Modern Liquid Chromatography

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1 Introduction

Liquid chromatography, (LC), was discovered nearly a century ago by a Russian botanist called Tswett who used a column packed with calcium carbonate to separate a number of plant pigments. The coloured bands produced on the adsorbent inspired the term chromatography to describe the separation process. Although colour has little to do with modern chromatography, the name has persisted and, despite its irrelevance, is still used to describe all separation techniques that employ a mobile and stationary phase. Unfortunately, the work of Tswett was not immediately developed to any significant extent, partly due to the original paper being in Russian, and partly due to the condemnation of the method by Willstatter and Stoll. Willstatter and Stoll tried to repeat the work of Tswett but did not heed the advice of Tswett to avoid 'aggressive' adsorbents and so their experiments failed. Inaccurate and careless experimental work has always been a threat to scientific progress and recent years have shown that contemporary science is by no means immune to such threats. However, the mistake of Willstatter and Stoll was particularly unfortunate as, not only did it retard the development of a very useful separation technique, it also inhibited progress in many other fields of chemistry.

It was not until thirty years had passed that Kuhn and his coworkers repeated Tswett's original work successfully and separated lutein and xanthine from a plant extract. Nevertheless, despite the success of Kuhn *et al.* and the validation of Tswett's experiments, progress continued to be slow and desultory. In 1941 Martin and Synge introduced liquid–liquid chromatography by supporting the stationary phase, in this case water, on silica in the form of a packed bed and used it to separate some acetyl amino acids. In the same paper Martin and Synge suggested that it would be advantageous to replace the liquid mobile phase by a gas to improve the rate of transfer between the phases and thus improve the separation. The recommendation was not heeded and it was left to Martin and James to bring the concept to practical reality in the early fifties. Thus, Gas Chromatography (GC) was born and a new and important era of chromatographic development began.

Gas chromatography grew from a laboratory novelty into a popular well established analytical technique in little more than a decade. During that time, the foundations of chromatography *column theory* were laid down which were to guide the development of LC in the sixties and seventies. As a result of the glamourous success of GC, LC became the Cinderella of chromatography and it was not until the major developments of GC were completed that scientists turned their attention to the development of LC. Today, LC is also a well established separation technique but its progress has been slow and arduous relative to that of GC. The difficulties encountered in the development of LC arose from two causes. Firstly, solutes have a very low diffusivity in liquids compared with a gas and thus, the kinetics of exchange between the phases are slow and special steps must be taken to achieve efficient separations. Secondly, very low concentrations of a solute in a liquid do not modify the properties of the liquid to the same extent that they do to a gas and this has made the development of LC detectors far more difficult.

2 Classification of Chromatography

The different forms of chromatography take their definitions from the physical nature of the mobile and stationary phases. Thus, in GC the mobile phase is a gas and in LC the mobile phase is a liquid. Furthermore, the stationary phase can be a liquid or a solid and thus there can be two forms of GC and LC; Gas Liquid Chromatography (GLC) and Gas Solid Chromatography (GSC) together with Liquid Liquid Chromatography (LLC) and Liquid Solid Chromatography (LSC). The classification of the different forms of chromatography are summarized in Table 1.

There are some sub-classifications of chromatography based on the physical shape of the chromatographic system; for example *column chromatography* where the phase system is contained in tubular form and *lamina chromatography* where the phase system takes the form of a flat sheet or strip as in thin layer chromatography (TLC). The technique of Critical Fluid Chromatography (CFC), where the mobile phase is a fluid operated above its critical temperature might also be considered a separate class. However, if the mobile phase is in its critical state, it can, in fact, still be classed as a gas, albeit a dense gas, and consequently is another form of GLC or GSC.

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Table 1 The classification of chromatography

MOBILE PHASE	STATIONARY PHASE	
GAS	LIQUID	SOLID
Gas chromatography GC	Gas–liquid chromatography GLC	Gas–solid chromatography GSC
LIQUID Liquid chromatography LC	Liquid–liquid chromatography LLC	Liquid–solid chromatography LSC

3 The Separation Process

Chromatography has been defined as:

'A separation process that is achieved by the distribution of substances between two phases, a stationary phase and a mobile phase. Those solutes distributed preferentially in the mobile phase will move more rapidly through the system than those distributed preferentially in the stationary phase. Thus, the solutes will elute in order of their increasing distribution coefficients with respect to the stationary phase.'

This definition is a little trite and, although it introduces the concept of a mobile and stationary phase which are essential characteristics of a chromatographic separation, it tends to obscure the basic process of retention in the term *distribution*. A solute is distributed between two phases as a result of the molecular forces that exist between the solute molecules and those of the two phases. The stronger the forces between the solute molecules and those of the stationary phase, the more the solute will be retained. Conversely, the stronger the interactions between the solute molecules and the mobile phase, the more rapidly will the solute pass through the column. Consequently, solute retention is controlled by molecular forces of which there are four basic types: *ionic forces, polar forces, dispersive forces*, and *chemical forces*. There could be considered a third type of molecular interaction, *hydrogen bonding*, but for the purpose of this discussion, forces due to hydrogen bonding will be classed as strong polar forces.

3.1 Ionic Interactions

Ionic interactions result from permanent electrical charges that exist on molecules when in the form of ions, for example organic acids, bases, and salts. Such interactions are exploited in ion exchange chromatography and it follows, that to retain *anionic* materials in a chromatographic system, the stationary phase should contain *cations*. Conversely, to retain *cationic* materials, the stationary phase should contain *anions*. The stationary phase can consist of an ion exchange resin or can take the form of an adsorbed ion exchanger on the surface of a reverse phase, such as an alkyl sulfonate.

3.2 Polar Interactions

Polar interactions between molecules arise from permanent or induced dipoles and do *not* result from *net* charges as in the case of ionic interactions. Alcohols, ketones, and aldehydes are examples of polar substances having permanent dipoles, aromatic hydrocarbons such as benzene or toluene are examples of polarizable substances with no permanent dipoles. When a molecule carrying a permanent dipole approaches a polarizable molecule, the field from the permanent dipole induces a dipole in the polarizable molecule and thus, electrical interaction can occur. It follows, that selectively to retain a polar solute, the stationary phase must also be polar and contain, perhaps, hydroxyl groups. If the solutes to be separated are *strongly* polar, then a polarizable substance such as an aromatic hydrocarbon might be adequate as the stationary phase. However, to maintain strong polar interactions *exclusively* in the stationary phase (as opposed to the mobile phase) the mobile phase must be relatively *non-polar* or dispersive in nature.

3.3 Dispersive Interactions

Dispersive interactions are more difficult to describe. Although electric in nature, they result from charge fluctuations rather than permanent electric charges on the molecule. Examples of purely dispersive interactions are the molecular forces that exist between hydrocarbon molecules. *n*-Heptane is *not* a gas due to the collective effect of all the dispersive interactions that hold the molecules together as a liquid. To retain solutes selectively, by dispersive interactions, the stationary phase must not contain polar or ionic substances but only hydrocarbon-type materials such as the reverse-bonded phases now so popular in LC. It follows, that to allow dispersive selectivity to dominate in the stationary phase, the mobile phase must be polar and significantly less dispersive. Hence the use of methanol–water and acetonitrile–water mixtures as mobile phases in reverse-phase chromatography systems.

3.4 Chemical Forces

Chemical forces are normally irreversible in nature and thus the distribution coefficient of the solute with respect to the stationary phase is infinite. Affinity chromatography is an example of the use of chemical forces in a separation process. The stationary phase is formed in such a manner that it will chemically react with one unique solute present in the sample and thus exclusively extract it from the other materials present.

In any distribution system, it is rare that only one type of interaction is present and if this occurs, it will certainly be dispersive in nature. Polar interactions are always accompanied by dispersive interactions and ionic interactions will, in all probability, be accompanied by both polar and dispersive interactions. However, it is not merely the *magnitude* of the interacting forces between the solute and the stationary phase that will control the extent of retention, but also the *amount* of stationary phase present in the system and it's *accessibility* to the solutes. If the stationary phase is a porous solid, some solutes, for example those of small molecular size, can penetrate and interact with more stationary phase than larger molecules which are partially excluded. Under such circumstances the retention is at least partly controlled by *size exclusion*. This type of chromatography is called Size Exclusion Chromatography (SEC) but is still included within the classification of LSC or LLC. However, even in SEC, retention is not exclusively controlled by the size of the solute molecule, it is still partly controlled by molecular interactions between the solute and the two phases.

4 The Stationary Phase

4.1 Silica Gel

The majority of stationary phases employed in LC are based on silica gel and, in fact, silica gel is probably the most important single substance involved in the technique. It is not only used as a stationary phase *per se*, but is also used as the matrix from which the so called 'bonded phases' are made. Silica gel has some unique properties that make it particularly useful in chromatography and these properties arise from the way it is produced. It follows that the process used for the manufacture of silica gel is of sufficient importance that it merits some detailed attention.

Silica gel is made in large batches by adding hydrochloric acid to a solution of sodium metasilicate. Initially, silicic acid is released but this quickly starts to condense with itself to form dimers, trimers, and eventually polymeric silicic acid. The polymer continues to grow until, at a particular size, the solution begins to gel. As a result of this process, primary particles of silica gel are formed which may have diameters ranging from a few ångströms to many thousands of ångströms. The size of the primary particles will depend, among other factors, on the temperature and pH of the mixture at the time of gelling. It is the formation of these primary particles that confers onto silica gel its high porosity and high surface area, which are so important in its use as a stationary phase or support in LC. Furthermore, it is the condensation of the surface silanol groups of the primary particles that causes their adhesion and the onset of gel formation. After the gel has become solid, it is allowed to stand for a few days while condensation between the primary particles continues and the gel shrinks and exudes water. This process is called sinerisis and the firm gel that is finally produced is called the *hydrogel*. The hydrogel is then heated for a few hours at 120 °C and the resulting product is called the *xerogel* which, after grinding to an appropriate particle size, is the material employed as the stationary phase or support for LC and is called *irregular*

silica gel. *Spherical* silica gel is made from an emulsion of appropriate silane esters by the addition of hydrochloric acid followed by sinerisis and heat treatment. The particle size of the spherical silica gel is adjusted by controlling the dispersion of the ester in the emulsion. The physical properties of irregular silica gel are difficult to control during manufacture and thus, appropriate material for LC is selected from the particular batch that provides the desired surface area and porosity. As the pore size is controlled by the size of the primary particles during the gelling process, the pores of the silica gel can also range from a few ångströms to many thousands of ångströms. The material can thus, act as an exclusion media, separating solutes on a basis of molecular size. If the mobile phase is chosen such that the interactions between the solutes and the two phases are closely similar, then the only effective mechanism for relative retention, will be size exclusion. The small molecules will enter all the pores and, therefore, will associate with all the stationary phase (or surface) and will be retained the most. The larger molecules will be excluded from some of the pores that are too small to enter and, therefore, associate with less stationary phase or surface and will be the least retained. However, even under conditions of normal solute retention, where there is a differential interaction of the solute between the two phases, the exclusion properties of the silica will still play a part. The restricted availability of the stationary phase to the larger molecules will reduce the extent of their interaction with it and their absolute retention will be proportionally reduced. For this reason, it is unwise to try to compare the chemical nature of two solutes from retention data obtained from a silica gel matrix. Such retention data would reflect true differences in molecular interaction *only* if both solutes were exposed to the *same amount* of stationary phase during elution.

4.2 Bonded Phases

Bonded phases, and in particular the *reverse phases*, are the most commonly used stationary phases in modern LC. The term reverse phase, though conventional, has no precise meaning. It originated in the work of Martin and Synge when they were investigating different phase systems. These authors replaced a system that employed water as the stationary phase and a hydrocarbon as the mobile phase by a hydrocarbon stationary phase and an aqueous mobile phase. This, was a form of phase reversal and, as the first stationary phase used was water, the alternative system employing a hydrocarbon stationary phase, was called the *reverse* phase. Today, it is a term given to stationary phases that are hydrocarbon in nature and, thus, retain solutes predominantly on the basis of dispersive interactions.

Bonded phases are made from silica gel by reacting the surface hydroxyl groups with appropriate reagents to attach an organic group, or chain, to the silica. It is this organic chain that acts as the interacting moiety, retaining the solutes and producing the required separation. The reagents used are usually organic chlorsilanes or esters. There are basically three types of bonded phase; 'brush' type phases, 'bulk' phases, and oligomeric phases.

Reacting silica, dried at about 150 °C, with dimethyloctylchlorosilane contained in a solvent at elevated temperatures causes a dimethyloctylsilyl group to be attached to the surface by silicon–oxygen–silicon bonds. This is a fairly stable bonding sequence (far more stable than the carbon–oxygen–silicon bond which was the first chemical link used in bonded phase synthesis) but is not very stable at extremes of pH. The product is a surface covered with dimethyloctyl chains like bristles of a brush–hence the term 'brush' phase. If the silica surface is saturated with water and octyl*trichlor*silane is used as the reagent, reaction occurs with both the hydroxyls of the silica surface and the adsorbed water, causing a crosslinking reaction and an octylsilanyl polymer to be built up on the surface. Due to the polymerization process, the stationary phase has a multilayer character and, consequently, is termed a 'bulk' phase. Finally, if methyloctyl*dichlor*silane is used as the reagent in a sequence of synthetic steps, an oligomeric phase can be built up on the surface that is far more stable than either the 'brush' or 'bulk' phases. The silica is first reacted with the methyloctyl*dichlor*silane to link methyloctyl*chloro*silyl groups to the surface. The bonded phase is then treated with water to generate methyloctyl*hydroxy*silyl groups which, in turn, are then reacted with more methyloctyldichlorsilane attaching another methyloctyl*chloro*silyl group to the previous group. This process can be repeated until eight or ten oligomers are linked to each other on the surface. The product is finally treated with trimethylchlorsilane to eliminate the last hydroxyl group. This final step is called capping. The oligomers are layered over the surface making the product extremely stable and exhibit almost no polar characteristics whatsoever. However, due to the complexity of the synthesis, oligomeric phases are expensive to manufacture and, consequently, are not often used. The most popular reverse phase appears to be the brush type phase with paraffin chains having, four, eight, or eighteen carbon atom chains attached. These types of reverse phase have been termed, C-4, C-8, and C-18 respectively. The C-8 and C-18 phases are mainly used for solutes having relatively low molecular weights whereas the C-4 phase is used for the separation of very large molecules. The C-4 reverse phase is also particularly useful in the separation of materials of biological origin that may be chemically labile or easily denatured.

Using appropriate organic chlorsilanes, polar or polarizable groups such as nitriles or aromatic rings can be bonded to the silica to provide stationary phases covering a wide range of polarities. Bonded ion exchange materials have also been synthesized, although the most common types of ion exchange media are ion exchange resins in the form of tiny polymer beads. An interesting carbon stationary phase, introduced by Knox, is obtained by filling the pores of appropriately sized silica particles with an organic polymer and carbonizing the product at elevated temperatures. The silica is removed from the product by treatment with strong alkali or hydrofluoric acid forming, perhaps, what might be termed, a true 'reverse phase'. It would be a 'reverse phase' in the sense that the pores are where the primary particles of silica existed and the solid matrix now replaces the pores. The product is too active for use in chromatography and so the carbon is graphitized by exposure to an argon plasma. This is a relatively new material and, due to the complexity of its manufacture, is expensive. Whether its performance relative to that of a conventional reverse phase merits the greater cost, remains to be established.

5 The Mobile Phase

The stationary phase determines the character of the mobile phase that must to be used in order to achieve the required resolution. Unfortunately, the type of detector employed also imposes limitations on the choice of mobile phase. If, for example, a UV detector is used, then the mobile phase must also be transparent to the UV light of the operating wavelength.

If silica gel, which is strongly polar, is employed as the stationary phase then a dispersive type solvent would be appropriate for the mobile phase. Normal paraffins such as n-hexane or n-heptane would constitute the more dispersive types of mobile phase and would also be transparent to UV light. If the solutes were retained too strongly, the hydrocarbon could be mixed with methylene dichloride which is also transparent to UV light. Progressively more polar solvents could be mixed with the n-paraffin to increase the magnitude of the polar interactions in the mobile phase and elute the more strongly-absorbed solutes. Tetrahydrofuran, propanol, and methanol are examples of polar solvents that are transparent to the UV light and that can be mixed in small concentration with the n-paraffin to elute more strongly-retained solutes.

Reverse phases, being dispersive in nature, require very polar mobile phases such as mixtures of water and methanol, acetonitrile, or tetrahydrofuran. To increase the magnitude of the interactions in the mobile phase, and thus help elute strongly retained solutes, the proportion of the organic solvent in the

mobile phase can be increased, relative to that of water. Very difficult separations may require subtle mixtures of all three solvents with water to optimize the separation. The need for such complex solvent mixtures, however, is rare in practice.

Ion exchange resins can be, weak or strong, anion or cation exchangers and will require aqueous buffer solutions as mobile phases. The pH of the mobile phase must be adjusted to complement the type of stationary phase employed. In general, a suitable mobile phase can be chosen on a rational basis, providing that the type of interactions that are occurring on the stationary phase are known and understood. Alternatively, the literature abounds with LC applications to help the novice choose an appropriate phase system for a specific separation problem.

6 The Liquid Chromatography Column

The column is the heart of the liquid chromatograph and is where the separation takes place. It is usually a stainless steel tube a few centimetres long and a few millimetres wide, packed with particles of stationary phase a few microns in diameter. In the column the separation is completed and, despite the complex and perhaps glamorous appearance of the rest of the chromatograph, the associated equipment is there merely to serve the column and help interpret the results. During the development of a chromatographic separation, two processes proceed progressively and simultaneously in the column. Firstly, the bands of the individual solutes in the sample are moved apart as a result of their different interactions with the stationary phase. Secondly, as the bands are moved apart, they spread or disperse and tend to merge together, blurring the separation that has been obtained. The column, by appropriate design, must minimize this dispersion, so that, having been moved apart and separated, the individual solutes enter the detector as individual bands. Thus, to obtain maximum resolution, the column must move the bands as far apart as possible but, at the same time, keep each band as narrow as possible. The capacity of the column to restrain the dispersion of the solute bands is called the column efficiency. Column efficiency is measured in 'theoretical plates' and the greater the number of plates provided by the column the greater the separating capacity of the column. The major difference between modern LC columns and those of Tswett are that the former have much greater efficiencies.

In order to design an efficient column the factors that control solute dispersion must be known and understood. A number of theories of band dispersion have been introduced and the one that is best supported by experimental results is that of Van Deemter *et al.* Van Deemter examined the different dispersion processes that could contribute to the total band variance per unit length of a column (H). The variance contribution of each process was then determined and the sum of all the variances provided a value for (H). The resulting equation would then show how (H) could be reduced and, consequently, the column made more efficient. Van Deemter *et al.* postulated that there were three basic dispersion processes that took place in a column the *multipath effect*, *longitudinal diffusion*, and *resistance to mass transfer*.

6.1 The Multipath Effect

As the solute molecules pass through the interstices of the packing some molecules will, on a random basis, follow longer paths than others. As a consequence, some molecules pass ahead of the bulk of the molecules while others will lag behind. This results in band spreading and was given the term the 'Multipath Effect'. Van Deemter *et al.* derived the following function for the variance contribution (σ^2_{mp}), from the multipath effect

$$\sigma^2_{mp} = 2\lambda d_p = A$$

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

6.2 Longitudinal Diffusion

Dispersion due to longitudinal diffusion is a band spreading process that results from the normal diffusion processes that occur in a liquid (mobile phase). Obviously, the longer the solute remains in the column, the longer this diffusion process continues and thus, the variance resulting from diffusion will be inversely proportional to the linear velocity of the mobile phase. Van Deemter *et al.* derived the following function for the variance contribution from longitudinal diffusion (σ^2_D)

$$\sigma^2_D = \frac{2\gamma D_m}{u} = \frac{B}{u}$$

where, (γ) is a constant, (D_m) is the diffusivity of the solute in the mobile phase and (u) is the linear mobile phase velocity.

6.3 The Resistance to Mass Transfer

Band dispersion from resistance to mass transfer is the major factor contributing to band variance at high mobile phase velocities. The dispersion results from the finite time required for the solute to diffuse through the stationary phase in order to enter the mobile phase and, similarly, to diffuse through the mobile phase to return to the stationary phase. Solute molecules that have diffused further into the stationary phase, will take longer to diffuse back to the surface and enter the mobile phase, than those that were closer to the surface. While this transfer is taking place, those molecules that entered the mobile phase earlier will be have been swept further down the column by the moving phase, thus, 'smearing' the band along the column. Exactly the same type of band broadening occurs when the solute molecules pass from the mobile phase and enter the stationary phase. Consequently, Van Deemter *et al.* derived two functions to describe this type of dispersion, one for the variance contribution from the resistance to mass transfer in the *mobile phase* and another for the *stationary phase*. Obviously, the higher the mobile phase velocity the greater will be this type of dispersion. The functions derived by Van Deemter *et al.* are as follows:

$$\sigma^2_M = \frac{f_1(k')d_p^2 u}{D_m} = C_1 u$$

where (σ^2_M) is the variance contribution from the resistance to mass transfer in the mobile phase and $f_1(k')$ is a function of the distribution coefficient of the solute between the two phases and the stationary phase/mobile phase ratio.

$$\sigma^2_s = \frac{f_2(k')d_f^2 u}{D_s} = C_2 u$$

where (σ^2_s) is the variance contribution from the resistance to mass transfer in the stationary phase and $f_2(k')$ is another function of the distribution coefficient of the solute between the two phases and the stationary phase/mobile phase ratio.

Summing the individual variances to obtain the total column variance per unit length (H)

$$H = \sigma^2_{mp} + \sigma^2_D + \sigma^2_M + \sigma^2_s$$

$$H = 2\lambda d_p + \frac{2\lambda D_m}{u} + \frac{f_1(k')d_p^2 u}{D_m} + \frac{f_2(k')d_f^2 u}{D_s} \quad (1)$$

or,

$$H = A + \frac{B}{u} + (C_1 + C_2)u$$

Now, in LC, $D_m \sim D_S$, and $d_p \gg d_f$, thus, the resistance to mass transfer in the stationary phase is very small, *i.e.* $C_1 \gg C_2$, and

$$H = A + \frac{B}{u} + C_1 u \quad (2)$$

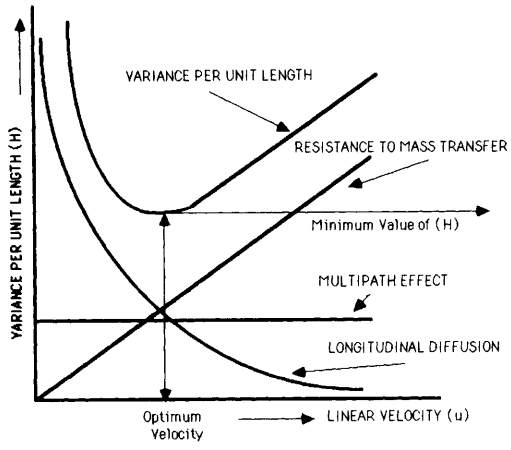

Figure 1 Curve relating variance per unit length to linear velocity.

Equation 2 is a hyperbolic function made up of a constant, a reciprocal function and a linear function. The relationship between (H) and the mobile phase linear velocity (u) is shown in Figure 1. It is seen that there is an optimum value of (u) that provides a minimum value for (H) which would correspond to a maximum column efficiency (n) where,

$$n = l/H$$

and (l) is the column length.

The optimum velocity, at which (H) will be a minimum can be determined by differentiating equation 2 and equating to zero when it is seen that,

$$u_{(opt)} = (B/C)^{0.5}$$

Furthermore, the minimum value of (H) can be obtained by inserting the expression for the optimum velocity in equation 2

$$H_{(min)} = A + 2(BC)^{0.5}$$

Thus, knowing the functions for A, B, and C from the Van Deemter equation, the particle diameter of the packing and the diffusivity of the solute in the mobile phase, the theoretical column efficiency can be calculated. Conversely, the particle diameter can be calculated that will achieve a desired efficiency to achieve a given separation. Chromatography column theory has progressed to a level where the dimensions and operating conditions necessary to achieve a given analysis in the minimum time can be calculated. A discussion on the design of optimum LC columns, however, is outside the scope of this review.

Examination of equation 1 indicates that the dominant factor that controls the minimum value of (H) is the particle diameter of the packing (d_p). It would appear to follow, that the maximum efficiency would be obtained from using the smallest possible particles. Unfortunately, it is not so simple, as reduction in the particle diameter also increases the column impedance to flow. Consequently, as the available column pressure is limited so, also, is the minimum particle diameter that can be used with a column of given length. Theory shows, that as a result of the dependence of both efficiency and flow rate on particle diameter, simple separations are best carried out on short wide columns packed with very small particles. Conversely, difficult separations require long narrow columns, packed with relatively large particles. The most common particle diameters that are commercially available for silica and bonded stationary phases are 3, 5, and 10 micron. A set of columns that would use these particle sizes to an advantage is given in Table 2.

Liquid chromatography column theory is now well developed and columns that can provide very fast separations and very high resolution can now be designed and fabricated. An example

Table 2 A practical set of columns employing readily available particle sizes

Column efficiency	Particle diameter	Column length	Column diameter
50 000	10 micron	100 cm	1 mm
15 000	5 micron	15 cm	2 mm
5000	3 micron	3 cm	3 mm

of a very fast separation obtained from a relatively short column is shown in Figure 2.

The separation was obtained employing a column 2.5 cm long, 3 mm in diameter packed with silica gel particles 3 μm in diameter. It is seen that the 5 components are separated in about 3.5 s. Separations as rapid as this are rarely required in general analysis, although they might be of use in following reaction kinetics. Nevertheless, the chromatogram shown in Figure 2 represents one of the fastest separations obtained with LC and also demonstrates the contribution that column theory has made to column design.

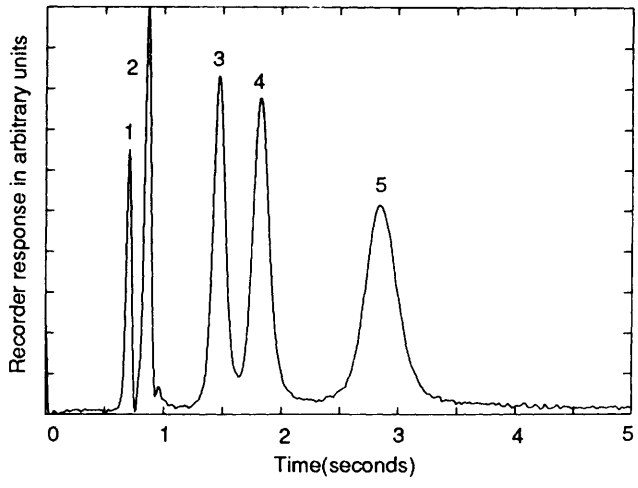

Figure 2 High speed isocratic separation of a five-component synthetic mixture.

Packing, Hypersil 3 μm; column i.d., 0.26 cm; column length, 2.50 cm; mobile phase, 2.2% methyl acetate in n-pentane; linear velocity, 3.3 cm/s.

1, *p*-xylene; 2, anisole; 3, nitrobenzene; 4, acetophenone; 5, dipropyl phthalate.

Figure 3 shows the separation of a group of aromatic compounds extracted from coal and was obtained from a column 14 metres long, 1 mm in diameter packed with reverse phase particles 10 μm in diameter. The column had an efficiency of over 500 000 theoretical plates and the elution time was over two and a half days. Unfortunately, very high efficiency columns must either be operated at exceedingly high pressures or a very long elution time must be tolerated. The inlet pressure used was 6000 p.s.i. and it was found that higher pressures were not possible due to leaks developing in the sample valve and excessive heat being generated in the column. The only alternative was to tolerate very long retention times. Such long elution times are rarely seen in normal LC analyses and would only be acceptable for very special samples. Nevertheless, the chromatogram shown in Figure 3 represents one of the highest column efficiencies obtained with LC and again demonstrates the value of column theory in helping column design. The insert in Figure 3 is the first part of the chromatogram presented by the computer in an expanded form to illustrate the excellent resolution achieved.

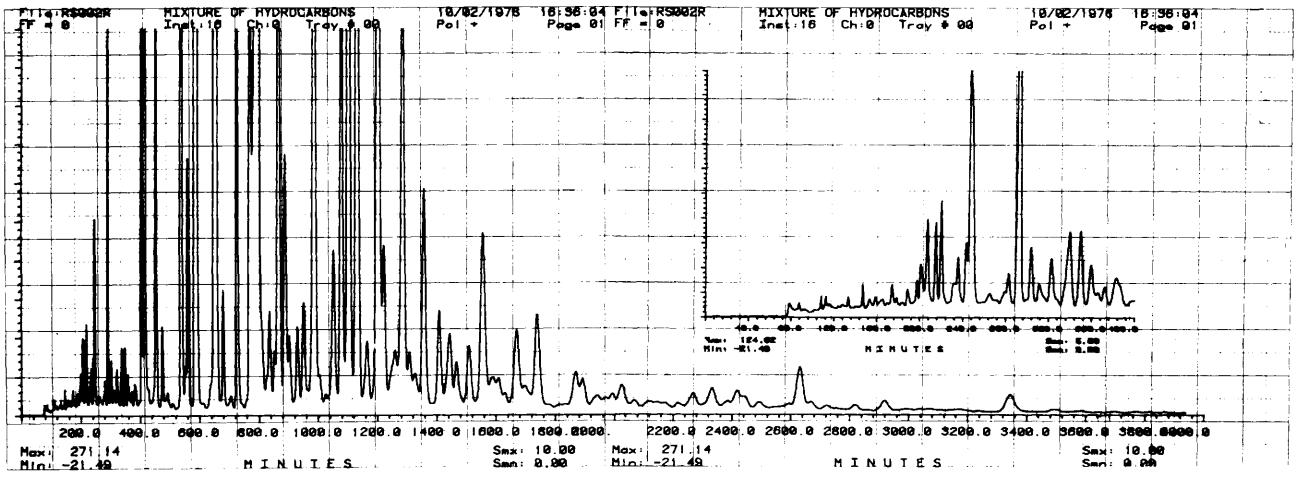

Figure 3 Chromatogram from a high resolution column.

7 The Basic Liquid Chromatograph

The basic liquid chromatograph is shown as a block diagram in Figure 4.

The chromatograph consists of seven major components, a solvent supply module, an optional solvent programmer, a pump, the sample valve, a column oven, the detector, and the data acquisition, processing, and display system.

7.1 The Solvent Supply System

The solvent supply system consists of a series of solvent reservoirs, usually four in number, from which one or more solvents can be selected. They are constructed of stainless steel or glass and normally have a capacity of about one litre and some degassing facility such as a supply of helium gas to each reservoir. The degassing system is essential as, on mixing, some solvents evolve dissolved air which adversely effects the column performance and causes serious detector noise.

7.2 The Solvent Programmer

The solvent programmer has two functions which are usually programmed from a keyboard associated with the programmer. One function is to select a particular mixture of solvents to be used to develop a separation isocratically. The second is to arrange the composition of the mobile phase to change regularly, in a pre-defined manner, during the development of the separation. Many mixtures contain solutes covering a wide range of polarities. Consequently, if the separation is carried out isocratically, some solute will elute very rapidly and others will be eluted very late in the chromatogram. The separation time can be shortened by changing the mobile phase composition during development, accelerating the strongly retained solutes through the column with solvent mixtures of greater strength. The majority of samples can be satisfactorily separated by employing two solvents only, although a few may require ternary mixtures. It is very rare indeed that four solvents are necessary. Thus, most programmers are designed to program two or three solvents and, although a number of four solvent programmers are available, they would only be useful for very special samples.

7.3 The Solvent Pump

Solvent pumps are usually piston operated and most contain two cylinders, operating alternately in parallel to reduce pressure pulses that can cause detector noise. All parts in contact with the solvent are made of stainless steel except for non-return valves, seats, and gaskets that may be made of sapphire or PTFE. Most pumps have a maximum pressures of 6000 or 10 000 p.s.i. Pumps can be made to operate at pressures above 10 000 p.s.i. but it is not the pump that limits the maximum pressure that can be employed in the chromatographic system. One of the problems associated with operating columns at extremely high pressures is the heat that is generated, which can seriously effect column performance. Flow rate ranges for analytical purposes should extend from a few μl per minute to about 10 ml per minute.

7.4 Sample Valves

Sample valves can have internal or external loops and a range of sample volumes should be available from about 0.5 μl to 10 μl. For most purposes the valve can also be made of stainless steel but for biochemical separations the material may need to be *bio-compatible*. Many labile materials of biological origin easily degrade or denature in contact with heavy metals. For this reason sample valves are often made of titanium, or have titanium liners, as this material is bio-compatible. Sample valves can be made to withstand pressures up to 10 000 p.s.i. but their lifetime at this pressure can be very limited. This is due to particulate contaminates in samples (which are almost impossible to eliminate in practice) causing continual wear on the sealing surfaces of the valve. A sample valve is best operated at a maximum pressure of about 3000 p.s.i. to ensure a reasonably long life.

7.5 Column and Column Oven

The column, as already stated can be of stainless steel, but for biochemical separations it, also, may need to be constructed from titanium. The column oven thermostats the column to ensure stability and helps dissipate the heat generated in the column. The retention time is linearly related to the distribution coefficient which, in turn, will depend on the operating temperature. Consequently, for consistent retention data the column *must* be thermostated. Provision should also be made to bring the temperature of the mobile phase to that of the column before it enters the sample valve and column. As the mobile phase has a fairly high specific heat it is of little use to employ an air bath as a thermostat. A liquid thermostating medium is strongly recommended.

7.6 Detectors

Books have been written solely on the subject of LC detectors so the treatment here must, of necessity, be somewhat cursory. An LC detector must respond to solute concentrations over the range of about 10^{-9} to 10^{-5} g/ml and have a linear response to concentration over at least three orders of magnitude. Prefera-

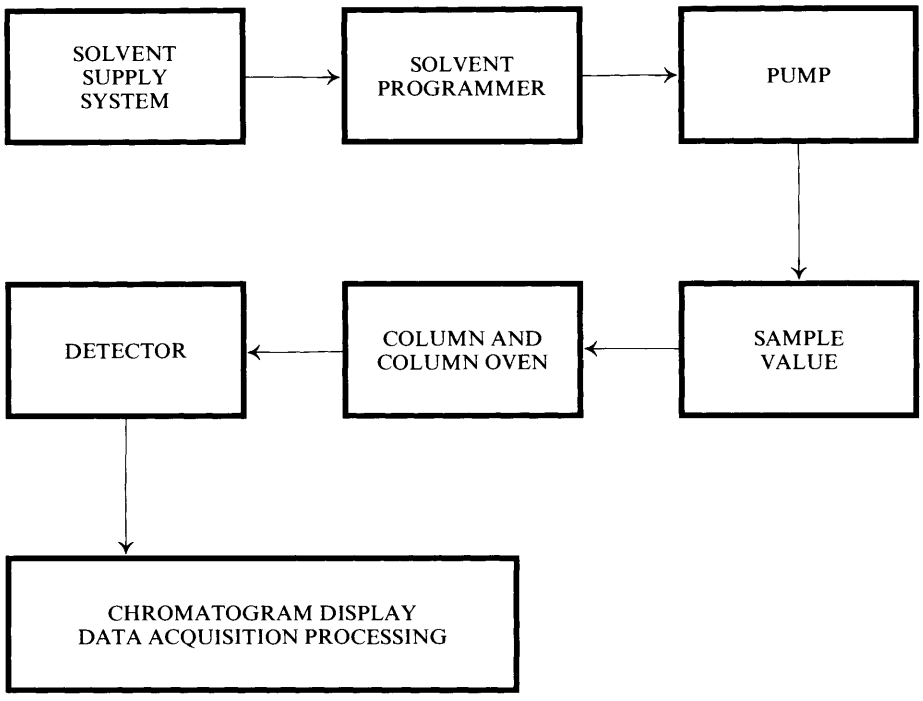

Figure 4 Block diagram of a liquid chromatograph.

bly, the detector should respond to solutes present in the mobile phase but be insensitive to the mobile phase solvents themselves or changes in solvent composition. The detector should also be insensitive to changes in pressure, temperature, and flow rate. Unfortunately, an LC detector with all these attributes does not exist but some approach this performance. If a group of different detectors is available, then one can be chosen to have the attributes necessary for the particular sample in hand. There are four LC detectors in common use. The fixed and variable wavelength UV detector, the refractive index detector, the electrical conductivity detector, and the fluorescence detector.

The fixed wavelength UV detector normally operates at 254 nm (the light emitted from a low pressure mercury lamp) although other wavelengths are available. It has a linear response of over three orders of magnitude extending from about 3×10^{-8} to 1×10^{-5} g/ml, but this will vary somewhat with the extinction coefficient of the solute. If designed correctly, it is relatively insensitive to changes in temperature, pressure, and flow rate and thus, can be used satisfactorily with gradient elution. It is the most useful general detector available but is not suitable for detecting paraffins, aliphatic alcohols, sugars, *etc.* nor any substances that do not adsorb significantly at 254 nm. The variable wavelength detector normally employs a deuterium lamp that emits light from about 180 nm to about 400 nm and can take two forms: the dispersive detector and the diode array detector. The dispersive detector contains a monochrometer prior to the detector cell by which the wavelength required is selected. The flow can be stopped at any time and the contents of the cell scanned and a UV spectrum obtained if so desired. The diode array detector has a diffraction grating subsequent to the cell and disperses the transmitted light across a diode array, the outputs from which are stored in a memory bank. Thus, the contents of the detector cell is monitored simultaneously over a range of wave lengths and, at any time, a spectrum can be printed out. This detector also has a linear response of about three orders of magnitude extending from about 1×10^{-7} to 3×10^{-4} g/ml but is slightly less sensitive than the fixed wavelength detector. In practice the variable wavelength detector is largely used to provide a choice of fixed wavelength detection using the specific wavelength that the solutes adsorb most strongly. With the exception of some aromatic compounds, UV spectra are not very informative relative to the IR or mass spectrum for structural elucidation. Consequently, the use of UV spectra obtained from variable wavelength detectors for solute identification is not very common.

The refractive index detector is far less sensitive than the UV detector and has a linear dynamic range of less than three orders of magnitude from about 5×10^{-7} to about 2×10^{-4} g/ml. It is very sensitive to temperature changes and thus should be thermostated. It is also sensitive to both changes in pressure and flow-rate and consequently is not suitable for use with gradient elution. Despite its disadvantages it is frequently used for detecting those solutes that do not adsorb in the UV and do not fluoresce.

The electrical conductivity detector, as its name suggests, measures the conductivity of the mobile phase and is frequently used in ion exchange chromatography. It is relatively insensitive to flow rate and thus can be employed for gradient elution, providing the gradient does result in a continuous change in the conductivity of the mobile phase. It has a linear dynamic range of about three orders of magnitude from about 1×10^{-8} to 1×10^{-5} g/ml but this range varies a little from detector to detector.

The fluorescence detector is the most sensitive detector in common use. It has a response ranging from about 1×10^{-9} to 1×10^{-5} g/ml but, unfortunately, a linear dynamic range of only just over two orders of magnitude. Nevertheless, its high sensitivity and other features make it a very useful detector. The fluorescence detector is insensitive to temperature changes, pressure changes, and changes in flow rate and, consequently, can be employed with gradient elution. It also has the advantage of being a selective detector and thus can pick out a specific component of a mixture that fluoresces, from a host of other unresolved undetected peaks which do not fluoresce.

7.7 Data Acquisition, Processing, and Display

The results obtained from a chromatographic separation, as depicted by the output from the detector, can be displayed at many levels of sophistication. The simplest way of displaying a chromatogram is to use a potentiometric recorder and many chromatographers still use this method of recording data. The heights of the peaks can be measured and used for quantitative analysis and the retention distance for peak identification. Most

modern chromatographs, however, have A/D converters and the chromatogram is digitized and stored on disc by a computer. A simple program can then present a table of results that prints out the retention times and peak heights or peak areas and, if previously calibrated, a complete quantitative analysis. Very subtle algorithms can be included in the program to obtain accurate quantitative results from partially resolved peaks, or from small peaks eluted on the tails of large peaks. Unfortunately, however clever the algorithm, it will always be a poor substitute for good chromatography.

8 Quantitative Analysis

Quantitative analysis is carried out using peak heights or peak areas. Most manual analyses are carried out using peak heights, or the product of the peak height and peak width at half height. Peak heights, although simpler to use, are very dependent on a constant mobile phase flow rate and thus computer processed data usually employ peak area measurements. Under some circumstances, a normalization procedure can be employed and the percentage of any given component is expressed as the percentage area of the peak of the total area of all the peaks. This procedure is only valid if the detector has the same response to all solutes. This condition is rare, but can be used, for example, in the analysis of high molecular weight polymers when a refractive index detector is employed.

Quantitative data are normally obtained using *internal* or *external* standards. If an internal standard is employed, the standard is added to the mixture and the ratio of the areas of the peaks of interest to that of the standard, corrected for their relative detector response, will give directly the percentage of each component. The response factors are obtained from calibration runs using known mixtures of the standard and the solutes of interest. If an external standard is used, it is run as a separate chromatogram and the areas of each peak in the sample chromatogram compared with that of the standard in the reference chromatogram. The external standard procedure is not as accurate as the internal standard, but eliminates the need to search for a substance to act as the internal standard that must be eluted in a position in the sample chromatogram where no other peaks occur. In fact, the external standard can be the same as the solute of interest thus eliminating the need for relative response factors. Experienced chromatographers, operating in a single laboratory, taking great care, can achieve a precision of about ± 3%. However, between-laboratory trials have shown that, in many cases, a precision of ± 10% might be considered very satisfactory. High accuracy and precision is *not easily* obtained in LC analyses and the presentation of the results on a computer screen in glorious colour does nothing to improve the situation. The extra cost might be more usefully spent elsewhere in the apparatus.

9 Tandem Techniques

Tandem techniques is the term originally given to combined instruments that included both a chromatograph and a spectrometer. These twin systems were provoked by the development of high efficiency columns in GC which were applied to the separation of complex mixtures such as essential oils. Compounds of hitherto unknown structure were separated and it was immediately apparent that a method was needed to identify them. As a consequence, the mass spectrometer was directly associated with the gas chromatograph providing mass spectra of each component as it was eluted. The same motivation resulted from the development of high efficiency LC columns, to provoke the association of the liquid chromatograph with different types of spectrometer. Books have been written on this subject so the treatment of tandem techniques in this review must also be somewhat cursory in nature. The association of the liquid chromatograph with a spectrometer proved to be far more difficult than for a gas chromatograph. The first LC tandem technique was introduced by McLafferty *et al.* who passed a fraction of the eluent directly from the column into the mass spectrometer using solvent vapour as the chemical ionization agent. The result was crude, in the sense that the solute bands were badly dispersed in the interface between the two instruments, and much of the resolution was lost, but the feasibility of the system was demonstrated. A transport system was then introduced similar in principle to the transport LC detector. This consisted of a moving wire, or band, that passed through the LC column eluent, leaving a coating of solvent containing the solute on the wire. The solvent was evaporated and the residual solute, coated on the wire, passed through two vacuum locks into the mass spectrometer. Inside the mass spectrometer, and close to the ion source, the solute was thermally volatilized from the wire and the spectrum produced. This method had the advantage of producing electron impact spectra which are more useful in structural identification than chemical ionization spectra. The most common and sensitive method of sample introduction for LC-MS today is the Thermospray interface. The eluent from the column is passed directly into the mass spectrometer through a tube, the end of which is heated, and the eluent is violently evaporated in spray form into the ion source. The solvent vapour is also used as the chemical ionizing agent. The Thermospray interface has proved very successful but expensive and a little cumbersome. The tandem technique LC-IR has also been developed, but has been found less useful than LC-MS. The system is less sensitive and IR spectra provide less information for structural identification than MS spectra. There is a problem with the column eluent as most solvents used in LC do not have a 'transmission window' over the wavelength range where the most useful data for structural information is obtained. There have been transport methods developed where the eluent is allowed to drip onto a KBr disc, the solvent evaporated and the disc then automatically placed in the light path of a IR spectrometer. However, this procedure appears to have been developed more as an act of desperation than a serious practical technique. The most useful spectroscopic technique for structure elucidation is, without doubt, NMR. However, NMR is a relatively insensitive technique and it is difficult to obtain enough sample from a liquid chromatograph to provide useful spectra. On line LC-NMR has been attempted by employing a stainless steel, small bore column that projects into the field of the NMR magnet. Surprisingly, this does not seriously disturb the homogeneity of the field and on-line spectra of good quality have been obtained. The liquid chromatograph has also been associated very successfully with the atomic absorption spectrometer and the combination has been used to follow heavy metal speciation in biological samples. The successful association of the Flame AA with the liquid chromatograph is a direct result of the high sensitivity of the spectroscopic technique and the simplicity of the interface.

It must be said, however, that tandem techniques are bulky, usually difficult to operate, and very expensive. The best approach in LC to the structural identification of eluted solutes is still by an off-line procedure. The peaks of interest should be collected and submitted for spectroscopic examination as a separate experiment. An off-line, high resolution mass or NMR spectrum of an unknown solute will provide far greater structural information than any tandem technique presently available.

10 The Present Status of LC

LC development by the extrapolation of present knowledge is now proceeding very slowly and, indeed, the technique has nearly reached the stable condition of GC. Column theory has progressed to the point where the limits of resolution and speed of analysis, attainable from packed or capillary columns, is known and the possibilities and impossibilities of the technique recognized. Instrumentation continues to become more sophisticated, impressive, costly, and unfortunately sometimes irrelevant, but the limits of column performance have not changed much in nearly a decade. This means that the separating

potential of LC has remained the same. There are, however, real needs in the biotechnology field to render the technique more amenable to samples of biological origin and in this area future developments may be exciting and rewarding. At any time, of course, a completely new concept could alter this relatively static condition dramatically, however, without such innovation, LC performance is likely to remain the same for some time to come.

11 Books Recommended for Further Reading

11.1 General LC

'High Performance Liquid Chromatography', ed. P. R. Brown and R. A. Hartwick, John Wiley and Sons, New York, Chichester, Brisbane, Toronto, Singapore, 1989.
'Practical High Performance Liquid Chromatography', ed. C. F. Simpson, Heyden and Son Ltd., 1976.
'Modern Practice of Liquid Chromatography', ed. J. Kirkland, John Wiley and Sons, New York, Chichester, Brisbane, Toronto, Singapore, 1989.

11.2 Chromatography Theory

R. P. W. Scott, 'Liquid Chromatography Column Theory', John Wiley and Sons, New York, Chichester, Brisbane, Toronto, Singapore, in press.
A. S. Said, 'Theory and Mathematics of Chromatography', Hüthig, Heidelberg, Basel, New York, 1981.
J. C. Berridge, 'Techniques for the Automated Optimization of HPLC Separations, 1985, John Wiley and Sons, New York, Chichester, Brisbane, Toronto, Singapore, 1985.

11.3 Quantitative Analysis

'Quantitative Analysis in Chromatographic Techniques', ed. E. Katz, John Wiley and Sons, New York, Chichester, Brisbane, Toronto, Singapore, 1987.

11.4 Liquid Chromatography Detectors

R. P. W. Scott, 'Liquid Chromatography Detectors', Elsevier, Amsterdam, Oxford, New York, Tokyo, 1986.
T. M. Vickrey, 'Liquid Chromatography Detectors', Marcel Dekker, Inc., New York and Basel, 1983.

11.5 Applications

J. F. Lawrence, 'Liquid Chromatography in Environmental Analysis', Himana Press, Clifton, New Jersey, 1984.
K. Gooding and F. Regnier, 'HPLC of Biological Molecules', Marcel Dekker, Inc., New York and Basel, 1990.