsorption by natural asymmetric materials.

Attempted resolution of Troger's base by Prelog and Wieland with optically active acids was only partially successful, for the diamine is a relatively weak base and it also rapidly reverts into the dl-racemic mixture in acid solution. When the

base was adsorbed on specially ground d-lactose hydrate and the column eluted with petroleum ether, initial fractions were dextrorotatory, intermediate fractions optically inactive, and terminal fractions levorotatory.

In a modified procedure, an optically inactive adsorbent (such as alumina) has been coated with a thin layer of an optically active substance. Resolution of dl-mandelic acid is an example of separation belonging to this category.

In another procedure the racemic mixture is converted into a mixture of diastereoisomers (by reaction with an optically active compound) which is then chromatographed on a column of an optically inactive adsorbent to separate the diastereoisomers.

Two such closely related compounds as *cis* and *trans* isomers of azobenzene have been separated by exploiting the difference in polarities of the two isomers. It has already been mentioned earlier that as the polarity of a sample component increases, its retention time on an LSC column also increases. Thus, *cis*-azobenzene (I), which is the more polar one of the two azobenzenes, is retained on the chromatographic column longer than *trans*-isomer (II) whereby separation of the two geometrical isomers results.

I

cis-Azobenzene

II

trans-Azobenzene
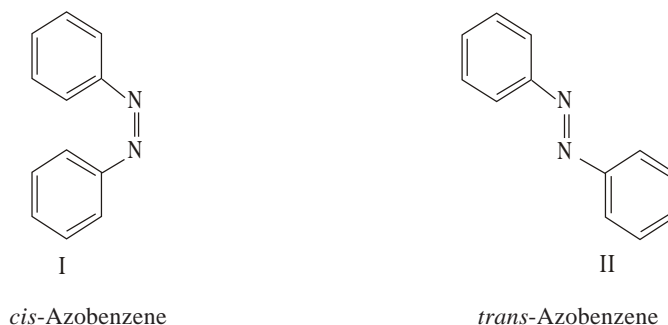
Compounds with higher molecular weights are retained on the column longer than those having lower lower molecular weights if a series is considered in which compounds have nearly identical polarities e.g. if the compounds have the same functional groups attached in the same way. This observation is in accordance with Traub's rule which states that the adsorption on an LSC column increases with increasing molecular weight for a homogeneous series of compounds. This is exemplified by the very important separation of mixtures of hydrocarbons in petroleum as given below.

In a typical separation of a petroleum fraction containing C_{18}–C_{25} hydrocarbons, an initial silica gel column is used to separate aromatics from paraffins and cycloparaffins. The aromatics are then chromatographed on an alumina column to separate them into groups containing one, two and three rings. Under appropriate conditions, silica gel can separate (i) olefines, paraffins, cycloparaffins

and aromatics, (ii) mono-olefines from di-olefines, (iii) mono-olefines from aromatics and (iv) some acyclic mono-olefines from cyclic mono-olefines.

Separation of mixtures of saturated hydrocarbons has been effected on silica gel or charcoal. Alcohols, phenols, aldehydes and ketones, acids, esters and lactones, amines, nitro compounds, azo compounds, halogen and sulphur compounds, sugars and glycosides, alkaloids, natural dyes, vitamins, harmones, antibiotics etc. have been separated under appropriate conditions.

Decreased adsorption of a sample group, X, which is close to a fairly large group, Y (probably due to the interference of Y with the close approach of X to an adsorbent site), has been exploited in some separations. For example, the *ortho* alkyl phenol derivatives are less strongly adsorbed on silica than para isomers and the difference in adsorption energies increases with the effective size of the alkyl group. An interesting reverse steric blocking effect has been reported in the adsorption of pyrrole derivatives on alumina. Bulky groups close to the nitrogen increase adsorption. This is attributed to the fact that acidity of these compounds is increased when the NH bond is strained.

Ortho di-substituted benzenes, capable of H bonding (e.g. di-amino or di-hydroxy benzene) are generally less strongly adsorbed on silica than their non-H bonding isomers.

The decomposition of some addition compounds of aromatic hydrocarbons (formed with picric acid, styphnic acid or trinitrobenzene) on an adsorbent column has been made use of for the preparation of pure hydrocarbons for spectral analysis. For example, chamazulene trinitrobenzene addition compound decomposes on alumina column. The pure hydrocarbon released is eluted with petroleum ether, leaving behind trinitrobenzene (which has high affinity for alumina) in the upper part of the column. This again can be eluted with more polar solvents.

Practical examples have also been reported in the use of specific adsorbents (e.g. preparation of silica gel with specific affinity for methyl orange). Such an approach involves preparation of the adsorbent in the presence of the concerned specific substance. Chromatography of a mixture of related substances has shown that there is a certain specificity for the substance in the presence of which the adsorbent was prepared.

Use has also been made of polar adsorbents (such as silica gel) modified by incorporating a complexing agent into the adsorbent. For example, separation of olefinic hydrocarbons from saturated hydrocarbons is improved when silica gel impregnated with $AgNo_3$ is used.

Localized adsorption, or the existence of discrete adsorption sites that are a 'fit' for the molecule being adsorbed, also plays an important part in selectivity, particularly in the separation of isomers. This high level of selectivity is exemplified by the separation of positional isomers e.g. *meta* and *para*

dibromobenzenes, on silica gel which has polar Si—OH adsorption sites. Because of the differences in molecular geometry, the *para* isomer is able to interact with two surface—OH groups, whereas the *meta* isomer can only interact with one and is less strongly retained.

Several cases of application of liquid-solid adsorption chromatography in the inorganic field have also been reported. The otherwise tedious separation in Inorganic Chemistry of the ions Li^+, Na^+, K^+ and Mg^{2+} when present together in a solution can be effected in the usual manner of adsorption column chromatography using purified silica gel G as the adsorbent and employing the mixed solvent, ethanol-acetic acid, for eluting the column. Similarly, separation of a mixture [e.g. $SbCl_3$, $AsCl_3$ and $Bi(NO_3)_3$], consisting of various cations and anions, has been carried out by liquid-solid column chromatography using alumina in the presence of tartaric acid. It is, however, to be stressed that these separations are not governed by adsorption effects alone. Effects such as fractional precipitation, hydrolysis or complex formation are possible.

Use of organic adsorbents such as 8-hydroxy quinoline has also been suggested for the separation of inorganic compounds.

■

<div align="center">

6

Partition Column Chromatography
[Liquid-Liquid Chromatography (LLC)]

</div>

Some mixtures do not respond well to the adsorption chromatographic technique and the partition process may be better suited to their separation. The partition chromatographic technique is the result of the historic discovery made by Martin and Synge in 1941. The stationary phase in liquid-liquid or partition column chromatography is a very thin film of liquid that is adsorbed on the surface of an inert solid material like kieselguhr, diatomaceous earth, cellulose powder or silica gel, the solid material constituting the column packing. The solid serves only a support for the liquid; it does not participate directly in the chromatography. A second liquid constitutes the moving phase.

6.1 Working of the Technique of Partition Column Chromatography

Consider the separation of a mixture of two constituents, A and B. The separation depends on what amounts to a large number of successive mini-extractions based on differences in distribution of components A and B in the two liquids. As the mobile phase passes through the column, substance A is preferentially extracted and moves downward more rapidly than B which is retained to a greater degree in the stationary phase.

In general, the mechanics of carrying out partition chromatography in a column are the same as those for adsorption, except for the preparation of the material for packing the column. The finely divided solid support as, for example, silica gel, is mixed with the liquid (water or other appropriate liquid) in a definite ratio by weight; the ratio used depends on the nature of the materials to be separated. The resulting mixture of stationary phase and solid support is actually a dry, free-flowing powder, not unlike the support material before treatment with the liquid. Thus silica gel absorbs about 70% by weight of water without becoming wet in the ordinary sense. The chromatographic tube, therefore, can be packed with this powder in the manner it is done for adsorption chromatography. Though dry packing is sometimes preferable to slurry packing, nevertheless dry packing is generally not applicable when the stationary phase is a liquid on an inert solid carrier.

The solvent pair constituting the stationary phase and the mobile phase must have low mutual solubility. Water is generally the stationary phase. Hydrophilic

organic solvents are the other liquids which can constitute the stationary phase in place of water. The solvent series for liquid-liquid chromatography is: Water > formaldehyde > methanol > acetic acid > ethanol > acetone > n-propanol > t-butanol > phenol > n-butanol > amyl alcohol > ethyl acetate > ether > butyl acetate > chloroform > benzene > toluene > cyclohexane > petroleum ether. This is also the order of decreasing capacity of these solvents for H-bonding.

For working of this technique, prediction of the optimum combination of stationary phase and mobile liquid phase is not always possible since the partitioning system may involve mixed solvents, solutions of salts, buffers, or complexing agents, and determination of the proper conbination of stationary phase and solvent system in partition chromatography is not unoften a matter of trial and error.

Collection of effluent fractions is followed by their analyses using the detection devices based on a physical property such as refractive index, condictivity, ultra violet or visible absorption.

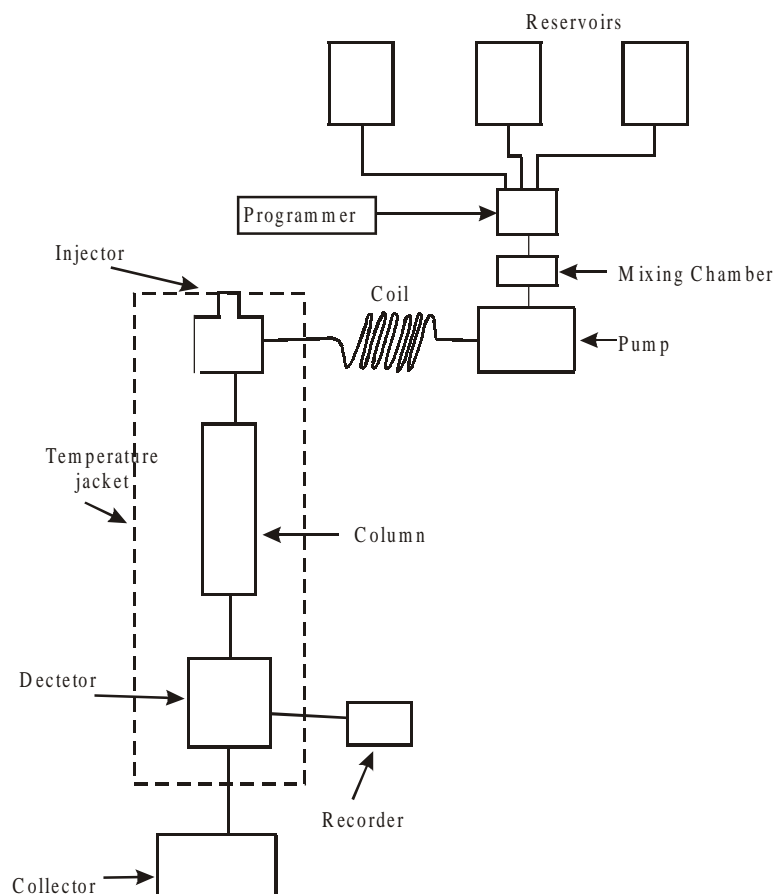Figure 6.1 gives design of a typical liquid chromatograph.

Figure 6.1 A typical design of a liquid chromatograph

In almost all cases of partition chromatography a mixed solvent system, sometimes requiring three solvents, is necessary, and a mixing chamber for solvents is an essential part of a liquid chromatograph. During the course of carrying out a particular chromatographic separation the need often arises for varying the composition of the mobile phase at different stages of the experiment. The operation is pre-programmed so that at any stage during the progress of the experiment only such quantities of the various solvents as are appropriate for that stage, are allowed to pass to the mixing chamber whose functioning brings about smoothly the needed variations in the composition of the mobile phase. The pump pulls the mobile phase from the reserviors, pushes the mobile phase through the column, maintaining all the while a constant rate of flow of the mobile phase during its passage through the column. A coil placed between the pump and the column dampens the pulsating action of the pump. Inserted above the column is the injection system. One type of the injection system, the syringe injector (Figure 6.2), involves a septum injection port where a syringe is used to inject the sample through an inert septum directly into the flowing phase. The second type of injection system involves a valve which can be rotated from the mobile phase tubing to a sample tubing. Thus the sample is injected into the sample line, the valve is rotated, and the mobile phase washes the sample into the main line. After this, the valve is returned to its original position. The sample mixture passes into the column and separation takes place, the effluent fractions being detected by one of the detection devices detailed below.
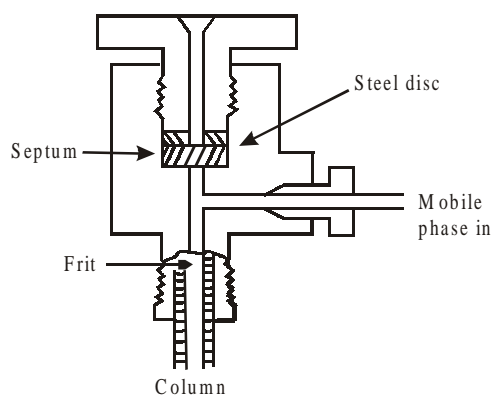
Figure 6.2 A syringe injector

6.2 LC Detectors

Two general type of detection devices are available. These are bulk property detectors and solute property detectors. The bulk property detector measures a change in some overall physical property in the mobile phase as it emerges from the column. Two typical examples are measurement of refractive index and conductance. The solute property detector is sensitive to changes in a physical property of the solute as it emerges from the column in the mobile phase; a typical example is the measurement of ultraviolet and / or visible absorption. In general,

the solute property detectors are more sensitive, particularly if the mobile phase does not contribute to the property to be measured.

Given below is the description of different types of detectors along with the underlying theory of working of each of them.

6.2.1 Absorption photometric detectors

When light of frequency υ is absorbed by a molecule, the electrons forming the chemical bonds are raised to higher energy levels (the molecule undergoes a transition from the ground state to one of the excited states with a higher energy content), the difference in energy level being given by the relation:

$$h\upsilon = E_1 - E_0,$$

where E_0 and E_1 are respectively the energies of the ground state and the excited state of the molecule.

Absorption of light energy may increase the vibrational energy (i.e. increase the amplitude of vibration of the atomic nuclei in the bonds), the rotational energy of the molecule as a whole (and parts of it about the chemical bonds) or cause the excitation of the outer valence electrons. To increase the rotational energy of the molecule, relatively small energy quanta are sufficient and, therefore, the corresponding absorption lies in the far infrared region—the region of longer wavelengths. The energies which are required to increase the vibrational energy of the molecule are provided by radiation in the near infrared region.

Higher energy quanta are required to excite the outer electrons of the molecule (absorption in visible and ultraviolet regions). The quanta required to change the electronic energy are about one or two orders of magnitude higher than those required to change the vibrational energy. Therefore, absorption in the ultraviolet region causes electronic, vibrational or rotational transitions; absorption in the long-wavelength infrared region is accompanied by the change of the rotational energy alone.

Absorption photometric detectors are based on the measurement of the relation between the intensity of absorption of light and the light frequency (or wavelength). For this measurement, a beam of monochromatic light (light of specific wavelength), whose wavelength is gradually changed with the aid of a special instrument called a monochromator, is allowed to pass through layer of a substance (more often in solution). The intensity of transmitted light, I, is measured and transmitted to the recorder which registers it in the form of a graph (spectrum), with the intensity I plotted as the ordinate and the wavelength or frequency as the abscissa.

The measurement procedure described above is characteristic of so-called single-beam instruments. The operation of current spectro-photometers is based on

the double-beam principle. The radiation detector and the recording device register not only the intensity of light, I, transmitted through the substance but also the incident intensity, I_0. The signal is recorded either as the difference I_0–I or as the ratio I/I_0; measures must be taken in order that both light beams—incident and transmitted—be optically equivalent. When absorption spectra of solutions are measured, one beam (I_0) passes through a cell (cuvette) full of pure solvent (or reference substance), and the other (I) through a cell containing a solution of the substance under study.

When working in the ultraviolet spectral region, use is made of quartz optics, and in the infrared region, salt prisms are employed (prisms made of Na Cl, LiF and KBr, depending on the wavelength); the common radiation sources are lamps or (in the infrared region) glowing rods made of specific materials.

There are two fundamental laws in spectrophotometry, which describe the absorption of light by matter. These are Lambert's and Beer's laws.

Lambert's law states that the intensity of transmitted light passing through a homogeneous medium decreases geometrically as the thickness of the layer increases arithmatically, that is, if a certain thickness absorbs half the light, then the thickness which follows the first one and is equal to it will not absorb the remaining half the light, but only the half of this half and will consequently reduce it to one-quarter.

Beer's law states that each molecule of solute absorbs the same fraction of light incident upon it, regardless of concentration in a non-absorbing medium, which stated in other words, means that the fraction of the incident light absorbed by a solute is proportional to the number of molecules of the solute in the path of the light (doubling of the concentration thus amounts to doubled thickness of the absorbing layer at the earlier concentration). Beer's law does not hold good over an entire concentration range and deviations from this law are accounted for by the different degrees of association, solvation and dissociation of molecules in solutions of different concentrations.

Combination of Lambert's and Beer's laws can be mathematically expressed by the relationship:

$$A = \log \frac{I_0}{I} = a \; b \; C,$$

where A is optical density or absorbance, a is the absorptivity, a constant dependent upon the wavelength of the radiation and the nature of the absorbing material whose concentration, C, is expressed in grams per litre, and b is the optical path length.

Light absorption may be registered as graphs (spectra) in terms of various quantities:

(i) $\dfrac{I}{I_0}$ = transmittance (transmission);

(ii) $100\dfrac{I}{I_0}$ = per cent transmittance;

(iii) $\dfrac{I_0 - I}{I_0}$ and $100\dfrac{I_0 - I}{I_0}$ = fraction of absorbed light and per cent absorbance;

(iv) $A = \log\dfrac{I_0}{I}$ = optical density (absorbance or extinction);

(v) $A_{1cm}^{1\%}$ = absorbance of 1 cm layer of solution which contains 1% by weight of absorbing solute.

In ultraviolet spectra, usually the wavelength [in millimicrons (mμ) or nanometre (nm), 10^{-9} m] is plotted on the abscissa and ε or log ε on the ordinate, ε being the molar extinction coefficient, which is the optical density of a 1 M solution for a layer thickness of 1cm i.e. $\varepsilon = \dfrac{A}{bC}$ where C is concentration in moles per litre under specified conditions of wavelength and solvent. Ultraviolet spectroscopy usually employs very dilute solutions so that the deviations from Beer's law are small.

When infrared spectra are recorded, the frequency [in reciprocal centimetres (cm^{-1})] or, less often, the wavelength [in microns (μ)], [in microns (μ)] is plotted as the abscissa, and the per cent absorbance or per cent transmittance is plotted as the ordinate.

The change of the energy of the molecule caused by absorption of the light energy (energy transitions) refers to a definite type of electronic or vibrational excitation (for each purely vibrational transition there is a progression of closely spaced rotational levels) of the molecule. The maxima in the absorption spectra correspond to the most probable transitions between the energy levels of the molecule, so that absorption spectra are intimately associated with the structure of the molecule. Certain maxima in the absorption spectra are so characteristic of some groups of atoms or bonds that the position of these maxima varies little from compound to compound containing the same functional group or the same bonds. Such are, for example, the valence vibrations of the bonds: C=O (about 1700 cm^{-1}), C $\equiv$ N (Ca, 2250 cm^{-1}), O–H (3550 – 3650 cm^{-1}) (see Table 6.1) shown in the IR spectra. They are called characteristic IR bands and enable determining the presence or absence in the molecule of the various groups of atoms or bonds. In UV spectra, points which represent wavelengths where absorption reachs maximum are called λ_{max} (where ε is represented as ε_{max}), and

these again can provide valuable aid for the detection of functional groups present in unknown molecules, though ultraviolet-visible spectra are not as useful as infrared spectra in this regard.

The detectors used most often for LC are absorption photometric detectors and these are exemplified by the following description of some particular UV and IR photometers.

6.2.1.1 UV Detectors

UV detectors have many positive characteristics including high sensitivity, small sample volume, linearity over wide concentration ranges and non-destructiveness of sample.

FIXED WAVELENGTH DOUBLE-BEAM UV PHOTOMETER

Shown schematically in Figure 6.3 is one of the more popular LC detectors, a simple fixed wavelength double-beam UV photometer. The source of light used is a low-pressure mercury lamp which gives most of its output at 254 nm. The optical system of the detector is designed so that enough radiant energy can be made to pass through the narrow absorption cells, which are limited to about 1 mm diameter to avoid dead space. The sample and reference cells consist of cylindrical channels drilled in a block of stainless steel or Teflon and closed by silica windows. UV radiation from the lamp, collimated by a lens, passes through the two cells. The sample cell contains the continuously flowing column effluent. The reference cell is
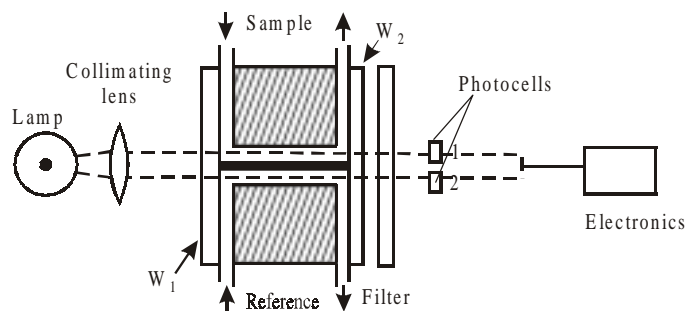
(W$_1$, W$_2$: UV transmitting windows)
Figure 6.3 A typical photometric LC detector.

usually filled with air. The intensity of light that passes through each cell depends upon the amount of light absorbed in the respective cell. The radiation passing through the reference and sample cells travels through a UV filter for removal of unwanted radiation and falls on two photocells i.e. detectors which are light-sensitive and can detect small amounts of light energy. The outputs of these two detectors are passed through a preamplifier to a log comparator that produces the electric signal for the recorder. The log comparator is necessary to convert the photons measured at the photodetector (transmittance) into absorbance units that are directly proportional to concentration.

This UV detector is inexpensive, sensitive to normal flow and temperature fluctuations, and well suited to gradient elution. It is, however, a selective detector. Only sample molecules which absorb at 254 nm can be detected.

Variable Wavelength UV Detector

Many analytes (i.e. the separated compounds as they emerge in the effluent from the column) do not absorb at 254 nm and a variety of UV/VIS detectors offering other wavelengths have become available.

A variable wavelength UV detector of this type, a spectrophotometer, produces light of a preselected wavelength, directs it through the sample cell, and measures the light transmitted by the cell.

Figure 6.4 shows a schematic spectrophotometer used as an LC detector.

For absorption measurements in the ultraviolet region, a high pressure hydrogen or deuterium lamp is used. These lamps produce radiation in the 200–320 nm
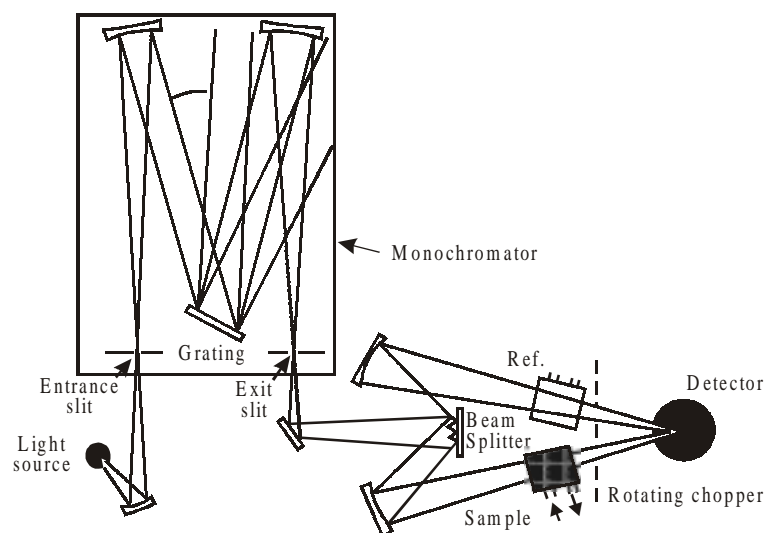
Figure 6.4 Schematic spectrophotometer used as an LC detector

range. The light source for visible region is the tungsten lamp, with a wavelength range of 320–800 nm. Instruments with both lamps have greater flexibility and can be used for the study of a wide variety of molecular species.

Both lamps discussed above produce continuous emissions of all wavelengths within their range. Therefore, a spectrophotometer must have an optical system to select monochromatic light. Modern instruments use a diffraction grating usually to produce the desired wavelengths.

Before the monochromatic light impinges on the sample, it passes through a system of necessary condensing optics which focusses it with sufficient intensity

towards the sample. By varying the position of the grating, the desired wavelength is focussed on the exit slit and then passed through sample and reference cells by means of a beam splitter. The detector measures the difference between intensities of light emerging from sample and reference cells. The detector signal is converted into absorbance by a logarithmic comparator. Direct readout of absorbance is given by a meter either in analogue form (i.e. indication by means of hands moving round a dial as time is indicated in a traditional clock) or digital form (i.e. display of measurements by means of changing numbers as in a digital clock).

To be generally more useful in terms of selectivity, a spectrophotometer, with its continuously variable wavelength, is desirable, even though there will be some loss of sensitivity. This spectrophotometer provides an additional degree of selectivity not possessed by other LC detectors, in being able to discriminate between various components of the column effluent. An *absorbance spectrum* of a compound is obtained by scanning a range of wavelengths and plotting the absorbance at each wavelength. Most double-beam spectrophotometers automatically scan the desired wavelength range and record the absorbance as a function of wavelength. If solvent is present in the reference cell and column effluent in the sample cell, the instrument will continuously and automatically subtract the solvent absorbance from the total (solvent plus sample) absorbance at each wavelength. A scan of wavelength vs. absorbance is given by a recorder. Some spectrophotometers are equipped with pen recorders, but ordinary strip chart recorders can be connected to most spectrophotometers.

The ultraviolet detector is suitable for compounds containing π-bonding and nonbonded electrons.

6.2.1.2 Infrared Photometers

Absorption in the infrared region can be used both as a general and as a specific detector. For use as a general detector a stopped-flow technique must be used. In

TABLE 6.1

Infrared Absorption Frequencies

Compound type	Functional group	cm^{-1}
Aliphatic hydrocarbons	$— C — H$	2800–3000
Olefine	$>C = C<$	1600–1670
Nitrile	$– C \equiv N$	2210–2260
Alcohol	$— CH_2\ OH$	3610–3640
Carbonyl	$CH_3 — \overset{\displaystyle O}{\overset{\displaystyle \parallel}{C}} — O — R$	1735
Ketone	$R — \underset{\displaystyle O}{\underset{\displaystyle \parallel}{C}} — R$	1715

this mode the sample can be held in the detector cell and the wavelength scanned to detect various functional groups. Alternatively, the sample may be trapped out and analysed.

As a specific detector the wavelength is set at a particular value for the detection of a single functional group. Table 6.1 lists some typical functional groups with their respective infrared absorption frequencies. Of course, a detector operating at 3.4 μ (2800 cm^{-1}) will respond to any organic compound exhibiting a C—H absorption and is essentially a universal detector.

An example of an infrared detector is the Miran Detector, which is a single-beam spectrometer with a continuously variable filter that scans from 2.5 to 14.5 μ (4000 to 690 cm^{-1}), with a resolution of 0.12 μ at 5 μ. Thus both stopped-flow with scan as well as single-wavelength monitoring is possible. The detector is not sensitive to temperature or flow-rate variations. The detector can be used with gradient elution, although shifts in base-line (i.e. the recorded detector response when only the eluent is passing through the detector) are experienced.

One of the major problems with the infrared detector is the choice of mobile phase, particularly when using the scan mode. Since IR detectors measure absorbance at selected wavelength the availability of 'spectral windows' in the spectrum of the mobile phase must be considered as well as the usual chromatographic requirements. A spectral window is commonly defined as a region of the spectrum where the solvent, in a sample cell of given path-length, shows at least 30% transmittance. Thus, solvents in a longer cell will have fewer windows e.g. with a l mm path-length cell acetonitrile has a window from 6–8 μ and from 8.5–11 μ; however, both of these regions are spectrally opaque in a 3 mm cell. This suggests that short path-length cells are advantageous. Indeed at a path-length of 0.1 mm, most common chromatographic solvents have substantial windows, but the shorter path-length reduces the sensitivity of the detection.

Since the magnitude of the absorption varies from one solvent to another and from one wavelength to another, base-line shifts are usually observed during gradient elution. These base-line shifts are often linear with concentration of the second solvent. The size of such shifts depends on the extent of the compositional change and on the absorbance characteristics of the mobile phase, the optical path-length and the detector sensitivity.

The principle of absorbance matching may be applied to IR detection to reduce these base-line shifts. Absorbance matching involves the blending of solvents to give mobile phase pairs with similar optical absorbance for use in gradient elution. For example, using acetonitrile as the primary solvent and a secondary solvent consisting of a mixture of methylene chloride and tetrahydrofuran that closely matches the absorbance of acetonitrile, carbonyl-containing compounds may be eluted under gradient conditions with minimal base-line shift, and can be monitored at 5.8 μ.

6.2.2 Fluorescence Detectors

As discussed earlier, interaction of photons with molecules gives molecules that are said to be in an excited state consequent upon the promotion of valance electrons from ground state orbitals to higher energy level orbitals. Molecules in the excited state do not remain there long, but spontaneously relax to the more stable ground state. With most molecules, the relaxation process is brought about by collisional energy transfer to solvent or other molecules in the solution. In the case of some excited molecules, however, this relaxation process, which is very rapid, leaves the molecule in the lowest vibrational level of the excited state from where the molecule may return to some vibrational level of the ground state by release of energy emitted as light; this phenomenon is called fluorescence.

Two important characteristics of the emitted light are: (1) It has wavelengths that are all longer (and, therefore, of lower energy) than the wavelength of the excitation light (this being so because part of the energy intially associated with the excited state is lost as heat energy, and because the energy lost by emission may be sufficient only to return the excited molecule to a higher vibrational level of the ground state); (2) the emitted light is composed of many wavelengths and the result is a fluorescence spectrum. This is due to the fact that fluorescence from any particular excited molecule may return the molecule to one of many vibrational levels in the ground state. Just as in the case of an absorption spectrum, a wavelength of maximum fluorescence is observed, and the spectrum is composed of a wavelength distribution centred at this emission maximum; of course, all emitted wavelengths are longer and of lower energy than the initial excitation wavelengths.
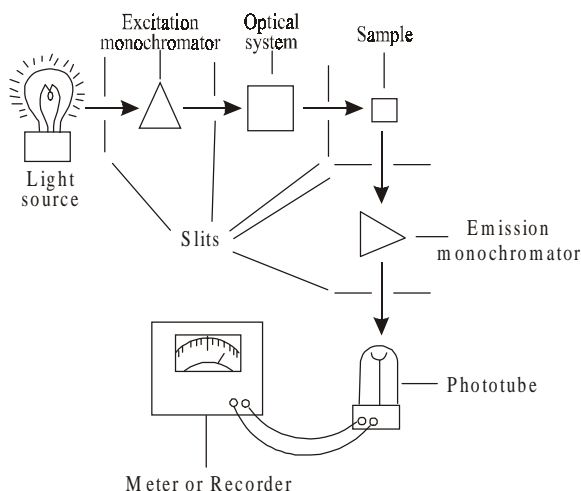
Figure 6.5 Set-up of a typical fluorometer

The basic instrument for measuring fluorescence is the spectrofluorometer. A typical experimental arrangement for fluorescence measure-ments is shown in Figure 6.5. The set-up is similar to that for absorption measurements with two significant differences: (i) There are two monochromators, one for selection of the wavelength, another for wavelength analysis of the emitted light; (ii) the detector is at an angle (usually 90º) to the excitation beam so as to eliminate interference by the light that is

transmitted through the sample. Upon excitation of the sample molecules, the fluorescence will be emitted in all directions and is detected by a photocell at right angles to the excitation light beam.

The light source used in most instruments is a Xenon arc lamp that emits radiation in the ultraviolet, visible, and near infrared regions (200 to 1400 nm). The light is directed by an optical system to the excitation monochromator, which allows either the preselection of a wavelength or scanning of a certain wavelength range. The exciting light then passes into a cuvette which is connected to the exit of the chromatographic column so that it contains the continuously flowing column effluent. Because of the geometry of the optical system, a special fluorescence cuvette with four translucent quartz or glass sides must be used instead of a typical fused absorption cuvette with two opaque sides. When the excitation light beam impringes on the sample cell, molecules in the solution are excited, and some will emit light.

Light emitted at right angles to the incoming beam is analysed by the emission monochromator. In most cases, the wavelength analysis of emitted light is carried out by measuring the intensity of fluorescence at a preselected wavelength (usually the wavelength of emission maximum). The analyzer monochromator directs emitted light of only the preselected wavelength toward the detector. A photomultiplier tube (PMT) serves as a detector to measure the intensity of the light. The output current from the photomultiplier is fed to some measuring device that indicates the extent of fluorescence.

Fluorescence measurements, unlike absorption, are temperature-dependent. All solutions, especially if relative fluorescent measurements are taken, must be thermostated at the same temperature.

Fluorescence detectors are becoming more popular due to their selectivity and sensitivity. Selectivity frequently means that the compounds of interest can be readily detected when present in a complex mixture of compounds that do not exhibit fluorescence. Sensitivity can be measured in terms of parts per billion. Both fixed wavelength and scanning fluorescence units are available.

Fluorescence is exhibited by molecules that contain multiple conjugated double bonds with an electron-donating group like $-NH_2$ or $-OH$ being present on the resonating nucleus. The fluorescence detector thus is widely used as an analytical tool for the determination of polycyclic compounds, aromatic amines and phenols. In Inorganic Chemistry, the detector finds its most frequent use in the determination of metal ions. Chromatographic separation of metal ions is carried out through their complexation with organic reagents and this complexation promotes fluorescence. The fluorescence detector is specially important in analyses of biomolecules. The amino acids with phenyl rings (phenylalanine, tyrosine and tryptophan) are fluorescent; hence proteins containing these amino acids are fluorescent. Similarly, nucleic acids and some coenzymes (NAD, FAD) are also

fluorescent because purine and pyrimidine bases (i.e. adenine, guanine, cytosine, uracil) are constituent units of their structures.

6.2.3 Refractometric Detector

Another type of important detector is the differential refractometer. This device continuously monitors the difference between the refractive index of pure solvent (mobile phase) and that of the mobile phase containing sample (column effluent), and depends for its sensitivity solely on the considerable difference in the index of solvent and that of solutes. Differences in the index of refraction can be measured to about 1 part in 10^7, which corresponds to a few parts per million of an organic solute in water, so that this detector is only moderately sensitive compared to the detectors discussed earlier. The advantage of refractive index detection is that it can be employed with solvents whose physical properties (such as UV absorption) might interfere with other modes of detection. The versatility of use of the refractometer detector is its major advantage. However, this advantage has a major limitation in that this type of detector cannot be used with gradient elution because the change in index due to solvent programming completely overwhelms any signals produced by the eluted components.
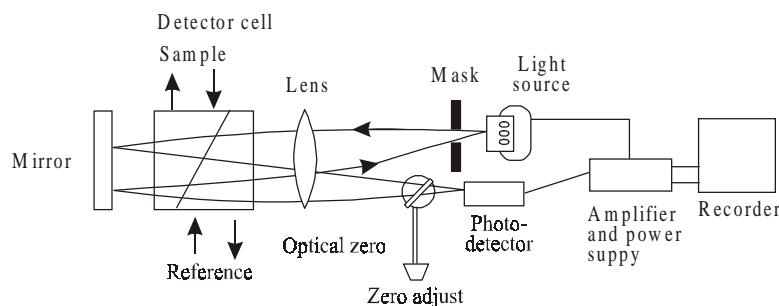
Figure 6.6 Schematic refractometric LC detector.

Figure 6.6 shows a refractometer detector whose working is based on the angular displacement undergone by a light beam on passing through two liquid-filled prisms. If the two liquids are identical, the displacement will be nil, but even a change of a few parts per million in the refractive index will make a detectable difference in the angle of the beam. The photodetector is sensitive to the location of the light beam rather than its intensity, so that its output changes as the beam moves across it. The zero-adjusting device is merely a glass plate that can be turned so as to displace the light beam slightly.

All the detectors described above are non-destructive to the samples so that column effluent can be collected for further physical/chemical characterization and any further use.

6.2.4 Other Detectors

(i) Reaction detector: In this detector, the liquid stream from the column is mixed continuously with a substance that will react with the expected species to form an absorbing or fluorescing product. The combined flow is then passed through an appropriate optical detection (i.e. a UV detector or a fluorescence detector). An example of the application of this principle is the determination of proteins and amino acids by using the polycyclic aromatic compound, fluorescamine (Fluoram), as the appropriate reagent. This reagent reacts with primary amines to give products that fluoresce strongly at 475 nm upon excitation at 390 nm.

(ii) The dielectric constant detector utilizes a narrow space between two metal electrodes as a capacitor. The column effluent, passing through this channel, causes a variation in the capacitance according to its dielectric constant. LC solvents vary greatly in values of their dielectric constants, from about 2 for symmetrical molecules such as benzene and cyclohexane to greater than 180 for such a highly polar compound as methyl formamide. Deviations seen by the detector can give either positive or negative peaks according to whether the solute component has a dielectric constant value greater or less than that of the solvent. This provides a detector of wide utility, somewhat more sensitive than that based on the refractive index.

(iii) Electrochemical detection finds application in reversed-phase chromatography (see page 101) wherein the components of the sample are electroactive. Components of the eluate from the column are subjected to oxidation or reduction on the surface of an electrode whereby a small current is produced. The current produced is proportional to the amount of material oxidized or reduced.

The potential between the electrodes in the electrochemical cell is adjusted to a value at which the sample components are electroactive. An amperometeric detection system keeps monitoring the current flowing through the cell as the effluent from the chromatographic column is continuously directed through the cell. As an alternative monitoring system, coulometry can be employed, this method of analysis being based on the application of Faraday's laws of electrolysis relating the equivalence between quantity of electricity passed and chemical change occurred.

Another instrument called the *transport detector*, used for detection of lipids, proteins or carbohydrates, requires the transport of the column eluent by a moving wire disc, chain or helix. The solvent is evaporated in a furnace and the non-volatile sample passes into a flame ionization detector (FID) which is detailed later under gas chromatography (GC) wherein FID counts amongst the major detectors.

(iv) Radioactive detectors: Detectors used for measuring the radioactivity of solutes separated chromatographically are based on (a) standard scintillation

systems and (b) Geiger counting. Sensitivities obtainable with flow-through systems are lower than those obtained in case of counting individual samples from a fraction collector. The sensitivity can be increased by increasing the counting time, but this can only be achieved by a lowering of mobile phase rates, or by using larger volume flow cells so that the residence time of the solute in the detector is increased. However, both of these possibilities lead to lower chromatographic efficiencies. A stopped-flow technique, however, allows for larger counting times without destroying the chromatographic separation. Radiochemical methods of detection are, therefore, best suited for use with relatively large columns and long analysis times.

(a) Liquid Scintillating Counting

Samples for liquid scintillation counting consist of three components viz. (1) the radioactive material, (2) a solvent, usually aromatic, in which the radioactive substances is dissolved or suspended, and (3) one or more organic fluorescent substances. Components (2) and (3) make up the liquid scintillation system. The β particles emitted from the radioactive sample (most of the radioisotopes used in biochemical research are β emitters) interact with the scintillation system, producing small flashes of light or scintillations. The light flashes are detected by a photomultiplier tube (PMT). Electronic pulses from the PMT are amplified and registered by a counting device called a scaler.

The scintillation process, in detail, begins with the collision of emitted β perticles with solvent molecules, S:

$$\beta + S \longrightarrow S^* + \beta \text{ (less energetic)}$$
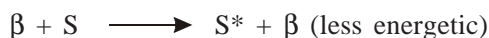
Contact between the energetic β particles and S in the ground state results in transfer of energy and conversion of an S molecule into an excited state, S^*. Aromatic solvents such as toluene or dioxane are most often used because their electrons are easily promoted to an excited state orbital. The β particle after one collision still has sufficient energy to excite several more solvent molecules. The excited solvent molecules normally return to the ground state by emission of a photon, $S^* \rightarrow S + h\upsilon$. Photons from the typical aromatic solvent are of short wavelength and are not efficiently detected by photocells. A convenient way to resolve this technical problem is to add one or more fluorescent substances (fluors) to the scintillation mixture. Excited solvent molecules interact with a *primary fluor*, F_1, energy being transferred from S^* to F_1, resulting in ground state S molecules and excited F_1 molecules, F_1^*:

$$S^* + F_1 \longrightarrow S + F_1^*$$
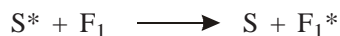
F_1^* molecules are fluorescent and emit light of a longer wavelength than S^*:

$$F_1^* \longrightarrow F_1 + h\upsilon_1.$$

If the light emitted during the decay of F_1^* is still of a wavelength too short for efficient measurement by a PMT, a *secondary fluor*, F_2, that accepts energy from F_1^* may be added to the scintillation system. The following two equations outline the continued energy transfer process and fluorescence of F_2:

$$F_1^* + F_2 \longrightarrow F_1 + F_2^*$$

and

$$F_2^* \longrightarrow F_2 + h\upsilon_2.$$
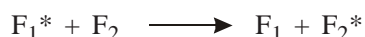
The light, $h\upsilon_2$, from F_2^* is of longer wavelength than $h\upsilon_1$ from F_1^* and is more efficiently detected by a PMT. Two widely used primary and secondary fluors are 2, 5-diphenyloxazole (PPO) with an emission maximum of 380 nm, and 1,4-bis-2–(5-phenyloxazolyl)-benzene (POPOP) with an emission maximum of 420 nm.

The most basic elements in a liquid scintillation counter are the PMT, a pulse amlifier, and a counter, called a scaler. This simple assembly may be used for counting; however, there are many problems and disadvantages with this set-up. Many of these difficulties can be alleviated by more sophisticated instrumental features. Some of the problems and practical solutions are outlined below.

Thermal Noise in Photomultiplier Tubes: The energies of the β particles from most β emitters are very low. This, of course, leads to low energy photons emitted from the fluors and relatively low energy electrical pulses in the PMT. In addition, photomultiplier tubes produce thermal background noise with 25 to 30% of the energy associated with the fluorescence-emitted photons. This difficulty cannot be completely eliminated, but its effect can be lessened by placing the samples and the PMT in a freezer at –5 to –8ºC in order to decrease thermal noise.

A second method to help resolve the thermal noise problem is the use of two photomultiplier tubes for detection of scintillations. Each flash of light that is detected by the photomultiplier tubes is fed into a coincidence circuit. A coincidence circuit counts only those flashes that arrive simultaneously at the two photodetectors. Electrical pulses that are the result of simultaneous random emission (thermal noise) in the individual tubes are very unlikely. A schematic diagram of a typical scintillation counter with coincidence circuitry is shown in Figure 6.7. Coincidence circuitry has the disadvantage of inefficient counting of very low energy β particles emitted from ^{3}H and [14]C.

Counting more than One Isotope in a Sample: The basic liquid scintillation counter with coincidence circuitry can only be used to count samples containing one type of isotope. Many experiments in Biochemistry require the counting of just one isotope; however, more valuable experiments can be performed if two radio-isotopes can be simultaneously counted in a single sample (double-labeling experiments). The basic scintillation counter previously described has no means of discriminating between electrical pulses of different energies.

The size of current generated in a photocell is nearly proportional to the energy of the β particle initiating the β pulse. β particles from different isotopes have

characteristic energy spectra
with an average energy.
Modern scintillation counters
are equipped with pulse
height analyzers that measure
the size of the electrical pulse
and count only those pulses
within preselected energy
limits set by discriminators.
The circuitry required for
pulse height analysis and
energy discrimination of β
particles is shown in Figure
6.7. Discriminators are
electronic 'windows' that can
be adjusted to count β
particles within certain
energy or voltage ranges
called *channels*. The channels
are set to a lower limit and an
upper limit, and all voltages
within these limits are
counted. Discriminator
number 1 is adjusted to
accept typical β particles
emitted by ^{3}H and channel

Figure 6.7 Block diagram of a typical scintillation counter, showing coincidence circuitry.

number 2 is adjusted to receive β particles of the energy characteristic of ^{14}C.

Practical Aspects of Scintillation Counting

I. Quenching

Any chemical agent or experimental condition that reduces the efficiency of the scintillation and detection process leads to a reduced level of counting, or *quenching*. There are four common origins of quenching.

Colour quenching is a problem if chemical substances that absorb photons from the secondary fluors are present in the scintillation mixture. Since the secondary fluors emit light in the visible region between 410 and 420 nm, coloured substances may absorb the emitted light before it is detected by the photocells. Radioactive samples must be treated to remove coloured impurities before mixing with the scintillation solvent.

Chemical quenching occurs when chemical substances in the scintillation solution interact with excited solvent and fluor molecules and, hence, decrease the

efficiency of the scintillation process. To avoid this type of quenching the sample can be purified or the concentration of the fluors can be increased.

Point quenching occurs if the radioactive sample is not completely dissolved in the solvent. The emitted β particles may be absorbed before they have a chance to interact with solvent molecules. The addition of solubilizing agents such as C_{ab}–O–Sil or Thixin decreases point quenching by converting the liquid scintillator to a gel.

Dilution quenching results when a large volume of liquid radioactive sample is added to the scintillation solution. In most cases, this type of quenching cannot be eliminated, but it can be corrected. Modern scintillation counters are equipped with a standard radiation source inside the instrument but outside the scintillation solution. The radiation source, usually a gamma emitter, is mechanically moved into a position next to the vial containing the sample, and the combined system of standard and sample is counted. Gamma rays from the standard will excite solvent molecules in the sample, and the scintillation process occurs as previously described. However, the instrument is adjusted to register only scintillations due to γ particle collisions with solvent molecules.

II. Sample Preparation

Many of the quenching problems discussed above can be lessened if an experiment is carefully planned. A wide variety of sample preparation methods and scintillation liquids are available for use. Some of the more common techniques are mentioned below.

Two major types of solvent are used, those that are immiscible with water and those that dissolve in water. Table 6.2 lists some of the more popular scintillation systems.

A mixture of solvent and fluor(s) [e.g. dansylchloride,]
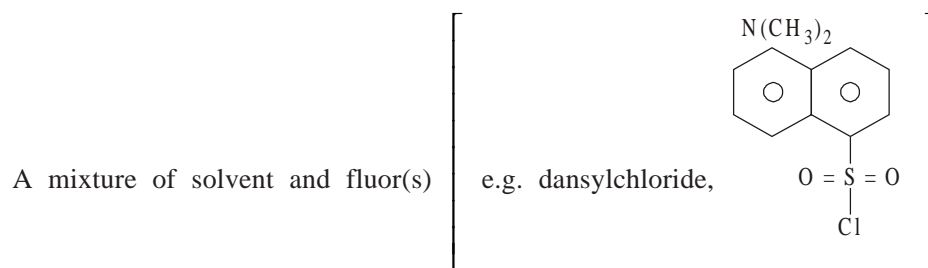
is popularly called a *scintillation cocktail*. Toluene-based cocktails are most extensively used for non-aqueous radioactive samples. Many biological samples, however, contain water or are not soluble in toluene solutions. Dioxane-based cocktails have been developed that dissolve most hydrophilic samples; however, dioxane causes some quenching itself. Bray's solution, containing the polar solvents ethylene glycol, methanol, and dioxane, is especially suitable for aqueous

samples. Toluene-based cocktails can be used with small amounts of aqueous samples if an emulsifying agent such as Aquasol, Bisolov, C_{ab}–O–Sil, or Triton is added.

TABLE 6.2

Useful Scintillation Systems

For Non-polar Organic Compounds:
1. Toluene-based: PPO, POPOP (or dimethyl POPOP) in toluene.

For Aqueous Solutions:
1. Toluene-based: PPO, POPOP, emulsifying agent (Aquasol, Biosolv, C_{ab}–O–Sil or Triton - X 100) in toluene.
2. Bray's solution: PPO, POPOP, naphthalene, methanol, ethylene glycol in dioxane.
3. Dioxane-based: PPO, p-bis (O-methylstyryl) benzene, naphthalene in dioxane.

For CO_2 and Acidic Substances:
1. Toluene-dioxane-based: PPO, POPOP, naphthalene in dioxane-toluene. Sample is prepared in a base such as hyamine hydroxide.

Radioactive samples which are insoluble in both toluene and water must be pretreated before analysis. Some insoluble samples can be chemically or physically transformed into a soluble form. A common method is to burn the sample under controlled conditions and collect the ^3H_2O and/or $^{14}CO_2$ in the scintillation solution. Alternatively, the radioactive substance can be collected on cellulose or fibreglass membrane filters. The filter with the imbedded radioactive sample is placed directly in a scintillation vial containing the proper cocktail. Acidic substances and CO_2 may be measured after treatment with a base such as hyamine.

The radioactive sample and scintillation solution are placed in a glass vial for counting. Special glass with a low potassium content must be used because natural potassium contains the radioisotope ^{40}K, a β emitter. Polyethylene vials are also in common use today.

III Background Radiation

All radiation counting devices register counts even if there is no specific or direct source of radiation near the detectors. This is due to *background radiation*. It has many sources including natural radioactivity, cosmic rays, radioisotopes in the construction materials of the counter, radioactive chemicals stored near the counter, and contamination of sample vials or counting equipment. Background count in a scintillation counter will depend on the scintillation solution used. Background counts resulting from natural sources cannot be eliminated; however, those due to contamination can be greatly reduced if the laboratory and counting instrument are kept clean and free of radioactive contamination. Since it is difficult to regulate background radiation, it is usually necessary to monitor its level. The

typical procedure is to count a radioactive sample and then count a background sample containing the same scintillation system but no radioisotope. The actual or correct activity is obtained by subtracting the background counts from the results of the radioactive sample.

IV Scintillation Counting of γ Rays

^{24}Na and ^{131}I are both β and γ emitters, and are often used in biochemical research. Gamma rays are more energetic than β particles, and denser materials are necessary for absorption. A gamma counter (Figure 6.8) consists of a sample well, a sodium iodide crystal as fluor and a photomultiplier tube. The high energy γ rays are not absorbed by the scintillation solution or vial, but they interact with a crystal fluor, producing scintillations. The scintillations are detected by photomultiplier tubes and electronically counted.
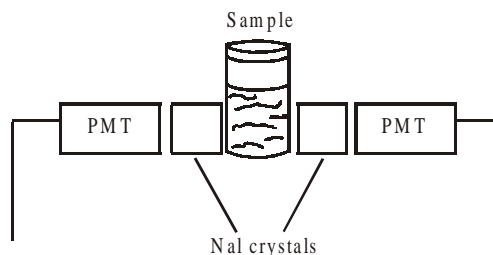
Figure 6.8 A gamma counter

(b) Geiger-Müller Counting of Radioactivity

Another method of radiation detection that has some applications in Biochemistry is the use of gas ionization chambers. The most common device that uses this technique is the *Geiger-Müller tube* (G-M tube). When β particles pass through a gas, they collide with atoms and may cause ejection of an electron from a gas atom. This results in the formation of an ion pair made up of the negatively charged electron and the positively charged atom. If the ionization occurs between two charged electrodes (an anode and a cathode), the electron will be attracted to the anode and the positive ion toward the cathode. This results in a small current in the electrode system. If only a low voltage difference exists between the anode and the cathode, the ion pairs will move slowly and will, most likely recombine to form neutral atoms. Clearly, this would result in no pulse in the electric circuit because the individual ions did not reach the respective electrodes. At higher voltages, the charged particles are greatly accelerated towards the electrodes and collide many times with unionized gas atoms. This leads to extensive ionization and a cascade or avalanche of ions. If the voltage is high enough (1000 volts for most G-M tubes), all ions are collected at the electrodes. A Geiger-Müller counting system uses this voltage region for ion acceleration and detection. A typical G-M tube is represented in Figure 6.9. It consists of a mica window for entry of β particles from the radiation source, an anode down the centre of the tube, and a cathode surface inside the walls. A high voltage is applied between the electrodes.

Current generated from electron movement toward the anode is amplified, measured, and converted to counts per minute. The cylinder contains an inert gas that readily ionises (argon, helium, or neon) plus a quenching gas (Q gas, usually butane) to reduce continuous ionisation of the inert gas. Beta particles of high energy emitted from atoms such as ^{24}Na, ^{32}P, and ^{40}K have little difficulty entering the cylinder by penetrating the mica window. Particles from weak β emitters (especially ^{14}C and ^{3}H) are unable to efficiently pass through the window to induce ionisation inside the chamber. Modified G-M tubes with thin mylar (a polyester) windows, called *flow window tubes* may be used to count weak β emitters.

The sample is prepared in metal disc called a *planchet*. Liquid samples are allowed to dry, leaving a radioactive residue for counting on the metal disc. Alternatively, a sample may be collected on a membrane filter and inserted into the planchet.

G-M counting has several disadvantages compared to liquid scintillation counting. The counting efficiency of G-M system is not as high. The response time for G-M tubes is longer than for photomultiplier tubes; therefore, samples of high radioactivity are not efficiently counted by the G-M tube. In addition, sample preparation is more tedious and time-consuming for G-M counting.

If a choice of counting methods is available, a researcher will usually decide on liquid scintillation unless the sample is not amenable to such measurement. Insoluble materials or the presence of strong quenching agents would probably indicate the need for G-M counting.

The following general observation may be made to conclude this account of the various detection systems employed in liquid chromatography.

Because of its high sensitivity, resproducibility, and ease of operation, the UV detector will be the natural choice for many analyses. For non-UV absorbing solutes the refractive index detector is the most probable choice.

6.3 Reversed-phase Chromatography

Separation of substances which are sparingly soluble in water cannot be successfully achieved if a hydrophilic phase like water were to be used as the stationary phase in the column, since such substances would move along with the solvent front. In the separation of such substances a water-repellant or hydrophobic
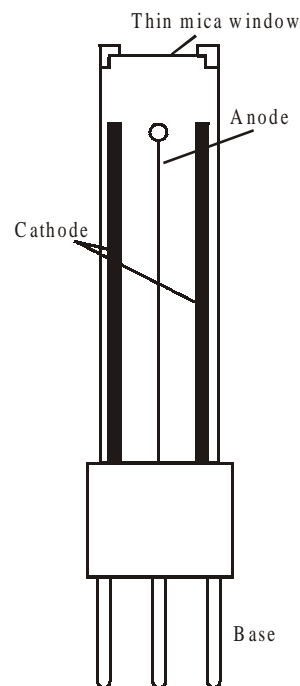

Figure 6.9
A Geiger-Müller tube

/

material constitutes the stationary phase, while the mobile phase is a hydrophilic one. This liquid-liquid partition chromatography is known as the reversed-phase partition chromatography, since the most polar molecules will now tend to elute first.

Preparation of the stationary phase for reversed-phase chromatography involves a special technique. The stationary support like silica gel, alumina, teflon or kieselguhr used for the purpose is first rendered hydrophobic by exposure to vapours of chlorosilane. This is followed by treatment of the stationary support with the appropriate hydrophobic solvent. This treatment of the stationary support may simply be carried out by immersing it in the hydrophobic solvent of choice; alternatively, this step is carried out employing a rotary vacuum evaporator which gives a uniform coating.

A great advantage of reversed-phase chromatography is that benefit can be taken of the previous knowledge available from batch extraction (Chapter 2) with regard to (i) suitable solvents that may be chosen to constitute the stationary phase and the mobile phase, and (ii) the variations required to be made in acidity or composition of the mobile phase during the course of elutions, in order to achieve a neat chromatographic separation. Ethyl acetate, tributylphosphate (TBP), tributyl phosphine oxide (TBPO), trioctylphosphine oxide (TOPO) and mesityl oxide are examples of the hydrophobic stationary phase used in the reversed-phase partition chromatography. For example, an excellent separation of metal ions Fe (III), Cr (VI), Ge (IV), Mo (VI), V (V), Au (III) and Hg (II), whose separation is not an easy job otherwise, has been achieved from multicomponent mixtures by using TBP as the stationary phase. Similarly, using TOPO as the stationary phase, bismuth and tin can be separated frm mixtures. Higher molecular-weight amines such as trioctylamine (TOA), triisooctylamine (TIOA) can also be used as the hydrophobic phase. Thus it is possible to separate cobalt from nickel and iron from manganese using TOA as the stationary hydrophobic phase. Chelating extracting ligands e.g. dinonyl sulphonate (DNS), are increasingly being employed now as the hydrophobic phase.

Reversed-phase partition column chromatography can be used as an improved substitute of the technique of batch extraction with solvents. Batch extraction gives unsatisfactory results where values of the relevant distribution constants are low. In such cases reversed-phase chromatography can prove successful, because this technique adds the advantage of column chromatography to that of solvent extraction. Implied in this added advantage is the repeated intimate contact established between the mobile hydrophilic phase and the stationary hydrophobic phase because of the numerous equilibria attained between the two phases on the large number of theoretical plates of the concerned chromatographic column. Additionally, reversed-phase column chromatography would also be less time-consuming than batch extraction with solvents.

6.4 Applications of Liquid-liquid Chromatography (LLC)

Because of the variety of stationary phases available, LLC, especially in its high performance version, HPLC (Chapter 10), is the most versatile mode of liquid chromatography, and a wide variety of sample types, both polar and non-polar, can be separated. Separation occurs on the basis of the nature and number of the substituent groups, and on differences in molecular weight.

Some classes of compound are best separated by normal phase LLC and some by reversed-phase LLC. Examples include:

Normal phase LLC—anilines, glycols, alcohols, phenols, aromatics, alkaloids, plasticizers, dyes, pesticides, steroids and metal complexes.

Reversed-phase LLC—alcohols, aromatics, expoxy, hydroxy and poly-unsaturated esters, alkaloids, oligomers, antibiotics, steroids and chlorinated pesticides.

Liquid-liquid chromatography is a more efficient separation technique than liquid-solid chromatography. Separation of similar compounds (e.g. members of a homologous series) is carried out more effectively and hence preferably by LLC. Also, LSC suffers from the drawback that usually the column cannot be regenerated for re-use, since the active sites can become poisoned by the irreversible adsorption of highly active compounds. On the other hand, in LLC the immobilized liquid film on the solid support can be essily washed off and replaced without the column having to be replaced.

■

<div style="text-align: center;">

7

Relationship of Theory to Practice of Liquid Chromatography

</div>

So far liquid column chromatography has been dealt with in respect of details of technique and equipment involved. The present chapter discusses theory that relates performance of an LC column to its various parameters. The aim of this discussion is to understand conditions which give high column efficiency, good resolution and quick analysis.

7.1 The key to achievement of separation in any form of chromatography is the proper combination of the differential migration of solutes and the control of band spreading. Fortunately, control of the migration rates and control of band spreading can be treated independently, since the former is governed by the thermodynamic (or equilibrium) considerations, while the latter is governed by kinetic considerations.

7.1.1 Relative Retention

The ability of a system consisting of a particular combination of stationary phase and solvent to produce separation is a function of the thermodynamics of the system.

The solvent efficiency i.e. the extent to which two substances can be separated is expressed by relative retention, α, for the two components, α being defined by the equation:

$$\alpha = \frac{k_2'}{k_1'} \qquad \qquad \text{... (7.1)}$$

in which k_2' and k_1', are terms called the k' values of component 2 and component 1 respectively. The k' value of a solute in its own turn is defined by the equation:

$$k' = \frac{n_s}{n_m},$$

in which n_s and n_m are respectively the number of moles of the solute in the stationary phase and the mobile phase. The k' value of a solute is a property of a particular combination of the stationary phase and the mobile phase (solvent) and hence is a fundamental chromatographic parameter. It is also known as the column capacity ratio. It can be shown that the k' value of a salute is given by the equation:

$$k' = \frac{t_r - t_m}{t_m} \qquad \qquad ... (7.2)$$

in which t_r is the retention time (the time elapsed between the injection of sample on to the column and elution of a component measured at its peak) of the solute, and t_m is the retention time of an unretained solute (i.e. one that has a distribution coefficient of zero, so that it is either non-absorbed or is insoluble in the particular stationary phase of the column and thus passes through the column at the same rate as the mobile phase). These two parameteres, t_r and t_m, are illustrated by Figure 7.1, which is a typical two-component chromatogram defining, besides t_r and t_m, various other parameters also, which are needed for the description of chromato-graphic peaks and will be referred to later. Equation 7.2 gives a direct means of determining the k′ values of solutes from their respective

Figure 7.1 A two-component chromatogram, showing the definitions of various parameters. The peak at t_m corresponds to an unretained (or mobile phase) component

peaks in the chromatogram. The use of k′ values is preferred to simply quoting retention times, since the latter can vary with flow rate variations from day to day, whereas k′ values remain constant.

$$\text{Since;} \qquad \alpha = \frac{k_2'}{k_1'} \qquad \qquad ... (7.1)$$

$$\text{and} \qquad k' = \frac{t_r - t_m}{t_m} \qquad \qquad ... (7.2)$$

$$\alpha = \frac{t_{r_2} - t_m}{t_{r_1} - t_m} \qquad \qquad ... (7.3)$$

The value of α can be determined from the chromatogram. Since the subscripts on the two k's in the equation: $\alpha = \dfrac{k_2'}{k_1'}$, are assigned in the order of elution, it follows that α will always be greater than unity. In effect, α is a measure of the difference in the thermodynamic distribution behaviour of the two components, this difference being the cause of separation brought about by chromatography.

7.1.2 Column Efficiency

Having achieved a separation, it is necessary to prevent mixing of the components, and the ability to achieve this is a function of the column geometry. The column efficiency is measured in terms of the number of theoretical plates (N) in the column.

7.1.2.1 Number of Theoretical Plates (N) and Height Equivalent to a Theoretical Plate (HEPT)

The concept of the 'theoretical plate' comes from fractional distillation and the term 'theoretical plate' has been used already in connection with evaluation of resolving power of fractionating columns (Chapter 2). In fractional distillation the condensed liquid percolates back into the distillation vessel by flowing over a series of plates that permit the liquid to come to equilibrium with the vapour that rises up the column. Of course, a chromatographic column has no 'plates' in it in a physical sense. The 'theoretical plate' is an imaginary zone or segment of the column in which the composition of the mobile phase flowing out of the segment is in equilibrium with the average composition of the fixed phase within the segment. The significance of the word 'average' has to be noted. In reality the composition of the fixed phase varies continuously along the length of the column. The moving phase is never in equilibrium with the fixed phase; its composition always lags behind the composition that would be in equilibrium with the fixed phase through which it is passing. However, the more closely it approaches equilibrium the shorter is the theoretical plate. The 'height equivalent of the theoretical plate', (HEPT), or plate height (H), is the length within the column that is able to achieve equilibrium between phases under a particular set of conditions viz. flow rate temperature etc.

The column length (L) divided by the plate height (H) gives the number of theoretical plates, N, of the column. This parameter is calculated from the equation:

$$N = 16 \left(\frac{t_r}{W} \right)^2 \qquad \qquad ...\ (7.4)$$

where t_r is the uncorrected retention time (i.e. in measuring t_r the retention time, t_m, of the unretained peak has been included) of the peak of width W at the base, both these parameters of the peak being measured in the same units (mm, ml, s etc.) from the chromatogram (Figure 7.1) W is obtained by extrapolation of tangents at the points of inflection to the base line as shown in Figure 7.1. The number of theoretical plates, N, calculated by substituting the values of t_r and W in Equation (7.4), is a measure of the efficiency of the column, that is, its ability to control the amount of spreading of a solute band as the latter travels down the column, and efficient systems are characterized by high values of N.

$$\text{Since} \quad N = \frac{L}{H},$$

$$\text{or} \quad H = \frac{L}{N},$$

$$\text{and} \quad N = 16\left(\frac{t_r}{W}\right)^2,$$

$$H = \frac{L}{16}\left(\frac{W}{t_r}\right)^2 \qquad \qquad .... \ (7.5)$$

This relationship shows that t_r and W must vary together, since H is a constant for a given system. This means that for several peaks in the same chromatogram, the longer the retention time, t_r, the larger will be W and broader the peaks.

It is clearly desirable that peak broadening, as represented by the magnitude of W, be as small as possible for attaining best separations of the sample mixture. W will be small only when H is small, since in Equation 7.5, L is fixed for a given column and, as already discussed, t_r and W vary together. In other words, best separations will be achieved if H is small under the set of experimental conditions employed.

7.1.2.2 Zone Broadening

Peak broadening for a particular solute in the chromatogram corresponds to 'zone broadening' for that solute on the chromatographic column. Obviously, zone broadening should be as small as possible for attaining best separations. This broadening of zones migrating through the chromatographic column is affected by the following processes:

(1) Axial or longitudinal molecular diffusion, and (2) mass transfer processes.

(1) *Axial or longitudinal molecular diffusion:* It is the migration of solute from regions of higher to those of lower concentrations in the direction of the column. Ordinary diffusion occurs when solute molecules randomly jump back and forth between successive collisions or equilibrium positions.

(2) *Mass transfer processes:* Contribution to band broadening made by mass transfer processes arises from slow equilibration between the mobile and stationary zones. Dispersion (or band broadening) arises because molecules in the stationary zone tend to get left behind when the main band passes over, while the molecules in the mobile zone move ahead. Dispersion due to slow equilibration increases directly with the flow velocity and the equilibration time. Mass transfer process may be conveniently divided into two terms:

(a) Stationary phase mass transfer, and (b) mobile phase mass transfer.

(a) *Stationary phase mass transfer:* The rate at which solute molecules transfer into and out of the stationary phase makes a significant contribution to zone

broadening and hence to efficiency of separation. This rate depends mainly on diffusion for liquid stationary phases and on adsorption-desorption kinetics for solid stationary phases. Solute molecules will reside in or on the stationary phase for varying lengths of time. Thus, for example, with a liquid phase some molecules will diffuse more deeply into the liquid layer. When these molecules are desorbed they will have been left behind the bulk of the molecules and the solute band will have been broadened. Likewise, in adsorption chromatography those solute molecules adsorbed on active sites will have a larger residence time on the surface and will again be left behind the main stream of molecules when they are finally desorbed. Hence, to reduce band broadening from stationary phase mass transfer effects, liquid layers should be as thin as is possible without introducing adsorption effects on the support material and solid surfaces should be of a homogeneous nature.

(b) Mobile phase mass transfer effects in liquid chromatography must be divided into contributions from (i) the 'stagnant' mobile phase, and (ii) the 'moving' mobile phase.

(i) 'Stagnant' mobile phase mass transfer: When porous stationary phases are used, the intra-particle empty volume is filled with mobile phase at rest. Solute molecules must diffuse through this stagnant mobile phase in order to reach the stationary phase. Molecules that diffuse only a short distance into the pore will rapidly regain the mainstream, whereas molecules that diffuse further and spend more time in the pore will be left behind the mainstream, again resulting in broadening of the band coming down the column. The adverse effect of stagnant mobile phase mass transfer has been instrumental in the development of specialized support materials for use in liquid chromatography.

(ii) 'Moving' mobile phase mass transfer: The flow of a liquid through a packed bed is complicated involving as it does flow inequalities due to eddy diffusion and lateral mass transport by diffusion and by convection.

Eddy diffusion arises from movement of the mobile phase (liquid in liquid chromatography and gas in gas chromatography) in a packed column in tortuous channels. The flow-paths available are tortuous and zig-zag, some of these being narrow ones and others wider ones. This is so on account of irregularities in the packing material (e.g. non-uniformity of particle size) and different qualities of the packing of the column. The zig-zag path taken by a part of the mobile phase makes some molecules of a particular solute take longer paths and they lag behind the bulk of the molecules of that solute, while some molecules of the same solute which are present in another part of the mobile phase take shorter paths and move ahead of the mainstream of the molecules of that particular solute. Also, the mobile phase moves more slowly in narrow flow paths and more rapidly in wider ones so that further inequalities are introduced in the transport of molecules of a particular

solute by the mobile phase. The eddy diffusion resulting from these effects broadens the relevant solute band.

The simple theory of eddy diffusion assumes that solute molecules are fixed in given flow paths. In practice, however, this is not true. The solute molecules may pass laterally by diffusion and by convection from one flow path into another where the flow velocity may be quite different. This 'coupling' of lateral mixing with the normal process of eddy diffusion results in a decrease in the amount of band broadening and hence an increase in efficiency of the chromatographic process.

7.1.2.3 Band Broadening and the Plate Height Equation

The degree of band-broadening actually occurring in a particular chromatographic technique is an overall result of the contributions made by each of the above processes. Since W (peak width) is a measure of the band-broadening and, as already shown, H is dependent on W, each of these band-broadening processes contributes to the overall plate height value (H) of the column. The relative contributions to the plate height by each of these processes depend upon the particular chromatographic technique under consideration, and even within a particular technique the relative importance of these processes may change with changing operating conditions like fluid flow velocity, viscosity of solvent, particle size of packing material of the column and temperature. Thus, the overall plate height equation obtained by summing the contributions from the various band-broadening processes is:

$$H = H_L + H_S + H_{SM} + H_M \qquad\qquad ... (7.6)$$

in which H_L, H_S, H_{SM} and H_M are the plate height contributions of longitudinal diffusion, stationary phase mass transfer effects, the 'stagnant' mobile phase transfer effects and 'moving' mobile phase mass transfer effects, respectively.

The plate height contribution of longitudinal diffusion H_L is given by:

$$H_L = \frac{2\gamma D_M}{v}$$

where γ is an obstruction factor which accounts for the fact that longitudinal diffusion is hindered by the column packing, D_M is the solute diffusion coefficient in the mobile phase and v is the velocity of the mobile phase. In packed columns, the value of γ is about 0.6. In liquid chromatography, because D_M is small, the contribution of H_L to the overall plate height is also small and in most cases it may be assumed to be zero.

The plate height contribution of stationary phase mass transfer, H_S, is given by:

$$H_S = 2\left(\frac{k'}{1+k'}\right)^2 v t_a,$$

where t_a is the time spent by a molecule in the mobile phase before adsorption occurs.

It is often more convenient to express H_S in terms of the mean desorption time t_d (the mean time that a molecule remains attached to the surface). The ratio, $\dfrac{t_a}{t_d}$, is therefore the ratio of times spent in the mobile and stationary phases, and therefore, $\dfrac{t_a}{t_d} = \dfrac{1}{k'}$, and $H_S = 2vt_d \dfrac{k'}{\left(1+k'\right)^2}$

Thus the smallest contribution to the plate height occurs at low mobile phase velocities and with rapid mass transfer. The function, $\dfrac{k'}{(1+k')^2}$, involving capacity factor, $\dfrac{k'}{1+k'}$, will show a maximum at $k' = 1$ and will then decrease with increasing k' value.

Mass transfer in a bulk stationary phase as in partition systems, is analogous to retention on an adsorptive surface in that a certain average time is required to absorb and desorb the molecule. However, whereas the mean desorption time is determined by the rate constant (k_d) for the desorption process, in a partition system the controlling factor is the solute diffusion coefficient in the stationary phase, D_S. The mean desorption time is replaced by the average diffusion time, t_D, the time taken for a molecule to diffuse a distance d in the liquid. Then:

$$t_D = \frac{d^2}{2D_S}$$

and the plate height contribution becomes:

$$H_S = \frac{k'}{\left(1+k'\right)^2} \cdot \frac{d^2v}{D_S} \qquad \qquad \text{.... (7.7)}$$

The distance d may be equated approximately with the thickness of the stationary liquid film, d_f, so that slow mass transfer is equated with high liquid loadings. It is also desirable to choose liquids within which the solute molecule has a large diffusion coefficient, D_S.

In a rigorous treatment of diffusion controlled mass transfer, the precise shape of the partitioning liquid should be considered. Equation 7.7 should thus be multiplied by a configuration factor, q. For uniform liquid film $q = \dfrac{2}{3}$. For diffusion in rod-shaped pore (as in paper chromatography) or in a spherically shaped body (an ion-exchange bead) the values of q are $\dfrac{1}{2}$ and $\dfrac{2}{15}$ respectively.

The plate height contribution from mobile phase trapped as 'stagnant' mobile phase in porous spherical particles is:

$$H_{SM} = \frac{\left(1-\theta+k^1\right)^2 d_p^2 \, v}{30(1-\theta)\left(1+k'\right)^2 \lambda D_M} \qquad \text{.... (7.8)}$$

where d_p is the particle diameter of the column packing, θ is the fraction of total mobile phase in the interparticle space and λ is a tortuosity factor inside the particle.

The plate height contribution, H_M, from moving mobile phase effects can be related to contributions from eddy diffusion (H_F) and lateral diffusion (H_D). The contribution from eddy diffusion is:

$$H_F = 2 \, \lambda \, d_p,$$

where d_p is the particle diameter and λ is a packing constant.

The term H_D may be written:

$$H_D = \frac{\Omega v d_p^2}{D_M} ,$$

where, again, Ω is a function of the packing structure.

As already mentioned, the eddy diffusion may be coupled with lateral diffusion. It was once assumed that H_M could be taken to be sum of the two effects:

$$H_M = H_F + H_D$$

However, a study of the quantitative aspects of the combined flow-diffusive exchange leads to the relationship:

$$H_M = \frac{1}{\dfrac{1}{H_F} + \dfrac{1}{H_D}} = \frac{1}{\dfrac{1}{2\lambda d_p} + \dfrac{D_M}{\Omega v d_p^2}}$$

At high mobile velocities, H_M approaches the constant value of H_F, and at low velocities it approaches H_D. In other words, at low mobile phase velocities lateral diffusion is the controlling factor whilst at high velocities eddy diffusion is most important.

In general, H_M increases with particle diameter and flow velocity, and decreases with solute diffusivity.

In order to optimize for a minimum plate height it is convenient to write the individual contributing components of the overall plate height Equation 7.6 in terms of those physical quantities which are most easily controlled e.g. d_f, d_p, D_M and D_S, thus:

$$H_L \;=\; C'_D \;(D_M/v)$$

$$H_S \;=\; C'_S \;(d_f^2 v/D_S)$$

$$H_F \;=\; C'_F \; d_p$$

$$H_D \;=\; C'_D \;(d_p^2/D_M)$$

$$H_{SM} \;=\; C'_{SM} \;(d_p^2 v/D_M)$$

from which

$$H = C'_D \frac{D_M}{v} + C'_s \frac{d_f^2 v}{D_S} + C'_{SM} \frac{d_p^2 v}{D_M} + \cfrac{1}{\cfrac{1}{C'_F} d_p + \cfrac{D_M}{C'_M v d_p^2}} \qquad \text{... (7.9)}$$

Equation 7.9 may be written in the from:

$$H = \frac{B}{v} + C_S v + C_{SM} v + \left(\frac{1}{A} + \frac{1}{C_M v} \right)^{-1}$$

or neglecting longitudinal molecular diffusion,

$$H = C_S v + C_{SM} v + \left(\frac{1}{A} + \frac{1}{C_M v} \right)^{-1} \qquad \text{... (7.10)}$$

In order to minimize H, it is necessary to use columns packed with small diameter particles, a slow-moving solvent of low viscosity, and high sepation temperatures, to increase diffusion rates.

In many LC columns, mobile phase effects predominate over the effects due to stationary phase. The extent of influence of k′ on H will be determined by the contribution made by H_{SM} compared to other terms.

7.1.2.4 Plate Height Curve

The very complex flow processes occurring in the liquid mobile phase in LC make plate height rise more flatly with mobile phase velocity than in GC (Figure 7.2, curves a and b).

The flat slope of plate height vs mobile phase velocity plot (Figure 7.2 a) in LC permits the use of high mobile phase velocity without much loss in column efficiency. In this respect, LC differs considerably from GC in which plate height varies more steeply with mobile phase velocity at large values of the latter (Figure 7.2 b).

Curves a and b of Figure 7.2, which are plate height vs mobile phase velocity plots, are known as plate height curves.

7.1.2.5 Reduced Plate Height and Reduced Velocity

Plate height curves provide means of comparing the efficiency of different columns and packing materials. However, since H depends on the particle size, d_p, a series of equally well packed columns of the same material in various size fractions will give a series of different plate height curves. To overcome this problem, it is sometimes preferable to use

Figure 7.2 Typical curves connecting HEPT and mobile phase velocity

'reduced' parameters, the reduced plate height, h, and the reduced fluid velocity, ν, respectively, in place of H and γ. The dimensionless parameters h and ν are respectively defined by the expressions:

$$h = \frac{H}{dp} \qquad \qquad ...\,(7.11)$$

$$\text{and} \quad \nu = \frac{\nu d_p}{D_M} \qquad \qquad ...\,(7.12)$$

The reduced plate height, h, is independent of the particle diameter. The reduced fluid velocity (υ), a concept conceived by Giddings, is a measure of the rate of flow over a particle relative to the rate of diffusion of solute within the particle. Since both reduced parameters, h and ν, are normalized for the particle diameter, when h is plotted against ν, the different size fractions of the same packing materials should give similar curves. This has been confirmed in practice, so that use of h vs ν curves is preferred over that of plate height curves (H versus mobile phase velocity) when comparison of the efficiency of different columns is to be carried out.

In terms of reduced plate height, an expression due to Knox et al. which is analogous to Equation 7.10 has the form:

$$h = A_\nu^{0.33} + \frac{B}{\nu} + C\nu \qquad \qquad ....(7.13)$$

The constants A, B and C are dimensionless, and for a good column (i.e. a column having packing with efficient materials possessing good mass transfer properties) have the approximate values A < 1, B ≈ 2, C < 0.1.

Figure 7.3 plots Equation 7.13 and includes lines representing the three terms of the equation. Since the dispersion caused by axial diffusion as represented by
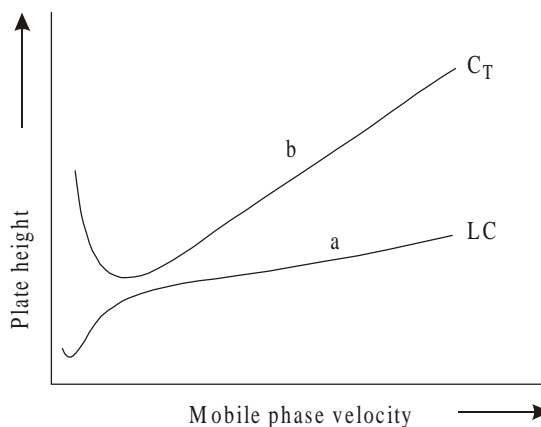
the term $\dfrac{B}{\nu}$ is minimum at high velocities, while dispersions caused by eddy diffusion, represented by the term $A\nu^{0.33}$, and mass transfer effects, represented by the term $C\nu$, are minimum at low velocities, lowest h is achieved at an intermediate reduced velocity when the contributions from terms $\dfrac{B}{\nu}$ and $C\nu$ are more or less equal.

The practical implication of the minimum in the curve marked h in Figure 7.3 is that maximum chromatographic efficiency (i.e. minimum value of the reduced plate height) will be obtained at a specific reduced velocity (and therefore at a particular operating pressure).

Although in gas chromatography the reduced velocity is not much higher than unity, in liquid chromatography the small D_M values mean that the υ values [order of magnitude of values of diffusion coefficient $(cm^2 s^{-1})$ in gas and liquid respectively is 10^{-1} and 10^{-5}] can be up to several thousand.

Figure 7.3 Logarithmic plot of reduced plate height (h) versus reduced velocity (ν) showing contributions from the three terms in equation, $h = A\nu^{0.33} + \dfrac{B}{\nu} + C\nu$

An idea of the magnitude of the value of N, the number of theoretical plates, generated in a typical modern column can be had from the knowledge that in well packed columns of modern efficient materials reduced plate heights of around 2 can be achieved. (In fact, a value of h greater than 10 may be taken as an indication of a poorly packed column or of poor column material.) An h-υ plot like the one shown in Figure 7.3, plotted for one such modern column, is used to find the value of υ corresponding to minimum reduced plate height. It is then possible to calculate the particle diameter necessary to give this reduced velocity. Knowing the particle diameter, d_p, the plate height, H, is calculated using the equation 1 : $h = \dfrac{H}{d_p}$, wherein h is the minimum reduced plate height in the h vs ν curve. Thus N can be calculated for a given column of length, L, since $N = \dfrac{L}{H}$. The magnitude
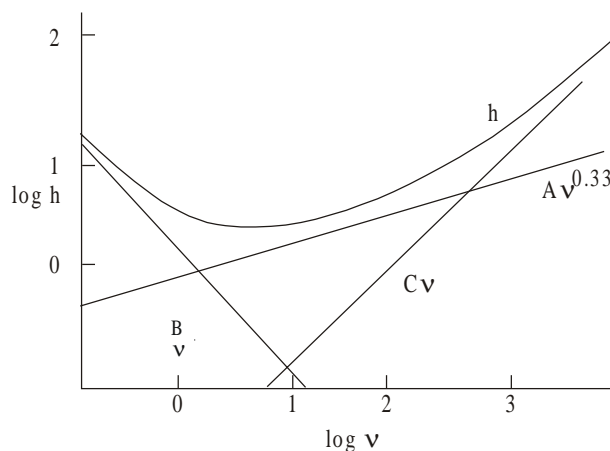
of N values in modern HPLC columns is of the order of 10^3 to 10^4. Thus for a typical modern column of length 125 mm, with packing of spherical silica of particle size, 7.5 μ, the plate height, H, was 16.1 μ (minimum reduced plate height in h vs ν curve being 2.14, for the case under consideration), and the 125 mm column generated 7800 plates.

7.1.2.6 *Extra-column Band-broadening*

The volume of the column available to the mobile phase or the mobile phase volume (also called dead volume) is dependent on the geometry of the column and the packing. A contribution to the mobile phase volume will also be made by the injection system, by the connecting tubing and by the detector. Thus the contributions may be separated into 'in-column' and 'extra-column' effects.

Extra-column band-broadening causes changes in H that generally result in lowering of column efficiency in LC. Extra-column effects become specially important when short columns are being used. This extra-column band-broadening can take place in the injection system, in connecting tubing, and in the detector. Good instruments are so designed as to keep volumes in these three extra-column regions of the liquid chromatograph to the minimum.

7.2 Resolution

The combined effects of solvent efficiency (i.e. the extent to which substances are separated on account of differences in their rates of migration with reference to a particular set of solvent and stationary phase) and column efficiency (i.e. ability to achieve prevention of remixing of the separated substances) is expressed in terms of the resolution, R_s, of the column :

$$R_S = \frac{2\left(t_{r_2} - t_{r_1}\right)}{W_1 + W_2} \qquad \qquad ...\ (7.14)$$

where t_{r_1}, and t_{r_2} are the retention distances and W_1 and W_2 are the peak base widths (in the same units as t_{r_1}, and t_{r_2}) of peaks 1 and 2 respectively (Figure 7.1).

For the two adjacent isosceles triangles, baseline resolution corresponds to $R_s = 1.0$.

Thus, in the simplest case of a mixture containing two components in the ratio 1:1, a value of $R_s = 1$ would give complete separation at the baseline. In practice, however, the peaks are Gaussian in shape, and effective beseline resolution is obtained for $R_s = 1.5$. Thus, a value of $R_s = 1.5$ would result in giving complete recovery of each component with 99.9% purity in the case of a two component mixture containing components 1 and 2 in equal concentration.

Though Equation 7.14 is useful in determining the degree of resolution, it is purely an empirical relationship being dependent upon the chromatogram that is

the result of an actual experiment. The equation does not give any guidance as to what sort of changes in experimental conditions could bring about an inprovement in resolution. A relationship which has been derived by Purnell and which involves substitution of W terms in Equation 7.14 in terms of N, shows that for column chromatography the resolution, R_s, can indeed be related to the column parameters k', α, and N. This relationship is given by the equation:

$$R_s = \frac{1}{4}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k_2'}{1+k_2'}\right)\sqrt{N_2} \qquad \dots (7.15)$$

in which the subscript 2 refers to the second component. The resolution can therefore be changed by making variations in the thermodynamic properties of the system to alter α and k', as also by changing the column conditions (particle size, flow rate etc.) to alter the value of N, the number of theoretical plates. The manner in which resolution changes by changing independently each of the three parameters α, k' and N is discussed below.

Equation 7.15 can be rewritten in each of the following forms:

$$R_s = C_1\left(\frac{\alpha-1}{\alpha}\right) \qquad \dots (7.16)$$

$$R_s = C_2\left(\frac{k_2'}{1+k_2'}\right) \qquad \dots (7.17)$$

$$R_s = C_3\sqrt{N_2} \qquad \dots (7.18)$$

Turnwise consideration of each of these forms of Equation 7.15 will lead to conclusions regarding change in resolution brought about respectively by independently varying α, k' and N. Factors $\left(\frac{\alpha-1}{\alpha}\right)$ in Equation 7.16 and $\left(\frac{k_2'}{1+k_2'}\right)$ in Equation 7.17 respectively represent terms called selectivity and capacity factor and N_2 (i.e. the number of theoretical plates) in Equation 7.18, of course, represents efficiency.

7.2.1 Dependence of R_s on Selectivity

In the equation:

$$R_s = C_1\left(\frac{\alpha-1}{\alpha}\right) \qquad \dots (7.16)$$

C_1 implies that two terms viz. capacity factor and efficiency, are being kept constant so that the variation of R_s with α may be obtained. When $\alpha = 1$, there will be no separation. However with systems having α as low as 1.01, at least

partial resolution have been achieved. A higher value for α is desirable to achieve a higher R_s and LC is capable of providing this.

The mobile phase in LC (unlike that in gas chromatography) contributes significantly to the α function by playing a significant role in the thermodynamic distribution process. Selective interaction of the mobile phase with solutes in the case of liquid-liquid chromatography, and competing of the mobile phase with the solutes for adsorption sites in the case of liquid-solid chromatography, enable the mobile phase to play this role of significant contributor to α.

The major reason for the remarkable success of classical LC, in spite of the highly inefficient columns employed, is the attainability of high values of α. In HPLC (High Performance Liquid Chromatography), this advantage of potentially high α values gets further combined with efficiencies and speeds which are comparable to those achieved in gas chromatography and hence the recent development of HPLC as a separation technique at such a fast pace.

7.2.2 Dependence of R_s on Capacity Factor

The equation:

$$R_s = C_2 \left(\frac{k_2'}{1 + k_2'} \right) \qquad \qquad \text{... (7.17)}$$

shows how R_s varies with k' when selectivity and efficiency terms are kept constant. Again, there will be no separation if $k_2' = 0$. The graph obtained by plotting experimental values of k' against relative resolution shows that R_s increases very markedly with increase in k' while k' has small values, but at larger k' values $\dfrac{k_2'}{1 + k_2'}$ tends towards unity and the k' term plays no further role in the resolution. Rearranging Equation 7.2 in the form: $t_r = t_m (1 + k')$, shows that large k' values also imply longer retention times leading to formation of diffuse bands which are difficult to detect. 1 to 10 is the optimum range of k' values.

7.2.3 Dependence of R_s on Efficiency

In the equation:

$$R_s = C_3 \sqrt[3]{N_2} \qquad \qquad \text{... (7.18)}$$

C_3 implies that the selectivity and capacity factor terms have been kept constant, so that the equation shows the dependence of R_s on N_2. Obviously, the resolution increases as N increases.

7.3 Effective Plate Number (N_{eff})

The number of theoretical plates, N, is not the best measure of the column efficiency since in measuring t_r the retention time (t_m) of an unretained peak has

been included and this is a function of both the column and the extra-column configuration. A better measure of column efficiency is the effective plate number, N_{eff}, where :

$$N_{eff} = 16\left(\frac{t_r - t_m}{W}\right)^2 \qquad \text{... (7.19)}$$

N_{eff} and N are related to each other by the expression:

$$N_{eff} = \left(\frac{k'}{1 + k'}\right)^2 N \qquad \text{... (7.20)}$$

Substituting N by N_{eff} makes Equation 7.15 take the much simpler form :

$$R_s = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)(N_{eff})^{\frac{1}{2}} \qquad \text{... (7.21)}$$

Relationship 7.21 expresses resolution in terms of two approximately independent factors and shows that R_s is proportional to N_{eff} for a constant value of α. Effective plate number is, therefore, a more useful parameter than N for comparing the resolving powers of different columns containing the same stationary phase.

7.4 Resolution Time for Analysis

The time of analysis is approximately equal to the retention time of the last component. For example, it is t_{r_2} in Figure 7.1, which is the chromatogram for separation of a mixture of two components. What we desire is that R_s/t_{r_2} must be as large as possible.

Equation 7.21 shows that for α being close to 1, small changes in value of α result in large decreases in N_{eff} required for achieving the same degree of resolution. As indicated by Equation 7.19, the required N_{eff} is roughly proportional to time, so that a proper choice of phases giving a larger value of α will lead to smaller separation time. In the rest of the discussion it will be assumed that the phases chosen give a high α value.

The retention time can be expressed in terms of the equation:

$$t_r = N(1 + k')\left(\frac{H}{v}\right) \qquad \text{... (7.22)}$$

Combining Equations 7.20 and 7.22 gives the relationship:

$$\frac{N_{eff}}{t} = \frac{v}{H} \cdot \frac{\left(k'\right)^2}{\left(1 + k'\right)^3} \qquad \text{... (7.23)}$$

Resolution and analysis time being interrelated, $\dfrac{N_{eff}}{t}$, *effective plates per second*, is sometimes a better criterion for comparing column performance.

The aim of high speed analysis is to maximize $\dfrac{N_{eff}}{t}$, which means the shortest analysis time. To achieve this aim, k' must be maximized for chosen values of ν and H to comply with the requirements of Equation 7.23. H can be assumed to be independent of k' when mass transfer effects in the mobile phase mainly decide the band broadening machanism; in such a case the optimum value of k' to maximize $\dfrac{N_{eff}}{t}$ is seen to be about 2. Higher k' values result in longer t_r and lower k' values give lower N_{eff}. In case major contributions to band broadening come from stationary-phase mass transfer processes or from stagnant mobile phase diffusion, H becomes a function of k' and the optimum k' value lies anywhere between 1.5 and 4.

Equation 7.22 shows that maximization of $\dfrac{H}{\nu}$ reduces t_r. By examining carefully Equation 7.10 it can be guessed that an increase in ν will decrease H/ν. In practice H/ν decreases rapidly in the beginning. At high ν, it continues to decrease (because of coupling of A and C_M terms), the slope, however, being small (Figure 7.4, curve a). Though this situation suggests the use of very high ν (to reduce t_r as much as possible), the excessive pressure drop (ΔP) developing across the column at very high ν (Figure 7.4, curve b) speaks against this approach. For high speed analysis, therefore, the ν at which the slope of the H/ν
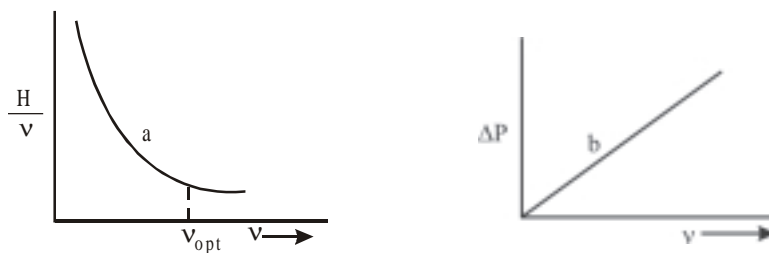
Figure 7.4 Variation of $\dfrac{H}{\nu}$ and Δp with ν.

vs ν curve ceases to change significantly, is generally taken as ν_{opt} (around 2–3 cm/sec for a column with 2-3 mm internal diameter).

7.5 Column Permeability (K^o)

Another criterion for comparing column performance is the column permeability, K^o, which is a measure of the facility with which the mobile phase

flows through the given chromatographic column. K° is defined by the expression:

$$K^{\circ} = \frac{v\eta L}{\Delta P} \qquad \text{... (7.24)}$$

where ΔP is the pressure drop through the column of length L and η is the mobile phase viscosity.

7.5.1 Relationship of K^{o} with d_p

In a regular packed column (a column in which the ratio of column diameter, d_c, to particle diameter, d_p, is greater than 10), K° is given by the equation:

$$K^{\circ} = \frac{d_p^2}{180} \times \frac{\varepsilon^3}{(1-\varepsilon)^2} \qquad \text{... (7.25)}$$

where ε is the interparticle porosity. $\varepsilon = 0.42$ for a regular packed column, so that Equation 7.25 can be written as:

$$K^{\circ} = \frac{d_p^2}{1000} \qquad \text{... (7.26)}$$

7.5.2 Relationship between K^{o} and t_r

Using the relationship between t_r, L and v expressed by the equation:

$$t_r = \frac{L}{v} \ (1 + k') \qquad \text{... (7.27)}$$

the relationship between permeability and retention time can be derived. Value of v derived from Equation 7.24 is $\dfrac{K^0 \Delta P}{\eta L}$. Substitution of this value of v in Equation 7.27 gives the relationship of t_r with K° as:

$$t_r = \frac{\eta L^2 \left(1 + k'\right)}{K^0 \, \Delta P} \qquad \text{... (7.28)}$$

Equation 7.28 shows that shorter retention times result with increasing permeable columns while values of L, k', n and ΔP are being maintained constant. Permeability can be increased by increasing the particle diameter, since $K^{\circ} \propto d_p^2$ (Equation 7.26).

Higher permeability attained by increasing particle size will give shorter retention time (t_r) if column length (L) is kept constant. Alternatively, if t_r is kept constant, a higher column permeability attainable by increasing particle size will allow for the use of a longer column as a result of which increase in N and hence better resolution will be achieved. However, in a regular packed column, column efficiency tends to decrease with increasing particle size, since H increases as the

particle diameter increases, H and d_p being related by the expression:

$$H \approx d_p^{\beta},$$

the value of β ranging between 1.8 to 1.3, but becoming increasingly larger as the particle size increases. As long as the column is 'well packed' smaller particles give more efficient columns regardless of column pressure or separation time. However, with particles less than 10 μ, efficient packing becomes increasingly difficult as the particle diameter decreases.

In an irregular packed column $\left(\text{viz. one with } \dfrac{d_c}{d_p} < 5 \right)$ H decreases (due to increased radial mixing possible in the mobile phase) and K^o increases by increased d_p. In LC such irregular packed columns are reported to be more advantageous than regular columns as $(1) t_r$ can be decreased by an order of magnitude, or (2) longer columns can be employed for a given ΔP. However, in practice, such columns are difficult to make reproducibly.

7.6 Influence of the Factor L/d_p on t_r and ΔP

Substituting the value of K^o from Equation 7.26 in Equation 7.28, the expression for t_r becomes:

$$t_r = \frac{1000\left(1 + k^{'}\right)\eta}{\Delta P} \times \left(\frac{L}{d_p} \right)^2 \qquad \qquad \text{... (7.29)}$$

Equation 7.29 describes the dependence of ΔP and t_r on $\dfrac{L}{d_p}$. N obviously increases with increase in $\dfrac{L}{d_p}$. However, t_r will also increase with increase in $\dfrac{L}{d_p}$. To increase efficiency at constant t_r, one must increase ΔP. The apparatus employed will, however, fix an upper limit (ΔP_{lim}) for the pressure employed. Once this ΔP_{lim} is attained, further increase in efficiency will increase t_r as well.

At constant $\dfrac{L}{d_p}$ ratio, a column of small L and d_p will give faster analysis than one having greater length and larger d_p.

7.7 Column Resistance Parameter

Another term of practical importance is the column resistance parameter, ϕ', where

$$\phi' = \frac{\Delta P \, d_p^2}{v\eta L} \qquad \qquad \text{... (7.30)}$$

ϕ' being dimensionless. It is a measure of the resistance to eluent flow caused by

the column and should have as low a value as possible. Typical values lie in the range 500-1000 for a column of porous particles.

7.8 Peak Capacity

Peak capacity is yet another criterion that may be employed for judging column performance. If a constant number of theoretical plates, N (independent of k′), is assumed, the peak capacity, ϕ, is defined as the number of peaks that can be placed within a certain time period in which all of the bands have a resolution of unity. The first band elutes at the time specified as zero and the final band has its peak maximum at the time specified as the end of the chromatogram, neglecting the small increment of time necessary for the final peak to return to the base line. For a given k′ for the final component, an increase in N increases ϕ. In addition, when there is insufficient ϕ for a given separation, an increase in k′ will improve ϕ for the column which has larger N. For a gas or liquid chromatographic system ϕ can be improved using one or more gradient techniques such as gradient elution or temperature programming.

7.9 Gradient Elution

For complex mixtures where components to be separated include a wide range of polarities, the k′ values are too dissimilar, and the peaks tend to crowd together near the start, while later peaks are broadened too greatly to be measured with accuracy (Figure 7.5A). This common difficulty encountered in the analysis of multicomponent samples is referred to as the *general elution problem.*

   The general elution problem is solved by the use of gradient elution. By changing the composition of the mobile phase, k′ values of the moving components of the mixture can be modified to satisfactory values. However, when such solvent changes during the column operation are planned, careful thought has to be given to column characteristics.

   Consider a column having water (plus an inert carrier) as the stationary phase, and chloroform as the mobile phase. If, during operation, water-saturated butanol is substituted for water-saturated chloroform as the mobile phase, the first butanol entering the column will become diluted with chloroform. This will make water less soluble in it, and water will separate out and waterlog the upper part of the column. This example amply emphasises the necessity of careful planning of solvent changes when these are required to be made. With gradient elution i.e. the procedure wherein the solvent change is made gradually, many of the difficulties are minimized. Gradient elution starts with a 'weak' solvent (i.e. one that gives large k′ values) and, as the chromatogram is developed, it proceeds progressively to a 'stronger' solvent (smaller k′). The result is a dramatic improvement in the chromatogram, with peaks well resolved. (Figure 7.5 B)
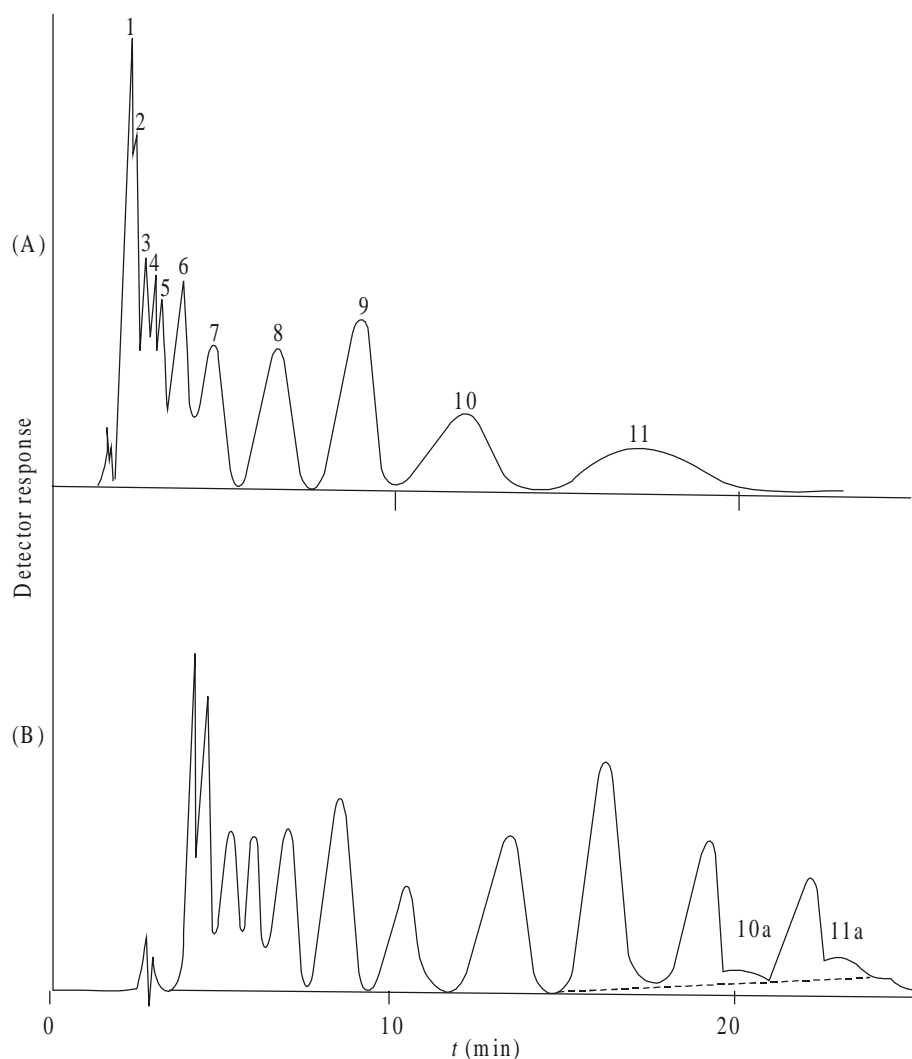
Figure 7.5 The general elution problem. (A) Normal elution, (B) Gradient elution.

7.10 Effect of Temperature on Resolution and
Utility of Temperature Programming

Temperature has only a small effect on retention and resolution in liquid chromatography, since the enthalpy of solution from the mobile phase to the stationary phase is quite small unlike it is the case for gas chromatography. Thus analysis by liquid chromatography is most often carried out at room or relatively low temperature.

Temperature management, however, helps in keeping k' values constant, in reducing viscosity of the mobile phase and improving mass transfer thereby, and

also in effecting some increase in the solubility of sparingly soluble substances in the mobile phase. However, in liquid chromatography, since k' is strongly dependent on the nature of the solvent, gradient elution is employed for improving resolution, instead of temperature programming, which is the means used in gas chromatography for improving resolution.

7.11 Overloading

In the discussion of the mechanism of chromatographic separation, it was tacitly assumed that the distribution coefficient is a constant, which, in other words, means occurence of a linear relationship between the concentration of the sample molecules in the stationary and mobile phases irrespective of the quantity of the sample. An elution peak with a Gaussian distribution in the chromatogram is taken to represent such a situation, namely, that the isotherm of the relationship:

$$\frac{\text{Concentration of the solute in the stationary phase}}{\text{Concentration of the solute in the mobile phase}}, \text{ is linear [Figure 7.6(a)].}$$
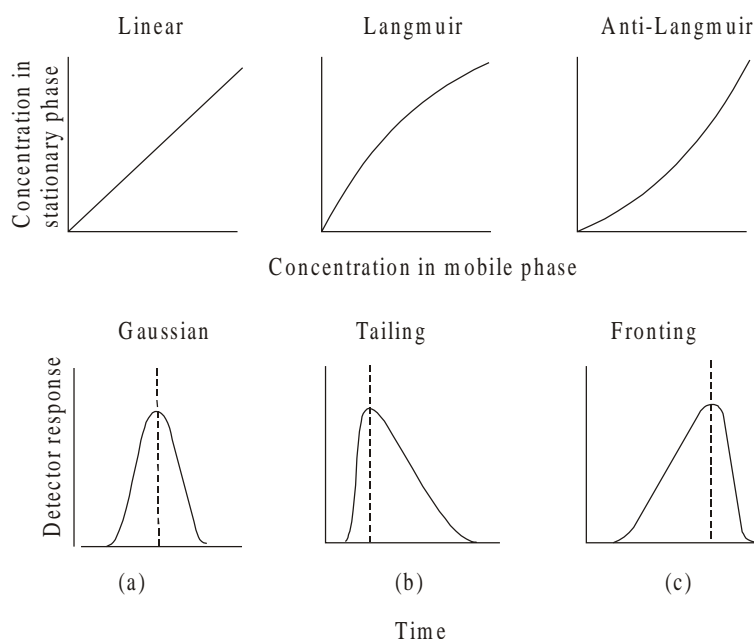
Figure 7.6 Three basic isotherm shapes and their effect on peak shape and retention time.

Under these conditions the retention time is independent of the sample size. When the distribution coefficient, as often happens, is not independent of sample concentration, abnormal behaviour of the column results. Skewed peaks are the result of non-linear distribution isotherms, as shown in Figure 7.6(b) and Figure 7.6(c), and the retention time will vary with sample concentration.

The leading and trailing edges of the solute band on the column contain solute at low concentrations, while at the centre of the band solute is present at high concentrations. Considering the case in which, at high concentrations, the stationary phase approaches saturation with solute, but the solvent does not, the centre of the band will move more rapidly than the leading edge and will tend to overtake it. The band will thus acquire a very steep leading edge. The trailing edge will travel more slowly than the band as a whole and will, therefore, form an elongated 'tail'. The band, as it issues in the effluent, will have a sharp leading edge and a long tail [Figure 7.6(b)]. Bands of this shape are frequently observed and are an indication of overloading, or, more accurately, of a distribution coefficient which changes markedly with concentration. Obviously, if the ratio:

$$\frac{\text{Concentration of the solute in the stationary phase}}{\text{Concentration of the solute in the mobile phase}},$$

increases at high solute concentrations, the centre of the band will move more slowly than either edge, and the band will have a sharp trailing edge and an elongated leading edge [Figure 7.6(c)].

Prior to 1950, non-linearity of distribution isotherms was considered very common in liquid column chromatography. However, the generally symmetrical spots obtained in thin-layer chromatography (TLC) clearly demonstrated that isotherm linearity was easily attainable in liquid column chromatography if only small sample loadings were employed, and it is only since the recent past that the practical advantages of linear isotherm separations in columns have been exploited. If the sample is sufficiently small, symmetrical Gaussian peaks can very often be obtained even for components with a non-linear isotherm, and retention times will again be independent of the sample size.

The maximum amount of sample that can be charged to a column without loss in the linearity of the isotherm is called *adsorbent linear capacity* denoted by $\theta^{0.1}$. In more precise terms, it is the weight of the sample per gram of the adsorbent at which a particular value of distribution coefficient deviates 10% from its linear isotherm value. The adsorbent linear capacity of an adsorbent is proportional to its *specific surface area* (i.e. the surface area in meter^2 of one gram of the adsorbent). Adsorbents used for chromatography are porous solids with high specific surface area which provides high sample capacity. Increase in porosity and decrease in the average pore diameter enhances the surface area.

7.12 Sample Volume

The volume in which the sample is added to the column should be kept small. As a first approximation, it may be stated that when the sample is added in a 10 ml volume rather than in a 1ml volume, we are producing on the column the equivalent of 10 solute bands, which will begin to appear in the effluent at 1 ml

intervals. The width of the effluent band will, therefore, be increased tenfold because of volume of the sample. If the actual observed width of the band is 100 ml, it is obvious that no great improvement could be effected by reduction of sample volume. If, however, the observed width is of the order of 20 ml, much better resolution could be obtained by reduction of sample volume. It is thus easy to determine, in an actual case, whether sample volume is having an appreciable effect on resolution.

7.13 Selection of the Appropriate Procedure of Sample Introduction

Preferably, the sample is introduced in a minimum volume of mobile phase. In some cases, when the mobile phase is an organic solvent, it is difficult to prepare such a solution. A number of other methods have been used with varying degrees of success. When the stationary phase is an aqueous solution held on a solid packing such as diatomaceous earth, the sample may in some cases be mixed as an aqueous solution, with a small amount of dry packing. The mixture is then added to the top of the column. Polar organic solvents have been used as solvents for sample addition.

Any unorthodox method of sample addition should be evaluated before adoption. Such evaluation is best done by comparing the resolution obtained by the new method with that obtained by the standard method i.e. addition in a small volume of mobile phase. The difficulties most often experienced are slow extraction of the sample from the top of the column, giving broad, poorly separated bands and interaction of the solvent used for addition of the sample with the mobile phase of the column, giving a non-uniform, poorly reproducible column.

7.14 Conclusion

The earlier discussion in the present chapter has detailed the theory that relates performance of an LC column to its various parameters. An appropriate conclusion to the theoretical discussions given above can be made in the form of the following summary of the essential improvements in equipment, materials and experimental procedures of liquid chromatography effected as a consequence of this better understanding, in recent times, of the underlying theory of chromatography.

7.14.1 Column Packing

In LC, mobile phase band-broadening plays a major role and column packing procedures play a very important role in obtaining reproducible columns. Spherical support particles are preferable to irregularly shaped ones for HPLC, because the former pack densely and reproducibly better than the latter. For regular packed columns, dense packing helps better radial mixing of the mobile phase through disruptions of the flow paths by intervening particles. Particle size distribution should also be as narrow as possible to prevent segregation of large and small particles taking place in the column. In its turn, occurrence of such a segregation

will create zones of different velocities in the column (since permeability $\propto d^2_p$). Segregation occurring in the entire column length will naturally create severe velocity inequalities, making it difficult for diffusion and convection effects to overcome this undesirable effect.

A uniform cross-sectional density of support material and dense packing are the two main desired features of any packing procedure. For particle size above 20 μ, dry packing works well. For particles of d_p < 20 μ, dry packing proves unsatisfactory and slurry packing is used instead. In general, it is not easy to pack particles of small d_p.
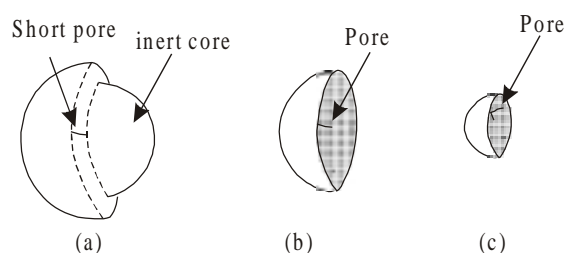

Figure 7.7 Types of HPLC packings (a) pellicular beads (25-50 μ), (b) microporous particles (20-40 μ) with longer pores (c) microporous particles (3-10 μ) with short pores.

Porous materials like silica and alumina are most commonly used for column packing in conventional liquid chromatographic techniques. These materials also possess large surface area necessary for adsorption. However, they become disadvantageous in HPLC, as C_{SM}^{ν} term in Equation 7.10 (slow mass transfer caused by solute diffusion in the mobile phase stagnant in the pores) becomes important, limiting the available speed which is consistent with reasonable efficiency. To meet the requirement of high speeds of HPLC, the migration distance or pore depth must be decreased. This has been achieved by use of two types of small-size porous materials (Figure 7.7) as stationary phase in HPLC: (i) Porous layer or pellicular beads (PLB), and (ii) microporous particles.

Porous layer or pellicular beads are composed of thin porous layers of adsorbent surrounding solid impregnable cores. The thin layer in PLB facilitates rapid mass transfer. Therefore, high ν can be achieved while conditions close to equilibrium can be maintained so that efficiency of separation is not harmed. Particle diameter ranges from 25 to 50μ and the layer thickness is about 1μ. PLB can be used in liquid-liquid chromatography as well as in liquid-solid chromatography.

In microporous particles, pore depths are decreased by decreasing d_p. Particles of 20–40 μ diameter with longer pores and of 3–10 μ diameter with short pores are available. Microporous beads are prepared from porous materials like silica or alumina.

In liquid chromatography mobile phase viscosities are some 100 times greater than those in gas chromatography. Thus pressure drops in liquid chromatography

will be 100 times greater than those in gas chromatography for a given column length and flow rate. The combined effect of viscosity and particle size accounts for the use of high pressures in liquid chromatography compared with gas chromatography.

Since the water content of absorbents like silica or alumina plays a critical role in liquid-solid adsorption chromatography in that it affects relative k′ values and band migration rates, adsorbent water content must be held constant to obtain repeatable separation with constant band migration rates in liquid-solid chromatography. Addition of some water to the adsorbent helps to (i) increase the linear capacity (addition of the right amount of water is reported to enhance $\theta^{0.1}$ by a factor of 5–100 relative to that of dry adsorbent), (ii) maximize separation efficiency, (iii) decrease the build-up of static charges during dry-packing (thereby favouring denser, more efficient columns), and (iv) decrease adsorbent-catalyzed sample reactions and irreversible sample adsorption (thereby favouring complete recovery of components). Constancy of adsorbent water content can generally be attained by adjusting the water content of the mobile phase.

Deactivation of adsorbents such as charcoal (which do not absorb water) can be achieved with certain high molecular weight organic compounds such as catyl alcohol or stearic acid.

The length required for a certain efficiency mainly decides the size of an analytical column. In preparative separations by liquid-solid adsorption chromatography, the quantity of the adsorbent required for a given load also comes into picture. The value of $\theta^{0.1}$ fixes the quantity of adsorbent required for a given sample size, and this translated into practical terms implies that the amount of sample that is placed on a given column does not cause overloading of the column. Also, it is to be kept in mind that H increases in an overloaded column. Sample size, therefore, should be less than $\theta^{0.1}$ of the column to achieve (i) constant sample migration rates and (ii) maximum separation efficiency.

In conventional columns the diameter is about 10 mm. In HPLC, narrow columns are preferred as (i) they exhibit greater efficiency (owing to the fact that the distance over which radial mixing needs to occur is less in these cases compared to wider columns; relaxation of inequalities in velocity, therefore, becomes easy), and (ii) they produce a higher υ for a given flow rate. In HPLC analyses, column diameters employed are in the range of 2–4 mm. Knox and Parcher described a phenomenon, the *infinite diameter column*, in which an injected band of solute never reaches the wall of the column before it has arrived at the column exit. They assumed that stream splitting is the important mechanism by which solute molecules placed in the centre of the column reach the walls, since diffusion of the solutes in liquids is very slow. Making this assumption, they described an expression relating column and packing dimensions to achieve the infinite diameter conditions:

$$A = \frac{d_c^2}{d_p L} \geq 2.4$$

where A is the Knox and Parcher coefficient and d_c is column internal diameter and d_p and L are, of course, particle diameter and column length respectively. A regular increase in column efficiency with increasing value of A up to 45 has been observed.

If the columns are used in an overloaded condition, the infinite diameter phenomenon does not apply.

7.14.2 Solvent Selection

Since the nature of the solvent chosen affects all the three factors viz. α, N and k′ which affect the resolution, the need of keeping this aspect in mind while making choice of an appropriate solvent (i.e. the one that maximizes resolution) cannot be over emphasized.

The criteria that minimally must be met by the chosen solvent have been mentioned in Chapter 5. These are: (*i*) Ability of the solvent for dissolving the sample and its non-interference with sample recovery; (*ii*) inertness of the chosen solvent towards the stationary phase used.

To achieve constancy of the water content of the stationary phase (needed for repeatable and reproducible operations) sufficient water must be added to the solvent so that solvent (mobile phase) and adsorbent (stationary phase) are in thermodynamic equilibrium with respect to the activity of water in each phase.

A primary requirement on the solvent for the present day techniques in LC is that the solvent must be compatible with the detector employed. For example, UV detectors cannot be used with solvents which exhibit very high absorption at the wavelength of interest.

A viscous solvent increases H (by reducing sample diffusion co-efficient and slowing mass transfer). Increase in solvent viscosity reduces permeability and requires a higher ΔP for a given solvent flow rate. The combined effect of both these factors will be to reduce N for an increase in η (other factors such as separation time being constant). For constant separation efficiency, increase in η increases separation time (other factors being equal). η can be kept below 0.4–0.5 centipoise (by proper blending of solvents of high and low viscosity). Use of solvents with very low η (pentane, ether—~0.2 cp) creates the undesirable side effect of formation of bubbles in the column and detector (owing to their low boiling points). Use of a short length of small diameter tubing (flow restrictor), after the detector, avoids this to some extent.

In a case where inadequacy of separation results from the general elution problem, the right solution is provided by gradient elution i.e. solvent programming. Temperature programming, flow programming, repeated separation under changed conditions and coupled columns i.e. stationary phase programming, may be mentioned as alternative solutions of the general elution problem.)

■

8

Ion-Exchange Chromatography

As its name suggests ion-exchange chromatography is used for the separation of ionic substances which range from simple inorganic and organic ions to polyelectrolytes such as enzymes, proteins, hormones, viruses, nucleic acids and other biologically important substances. Separation of inorganic mixtures, especially of metals with similar chemical characteristics e.g. lanthanides, is among the important applications of this technique. Ion-exchange methods have, in some respects, marked advantages over the adsorption and partition techniques. The technique can readily handle quite large quantities (0.1g or more) of the starting material. Also, recovery from the column is virtually complete, which is, of course, very important in quantitative work. Columns packed with an ion-exchange material can be used repeatedly. Conditions can readily be manipulated to achieve almost any desirable value of distribution co-efficient for almost any ion. Construction of high efficiency columns is readily possible. Disadvantages of the technique are: (i) Gravity separations are slow; (ii) separated components are accompanied by a large excess of eluting electrolyte.

Ion-exchange chromatography is a liquid-solid technique of column chromatography and comprises of an electrolyte solution as the mobile phase and an ion-exchanger as the staionary phase.

8.1 Ion-exchangers

Ion-exchangers are insoluble substances containing reactive groups possessing ions which are capable of exchanging with ions in the surrounding medium. Ion-exchangers have a porous structure so that liquid can percolate through them as well as around them.

Ion-exchangers may be naturally occurring substances or they may be synthetic. Both natural and synthetic ion-exchangers may be inorganic or organic in chemical composition. For use in chromatography, man-made organic synthetic ion-exchangers are the most important.

Zeolites (sodium aluminosilicates) are the most well-known naturally occurring inorganic ion-exchangers. The first inorganic synthetic exchangers were the alkali metal aluminosilicates used in water softening.

Humus in the soil and some kinds of lignite (brown coal) are examples of organic ion-exchangers found in nature. After discovery that ion-exchange could be performed in aqueous solution on finely ground gramophone records, Adams

and Holmes attempted preparation of organic ion-exchange resins and succeeded in getting the first synthesis of such resins in 1935. Since then a number of classes of organic ion-exchanger have been synthesised and put into use in ion-exchange chromatography.

Organic ion-exchangers used in chromatography have been classified on the basis of the nature (i.e. acidic or basic) and the individual microstructure that each has. Getting well acquainted at this stage with this classification and with the details of the basis for it, will help in acquiring a better understanding of the succeeding discussion of the theory underlying ion-exchange chromatography.

8.1.1 Classification of Ion-exchangers

Now-a-days, three types of ion-exchange materials are commonly encountered: (i) Ion-exchange resins, (ii) Ion-exchange celluloses, and (iii) Ion-exchange gels. As will be seen from the following account of these three types of ion-exchanger, the difference between them lies partly in the nature of the exchanging groups which are incorporated into each, but mostly in the individual microstructures that each has.

(i) Ion-exchange Resins: These resins are products either of polycondensation or of polymerisation. The first synthetic ion-exchangers, as said earlier, were made by Adams and Holmes, who prepared these by polycondensation of phenols with formaldehyde. A typical example of such a polycondensation leading to the formation of a long linear chain can be represented as:
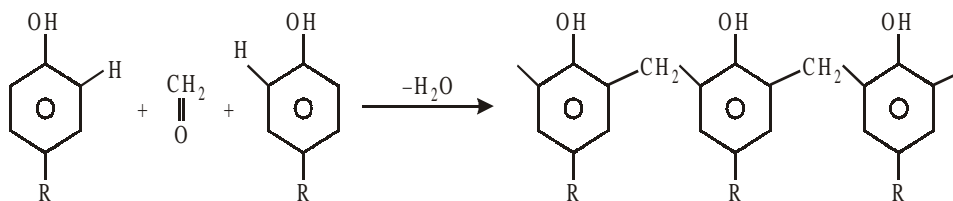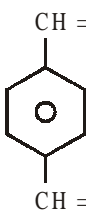

The chain can be cross-linked if some unsubstituted phenol and additional formaldehyde are added to the reaction mixture, the polycondensation under these conditions being represented by the equation:
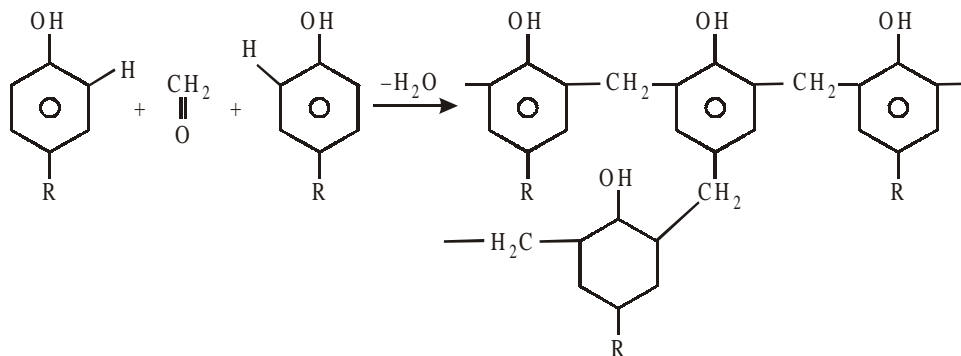
The degree of cross-linking in the resulting polycondensate can be controlled by varying the ratio of substituted and unsubstituted phenols. If there are no cross-links, the product may be soluble and consequently of little use as an ion-exchanger; if too many cross-links are present no ion-exchange may be possible with the product. The phenol may be replaced by an aromatic amine.

Ion-exchangers obtained by polymerisation are generally made from styrene, which ordinarily polymerises to give a linear polymer as represented by the following equation:
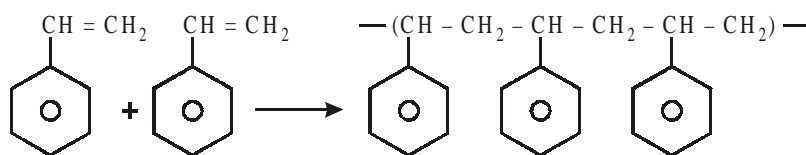
However, in the presence of divinylbenzene, , styrene gives a cross-

linked polymer which may be represented as :
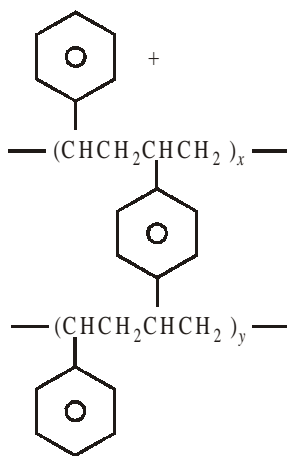
The degree of cross-linking is controlled by the ratio of the components.

Polymerisation of methacrylic esters with divinylbenzene also yields copolymers meant for the commercial preparation of polymer-type ion-exchangers.

It is the aforesaid methods that are most generally used to prepare the cross-linked network. It is this network that carries the ionogenic centres, designated as the functional groups of the resins, which give the ion-exchangers their typical

properties. The functional groups are introduced either by subsequent treatment of the resin or by incorporating the functional groups into the starting material.

If the ion-exchangers liberate and exchange cations they are called cation-exchangers or catexes. Anion-exchangers or anexes set free and exchange anions. Each type of exchanger is also classified as strong or weak according to the ionising strength of the functional group. A *strongly acidic cation-exchanger* contains the sulphonic acid group, whereas the weakly acidic carboxylic and phenolic groups would lead to a *weakly acidic cation-exchanger*. The functional groups all dissociate to release hydrogen ions, which are exchanged for other cations e.g.

$$R - SO_3H \rightleftharpoons RSO_3^-H^+ \underset{H^+Cl^-}{\overset{Na^+Cl^-}{\rightleftharpoons}} R - SO_3^-Na^+$$

(R = organic polymeric matrix)
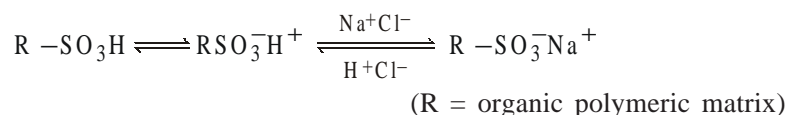
Similarly, an exchanger with a quaternary basic group is a *strongly basic anion-exchanger*, whereas presence of a primary or secondar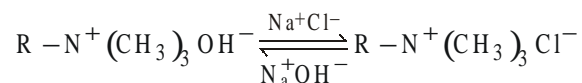y aromatic or aliphatic amino group will give a *weakly basic anion-exchanger.* A typical anion-exchange reaction with a strongly basic anion-exchanger (a quaternary base) is given by the equation:

$$R - N^+(CH_3)_3 OH^- \underset{N_a^+OH^-}{\overset{Na^+Cl^-}{\rightleftharpoons}} R - N^+(CH_3)_3 Cl^-$$

The preparation of polycondensates has been gradually abandoned and today ion-exchangers produced mainly by polymerisation reactions are used. Polymeric ion-exchangers possess higher chemical stability than polycondensates; also it is easier to make ion-exchangers with the necessary outer shape, porosity and chemical composition from polymer resins.

Polymer resin beads may be of two types (Figure 8.1): (i) Microreticular (gel) [Figure 8.1(a)], and (ii) macroreticular (macroporous) [Figure 8.1(b)]. The gel type becomes porous on swelling, with the pore size dependent on the degree of cross-linking.

(ii) Ion-exchange Celluloses : Derivatives of cellulose with ion-exchanging groups play an important role in chromatography of biochemical mixtures. Carboxy-methyl cellulose [known as CM — cellulose, represented in Figure 8.2(a)] and diethylaminoethyl cellulose [DEAE — cellulose, represented in Figure 8.2(b)] are the two ion-exchange celluloses
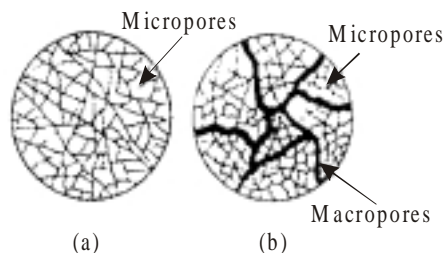

Figure 8.1 Ion-exchange packings
(*a*) Microreticular ion-exchanger with small pores.
(*b*) Macroreticular ion-exchanger with macropores

mainly used at present. CM — cellulose represents a cation-exchange type with weakly acidic groups, and it is produced by reaction of cellulose (Cel – OH) with chloroacetic acid in alkaline medium:

$$Cel\text{–}OH \xrightarrow{ClCH_2COONa} Cel\text{–}OCH_2\ COO^-Na^+$$

(a)   (b)
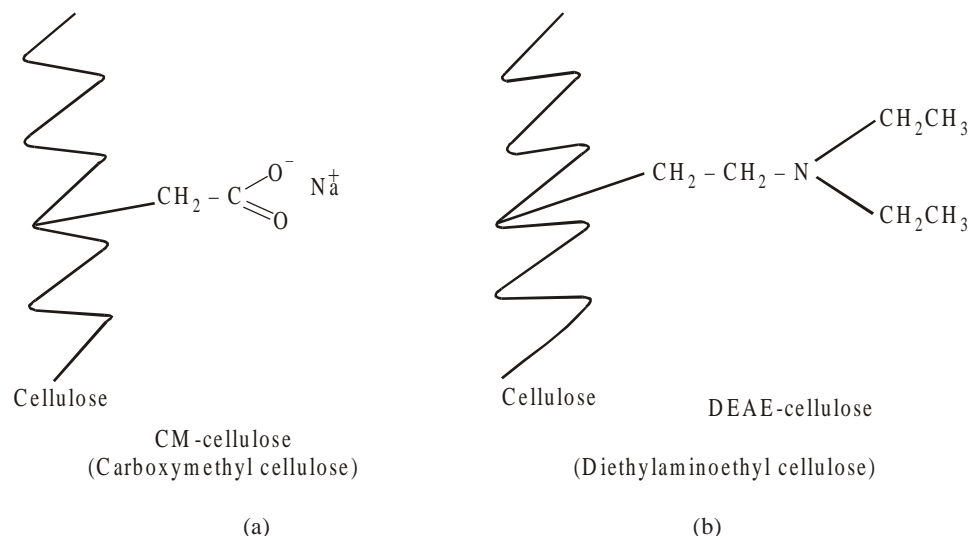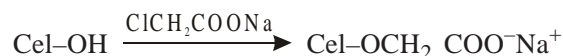
Figure 8.2 Common ion-exchange materials used in biochemical separations

DEAE-cellulose is an example of an anion-exchange type with moderately basic groups and it is prepared by reaction of cellulose with N, N-diethylaminoethyl chloride in the presence of alkali:

$$Cel\text{–}OH \xrightarrow{Cl\ CH_2\ CH_2N(C_2H_5)_2} Cel\text{–}OCH_2CH_2N\ (C_2H_5)_2.$$
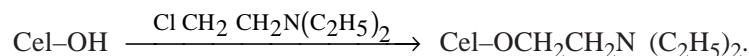
In the preparation of ion-exchange celluloses main attention is paid to their physical structure and controlled distribution of exchange groups in their mass.

Ion-exchange celluloses possess a characteristic physical structure with large pores, and they can be used for work with biopolymers of molecular weight upto 10^6. Owing to the presence of a large number of –OH groups in the structure of ion-exchange celluloses, these ion-exchangers are highly hydrophilic and are better suited for work with biopolymers than ion-exchange resins having hydrocarbon skeleton. In contrast to other types of ion-exchanger, ion-exchange celluloses are prepared in fibrous form or as so-called microgranules, in the form of short rolls, though recently chromatographic cellulose derivatives have been prepared in spherical form as well.

(iii) Ion-exchange Gels: Ion-exchange derivatives of the polysaccharide, dextran, with carboxymethyl ($-CH_2\ COO^-$), diethylaminoethyl [$-C_2H_4N^+H$

$(C_2H_5)_2$], sulphoethyl ($-C_2H_4SO_3^-$), sulphopropyl ($-C_3H_6SO_3^-$) and quaternary basic groups like $- N^\oplus R_3$ or $> N^\oplus R_2$ have been prepared. Because of the high content of hydroxyl groups of dextran, the ion-exchangers based on this polysaccharide are highly hydrophilic and they, therefore, swell up in water or electrolyte solutions to give semi-transparent gel particles. These can be packed in the normal way into columns for chromatography.

Dextran ion-exchangers differ from the cellulose ones primarily in their physical structures. Though having much larger pores, dextran ion-exchangers are similar in their physical structure to the microporous type of ion-exchange resins, the porosity of which is a consequence of swelling. Like ion-exchange resins, dextran

TABLE 8.1

Examples of some commonly used ion-exchangers

Type	Polymer	Functional group	Examples of commercial products
Weakly acidic (cation exchanger)	Polyacrylic acid	$-COO^-$	Ambarlite IRC 50 Bio-Rex 70 Zeocarb 226
	Cellulose or dextran	$-CH_2COO^-$	CM–Sephdex Cellex CM
	Agrose	$-CH_2COO^-$	CM–Sepharose
Strongly acidic (action exchanger)	Polystyrene	$-SO_3^-$	Amberlite IR 120 Bio-Rad AG 50 Dowex 50 Zeocarb 225
	Cellulose or dextran	$-CH_2CH_2CH_2SO_3^-$	SP-Sephadex
Weakly basic (anion-exchanger)	Polystyrene	$-CH_2N^+HR_2$	Amberlite IR 45 Bio-Rad AC = 3 Dowex WGR
	Cellulose or Dextran	$-CH_2CH_2\overset{+}{N}H(CH_2CH_3)_2$	DEAE-Sephadex Cellex D
	Agrose	$-CH_2CH_2\overset{+}{N}H(CH_2CH_3)_2$	DEAE-Sepharose
Strongly basic (anion exchanger)	Polystyrene	$-CH_2\overset{+}{N}(CH_3)_3$	Amberlite IRA 401 Bio-Rad AG1 Dowex 1
		$-CH_2\overset{+}{N}(CH_3)_2$ $\mid$ CH_2CH_2OH	Amberlite IRA 410 Bio-Rad Ag-2 Dowex 2
	Cellulose or dextran	$-CH_2CH_2\overset{+}{N}(CH_2CH_3)_2$	Cellex T

ion-exchangers are also prepared in spherical form. Having a highly hydrophilic character and a structure with pores of sufficiently large size, dextran-based ion-exchangers can be advantageously used for work with natural biopolymers.

Quite recently ion-exchange derivatives of agarose, which has been cross-linked to have more or less uniform 3-dimensional network, have become available. These CM (i.e. carboxymethyl) and DEAE (i.e. diethlaminoethyl) — derivatives are superior to ion-exchange dextrans in that they have greater stability and can be used for biopolymers having molecular weight upto 10^6.

Table 8.1 lists the various types of some commonly used ion-exchangers.

8.2 Theory of Ion-exchange Chromatography

The five successive steps comprising the over-all ion-exchange process are as follows:

 (i) Diffusion of the ion from the solution to the surface of the ion-exchanger; it is a very fast process in homogeneous solutions.

 (ii) Diffusion of the ion through the matrix structure of the ion-exchanger to the exchange site; this is considered to be the rate-controlling step of the over-all ion-exchange process.

 (iii) Exchange of ions at the ion-exchange site.

 (iv) Diffusion of the exchanged ion through the exchanger to its surface.

 (v) Selective desorption by the eluent and diffusion of the molecule into the external solution; the selective desorption of the bound molecule is achieved by changes in pH and/or ionic concentration or by using a complexing agent in the mobile phase or by affinity elution in which case an ion which has greater affinity for the exchanger than has the bound molecule, is introduced into the system.

Ion-exchangers have a porous structure so that liquid can percolate through them, thereby bringing about swelling of the beads of the ion-exchanger. A two-phase system is constituted by putting swollen particles of an ion-exchanger in contact with an aqueous solution of a mixture of several components. If the components form ions in solution, then electrostatic interaction of ions takes place with the ionogenic (ion-bearing) site of the ion-exchanger and exchange of ions results. To a first approximation Coulomb's law expressed by the equation:

$$F = \frac{q_1 q_2}{r^2} = \frac{q_{ion} \times q_{(ion-exchanger)}}{r^2_{(hydrated\ ion)}}$$

can be used to predict the force F of attraction between an ion-exchange site of charge $q_{(ion-exchanger)}$ and appositely charged ion with a charge of $q_{(ion)}$ which are separated by distance r.

If the charge of the ion is assumed to be located in the centre of the hydrated ion in solution, then the distance of closest approach between the ion-exchange site

and the ion is approximately equal to the radius of the hydrated ion, $r_{(hydrated\ ion)}$. It is readily apparent from the equation that the force of attraction between a specific ion-exchange resin and an ion increases as the charge of the ion increases but decreases with increasing radius of the hydrated ion. Consequently, an ion-exchanger has greater attraction or affinity for ions with greater charge and smaller diameter. As the charge increases and/or as the radius of the hydrated ion decreases the retention time of the ion on an ion-exchange column increases.

The effect of charge on retention on an ion-exchange column is demonstrated by the relative retentions of Na^+, Ca^{2+}, La^{3+} and Th^{4+} on cation-exchange columns. All four ions have nearly the same radius but the order of attraction to an ion-exchanger is $Th^{4+} > La^{3+} > Ca^{2+} > Na^+$. That is, the ion with greatest charge is most strongly attracted. Generally the degree of attraction of an ion to an ion-exchanger increases in going down in a group in the Periodic Table. For the group IA elements the order of attraction on a cation-exchange column is $Li^+ < Na^+ < K^+ < Rb^+ < Cs^+$; for the group II A elements the order of attraction is $Be^{2+} < Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+}$; and for the group VII A elements the order of attraction to an anion-exchange column is $F^- < Cl^- < Br^- < I^-$. In each case the degree of attraction to the ion-exchanger apparently decreases with increasing radius of the hydrated ion.

The process of exchange of ions taking place at the ionogenic site of the ion-exchanger which has been mentioned above is a reversible process and can be expressed by the equation (R = ion-exchanger):

R – X + Y = R – Y + X where X and Y are ions with charges of the same value. How the direction of this reversible reaction is affected by a change in the ionic concentration of the mobile phase or by a change of the eluent ion, will become clear from the following discussion illustrated by taking the case of a column packed with a strongly acidic cation-exchanger. Consider the case of a column packed with a typical strong acid resin, Dowex 50 × –8 in Na^+ form. Suppose that the mobile phase is lM NaCl. Properties of the above column are tabulated below.

Grams dry resin per ml of column 0.4

Milliequivalants resin per ml of column 1.94

Ml aqueous solution per ml of column 0.73

One ml of column then contains 0.73 meq of Na^+ in the mobile phase and 1.94 meq of Na^+ in the stationary phase. The sodium ions on the column continuously and rapidly exchange between the two phases. Any individual sodium ion spends 0.73 second in the mobile phase for every 1.94 seconds it spends on the stationary phase.

A term which is very conveniently applicable in chromatography is *effective distribution coefficient* (B). This may be defined as the total amount of substance present in the mobile phase divided by the total amount present in the stationary phase.

The B value for sodium ions for the case of the column considered above is 0.73/1.94 or 0.376. The B value for any other cation used in lM concentration as the mobile phase as an eluent cation would also be 0.376. If the concentration were other than lM, the B value would be 0.376 times the molarity. What is of primary interest for chromatography is the B value of a sample cation, added to the column in small amounts. Suppose that the eluent cation is Na^+ and that the sample cation is NH_4^+. The ammonium ions will equilibrate with the stationary phase according to the following reaction:

$$R^-NH_4^+ + Na^+ \rightleftharpoons R^-Na^+ + NH_4^+$$

$$K_{eq} = \frac{\left[NH_4^+\right]\left[R^-Na^+\right]}{\left[R^-NH_4^+\right]\left[Na^+\right]}$$

$$\text{or} \quad \frac{\left[NH_4^+\right]}{\left[R^-NH_4^+\right]} = K_{eq}\frac{\left[Na^+\right]}{\left[R^-Na^+\right]}$$

It will be noted that if the equilibrium constant, K_{eq}, is equal to one, the B value for ammonium ion, $\dfrac{\left[NH_4^+\right]}{\left[R^-NH_4^+\right]}$, is equal to the B value for sodium ion. Regardless of the value of K_{eq}, the B value for ammonium ion is proportional to that for sodium ion and can be varied over a wide range by changing the concentration of the sodium ion in the mobile phase. The B value of the sample ion, NH_4^+, may be adjusted to an optimum value by selecting the proper concentration of the eluting ion, Na^+.

It is evident from the equation:

$$\frac{\left[NH_4^+\right]}{\left[R^-NH_4^+\right]} = \frac{K_{eq}\left[Na^+\right]}{\left[R^-Na^+\right]}$$

that K_{eq} is the B value of ammonium ion relative to that for sodium ion. The value of K_{eq} for an ion depends on its affinity for the ion-exchange resin. Ions of high affinity have low K_{eq} values.

As given above, the B value for Na^+ is 0.376, when lM Na^+ is used as an eluent in a column packed with Dowex 50×–8 resin and the sample is ammonium ion. In the same column, the B value of NH_4^+ is 0.376 multiplied by 0.78 (the value of NH_4^+ relative to that of Na^+) or 0.293. If, however, 1 M K^+ is used as the eluent, the B value of K^+ is also 0.376, but the B value for NH_4^+ is now

$$0.376 \times \frac{0.78}{0.69 \left(\text{the value of } k^+ \text{ relative to that of } Na^+ \right)} \text{ or } 0.425.$$

The cations viz. Na^+, NH_4^+ and K^+, referred to above, are all monovalent ions. The B value of a polyvalent cation on a column where the mobile phase is a sodium chloride solution does not vary linearly with the sodium chloride concentration. The equilibration of a divalent cation in the column may be represented thus:

$$R_2^- Mg^{2+} + 2 Na^+ \rightleftharpoons 2R^-Na^+ + Mg^{2+}$$
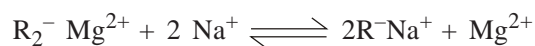
$$K_{eq} = \frac{\left[Mg^{2+} \right]\left[R^-Na^+ \right]^2}{\left[R_2^-Mg^{2+} \right]\left[Na^+ \right]^2}$$

whence

$$\frac{\left[Mg^{2+} \right]}{\left[R_2^-Mg^{2+} \right]} = K_{eq} \frac{\left[Na^+ \right]^2}{\left[R^-Na^+ \right]^2}$$

It will be noted that the B value for Mg^{2+} is proportional to the square of the Na^+ concentration. In the case of a cation with many charges, for example, a protein or polypeptide cation, the B value will be an extremely steep function of the Na^+ concentration.

Effect of pH on attraction to ion-exchange columns

If one or more of the ions in an ion-exchange column is the conjugate acid or base of a weak base or weak acid, the pH of the solution will have an important effect on the degree of dissociation of the weak acid or base. Since it is ions that are retained on ion-exchange columns and since the pH of the solution can affect the relative number of ions in the column because of the dependence of the dissociation constant (or, in other words, pK values) of the ionogenic group on pH, the pH can have a large effect on the retention of the species on the column.

Consider the equation:

$$HA \rightleftharpoons H^+ + A^-,$$
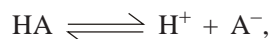
illustrated by the case of the hypothetical weak acid HA. As the pH of the mobile phase passing through an anion-exchange column containing A^- or HA decreases, the concentration of H^+ in the solution increases and the equilibrium of the above equation is shifted to the left. This results in a decrease in the amount of A^- retained on the ion-exchanger. Likewise, as the pH is decreased in a cation-exchange column containing a weak base or its conjugate acid, relative number of

cations increases and consequently the degree of retention increases. It is thus evident that stepwise or gradient adjustment of the pH of the eluting solution can be used as a tool for separating weak acids or weak bases whose pK values are significantly different. Besides the concentration of the solution, the degree of cross-linking of the ion-exchange resin and the effective diameter of the hydrated ions determine the rate of diffusion of the ions through the matrix structure of the ion-exchanger. The higher the degree of cross-linking, the more difficult such penetration is. To be held by the resin, an ionized molecule must penetrate the particle, and since practically all of the ionized groups in a particle of the ion-exchange resin are inside the particle, this diffusion of the ions through the matrix structure of the ion-exchanger is considered to be the rate determining feature of the over-all ion-exchange process.

From the above discussion it can be concluded that the various factors which are responsible for the varying degree of distribution of the components between the phase constituted by the ion-exchanger and the solution phase are: (i) Differing ionic change of the components, (ii) differing dissociation constant of the ionogenic groups of the components, (iii) difference in ionic size of the components in solution. In the case of some components distribution between the two phases is also affected by the different degree of adsorption on to the matrix of the ion-exchanger. Varying degree of distribution of the components between the two phases that results from the play of the aforesaid factors brings about separation of the components when there is relative movement between the solid and liquid phases just as in other types of chromatography.

For the chromatography of lower molecular weight ionogenic organic substances (amines and other bases, acids, amino acids, peptides, nucleosides, nucleotides) ion-exchange resins should be chosen. However, these are not suitable for the chromatography of proteins. Biopolymers (proteins, nucleic acids and their higher molecular weight fragments) may be separated successfully on ion-exchange derivatives of cellulose, dextran and agarose. Basic substances are chromatographed in the form of cations on cation-exchangers. Acid substances are chromatographed in the form of anions on anion-exchangers. For chromatographic separation of cations or anions preference is given to strongly acid cation-exchangers or strongly basic anion-exchangers. Amphoteric biopolymers or their fragments can be chromatographed both on strongly and weakly acid cation-exchangers and on strongly and weakly basic anion-exchangers. For chromatography on ion-exchange resins preference is given to monofunctional ion-exchangers. They are usually styrene-divinylbenzene type resins. A single type of functional groups gives hope of a sharper separation. An important factor for the choice of an appropriate ion-exchanger is the degree of cross-linking. It is selected so that the substance may just permeate into the ion-exchanger. Small pores do not permit the entrance of the substances to the inner functional groups, and excessively large pores decrease the separation efficiency. The parameters of the

chromatographic process should enable equilibrium to be attained between the mobile and the stationary phase during the flow through the column. Here the grain-size of the ion-exchanger is a decisive factor. The smaller the particles the more rapidly equilibrium is attained i.e. a more rapid flow may be used. However, the resistance to flow in the column increases with decreasing particle size. In case of sufficiently hard particles (ion-exchange resins) pressure may be applied. Some softer ion-exchangers (e.g. dextran type) cannot stand increased pressure. For chromatography the uniformity of particle size is of great importance.

In regard to the choice of the buffer, the decision includes not just the buffer substance, but also the pH and the ionic strength. Buffer ions with a charge opposite to that on the ion-exchanger will compete with solute for binding sites and greatly reduce the capacity of the column. The following guidelines should be used: (i) Cationic buffers should be used with anionic exchangers; (ii) anionic buffers should be used with cationic exchangers.

The buffer pH should be chosen so that the desired molecule will bind to the ion-exchanger. When one is dealing with large bionolecules e.g. proteins, the pH 'range of stability' must be kept in view. The 'range of stability' refers to the pH range in which the biomolecule is not denatured. In addition, the ionic strength should be relatively low to avoid 'damping' of the interaction between solute and ion-exchanger. Buffer concentrations in the range of 0.05 to 0.1 M are recommended.

As regards monitoring of the separated components emerging in the column effluent, it is carried out by means of a physical measurement, for example, UV or visible absorbance, refractive index or conductivity, as is routinely done in liquid column chromatography. Alternatively, separate fractions can be collected automatically and subjected to further analysis.

8.3   Separation on Ion-exchange Columns

8.3.1 Pretreatment of Ion-exchanger

Pretreatment of the chosen ion-exchanger precedes its use in the column. Failure to pretreat ion-exchangers will greatly reduce the capacity and resolution of a column. The commercial suppliers of ion-exchangers provide detailed instructions for the pretreatment of the adsorbents. As an example, a suitable method of preconditioning of cellulose ion-exchangers, which are extensively used in biochemical research, will be described here. Most cellulosic ion-exchangers are supplied in a dry power form and must be swollen in aqueous solution to 'uncover' ionic functional groups. The two most common cellulosic resins are diethylaminoethyl (DEAE) cellulose and carboxymethyl (CM) cellulose. To pretreat the anion-exchanger, DEAE cellulose, 100g, is first suspended in 1 litre of 0.5 M HCl and stirred for 30 minutes. The cation exchanger, CM cellulose, is suspended in 1 litre of 0.5 M NaOH for the same period. This initial treatment puts

a like charge on all the ionic functional groups, causing them to repel each other; thus, the exchanger swells. The cellulose is then allowed to settle (or filtered) and washed with water to a neutral pH. DEAE cellulose is then washed for 30 minutes with 1 litre of 0.5 M NaOH (CM cellulose with 0.5 M HCl), followed by water until a neutral pH is attained. This treatment puts the ion-exchanger in the free base or free acid form, and it is ready to be converted to the ionic form necessary for the separation. In the next step trace metal ions are removed by treatment of the swollen cellulose with 0.0lM EDTA.

The above washing treatments do not remove small particles that are present in most ion-exchanger materials. If left in suspension, these particles, called fines, will result in decreased resolution and slow column flow rates. Removing the fines from an exchanger is done by suspending the swollen adsorbent in a large volume of water in a graduated cylinder and allowing at least 90% of the exchanger to settle. The cloudy supernatant containing the fines is decanted. This process is repeated until the supernatant is completely clear. The number of washings necessary to remove most fines is variable, but for a typical cellulose exchanger, washing 8 to 10 times is probably sufficient.

The washed, metal-free cellulose ion-exchanger is now ready for equilibration with the starting buffer. The equilibration is accomplished by washing the exchanger with a large volume of the desired buffer, either in a packed column or batchwise.

8.3.2 Using the Ion-exchange Resin

We have seen that in a column containing a strongly acidic ion-exchange resin, the B value of cations depends on the affinity of the cation for the resin, on the concentration of eluting ion, and on the pH. Methods of treating these variables in column design are illustrated below by taking two examples viz. (i) separation of glycine from sarcosine and (ii) separation of constituents of a mixture of lysine, alanine and aspartic acid.

The separation of amino acids involves careful preparation of the cation-exchange medium as the first step. The resin in the H^+ form is washed with 4N HCl to remove contaminating metal ions. The resin is then washed with 2N NaOH to convert the H^+ form to the Na^+ form, as shown below. Now the resin can function as a cation exchanger.
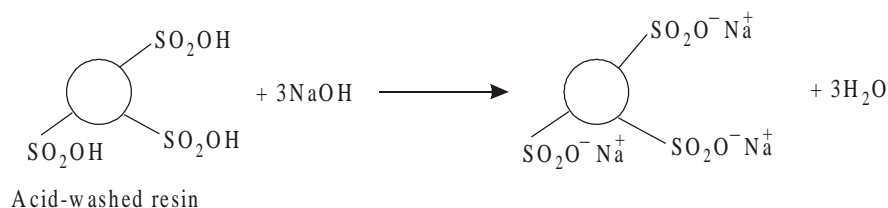
(i) Separation of glycine and sarcosine: The pK cf glycine is 2.35 and that of sarcosine (N-methylglycine) is 2.15. That is, their difference in pK is only 0.2 units. Organic ions bearing non-polar substituents have a high affinity for the resin, the non-polar protion of the molecule being attracted by the non-polar polystyrene matrix of the resin; it can, therefore, be reasonably assumed that the cationic forms of glycine and sarcosine have the same affinity for the resin because of their similarity in structure, and this, in other words, means that the two compounds have identical K_{eq} values. The operating pH should be such that ratio of B values for the two compounds is as large as possible. The higher the pH, the greater this ratio is, as is shown by Table 8.2.

TABLE 8.2

Ionic Forms of Glycine and Sarcosine at various pH values

pH	Fraction cationic		Ratio
	Glycine	*Sarcosine*	
2.0	0.69	0.59	1.17
2.5	0.415	0.31	1.34
3.0	0.183	0.124	1.47
3.5	0.066	0.043	1.54
4.0	0.022	0.014	1.56

If the pH is too high, however, both compounds will be only slightly cationic and will have very high B values. By reference to, Table 8.2, it can be seen that a value of pH = 3 is a good compromise. A pH = 3, the ratio of B values is not far from maximal and at this pH an appreciable fraction of the compounds is cationic. At pH = 3, with lM Na^+ as the eluent, in a Dowex 50 column, the B value for glycine will be the B value for Na^+ (0.376) times K_{eq} for glycine (0.43) divided by 0.183, which is the fraction of the glycine in cationic form (only the cationic form can be held on the resin). This calculation gives the B value of 0.88 for glycine. A similar calculation for sarcosine (assuming the K_{eq} values to be identical) gives the B value of 1.30 for sarcosine. In what manner B values affect the degree of resolution will become clear from the following discussion which pertains to elucidation of relationship between the resolving power of a column and magnitudes of B values of the components being separated.

For the purpose of the present discussion, two new terms must be introduced. The first of these is the holdup volume, v, which is the volume of mobile phase present at any time in the column. The second term is V, the volume of effluent which must be collected before the peak of the solute band appears in the effluent. V varies with the B value of the compound in question, but v is constant for a given column. The expressions relating V, v and the B value are:
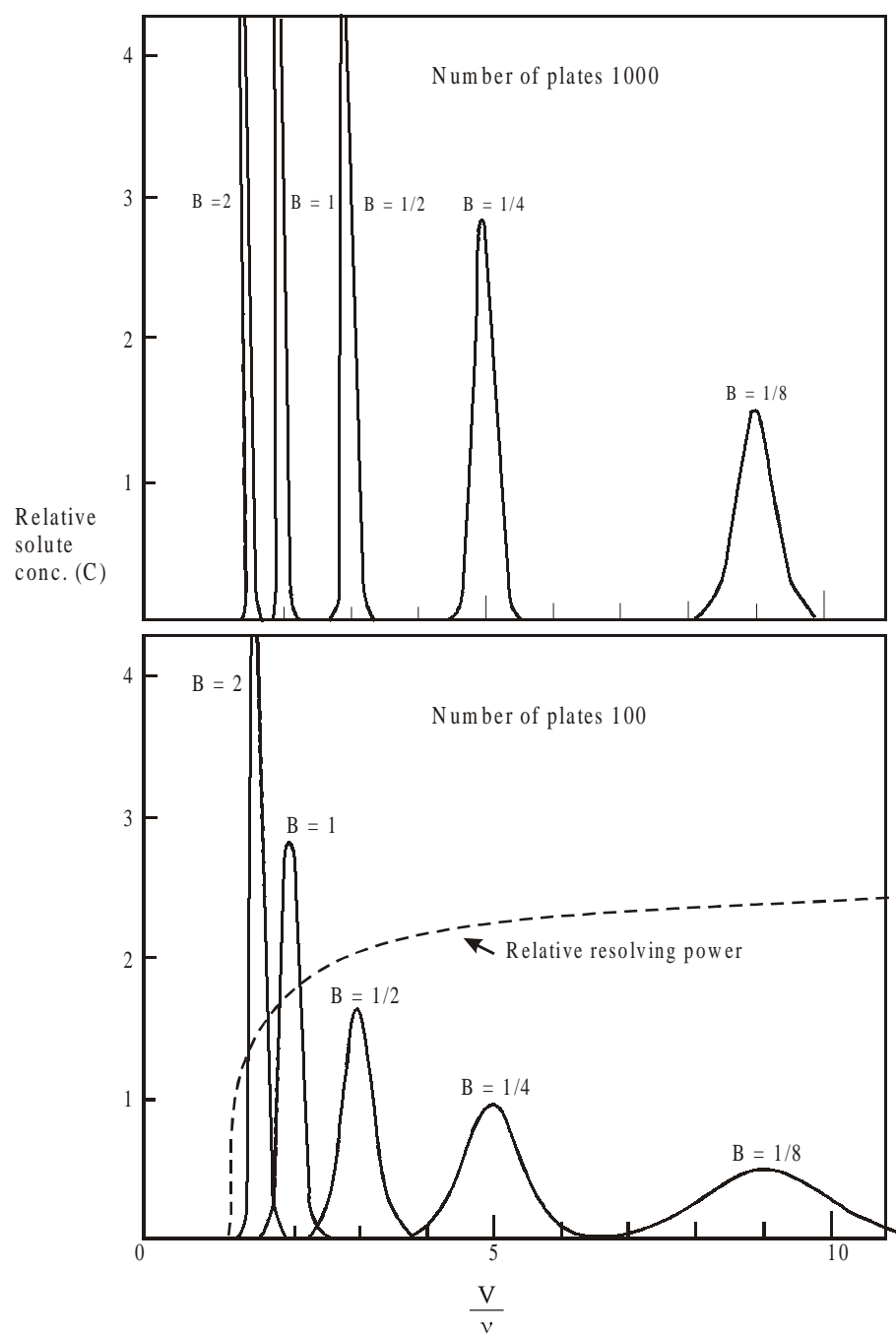
Figure 8.3 The effect of B (effective distribution coefficient) and number of plates on band position and band shape.

$$\frac{V}{\nu} = \frac{B+1}{B} \text{ or } B = \frac{V}{V-\nu}$$

It is apparent from this relation that $\frac{V}{\nu}$, or the number of holdup volumes necessary to elute the peak of a given compound, depends only on the B value of the compound and not on the length or diameter of the column being used. The relation between band position in the effluent and the effective distribution co-efficient (B) is illustrated, for a 100-plate and 1000-plate column in Figure 8.3. It will be noted that the volume of effluent necessary for elution increases rapidly as B decreases. The symbol C used for peak concentration of effluent is a measure of the steepness of the solute band, C being expressed as fraction of total solute present in unit volume of effluent, the same volume units being used for ν, V, and C.

The relative resolving power is plotted as a dotted line in Figure 8.3. It will be seen that it increases with increasing effluent volume but rapidly approaches a maximum.

It should be emphasized that although compounds of low B value issue from the column as diffuse bands, this does not indicate that separations are unsatisfactory. The separation between bands increases somewhat more rapidly than the width of the bands. The net resolving power is as indicated by the dotted line in Figure 8.3. The disadvantage of low B values lies in the inordinately long times necessary for the bands to pass through the column.

The above discussion, which pertains to elucidation of the relationship between resolving power of a column and magnitudes of B values of the components undergoing separa-tion, leads to the conclusion that high B values do not give good resolution. In the context of the separation of glycine and sarcosine to which we can now revert, the B values of 0.88 and 1.30 for glycine and sarcosine
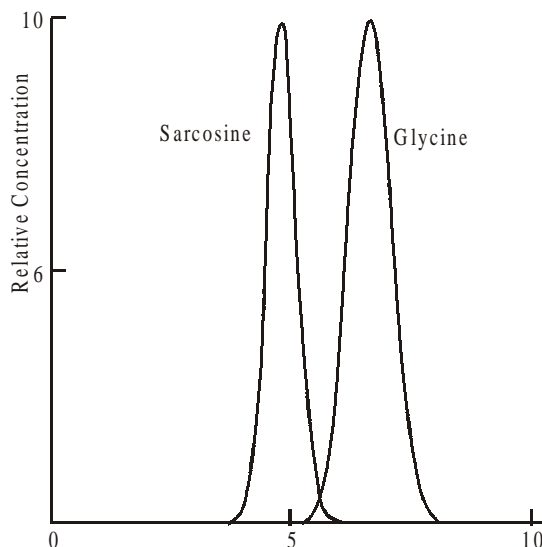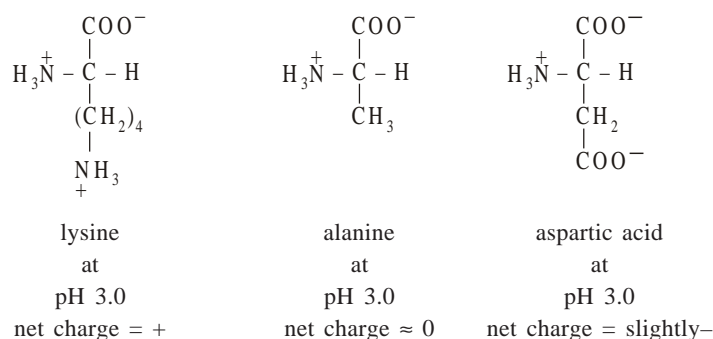

Figure 8.4 Separation on a 200-plate ion-exchange column, of 2 compounds differing in pK by 0.2 units.

(assuming the K_{eq} values for these compounds to be identical) are too high to give good resolution at pH = 3 on a Dowex 50 column when lM Na^+ is used as the eluent. This only means that the optimal eluting ion concentration should be less than lM. If the Na^+ concentration in the mobile phase is reduced to one-fifth, that is, to 0.2M, the B velues are also reduced by the same proportion, and $\dfrac{V}{v}$ for glycine becomes 6.68, and for sarcosine 4.85. These values are in the region of maximum resolution. Under these conditions, a 200-plate column (which will be from 3 to 10 cm long, depending on the resin particle size and uniformity and on the care taken in packing) will give adequate separation, as shown in Figure 8.4. Each component will be contaminated with about 0.7 per cent of the other.

The above example is intended to illustrate how necessary the adjustment of pH and eluent concentration is to get optimal column operation. The adjustment of variables is similar in the case of separation of weak acids on a strongly basic resin.

(ii) Separation of a mixture of lysine, alanine and aspartic acid: The mixture of the three components, lysine, alanine and aspartic acid, is dissolved in a pH 3.0 buffer. The predominant ionic form of each is shown below:

$$
\begin{array}{ccc}
COO^- & COO^- & COO^- \\
| & | & | \\
H_3\overset{+}{N}-C-H & H_3\overset{+}{N}-C-H & H_3\overset{+}{N}-C-H \\
| & | & | \\
(CH_2)_4 & CH_3 & CH_2 \\
| & & | \\
N\underset{+}{H_3} & & COO^- \\
\end{array}
$$

lysine	alanine	aspartic acid
at	at	at
pH 3.0	pH 3.0	pH 3.0
net charge = +	net charge ≈ 0	net charge = slightly–

Since the stationary resin, a cation-exchanger in the Na^+ form, is negatively charged, only cations will favoruably interact. Aspartic acid with a negative charge, will therefore percolate through the column with buffer solution and elute first. Both lysine and alanine are cationic and will interact with the resin by exchanging with bound sodium ions. The rate of elution of lysine and alanine from the column will depend upon the strength of their interaction with the resin. Lysine has a greater positive charge and will bind more tightly to the resin at pH3. Alanine, with a slightly positive charge, will also remain bound to the resin. At this point, one amino acid (aspartic acid) has eluted and, therefore, separated from the mixture. Increasing the pH of the buffer should decrease the positive charge on the two remaining amino acids, lysine and alanine, and weaken the ionic interactions between the bound amino acids and the resin. At pH values greater than 6.0,

alanine becomes negative and hence would repel the resin and be eluted. At pH values greater than the pH_I (isoelectric pH, that is, the pH at which the amino acid has no net charge) of lysine (9.74) it, too, becomes negative. Changing from a buffer of pH 3.0 to pH 6.0 would elute alanine, and then elution with a buffer of pH 9.0 would elute lysine. The separation of the three amino acids can thus be carried out by a step-wise increase in pH.

The amino acids as they emerge from the column in either of examples (i) and (ii), are detected by the colorimetric reaction with ninhydrin. The coloration may be used to give quantitative estimate of the relative amount of a particular amino acid.

The separation of amino acids has become a standard laboratory method of analysing protein hydrolysates, which are routinely prepared in the study of chemistry of proteins, and amino acid analyzers based on considerations discussed above are commercially available. Figure 8.5 is a schematic diagram of an amino acid analyzer using the ninhydrin reagent for quantitative determination.

The instrument permits buffers of varying pH or ionic strength to be pumped through a thermostatically controlled resin column.

The separation takes place in a column of sulphonated cross-linked polystyrene resin, which is a strong cation exchanger. The matrix of the resin is strongly
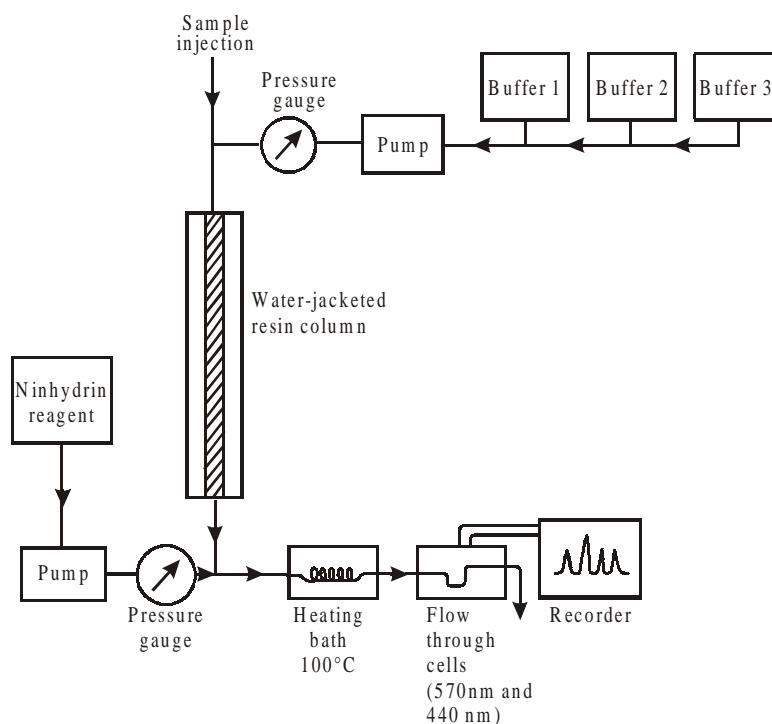

Figure 8.5 Schematic diagram of an amino acid analyser using the ninhydrin reagent for quantitation

anionic in nature (SO_3^-) and at the low pH used for separating, the positively charged amino acids will be attracted to the negatively charged sulphonated groups. As the pH of the buffer passing through the column is raised, the amino acids will be differentially eluted and they will emerge from the column in a sequence.

The sample may be applied in pH 2.2 buffer directly to the top of the column. The analysis sequence is generally started with the initial buffer pH value of around 3.0. The composition and pH of the buffer should be accurate to 0.001 mol l^{-1} and 0.01 pH unit. Sodium citrate or, more recently, lithium citrate buffers are used which incorporate a detergent (BRIJ 35), an antioxidant (thiodiglycol) and a preservative (caprylic acid) and may be used to perform either stepwise or gradient elution.

When stepwise elution is carried out, each buffer is pumped through the column for varying lengths of time and these are chosen with reference to the types of amino acid to be separated and the column dimensions.

When gradient elution is employed, the buffers are mixed in a predetermined manner to give gradual changes in pH and ionic strength. Gradient elution gives improved separation with decreased analysis time. A less common practice is to use an 'Iso –pH' system which employs a stepwise or gradient elution using buffers with increasing cation concentration which may be from 0.2 mol l^{-1} to 1.6 mol l^{-1} and an almost constant pH value of between pH 3.25 and pH 3.6.

The temperature of the resin column must be carefully maintained to avoid changes in both the pH of the buffers and ionisation of the amino acids. Although increasing temperature usually results in faster elution, the effect may be variable for different amino acids and the relative elution positions can be altered, making interpretation of results difficult. The temperature often chosen is 60ºC although lower temperature are sometimes required to resolve two similar amino acids. Temperature programming involving an alteration in temperature at a specified time during the separation is widely employed.

Successful and reproducible separations require the use of a pump that delivers the buffer at a constant flow rate independent of the resistance to the flow. The choice of flow rate is dependent upon the type of resin, the diamensions of the column and the overall design.

For detection, ninhydrin is the most widely used reagent. A second pump is required to deliver a constant flow of the reagent to meet the column effluent. The ninhydrin reaction requires heating and, therefore, the stream of column eluate plus ninhydrin must pass through a coil of narrow bore tubing held in a 100ºC heating bath. When the reaction has taken place the stream is monitored continuously using a flow-through cell in a colorimeter, the output of the latter being traced by the instrument recorder in the form of a chromatogram.

It is to be noted here that the sequence of elution of amino acids is determined not only by the composition and pH of the eluting buffer but that the sequence is

also affected by the following other factors whose influence on the elution process has also been already discussed.

(i) Non-ionic interactions between the amino acids and the resin, (ii) the degree of cross-linking of the ion-exchange resin because it influences the rate of diffusion of the charged species to the exchange sites. Thus, it is difficult to make an accurate prediction of the positions of the amino acids on the actual chromatogram, traced by the instrument recorder, by just taking into consideration the composition and pH of the eluting buffer.

For quantitative determination, consideration has to be given to the fact that the colour produced per mole of amino acid varies slightly for different amino acids. The allowance to be made for each amino acid on this account is determined by loading a mixture of amino acids containing the same concentration of each amino acid (for example, 200 nmol ml^{-1}) and calculating each *colour value* from the areas of the peaks on the recorder trace. This procedure is carried out manually or is done automatically if an integrator and computer is a feature of the analyzer. The value is noted and used in subsequent calculations of sample concentration.

An internal standard should always be used for every analysis carried out. Such an internal standard is an amino acid which is known to be absent from the sample under investigation. For instance, in blood plasma analysis a known amount of either of the non-physiological amino acids, nor leucine or α-amino-β-guanidinobutyric acid can be used. From the known amount of internal standard, the concentration of the unknown amino acid can be determined using peak area relationships, the difference in colour values for the unknown and the internal standard having been duly taken care of while doing calculations.

With the advent of high quality resins, sophisticated automation and increased sensitivity of the detection systems, a reduction in analysis time from days to hours and an extended analytical range to below the nanomole (10^{-9} mol) level have been achieved. The evolution of

1. Cysteic acid
2. Aspartic acid
3. Methionone sulphate
4. Threonine
5. Serine
6. Glutamic acid

7. Proline
8. Glycine
9. Alanine
10. Cystine
11. Valine

12. Methionine
13. Isolencine
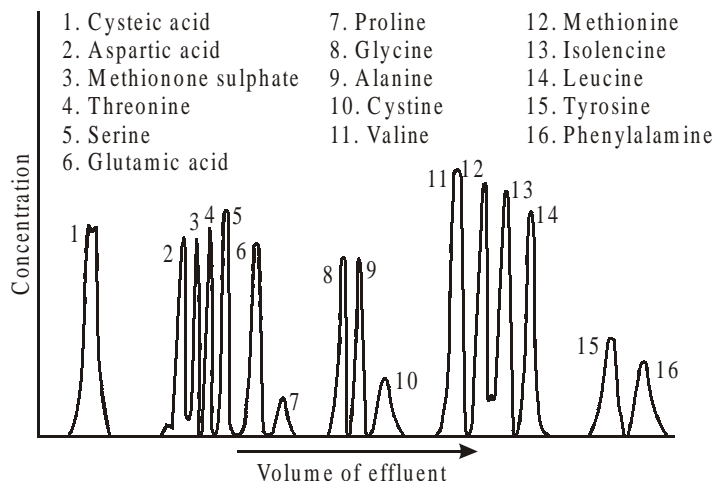14. Leucine
15. Tyrosine
16. Phenylalamine

Figure 8.6 Amino acid separation on ion-exchange column, with automatic recording of the effluent composition.

such refined instruments has played a major role in the successful amino acid sequence analysis of many proteins and peptides.

Figure 8.6 illustrates an amino acid separation achieved with automatic recording of the effluent composition.

8.4 Batch Separation using Ion-exchangers

Ion-exchangers are most commonly used in a column form. An alternate method of ion-exchange is *batch separation.* This involves mixing and stirring equilibrated exchanger directly with the solute mixture to be separated. After an equilibration time of approximately one hour, the slurry is filtered and washed with buffer. The ion-exchanger can be chosen so that the desired solute is adsorbed onto the exchanger or remains unbound in solution. In the latter case, the desired material is in the filtrate. If the desired solute is bound to the exchanger, it can be removed by suspending the exchanger in a buffer of greater ionic strength or different pH. Batch processes have some advantages over column methods. They are rapid, and the problems of packing and channeling are avoided.

8.5 Chromatofocussing

A new development in ion-exchange column chromatography allows for the separation of proteins according to their isoelectric points. This technique, chromatofocussing, involves the formation of a pH gradient on an ion-exchange column. If a buffer of a sepecified pH is passed through an ion-exchange column that was equilibrated at a second pH, a pH gradient is formed on the column. Proteins bound to the ion-exchanger are eluted in the order of their isoelectric points. In addition, protein band concentration (focussing) takes place during elution. The requirements for chromatofocussing include appropriate column equipment and specialized exchanger buffers.

8.6 Ligand-exchange Chromatography

Ligand exchange chromatography can be described as a process in which interaction between the stationary phase and the species to be separated occurs during the formation of coordination bonds inside the coordination sphere of the complex-forming metal ion. When a stationary phase that contains a chiral ligand metal complex interacts with enantiomeric mobile ligands, a discrimination of the latter can occur due to enantioselectivity in the formation of mixed-ligand sorption complexes. To take advantage of this difference in thermodynamic stabilities, the kinetics of the complex formation/dissociation reaction must be rapid. These criteria have been met for copper proline or copper hydroxyproline complexes, immobilised via chemical attachment to various polymeric resins, for the separation of enantiomeric amino acids and peptides. These special resins are prepared by coupling chloromethylated polystyrene with optically active amino acids. The

resins are then loaded with copper ions. The resin with bound proline will illustrate what happens. The structure of the coupled resin is:
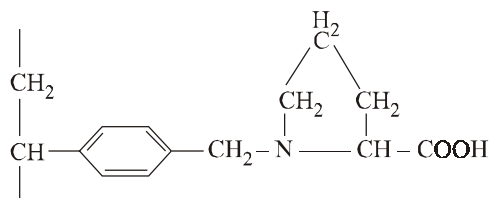
With a copper ion and a molecule of absorbed (or mobile) proline it forms a complex:
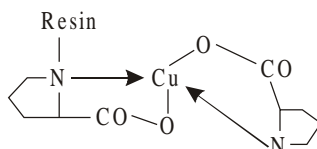
The absorbed proline may, in turn, be displaced from the copper ion by two molecules of water or ammonia. Now, the stability of the complex depends on its stereochemistry. The complex formed from l-proline (fixed) and d-proline (mobile) is more stable than the one formed from l-proline (fixed) and l-proline (mobile). When a racemic mixture is absorbed, l-proline may be eluted by water or 0.1M ammonia, whereas 1-2M ammonia is needed to elute d-proline.

8.7 Separation of Uncharged Molecules using Ion-exchange Resins

Ion-exchange resins can also be used for chromatographic separations of mixtures of substances whose molecules are uncharged.

There are at least three ways in which an ion-exchange resin can hold uncharged molecules. One is by 'matrix affinity', comprising of the forces between absorbed molecules and the polymer matrix. These forces, in turn, can be of different kinds, an important example of which is π-electron overlap responsible for the strong binding of aromatic solutes to an aromatic polymer matrix like cross-linked polystyrene. Another effect, important in mixed solvents, comes from the fact that solvent composition inside the resin is different from that outside. The more polar solvent component tends to be more abundant inside the resin, with respect to the less polar component, because the ions of the resin solvate polar molecules. In mixtures of water and alcohol, for example, the proportion of water to alcohol is higher inside the resin than outside. The resin, therefore, absorbs highly polar compounds, like sugars, from their solutions in aqueous alcohol. The third kind of force that causes uncharged organic molecules to be absorbed by ion-exchange resins is the association of these molecules with the counter-ions of the resin. Borate ions, for example, form cyclic complexes with 1, 2-diols, including

sugars. Thus a strong base anion-exchange resin loaded with borate ions absorbs sugars. Likewise an ion-exchange resin carrying bisulphite ions could absorb aldehydes and ketones, which form charged bisulphite complexes.

Riemann, in 1957, described two processes that are called *salting-out chromatography* and *solubilization chromatography*. The first one is used with water-soluble compounds like the lower alcohols, esters, ethers, aldehydes and ketones. These are absorbed from concentrated salt solutions, like 4M ammonium sulphate, into cation- or anion-exchange resins and then eluted with aqueous salt solutions of decreasing concentration. The more water-soluble, that is, the more hydrophilic, substances like glycerol and ethyl alcohol are eluted first, and the more hydrophobic, that is, the less water-soluble, substances like amyl alcohol emerge later.

'Solubilization chromatography' is used with compounds which are sparingly soluble in water, like longer-chain alcohols, esters and ketones, phenols and aromatic hydrocarbons. These compounds are absorbed from water mixed with just enough organic solvent to keep them in solution, then eluted with solvent mixtures containing increasing proportions of methanol, ethanol or acetic acid. The more hydrophilic compounds, or, those of lower molecular weight, are eluted first, and the less water-soluble compounds come out later. For example, aromatic hydrocarbons are eluted in the order benzene, toluene, xylene, naphthalene, methylnaphthalene.

A combination of salting-out and solubilization chromatography may be employed. Thus separation of acetanilide and the three compounds derived from acetylation of p-aminophenol was successfully carried out by using solutions of potassium phosphate in aqueous methanol.

8.8 Applications of Ion-exchange Chromatography

Some typical separations carried out by ion-exchange chromatography in the field of Organic Chemistry have already been cited earlier in the present chapter as illustrations in aid of the discussion of the theory and practice of this separation technique. The use of ion-exchange chromatography in the realm of Inorganic Chemistry may be illustrated with some typical separations belonging to that field.

Separation of chlorides of three closely related transition metals—iron, cobalt and nickel is carried out by ion-chromatography via formation of metal complexes carrying a charge e.g. $FeCl_6^{3-}$ and $CoCl_4^{-}$.

For example, when a salt containing the hydrated ferric ion is dissolved in 9 MHCl, a complex ion is formed:

$$Fe(H_2O)_6^{3+} + 6Cl^- \rightleftharpoons FeCl_6^{3-} + 6H_2O$$
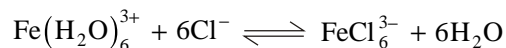
An anion-exchange resin containing the grouping—N $(CH_3)_3{}^+Cl^-$ is used in the experiment and the complex anions like Fe Cl_6^{3-}, formed as shown above, will be exchanged for the chloride ions of the resin. If the concentration of the chloride ion in the ion-exchange chromatographic column is then decreased by using 5M HCl and finally lM HCl, the chloride complexes will successively shift their equilibria to form the hydrated metal cations, which are no longer retained by the resin and can be washed through the column. The chloride complex which constitutes the least stable complex will be eluted even at the higher concentration of the chloride ion (9 M HCl), while the most stable complex will not be eluted until the chloride ion concentration has been reduced to that of lM HCl. Each of the three metal ions has a characteristic colour in solutions containing chloride ions and thus is readily distinguished as those ions are washed off the resin. For the purpose of this separation the original solution containing the three chlorides may be assumed to have been prepared by dissolving the salts in 12M HCl.

Ion-exchange methods are prominent in separation, purification and analysis of fission products. Thus, rare earth fission products have been separated and determined by ion-exchange. Similarly, the separation of transplutonium elements has been studied using different anion-exchangers. Weakly basic ion-exchange resins like Amberlite XE-270 have long been used successfully for kilogram quantities of ^{237}Np.

Inorganic exchangers are finding applications in fission product separations due to their high specificity and stability to intense radiation fields. In laboratory experiments, undiluted fission product waste solutions, 2-3M in HNO_3, when passed through NH_4^+ molybdophosphate/NH_4^+ tungstophosphate, undergo losses of Cs, Rb, and small amounts of Zr due to uptake by the resin. After oxidation of the resulting effluent with NH_4 persulphate in the presence of Ag NO_3 as a catalyst, the solution is passed through a column of MnO_2 exchanger, which retains Ce^{4+} along with the remaining Zr^{4+} and Ru^{4+}. Strontium and sodium can then be removed with hydrated antimony pentoxide.

Rapid automated and semiautomated process schemes are being developed for the separation of nuclear corrosion and fisslon products using combinations of inorganic and chelating resins. Mo (ferrocyanide), ZrP, and Chelax 100 resins have been examined for the selective separation of the radionuclides, ^{46}Sc, ^{98}Zr, ^{95}Nb, ^{137}Cs, and ^{152}Eu.

For future nuclear energy production, considerable promise is held out by the fast breeder reactor techniques used to produce fissional materials from non-fissional ones. There is the corresponding problem of substantially increasing the levels of nuclear wastes that must be handled in such a way as to render them as harmless as possible to the environment. In this area of processing of nuclear fuels, ion-exchange techniques again are useful. After the production of ^{233}U via the reaction:

$$^{232}Th_{(n, r)} \longrightarrow {}^{233}Th \xrightarrow{-\beta^-} {}^{233}Pa \xrightarrow{-\beta^-} {}^{233}U,$$
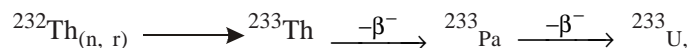
the fissionable product ^{233}U must be separated from the thorium matrix. Selective removal of traces of ^{233}U from Th and from other macroimpurities except Fe was carried out by passing the solution through AGl-X8 anion exchange resin in the Cl^- form. Further purification of U for removal of Fe was based on a subsequent passage of the solution through another column of the same resin in the NO_3^- form.

Medicinal radioisotopes for clinical diagnostics and highly specific radioisotopes for use in tracer and other analytical applications are produced via accelerator cyclotron, and neutron activation techniques, some examples of theses radioisotopes being ^{28}Mg, ^{47}Sc, K 42, 43, 38, ^{201}Tl, ^{123}I, ^{99}Mo, Br $^{76, 77, 97}Ru$, ^{18}F, ^{13}N, ^{11}C, ^{15}O. In almost every case of the generation of the desired isotope via electron, photon, proton, neutron, or charged particles activation of the target nuclide, the produced isotope must be chemically separated from matrix isotopes. The production of reasonably pure isotopes has been based on selective ion-exchange separations following the activation of the target nuclide. For example, ^{99}Mo is recovered from nuclear radiated MoO_3 following its dissolution in 10M sodium hydroxide, reduction with 0.2M I_2 in alkali solution, acidification to 0.1—6M with acid, and formation of $[Mo (SCN_6]^{3-}$ complex, which is then separated by an anion exchanger with nitrilodiacetate groups. The anion was then eluted from the column with mineral acid. Any straightforward adoption of conventional chemistry with an appropriate ion-exchange separation can be used to isolate and recover the produced radioisotope.

This illustrative account of the applications of ion-exchange chromatography can be concluded with Table 8.3, which shows the potential of this technique for effecting separation of constituents of complex mixtures which are not easily amenable to resolution by other chromatographic techniques.

TABLE 8.3

Separations	*Resin used*	*Elution method*
Aldehydes, ketones, alcohols	Anion	Ketones and aldehydes held as bisulphite addition compounds. Eluted with hot water and NaCl respectively.
Carboxylic acids	Anion	Gradient pH elution
Phosphate mixtures e.g. ortho-, pyro-, tri–, tetra– etc.	Anion	lM to 0.005M KCl
Lanthanides	Cation	Stepwise or gradient elution with citrate buffers.
Transition metals e.g. Ni^{2+}, Co^{2+}, Mn^{2+} Cu^{2+}, Fe^{3+}, Zn^{2+}	Anion	Stepwise elution 12M to 0.005M HCl

9

Gel Chromatography

This technique is particularly valuable in the separation of molecules of similar polarity but of different sizes, traditional methods of chromatography being less effective in these circumstances. The separation depends not only on the size but also on the shape of the molecules. There are several aspects by which separation effected by this technique can be viewed and this type of chromatography is variously named according to the particular separation aspect stressed upon. Accordingly, this technique is called gel filtration, gel permeation, molecular sieve filtration, molecular sieve chromatography, size-exclusion or simply exclusion chromatography.

9.1 Xerogds

Gel chromatography brings about separation on a xerogel, which may simply be defined as a polymeric material that swells greatly in a solvent to produce a gel system. A definite degree of swelling must be maintained for the gel, since the pore structure of the gel depends on this factor. The phenomenon of molecular sieve action is frequently observed with materials like dextran and agarose which constitute the xerogel matrix. Since Porath and Flodin's observation of the molecular sieving effect of these xerogels and the simultaneous introduction of a cross-linked dextran, named Sephadex, on the market in 1959, this chromographic technique has been rapidly accepted. Though of comparatively recent origin, owing to its simplicity and speed, this method has become one of those separation methods which find the widest application in the chemistry of polymers.

9.2 Principle underlying Gel Chromatography

The principle of gel chromatography can be explained by a simple model illustrated by Figure 9.1. The stationary phase is formed by the xerogel saturated with a liquid, usually water. The same liquid is also used as the mobile phase or eluent. The inert polymer matrix of the swollen xerogel contains pores which have various dimensions according to the type of gel used. Consider the case of a mixture of small medium and large molecules being eluted through the gel. It will be seen from Figure 9.1 that, at each level in the passage through the system, the small molecules penetrate almost completely into the gel system, the medium-sized molecules penetrate partially, and the large molecules do not penetrate at all. Thus, the small molecules are retained longest on the column, the medium-sized
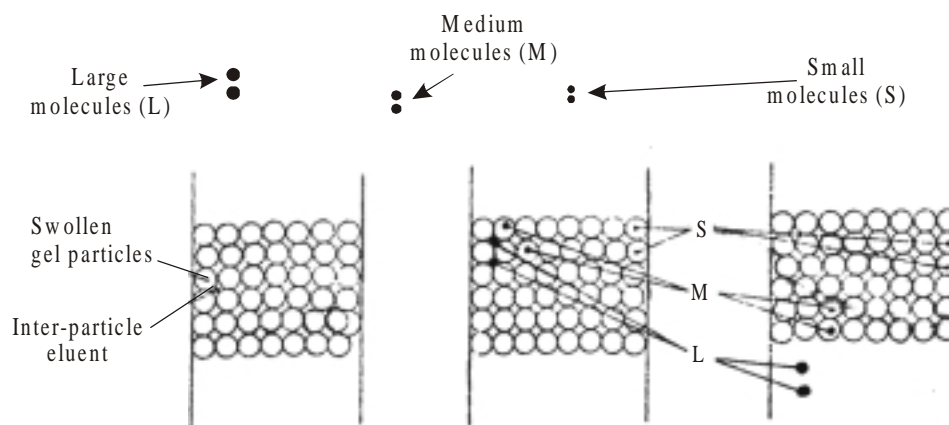
Figure 9.1 Diagrammatic representation of the gel-filtration process.

molecules for less time and the large molecules pass straight through in the eluent surrounding the gel system. To give some insight into the relationship of pore size and molecular size, it may be mentioned that the size exclusion effect is eliminated when pore size is 2-10 times the size of the molecule.

Figure 9.2 is a calibration curve for an exclusion separation. Molecules with a molecular weight larger than the value represented by the point 'A' are totally excluded from the pores and elute as a single peak (1) of with retention volume (i.e. the volume of the eluate that emerges from the column outlet in the retention time) which has the value V_o, that is the same as the intestital, interparticular or the void volume of the column in the case. Point 'B' represents the molecular weight at which total permeation of the packing occurs. Thus all molecules with a lower molecular weight than that represented by 'B' will again elute as a single peak (4) with a retention volume V_t (the total permeation volume). Compounds with molecular weights between the limits represented by 'A' and 'B' may be separated, this separation being represented by peaks 2 and 3. As a rule, it is found that unless there is a difference between molecular weights of compounds of at least 10%, no resolution will be achieved.
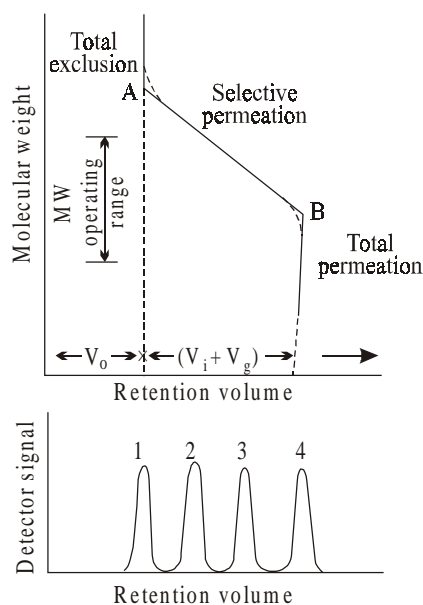

Figure 9.2 Relationship between solute molecular weight and retention volume for 'ideal' exclusion chromatography.

The total permeation volume, V_t, is the sum of the volume outside the gel grains i.e. the interparticle volume (V_o), the volume of the liquid inside the gel grains (V_i) and the volume of the gel matrix (V_g), that is,

$$V_t = V_o + V_i + V_g.$$

V_o may be easily measured by applying to the system a molecular species completely excluded from the gel system. V_i is the product of multiplication of the number of grams (a) of dry xerogel and the solvent regain, W_r, which is the amount of solvent taken up by one gram of dry xerogel.

It is possible to define a parameter in gel filtration analogous to the partition coefficient in liquid-liquid or gas-liquid chromatography. In this case the distribution coefficient, K_D, relates to the fraction of inner volume that is accessible to a particular species. If V_e is the elution volume in which a particular molecular species is eluted, then

$$V_e = V_o + K_D V_i$$

$$\text{whence} \quad K_D = \frac{V_e - V_o}{V_i} = \frac{V_e - V_o}{\alpha W_r}$$

For a molecular species completely excluded, $V_e = V_o$ and K_D is zero. For small solute molecules which can enter all the pores, $K_D = 1$. Separation, therefore, occurs only where solute molecules obey the condition $0 < K_D < 1$.

The K_D value of a solute molecule depends upon its effective molecular size which in its own turn is determined by the radius of gyration of the molecule in the solvent and hence by such factors as the geometric shape (e.g. spherical, coiled or rod-like), solute-solute association and solvation. K_D is independent of the size and geometry of the column within a given gel-solvent system, but the change in the nature of the gel which implies change in the pore size, and change of the solvent or the temperature would change the K_D value of the solute. If the value of $K_D > 1$, then adsorption, partition, ion-exchange and charge transfer effects may be contributing to the retention in addition to exclusion.

The efficiency of a gel permeation column is markedly influenced by the mobile phase flow rate and working at high flow rates is disadvantageous.

Gel chromatography has several advantages:

(1) Analysis times are short and gradient elution is not required.
(2) Band widths are narrow so that detection is easier.
(3) Column life is long.
(4) Because gels are inert in nature, sample loss or reaction on the column does not occur.
(5) Retention times, being dependent on molecular size, are predictable.

9.3 Experimental Technique

Grains of the selected gel are slurried with the appropriate liquid, which is the one to be used for the elution of the column later on. The desired length of the column is filled with this suspension. After the gel slurry has been added to the column, the bed is left to settle for 5-10 minutes. When a bed of sedimented gel about 5 cm high has formed, the column outlet is opened so that further sedimentation of the gel occurs under liquid flow. When all the gel is settled in the coloumn, it is finally washed with a fresh quantity of the eluent so that the bed stabilizes completely before the sample solution is introduced into the column.

The sample to be chromatographed is applied in the form of its solution from a pipette along the walls of the tube carefully so that the surface of the gel is not disturbed. The sample is then allowed time to soak into the bed before the eluent is introduced into the column.

The eluent flow through the column is arranged, in principle, in two ways : Either by gravity flow in which case the inlet to the column is at a higher level than the outlet, or by pumps. Sometimes the use of a pump is indispenable for the regulation of eluent flow. In the set-up for gel chromatography in which the eluent flows from the top downwards, it happens sometimes that the gel used, which may happen to be a soft one, packs and the original bed height thereby decreases. This results in slowing down the eluent flow through the column. This situation can be avoided by arranging an upward flow of the eluent by pumping it from the bottom upwards. The apparatus used in the upward-flow gel chromatography is shown schematically in Figure 9.3.

For the case illustrated by Figure 9.3, in which a pump is used to maintain a regulated eluent flow, it becomes unnecessary to apply the sample soultion at the top of the column through a pipette, as detailed above. Instead, the solution of the substances to be separated is gradually sucked by the pressure pump through the column from bottom to
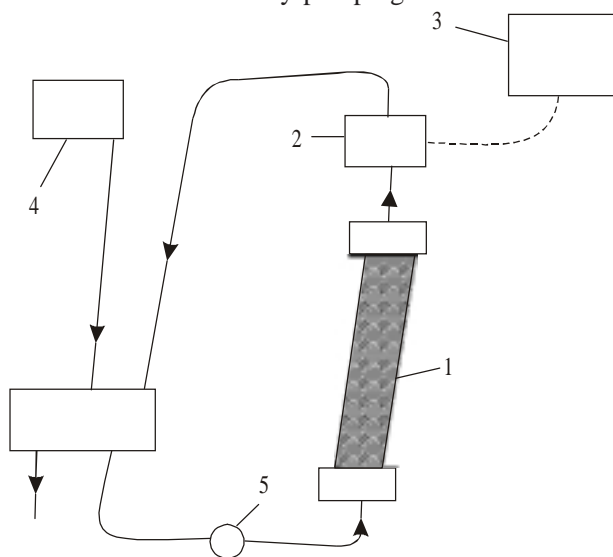

Figure 9.3 Schematic of circulation apparatus for upward-flow gel chromatography.
1. Column; 2. Spectrophotometer; 3. Recorder; 4. Eluent reservoir; 5. Pump.

top. When the sample has been completely sucked into the system, the pump is connected back to the eluent reservoir. Figure 9.3 depicts a set-up which also includes a suitable instrumental device, for example, a spectrophotometer, to monitor the column effluent continuously with regard to the nature of its contents and their concentration.

## 9.4 Gels used for Separation by Exlcusion Chromatography

Gels to be used for exclusion chromatography should fulfil some general requirements. Gels must be chemically inert to the constituents of the mixture being separated. They should have chemical stability, that is, they should not undego chemical changes over a wide range of pH and temperature. The gel material must not decompose during chromatography, otherwise leaching of the decomposition products would contaminate the separated substances. The gel should not contain ionizable groups, so that exchange does not take place during gel chromatography. Chromatographic properties of a gel are determined by the degree of cross-linking in it. The lower the cross-linking the more the gel swells and the higher is its accessibility to larger molecules. Commercial gels produced now-a-days provide a wide choice of fractionation (M.W. from 10^3 to 10^7). A very important property of a gel is its mechanical strength and during its use in the chromatographic column the packed gel bed should not get deformed. The size of the particles and uniformity of this size are important factors on which quality of separation effected by the gel depends. Small particles give an excellent separation of the mixture into its constituents; however, with fine particles flow-rates through the column are rather low. Large particles permit high flow-rate, but their use often leads to unsatisfactory separations.

Amongst the commercially available gels meant for exclusion chromatography are Sephadex (cross-linked dextran), agarose gels, polyacrylamides, hydroxyalkyl-methacrylates and cross-linked polystyrene. The advantage of these materials compared with a natural xerogel (for example, starch) is that gel system with definite and reproducible characteristics can be obtained by controlling the degree of cross-linking. There is also a wide distribution of pore sizes in each gel type on account of random distribution of cross-linking. This means that molecules of a size below the limit where complete exclusion occurs are either partly or fully able to enter the gel.

The dextran gels are obtained by cross-linking the polysaccharide, dextran, with epichlorhydrin. In this way the water soluble dextran is made water insoluble, but it retains its hydrophilic character so that it swells rapidly in aqueous media thereby rendering gel particles suitable for gel filtration. By varying the degree of cross-linking, several types of Sephadex have been obtained. They differ in porosity and consequently are useful over different molecular size ranges. Each type of Sephadex is characterized by its water regain.

Agarose gels, which are produced from agar, are linear polysaccharides and their gelling properties are attributed to hydrogen bonding of both inter- and intra-molecular type. Due to their hydrophilic nature and the nearly complete absence of charged groups, agarose gels, like dextran gels, cause very little denaturation and adsorption of sensitive biochemical substances. By virtue of their greater porosity they complement the dextran gels. Whereas the latter allow fractionation of spherical molecules such as globular proteins, of dimensions corresponding to molecular weights of upto 800,000, or randomly coiled polymers like dextran of molecular weights upto 200,000, the agarose gels may be used to separate molecules with molecular weights of several millions. They have, therefore, been widely used in the study of viruses, nucleic acids and polysaccharides.

Polyacrylamide gels are prepared by copolymerisation of acrylamide monomer ($CH_2=CHCO\ NH_2$) with a cross linking agent, usually N, N'-methylene bisacrylamide, $CH_2(NHCOCH = CH_2)_2$, in the presence of a catalyst accelerator-chain initiator mixture. This mixture may consist of freshly prepared ammonium persulphate as catalyst (0.1 to 0.3% w/v) together with about the same concentration of a suitable base, for example, dimethylamino propionitrile (DMAP) or N, N, N', N' tetramethylene diamine (TEMED) as initiator. TEMED is most frequently used and proportional increases in its concentration speed up the rate of gel polymerisation. Photochemical polymerisation may be brought about by riboflavin in the presence of UV radiation. Gelation is due to vinyl polymerisation as shown below:

$$CH_2 = CHCONH_2 + CH_2\ (NHCOCH = CH_2)_2$$

acrylamide   N, N' methylene bisacrylamide

free radical catalyst
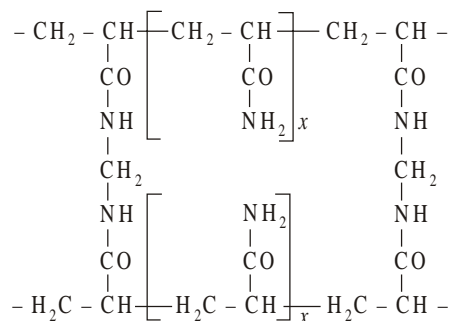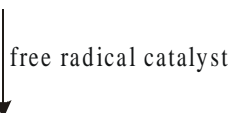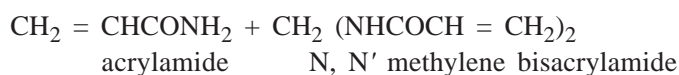
(Polyacrylamide gel formation)

The synthetic xerogels have been marketed under various trade names, and in recent years their use in Biochemistry has been very extensive. Table 9.1 lists some commonly used gels under their respective trade names along with their respective exclusion limits.

TABLE 9.1

Polymer	Trade name	Exclusion limit (approximate)
Dextran	Sephadex G-10	700
	G-15	1,500
	G-25	5,000
	G-50	10,000
	G-75	50,000
	G-100	100,000
	G-150	150,000
	G-200	200,000
Agarose	Sepharose 2B	4,000,000
	4B	20,000,000
	6B	40,000,000
Agarose	Bio-gel A5m	5,000,000
	A 15m	15,000,000
	A 50m	50,000,000
	A 150m	150,000,000
Polyacrylamide	Bio-gel P2	1,800
	P6	6,000
	P30	40,000
	P 100	100,000
	P 300	400,000

9.5 Applications of Gel Charomatography

Applications of gel chromatography range from simple desalting to fractionation of complex mixtures of proteins and nucleic acids. A highly cross-linked xerogel e.g. G-25 (exclusion limit ≈ 5,000) would be used for desalting and one of the less cross-linked xerogels for the fractionation of proteins. Xerogels with exclusion limits higher than that of G-200 (exclusion limits ≈ 200,000) are available for the fractionation of nucleic acids and viruses.

The xerogels mentioned above have been used for the separation of hydrophilic molecules, and these xerogels swell significantly only in water. The technique of gel filtration has now been extended by the introduction of xerogels which swell in polar organic solvents. Sephadex LH is such a material, swelling in alcohol and chloroform to give a gel system approximating to G-25. Using such a system the separation of lipids is possible.   ∎

10

High Performance Liquid Chromatography (HPLC)

The decision to use conventional low pressure liquid chromatography or HPLC, the more modern high pressure mode of LC, for a particular separation, depends on many factors including the availability of apparatus, cost, qualitative or quantitative assay and the procedure adopted in successful separations recorded in literature. The modern trend is to select HPLC, which is certainly capable of giving fast accurate and precise data. Over gas chromatography, which is its main rival in being capable of giving fast, accurate and precise data, HPLC enjoys the obvious advantage in that apart from the primary equilibrium established between the mobile and stationary phases, secondary chemical equilibria take place in HPLC between solute molecules and components present in either mobile or stationary phase that can be exploited by the selection of appropriate chromatographic conditions. Further, considering that of all known substances 85% are non-volatile, the scope of application of this version of liquid coloumn chromatography vis-a-vis gas-liquid chromatography, is immediately obvious.

10.1 Working of HPLC

In the context of the working of HPLC, some of the conclusions earlier derived under the detailed sicussion entitled 'Relationship of Theory to Practice of Liquid Chromatography' (Chapter 7) may be recapitulated here. Low flow rates of the mobile liquid phase over the stationary phase are necessary for achieving a satisfactory separation in the conventional liquid chromatography. This requirement is directly related to the slow rates of diffusion prevailing in liquid phases. If flow rates are high, the desired chromatographic equilibration of the components of the sample between the two phases is neither attained nor maintained, and the efficiency of separation of the components suffers. Thus the conditions required for efficient separations generally result in rather time-consuming experiments. One approach to the solution of this problem is *to decrease* the distance through which molecules must diffuse. This may be accomplished by using much smaller particles for column packing. The use of smaller particles will increase considerably the total surface area of the stationary phase, and further, because smaller particles pack more tightly, it will bring about significant reduction of the interstitial volumes of liquid between the particles. Under these conditions equilibration between phases is established in a much

shorter time so that higher flow rates become possible without efficiency of separation getting adversely affected thereby. However, the much more tightly packed solid leads to a restriction of flow and the use of greatly increased inlet pressure at the head of the column as a counter-balance becomes a requirement.

A combination of high pressure and adsorbents of small particle size leads to high resolving power and short analysis times characteristic of the modern high pressure liquid chromatography known as high performance liquid chromatography (HPLC). The two types of small-size porous packing material used as stationary phase in HPLC have already been described in Chaper 7, these being (i) porous layer beads or pellicular beads, and (ii) microporous particles.

The fit-up shown in Figure 6.1 can be taken to represent a typical high performance liquid chromatograph also, with the necessary modification that a high pressure pump is used.

The pump used in HPLC should meet the following requirements :–

(1) It should have a controlled reproducible flow delivery.
(2) It should yield pulse-free solvent flow.
(3) It should have a small hold-up volume.

Although neither of the two types of pumps available—the constant pressure type and the constant volume type, meets all these criteria, constant volume pumps maintain a more accurate flow rate and give a more precise analysis.

In order to achieve good separation, the sample must be injected into the column in one small dose, so that all components start their trip through the column at the same instance. One of the most popular injectors is the 'syringe injector' (see Figure 6.2) whereby the sample, in a microlitre syringe, is injected through a neoprene/Teflon septum.

10.2 Comparison of Analytical Scale HPLC with Preparative Scale HPLC

HPLC can be used on the analytical sacle (both quantitatively and qualitatively) or on a preparative scale.

In analytical HPLC, resolution is the prime requistie, with speed of analysis another important variable. The scope of the system i.e. its ability to separate mixtures of wide polarity range, is also important and can be varied by altering the mobile phase. Capacity is sacrificed in analytical HPLC. For preparative HPLC, capacity and scope are more important and speed is sacrificed to provide the maximum capacity.

Preparative HPLC may involve either scale-up operations where analytical chromatographic conditions are used but with slightly larger sample loads, or large scale operations where the column is used under overloaded conditions.

There are three basic situations involved when HPLC is used on apreparative scale:

(i) The easist situation is where there is only one major constituent in the mixture and it is the component that has to be obtained in good yield and high purity.

(ii) In the second case, the mixture has two major components, both of which need to be isolated.

(iii) The mixture in the third situation has several constituents, all of which are present in similar quantities.

The steps involved in successful preparative HPLC are: (a) Recognition from the chromatogram as to which one amongst the aforesaid three situations is presented by the given mixture; (b) preparation of the sample in a form suitable for HPLC; (c) development of a separation on an anlytical scale; (d) alteration of the variables to permit a large scale preparation.

Having defined the problem as required under (a) above, the sample preparation as required by step (b) has to be undertaken. Step (b) implies the removal of any impurities that could create problems for successful chromatographic performance, as for example, it may be necessary to remove carboxylic acids from a mixture containing esters by extraction of the acids from an etheral solution of the solutes with aqueous sodium bicarbonate. Monitoring of the column eluent with an ultraviolet detector may represent another exigency where removal of impurities (e.g. aromatics) from a reaction mixture by a preliminary crystallisation may become a requirement. Occasionally, a derivative has to be made that is suitable for one type of detector, this derivation making the solute molecule absorb in the UV region.

The next stage i.e. the step (c) mentioned above, is to consider the analysis as a purely analytical problem to be solved, with the best resolutions, in as short a time as possible. Normally, this analytical separation will be performed on a small microporous particle packing.

Alteration of the variables to permit a large scale preparation, mentioned as step (d) above, implies that it is essential to improve the resolution even if it means sacrifice in time. For the single component system, it is advisable to try to get as big a difference as possible in time between the preceding peaks and the major component, and between the major component and the succeeding peaks. When the major component is well separated from the other solutes, it is possible to introduce larger quantities of the sample on to the column. It is sometimes recommended that the solutes should have k' values > 5 so as to increase sample loadability.

As already discussed earlier under "Relationship of Theory to Practice of Liquid Chromatography", the sample volume and the solute amount thereby injected on to the column influence the efficiency of the column considerably.

It is usual to inject a fairly concentrated solution on to the column (there are occasions, however, when solubility of the solute requires using a fairly dilute solution). In one trial, the analysis of a sterioid (40 μg) on a 28.5 cm × 18 mm column showed that a twenty-fold increase of sample volume (0.1 ml to 2.0 ml) led to a decrease in efficiency from 34.5 μ height equivalent to a theoretical plate, to 49.5 μ. In any event, the sample volume should not exceed one-third of the mobile phase volume needed to elute the solute.

As table 10.1 shows, the increase in column diameter enables substantially more material to be analyzed.

TABLE 10.1

Typical sample sizes for preparative liquid-liquid and liquid-solid chromatography

Column internal diameter (mm)	Relative internal cross-sectional area	Typical sample load		Injection volume	Solvent flow rates (ml min^{-1})
		Easy separation	Difficult separation		
2.1	1.0	10-50	1	5-100μ1	0.3-6.0
6.2	8.0	100-500	10	0.5-5 ml	1.0-10.0
23.5	140.0	1000-5000	100	4-40 ml	5.0-90.0

More support provides greater surface area with which the solutes can interact, and since it does not increase the length of the colum it does not increase the retention times. In the single major component system separation is relatively easy so that, even on a 2.1mm i.d. (internal diameter) column, 50 mg of sample could be separated when the column is overloaded. Using Particle 10 (see Table 10.3) to obtain the same separation in the same time, a 1 ml/min flow rate for an analytical column, 4.6 mm i.d × 25 cm, must be changed to 4 ml/min flow rate on a 9.4 mm i.d. × 25 cm column and to 20 ml/min on a 22 mm i.d. × 25 cm column.

Where the resolution is large, wide-bore columns can be used and overload samples can be analyzed, for example, on a 1 inch column, samples in quantities upto 5 grams can be injected.

With increasing solute amount values of the height equivalent to a theoretical plate increase and k' values decrease if other parameters are fixed. Estimate of the maximum sample amount that can be charged to a column so as to avoid loss of column performance occurring due to overloading, can be made from the value of the adsorbent linear capacity, $\theta^{0.1}$. On this basis, it is found that the maximum sample size for porous silica is about 2×10^{-4} g of sample per gram of silica.

The weight of the stationary phase (and thus, sample size) can be increased by increasing either the column diameter or the column length. However, if the column is too short (less than 4 cm) the inlet and the outlet cause irregular movements in the solvent flow leading to band broadening. If the column is too

long, the packing at the inlet becomes overloaded, and there is a loss of resolution. If the internal diameter is increased, it becomes difficult to ensure that the solutes are spread evenly over the cross-section of the column at the inlet.

As important as the amount of packing in the column is the dead volume of the column since this indicates how much solvent is needed to equilibrate and to elute. In an analytical column where the dead volume is 3 ml and a solute has a k' value of 10, 30 ml of solvent has to be used to elute the solute. Equilibration can be achieved with 10-20 column volumes i.e. 50 ml. With a 22 mm × 50 cm preparative column where the dead volume is 130 ml, 1300 ml of solvent would be needed to elute a solute with $k' = 10$ and 1.5–2.0 litres of solvent for equilibration.

When the sample is overloaded there may be difficulties with the detection system. Detectors that operate at high flow rates are required. In contrast to the analytical detectors, where special small-bore tubing is used to provide minimum dead volume, preparative scale refractometers have large-bore tubing to carry the high flow rates. Alternatively, a stream splitter can take only a small aliquot of the column eluent into the detector, thus eliminating flow restrictions. The most popular detector for preparative scale HPLC is the refractometer, with the moving wire detectors a good second.

Having separated the components of a mixture, it is essential to have a good fraction collector, so that resolution will not be lost by fraction mixing beyond the detector. In order to isolate the separated solutes from HPLC analyses it is necessary to remove the solvent.

It is usual to remove the solvent by vacuum distillation. If the solvent has a very high boiling point, there is a danger that the solutes may decompose during the distillation. Low boiling solvents are therefore best for preparative HPLC.

A novel UV detector has been reported for preparative scale HPLC in which the solvent is allowed to flow over a supporting plate where it assumes the thickness characteristic of the flowing liquid film. The UV radiation falls on the film a little below the delivery tube from the column. The detector has been used for concentrations from 1mg to 30 g and for flow rates from 1ml min^{-1} to 400 ml min^{-1}.

The second type of problem is where there are two major components, say, 1 and 2, which, on overload conditions, become one very broad band. To obtain either solute in pure form, it is necessary to trap the front edge or the rear edge of the combined band without collecting the material near the centre. The front edge is often pure component, say, 1, whereas the middle of the band is contaminated with both solutes 1 and 2.

This situation can also be tackled by using the *re-cycle technique*. Partly resolved peaks can be returned to the column for further separation. The solutes pass through the column normally until the two partially resolved peaks are noted by the detector; then these two peaks are returned to the pump by appropriate

valve switching, for subsequent entry to the column for a second separation. Thus, resolution of a 100 mg mixture of the diastereoisomers of abscisic acid was achieved using a fully porous silica in a 9 ft $\times \frac{3}{8}$ inch column using the re-cycle technique. The first time the mixture was eluted from the column in 900 ml of 1% propan-2-θ1 in hexane, the components were not resolved. The mixture was re-cycled five times, each time improving the resolution until complete resolution was achieved. By overloading the column with a 250 mg sample, satisfactory resolution was obtained after six passes through the column.

In the third case, the mixture has several constituents and this situation requires that preliminary fractions should be taken around the trailing edge of the large band to allow a cut to be obtained which has a higher concentration of the desired product. Threatment of the original mixture in this way should enrich the fraction with respect to the desired product until the problem reduces to the first example considered.

Another approach to preparative liquid chromatography is to use medium pressure system (upto 300 psi, max), with shatter-proof glass columns and Merck silica gel 60 (230-400 mesh). These column can be dry packed with 100 g silica gel and will separate 5 g of solute in 2-3 hours. Solvent used does not need to be of analytical grade so long as it is distilled.

10.3 Trace Analysis

One very important area where HPLC can be employed is trace analysis, where small amounts of the compound of interest have to be analysed in the presence of a large amount of interfering matrix. Typical trace anlysis problems that can be dealt with by HPLC include the determination of drug metabolites in body fluids, the analysis of pesticide residues in environmental samples or the determination of unreacted intermediates or of by-products in various industrial products.

Two techniques that may be of interest in trace analysis of mixtures of wide-ranging polarities are *column switching* and *column back-flushing*. In the former, a relatively large volume of sample is injected into a short analytical column I (Figure 10.1). The early-eluting components of interest pass into the longer analytical column II. The valves
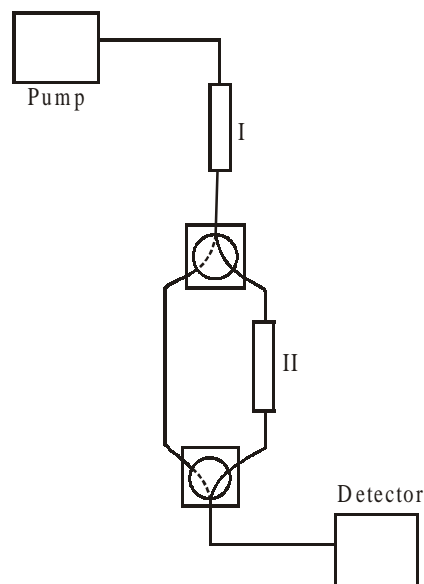

Figure 10.1 Schematic diagram of system for column switching.

are then switched so that these components are held at the top of column II while the later-eluting components from column I are analyzed directly. The flow is then once again diverted through column II and the trace component can be analyzed.

Column back-flushing is used to analyse a trace component in the presence of a sample that is strongly retained in the chosen chromatographic system. After elution of the solute of interest the eluent flow is reversed via a series of valves. (Figure 10.2) and the strongly retained components stripped from the top of the column.
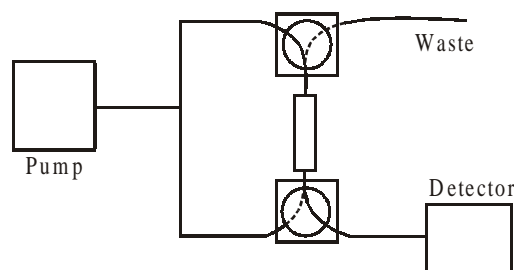
Figure 10.2 Schematic diagram of system for column back-flushing.

10.4 Modes of Working of HPLC and Their Applications

HPLC can function in several chromatographic modes illustrated below.

10.4.1 Liquid-solid (Adsorption) HPLC

10.4.1.1 Column Packing Materials

Both microporous and pellicular bead silica and alumina can be used for HPLC in the adsorption mode (liquid-solid chromatography). Pellicular beads, however, are rarely used now. While the pellicular beads (Table 10.2) mostly are spherical in shape, microporous particle column materials (Table 10.3) can be either irregular in shape or spherical. Theoretically irregular particles should give higher efficiencies but spherical materials pack together better.

TABLE 10.2

Pellicular Bead Packing Materials for HPLC

Type	Name	Particle size (μ)	Surface area (m^2g^{-1})	Shape
Silica (active)	Corasil I	37-50	7	Spherical
	Corasil II (has a thicker silica coating than Corasil I)	37-50	14	Spherical
	Pellosil HC (high capacity) [the material has a thicker coating than the HS (high speed) material]	37-44	8	Spherical

Contd.

Type	Name	Particle size (μ)	Surface area ($m^2 g^{-1}$)	Shape
	Pellosil HS	37-44	4	Spherical
	Perisorb A	30-40	14	Spherical
	SIL-X-II	30-40	12	Spherical
	Vydac	30-44	12	Spherical
Silica (inactive)	Liqua-Chrom (silica glass, and it has higher capacity than others)	44-53	<10	Spherical
	Zipax (inactive surface; precoated packings available)	25-37	~1	Spherical
Alumina	Pellumina HC (high capacity)	37-44	8	Spherical
	Pellumina HS (high speed)	37-44	4	Spherical

TABLE 10.3

Porous Particle Packing Materials for HPLC

Type	Name	Particle size (μ)	Surface area ($m^2 g^{-1}$)	Shape
Porous silica	Bio-Sil A	20-44	>200	Irregular
	Hypersil	5	200	Spherical (average pore diameter, 10 nm)
	Chromosorb LC-6	5-10	400+	Pore diameter is 120 Å, density 0.40 g cm^{-3}
	Li Chrospher SI 100	10	25	Spherical
	Li Chrosorb SI 60 (formerly marketed as Merckosorb)	5,10,30	500	Irregular (average pore diameter 6 nm)
	Li Chrosorb SI 100 (formerly marketed as Merckosorb)	5,10,30	400	Irregular (average pore diameter, 10 nm)
	Nucleosil	5-10	—	Spherical

Contd.

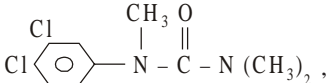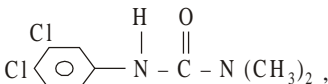| Type	Name	Particle size (μ)	Surface area (m^2g^{-1})	Shape
	MicroPak SI5, SI10	5, 10	500	Irregular
	Partisil 5,10,20	5,10,20	400	Irregular (average pore diameter, 5,5–6 nm)
	μ Porasil	10	400	Irregular
	Porasil A	37-75 or 75-125	350-500	Spherical (average pore diameter, <10 nm)
	Porasil B	37-75 or 75-125	125-250	Spherical (average pore diameter, 15 nm)
Porous silica	Porasil C	37-75 or 75-125	50-100	Spherical
	Porasil D	37-75 or 75-125	25-45	Spherical
	Porasil E	37-75 or 75-125	10-20	Spherical
	Porasil F	37-75 or 75-125	2-6	Spherical (average pore diameter, r >150 nm)
	Porasil T	15-25	300	Irregular
	SIL-X	32-45	300	Irregular
	SIL-X-I	13	400	Irregular
	Spherisorb S5W S10W S20W	5,10,20	200	Spherical (average pore diameter of S5W and S10W, 8 nm)
Porous Silica	Spherosil XOA-400	100-150	350-500	Irregular (average pore diameter, 8 nm)
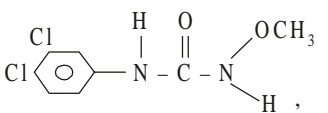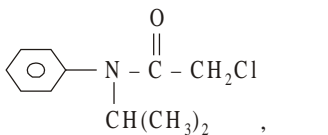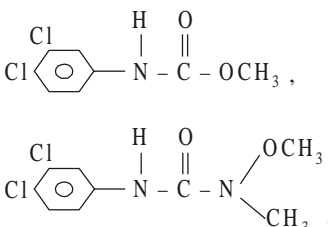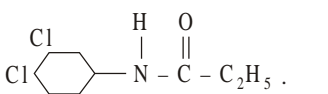
Type	*Name*	*Particle size (μ)*	*Surface area (m²g⁻¹)*	*Shape*
	XOA-200	100-150	125-250	Spherical (average pore (diameter, 15 nm)
	XOA-075	100-150	50-100	Spherical
	XOB-030	100-150	25-45	Spherical
	XOB-015	100-150	10-20	Spherical (average pore diameter, 125 nm)
	XOB-005	100-150	2-6	Spherical
	XOA-800	5	800	Spherical
	XOA-600	5	600	Spherical
	Vydac TP silica (TP = totally porous)	10	200	Irregular
	Zorbax SIL	5	300	Spherical (average pore diameter, 7.5 nm)
Porous alumina	Bio-Rad AG Li Chrosorb (formerly marketed as Merckosorb)	<75	>200	Irregular
	Alox T	5,10,30	70	Irregular
	MicroPak Al-5, Al-10	5,10	70	Irregular
	Spherisorb A5W, A10W, A20W	5,10,20	95	Spherical
	Woelm Alumina	18-30	>200	Irregular

10.4.1.2 Applications of Liquid-solid (Adsorption) HPLC

Examples of the application of HPLC in the liquid-solid (adsorption) mode in the fields of Organic Chemistry, Inorganic Chemistry and Biochemistry are listed in Table 10.4.

TABLE 10.4

Applications of Liquid-solid (Adsorption) HPLC

Sample	*Stationary Phase*	*Mobile Phase*
Organic Nitrobenzene, methyl benzoate, L, L-dimethylbenzyl alcohol, cinnamyl alcohol	Zorbax SIL 6 μ	Gradient: 0.1% propan -2-01 in hexane to 1.0% propan-2-01 in dichloro-methane
1,3- Dichlorobenzene and chlorobenzene	7μ Li Chrosorb SI 60	Heptane
Mixture of the following substitution products of urea and carbamic acid along with amides:–		

$$Cl\text{—}C_6H_3(Cl)\text{—}\underset{\underset{CH_3}{|}}{N}\text{—}\overset{\overset{O}{\|}}{C}\text{—}N(CH_3)_2 \; ,$$

$$Cl\text{—}C_6H_3(Cl)\text{—}\underset{\underset{H}{|}}{N}\text{—}\overset{\overset{O}{\|}}{C}\text{—}N(CH_3)_2 \; ,$$

$$Cl\text{—}C_6H_3(Cl)\text{—}\underset{\underset{H}{|}}{N}\text{—}\overset{\overset{O}{\|}}{C}\text{—}N\underset{\diagdown H}{\overset{\diagup OCH_3}{}} \; ,$$

| | 8-9 μ poros silica microspheres, surface area 250 m^2 g^{-1} | Dichloromethane (50% water saturated) |

$$C_6H_5\text{—}\underset{\underset{CH(CH_3)_2}{|}}{N}\text{—}\overset{\overset{O}{\|}}{C}\text{—}CH_2Cl \; ,$$

$$Cl\text{—}C_6H_3(Cl)\text{—}\underset{\underset{H}{|}}{N}\text{—}\overset{\overset{O}{\|}}{C}\text{—}OCH_3 \; ,$$

$$Cl\text{—}C_6H_3(Cl)\text{—}\underset{\underset{H}{|}}{N}\text{—}\overset{\overset{O}{\|}}{C}\text{—}N\underset{\diagdown CH_3}{\overset{\diagup OCH_3}{}} \; ,$$

$$Cl\text{—}C_6H_3(Cl)\text{—}\underset{\underset{H}{|}}{N}\text{—}\overset{\overset{O}{\|}}{C}\text{—}C_2H_5 \; .$$

Contd.

Sample	Stationary Phase	Mobile Phase
Four isomers (i.e. 1-chloro, 2-chloro, 3-chloro, 4-chloro) of chloropheno-thiazine-5, 5-dioxide	7.5 μ Spherisorb Al	Hexane-dioxan (50:50)
Inorganic		
$1\text{-}C_5H_5\text{-}Co\text{-}2,3\text{-}C_2B_9H_{11}$, $1\text{-}C_2H_5\text{-}C_5H_4\text{-}Co\text{-}2,3\text{-}C_2B_9H_{11}$, $1\text{-}C_2H_5\text{-}Co\text{-}2,8\text{-}C_2B_9H_{11}$	Silica 13 μ	Heptane-dichloro-methane-isopropanol (89.8 : 10.0 : 0.2)
Chromium hexafluoroacety-lacetonate, tris- (2′-hydroxy-acetophenono) chromium, chromium acetylacetonate.	Porasil A silica; large porous spherical particles	0.5% pyridine-tolune
C_2H_5HgCl, CH_3HgCl	Corasil I	n-Hexane
Biochemical		
Peptides: N-Bz-Val Val-OMe, N-Bz-Val Gly-OMe, N-Bz-Gly Val-OMe.	Pellosil HC	Dichloromethane +1% methanol
Carbohydrates: β-Methyl D-allopyranoside 2,4,6-tribenzoate, β-Methyl D-glucopyranoside 2,3,6-tribenzoate, β-Methyl D-galactopyranoside 2,3,6-tribenzoate.	Pellosil HC	Dichloromethane
β-D-galactose penta-acetate, Phenyl β-D-glucopyranoside, Methyl α-D-glucopyranoside.	Porasil A	Methyl ethyl ketone-water-acetone (85:10:5)
Fructose, glucose, mannose	Li Chrosorb-Si60 5μ	0.1% Water in acetonitrile
Lipids: Methyl oleate, Methyl stearate, Methyl arachidate, Methyl behenate, Methyl lignocerate	Vydac 35-44 μ	Methanol-water (90:10)

Contd.

Sample	Stationary Phase	Mobile Phase
cis-Methyl farnesoate, *trans*-Methyl farnesoate	MicroPak Si-10	Hexane-dichloro-methane-isopropanol (99.2:0.7:0.1)
Squalene, Cetyl oleate, Methyl oleate, Triolein		Programmed: Heptane-diethyl ether (99:1); chloroform-dioxan (92:8);
Glycerol-1-oleate-2-3-diacetate 1, 3-Diglyceride, 1, 2-Diglyceride cholesterol, Mono-olein	Spherisorb S10W	heptane-diethyl ether-dioxan-propan-2-01-water (20:20:30:30:1)
Methyl 11-hydroperoxyoctadec-9c-enoate, Methyl 11-hydroperoxyoctadec-9t-enoate.	Partisil 5 5μ	75% Ethanol in hexane
Vitamins: Vitamin A acetate, Vitamin E, Vitamin D_3, Vitamin A alcohol.	Corasil II	25% Chloroform in iso-octane
Vitamin A palmitate, Vitamin A acetate.	Li Chrosorb Si 60 5 μ	Heptane-di-isopropyl ether (95:5)
cis-Vitamin K_1, *trans*-Vitamin K_1, Vitamin K_1 epoxide,	5μ Silica	0.2% Acetonitrile in n-hexane
Carboxylic acids: Oxalic acid, lactic acid, L-ketoglutaric acid, *trans*-aconitic acid.	Corosil II	Isopropanol -n-hexane (1:50)

10.4.2 Liquid-liquid (Partition) HPLC

10.4.2.1 Column Packings

Liquid-liquid or partition chromatography, either normal or reversed-phase, requires a column of more or less inert packing material that has been layered with a stationary phase.

Mechanically held stationary phases were used when HPLC was first applied to liquid-liquid chromatography. Their preparation involved coating of solid

adsorbents with 10-20% of stationary phase. These high percentage loading phases on porous supports are difficult to pack and produced mass transfer problems resulting in band broadening. Pellicular bead materials used with 1% stationary phase such as β, β′-oxydipropionitrile or polyethylene glycol gave more easily packed, more efficient and more homogeneous beds than large porous particle material.

Liquid-liquid chromatography can also be performed on the microporous particles (Table 10.3) but since packing of these small particle materials with added liquid phase is difficult, it has been necessary to utilize *in situ* coating methods. Low viscosity stationary phases can be loaded by pumping them through the dry packed bed or they can be injected with a syringe into a column allowing a presaturated mobile phase to distribute the coating. High viscosity phases have to be dissolved in a suitable solvent and pumped through the column, after which the solvent is removed by a purge gas.

Additionally liquid-liquid (partition) HPLC can be performed with packings which are polymer-coated materials such as those illustrated in Table 10.5.

A special type of partition system employs a two-phase ternary mixture formed, for example, by mixing methylene chloride, ethanol and water or isooctane,

TABLE 10.5

Polymer-coated Materials for HPLC

Type	Name	Polymer constituting the coating	Particle size (μ)
Polymer coated glass beads (or superficially porous glass beads)	Pellidon	Nylon	45
	Periosorb	Polycaprolactam	30-40
	Zipax-ANH	Cynoethylsilicone	37-44
	Zipax-HCP	Saturated hydrocarbon	25-37
	Zipax-PAM	Nylon	25-37

ethanol and water in appropriate proportions. The three-component mixture is stirred at constant temperature until equilibrium is reached. The aqueous layer is separated and used as the stationary phase, while the organic-rich layer is used as mobile phase. Strict temperature control is necessary. Excellent separations have been carried out and good selectivity for similar compounds can also be achieved.

Since the two phases have to be essentially immiscible, the polarity range of sample that can be separated by any column is relatively small. This is so because the sample has to be soluble in both phases and to a certain extent this is an incompatible requirement. However, the wide range of phase systems that can be and have been used does allow small differences is solute properties to be exploited to achieve separation.

The main disadvantages of partition chromatography are the necessity for saturating the eluent with stationary phase and the closely related disadvantage of not being able to use gradient elution. Nevertheless, the advantage of selectivity inherent in the range of stationary phases available for liquid-liquid partition chromatography should always be borne in mind when selecting the mode of HPLC to be used for the separation in question.

Molecules which are highly polar give rise to problems of long retention times and peak tailing in adsorption chromatography and the solution to this problem lies in the use of reversed-phase chromatography, which employs a non-polar stationary phase in conjunction with a polar mobile phase; the polar molecules now have little affinity for the hydrophobic support and are eluted relatively quickly by a polar eluent such as a methanol/water mixture.

The stationary phases used in reversed-phase chromatography, when it was first introduced, comprised of a non-polar substance (e.g. squalene) coated on to a silica-based support. These are now seldom used. The stability of such systems is low, because the forces holding, say, squalene to even a silylated silica are so weak that the stationary phase is easily washed from the column. A compromise reversed-phase packing material was developed, which had a polymeric hydrocarbon stationary phase on the support, but although quite successful it has now been superseded by a chemically bonded stationary phase of which some examples are discussed below.
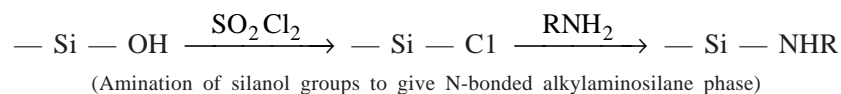
Chemically Bonded Stationary Phases

A number of bonded stationary phases are available and they differ in particle shape, particle and pore size distribution, the nature of the bonding material, the texture of the bonded surface layer and the extent of surface coverage.

The first chemically bonded stationary phases for HPLC were prepared by reacting the surface silanol groups of silica with an alcohol as shown below:

$$— Si — OH \xrightarrow{ROH} — Si — O — R$$

(Esterification of siland groups to give alkoxy silane phase).
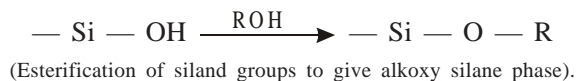
Other materials were prepared by converting silica to silica chloride by treatment with thionyl chloride, and then reacting the surface chloride groups with amines as shown below:

$$— Si — OH \xrightarrow{SO_2Cl_2} — Si — Cl \xrightarrow{RNH_2} — Si — NHR$$

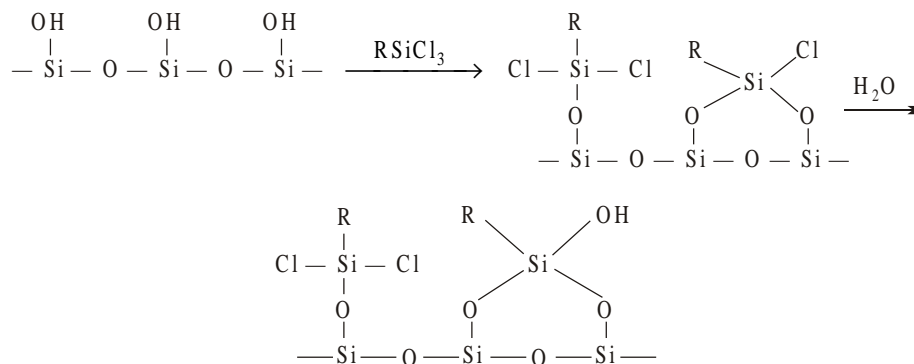(Amination of silanol groups to give N-bonded alkylaminosilane phase)

These bonded phases are commercially available as Dupak supports. These new phases had strands of organic chains pointing away from the silica surface so that they offer increased accessibility of the stationary phase to solute. However, they suffer the known drawback of silicate esters in that they have limited hydrolytic

stability within the pH range 4-7 only, due to hydrolysis of the Si—NR bonds as is the case with the Si—OR bonds in silicate esters.

The great majority of modern commercially available bonded materials are derived from reactions between organochlorosilanes or alkoxysilanes with the surface silanol groups. The two general approaches to the bonding of organosilanes to silica are described below.

In the first approach, the reaction is carried out under conditions that exclude, as far as possible, water from the reaction mixture. Typical reaction conditions might involve heating dry silica under reflux with, for example, octadecyl-trichlorosilane in toluene. In the absence of moisture, no hydrolysis of the Si—C1 bonds in the chlorosilane takes place and therefore no polymerisation of the silane occurs. Bonding takes place by elimination of HC1 between the organosilane and one or more of the surface silanol groups. After removal of any excess of silane, the product is hydrolysed to convert unreacted Si—C1 groups to silanol groups as depicted below:

(Reaction of silanol groups to give an alkyl silyl bonded phase using an alkyltrichlorosilane)

In the second approach of reacting organosilanes with siliceous surfaces, the organochloro or alkoxysilane is first hydrolysed to the silanetriol which polymerises partially. The polymer is then bonded to the support surface by multiple attachments, again via stable siloxane linkages. The reaction sequence is shown below:
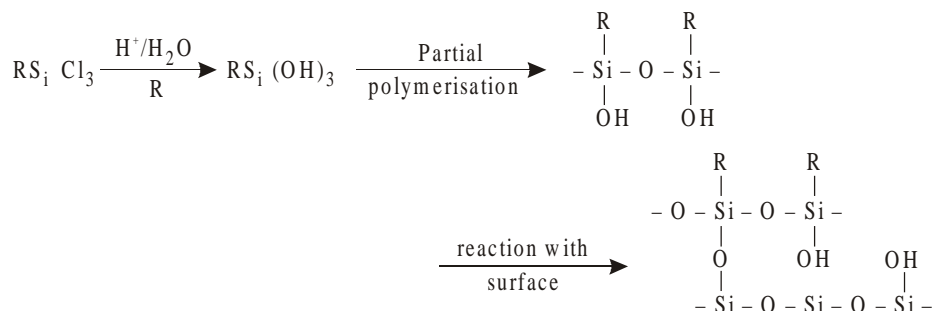

(Reaction of silanol groups with a partially polymerised silane to give a ploymeric stationary phase).
Complete cross-linking is achieved by–final heat treatment.

Bonded phase coverage depends on the number of silanols available and on the type of reaction. Low surface area silicas (e.g. Zipax) require polymerisation but for high surface area silicas (e.g. Li Chrosorb) direct reaction without polymerisation is satisfactory.

The alkyl phase loading (as a % carbon by weight) of the commercially available bonded phase materials can range from 5% to 22%. When the loading is low (5-10%) there is probably a monolayer coverage whilst the higher loading suggests a polymeric coverage. The monomeric phases ought to have a higher separation efficiency since the mass transfer rate is slower on the polymeric layer. Against this, any residul silanols which may act as adsorption sites are more likely to be blocked with the polymeric layer reducing the chance of tailing.

Reaction of silica chloride with Grignard or organolithium reagents leads to the bonding of organic groups to the surface of silica via direct Si–C bonds, as depicted below:

(Formation of an alkyl silyl bonded phase using
a Grignard reagent or an alkyl lithium).
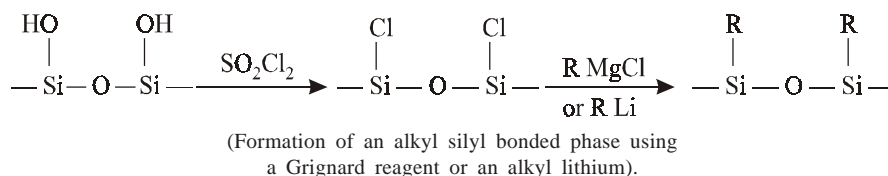
The advantage of supports formed in this way is that the Si — O — Si and Si — C bonds formed during the reaction are relatively stable to hydrolysis. These bonded phases are regarded as being generally stable in the pH range 3-8. The organic groups begin to be cleaved from the support at pH below 2 or 3 and silica itself starts to dissolve at about pH 8. The stability of the bonded phase is also governed by the nature of the bonded group, the composition and the ionic strength of the eluent, and the column operating temperature.

The non-polar pellicular beads with their long hydrocarbon chains are especially useful for the reversed-phase mode. Samples that are insoluble in water but soluble in alcohol or other water-miscible organic solvents e.g. dioxan, acetonitrile and tetrahydrofuran, are candidates for reversed-phase chromatography.

The most common bonded hydrocarbon phase is that formed by bonding octadecylsilyl ($C_{18} W_{37} Si$ —) groups to silica, although shorter chain lengths are commercially available. The efficiencies obtained with reversed-phase materials can be similar to those obtained with the unbonded silica adsorbent. The mechanism of reversed-phase chromatography has not been satisfactorily worked out. One of the problems is that most commercially available materials may contain a high proportion of residual silanol groups and retention on such supports will almost certainly be a mixed adsorption/partition mechanism. (The residual silanol groups may be silanol groups originally present on the silica surface which have not reacted for steric or other reasons, or they may be formed on a di- or

trichlorosilyl reagent at the end of the reaction by hydrolysis of unreacted Si – C1 bonds). It seems reasonable to suggest that use of a 'capped' stationary phase obtained by blocking of resudual silanol groups with, for example, trimethylsilyl groups should reduce the tailing of polar solutes on the silanol sites in reversed-phase chromatography. It has been suggested that the role of the bonded hydrocarbon is to extract from a binary mobile phase the more lipophilic component. This results in an organic-rich layer at the particle surface where the chromatographically useful partitioning takes place.

Regarding the chain length of reversed-phase materials, the longer the chain length the greater the solute retention with a given eluent. Selectivity has been found to increase with chain length, to remain constant and also to show an initial increase (C_{12} chain compared with a C_6 chain) followed by a levelling off (comparing C_{12} and C_{18} chains). In practice, many separations obtained on long chain materials can be reproduced on short chain materials by increasing the amount of the aqueous component in the eluent.

The polar phases can be used with normal LLC mode (e.g. polar stationary phase or a non-polar mobile phase) or with reversed-phase mode. These polar phases have either nitrile, ether or amino functional groups.

The commonly used chemically bonded stationary phases for HPLC, marketed under their trade names by various suppliers, are listed in Table 10.6 (Commercial products presently available under these trade names may have specifications which are different from those given in the Table).

TABLE 10.6

Bonded phase	Name	Support type	Particle size (μ)
Octadecyl hydro-carbon ($C_{18} H_{37}$ Si-)	Bondapak C_{18}/Corasil	Pellicular	37-50
	Co : Pell ODS	Pellicular	41
	ODS-SIL-X-II	Pellicular	30-40
	Perisorb RP	Pellicular	30-40
	Permaphase ODS	Pellicular	25-37
	Vydac RP	Pellicular	30-44
	Bondapak C_{18}/Porasil	Porous	37-75
	μ Bondapak C_{18}/Porasil	Porous	10
	ODS-Hypersil	Porous	5
	Li Chrosorb RP-18 (carbon loading, 22%)	Porous	5, 10
	MicroPak CH (carbon loading, 22%)	Porous	10
	Nucleosil C_{18}	Porous	5, 10
	Partisil-10 ODS (carbon loading, 5%)	Porous	10

Bonded phase	Name	Support type	Particle size (μ)
	Partisil-10 ODS-II (carbon loading 16%)	Porous	10
	ODS-SIL-X-I	Porous	8-18
	Spherisorb S5 ODS (S100DS)	Porous	5, 10
	Vydac TP Reversed phase	Porous	10
	Zorbax ODS	Porous	5
Short Chain Hydrocarbon	Li Chrosorb RP-8 (contains bonded octyl groups)	Porous	5, 10
	Li Chrosorb RP-2 (support treated with dimethyl-dichlorosilane)	Porous	5, 10, 30
	Nucleosil C_8 (contains bonded octyl groups)	Porous	5, 10
	SAS - Hypersil	Porous	5
Phenyl	Bondapak Phenyl/Corasil	Pellicular	37-50
	Bondapak Phenyl/Porasil	Porous	37-75
	Phenyl-SIL-X-I	Porous	8-18
Allylphenyl	Allylphenyl-SIL-X-I	Porous	8-18
Fluoroether	FE-SIL-X-I	Porous	8-18
Ether	Permaphase ETH	Pellicular	25-37
Nitro	Nucleosil NO_2	Porous	5, 10
Cyano	Co : Pell PAC	Pellicular	41
	Vydac Polar Phase	Pellicular	30-44
	μ Bondapak CN	Porous	10
	Cyano-SIL-X-I	Porous	8-18
	Durapak OPN/Porasil	Porous	37-75
	(Durapak supports are silicate esters i.e. formed via Si-O-C linkages)		
	Nucleosil CN	Porous	5, 10
	MicroPak CN	Porous	10
	Partisil-10-PAC	Porous	10
	Spherisorb S5CN	Porous	5
	Vydac TP Polar Phase	Porous	10
Hydroxyl (as in Carbowaxes which are poly-ethylene glycols)	Durapak Carbowax 400/Corasil	Pellicular	37-50
	Durapak Carbowax 400/Corasil		

Bonded phase	Name	Support type	Particle size (μ)
		Porous	37-75
	Li Chrosorb Diol	Porous	10
Amino (function as polar bonded phases or weak anion exchangers)	APS-Hypersil	Porous	5
	Amino-SIL-X-I	Porous	8-18
	μ Bondapak NH_2	Porous	10
	Li Chrosorb NH_2	Porous	10
	MicroPak NH_2	Porous	10
	Nucleosil NH_2	Porous	5-10
	Nucleosil $N(CH_3)_2$	Porous	5-10

10.4.2.2 Applications of liquid-liquid (Partition) HPLC

Applications of HPLC in the liquid-liquid mode are illustrated in Table 10.7.

TABLE 10.7

Sample	Stationary Phase	Mobile Phase
Biochemical		
Amino acids:		
As dansyl amino acids		
Asparagine		
Glutamate		
Dansyl hydroxide		
Serine		
Threonine		
Alanine		
Proline	μ Bondapak C_{18}	Initial 30% methanol-
Valine	(30 cm × 4 mm internal	70% 0.01M
Methionine	diameter) 2 in tandem	K_2HPO_4
Cystine ⎫ retention time		
Isoleucine ⎭ of each, 26.8 min		Final 70%
Histidine ⎫ retention time of		methanol-
Arginine ⎬ each,		30% 0.01M
Leusine ⎭ 27.6 min		K_2HPO_4
Lysine ⎫ retention time of		(flow rate, 1.2 ml
Phenylalanine ⎭ each, 28.8 min		min^{-1} temp., 25ºC)
Dansylamide		
Lipids:		
Methyl linolenate	Bondapak C_{18}/Porasil	100%

Sample	Stationary Phase	Mobile Phase
Methyl linoleate Methyl oleate Methyl palmitate Methyl stearate		acetonitrile
Naphthacyl linolenate Naphthacyl linoleate Naphthacyl oleate	Corasil-C_{18}	Methanol- water (85:15)

p-Bromophenacyl esters of
  Prostaglandin F_2
    Prostaglandin E_2
    Prostaglandin D_2
    Prostaglandin A_2
    Prostaglandin B_2
	Bondapak C_{18}	Acetonitrile
		-water
		(50:50)

Triacylglycerols from cotton seed	μ Bondapak C_{18}	Acetonitrile- acetone (42:58)
Chlorophyll b Chlorophyll a	Spherisorb ODS 5μ	5.5% water in methanol
Cytosine Uridine diphosphoglucose Uracil	Bondapak C_{18} 10μ	Sol A 0.02 mol l^{-1} KH_2PO_4 (pH 5.6);
Guanosine monophosphate Thymine Guanosine + 30 other nucleoside bases		Sol B 60% methanol; (gradient, 0-60% methanol in 87 min)

Vitamins:
    Vitamin D_2
    Vitamin D_3
	Spherisorb ODS	95% methanol-
		5% water

Ascorbic acid Isoascorbic acid	Li Chrosorb NH_2 10 μ	75% acetonitrile in 0.005M- KH_2PO_4 (pH 4.4-4.7)

Plant growth factors:
    trans-Zeatin
    Zeatin riboside
    Indoleacetic acid
    Abscisic acid
	μ Bondapak C_{18}	12.5% methanol
		(pH 2.8) for
		20 min, then
		linear to 50% methanol
		(pH 2.8)
		over 30 min

Contd.

Sample	Stationary Phase	Mobile Phase
Gibberellin A8 Gibberellin A29 Gibberellin A23 Gibberellin A$_1$	Bondapak C$_{18}$/Porasil B	Solvent A, 1% aqueous acetic acid Solvent B, 95% ethanol

Inorganic
Metaldiethyldithiocarbamates
   Pb
   Ni
   Co
   Cu
   Hg
	Li Chrosorb RP-18	Acetonitrile-
		water (65:35)

Organic Dinitrophenyl hydrazones of: n-Pentanal n-Butanal ⎱ same retention times Acetone ⎰ for the column used Acetaldehyde Formaldehyde o-Hydroxyphenylacetic acid 6-Hydroxycoumarin 7-Hydroxycoumarin ⎤ same retention 8-Hydroxycoumarin ⎦ times for the column used	Zipax + 1% tri- (2-cyanoethoxy) propane	Hexane saturated with stationary phase
5-Hydroxycoumarin o - Coumaric acid Coumarin 3 - Hydroxycoumarin Pre-column of Co : Pell ODS	Partisil 10 ODS	Methanol– water– acetic acid (400:600:1)

10.4.3. *HPLC using Chiral Stationary Phases*

Use of chiral stationary phases can brings about separation of enantiomers by HPLC. These stationary phases can be prepared simply by impregnating an adsorbent such as silica gel with a chiral reagent. The solvent for development is chosen such that the chiral reagent is not eluted from the column. For example, the separation of a mixture of recemic carbohelicenes was obtained using a silica gel column impregnated with 25% w/w R(–)–2–(2,4,5,7-tetranitro-9-fluorenyli-deneaminooxy) propionic acid (TAPA) [see figure 10.3 (IV)] TAPA is a well known resolving agent for optically active aromatic and unsaturated hydrocarbon compounds capable of interacting with the former through charge transfer complexation. Since these charge transfer complexes are really diasteromeric complexes, they have different equilibrium constants for their formation and dissociation on the silica gel support. The need for the resolving agent to be completely insoluble in the mobile phase is an unnecessary limitation on the selection of mobile phase for the separation. Permanently bonded phases offer

greater versatility and can be conveniently prepared by condensation reactions employing the commercially available 3-aminopropyl silica phase as substrate. TAPA can be chemically bonded to this phase for the resolution of carbohelicenes and aromatic diols. The structures of some representative bonded chiral stationary phases, all prepared from 3-aminopropylsilica, are shown in Figure 10.3. The chiral phase, shown in Figure 10.3 (I) is probably the most widely investigated phase and has wide applicability, particularly for samples containing π-acid substituents. Solutes lacking π-acid substituents can often be derivatized to incorporate such a functionality. For example, amines, alcohols and thiols can be derivatized with 2,4-dinitrofluorobenzene or 3, 5-dinitrobenzoyl chloride to provide derivatives with better separation characteristics than the parent solute. The chiral phase, Figure 10.3 (II), can be conveniently prepared by passing a
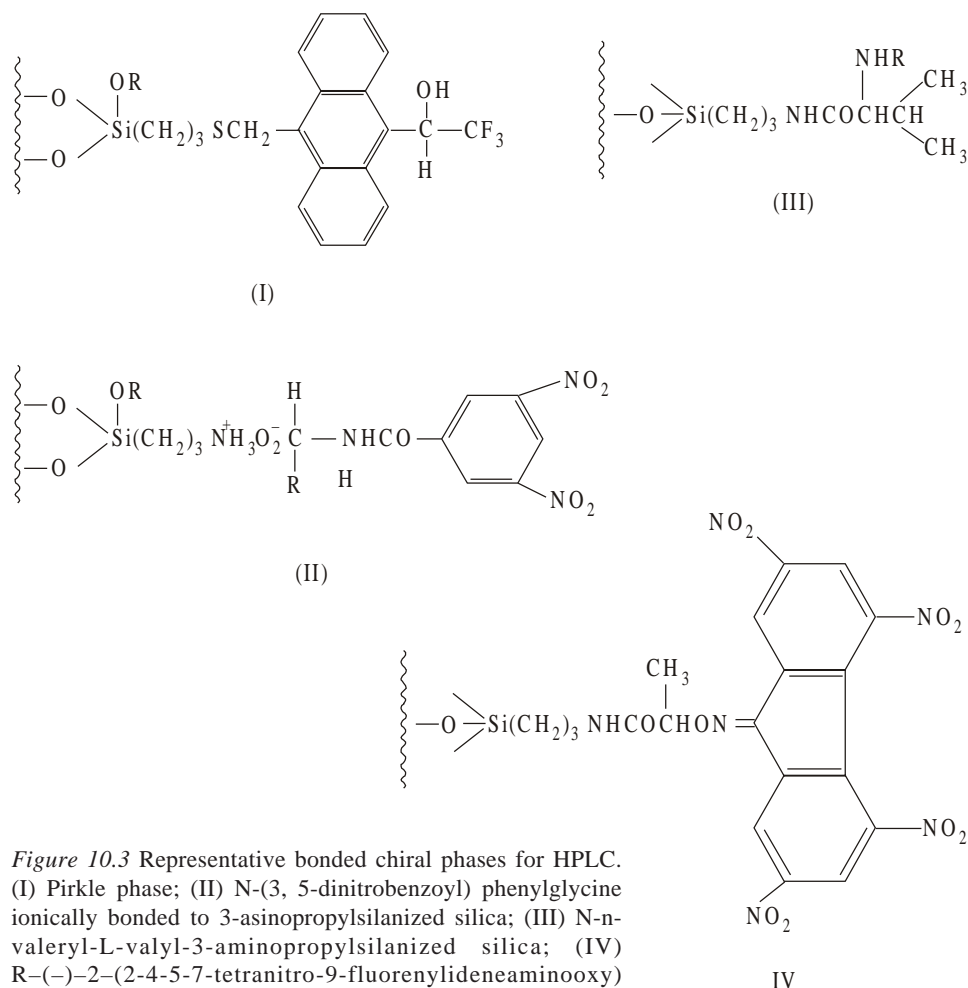
Figure 10.3 Representative bonded chiral phases for HPLC. (I) Pirkle phase; (II) N-(3, 5-dinitrobenzoyl) phenylglycine ionically bonded to 3-asinopropylsilanized silica; (III) N-n-valeryl-L-valyl-3-aminopropylsilanized silica; (IV) R–(–)–2–(2-4-5-7-tetranitro-9-fluorenylideneaminooxy) propionamidepropyl silanized silica.

solution of (R)-N-(3.5-dinitrobenzoyl) phenylglycine through a prepacked column of 3-aminopropyl bonded silica. This ionically bonded phase is suitable for use with relatively non-polar solvents. The N-n-valeryl-L-valyl-aminopropylsilica phase, Figure 10.3 (III), was used to separate racemic N-acetyl-α-amino acid methyl esters. The mechanism of chiral recognition was assumed to involve selective complexation between the phase and the amino acid derivative amide group, and to some extent the steric effect of the chiral alkyl group in the localized solute molecule.

In practice, separation of enantiomers by the use of chiral stationary phases is not free from problems. Chiral stationary phases are difficult to prepare reproducibly, are sometimes of lower chromatographic efficiency than expected, and optimization of separation conditions is restricted by the fixed nature of the chiral centres. Chiral mobile phases are free from many of these problems, optimization of the separation is more convenient, and conventional reversed-phase columns may be used. Thus N-(2, 4-dinitrophenyl)-L-alanine-n-dodecyl ester has been used as a non-ionic chiral mobile phase additive for the resolution of 1-azahexahelicenes by reversed-phase chromatography. The resolution obtained was found to be a function of the mobile phase polarity and the concentration of chiral additive used.

10.4.4 Complexing HPLC

The rapid and reversible formation of complexes between some metal ions and organic compounds that can function as electron donors can be used to adjust retenition and selectivity in gas and liquid chromatography. Such coordinative interactions are very sensitive to subtle differences in the composition or stereochemistry of the donor ligand, owing to the sensitivity of the chemical bond towards electronic, steric and strain effects. Mixtures of constitutional, configurational and isotopic isomers in whose case separation is difficult, may be separated by complexation chromatography. Perhaps the most widely known example is the use of silver ions to complex organic compounds containing π-electrons in various kinds of double and triple bonds, and heteroatoms such as N, O, and S with lone pairs of electrons. The selectivity of the silver nitrate-containing phases results from the marked effect that the relatively small structural or electronic changes in the donor ligand have on the stability constants of the complexes. Some of these trends are:

Substitution at the double bond decreases the retention volume.

A 1-alkyl compound has a lower retention volume in comparison to the 3- and 4-alkyl isomers.

Olefines having a substitutent in the 3-position have higher retention volumes than those of the 4-isomers.

Cyclobutenes have less tendency to form complexes than the corresponding 5- and 6- membered cyclo-olefines.

Cyclopentene derivatives have higher retention volumes than those of the corresponding isomeric cyclohexenes.

A conjugated double bond system has a lower complex-forming capacity than a simple double bond.

In reversed-phase HPLC, the use of silver ions to complex organic compounds containing π-electrons has been made for separation by complexation chromatography. The formation of silver complexes with unsaturated ligands results in a decrease in retention due to an increase in the hydrophilic character of the complex compared to the parent ligand. Varying the concentration of silver nitrate in the mobile phase enables the retention of the complexed species to be changed over a wide range, facilitating method development.

Separation of nucleotides and nucleosides has been carried out in the presence of Mg (II) ions using silica bonded with dithiocarbamate ligand as the stationary phase of the chromatographic column. The diphosphate and triphosphate groups of the nucleotides bind Mg (II) very strongly and compete with the dithiocarbamate ligand of the stationary phase for the metal cation. The retention of the nucleotides was found to vary inversely with the magnesium ion concentration in the mobile phase and also exhibited a strong pH dependence. Likewise, the competition between the amino groups of an amino-propylsilica bonded phase and the amino groups of aminosugars or peptides for cadmium or zinc ions was used to enhance their resolution in a reversed-phase chromatographic system.

A relatively hydrophobic chelating agent, namely, 4-dodecy-diethylene triamine, has been employed in the presence of Zn (II) ions to achieve the reversed-phase separations of dansyl amino acids, dipeptides, and aromatic carboxylic acids. The metal-derived selectivity results from the formation of outer sphere complexes. The formation of these complexes is rapid, contributing to the high separation efficiencies observed.

10.4.5 HPLC utilising the Formation of Crown Ethers

Most recently HPLC separations of metal ions have been carried out utilising the formation of stable complexes of metal cations with large cyclic ethers called 'crown ethers' (from the geometry of their appearance), though the theoretical plate numbers usually characteristic of HPLC have not yet been achieved with the development stage attained by this complexing technique.

Silica gel (Wako-gel LC–10H, 10μ, irregular) was reacted with (3-aminopropyl) triethoxysilane. The NH_2-modified silica gel was reacted with methacrylic anhydride to form a vinyl-modified silica gel, which then was co-polymerised with a vinyl crown ether. Two columns, A and B, each with internal diameter of 4 mm and length 30 cm, were studied: A—poly (benzo—15 crown—5)—modified silica and B–bis (benzo—15 crown—5)—modified silica.

Investigations carried out with the columns using alkali and alkaline earth metal ions showed the differences between ion-exchange resin columns and these crown ether columns.

With cation exchange resins having sulphonic acid or carboxylic acid as exchange sites, retention is typically dependent on mass/charge ratios:

$$Li^+ < Na^+ < K^+ < Rb^+ < Cs^+; \; Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+}; \; Na^+ < Ca^{2+} < Al^{3+}.$$

With crown ethers, retention is dependent upon the relative size of the cation and the ring of crown ether. The ring in this investigation was $(- C - O - C -)_5$ and the orders of elution were $Li^+ < Na^+ < Cs^+ < Rb^+ < K^+$ and $Mg^{+2} < Ca^{2+} < Sr^{2+} < Ba^{2+}$. Flow rates were 1.0 ml/min and pressure drops were 40 to 140 kg/cm^2. The effect of solute-solvent interactions upon the separations also was investigated by changing solvents (water/methanol ratios) and counterions. Decreasing water content increases retention time, but differently for each cation. For example, in one experiment when water content was decreased to 50% by using methanol, the retention times for Li^+, Na^+, Cs^+, Rb^+, and K^+ were 1.11, 1.63, 1.85, 4.09 and 6.64 times higher. When counterions are compared, in retention KC1 < KBr < KI.

10.4.6 Ion-exchange HPLC

Conventional resin beads which are prepared by copolymerisation of styrene and divinylbenzene have been discussed earlier in detail. Although these beads possess a degree of mechanical stability imparted to them by the extent of cross-linking of the resin and, as described earlier, analysis of amino acids can be carried out using ion-exchangers based on these polymeric resin beads, poor mass transfer (exchange equilibria) can be a problem in their use. To overcome this problem the conventional resins have been modified by using porous surfaces having ion-exchange properties layered on inert cores. These modified packings developed for HPLC are: (i) Pellicular bead exchangers [Figure 10.4 (a)]; these have been produced either from a divinylbenzene-polystyrene polymer resin core which has ion-exchange resin only on the surface, or from glass beads which are treated so as to acquire a skin of ion-exchange material; (ii) porous beads, which are constituted of an exchanger-coated porous surface on an inert core [Figure 10.4 (b)].
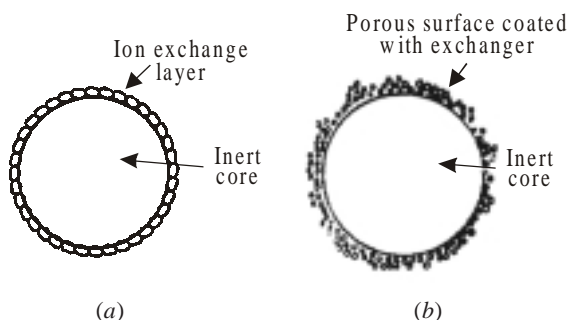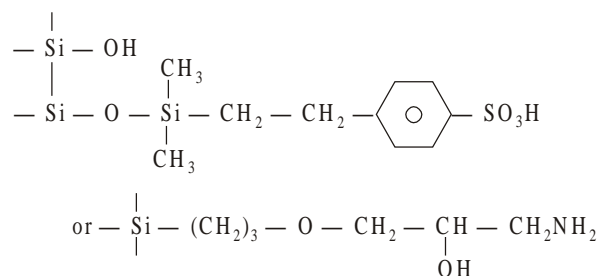

Figure 10.4 Ion-exchange packings: (a) Thin layer of ion- exchanger on solid inert core; (b) porous surface coated with exchanger on an inert core.

Although pellicular bead material is not used for many applications in liquid-liquid and liquid-solid chromato-graphy, it has retained its popularity for ion-exchange chromatography, since the pellicular beads can give significant differences in retention behaviour compared with conventional resin beads. The fast mass transfer in pellicular exchangers permits analysis time to be reduced by factors of 10. They show lower pressure drops with aqueous solutions though their larger particle sizes cause lowring of efficiencies. They have lower ion-exchange capacity so that the buffer solutions need to be of lower strength. The larger particle size allows them to be dry-packed.

Recently, silica based chemically bonded ion-exchangers have been developed. Here, functional groups can be attached to the silica surface, as is schematically shown below:

$$
\begin{aligned}
&- \overset{|}{\underset{|}{Si}} - OH \\
&\qquad\qquad\qquad\quad CH_3 \\
&\qquad\qquad\qquad\quad | \\
&- \overset{|}{\underset{|}{Si}} - O - \overset{|}{\underset{|}{Si}} - CH_2 - CH_2 - \langle\bigcirc\rangle - SO_3H \\
&\qquad\qquad\qquad\quad CH_3
\end{aligned}
$$

$$
or - \overset{|}{\underset{|}{Si}} - (CH_2)_3 - O - CH_2 - \underset{\underset{OH}{|}}{CH} - CH_2NH_2
$$

Various acidic, basic and amphoteric groups can be attached to the silica surface in this way (Table 10.8).

TABLE 10.8

Some types of functional groups which can be chemically bonded to the surface of silica

Acidic groups $- SO_3H; - COOH; - CH_2 - COOH;$

$$- \underset{\underset{OH}{|}}{CH} - \underset{\underset{OH}{|}}{CH_2}$$

Basic groups $- CH_2\,N\,(CH_3)_3\;Cl;$

$- CH_2N\,(CH_3)_2\,(C_2H_4)\;OHCl;$

$- CH_2NH_2; - N(CH_2CH_3)_2;$

$- OCH_2\,N\,(C_2H_5)_2.$

Amphoteric groups $- \underset{\underset{COOH}{|}}{CH} - CH_2 - CH_2 - NH_2.$

Some of the chemically bonded ion-exchangers marketed by suppliers under various trade names are tabulated below.

TABLE 10.9

Pellicular ion-exchange resins

Type	Name	Particle size (μ)	Base
Anion	Bondapak/AX/Corasil	37-50	BP
	Perisorb-AN	30-40	BP
	Permaphase-AAX	37-44	BP
	Vydac SC Anion	30-44	PS-DVB
	Zipax-SAX	25-37	Lauryl methacrylate polymer
	Zipax-WAX	25-37	PAM (polyamide)
Cation	Bondapak CX/Corasil	37-50	BP
	Perisorb-KAT	30-40	BP
	Zipax-SCX	25-37	FC
	Vydac SC Cation	30-44	PS-DVB

PS-DVB = polystyrene-divinylbenzene copolymer
PS-AE = polystyrene-aliphatic ester copolymer
FC = flouropolymer base
BP = bonded phase through siloxane base

Note : All anion exchangers have NR_3^+ except Zipax-WAX which has NH_2 functionality; all cation-exchangers have SO_3^-.

TABLE 10.10

Microporous particle ion-exchangers

Type	Name	Particle size (μ)	
Anion—based on divinyl-benzene-polystyrene	Aminex A-series	A-14	20 ± 3
		A-25	17.5 ± 2
		A-27	13.5 ± 1.5
		A-28	9 ± 2
	AN–X	11	
	Benson BA-X	7-10	
	Benson BWA	7-10	
	Chromex	11 ± 1	
	Ionex 5B	5-20	
Anion—Silica based	μ-Bondapak-NH_2	10	
	Li Chrosorb AN	10	

Contd.

Type	Name	Particle size (μ)
	MicroPak-NH$_2$	10
	MicroPak SAX	10
	Microsil SAX	10
	Nucleosil-NH$_2$ or N(CH$_3$)$_2$	5-10
	Nucleosil-SB	5-10
	Partisil 10SAX	10
	Vydac 301TP	10
	Zorbax SAX	7
Cation—based on divinyl-benzene-polystyrene	Aminex A-series	A – 4 20 $\pm$ 4 A – 5 13 $\pm$ 2 A – 6 17.5 $\pm$ 2 A – 7 7 – 11
	Aminex HPX-87	9
	Benson BC-X	7-10, 10-15
	Chromex Cation	11
	Dionex DC	–1A 14 $\pm$ 2 –4A 9 $\pm$ 0.5 –6A 11 $\pm$ 1
	Ionex SA	10 $\pm$ 2 15 $\pm$ 2.5 20 $\pm$ 3
Cation-Silica based	Li Chrosorb KAT	10
	Microsil SCX	10
	Nucleosil SA	5, 10
	Partisil-10 SCX	10
	Vydac 401 TP	10
	Zorbax SCX	6-8

Ion-exchange columns are generally less efficient than other column types used in HPLC. To improve solute diffusion and mass transfer, ion-exchange columns are often operated at elevated temperatures. As well as increasing column efficiency, an increase in column temperature usually results in low capacity factor values. Small changes in column temperature often result in large changes in separation selectivity, particularly for structurally dissimilar compound types. Ion-exchange columns are also often operated at low mobile phase flow rates to maximize column efficiency. For most separations the mobile phase is usually

completely aqueous. Water-miscible organic solvents may be added to the mobile phase to increase column efficiency and to control solvent strength. The presence of an organic modifier in the mobile phase has greatest influence on the separation when solute retention is at least partly controlled by a reversed-phase mechanism. When this is the case, solvent strength and selectivity can be adjusted.

The utility of ion-exchange HPLC for the separation of biomolecules is specially noteworthy and some applications of the technique in this field are listed in Table 10.11.

TABLE 10.11

Sample	Stationary Phase	Mobile Phase
Carbohydrates: Rhamnose, xylose, fructose, dextrose, sucrose, lactose and melibiose	Bondapak AX/Corasil	Water–ethyl acetate– propan–2-01
Rhamnose, xylose, arabinose, glucose, galactose, maltose	Aminex A6 Li$^+$	85% Ethanol-water
Nucleic Acid Constituents: Uracil, cytidine, guanine, cytocine and adenine	Zipax SCX	0.01M HNO_3 + 0.05M NH_4NO_3
Vitamins: Riboflavin	Zipax SCX	Water
Nicotinamide Riboflavin Pyridoxine	Zipax SCX	0.05M NaH_2PO_4 + 0.05M KH_2PO_4 at pH 4.4

Ion-exchange Chromatography with Detection by Atomic Absorption

A mixture of several compounds of arsenic like arsenites $\left(AsO_3^{3-}\right)$, arsenates $\left(AsO_4^{3-}\right)$, monomethyl arsonates $\left(CH_3AsO_3^{2-}\right)$, dimethylarsenates $\left[\left(CH_3\right)_2 AsO_2^{-}\right]$ and p-aminophenylarsonates $\left[p-NH_2\left(C_6H_4\right)AsO_3^{2-}\right]$ has been separated into its constituents using an ion-exchange column. Detection of the separated constituents was carried out by *atomic absorption* after reduction to arsine, AsH_3.

The basis of the method of determination by atomic absorption is the measurement of the light absorbed at the wavelength which is defined by sharp maxima in intensity of absorption by unexcited atoms of the element.

Elements not themselves excited to emission by a flame may be determined in a flame by absorption in case the atomic state is capable of existence. At the

temperature of a normal air-acetylene (or similar) flame, only a very small fraction of all atoms of such an element is excited to emission in a flame and almost 99 per cent are not excited to emission; the absorption due to a transition from the ground electronic state to a higher energy level is thus virtually an absolute measure of the number of atoms in the flame, and hence the concentration of the element in a sample.

To realise the full potentialities of the method, the strongest absorption line must be used. For elements with simple spectra, the resonance line arising from the lowest excited state is usually the line exhibitin strongest absorption.

In the general arrangement of an *atomic absorption detector*, an aerosol is introduced into a flame which is placed on the optical axis between the entrance slit of the monochromator and the monochromatic light source. Energy of the wavelength absorbed by the sample is provided by a source lamp whose emitting cathode is made of that element. This energy is passed through the flame and then through the dispersing device. A detector measures the absorbed exciting radiation.

The atomic-line source is the critical component. Because the width of absorption lines is extremely small, it is difficult to measure the absorption accurately against the background of a continuous spectrum. The source must therefore be sensibly monochromatic. For metals such as alkali metals and mercury, vapour lamps are satisfactory. For other elements a hollow cathode lamp is the most useful source for sharp resonance lines. These lamps can be used for a wide range of elements. The hollow-cathode discharge tube consists of an anode and a cylindrical cathode enclosed in a gas-tight chamber. An inert carrier gas (helium or argon) is introduced at 1-2 mm pressure. When a potential of 600-1000 volts is applied to the electrodes, a discharge is created which fills the cathode cavity. The inert gas ions formed by this discharge are accelerated toward the cathode and upon collision with the element in the cathode cavity, sputter it into the discharge zone. The highly energetic carrier gas ions then excite the sputtered atoms to emission through collisions. Lamps are operated at low currents to improve linearity of response and maintain narrow emission lines.

Constant feed rate of sample into the burner is critical. Although solids have been volatilised directly using induction heating, the most common practice involves use of a solution which is introduced into a flame by an atomiser. The burner has two principal functions to perform: (i) It must introduce the sample into the flame and (ii) it must reduce the metal to the atomic state. Flame shape is important. The flame should have a long path length (but a narrow width, such as a fishtail flame) so that the source traverses an increased number of atoms capable of contributing to the absorption signal. The effective length of the flame may be increase by alignment of several burners in series. To perform adequately its second function, the flame temperature need only be high enough to dissociate molecular compounds into the free metal atoms.

The dispersing device has two tasks: (i) To reject resonance lines of other elements, and (ii) to prevent the detector from being overloaded with light e.g. from the carrier gas in the discharge tube. Usually the spectrum line is isolated with a monochromator and its intensity measured by a photomultiplier tube and conventional amplifier. For some purposes the arrangement of an appropriate filter and photocell is adequate. The resolution of the method is implicit in the extremely small width of the emission and absorption lines.

The present account of 'Ion-exchange Chromatography with Detection by Atomic Absorption" began with exemplification of the applicability of this method by taking up the example of determination of constituents of the mentioned mixture of several arsenic compounds. The results of this determination are illustrative of the sensitivity that the method can show in certain cases of analysis of mixtures which may be amenable to resolution and analysis by this technique. In the case cited, the method has a sensitivity of at least 3 ng/ml and 20 ng/ml respectively for the arsonate and the arsenate derived from methane.

10.4.7 Exclusion HPLC

Mention has been made earlier (Chapter 9) of xerogels which were the first supports used in exclusion chromatography. These xerogels, cross-linked dextran (Sephadex) and cross-linked polyacrylamide (Biogel P), were used with aqueous media. Additionally, Sephadex LH 20, the hydroxypropyl ether of cross-linked Sephedex, and Enzacryl, cross-linked polyacrylolymorpholine, were developed to permit chromatography in organic solvents. These soft xerogels have poor mechanical stability which deteriorates as pore size increases, and these soft gels cannot be used at pressures of HPLC.

The packings available for use in HPLC are either of the cross-linked polystyrene type or of the controlled pore size glasses or silica.

Moore, in 1964, developed cross-linked polystyrene gels, covering a wide range of pore sizes, which are suitable for separation of polymers in organic media. These gels may be classified as *aerogels* which undergo limited or no swelling with the solvent. The cross-linked polystyrene gels are compatible with a wide range of organic eluents. Possible disadvantages of rigid organic gels are a susceptibility to thermal degradation and a decrease in mechanical stability at the elevated temperatures which are required in separations of polyolefines and some condensation polymers. In order to obtain compatibility with aqueous eluents, some manufacturers have modified the surface of cross-linked polystyrene gels, for example, by sulphonation.

The excellent mechanical stability that inorganic gels exhibited led to the development of microporous silica and glass particles for polymer separations with both aqueous and organic eluents. Columns of inorganic packings can be used at

high flow rates and high pressures. These packings are particularly suitable for separations of polyolefines at high temperature, because of the excellent mechanical and thermal stability of the gel particles. The main deficiency of inorganic packings is the presence of surface sites which may facilitate the adsorption of sample constituents on to the packing. Adsorption may be irreversible, when all the polymer is retained in the particles, or reversible, when chromatograms are broadened. For polar samples in both organic and aqueous phases, it is advantageous to coat inorganic packings with a surface-bonded phase in order to minimize solute-gel interaction effects. Thus for example, packings produced by reaction of porous silica or porous glass with γ–glycidoxy propyltrimethoxysilane have given size exclusion separations for a range of water-soluble polymers.

Some commercially available rigid packings are listed in Table 10.12.

TABLE 10.12

Rigid Microparticulate Packings

Type	Name
Cross-linked polystyrene	PL gel
	μ-Styragel
	Shodex A
	HSG
	TSK Type H
Porous silica	Zorbax PSM
	Li Chrospher
	Li Chrospher Diol
	Synchropak GPC
	Chromegapore
	Spherosil
	μ-Bondagel E
	Protein Column
	TSK Type SW
Porous glass	CPG
	CPG Glycophase G
Hydrophilic polymer gel	TSK Type PW
	Ionpak
	OH pak

The most common eluents in exclusion chromatography are tetrahydrofuran for polymers which dissolve at room temperature, o-dichlorobenzene and trichlorobenzene at 130-150ºC for crystalline polyolefines, and o-chlorophenol at

90ºC for crystalline condensation polymers such as polyamides and polyesters. For more polar polymers, dimethylformamide and aqueous eluents may be employed, but care is required so as to avoid solute-gel interaction effects. Adsorption and partition effects are always likely to occur when polymor-solvent interactions are not favourable, when polar polymers are separated with less polar eluents and when packings have surface active sites. If the solvent has considerable affinity for the surface, then no polymer is adsorbed. Also, since adsorption is more prevalent with poor solvents, good solvents must be used to prevent occurrence of preferential solvent—adsorbent interactions. The choice of eluent may be restricted because of sample solubility considerations and small quantities of an adsorption active substance may be added to the eluent in order to suppress sample adsorption.

The resolution should first be improved by using the flow rate corresponding to maximum efficiency. An increase in column length will also improve resolution since the degree of separation is directly related to column length. This can either be achieved by adding to the actual column length by using longer columns or coupling columns in series, or by increasing the effective column length by re-cycling the partially resolved sample through the same column until the desired resolution is obtained.

The main advantages of recycling are that lower pressures may be used and, since no extra columns are required, costs in terms of column packings and solvent are minimized.

Resolution can also be increased by using a smaller diameter packing with a narrow particle size distribution.

If adequate resolution is still not obtained it means that the molecular size differences are too great to be dealt with on a single column, and two or more columns of varying pore sizes should be connected in series, the columns being placed in order of increasing pore size so that the sample first enters the column of smallest pore size.

10.4.7.1 Applications of Exclusion HPLC

With the development of a wide range of macroparticulate packings starting in early seventies, high speed and high resolution separations of small molecules and synthetic polymers such as plastics, fibres, elastomers and coatings have been carried out by high performance gel permeation chromatography (HPGPC). Similar HPGPC separations of water-soluble polymers and biopolymers are superior to those accomplished by the traditional analytical gel filtration method. Some of the illustrative examples of such separations are:

  (i) Resolution of a mixture of molecules of dioctyl phthalate, dibutyl phthalate, diethyl phthalate and dimethyl terephthalate.
 (ii) Resolution of constituent oligomers of an epoxy resin.
(iii) Resolution of polystyrene 600 standard into its components.

(iv) Resolution of a mixture of biopolymers comprising of deoxyribonucleic acid, fibrinogen, bovine serum albumin, chymotyrypsinogen, cytochrome and glycyltyrosine.

In the field of Inorganic Chemistry, size exclusion chromatography (SEC) has been used out only for separating complexes for analyses but also for purifying complexes; a note-worthy example in the latter context is the separation of the labile nickel complexes of the phosphorous esters like

$$\text{Ni}\left[\text{P(O}-\text{p}-\text{tolyl)}_3\right]_4, \text{Ni}\left[\text{P(O}-\text{C}_6\text{ H}_5)_3\right]_4,$$

$$\text{Ni}\left[\text{P(O}-\text{CH}_2\text{ CH}_3)_3\right]_4 \text{ and } \text{Ni}\left[\text{P(O}-\text{CH}_3)_3\right]_4,$$
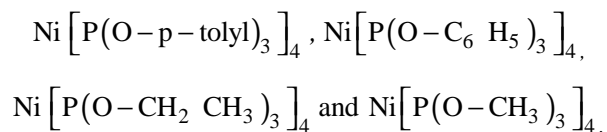
An interesting example of the application of SEC is the separation of free Cd (II) and the complex with fulvic acid (FA), which is the soil organic acid considered to be responsible for metal ion transport in the environment. Cadmium is of increasing concern as a heavy metal environmental pollutant and the understanding of cadmium transport requires knowledge of the equilibrium constant for

$$\text{Cd}\left(\text{II}\right) + \text{H}_x\text{ FA} \rightleftharpoons \text{CdFA} + x\text{ H}^+$$

The free and complexed Cd (II) are separated by two 25 cm HPLC columns of Sephadex G-10 (a cross-linked dextran gel of 40-120 μ bead diameter). The mobile phase was distilled deionized water. Sephadex G-10 xerogel has an exclusion limit ≈ 700, that is, it can be used to fractionate species of molecular weight less than 700. The larger Cd-fulvic acid complex is unretained and elutes before hydrated Cd (II). As with the phosphorus esters above, SEC is a viable method not only for separating these complexes for analysis but also for purification.

Use of exclusion chromatography has been extended from molecules to molecular clusters of inorganic colloids in the 1.50μ range. Thus, separation of sols like aluminosilicate sols, can be effected rapidly by exclusion chromotography and SEC is suggested to be a useful technique for characterizing inorganic colloids.

10.4.8 Ion-pair Chromatography (IPC)

Factors like the limited selection of ion-exchange packings available and the ion-exchange technique not always performing with good column efficiencies rapidly promoted general interest in ion-pair partition chromatography (IPC) as an alternative to ion-exchange chromatography. IPC also offers the advantages of longer column life and greater reproducibility. The technique of ion-pair chromatography, which is also known as paired-ion chromatography, can bring about simultaneous separation of the constituents of a sample of ionised and

ionisable molecules through the use of an ion-pair reagent which plays counterion to the ionic species in the sample. The technique owes its development largely to Schill and co-workers.

10.4.8.1 Types of Ion-pair Chromatography

The following three fairly distinct types of ion-pair partition chromatography have evolved.

(i) Normal phase ion-pair partition chromatography: In this mode silica is used as the support which is coated with an aqueous stationary phase containing a pairing ion (an anion e.g. perchlorate ion, for the separation of bases, or a cation e.g. tetraalkyl ammonium ion, for the separation of acids); an organic mobile phase is used, a typical example being butanol/dichloromethane. The potential for varying retention and selectivity by changing the organic solvent composition in the normal phase ion-pair partition chromatography may be illustrated by taking the example of separation of the constituents of a mixture comprising of toluene and a number of carboxylic acids viz. vanilmandelic acid (VMA), indoleacetic acid (IAA), homovanillic acid (HVA), and 5-hydroxyindole-3-acetic acid (HIAA). The separation used 0.1 M tatrabutylammonium hydrogen sulphate + phosphate buffer as the stationary phase. In separation (a) the mobile phase consists of 4% butanol in dichloromethane. The separation is quite good and excellent column efficiency is obtained. The peak positions are in the sequence : Toluene, VMA, IAA, HVA and HIAA. By using the same stationary phase in separation (b) but now having a mobile phase consisting of butanol- $CH_2 Cl_2$-hexane (20:30:50) (which means a higher percentage of butanol and hence an increased solvation ability of the eluent), a completely different separation is achieved, with better resolution, faster separation and also some alternation in peak positions, the sequence of peak positions now being: Toluene, IAA, HVA, VMA and HIAA.

(ii) Reversed-phase ion-pair partition chromatography: In practice, normal phase ion-pair partition chromatography is rather troublesome due to the stripping of the stationary phase. The problems of reproducibly coating the support with aqueous phase and the associated problems of bleeding (the elution of liquid phase from the solid support by the flow of the eluent) are overcome in the reversed-phase ion-pair partition chromatography. In the reversed-phase columns, silica gel coated with, for example, butanol constitutes the stationary phase and such a stationary phase behaves as a bulk liquid. Reversed-phase systems employing chemically bonded stationary phase (e.g. ODS-silica), however, are the most convenient to use.

Retention and separation selectivity are controlled by adjusting the composition of the mobile phase by making changes in the nature and concentration of the ion-pair reagent, the buffer composition and pH value, and the type and amount of organic modifier in the mobile phase.

The usual mobile phase used in reversed-phase ion-pair partition chromatography is methanol/water or acetonitrile/water containing the required acidic or basic counter-ions. Here it is an easy matter to change the nature or concentration of the counter-ions, as compared with normal phase ion-pair partition chromatography. The main limitation on the choice of mobile phase is the solubility of the ion-pairing agent. When using acetonitrile the solubility may be increased by using 90/10: acetonitrile/H_2O as the organic modifier. If the ion-pairing agent is of low solubility it may precipitate out and cause plugging of tubing. The ion-pairing agent is usually a large ionic or ionisable organic molecule (e.g. quaternary amines and alkyl or aryl sulphonates) although perchloric acid is also used.

(***iii***) *Soap chromatography:* Soap chromatography is the term given to the form of ion-pair partition chromatography where the counter-ion of the ionizable components to be separated is a detergent. For example, certimide (C_{16} H_{33} N^+ Me_3) has been used for acids and lauryl sulphonic acid (C_{12} H_{25} SO_3^-) for bases.

10.4.8.2 Mechanism of IPC

The mechanism by which separation takes place in IPC is subject to debate. Each of the models discussed below represents, in effect, an extreme situation, and it is possible that separation is effected by a simultaneous operation of alternative mechanisms.

The *ion-pair moeld*, also known as the partition model, assumes that counterions (denoted by C) react with the sample ions (denoted by S) to form neutral, undissociated species (CS or SC) in the mobile phase, as shown in the following equations for anionic and cationic sample ions, respectively.

$$C^+ + S^- \rightleftharpoons CS\text{(mobile phase)} \xrightleftharpoons{K_D} CS \text{ (stationary phase)}$$

$$S^+ + C^- \rightleftharpoons SC \text{ (mobile phase)} \xrightleftharpoons{K_D} SC \text{ (stationary phase)}$$
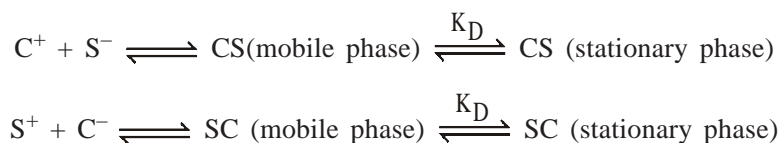
The neutral species are partitioned between the liquid stationary and mobile phases (K_D is the relevant distribution constant). Separation is based upon the relative values of the distribution coefficient of the different neutral species. This model most closely explains the experimental results obtained with non-bonded reversed-phase columns (e.g. n-pentanol coated onto silica gel), in which the stationary phase behaves as a bulk liquid. The ion-pair model is, however, unable to explain ion-pair interactions with chemically bonded reversed-phase columns, and the working of these clumns is more appropriately explained by a dynamic ion-exchange model.

The *ion-exchange model* assumes that the ion-pairing reagent is adsorbed onto the stationary phase surface where it behaves as a liquid ion-exchanger. Retention

and separation are due to ionic interactions between the ionized solute molecules and the counterions adsorptively bound to the stationary phase.

10.4.8.3 Choice of Column Parameters in IPC

There are many parameters which can influence retention in ion-pair chromatography. Decisions regarding choice of parameters that influence the chance of success need to be made before attempting a separation.

The first decision is with regard to the choice of column type. While making the decision in this regard the considerations to be kept in view are: Chemically bonded reversed-phase columns are commercially available and they can be used without any further treatment. Another great advantage of using there columns is that they can be eluted employing gradient elution. Normal phase and non-bonded reverse-phase columns have to be prepared in the laboratory prior to use. The main limitation in the normal phase chromatography is that whereas the sample is needed to be transferred to the organic phase prior to analysis ionic hydrophilic substances have low solubility in lyophilic solvents; also it is difficult to optimize the counterion concentration or pH of the stationary phase. However, the major advantage that can be taken from the normal phase chromatographic technique is that it enables making use of counterions with a high detector response which, in turn, enhances the detection capabilities of solutes having poor detector responses. For example, the very UV-active naphthalene-2-sulphonate is used as the ion-pair reagent in the separation of dipeptides; the eluted dipeptides as they leave the column paired with this ion are sucessivily detected because of the chromophoric properties of the pairing ion.

A convenient starting point to make decision regarding column choice is to select a chemically bonded reversed-phase column with optimization of separation being attained via changes in the composition of the mobile phase.

The next decision to be made is regarding the selection of a counterion for a particular separation. For this, the most important consideration is charge compatibility. The counterion should ideally be univalent, aprotic, soluble in the mobile phase (reversed-phase methods), non-destructive to the chromatographic system, and it should not undergo aggregation or other secondary equilibria. For the separation of anionic solutes such as carboxylic acids and sulphonates, the tetraalkylammonium salts in the chloride or phosphate forms are generally used. Protonated amines can also be used, but these are generally less useful than the tetraalkylammonium salts. Inorganic anions, alkyl or aryl sulphonates, and alkyl sulphates are generally used for the separation of cationic solutes. Hydrophobic amino compounds are best separated with small hydrophilic counterions such as dihydrogen phosphate, bromide or perchlorate. For cations of intermediate polarity, hydrophobic counteions such as naphthalene sulphonate, picrate are used. The reversed-phase separation of small or polar cations requires the use of lipophilic counterions such as alkyl sulphonates, sulphamates, or sulphates. With these

counterions, increasing the length of the alkyl chain leads to an increase in retention of the solute.

There is no general rule for predicting the optimum concentration of a counterion and, for any particular separation, the optimum concentration of the counterion is established by experiment. At low counterion concentrations, increasing concentration usually increases retention of the solute, but upto a limit. For large counterions, concentration of 0.005M of the mobile phase with respect to the counterion is fairly common. With small counterions much higher concentrations (e.g. 0.10–0.50 M) are generally used.

The pH of the mobile phase is usually used to give the maximum concentration of the ionic form. However, it may also be used to suppress ionisation and to reduce k′; for example, if the solute is a strong acid ($pK_a < 2$), or a strong base ($pK_b > 8$), the solute will be completely ionised in the usual working pH range of 2-8. However, the pH may still be adjusted in this range to give selectivity if weak acids or weak bases are also present. Weak acids will be ionised in the pH range 6-7.4 and their k′ values will be dependent on the ion-pair formed. Below pH6 they will not be ionised and their retention will depend on their lipophilic character. Similarly, weak bases will be ionised below pH6 but will undergo ion suppression above this pH.

Adjusting the concentration of the organic modifier in the mobile phase is the most convenient and useful method for controlling retention and selectivity in reversed-phase chromatography, and this holds true for ion-pair chromatography also. Generally, either methanol or acetonitrile is used as the organic modifier in such concentration that under gradient-elution conditions the least polar solvent used is able to completely dissolve the ion-pair reagent.

The retention will be expected to decrease as the temperature increases and there may also be an increase in efficiency.

From the discussion given above, it would seem that separation by IPC will imply facing the practical problem of optimizing many parameters simultaneously. However, this is not a problem for simple separations, because changes in just one or two or the foresaid parameters while maintaining the others within sensible ranges will yield the desired results. For the analysis of complex mixtures of ionised and ionisable molecules, the added degree of flexibility of IPC arising form its many adjustable parameters makes this separation technique more efficient than ion-exchange chromatography.

10.4.8.4 *Applications of IPC*

Typical applications of IPC in Organic Chemistry along with the corresponding ion-pairing agents used are given in Table 10.13.

Ion-pair formation with a chiral counterion has also been used to separate enantiomers. Thus, separation of enantiomeric amines has been achieved by ion-

pair formation with the chiral counterion, (+)–10–camphosulphonate. Diastereomeric ion-pairs have structural differences resulting in different distribution coefficients between the mobile and stationary phases.

In the field of Inorganic Chemistry, separation of metal ions has been carried out efficiently by paired-ion chromatography. Cassidy and Elchuk have combined the principles of paired-ion chromatography and the performance of modern 5- and 10µ C_{18} phases bonded to silica. Table 10.14 gives the ion-pairing reagents commonly used.

TABLE 10.13

Application	Type of ion-pairing agents used
Strong or weak acids, Sulphonated dyes, carboxylic acids,	Quaternary amines e.g. $(CH_3)_4 \overset{+}{N}$, $(C_4H_9)_4 \overset{+}{N}$,
Sulphonates Strong and weak bases, catecholamines	Tertiary amines e.g. trioctylamine Alkyl and aryl sulphonates e.g. camphor sulphonic acid, and alkylsulphates (e.g. lauryl sulphate) which show different selectivity
A wide range of basic solutes e.g. amines	Perchloric acid

TABLE 10.14

Ion-pairing Reagents

Name	Structure	No. of Carbon atoms
Cation-exchange reagents:		
1-Hexane sulphonate	$CH_3 (CH_2)_5 SO_3{}^-$	C_6
1-Octane sulphonate	$CH_3 (CH_2)_7 SO_3{}^-$	C_8
1-Dodecyl sulphate	$CH_3 (CH_2)_{11} SO_4{}^-$	C_{12}
1-Eicosyl sulphate	$CH_3 (CH_2)_{19} SO_4{}^-$	C_{20}
Anion-exchange reagents:		
Tetraethylammonium-salt	$(CH_3 CH_2)_4 N^+$	C_8
Tetrabutylammonium-salt	$[CH_3 (CH_2)_3]_4 N^+$	C_{16}
Trioctylmethylammonium-salt	$CH_3 [CH_3 (CH_2)_7]_3 N^+$	C_{25}
Tetraoctylammonium-salt	$[CH_3 (CH_2)_7]_4 N^+$	C_{32}
Tridodecylmethylammonium-salt	$CH_3 [CH_3 (CH_2)_{11}]_3 N^+$	C_{37}

At high carbon number, the reagents are effectively permanently bound to the HPLC column which acts to give high performance. For example, using a 30-cm

Varian 10μ C_{18} column coated with $C_{20} H_{41} SO_4$ Na, efficient seapration of Cu (II), Co (II), and Mn (II) occurred in 200 seconds at a flow rate of 2ml/min of the eluent, 0.075 mol/1 tartrate, pH 3.4. Similar column efficiencies were found for anion IPC/HPLC columns. For example, a 25cm Whatman C_{18} column, coated with tridodecylmethylammonium iodide, efficiently separated IO_3^-, NO_2^-, NO_3^-, and I^- in 400 seconds, the height equivalent of theoretical plate for these ions being 0.07, 0.12, 0.08 and 0.14 mm, respectively.

The eluted metal ions were detected by absorption spectrophotometry at 530-540 nm after post-column reaction with 4-(2-pyridylazo) – resorcinol (PAR). Anion detection was by ultraviolet absorption spectrophotometry at 215 nm and by conductivity to detect such anions as Cl^-, F^-, SO_4^{2-}, and PO_4^{3-}, which do not absorb light sufficiently at 215 nm. HPLC separation of Fe (II), Ni (II), Ru (II), as the 1, 10-phenanthroline complexes has been carried out using μ-Bondapak/C_{18} and μ-Bondapak—CN columns. The cationic chelates Fe $(Phen)_3^{2+}$, Ni $(Phen)_3^{2+}$ and Ru $(Phen)_3^{2+}$ form ion-pairs with alkyl sulphonic acids. Separation of these ion-pairs by reversed-phase HPLC are dependent on the reagents, pH, methanol/water, or acetonitrile/water ratios. The optimum composition of the mobile phase was 20% of the organic component. At lower organic component ratio, resolution increased but retention volumes are too high. Plate counts and resolution similar to organic HPLC separations are obtained by forming ion-pairs of inorganic complexes.

Separations such as those enumerated in Table 10.15 are some typical applications of IPC in the field of Biochemistry.

TABLE 10.15

Sample	Stationary phase	Mobile phase
(i) *Biogenic amines:* Nephrine Tyramine 3-Methoxytramine Dopamine Normetanephrine Methanephrine Noradrenaline Adrenaline Dipeptides	0.1M $HClO_4$ + 0.9M $NaClO_4$	Ethyl acetate-tributylphos-phate- hexane (72.5:10:17.5)
(ii) *Dipeptides:* Leu-Leu Phe-Val Val-Phe Leu-Val Met-Val	0.1M naphthalene-2-sulphonate pH = 2.3	$CHCl_3$-pentanol (9:1)

Sample	Stationary phase	Mobile phase

(iii) Nucleobases and nucleosides:
 	
    Urd
    Ura
    Thy
    Xao
    Ino
    Xam
    Guo
    Hyp
    Cyt
    Ada
    Gua
    5-Cyt
    Ade
	C_{18}^- bonded silica (Hypersil ODS)	0.1M $HC1O_4$–ethanol (9:1) + 0.1% SDS (sodium dodecyl sulphate)

10.4.9 Ion Chromatography with Eluent Suppressed Conductivity Detection

10.4.9.1 Working of the Technique

The technique involves two ion-exchange columns known as the separator column and the suppressor column respectively, coupled together in series followed by a conductivity monitor. Whereas the separator column separates the ions of the given sample, the scond column viz. the suppressor it column suppresses the conductivity of the electrotyte.

The principal problem in the development of ion chromatography (IC) was one of providing a suitable on-line detection system. Most common ions cannot be detected photometrically and a method is needed which can detect the separated ions in the background of a highly conducting eluent. Since conductivity is a universal property of ions in solution and can be simply related to ion concentration, it could be considered a desirable detection method. But conductivity detection will require elimination of the contribution to conductivity from the eluent background, and it is to meet this requirement that the suppression column is introduced in succession to the separator column. The inclusion of this second column suppresses the conductance of the electrolyte in the eluent and it enhances the conductance of the separated ions since these are converted to the highly conducting base or acid as illustrated by the examples given below.

Consider the separation of a mixture of Li^+, Na^+ and K^+ on a cation-exchange column using dilute hydrochloric acid as the mobile phase. The three cations are separated on the first column according to their strength of interaction with the column packing and they leave the column in the form of their chloride salts dissolved in the mobile phase. As the eluent enters the suppressor column, the following two reactions occur:-

$$HCl + Resin^+\ OH^- \rightarrow Resin^+\ Cl^- + H_2O$$

$$MCl + Resin^+\ OH^- \rightarrow Resin^+\ Cl^- + MOH$$

$$M = Li^+,\ Na^+,\ K^+$$
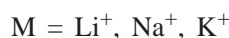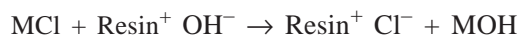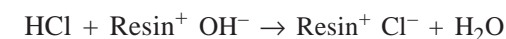
The eluent leaving the suppressor column contains the sample ions as their respective highly conducting hydroxides in a weakly conducting background of water. The suppression reaction simultaneously serves two functions. First, it maximizes the detector sensitivity to the analyte cation by converting it to its hydroxide because the mobility of the hydroxide anion is 2.6 times that of the chloride ion. Second, the suppression reaction minimizes the detector sensitivity to the eluent.

Consider an anion chromatography system. In the simplest case, an eluent consisting of sodium hydroxide is used to displace anions from the anion-exchange sites in the separator column. In such a case, the anion, A^-, is eluted from the column as its sodium salt. The column eluent is passed through a suppressor column consisting of a high-capacity cation-exchange resin in the hydrogen form. The following two reactions occur as the eluent enters the suppressor.

$$Resin^-\ H^+ + Na^+\ OH^- \quad \rightarrow \quad Resin^-\ Na^+ + H_2O$$

$$Resin^-\ H^+ + Na^+\ A^- \quad \rightarrow \quad Resin^-\ Na^+ + H^+A^-$$

The effluent of the combined separator-suppressor system contains the sample ion as its highly conducting acid in a weakly conducting background of water. Again, the effect of the suppression reaction is to minimize detector sensitivity towards the eluent while simultaneously increasing the detector sensitivity to the analyte anion by converting it into the acid form, mobility of the hydrogen ion being 7 times that of the sodium ion.

The separation column and the suppressor column are discussed below in detail.

(i) The Separation Column: The use of suppression reaction necessitates the use of a properly designed separator column which meets the requirements of successful working of the suppressor device. There is an upper limit to the quantity of inflowing ions which a suppressor device can process with minimal dispersion of solute bands, and, therefore, it becomes necessary that the ion-exchange capacity of the separating column be minimized. Hence, low ion-exchange capacity separator columns that allow the use of dilute eluents and permit only low ion-outflow rates are used in conjunction with the suppression reaction. However, the reduction of the ion-exchange capacity has to be reasonable, since it is necessary for the separator column to handle significant sample loads as well as sample contaminants.

The other consideration in the choice of separator column is the operating pH range of the eluent used for chromatography. Generally, the pH of cation eluent will be between 2 and 5 and the pH of the anion eluent will be between 8 and 12. The proper separator column material must then be able to handle such extremes

of pH. Because of this, the materials of choice in separator columns are low-capacity organic ion-exchange resins, rather than silica-based materials.

For anion chromatography, a binary pellicular resin (Figure 10.5) is used in the separator column. The core of the resin particle is polystyrene-divinylbenzene polymer. Surrounding this core is a layer of sulphonated polystyrene-divinylbenzene. The purpose of the sulphonated layer in this resin is to provide a surface to which the outer anion-exchange layer is bound via ionic bonding interactions. The outer layer consists of uniformly sized anion-exchange latex particles which are deposited on the sulphonated layer in a uniform monolayer. Due to the large number of ionic interaction sites between each latex particle and the resin surface, the attachment of the latex to the surface of the sulphonated layer is essentially an irreversible process (for example, none of the latex particles is removed from the surface of the anion resin on treatment of the resin with IM NaOH).
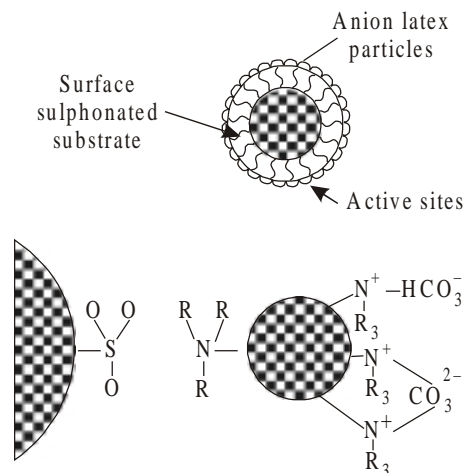
Figure 10.5 Composition of the IC anion-exchange particle.

The thinness of the anion-exchange layer and the Donan exclusion (i.e. the inability of ions present in low concentrations to enter ion-exchange materials containing a higher density of ion-exchange sites of the same charge type as the ions of interest) effected by the intermediate sulphonated layer provide excellent mass transfer properties by ensuring that sample penetration is confined to the outer lates layer.

For cation chromatography, separator resin is surface sulphonated ion-exchange resin (Figure 10.6), or a binary pellicular resin with cation exchangers attached to the surface of the resin spheres, analogous to the anion-exchange particles (Figure 10.5). The inert polystyrene-divinylbenzene core of the resin particle provides a rigid support for the sulphonated outer layer.
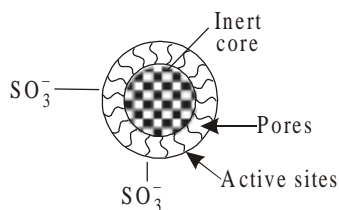
Figure 10.6 Composition of the surface sulphonated IC cation-exchange particle

(ii) The Suppressor Column: The suppressor column has an ion-exchange packing opposite in type to the separator column packing and of higher capacity since it must neutralize a large volume of column eluent. The length of time for which the suppressor will function before needing

to be regenerated is a primary consideration in regard to the suppressor column. Maximum suppressor life is achieved with high-capacity ion-exchange resins of strong acid type for anion chromatography and of the strong base type for cation chromatography.

The chromatographic behaviour of *weakly acidic* anions in anion chromatography or *weakly basic* cations in the cation chromatography in the suppressor column is also to be kept in mind while making decision regarding the suppressor resin packing. While retention of weak electrolytes in the suppressor column represents only a minor inconvenience, it is possible to minimize the phenomenon with proper choice of suppressor resin. In high-capacity ion-exchange resins, the micropore volume is inversely proportional to the degree of crosslinking. Thus, by maximizing the crosslinking of the suppressor resin, it is possible to minimize the effect of micropore retention of weak electrolytes in the suppressor column. Unfortunately, adsorption of weak electrolytes in the resin micropore also is directly proportional to the crosssslinking of the suppressor resin. Thus, the proper choice of crosslinking for suppressor resins is a compromise between maximal crosslinking (to minimize pore volume and maximize the physical integrity of the resion) and minimal crosslinking (to minimize adsorption of weak electrolytes in the micropores). In practice, this means that suppressor resins have crosslinkings in the range of 8-12%. The moderate degree of crosslinking, besides minimizing adsorption also reduces swelling effects. The column packing should have a small bead diameter to minimize band dispersion and a minimal total volume to minimize the effects of dispersion and the retention of weak electrolytes.

A relatively recent development in ion chromatography is the replacement of the suppressor column by the more efficient and convenient fibre suppressor. In the fibre suppressor device, the same basic function originally served by the packed bed suppressor is accomplished with an ion-exchange fibre. Since the exterior of the fibre is continuously bathed in regenerating solution, there is no need to interrupt analyses for regeneration in this case as is needed with suppressor columns. Another advantage of this type of device is that Donnan exclusion phenomenon commonly prevailing in resin-type suppressors is not much of a problem here because of the low concentration of the regenerant present on the exterior of the fibre; this results in analytical improvement as shown by improved ion peak height and peak shape of an ion in the chromatogram.

Eluents for Suppressed Conductivity Detection

The general requirement for an anion chromatography eluent is that the eluting anion must have useful affinity for anion-exchange resins. Since it is important to minimize the conductivity of the eluent, the preferred anion is one which has a pK_a

value of 6 or greater. Only hydroxide, borate, carbonate and phenate meet the acidity requirements stated above. Of the four anions, phenate is generally not useful because of the ease with which it is oxidized in alkaline solution. The eluent use has to be effective at low concentrations necessary for long suppressor life.

For cation chromatography there are basically two eluent systems used. In the case of monovalent cations, the normal eluent is 0.005M HCl. However, the concentrations of hydrogen ion required for divalent cations are such that hydrogen ion is impractical eluent for divalent cations. Instead, the preferred cation for divalent cations like the alkaline earth cations is m-phenylenediamine dihydrochloride. The divalent nature of m-phenylenediamine makes it an efficient eluent for other divalent cations, while its weakly basic character results in very little conductivity when it is converted to the free base form in the suppressor. Figure 10.7 shows ion chromatographic separation of some divalent cations using m-phenylenediamine eluent.
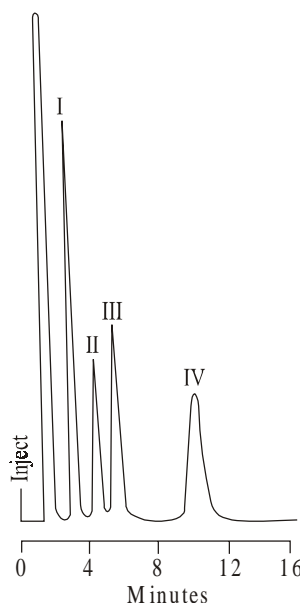
Figure 10.7 Separation of alkaline earth elements. Mobile phase 2.5 mM m-phenylenediamine dihydrochloride-2.5 mM HNO_3, flow rate 3.5 ml/min. The separator column was a surface sulphonated 35-55μ macroporous resin with a conventional suppressor and conductivity detector. I, Mg^{2+}; II, Ca^{2+}; III, Sr^{2+}; IV, Ba^{2+}.

10.5 Applications of HPLC in the Day-to-day Life

If the foregoing account of the applications of HPLC through its various modes of functioning clearly brings out the wide utility of this separation technique in the realms of pure Chemistry and Biochemistry, no less significant are the contributions made by HPLC to the fields of applied Chemistry and Biochemistry e.g. pharmaceutical Chemistry, Clinical Chemistry, Forensic Chemistry, Food Chemistry, pollution management, chemical industry or chemistry of naturally occurring plant products (which may be of pharmaceutical interest or may be the colour, flavour or aroma constituents of commercial products), each being of direct conern to man in the day-to-day life. Their utilization of this separation technique, especially in its version of trace-analysis HPLC, is indeed extensive. An account of typical examples of the use of HPLC in these areas is given below.

Application of HPLC in Pharmaceutical Chemistry

The commonly used chromatographic methods for drug analysis are thin-layer chromatography (TLC), gas liquid chromatography (GLC) and HPLC. Thin-layer chromatographic methods are inexpensive but lack resolution and also present difficulties of quantitative assaying, although using multiple spotting techniques several assays can be performed simultaneously. As compared with GLC, one important advantage of HPLC is that extraction procedures and sample clean-up prior to injection are greatly reduced. Thus in the analysis of drugs in formulated products it is forequently sufficient to crush or mix the product with a solvent, filter and inject. Contaminants move with the solvent front or are separated chromatographically from the drug of interest. Many a time when untreated urine or plasma samples are injected onto the column, no marked deterioration in column performance occurs. The capability of HPLC to handle directly polar drugs and their metabolites and conjugates in body fluids is another major advantage of the method. Rapid sample clean-up by passage down an exclusion column to achieve a molecular weight separation may also be useful. Since analyses can be performed at room temperature, thermal degradation is extremely rare. Also, the high resolving power of the system means that individual compounds belonging to the same class can be discriminated. Thus toxic metabolites or by-products can be analysed. Automated injection systems are available for HPLC systems so that in quality control situations the method, once established, is not operator intensive. Analyses are usually completed in 10-30 min. Quantitative analysis of components is usually achieved by the inclusion of a suitable internal standard in the sample. Preparative HPLC has also shown promise for the collection of the separated components in amounts sufficient for identification.

The disadvantages of HPLC centre around the detection systems available. Ultraviolet spectrometers are most commonly used detectors but they require that the compound has a UV absorbing chromophore. Variable wavelength UV spectrophotometers offer reasonable versatility, but some steroids and other drugs must be derivatized before UV detection is possible. With the introduction of electrochemical and spectrophotometric detection in region other than the ultraviolet, and the rationalization of mobile phase delivery systems by the use of microprocessor control, capabilities of HPLC have significantly increased.

Another slight disadvantage may be that the chemically bonded stationary phases which are most applicable to drug analysis should only be used within the pH range 3-7 to ensure long term stability.

Table 10.16 summarizes some applications of HPLC in Pharmaceutical Chemistry which encompass drug stability studies, the determination of trace impurities or decomposition products in bulk drug samples, and the assay of drugs and metabolites in body fluids.

TABLE 10.16

Application of HPLC in Pharmaceutical Chemistry

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
Antibiotics Penicillins G and V and synthetic penicillins such as ampicillin, oxacilin, methicillin and nafcillin	Partition	C_{18}/Poracil B	0.05M ammonium carbonate/methanol (70:30)
Ampicillin, Penicillin G and Pencillin V	Ion-exchange	Vydac P150 AX	0.02M sodium nitrate in 0.01M pH 9.15 borate buffer
Cephalosporin antibiotics	Partition	ODS-SIL-X-II	0.95M ammonium carbonate/methanol (95:5)
Drug stability studies (i) in aged solutions of potassium penicillin G : Potassium penicillin G in the presence of five of its degradation products, DL-penicillamine, benzyl penilloic acid (two isomers), benzylpenamaldic acid, benzylpenicilloic acid and benzylpenillic acid	Partition	Bondapak AX/ Corasil	pH 3.8 buffer
(ii) Ampicillin and its degradation products (after aging of ampicillin trihydrate bulk powders at 55ºC for one month, the HPLC assay showed 6.8 - 12.5 per cent reduction of potency)	Ion-exchange	Vydac P150 AX	(Borate buffer at pH 9.15
Metabolism studies Caphalothin and its deacylated metabolite, de-acetylcephalothin (in urine samples collected before and at intervals after dosing healthy humans)	Ion-exchange	Zipax SAX Permaphase	0.25M sodium acetate at pH 5.0

Contd.

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
Tetracycline, 4-epitetra-cycline, chlortetracycline, epi-anhydrotetracycline, and anhydrotetracycline	Ion-pair chromatography	Microparticulate silica whose surface silanol sites had been fully silanized.	Parchloric acid/ acetonitrile
β-Cetotetrine in biological fluids	Partition	Bondapak Phenyl/Corasil	Potassium hydrogen phosphate (0.025M), sodium EDTA (0.1 per cent) buffer, pH 7.8/ethanol (93:7)
Separation of intermediates from by-products formed during the preparation of the widely used antibiotic, chloramphenical, whose purity is greatly dependent on the purity of the intermediates.	Partition	ODS-SIL-X-II	0.5M ammonium carbonate
Antibacterials Sulphamerazine, sulpha-diazine and sulphadimidine in pharmaceutical formu-lations.	Ion-exchange	Zipax SCX	0.2M disodium phosphate solution adjusted to pH 6.0 with 85% phosphoric acid.
Sulphasalazine in the presence of its precursors or degradation products.	Partition	C_{18}/Corasil	Phosphate buffer (pH 7.7)/isopropanol (9:1)
Sulphacetamide and its principal hydrolysis product, sulphanilamide, in ophthalmic solutions	Adsorption	Corasil II	Dichloromethane/ isopropanol/ conc. ammonia (26:13:0.5)
Assay in body fluids and tissue: (i) Sulphadimidine in bovine kidney, liver, muscle and fat tissue.	Partition	Bondapak C_{18} /Porasil B	2.5% isopropyl alcohol in phosphate buffer (pH 7.7)
(ii) Nalidixic acid and its metabolite, hydroxynalidixic acid, in plasma and urine extracts.	Ion-exchange	Zipax SAX	0.05M sodium sulphate/0.02M boric acid at pH 9.

Contd.

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
Antidepressants Trimipramine chlorimipramine, imipramine and opipramol.	Adsorption	LiChrosorb SI 60	Dichloromethane/ isopropanol (90:10) saturated with aqueous ammonia
Caffeine, theobromine, theophylline and xanthine.	Ion-exchange	Zipax SAX	0.01M sodium borate
Amphetamine type of stimulants: Methylamphetamine, 2, 5-dimethoxy-4-methyl amphetamine, methylene dioxyamphetamine	Adsorption	Corasil II	Gradient system of ethanol/dioxan/ cyclohexylamine in hexane
Central nervous system depressants Hypnotics and sedatives: Barbiturates in pharmaceutical preparations	Ion-exchange	Zipax SAX	Alkaline (0.01M sodium borate/ 0.03M sodium nitrate) or acid (0.01M acid) mobile phases
Barbitone, allobarbitone, aprobarbitone, phenobarbitone	Ion-pair chromatography	LiChrosorb RP-z loaded with butyronitrile	0.01M tetrabutyl-ammonium (TBA), pH 7.7
Anticonvulsants: Phenobarbitone, phenytoin primidone, ethosuximide and carbamazepine in serum.	Partition	ODS-SIL-X-I	Water/acetonitrile (83:17)
Phenobarbitone, phenyltoin nitrazepam, carbamazepine and its metabolite, carbamazepine-10, 11-epoxide	Adsorption	Lichrosorb SI 100	Dichloromethane/ tetrahydrofuran
Tranquillizers: Benzodiazepines	Adsorption	Silica (surface deactivated with water-saturated dichloromethane/ isopropanol)	1% methanol in chloroform

Contd.

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
Chlorodiazepoxide (Librium) in capsules (solution prepared in 4% diethylamine in chloroform)	Adsorption	SIL-X-II	Chloroform/ methanol (98.5:1.5)
Triflubazam and its primary metabolites in blood and urine	Adsorption	Pellicular silica	Isooctane/dioxan (85:15)
Thioridazine and its metabolites (northioridazine, thioridazine-2-sulphone, thioridazine-2-sulphoxide, thioridazine-5-sulphoxide, northioridazine-2-sulphoxide) in blood	Adsorption	Silica	Isooctane/ acetonitrile/ 2-aminopropane (95.87:2.69:0.96)
Analgesic and anti-inflammatory drugs Analgesics: Salicylic acid, aspirin, caffeine, butalbarbitone, phenacetin, p-chloro-acetanilide, in a multi-component analgesic product	Partition	μ Bondapak C_{18}	0.01% aqueous ammonium carbonate/ acetonitrile (60:40)
Propoxyphene in admixture with both thermal degra-dation products and decomposition products formed in the presence of paracetamol in tablet and capsule samples	Adsorption	Microparticulate silica	Basic isopropanol/ hexane
Cough-cold preparations: Paracetamol, dextromethor-phan hydrobromide and chlorpheniramine maleate in antitussive syrups	Partition	Permaphase ODS	Methanol/buffer solution
Two- or three-component mixtures from decongestant-antihistamine combinations ingredients of which included phenylpropanol-			

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
amine hydrochloride, chlorpheniramine maleate, pseudoephedrine hydro-chloride, pheniramine maleate and mepyramine maleate	Ion-exchange	Zipax SCX	Phosphate buffter dioxan
Anti-inflammatories: Fluocinolone acetonide in creams and fluocinolone acetonide acetate in ointments	Partition	Permaphase ODS	Water/methanol (92.5:7.5 and 70:30 respectively)
Triamcinolone acetonide in its formulations	Ion-exchange	Zipax	Water-saturated dichloromethane
Dexamethasone disodium phosphate in tablets	Ion-exchange	Zipax WAX	Water/ethanol (90:10)
Analysis in body fluids: Phenylbutazone and its metabolite, oxyphenbuta-zone, in human plasma	Adsorption	SIL-X	Hexane/ tetrahydrofuran/ acetic acid (77:23:0.002)
Phenacetin in the blood, urine and tissues of treated dogs	Adsorption	Porasil A	Isooctane/ dioxan (70:30)
Diuretics Thiazide diuretics in tablet formulations	Partition	Phenyl/Corasil	Water/methanol
Canrenone in pharmaceu-tical preparations containing the degradation products of the drug	Adsorption	SIL-X	Hexane/isopropanol (96:4)
Determination in body fluids: Hydrochlorothiazide in extract of that fraction of the serum sample which has been previously freed from the protein content by exclusion chromatography	Partition	Spherisorb ODS	Water/methanol (85:15)
Acetazolamide in plasma extract (freed from lipids	Partition	ODS-silica	0.05M sodium

<div align="right">*Contd.*</div>

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
with dichloromethane), the method being used for measuring the comparative bioavailability of the drug in different formulations.			acetate buffer at pH 4.5/methanol (98:2)
Alkaloids (most alkaloids are pharmacologically active and many are of therapeutic value)			
Tropane alkaloids: Scopolamine, apoatropine and atropine	Adsorption	Partisil	Diethyl ether/ methanol (90:10) plus one per cent diethylamine
Scopolamine and atropine in antispasmodic mixutres	Partition	Phenyl/Corasil	Methanol/water buffered with ammonium dihydrogen orthophosphate (1 per cent)
Hyoscyamine, scopolamine and ergotamine in pharmaceutical products	Ion-pair chromatography	LiChrosorb SI 100 loaded with 0.06M picric acid in pH6 buffer	Chloroform saturated with 0.06M picric acid in buffer pH6.
Indole alkaloids: Spiro isomers of the oxindole alkaloids from the genus Mytragyna	Partition	C_{18}/Corasil	Methanol/water (80:20)
Ergot alkaloids: Ergotamine, and its isomerization and hydrolysis breakdown products viz. ergotaminine, aci-ergotamine, aci-ergotaminine lysergic acid amide, isolysergic acid amide, lysergic acid, iso-lysergic acid.	Partition	μ Bondapak C_{18}	Solvent gradient of 0.01M aqueous ammonium carbonate/acetonitrile (37:3) to 0.01M aqueous ammonium carbonate/acetonitrile (50:50) in 15 min.
Strychnos alkaloids: Strychnine and brucine	Adsorption	Silica gel	Diethyl ether/ methanol (90:10)

Contd.

Sample	*Mode of HPLC used*	*Stationary Phase*	*Mobile Phase*
			containing 1% diethylamine
Cinchona alkaloids: Quinine and cinchonine	Partition	Corasil II coated with Poly G-300	Heptane/ethanol (10:1)
Miscellaneous alkaloid separations: Dansyl derivatives of cephaeline, emetine, ephedrine and morphine obtained by direct deriva-tization of syrups and aqueous slurries of capsules having a complex excipient and drug composition	Adsorption	Silica gel	Di-isopropyl ether/isopropanol/ conc. ammonia (48:2:0.3)
Miscellaneous drugs Hypoglycaemic agents: Sulphonylurea, antidiabetic drugs like chlorpropamide or tolbutamide, in the presence of the respective metabolites of either extracted in ether from acidified plasma.	Partition	μ Bondapak C_{18}	0.05M ammonium formate/acetonitrile (83:17)
Sympathomimetics: Imidazoline derivatives and their degradation products in the intact form of these drugs or in their pharma-ceutical preparations.	Ion-exchange	Zipax SCX and SAX	Aqueous mobile phase buffered at pH 11.4–12.4
Cardiovascular drugs: Pentaerythritol tetranitrate (used in the treatment of anginal attacks) extracted from blood after conversion of the compound into its tetra-p-methoxy benzoate derivative	Adsorption	Corasil II	Heptane/chloroform

Contd.

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
Disopyramide, an antiarrythmic agent, and its mono-N-dealkylated metabolite present in the extracts derived from plasma and urine	Ion-pair chromatography	ODS-silica	Methanol/water (53:47) containing heptanesulphonic acid
N-acetylprocainamide and procainamide present in extracts derived from plasma	Partition	μ-Bondapak C$_{18}$	0.4 per cent sodium acetate/acetic acid/ acetonitrile (100:4:5)
Anaesthetics: Procaine in different dosage forms	Partition	μ-Bondapak C$_{18}$	Acetonitrile/water (60:40) containing 0.01 per cent con- centrated ammonia

Application of HPLC in Clinical Chemistry

Two examples being taken below to illustrate the application of HPLC in the clinical laboratory are:

(a) Monitoring of urinary oestriol levels in pregnancy, and (b) routine analysis of blood, urine and faeces with regard to porphyrins in the diagnosis of disorders in porphyrin metabolism.

(a) Knowledge of the urinary oestriol levels is of considerable use in the management of high risk pregnant women facing complications such as fetal growth retardation or diabetes. Normally, oestriol levels rise during pregnancy and a decline is indicative of placental malfunction. Large differences in values for plasma and urine levels in basal and pregnant states occur between individuals. It is, therefore, necessary to perform several tests on a given individual. A wide range of reversed-phase columns are capable of providing excellent resolution of most biologically active steroids. Previous methods of detection of oestriol have relied on HPLC with UV or fluorescence detection. But these detection systems are relatively insensitive and alternative detection techniques (which may also be employed for the detection of other steroids such as oestrone, oestradiol and several of their metabolic derivatives) are in use now. Electrochemical detection is an example of such alternative detection techniques. Electrochemical detection relies upon the oxidation of the phenolic ring of the steroid at a carbon paste electrode, the technique having the sensitivity to detect the oestriol in as little as 1 ml of a urine sample.

(b) Diagnosis of disorders in porphyrin metabolism necessitates the routine identification and determination of porphyrins in blood, urine and faeces. Urinary

porphyrins alone are not definitive with respect to porphyrias but consideration of total urinary porphyrins and the pattern of the faecal porphyrins on the chromatogram allows differentiation of the diseases. The porphyrins may be analyzed as their methyl esters by adsorption chromatography on a μ-Porasil column using a mobile phase of n-heptane/methyl acetate (3:2) with flow programming.

Application of HPLC in Forensic Chemistry

Sensitive and rapid analyses of drugs of abuse are needed by law enforcement agencies and in toxicology and criminology laboratories. The analyses are important in determining the composition of seized substances, and the quantity of drug in a specific sample. Since many of the samples are mixtures or impure, identification or quantitative determination by spectroscopic methods usually requires preliminary clean-up. The high polarity and low volatility of many of the drugs restrict the use of GLC, and TLC is of limited use for quantitative analysis. HPLC is inherently suitable for dealing with these samples.

Table 10.17 illustrates application of HPLC in assaying drugs of abuse.

TABLE 10.17

Application of HPLC in Forensic Chemistry

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
Phenethylamines: Methamphentamine, metho-xyphenamine and ephedrine as their free bases	Adsorption	Corasil II	Chloroform/ methanol
Benzphetamine, phendime-trazine, phenmetrazine, dexamphetamine, N-methyl-ephedrine, ephedrine, methylamphetamine and mephentermine	Adsorption	Microparticulate silica	Methanol/2N ammonia/1N ammonium nitrate (27:2:1)
Barbitone, caffeine, morphine, 6-0-acetyl-morphine, strychnine, heroin (diamorphine), quinine and cocaine in illicit heroin samples.	Ion-exchange	Zipax SCX	Gradient from 0.2M boric acid, borate buffer adjusted to pH 9.3 with 40 per cent sodium hydroxide to 0.2M boric acid/

Contd.

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
			acetonitrile/n-propanol (86:12:2) adjusted to pH 9.8 with 40 per cent sodium hydroxide
Narcotine, thebaine, codeine and morphine present as the major components in an opium sample	Adsorption	Partisil	Methanol/2N ammonium hydroxide/1N ammonium nitrate (27:2:1)
Caffeine, heroin, mono-acetylmorphine, morphine and strychnine in a 'Chinese heroin' sample.	Adsorption	Partisil	Methanol/2N ammonium hydroxide/1N ammonium nitrate (27:2:1)
Cannabis samples: Cannabinol (CBN), Cannabidiol (CBD), Δ^8–and Δ^9–tetra-hydrocannabinol (Δ^8–and Δ^9–THC) in a Cannabis extract	Partition	ODS-bonded silica	Methanol/0.02N sulphuric acid (80:20)
Lysergic acid diethylamide (LSD): LSD in illicit preparations containing ergot alkaloids like iso-LSD, ergometrine maleate, ergocryptinine base and ergotamine tartrate	Partition	μ-Bondapak C_{18}/ Corasil	Methanol/0.1 per cent ammonium carbonate (60:40)
Miscellaneous: Separation of constituents of a mixture of sixteen street drugs viz. phency-clidine, methadone, cocaine, tetrahydrocanna-binol (THC), methylam-phetamine, 2,5-dimethoxy-4-methylamphetamine (STP), methylenedioxyam-			

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
phetamine (MDA), heroin, N, N-dimethyltryptamine (DMT), lysergic acid di-ethylamide (LSD), diazepam (Valium), mescaline, secobarbitone, amylobarbi-tone, phenobarbitone and di-phenylhydantoin (Dilanton)	Adsorption	Corasil II	Gradient system of ethanol/dioxan/cyclohexylamine in hexane.
Separation of a wide range of drugs of abuse present in mixtures	Adsorption	Partisil 'fines'	Methanol/2N ammonia/1N ammonium nitrate (27:2:1)

Application of HPLC in Food Chemistry

Examples of the application of HPLC for the analysis of certain compound types (e.g. vitamins, carbohydrates) in foodstuffs have already been described in the earlier sections of this chapter during discussions on working of the individual modes of HPLC. Table 10.18 further illustrates application of HPLC in food industry.

TABLE 10.18

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
Grape juice: Citric acid, malic acid and tartaric acid	Ion-exchange	Aminex A25 anion-based on divinyl-benzene	1.0M sodium formate
Food additives: Benzoate, saccharin	Ion-exchange	Zipax SAX	0.01M sodium tetraborate + 0.02M sodium nitrate
Lecithin	Ion-exchange	MicroPak-NH$_2$	Isopropanol-hexane (30:70)
Phosphatidylcholine	Adsorption	μ Porasil	Acetonitrile –methanol–water
Antioxidants: Butylated hydroxytoluene, triphenyl phosphate and butylated hydroxyanisole	Adsorption	MicroPak Al-5 (Alumina)	Hexane-dichlorome-thane (programmed)

Applications of HPLC in Pollution Management

The urgency of monitoring the levels and effects of hazardous pollutants in our air, water and food supplies cannot be overemphasized. Analytical surveys are

undertaken to evaluate the persistence of pollutants such as pesticides and carcinogens and their metabolites in the environment with a view to defining acceptable daily intakes of pesticide residues and food additives. Two of the problems involved in this complex analytical undertaking centre firstly on the severe demands placed on the sensitivity and selectivity to be achieved by detection systems monitoring trace components, and secondly on the large number of samples which must be analyzed in pesticide screening projects.

Although the detectors currently available for HPLC do not usually match the sensitivity of GLC detectors [e.g. electron capture detector (Chapter 13) and GLC/ mass spectrometer (MS) combination (Chapter 13)], the practical detection limits of the two methods are about equal since large sample volumes can be injected into HPLC columns. Thus monitoring of solutes in picogram amounts, as is achieved by GLC, has now been attained by HPLC also in several cases. Further, selective HPLC detectors such as spectrofluorimeters and electrochemical detectors can significantly increase sensitivity or reduce sample clean-up for suitable analytes. The advantage of wave length variation in the UV monitoring of pesticides has also been made use of. Further advances in the HPLC/MS link-up can also be expected to improve detection limits and specificity in environmental analysis. The use of non-destructive detectors in HPLC also allows samples to be collected for structure elucidation or confirmation or, on the preparative scale, for biological testing or further chemical modification.

The solution to the problem of analyzing large numbers of samples in environmental screeing projects has been found in the development of automated pesticide analyzers. Also, the range of automatic injection devices for HPLC now available reduces operator involvement in analyzing large numbers of samples.

As in other areas of analysis, HPLC is a useful complementary technique to GLC in analyzing pollutants in the environment. An advantage of HPLC in environmental analysis is that molecules of varying polarity (e.g. pesticide + metabolite mixtures) can be analyzed in one chromatographic run. Since aqueous mobile phases can be used in reversed-phase HPLC (including ion-pair partition modes) sample preparation is often less extensive than in GLC. The number of clean-up steps can also be reduced by the use of a precolumn to protect the analytical column or by a preliminary size separation of a crude extract on an exclusion column.

Pesticides

Pesticides can be classified as insecticides, herbicides, fungicides and rodenticides. Application of HPLC for monitoring pesticides is illustrated below by taking examples of each of these types.

Insecticides

Pyrethrins and insect juvenile harmones provide examples of naturally occurring compounds which are of interest as alternatives to chemical insecticides. The class

of chemical insecticides consists of chlorinated insecticides, organophosphorus insecticides, esters of carbamic acid, and this class of insecticides also includes larvicides e.g. Thompson-Hayward 6040 which is a substituted area.

Pyrethrins: These have low mammalian toxicity. Separation of constituents of pyrethrum extracts has been achieved by chromatography on permaphose ODS.

Insect juvenile harmones: These act against insects by retarding their maturation. Steroidal insect moulting hormones such as β-ecdysone have been analyzed by HPLC. A study of the persistence of the synthetic juvenile hormone JH-25 [7-ethoxy - 1 (p-ethylphenoxy)-3, 7-dimethyl-2-octene] in flour samples has been carried out by HPLC.

Chlorinated insecticides: The analysis of chlorinated pesticides in residue samples in complicated by the fact that they usually occur along with polychlorinated biphenyls (PCBs). The latter compounds occur widely in the environment due to their use as plasticizers, dye stuff additives and hydraulic oils, and both chlorinated pesticides and polychlorinated biphenyls are persistent in the environment. Since both compound classes include non-polar aromatic molecules, adsorption chromatography has been the mode of choice for the HPLC separation of these compounds.

Using a 250×3 mm i.d. column of 5 μ LiChrosorb SI 60 silica and dry hexane as the mobile phase, hexachlorobenzene, aldrin, p, p′–DDE, o-p′–DDE, o, p′-DDT, p, p′–DDT and p, p'–DDD were separated from each other and from decachlorobiphenyl and biphenyl in 8 min, at a column temperature of 27ºC and a flow rate of 4 ml min^{-1}. A 10- to 80- fold increase in sensitivity was btained for all pesticides except the DDE isomers (metabolites of DDT) by using the UV detector at 205 nm rather than 254 nm. Using the LiChrosorb SI 60 silica/dry hexane system chlorinated PCBs such as Aroclor 1254, 1260 and 1268 (where 12 represents biphenyl and the remaining two digits represent the per cent by weight of chlorine in the mixture) elute before the less highly chlorinated PCBs (Aroclors 1232, and 1248) and are quantitatively separated from longer-retained pesticides such as the DDT isomers, o, p′–DDE and p, p′–DDD. Pesticides with even longer retention times (lindane ≡ γ-benzene hexachloride ≡ γ – BHC, endosulfan, endrin, dieldrin and methoxychlor) were well separated from all of the Aroclors.

Organophosphorus insecticides: Separation of insecticides like phosphamidon, monocrotophos, CGA 18809 (an experimental insecticide) and dicrotophos has been achieved by chromatography on Permaphase ETH columns.

In a study of the analysis of organophosphorus pesticides, fenchlorphos, cruformate and fenthion were hydrolyzed to the corresponding phenols which were reacted with dansyl chloride to form fluorescent derivatives. Separation of the three derivaties was achieved by adsorption chromatography on silica. Separation of the *cis* and *trans* isomers of three organophosphorus pesticides, dimethyl-

vinphos, temivinphos and chlorphenvinphos, has been carried out by adsorption chromatography on silica.

Esters of Carbamic acid: The carbamates, esters of carbamic acid (NH_2-COOH), constitute an important family of insecticides of which cabaryl is the
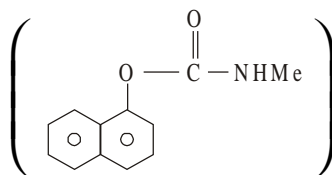
member most commonly used. The 2-isomer of carbaryl (2-naphthyl-N-methylcarbamate) shows carcinogenic effects not exhibited by carbaryl itself, and hence this isomer must be absent from carbaryl formulations. A method for the analysis of the 2-isomer in the presence of carbaryl involved hydrolysis of the carbamates to 1- and 2- naphthol and analysis of the naphthols in the presence of unhydrolyzed carbamate on a Corasil II column with a hexane/chloroform (80:20) eluent.

Recently separation of around 30 carbamate pesticides by adsorption and reversed-phase chromatography were reported. For many of the Carbamates studied, improved detection limits (1-10 ng) were achieved by monitoring at 190-220 nm.

Substituted urea larvicide: The substituted urea, [N-(4-chlorophenyl)-N′-(2, 6-difluorobenzoyl-urea], is a larvicide patented as Thompson–Hayward 6040. It has been analyzed in cow's milk following extraction and chromatography on Permaphase ODS with a methanol/water (50 : 50) eluent.

As mentioned earlier, pesticides comprise of compounds classified as herbicides, fungicides and rodenticides besides those classified as insecticides. Herbicides or fungicides or rodenticides are far less numerous than insecticides, and examples of the use of HPLC in the monitoring of pesticides belonging to the three former classes are all considered together in Table 10.19.

TABLE 10.19

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
Herbicides Substituted urea herbicides: Diuron, neburon, phenobenzuron and isoproturon	Adsorption	LiChrosorb SI 60	Water saturated with dichloromethane (the role of water being to block the most active silanol sites thereby

Contd.

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
			providing a less active but more homogeneous separating surface)
Quanternary ammonium herbicides:			
Paraquat in the urine samples obtained from patients who had ingested the compound	Partition	Alumina containing bonded aminopropyl groups.	Methanol/acqueous acid
Paraquat and diquat (which is often found in combination with paraquat)	Partition	Alumina containing bonded amino-propyl groups.	Methanol/aqueous acid (paraquat and diquat monitored by measuring the UV absorbance respectively at 258 nm and 310 nm)
Paraquat in its commercial formulations	Ion-exchange	Vydac cation-exchange resin	0.2M dimethylamine in methanol
Phenoxyacetic acid herbicides: Mixture of several phenoxyacetic acid herbicides	Adsorption	Perisorb A pellicular adsorbent	Hexane/acetic acid (92.5 : 7.5)
Fungicides Benomyl (estimated as MBC, methyl-2-benzimidazole carbamate, the principal degradation product of benomyl), MBC and 2-AB (2-aminobenzimidazole, a second degradation product of benomyl) present in extracts from soils or tissues of plants e.g. cucumber	Ion-exchange	Zipax SCX pellicular strong cation-exchanger	0.025N tetramethyl-ammonium nitrate/ 0.025N nitric acid; pressure, 300 psi; flow rate, 0.5 ml second min^{-1}; column temperature, 60ºC
Vitavax, an anilide fungicide, and its two oxygenated derivatives produced by photolysis of vitavax,	Partition	Bondapak C_{18}/ Corasil	Water/acetonitrile (80 : 20)

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
present in the dichloro-methane extract of a forti-fied sample of lake water			
Rodenticide Warfarin, an anti-coagulant rodenticide, in animal tissues, stomach contents, body fluids and feedstuffs	Adsorption	Corasil II	Isooctane/isopropyl alcohol (98 : 2)

Carcinogens

Monitoring of carcinogens by HPLC is illustrated in Table 10.20.

TABLE 10.20

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
Aflatoxins (metabolites of *Aspergillus flavus* which are among the most potent known inducers of tumours) : Aflatoxins in extracts from grain samples infested with *Aspergillus flavus*	Adsorption	SIL–X silica	Isopropyl ether/ tetrahydrofuran (88 : 12)
Aflatoxins B_1, B_2, G_1 and G_2	Adsorption	Partisil-5 silica	Water-saturated dichloromethane/ methanol (99.7 : 0.3)
Nitrosamines: The *syn-* and *anti-*confor-mers of N-nitrosonor-nicotine (the carcinogen found unburned in U.S tobacco at levels of 0.3-90 ppm)	Partition	µ Bondapak C_{18}	Acetonitrile/water (50 : 50)
Fluorenylhydroxamic acid esters: Mixture of carcinogenic-o-acetates of fluorenyl-hydroxamic acids	Adsorption	Corasil II	Hexane/ethyl acetate (50 : 50)

Contd.

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
1, 2-Dimethylhydrazine: Separation of 1, 2-dimethyl-hydrazine, a potent colon carcinogen in rats, from azomethane, azoxy-methane and methylazoxymethanol, formaldehyde and methanol (probable metabolites of 1, 2- dimethylhydrazine)	Partition	μ Bondapak C_{18}	1% aqueous ethanol eluent gave good resolution of all the compounds except 1, 2-dimethylhydra-zine itself, which was excessively retained and badly tailed, and this compound could be eluted in the proto-nated form by changing the eluent to 0.05M acetic acid.
1, 2-Dimethylhydrazine, azomethane, azoxy-methane and methylazoxy-methanol, formaldehyde and methanol	Ion-exchange	Aminex A-27 (a strong anion-exchange polystrene based resin)	With eluent having pH 5.6, 1, 2-di-methylhydrazine could be eluted rapidly through the column, with rea-sonable resolution of the other metabolites (except methanol and formaldehyde). (Azo and azoxy com-pounds were detected at 205 nm and radioactive, 1, 2-di-methylhydra-zine and formal-dehyde were detected by liquid scintillometry of collected fractions.)
Polynuclear hydrocarbons: Separation of benzo [a]			

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
pyrene (a powerful hydrocarbon carcinogen which is emitted into the atmosphere as a consequence of the combustion of fossil fuels, the compound being metabolized to oxygenated derivatives from 8 metabolites viz. three dihydrodiols (9, 10; 7, 8; and 4, 5), three quinones (benzo [a] pyrene-1, 6, -3, 6-; and -6, 12-dione) and two phenols (3- and 9-hydroxybenzo [a] pyrene)	Partition	Permaphase ODS	Methanol/water gradient
Phenathrene, anthracene, fluoranthene, pyrene, triphenylene, benzo [ghi] fluoranthene, chrysene, benzo [a] anthracene, benzo [j] fluoranthene, benzo [b] fluoranthene, benzo [e] pyrene, perylene, benzo [k] flouranthene, benzo [a] pyrene, benzo [ghi] perylene, indeno [123 cd] pyrene, anthanthrene, coronene, in an air sample.	Partition	Zorbax ODS	Methanol/water (65 : 35) gradient of 1 per cent methanol min^{-1} initiated after 70 min; pressure, 1200 psi; flow rate, 0.21ml min^{-1}; column temperature, 60ºC

Analysis of Industrial Pollutants in Environment

Analysis of phenols in complex mixtures and in industrial wastes using UV detection and monitoring of trace phenols in aqueous environmental samples with the use of fluorimetric detectors have been achieved by HPLC. Chlorophenols, which can be formed during the disinfection of industrial waste by chlorination, can be analyzed as quinones after oxidation by ruthenium tetroxide.

HPLC has been applied to the analysis of nitro compounds e.g. nitroglycerine in waste waters, and trinitrotoluene in munition waster waters.

HPLC has also been applied to the analysis of trimethylolpropane and pentaerythritols in industrial synthesis solutions, 2-mercaptobenzothiazole in waste dump effluent, polythionates in mining waste water, isocyanates in working atmospheres, carcinogenic amines, aniline in waste water, polyethylene oxide fatty acid surfactants in industrial process waters and phthalate esters in river water.

Application of HPLC in Chemical Industry

Some examples of the application of HPLC in the chemical industry have been given earlier in this chapter during the course of discussion of working of the individual modes of HPLC. Table 10.21 lists some more examples of the application of HPLC in the chemical industry.

TABLE 10.21

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
Benzene, naphthalene and phenanthrene	Partition	Merck SI 60 + added water	n-Hexane
Anthracene, pyrene, chrysene, perylene, and anthanthrene	Partition	Durapak OPN	Isooctane
Benzene, biphenyl, m-terphenyl, m-quaterphenyl, m-quinquephenyl	Adsorption	Perisorb A	n-Heptane
Phenanthrene, pyrene, chrysene, and benzo [a] pyrene	Partition	Li Chrosorb RP – 8	Water-methanol (20 : 80)
Polymers:			
Polystyrene— mol.wt. 1,800,000 mol. wt. 92,000 mol. wt. 4,00	Exclusion	Three in series: CPG 75, CPG 240, CPG 700 (porous glass)	Dichloromethane
Surfactants: Non-ionics surfactant Igepal 430 $n = 2$ $n = 3$ $n = 4$ $Me[CH_2]_8 \langle \bigcirc \rangle - [OCH_2CH_2]_n OH$	Ion-exchange	MicroPak-NH_2	Non-linear gradient 0 to 100% isopropanol in hexane
Explosives and propellants: TNT, tetryl and RDX	Adsorption	Corasil II	30% dioxan + 70% cyclohexane
Trinitroglycerine, 1, 3-dinitroglycerine, 1, 2-dinitroglycerine	Adsorption	Vydac	Dichloromethane – hexane (60 : 40)

Contd.

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
Nitroglycerine, diethyl phthalate, ethyl centralite, acetanilide	Adsorption	Vyadac	1, 1-dichloro-ethane
HMX, RDX, m-nitrophenol and TNT	Partition	Bandapak C_{18}	40% methanol in water
Dyestuffs: Azodyes viz. Du Pont Oil Red, CI Disperse Red 65, CI Disperse Orange 3, CI Disperse Yellow 3,	Partition	Zipax/1% dipro-pionitrile	Hexane
Azobenzene, N, N-diethyl p-amino-azobenzene, N-ethyl p-amino azobenzene, p-amino-azobenzene	Adsorption	MicroPak A1-5	1% dichloro-methane-hexane

Application of HPLC in Monitoring of Miscellaneous Plant Products

Examples of the application of HPLC in this field can be found dispersed in the earlier sections of this chapter wherein working of the individual modes of HPLC has been dealt with. Use of HPLC in monitoring of plant products, which may be of pharmaceutical interest or may be the colour, flavour, or aroma constituents of commercial products, is further illustrated in Table 10.22.

TABLE 10.22
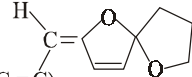
Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
Several methylated naturally-occurring xanthones	Partition	MicroPak CN	Hexane/chloroform (65 : 35)
Four anthraquinones viz. chrysophanol, physcion, emodin and aloe emodin from plant extracts	Adsorption	Corasil II	Gradient from hexane to ethyl acetate
A series of phenolic acids, cinnamic and bonzoic acids	Partition	μ Bondapak/C_{18}	Water/acetic acid/ methanol
A variety of flavonoid compounds including	Partition	μ Bomdapak/C_{18}	Water/acetic acid/

Contd.

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
flavones, flavonols and flavonones as well as glycosylated flavone derivatives			methanol
O-glycosides of glyco-flavones differing only in the position of the hydro-lyzable sugar in the flavone skeleton	Partition	LiChrosorb RP8 and LiChrosorb NH$_2$	Acetonitrile/water gradient
Organic acids from a crude extract of maize leaves	Partition chromatography	Bio-Sil A (as aqueous stationary phase)	Gradient of tertiary amyl alcohol in chloroform/cyclo-hexane (50 : 50)
Constituents of essential oils from *Lindera umbellata* and *L. sericea*	(i) Exclusion chro-matography, as the first step for preliminary separation (five peaks were obtained in this step), followed by separation of these samples by (ii) partition chromatography as the second step (this step yielded a total of more than 20 peaks)	μ-Styragel μ Bondapak/C$_{18}$	Tetrahydrofuran Methanol/water (50 : 50)
Cinnamon oils	Adsorption	Corasil II	Cyclohexane/ethylacetate (99 : 1)
The spiro ethers I and II (isolated from chamomile) : CH$_3$ – (C ≡ C)$_2$ I (*Cis*)	Adsorption	Silica	Hexane/chloroform (60 : 40)

Sample	Mode of HPLC used	Stationary Phase	Mobile Phase
(only this isomer has anti-spasmodic and anti-infla-mmatory properties) II (*Trans*)			
The large number of metabolites (of which some are known carcinogens) produced by fungus, *Aspergillus versicolor*	Adsorption	μ-Porasil	Hexane/n-propanol/ acetic acid (99.3 : 0.7 : 0.1)
Mixtures of metabolites (e.g. alternariol monomethyl ether, alternariol and altenuene) of the fungus, *Alternaria*, extracted from grain sorghum or grain cultures with methanol (after having subjected the extract to a clean-up procedure)	Adsorption	Corasil II or Zorbax SIL	Either Isooctane/ tetrahydrofuran or petroleum ether/ tetrahydrofuran, with a system of solvent programming

$CH_3 - (C \equiv C)_2$

■

11

Paper Chromatography (PC)

The stationary phase used in this procedure is a sheet of filter paper and hence the name 'Paper Chromatography' for the method. The mobile used is liquid. Since the stationary phase is in a plane rather than in a column, the technique is categorized under 'Plane Chromatography'.

The discovery of paper chromatography (PC) in 1944, which tremendously increased the possibility of detection, identification and separation of small amounts of substances, meant a true revolution in Chemistry, and mainly in Biochemistry. This method was discovered by Conden, Cordon, Martin and Synge, who developed it in connection with the need to analyze protein hydrolyzates i.e. to analyze mixtures of amino acids. The last two of the authors mentioned were awarded the Nobel Prize for the discovery. During the following ten years the method devloped and spread to such an extent that no chemical or biochemical laboratory would imagine working without it. The extreme sensitivity with which compounds can be located after separation is one of the most important features of paper chromatography. In case of some compounds, amounts as little as 0.1 microgram can be detected by this chromatographic technique. On account of its such high degree of sensitivity for detection, paper chromatography has played a very important role in elucidating the chemistry of many naturally occurring organic substances. Many a time these natural products are isolable in quantities of the order of a few milligrams; but even with such minute amounts many reactions can be carried out and products of such reactions can be separated and identified by paper chromatography.

11.1 Theory underlying Working of Paper Chromatography

The working of this technique is based on two phenomena:

(i) Molecules of different substances will generally have different attractive forces of adsorption to the cellulose fibres of which paper is made. (ii) Even 'dry' paper generally has about 15% water, by weight, on its fibres, and in paper chromatography molecules of different constituents of a mixture dissolved in a water-immiscible solvent will partition themselves in differing degrees between this adsorbed water of the cellulose fibres and the solvent. The partition effect usually plays the major role in paper chromatography.

The mechanism which effects separation of a mixture into its constituents can be explained in the following manner. A suitable solvent, which constitutes the

mobile phase of the system, is made to flow by capillary action through a filter paper (stationary phase) past a dried spot of the solution prepared by dissolving the given mixture in an appropriate solvent. The mobile phase, as it reaches the chemical spot, will have the tendency to dissolve and carry along the constituents say, A and B, of the original mixture, which is available in a very small amount on the filter paper in the form of the said spot. However, this tendency is offset by the fact that the individual molecules of A and B are attracted to the stationary phase. The case with which moleucles of say, A, can be detached from the stationary phase and dissolved in the solvent depends on the magnitude of the factors which are responsible for retention of A on the stationary phase. These two factors are: (i) The partition coefficient of A between water (held on the fibres of the filter paper) and the solvent, and (ii) adsorption of A on the paper due to the adsorption properties of the cellulosic fibre network itself. Thus degrees of retention of A and B by the stationary phase will have their own different values. If it is assumed that this magnitude of retention on the stationary phase is higher for A than for B, A will be held more strongly and it will move along with the mobile phase more slowly than B. The molecules of A and B are carried along a little way from the chemical spot by the moving solvent until they chance to become bound again to the paper fibres a little farther on in the direction of the solvent flow. The molecules of A and B will move along the paper with the solvent, alternately binding to the fibres and then redissolving into the solvent, which moves them a little farther along until they attach to the surface again. As already explained, A, which is held to the paper more strongly, will move along more slowly than B; it is released from the surface into the solution more slowly and is quicker to reattach itself to the surface. So, as the molecules are carried along by the solvent, molecules of A lag farther and farther behind the molecules of B. Eventually, A becomes completely separated from B. Detailed below are various experimental techniques of separation by paper chromatography.

11.2 Experimental Techniques of Paper Chromatography

11.2.1 Ascending Paper Chromatography

A typical set-up for ascending paper chromatography is shown in Figure 11.1 A sheet of the selected size and grade of special filter paper is taken. A thin pencil line is drawn across this sheet a few centimeters from the bottom. This line serves as a future reference. Figure 11.2 shows methods of applying the sample. It may be applied as a spot (1), or as a band (2). The latter procedure has the advantage of increasing the capacity and thus minimizing overloading difficulties, but in some systems the solvent tends to flow faster in the centre of the strip than at the edges thereby causing bending of the band. Often, a wide strip is used (3), on which a number of samples are spotted on the pencil line with the help of a

capillary pipette. For comparison, drops of solutions of known substances (standards) are also applied to the filter paper on the pencil line. When increased capacity is desired, one sample may be streaked across the whole width of a wide strip (4). The solvent is then allowed to dry.

The filter paper is placed in the chamber and the vessel is closed by placing the lid securely in its top. Ample time for vapour equilibration is allowed before the strip is lowered (by some means, not involving opening the chamber) until its lower end dips into the solvent. Choice of solvent most suitable for a particular substance is made by reference to handbooks on chromatographic techniques. (Among the solvents usually employed for chromatographic techniques are, for example, water-saturated 1-butanol, 2-butanol, 1-hexanol, cycohexanol, cyclohexanone, phenol,

Figure 11.1 Set-up of ascending paper chromatography.
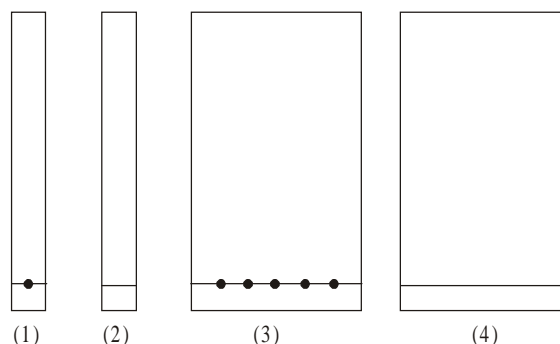
Figure 11.2 Methods of applying the sample.

butyl acetate). Solvent rises along the filter paper strip by capillary action. The height to which the solvent will rise is limited, usually, to 50 cm or less. The sheet is then removed from the vessel and dried.

If the substances being separated are coloured, the location of the spots of the adsorbed substances that have ascended the paper by capillary action is easily achieved. In the case of colourless substances, the spots have to be located by either conversion to a coloured derivative by reaction with a suitable detecting agent or by some suitable detecting device.

The use of a detecting agent can be exemplified by that of ninhydrin. This reagent is used for the location of amino acids with which it gives purple spots on warming, through the following reaction sequence.

Chemistry of the Ninhydrin Colour Test

Ninhydrin
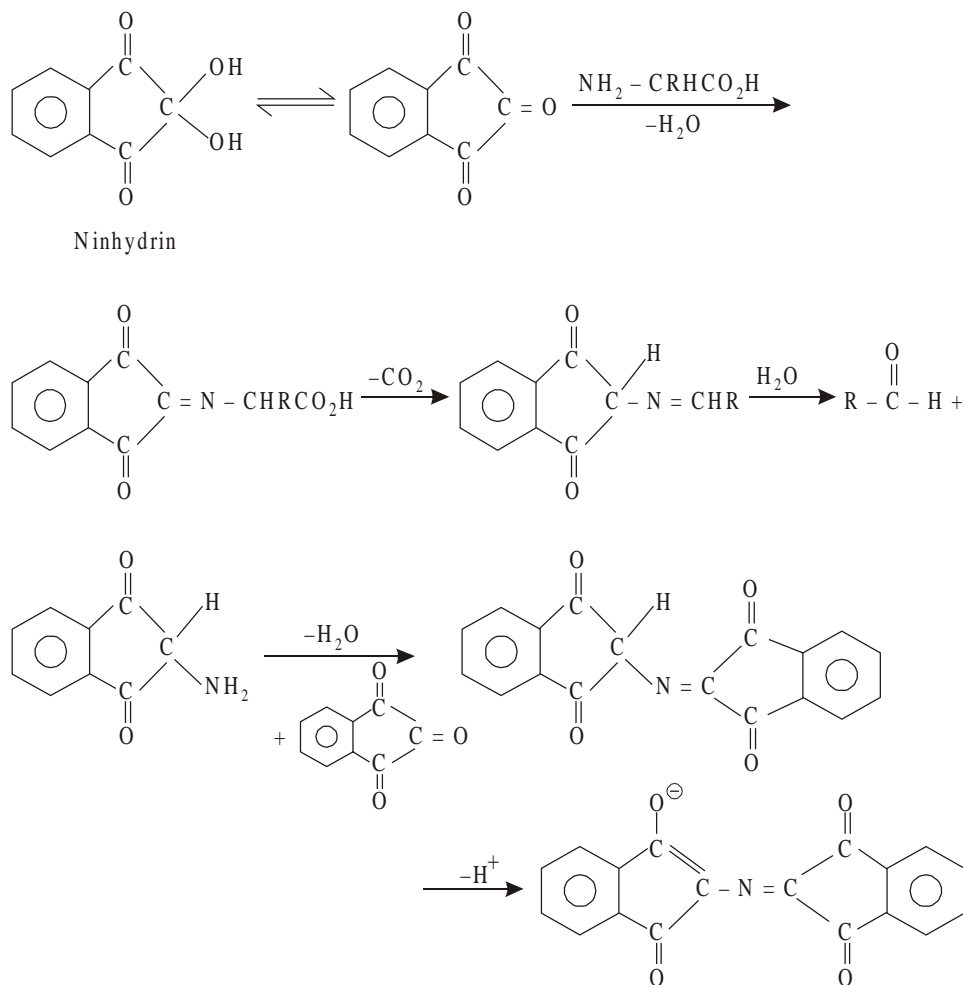
[Coloured product formed is the same (blue-violet)
with most of the amino acids, $H_2N–CHRCO_2H$]

Dithiooxamide provides an example of a detecting agent used in paper chromatography in Inorganic Chemistry. Its use can be illustrated by taking the example of separation of chlorides of copper, cobalt and nichek. The developer employed for the purpose is a solution made up of HCl-water-ketone mixture which is prepared just before it is to be used. Spot locations are revealed by spraying with 0.1% dithiooxamide in 60% ethanol. Colours produced are green, yellow and blue with copper, cobalt and nickel respectively.

Table 11.1 lists important examples of chromogenic reagents used as detecting agents in paper chromatography.

TABLE 11.1

Chromogenic Reagents for visualizing Paper Chromatogram

Reagent	*Applications*
Ninhydrin	Amino acids
2, 4-Dinitrophenyl hydrazine	Carbonyl compounds
Phosphomolybdic acid	General organic
Fluorescein/bromine	General organic
Dithiooxamide	Metals
H_2S water, diphenyl carbazide	Metals
or rubeanic acid	Metals
Aniline phthalate	Sugars
Chloroplatinic acid	Alkaloids
Bromothymol blue	Lipids
Antimony trichloride	Steroids, essential oils

Fluorescent substances can be located by expousre to ultraviolet radiation.

If the spot of the substance being analysed has travelled the same distance as the known compound, the identity of the substance as being the same as the known compound stands established with sufficient certainty.

If no simultaneous run of the reference compounds has been carried out during the chromatographic separation, which more usually is the case, identities of the constituents of the mixture after their separation on the chromatographic paper are established by measuring the experimental value of R_f of each constituent. The quantity R_f (retardation factor) is defined as the ratio of the distance travelled by the compound to the distance travelled by the solvent front for the same time interval and is a characteristic constant of the substance under complete uniformity of experimental conditions (standard paper, constant temperature, definite solvent specified by the procedure). The procedure then is to run a chromatogoram of the unknown substances and compare the R_f of the compounds thus experimentally determined with R_f values obtained from chromatogram, made under identical conditions, of known standard compounds. The R_f values of the substances are not affected by the detecting agent, the transport having been achieved prior to detection.

11.2.2 Two-dimensional Paper Chromatography

When many substances are to be separated or when the R_f values of the constituents in a given solvent are smilar, straight-forward one-dimensional chromatography gives incomplete separation, and use of two-dimensional paper partition chromatography becomes necessary to achieve satisfactory separation of the given mixture into its components. In this variation of the technique the initial spot is placed near one corner of a square sheet, and developed in the usual way

with solvent X [Figure 11.3 (a)]. X is then dried off, the paper turned through 90°, and developed with another solvent Y. By suitable choice of X and Y, a complete two dimensional separation may be
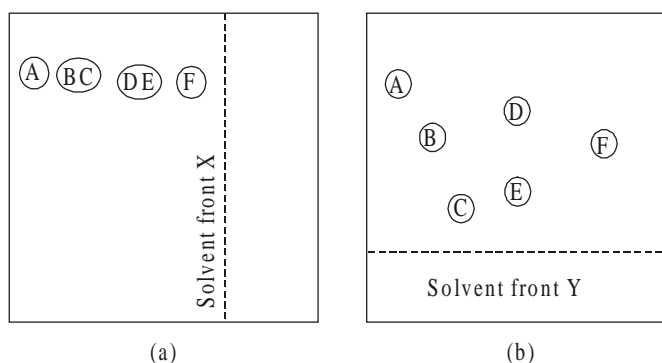
Figure 11.3 Two-dimensional paper chromatography

obtained, as in Figure 11.3 (b). An outstanding example of the application of this technique is provided by the sucess that Dent achieved in separating all the individual constituents of a mixture containing no less than sixty two amino acids.

11.2.3 Descending Paper Chromatography

A disadvantage of the ascending technique described earlier is that the solvent can ascend only until it reaches the top edge of the paper at which stage the flow of the solvent ceases, and compounds with low R_f values are often incompletely separated. With the descending technique the developing solvent can be allowed to run off the end of the paper under the influence of gravity so that one is able

(a) Layout of the tank

(b) Insertion of Paper
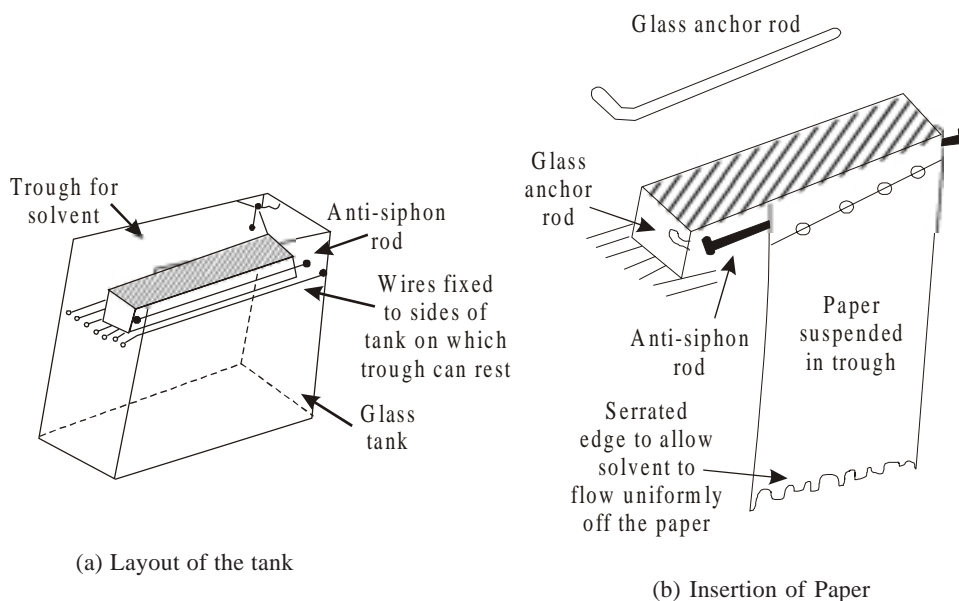
Figure 11.4 Descending paper chromatography

to increase considerably the effective length of the run and thus improve the separation.

In the descending technique solvent is placed in a glass trough which is set in the chromatographic tank as shown in Figure 11.4 (a). Some of the solvent is poured into the bottom of the tank so as to saturate the atmosphere with its vapour. The paper, prepared as described under 'Ascending Paper Chromatography' is then suspended in the solvent and the lid placed securely on the tank. The paper is anchored in the trough by means of a glass rod and passes over a second rod slightly higher than the edge of the trough to prevent siphoning of the solvent, the arrangement being shown in Figure 11.4 (b).

11.2.4 Chromatography on Circular Papers

This method has proved particularly useful in separating mixtures of dyestuffs. A circular paper is held between two discs of glass or plastic, the upper one of
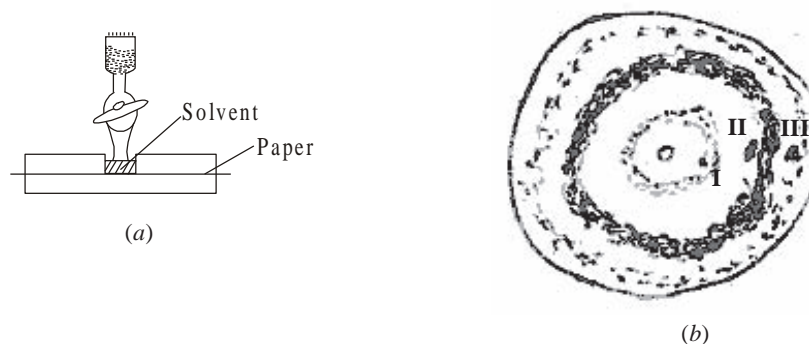
Figure 11.5 : Circular paper chromatography

which has a hole in its centre [Figure 11.5 (a)]. The mixture (in solution) is placed in the centre hole followed dropwise by solvent, which spreads radially by capillary attraction carrying the components, I, II, III, into concentric rings as in Figure 11.5 (b). With coloured substances a circular trough may be cut in the lower disc and elution continued until each ring is washed to the edge, collected, and drained off for subsequent recovery. The circular method has the following advantages over the others:

(1) The apparatus is even simpler, no air-tight container being need. (2) The run-out is very rapid. (3) Separations are usually sharper. (4) Much larger quantities (upto 100 mg) of mixture can be handled.

11.3 Factors affecting Resolution on Paper Chromatography

In a paper chromatogram, the mechanism of distribution of solute which takes place between the mobile phase (developing solvent) and the stationary phase may be distribution between the solvent and an immiscible liquid phase which moistens

the paper; it may be adsorption of solute by the paper; it may be reaction of the solute with a salt or other compound present on the paper or, it may be a combination of two or more types of distribution. In any case, the behaviour of a paper 'column' is the same as that of a packed column, and the same factors influence resolution on both. Resolution is best at the centre of a chromatogram. Good paper chromatograms have resolving powers equivalent to 30 or more plates per centimeter.

The kind of paper selected may influence resolution. A coarse-grained paper will, in general, give poorer resolution than a fine grained paper. Thin paper is, of course, more likely to become overloaded than thick paper. When chromatographic strips are cut from large sheets, they should always, for reproducible results, be cut in the same direction relative to the grain of the paper (the direction of movement in the paper-making machine). Filter paper-strips, cut in various widths, are commercially available in the form of rolls. These, of course, are always cut parallel with the grain.

The rate of solvent flow can influence resolution. Since flow rate is not as readily controlled on paper chromatograms as in packed columns, there are few data available on the effect of flow rate. It is probable, however, that the flow rates usually obtained are not fast enough to decrease resolution appreciable. Flow rates can be decreased, when desired, by making the section of the filter paper strip which dips into the solvent narrower than the rest of the strip, the narrow portion extending for some distance above the solvent.

A very important factor influencing resolution, and one which is often given too little attention, is the control of the amount of liquid stationary phase on the paper. In a packed column in which the packing is moistened with an aqueous solution, a mobile phase is used which is saturated with this solution. Conside, for example, a column containing an inert packing which is moistened with water and which is developed with water-saturated butanol. The amount of water in the packing is fixed at the outset, and does not change during the course of development of the chromatogram. Consider a corresponding paper chromatogram. A strip of paper is developed with water-saturated butanol after being equilibrated in an atmosphere saturated with respect to both water vapour and butanol vapour. The paper may absorb water from the atmosphere or from the solvent during development. The amount of such absorption cannot readily be controlled. A strip of paper containing a very thin film of water around each fibre is in equilibrium with a water-saturated system, but a strip of paper which is dripping wet is also in exact equilibrium with a water-saturated system. It is very difficult to operate a paper chromatogram under such conditions, and the *use of water-saturated systems should be avoided*. In the literature, however, specifications for water-saturated systems are very common. Such systems never operate with good reproducibility but can be made to function if saturation of the paper with water can be avoided. Such lack of

equilibration can be achieved by a number of devices, such as use of an atmosphere not quite saturated with water vapour, use of temperature somewhat higher than that at which the solvent was saturated with water, or limitation of the development time so that there is not sufficient time for too much water absorption. Since such devices are likely to be poorly reproducible it is advisable to use an unsaturated system from the outset. In our example, if the solvent had been, for example, a mixture of 9 parts water-saturated butanol and one part anhydrous butanol, the amount of water adsorbed by the paper at equilibrium would be definite and reproducible.

When buffer salts are present on the paper, the salts may determine the amount of water taken up by the paper. Suppose, for example, that the paper prior to use, is dipped in a 10% solution of potassium phosphates. After dipping, a part of the solution is removed by blotting, and the paper dried. If this paper is then developed with butanol which has been shaken with a large volume of 10% buffer, the paper will continue to absorb water until the buffer concentration on the paper is 10%. If the developing solvent, on the other hand, is saturated with water, the paper will continue to basorb water from the developing solvent until the buffer on the paper is infinitely dilute. It is obvious that, when a paper chromatographic procedure is designed, consideration must be given to the amount of water which will be taken up by the paper under equilibrium conditions.

Another factor of great importance in paper chromatography is the means taken to insure complete equilibration of the atmosphere in the chromatographic vessel with the developing solvent. Suppose that a solvent used for chromatography of polar substances, such as amino acids or sugars, is a mixture of water, acetic acid and acetone. Suppose that when this solvent was first tried, a rather large vessel was used and only a small surface of solvent was exposed to the atmosphere of the vessel. Equilibrium, therefore, was not attained before the beginning of development. Acetone, the most volatile constituent of the mixture, was lost from the strip by evaporation during development. Therefore, the composition of the solvent on the strip was richer in water and acetic acid and poorer in acetone in comparison with that of the original solvent. This modified solvent caused polar compounds to migrate more rapidly. The R_f values thus obtained, let us say, were approximately optimal for the compounds of interest. However, because the R_f values obtained depended upon the degree of disequilibrium present, they were poorly reproducible, particularly when the type of vessel was changed. Neeless to say, it would be much better to use an original solvent containing less acetone and to take steps to insure equilibration. To insure equilibration, the chromatographic vessel should have in it a container of solvent which exposes a large surface of solvent and which contains enough solvent to saturate the atmosphere without causing an appreciable change in solvent composition due to greater evaporation of the more volatile constituents. Sufficient time (several hours

for larger vessels) should be allowed to elapse between the time the vessel, containing the strip, is closed and the time development is started.

The considerations governing overloading discussed for packed columns also hold for paper strips. The tailing caused by overloading is readily detected on paper chromatograms. An effect which will be called physical overloading is often encountered in paper chromatography of crude substances. Suppose, for example, 10 microlitres of a crude sample contains 10 μg of amino acids that need to be separated and 100 μg of protein. We spot the sample on the paper, and it dries, with the amino acids encased in a layer of protein which is insoluble in our solvent and from which the amino acids are only very slowly extracted by the solvent. After development, some of the amino acids will still be at the origin, and the remainder will be well spread out over the whole strip because they left the point of origin continously throughout the development period. Such physical overloading occurs whenever too much material (organic or inorganic), insoluble in the developing solvent, is present as a contaminant in the sample. The cure, of course, is some rough preliminary purification step.

The control of ionic form of the sample is important in paper chromatography as in column chromatography. When the substances to be separated have acidic or basic properties, it is essential that either the developer or the paper carries a constituent that controls the degree of ionisation of the compound being separated. A solvent can be used which contains an acid or a base, or the paper may be impregnated with a buffer. Sometimes buffering action is provided by the solvent. For example, if a solvent contains both an organic amine and an organic acid, it is possible, by varying their proportions, to vary the pH of an aqueous phase adsorbed on the paper. It is appropriate to mention that solvent mixtures containing an organic acid and an alcohol will undergo esterification on standing. Such mixtures should always be made immediately before use. A better solution to the difficulty is to avoid the use of such mixtures. Substitution of a ketone or an ether for the alcohol is one possibility.

The best resolution is obtained in the centre of the chromatogram, and changes in solvent should be made with this in mind. Often, with solutes of low R_f values, development is continued much longer than is required for the solvent front to reach the bottom of the paper. This gives better resolution of compounds of low R_f, since the effective column is much longer than the actual strip.

11.4 Reversed-phase Paper Chromatography

The technique of paper chromatography can be extended to compounds which are sparingly soluble in water, by drying the paper and impregnating it with olive oil, silicone oil, paraffin or rubber latex.

The impregnated paper absorbs the relevant component of the solution and the component is partitioned between the impregnated paper and the solvent that

consists of an organic liquid containing a little water, and the large number of repetitions of this process brings about the desired separation. This is an example of reversed-phase chromatography because the stationary phase used is less polar than the mobile phase.

11.5 Ion-exchange Chromatography on Paper

Paper can be employed for carrying out ion-exchange chromatography. Paper can be treated with chemical reagents to convert the hydroxyl groups into ionic groups like carboxyl, –COOH, and diethylaminoethyl, $-C_2H_4N(C_2H_5)_2$.

Resin-inpregnated papers are also commercially available, for example, Amberlite SA-2 (strongly acidic) and Amberlite SB-2 (strongly basic).

∎

<div align="center">

12

Thin-layer Chromatography (TLC)

</div>

Modern chromatographic methods which have caused such a tremendous development in chemical and biochemical analysis and in preparative separations, are unthinkable today without thin-layer chromatography (TLC), which is the subject of discussion of the present chapter. Its rapid development began in about 1958 mainly due to the work of Stahl who elaborated this method and standardized it in its present form.

Included in this chapter is also a discussion of the modernised versions of liquid-solid column chromatography which have enabled this technique to hold its own against the competition offered by TLC, the latter having the advantage of being a convenient and rapid chromatographic technique.

12.1 Working of TLC

This technique may, in principle, be based on adsorption or partition, but usually its adsorption version is exmployed. Steps involved in TLC for separation and subsequent analysis (qualitative and quantitative) of the constituents of a given mixture are detailed below:

12.1.1 Preparation of a Chromatoplate

Like paper chromatography, thin-layer chromatography is a form of 'plane chromatography' in that the stationary phase is held on a plane rather than in a column. Table 12.1 lists important stationary phases used in TLC along with the respective predominant sorption process operative with each of them. The solid phase is supported on to glass, metal or a 'plastic' substance. (Microscope slides

<div align="center">

TABLE 12.1

Stationary Phases for Thin-layer Chromatography

</div>

Stationary Phase	Predominant sorption process
Alumina	Adsorption or partition
Silica gel	Adsorption or partition
Modified silica gel	Adsorption, partition
Kieselguhr	Partition
Cellulose powder	Partition
Modified cellulose, e.g. D E A E and C M	Ion-exchange
Sephadex gels	Exclusion
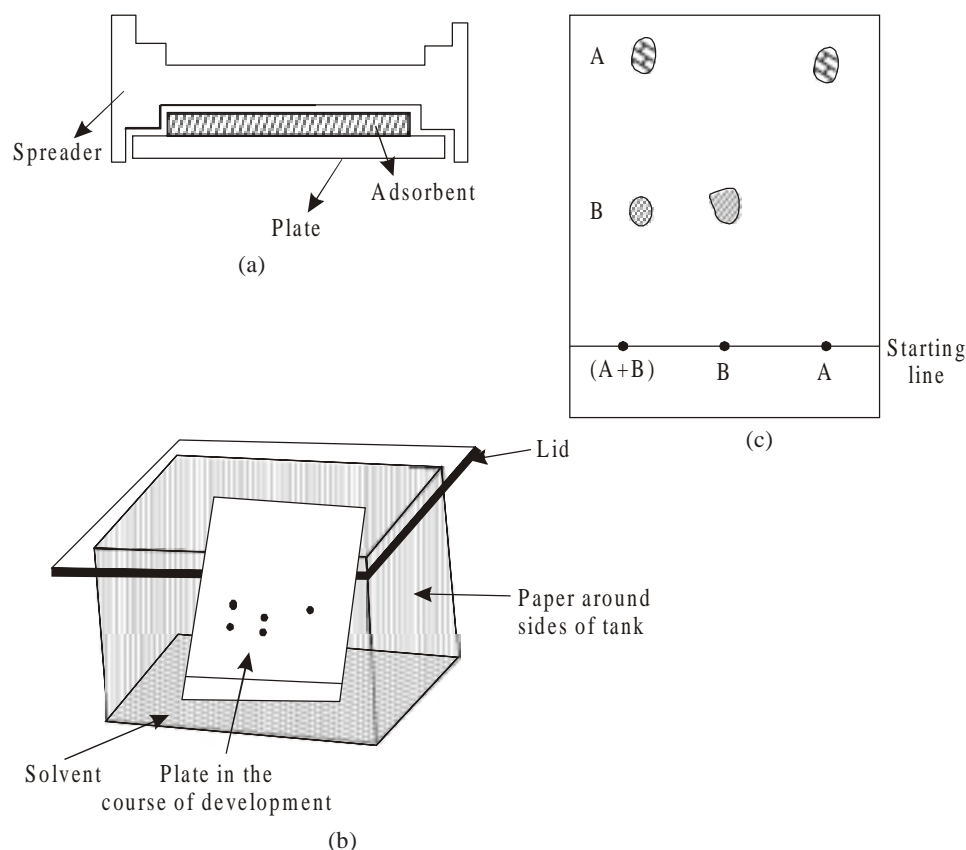
Figure 12.1 Thin-layer Chromatography (a) Device for spreading a layer of adsorbent; (b) Chamber for chromatographing with a solvent (aluent) at the bottom; (c) A developed plate showing mixture (A+B) having separated into its constituents B and A; spots marked (A+B), B and A on the starting line refer to those of the mixture and known compounds B and A respectively.

are excellent supports; commercial plates are prepared on metal and polymer foils and can be cut to size). The solid e.g. alumina, chosen as the stationary phase is mixed with liquid (usually, water) to obtain a slurry, which is spread uniformly on the plate. An excess of slurry (over the desired thickness) is removed with the aid of a roller [Figure 12.1 (a)], and the layer is dried in an oven at 100-150ºC. If there is poor adherence of the layer to the plate, a binding agent such as calcium sulphate may be added. For analytical purposes, a 0.25 mm layer is spread, and for preparative purposes, a layer from 0.75 to 2 mm. The plate thus prepared is referred to as a chromatoplate.

12.1.2 Running a Thin-layer Chromatogram

   Test solutions are applied to the chromatoplate as described under paper chromatography, except that the greater delicacy of the adsorbent layer on the

plate makes it necessary to take much more care. The test solution is applied from a micro-pipette on the adsorbent layer at a point on a line (the starting line) parallel to one end of the plate at a distance of 1 cm from that end. A known substance is applied as a spot at the same starting line at a distance of about 2 cm from the first point, and the other control substances are each placed 2 cm farther along the starting line. Development of the chromatoplate is usually carried out by the ascending method [Figure 12.1 (b)]. The end of the plate near the starting line is dipped in an inclined position into the chosen solvent (typica1 mobile phases used for TLC on silica gel, for example, are listed in Table 12.2) which is at the bottom of a glass chamber.

TABLE 12.2

Typical Mobile Phases used in Thin-layer Chromatography on Silica Gel

Mobile Phase	*Applications*
Chloroform/acetone	Steroids e.g. bile acids, estrogens, sterols
Chloroform/ethanol	Alkaloids e.g. belladona, morphine, opium, purine
Water	Vitamins A, D and E
Hexane/acetone	Vitamins B and C
Light petroleum/ether	Hydrocarbon oils, ester oils
Hexane	Chlorinated insecticides e.g. Aldrin, DDT, Heptachlor, Endrin

The air in the chamber is saturated with the solvent vapour. For keeping an approximately constant temperature, the walls of the chamber and its bottom are covered with strips of filter paper and the chamber is covered with close-fitting lid. Usually, the solvent front is allowed to ascend about 10 cm above the origin before the plate is removed. The solvent front is carefully marked with a sharp pencil. The solvent in the layer evaporates within a few minutes. Heat can be applied if necessary, and then the plate is ready for location of the compounds.

Though the usual and easier way to running a thin-layer chromatogram is the ascending method, various other techniques to suit individual needs or problems have been tried. As in paper chromatography, it is possible to carry out *descending* and *circular* chromatography. The flow of solvent from the reservoir to the layer is through a filter paper strip. In circular TLC it is possible to put the glass plate in such a way that the sorbent layer is either facing upwards or downwards.

In *horizontal TLC* also the sorbent layer could be facing upwards or downwards. The solvent is brought to the sorbent layer through filter strip of the same width as the glass plate.

Continuous TLC is useful for substances having small and close R_f values. It could be carried out in sandwich or specially constructed chambers. Continuous

TLC could also be done on horizontal plates. The principle is the same as that for horizontal TLC with the difference that the solvent at the other end is allowed to evoporate. Continuous TLC using the descending technque has been accomplished too. A new method has been developed where the mixture to be separated into its constituents is applied near the apex on thin-layer prepared on a triangular glass plate. Two different solvent mixtures are fed from different sides to the thin-layer and fractions collected at the base.

For the separation of substances having small R_f values, it is advisable to run the TLC plate more than once, drying the layer before it is developed again. If the solvents used for subsequent runs are the same as in the first run it is called *multiple* or *repeated development*, but when the solvents are different in the first and later runs, the technique is called *stepwise development*. These techniques are specially useful for mixtures which contain polar as well as non-polar substances. Polar solvent systems are usually employed for the first run and a non-polar solvent system for the later runs.

Multiple-dimensional TLC is a variant of multiple-development chromatography. For example, in one experiment the chromatoplate was devloped with propanol-ammonia (2 : 1) which carried fatty acids, cholesterol and their esters to the solvent front and resolved lecithins and polar lipids. The second run with chloroform-benzene (3 : 1) separated fatty acids and free cholesterols and carried the esters to the solvent front. The TLC plate was turned at an angle of 180º and developed with carbon tetrachloride which resolved the cholesterol esters. Using more than one plate and the same solvent for the first development but different solvents for the subsequent developments after rotating the plates at an angle of 90º, a large number of amino acids could be successfully resolved.

12.1.3 Location of the Spots

If coloured substances are involved, location of the spots which are visible is easily achieved. Procedures for locating the invisible spots in the case of colourless compounds are similar to those used in paper chromatograms, and Table 11.1, 'Chromogenic Reagents for visualizing Paper Chromatograms' holds good for TLC also.

Invisible spots of fluorescent substances can be located by exposure to ultra-violet light. The visualization is done by looking at the chromatogram under UV lamp when the substances show up as dark spots against a fluorescent background.

An advantage of most thin-layers is that, being of a purely inorganic nature, very corrosive reagents can be used for the location of compounds on them. An all-purpose reagent that can be used with thin-layer plates, but not with paper, is a dilute solution of potassium dichromate in concentrated sulphuric acid. Dichromate (yellow) is reduced to chromic sulphate (green) by most organic compounds and it is particularly useful for sugars. The vapour of sulphur trioxide, produced on warming fuming sulphuric acid, chars organic compounds and makes

them visible as dark spots on thin-layer plates. Strong oxidising agents may also be employed for locating the invisible spots. Application of a solution of potassium permanganate, for example, reveals the invisible spots of the colourless substance as spots of manganese dioxide on the developed chromatogram. Another very useful reagent is iodine vapour. The developed chromatogram is placed in a tank with a few crystals of iodine on the bottom and left for a few minutes. The iodine tends to accumulate at locations of the separated organic compounds so that one gets drak brown spots on a pale yellowish background.

In Inorganic Chemistry, typical spray reagents for cations include potassium iodine (0.2%, aqueous), hydrogen sulphide (saturated aqueous solution), ammonium sulphide (0.2 N, aqueous), quercetin (0.1%, alcoholic), 1-(2-pyridylazo)-2-naphthol (PAN) (0.2%, methanolic), oxine (8-hydroxyquinoline) (1% methanolic, view under visible and UV light), and sodium rhodizonate (0.5%, aqueous). Reaction with dithizone to produce coloured dithizonate chelates of many metals is particularly suitable if quantitative spectrometric analysis (*in situ* or after elution) is to be carried out. Anions are detected with bromocresol purple (0.1%, alcoholic), 1% ammoniacal silver nitrate + 0.1% alcoholic fluorescein/UV light, zirconium alizarin lake (0.1% in HCl solution), and ammonium molybdate (1%, aqueous) followed by $SnCl_2$ (1%) and HCl (10%). Typical detection limits range from 10 ng (10^{-9}g) to several μg (10^{-6}g).

Labelling enables species to be detected using radioactivity scanners and detectors with counting equipment. This is an especially valuable method for detection and quantitation of very low levels of inorganic material.

After carrying out visualization of invisible spots by any of these procedures, comparision of the distance travelled by each component of the test mixture with the distance travelled by known substances can be made, and the unknown substances are identified on the basis of the R_f values thus measured. Once this distance and hence the position of the spot of each componant of the mixture has been determined, the corresponding areas of the adsorbent can be separated without subjecting these to the aforesaid spot-location techniques and each componant eluted separately. The simplest method of elution consists in scraping off the appropriate area of the thin-layer with the tip of a spatula, followed by extracting the substance from this powder with a solvent.

12.1.4 Quantitative Analysis through TLC

TLC (PC, as well) does not provide quantitative information of the highest precision and accuracy. Quantitative thin-layer chromatography can be performed by applying a *known* volume of the sample to a chromatoplate and developing the plate, followed by locating and recovering the separated substance by elution as described above. This isolated material can then be quantitatively determined by any of the usual methods of quantitative analysis such as titration, microgravimetric procedures and colour reactions.

Estimation can also be made directly on the layer.

Estimation on the layer can be carried out by, amongst other methods, densitometry. A densitometer is a modified photometer in which the chromatogram replaces the usual glass cuvette. The chromatogram is slowly moved across the light path and the signal from the photoelectric detector is plotted by a pen recorder, the chart speed of which is synchronized with the movement of the chromatogram. The result is a trace which plots the absorbance value against the distance along the chromatogram. From the area under the relevant peak, the relative proportion of each of the constitutents of the separated mixture can be calculated.

Alternatively, direct quantitation on the layer is carried out by direct evaluation of the areas of the various spots.

The main dfficulties with area and density measurements lie in defining the boundaries of spots and controlling chromogenic reactions in a reproducible manner. Relative precision can be as good as 1 to 2%, but is more usually 5 to 10%.

Other methods of making quantitive estimation directly on the layer are: (i) Fluorimetry, (ii) spectrophotometry, (iii) radiation counting etc. or, in the simplest prcedure, by direct visual comparison.

12.2 Factors affecting R_f Values and Resolutions

Basically the R_f value of a solute is determined by its distribution ratio which in turn is dependent on relative solubilities for partition systems or relative polarities for adsorption systems. For example, if adsorption TLC is used to separate mixture of squalene, methyl oleate, cholesterol and α-tocopherol (vitamin E), then squalene, being the least polar, will move furthest and cholesterol, being the most polar, will remain close to the origin. Methyl oleate is less polar than α-tocopherol and will therfore be found between it and squalene.

Increasing the temperature increases R_f. Although the separation proceeds more quickly at elevated temperatures, resolution suffers because of increased rates of diffusion. In paper chromatography R_f values increase significantaly with an increase in the development temperature, whereas in adsorption TLC only small increases in R_f values are observed even with 20ºC rise. Strict temperature control is not necessary if samples and standards are run at the same time, although large fluctuations should be avoided. The quality of paper and thin-layer materials, and in particular the presence of impurities in them, determine the extent to which partition, adsorption, ion-exchange and exclusion participate in the sorption process. These factors affect R_f values in an unpredictable manner. Paper and thin-layers should be of uniform thickness, a condition readily achieved during paper manufacture but more difficult to attain in coating a plate. The overall thickness

of a thin layer should be between 0.2 and 0.3 mm; with thinner layers, local variations in thickness can result in appreciable variations in R_f values.

A stable atmosphere saturated with the vapour of the mobile phase is required to ensure reproducible R_f values. Unless saturation conditions prevail, solvent will evaporate from the surface of the paper or thin layer causing an increased solvent flow but slower movement of the solvent front; R_f values consequently increase. In practice, chromatograms are best developed in a sealed galss tank in which a saturated atmosphere has been produced by lining the walls with filter paper soaked in the appropriate solvent or solvent mixture. The range of the optimum amount of sample required to produce detectable spots with a minimum of spreading due to overloading has an upper limit of about 50 μg; with samples amount much larger than this, isotherms become non-linear and the R_f values alter significantly with sample size and resolution suffers because of increased tailing or fronting.

12.3 Advantages of TLC as a Separatory Technique

The relatively rapid spread of TLC is due mainly to the fact that it permits a relatively efficient separation in very short time and by simple and inexpensive means. Another advantage is the possibility of extensive analytical (qualitative and semi-quantitative) and preparative application, ranging from the detection of trace substances up to a separation of one gram of substance in a single operation, using readily available adsorbents, solvents and detection reagents. No less important is the fact that it may be used for monitoring of other methods of separation (e.g. distillation, column chromatography) and for aiding purification procedures like recrystallization etc. A large number of samples can be examined simultaneously. Further, its versatility (electrophoresis in a thin-layer, ion-exchange chromatography in thin-layer, gel filtration) has greatly enhanced its applicability. Owing to these advantages, the technique has overshadowed and, in many instances, supplanted paper chromatography, which is the other method that employs a planer arrangement. The resolutions on thin-layer chromatograms are often better than those obtainable on paper. The efficiency of a conventional TL chromatographic separation is around 1000 theoretical plates. Mention has already been made of the feasibility of the use of aggressive spray reagents like sulphuric acid for spraying of TLC plates with advantage for identification purposes. Spreading of spots by diffusion is reduced, as fibres are absent in the adsorbent used. One also obtains in TLC more compact spots with shorter solvent migration than that occurring in PC. This is the reason why TLC can be performed on a microscopic slide. Thin-layer chromatography has the unique advantage of allowing analysis in parallel. All the other chromatographies offer only serial analysis. A thin-layer chromatographic plate may contain strips of stationary phase along the length of the plate.

Paper chromatography, however, is advantageous in some respects. Chromatoplates are more difficult to prepare and handle in comparison with sheets of paper. Also it is usually easier to elute a compound from a piece of paper cut from a paper chromatogram than it is to scrape out an area of a thin-layer chromatogram for elution. Further, since thin-layer chromatograms cannot readily be run by the downflow technique, the practical length of thin-layer chromatogram is severely limited.

A series of precoated plates prepared from a variety of single or mixed adsorbants on glass, plastic, aluminium, and other supports are now commonly available. These precoated plates greatly simplify the use of thin-layer chromatography.

Thin-layer chromatography can also be employed for carrying out ion-exchange or gel filtration for which purpose thin-layer plates are coated with ion-exchange resins or cross-linked dextran gel.

By using somewhat thicker layers and wider plates, thin-layer chromatography can be used for preparative purposes. By developing several plates together, quite large amounts of material can be purified.

In conclusion, it can be said that both paper chromatography and thin-layer chromatography are useful techniques, and choice of procedure in a given case will depend on more than one factor.

12.4 Comparison of TLC and Liquid-solid Column Chromatography

The following observations can be made with regard to the experience with TLC and liquid-solid column chromatography.

(i) While thin-layer chromatography has the advantage of being a convenient and rapid analytical technique, it has the disadvantage of not being readily adaptable to preparative-scale separations involving, as it does, developing several plates together; (ii) though liquid-solid column chromatography can be employed effectively for large-scale searations, the technique suffers from the disadvantage that it is commonly quite time-consuming, four hours or more sometimes being required to elute the column.

Though, thin-layer chromatography earlier enjoyed a greater popularity over liquid-solid column chromatography because of the former being a convenient and rapid analytical technique, it is to be emphasized that liquid-solid column chromatography in its modernised versions viz. flash chromatography, dry-column chromatography, dry-column flash chromatography, which are detailed below, has gained its rightful place among chromatographic techniques in recent decades. These modified versions of liquid-solid column chromatography give separations with the efficiencies and economy in time and solvents as are attained with thin-layer chromatography combined with the advantages of the scale offered by column chromatography and minimal material loss.

Flash Chromatography

This technique has been described by W.C. Still *et al.* and allows for the rapid separation (10 to 15 minutes) of mixtures of components having a difference (ΔR_f) in R_f values greater or equal to 0.15 and with a sample loading of 0.01-10.0 g, although the latter is not regarded as being a limiting value. The essential features are the use of silica gel 60, 40-63 μ (40-230 mesh), in a column through which the solvent is passed by the application of positive air pressure applied to the column head; resolution is sensitive to eluent flow rate, and this is 50.8 ± 2.5 mm/minute. The solvent systems used are ethyl acetate/light petroleum, acetone/light petroleum or acetone/dichloromethane.

The apparatus consists of a chromatography column and a flow controller. The flow controller [Figure 12.2 (a)] is a simple variable bleed device for precise regulation of the elution rate and is constructed from a glass/Teflon needle valve. Eluate fractions are collected in test tubes (20 × 150 mm), and separated components detected by TLC.

The general procedure is as follows.

(a)
Flow controller

(b) Chromatography column

Figure 12.2 Flash chromatography

First a low viscosity solvent system (e.g. ethylacetate/light petroleum b.p. 30-60ºC) is found which separates the mixture and moves the desired components on analytical TLC to an R_f of 0.35. If several compounds are to be separated which run very close on TLC, the solvent is adjusted to put the midpoint between the components at R_f 0.35. If the compounds are widely separated, the R_f of the less mobile component is adjusted to 0.35.

Having chosen the solvent, a column of appropriate diameter [Figure 12.2 (b)] is selected and a small plug of glass wool is placed in the tube (A) connecting the stopcock to the column body.

Next a smooth 3-mm layer of 50-100 mesh sand is added to cover the bottom of the column and dry 40-63 μ silica gel is poured into the column in a single portion to give a depth of c 140 mm. With the stopcock open, the column is gently tapped vertically on the bench top to pack the gel. Next a 3-mm layer of sand is carefully placed on the flat top of the dry silica-gel bed and the column is clamped for pressure packing and elution. The solvent chosen as above is then poured carefully over the sand to fill the column completely. The needle valve (B) of the flow controller is opened all the way and the flow controller is fitted tightly to the top of the column and secured with strong rubber bands. The main air-line valve leading to the flow controller is opened slightly and a finger is placed fairly tightly over the bleedport (C). This will cause the pressure above the adsorbent bed to climb rapidly and compress the silica gel as solvent is rapidly forced through the column. It is important to maintain the pressure until all the air is expelled and the lower part of the column is cool; otherwise the column will fragment and should be repacked unless the separation is a trivial one. Particular care is necessary with large diameter columns. The pressure is then released and excess eluent is forced out of the column above the adsorbent bed by partially blocking the bleedport (C). The top of the silica-gel column should not be allowed to run dry. Next the sample is applied by pipette as 20-25 per cent solution in the eluent to the top of the adsorbent bed and the flow controller is briefly placed on top of the column to push all of the sample into the silica gel. The solvent used to pack the column is ordinarily reused to elute the column. The walls of the column are washed down with a few millilitres of fresh eluent, the washings are pushed into the gel as before and the column is carefully filled with eluent so as not to disturb the adsorbent bed. The flow controller is finally secured to the colunm and adjusted to cause the surface of the solvent in the column to fall 51 mm/minute. This seems to be the optimum value of the flow rate for most low-viscosity solvents for any diameter

TABLE 12.3

Column diam. (mm)	Vol. of eluent (ml)	Sample typical loading (mg)		Typical fraction size (ml)
		$\Delta R_f \geqslant 0.2$	$\Delta R_f \geqslant 0.1$	
10	100	100	40	5
20	200	400	160	10
30	400	900	360	20
40	600	1600	600	30
50	1000	2500	1000	50

with the 40-63 μ silica gel. Fractions are collected until all the solvent has been used (Table 12.3 is used to estimate the amount of solvent and sample size). It is best not to let the column run dry since further elution is occasionally necessary. Purified components are identified as described above by TLC. If the foregoing instructions are followed strictly, there is little probability of separation failing.

Dry-column Chromatography

The technique of dry-column chromatography involves the use of a dry column. Glass or nylon columns are used. The dry adsorbent is slowly poured into the column while gently tepping the column to ensure uniform packing. Generally speaking, about 70 grams of adsorbent are required per gram of mixture for 'average' separations and about 300 grams of adsorbent per gram of mixture for 'difficult' separations. This can be contrasted to the approximately 30 grams of adsorbent per gram of mixture for 'wet' column chromatography. The mixture to be separated is adsorbed on a separate portion of adsorbent and this is added to the top of the column. Solvent is then allowed to flow down the column until it has just reached the bottom of the column. Further development of the column is now stopped, the adsorbent is removed from the column, and the bands of separated components are isolated to be followed by extraction of each band of the adsorbent with an appropriate solvent.

In this technique it is preferable to use a pure solvent or a single mixture of solvents rather than using a series of solvents. The choice of solvent is greatly facilitated by carrying out a prior separation of the components of the given mixture by TLC. Thus, a number of pure solvents and mixtures of solvents can be tested rapidly on TLC plates to find out the solvent of choice for the dry-column chromatography—the solvent that gives the best results on a TLC plate will also give the best separation with a column.

An interesting example of application of the technique is that of separation of the isomeric compounds I and II reported in 1975 by T.C. Morill and co-workers, who used a silica-gel dry column for the purpose. The achievement is quite impressive considering the very slight structural differences of compounds I and
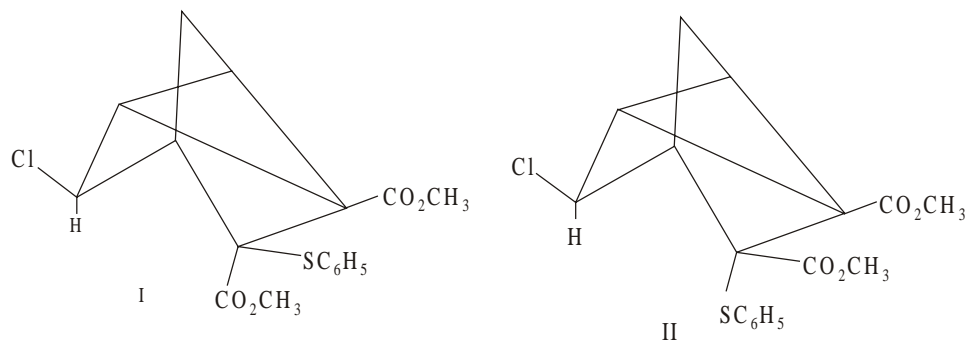
II. Thus only the slightly different dipolar attractions of I and II to the silica gel of the dry column result in the separation of the two components.

Dry-column Flash Chromatography

This technique has been developed from flash chromatography by L.M. Harwood. The principal feature is that suction is applied to the column packing, and eluting solvents are added in predetermined volumes with the column being allowed to run dry before the next fraction is added. The apparatus is simple and easy to operaters.

The general procedure is as follows.

The apparatus (Figure 12.3) is set up for filtration using a porosity 3 cylindrical sinter. Table 12.4 gives guidlines for the choice of sinter size and amounts of silica, sample and solvent. Columns longer than 55 mm are neither practical nor necessary, since reduction in efficiency may be observed on large-scale set-ups.

The sinter is filled to the lip with silica gel 60 (400-230 mesh). Suction is applied while pressing the silica with extra care at the circumference. Still pressing the silica and by tapping the sides of the sinter firmly, a totally level silica surface is obtained leaving a head space for the addition of solvents.

To water aspirator
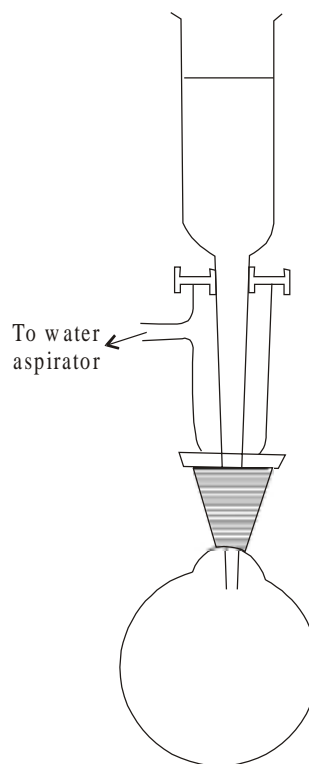
Figure 12.3 : Dry-column flash chromatography

TABLE 12.4

Sinter size, diam-/ length (mm)	Silica (wt. g)	Sample	Solvent fraction (ml)
30/45	15	15-500 mg	10-15
40/50	30	0.5-3 g	15-30
70/55	100	2-15 g	20-50

The column is pre-eluted under vacuum, using the least polar combination of the required solvents in which the product mixture is readily soluble. If possible, a single least polar component (e.g. pentane for pentane/ether gradient elution) is used. Combinations of pentane (hexane), ether, ethyl acetate and methanol are adequate for most separations. If the silica has been packed correctly, the solvent front will be seen descending in a horizontal line. If channelling occurs, the

column is sucked dry and the packing procedure repeated. The surface of the silica gel is kept covered with solvent during pre-elution until solvent is seen passing into the receiver. Then the silica is allowed to dry under suction. The sample mixture (dissolved in minimum amount of the pre-elution system) is loaded evenly on to the surface of the silica and the constituents eluted by adding successive portions of increasing-polarity solvent mixtures, allowing the column to be sucked dry after each addition (the silica surface being only slightly disturbed on addition of solvent). Generally a solvent gradient whereby the more polar component is increased by 5-10 per cent is the most convenient. Under these conditions, the product is usually eluted by that solvent mixture in which it would have R_f 0.5 on TLC. For quantities greater than 100 mg, elution is often accompained by frothing on the underside of the sinter.

12.5 High Performance Thin-layer Chromatography (HPTLC)

Recent changes in the practice of TLC have led to its wider acceptance as a powerful separating tool for multicomponent trace level analysis of which a typical example is the analysis of more than 20 common metals on silica gel S with three developing solvents viz. (i) acetone 3N HCl (99:1) for separation of Ni, Co, Cu, Fe, Pb, Mn, Cr and As; (ii) methanol-butanol 35% HCl (8:1:1) for Ba, Sr, Ca, Mg, Al, NH_4^+, Na, K and Li; and (iii) butanol-benzene–IN HNO_3 – INHCl–(50 : 46 : 2.6 : 1.4) for Sb, As, Cd, Sn, Bi, Zn and Hg. The expectations accompanying these modifications in the practice of TLC have given birth to a new expression—high performance thin-layer chromatography (HPTLC), analogous to the change of name applied to liquid chromatography from LC to HPLC when liquid chromatography underwent a similar expansion in performance capabilities. The performance breakthrough in TLC was not a result of any specific advance in instrumentation or materials, but was rather a culmination of improvements in practically all of the operations of which TLC is comprised.

Improvement in the quality of the adsorbent layer and methods of sample application, and the availability of scanning densitometers for *in situ* quantitative analysis were all important developments in the evolution of HPTLC. The new HPTLC plates are prepared from specially purified silica gel with average particle diameters of between 5 to 15 micrometers with a narrow particle size distribution. These plates give HETP values of about 12 micrometers and a maximum of about 5000 usable theoretical plates in any separation. By contrast, conventional TLC plates prepared from silica gel with average particle diameters of 20 micrometers and a rather broad particle size distribution gave HEPT values of about 30 micrometers and a maximum of approximately 600 usable theoretical plates. The new HPTLC plates can provide an increase in performance approaching an order of magnitude over conventional TLC plates. Thus, it is possible to carry out separations on HPTLC plates that were not posible by conventional TLC, and also

to carry out those separations which are presently carried out by conventional TLC in much shorter times.

Due to the lower sample capacity of the HPTLC layer the amount of sample applied to the layer is reduced. Sample volumes of 100-200 nl (10^{-9}1), which give starting spots of 1.0-1.5 mm diameter, are typical. After developing, separated spots distanced at 3-6 cm and with diameters of 3-6 mm are obtained. As spots are more compact than those obtained in conventional TLC, the lower sample capacity of the plate does not present any problems and, on the contrary, results in detection limits ten times better than those for conventional TLC. The compact starting spots in HPTLC also allow an increase in the number of samples which may be applied along the edge of the plate. Depending on the method of development and the size of the plate, as many as 18 samples on a 10×10 cm plate or 36 samples on 10×20 cm plate can be simultaneously separated. Linear development from two directions (i.e. simultaneously from opposite edges towards the middle) doubles the number of samples which can be separated. Although the mobile phase velocity is greater for conventional TLC plates than for HPTLC plates, still analysis times are considerable shorter for HPTLC since longer migration distance are needed in TLC for an equivalent separation. Typical migration distances in HPTLC are 3-6 cm while development times are an order of magnitude lower than those in conventional TLC.

In summary, HPTLC is a more rapid, efficient and sensitive technique than conventional TLC and thus represents a considerable advance in the practice of thin-layer chromatography.

12.6 Comparison of High Performance Liquid Chromatography and HPTLC/TLC

If the comparison between the two techniques were to be based on the criterion of HETP (height equivalent to theoretical plate) values, HPLC can easily be shown to be a more efficient technique than HPTLC. The conventionally packed HPLC columns used in most analytical laboratories are capable of providing approximately 10,000 theoretical plates. Special HPLC columns, such as capillary columns, are capable of even greater efficiency. However, HPTLC gains certain advantages, which are outlined below, because it is an open-bed system while HPLC is a closed-bed system.

In HPLC the mobile phase velocity is, upto a limit, controllable electronically. This limit is established by the maximum pressure gradient that can be maintained across the colunm. For HPTLC, the mobile phase velocity is governed by the capillary forces which transport the solvent through the sorbent bed. This disadvantage is partially offset by the fact that the development process is different for the two techniques. In HPLC the development occurs by elution chromatography, that is each sample component must travel the complete length of the column bed and the total separation time is determined by the time required

by the slowest moving component to reach the detector. The HPTLC process is governed by development chromatography. The total time for the separation is the time required for the solvent to migrate a fixed or predetermined distance and is independent of the migration distance of the sample components. For most analyses, only a few components of the sample are of interest and the mobile phase is selected so as to provide the necessary separation. The remainder of the sample material can be left at the origin or moved away from the region of maximum resolution. This results in considerable saving of time. 'Poisoning' in an HPLC column occurs only too easily. There is no possibility of such an occurrence in the case of a TLC plate. Such poisining results in a considerable loss of time while components accumulated at the head of the column are being completely eluted. This may result in permanent damage (i.e. loss of resolution) to the column if these samples cannot be removed. TLC plates are disposed of at the conclusion of each separation and are thus immune to the aforesaid problems.

Detection in HPTLC, unlike that in HPLC, is a static process, being completely separated from chromatographic development. Consequently, the selection of the mobile phase does not limit the choice of the detector. For example, UV absorbing solvents cannot be used with UV detectors in HPLC. In HPTLC the solvent is completely evaporated between development and measurement so that it does not influence the detection process.

Perhaps the most important feature of development chromatography is that the sample is separated by distance rather than time. This freedom from time constraints permits the utilisation of any of a variety of techniques to enhance the sensitivity of detection, such as reactions which increase light absorbance or fluorescence emission and wavelength selection for optimum response of each compound measured. The separation can be scanned as many times as desired, at a variety of wavelengths, and a complete UV visible or fluorescence spectrum can be easily plotted out for each component. Thus, the detection process in HPTLC is more flexible and variable than that for HPLC. Detection limits under optimum conditions are approximately the same for the two techniques.

Because of the nature of the method of development, analysis in HPLC is by necessity performed in a sequential manner. Each sample must individually undergo the same sequence of injection, separation, detection and column re-equilibration. For a series of n samples, the total time for the analysis will be n multiplied by the time for each individual analysis. HPTLC techniques permit simultaneous sample analysis with the possibility of substantially reducing the time required for the analyses of a large group of samples.

In conclusion, for individual samples HPLC can provide greater separating power than HPTLC. However, this disadvantage is offset to some extent by the greater simplicity of performing the actual experiment in HPTLC in view of the availability of a larger number of operating variables to choose from, and detection flexibility of HPTLC When a high throughput of similar samples is required, then

HPTLC can provide considerable savings in time. In essence, the two techniques complement one another and, therefore, selection should be based on the type of problem to be solved.

12.7 Applications of TLC/HPTLC

Table 12.2 exexmplified applications of thin-layer chromatography in the realms of Organic Chemistry and Biochemistry and separations of Inorganics carried out by TLC or its high performance version are illustrated below in Table 12.5

TABLE 12.5

Typical Separations of Inorganics by TLC/HPTLC

Layer	Mobile phase	Detection spray agent	Applications
Silica gel G	Ethanol-acetic acid (100 : 1)	1.5% violuric acid	Alkali metals (Li^+, Na^+ . K^+)
Silica gel G	Methanol-conc. NH_4OH–10% trichloroacetic acid –H_2O (50 : 15 : 5 : 30)	1% aqueous ammonium molybdate followed by 1% Sn Cl_2 in 10% HCl	Phosphates, $H_2PO_2^-$, $H_2 PO_3^-$, $H_2PO_4^-$, $H_2P_2O_7^-$
Silica gel G	Butanol–1.5 NHCl–2, 5-hexanedione (100 : 20 : 0.5)	2% KI, NH_3 vapour, H_2S gas	Hg^{2+}, Cd^{2+} Pb^{2+}, Cu^{2+}
Cellulose	Dioxane-conc. HCl–H_2O (58 : 12 : 30) and methanol-conc. HCl–H_2O (73 : 12 : 15)	Conc. NH_4OH followed by 2% oxine in ethanol and viewing under 366 nm UV light	Alkaline earths (Be^{2+}, Mg^{2+}, Ca^{2+} Sr^{2+}, Ba^{2+}, Ra^{2+})
Dowexi-cellulose (D.1)	1M aqueous $NaNO_3$	Detection via radio-activity	Halogens (Cl^-, Br^-, I^-)
Silica gel G	Acetone-conc. HCl-2, 5-hexanedione (100 : 1: 0.5)	NH_3 vapour, 0.5% oxine in 60% ethanol and viewing under 366 nm UV light	Fe^{3+}, Zn^{2+}, Co^{2+}, Mn^{3+} Cr^{3+}, Al^{3+}, Ni^{2+}
Silica gel G	(A) Methanol– n-propanol-conc. NH_4OH–H_2O (10 : 10 : 1 : 2)	1% Aqueous bromocresol green	Sulphates and polythionates (A) $S_2O_8^{-2}$, SO_3^{2-} $S_2O_3^{2-}$, SO_4^{2-}

Contd.

Layer	Mobile phase	Detection spray agent	Applications
	and (B) methanol-dioxane-conc. NH_4OH–H_2O (3 : 6 : 1 : 1)		and (B) $S_5O_6^{2-}$, $S_4O_6^{2-}$, $S_3O_6^{2-}$ $S_2O_6^{2-}$, $S_2O_3^{2-}$
Silica gel H	H_2O-glycol mono-methyl ether-methylethyl ketone-acetone-NH_4OH (40:20:20:20:0.15)	Natural colour 20% ammonium peroxydisulphate solution	EDTA complexes of Co, Cu, Ni, Mn, Cr, Fe
Silica gel G	Benzene-methylene chloride (5:1)	Detection by natural colour	Dithizonate complexes of Hg^{2+}, Zn^{2+}, Cu^{2+} Bi^{3+}, Pb^{2+}, Cd^{2+}

■

13

Gas Chromatography (GC)

Gas chromatography is an analytical technique for rapidly separating mixtures of volatile substances with an inert gas stream flowing over an adsorbent. The technique was introduced by James and Martin in 1952 and they demonstrated the advantages of this new method on their study of the separation of volatile fatty acids. Almost simultaneously Janak published his paper on the separation of hydrocarbons by gas-liquid chromatography.

Usefulness of GC as a separatory technique cannot be over-emphasized in view of its several other advantages, besides the one already referred to above, namely the rapidity with which it brings about separations. The amount of sample used needs to be indeed small. With the elution technique used in gas chromatography, the column is ready for the next sample as soon as the previous one has passed through. Columns ordinarily have long life and it is not uncommon to have a column perform satisfactorily for a year or two. The usual apparatus employed is essentially simple and inexpensive but for the electronic recorder needed for the automatic recording of the chromatograms which certainly is an expensive component.

13.1 Underlying Theory of the Technique

Gas chromatography is essentially similar to liquid chromatography using columns where the mobile liquid phase is replaced by a moving gas phase. Gas chromatography is divided into two major categories:

Gas solid chromatography (GSC) where the adsorbent is a solid of large surface area, and *gas-liquid chromatography* (GLC) where the adsorbent is a non-volatile liquid coated on an inert solid support. The moving gas phase or carrier gas in each method is either nitrogen, argon, hydrogen or helium, the choice being determined by availability, purity and the type of detector employed. The gas is made to flow at a constant rate through a packed column consisting of a tube of small diameter containing the adsorbent. The technique of *gas-liquid* (partition) chromatography is the more important one because of its greater utility.

When a mixture is injected into the carrier gas stream flowing through the packed column, the components move through the column at rates dependent on their respective volatilities and interaction with the non-volatile liquid phase. The various molecules dissolve and revaporize as they pass down the column. Each

particle of the column packing acts as a *partitioning unit*, so that many thousands of individual partitions occur during the passage of the mixture through the column. Each partition is governed by the distribution law i.e. the ratio:

$$\frac{\text{Concentration of one component in the gas phase}}{\text{Concentration of the same component in the liquid phase}}$$

is a constant, the value of which depends on the nature of the species and the temperature. Thus, although there may be only a very small difference in the distribution constants of the components of a mixture, a complete separation is achieved because so many partitions occur.

In *gas-solid* chromatography *adsorptive processes* on the solid phase perform the function of retarding the various components in analogy with the retardation arising from the partitioning process occurring in gas-liquid chromatography.

Gas chromatography brings about separation of constituents of complex mixtures much more rapidly in comparison with liquid chromatography, and qualitative and quantitative analyses (if required) are usually accomplished by gas chromatography in a few minutes. This is so on account of the following two factors:-

(i) Low viscosity of the gas compared with a liquid mobile phase.
(ii) Difference between diffusion rates in a gas and in a liquid, this difference being very marked as is illustrated by Table 13.1, which compares gaseous and liquid diffusion coefficients for a few compounds.

TABLE 13.1

Compound	Diffusion coefficient, cm^2/sec		Ratio, air/H_2O
	In air	In water	
Ethyl alcohol	0.119	1.0×10^{-5}	1.19×10^4
Butyl alcohol	0.090	0.77×10^{-5}	1.17×10^4
Acetic acid	0.133	0.88×10^{-5}	0.66×10^4
O_2	0.206	1.80×10^{-5}	0.875×10^4
CO_2	0.164	1.77×10^{-5}	0.97×10^4

It is apparent that diffusion is approximetely 10,000 times as fast in air as in water. Since the rate at which a chromatogram can be developed is dependent on the rate at which diffusional equllibrium can be established between the mobile phase and the stationary phase, the rate at which gas chromatograms can be run is much greater than the rate at which liquid chromatograms can be run. Development rates for liquid chromatograms vary from 0.01 to 1.0 ml per min per sq.cm. In gas chromatography, flow rates range from 50 to 1,000 ml per min per sq cm. Because of these rapid flow rates, it is possible to obtain rapid elution of

compounds having very low values of effective distribution coefficient (meaning that the solute moves slowly compared to the solvent front).

The most important aspect of gas chromatography is the fact that under a given set of operating conditions, a particular compound has a constant value of the retention time. The retention time (t_r) is measured in terms of the distance along the abscissa from the injection pip to the peak of the component band. The retention time is the principal constant utilized for the identification of compounds in gas chromatography. Another constant that may be used is the retention volume (V_R), which is carrier gas volume at column temperature which emerges from the column at outlet pressure in the retention time, t_r. On a given column the peak width is proportional to the retention volume i.e. peaks gradually broaden from beginning to end of the chromatogram.

13.2 Factors contributing to the Efficiency of GC Columns

A somewhat detailed discussion of these factors follows.

(1) The flow of gas through the column and the diffusion of the solute in both gas and liquid all influence the solute band-width and, hence, efficiency and resolution. Early in the history of GC it was shown (by van Deemter and co-workers for packed columns, and by Golay for capillary columns) that H, height equivalent to a theoretical plate (HEPT) depends on the average linear gas velocity ($\bar{u}$) through the column according to Equation 13.1:

$$H = A + \frac{B}{\bar{u}} + C_G \cdot \bar{u} + C_S \cdot \bar{u} \qquad \qquad ...(13.1)$$

The term A represents the plate height contribution arising from the effect of the multiple gas pathways through a packed column bed. This term is given by

$$A = 2 \lambda d_p$$

where λ is the packing uniformity, and d_p is the particle diameter. For the capillary column A = O, because of the absence of a solid support.

The term B represents plate height contribution from longitudinal diffusion in the gas phase, this term being proportional to the diffusion coefficient (D_G) of the solute in the gas.

The C terms, namely C_G and C_S, in Equation 13.1 express the effect of resistance to mass transfer in the gas and liquid phases respectively.

$$C_G \propto \frac{d_p^2}{D_G} \left(\text{packed column}\right)$$

or
$$\left[\propto \frac{r^2}{D_G} \left(r, \text{capillary column radius}\right) \right]$$

For rapid diffusion that occurs through the gas phase and with smaller particles used as column packing, transfer through the gas phase will be rapid enough to reduce band broadening, but owing to the difficulty in packing such columns uniformly, the eddy diffusion term increases with a resultant lowering of the column efficiency.

Slow diffusion in the liquid spreads out the band by leaving solute behind, and

$$C_S \propto \frac{d_f^2}{D_S}$$

where d_f is the liquid film thickness and D_S is the diffusion coefficient for the solute in the liquid. Since the diffusion coefficient of the solute in the liquid phase varies inversely with viscosity, the column efficiency is improved by the use of low viscosity liquid phase.

The relative magnitudes of the different terms in Equation 13.1 for GC are shown in Figure 13.1. At low $\bar{u}$, the B term is large, but quickly diminishes with increasing $\bar{u}$; and C_G and, to a lesser extent, C_S then dominate.

In gas-solid chromatography, the kinetics of adsorption and desorption determine the C terms in Figure 13.1.

The overall curve of Figure 13.1 is a hyperbola—the so-called van Deemter curve. The smallest value of H is H_{min}, at which $\bar{u}$ is optimum, $\bar{u}_{opt}$. The greater $\bar{u}_{opt}$, the faster a sample can be analysed and, in general,

Fig. 13.1 : Relative magnitudes of the different terms in the van Deemter equation in GC

$\bar{u}_{opt}$ will be higher for a low density gas, such as helium, but H_{min} will be a little more favourable for a denser gas such as nitrogen.

(2) The value of the partition ratio of the solute between the gas and liquid phases varies inversely with temperature and, and as both C_G and C_S are functions of the partition coefficient, it is these terms which are principally responsible for the change in the column efficiency with a change in temperature. There will also be small change in the value of the term B as a consequence of an increase in temperature. The overall effect of temperature upon column efficiency is extremely complex but, in general, the efficiency is increased by lowering the temperature of

the column. However, as a result of the lower temperature, the retention of the solute on the column increases and thereby gives a greater opportunity for the band to broaden.

(3) The temperature of the sample injector is also important for high column efficiency. To obtain a narrow input distribution of sample on to the column, it is necessary to have the injection temperature higher than the boiling point of the least volatile component of the sample, thus ensuring rapid and complete vaporization. As the temperature of the injector block is further increased, the column performance improves until a temperature is reached at which the breadth of the input distribution is small compared with the broadening effect of the column. Beyond this critical temperature no further improvement in column efficiency can be attained by altering the injection temperature.

(4) The introduction of large samples at low injection temperatures results in considerable band broadening since complete vaporization of the sample takes longer and the total heat of vaporization is high. Hence the amount of sample which can be efficiently separated is limited. The sample capacity of a column is the amount of sample which can be applied without more than 10% loss in column efficiency and is directly proprotional to the amount of stationary phase per unit length of column. It is consequently proportional to the square of the column diameter. The effect of overloading the column is observed as a loss of resolution with band broadening and an asymmetry in the band shape with a resultant apparent change in the retention of the solute by the column. This loss in column performance originates in two factors. *Firstly*, because of the finite volume of the vapour injected the input distribution even at high temperatures is broadened by large samples, and *secondly*, it is more difficult for the vapour zone of a large sample to attain a dynamic equilibrium between the gas and stationary phases. If it is assumed that the sample is completely vaporized i.e. the column temperature is above the boiling point of the sample, then the excess of sample beyond its
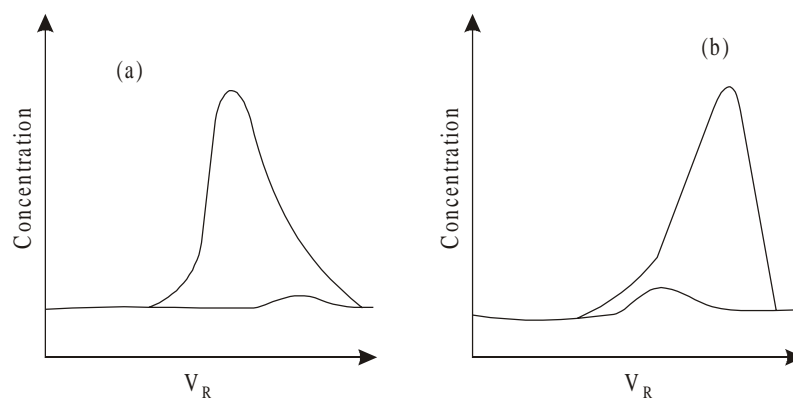
Figure 13.2 Peak shapes and retention volumes in non-linear chromatography

solubility limit in the stationary phase moves at the same velocity as the carrier gas. This produces a band [Figure 13.2(a)] with a sharp profile and a diffuse tail. The converse effect is observed if the column temperature is below the boiling point of the sample. The excess of sample remains in a condensed state and its velocity through the column depends entirely upon its solubility in the stationary phase. The bulk of the sample therefore travels more slowly than the carrier gas and the eluted band has a diffuse profile and a sharp tail [Figure 13.2(b)]. In both situations the observed position of the band maximum differs from the true value as measured for an infinitely small sample. As is to be expected, the column temperature has a considerable effect upon the band shapes. There is a decrease in the asymmetry of the band as a result of the higher solubility of the solute in the stationary phase at the higher temperature.

(5) For a given number of plates the retention volume and the peak width are proportional to the effective volume of each plate i.e. for two columns of identical plate number, the column with the smaller HEPT, H, that is, the shorter column will have the smaller peak width. The amount present at the peak maximum will be unchanged but the volume of gas required to elute the peak will be less, the concentration in that gas will be higher, and the recorded peak height will be greater. In assessing the efficiency of a column the HEPT is important in addition to the plate number. Since H decreases with decrease in d_f (liquid film thickness), as is shown by Equation 13.1 and d_f in its own turn is dependent upon the ratio of the liquid phase to solid support, a small proportion of stationary phase (so that the film thickness is reduced) improves the column efficiency.

(6) For a given retention volume, the peak width is proportional to $\dfrac{1}{\sqrt{n}}$ so that of two columns, the one with the higher plate number gives the sharper peak and a better separation between substances with retention volumes close together. It is therefore advantageous to increase the length of the column for a difficult separation.

(7) The variation in velocity should be small i.e. the ratio $\dfrac{p_i}{p_o}$ (p_i = inlet pressure, p_o = outlet pressure) must be as small as possible. An increase in the column length leads to an increase in the ratio, for example, if the outlet is at atmospheric pressure, a larger column requires a higher inlet pressure. For a given flow-rate, however, the pressure drop, p_i–p_o, is not very dependent upon the actual pressure, and it may therefore be advantageous to reduce the ratio $\dfrac{p_i}{p_o}$ by increasing the outlet pressure p_o.

(8) A compromise has to be made between the improved column performance which is theoretically possible using smaller diameter particles, and the loss of

performance observed in practice, resulting from the difficulty encountered in packing the smaller particles evenly. However, whatever size is chosen, the range of particle sizes should be as narrow as possible to prevent segregation of large and small particles taking place in the column. In its turn, occurrence of such a segregation will create zones of different velocities in the column whereas efficient performance by the column requires that variation in gas velocity be small and also the column be operated at the optimum gas velocity.

The results of the survey runs may show that, irrespective of the type of column used or of the operational parameters, not all of the components of the mixture are eluted. It may also be found that, as a result of strong interaction between the stationary phase and the solute, tailing of certain bands is prevalent or that the resultant extended retention time on the column has caused excessive thermal or catalytic decomposition of the solute. These undersirable effects can often be obviated by the conversion of the components into more volatile and usually less polar derivatives. Some examples of such conversions are given below in Table 13.2.

TABLE 13.2

Illustrative Derivations for GLC

Class of compound	Reagent	Derivative
Carboxylic acids	CH_3OH : HCl or BF_3 CH_2N_2 $ClCH_2CH_2OH$: BF_3 or HCl	Methyl ester Methyl ester β-chloroethyl ester (used for low MW acids)
Alcohols and phenols	Hexamethyldisilazane (HMDS) + trimethyl chlorosilane (TMCS) N, O-bis (trimethylsilyl) acetamide (BSA) or N, O-bis (trimethylsilyl) trifluoroacetamide (BSFA) $(CH_3CO)_2O$: pyridine $(CF_3CO)_2O$ $(CF_3CF_2CO)_2O$ Trifluoroacetylimidazole	Trimethylsilyl ether Trimethylsilyl ether Acetate Trifluoroacetate Pentafluoropropionate Trifluoroacetate
Carbohydrates	(i) MeOH : HCl (ii) HMDS : TMCS (i) $(CH_3CO)_2O$: pyridine (ii) MeOH : HCl	Polytrimethysilyl ethers of methyl acetal Polyacetyl esters of the methyl acetal

Contd.

Class of compound	Reagent	Derivative
Amino compounds: Primary and secondary amines	HMDS + pyridine	Monotrimethysilyl amine
	BSA	Mono- and bis-trimethylsilylamine
	CF_3COCH_3	Schiff's base
	$(CH_3CO)_2O$: pyridine	Acetylamino compound
	$(CF_3CO)_2O$	Trifluoroacetylamino compound
	$(CF_3CF_2CO)_2O$	Pentafluoropropionyl amino compound
	Trifluoroacetylimidazole	Trifluoroacetylamino compound
Amino acids	(i) $(CF_3CO)_2O$	
	(ii) CH_2N_2	Trifluoroacetylamino methyl esters
	TMCS on Na salt	Trimethylsilylamino trimethylsilyl ester
	Trimethylsilyldiethylamine (TMDA)	Trimethylsilylamino trimethylsilyl ester
Amino alcohols	(i) HMDS + TMCS	
	(ii) $(CF_3CO)_2O$	Trifluoroacetylamino trimethylsilyl ether
	(i) HMDS	
	(ii) CH_3COCH_3	Trimethylsilyl ether of the imine

The preceding discussion may be translated into the following summary in respect of the factors that contribute to the high efficiency of GC columns.

(1) Low ratio of inlet pressure to outlet pressure.
(2) Operation at the optimum practical gas velocity (found by determination of column performance in terms of HEPT which in turn is a function of gas flow-rate).
(3) The use of a dense carrier gas (although there may be practical reasons why a light gas is preferred even though high efficiency is sought).
(4) Small column dimensions.
(5) A small proportion of stationary phase.
(6) A small sample size—for example, it is suggested that when less than 5% of stationary phase is used, the sample should not contain more than 5μg of any one component.
(7) A small range of particle sizes of the support.
(8) A study of the apparatus and technique to ensure that their contributions to zone spreading are minimal—correct injection technique is most

important. [Regarding designing of apparatus for gas chromatography, it may be commented that band-broadening other than that in the column is negligible in GC, unlike it is in LC wherein the apparatus has to be designed so as to keep volumes in the extra-column regions (namely, the injection system, the connecting tube and the detector) of the liquid chromatograph to the minimum].

13.3 Working of the Technique

13.3.1 Columns for GC

The column consisting of tube loaded with the appropriate packing is surrounded by a suitable jacket for maintaining constant temperature environment.

Packed columns are usually constructed from glass, copper, stainless steel, or aluminium tubing and those used for routine analysis usually have an internal diameter of between 1/12 in and 3/8 in (2 to 9 mm). Glass as column tubing has the advantages of being chemically inert, and of allowing inspection of the state of packing both during the preparation of the column and subsequently after use—it has been extensively used in laboratory-built equipment. Copper tubing may sometimes react with components of samples being analyzed or exert catalytic effect on decomposition, and in such circumstances stainless steel tubing is usually to be preferred—it is the material most frequently used for columns in commercial equipment.

13.3.1.1 Column Packings

(a) Packings for GSC: If the column is to achieve separation by adsorption on a solid (gas-solid chromatography) rather then by solution in the oil (gas-liquid chromatography), suitable packing materials are alumina, silica gel and activated charcoal. In gas-liquid chromatography, although the support material is commonly thought of as being inert and non-active in the separation process, interaction between the solute and support material does occur, and this interaction becomes increasingly evident as the solutes become more polar, as the weight of liquid phase is decreased and as the sample size is decreased. Seen in this context the distinction drawn between gas-liquid chromatography and gas-solid chromatography gets blurred and it will become more so because wrok on adsorbents may well produce materials which will displace diatomaceous earth from its pre-eminent position as a support. Materials like silica gel or graphitised carbon black are often used with the addition of a liquid stationary phase so that they are active supports for gas-liquid chromatography as much as adsorbents for gas-solid chromatography.

The aim in the development of new adsorbents is to find materials for which the adsorption isotherms are more nearly linear. Tailing on adsorbents is caused by the

presence of sorption centres which have differing sorption forces, and a homogeneous surface free from geometrical irregularities is needed for the elimination of these differences. Improvements in homogeneity may be obtained in three ways: By the preparation of materials, such as molecular sieves, which have perfect lattices; by blocking the highly adsorptive centres, for example, by modifying the adsorbent surface with an adsorbed liquid or by chemical combination of the adsorbent with molecules containing active groups, and by blocking the highly adsorptive centres by selection of a carrier gas of high inherent adsorption such as carbon dioxide or ammonia. Development of some specific adsorbents discussed below illustrates the application of one or the other of these three ways of effecting improvement in the quality of materials for their use as adsorbents that are employed most commonly in gas-solid chromatography these days.

Molecular Sieves: If the water of crystallisation of certain synthetic zerolites is driven off by the action of heat, a network of empty holes remains. These holes are of molecular proportions, and of a uniform diameter which is determined by the crystal structure of the parent zeolite. The name *molecular sieves* has been given to zeolites which have been developed especially for this sieve like property. They are now commercially obtainable as types 3A, 4A, 5A 10X and 13X, the numbers signifying the sizes of the holes. The size of the holes determines the sieve-like properties of the respective type so that each type is a highly specific adsorbent for those molecules which are of such a size and shape that they can enter the holes of that particular type of sieve. Molecular sieves can be used as the packing in an adsorption column and they behave in a manner similar to that shown by any other adsorbent material, selectively adsorbing the components of a mixture and then releasing them again, according to their concentrations in the gas phase.

All types of sieve readily adsorb water, which reoccupies the cavities from which it was driven off during manufacture, and is held more tenaciously than any other substance. As a consequence, molecular sieves can act as powerful drying agents. Carrier gases must therefore be dry, and water must be removed from samples before they are analyzed. Water may be taken up during handling processes and sieve should be reactivated before use by heating (conveniently in column itself) to between 150 and 300ºC (the temperature of activation affects the separations obtained). High concentration of water may damage molecular sieve, and if any large quantity of water has been taken up the bed should be purged with air or nitrogen during reactivation or the reactivation may be done under vacuum. Reactivation of sieve in the column should always be carried out in a flowing stream of carrier gas.

Molecular sieves may be available in the form of cylindrical pellets or of beads which are suitable for drying liquids or gases; for chromatographic purposes the

sieve must be ground and graded in fractions (and can be bought ready prepared in this form).

Silica Gel: The silica gel skeleton consists of intergrown silica globules which are not very large (usually less than 100Å), are heterogeneous, and are not ideally packed, the pores between the globules being consequently very small and irregular. When such a gel is treated with steam at pressure upto 250 atm (345ºC) the globules grow so that the surface area decreases and the pore size increases. With very large pore sizes the geometrical irregularities at the points of contact become insignificant and improvement in the linearity of isotherms results. Thus symmetrical elution peaks are obtained from this material, for example, for alkanes, but marked tailing is still shown by benzene. Further improvement is obtained by treatment with trimethylchlorosilane which provides a non-polar layer on the surface stable up to 350–400ºC. Polar surfaces can be produced by the use of chlorosilanes containing functional groups like –OH, –COOH, $-CH_2=CH_2^-$, for the treatment.

Graphitised Carbon Black: Particles of carbon blacks which are heated at 2500–3000ºC are converted into polyhedra with homogeneous graphite faces, and this material forms a non-specific adsorbent which can be supported on the chemically modified silica gel described above. Peaks obtained for polar compounds with carbon black are of good shape and this material is in fact the only adsorbent which shows good promise for the separation of polar compounds. The retentions of different compounds have little relation to their boiling points or molecular weights, but are more closely related to their polarisabilities (but not dipole moments) and are also affected by the spatial structure of the molecules (branching, *cis-* and *trans*-isomerism).

Carbon black has also been supported on polythene moulding powder by heating a mixture of the black with polythene in air to 160–170ºC so that the polythene softened sufficiently for the black to adhere to it, and on the walls of glass capillary columns and on glass beads, Carbon black may with advantage be modified by addition of a liquid such as squalane.

Modification of Adsorbents by Organic Compounds: Addition of an organic liquid to the adsorbent was the earliest method used for the modification of adsorbents, the organic liquid acting as a tailing reducer. In the separation of the C_5 and C_6 hydrocarbons, for example, it was found that 2, 3-dimethylbutane and 2–methylpentane could not be separated with any of a wide variety of liquids and all partition columns retarded naphthenes relative to paraffins, so that cyclohexane and sometimes methylcyclopentane had greater retention volumes than the first C_7 paraffins. Solid adsorbents, on the other hand, retarded paraffins relative to naphthenes, and all the C_6 members were eluted before any of the C_7 members on such columns. Some of the solids resolved 2, 3-dimethylbutane and 2-methylpentane, but tailing led to mixing of the peaks. This tailing was reduced

(and sometimes completely eliminated) by adding small amounts of a high-boiling liquid to the adsorbents. In small quantities the added liquid did not have any significant effect on the elution order, but the larger the amount added the less was the effect of the adsorbent and the nearer the column approached the behaviour of a partition column. The best column packing was found to be 1.5% squalane on Pelletex (a furnace black). The presence of squalane in Pelletex led to a marked improvement in the shapes of the peaks, reduced the relative retentions, and permitted a lower temperature to be used.

Modification of molecular sieve with 3% of silicone elastomer SE30 has also been found useful in the separation of C_7–C_{20} hydrocarbons.

Modification of Adsorbents by Inorganic Compounds: The properties of adsorbents are affected by the amount of water adsorbed on them, and if the amount is controlled, selectivity can be controlled in a manner useful in hydrocarbon gas analysis. They have also been modified with other inorganic compounds such as sodium hydroxide, iodide and chloride, silver nitrate and cuprous chloride, and sodium sulphate, molybdate and phosphate. If alumina is treated with an aqueous solution of the modifier and then heated to 100ºC, an adsorbent is obtained which not only causes less tailing than the unmodified alumina, but is also usable at lower temperatures (which are nevertheless high relative to those normal for gas-liquid operation). Symmetrical peaks are obtained for hydrocarbons and halogenated hydrocarbons and appropriate choice of adsorbent and modifier gives a considerable range of selectivity. Thus, for example, the relative retention of benzene to isooctane is less with sodium chloride as modifier for alumina than it is with sodium iodide. The separation of *cis*– and *trans*–alkenes is better than that obtained on most liquid columns. The columns, however, have the disadvantage that they are not suitable for separation of polar compounds. Alumina modified with cuprous chloride and silver nitrate may also be used for the subtraction of alkenes and aromatics respectively as these compounds are retained indefinitely and can be recovered by displacement.

Other Adsorbents

Mention of *Porasil*, a special form of silica gel, available as porous spherical beads, has been made earlier (Chapter 10). This material may be used modified or unmodified in gas chromatography for the separation of hydrocarbons and oxygenated compounds. Modification with a liquid such as polyethytlene glycol reduces tailing and, for example, has accomplished chromatographic separation of *m*- and *p*-xylenes. Addition of Carbowax (polyethylene glycols) to Porasil causes the retentions of esters to decrease and of hydrocarbons to increase, that is, modification by this liquid can yield considerable change in selectivity. Liquid modifiers can be chemically bound to Porasil so that there is no bleed from the column, and this material sold under the name of *Durapak*, has also been earlier, mentioned in the context of its use in HPLC (Chapter 10) as a bonded phase.

Colloidal Alumina: Alumina is available in a colloidal form which can be deposited on firebrick or glass beads, or on the wall of an open tubular column, to give a porous adsorptive layer which may be modified by addition of a liquid or other adsorbent such as colloidal silica. This way a wide variety of selective separating layers can be prepared.

Montmorillonite Clays: Bentone-34, dimethyldioctadecyl ammonium bentonite, a derivative of montmorillonite clay, shows selectivity for positional isomers, but as the peaks tail badly it is usually employed with a high proportion of stationary liquid phase. For example, ethylbenzene and the xylenes may be separated on a column containing Bentone-34 and isodecyl phthalate in equal amounts or containing Bentone-34 and silicone.

Inorganic Salts: Sodium chloride has been used as a support, but alkali-metal chlorides and nitrates can behave as active solids. Polynuclear aromatic hydrocarbons (of two to seven rings, boiling range 180-600ºC) have been separated on columns containing lithium chloride supported on Chromosorb P with the temperature programmed from 80ºC to 300ºC. Biphenyl and terphenyls have been separated on caesium chloride supported on Chromosorb at 230-300ºC (and on graphitised carbon black). A range of compounds, including esters, ketones, and butyl alcohols besides hydrocarbons, has been separated on copper amine complexes supported on Chromosorb W.

Glass: Glass surfaces may be modified. The walls of glass capillary columns treated with 20% sodium hydroxide in water for six hours at 100ºC are left uniformly white after the alkali has been thoroughly washed away. In this state good separations of low-boiling hydrocarbons have been obtained at room temperature; the columns, when treated with squalane in various thicknesses, gave separations varying from those typical of adsorption columns to these typical of partition columns.

Porous Aromatic Polymer Beads: Most rapid current advance is probably being made in the use of microporous aromatic hydrocarbon polymer beads marked under the names of Porapak, Polypak, Phasepak and various grades of Chromosorb, and this class of material may well replace diatomaceous earth supports in many applications. The special structure of these materials has led to the use of the term *gas-gel chromatography* to characterize the process in which they are used. They are complete packings in bead form with large surface area and strong physical structure which are available in suitably graded sizes, and are usable at temperatures upto 250ºC or higher for short periods during programmed-temperature operation. After conditioning to remove polymers of low molecular weight, there is no column bleed to affect baseline position until the onset of decomposition, but the outstanding property of these materials is the minimal tailing of the peaks, either of polar or of non-polar compounds. On a polymer-bead column water is merely one of the components of any mixture in which it is present, and the column has a relatively low affinity for all compounds containing

hydroxyl groups. Water is eluted as the first of the alcohols before methanol, but also before butane, and, from some grades, before propane. An alcohol is eluted before a hydrocarbon of the same boiling point and the dihydric alcohol ethylene glycol (b.p. 197ºC) is eluted before the monohydric n-pentyl alcohol (b.p. 137ºC). These column packings have therefore, apart from their other advantages, provided a type of selectivity available on no other column. In one example of their use, glycols and glycol ethers were separated at 200ºC; the columns were stable and the peak shapes were good, in contrast to the behaviour of these compounds on other columns where badly shaped peaks were obtained even from silanised Chromosorb G coated with polyethylene glycol adipate.

Polymer beads may be modified by addition of a liquid such as Apiezon L (high molecular weight hydrocarbons); the effect of this is apparently not to alter the selectivity of the packing but only to reduce retention times. Some tailing is observed with ammonia and amines, and improvement in performance of these compounds had been obtained by addition of tetraethylenepentamine or polyethylenimine.

Use of the dextran polymer, Sephadex, as a stationary phase in gas chromatography has been reported to yield well-shaped peaks for water and ethanol.

Besides blocking the highly adsorptive centres by modifying the adsorptive surface with an adsorbed liquid or by chemical combination of the adsorbent with molecules containing active groups as illustrated by the examples given above, blocking the highly adsorptive centres can be achieved by selection of a carrier gas with a high tendency for adsorption. This effect due to the properties of the carrier gas has been demonstrated in the separation of gases on a column containing activated carbon—peaks obtained with air, nitrogen or argon as carrier were more symmetrical than those obtained with hydrogen or helium which are less strongly adsorbed than the first three gases named.

(b) Packings for GLC: In GLC, which is the more important one of the two gas chromatographic techniques, the liquid stationary phase is absorbed on a porous solid support. The ideal features of this support are that it should consist of inert uniformly spherical particles having a large surface area per unit volume and that it should be mechanically strong over a wide temperature range. The most commonly used supports are diatomaceous earths, either kieselguhr, which is sold under the trade names of Celite, Chromosorb W, Embacel and Celatom, or crushed fire brick, which has the trade name of Sterchamol, C_{22} and Chromosorb P. The crushed fire brick has a higher mechanical strength than the kieselguhr support but the former has the disadvantage of not being completely inert and will adsorb polar samples, this adsorption resulting in tailing of the peaks. Adsorption appears to be due to hydrogen-bonding of the sample components with silanol sites, –Si–OH–, present on the surface of the support. Undesirable isomerization and

decomposition of the sample is also prevalent on the firebrick support. Conversely, kieselguhr is comparatively inert but is extremely fragile. With the recent introduction of Chromosorb C, however, the desirable high mechanical strength of the firebrick support and inertness of the kieselguhr support have been combined.

Reduction of the aforesaid undesirable adsorption of samples by the firebrick support has been attempted by removal of the adsorption sites in the following ways:—

(1) By choice of a stationary phase which itself contains suitable functional groups for the formation of hydrogen bonds with the support. This is frequently achieved by the incorporation of a *tailing reducer* in the stationary phase—for example, the presence of 0.1% of a polar material such as Alkaterge T in squalane eliminated tailing of alcohols and ketones. For nitrogen compounds tailing reducers must apparently be alkaline—successful elimination of tailing of aliphatic amines was achieved by coating the support with potassium hydroxide or carbonate before Carbowax was added as the stationary phase and a high-boiling amine was satisfactory for methylamines.

(2) By removal of the adsorption sites carried out by chemical reaction on the silanol group using reactive silyl compounds, such as trimethylchlorosilane (TMCS), dimethyldichlorosilane (DMCS), or hexamethyldisilazane (HMDS), to give inert silyl ethers. For example, the reaction of the active silanol sites with hexamethyldisilazane proceeds in the following manner:-

$$
\begin{array}{l}
-\overset{\mid}{S}i\,OH \\
\quad | \\
\quad O \qquad + (CH_3)_3\,Si\,.\,NH\,.\,Si(CH_3)_3 \longrightarrow \\
\quad | \\
\quad Si\,OH \\
\quad |
\end{array}
\qquad
\begin{array}{l}
-\overset{\mid}{S}i\,.\,O\,.\,Si(CH_3)_3 \\
\quad | \\
\quad O \qquad\qquad + NH_3 \\
\quad | \\
-\overset{\mid}{S}i\,O\,.\,Si(CH_3)_3 \\
\quad |
\end{array}
$$
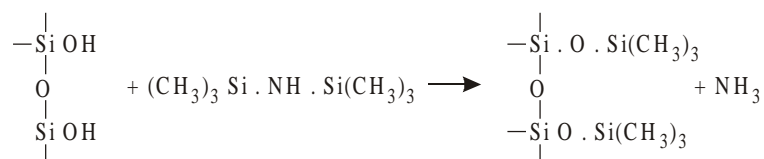
The reagent is most conveniently used by injection on to the column at about 80 to 100°C with a slow carrier gas flow-rate. The procedure may be repeated until the required performance is obtained from the column. It should not of course be used if the solid support is coated with a polar liquid phase having reactivity dependent upon hydroxyl or other similarly reactive substituents. The alternative procedure involves treatment of the solid support prior to coating with the liquid phase.

Dimethyldichlorosilane reacts with the active sites of the solid support giving both the cyclic silyl ether, formed by the reaction of one molecule of DMCS with two silanol groups, and also the monochlorosilyl ether. Consequently, it is necessary to treat the solid support further with methanol in order to convert the chlorosilyl group into its methyl ether derivative.

$$
-\overset{\mid}{S}i\,OH \xrightarrow{(CH_3)_2\,SiCl_2} -\overset{\mid}{S}i-O-\overset{\overset{\displaystyle CH_3}{\mid}}{\underset{\underset{\displaystyle CH_3}{\mid}}{S}i}\,Cl \xrightarrow{CH_3OH} -\overset{\mid}{S}i-O-\overset{\overset{\displaystyle CH_3}{\mid}}{\underset{\underset{\displaystyle CH_3}{\mid}}{S}i}\,.\,O\,.\,CH_3
$$
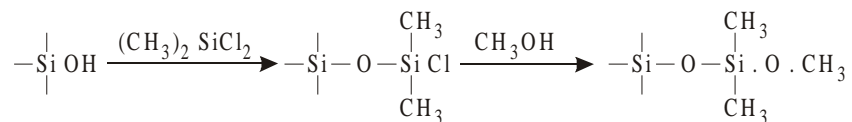
(3) By removal of the adsorption sites with acid or alkali. The support may be washed with hot hydrochloric acid or hot nitric acid and then with water. An alkaline wash is carried out with sodium hydroxide in methanol, followed by methanol and then water. These treatments, both separately and successively, have a long tradition in gas chromatography but it is not certain what they do. Acid is supposed to remove iron (and washing is usually continued until the thiocyanate test shows that the wash water is free from iron) but there no longer appears to be agreement that either acid- or alkali- washing is generally beneficial and some studies have suggested that the reverse is true. In view of the difficulty of removing the reagents, their use seems best deferred until other ways of reducing adsorption have been found inadequate.

(4) By coating the support with a solid which will cover the adsorption sites. The example given under (1) of coating the support for amine separations with potassium hydroxide or carbonate should strictly be classified under this heading. A more troublesome procedure is silver-plating the support, and by this means improved separations of alcohols have been obtained from columns containing Apiezon stationary phase.

Other supports: Of other support materials, perhaps polytetrafluoroethyelne (PTFE) is the most important because, although so far the column packings made from it are not as efficient as those made from kieselguhr, its adsorptive capacity is lower than that of any other suitable material. Consequently, symmetrical peaks can be obtained on PTFE-based columns for water, alcohols and amines. The chemical inertness of PTFE is of value for separation of halogens and inter-halogen compounds.

Glass beads, common salt, sand and unglazed tile have all been used as support materials, but in general they give columns of low efficiency. The household detergent, Tide, is used quite frequently. Tide consists of about 20% alkylaryl sulphonate on an inorganic base consisting of sodium sulphate, chloride, phosphate and silicate. After removal of water at 100ºC this provides a complete column packing in which the sulphonate acts as stationary phase suitable for use upto a temperature of 245ºC. If the organic component is removed by solution in light petroleum, the inorganic matrix can act as support for other stationary phases.

Whatever particle size of the column packing is chosen, mesh range of particles should be as narrow as possible, and care must be taken to remove the fines from any graded fraction.

In choosing the stationary phase, the following two major factors are to be considered:—

 (a) The chemical character of the sample to be analysed, the general rule in selecting the liquid phase being that it should be similar to the components of the mixture. Thus, hydrocarbons can be conveniently separated on silicone oil or dinonyl phthalate; polyethyleneglyol is more suitable for polar substances such as fatty acids.

(b) The upper and lower temperature limits at which the column may be operated and their relation to the optimum temperature for the separation of compounds.

Some typical liquid phases used in GLC alongwith their respective suitability for solute type and their temperature limit of working are given in Table 13.3.

TABLE 13.3

Typical Liquid Phases for GLC

Stationary Phase	*Solute Type for which suitable*	*Upper Temperature Limit (°C)*
Paraffin oil (Nujol)	Paraffin, olefine, halide	150
Squalene	Paraffin, olefine	140
Silicone oils	Paraffin, olefine, ester	200 (MW dependent)
Silicone greases	Paraffin, olefine, ester, ether	350
Polyglycols (Carbowaxes, Ucon)	Amine, nitrile, ketone, ester alcohol, ether	100–200 (MW dependent)
Dialkyl phthalates e.g. dinonyl phthalate, di-isodecyl phathalate, di (3,3,5–trimethylhexyl) phthalate	Alkanes, alkenes, arenes, ketones, aldehydes, esters, phenols, chloro compounds, mercaptans, sulphides	130 < 175
β, β′–Oxydipropionitrile	Alkenes	100
Apiezone (high molecular weight hydocarbons)	Useful for high temperature work	300
Silver nitrate— ethylene glycol	Specific for *cis* and. *trans* alkenes	50
Silver nitrate—benzyl cyanide	Specific for *cis* and *trans* alkenes	50
Thallium nitrate— polyethylene glycol	Specific for cis and trans alkenes	100
N,N,N′,N′–Tetrakis (2–hydroxypropyl) ethylene diamine (Quadrol)	Ketones, aldehydes, sulphur compounds and for the separation of gases	150
Squalane	Alkanes, arenes, sulphides, separation of enantiomers and	150

Stationary Phase	Solute Type for which suitable	Upper Temperature Limit (ºC)
	diastereoisomers e.g. diastereoisomeric alkanes and diastereoisomeric α-alkanoyloxypropionic esters	
Tricresyl phosphate	Hydrocarbons, esters, ethers, mercaptans	125
Cyanosilicones	Ketones, aldehydes, esters, alcohols, phenols, amines, essential oils, steroids, alkaloids	200
Fluorosilicone	Ketones, aldehydes, halogeno compounds, steroids, alkanoids	250

13.3.1.2 Packing the Column

Before the stationary phase is added to the support, the latter should be dried at 100-150ºC. If the stationary phase is a liquid at *room temperature*, the required proportion of the liquid and support may be stirred together, the liquid getting absorbed by kieselguhr to give a fairly free-flowing powder, though this method is not likely to give the most even distribution. A higher efficiency will be obtained if the liquid is dissolved in sufficient volatile solvent to form a slurry with the support and the slurry is then poured into a flat dish as a thin layer with the solvent then gething gently evaporated; alternatively, the evaporation may be carried out in a large filter flask which is warmed while a current of dry air is passed through it. In making up the packing, adding solution to the support may be preferrable rather than vice versa; either way it is important that the stationary phase be completely dissolved before it is blended with the support. After the solvent has been evaporated the packing may with advantage be heated under vacuum for some hours at a temperature slightly higher than the intended maximum operating temperature as this assists the conditioning process described later.

Before being packed, the empty tubing should be cleaned to remove any residual contamination from the manufacturing process or the previous packing. In loading the column an even packing is desirable so that the gas flow does not vary either across the column or irregularly along its length. A funnel is attached to one end of the column by rubber tubing, the other end is plugged with a wad of glass or silica wool, and sufficient packing to fill 5-10 cm of the column is placed in the funnel after a final sieving, and is agitated so that it moves down into the column. This agitation may be brought about normally with a hammer consisting of a

rubber bung on a rod, by bumping a straight or U-shaped column (i.e. by allowing it to fall freely 1-2 cm on a wooden block), by means of an electric massager, or by holding the column against the shaft of an electric motor on which a flat has been filed. It is sometimes convenient to apply suction, or a pressure (about 1 atom) to the appropriate end of the column while it is being agitated. The packing should be carried near to the end of the column and then plugged with glass or silica wool so that no dead space is left, but care must be taken that the wool plugs do not introduce any undue resistance to flow.

Impregnation with the stationary phase may be deferred until after the column has been packed with dry support. A solution of the stationary phase is then forced by gas pressure through the column, the volatile solvent remaining being removed by a stream of gas from that part of the solution which has been absorbed by the support. This method would seem to offer most advantage for packings such as those made up from glass beads, which do not flow freely when coated.

The proportion of stationary phase to support in use varies from 5:95 to 30:70 by weight. The most commonly used ratio is 20% by weight. Low ratios of 5% have the advantage of being less susceptible to 'bleeding' and also the elution times are usually shorter with a higher column performance than those obtained from the more highly loaded columns. They have the disadvantage, however, that the solute is more readily adsorbed by the solid support resulting in tailing of the eluted bands. Thus, whereas the length of the column does not affect the column performance when there is a high percentage of liquid phase on the solid support (about 20-30%), it has been found that the column performance is proportional to the column length for the lower ratios of liquid phase.

As so many types of columns are commercially available, the packing of an individual column may indeed be not needed many a time. It is often the case that in laboratories which are concerned with the routine analysis of only one or two classes of compound it may be found that two or three carefully chosen columns will suffice for all separations.

In spite of the enormous number of stationary phases available, it is often found that no one phase will effect an efficient separation of a complex mixture. In such instances it may be possible to collect the components which are not well resolved and to rechromatograph them on a different type of column. This procedure is laborious and often, due to the low concentrations of the components, it is also impracticable. Essentially the same procedure may be adopted, however, by the use of a mixed-phase column. Such a system consists of either two lengths of column containing different stationary phases connected in series or a single column containing a mixture of the two stationary phases. In the latter case, such a column is constructed by coating the solid support with the two phases either simultaneously or sequentially, or alternatively, by constructing the column from two batches of solid support which have been coated independently with one or the other of the liquid phases.

Newly packed columns and also those columns which are available commercially should be conditioned before being put into use by passing carrier gas through each such column at the highest intended operating temperature so as to remove volatile contaminants (water, solvent used in making up the packing, low-boiling components of the stationary phase) which otherwise would affect the detector and cause movement of the baseline. During the column conditioning processes the detector is not connected to the column but from time to time it is reconnected and the baseline is monitored. When a steady baseline is obtained on the recorder all the volatile impurities have been removed. It has been advocated, however that the conditioning process should be continued further until the baseline begins to drift again as a result of volatilization of the liquid phase. The conditioning process has the added attraction of producing more even distribution of the liquid phase on the solid phase. Conditioning may also lead to chemical reaction between components of the stationary phase which will help to stabilise its performance.

13.3.2 Procedure for Separation by GC and Detection and Quantitation of Separated Constituents

13.3.2.1 A schematic diagram of a gas chromatograph is shown in Figure 13.3.

A source of compressed carrier gas is required to supply a gas of constant purity to the flow or pressure controller. As the operating efficiency of a chromatograph is directly dependent on the maintenance of a highly constant carrier gas flow-rate, the flow of the carrier gas must be maintained constant to within a few per cent for most purposes.

The most exacting problem in gas chromatography is presented by the sample injection system. This device must introduce the sample in a reproducible manner and, if the sample is a liquid, vaporize it, instantaneously. Tremendous amounts of heat are required but still the sample must not get
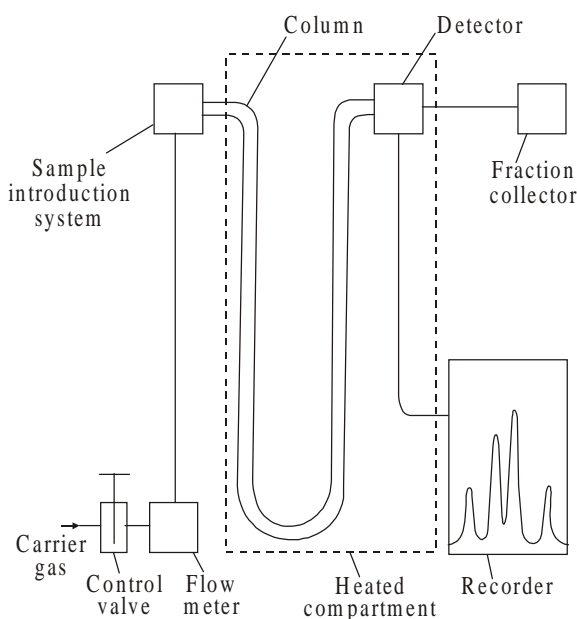
Figure 13.3 Arrangement of apparatus for gas chromatography

decomposed nor pressure surges created. A precise amount of sample has to be metered and transferred to the column without fractionation, condensation or adsorption of components. Liquid samples are injected by hypodermic syringes through self-sealing, silicone rubber septum (compressed by means of a threaded nut) into a heated metal block flash-evaporator. A typical arrangement is shown in Figure 13.4(a). The metal block which encloses the capillary is heated by a controlled resistance heater. Here the sample is vaporized as a 'plug' and carried into the column by the carrier gas. Every effort should be made to get the needle
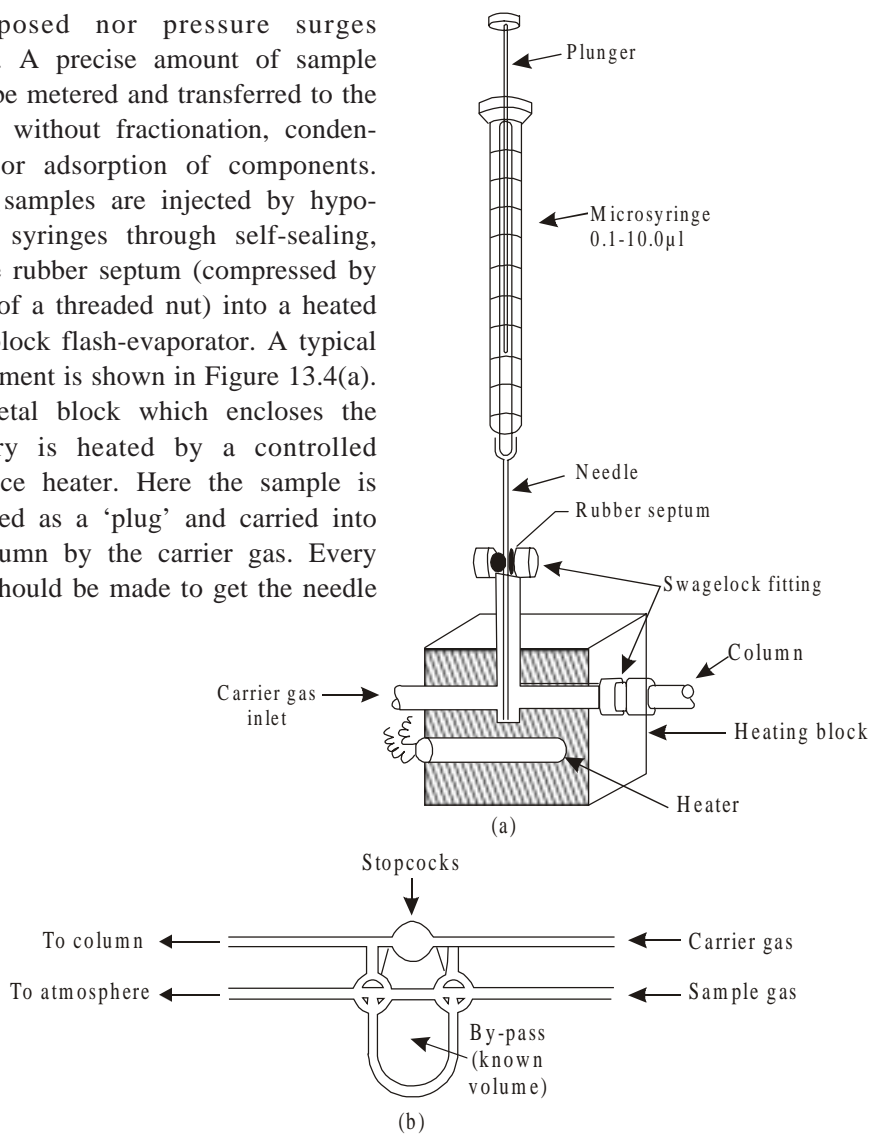
Figure 13.4 Sample inlet systems: (a) Hypodermic needle syringe and heater block for liquids, (b) gas sample introduction.

tip close to the packing or well down into the heated block ahead of the column. Insertion, injection, and removal of the needle should be performed quickly. For maximum efficiency the smallest possible sample (1 to 10 μl) consistent with detector sensitivity should be used.

Solids are dissolved in volatile liquids, or temporarily liquefied by exposure to infrared heat.

Gas samples are injected by a gas-tight syringe or gas-sampling valve called a stream-splitter [Figure 13.4(b)]. In the simplest form, the stream-splitter is merely

a glass system of three stopcocks between two of which there is a standard volume in which gas is trapped. Gas from this bypass capillary loop is introduced into the column by sliding or rotating a valve to connect the loop with the stream of carrier gas. A stream-splitter serves also to obtain minute (< 0.01 µl) liquid samples for capillary columns and small-bore packed columns. The liquid sample is introduced by standard techniques, vaporized, and mixed with the carrier gas, and then the gas flow is divided before entering the column. The stream-splitter must be highly non-discriminatory and separate each component of the sample mixture in exactly the same ratio.

The next component of the chromatograph after the column is the detector which determines the sequence and amount of the separated sample components leaving the column, these being recorded by the pen recorder coupled to the detector to yield the chromatogram.

Various detectors used in GC are described below.

13.3.2.2 GC Detectors

Amongst the wide range of detectors available, the *flame ionisation detector* (Figure 13.5) is effective with the easily ionisable organic molecules. The principle involved in the working of flame ionisation detector (FID) is: A flame of burning hydro-gen has a small electrical conducti-vity due to the formation of gaseous ions such as HO_2^+. A potential is applied between the electrode placed above the flame and the electrode at the base of the flame. A very small amount of a carbon compound introduced into the flame by the carrier gas (normally nitrogen) coming from the column gets combusted in the flame. During the combustion of the
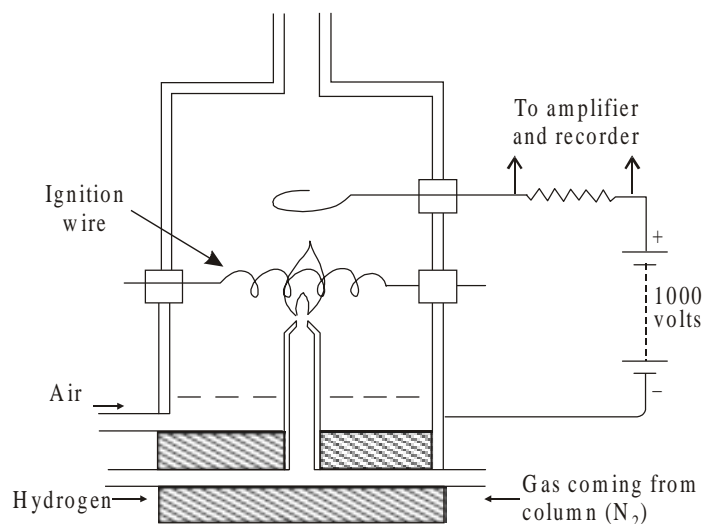
Figure 13.5: Flame ionisation detector

organic compound, many more ions are produced and the current passing through the arrangement greatly increases. It is a very sensitive detector and it has the widest range in comparison to other detectors in use. The combination of high sensitivity and wide linear range makes the FID an excellent detector for quantitative trace analysis.

Nitrogen-phosphorus detector (NPD): Figure 13.6 is a schematic digram of a typical nitrogen-phosphorus detector (also known as the *thermionic detector*). An electrically heated silicate bead doped with an alkali (such as rubidium) salt is mounted between the jet and the collector. Hydrogen at very low flow-rate (typically 2 ml/min) is mixed with the carrier gas and burns as a plasma (very hot gas) flame as it makes contact with the heated bead. The collector is maintained at a positive electrical polarity with respect to the bead and jet.

As to the mechanism for producing response by the NPD, the most widely accepted theory in this regard is that due to Kolb. According to this theory, the bead substrate is electrically conductive at the operating temperature and some of the alkali ions are able to acquire an electron and are thus converted to the atomic form. These atoms are relatively volatile and are emitted into the plasma, where they quickly react with combustion products and are ionised again and recollected on the negatively polarized bead. This cyclic process gives rise to the background signal and explains why the bead continues to function over an extended time period. If a compound containing nitrogen or phosphorus elutes from the column into plasma, these molecules will burn and react with the excited atomic alkali to form cyan or phosphorus oxide anions, respectively. These reactions disturb the alkali equilibrium in the plasma and additional alkali is released into the plasma,
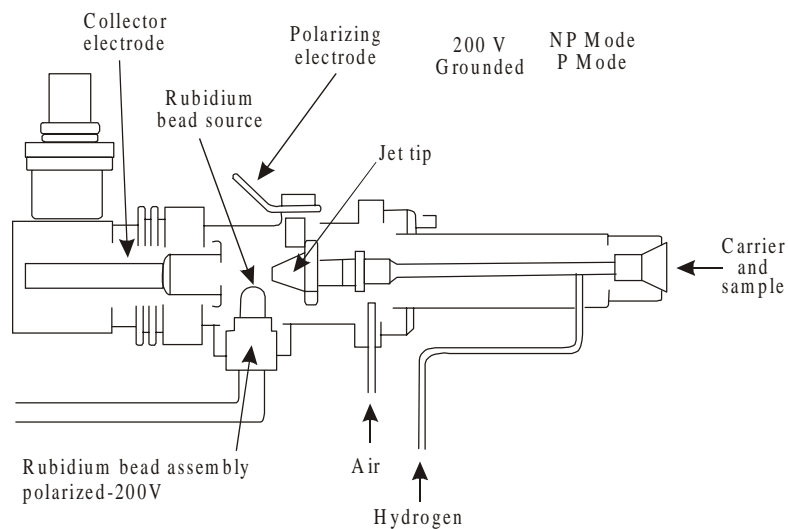
Figure 13.6 Schematic diagram of a typical NPD

thus promoting further ion formation and increasing the signal. Cyan anions are only formed from molecules containing nitrogen bonded to a covalently bonded carbon atom, and so a poor response is produced from nitrogen gas (thus this can be used as a carrier gas) and compounds such as carbamates, ureas, barbiturates etc.

The formation of cyan anions can be suppressed by increasing the reaction temperature with an increased flow of hydrogen (electrical heating of the bead is unnecessary under these conditions) allowing a phosphorus selective mode of operation. The jet is usually grounded under this mode.

The *thermal conductivity detector* or *katharometer* is based on the variation in the thermal conductivity of the carrier gas when the components of the mixture are eluted. This detector is diagramatically represented in Figure 13.7(a). The katharometer consists of two identical cells made in the form of two cylindrical cavities machined in a metal box. Along the axis of each cell is mounted an inert electric wire (e.g. tungsten) in the form of a spiral. Passage of an electric current through each wire raises its temperature. Pure carrier gas is passed through one cell and the gas flowing out of the column is led into the other cell. The usual arrangement in to pass the carrier gas through one cell before it enters the column and before the sample is injected, then to lead it through the sample injection
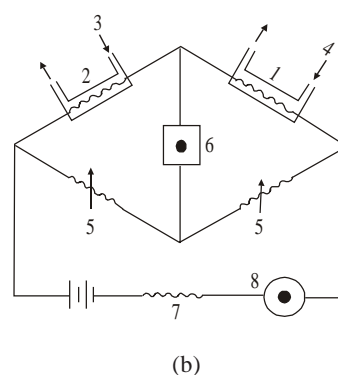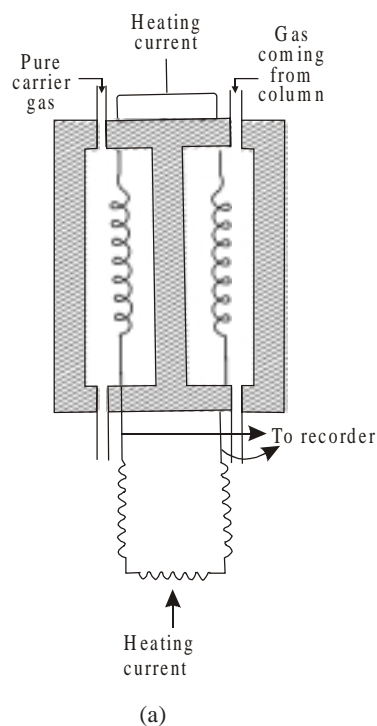
Simple katharometer circuit. (1) Reference cell; (2) Detecting cell; (3) Flow of carrier gas; (4) Effluent gas from column; (5) Variable resistance; (6) Recording unit; (7) Power supply for the Wheatstone bridge circuit; (8) Ammeter

Figure 13.7 Thermal conductivity detector

system and the column, then back into the detector through the second cell. Since the wires in the two cells are similar and heated by equal currents, and everything else is the same as long as the pure carrier gas is passing through each cell of the detector, the two wires will be heated to the same temperature and their resistances will be the same. This will be shown to be so by means of the Wheatstone bridge which forms part of the circuit which contains the recorder [Figure 13.7(b)]. As soon as the vapour of an organic compound coming out of the chromatographic column enters the second cell, thermal conductivity of the gas inside that cell will be lowered and the wire in this cell will not be able to lose its heat as fast as it did before, and the temperature of the wire will rise. As the temperature of the wire rises, so does its electrical resistance so that the balance of Wheatstone bridge will suffer disturbance to which the recorder pen will correspondingly respond. By proper circuitry it can be arranged that the movement of the recorder pen is proportional to the concentration of the concerned constituent of the mixture. Thermal conductivity detectors work best with helium as carrier gas, because helium has sufficiently high conductivity, but these detectors give satisfactory working with nitrogen also. The products are not destroyed by this method and, so, can be collected in preparative work.

Another detector uses a fundamentally different principle. Using hydrogen as the carrier gas, the column effluent is burnt in a small flame just above which is suspended a thermocouple. When an organic compound emerges from the column the flame temperature increases somewhat, causing a change in the thermocouple signal, proportional to the vapour concentration.

In another commercially used device, the *argon detector*, argon is used as the carrier gas and it flows through an ionisation chamber which is provided with a radioactive source. Electrons are ejected which are accelerated by an electric field, and by collisions produce large numbers of metastable argon atoms. When an organic vapour reaches the detector its molecules collide with these metastable atoms, producing many more electrons, and causing the ionisation current to increase. The device is very sensitive and can detect as small a quantity as 10^{-13} mol of most organic compounds.

The electron capture detector (ECD) (Figure 13.8) measures the loss of signal rather than an increase in electrical current. As the nitrogen carrier gas flows
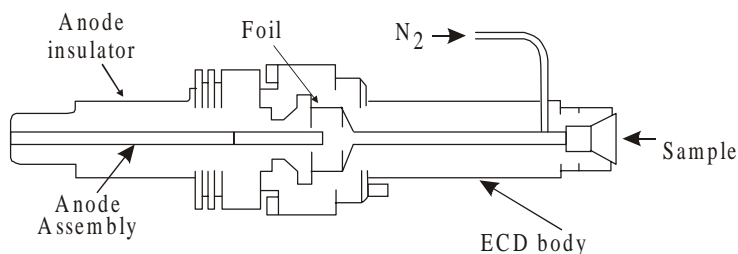
Figure 13.8 Schematic diagram of a typical ECD.

through the detector, a radioactive Ni^{63} foil (which emits β-particles) ionises the gas, and slow electrons are formed. These electrons migrate to the anode, which has a potential of 90 volts positive. When collected, these slow electrons produce a steady current of about 10^{-8} amps, which is amplified by an electrometer. If a sample containing electron-capturing molecules is then introduced to the detector, this current will be reduced.

Recent EC detectors have pulsed-voltage power supplies that maintain a constant current. With no sample, the pulse frequency is very low; as the sample enters the detector, the frequency increases to offset the current loss due to the electron-capturing species. The pulse frequency is proportional to the sample concentration and can be used for quantitative analysis.

The electron capture detector is extremely sensitive to certain molecules, such as alkyl halides. conjugated carbonyls, nitriles, nitrates and organometals. It is virtually insensitive to hydrocarbons, alcohols and ketones. In other words, the detector selectively responds to molecules containing electronegative atoms, since these atoms easily attach or attract an electron and thus produce an electrical signal. Quantities of halides at picogram (pg; 10^{-12} gm) levels can be detected by the electron capture detector.

The *gas density balance* (GDB), one of the chromatographic detectors that first became available, has the advantage that it can be used with corrosive gases as the sample does not pass over filaments. The schematic of this detector is shown in Figure 13.9.

Reference gas enters at A while gas from the column enters at C; both gas streams exit at D. Measuring elements are mounted in the reference stream at R_1 and R_2 and connected in opposite arms of a Wheatstone bridge. The

Figure 13.9 Schematic of GDB

sample never contacts the measuring elements, making the GDB. useful for the analysis of corrosive materials. If the gas eluted at C has the same density as the reference gas, the gas flows are at equilibrium and no unbalance is detected by the bridge.

If the gas entering at C carries a sample component of higher density, gravity pulls more of the gas stream down, and this increased flow from C retards the lower gas flow AR_2 while the upper flow increases. This flow imbalance causes a variation in resistance of the measuring elements R_1 and R_2, causing an unbalance in the bridge. Solutes of density lower than that of the carrier gas tend to rise, producing the opposite effect on the flow paths.

Sensitivity depends, in part, on the difference in density of the carrier gas and that of the sample component. Nitrogen is the preferred carrier gas except when CO, C_2H_4 and C_2H_2 are being determined; then, carbon dioxide or argon should be used as carrier. Sulphur hexafluoride has been preferred as a carrier gas.

Another detector available for use in gas chromatography is the *flame photometric detector (FPD)*. Detection of emission of the characteristic radiation by an element and the correlation of the emission intensity with the concentration of that element form the basis of this detector.

Figure 13.10 shows the schematic of the flame photometric detector. In this detector, the column effluent is mixed with oxygen and combusted in a flame surrounded by an envelop of hydrogen. The flame is seated within a high-walled cup to avoid background emission. Organic species in the sample are fragmented and excited to higher energy states in the reducing atmosphere of the flame. A high ratio of hydrogen to oxygen assures a reducing atmosphere. The plasma above the cup is viewed by a conventional photomultiplier tube through a narrow bandpass filter of the appropriate wavelength.
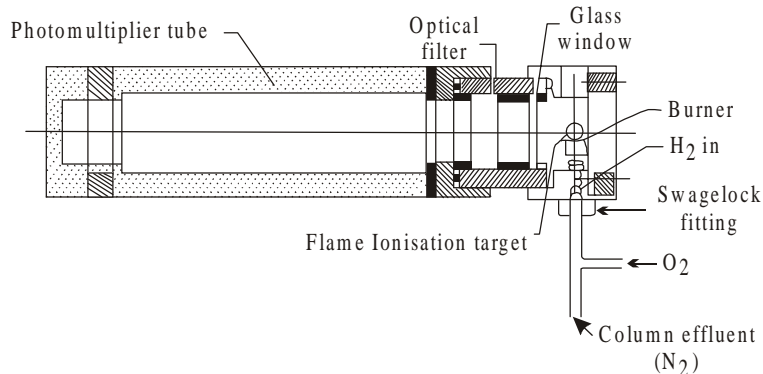
Figure 13.10 Schematic of FPD.

The detector is very sensitive and selective for compounds of S and P. Filters are chosen to have maximum transmittance at 536 nm in case of phosphorus compounds, and at 394 nm for the determination of sulphur compounds. The detection limit of the FPD is 10 pg of phosphorus and 40 pg of sulphur.

Yet another detector, *the mass spectrometer (MS)*, is especially suitable for use in gas chromatography because its mode of action is based on the same process— ionisation—which has provided gas chromatography with its most sensitive detectors. It is especially suitable for work with open tubular columns because the size of sample used with these columns is so small that other methods for the examination of the separated constituents of a given mixture can only be applied with difficulty.

In mass spectrometry, the molecule of an organic compound, which is in the gaseous state under conditions of high vacuum (10^{-7} to 10^{-9} mm Hg), is subjected to bombardment with a beam of medium-energy electrons (25-70 electron volt). Under these conditions a highly excited positively charged ion, called the molecular ion (M^+), is formed from the starting molecule as a result of removal of an electron from the latter. As a rule, electron bombardment knocks out one of the electrons of the unshared electron pair of a heteroatom or one of π-electrons of an aromatic system or a multiple bond. The molecular ion being quite labile due to the high degree of excitation, undergoes a number of successive breakdowns with the formation of positively charged ions (fragments) and neutral molecules or radicals.

In the next step of its operation, the mass spectrometer sorts the ions formed by the aforesaid fragmentation according to their mass/charge ratio $\left(\dfrac{m}{e}\right)$. The ions after being accelerated out of the ion source by an electrostatic field enter a perpendicularly directed magnetic field in which they change their initial direction. The following mathematical derivation shows that the ions will change their initial direction differently according to their $\dfrac{m}{e}$.

Let the applied potential be V, then the applied electric energy is Ve, which is imparted to the particle as kinetic energy, that is,

$$Ve = \frac{1}{2}\, mv^2 \qquad\qquad \text{...(13.2)}$$

where e = the charge (usually unit charge e) carried by the ion,

  m = mass of the ion,

and v = the velocity of the ion.

As a result of its passage through the applied magnetic field, the positively charged particle takes a circular trajectory of radius r. Intensity of the magnetic field being H, the force on the particle due to this magnetic field is Hev, which equals the centripetal force $\left(=\dfrac{mv^2}{r}\right)$.

Hence, $Hev = \dfrac{mv^2}{r}$ $\qquad\qquad$ ...(13.3)

or $H^2 e^2 = \dfrac{m^2 v^2}{r^2}$ $\qquad\qquad$ ...(13.4)

Since $\qquad$ $V e = \dfrac{1}{2} m v^2$ $\qquad$ ...(13.2)

$$\frac{H^2 e^2}{Ve} = \frac{m^2 v^2}{r^2} \times \frac{2}{mv^2} = \frac{2m}{r^2} \qquad ...(13.5)$$

or $\qquad$ $\dfrac{m}{e} = \dfrac{H^2 r^2}{2V}$ $\qquad$ ...(13.6)

Rearranging Equation 13.6 gives

$$r = \left(\frac{2V}{H^2} \cdot \frac{m}{e} \right)^{\frac{1}{2}} \qquad ...(13.7)$$

Thus, r is a function of $\dfrac{m}{e}$ of the charged particles, which in physical terms means that the ions are segregated by the magnetic field into beams each of a different $\dfrac{m}{e}$.

Equation 13.7 implies that, under a specified set of values of H and V, only those ions which follow the path that coincides with the arc of the analyzer tube in the magnetic field will pass through the resolving slit (at the exit of analyzer section) and enter into the collector which is the next component of the detector immediately following that slit.

The ion species which will be collected under a specified set of operating conditions is given by Equation 13.6:

$$\frac{m}{e} = \frac{H^2 r^2}{2V}$$

To obtain a mass spectrum, particles of different $\dfrac{m}{e}$ can be collected successively either by accelerating voltage or by varying the magnetic field continuously at a constant rate; usually the voltage is varried keeping the magnetic field constant.

As the ions enter the collector, they impinge upon a photometer tube where a minute current is produced. The magnitude of this current is proportional to the intensity of the ion beam. The current produced is amplified and fed into a recorder. The recorder must include provision for automatically recording peaks of widely varying amplitude. By the use of five separate galvanometers (a recording oscillograph) with relative sensitivities of 1, 3, 10, 30 and 100, spectrum peaks varying in height from 0.2 to 10,000 arbitrary divisions may be significantly read on 8-in photographic paper. This enables the height of any peak to be recorded

within better than 1 per cent accuracy over a range of magnitude of 1 to nearly 1000.

The mass spectrometer in an expensive equipment and there are some difficulties in the way of direct coupling of the gas chromatograph and the mass spectrometer. Coupling of the two instruments requires a change from the atmospheric pressure of the chromatographic column outlet to the vacuum conditions required in the mass spectrometer. The pressure reduction may be obtained by use of a capillary leak consisting of a stainless steel tube 100 cm long and 0.1 mm in diameter which can be heated by passing an electric current through it. Also, reduction in the amount of carrier gas entering the mass spectrometer is desirable, and a suitable separator is used for bringing it about.

The mass spectrum reflects sufficiently precisely the identity of the molecule. The relative numbers of each of the kinds of ionic fragments are characteristic for every compound so that the mass spectrum becomes a sort of 'fingerprint' for each compound. Differences in these various molecular fingerprints are noticeable enough to allow identification of the individual constituents of even a complex mixture.

13.3.2.3 Interpretation of a Gas Chromatogram

The harvest of information about a mixture that can be reaped from the interpretation of a chromatogram is illustrated with reference to a typical gas chromatogram shown in Figure 13.11.
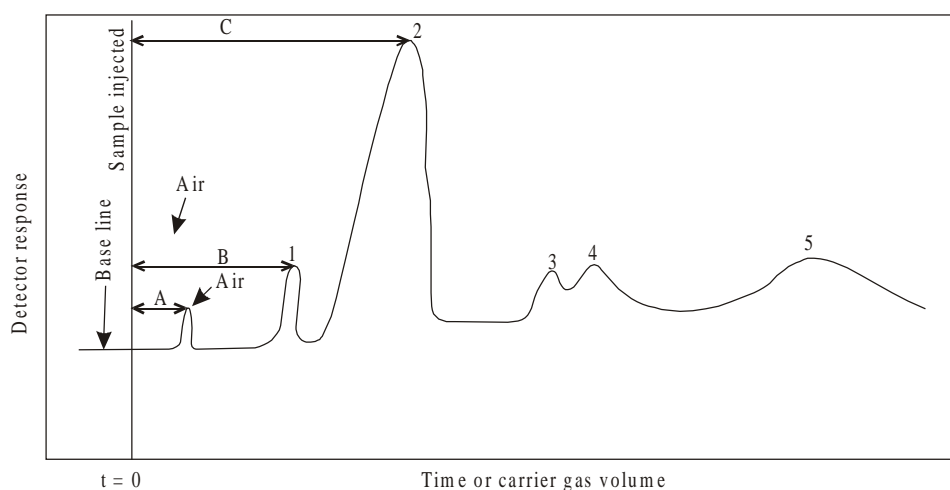
Figure 13.11 A typical gas chromatogram

Suppose that the sample was a mixture of saturated aliphatic hydrocarbons and was separated on a paraffin oil column. Since it requires a finite time for any

component to traverse the column, the recorder pen continues to trace a zero base line for some time after the sample introduction. However, after a few seconds or minutes (A), a small peak may appear on the chart paper. This is the so-called air peak. Since oxygen and nitrogen are only very slightly soluble in most liquid phases, air (either dissolved in the sample or introduced during the sample injection) will traverse the column with the same velocity as the carrier gas. The presence of five peaks in the recorder tracing, excluding the air peak, shows that the minimum number of components is five. The height and area of peak 2 are greater than the heights and areas of the other peaks. This is then the major component with a retention value (time or volume) of C. It is a good guess that, since the component of peak 1 had a smaller retention value (B), its boiling point is lower than that of the major component represented by peak 2. Similarly, the longer retention times of peaks 3, 4, and 5 indicate that the three components represented by these peaks should have boiling points which are higher than the boiling point of the major component. The non-resolution (non-separation) of components, such as shown by peaks 3, and 4, also is sometimes encountered in gas chromatography. One must also be aware that two or more components can appear as a single peak.

Information regarding the identity of the individual constituents of the mixture as also a quantitative estimate of each such constituent can be derived from the gas chromatogram in the manner detailed below.

Suppose that one has, from chemical evidence, a fair idea of what the structure of the major component represented by peak 2 in Figure 13.11 may be. The identity of the compound then becomes established as soon as one finds which member of its class has the literature value of retention volume equal to C, within experimental error. But this is not so simple as that, if one bears in mind that the usefulness of retention volume as read from Figure 13.11 is limited, in that it applies only to a particular column packing at a given temperature. Values of retention volumes are very sensitive to temperature—a temperature change of 1ºC frequently causes a 5% change in these values. For this reason it is now usual to deal with retention volumes measured relative to some given pure substance. This substance is then added to a mixture in which unknowns are desired to be studied, retention volumes of the unknowns are measured relative to the retention of the added substance. This eliminates the need for accurate control of column temperature and flow-rate.

In the analysis of compounds which form an homologous series, for example, the methyl esters of saturated fatty acids, there is a linear relationship between the logarithm of the retention time and the number of carbon atoms. This can be exploited, for example, to identify an unknown fatty acid ester in a fat hydrolysate. A widely used system in this context is the *Retention Index* (RI) which is based on the retention of a compound relative to n-alkanes. The compound is

chromatographed with a number of n-alkanes and a semi-logarithmic plot of retention time against carbon number is constructed. Each alkane is assigned an RI of 100 times the number of carbon atoms it contains (pentane therefore has an RI of 500) allowing the RI for the compound to be calculated. Many commercially available GLC systems with data processing facilities have the capacity to calculate RI values automatically.

Identification of the constituents of a mixture by gas chromatography can be more cheaply carried out by chemical tests. If samples are large, these tests can probably give most of the answers which can be obtained by instrumental

TABLE 13.4

Functional Group Classification Tests

Compound type	Reagent	Type of positive test	Compounds tested
Alcohols	$K_2Cr_2O_7$–HNO_3 Ceric nitrate	Blue colour Amber colour	C_1–C_8 C_1–C_8
Aldehyde	2, 4 DNP Schiff's	Yellow ppt Pink colour	C_1–C_6 C_1–C_6
Ketones	2, 4 DNP	Yellow ppt	C_3–C_8 methyl ketones
Esters	Ferric hydroxamate	Red colour	C_1–C_5 acetate
Mercaptans	Sodium nitroprusside	Red colour	C_1–C_9
	Isatin	Green colour	C_1–C_9
	Pb $(OAc)_2$	Yellow ppt	C_1–C_9
Sulphides	Sodium nitroprusside	Red colour	C_2–C_{12}
Disulphides	Sodium nitroprusside	Red colour	C_2–C_6
	Isatin	Green colour	C_2–C_6
Amines	Hinsberg Sodium nitroprusside	Orange colour Red colour, 1° Blue colour 2°	C_1–C_4 C_1–C_4 Diethyl and diamyl
Nitriles	Ferric hydroxamate-propylene glycol	Red colour	C_2–C_5
Aromatics	HCHO–H_2SO_4	Red-wine colour	ϕH–ϕC_4
Aliphatic unsaturation	HCHO–H_2SO_4	Red-wine colour	C_2–C_8
Alkyl halide	Alc. $AgNO_3$	White ppt	C_1–C_5

methods, though possibly more laboriously. The effluent gas is bubbled through tubes containing reagents such as those listed in Table 13.4.

The minimum detectable quantity (for C_1–C_9 compounds) is in the range of 20–100 µg. Tests of this type have the advantage that no special equipment is needed in the chromatographic laboratory and they can be applied rapidly whenever any unexpected problem arises.

Gas-liquid chromatography provides a convenient method for quantiative analysis of small quantities provided all the components are known. This application of GLC uses the strength of the chromatogramic bands. Strength of the bands is given by their peak height, or, if high accuracy is required, by the area under the peak, which in turn is given fairly closely by the product of the height times the width at half height. In chromatograms obtained by using detectors that are commonly employed, strength of a peak is proportional to the quantity of the corresponding component and is independent of the substance giving the peak. Analyses can, therefore, be performed by direct comparison of the peak strengths observed in a mixture, with those obtained when known amounts of each component are passed separately through the column. However, there is a practical difficulty, since the requirement of very accurate reproduction of the column conditions between measuring the samples and the pure components must be fulfilled besides meeting the requirement, implied in such a method, of accurate measurement of the very small quantities used (about 1 mg). The problem posed by these practical difficulties has been overcome by using a normalisation method in place of direct comparison of the peak strengths observed in the case of the mixture under analysis with those obtained for the mixture which has known amounts of each component. In the normalisation method the peak heights (or areas) of all the peaks are added up and the proportion, x, of compound Y may be obtained from the equation:

$$x = \frac{\text{Height (or area) of peak Y}}{\text{Total height (or area) of all peaks}}$$

The applications described so far are usually carried out with an analytical scale column, of diameter about 5 mm and length about 2 m, capable of handling quantities in the range of 50 mg. In order to perform further physical or chemical experiments on an isolated component of a mixture, the compound may be needed in quantities of the order of a gram or more. In such situations, *preparative scale* gas-liquid chromatography may have *distinct* advantages over distillation. In the first place, gas-liquid chromatography is basically a more efficient separatory technique. Also the problem posed by formation of an azeotrope, encountered occasionally in distillation, has no counterpart in chromatography. Whereas no choice of set of conditions is possible in the case of distillation, choice of varying

more than one column parameters is available to obtain the best chromatographic separation. Further, an efficient separation by distillation requires at least 100 ml of a mixture since the whole of the fractionating column must be filled with the vapour-liquid mixtute, whereas gas-liquid chromatography can effect separation of as small a quantity as 0.5 ml of a given mixture.

Scaling up separations requires employing larger columns and there is no particular difficulty created by use of such columns. However, rapid volatilization of the sample must be ensured so that it all enters the column at once. Use of a preheater achieves this. Collection of the components of the mixture as they emerge from the column has to be dealt with effectively. This can be carried out by use of carefully designed fraction collectors which ensure that a large cold surface is presented to the gas. Packing the traps with glass beads may achieve the fulfilment of this requirement. Depending upon the nature of the constituents of the given mixture, an appropriate coolant in which the fraction collectors are kept or immersed may be ice or acetone–dry ice or liquid nitrogen, the last one being by far the most efficient coolant.

13.3.3 Developments in the Technique of GC which have enhanced its Efficiency and Applicability

The following modified forms in which gas chromatography is now being widely practised have added to the effectiveness of the technique and the area of its applicability as a separating method.

  (i) Programmed-temperature gas chromatography.
 (ii) Programmed-pressure gas chromatography.
(iii) Gas chromatography with capillary columns.

 (i) Gas-liquid chromatographic separations are not only carried out isothermally (i.e. at a fixed temperature) but also with temperature variation during the analysis, the technique in the latter case being known as *programmed-temperature gas chromatography*. Because the mechanism of distribution of a compound between the mobile and stationary phases involves the vapour pressure of the compound, the effective distribution coefficient (B) values may conveniently be adjusted to desired values by changing the temperature of the column. Because B values of compounds rise with temperature, the gas-chromatographic equivalent of gradient elution in liquid chromatography is temperature programming, that is, an arrangement where the column temperature is raised during development according to a predetermined pattern. By this device, mixtures containing both very volatile and very non-volatile compounds may be separated.

Since B values are temperature dependent, a column for gas chromatography must be in an enclosure maintained at a controlled temperature. Very long columns may be used in a relatively small enclosure, because columns (packed metal tubes) may readily be coiled. Although the usual gas chromatographic column does not

give as many plates per centimeter as a good LC column (5 plates per cm is typical in gas chromatography), the ease with which very long columns can be made and used and the ease with which B values can be varied by changing temperature, make good separation of complex mixtures by gas chromatography possible.

(ii) Increase in the flow-rate during an isothermal chromatography (programmed-pressure gas chromatography) can provide the same improvements for wide-boiling-range samples as are obtained by increase in the column temperature. It should have the additional advantages that these improvements are obtained at low temperatures (where the separation factor is usually greater) and will be particularly useful for thermally labile materials, and that when ionisation detectors are used baseline control is simpler than it is under conditions of programmed temperature. If high temperatures must be used for the separation, the addition of flow-programming at the end of a temperature-programmed run will extend the range of volatility thereby adding to the number of the components which are amenable to separation by gas chromatography.

(iii) Introducing the use of capillary or Golay columns of 30–300 m length with internal diameter of 0.25–0.50 mm replacing standard packed columns has resulted in great improvements of degree of separation.

A capillary column cannot be packed normally and, in fact, requires no solid support. A dilute solution of the stationary liquid in a volatile solvent is forced into the tube, and the solvent carefully pumped out, leaving a thin layer of liquid on the capillary walls. Because of the low proportion of the liquid stationary phase in the capillary column, the volume of sample introduced is critical if overloading is not to occur. The low concentration of the eluted solute requires the use of a highly sensitive detector with a short response time. It was fortunate, therefore, that the capillary column and the flame ionisation detector were developed almost simultaneously.

The main feature of capillary columns or, as they are often called, the
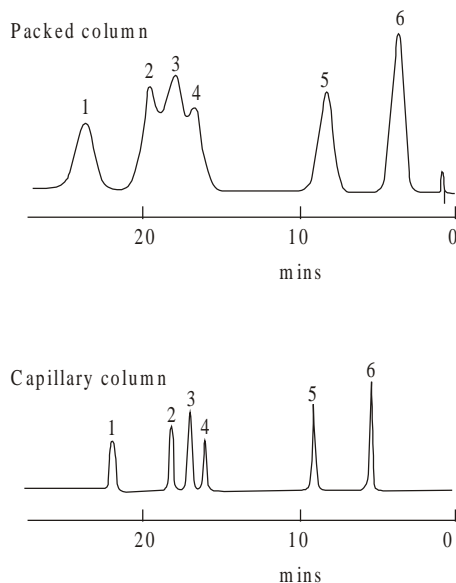
Figure 13.12 Comparison of the column efficiencies of a packed and a capilary column. Packed column : 120×0.4 cm 20% 7,8-Benzoquinoline on celite at 78.5°. N_2 flow-rate 42 ml/min. Column efficiency 1.600 theoretical plates (O-xylene). Capillary column : 12 m $\times$ 2.5 mm 12 mg 7,8-Benzoquinoline at 78.5°. N_2 flow-rate 0.7 ml/min. Column efficiency 146,000 theoretical plates (*O*-xylene). 1, O-xylene; 2, *m*-xylene; 3, *p*-xylene; 4, ethylbenzene; 5, toluene; 6, benzene.

open tubular columns, is the low resistance to the carrier gas flow with a consequent low pressure drop along the length of the column. This effectively results in narrower peaks and a higher resolution and also facilitates faster analysis without loss of column performance (Figure 13.12). It also permits the use of considerably longer columns. Typical dimensions are 0.1–0.53 mm i.d. and 10–50 m length.

Column bleed, hence detector noise, are reduced compared to packed columns because (i) the amount of stationary phase is low, (ii) the phase is chemically bonded to the wall of the capillary. Thermal mass is low and thus temperature programming is facilitated. More importantly, interfacing to MS is simplified because carrier gas flow-rates are also low. A further advantage with respect to packed columns is that when using polysiloxane stationary phases accumulation of siliceous deposits on NPD/ECD is minimized. On the other hand, one complication is that GC detectors other than the MS require higher flow-rates than normally used with capillaries and thus a 'make-up' carrier gas supply is needed (e.g. make-up gas of nitrogen or argon/methane must be used—enabling the use of helium or hydrogen as the carrier gas).

The standard capillary column can be modified to give a higher ratio of liquid phase to gaseous phase without further reducing the diameter of the capillary. This is accomplished either by chemical treatment of the internal surface of the capillary, so that it retains a higher proportion of the liquid phase or by deposition of a support for the liquid phase on the internal surface of the capillary. These porous layer columns combine the high permeability of the simple capillary column with the higher sample capacity of the packed column.

Packed capillary columns have also been described which have an internal diameter of < 0.5 mm with a solid support particle size of between 100 and 200 μ. The characteristics of these columns are more akin to those of simple capillary columns than to the characteristics of packed columns. Their permeability is higher than that of the standard packed column and, therefore, they give fast analysis with a high separation power. The sample loading is considerably higher than what can be introduced without overloading onto a capillary column and, as wider choice of stationary phases is available, packed capillary columns have a wide range of application.

The efficiency of gas-liquid chromatography with capillary columns is exemplified by its accomplishing the separation of meta- and- para- xylenes, which had proved to be an extremely difficult separation in earlier attempts made with conventional columns. Various mixtures of acids and esters were resolved chromatographically for the first time making use of capillary columns, a noteworthy example being the resolution of a mixture of methyl elaidate and methyl oleate which are *cis*- and *trans*-isomers.

13.3.4 *Applications of Gas Chromatography*

Gas chromatography can handle a wide range of materials with boiling points ranging from below –180ºC to over 300ºC.

Application of GC for effecting separations in the realm of Organic Chemistry is amply illustrated by Tables 13.2, 13.3 and 13.4. Accomplishment of difficult separations like those of m-xylene from p-xylene and of methyl elaidate from methyl oleate, just referred to above, illustrates the importance of GC for the chemist. Mixtures of constitutional, configurational, and isotopic isomers whose separation is difficult, may be separated by complexation chromatography. Perhaps the most widely known example is the use of silver ions to complex organic compounds containing π-electrons in various kinds of double and triple bonds, and heteroatoms such as N, O, and S with lone pairs of electrons. The selectivity of the silver nitrate-containing phases results from the marked effect that the relatively small structural or electronic changes in the donor ligand have on the stability constants of the complexes. Some of these trends are summarised in Table 13.5.

A limitation of silver ion complexation chromatography is the low upper temperature limit of the columns, 65ºC or 40ºC, as variously reported. Metal camphorate complexes have been used for both isomeric and enantiomeric

TABLE 13.5

The Influence of Olefine Structure on Chromatographic Retention using Silver nitrate-containing Stationary Phases

Substitution at the double bond decreases the retention volume.
A l-alkyl compound has a lower retention volume in comparison with the 3- and 4- alkyl isomers.
Olefines having a substituent in the 3-position have higher retention volumes in comparison with the 4- isomers.
Cyclobutenes have less tendency to form complexes than the corresponding 5- and 6- membered cyclo-olefines.
Cyclopentene derivatives have higher retention volumes than those of the corresponding isomeric cyclohexenes.
A conjugated-double-bond system has a lower complex-forming capacity than a simple double bond.

separations. Using either dicarbonyl rhodium-trifluoroacetyl -d-camphorate or the dimeric 3-trifluoroacetyl-or 3-heptafluorobutyryl-(IR)-camphorate of Mn^{2+}, Co^{2+}, Ni^{2+} or Cu^{2+} dissolved in the noncoordinating stationary phase squalene, separations can be obtained for $\leqslant$-donor ligands. Resolving power of these chiral complexing agents may, for example, be illustrated by the separation of racemic 2, 2-dimethyl-chloroaziridine (Figure 13.13 A) carried out on a capillary column

containing 0.113 M nickel (II)-bis-3-heptafluorobutyryl-(IR)-camphorate (Figure 13.13 B) in squalene. The remarkable resolution of the recemate (Figure 13.13 C) is accounted for by the direct participation of the lone electron-pair of the chiral nitrogen at the enantio-specific coordination site of the nickel camphorate complex.

Another approach to achieving the separation of enantiomers by gas chromatography makes use of a chiral stationary phase. Separation occurs because interactions between the enantiomers and the stationary phase results in the formation of diastereomeric association complexes having different solvation enthalpies and hence different retention

Figure 13.13 A, enantiomeric forms of 2, 2-dimethylchloroaziridine; B, nickel (II) bis-3-heptafluorobutyryl-(IR)-camphorate; C, resolution of mixture A on a 100 m capillary column coated with B dissolved in squalane.

characteristics. A number of chiral phases, including amides ($RCONHCHR_1R_2$), diamides, ($RCONHCHR_1CONHR_2$), dipeptides (N-trifluoroacetyla-minoacid-aminoacid - R), and polysiloxane polymers with chiral substituents are commercially available. Some examples are shown in Figure 13.14. The chiral phase in Figure 13.14(I) has been used to separate the enantiomeric N-trifluoroacetylamines, N-trifluoroacetylamino acid esters, and α-alkylcarboxylic acid amides. The diomide phases of which Figure 13.14(II) is an example, show high stereoselectivity for the separation of enantiomeric amides derived from amino acids, amines, amino alcohols, α-hydroxy acids and esters of aromatic diols. Many dipeptide stationary phases of which Figure 13.14(III) is an example, have been evaluated for the separation of enantiomeric amino acid derivatives. Phases providing the highest resolution of enantiomeric pairs contain a trifluoroacetyl group, bulky side groups and similarly bulky ester groups, and are operated at the lowest feasible temperature (different phases are stable at 110–170ºC). Polymeric chiral phases exemplified by Figure 13.14(IV), have been synthesized to provide chiral phases with good chromatographic performance and higher operating temperature limits. All phases are prepared from a polycyanopropylmethyl-dimethylsiloxane or polycyanopropylmethylphenylmethyl-siloxane by hydrolysis

$$CH_3(CH_2)_{10}CONHCHCH_3$$
$$|$$
$$C_{10}H_8$$

(I)

$$CONHC(CH_3)_3$$
$$|$$
$$CH_3(CH_2)_{20}CONHCH$$
$$|$$
$$CH(CH_3)_2$$

(II)

$$(CH_3)_2 CH \quad\quad CH (CH_3)_2$$
$$| \quad\quad\quad\quad |$$
$$F_3CCONHCHCONHCHCHCOOC_6H_{11}$$

(III)

$$-Si-O \left(Si-O \right)$$
with CH_3, C_6H_5, $(CH_2)_3$, $C=O$, $NH\ CHCH\ (CH_3)_2$, $CONHC(CH_3)_3$

(IV)

Figure 13.14 Some representative chiral stationary phases for gas chromatography. (I), N-lauroyl-S-α-(1-naphthyl) ethylamine; (II), N-docosanoyl-L-valine-t-butylamide; (III), N-trifluoroacetyl-L-valyl-valine cyclohexyl ester; (IV), polymeric chiral phase prepared from polycyanopropylmethylphenylmethyl silicone and L-valine-t-butylamide.

of the cyano group to the carboxylic acid, which is then coupled with L-valine–t-butylamide on L-valine-S-α-phenylethylamide to introduce the chiral centre. Separation of each chiral centre by several dimethylsiloxane or methylphenylsiloxane units (> 7) seems to be essential for good enantiomeric resolving power and thermal stability. The upper temperature limit of these phases is about 230ºC; at the higher temperature racemization of the chiral centres becomes significant. Nearly all the enantiomeric protein amino acid N-pentafluoropropionylamide isopropyl ester derivatives can be separated in a single analysis. Racemic mixtures of α-amino alcohols, glycols, aromatic and aliphatic α-hydroxy carboxylic acids, and amines can also be separated on these phases. Racemic hydroxy acids and carbohydrate derivatives were separated on the phase containing L-valine-S-α-phenyl ethylamide.

GC Separations in Inorganic Chemistry

Inorganic GC separations have relied on both adsorption and partition processes.

There is often considered to be a minimal group of chemical properties which an inorganic compound is to possess for gas chromatography to be successfully applied to that compound. Besides, of course, adequate volatility and thermal stability, these properties are: Monomeric form, neutrality, comparatively low molecular weight, coordinative saturation and, perhaps, most importantly, adequate structural shielding of the metal atom (s) by bulky and inert organic functional or

coordinating groups. The chemical reactivity of volatile inorganic compounds has frequently necessitated the most rigorous control of columns and substrates in order to prevent undesirable on-column decomposition or unfavourable interactions.

Adsorption GC has been widely employed for the separation of gases. A wide variety of adsorptive column packings have been investigated. Silica, alumina, other metal oxides, and inorganic and carbon molecular sieves have predominated for the separation and quantitative estimation of stable permanent atomic and molecular gases. Organic polymers, notably polystyrene copolymer-based materials find favour for separation of mixtures containing more reactive and polar components such as hydrides, low molecular weight organometallics and more polar compounds such as water and ammonia. Inorganic materials ranging from quartz and diamond to metal salts and eutectics have been used for GC of reactive species at very high temperatures.

Methyl silicone oils and waxes have found the widest application as partitioning stationary phases, although other substituted silicones such as phenyl, cyano, and particularly fluoroalkyl have also been favoured.

Elution problems and nonideal chromatography have always been troublesome in GC, and many approaches have been necessary to reduce them. All chromatographic parameters have been varied, including column material, solid support, treatment and deactivation, injection port design, and system pretreatment. Most studies have been conducted with glass or stainless steel columns and the former are preferred for most inorganic separations. Since metals present on internal column surfaces or supports are often responsible for on-column catalytic decomposition, there has always been danger of increasing sample degradation with continued column use as more metals and oxides are deposited.

The increasingly rapid adoption of capillary column GC techniques promises an advance for inorganic separations as much by virtue of the absence of decomposition sites on supports or columns as by high resolution capabilities. This is particularly true for fused-silica capillary columns, which are characterized by extremely low residual trace metal concentrations in the silica matrix.

Keeping in view the foregoing general observations made in the context of the use of GC as a separatory technique for inorganic substances, application of this technique in Inorganic Chemistry is exemplified by the following separations of various classes of inorganics.

Gas Chromatography of Inorganic Gases

Gas-liquid partition chromatography has very limited application for the analysis of permanent gases since their solubilities in liquid stationary phases are minimal at ambient and subambient temperatures. This, in combination with their low boiling points, minimizes retention. Although there have been examples of GLC

of some polar gases, such as ammonia with ester and silicone oil phases, gas-solid adsorption (GSC) is the preferred method for such analyses as well as those of non-polar atmospheric gases. Inorganic zeolite-based molecular sieves possessing concentrated surface charge generate surface adsorption based on polarizability of adsorbed molecules, and have found most favour for atmospheric gases. Use of capillary columns wherein the adsorbent is coated as small particles on the inner column walls (the support-coated open-tubular column—SCOT), may be promising for GSC of gases.

Excepting oxygen and nitrogen all the components of a mixture of hydrogen, oxygen, nitrogen, carbon monoxide, methane, kryton and xenon are amenable to separation with silica. The full resolution of the mixture may be achieved by setting silica and molecular sieve columns in series separated by a low-volume, non-destructive detector. Another noteworthy application of silica has been for the separation of sulphur gases viz. COS, H_2S, CS_2 and SO_2 present together as a mixture.

Porous polymers of the Porapak^{TM} and Chromosorb Century^{TM} types of polystyrene or similar polymer bring about separation of polar compounds such as water, ammonia, hydrogen cyanide and sulphur dioxide from their admixture with low-molecular weight organic compounds because these inorganic polar compounds are eluted more rapidly. These polymers may also be used advantageously in series with molecular sieves or as column pairs at different temperatures. Two Porapak Q^{TM} columns in series at 75ºC and –65°C gave a complete resolution of CO_2, H_2S, H_2O, COS, SO_2, N_2, O_2, Ar and CO. Helium carrier gas containing 100 ppm of SO_2 was employed for this separation.

Detection for inorganic gas analysis has been almost entirely carried out by means of thermal conductivity since ionisation detector response is minimal.

Gas Chromatography of Binary Metal and Metalloid Compounds

The principal simple binary inorganic compounds that have adequate vapour pressures and thermal stabilities at normal gas chromatographic temperatures are main group hydrides and halides. If high temperature applications with temperatures upto 1000ºC or above are also considered, a number of metal oxides also show adequate physical characteristics for undecomposed elution.

Amongst the hydrides of elements of Groups IIIA, IVA and VA, boron hydrides were among the earliest of inorganic compounds to be successfully gas chromatographed.

Amongst the earliest separations of hydrides of boron carried out in the 1950s and 1960s was the separation of the borane homologues from diborane to pentaborane. Low column temperature and an inert system were needed to minimize the on-column degradation, but some interconversion of species was seen due to catalysis by the substrate. Separation of boron hydrides have also been

carried out successfully with programmed temperature GC and it seems possible that capillary columns using inert materials like Teflon[TM], fluorocarbon oils and paraffins may further aid such separations. Subambient capillary GC may be the only feasible approach for the separation of hydrides of aluminium, gallium and indium, because of the reactivity of these hydrides.

Separations of hydrides of silicon and germanium have been achieved by GC on ester and silicone phases. Separation of alkyltin mono- and dihydrides by GC requires the use of most inert stationary phases to preclude on-column reaction. Among the Group IVA elements, lead alone has hydrides for which GC has not proved possible.

An early example of separation of Group VA hydrides was the resolution of arsine and phosphine along with silane and germane on a packed 8 m column with a stationary phase of silicone oil coated on alumina, column temperature being 30ºC. Hydrides of arsenic and antimony along with those of germanium and tin have been separated on porous polymer column phases at or above ambient temperatures, temperature programming being very important in gaining complete resolution.

The other major group of binary metal and metalloid compounds to be gas chromatographed at normal temperatures is that of halides. The major practical difficulty for these compounds often lies in their high reactivity and extensive precautions have to be taken to ensure maximum inertness of the whole chromatographic and sample handling system. In particular, care must be exercised to avoid hydrolysis during injection.

Among the chlorides that have been gas chromatographed are those of titanium, aluminium, mercury, tin, antimony, germanium, gallium, vanadium, silicon, arsenic and phosphorus. Problems arise from reaction of the chlorides with even such unreactive stationary phases as methyl silicone oils; frequently, inert fluorocarbon packings have been favoured for reactive chlorides and oxychlorides including $VOCl_3$, VCl_4, PCl_3 and $AsCl_3$. Porous polymer stationary phase Porapak P has been used for the separation of chlorides of silicon, tin, germanium, vanadium, arsenic, antimony, titanium and phosphorus. Low-melting inorganic salts, eutectics, and metal phases have also been applied in this field with considerable success. Thus, separation of $TiCl_4$ and $SbCl_3$ has been achieved on a column of $BiCl_3$ - $PbCl_2$ eutectic at 240ºC and that of $NbCl_5$ and $TaCl_5$ on a LiCl-KCl eutetic at 444ºC. A study of eutectic and single chloride phases at column temperature from 450ºC–1000ºC yielded elution of chlorides of bismuth, cadmium, zinc, thallium[+] and lead[2+].

The more volatile silicon tetrachloride and silicon tetrabromide may be effectively separated on silicone oils coated on a polytetrafluoroethylene supports. Less volatile metal bromides generally require high-temperature stationary phases. Thus, the use of alkali bromide salts coated on silica as stationary phases with the use of bromine/nitrogen and boron tribromide/bromine/nitrogen mobile

phases enabled quantitative elution and separation of the bromides of zinc, niobium, molybdenum, technitium, indium, antimony, tin, bismuth, tellurium and iodine.

Some metal fluorides have low boiling points and for these low column temperatures are feasible. Thus, good separation of tungsten fluoride (B.P., 17.5ºC), molybdenum fluoride (B.P., 35ºC), rhenium fluoride (B.P., 47.6ºC) and uranium fluoride (B.P., 56.2ºC) has been achieved at 75ºC on a column of Kel-F oil^{TM} coated on Chromosorb T^{TM} (Teflon^{TM}). The determination of alloys and metal oxides, carbides etc. after conversion to their analogous fluorides by fluorination appears feasible. Separation of a range of fluorides comprising ClF, ClF$_3$, OF$_2$, ClO$_4$F, ClO$_3$F and UF$_6$ has been carried out by gas chromatography again using Kel-F as a stationary phase.

An extreme modification of gas chromatography known as 'thermochromatography', employs columns maintained at very high temperatures. The most striking examples of its application are separations of metal oxides, hydroxides and oxychlorides brought about by this technique. Gas chromatographic separation of oxides and hydroxides of technetium, rhenium, osmium and iridium employed column temperatures as high as 1500ºC. Quartz granules were used as column packing and oxygen and oxygen/water mixture as carrier gases with the necessary equipment modifications. Mechanism of these separations may involve differential volatilization/distillation process predominating over adsorption or partition processes.

Gas Chromatography of Metal Chelates

The preparation of metal chelates for separations by gas chromatography has been studied for many years by now. The principal limit to the success of this approach has been the paucity of suitable reagents which can confer the necessary volatility and thermal stability and an adequate shielding of the metal atom from unwanted column interactions.

The class of metal chelating reagents which have been most studied for separations in Inorganic Chemistry are the β-diketones, including their halo - and alkyl-substituted analouges as well as the corresponding thio and amine forms.

Enolization and subsequent ionisation of β-diketones occurs as indicated below:

$$R-\underset{\underset{O}{\|}}{C}-CH_2-\underset{\underset{O}{\|}}{C}-R' \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} R-\underset{\underset{O}{\|}}{C}-CH=\underset{\underset{O^-}{|}}{C}-R'$$
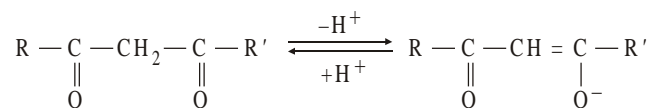
In many instances the complexing enolate anion forms neutral chelates with metals whose preferred coordination number is twice their oxidation state; the resulting complexes are effectively coordinatively saturated, thus precluding further

adduction by solvent or other ligand species. Stable complexes are formed with ions such as Be^{2+}, Al^{3+} and Cr^{3+}, which conform to the above rule. Other ions such as Ni^{2+}, Co^{2+}, Fe^{2+}, and La^{3+}, which readily adduct additional neutral ligands and assume a coordination state greater than twice their oxidation state, are difficult to gas chromatograph. Formation of hydrates lowers the volatility and increases the polarity of the derivative and this results in undesirable column behaviour. Acetylacetonates of metals such as Cu^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+} and La^{3+} which are not coordinatively saturated, however, tend to solvate, polymerise, or may undergo on-column reaction with active sites thereby giving excessive peak broadening or even irreversible adsorption on column or in the GC system. In fact, the non-fluorinated beta-diketonates are generally of marginal thermal and chromatographic stability; they usually require column temperatures that are too high for thermal degradation to be completely absent.

The major breakthrough that transformed metal chelate GC into a useful analytical technique was the introduction of fluorinated beta-diketone ligands, which formed complexes of greater volatility and thermal stability. Trifluoroacetylacetone (1,1,1-trifluoro-2,4-pentanedione—HTFA) and hexafluoro-acetylacetone (1,1,1,5,5,5-hexafluoro-2,4,-pentanedione—HHFA) are the fluorinated ligands most frequently employed. HTFA extended the range of metals that may be gas chromatographed with little or no evidence of decomposition to include Ga^{3+}, In^{3+}, Sc^{3+}, Rh^{3+} and V^{4+}. An example of a recent application is the analysis for beryllium in ambient air particulates. After filter sampling and extraction/chelation, packed column GC with electron capture detection allowed ppm level beryllium quantitation in collected particulates which corresponded to levels of $2\text{-}20 \times 10^{-5}$ μg/m^3 in the sampled air.

The great electron-capturing ability of HHFA complexes e.g. Cr (HFA)$_3$, has permitted extremely low detection limits for these complexes.

Since modifications of the basic beta-diketone structure may be made readily, various modified chelating ligands have been evaluated for GC applications. The widest development in ligand modification has been in the case of diketones containing a tert-butyl group and a long-chain fluoroalkyl moiety. Thus, 1,1,1,2,2,3,3,-haptafluoro-7,7-dimethyl-4,6-octanedione [heptafluoropropanoyl-pivalylmethane (HFOD or HHPM)] has been used for lanthanide separations. This and other similar ligands with different fluoroalkyl substituents have been used for a range of analytical procedures and preparative separations.

Other ligands studied were those in which donor oxygen atoms of the beta-diketones were substituted by sulphur or nitrogen atoms. Example of such ligands are:

$$R - C = CH - C - R'$$
$$\quad\ \ |\qquad\qquad\ \|$$
$$\quad\ SH\qquad\qquad O$$
β- thioketone

and $R - C = CH - C - R'$
$\qquad\qquad\ \ |\qquad\qquad\ |$
$\qquad\qquad SH\qquad\qquad S$

β = thio-ketone

$$R - C - CH_2 - C - R'$$
$$\quad\ \|\qquad\qquad\ \|$$
$$\quad\ O\qquad\qquad\ O$$
Parent
β - diketone

H_2S
H^+

$$R - C = CH - C - R'$$
$$\quad\ \ |\qquad\qquad\ \|$$
$$\quad\ OH\qquad\qquad O$$

$$R - C = CH - C - R'$$
$$\quad\ \ |\qquad\qquad\ \|$$
$$\quad\ NH\qquad\qquad O$$
$$X - NH\qquad\qquad O$$
$$\quad\ \ |\qquad\qquad\ \|$$
$$R - C = CH - C - R'$$
tetradentate β-ketoamine

2 molecules of
β-diketona +
diamine
$NH_2 - X - NH_2$

XNH_2 (primary amine)

$$R - C = CH - C - R'$$
$$\quad\ \ |\qquad\qquad\ \|$$
$$\quad\ NHX\qquad\qquad O$$
bidentate β- ketoamine
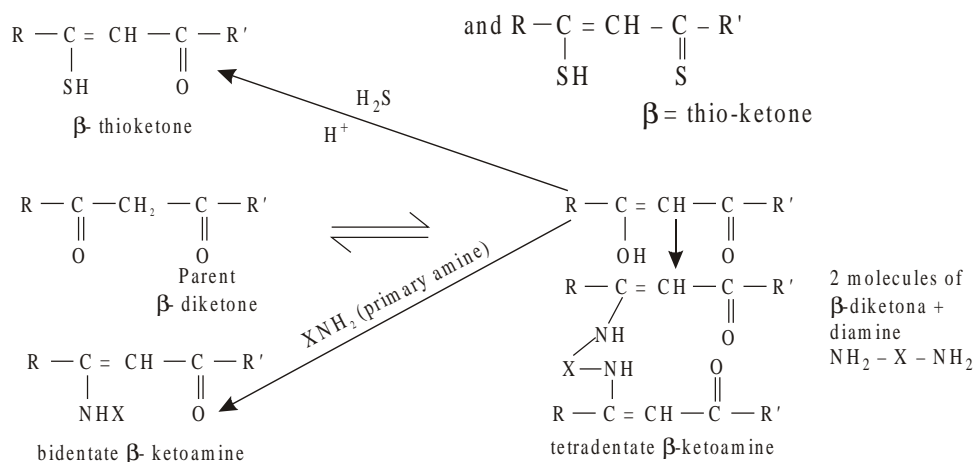
(Formation of major beta-difunctional ligands for metal complex GC)

Gas Chromatography of Organometallic Compounds

Organometallic compounds are defined as compounds which contain at least one metal to carbon bond, other than metal carbides. Resolution of organometallic compounds by gas chromatography can be illustrated by citing the remarkable separation of ferrocene and its analogues, ruthenocene and osmocene.

High sensitivity of alkylmercurial compounds to electron–capture detection has prompted conversion of inorganic mercury into its thermally stable alkyl derivatives for gas chromatographic analysis. Among the more commonly used alkylating reagents are sodium tetraphenylborate, tetramethyltin and the arylsulphinates.

$$Na\ B(C_6H_5)_4 + 2HgCl_2 + 3H_2O \longrightarrow 2Hg(C_6H_5)_2 + 3HCl + NaCl + B(OH)_3$$

$$(CH_3)_4Sn + Hg^{2+} \xrightarrow{\ 2Cl^-\ } CH_3\ HgCl + (CH_3)_3SnCl$$

$$C_6H_5SO_2H + HgCl_2 \xrightarrow{\ HCl\ } C_6H_5\ HgCl + SO_2 + HCl$$
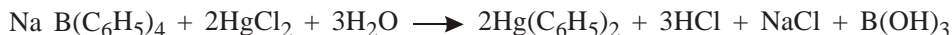
The electron-capture detector responds to ppb concentrations of the derivatives.

Gas Chromatography of Derivatized Anions

Alkylation has also been used for the simultaneous determination of trace quantities of bromide and chloride by gas chromatography with electron - capture detection. The anions were derivatized with dimethyl sulphate.

The most general method for the simultaneous analysis of oxyanions by gas chromatography is the formation of trimethylsilyl derivatives.

$$AsO_3^{3-} \longrightarrow (TMS)_3\ AsO_3$$

$$BO_3^{3-} \longrightarrow (TMS)_3\ BO_3$$

$$CO_3^{2-} \longrightarrow (TMS)_2\ CO_3$$

$$SO_4^{2-} \longrightarrow (TMS)_2\ SO_4$$
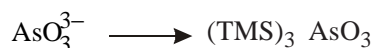
Fluoride can be derivatized in aqueous solution and extracted by trimethyl-chlorosilane into an immiscible organic solvent.

$$(C_2\ H_5)_3SiOH + F^- \longrightarrow (C_2H_5)_3SiF$$
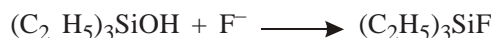
A number of methods are available for the derivatization of cyanide and thiocyanate.

$$CN^- + Chloramine - T \longrightarrow ClCN + products$$

$$CN^- + Br_2 \longrightarrow BrCN + Br^-$$

$$SCN^- + 4Br_2 + 4H_2O \longrightarrow BrCN + SO_4^{2-} + 7Br^- + 8H^+$$

The cyanogen halides can be detected at trace levels using gas chromatography with electron-capture detection. Thiocyanate can be quantitatively determined in the presence of cyanide by the addition of an excess of formaldehyde which converts the cyanide ion to the unreactive cyanohydrin.

Iodine, in acid solution, reacts with acetone to form monoiodoacetone, which can be detected at low concentrations by electron-capture detection. The reaction is specific for iodine. Iodide can be determined after oxidation with potassium iodate. The other halide anions are not oxidized by iodate to the reactive molecular form and thus do not interfere in the derivatization reaction.

The nitrate anion can be determined in aqueous solution by reaction with a mixture of benzene and sulphuric acid. Nitrobenzene is formed in 90 ± 8% yield for nitrate concentrations in the range 0.12–62 ppm. The nitrite anion can be determined after oxidation to nitrate with potassium permanganate.

13.4 Contributions of GC in the Day-to-day Life

Contributions made by GC in the fields of applied Chemistry and Biochemistry e.g. petroleum industry, environmental chemistry, clinical biochemistry and toxicology are of direct concern to man's daily life. By 1963, that is, only 11 years after the first paper on gas chromatography had been published, separation and identification of all the 39 hydrocarbons comprising the petroleum fraction boiling in the range of 28-140ºC had been accomplished by using this technique. Precise analyses of pollutants in air present in as low concentration as parts-per-billion of pesticide residues in soil, or hydrocarbons spreading on seas and beaches from spillovers from oil tankers are carried out by gas chromatography. The sort of separation that can be achieved by gas chromatography in the clinical laboratory may be illustrated by the fact that volatiles extracted from urine have been shown

to be comprised of almost as many as 300 compounds and out of these as many as 40 have been identified. Identity of a person as being a male or a female becomes known by studying the gas chromatogram obtained from effluents carried along by a stream of air passing over the body of the person concerned! The following account with the accompanying Tables is an adequate representation of the numerous applications of gas chromatography in the aforesaid fields.

GC in Clinical Biochemistry

Human breath, body fluid, urine and saliva contain a vast number of 'organic volatiles' which consist of essential nutrients, metabolic intermediates and waste products, environmental contaminants, and low molecular weight substances involved with various metabolic processes. Knowledge of the composition of these complex mixtures offers a considerable potential for the recognition of biochemical finger prints characteristic of the aetiology, pathogenesis or diagnosis of diseases. For such analyses, GC, especially in combination with mass spectrometry (MS), provides the most effective analytical tool. In view of their large number and chemical diversity, the study of organic volatiles poses special problems with regard to sample preparation for chromatography, resolution, sensitivity of detection, and the processing of the vast quantity of data generated from such measurements. In this field, as in most other biochemical applications of GC, the use of capillary columns and MS detection systems have become essential features of standard methodology. The preparation of samples involves procedures of varying complexity and efficiency, depending on the nature of the biological specimen and the analysis under consideration.

The methods of preparation of samples for GC analysis of biological specimens for organic volatiles may be classified into three broad categories:

  (i)   solvent extraction,
 (ii)   headspace techniques,
(iii)   direct chromatography.

Extraction of specimens with a suitable organic solvent and concentration of the extract prior to chromatography has been used for urine, plasma, blood, breast milk, amniotic fluid and saliva.

In direct headspace analysis, the sample e.g. serum or urine, is equilibrated with the headspace in a suitable container. A protion of headspace gas is then injected for analysis. More elaborate headspace trapping devices combine separation of the volatiles from the sample matrix with subsequent enrichment of the constituents. Such a system, suitable for small volumes of body fluids, is known as the transevaporator sampling technique. It contains a microcolumn packed with Porasil E (pore silica gel), into which the sample is injected. In one mode of use, helium is passed through the column to remove the volatiles which are then collected in a trap (Tenax-GC, a porous polymer, 2,6-diphenyl-p-phenylene oxide).

Alternatively, the volatiles in the specimen may be extracted by the vapour of an appropriate solvent e.g. 2-chloropropane and collected on a suitable adsorbent, such as Tenax-GC or glass beads. The substances in this adsorbent trap are thermally desorbed in a stream of helium, condensed in a cooled short pre-column and finally 'flashed' on to the main chromatographic column. Similarly, in the methods for breath analysis the sample is passed through a device in which organic compounds are retained, while nitrogen and oxygen present pass through unhindered. This process can be accomplished by cold trapping, chemical interaction or adsorptive binding.

Suitably diluted samples of body fluids have been used directly for the chromatography of, for example, ethanol, acetone and other metabolites. If needed volatiles can also be trapped and concentrated on a small, cooled pre-column of an adsorbent or a stainless steel capillary tube immersed in liquid nitrogen.

Some specific examples given in Table 13.6 are illustrative of the application of GC in Clinical Biochemistry.

TABLE 13.6

Applications of GC in Clinical Biochemistry

Sample	Preparation of sample for GC analysis and column conditions employed	Remarks
(a) Alkanes e.g. ethane, propane, butane and pentane in human breath	Sampling usually involves collection of breath for a period in a gas-tight bag and then passing a known volume of the sample through a liquid-nitrogen-cooled adsorbent pre-column. The adsorbed alkanes are desorbed thermally at the time of analysis. Column: 2 m (stainless steel) Packing: Chromosorb 102 Oven temperature: 50ºC for 1 min 50ºC/ min to 100ºC 15ºC/ min to 190ºC Injector temperature: 150ºC Detector: FID Temperature: 225ºC (tr: ethane 2.7 min, propane 4.56 min, butane 6.74 min, pentane 8.85 min)	The measurement of pentane in breath was proposed as a good index of lipid peroxidation, thus providing a sensitive test for assessing vitamin E status.

Contd.

Sample	*Preparation of sample for GC analysis and column conditions employed*	*Remarks*
(b) Volatile metabolites, methyl mercaptan, acetone 2-butanone, chloroform benzene, toluene, dipropylketone, 4-heptanone, cyclohexanone in plasma	A standard beadspace is employed for sample preparation. An aliquot of vapour phase obtained by incubation of a blood sample (0.5 ml) mixed with distilled water (2 ml) in a sealed vial at 55ºC for 20 min, is used for chromatography. Column : 2.1 m × 2.6 mm glass tube with 100% OV-17 on Chromosorb WHP, 80-100 mesh or Porapak P. Detector : FID	Raised levels of these indicate the condition of uraemia.
(c) Organic acids : Fatty acids excreted in faeces, organic acids in urine and plasma fatty acids.	For the isolation of short-chain fatty acids from biological materials such as faeces, steam distillation has been used. Body fluids e.g. serum, need deproteinizing by precipitation or processing by dialysis, ultra filtration or precipitation, before G.C. analysis may be undertaken. A biological sample of small size may be processed with a relatively larger volume of organic solvents e.g. methanol or chloroform-methanol mixture, thereby combining precipitation of proteins with the isolation of acid analytes. Urine is generally acidified before organic acids are isolated by solvent extraction or by some form of column chromatography. Inorganic acids in the sample are removed by precipitation with barium hydroxide. Some special adjuncts to isolation and pre-fractionation of acidic substances in body fluids are TLC, ion-exchange resins. Organic acids are normally made into alkyl or halogenated alkyl, halogenated arylalkyl or trimethylsilyl (TMS) esters before chromatography.	Short-chain fatty acids, excreted in faeces, are of interest in disorders of the large bowel because they are considered to be markers of fermentation in proximal part of the colon. Studies of the profiles of organic acids in body fluids have helped in the detection and characterization of the biochemical defects underlying organic acidaemias and acidurias found in some hereditary diseases, acquired metabolic disorders, such as diabetes mellitus, and other diseases e.g. those of the liver and kidneys.

Contd.

Sample	Preparation of sample for GC analysis and column conditions employed	Remarks
	Hydroxyl groups present are changed to TMS-ethers, amino groups to N-acyl derivatives, and carbonyl groups are stabilized by conversion to O-methyl or ethyl oximes. Column conditions employed for analysis of fatty acids from biological materials are illustrated by the following two examples: (i) *Measurement of plasma fatty acids* Column : 50 m × 0.22 mm WCOT CP-Sil 88 d_f 0.2 mm Oven temperature 180ºC and 225ºC Carrier gas: Helium; Flow-rate : 30 cm/s Detector: FID (ii) *Analysis of metabolites of thromboxane A_2 in urine* (Recoveries of 2, 3-dinorthrom-boxane B_2 and 11-dehydrothrom-boxane B_2 at 100-150 pg/ml were between 93-95%) Column: 30 m × 0.25 mm, DB-1 Inital temperature: 2 min at 100ºC; Temperature rise: 100 to 250 at 30ºC/min; 250 to 300ºC at 5ºC/min; Final temperature 300ºC for 10 min Carrier gas: Helium Detector: MS	
(d) Amino acids e.g. tyrosine, glycine alanine, glutamic acid, glutamine, aspartic acid, ornithine, phenylalanine, lysine, leucine, isoleucine, methionine, proline, tryptophan, valine, in body fluids.	Amino acids have to be converted to suitable derivatives for G.C. analysis. Before carrying out reactions for preparing derivatives, samples require some form of clean-up, such as deproteinization or more commonly the use of a cation-exchange resin. Both the amino and carboxyl groups may take part in the	Body fluid amino acid analysis is often a part of research on patho-physiology of diseases concerning e.g. nutri-tion, neuro-trans-mitters, muscles, and endocrine systems.

Contd.

Sample	Preparation of sample for GC analysis and column conditions employed	Remarks
	preparation of derivatives and some examples of the acylation and esterificaton products are : N-fluoroacetyl butyl esters, N-heptafluorobutyryl (HFB) -n-propyl esters, HFB-isoamyl esters, HFB-isobutyl esters, and pentafluoropropionyl, hexafluoroisopropyl esters. Overall, the choice of the entire procedure—sample isolation and preparation of derivatives—has to be made and validated with respect to the individual amino acids at hand. Columns conditions are: Column: 30 m × 0.25 mm, DB–5 Inlet temperature: 180ºC Initial temperature: 60ºC for 3 min Temperature rise: 5ºC/min to 180ºC Derivatives: Pentafluoropropionyl (PFP) and hexafluoroisopropyl (HFIP) esters Detector: MS	
(e) Cholesterol, metabolites, and bile acids in body fluids.	Sample preparation for analysis of cholesterol in body fluids generally begins with a hydrolysis step, chemical or enzymic, to convert cholesterol esters to cholesterol. Dilution of body fluids with, say, 0.1M NaOH releases bile acids from albumin. Bile acids and their conjugates can be extracted by anion-exchange resins e.g. Amberlite XAD-2. Cartridges packed with materials such as octadecylsilane-bonded silica provide an easy and effective way of retaining bile acids and steroids which can then be eluted with organic solvents. The conjugated metabolites of steroids and bile acids have to be hydrolyzed	Body fluid steroid profile may be of diagnostic significance in certain special disorders e.g. inborn errors of steroid metabolism. GC is valuable for analyzing bile acids and their metabolites in bile, intestinal content, and serum in research on hepatic, hepatobiliary and intestinal diseases.

Contd.

Sample	Preparation of sample for GC analysis and column conditions employed	Remarks
	first by chemical or enzymic means, producing the appropriate alcohol or acid before the sample may be further processed for GC. The acids are converted to methyl, ethyl, or some fluoroalkyl esters. The hydroxyl group in the steroid nucleus may be changed to acetate or fluoroacetate or silyl or methyl ether; the carbonyl group can be made into an oxime. The following two examples viz. assay of cholesterol in body fluids and the determination of serum bile acids, illustrate the column conditions employed in analysis of fluids for cholesterol metabolites and bile acids. *Assay of cholesterol in body fluids* Column : 5 m × 0.53 mm, HP–1 methylsilicone megapore Isothermal temperature: 253ºC Carrier gas: Methane/argon Flow rate: 6 ml/min Detector: ECD *Determination of serum bile acids* Column: 20 m × 0.2 mm, HiCap–CBPI Isothermal temperature: 275ºC Carrier gas: He Linear velocity: 40 cm/s Detector: FID and MS	
(f) Amines and related compounds e.g. catecholamines, polyamines, 5-hydroxytryptamine and their metabolites in body fluids.	Catecholamines in body fluids can first be extracted with diethyl ether or ethyl acetate and then back-extracted into an acidic aqueous phase. Solid phase matrices used for extraction include activated alumina, cation-exchange resins and boric acid-bound affinity gel. For acidic meta-bolites, samples are acidified before extraction with organic	Studies of biogenic amines are mostly connected with research relating to psychiatric disorders, regulation of sympathetic activity, blood circulation, harmonal control and diabetes mellitus. Altered

Contd.

Sample	Preparation of sample for GC analysis and column conditions employed	Remarks
	solvent. Amines and their metabolites have to be converted to suitable derivatives for analysis by GC. For example, determinations of acidic and alcoholic products of catecholamines in plasma and urine can be conducted with good precision using acetylation or silylation of phenolic hydroxyl groups, esterification of carboxyl group (pentafluorobenzyl ester) and acylation of aliphatic hydroxyl groups. Column conditions employed for the analysis of amines and related compounds are illustrated below by taking the case of the chromatography of acidic metabolites of catecholamines. Column: 20 m × 0.22 mm, CPSIL–19 Initial temperature: 260ºC for 2 min Temperature rise: 5ºC/min Final temperature: 300ºC Carrier gas: He Flow rate: 0.55 ml/min Detectors: ECD and MS (Recoveries of homovanillic acid and vanillylmandelic acid and dihydroxyphenyacetic acid are between 86 and 94%) Simultaneous measurement of polyamines such as 1,3-diaminopropane, putrescine cadaverine, spermidine and spermine in body fluids or erythrocytes is possible by GC using capillary columns and FID, ECD, NPD or MS detector. Urinary metabolites have to be first hydrolyzed in acid. Most procedures involve adsorption of analytes on a solid phase e.g. silica gel, followed by the preparation of	patterns of erythrocyte polyamines were reported in chronic renal failure, sickle cell anaemia and liver diseases, but they appear more promising for estimating the extent of chemo- and radiotherapeutically induced tumour cell death in cancer patients.

Sample	Preparation of sample for GC analysis and column conditions employed	Remarks
	halo-acyl derivatives e.g. heptafluorobutyryl amine. The column and detectors employed for GC of polyamines and metabolites are illustrated below for the case of analysis of methyl heptafluorobutyryl derivatives of polyamines and metabolites from acid-hydrolyzed urine. Column: 35 m × 0.2 mm fused silica capillary coated with cross-linked methyl silicone gum, 0.11 μ film thickness Detectors : FID or NPD	
(g) Aldo and keto sugars and polyols e.g. mannose, fructose, glucose, threitol, erythreitol, arabitol, ribitol anhydroglucitol, glucitol, myoinositol, from body fluids e.g. cerebrospinal fluid or plasma.	Plasma and cerebrospinal fluid (CSF) with added internal standard e.g. rhamnose, may be processed by shaking with organic solvent such as methanol. After drying the liquid phase *in vacuo*, the common practice is to convert polyols to silyl ethers prior to GC. Column: 25cm × 0.31mm coated with 0.52 μm film of cross-linked methyl silicone Temperature programming: 50ºC to 140ºC at 10ºC / min, then 140ºC to 190ºC at 1ºC / min, and finally to 260ºC at 30ºC / min Detectors : FID and MS	Polyols, the reduction products of sugars, are known to play important roles in processes associated with human physiology and pathology. GC methods of analysis of polyols in body fluids are applied to the study of brain carbohydrate metabolism in health and disease.

GC IN ANALYTICAL TOXICOLOGY

Drugs

The requirement for drug screening is to have the ability to detect as wide a range of compounds as possible in as little sample (plasma/serum/whole blood, urine/vitreous humour, stomach contents/vomit or tissues) as possible at a high sensitivity but with no false positive(s). Ideally some sample should be left to permit confirmation of the results using another technique such as paper chromatography or TLC, and quantification of any poison(s) present to aid clinical interpretation of the results.

As is the case with GC in Analytical Biochemistry, there is need for some form of sample pretreatment for GC in Analytical Toxicology, and the sample preparation procedures already discussed under 'GC in Analytical Biochemistry' are used to meet the aforesaid need as well.

In drug-screening, low-polarity phases such as SE-30/OV-1/OV 101 are used. These are dimethylpolysiloxane stationary phases which are regarded as broadly equivalent. Polar interactions are minimal with these phases and thus separations occur largely on the basis of molecular weight. However, a disadvantage is that peak tailing may occur with polar compounds. In general, peak tailing is reduced on more polar phases; SE–54, OV-7, OV-17, and OV-225 are amongst the more polar polysiloxane-based phases available. Carbowax 20 M (polyethylene glycol of average formula weight 20,000) is a high-polarity phase but has a relatively low maximum operating temperature. However, precoating the support with KOH effectively minimizes peak-tailing of strong bases and the maximum operating temperature is unaffected. Polyester phases such as cyclohexanedimethanol-succinate (CHDMS) and also polyamides such as poly A103 have been advocated for specific separations of polar compounds such as barbiturates. Mixtures of stationary phases have also been advocated for specific problems. For example, a commercially available mixture of SP-2110 and SP–2510-DA (Supelco) has been widely used in the analysis of common anticonvulsants.

Use of low polarity phases such as SE-30/OV-1/OV-101 have the advantage that retention data for a large number of drugs and other compounds of interest generated on packed columns are available—such data are generally directly transferable to capillary columns. However, use of a 25 m × 0.32 mm i.d. fused silica capillary coated with cross-linked HP-5 (0.52 μ film) is advantageous when screening for basic drugs (HP-5 is a 5% phenyl, 95% methysilicone phase).

Examples of screening of some classes of drugs are listed in Table 13.7.

TABLE 13.7

Applications of GC in Analytical Toxicology

Sample	Column employed	Remarks
(i) Amphetamines and related compounds.	Capillary GC-NPD.	Dexamphetamine [(S)-α-methyl-phenylethylamine] and methyl-amphetamine [(S)-N, α-dimethyl-phenylethylamine] are commonly-abused central nervous system (CNS) stimulants. Other drugs such as chlorphentermine, diethylpropion, ephedrine, fenfluramine, phenter-mine, and phenylpro-panolamine (nor-ephedrine) may be encountered.

Contd.

Sample	Column employed	Remarks
(ii) Anticholinergics	Capillary GC-NPD and GC-MS	Atropine is used as a preanaesthetic medication in ophthalmic proce-dures and as an antidote in poison-ing due to cholinesterase inhibitors. The related compound, hyoscine (scopolamine), is used as a premedi-cation and to prevent travel sick-ness. Further, another com-pound, orphenadrine, is used primarily as an anti-Parkinsonian agent.
(iii) Anticonvulsants	Capillary or packed column GC-NPD and GC-FID A mixed SP-2110/ SP–2510–DA (Supelco) phase permits the isothermal analysis of 11 common anticonvulsants and asso-ciated internal standards by GC on a packed column.	Amongst the commonly-used anticonvulsants are carbamazepine, ethosuximide, phenobarbitone, phenytoin (diphenyl hydantoin) primidone and sodium valproate.
(iv) Antihistamines and N-demethylated and other metabolites	Capillary GC-NPD	This is a heterogeneous group and includes chlorpheniramine, cycli-zine, diphenhydramine, doxylamine, pheniramine, terfenadine, and tripelennamine; many phenothia-zines are used as antihistamines.
(v) Barbiturates and related hypnotics	Capillary GC. On-column methylation by injection with 0.2 mol/l trimethylanilinium hydro-xide in methanol has been employed in packed column GC or GC-MS of barbi-turates	Barbiturates are 5, 5′-disubstituted derivatives of barbituric acid. Substitution of sulphur for oxygen at position 2 gives thiobarbiturates such as thiopentone. This latter compound and phenobarbitone are used as anticonvulsants. Barbitu-rates with relatively short half-lives such as amylobarbitone, butobar-bitone, pentobarbitone and quinal-barbitone are now rarely used as hypnosedatives in clinical practice, but these compounds are widely abused either directly or when used to 'cut' other substances, and are subject to Regulations on Misuse of Drugs.

Contd.

Sample	Column employed	Remarks
(vi) Benzodiazepines e.g. diazepam, tema-zepam alprazolam, flunitrazepam, keta-zolam, lorazepam, midazolam, and triazolam.	GC-ECD is commonly used in analysis of this group of compounds.	Benzodiazepines are used as tranquillizers and hypnotics, and in some cases as anticonvulsants and short-acting anaesthetics; some 60 compounds have been marketed. Temazepam (3-hydroxydiazepam) especially has been abused, often together with other drugs. Most benzodiazepines are extensively metabolized. Indeed many are metabolites of other compounds. Thus diazepam gives nordiazepam, temazepam, and oxazepam (3-hydroxynordia-zepam); the latter compounds are excreted as glucoronides and sulphates.
(vii) Cannabinoids	GC or capillary GC-MS	Cannabis preparations in their various forms are widely abused. Over 60 active constituents (cannabinoids) are known.
(viii) Monoamine oxidase inhibitors (MAOIs)	Capillary GC-NPD	This group includes phenelzine and tranylcypromine. Plasma concentra-tions are low, since binding to monoamine oxidase occurs rapidly *in vivo* and unbound drug is excreted.
(ix) Narcotic analgesics	GC-MS	This is a complex group and includes opiates such as morphine and codeine, heroin (diamorphine, diacetylmorphine) produced by treating morphine (or opium in the case of illicit preparations) with acetic anhydride, and synthetic analogues such as buprenorphine and dihydrocodeine. All are strictly controlled, but compounds with very similar structures such as the antitussive pholcodine (morpho-linylethylmorphine) have virtually no opioid agonist activity and are available without prescription.

Contd.

Sample	Column employed	Remarks
		Other compounds with similar structures (nalorphine, naloxone) are potent opioid antagonists. Metabolism is complex, morphine being a metabolite of both heroin and codeine, for example. HPLC is important in the analysis of this group of compounds since many analytes are hydrophilic, thermally labile, or otherwise unsuited to GC unless derivatized.
		Many other synthetic compounds such as dextropropoxyphene (D-propoxyphene) and methadone, for example, are potent narcotic analgesics (opioid agonists) and their use is controlled in most countries. Dextropropoxyphene is extensively metabolized by N-demethylation and by other routes. Methadone is metabolized largely by N-demethylation and hydroxylation. Dextropropoxyphene is thermally labile but may be measured together with nordextro-propoxyphene by HPLC with electrochemical oxidation detection; methadone and some metabolites may also be measured using this same system.
(x) Paracetamol	GC-FID after derivatization	Paracetamol (acetaminophen) is a widely used analgesic and sometimes occurs together with other drugs such as dextropropoxyphene. The detection and measurement of paracetamol is of great importance in clinical toxicology, since clinical management of cases of acute poisoning is guided by the result.
(xi) Antidepressants	GC-NPD and GC-MS	Amititriptyline, clomipramine, dothiepin, doxepin, imipramine, protriptyline and trimipramine are examples of tricyclic

Contd.

Sample	Column employed	Remarks
		anti-depressants. N-dealkylated metabolites are common and some e.g. nortryptyline and desipramine, are also used as drugs. Related compounds include the tetracyclic antidepressants maprotiline and mianserin, and the isoquinoline derivative nomifensine.
(xii) Pesticides (a) Organochlorines	Solvent extraction followed by GC-ECD remains the method of choice for the analysis of organochlorines in biological specimens. The phenolic metabolites of lindane may be measured by GC-ECD after acid hydrolysis of conjugates followed by acetylation. Analysis of the insecticide pentachlorophenol is conveniently carried out by solvent extraction followed by GC-ECD after acetylation of the compound.	Organochlorine pesticides include aldrin, chlordane, dichophane (DDT), dieldrin (also a metabolite of aldrin) endrin heptachlor, hexachlorobenzene (HCB), 1, 2, 3, 4, 5, 6-hexachlorocyclohexane (HCH = benzenehexachloride, BHC), and lindane (gamma - HCH). These compounds were widely used but persist in the environment, and lindane, which has a relatively short half-life *in vivo*, is now the only member of this group still in common use. Pentachlorophenol is used as a contact herbicide. This compound is still widely used in wood preservatives and disinfectants.
(b) Organophosphates (OPs) and carbamates	OPs themselves can be measured after solvent extraction by GC-NPD or by GC-FPD. Chlorinated OPs such as chlorpyriphos and its major metabolite have also been measured by GC-ECD after gel permea-tion chromato-graphy. OPs such as parathion which contain a nitro moiety have also been measured by GC-ECD. Carbamates such as carbaryl have been measured by GC-ECD after derivati-zation	Some organophosphates and carba-mates are used as herbicides and fungicides and are relatively non-toxic to man. OP and charbamate insecticides, however, inhibit acetylcholinesterase and some are extremely toxic.

Contd.

Sample	Column employed	Remarks
	with heptafluoro-butyric anhydride.	
(xiii) Gases, solvents and other poisons		
(a) Carbon monoxide	Liberation of CO from carboxyhaemoglobin (present in the blood sample) by mixing with ferricyanide in a headspace vial followed by GC on a column packed with 60/80 mesh 0.3 nm molecular sieve, is a conve-nient method of analysis. CO itself does not respond on the FID, but reduction to methane by reaction with hydrogen in the presence of a nickel catalyst allows an FID to be used.	Carbon monoxide (CO) poisoning remains the single most common cause of fatal poisoning in developed, Western countries and most probably in the rest of the world as well. Motor vehicle exhausts, defective heating systems and smoke from all types of fires are common sources. Some 40% of an absorbed dose of dichloro-methane is also metabolized to CO.
(b) Cyanide	A headspace GC method has been used, the method being based on NDP after liberation of HCN and employs acetonitrile as the internal standard.	Cyanide (CN) poisoning may be encountered after the inhalation of hydrogen cyanide (HCN), or after the ingestion of hydrocyanic acid or of potassium or sodium cyanides. Cyanide solutions used in electroplating may release HCN if acidified, while a number of naturally occurring nitriles are metabolized to cyanide ion *in vivo*. Thioyanate insecticides also give rise to cyanide *in-vivo*. In addition, cyanide is often present in the blood of fire victims due to the inhalation of HCN from the partial combustion of wool, silk, and synthetic polymers such as polyurethanes and polyacryloni-triles; carbon monoxide is usually also present.
(c) Alcohols	GC-FID is the method of choice if a range of compounds of interest (primarily methanol,	Despite the introduction of evidential breath ethanol instruments, the measurement of blood ethanol in road traffic

Contd.

Sample	Column employed	Remarks
	isopropanol and acetone) are to be measured. (Direct injection or headspace methods have been employed for ethanol and are often applicable to the other compounds).	accidents remains an important analysis. In addition, ethanol from alcoholic drinks is very frequently encountered in hospital admissions due to acute poisoning. However, poisoning with industrial alcohol (methylated spirit) containing various denaturants, notably methanol, still occurs. Ethanol may also be given to treat poisoning with methanol or with ethylene glycol. Methanol itself is used as a general and laboratory solvent, in car radiator antifreeze, and in wind-screen washer additives. Serious outbreaks of acute methanol poisoning have occurred in several countries in recent years due to the sale of illcit 'alcoholic' drinks based on methanol. Isopropanol used in lotions for topical administration, window and windscreen washers, and as a solvent for toiletries, is metabolized to acetone and is much less toxic than methanol.
(d) Glycols	A column packed with a porous polymer such as Chromosorb 101 or, after derivatization of the glycols as the corresponding phenyl boronates, on a OV-101 packed column.	Ethylene glycol is used mainly in car radiator antifreeze as a concentrated (20-50%, v/v) aqueous solution, sometimes together with methanol. Propylene glycol is used as a solvent in pharmaceutical and food industries and is relatively non-toxic.
(e) Solvents and volatiles	Separation of many volatiles in blood or in tissue digests is often conveniently carried out with headspace GC using a packed column [e.g. a 2 m × 2 mm i.d. 0.3% (w/w) Carbowax 20 M on Carbopack C column] programmed from 35ºC to	Today, if anaesthesia is excluded, acute poisoning with solvents and other volatile substances usually follows deliberate inhalation of vapour in order to become intoxicated [volatile substance abuse (VSA)]. Patients who ingest solvents or solvent-containing products either by accident or

Contd.

Sample	Column employed	Remarks
	175°C, and split-flame ionisation/electron capture detection (FID/ECD). Good peak shapes are obtained. Use of a 60 m × 0.53 mm i.d. fused silica capillary coated with the dimethylpolysiloxane phase SPB - 1(5 μm film thickness) is more advantageous than the employment of the packed column in that good peak shapes result for the most commonly abused compounds such as bromo-chlorodifluoromethane (BCF), dichlorodifluoro-methane (FC 12), DME, fluorotrichloromethane (FC 11).	deliberately, and the victims of domestic and industrial mishaps, provide further groups which may suffer acute poisoning by these compounds. Toxicity due to fumigants such as bromo-ethane or due to compounds used primarily as chemical intermediates, on the other hand, is more commonly associated with occupational exposure. Concern as to the consequences of the release of massive quantities of organochlorine and organobromine compounds such as chlorofluoro-carbons (CFCs) into the atmosphere has led to the planned phased withdrawl of many of the volatile halogenated compounds in current use. Commercial 'butane' (liquified petroleum gas, LPG) and dimethyl ether (DME) have already largely replaced CFCs as propellants in aerosols, for example, in many countries. In the case of refrigerants the trend is towards the use of polyfluori-nated compounds such as 1, 1,1,2-tetrafluoroethane.
(f) Nicotine	Capillary GC-NPD	Nicotine is commonly encountered in tobacco, although usually in concentrations insufficient to cause acute poisoning except when ingested by young children. However, nicotine occurs in higher concentrations in some herbal medicines and may also be used as a fumigant in horticulture. Nicotine is metabolized by N-demethylation to give cotinine. The need for the measurement of nicotine and/or cotinine in biological specimens is most often occasioned in the context of studies of smoking behaviour.

Environmental Analysis

Before any GC separation and quantification can be carried out on an environmental sample, the target compounds must first be extracted from the sample matrix and interfering compounds removed by a clean-up procedure.

The environmental samples that may be collected are of the following types:

water—river water, sea water or potable supply

effluent—industrial or sewage

sewage sludge

sediment—fresh water or marine

biological tissue—from a variety of organisms such as fish invertebrates, birds

gases—from stack emission, landfill of industrial and domestic wastes, or workplace environments.

oil—from pollution incident or from suspected source

Apart from gases and oil, the most likely pollutants that will need to be determined are pesticides, solvents, polyaromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs). The pesticides may be either insecticides or herbicides and are usually organochlorine, organophosphorus or organonitrogen compounds.

These substances will generally be present at low concentrations in environmental samples typically in the pg to μg range (10^{-12}–10^{-6} g/litre), and rarely at the mg (10^{-3} g/litre) level. Gas chromatography is an extremely sensitive technique, especially when an ECD is used as the detector.

Because of its sensitivity, the technique is very responsive to contamination and high background interference. Any material that contains a semi-volatile organic compound which could come in contact with the sample will cause interference. Such materials include plastic tubing, plastic bottle tops, liners in bottle caps, GC vial septa, rubber components in gas regulators, impure carrier gas, and impurities dissolved in extractant solvents from the laboratory atmosphere among others.

In any extraction technique, it is quite likely that the extractant solvent will contain interfering substances which will produce additional peaks in the chromatogram. This occurrence is particularly so in the extracts from samples of effluent, sediment and tissue which contain fats, oils and other naturally occurring substances. The long-established procedure for the removal of these substances has been to pass the extract through an alumina column and then to separate the target compounds into different batches by passing the clean-up extract through a silica gel column. In a modified form of this clean-up and separation, the concentrated extract is cleaned up on an alumina/silver nitrate column followed by work-up with silica gel.

Recently, the process of clean-up and separation has been streamlined by the use of commercially available solid-phase extraction cartridges or discs.

There are a variety of cartridges available (e.g. Bond Elut). Each is filled with a chemically modified silica adsorbent, the appropriate one being selected

according to the nature of the material to be extracted. The adsorbed compounds are next eluted with suitable solvents.

Extraction discs are suitable only for clean water samples. The organic compounds are removed from the water as it is filtered through the extracting disc and then the compounds are eluted with a suitable solvent.

Typical examples of analysis of environmental samples of water, effluent etc. using gas chromatography are listed in Table 13.8.

TABLE 13.8

Applications of GC in Analysis of Environmental Samples of Water, Effluent, Sediment, Tissue

Sample	*Preparation of the sample extract and its work-up prior to its GC analysis*	*GC column conditions*
A: Organochlorine compounds present in (i) Samples of water	Two internal standards, octachloro-naphthalene and ε-hexachlorocyclo-hexane (ε-HCH), are added to a measured volume of the water sample and extraction of the organochlorine compounds is carried out by shaking with hexane. The hexane extract is dried over anhydrous sodium sulphate and the extract is filtered. A little quantity of isooctane is added to the hexane extract as 'keeper' to prevent evaporation losses. Concentration of the extract has to be brought about as the next step. A special procedure for carrying out the concentration of the hexane extract is needed to avoid loss of organo-chlorine compounds. The volume of the hexane extract is first reduced to about one-tenth of its original volume by using a rotary evaporator under vacuum and then to about one-hundredth of its original volume by evaporating it slowly (i.e. small disturbance on surface only) in a fume cupboard using the nitrogen or compressed air line.	With the introduction of capillary columns, the choice of stationary phase is not so critical as with packed columns and most components can be satisfactorily separated on either a non-polar (OVI) or a polar (Carbowax 20M) column. Examples of narrow-bore capillary columns which are widely used include 30 m × 0.25 mm DB 5 and SP 608. For organic chlorine compounds the following. GC conditions are typical: Detection mode — ECD Carrier/ make-up gas; flow-rate — helium, 1 ml/min nitrogen, 60ml/min Temperature — 60°C held

Contd.

Sample	*Preparation of the sample extract and its work-up prior to its GC analysis*	*GC column conditions*
	The concentrated hexane extract is passed through an alumina/silver nitrate column for the removal of interfering substances. Elution of this column is then carried out using hexane as the eluent. The eluate is first concentrated to about one-third of its original volume in a rotary evaporator and then the volume of the concentrate is further reduced tenfold by blowing slowly. This concentrated hexane eluate from the alumina/silver nitrate column is added to the top of a silica gel column and allowed to be adsorbed. The elution of the silica column is carried out first by using hexane to obtain eluate 1, and subsequently by using diethyl ether/hexane mixture (20:80, v/v) to obtain eluate 2. Each eluate is concentrated down to about one-tenth of its volume using a blower and is kept in sealed vial until injection into the gas chromatograph. The possible compounds in eluates 1 and 2 are listed below: *Eluate number*	for 1 min upto 140°C at 20°C/ min, held for 1 min programme upto 190ºC at 3ºC/min, held for 1 min upto 28ºC at 15ºC/min, held for 1 min Injection volume 1 µl Injection mode cold on-column Although wide-bore capillary columns are not as widely employed as the narrow or medium-bore variety, these columns can be used, in general, for pesticides and priority pollutants (where fast analysis is essential) as an alternative to packed columns, and examples are : 30 m × 0.53 mm DB 5 and DB 608. The following GC conditions are typical:

1	2
Aldrin	Dieldrin
pp-DDE	Endrin
op-DDT	Chlorpyrifos
PCBs	HCH
Endosulphan B	Heptachlor
HCB	epoxide
HCBD	op-DDT
Heptachlor	pp-TDE
	Endosulphan A

Alternatively, clean-up of the sample extract can be carried out using a solid phase extraction (SPE) cartridge.

Carrier gas;   helium;
flow-rate   6 ml/min
Make-up gas;   nitrogen;
flow-rate   60 ml/min

Temperature programme
    140ºC, held for 4 min
    upto 225ºC at 5ºC/min
    upto 250ºC at 2ºC/min,
        held for 10 min
Injection volume   4 µl

Though the preparation of packed columns is less

<div align="right">*Contd.*</div>

Sample	*Preparation of the sample extract and its work-up prior to its GC analysis*	*GC column conditions*
	The most appropriate one for use with organochlorine compounds is the aminopropyl cartridge. The hexane extract is added to the top of a Bond Elut aminopropyl cartridge (filled with a chemically modified silica adsorbent) and allowed to pass through it. The clean extract is collected in a glass test-tube. A further quantity of the solvent, hexane, is passed through the cartridge and the eluate collected in the same test-tube. The total eluate is concentrated by blowing down. The cleaned-up eluate is put on top of a silica gel column and allowed to be adsorbed. This column is eluted first with hexane and then with ether/hexane mixture (20:80 v/v), collecting the first and second eluates separately. Eluates 1 and 2 are then concentrated by using a blower. Each eluate is now ready for GC analysis. The possible compounds in eluates 1 and 2 are listed below.	technically difficult than the preparation of capillary columns, the former can be used only selectively and such columns would not be recommended if high performance and efficient separation are to get priority as, for example, is the case with complex mixtures. For the separation of organ-chlorine compounds using packed columns, The appropriate GC column conditions are : Glass column, 1.5 m × 2mm, packed with 80–100 mesh Chromosorb WHP containing 5% OV–1–1

Eluate number

1	2	
	HCH	Carrier gas; nitrogen flow-rate 60 ml/min
HCB	Endosulphan A	Injector temperature 240ºC
HCBD	Dieldrin	Oven temperature 200ºC
Aldrin	Endrin	
Heptachlor	Heptachlor epoxide	Detector temperature 320ºC
pp-DDE	op–DDT	
op-DDE	pp–TDE	Injection volume 5 µl
Endosulphan B	Chlorpyrifos	
PCBs		

| (ii) Samples of effluent | The procedure of preparation of the sample extract is essentially the same as that for the preparation of clean | The same column condi-tions for the GC analysis of the organochlorine (OC) |

Contd.

Sample	Preparation of the sample extract and its work-up prior to its GC analysis	GC column conditions
	water samples. An emulsion is more likely to form with these types of sample. Its formation can be discouraged by adding Analar grade sodium chloride along with the internal standards before starting extraction with hexane.	com-pounds in this case are applied as those detailed under the clean water-sample analysis for OC compounds.
	Effluent samples, however, may contain sulphur compounds which interfere with the chromatography by producing extra peaks. These compounds can be removed using either mercury or tetrabutylammonium sulphate (TBAS). Though both procedures work satisfac-torily, the TBAS method is preferred because of the hazards associated with mercury and the difficulties with the disposal of the metal. The following procedure is used for the removal of sulphur compounds:—	
	Appropriate quantities of propan-2-ol, TBAS solution and solium sulphite are added to the concentrated effluent sample extract (prepared in the manner employed for clean water samples), and its extraction is then carried out with hexane. The hexane extract, after being dried by shaking with anhydrous sodium sulphate, is filtered. The filtered extract is now ready for further processing in the same manner as that given earlier for the dried hexane extract of a clean water sample.	
(iii) Sediment samples	Extraction of organochlorine compounds from sedimentary material involves the use of soxhlet extraction equipment. Any supernatant water from the sample is removed with a Pasteur	Analysis for organochlorine compounds is carried out by any of GC column conditions already detailed under the GC analysis for OC compounds present in clean water samples.
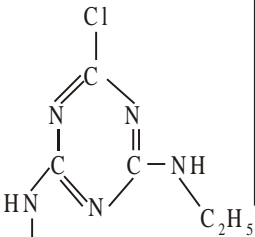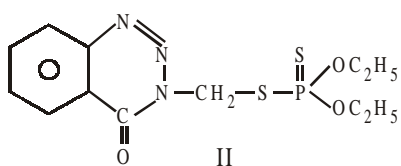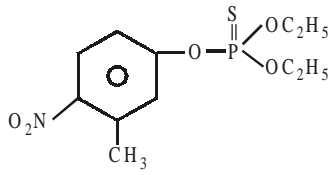
Contd.

Sample	*Preparation of the sample extract and its work-up prior to its GC analysis*	*GC column conditions*
	pipette. A weighed quantity of the wet sediment is ground with enough anhydrous sodium sulphate until a dry, free-flowing, powder is produced. An appropriate quantity of an internal standard such as ε-HCH is added to the mixture. The powder is transferred to the soxhlet thimble which is then placed in the extraction apparatus charged with ether/hexane mixture (20:80, v/v). The sample is allowed to be extracted for at least 10 cycles. The extract is transferred to a rotary evaporator and concentrated to about one-tenth of its volume. Removal of sulphur compounds from the concentrated extract and its subsequent work-up is a repeat of the procedure detailed under preparation of the extract of effluent samples.	The amount of organo-chlorine compounds in the sediment needs to be related to the amount of dry matter and to the organic content of the sample. These should be determined separately by determining the moisture content of the sediment by drying a weighted amount in an oven at 105ºC, while the total organic content is determined by igniting in a muffle furnace or chemical oxidation with chromic acid.
(iv) Tissue samples	The procedure for extraction is the same as that described for sediment samples i.e. soxhlet extraction, except for the preparation of the material. The amount of material extracted depends upon the lipid content because the more the fatty material in the tissue the more pre-injection clean-up is required. Thus, for a fish liver sample, about 2g of material is extracted. For muscle tissue, which contains less fatty material, the sample weight can be increased to about 5g. The lipid content is determined on a separate amount of sample by extracting with petroleum ether and evaporating the extract to constant weight over a steam bath. The tissue sample for extraction is weighed wet after it has been broken	The same column conditions for the GC analysis of organochlorine compounds in this case are applied as are given under the clean-water sample analysis for OC compounds.

Sample	Preparation of the sample extract and its work-up prior to its GC analysis	GC column conditions
	up by homogenizing, using a suitable homogenizer such as Ultraturex. An alternative method for preparing the biological tissue for extraction is to freeze-dry the material and then grind it to a fine powder. The subsequent work-up of the tissue sample extract is carried out by following the procedure applied to the work-up of a sediment sample extract is carried out by following the procedure applied to the work-up of a sediment sample extract.	
(v) Water samples containing pentachlorophenol (PCP). (Pentachlorophenol is a common organochlorine compound that is found in environmental samples. It is used as a preservative for wood and glue as well as an insecticide, herbicide and as a defoliant. It has been found in effluent from the paper industry, tanneries and textile plants.	Before pentachlorophenol can be extracted from water samples by a solvent, the compound is converted into acetate by shaking with acatic anhydride in the presence of borax. α-HCH (as its 10 μg/litre isooctane solution) is added as an internal indicator. The PCP acetate is extracted with hexane. About 1ml of the hexane layer transferred to a sample vial is ready for GC analysis.	Column conditions employed are the same as detailed above for the analysis of OC compounds.
B: Non-persistent pesticides present in other samples (i) Orga-nophosphorus (OP) and organo-nitrogen (ON) compounds in water. (In the light of the accumu-lating evidence that some organo-chlorine pesticides persist in the	The water sample is extracted with dichloromethane in the presence of sodium chloride. The dichloromethane extract is dried by passing it through a No. 1 filter paper containing anhy-drous sodium sulphate, the filtrate being collected in a rotary evaporator flask. The extract is reduced to about one-tenth of its original volume in the rotary evaporator, under vaccum. This	Narrow bore capillary columns which are widely used for OP and ON pesticides include 30 m × 0.25 mm DB 5 and SP 608. The appropriate GC conditions are: Detection mode NPD Carrier/make-up gas flow rate

Contd.

Sample	Preparation of the sample extract and its work-up prior to its GC analysis	GC column conditions
environment and bioaccumulate in the tissue of some target organisms, 'environment friendly' new pesticides such as those illustrated below by structures I to IV, which are based on nitrogen and phosphorus, have been manufac-tured more recently).	concentrated extract is transferred to a graduated test-tube and further concentrated to one-tenth of its volume by carefully blowing a stream of clean air or nitrogen. The extract is now ready for analysis by GC.	helium, 1ml/min nitrogen 60 ml/min gas flow-rate nitrogen 60 ml /min Temperature 35ºC held programme for 1 min upto 160ºC at 10ºC/min, held for 2 min upto200ºC at 2ºC/min, held for 1 min upto 300ºC at 10ºC/min, held for 5 min Injection 3 µl volume
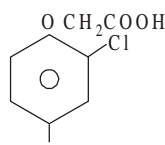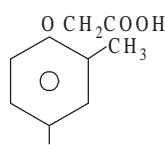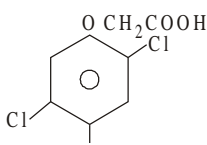
I
(simazine)

II
(azinphosethyl)

III
(fenitrothion)

Sample	Preparation of the sample extract and its work-up prior to its GC analysis	GC column conditions
IV (dichlorvos) (ii) Permethrin (Pyre-throids are another group of 'environment friendly' pesticides. They are extracted from the pyrethreum plant and are used as insecticides. An example of a synthetic analogue pyrethroid is permethrin: permethrin		
Permethrin is increas-ingly being used to tackle a variety of problems requiring an effective insecticide that will not cause adverse effects in the receiving environment. It is used, for example, as a moth-proofing agent in carpet manufacture, and in garden sprays to combat a variety of pests.)	Clean water samples are extracted with hexane. The hexane extract is dried and then concentrated before quantification by GC. The dirtiet samples are processed in the following manner which involves the use of solid-phase extraction cartridges. Extraction of a specific volume (say, 500 ml) of the dirty water sample with hexane is carried out after bringing the pH to 2 by acidification of the sample. The hexane extract is dried by passing it through No. 1 filter paper containing anhydrous sodium sulphate. The extract is then blown dry to about 1 ml. This concentrated extract is adsorbed on a Bond Elut C_{18} octyl (No. 606303)	

Sample	Preparation of the sample extract and its work-up prior to its GC analysis	GC column conditions
	cartridge. Hexane (5 ml) is forced through the Bond Elut tube with the help of a syringe colleting only the first 2 ml of the eluate into a 5 ml tube. The 2 ml eluate is evaporated down to l ml. The l ml eluate is added to the top of a Bond Elut silica (No. 601303) tube and allowed to be adsorbed. The tube is washed with hexane (10 ml), discarding the eluate. Eher/hexane (5:95, v/v) mixture is then forced down the tube with the help of a syringe, using 6 ml of the solvent mixture. First 2.5 ml of the eluate is discarded, collecting the next 3 ml which will contain any perme-thrin that may be present. This latter eluate (3 ml) is concentrated slowly to l ml using a blower and this extract is now ready for GC quantification.	Narrow-bore capillary colu-mns are used employing column conditions detailed earlier under GC of organo-chlorine compounds.
(iii) Phenoxy acetic acid type of pesticides A third group of non-persistant pesticides are herbicides based on phenoxyacetic acid. [They have been widely used as selective weed killers for many years and act by stimulating rapid growth in the weeds causing them to go through their life cycle in a shorter time. Some compounds in this group are illustrated below (these com-pounds are normally present as their sodium salts in weed-killing formulations).	The herbicide is derivatized to its butyl ether for quantification by GC by the procedure detailed below: To the water sample is added enough 5M sulphuric acid so that the pH is less than 2. Extraction of the sample is then carried out with diethyl ehter. The ether extract is dried by passing it through a filter funnel containing anhydrous sodium sulphate in a filter paper, collecting the filtrate in a round-bottomed flask. The filtrate is evaporated down to about 5 ml by connecting the flask to a rotary evaporator. The concentrated extract is transferred to a test-tube and blown to dryness with the blower drier. The residue in the test-tube is mixed with 5 ml 5% potassium hydroxide solution by swirling and then the tube is placed in a beaker of hot water in a water bath for about 1 hour. The aqueous solution is	

Contd.

Sample	Preparation of the sample extract and its work-up prior to its GC analysis	GC column conditions
 2, 4, D MCPA 2, 4, 5-T	cleaned by extracting with hexane using a separating funnel, the hexane layer being discarded. About 2 ml of concentrated sulphuric acid is added to the funnel with mixing to get the acidic form of the herbicide. This is followed by extraction with dichloromethane by shaking the contents of the separating funnel with about 20 ml of the extracting solvent. The separated dichloromethane layer is dried by filtering through anhydrous sodium sulphate in a filter funnel, collecting the dried extract in a round-bottomed flask. The extract is evaporated down to 5 ml, transferred to a test-tube and blown to dryness. After the addition of about 1 ml butan-1-ol and 2 drops of concentrated sulphuric acid, the tube is placed in a beaker of hot water in a water bath for 1 hour. The reaction mixture is then cooled. A little distilled water is added to the cooled mixture and this is followed by the addition of about 1 ml hexane. The reaction mixture is shaken and allowed to separate into layers. The hexane layer is pipetted off into a stoppered tube for quantification by GC.	Appropriate GC column conditions are : Glass column — 1.5 m × 3 mm packed with 80–100 mesh Chromosorb WHP containing 4% DC 200 stationary phase Carrier gas; nitrogen flow-rate — 60ml/min Injector temperature — 250ºC Oven temperature — 200ºC Detector temperature — 210ºC Injection volume — 5µl

Analysis of Environmental Gases and Vapours

Environmental samples of gases and vapours arise from three main sources:
  (i) Atmosphere at the work place or in homes (e.g. gas leaks),
 (ii) stack emissions from incinerators, power stations, factory effluent,
(iii) gases from landfill sites, spillages, deliberate emissions (e.g. use of chemical weapons against the enemy in wars).

Each of these sources requires the employment of special sampling equipment before any analysis is carried out. For some atmospheres, the pollutant may be present at very low concentrations and a pre-concentration technique is used.

In order to obtain a representative sample of the atmosphere, the usual sampling device is a gas bulb typically of 250 ml capacity. This apparatus is fitted with two valves (A and B), and has a sampling septum (C) in the centre (Figure 13.15). The gas bulb, with its valves open, is connected to a small vacuum pump. Sufficient of the atmosphere is passed
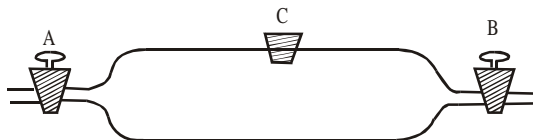
Figure 13.15 Gas bulb for sampling atmosphere.

through the bulb to give at least a 5 vol. exchange in order to obtain a representative sample of the atmosphere. The time required to achieve this condition is calculated from the extraction rate of the vacuum pump. The valves are closed and the bulb is taken to the laboratory for analysis. A sample for GC analysis is obtained by pushing a gas syringe through the septum and extracting 1-2 ml. In cases where concentrations of pollutants are low, the volatile component has to be concentrated by being adsorbed on to a suitable adsorbent material.

There are three main types of adsorption tube:

(a) Activated carbon from which the adsorbed material is removed by solvent extraction,
(b) Tenax tubes which are thermally desorbed thus making their use advantageous in that no solvent peak is produced, and
(c) graphite tubes, also thermally desorbed.

The atmosphere may also be sampled using cryotropic traps which are very effective at trapping the most volatile compounds but their operation is complicated.

Depending on the concentration of the target compound, the adsorption tube may be used to collect the pollutant either by passive diffusion or by pumping the atmosphere through the tube. The higher concentrations are measured by diffusion. If a work place atmosphere is being monitored, the worker will have an adsorption tube (Figure 13.16) attached to a lapel during the working day.

Figure 13.16 Adsorption tube for sampling atmospheres.

For lower concentrations, the atmosphere is sampled by drawing it through the tube fitted with a pump. The amount of air sampled can be calculated by measuring the pump rate. If an individual worker's exposure is being assessed, portable pumps are available that are connected to a lapel-worn adsorption tube. Sampling of stack emissions is a specialized technique as the requirement here is to sample the fumes in the chimney at the same rate at which

they are being ejected and the various techniques used are to ensure this. The sample is cooled by passing it through condensers and, if it is a gas, it is passed into a gas bulb whilst vapour is adsorbed on to Tenax-GC or other adsorbent material.

Landfill gases, particularly methane, C_2-C_4 hydrocarbons, hydrogen, and oxygen, are sampled via a sampling probe pushed into the deposited refuse (Figure 13.17). The gases at the required depth are evacuated via a vacuum pump and a sample put into a gas bulb for GC analysis.

For vapours present in the landfill emissions in the range of 0.1 to 400 mg/m^3, the preferred method is adsorption on to Texax-GC. Usually 25 ml sample is sufficient and this is adsorbed on the first part of the adsorption material. To ensure that no material is lost in transit to the

Figure 13.17 Sampling of gases in a landfill.

laboratory, the Tenax-GC should be securely sealed with swagelok caps. The tubes should be cooled before removing the caps prior to GC analysis after heat adsorption.
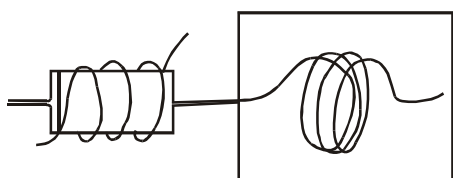
Figure 13.18 Thermal desorption of target compounds from an adsorption tube.

As for desorption of compounds, the trapped atmospheric pollutants are released from the adsorption tube into the carrier gas stream by heating (Figure 13.18). In case activated carbon has been used it is eluted with a suitable solvent. For most conditions Tenax-GC is the preferred adsorbent and is suitable for a wide range of volatile compounds. The amount of heat that is applied to the tube depends upon the volatility of the adsorbed compounds.

A more satisfactory chromatogram can be obtained by holding the desorbed compounds in a cold trap prior to GC analysis (Figure 13.19). The cold trap is cooled either with ice, solid carbon dioxide, or liquid nitrogen, depending

Figure 13.19 Thermal desorption and cold trapping of target compounds from an adsorption tube.

upon the volatility of the compound. Once in the cold trap, the compounds are then released into the carrier has stream by rapidly heating the trap up to 300ºC.

Tenax-GC tubes are unsuitable for alcohols, acid, and amines in the C_1-C_4 range. The preferred method for these compounds is cryogenic cooling of about 30 litres gas to obtain about 1 ml condensate. The level of these substances is in the range of 0.1-2000 mg/m^3.

Quality control of the GC data is very important and for atmospheric sampling, an internal standard should be used to check on recoveries and the performance of the sampling equipment. A suitable compound for atmospheric work is anisole. A known concentration dissolved in methanol should be injected into the adsorption tube before sampling is started. The method is standardized by sampling a prepared standard atmosphere.

The analysis of the sample prepared by any appropriate technique detailed above is carried out using the following GC column and conditions:—

Column	15 m × 0.32 mm i.d. silica capillary with OV-1701
Temperature	20ºC for 5 min, then up to 32ºC followed by a rise to 200ºC at 4ºC/min
Detector	MS

Oil Fingerprinting

Oil is a common pollutant in waterways and the sea and originates from many sources e.g. tanker spillages, storage tanks overflowing or puncturing, cracks in pipelines. The various constituents in the oil can be separated by GC and an examination of the overall appearance of the chromatographic trace (the oil fingerprint) gives an indication of the type of oil involved in the pollution. The GC trace of the pollution sample can be matched against GC traces of possible sources of the oil in the vicinity of the incident. The source giving the trace of the greatest similarity of appearance with the sample trace is likely to be the cause of the pollution. The oil may be exposed to the atmosphere for some time before it is discovered and many of the more volatile components will by then have evaporated into the atmosphere. For these weathered samples, the GC trace will be markedly different when compared with the traces of possible sources because of the absence of the light low molecular weight fractions from the chromatogram. However, an examination of the ratio of some of the normal hydrocarbon peaks to those of adjacent isoprenoid peaks, for chromatograms run on capillary columns, can often be used to track down a source of pollution.

An oil fingerprint is best obtained using a packed column coupled to a flame ionization detector (FID), whilst a capillary column linked to FID will provide the details in the trace to discriminate between peaks for the normal hydrocarbons and those for the isoprenoid.

In general, the oil is injected directly into the chromatographic column from the sample. For some samples, however, the oil may need to be removed from solid

material e.g. birds' feathers or stones, or other samples, such as surface water, which may contain only a trace. In these instances, the oil is removed by either petroleum ether or hexane and the solvent solution of the oil injected into the column.

For analysis using capillary GC, the column and GC conditions are:

Column	30 m × 0.25 mm DB5
Carrier gas; flow-rate	helium; 1 ml/min
Make-up gas; flow-rate	nitrogen; 25ml/min
Fuel gas; flow-rate	hydrogen; 25 ml/min with air, 250 ml/min
Temperature programme	100ºC upto 220ºC at 4ºC/min
Injection volume	0.3 μl

Capillary columns are suitable for analysis of lubricating and fuel oils, and the column and GC conditions are:

Short capillary column	15 m × 0.25 mm DB5
Carrier gas; flow-rate	helium; 1 ml/min
Make-up gas; flow-rate	nitrogen; 25 ml/min
Fuel gas; flow-rate	hydrogen; 25 ml/min with air, 250 ml/min
Temperature programme	100ºC upto 290ºC at 4ºC/min
Injection volume	0.3 μl

For packed columns used in analysis of lubricating and fuel oils, the column and GC conditions are:

Stainless steel column	5% OV-101 phase on Chromosorb WHP 80-100 mesh
Carrier gas; flow-rate	nitrogen; 25 ml/min
Fuel gas; flow-rate	hydrogen; 25 ml/min with air, 250 ml/min
Temperature programme	75ºC upto 280ºC at 10ºC/min
Injection volume	0.3 μl

■

<div align="center">

14

</div>

Capillary Supercritical Fluid Chromatography

Though mixtures of less volatile compounds can be analysed by HPLC, efficient separations by HPLC require the use of large number of theoretical plates which can only be obtained at the expense of long analysis time. Maximizing the number of theoretical plates through achievement of low plate heights demands the use of capillaries with extremely small internal diameters in the range of 10 μm. This requirement is a consequence of the order of magnitude of solute diffusivities and mobile phase viscosities prevalent in chromatographic systems using liquid mobile phase, and it is often difficult for the available equipment to fulfil this requirement.

While gas chromatography using capillary columns has high resolving power and allows separation, detection and quantitation of hundreds of compounds contained by a sample in a single chromatogram, the application of the technique is somewhat restricted by the limited volatility and stability of many organic compounds.

In the light of the aforesaid limitations of the application of gas chromatography or HPLC for the separation of compounds having limited volatility and thermal stability it is too obvious that there is need to use other alternatives for separating such compounds. Capillary supercritical fluid chromatography is such an alternative and although of comparatively recent origin, meets the said need adequately.

14.1 Theory and Working of Capillary Supercritical Fluid

Chromatographic Technique

Rapid and efficient separations of non-volatile solutes can be carried out using capillary columns in conjunction with supercritical fluids. Supercritical fluids constitute mobile phases that have intermediate viscosities and solute diffusitvities between those of liquids and gases. Thus, compared to HPLC, the present technique gives higher mobile phase linear velocities (and hence shorter analysis times) and better separation efficiencies per unit time. Further, fluids compressed to their critical points and above exhibit and 'extraction effect' on chromatographic samples similar to solvation in HPLC and, therefore supercritical fluid chromatography should provide superior migration of labile and less volatile substances through a capillary column when compared to GC.

While ordinary gases can be compressed to produce the aforesaid effects, similar conditions can be met with some other fluids at pressures and temperatures well within the limits of ordinary HPLC. The noteworthiness of this point in terms of its practical usefulness needs emphasis. Some examples of potentially useful mobile phases are given in Table 14.1.

TABLE 14.1

Potentially Useful Mobile Phases

Mobile phase	*Critical Pressure (atm)*	*Critical Temperature (°C)*	*Corresponding Density (g/ml)*
n-Pentane	33.3	196.6	0.232
Dichlorotetrafluoroethane	35.5	146.7	0.582
Isopropanol	47.0	253.3	0.273
Carbon dioxide	72.9	31.3	0.448
Sulphur hexafluoride	37.1	45.6	0.752

Such commonly available fluids as n-pentane, isopropanol or carbon dioxide, allow the migration of relatively heavy molecules under reasonable pressures and temperatures. These mobile phases combined with the small volume requirements of capillary techniques make safety hazards almost negligible.

Like gas chromatography, supercritical fluid chromatography can be practised both in packed and capillary column techniques. Observations made earlier under gas chromatography in regard to column performance of capillary columns compared with that of packed columns, are also valid for supercritical fluid chromatography.

Although, greater sample capacities are attained with the use of packed columns in supercritical fluid chromatography as in all other forms of chromatography, the pressure drop across the column limits the length and hence the total chromatographic efficiency (total number of the theoretical plates) obtainable.

In comparison, capillary columns offer low resistance to fluid flow with a consequent low pressure drop along the length of the column. This effectively results in higher separation efficiencies and facilitates faster analyses.

The three requirements for achieving optimal results using capillary supercritical fluid chromatography for separation of mixtures are:

(i) Use of stationary phase films of optimum thickness permanently bonded to the capillary wall in a manner that ensures that the resistance to mass transfer in the stationary phase is small and that the passing fluid does not strip off the stationary phase. (Glass capillary columns with wall thicknesses similar to those used in conventional gas chromatography can be usually used; advantage can also be taken of recently developed bonded phases.)

(ii) Operation of the entire column and the detector at the same high pressure.

(iii) Ensuring minimal instrumental contribution to band broadening.

Figure 14.1 is a block diagram representing the basic features of a capillary supercritical fluid chromatograph.

A high-pressure syringe pump operating at room temperature delivers (at constant pressure) a liquid (e.g. n-pentane) or compressed gas (carbon dioxide) that is converted into supercritical fluid in a pre-heating column. The fluid subsequently sweeps through a loop valve where the sample is introduced directly into the glass capillary column. The column is heated to temperatures consistent with the desired fluid conditions in a thermostat. The thermostat also contains a flow-through cell (either a UV or spectrofluorometric detector) used to monitor chromatographic effluents under high pressure. A length of 50μ glass capillary after the detection cell in used to maintain system pressure. Increasing the pressure is the most convenient means to influence solute retention.

Figure 14.1 Block diagram showing the components of a capillary supercritical fluid chromatograph.

14.2 Conclusion

The discussion of supercritical fluid chromatography given above may be concluded by highlighting the distinct usefulness of this technique.

For separations where both selectivity and high column efficiency are needed (e.g. resolution of certain isomeric substances) it may be of advantage that the interaction of solute and mobile phase molecules can be sensitively adjusted by pressure. Different fluids and pressure regions can be explored to achieve chromatographic migration of various non-volatile and unstable molecules.

While gradient elution techniques are needed to 'programme' solute retention in liquid chromatography, pressure programming in supercritical fluid chromatography can be used to meet similar goals. As the former programming technique is incompatible with certain detection systems, fewer problems are anticipated with supercritical fluids under increasing pressure. For example, one can envision a complex mixture of non-volatile substances in supercritical carbon disulphide being separated by pressure programming, while the resolved components are introduced into a high-pressure cell of an infrared detector. ∎

15

Chromatographic Techniques used more Specifically for the Separation of Biomolecules

Chromatographic techniques discussed in the earlier chapters find application in the separation and isolation of biomolecules as also molecules of other types. This chapter deals with techniques which are used more specifically for separation of *biomolecules*, these methods being affinity chromatography, dye-ligand chromatography, covalent chromatography, hydroxylapatite chromatography and hydrophobic interaction chromatography.

15.1 Affinity Chromatography

The year 1967 may be considered as marking the beginning of the currently most modern chromatographic method—bioaffinity chromatography—introduced in that year by Porath, Axen and Ernback. While other chromatographic methods described in the earlier chapters have achieved widespread usage in the separation of sensitive molecules such as proteins, they suffer from the disadvantage that repeated fractionation is necessary to achieve a high degree of purity. In many cases, the required protein may be present at levels less than one per cent in a complex mixture which contains other species of protein of similar size and structure. In the repeated separations necessary to achieve a high degree of purity of the required protein, some denaturation as well as loss of material may arise. Bioaffinity chromatography, later named as affinity chromatography by Cuatrecasas, Wilchek and Anfinsen in 1968, affords a method in which the required protein may be recovered in good yield in a single operation under conditions well suited to maintaining its natural conformation and biological activity.

15.1.1 Theory underlying the Working of the Technique

The chromatographic procedures so far discussed rely on rather non-specific physico-chemical interactions between stationary support and solute. The molecular characteristics of net charge, size and polarity do not provide a basis for high selectivity in the separation and isolation of biomolecules. Desirability of greater specificity in chromatographic separations led to the discovery of affinity chromatography. This technique offers the ultimate in specificity—separation on the basis of biological interactions. The biological function displayed by most

macromolecules (proteins, enzymes, nucleic acids, polysaccharides etc.) is a result of a recognition and interaction with specific molecules called ligands.

In affinity chromatography a column is prepared in which a ligand appropriate for the required macromolecule is covalently bonded to an insoluble matrix. When a solution containing this macromolecule e.g. a protein, is passed through the column, it becomes bound to the column while all other species pass through. Even slightly modified or denatured molecules of the protein do not bond to the ligand groups and are thus removed. When the column has been washed free of all contaminants, the required protein is eluted in a very pure form by either changing the pH or ionic strength of the eluting buffer or by the addition of a free ligand to the eluent, and only occasionally by the addition of a chaotropic agent to the buffer (see 15.1.2).

The support or matrix to which the ligand is bound must conform to stringent criteria; for example, it must consist of spherical gel particles with good flow properties, a chemically inert macromolecular network with very large-sized pores must be present through which unbound protein molecules may freely pass, and suitable functional groups must be present on matrix to which the ligand can be bonded.

Four types of media possess most of these desirable characteristics: Agrose, polyvinyl, polyacrylamide and controlled porosity glass (CPG) beads. Highly porous beads such as Sepharose 4B and Bio-Gel A-150 m possess virtually all the above characteristics and are the most widely used matrices. Polyacrylamide gels such as Bio-Gel P-300 display many of the above recommended features; the porosity is not especially high, however. Fractogel HW-65F, a vinylpolymer, has many of the above characteristics and a protein fractionation range of 50,000 to 5,000,000. CPG beads have the advantages of their mechanical strength and chemical stability, but these beads have not found wide acceptance primarily because of extensive non-specific protein adsorption by them and on account of their having small numbers of reactive functional groups for ligand attachment.

The chemical nature of the ligand is determined by the prior knowledge of the biological specificity of the compound to be purified. It is essential that the ligand possesses a suitable chemical group which will not be involved in the reversible binding of the ligand to the macromolecule, but which can be used to attach the ligand to the matrix. The most common such groups are:

$-NH_2$, $-COOH$, $-COBr$, $-SH$ and $-OH$ (phenolic and alcoholic).

It should be recognized that several types of ligands may be used for affinity purification of a particular macromolecule. Of course, some ligands will work better than others and empirical binding studies can be performed to select an effective ligand.

Several procedures have been developed for the covalent attachment of the ligand to the stationary support. All procedures for gel modification proceed in two

separate chemical steps: (1) Activation of the functional groups on the matrix and (2) joining the ligand to the functional group on the matrix.

The most common method of attachment of the ligand to the matrix involves treatment of the matrix with cyanogen bromide (CNBr) at pH 11. The reaction conditions and the relative proportion of the reagents will determine the number of ligand molecules which can be attached to each matrix particle. The procedure for CNBr activation and ligand coupling is outlined below.

Agrose and ligand bonding in affinity chromatography
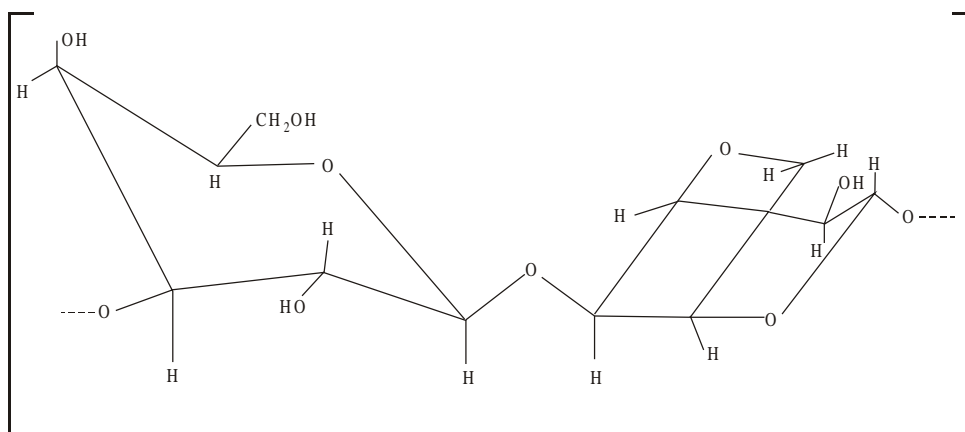
(a)
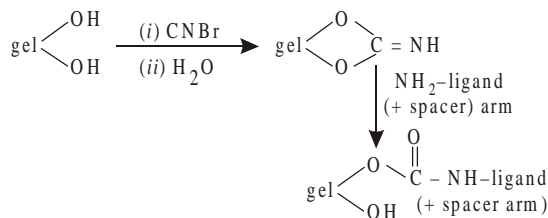
(b)

(a) The repeating disaccharide unit of agarose
(D-galactosyl-β-(1→4) 3, 6 anhydro-L-galactose)

(b) The use of cyanogen bromide for bonding the ligand to agarose.

One disadvantage of CNBr activation is that small ligands are coupled very closely to the matrix surface; macromolecules, because of steric repulsion, may not be able to interact fully with the ligand, and this problem is overcome by interposing a spacer arm between the ligand and the matrix. The importance of the spacer arm holding the ligand is illustrated in Figure 15.1.

Many different spacer arms are used. The optimum length of this spacer arm is six to ten carbon atoms or their equivalent. Spacers must possess a second functional group to which the ligand may be attached by conventional organo-synthetic procedures. A number of supports of the agrose, dexran and polyacrylamide type are commercially available with a variety of spacer arms pre-attached.
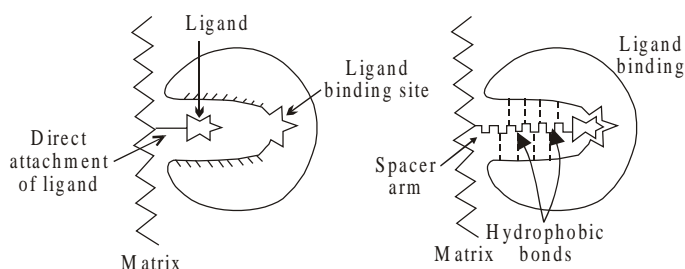
Figure 15.1 Ligand-protein interaction in affinity chromatography. A diagrammatic representation of the interaction between matrix-bound ligand and protein showing the necessity of a 'spacer arm' joining the ligand to the matrix.

Attachment of the ligand to the cyanogen bromide-activated agrose has been outlined earlier. The following reactions illustrate ligand attachment to some of the most widely used activated gels, which are commercially available and have pre-attached spacer arms:—

(1) $gel - NH - (CH_2)_5 COOH + ligand - NH_2 \longrightarrow gel - NH - (CH_2)_5 - \overset{\overset{O}{\|}}{C} - NH\ ligand$

CH – Agarose

[6-Aminohexanoic Acid (CH) – Agarose]

(2) $gel - NH - (CH_2)_6 - NH_2 + ligand - COOH \longrightarrow gel - NH - (CH_2)_6 - NH - \overset{\overset{O}{\|}}{C} - ligand$

AH – Agarose

[1, 6 – Diaminohexane (AH) – Agarose]

(3) $gel - O - CH_2 - \underset{\underset{O}{\diagdown\diagup}}{CH - CH_2} + ligand - OH \longrightarrow gel - O - CH_2 - \underset{OH}{\overset{|}{CH}} - \underset{O - ligand}{\overset{|}{CH_2}}$

Epoxy-activated Agarose
[provides for the attachment of ligands containing hydroxyl, thiol or amino groups]
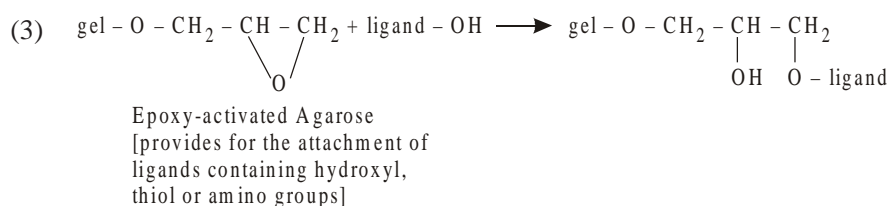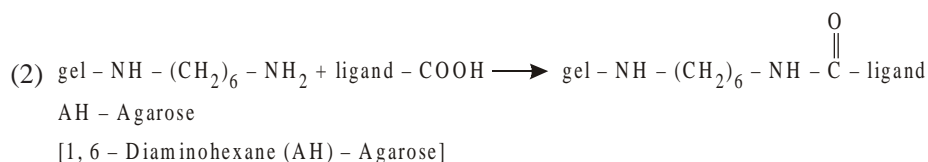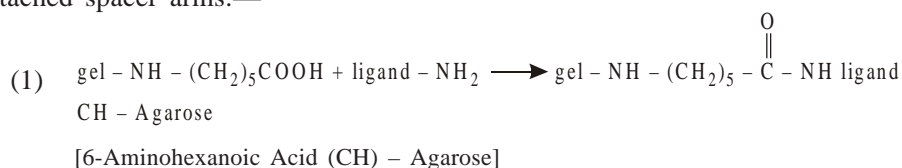
Group-specific Adsorbents

The affinity materials described up to this point are modified with a ligand that displays specificity for a particular macromolecule. Therefore, each time a biomolecule is to be isolated by affinity chromatography, new adsorbent must be designed and prepared. Ligands of this type are called substance-specific. In contrast, group-specific adsorbents contain ligands that have affinity for a class of biochemically related substances. Table 15.1 gives several commercially available group-specific adsorbents and the specificity of each.

TABLE 15.1

Several Group-specific Adsorbents useful in Biochemical Applications

Group-specific Adsorbent	Group Specificity
5′-AMP-agarsoe	NAD^+- dependent dehydrogenases and ATP-dependent kinases
Protein A-agarose	IgG-type antibodies
Poly (U)-agarose	m-RNA containing a poly (A) tail; poly (U)-binding proteins
Poly (A)-agarose	Ribonuleic acids containing poly (U) - sequences; RNA-specific proteins such as nucleic acid polymerases
Concanavalin A-agrarose	Macromolecules with glucopyranosyl and other carbohydrates (glycoproteins and glycolipids)
Boronic acid-agarose	Compounds with *cis*-diol groups; sugars; catecholamines; ribonucleotides.

15.1.2 Experimental Procedure

The experimental procedure for affinity chromatography is similar to that used in other forms of liquid chromatography. The major difference however, is the use of shorter columns. Most affinity gels have high capacities, and column beds less than 10 cm in length are commonly used. The ligand-treated matrix is packed into a column in the normal way for the particular type of support. A buffer is used which will encourage the binding macromolecules to be strongly bound to the ligand. The buffer generally has a high ionic strength to minimize non-specific adsorption of polyelectrolytes onto any charged groups in the ligand. Once the sample has been applied and the macromolecule bound, the column is eluted with more buffer to remove non-specifically bound contaminants. The purified comound is finally recovered by elution by an appropriate procedure as discussed below.

Ligand-macromolecule complexes immobilized on the column are held together by hydrogen bonding, ionic interactions, and hydrophobic effects. The choice of elution method depends on many factors, including the types of forces responsible for complex formation and the stability of the ligand matrix and isolated

macromolecule. The common methods of elution are change of buffer pH or buffer ionic strength, affinity elution, and use of chaotropic agents.

Non-specific Elution (Change of buffer pH or buffer ionic strength):

If ionic interactions are important for complex formation, a change in pH or ionic strength weakens the interaction by altering the extent of ionisation of ligand and macromolecule. In practice, either a decrease in pH or a gradual increase in ionic strength (continual or stepwise gradient) is used.

Affinity Elution

In this method of elution, a selective substnace added to the eluting buffer competes for binding to the ligand or for binding to the adsorbed macromolecule. An example of the first case is the elution of glycoproteins from a boronic acid-agarose gel by addition of a free sugar, sorbitol, to the eluting buffer. The latter type of affinity elution is illustrated by the elution of lactate dehydrogenase isoenzymes from a 2, 5-ADP agarose gel with a gradient of NADH.

Chaotropic Agents

If gentle and selective elution methods do not release the bound molecule, then mild denaturing agents can be added to the buffer. These substances deform protein and nuclaic acid structure and decrease the stability of the complex formed
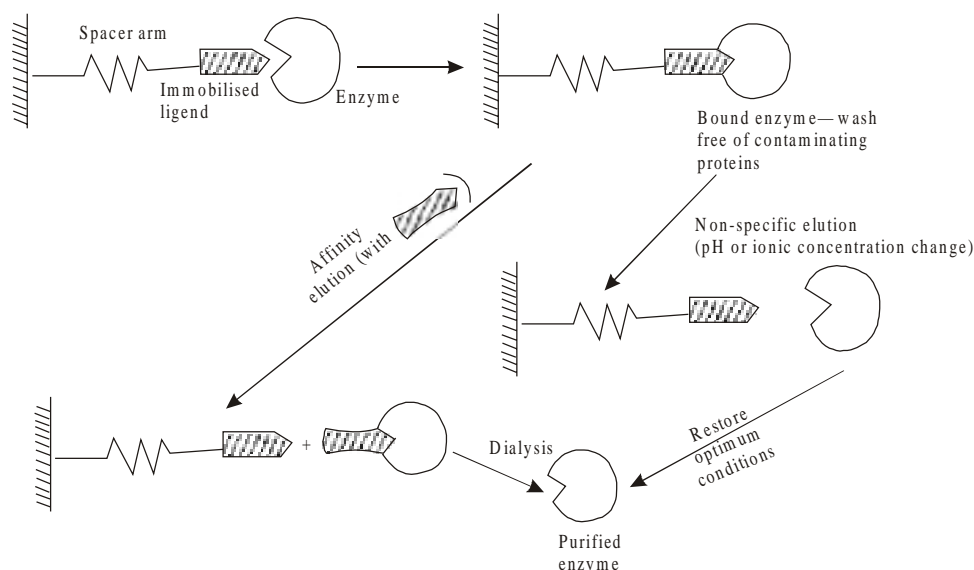
Figure 15.2 Diagrammatic representation of purification of an enzyme by affinity chromatography.

on the affinity gel. The most useful agents are urea, guanidine. HCl, CNS^-, ClO_4^-, and $CCl_3 COO^-$. These substances should be used with care, because they may cause irreversible structural changes in the isolated macromolecule.

Elution carried out by any of the above procedures eventually yields the purified material in a buffer solution which may be contaminated with specific eluting agents or high concentration of salts and these must finally be removed before the isolation is complete.

Figure 15.2 is a diagramatic representation of purification of an enzyme by affinity chromatography.

15.2 Dye-ligand Chromatography

In dye-ligand chromatography, a dye is immobilised and used as a ligand. The dyes used contain ionic groups and a conjugated ring system which have the ability to bind to the catalytic or effector site of some proteins. Thus, Cibacron-Blue, which is one of a number of triazine dyes which can be immobilised, illustrates the use of a dye as a ligand to purify proteins. In this technique, the interaction between the macromolecule and ligand is not genuinely specific, so that this technique is preferably termed as dye-ligand chromatography. Examples of proteins purified by this procedure are provided by interferon and plasminogen.

15.3 Covalent Chromatography

It differs from affinity chromatography in that it involves covalent bond formation between the bound ligand and the compound to be separated (most commonly proteins). The most common form involves the formation of a disulphide-bond between thiol groups in the compound and ligand. Commercially available ligands include thiopropyl—Sepharose and thiol—Sepharose. Elution is carried out with dithiothreitol or cysteine, the success of the technique depending on the number of thiol groups in the protein and the case with which the disulphide bonds are broken by the eluent. Papain and urease which have many thiol groups are both readily purified by the technique and newly synthesized m-RNA can be separated from other RNA and DNA by a similar procedure.

15.4 Hydroxylapatite Chromatography

Cystalline hydroxylapatite $[Ca_{10} (PO_4)_6 (OH)_2]$ is an adsorbent used to separate mixtures of proteins or nucleic acids. The mechanism of adsorption is not fully understood but is thought to involve both the calcium ions and phosphate ions on the surface and to involve dipole-dipole interactions and possibly electrostatic attractions. One of the most important applications of hydroxylapatite chromatography is the separation of single-stranded from double-stranded DNA. Both forms of DNA bind at low phosphate buffer concentrations but as the buffer concentration is increased further, double-stranded DNA is released. The affinity

of hydroxylapatite for double-standed DNA is so high that the latter can be selectively removed from RNA and proteins in cell extracts by use of this form of chromatography. The forms in which hydroxylapatite is available include crystalline or spheroidal hydroxylpatite and forms bonded to an agarose matrix. The adsorption capacity of these forms is maximum around neutral pH and such conditions are usually employed using 20 mM phosphate buffer for the adsorption process. Elution is achieved by increasing the phosphate buffer concentration to 500 mM.

15.5 Hydrophobic Interaction Chromatography (HIC)

This form of chromatography was developed to purify proteins by exploiting their surface hydrophobicity, which is related to the presence of non-polar amino acid residues. Groups of hydrophobic residues are scattered over the surface of proteins in a way that gives characteristic properties to each protein. In aqueous solution these hydrophobic regions on the protein are covered with an ordered film of water molecules that effectively masks the hydrophobic groups. These groups can, however, by exposed by the addition of salt ions, which preferentially take up the ordered water molecules. The exposed hydrophobic regions can then interact with each other and this is the basis of salting-out using ammonium sulphate. In HIC, rather than facilitating protein-protein interaction by the exposure of the hydrophobic groups, the presence of hydrophobic groups attached to a suitable matrix facilitates protein-matrix interaction. The most commonly used stationary phases are alkyl (hexyl), octyl) or phenyl groups attached to an agarose matrix. Commercial materials include Phenyl Sepharose and Phenyl SPW. Bio-gel TSK Phenyl and Spherogel YSK Phenyl are empolyed for HIC in HPLC.

Since HIC requires the presence of salting-out compounds such as ammonium sulphate to facilitates the exposure of the hydrophobic regions on the protein molecule, it is commonly used immediately after fractionation with ammonium sulphate as ammonium and sulphate ions are already present in the protein sample. To maximize the process, it is advantageous to adjust the pH of the protein sample to that of its isoelectric point. Once the proteins have been adsorbed to the stationary phase, selective elution can be achieved in a number of ways, including the use of an eluent of gradually decreasing ionic strength or of increasing pH (this increases the hydrophilicity of the protein) or by selective displacement by a *displacer* that has a stronger affinity for the stationary phase compared with that of the protein. Examples include non-ionic detergents such as Tween 20 and Triton X-100, aliphatic alcohols such as butanol and ethylene glycol, and aliphatic amines such as butylamine. One of the potential problems with HIC is that some of these elution conditions may cause protein denaturation. The other practical problem with the technique is its non-predictability in that it works well for some proteins but not for others and a trial study is invariably necessary. Proteins purified by the technique include aldolase, transferrin, and thyroglobulin. ■

This page intentionally left blank

PART IV

Other Diverse Techniques of Separation and Purification

16

Electrophoresis

Separations by electrophoresis depend upon differences in rates of migration of the components in a mixture in an applied electric field. Provided the electric field is removed before ions in the sample mixture reach the electrodes, the components may be separated according to their electrophoretic mobility. Electrophoresis is thus an incomplete form of electrolysis. Electrophoresis is especially useful for analysis and separation of amino acids, peptides, proteins, nucleotides, nucleic acids and carbohydrates.

The development of this experimental technique is largely the result of the work carried out by A. Tesilius and W. Grassmann in the ninteen fifties. Although a solution of an electrolyte held on a supporting medium is required, this method of separation does not require a mobile phase. Since the sample ions are not partitioned between a stationary phase and a mobile phase, electrophoresis is not a chromatographic technique.

16.1 General Principles

The movement of a charged molecule subjected to an electric field is expressed by Equation 16.1:

$$Eq = fv \qquad ...(16.1)$$

where

$E =$ the electric field in volts/cm,
$q =$ the net charge on the molecule,
$f =$ the frictional coefficient, which depends on the mass and shape of the molecule,
$v =$ the velocity of the molecule.

The charged particle moves at a constant velocity that depends directly on the electrical force (Eq) but inversely on a counteracting force, *viscuous drag* (f). The velocity of the charged molecule is defined by Equation 16.2:

$$v = \frac{Eq}{f} \qquad ...(16.2)$$

The applied field (voltage or current) represented by E in Equation 16.2 is usually held constant during electrophoresis. Under these conditions, the movement of the charged molecule actually depends on the ratio, $\frac{q}{f}$.

The movement of a charged particle in an electric field is often defined in terms of *electrophoretic mobility*, μ, the velocity per unit electric field, that is, by Equation 16.3:

$$\mu = \frac{q}{f} \qquad \qquad ...(16.3)$$

Each molecule in a mixture is expected to have a unique charge and size, and its mobility in an electric field will therefore be unique; it is this expectation which forms the basis of the electrophoretic technique.

In theory, if the net charge, q, on a molecule is known, it should be possible to measure the viscous drag, f, in Equation 16.3:

$$\mu = \frac{q}{f}$$

and, therefore, obtain information about the hydrodynamic size and shape of that molecule by investigating its mobility in an electric field. Attempts to define f by electrophoresis have not been successful, primarily because Equation 16.3 does not adequately describe the electrophoretic process. Althoug the supporting medium as implied in Equation 16.3 is relatively inert, the precise composition of the medium may cause adsorption, electro-osmosis and molecular sieving, each of which may influence the migration rate of compounds. A consideration of these factors and how sample ions will be influenced by them will determine the choice of supporting medium for a particular separation.

Adsorption: This is the retention of samples molecules by the supporting medium, as shown in adsorption chromatography. Adsorption causes *tailing* of the sample so that it moves in the shape of a comet rather than as a distinct compact band, thus reducing both the rate and the resolution of the separation.

Electro-osmosis (electro-endosmosis): This phenomenon results from a relative charge being produced between water molecules in the buffer and the surface of the supporting medium. The charge may be caused by surface adsorption of ions from the buffer and the presence of stationary carboxyl groups on paper or sulphonic groups on agar. This generates a motive force for the movement of fixed anions to the anode and results in the movement of hydronium ions (H_3O^+) in the buffer to the cathode carrying along neutral substances by solvent flow.

The electro-osmosis (osmosis is diffusion of fluid through porous partition into another fluid) will accelerate the movement of cations but retard anion transference.

Molecular sieving: This feature is shown by gels being used as supporting medium. It is to be noted that there is a major difference between gel filtration and gel electrophoresis in that whereas in the former separation method the rate of movement of large molecules is greater than that of small molecules, the opposite is the case for gel electrophoresis. In gel electrophoresis, there is only a continuous matrix network throughout the gel so that there is no void volume in the system.

The electrophoresis gel is comparable to a single bead in gel filtration. Therefore, large molecules do not move easily through the medium.

The Supporting Medium: There are many different types of supporting media available such as sheets of adsorbent paper or cellulose acetate, a thin layer of silica or alumina or a gel of strach, agar or polyacrylamide. Each may offer some advantage over the others for a particular separation. All supporting media have a capillary structure which has good anticonvectional properties. Sometimes the medium used may be designed specifically to interact with the sample ions being separated so as to exploit differences in the charge/mass ratios and to introduce special retardation forces to suit the separation.

Buffer: Careful choice of buffer is required for the best results. The buffer functions firstly by wetting the paper with the electrolyte so that current will flow and, secondly, by maintaining the correct pH throughout the experiment so that the conditions of migration remain as constant as possible. The buffer can also affect migration rates of the various comounds being separated in a number of ways. These ways comprise of roles played in this regard by composition, concentration and pH of the buffer, as discussed below.

Composition: The buffers in common use are formate, citrate, barbitone, phosphate, Tris (2-amino-2-hydroxymethyl propane-1, 3-triol), EDTA and pyridine. The buffer should be such that it does not bind with the compounds being separated as this may alter the rates of migration. In some cases, however, binding can be advantageous, for example, borate buffers are used to separate carbohydrates since they produce charged complexes. Since the buffer acts as a solvent for the sample, some diffusion of the sample is inevitable, being particularly noticeable for small molecules such as amino acids and sugars. The extent of diffusion can be minimized by avoiding overloading of the sample, by applying samples as narrow bands, using a high voltage for as short a time as possible and by rapid removal of the supporting medium after the separation has been completed.

Concentration: As the ionic strength of the buffer increases, the proportion of current carried by the buffer will increase and the share of the current carried by the sample will decrease, thus slowing its rate of migration. High ionic strength of the buffer will also increase the overall current and hence heat production. At low ionic strengths the proportion of current carried by the buffer will decrease and the share of the current carried by the sample will increase, thus increasing its rate of migration. A low ionic buffer strength reduces the overall current and results in less heat production, but diffusion and the resulting loss of resolution are higher. Therefore, the choice of ionic strength must be a compromise and this is generally selected within a range of ionic strength of 0.05 M to 0.10M.

pH: This has little effect on fully ionised compounds such as inorganic salts, but for organic compounds pH determines the extent of ionisation. The ionisation of organic acids increases as pH increases whereas the reverse applies for organic

bases; therefore, the degree of migration of acids and bases will be pH dependent. Both effects can apply to compounds such as amino acids that are ampholytes (i.e. have basic and acidic properties).

$$H_3\overset{+}{N} - \overset{\overset{\displaystyle R}{|}}{C}H - COOH \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} H_3\overset{+}{N} - \overset{\overset{\displaystyle R}{|}}{C}H - COO^- \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} H_2N - \overset{\overset{\displaystyle R}{|}}{C}H - COO^-$$

pH :   Acidic   Isoionic point   Alkaline
                            (i.e. the hydrogen ion
                            concentration express-
                            ed usually as the pH
                            value at which the
                            ionisation   of   an
                            amphoteric substance
                            as an acid equals the
                            ionisation of a base and
                            becomes identical with
                            the isoelectric point in
                            the absence of foreign
                            inorganic ions)

Ionic form : Cation   Zwitterion (species   Anion
                            on which total charge
                            is nil)

Migration : Towards cathode   Stationary   Towards anode

The direction and also the extent of migration of ampholytes are thus pH dependent and buffers ranging from pH1 to pH11 can be used to produce the required separations. The correct buffer, that is the one which has the most apprporiate value of pH and ionic strength for the particular separation in hand, is found by experimentation.

16.2 Experimental Techniques

Various experimental techniques employed for electrophoresis are detailed below.

16.2.1 Zone Electrophoresis

In this technique, which is the simplest one amongst the various electrophoretic techniques, solution of the materials to be separated is placed as a spot or thin band on a paper moistened with a suitable buffer solution, the ends of the paper dipping into electrode vessels. On applying an electric potential from a power supply, positive ions in the sample mixture migrate towards the cathode and the negative ions migrate towards the anode, the paper serving as porous support. Remixing by

diffusion of the components, which separate by virtue of moving at different speeds in the applied electric field, is prevented by the paper support.
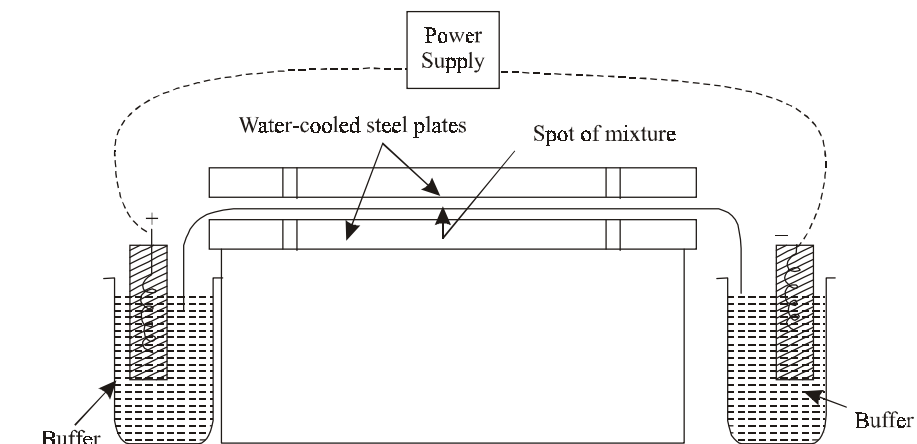
Figure 16.1 (a) Apparatus for high-voltage electrophoresis

The usual experimental arrangement is shown in Figure 16.1 (a). Most often Whatmann filter paper No. 1 or 3 MM paper is use in the form of strips. The paper strip should be just moist enough to conduct, so as to avoid diffusion. As already discussed, it is better to work with higher voltages (about 200 volts/cm and upwards) so that diffusion can be minimized. Also, in high-voltage electrophoresis, salts and other substances which may be present in the sample affect the quality of electrophoretogram to a lesser extent. Use of such higher voltages, however, introduces a serious experimental difficulty, since the heat generated in the strip increases with the square of the voltage with resultant risks of evaporating the solvent. The difficulty is best overcome by enclosing the strip between water-cooled steel plates or by performing the experiment in a refrigerated chamber. After the sample ions have migrated towards the electrode of opposite charge, but before the ions reach the buffer solution in the vessel containing the electrode, the power supply is turned off and the paper strip is removed and electrophoretogram dried.

Identification of the separated components of unknown mixtures on the electrophoretogram is achieved by comparing the migration characteristics of the resolved mixture with those of a mixture of known compounds resolved by electrophoresis under identical conditions. In this respect, electrophoresis is exactly similar to chromatography. Individual compounds are usually detected and identified in *situ*. If substances naturally absorb in ultraviolet region or fluoresce under ultraviolet light and the supporting medium does not, this may be used as a means of detection. Ultraviolet absorbance can also be exploited if compounds

form complexes with agents which then induce fluorescence under ultraviolet light, as, for example, in the case of amino acids, peptides and proteins with fluorescamine or dansyl chloride.

Most biological molecules are colourless and have to be treated with reagents which specifically produce stable coloured compounds. Thus Coomassie Brilliant Blue R-250, for example, is the commonly used dye solution for staining protein zones. Staining is followed by several rinses in a destaining solution (ethenol-acetic acid-water) to remove excess background dye. The paper strip is then dried. Figure 16.1 (b) shows the nature of the final electrophoretogram.

Quantitative estimation of the zones may be carried out with moderate accuracy by dipping the stained strip in oil to render the paper transparent and passing it through a photometric scanner. Alternatively, the coloured zones may be cut out, the coloured material extracted and the colour measured in solution.

1. 3, 4 Dihydroxy-phenylalanine	6. Serine
2. L-Aspartic acid	7. α-Aminobutyric acid
3. L-Glutamic acid	8. Alanine
4. L-Methionine	9. Glycine
5. Threonine	10. γ-Aminobutyric acid

Figure 16.1 (b) Aminoacid separation by electrophoresis (after treatment to render the strips visible)

Table 16.1 lists examples of reagents used for staining of electrophoretograms.

TABLE 16.1

Staining Reagents used for Visualizing Compounds on Electrophoresis Strips

Compound	Reagents	Remarks
Nucleic acids	Methyl green-pyronine	RNA—red DNA—blue
	Ethidium bromide	Fluoresces under UV when bound to DNA

Contd.

Compound	Reagents	Remarks
	Silver stains	Very sensitive for DNA and RNA
Peptides	C1O$_2$ or NaOCl chlorination followed by KI-starch or benzidine-acetic acid.	Reacts with all—NH$_2$ compounds
Proteins	Nigrosine in acetic acid or trichloroacetic acid	Very sensitive
	Bromophenol-ZnSO$_4$-acetic acid	Quantiative
	Lissamine Green in aqueous acetic acid	Quantitative
	Coomassie Brilliant Blue R-250	Quantitative and very sensitive
	Silver stains	Ultrasensitive but not quantitative
Glycoproteins	Periodic oxidation followed by treatment with Schiff's reagent	Quantitative
Lipoproteins	Sudan black in 60% ethanol	
Polysaccharides	Iodine	
Acid mucopolysaccharides	Toluidine Blue in methanol-water	

16.2.2 Continuous-flow Paper Electrophoresis (Electrochromatography or Curtain Electrophoresis)

In the second form of paper electrophoresis, known as *continuous-flow paper electrophoresis*, the sample solution of the ions to be separated and the buffer-electrolyte solution are continuously added to the top of the sheet of paper (Figure 16.2). The electrodes are attached to the vertical edges of the paper. As the sample descends through the paper, ions migrate horizontally toward the electrode of opposite charge and eventually drip from the bottom of the paper after having been horizonatally displaced from the position at which the sample was added. Notches which are cut in the bottom edge of the paper act as drip points
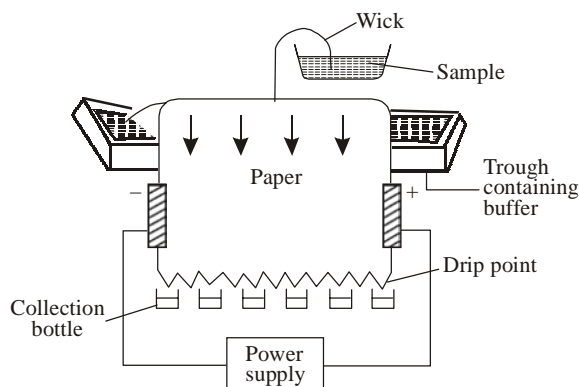
Figure 16.2 Diagram of the apparatus used for continuous-flow paper electrophoresis.

at which the separated ions can be caught. The paper is sometimes sandwiched between two plates as in the type of paper electro-phoresis described earlier.

The apparatus used for continuous-flow paper electrophoresis can be used for separation with an elution rather than a continuous-flow method. The sample is spotted at the top of the paper and only the buffer solution is continuously added. If the electrophoresis is stopped before the sample components drip from the bottom of the paper, the technique combines the separating ability of descending paper chromatography (separation occurring vertically) with that of electrophoresis (separation occurring horizontally) to yield spots simultaneously developed in two dimensions.

Paper electrophoresis has been applied widely for separations of amino acids, peptieds, proteins, carbohydrates, purines, biological acids and alkaloids.

Electrophoresis and paper chromatography can sometimes be profitably combined. For example, products of protein hydrolysis were separated by paper electrophoresis into glutamic acid, aspartic acid, cysteic acid, a group of neutral amino acids, and a group of basic amino acids. Each of the groups was then resolved by normal paper chromatography.

16.2.3 Electrophoresis on Cellulose Acetate Strips

The many hydroxyl groups on cellulose provide for extensive interaction between polar macromolecules and the paper electrophoresis strip. Therefore, hydrophilic proteins and nucleic acids tend to have low electrophoretic mobility. Acetylation of the cellulose hydroxyl groups produces a medium (cellulose acetate) that greatly speeds up electrophoreses. For cellulose acetate electrophoreses 0.IM barbital buffer is the standard one.

Cellulose acetate strips have some other distinct advantages over paper strips as support media in electrophoresis. These strips greatly improve electrophoretic separations of polar molecules. Cellulose acetate may be cleared more easily than paper to produce transparent strips for automatic scanning. Further, cellulose acetate strips are thinner, more homogenous and chemically purer than paper strips.

16.2.4 Ionophoresis

Another modification in which form thin sheet electrophoresis is employed is known as *ionophoresis*. In this method, devised by Sanger and his co-workers, high voltage electrophoresis is carried out on ion-exchange paper. It is a rapid method of great resolution and sensitivity and is used in Biochemistry for the separation of constituents of a highly complex mixture e.g. a mixture of oligoribonucleotides produced by partial enzymic hydrolysis of RNA. For separation of the constituents of this hydrolysate mixture, a two-dimensional technique is used in which the mixture is subjected to electrophoresis on cellulose acetate and then the partially separated mixture is transferred to a DEAE-cellulose

paper and a further electrophoretic separation performed. Separation occurs through a combination of differences in electrophoretic mobility and ion-exchange binding of the negatively charged constituents of the hydrolysate mixture. In the electrophoretic migration, the more anionic components move faster and their progress is slowed on the DEAE-cellulose by binding to the positive charges in the paper.

16.2.5 Gel Electrophoresis

Electrophoresis using support media like starch gel or polycrylamide gel has now largely superseded thin sheet electrophoresis systems for the separation of biomolecules such as proteins. The gel methods have the great advantage that besides their useful chracteristics purely as support media, gels act as molecular sieves so that the movement of components is controlled by the size and shape of molecules in addition to charge and this results in more efficient electrophoretic separations.

Starch Gels: The first gel media to receive attention was starch. Starch gels are prepared by heating and cooling a mixture of partially hydrolyzed starch in an appropriate buffer. This causes the branched chains of the amylopectin component of the starch to intertwine and form a semi-solid gel.

Polyacrylamide Gels: Though starch gels are still used for some protein and isoenzyme analyses, these gels have rather limited use because pore sizes in the matrix cannot be controlled. As polyacrylamide gels can be prepared with different pore sizes, electrophoresis through these gels leads to enhanced resolution of sample components. Besides the reproducible preselected pore size provided by polyacrylamide gels, their use has other advantages over starch, namely, minimal protein adsorption, rapid analysis and ease of preparation and staining.

Preparation of polyacrylamide gels by copolymerisation of acrylamide monomer with the cross-linking agent methylene bisacrylamide, has been discussed earlier (Chapter 9). The porosity of a gel is determined by the relative proption of acrylamide monomer to cross-linking agent. Gels may be defined in terms of the total percentage of acrylamide present. Gels may be prepared containing from 3% to 30% acrylamide, corresponding to pore sizes of 0.5 nm and 0.2 nm diameter respectively. The standard gel is 7.5% polyacrylamide. It can be used over the molecular weight range of 10,000 to 1,000,000; however, the best resolution is obtained in the range of 30,000 to 300,000. Gel that is 3.5% polyacrylamide can be used for macromolecules with molecular weights in the range of 1,000,000 to 3,500,000. For macromolecules of molecular weights above 3,500,000 agarose gels are used, and thus the standard method used to characterize RNA and DNA, for example, is electrophoresis through agarose gels.

The experimental arrangements of gel electrophoresis use either a horizontal slab gel or a vertical column gel. These are prepared from a mixture of the selected

gel medium e.g. polyacrylamide in the buffer chosen for the experiment. Tris-glycine, Tris-phosphate, Tris-borate in concentrations of 0.05M are examples of buffer systems appropriate for gels.

Gels are prepared in the glass containers in which they are to be used. In the case of slabs, the gels are cast betweent two clean glass plates which are clamped together but held apart by plastic spacers. Gel dimensions of upto 12×25 cm are used for starch and agrose gels, with thickness upto 6 and 3 mm respectively. For polyacramide gels typical dimensions are 12×14 cm, with thickness ranging from 1 to 3 mm. Vertical slabs are run with the glass plates left on both sides of the gel. For horizontal slabs, the plate above the gel is removed before the run.

For electrophoresis by horizontal system dissolved samples are applied to the surface of the horizontal gel slabs via filter paper strips, but more commonly by injection from a microsyringe into slots or wells made in the gel by a comb-like template having been inserted into the gel before it set. The buffer in which the sample is dissolved usually contains sucrose or glycerol (10 to 15%) to increase its density and ensure that the solution sinks into the well. Also, a 'tracking dye' such as bromophenol blue is often added. It aids observation of loading and monitoring the migration, as the dye, which is of small molecular size and is anionic, moves rapidly through the gel ahead of most proteins or nucleic acids. For horizontal systems, electrical contact between the gel and the buffer in the electrical compartments can be mintained by wicks. Alternatively, the gel can be submerged in the buffer, thus allowing the current to pass directly through it, with the added benefit of the buffer dispersing heat from the gel. Upto 20 samples can be run simultaneously.

After electrophoresis, the protein bands have the tendency to widen by diffusion processes. Because this may decrease resolution, the electrophoretogram is treated with a fixative to reduce zone spreading. This treatment is carried out immediately after the run is complete by soaking the gel in a fixing solution (e.g. 10% trichloroacetic acid) for 30 minutes. The samples can then be stained by immersing the gel in a suitable staining solution (Coomassie Brilliant Blue R-250, as 0.25% aqueous solution, is particularly suitable for proteins) for 10 minutes. Several rinses in a destaining solution (an aqueous solution containing 25% ethanol and 8% acetic acid) are required to remove excess background dye. Alternatively, gel may be stained by soaking in 0.25% dye in water-methanol-acetic acid (5:5:1) followed by repeated washings with the same solvent. After partial drying, the stained gel can then be preserved between two layers of plastic film.

For vertical systems the gel column is inserted between two separate buffer reservoirs; the upper reservoir contains the cathode, and the lower one contains the anode. Gel electrophoresis is usually carried out at basic pH, where most biological polymers are anionic and hence they move down toward the anode. The sample to be analyzed is layered on top of the gel and voltage is applied to the system.

The gel completes the electrical circuit between the electrodes in the upper and lower compartments (Figure 16.3). Although the buffer surrounding the gel helps to disperse heat generated by the current, additional cooling may be required during long runs. This can be achieved by carrying out the run in a cold room or by circulating the buffer through a cooling system. A 'tracing dye' such as bromophenol blue is also applied. When the dye band has moved to the opposite end of the column, the voltage is turned off.

The precise voltage and time required to obtain optimal separations will depend on the nature of the samples and the type of gel used, but several hours at a few hundred volts are generally required. On the completion of the electrophoresis, the gel is removed from the column and

Figure 16.3 A column for polyacrylamide gel electrophoresis.

processed in the same manner as the gel run on the horizontal slab system.

Quantitative analysis of gel electrophoretograms can be carried out by removing the separated compounds from the supporting medium. The most common method of recovery is to divide up the supporting medium into standard uniform lengths and to treat the supporting medium in a way that releases the test compounds. Starch gel is readily eluted following either mechanical maceration or maceration by alternate freezing and thawing. Macromolecules may be recovered from starch gels by electrodialysis (enclosing the gel inside a dialysis membrane which, in turen, is placed inside an electrophoresis unit). Polyacrylamide gels will not breakdown on freezing and thawing and they can be sliced up only when semi-frozen, but they can be eluted by electrodialysis (Chapter 17).

Recovery of compounds by electrophoresis, continued until the sample migrates off the end of the supporting medium, can also be achieved for some forms of electrophoretogram, for example, preparative polyacrylamide rods, although this requires a special attachment at the base of the column to bleed off the sample as it emerges.

Two modifications of polyacrylamide gel electrophoresis have greatly increased its versatility. These are: (i) Discontinuous gel electrophoresis, and (ii) sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis.

Figure 16.4 The process of disc gel electrophoresis. (A) Before electrophoresis. (B) Movement of chloride, glycinate, and protein through the stacking gel. (C) Separation of protein samples by the resolving gel.

16.2.5.1 Discontinuous Gel Electrophoresis

The experimental arrangement for 'disc' gel electrophoresis is shown in Figure 16.4. The three significant differences in this method are:

(i)   There are now two gel layers, a lower or *resolving gel* and an upper or *stacking gel*.

(ii) The buffers used to prepare the two gel layers are of different ionic strength and pH.

(iii) The stacking gel has a lower acrylamide concentration, so that its pore sizes are larger.

These three changes in the experimental conditions cause the formation of highly concentrated bands of sample in the stacking gel and greater resolution of the sample components in the lower gel in the following manner.

The sample is usually dissolved in glycine-chloride buffer having the pH range of 8 to 9, before being loaded on the gel. Glycine exists primarily in two forms at this pH, zwitterion and an anion, as shown by the equation:

$$H_3NCH_2COO^- \rightleftharpoons H_2NCH_2COO^- + H^+$$
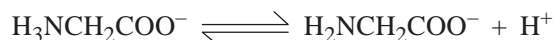
The average charge on glycine anions at pH 8.5 is about –0.2. Upon turning on the voltage, buffer ions (glycinate and chloride) and protein or nucleic acid sample move into the stacking gel, which has pH of 6.9. This entry into the upper gel causes the equilibrium of the above reaction to shift toward the left, increasing the concentration of glycine zwitterion, which has no net charge and hence no electrophoretic mobility. In order to maintain a constant current in the electrophoresis system, a flow of anions must be maintained. Since most proteins and nucleic acid samples are still anionic at pH 6.9, they replace glycinate as mobile ions. Therefore, the relative ion mobilities in the stacking gel are chloride > protein or nucleic acid sample > glycinate. The sample will tend to accumulate and form a thin, concentrated band sandwiched between the chloride and glycinate as they move through the upper gel. Since the acrylamide concentration in the stacking gel is low (2 to 3%), there is little impediment to the mobility of the large sample molecules.

Now, when the ionic front reaches the lower gel with buffer of pH 8 to 9, the glycinate concentration increases and anionic glycine and chloride carry the major proportion of the current. The protein or nucleic acid sample molecules, now in a narrow band, encounter both an increase in pH and a decrease in pore size. The increase in pH would, of course, tend to increase electrophoretic mobility of the sample molecules, but the smaller pore size will decrease their mobility. The relative rate of movement of anions in the lower gel is chloride > glycinate > protein or nucleic acid sample. The separation of sample components in the resolving gel occurs as already described under gel electrophoresis. Each component has its own value of charge/mass ratio and a discrete molecular size and shape, which will directly influence its mobility.

Disc gel electrophoresis yields excellent resolution and is the method of choice for analysis of proteins and nucleic acid fragments. Mixtures of proteins or nucleic acids in quantities as little as 1 or 2 μg can be separated. Detection of separated bands is carried out by staining the gels on completion of electrophoresis. Disc gel is not limited to the column arrangement, but is also adaptable to slab gel electrophoresis.

16.2.5.2. *Sodium Dodecyl Sulphate (SDS) Polyacrylamide Gel Electrohoresis*

This form of polyacrylamide gel electrophoresis is one of the most widely used methods for separating proteins from mixtures and for determining their molecular weights as well. Sodium dodecyl sulphate (SDA) is an anionic detergent which binds strongly to proteins in the presence of a disulphide reducing agent, mercaptoethanol, that assists denaturation of proteins by reducing all disulphide bonds. Each gram of protein binds to about 1.4 g of the detergent, thereby the protein acquiring a constant negative charge per unit mass. During electrophoresis, therefore, protein-SDS complexes will all move towards the anode, and the electrophoretic mobility of these protein-SDS complexes will be influenced primarily by molecular size. The larger molecules will be retarded by the molecular sieving effect of the gel, while the smaller molecules will have greater mobility. Empirical measurements have shown a linear relationship between the $\log_{10}$ molecular weight and the electrophoretic mobility.

The application of this relationship enables determination of the molecular weights of the sample proteins, if standard proteins of known molecular weights are also run.

16.2.6 *Isoelectric Focussing (IEF)*

This technique, sometimes called electrofocussing, is based on moving boundary rather than zone electrophoresis. Amphoteric substances such as amino acids, peptides and proteins are separated in an electric field across which there is a *pH gradient*, the anode region being kept at a pH which is lower than that of the cathode region. The pH range chosen is such that the sample constituents being separated will have their isoelectric points within this range. Substance which are initially at pH regions below their respetive isoelectric points will be positively charged and will migrate towards the cathode, but as do so, the surrounding pH will be steadily increasing until it corresponds to their respective isoelectric points. They will then be in the zwitterion form with no net charge so that their movement wil cease. Likewise, substances which are initially at pH regions above their respective isoelectric points will be negatively charged and will migrate towards the anode until they reach their respective isoelectric points and become stationary. Amphoteric substances thus become focussed into narrow stationary bands. This is illustrated in Figure 16.5.

Sample applied at
pH region below
isoelectric points

Lower pH

$A^+ B^+ C^+$

A^+

Sample components
migrate as cations

B^+

C^+

Increasing pH

A

Sample components
focussed as zwitterions
at their respective
isoelectric point

B

C

A^-

Sample components
migrate as anions

B^-

C^-

Sample applied at pH
region above isoelective
points

$A^- B^- C^-$

Higher pH
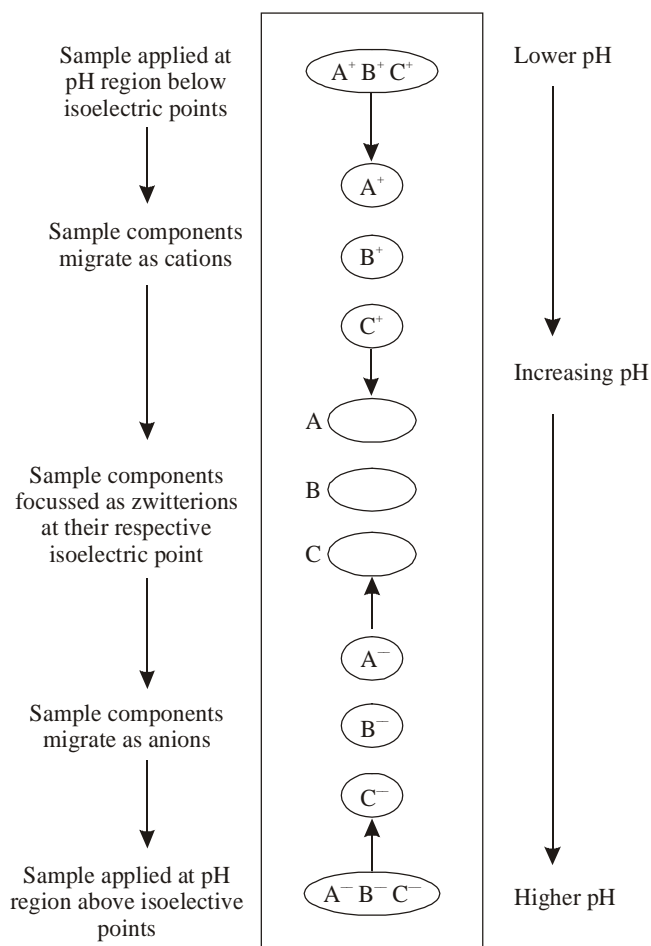
Figure 16.5 Illustration of the principle of isoelectric focussing.

As the sample constituent will always move towards their respective isoelectric points, it is not crucial where the samples are applied. Isoelectric focussing gives very high resolutions of samples, and it is particularly suitable for separating isoenzymes, as differences between isoelectric points of only 0.01 pH units are sufficient for separation by this technique.

Special precautions must be taken so that the pH gradient remains stable and is not disrupted by diffusion or convective mixing during the electrophoresis experiment. The most common stabilising technique is to form the gradient in a polyacrylamide, agrose or dextran gel. The pH gradient is formed in the gel by electrophoresis of synthetic polyelectrolytes, called ampholytes. These materials are low molecular weight polymers that have a wide range of isoelectric points as the result of these polymers containing numerous amino and carboxyl or sulphonic

acid groups. The polymer mixtures are available in specific pH ranges (pH 5-7, 6-8, 3.5-10, etc.) under the trade names LKB-Ampholine, Bio-Rad-Lyte, and Pharmacia-Pharmalyte. It is critical to select the appropriate pH range for the ampholite so that all the sample constituents have their respective isoelectric pH values (pH$_I$, pH at which a particular constitutent has zero net charge) in that range. The best resolution is, of course, achieved with an ampholyte mixture over a small pH range (about two units) encompassing the pH$_I$ for the sample constituents. If the pH$_I$ values for the sample constituents under study are unknown, an ampholyte of wide pH range (pH 3-10) should be used first and then a narrower pH range selected for use.

The gel medium is prepared as previously described except that the appropriate ampholyte is mixed prior to polymerisation. The gel mixture is poured so as to set into the desired form e.g. column tubes, horizontal slabs etc. Immediately after casting of the gel, the pH is constant throughout the medium, but application of voltage will induce migration of ampholyte molecules to form the pH gradient.

Loading the sample on the gel can be done by one of two alternative methods. A concentrated, salt-free sample can be layered on top of the gel as previously described under traditional gel electrophoresis. The second method is simply to add the protein to the gel preparation, resulting in an even distribution of the sample throughout the medium. The molecules of the sample constituents move more slowly than the ampholyte molecules, whereby the pH gradient is set up before significant migration of the sample constituents occurs. Samples in quantities as small as 10 to 50 µg can be analysed by IEF. Larger sample quantities (up to 20 mg) may be used for preparative purposes.

Electrofocussing is carried out under an applied current of 2 mA per tube and usually takes from 30 to 120 minutes. The time period for electrofocussing is not as critical as for traditional electrophoresis. Trial and error can be used to find a period that results in an affective separation; however, longer periods do not move the sample constituents out of the medium as is the case in gel electrophoresis.

After IEF electrophoresis, ampholytes have to be removed from gels before staining can be carried out, because the staining agents commonly used are bound by the ampholytes. Removal of the ampholytes is effected by soaking gels in 5% trichloroacetic acid, and staining is carried out with Coomassie Blue dye.

Electrofocussing is now established as the best method for resolution and analysis of proteins, including enzymes, and is considered as the standard criterion of purity for proteins.

16.2.7. SDS-Isoelectric Focussing Gel Electrophoresis

Separation of amphoteric substances by IEF is based on their respective pH$_I$ values. SDS-gel electrophoresis separates molecules on the basis of the molecular

size. A combination of the two methods should lead to enhanced resolution of mixtures of constituents such as proteins. This comibnation has, indeed, now become a routine and powerful technique for separation of proteins. First such experiment by which O'Farrell successfully carried out the analysis of total *E.coli* protein was reported in 1975. O'Farrell subjected the sample to separation first by isoelectric focussing, and in the next step the protein bands were transferred to an SDS gel. At least 1000 discrete protein spots resulted.

16.2.8 Grdient Gel Electrophoresis

The procedures for preparation of the slab or column gel earlier outlined are the ones which are used to form the gels needed for the various gel electrophoresis techniques discussed so far. A gel prepared by any of those procedures has more or less uniform pore sizes. Where molecular weights of the sample constituents to be separated by gel electrophoresis are similar, *gradient gels* give better resolution than that achieved by the use of a uniform (i.e. non-gradient) gel. Gradient gels contain concentration gradient of acrylamide increasing from 5 to 25%, with a corresponding decrease in pore size. The gradients are formed by running high and low concentrations of acrylamide solutions between the gel plates via a gradient mixer. The migration of the sample constituents in the gel prepared in this manner will be impeded once the pores become too small. This will result in formation of narrower bands and hence increased resolution. Separation by gradient gel electrophoresis is therefore primarily based on differences in molecular size of the sample constituents. This means that samples in which there are wide ranges of molecular weights can be run as well, besides those containing constituents with similar molecular weights. Although gradient gels can be used without SDS and stacking gels, these latter can be gainfully employed for optimal separations by gradient gel electrophoresis.

16.2.9 Two Dimensional Gel Electrophoresis

Use of two-dimensional gels achieves high resolution in the case of even a complex mixture. The experiment in its first step brings about partial separation of sample components by isoelectric focussing on the basis of differences in isoelectric points, using a cylindrical column of gel. In the next step of the experiment, this gel is applied along the top of the stacking gel for SDS gel electrophoresis in the second dimension for separation to be completed on the basis of differences in molecular size. Either gradient or SDS gels may be used, depending on requirements.

16.2.10 Isotachophoresis

The name of this technique derived from Greek, refers to the fact that the ions being separated all travel (phoresis) at the same (iso) speed (tacho).

Separation of the ionic components of the sample by this technique is achieved through stacking them into discrete zones in order of their mobilities, producing very high resolution.

For the separation of a mixture of anions, a leading anion (e.g. chloride) is chosen which has a mobility higher than that of the sample ions and a tailing (or terminating) anion (e.g. glutamate) whose mobility is lower than that of the sample ion. All the anions must have a common cation. Likewise, for the separation of cations, such as metal ions, there are requirements for leading and tailing cations and a common anion. When the current is switched on, the leading ions will move towards the appropriate electrode, the sample ions will follow in order of their mobilities, and the tailing ion will follow behind the sample ions. Once the equilibrium is achieved, the ions will move in discrete bands in the order of their mobilities (Figure 16.6).

Solution in separation tube containing
high-mobility leading ion (L^-) and
low $\times$ 2 mobility trailing ion (T^-)

Sample mixture containing ions of
intermediate mobility (A^-, B^-, C^-) added
and current switched on.
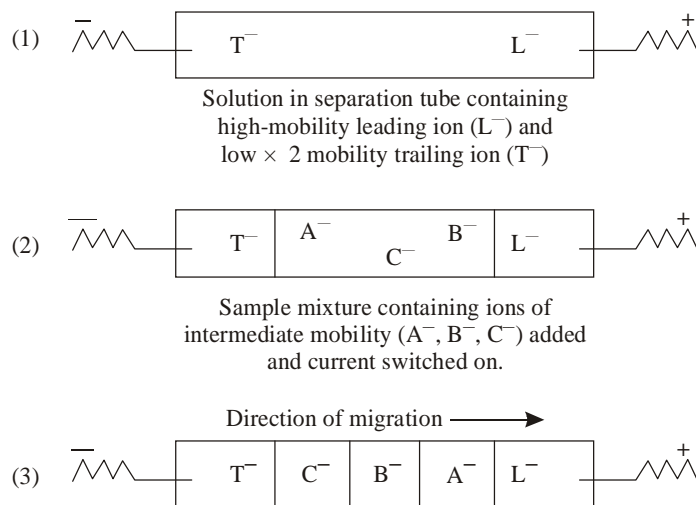
Separation now complete as ions move in order of their mobilities
at the same velocity.
Figure 16.6 Illustration of the principle of isotachophoresis.

Separation is achieved in a capillary tube, into which the samples are injected between the leading and tailing ions. Isotachophoresis is carried out in an aqueous environment, the only solution being the leading and tailing electrolytes and the samples. As voltages upto 30 kv are used, a thermostatically controlled cooling bath is used. Separations are achieved in 10 to 30 minutes. Length of each separated band is proportional to the amount of the respective ion present. Therefore, besides achieving resolution of mixtures, quantitative estimation (e.g.

by measuring ultraviolet absorption) can also be carried out through separation by isotachophoresis.

Where the mobilities of the sample ions are very similar, their resolution may be enhanced by including, with the sample, synthetic ampholytes called *spacer ions*. These have mobilities intermediate to those of the sample ions and hence help to separate them by taking up positions between the sample ions. The spacer ions are similar to the ampholytes used in isoelectric focussing.

Isotachophoresis can be used for separating charged species e.g. inorganic ions, and organic acids or proteins and nucleic acids in quantities of samples which may be as small as a few micrograms. In this context it can be seen that the phenomenon of isotachophoresis forms the basis of the excellent resolution of proteins and nucleic acids by disc gel electrophoresis discussed earlier. As well as being used extensively in research laboratories, isotachophoresis is now finding industrial applications in pollution control (detecting detergents and inorganic ions in effluent water) and in quality control in the food, brewing and pharmaceutical industries.

■

17

Membrane-based Methods

Methods dealt with in this chapter are: Dialysis, electrodialysis and ultrafiltration. Use of porous membranes forms the basis of each of these techniques.

17.1 Dialysis

Dialysis is a technique which permits separation of low-molecular weight solutes (crystalloids) from colloids (high-molecular weight solutes) by making use of a membrane.

17.1.1 Working of the Technique

The phenonmenon of dialysis can be illustrated with the help of a simple model comprising a vessel which is divided by a membrane into two compartments, of which one is occupied by an aqueous solution, molecular or colloidal, and the other by water. Owing to the concentration gradient across the membrane the water will tend to diffuse into the solution, and the solute into the water compartment. If the membrane pores are large compared with the diameters of all solute particles present, so that no specific steric hindrance is offered to these particles, both processes take place at relative rates which are the same as in *free* diffusion. Of course, the presence of membrane reduces the area through which diffusion can occur; also, in case the pores do not run perpendicular to the membrane surface, the presence of membrane will cause prolongation of the path traversed by the diffusing molecules. If, however, the pore sizes are of the same order of magnitude as the solute particle sizes, the solute particles encounter varying resistance to their passage from one compartment to the other. Such diffusion may be termed impeded to differentiate it from the free diffusion, referred to earlier, through a membrane having large pores. Dialysis is a differential diffusion, employing a membrane impermeable to the colloidal solute, but permeable to the crystalloidal ones; the latter diffuse into the water, while the water diffuses into the solution.

Just as in filtration employing an ordinary filter paper, the term 'residue' is used for the filter paper-retained material and 'filtrate' stands for the materials passing out through the filter paper, in dialysis the term 'retentate' is used for the membrane-retained components while materials permeating the membrane are called 'diffusate'.

Figure 17.1 illustrates a typical set-up for dialysis. The solution to be dialysed is contained with the membrane and pure solvent is placed outside the membrane. This solvent is changed periodically or continuously until the concentration of diffusible solutes is reduced to near zero in the solution in the membrane.
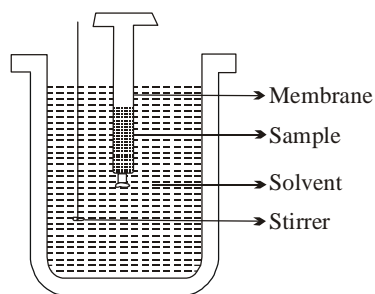
17.1.2 Membranes

Figure 17.1 Equipment for dialysis

Membranes suitable for dialysis include vegetable parchment, animal parchment, gold beater's skin (peritoneal membrane of cattle), fish bladders, collodion and cellophane.

Collodion sacs were the first artificial membranes to be generally adopted (Schumacher, 1860), and have been used very extensively, especially in biological research. A test tube is filled with a solution of collodion (cellulose nitrate containing about 11% nitrogen) in ether and alcohol, and is inverted and drained leaving a film clinging to the interior. After evaporation from this film has proceeded for a given time (sufficient for the collodion to set to a gel) the tube is plunged into water; the sac is loosened, removed, and washed free of the remaining solvents.

The porosity of the collodion sacs is varied by adjusting the ratio of alcohol to ether in the solvent, and varying the time of draining and the duration of evaporation, as also by adding small quantities of other reagents to the solution, though these added substances, like amyl alcohol, acetone, glycerol, water, lactic acid, ethylene glycol, also affect the mechanical properties like strength of the membrane.

The collodion sac is particularly popular because of the ease of preparation and the large area available for filtration, and because it acts as the container by itself.

Cellophane (regenerated cellulose from cellulose xanthate) is another material frequently used for dialysis. It has a pore size of approximately 4-8μ and is impermeable to molecules with a relative molecular mass in excess of about 10,000.

Separations of crystalloids from colloids reported in 1861 by Graham, the discoverer of dialysis, pertained to molecules with large differences in molecular size. In recent times smaller differences in molecular size have been found adequate to achieve highly satisfactory separations, and in some cases size has been an inadequate parameter to represent the separations. The earlier simple theory to explain dialysis which was based on the concepts of the membrane being a rigid sieve and the diffusible solute speceies being hard objects of definite dimension has been replaced by one based on mebrane-solution interactions, involving electrostatic effects, ionic covalent, and hydrogen bonding, chemisorp-

tion, physical adsorption, and the concept of the membrane as a flexible matrix whose permeable pathways change dimensions randomly over time. New dialysis membranes have contributed to development of this more comprehensive view and of a broader range of industrial applications for dialysis. Many of the new membrane materials have membrane pore size which can be adjusted to any desired value during the synthesis of the polymer and the forming of the membrane. These synthetic membranes can also withstand many chemical environments that were entirely destructive to classical simple cellulose materials as, for sample, membranes made from vinyl polymers are acid-resistant.

17.1.3 Gerneral Considerations of Diffusion

17.1.3.1 Fick's First Law

The mathematical theory of diffusion is based upon the hypothesis that the rate of transfer of diffusing substance through unit area of a section is proportional to the concentration gradient measured perpendicular to the section. This hypothesis is expressed mathematically as

$$J = -D\frac{\partial C}{\partial x} \qquad \text{...(17.1)}$$

where J is the rate of transfer per unit area of section, C is the concentration of the diffusing substance, x is the space coordinate measured perpendicular to the section, and D is called the diffusion coefficient or diffusivity. The value of D for the glucose-water system, for example, is 0.52×10^{-5} cm^2/sec at 15ºC. The minus sign in the equation indicates that the diffusion occurs from a higher to a lower concentration. Equation (17.1) is the mathematical expression of Fick's first law enunciated in the year 1855.

For dialytic diffusion, Fick's first law is usually rewritten with the following substitutions: The diffusion coefficient D is replaced by the product of k, a permeability constant, and A, the area of the membrane.

$$D = kA \qquad \text{...(17.2)}$$

The concentration gradient can be replaced by the difference between the two specific concentrations, C_0 and C_i :

$$\frac{\partial C}{\partial x} = C_o - C_i \qquad \text{...(17.3)}$$

On integration, Fick's first law becomes

$$N = kA (C_0 - C_i)t \qquad \text{...(17.4)}$$

This equation shows that the amount of material diffusion, N, is directly proportional to the area A, the concentration difference $(C_0 - C_i)$, and to the time t.

The permeability coefficient, k, is a characteristic of the membrane. Equation (17.4) is valid for a constant temperature. Furthermore, it assumes that the substance in question is small enough to pass through the pores of the membrane and that it is non-ionic.

17.1.3.2 Donnan Membrane Equilibrium

With regard to the previous discussion, it is interesting to observe the consequences of diffusion of ionic substances when one of them is too large to pass through a membrane.

Suppose a vessel is separated into two compartments by a semipermeable membrane which permits water and crystalloids, but not colloidal particles, to pass through. If water is placed in both the compartments and then some NaCl is added to one compartment, the NaCl will diffuse through the membrane and after a time become equally distributed in the water of both the compartments. However, if an ion which cannot pass through is placed on one side of the membrane, the distribution of a freely diffusible electrolyte like NaCl, becomes unequal in the solutions on the opposit sides of the membrane. This observation made in 1911 by Donnan is known after his name as Donnan's equalibrium theory. A theroretical derivation of this generalisation based on considerations of kinetics is given below.

Sodium salts of non-diffusible ions such as Congo Red which Donnan used, may be represented by the general formula NaR. The membrane is impereable to R^- and also to undissociated NaR, but freely permeable to NaCl. Suppose we have a solution of NaR on one side of the membrane and that the concentration of NaR is equal to a. Suppose a solution of NaCl of concentration b is on the other side of the membrane. The initial situation may be shown diagrammatically as follows:—

(1)			(2)	
a	Na^+		Na^+	b
a	R^-		Cl^-	b

Na^+ and Cl^- ions diffuse in pairs to maintain electrical neutrality of the solution, from solution (2) through the membrane to solution (1) Upon passing into (1), they may reversibly diffuse back into (2) After a certain period of time, the rate of diffusion from (1) to (2) equals the rate of diffusion from (2) to (1), and the system is at equalibrium. The net process involves the loss of NaCl from (2) and its addition to (1). Let x represent the net concentration of NaCl which has passed from (2) to (1) at equilibrium. Also, x equals the concentrations of Na^+ and Cl^- ions which have passed from (2) to (1) (x NaCl = x Na^+ + x Cl^-). Then (b–x) represents the concentrations of NaCl and Na^+ and Cl^- remaining in (2) at equilibrium. The ionic distribution and concentration at equilibrium are:

	(1)		(2)	
a + x	Na^+		Na^+	b – x
a	R^-			
x	Cl^-		Cl^-	b – x

In order that Na^+ and Cl^- ions may pass through the membrane together, they must arrive simultaneously at a given point on the membrane. The probability that they will do this is directly proportional to the product of the ion concentraions. The rate of diffusion of Na Cl from (2) to (1) is thus proportional to the product of the concentrations of Na^+ and Cl^- in (2) that is, $(b – x)^2$. The rate of diffusion of NaCl from (1) to (2) is proportional to the product of the concentrations of Na^+ and Cl^- in (1), that is, $(a + x)x$. Since the rates of diffusion are equal at equilibrium, we have

$$(b – x)^2 = (a + x)x \qquad\qquad ...(17.5)$$

This is the fundamental Donnan equation and may be written for the above example in the form:

$$[Na^+]_2\,[Cl^-]_2 = [Na^+]_1\,[Cl^-]_1 \qquad\qquad ...(17.6)$$

It states that at equilibrium the product of the concentrations of Na^+ and Cl^- ions in compartment (2) equals the product of concentrations of Na^+ and Cl^- ions in compartment (1), which contains the nondiffusible ion.

As is evident,

$[Na^+]_1 = [Cl^-]_1 + [R^-]_1$ with reference to compartment (1), and

$[Na^+]_2 = [Cl^-]_2$ in compartment (2), so that Equation (17.6):

$[Na^+]_2\,[Cl^-]_2 = [Na^+]_1\,[Cl^-]_1$ can be rewritten as:

$$[Cl^-]_2^2 = [Cl^-]_1\,([Cl^-]_1 + [R^-]_1)$$

$$= [Cl^-]_1^2 + [Cl^-]_1\,[R^-]_1 \qquad\qquad ...(17.7)$$

So

$$[Cl^-]_2 > [Cl^-]_1$$

These ionic relations in the Donnan equilibrium may be summarised as:
  (i) The concentration of a diffusible positive ion is greater on the side of the membrane containing a nondiffusible negative ion, $[Na^+]_1 > [Na^+]_2$.
  (ii) The concentration of a diffusible negative ion is greater on the side of the membrane not containing the nondiffusible negative ion,

$$[Cl^-]_2 > [Cl^-]_1.$$

(iii)   An examination of the equation:

$$[Cl^-]_2^2 = [Cl_1^-] \, ([Cl^-]_1 + [R^-]_1),$$

shows that by keeping the concentration of the nondiffusible ion high in compartment (1), we can prevent the diffusion of the diffusible species from (2) to (1).

The above distributions are reversed when the nondiffusable ion is positive, as in the case of protein chloride.

17.1.4 Applications

The two prerequisites that render dialysis an attractive choice as a method of separation are: (i) The existence of a large concentration difference of the substance to be diffused between the two process streams on the two sides of the membrane, and (ii) a large molecular weight (and hence permeability) difference between the species to be separated.

Amongst the applications of dialysis in industry the most outstanding one over a long period of time has been the recovery of caustic soda in the pulp industry. This recovery amounts to a great saving in caustic soda and it greatly reduces the disposal problem of the waste liquor. This industrial application is an example wherein the two prerequisites listed above are eminently met to make dialysis the chosen method for attaining the purposeful recovery of caustic soda.

The importance of dialysis in the recovery and purification of materials in the chemical, food, biological and pharmaceutical fields cannot be overemphasized. In general, application of dialysis is needed when salts must be removed from colloidal suspensions, or when low-molecular weight, but water-soluble, organic compounds are to be separated from higher-molecular weight substances. In this context, the role that dialysis is going to play in the foreseeable future in the development of biotechnology can be easily visualized. In biotechnological projects like 'fuels from biomass', 'foods—nonconventional', single-celled organisms are cultured, suspended within large liquid masses, either to augment natural photosynthesis or to convert its products into more useful forms e.g. cellulose into sugars, organic acids or aldehydes and alcohols. The organisms are often inhibited in activity by these products which must be continuously removed with minimum trauma to the suspended organisms. Dialysis provides the gentleness that is needed.

Examples of application of dialysis involving use of chemical-resistant membrane are the recovery of copper and nickel sulphates from sulphuric acid, and the recovery of iron sulphate from steel pickling liquor. In each case all components will permeate the membrane; separations are effected because of the differences in respective diffusion rates—for example, copper transfers at only one-tenth the rate of sulphuric acid, and nickel is slightly slower.

At present, the most important application of dialysis is in haemodialysis, that is, the treatment of the blood of persons with end-stage renal diseases in which the kidneys are no longer capable of removing the products of metabolism from the blood and excreting them. The application of dialysis is essential here because of two requirements. Firstly, the processing has to be gentle and, secondly, separation has to be carried out between quite large and quite small molecules—in this case the separation involved is between quite bulky molecules of proteins and the quite small molecules of urea in which form catabolic nitrogen is principally eliminated.

In Analytical Chemistry, dialysis is frequently employed as a means of removal of interfering colloids before carrying out quantitative determination of the crystalloid that is present or *vice versa*.

17.2 Electrodialysis

The combined process of dialysis and electrophoretic transport of solutes through a membrane by applying an electric field is known as electrodialysis.

Electrical energy is supplied to the system and it becomes possible to drive electrolytes from a dilute solution to a more concentrated solution across the membrane separating the two solutions.

17.2.1 Membranes

Ions do not migrate readily through membranes that carry in their pores charges of the same sign as the ion; ions of charge opposite to that of the membrane are not prevented from passing through the membrane. Such a selective permeability to ions has been used to improve the efficiency of electrodialysis. Reference has earlier been made to membranes of vegetable and animal origin besides membranes of collodion and cellophane. In the present context it may be added that membranes of collodion, vegetable parchment and cellophane carry negative charges in contact with aqueous solutions. Animal membranes show positive charges at low pH and negative charges at high pH. Again, mention was also made of the newer polymeric synthetic membranes now available for dialysis. Compared to dialysis, a wider selection of membranes of man-made polymer materials is possible for electrodialysis. Generally, those membranes are ion-selective membranes and, in particular, ion-exchange membranes.

Membranes of ion-exchange resins are commercially available in cationic and anionic forms. The ion-exchange membranes show a high selectivity for ions of one charge type. The ion-selective membranes commercially available before 1950 suffered from practical limitations; those that had high selectivity also had high electrical resistance; those that had low electrical resistance also had low selectivity. None was sufficiently mechanically strong or chemically stable for practical purposes. In 1950 ion-selective membranes having high selectivity, low

electrical resistance, good mechanical strength and good chemical stability were described. These were essentially insoluble, synthetic, polymeric organic ion-exchange resins in sheet form. Typical examples of modern homogeneous membranes are represented by structures I and II. These cation- and anion-exchange resins have already been discussed and the methods of their preparation described under Ion-exchange Chromatography (Chapter 8).

I. $R - SO_3^- M^+$

II. $R - \overset{+}{N}(CH_3)_3\ X^-$

I. Cation-exchange resin
II. Anion-exchange resin

Organic polymeric matrix, R
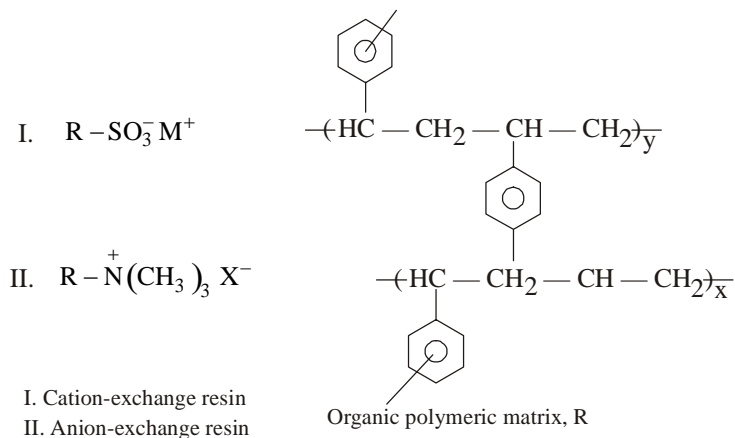
When in contact with electrolyte solution of low or moderate concentrations, an ion-exchange membrane contains a large number of dissociated counterions in the water imbibed by the membrane. The high concentration of the mobile counterions in ion-exchange resins (i.e. M^+ in the case of cation-exchange resin I and X^- in the case of anion-exchange resin II) is responsible for the low electrical resistance of the membrane. The high concentration of the bound charged groups i.e. negatively charged ones in the case of cation-exchange resins and positively charged ones for anion-exchange resins, tends to exclude mobile co-ions (i.e. ions carrying the same charge as the bound groups possess) from surrounding solution and is responsible for the high ion-selectivity of the membranes.

17.2.2 Electrodialysis Cells

The earlier electrolytic cells used for dialysis are now of historical interest only. These were three-compartment cells, the compartments being separated from one another by membranes. The end compartments contained electrodes. Thes cells used membranes which did not have much of ion-selectivity.

Multi-compartment electrodialysis equipment using ion-selective membrane was introduced in 1940. Figure 17.2 illustrates the working principle of such a cell. In the cell anion-selective membranes (a) alternate with cation-selective membranes (c). Under an applied d-c potential, cations M^+ tend to move towards the cathode. These cations are able to permeate the cation-selective membranes but not the anion-selective membranes. Similarly, anions X^- tend to move towards the

anode. They are able to permeate the anion-selective membranes but not the cation-selective membranes. As a result, the odd-numbered compartments in Figure 17.2 become depleted in electrolyte and the even-numbered compartments enriched.
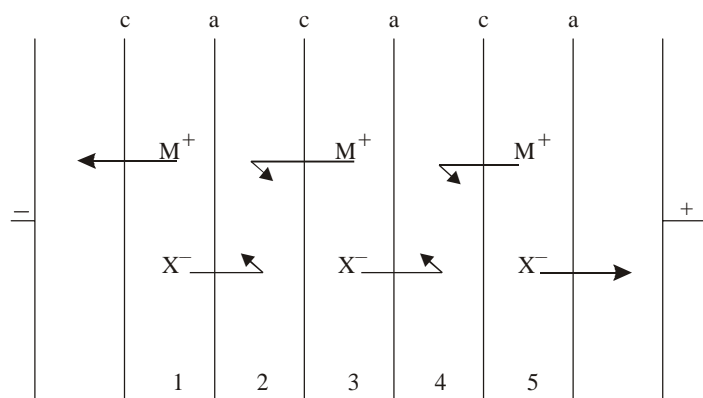
Figure 17.2 Principle of multicomponent electrodialysis

In the electrodialysis apparatus the membranes are separated from each other by gaskets which form fluid compartments. Compartments that have anion-selective membranes on the side facing the anode are electrolyte-depletion compartments and the remaining compartments are electrolyte-enrichment compartments.

17.2.3 Applications

(a) An important application of electrodialysis using membranes which are not ion-selective is the removal of the last traces of electrolytes from colloids.

(b) The ion-selective membranes which had become available by 1940, when the multi-compartment cell was first introduced, were not commercially useful, because those which possessed high ion-selectivity also had high electrical resistance. As mentioned earlier, such deficiencies have been removed in the improved membranes that have become available since 1950, and with the introduction of these highly ion-selective membranes, electrodialysis now-a-days plays a deciding role in several important commercial activities as illustrated below.

(*i*) *Especially pure water:* High purity water is needed for several industrial purposes e.g. for use in high pressure boilers, in the manufacture of pharmaceuticals and in the electronic industry. To get water of the required degree of purity for such uses, electrodialysis is usually the first step of the purification process. This step brings down the electrolyte concentration of the water being processed. Further deionisation is then achieved by chemically regenerated ion-exchange. Next, organic matter is removed

using activated carbon. Finally the particulate matter is removed by ultrafiltration (see 17.3).

(*ii*) *Potable water from brackish water:* One of the major uses of electrodialysis is the application of this purification method for production of potable water from brackish water. Several electrodialysis plants are successfully operating to give potable water from sea water.

(*iii*) *Salt from sea water:* Another principal application of electrodialysis is the production of common salt from sea water. In the manufacture of salt from this source, generally sea water is first filtered and then warmed with waste heat. Next, this water is slowly passed through the depletion compartments of the multicompartment electrodialysis equipment. Concentration of solids reaches about 20% by weight in the brine collecting in the enrichment compartments. Further processing of this concentrated brine by evaporation yields the salt in solid crystalline state.

(*iv*) *Protein from whey:* In the manufacture of cheese, the whey which is left behind after the removal of cheese from milk still contains about half of the solids originally present in milk. The protein component of these solids in the whey has a rich food value. Recovery of this protein involves removal of the high content electrolytes from whey by electrodialysis. The resulting product is then concentrated and generally spray-dried. Recovery of protein by this application of electrodialysis reduces the extent of denaturation of the protein.

17.3 Ultrafiltration

Ultrafiltration is a pressure-driven filtration resulting in separations which take place on a molecular scale.

17.3.1 Working Principle

The model that was earlier employed to illustrate dialysis can also be used for understanding ultrafiltration. If the membrane which divides the vessel into the two compartments, of which one contains water while the other one is occupied by the solution to be processed, is impermeable to all solutes, the only diffusion that takes place is that of water into the solution and the process is ordinary osmosis. At osmotic equilibrium, the solution is under a hydrostatic pressure (the osmotic pressure) which causes sufficient flow out of the solution to balance the diffusion of water molecules into it. Now, if the hydrostatic pressure is increased beyond the osmotic pressure, there is a net flow of water out of the solution compartment, resulting in concentrating of the solution with respect to the solute, and the phenomenon is ultrafiltration. Similarly, in dialysis (wherein the membrane used is such that it is permeable to the crystalloidal solutes) when carried out under

pressure, if the pressure is increased so that both water and diffusible solutes flow out of the solution, the process becomes one of ultrafiltration. Evidently, the driving force in ultrafiltration is the energy due to pressure difference on the two sides of the membrane. Material transport takes place by convection, and the amount passing through the cross-section of the membrane depends on the pressure applied.

Ultrafiltration separations range from 2 to 20 mμ. Below *ca* 2 mμ, interactions between the membrane material and the solute and solvent become significant. That process is called *reverse osmosis* and is best described by solution-diffusion mechanisms rather than by the simpler model of impeded diffusion discussed earlier. Separation in reverse osmosis takes place at the membrane as the result of different solubilities in the homogeneous polymer membrane (solubility membrane). The permeation of a solute through a solubility membrane is determined by the concentration gradient and the coefficients of diffusion in the membrane. The separating capacity is therefore hardly influenced by the operating pressure. Criteria for distinguishing between ultrafiltration and reverse osmosis are summarised in the form of Table 17.1.

TABLE 17.1

Criteria for distinguishing between Ultrafiltration and Reverse Osmosis

Criterion	*Reverse osmosis*	*Ultrafiltration*
Size of the dissolved particles retained	MW < 500 – 1000	MW > 1000
Osmotic pressure	considerable, may rise to 800 – 1000 N/cm^2 (N = kg ms^{-2})	negligible
Working pressure	greater than 1000 N/cm^2 and upto 1500 N/cm^2	upto 100 N/cm^2
Principal processes	transport by diffusion; the material of the membrane may affect transport properties	separation according to molecular size; sieve effect; the material of the membrane has no influence, the important characteristic being the pore size

In all membrane filtration systems, the flow of liquid in the direction of the membrane and the retention of molecules at the surface of the membrane result in an increase in the concentration of these molecules at the surface of the membrane, an effect which is known as concentration polarization. The concentration of a substance increases with approach to the membrane and then behind the membrane, falls suddenly. With rising concentration at the surface of the membrane, a

concentration-dependent back-diffusion of molecules into the solution begins. An equilibrium is set up which results in a layer of raised concentration at the surface of the membrane of certain thickness. Consequently, in this area the osmotic pressure rises and the filtration capacity of the membrane falls. In addition to the decrease in capacity, the separation characteristics may change because of the 'secondary membrane' located in front of the membrane. In a membrane filtration, the concentration polarization and the back-diffusion must be limited. Technically, this is ensured by stirring the solution or by the use of so-called 'cross-flow systems' i.e. by the enforcement of turbulent flow in the case of relatively large cross-sections or laminar flow in thin channels or 'hollow fiber' systems.

Ultrafiltration necessitates a certin minimum pressure which is slightly greater than the osmotic pressure of the colloid system which is filtered. The required pressure varies from the hydrostatic pressure of the colloid solution proper, upto values which may range from 135 to 300 atmospheres. Ultrafilters must, therefore, have sufficient strength.

17.3.2 Ultrafiltration Membranes

Most ultrafilter membranes are gelatinous, and in the great majority of cases the gel consists of collodion. The other type is the non-gelatinous one.

17.3.2.1 Gel Membranes

(i) Collodion membranes impregnated in filter paper were introduced by Bechhold (1907). A piece of hardened filter paper is soaked in a solution of nitrocellulose in glacial acetic acid. The excess solution is drained from the paper, and the membrane is gelled by immersion in water. Acetic acid is removed by prolonged washing, leaving a film of nitrocellulose (with perhaps some cellulose acetate) imbedded in the filter paper. The higher the concentration of nitrocellulose in the original solution, the lower the porosity of the membrane.

The chief advantage of the Bechhold membranes lies in the relative simplicity of preparation and the wide range of porosities obtainable (pore diameter from 1 to 5μ down to less than 10mμ). However, in a given filter paper, the pore sizes vary over considerable range, and the limited reproducibility in average pore diameter from one membrane to the next makes comparative experiments difficult.

(ii) Impregnation of collodion in a cloth support was patented in 1928 by Duclaux, who has also impregnated cloth with cellulose acetate, forming a gel suitable for ultrafiltrations with some organic solvents like benzene.

(iii) More rigid supports for the ultrafilter gel are porcelain, alundum or metal. Of this type were the earliest impregnated filters made by Martin (1896)

by filling the pores of unglazed porcelain with gelatin or silicic acid. The semipermeable membranes introduced by Pfeffer (1877), and developed by Berkelay and Hartley (1906) and Morse and Frazer (1914) for their experiments on osmotic pressure measurements were made by depositing copper ferrocyanide in unglazed porcelain.

Ultrafilters of the Bechhold-König type consist of crucibles, evaporating dishes and other vessels with unglazed bottoms, impregnated with acetic collodion. Ultrafilters of this type have been prepared for filtration of non-aqueous solutions. Bechhold and Szidon (1925) studied the gelation of collodion and cellulose acetate in different organic solvents and found the most satisfactory combination to be the one involving impregnation by a solution of collodion in ether, followed by coagulation in toluene. Alundum thimbles have been employed as support for ether-alcohol collodion ultrafiltration. Wire gauze impregnated with collodion forms strong ultrafilters for use under pressure.

Ultrafilters with these rigid supports have the advantage of mechanical convenience and mechanical strength. They are, however, excessively thick, and can remove large quantities of material from filtrates by adsorption. They should be used only for filtration of large volumes of materials, where rigid control of membrane porosity is not required.

(iv) Membranes of very low porosity have been prepared using support of cellophane or collodion. Cellophane, which in itself acts as a self-supporting ultrafilter, can be given still smaller porosity by depositing on it a film of cellulose or collodion (McBain and Kistler, 1928). By filtering through cellophane a solution of cellulose in Schweitzer's reagent, (solution of cupric hydroxide in ammonia), or of ether-alcohol collodion, membranes are obtained which, in filtration of an aqueous solution of sucrose, retain sugar in varying degrees and behave as molecular sieves. Collodion films impregnated with copper ferrocyanide constitute ultrafilters of very low porosity.

17.3.2.2 Non-gelatinous Membranes

The following examples illustrate ultrafilter membranes which do not have a gelatinous structure.

Blanc obtained a porous structure of silica by leaching leucite ($KAl\ Si_2\ O_6$) with strong acid. Ultrafilters of sintered glass have been reported which have a pore diameter of about 1.5μ. Zeolite crystals have been suggested as molecular sieves. Manning plated nickel on wire gauze of nickel and bronze to get pore size of 50 mμ to 300 mμ.

17.3.3 Applications

Ultrafiltration processes are used to recover or concentrate a particular species in the retentate, as is exemplified by latex concentration, or to obtain a purified permeate as in sewage treatment. Ultrafiltration is a means of cold sterilization for many heat-sensitive liquids whose sterilization could not be carried out earlier. Thus, in the food industry draft beer provides an interesting example of a product whose feasibility as a commercial beverage has been an outcome of ultrafiltration; cold sterilization by ultrafiltration retains the esters on account of which the beer has its 'draft' flavour. Similarly, ultrafiltration plays a key role in the pharmaceutical industry for the commercial-scale preparation of sterile water and antibiotics.

17.4 Dialysis compared with other Membrane-separation Methods

In all these membrane-separation methods the distinguishing feature responsible for effecting the desired separation is a membrane which has selective permeability.

Amongst the membrane-separation methods dialysis is old enough and standardized equipment for this process has long been available. The concentration gradient existing across the membrane is the internally available driving force in the system which is responsible for the phenomenon of dialysis.

The most recent membrane-separation methods competitive with dialysis are essentially ultrafiltration for separation and concentration of solutes, reverse osmosis for solvent purification and electrodialysis for the separation of charged species from solvents and other solutes or from each other according to charge and mobility. Standardization of membranes and equipment for these newer methods also has more or less been accomplished during the past several decades. In all these methods it is an external driving force or source of energy which is essentially responsible for effecting the desired separation.

The newer membrane-separation techniques have two distinct advantages. They not only achieve separations quickly but also give sharper separations, because each of these techniques can make use of membranes with pores of uniform size and any desired diameter. Because of these advantages in the use of the new methods these are increasingly gainging in importance. However, in the use of each of these active processes there is the lurking probability of the development of undesired precipitation or chemical change at some points in the system consequent to the development of high solute concentrations or changes in solution composition such as pH shifts. In this regard dialysis has superiority over its competitors and dialysis has to be the chosen method where gentleness of processing is of supreme importance as in haemodialysis and in the recovery and prufification of materials in the food, biological and pharmaceutical fields.

■

18

Centrifugation

Separations by centrifugation techniques are based upon the behaviour of particles in an applied centrifugal field. Particles that can be subjected to separation by centrifugation may, of course, mean entities belonging to the realm of 'matter in bulk' as, for example, a precipitate. However, in the present context, particles may as well mean subdivisions of matter as small as biochemical macromolecules.

18.1 Working Principle

A particle, whether it is a macromolecule, a precipitate or a cell organelle, is subjected to a centrifugal force when it is rotated at a high speed, the latter being commonly expressed in terms of revolutions per minute. The particles are normally present in a specified liquid medium held in tubes or bottles, which are loacted in the rotor of a centrifuge. The rotor is positioned centrally on the drive shaft of the centrifuge. Particles which differ in density, shape or size can be separated since they sediment at different rates in the centrifugal field.

The rate of sedimentation of particles is dependent upon the applied centrifugal field (G). The field, which is directed radially outwards, is determined by the square of angular velocity of the rotor (ω, in radians per second) and the radial distance (r, in cm) of the particle from the axis of rotation, according to the equation :

$$G = \omega^2 r \qquad \qquad ...(18.1)$$

Since one revolution of rotor is equal to 2π radians, its angular velocity, in radians per second, can readily be expressed in terms of revolutions per minute (rev min^{-1}) :

$$\omega = \frac{2\pi \, \text{rev min}^{-1}}{60} \qquad \qquad ...(18.2)$$

The centrifugal field (G) in terms of rev min^{-1} is then :

$$G = \frac{4\pi^2 \left(\text{rev min}^{-1} \right)^2 r}{3600} \qquad \qquad ...(18.3)$$

The centrifugal field is generally expressed as a multiple of the earth's gravitational field ($g = 980$ cm sec^{-2}) i.e. the ratio of the weight of the particle in the centrifugal field to the weight of the same particle when acted on by gravity alone, and is then referred to as the relative centrifugal field (RCF) or more commonly as the 'number times g'.

$$\text{Hence, RCF} = \frac{4\pi^2\left(\text{rev min}^{-1}\right)^2 r}{3600 \times 980} \qquad ...(18.4)$$

which may be shortened to give:

$$\text{RCF} = (1.119 \times 10^{-5})\ (\text{rev min}^{-1})^2\ r \qquad ...(18.5)$$

When conditions for the centrifugal separation of particles are reported, rotor speed, radial dimensions and time of operation of the rotor must, therefore, all be quoted.

Since experiments are usually conducted with particles being present in liquid medium, the rate of sedimentation of a particle is dependent not only upon the applied centrifugal field but also upon the density and size of the particle, the density and viscosity of the medium in which it is sedimenting and the extent to which its shape deviates from that of a sphere. When a particle sediments it must displace some of the liquid in which it is present, and this results in an upthrust on the particle equal to the weight of the liquid displaced. If a particle is assumed to be spherical and of known volume and density, then the net force (F) it experiences when centrifuged at an angular velocity of ω radians per second is given by:

$$F = \frac{4}{3}\pi r_p^3\ (\rho_p - \rho_m)\omega^2 r \qquad ...(18.6)$$

where $\dfrac{4}{3}\pi r_p^3$ = volume of a sphere of radius r_p,

$\quad\quad\quad\quad \rho_p$ = density of the particle,

$\quad\quad\quad\quad \rho_m$ = density of the medium,

and $\quad\quad\quad r$ = distance of the particle from the centre of rotation.

Particles, however, generate friction as they migrate through liquid. If a particle is spherical and moving at a known velocity, the frictional force opposing motion is given by Stokes' law:

$$f_o = 6\pi\eta r_p v \qquad ...(18.7)$$

where $\quad\quad\quad f_o$ = frictional coefficient for a spherical particle,

$\quad\quad\quad\quad \eta$ = viscosity coefficient of the medium,

and $\quad\quad\quad\quad v \quad = \quad$ velocity or sedimentation rate of the particle.

A particle of known volume and density and present in a medium of constant density will, therefore, accelerate in a centrifugal field until the net force on the particle equals the force resisting its motion through the medium i.e.

$$F = f_o \quad\quad ...(18.8)$$

or $$\frac{4}{3}\pi r_p^3 \ (\rho_p - \rho_m)\omega^2 r = 6\pi\eta r_p v \quad\quad ...(18.9)$$

In practice the balancing of these forces occurs quickly with the result that the particle sediments at a constant rate. Its rate of sedimentation (v) is then

$$v = \frac{dr}{dt} = \frac{2 r_p^2}{9} \frac{\left(\rho_\rho - \rho_m\right)\omega^2 r}{\eta} \quad\quad ...(18.10)$$

It can be seen from equation (18.10) that the sedimentation rate of a given particle is proportional to its size and to the difference in density values of the particle and the medium. It will be zero when the density of the particle and that of the medium are equal; it will decrease when the viscosity of the medium increases, and increase as the force of the centrifugal field increases. However, since the equation involves the square of the particle radius, it is apparent that the size of the particle has the greatest influence upon its sedimentation rate. Particles of similar density but only slightly different in size can, therefore, have large differences in their sedimentation rates. Integration of Equation 18.10 yields Equation 18.11, which gives the sedimentation time for a spherical particle in a centrifugal field as a function of the various variables and in relation to the distance of travel of the particle in the centrifuge tube.

$$t = \frac{9}{2} \frac{\eta}{\omega^2 r_p^2 \left(\rho_\rho - \rho_m\right)} \ln \frac{r_b}{r_t} \quad\quad ...(18.11)$$

where $\quad t \quad = \quad$ sedimentation time in seconds,

$\quad\quad r_t \quad = \quad$ radial distance from the axis of rotation to liquid meniscus,

$\quad\quad r_b \quad = \quad$ radial distance from the axis of rotation to bottom of tube.

It is thus clear that a mixture of heterogeneous, approximetely spherical particles can be separated by centrifugation on the basis of their densities and/or their size, either by the time required for their complete sedimentation or by the extent of their sedimentation after a given time. These alternatives form the basis for the separation of biological macromolecules and cell organelles from tissue homogenates. The order of separation of the major cell components is generally whole cells and cell debris first, followed by nuclei, choloroplasts, mitochondria,

lysomes (or other micro bodies), microsomes (fragments of smooth and rough endoplasmic raticulum) and ribosomes.

Considerable discrepancies exist between the theory and practice of centrifugation. Complex variables not accounted for in Equations 18.10 and 18.11, such as concentration of the suspension, nature of the medium, and characteristics of the centrifuge, will affect the sedimentation properties of a mixed population of particles. Moreover, the frictional coefficient, f, in the case of an asymmetrical molecule (e.g. a protein such as myocin) can be several times the frictional coefficient (i.e. f_o) of a sphere. This results in particles sedimenting at a slower rate. Equation 18.10 can, therefore, be modified to give Equation 18.12:

$$\nu = \frac{dr}{dt} = \frac{2 r_p^2}{9} \frac{\left(\rho_p - \rho_m\right)\omega^2 r}{\eta\left(f/f_o\right)} \qquad \text{...(18.12)}$$

which takes into account the effect of varying size and shape on the sedimentation rate of a particle. The frictional ratio, f/f_o, is approximately 1 for spherical molecules, larger values being observed for non-spherical molecules. Hence particles of a given mass, but different shape, sediment at different rates. This point is exploited in the study of conformation of molecules by analytical ultracentrifugation.

Though it is convenient to consider the sedimentation of particles in a uniform centrifugal field, this is not attainable whilst a preparative rotor is being actually operated. Due to the nature of rotor design the effective sedimentation of a given particle will change according to its position in the same contrainer and will vary between r_{min} and r_{max} of Figure 18.1, for the case of a fixed angle rotor taken as an example. Since the centrifugal field generated is proportional to $\omega^2 r$, a particle will experience a greater field the further away it is from the axis of rotation. It is evident from figure 18.1 that there will be a marked difference in the centrifugal fields operative at the top and bottom of the centrifuge tube. Consequently, the sedimentation rate of particles at the bottom of the tube will be markedly higher than that of identical particles
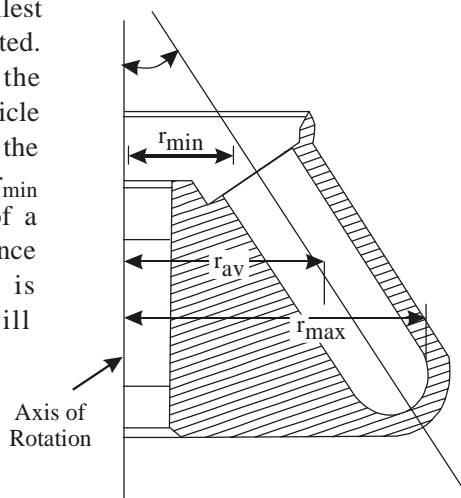
Figure 18.1 Diagram illustrating the variation of RCF with r, the distance of the sedimenting particle from the axis of rotation.

near the top of the tube. As a result, particles will tend to move faster as they sediment through a non-viscuous medium. It is normal, therefore, to record the relative centrifugal field calculated from the average radius of rotation (r_{av}) of the column of liquid in the tube (i.e. the distance from the centre of rotation to the middle of the liquid column in the centrifuge tube). The average relative centrifugal field (RCF_{av}) is therefore the numerical average of the values exerted at r_{min} and r_{max}. If the sample container is only partially filled, then, in the case of fixed angle and swinging bucket rotors, the minimum radius (r_{min}) is effectively increased and so the particles will start to sediment in a higher gravitational field and have a reduced path length to travel. Consequently, sedimentation will be quicker.

The sedimentation rate or velocity (v) of a particle can be expressed in terms of its sedimentation rate per unit of centrifugal field, commonly referred to as its sedimentation coefficient, s, that is,

$$s = \frac{\nu}{\omega^2 r} \qquad \qquad ... (18.13)$$

From Equation 18.12 it can be seen that, if the composition of the suspending medium is defined, then the sedimentation rate is proportional to the centrifugal field, and Equation 18.12 simplifies to:

$$s = \frac{dr}{dt} . \frac{1}{\omega^2 r} \qquad \qquad ... (18.14)$$

Since sedimentation rate studies may be performed using a wide variety of solvent-solute systems, the measured value of the sedimentation coefficient which is affected by temperature, solution viscosity and density, is often corrected to a value that would be obtained in a medium with a density and viscosity of water at 20°C, and expressed as the standard sedimentation coefficient or s_{20}, ω. For many macromolecules including nucleic acids and proteins the sedimentation coefficient usually decreases in value with increase in the concentration of solute, this effect becoming more severe with increase both in molecular weight and the degree of extension of the molecule. Hence s_{20}, ω is usually measured at several concentrations and extrapolated to infinite dilution to obtain a value of s_{20}, ω at zero concentration, s_{20}°, ω. The sedimentation coefficients of most biological particles are very small, and for convenience its basic unit is taken as 10^{-13} seconds which is termed as one Svedberg unit (S), in recognition of Svedberg's pioneering work in this type of analysis. Therefore, a ribosomal RNA molecule possessing a sedimentation coefficient of 5×10^{-13} seconds is said to have a value of 5S. Sedimentation coefficients of some of the entities of interest to the biochemist are as given below against each:

Enzymes, peptide hormones and soluble proteins	2 to 25 S
Nucleic acids	3 to 100 S
Ribosomes and polysomes	20 to 200 S
Viruses	40 to 1000 S
Lyosomes	4000 S
Membranes	100 to 100×10^3 S
Mitochondria	20×10^3S to 70×10^3S
Nuclei	4000×10^3S to $40,000 \times 10^3$S

18.2 Centrifuges

The basic centrifuge consists of two components:

  (i) An electric motor with drive shaft to spin the sample.

 (ii) A rotor to hold tubes or other containers of the sample.

The centrifuges are available in a wide variety and may be classified into three major groups: (1) Bench or clinical centrifuges, (2) high-speed refrigerated centrifuges and (3) ultracentrifuges.

Bench centrifuges: Most laboratories have a standard low-speed bench centrifuge used for routine separation of relatively heavy particles. The common centrifuge has a maximum speed in the range of 4000 to 5000 rev min^{-1}, with RCF values up to 3000 x g. These instruments usually operate at room temperature with no means for temperature control of the samples. Two types of rotors, *fixed angle* and *swinging bucket*, may be used in the instrument. Centrifuge tubes or bottles that contain 12 to 50 ml of sample are commonly used.

High-speed centrifuges: For more sensitive biochemical separations, higher speeds and temperature control of the rotor chamber are essential. Refrigerated high-speed centrifuges are capable of speeds up to 25,000 rev min^{-1}. The operator of this instrument can carefully control speed and temperature. Temperature control is especially important for carrying out reproducible centrifugations of temperature-sensitivie biological samples. Rotor chambers in most instruments are maintained at or near 4ºC. Both fixed-angle and swinging-bucket rotors may be used; however, the former are more common. Rotors used are capable of generating RCF of 20,000 to 30,000 x g. Centrifuge manuals normally provide details of the maximum permitted speed for a rotor, maximum relative centrifugal fields generated and graphs which enable the ready conversion of RCF to rev min^{-1} at r_{min}, r_{av} and r_{max}.

Ultracentrifuges: The most sophisticated of the centrifuges are the ultracentrifuges. These instruments are capable of working at speed of upto 75,000 rev min^{-1} with RCF values up to 500,000 x g. Because these high speeds will generate intense heat in the rotor, the chamber is refrigerated and placed under a high vacuum to reduce friction.

The sample in a cell or tube is placed in an aluminium or titanium rotor, which is then driven by an electric motor. Although it is relatively uncommon, these rotors, when placed under high stress, sometimes break into fragments. The rotor chamber on all ultracentrifuges is covered with protective steel armour plate. The drive shaft of the ultracentrifuge is constructed of a flexible material to accomodate any 'wobble' of the rotor due to imbalance of the sample. It is still important to counterbalance samples as carefully as possible.

The two centrifuges discussed earlier—the benchtop and high-speed types—are of value for preparative work, that is for the isolation and separation of precipitates and biological samples. Ultracentrifuges can be used both for preparative work and for analytical measurements. Thus, two types of ultracentrifuges are available, *preparative models* primarily used for separation and purification of samples for further analysis, and *analytical models*, which are designed for performing physical measurements on the sample during sedimentation and for this purpose they are equipped with optical systems to monitor directly the sedimentation of the sample during centrifugation.

18.3 Techniques of Separation by Centrifugation

18.3.1 Velocity sedimentation centrifugation

This technique separates relatively heavy precipitates in low-speed benchtop centrifuges and is used most often for preparative scale separations. The technique is quite straightforward and consists in spinning the sample for a fixed period, the sample being contained in a tube or a bottle which has been inserted in the rotor of the centrifuge. After the spinning period is over, the sample tube is removed and the resulting two phases, pellet and supernatant (which should be readily apparent in the tube), may be separated by careful decantation. Further characterization or analysis is usually carried out on the individual phases.

18.3.2 Differential centrifugation

The specific method of separation, called differential centrifugation, consists of centrifugation in stages, each successive stage being carried out at an increased successive rotor speed. The material to be separated (e.g. a tissue homogenate) is thus divided into a number of fractions. The rotor speed in each successive stage is chosen and centrifugation for a predetermined period of time at that rotor speed yields a supernatant containing unsedimented material and a pellet of a particular set of particles which sedimented through the solution during that time period. Any type of particle originally present in the homogenate may be found in the pellet or the supernatant or both fractions depending upon the time and speed of centrifugation and the size and density of the particles. At the end of each centrifugation stage, the pellet is separated from the supernatant.

The separation achieved by differential centrifugation may be improved by repeated (2 to 3 times) resuspension of the pellet in homogenisation medium and recentrifugation under the same conditions as were used to get the pellet originally, but each successive recentrifugation will inevitably reduce the yield obtained. To appreciate why, however, the pellet is never absolutely pure (homogeneous), it is necessary to consider the conditions prevailing in the centrifuge tube at the beginning of each stage.

Initially all particles of the homogenate are homogeneously distributed throughout the centrifuge tube [Figure 18.2 (a)]. During centrifugation particles move down the tube at their respective sedimentation rates [Figure 18.2 (b) to (d)], and start to form a pellet at the bottom of the tube. Ideally, centrifugation is continued long enough to pellet all the
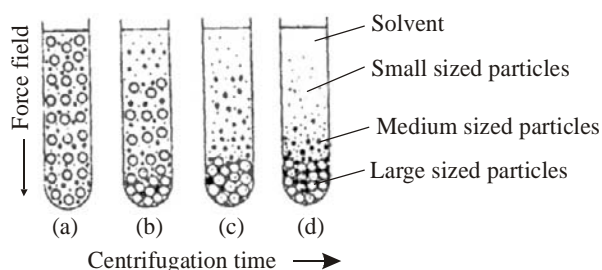
Figure 18.2 Differential sedimentation of particulate suspension in a centrifugal field. (a) Particles uniformly distributed throughout the centrifuge tube; (b) to (d) sedimentation of particles during centrifugation dependent upon their size and shape.

largest class of particles. However, since particles of varying sizes and densities were distributed homogeneously throughout the centrifuge tube at the commencement of centrifugation, it is evident that the pellet will not be homogeneous on account of the fact that some of the lighter and medium sized particles originally suspended near the bottom of the tube, will also have sedimented during the time required for the complete sedimentation of heavier particles. Pure preparations of the pellet of the heaviest particle cannot, therefore, be obtained in one centrifugation step. It is only the most slowly sedimenting component of the mixture that remains in the supernatant liquid, after all the larger particles have been sedimented, which can be purified by a single centrifugal step but its yield is often very low.

After each centrifugation run, the supernatant is poured into another centrifuge tube, which is then rotated at the next higher speed. Such further centrifugations of the supernatant in successive gradually
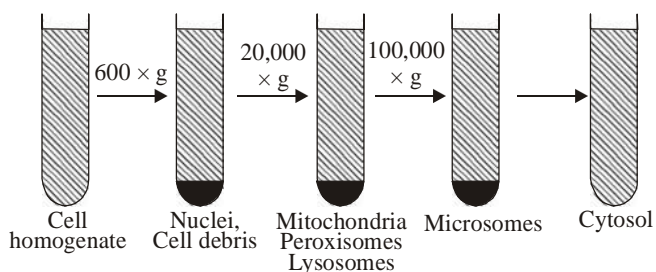
Figure 18.3 Differential centrifugation of a cell homogenate.

increased centrifugal fields result in the sedimentation of the intermediate and finally the smallest and least dense particles.

Figure 18.3 illustrates the differential centrifugation of a cell homogenate leading to the separation and isolation of the common cell organelles. For most biochemical applications, the rotor chamber must be kept at low temperatures to maintain the native structure and function of each cellular organelle and its component biomolecules. A high-speed centrifuge equipped with a fixed-angle rotor is most appropriate for the first two centrifugations at $600 \times g$ and $20,000 \times g$. The final centrifugation at $100,000 \times g$ to sediment microsomes and ribosomes must be done in an ultracentrifuge. The $100,000 \times g$ supernatant, *cytosol*, is the soluble portion of the cell and consists of soluble proteins and smaller molecules.

18.3.3 Density gradient centrifugation

This technique permits the separation of components of multicomponent mixtures of macromolecules. The initial concentration of the sample is the same throughout the length of the centrifuge tube in differential centrifugation, and clearcut separations of macromolecules are seldom obtained when differential centrifugation is applied to samples with more than one component, since large particles that sediment faster pass through a medium consisting of solvent and particles of smaller size. This is avoided in density gradient centrifugation in which the sample is centrifugated in a fluid medium that gradually increases in density from top to bottom. There are two methods of density gradient centrifugation, the *rate zonal technique* and *isopycnic technique* and either of the two can be used when a quantitative separation of all the components of a mixture of particles is required.
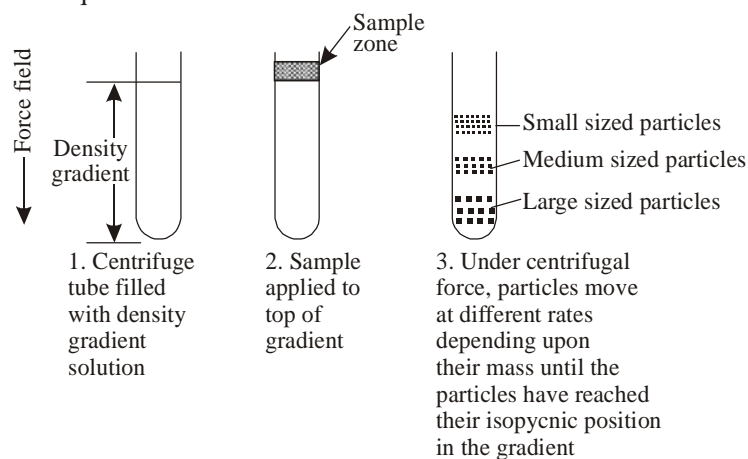
Figure 18.4 Rate zonal centrifugation in a preformed liquid density gradient.

Figure 18.4 outlines the procedure for *rate zonal centrifugation* of mixture of macromolecules. A liquid density gradient is prepared in a tube prior to centrifugation such that the highest density of the gradient does not exceed that of the densest particles to be separated. This is accomplished with the use of an automatic gradient mixer. Solutions of solutes of low molecular weight, such as sucrose or glycerol, are allowed to flow into the centrifuge tube. The sample is layered on top of the gradient and placed in a swinging bucket rotor. Sedimentation in an ultracentrifuge results in movement of the sample particles at a rate dependent on their individual s values. As shown in Figure 18.4, the various types of particles sediment as zones and remain separated from the other components. The centrifuge run is terminated before any of the separated zones pellet at the bottom of the tube. The various zones in each of the centrifuged tubes are then isolated and analyzed for the presence of macromolecules. The zones of separated macromolecules are relatively stable in the gradient because it slows diffusion and convection. The gradient conditions can be varied by using different ranges of sucrose concentration. Sucrose concentrations up to 60 per cent can be used, with a density limit of 1.28g/cm^3.

The zonal method can be applied to the separation and isolation of macromolecules (preparative ultracentrifugation) and to the determination of s (analytical ultracentrifugation).

The method has been used for the separation of enzymes, hormones, RNA–DNA hybrids, ribosomal subunits, subcellular organelles, for the analysis of distribution of samples of polysomes and for lipoprotein fractionation.

Isopycnic centrifugation depends solely upon the buoyant density of the particle and not its shape or size and is independent of time. Hence soluble proteins, which have a very similar density (e.g. $\rho = 1.3 \text{ g cm}^{-3}$ in sucrose solution) cannot be usually separated by this method, whereas subcellular organelles (e.g. mitochondria, $\rho = 1.19 \text{ g cm}^{-3}$, and peroxisomes, $\rho = 1.23 \text{ g cm}^{-3}$, in sucrose solution) can be effectively separated.

The sample is layered on top of a continuous density gradient which spans the whole range of the particle densities which are to be separated. The maximum density of the gradient, therefore, must always exceed the density of the most dense particle. During centrifugation, sedimentation of the particles occurs until the buoyant density of the particle and the density of the gradient are equal. At this point no further sedimentation occurs, irrespective of now long centrifugation continues, because the particles are floating on a cushion of material that has a density greater than their own. Isopycnic centrifugation, in contrast to the rate zonal technique, is an equilibrium method, the particles banding to form zones, each at their own characteristic buoyant density. In cases where, perhaps, not all the components in a mixture of particles are required, a gradient range can be selected in which unwanted components of the mixture will sediment to the bottom of the centrifuge tube whilst the particles of interest sediment to their respective

isopycnic (equal density) positions. Such a technique involes a combination of both the rate zonal and isopycnic approaches.

As an alternative to the use of a preformed density gradient, the density gradient may be created during the centrifugation. In this method (referred to as the *equilibrium isodensity method*) use is generally made of the salts of heavey metals (e.g. caesium or rubidium), sucrose, colloidal silica or Metrizamide. The sample (e.g. DNA) is mixed homogeneously with, for example, a concentrated

—Small sized particles
—Medium sized particles
—Large sized particles

(a) Uniform mixture of sample and gradient

(b) Under centrifugal force, gradient redistributes and sample particles band at their isopycnic positions

Force field
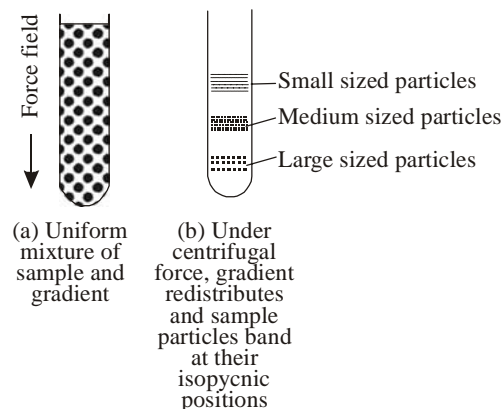
Figure 18.5 Isopycnic centrifugation; the density gradient forms during centrifugation.

solution of caesium chloride [Figure 18.5 (a)]. Centrifugation of the concentrated caesium chloride solution results in the sedimentation of the CsCl molecules to form a concentration gradient and hence a density gradient. The sample molecules (DNA), which are initially uniformly distributed throughout the tube now either rise or sediment until they reach a region where the solution density is equal to their own buoyant density i.e. their isopycnic position, where they will band to form zones [Figure 18.5 (b)]. This technique suffers from the disadvantage that often very long centrifugation times (e.g. 36 to 48 hours) are required to establish equilibrium. However, it is commonly used in analytical centrifugation to determine the buoyant density of a particle, the base composition of double stranded DNA and to separate linear from circular forms of DNA. Many of the separations can be improved by increasing the density differences between the different forms of DNA by the incorporation of heavey isotopes (e.g. ^{15}N) during biosynthesis, a technique used by Meselson and Stahl to elucidate the mechanism of DNA replication in *Escherichia coli* or by the binding of heavy metal ions or dyes such as ethidum bromide. Isopycnic gradients have also been used to separate and purify viruses and analyze human plasma lipoproteins.

Recovery and Monitoring of Gradients from Centrifuge Tubes

After particle separation has been achieved, it is necessary to remove the gradient solution in order to isolate the bands of separated material. Removal of gradients from centrifuge tubes can be achieved by a number of techniques. If the bands can be visually detected, recovery can be achieved using a hypodermic needle or syringe. A common method, however, is that of *displacement*. A dense

liquid, for example 60 to 70% (w/w) sucrose solution, is pumped to the bottom of the centrifuge tube through a long needle. The gradient is displaced upwards and the fractions removed in sequence using a syringe or pipette, or by being channelled out through a special cap to which is attached a collection pipe either leading to a fraction collector or directly into a flow cell of an ultraviolet spectrometer.

Alternatively, the centrifuge tube may be punctured at its base using a fine hollow needle. As the drops of gradient pass from the tube through the needle they may be collected using a fraction collector and further analyzed. Analysis of the contents of the displaced gradient can be achieved by ultraviolet spectrophotometry, refractive index measurements, scintillation counting or chemical analysis.

Nature of Gradient Materials and Their Use

There is no ideal all-purpose gradient material and the choice of the material depends upon the nature of the particles to be fractionated. The gradient material should permit the desired type of separation, be stable in solution, inert towards biological materials, should not absorb light at wavelengths appropriate for spectrophotometric monitoring (visible or ultraviolet region), or otherwise interfere with assaying procedure, be sterilisable, non-toxic and non-inflammable, have negligible osmotic pressure and cause minimum changes in ionic strength, pH and viscosity, be inexpensive and readily available in pure form and capable of forming a solution covering the density range needed for a particular application without overstressing the rotor.

Gradient-forming materials which provide the densities required for the separation of subcellular particles include salts of alkali metals (e.g. caesium and rubidium chloride), small neutral hydrophilic organic molecules (e.g. sucrose), hydrophilic macromolecules (e.g. proteins and polysaccharides), and a number of miscellaneous compounds more recently introduced and not included in the above group, such as colloidal silica (e.g. Percoll) and non-ionic iodinated aromatic compounds (e.g. Metrizamide, Nycodenz and Renograffin).

Sucrose solution whilst suffering from the disadvantages of being very viscuous at densities greater than 1.1 to 1.2 g cm^{-3} and exerting very high osmotic effects even at very low concentrations (i.e. at approximately 10% w/ν concentration) has been found to be the most convenient gradient material for rate zonal separation. Ficoll (a copolymer of sucrose and epichlorhydrin) has been successfully used instead of sucrose for the separation of whole cells and subcellular organelles by rate zonal and isopycnic centrifugation, but whilst being relatively inert osmotically at low concentrations, both osmolarity and viscosity rise sharply at higher concentrations (i.e. above 2% w/ν). Caesium and rubidium salts have been

most frequently used for isopycnic separation of high density solutes such as nucleic acids.

18.3.4 Centrifugal elutriation

In this technique the separation and purification of a large variety of cells from different tissues and species can be achieved by a gentle washing action using an elutriator rotor. The technique is based upon differences in the equilibrium, set up in the separation chamber of the rotor, between the opposing centripetal liquid flow and applied centrifugal field being used to separate particles mainly on the basis of differences in their size. The technique does not employ a density gradient. Any medium in which cells sediment can be used, and since pelleting of the particles does not occur, fractionation of delicate cells or particles of 5 to 50 mμ diameter can be achieved. Separations can be achieved very quickly giving high cell concentrations and a very good recovery with minimum damage to the cells so that they retain their viability.

■

19

Miscellaneous Methods

This chapter deals with a number of separation and purification methods so far remaining uncovered; each one of these has different underlying basis for its functioning and belongs to the category of methods being discussed under Section IV of this book. Separation and purification techniques discussed in this chapter are: Zone Refining, Fractional Melting, Reversible Adsorption, Inclusion-compound Formation, Foam Separation, Fractional Diffusion, Thermal Diffusion, Electro-magnetic Separation, Fractional Electrolysis and Electrorefining.

19.1 Zone Refining

Traditionally the purification of a crude solid is carried out by fractional crystallisation and satisfactory results can very often be obtained by this method. However, effecting purification by fractional crystallisation is a tedious and time-consuming process; also, serious reduction of yields of the desired products may occur. Should it be necessary also to isolate or concentrate the impurities of the crude for their identification, crystallisation has the disadvantage that these substances end up in a solvent in which they are very soluble, and from which their isolation is difficult. For these reasons an alternative to fractional crystallisation must sometimes be sought. Zone refining is one alternative and chromatography is another. Zone refining has the advantage that it can handle large quantities whereas chromatographic techniques, with the possible exception of gas-liquid chromatography of fairly volatile samples, cannot handle large amounts.

Zone fefining is a thermal technique that is used for preparing ultrapure metals and compounds and it was originally developed in 1952 by Pfann for the purification of materials like germanium used as semi-conductors which need to possess purity of an extremely high order. By 1956 applications of this purification technique to Organic Chemistry were being made by Herington, Handley and Cook. Since the publication of the work of Beynon and Saunders in 1960, the use of this technique in the field of Organic Chemistry has been steadily increasing. Provided that a solid satisfies the basic requirement that it is extremely stable at its melting point, zone refining is most convenient technique for preparing a very pure sample of the compound. In fact, the method is used for obtaining ultrapure samples of compounds for use as melting point, microanalytical, mass, ultraviolet and infrared standards, where purities greater than 99.9 per cent are required. Zone

refining is usually of particular value and most effective when applied to samples that are already of 99 per cent or grater purity. The starting material for zone refining has, therefore, often undergone preliminary purification by gas-liquid chromatography, fractional distillation or some other chemical separation method.

The principle involved in zone refining is the utilization of the difference in solubility of an impurity in the liquid and in the solid main component when an equilibrium is reached at the interface of a cooling solid in contact with its melt —the impurities will remain in the melt and the major component will freeze out in a purer state.

The impure sample is packed into a column. This is done by pouring it in the molten state. The column is set vertically. A zone refiner, which is a ring-shaped heater, is placed at the top and it is moved by a mechanism that allows it to travel down the tube very slowly. Depending on the rate of crystallisation the speed of traverse will be of the order of one inch per hour. A small molten zone forms and travels slowly along the column of solid (Figure 19.1). Melting occurs in front of the zone, solidification from an air blast at its rear, impurities usually falling. If the reverse is the case, the melting must be started at the bottom. What occurs is a continuous recrystallisation. The heater may be returned to the starting point and the process repeated as many times as necessary.
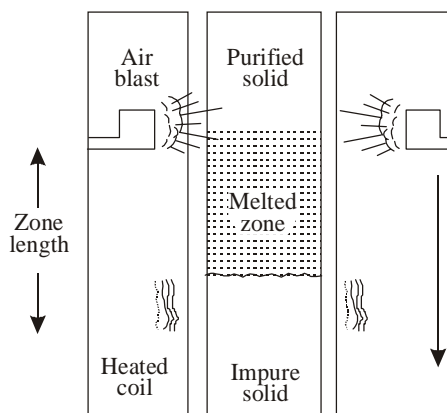
Figure 19.1 Schematic of a zone refiner.

Although the separations by zone refining are not rapid, the cost in man-hours is small because the process requires little attention since automation of the apparatus is easily achieved. A valuable feature of the technique is that relatively simple and cheap apparatus is used. No reagents are added and hence contamination of the sample from such an addition does not arise. The method has sufficiently wide applicability in that it can be used for nearly any organic or inorganic substance whose melting point is in the range or 50°-300°C.

Amongst the disadvantages of this purification technique is the occurrence of some secondary processes such as volatilization or oxidation of particular impurities, which may influence the course of the concentration process. Also, the fact that values of partition coefficient between the solid and liquid phases for various elements and compounds will differ, detracts from facility with which purification can be effected by zone refining.

19.2 Fractional Melting

Isolation of the major component in the pure state from a mixture can be carried out by fractional crystallisation, if only small amounts of that component need to be crystallised out from a system involving liquid-solid equilibrium in which the contaminant tends to concentrate in the liquid phase. The reverse process of fractional melting, however, can sometimes be used to achieve the same objective with greater advantage. Purification by fractional melting requires less effort than that required by repeated crystallisation and it gives the purified product in comparatively high yields also.

Fractional melting is based on the principle that at any temperature a contaminant is concentrated in the liquid phase, if its addition depresses the freezing point. One specific example will suffice to illustrate how fractional melting makes use of this underlying principle to bring about purification. The example under reference is the isolation of pure isooctane (2, 2, 4-trimethyl pentane) from its mixture with the eutectogenic contaminant, n-pentane. This mixture constitutes a system which involves liquid-solid equilibrium in which the impurity tends to concentrate in the liquid phase.

A eutectic is an intimate mixture of very small crystals of each of its components, all of which have crystallised together. The components representing the eutectic composition are completely miscible with one another in the liquid state; this liquid solution can undergo a reversible transformation at a constant pressure and at a constant temperature (known as the eutectic temperature) to yield simultaneously solid components as the microstructure that constitutes the eutectic. The progress of isolation of the major component, isooctane, in the pure state from the eutectic mixture under consideration (i.e. isooctane—n-heptane), carried out by fractional melting under equilibrium conditions throughout, is shown below:

Equilibrium temperature (^{o}K)	161.05	163.36	164.15	165.48	165.71
Per cent purity	94.4	99.1	99.40	99.91	100.00
Per cent yield	100	94.4	81.6	77	69.6

Measurement of temperature enables one to keep track of the progress of purification, since the liquid fraction is separated from the solid fraction.

Factors such as chemical instability of the melt, high viscosity of the melt and difficulty of revoval of mother liquor limit the applicability of fractional melting as a purification method, but this technique can be used with effectiveness when purification is to be carried out from solid-liquid systems involving non-viscous and chemically stable melts. Besides isooctane, other hydrocarbons like cyclohexane, n-heptane and cis-2-butene have been freed from eutectogenic constaminants by fractional melting.

Liquation is another process which also involves formation of a melt of a fraction of the starting impure material and can occasionally be successfully employed for effecting purifications. But here the melt represents the liquid form

of the desired substance, in a more or less pure state, and this melt is not a liquid mixture representing the composition of a eutectic at the temperature employed as is the case in fractional melting discussed above. Liquation is used to carry out the purification of metals like bismuth, tin and lead whose melting points are lower than those of the concerned impurities. The impure metal is placed on the sloping hearth of a reverberatory furnace which is then heated. At a temperature slightly higher than the melting point of the metal, the latter flows down the sloping hearth leaving the impurities behind on the hearth.

19.3 Separation of Gases through Reversible Adsorption on a Solid

This adsorption occurs at low temperatures and is due to van der Waals forces of attraction.

Application of this method may be exemplified by taking the case of separation of noble gases viz. He, Ne, Ar, Kr and Xx. These gases are obtained in the form of the residual mixture of these noble gases after the removal (by suitable chemical reactions) of nitrogen, oxygen, carbon dioxide and water vapour from air. The separation of these gases is carried out by the Dewar's charcoal method. This method is based on the fact that coconut charcoal adsorbs different noble gases at different temperatures.

In the Dewar's method, the mixture of noble gases is introduced from a gasholder into a bulb filled with coconut charcoal. This bulb is placed in a cold bath (–100ºC). The mixture of gases is allowed to remain there for about half an hour during which time Ar, Kr and Xe get adsorbed. He and Ne, which remain unadsorbed, are pumped out of the bulb and kept in contact with another lot of charcoal maintained at –180ºC whereby Ne is adsorbed, leaving He in the free state and the latter is pumped out. Ne is recovered by heating the charcoal.

The first lot of charcoal which had adsorbed Ar, Kr and Xe is placed in contact with still a different (i.e. third) lot of charcoal that is cooled to the liquid air temperature. Ar diffuses into this charcoal and heating the latter liberates Ar in the free state.

The temperature of the first charcoal which, by now, would contain the adsorbed Kr and Xe, is next raised to –90ºC whereby Kr is released as free gas and is collected. The remaining noble gas viz. Xe, still left behind adsorbed in the charcoal in the first bulb, is recovered as free gas on heating that charcoal.

19.4 Inclusion-compound Formation

An inclusion-compound is a combination of two molecular species held together by operation of van der Waals forces, one of these species acting as the 'host' in that it provides space in its structure to accomodate molecules of the other one, the 'guest' species. If in a mixture of two substances, A and B, molecules of only A have the proper size and geometry to form an inclusion-compound with a

particular host material, A can be easily isolated from the mixture in a pure state through the formation of its inclusion-compound and subsequent release from it of A. Types of inclusion-compound formed are: (i) Clathrates, (ii) Channel inclusion-compounds, and (ii) Layer inclusion-compounds.

Clathrates: In clathrate type of molecular combinations, guest molecules fit into cavities or 'cages' formed by the host molecules. Clathrates are formed by crystallising the structure from a solution of unassociated host and guest molecules, the solvent used being one whose molecules cannot be trapped by the host. The guest material is recovered on breaking down the host structure of the clathrate by such methods as melting, sublimation or dissolution in a suitable solvent such as alcohol.

Some of the noteworthy examples of separations which have been effected by clathration are:

   (i)   In the separation of noble gases, Ne has been separated from Ar, Kr and Xe, making use of the fact that Ne is the only gas that does not form a clathrate with quinol.

  (ii)   Removal of thiophene from benzene by the formation of the clathrate of benzene and monoammine nickel (II) cyanide.

 (iii)   Using tetra- (4-methyl pyridine) nickel dithiocyanate as the clathrating agent each one of the following mixtures of pairs of isomers had been separated into the individual components.

         (a) Anthracene and phenanthrene,

         (b) Naphthalene and diphenyl,

         (c) p-Xylene from o- or m-xylene.

Resolution of a recemate can be achieved in some cases through clathration to get either the d-isomer or the l-isomer, and thus an optically active compound is obtained by this means without use of an optically active resolving agent.

Tri-o-thymotide,
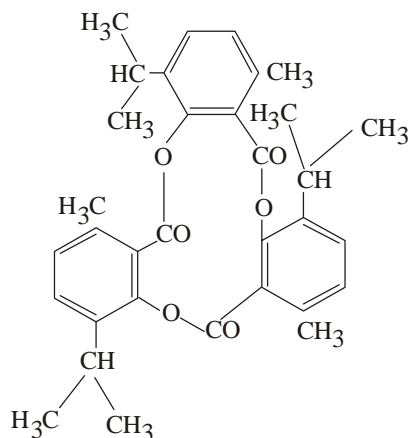
crystallises as a (+) mixture or racemate. By clathration with n-hexane, benzene or chloroform, resolution of the racemate can be achieved to obtain either the l-form or the d-form. Addition of a (+)-seed crystal to a saturated solution of the (+) mixture of tri-o-thymotide in one of those solvents and slow growth of the crystal causes eventual crystallisation of the entire lot as the (+) enantiomer.

Channel Inclusion-compounds: In this type of molecular complex, the host molecules form a *channel* structure and the guest molecules are enclosed in this cylindrical channel. Stable crystalline complexes obtained from the combination of urea with straight-chain alkanes containing seven or more carbon atoms were first reported by M.F. Bengen in 1941. Their structure as determined by X-ray analysis shows that these complexes are inclusion-compounds. Urea molecules spiral in the crystal lattice in a manner which results in the formation of the wall of a hexagonal cylindrical channel. This channel is formed on account of three interpenetrating spirals of urea molecules holding together by hydrogen bonding. This channel accomodates the guest hydrocarbon molecules to complete the crystal lattice. Stabilization of the lattice results from hydrogen bonding between urea molecules and operation of van der Waals forces between urea molecules themselves and between urea and hydrocarbon molecules. The guest component i.e. the hydrocarbon, is not bonded to the host (urea) but merely trapped in the channel. The channel diameter is large enough to accomodate a zig-zag normal alkane molecules but not large enough for a thick branched-chain hydrocarbon to be accomodated in it.

Since a terminal functional group does not appreciably alter the space requirement, urea forms inclusion-compounds with alcohols, aldehydes, carboxylic acids and amines derived from n-C_7 and higher hydrocabons. For molecules with cross section too small, the introduction of functional groups of adequate size leads to inclusion.

The guest hydrocarbon can be recovered from the urea inclusion-compound by shaking it with water which dissolves out the urea; alternatively, treatment with ether can be carried out, in which case the hydrocarbon will go into solution leaving a residue of urea.

Formation of urea complexes can be used for separation of straight-chain from branched-chain hydrocarbons, saturated from unsaturated hydrocarbons and even for separating two hydrocarbons differing just in chain-length.

The urea channel is about 6Å at its widest part and about 5Å at its narrowest. Straight-chain hydrocarbons have a cross section of about 4.1 Å and, as already mentioned, form urea inclusion-compounds readily Hydrocarbons with a single methyl branch require a channel diameter of about 5.5 Å. The urea inclusion-compound of a singly branched hydrocarbon can also form if the straight-chain part of such a hydrocarbon is long enough, but a singly branched hydrocarbon like 3-methyl heptane, in which the straight-chain portion is relatively small, does not

form an inclusion-compound with urea. Compounds with two branches on the some carbon atom require a channel diameter of about 6 Å and are not known to form a urea inclusion-compound. Cyclic compounds, similarly, cannot give uarea inclusion-compounds. Thus it becomes possible that by taking the aid of urea, compounds with a normal chain of carbon atoms (not only hydrocarbons but also a number of their derivatives) can be separated from branched-chain compounds. In actual practice the complex of a liquid hydrocarbon e.g. n-haptane can be prepared by shaking with a suspension of finely powdered urea for several hours. Urea inclusion-compound of a solid hydrocarbon can be prepared by first bringing it into solution in isooctane which does not complex with urea. An alternative procedure uses a saturated solution of urea in methanol into which is stirred the guest material which may by an alkane, alcohol or acid. The urea process finds a commercial application in the pertroleum industry. Improvement in quality of fuels meant for specific uses is brought about by treatment with urea. Straight-chain components of gasoline cause engine knocking and undesired quantities of these are removed by the urea process before marketing gasoline. Straight-chain hydrocarbons have higher freezing points than branched chain hydrocarbons and removal of the former from fuel for jet planes becomes necessary to prevent freezing of the fuel at low temperatures prevailing at high altitudes. Formation of urea complexes by n-alkanes is made use of in the removal of these straight-chain hydrocarbons from fuels meant to be used in jet engines.

At a given length, a saturated hydrocarbon present in a mixture will form urea inclusion-compound preferentially to the mono-unsaturated component, this trend continuing with increasing degree of unsaturation of the components of the mixture. Advantage is taken of this fact for separation of saturated from unsaturated components of a given mixture. If insufficient host material is employed to combine with all the constituents of a mixture, the least unsaturated ones will crystallise out in the form of inclusion-compounds. Isolation of the di-unsaturated *cis*, *cis* dienic acid, linoleic acid, is based upon the finding that it does not form a stable urea inclusion-complex. The acidic fraction obtained on saponification of safflower seed is added to a solution in methanol of an amount of urea sufficient to precipitate the saturated and mono-unsaturated acidic components. Fractionation of the material recovered from the mother liquor yields pure linoleic acid.

In separations based on differences in chain-length, the preferential formation of inclusion-compounds of components having longer chains is exploited. For achieving satisfactory results, the components to be separated should differ in chain-length by at least four carbon atoms.

1-, 2-, 3-Nonyne and 2- and 3-decyne are among the acetylenic hydrocarbons which give urea inclusion-compounds; among those of alkynes which do not yield complexes with urea are 4-nonyne, 1, 8-nonadiyne and 5-decyne.

Formation of urea inclusion-compounds has also been successfully applied to the resolution of mixtures of d-and 1-isomers. The hexagonal spiralling urea crystal lattice can assume either a right-handed or a left-handed form, and when urea crystallises in the presence of a dl-mixture of a hydrocarbon or of a derivative of a hydrocarbon which can complex with urea, the crystallisate will have one inclusion-compound arising from the complexing of right-handed urea with the dextrorotatory component of the starting dl-mixture and another inclusion-compound formed by complexing of left-handed urea with the levorotatory component of the said mixture. These inclusion-compounds which differ in their properties, say solubilities, are isolated from the crystallisate by separation from each other by a process of repeated crystallisation. Each of these purified urea inclusion-compounds is then processed separately, in the manner indicated earlier for the recovery of the d-isomer and the l-isomer of the starting racemate. Amongst the reacemates which have been thus resolved into their respective optically active pure components are: dl-3-Methyloctane, dl-3-methylnonane, dl-2-chlorooctane.

Thiourea also forms inclusion-compounds. The presence of a sulphur atom instead of an oxygen atom in thiourea accounts for an increase in the size of the thriourea molecule and of the crystalline structure as a whole so that the central channel has a diameter of about 6.5Å, Branched-chain hydrocarbons become trapped in the channel to form complexes. Thus 2, 2, 4-trimethyl pentane fits into the channel and yields thiourea inclusion-compound. Normal hydrocarbons do not fill the available space to an extent sufficient for stability and do not form complexes.

Layer Inclusion-compounds: In this type of complex formation, the layer structure of the host is made use of. Certain clays, such as montmorillonite and halloysite, have a sandwich or layer structure that will readily include organic compounds of a polar nature. Alcohols, ethers, nitriles and amines are among the guests included by these clays. The layer width may vary, depending upon the compound included. Ionic attraction and van der Waals forces are involved in this type of inclusion.

19.5 Foam Separation

Foam separation is the method of separating substances from solution by employing a stable foam as the second phase which, during the process of its formation in the system, carries along appreciable quantities of certain types of solutes from the bulk of the solution. As distinct from froth floatation which removes solids in suspension, foam separation is used with true solutions or colloidal solutions.

Foam is a material constituted of gas bubbles separated from one another by films of liquid. Pure liquids do not foam as they cannot produce films of any permanance. The presence of a surface-active substance in the liquid imparts to the

latter the property of foaming. Molecules of a surface-active substance have hydrophobic and hydrophilic segments, the former part directed out of, and the latter part directed into, the liquid phase. The implication of this situation in physical terms is that surface layers of the added substance are produced. The reluctance of the substance so adsorbed at the surface to enter the bulk of the liquid preserves the surface and, hence, the stability of the foam. Since foam is a collection of a very large number of bubbles separated from one another by liquid films, foaming is a practical device of producing and collecting large quantities of gas-liquid interface that is associated with appreciable amounts of the solute desired to be separated from the solution which is under processing.

The set-up of the foam separation equipment is essentially similar to an industrial distillation column and consists of a column on the top of a reservoir containing the liquid to be foamed. Air or another suitable gas is dispersed in the liquid through a device of which fritted glass disc is an example, that breaks the continuous gaseous stream into small bubbles. The liquid is kept stirred by the bubbles during their passage through the bulk of the liquid. Foam thus produced collects in the receiver at the top. Collapsing of the collected foam yields a liquid which is rich enough in the component whose separation from the original solution was desired. If so required, the process can be made a continuous one by steadily introducing the feed solution into the system and withdrawing the foam likewise.

Separation of ionic species which are nonsurface-active can also be achieved by adding a surface-active agent which carries a charge opposite to that of the metal ion to be separated from its aqueous solution. The added surfactant serves both as a foaming agent and an extracting agent in that it withdraws the metal ion from the solution by complexing with the latter and carrying the metal ion along into the foam. Removal of radioactive strontium from nuclear process streams, for example, has been accomplished in this manner by using polyaminopolycarboxylic acids.

19.6 Fractional Diffusion

The rate of diffusion of a gas is inversely propotional to the square root of its vapour density (Graham's law). Diffusion through a porous partition, therefore, affords a method of separating the constituents of a mixture of gases even, say, of isotopic gases.

Theoretically, diffusion should effect the difficult separation of isotopic gases from one another because of the difference in rates of diffusion of the constituent gaseous isotopes. However, the problem faced in such a separation by fractional diffusion is the one that arises from the fact that difference in respective densities of isotopes is generally very slight, so that the method in practice is a very laborious one. It is obvious that if diffusion were allowed to proceed for a long time, the composition of the gas mixture would become identical at both sides of the partition. If, however, only half of the gaseous mixture is allowed to diffuse

through, there would be an appreciable enrichment of the lighter isotope in the diffusate.

The method became a practical success for the first time in 1932 when Hertz separated ordinary neon of mass 20.2 to obtain a lighter fraction containing 99 per cent of ^{20}Ne and a heavier one containing over 70 per cent of ^{22}Ne.

Hertz made use of the principle of fractional diffusion under reduced pressure. Figure 19.2 illustrates the working principle of the apparatus used by Hertz. The
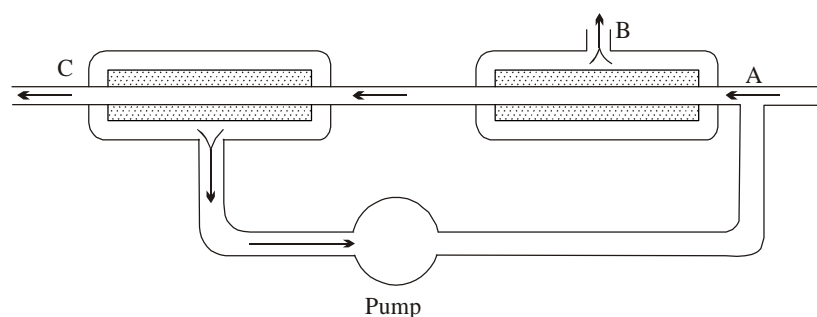
Figure 19.2 Illustration of working principle of Hertz apparatus.

mixture of isotopic gases to be separated enters the diffusion apparatus under reduced pressure at A, and, as it passes through the first diffusion unit, the lighter isotope diffuses out quicker than the heavier one. The first diffusate, containing a higher proportion of the highter isotope, is removed at B. The residual gas passes on through the second diffusion unit, arrangement made being such that the second diffusate has practically the same isotopic composition as the gas entering at A. It is pumped back to the gas stream entering at A. The gas leaving the second diffusion unit at C is made to pass on to a third diffusion unit (not shown in Figure 19.2). The apparatus consists of several such diffusion units connected in series so that from the nth unit the fraction enriched with respect to the lighter isotope is always returned to the (n–1)th unit and the one enriched with respect to the heavier isotope is passed on to the (n+1)th unit with the result that there is progressive enrichment of one isotope at one end of the chain and of the other isotope at the other end. In the example of separation of ordinary neon into ^{20}Ne and ^{22}Ne enriched fractions cited above, Hertz used a 24-unit plant working for 8 hours.

The method has been successfully employed for the separation of isotopes of argon and nitrogen. The Hertz procedure has brought about separation of deuterium (spectroscopically pure) from ordinary hydrogen.

The most noteworthy separation effected by the fractional diffusion method is that of ^{235}U (the fissionable material used in the atom bomb during World War II) from natural uranium, which contains less than 1 per cent of this isotope, the rest being ^{238}U. Volatile UF_6 was used as the diffusion gas. Enrichment as high as 99

per cent with respect to ^{235}U was achieved as against its original 0.72 per cent content in natural uranium. The processing involved as many as 4000 diffusion stages which were carried out in the huge plant constructed for the purpose at Oak Ridge (USA).

19.7 Thermal Diffusion

Thermal diffusion is the phenomenon of subjecting a homogeneous mixture of fluids to a temperature gradient in consequence of which a partial separation of the mixture into its constituents results.

From considerations of kinetic theory S. Chamnan (1916) showed that a termperature gradient imposed on a gaseous mixture would cause a flow of one component relative to the mixture as a whole. This finding was verified experimentally by L. Champman and F.W. Doosten. Thus, when a vessel containing a homogeneous mixture of two gases was heated at one end and cooled at the other, a relative motion of two components was induced in the mixture, one component tending to concentrate in the hotter region, the other in the colder region. A similar effect had been reported earlier for liquids by Ludwig in 1856 and by J.L. Soret in 1879, and is called the Soret effect which depends more upon the shape differences between molecules than upon any other property when the fluid mixture is liquid.

Specific equipment and processes have been developed to bring about the desired enrichment with respect to the individual components of a mixture by thermal diffusion.

In the single-stage cell for liquid-phase thermal diffusion, a stationary fluid mixture is subjected to a vertical temperature gradient, the top of the cell being kept at the high temperature so that thermal convection currents do not set up. It is the lighter component of the mixture which usually, but not always, gets enriched at the top of the thermal diffusion unit. For achieving chrichment of the individual components of a gaseous mixture by partial separation through thermal diffusion, the usual single-stage fit-up comprises two bulbs, one maintained at a much higher temperature than the other, the two bulbs being connected by a capillary tube to prevent convective mixing between their contents. Magnitudes of the enrichment obtained by a single-stage cell, however, are low, since practical considerations put a limit on the temperature difference to which the system can be subjected.

The multistage thermal diffusion equipment, now called thermal diffusion column or thermogravitational column, was first introduced in 1938, and the credit for this goes to Clausius and Dickel. Unlike the vertical temperature gradient to which the single-stage cell is subjected, in the thermal diffusion column a horizontal temperature gradient is applied to the system whereby natural thermal convection currents are set up with the flow being upward in the neighbourhood of the hot wall and downward near the cold wall. This convective flow induced by the thermal gradient in the thermogravitational column effects cascading

(analogous to the refluxing effected by a fractionating column in fractional distillation), which multiplies the separation that would have been otherwise achieved. As a consequence of this, the component which diffuses preferentially toward the hot wall gets enriched at the top of the column, while the component which diffuses preferentially toward the cold wall gets concentrated at the bottom. Equipment used in thermogravitational separations actually comprises two verticle concentric pipes, the inner one electrically heated and the outer one coold, thermal diffusion being carried out in the annular space between the two pipes.

Figure 19.3 is a schematic showing distin-guishing features of working of a single-stage thermal diffusion cell [Figure 19.3 (a)] and a thermogravitational column [Figure 19.3 (b)].

(a) Single-stage cell   (b) Thermogravitational column
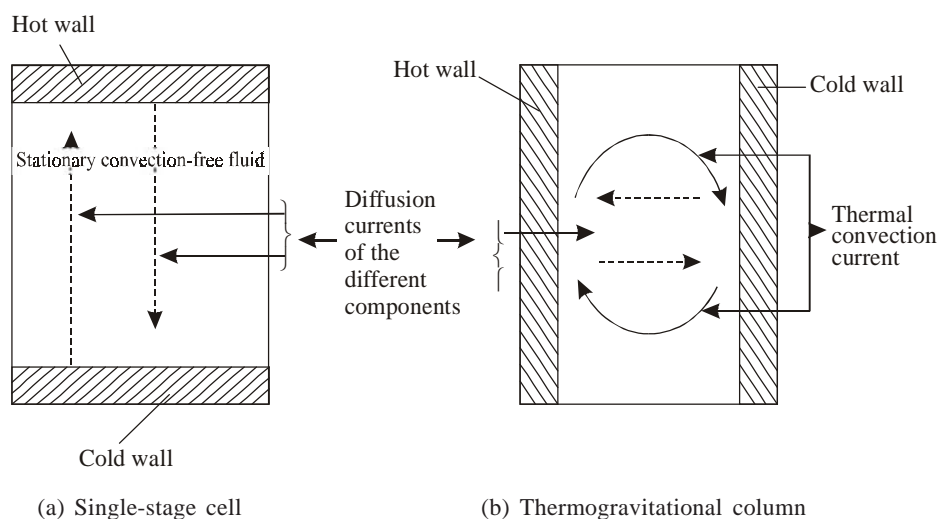
Figure 19.3 Schematic illustrating working of thermal diffusion equipment.

With the development of specific equipment and processes by which thermal be diffusion is now carried out, separation of substances from their mixtures can often carried out more cheaply by this method, if applicable, than by other separation techniques. Amongst the successful separations effected by thermal diffusion are those of the isotopes of helium and the isotopes of chlorine gas. The method had also been used to effect separation of the isotopes of uranium during the years of World War II in the U.S.A. Constituent hydrocarbons can easily be separated from their mixture by liquid-phase thermal diffusion, because interaction between molecules of different hydrocarbons is practically non-existent and, consequently, each hydrocarbon molecule of the mixture acts independently under the influence of the applied temperature gradient. Another use of thermal diffusion of special interest is its applicability to the separation of mixtures of liquids of close boiling points and of mixtures of isomers, into their respective components.

19.8 Electro-magnetic Separation

Electro-magnetic separation is the only method by which the isotopes of an element can be completely separated from one another. The separation is carried out by the mass spectrograph which works on the principle elaborated earlier for the mass spectrometer (Chapter 13). Working of the Dempster's mass spectrograph diagramatically represented by Figure 19.4, illustrates the basics of electromagnetic separation of isotopes.

A small quantity of the solid salt of the concerned element is placed on a filament which is heated electrically. The vapour is ionised by bombardment with electrons. The positive ions so produced are passed through slit S_1 and here accelerated by passage through the electric field between plates A and B to which a potential difference (V) of 1000-2000 volts is applied. The accelerated ions emerge through slit S_2 as a thin stream of positive rays. This stream of accelerated ions is next subjected to a uniform magnetic field M (being applied between two

Figure 19.4 Electro-magnetic separation.

semicircular poles of an electromagnet) of strength H and acting in a direction perpendicular to the motion of the positively charged particles. Under the influence of this magnetic field the positive ions take a circular path of radious r, where r has the value given by the equation:

$$r = \left(\frac{2V}{H^2} \cdot \frac{m}{e} \right)^{\frac{1}{2}}$$

The mass/charge ratio, $\frac{m}{e}$, is different for charged ions formed from each of the various isotopes of the concerned element. Thus the ions get separated by the magnetic field into beams each of a different $\frac{m}{e}$ and each beam travelling in a different trajectory ending in its getting collected separately (Figure 19.4).

The method can be successfully used to get separation of appreciable quantities of isotopes of an element. Perhaps, the most important case of separation by the electromagnetic method was that of the separation of ^{235}U from ^{238}U carried out

by Dempster. The separation was one of the important steps involved in the manufacture of the atom bomb. Since December 1945, concentration of a wide variety of stable isotopes has been carried out at Oak Ridge by using electro-magnetic separators.

19.9 Fractional Electrolysis

During electrolysis isotopic ions have slightly different rates of migration and, therefore, a view could be formed that separation of isotopes by electrolysis may be achievable in actual practice. Practical confirmation of the validity of this view came only when Washburn and Urey discovered in 1932, that a higher proportion of D_2O was present in water from some commercial electrolytic cells which had been working for some years. Following this discovery, Lewis and Macdonald, in 1933, electrolysed 20 litres of water (alkalified with NaOH) until the volume of the residual liquid was reduced to 1.5 ml. Distillation of this residual liquid yielded water containing 65 per cent of D_2O. Further electrolysis of this concentrate in D_2O finally gave almost pure heavy water. This was the first case of having achieved an almost complete separation of isotopes by a method other than the electro-magnetic method of separation of isotopes. The present-day preparation of heavy water uses the method given by Lewis and Macdonald.

Separation of isotopes of Li is another example of the application of fractional electrolysis for separating isotopes. Taylor and Urey (1933) carried out repeated electrolysis of a solution of LiOH using mercury as the cathode, when an amalgam rich in ^6Li was obtained.

19.10 Electrorefining

In this method purification of metals is brought about by electrolysis. An electrolytic cell is used in which the anode is of the crude metal to be purified, the cathode being a strip of pure metal coated with a thin layer of graphite from which deposited pure metal can be removed, and the electrolytic bath is an aqueous solution of a soluble salt of the metal. On electrolysis the impure metal goes into solution at the anode, and on the surface of the cathode is deposited the pure metal as only its ions get reduced at the cathode because the voltage between the electrodes is regulated. The process of electrorefining can be illustrated by taking the example of purification of copper, which is a particularly important application of this method.

Impure copper as obtained by the reduction of its ores contains small amount of other metals like iron, zinc, nickel, silver and gold with which the starting copper ores are associated. A slab of this impure copper is made the anode of an electrolytic cell in which a graphite-coated sheet of pure copper constitutes the cathode, and an aqueous solution of copper sulphate containing sulphuric acid is the electrolyte. Electricity is passed through the cell from a source of direct electric

current. At the potential applied to the cell, impurities of silver and gold present in the anode do not dissolve, and the two precious metals fall to the botton of the vessel giving anode mud as the atoms of the other metals around them dissolve; copper and the other metals viz. iron, zinc and nickel dissolve giving the ions Cu^{2+}, Fe^{2+}, Zn^{2+} and Ni^{2+} respectively and all these migrate to the cathode. On migration of all these different cations to the cathode only Cu^{2+} ions get reduced to metal atoms which deposit at the cathode leaving other cations in solution, because conversion of each of these other cations back to neutral atoms requires a higher voltage than the regulated voltage at which the electrolysis is being carried out. (Electrode potentials of the relevant couples are : Au^{3+}/Au, +1.42V; Ag^+/Ag, + 0.8V; Cu^{2+}/Cu, +0.34V; Fe^{2+}/Fe, – 0.44V; Zn^{2+}/Zn, –0.76V; Ni^{2+}/Ni, –0.25V).

Only weakly electropositive metals can, obviously, be purified by electrorefining, since the concerned metal must not react with water and must be easily oxidized (at anode) and reduced (at cathode) relative to hydrogen. Beside copper, some other metals for which electrorefining has been employed are: Silver, gold, tin and lead. In the electrolytic purification of these metals the relevant electrolyte is the one listed below against each metal:

Ag—Silver nitrate solution containing 10% nitric acid.

Au—Gold chloride solution acidified with hydrochloric acid.

Sn— Hydrofluosilicic acid (H_2SiF_6) containing tin sulphate acidified with sulphuric acid.

Pb— Lead fluosilicate ($PbSiF_6$) solution containing 8-10% hydrofluosilicic acid.

A slab of the concerned impure metal constitutes the anode while a sheet of the concerned pure metal acts as the cathode, in each case.

■

Index