INTRODUCTION TO HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Second Edition

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Introduction to high performance liquid chromatography

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to our wives Shiela and Pat

Preface

Since the first edition of this book the major advances have been in column packings, where over ninety per cent of separations are now performed using chemically bonded microparticulate packings, and in instrumentation.

The use of microprocessor control has brought about a rationalization of mobile phase delivery systems and in detectors, the introduction of electrochemical and spectrophotometric detection other than in the ultra-violet region, has widened the field of applications and the sensitivity of the technique. The use of ion-pair chromatography has increased at the expense of ion-exchange and this together with the improvements in detectors has greatly increased the application of the technique in the biomedical field.

These advances are described together with the established methods to enable the beginner to carry out a satisfactory separation and to gain the experience necessary for the full exploitation of the technique.

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Liverpool, 1981

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Introduction to high performance liquid chromatography

1.1 Introduction

Chromatography in its many forms is widely used as a separative and an analytical technique. Gas chromatography since its introduction by James and Martin [1] has been pre-eminent in the field. Liquid chromatography in the form of paper, thin-layer, ion-exchange, and exclusion (gel permeation and gel filtration) chromatography had not been able to achieve the same success, mainly because of the poor efficiences and the long analysis times arising from the low mobile phase flow rates. The emergence of liquid chromatography on a basis comparable to gas chromatography is usually considered to start with the publication by Huber and Hulsman [2] in 1967, although Giddings [3] had already shown the potential advantage, in terms of column efficiencies and speed of analysis, of liquid chromatography over gas chromatography.

Various names have been used to describe the main attributes of this 'new' liquid chromatography: high speed (HSLC), high efficiency (HELC), and high pressure or high performance (HPLC). The generally accepted name is now ***high performance liquid chromatography*** (*HPLC*). It should be made clear that these names refer to the analogue of gas chromatography where the stationary phase, be it a solid surface, a liquid, an ion exchange resin, or a porous polymer, is held in a metal column and the liquid mobile phase is forced through under pressure. Open bed chromatography (paper and thin-layer chromatography) is not included although claims are being made for so-called high performance thin-layer chromatography (HPTLC). These forms of chromatography, together with the 'classical' low pressure column chromatography, will continue as part of the analysts armoury and their utility should not be overlooked. Affinity chromatography, using specific biological interactions, is used increasingly for the separation of high-molecular-weight biological substances, but this too is beyond the scope of this book.

Both gas and high performance liquid chromatography have their place in the analytical laboratory, and there will obviously be an area of overlap where either technique could be used. In general, however, capital costs for liquid chromatography equipment, and the running costs of column packings and mobile phases,

are far higher than for gas chromatography, so gas chromatography will probably remain the preferred technique in these areas of overlap.

However, a large number of organic compounds are too unstable or are insufficiently volatile to be handled by GC without prior chemical modification, and liquid chromatography would be the first choice for such compounds. It is ideally suited for the separation of a wide range of pharmaceuticals, food, heavy industrial, and bio-chemicals.

Because lower temperatures can be used, and because there are two competing phases (mobile and stationary) compared with one phase (the stationary phase) in GC, liquid chromatography may often achieve separations that are impossible by GC. Furthermore, there is a wide choice of detectors available for use in LC, many of which are selective, so a complete separation need not necessarily be made on the column but a detector can be chosen that will monitor only species of interest.

Finally, recovery of the sample in LC can be achieved more easily and quantitatively than in GC. Although the mobile phase in LC has to be removed by distillation or some other means, this does not usually present any difficulty because of the wide difference in volatility between the mobile phase and the sample.

The advantages of HPLC over other forms of liquid chromatography may be summarized thus: (i) the HPLC column can be used many times without regeneration; (ii) the resolution achieved on such columns far exceeds that of the older methods; (iii) the technique is less dependent on the operator's skill, and reproducibility is greatly improved; (iv) the instrumentation of HPLC lends itself to automation and quantitation; (v) analysis times are generally much shorter; (vi) preparative liquid chromatography is possible on a much larger scale.

1.2 Nomenclature

Because of its relationship both to older 'classical' forms of liquid chromatography (column, thin-layer, and paper) and to gas chromatography, some confusion may exist in the language of high performance liquid chromatography.

In classical forms of liquid–solid chromatography the *sample* (or *solute*) was dissolved in a *solvent* and was *eluted* from a packed column containing silica gel or alumina. In gas–liquid chromatography the sample is carried through the column by the *carrier gas* (or *mobile phase*) and retention occurs on the *stationary phase*. Because the thermodynamics of the separation involves a simple two-component process, sample and stationary phase are often equated with the terms *solute* and *solvent*. Hence the term 'solvent' has two different meanings in the context of the two techniques.

In this book we shall use the term *solute* or *sample* to represent the components of the mixture to be separated, *stationary phase* or more specifically *adsorbent* or *absorbent* to represent the column packing on which the separation takes place, and *solvent, mobile phase*, or *eluent* to represent the eluting agent.

1.3 Liquid Chromatography Modes

One of the major advantages of liquid chromatography over other separation techniques is to be found in the several different mechanisms by which the chromatographic separation may be achieved. These mechanisms or modes of operation make it possible to achieve separations by liquid chromatography within such diverse sample types as solvents, ionic compounds and polymers. Although the modes of chromatography shall be discussed individually in practice one or more modes may be responsible for effecting the separation. This of course may add another dimension to a separation, but it also makes the prediction of retention behaviour more speculative.

1.3.1 Liquid-liquid (Partition) Chromatography (LLC)

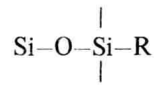
Liquid-liquid or partition chromatography was developed by Martin and Synge [4] in 1941 for the separation of acetylated amino acids using a stationary phase of water on silica gel with chloroform as mobile phase. LLC involves the use of a liquid stationary phase, which is either coated on to a finely divided inert support or chemically bonded to the support material, and a liquid mobile phase. The sample to be analysed is dispersed in the mobile phase and its components are partitioned between the stationary and mobile phases according to their partition coefficients $K_1, K_2 \cdots K_n$. This partitioning leads to a differential rate of migration and separation occurs.

The early workers in high performance liquid-liquid chromatography had their origins in 'classical' and in gas chromatography where the coating procedure for preparing the stationary phase is standard practice. The use of stationary phases prepared in this way in high performance liquid chromatography is unsatisfactory because the liquid stationary phase is stripped off the column, either by solubility effects or by mechanical shear forces, resulting in changes in retention times on the column. To prevent 'stripping', due to solubility effects the mobile phase could be passed through a pre-column containing the same stationary phase as the analytical column, so that it becomes saturated with respect to the stationary phase, and no further stationary phase is removed from the analytical column. However, the presence of a pre-column excludes the use of the gradient elution technique, since the phases would not be in equilibirum, an essential requirement in chromatography.

The development of chemically bonded stationary phases or 'brushes' by Halasz and Sebastian [5] eliminates the problem of stripping of the stationary phase and allows the use of gradient elution in the normal way.

The original bonded phases were prepared by reacting the surface –OH groups of silica with an alcohol to give an Si–O–R grouping. Due to the easy hydrolysis of this grouping these stationary phases could only be used in the pH range 4-7.

The majority of modern bonded phases are prepared from silica by reacting the surface silanol groups with an organochlorosilane or alkoxysilane to give an



linkage which is hydrolytically stable. The R group may be a hydrocarbon (e.g. C_8 or C_{18}) or a hydrocarbon with a polar terminal group (e.g. R–CN, R–NH_2). The ability to prepare bonded phases with functional groups of varying polarity now means that bonded phases may range from polar to non-polar. Furthermore, by introducing a polar group, such as nitrile, a stationary phase with many of the characteristics, but none of the disadvantages, of silica may be produced.

The success of bonded phases has virtually eliminated the use of coated stationary phases.

The term *normal phase* liquid-liquid chromatography originally referred to a system with a polar liquid stationary phase, e.g. water, glycol or β,β' -oxydipropionitrile, while the mobile phase is relatively non-polar, e.g. hexane, benzene, or chloroform. This mode of operation was used to separate polar compounds which would be distributed preferentially in the polar stationary phase.

If the stationary phase is non-polar, e.g. a hydrocarbon, and the mobile phase is polar, e.g. water, the technique is referred to as *reverse-phase* liquid chromatography. In reverse-phase chromatography the non-polar stationary phase may be displaced from a polar support by virtue of the preferential adsorption of the polar mobile phase. This may be prevented by de-activating the support material by silanization before coating with the stationary phase.

With the advent of bonded phases the distinction between normal and reversed-phase chromatography has been redefined. Normal phase refers to a system where the stationary phase is more polar than the mobile phase and reversed phase where the stationary phase is less polar than the mobile phase. Since the majority of separations are now carried out in the reversed-phase mode, the choice of name is a little unfortunate.

Ion pair chromatography (IPC) is a special form of LLC used for the separation of ionic or ionizable compounds, e.g. quaternary ammonium salts, sulphonates, amino acids, and amino-phenols [6]. In its most usual form it is used in the reverse-phase mode with a hydrocarbon bonded stationary phase. The mechanism of retention is subject to debate [7–12], two mechanisms having been proposed. In the partition mode in the ionic or ionizable sample molecule, which has insufficient lipophilic character to be retained, forms an ion-pair with a suitable counter-ion (or ion-pair agent) added to the mobile phase. The formation of the ion-pair increases the lipophilic character of the sample and increases its affinity for the stationary phase.

In the ion-exchange mode the polar counter-ion is assumed to be absorbed by the hydrocarbon stationary phase thus creating an ion-exchange site on to which the polar sample molecule is adsorbed much as in ion-exchange chromatography.

However, these two views are certainly an oversimplification, but many of the

factors controlling retention may be understood using these hypotheses.

IPC can be applied to the separation of samples containing both ionic and non-ionic compounds. Strong acids (sulphonated dyes) and bases (quaternary amines) are completely ionized in the pH range 2–8 and in the absence of an ion-pairing reagent will be poorly retained on a hydrocarbon stationary phase. However, the ionization of weak acids (amino acids, carboxylic acids) and weak bases (catecholamines) can be controlled by choice of pH in this range so that the equilibrium either lies to the left of the relationship:

$$\text{non-ionic} \rightleftharpoons \text{ionic}$$

in which case they elute as non-ionic components on a non-polar stationary phase, or to the right, in which case they can interact with the ion-pair agent as for strong acids or bases. As well as pH control, the nature and size of the counter-ion and, to a lesser extent, the nature of the solvent and stationary phase all have an effect on the degree of selectivity.

IPC is an alternative to ion-exchange chromatography but offers the advantages of longer column life and greater reproducibility.

1.3.2 Liquid–Solid (Adsorption) Chromatography (LSC)

Liquid–solid chromatography as developed by Tswett in 1906 using 'classical' column chromatography is the original form of liquid chromatography.

The separation is carried out with a liquid mobile phase and a solid stationary phase which reversibly adsorbs the solute molecules. The stationary phase may be either polar (e.g. silica gel, porous glass beads or alumina) when the mobile phase would be relatively non-polar (e.g. hexane or chloroform), or non-polar (e.g. polymer beads) when a polar mobile phase (e.g. water or ethanol) would be used. This latter mode is known as *reverse-phase adsorption*.

The retention of samples in LSC is more predictable than in LLC. since the elution order follows the polarity of the solutes. Furthermore, there is a vast literature on thin-layer chromatography (TLC), the results of which can usually be transferred more or less directly to HPLC. Because of the simplicity and flexibility of TLC it is often more convenient to establish the best combination of mobile and stationary phase using this technique before transferring the separation to HPLC to utilize its greater speed and efficiency. An example of this is illustrated in Fig. 1.1.

A feature of LSC is the degree of selectivity that can be introduced into the technique. The method is less sensitive to molecular weight differences between the solute species than is LLC but it is highly sensitive to compound type. Complex samples can therefore be separated into classes of compounds having the same functional groups, whereas molecules that differ only in alkyl chain length would be poorly separated.

The explanation of this selectivity lies in the nature of the adsorption process. Solvent molecules in the mobile phase compete with the solute molecules for sites on the adsorbent. A set of equilibria is involved:

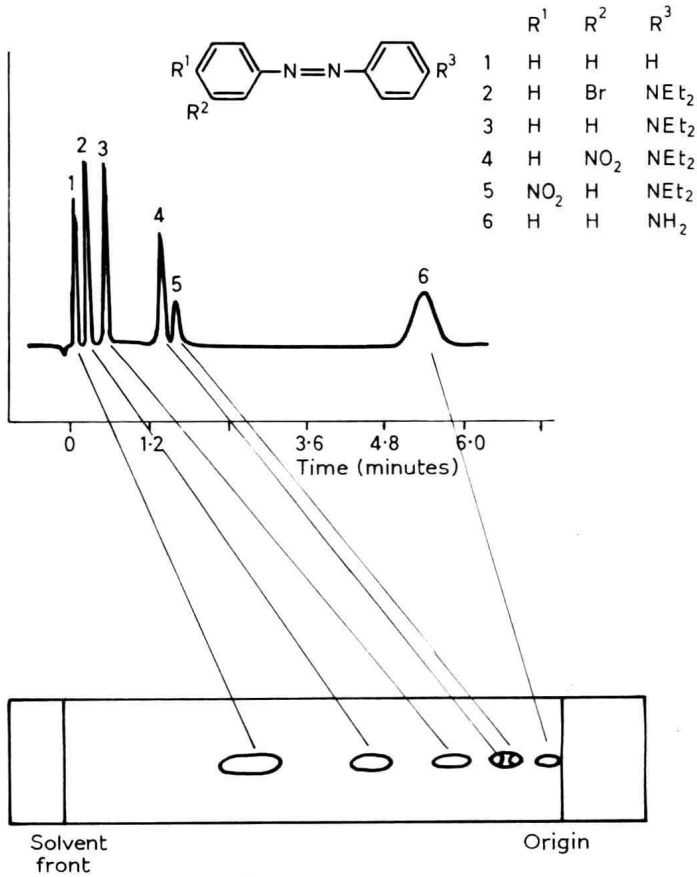
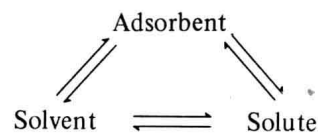


Fig. 1.1 TLC and HPLC of a dyestuff mixture. TLC conditions: silica gel F 254; 10% CH_2Cl_2 in hexane; development time 50 min. HPLC conditions: 15 cm × 2 mm i.d. MicroPak Si 10; 10% CH_2Cl_2 in hexane at 132 cm^3 per hour; 350 lbf in^{-2}; u.v. Detector; 0.2 mg cm^{-3} of each dye (1 μl injected).



In order that a solute molecule can be adsorbed on to the stationary phase, a solvent molecule must first be displaced from the surface. If it is assumed that the adsorbent possesses a polar surface (e.g. silica or alumina), non-polar groups (e.g. hydrocarbons) will have little affinity for the surface and will not displace the solvent molecules; they will not therefore be retained. Polar functional groups or groups capable of hydrogen-bonding will have a strong affinity for the surface and will be strongly retained. Polarizable molecules (aromatic molecules and high-molecular-weight compounds) will exhibit dipole-induced dipole

interactions with the adsorbent surface and will therefore also be retained, the degree of retention depending on the ease of polarization of the functional group or molecule.

For non-polar adsorbents (e.g. charcoal) the dominant intermolecular forces are the London (dispersion) forces, and polar and polarizable molecules will be less strongly retained.

Localized adsorption, or the existence of discrete adsorption sites that are a 'fit' for the adsorbing molecule, also plays an important part in this selectivity, particularly in the separation of isomers. This high level of selectivity is exemplified by the separation of positional isomers, e.g. *m*- and *p*-dibromobenzene. 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.

Although the new generation of adsorbents are an improvement on those introduced for use in gas–solid chromatography, batch-to-batch reproducibility of adsorbents can still be a problem. Some workers have attempted to standardize batch-to-batch adsorbents by adjusting the water content to give duplicate k' values for a given solute. However, since a single column may, if properly treated, be used for a hundred or more separations, the initial purchase of a 'stock' of adsorbent can minimize the problem of batch-to-batch reproducibility.

Catalytic reactions induced by the adsorbent are not the problem in liquid chromatography as they sometimes are in gas chromatography, because of the lower temperatures employed.

1.3.3 Ion-Exchange Chromatography

Ion-exchange chromatography, which is a form of adsorption chromatography, has been used as a separative technique for over 30 years.

Ion exchange involves the substitution of one ionic species for another. The stationary phase consists of a rigid matrix, the surface of which carries a net positive charge to give an ion-exchange site (R^+). If a mobile phase containing anions is used, the exchange site will attract and hold a negative counter-ion (Y^-). Sample anions (X^-) may then exchange with the counter-ions (Y^-).

The process can be represented in terms of the equilibrium:

$$R^+Y^- + X^- \rightleftharpoons R^+X^- + Y^-$$

Since the process involves the exchange of anions it is known as *anion exchange*. The complementary process of *cation exchange* occurs when the surface carries a net negative charge to give an exchange site (R^-). The counter-ions (Y^+) and the sample ions (X^+) are then both cations and their exchange may be represented by:

$$R^-Y^+ + X^+ \rightleftharpoons R^-X^+ + Y^+$$

The separation is thus based on the strength of the interactions between the sample ions and the exchange site. Ions that interact only weakly with the exchange site will be poorly retained and will have small k' values, whilst ions that have strong interactions will be strongly retained and will have high k' values.

This simple picture of ion exchange can be supplemented by additional processes which may occur under given conditions. The presence of complexing ions may have a marked effect on the process; heavy-metal cations are often complexed with Cl^- ions to give a complex anion and then separated by anion exchange, and the lanthanons are separated on cation exchangers having first complexed the lanthanon cation with citrate ions. Non-ionic species, e.g. sugars, can be separated by ion exchange following the formation of an ionic complex with borate anions [13].

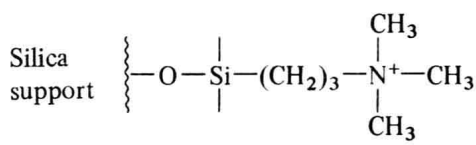
Ligand exchange involves the separation of ligands by virtue of their complexing strength for a metal ion sorbed on the exchange resin, the counter-ion again being displaced. The separation of amino acids on a zinc or cadmium modified resin with ammonium counter-ions is an example of this technique [14].

The existence of one or more of these additional processes makes it difficult to predict the selectvitiy of separations using ion-exchange systems.

Ion exchangers can be further divided into *weak* or *strong*, *anion* or *cation exchangers* according to the nature of the functional groups in the resin. Cation exchangers contain acidic groups (e.g. $-SO_3^-$, strong; or $-COO^-$, weak), whilst anion exchangers contain basic groups (e.g. $-NR_3^+$, strong or $-NH_2$, weak).

The classical ion-exchange resin, e.g. Dowex and Amberlite, is usually a styrene–divinylbenzene copolymer which has been cross-linked to provide mechanical rigidity, with the functional groups built into a matrix. These resins swell when placed in water and, to a lesser extent, in organic solvents, the degree of swelling depending on the degree of cross-linking and the nature of the exchange groups. These materials are also porous, so separation by size can also occur. Furthermore, slow mass transfer effects reduce the efficiency of such columns (300 plates/metre except for microparticle resins).

Chemically bonded exchangers have not the above-mentioned disadvantages of conventional exchange resins but they possess other disadvantages. These exchangers consist of a non-porous silica matrix to which the functional groups are bonded by a covalently bonded cross-linked silicone network:



Efficiences up to 25000 plates per metre are obtained on microparticulate packings and the exchangers do not swell in solvents. However, these exchangers have limited ion-exchange capacities and some can be used only over a limited pH

range. The relative merits of the two types of ion exchangers will be discussed in greater detail in Chapter 4.

Ion exchange rarely causes the degradation of a sample. If, however, it should occur, a change to a lower temperature or a change in pH or ionic strength of the carrier is usually sufficient to prevent degradation. Failing this, a change from a strong to a weak ion exchanger may be necessary.

Deactivation of the exchange resin may occur, either by the preferential adsorption of retained sample components, or by the physical blocking of the exchange sites by particulate matter.

The effect of temperature on the selectivity of the resin is difficult to predict, and this factor is usually disregarded in selecting the operating temperature; however, the stability of the exchange resin may be affected by temperature, and the manufacturers' recommendations should be followed. Higher temperatures are an advantage because the decrease in viscosity of the mobile phase with increase in temperature leads to improved mass transfer of the sample components with the consequent increase in column efficiency. Higher temperatures also usually lead to shorter analysis times.

Batch-to-batch reproducibility of ion-exchange resins is often poor and some form of standardization is desirable.

1.3.4 Exclusion Chromatography

Exclusion chromatography, also referred to as *gel filtration, gel permeation chromatography*, or *gel chromatography*, dates from 1959 with the introduction of a dextran gel in bead form marketed under the name of Sephadex. In modern exclusion chromatography a wide range of stationary phases is available and these can be divided into three classes: (i) the aerogels (porous glass or silica); (ii) the xerogels, e.g. cross-linked dextran and polyacrylamide; (iii) xerogel-aerogels, e.g. cross-linked agarose, polystyrene, and polyvinylacetate.

Exclusion chromatography separates substances according to their molecular size and shape. Small molecules that can enter freely into the pores of the stationary phase are said to have a distribution coefficient $K = 1$, and large molecules which are completely excluded from all pores have a distribution coefficient $K = 0$, whilst molecules of an intermediate size will have distribution coefficients between 0 and 1. Thus large molecules will move more rapidly through the column than will the smaller molecules and they are eluted first. Molecules are therefore eluted in order of decreasing molecular size. The process is shown diagrammatically in Fig. 1.2.

Since the solvent molecules are usually very much smaller than the molecules being separated by this method, they are eluted last (at t_m). Hence, contrary to other forms of chromatography, the sample is eluted before t_m.

The mechanism of separation in exclusion chromatography is complex, but the overriding mechanism appears to be that of steric exclusion [15]. Diffusion [16-18] may also play some part, as may adsorption on to the gel, but in properly designed systems the effect of these should be minimal.

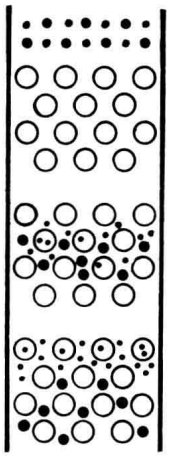


Fig. 1.2 Schematic diagram of three stages in a separation by exclusion chromatography. Empty circles represent the gel bed, filled circles the solute molecules.

Exclusion chromatography possesses several advantages in its application. Because all of the sample is eluted in a relatively short elution time (before t_m), gradient elution facilities are not required. The short elution times also lead to narrow solute bands which are easier to detect and do not give rise to the problems of detection limits often encountered in other forms of liquid chromatography where the solute bands may become more diffuse. Hence, the less sensitive refractometer detector is usually used in exclusion chromatography.

The retention time is a function of molecular size and can be predicted for a compound of known molecular structure. The beginning of the chromatogram is therefore determined by the retention time predicted for the compound of largest molecular size, and the end by the solvent retention time (t_m). A series of different samples can therefore be injected at predetermined intervals without fear of the chromatograms overlapping each other, and automatic injection systems are widely used in exclusion chromatography. The correlation of retention times with molecular structure also aids the identification of unknown substances.

Because intermolecular forces are absent in the separation process, sample loss or chemical interaction is kept to a minimum and the column does not accumulate strongly retained molecules. This results in a longer than usual column life, and pre-columns are not normally required for clean-up.

The main disadvantages in exclusion chromatography are its inability to resolve compounds with similar molecular size distributions and its limited peak capacity. Molecular weight differences of 10% or more are usually required to achieve separation, so the technique does not lend itself to complete separations of complex mixtures but is used mainly as a preliminary separation method or as a method of obtaining the molecular weight distribution of a polydisperse polymer.

Limited peak capacity arises from the relatively short time required to run the chromatogram (maximum time = t_m), and chromatograms with more than ten peaks are rarely seen.