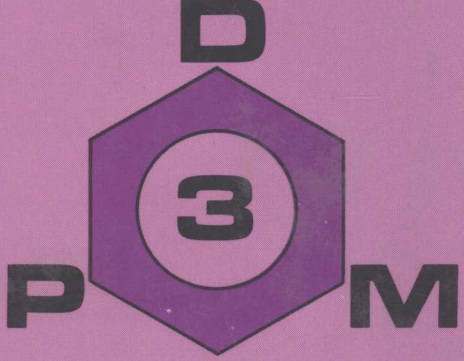
PROGRESS IN DRUG METABOLISM



Edited by
J. W. Bridges
and L. F. Chasseaud

Progress in Drug Metabolism

Volume 3

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J. W. Bridges

Institute of Industrial and Environmental Health and Safety, University of Surrey

L. F. Chasseaud

Department of Metabolism and Pharmacokinetics, Huntingdon Research Centre

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Contributors to Volume 3

S. BRECHBÜHLER — *Ciba–Geigy AG, Basel, Switzerland*

G. T. BROOKS — *Agricultural Research Council, Unit of Invertebrate Chemistry and Physiology, University of Sussex, Brighton, Sussex*

I. C. CALDER — *School of Chemistry, University of Melbourne, Victoria, Australia*

J. P. DUBOIS — *Ciba–Geigy AG, Basel, Switzerland*

D. H. HUTSON — *Shell Toxicology Laboratory (Tunstall), Sittingbourne Research Centre, Sittingbourne, Kent*

F. OESCH — *Institute of Pharmacology, University of Mainz, Mainz, West Germany*

G. B. QUISTAD — *Biochemistry Department, Zoecon Corporation, Palo Alto, California, USA*

W. RIESS — *Ciba–Geigy AG, Basel, Switzerland*

D. A. SCHOOLEY — *Biochemistry Department, Zoecon Corporation, Palo Alto, California, USA*

Preface

The two recent major advances in instrumentation useful to workers in drug metabolism have been those connected with mass spectrometry and liquid chromatography. Mass spectrometry was reviewed in Volume 1 and the use of stable isotopes in mass spectrometry in Volume 2. Liquid chromatography and its applications are now covered in the present volume.

The thinking behind the analytical needs of drug metabolism has received scant attention in the literature. Because of the heavy commitment of those working in drug metabolism to analysis, an examination of the principles underlying assay selection is included in the present volume.

As illustrated by this volume, reviews in this series are not confined to subjects connected with only pharmaceuticals. The term 'drug metabolism' is used in this series to encompass all classes of compounds and their fate in any biological system *in vivo* or *in vitro*.

Epoxides as potentially toxic intermediates in the metabolism of many compounds were discussed in Volume 1. This theme is developed in the present volume through a review on epoxide hydratase.

J. W. BRIDGES
L. F. CHASSEAUD

Contents

CHAPTER 1

High-pressure, high-resolution liquid chromatography and its application to pesticide analysis and biochemistry

D. A. Schooley and G. B. Quistad

INTRODUCTION

The development of liquid chromatography has been characterized by two periods of dormancy. This technique is generally acknowledged to have been discovered by Tswett, who in 1903–6 separated plant pigments by liquid column chromatography with calcium carbonate and other adsorbents. As discussed by Ettre and Horvath (1975) in an historical review, chemists of Tswett's era felt that his 'chromatographic analysis' was unsuited for preparative work, in an

era when preparative isolation was accomplished by extraction, distillation, or crystallization. Column adsorption chromatography saw little use in the ensuing decades, but in 1931, Kuhn, Winterstein, and Lederer triggered a popularization of the technique with a preparative isolation of xanthophylls by bed development in a column. The technique was soon widely adopted; modifications were made such as the extension from bed development to elution chromatography (Reichstein and Van Euw, 1938). The popularity of column chromatography in the 1930s inspired the development of liquid–liquid partition chromatography (Martin and Synge, 1941), paper chromatography (Consden, Gordon, and Martin, 1944), reversed-phase chromatography (Howard and Martin, 1950), and gas–liquid chromatography (James and Martin, 1951).

However, further development of adsorption chromatography was relatively modest from the late 30s until about 1968–9, when several firms introduced 'high pressure liquid chromatographs' as integral units. Probably the rapid maturation of gas–liquid chromatography (g.l.c.) as a tool for qualitative and quantitative analysis, and the rapid popularization of thin-layer chromatography (t.l.c.), contributed to the neglect of technological advances in column liquid chromatography during the 50s and early 60s. It remains curious that this oldest of chromatographic techniques was the slowest to mature.

The renaissance in liquid chromatography (l.c.) has been accepted most eagerly by researchers in biochemistry, natural products chemistry, pharmaceuticals (reviewed by Wheals and Jane, 1977), pesticide chemistry, and related areas where the chemicals of interest are frequently too non-volatile or unstable for g.l.c. separation. The early dogma that liquid chromatography would not be useful for preparative purposes has been clearly refuted, and its superiority in this respect over g.l.c. is becoming widely recognized. Rapid improvements have been made in l.c. instrumentation—especially in pumping systems, injectors, and columns—so that rapid analyses can be made with high resolution. Detection of samples by l.c. remains more of a problem than in g.l.c., especially with respect to the lack of a high-sensitivity, 'universal' detector (like the hydrogen flame ionization detector in g.l.c.) and also with respect to the relatively immature technology of most types of selective, high-sensitivity l.c. detectors. The latter factor may explain why adoption of modern l.c. for detection and quantitation of pesticide residues has been comparatively slow.

Nevertheless, liquid chromatography is playing a rapidly increasing role in pesticide analysis. Prior to presenting an overview of applications of modern l.c. to pesticide analysis (metabolism studies, residue analysis, and formulations), we shall present a general discussion of this technique, especially those facets most relevant to these applications. A number of texts are available (Snyder and Kirkland, 1974; Brown, 1973; Perry *et al*, 1972; Kirkland, 1971a) which discuss in detail, theory, instrumentation, and general applications of the various modes of liquid chromatography. We present a condensed, largely non-mathematical discussion of chromatographic theory, with comparisons between g.l.c. and l.c., since basic understanding of theory is essential for efficient use of l.c. methods. We discuss apparatus only in general terms, because of previous

coverage and the rapid evolution of commercial instruments. Because of the importance of specific, sensitive detectors in pesticide analysis, special emphasis is given to this subject. Exact conditions for analysis of over 220 pesticides are presented in tabular form, and general discussion of preferred techniques for l.c. analysis of pesticides is arranged according to structural classification. Residue analysis of pesticides was reviewed by Horgan (1973) and Moye (1975), so our emphasis in this area is on subsequent work. Reviews of l.c. applications to multiresidue analysis have been presented by Sidwell (1977) and Ishii (1976, in Japanese). We have attempted as thorough a literature coverage as possible until January 1978. We have not reviewed use of classical column chromatography for clean-up of pesticide residues or as a purification technique in synthetic chemistry.

Finally, there is no standard abbreviation for this technique. Most widely used is h.p.l.c. which can represent a choice between high pressure l.c., high performance l.c., or perhaps even high price l.c.! Also used are high resolution liquid chromatography, h.r.l.c.; high efficiency l.c., h.e.l.c.; and high speed l.c., h.s.l.c. In the authors' opinions, resolution, efficiency, and speed are more evocative of the results obtained with this method than 'pressure' or 'performance'. To compromise, we shall use l.c. (the abbreviation favoured by Snyder and Kirkland, 1974) and allow the readers to append prefixes of their choice if desired.

THEORY

In this section chromatographic theory is reviewed briefly. For newcomers to this field, supplementary discussion can be found in the texts referenced previously.

Band Broadening and Column Efficiency

Column efficiency is determined according to the theoretical plate model advanced by Martin and Synge (1941). Thus, the column is imagined to consist of discrete regions termed theoretical plates, each of which corresponds to a single step of a counter-current distribution apparatus (or a separatory funnel). When the number of theoretical plates (N) is sufficiently large, the band shape of a discrete species of retarded solutes moving through a chromatography column should approximate a Gaussian distribution. The value of N can be determined from the retention volume (V_R) and the bandwidth ($w = 4\sigma$) as shown in figure 1. This model requires the unrealistic assumption that a chromatographic system consists of discrete regions. Actually, in moving through a chromatographic bed, a solute is either adsorbed to the support and stationary, or in solution and moving with the velocity of the mobile phase until it is next adsorbed. Such motion corresponds to a 'random walk' mathematical model. Statistical analysis of a sharp band of solute molecules so moving through a column also reveals that the molecules will gradually assume a Gaussian distribution, with the band broadening as migration increases.

Such models assume instantaneous equilibrium of solute between mobile and stationary phase, and ignore rates of diffusion, mobile phase velocity, packing

particle size, and other factors. Consideration of the dynamics of the chromatographic process is necessary to describe quantitatively the parameters that contribute to band spreading in chromatography. The first comprehensive theory to relate these parameters was provided by van Deemter *et al* (1956). Data on which their theoretical interpretations were based were derived from g.l.c., due to convenience of measurement at that time. Studies of the effect of changes in mobile phase velocity on the height equivalent to a theoretical plate, H (= number of plates N/column height in mm), revealed curves that are

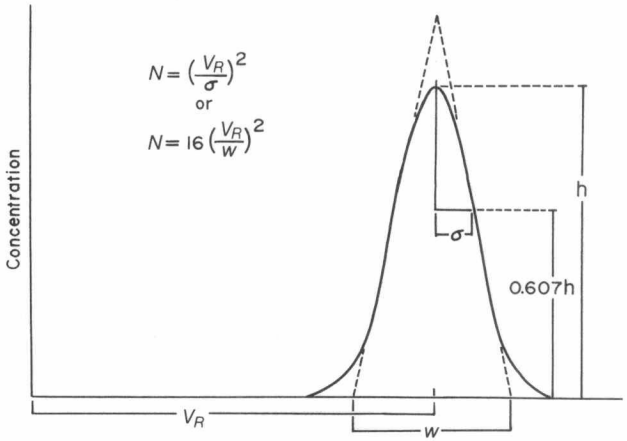

Figure 1 Definition of parameters and equations used in determining the efficiency of a chromatographic column. An idealized chromatographic peak is Gaussian and the peak width (w) is equal to four standard deviations (4σ). These parameters are measured by (a) determining peak width at $0{\cdot}607h$ to obtain 2σ, or (b) drawing tangents at the inflection points ($0{\cdot}607h$) to obtain w. Measurement of peak width at halfheight gives $2\sigma \times \sqrt{(2 \ln 2)}$, requiring use of a different formula: $N = 5{\cdot}54\ (V_R/\text{halfheight width})^2$. From Schooley and Nakanishi (1973), reproduced by permission of Academic Press

roughly hyperbolic with a pronounced decrease in efficiency (increase in H) found below certain values of the carrier velocity (figure 2). The optimum carrier velocity for minimum H (maximum efficiency) and the curve shape are quite dependent on the type of carrier gas, so their consideration is thus important for achieving maximum efficiency in g.l.c. The van Deemter theory is summarized in equation (1).

$$\underset{(A)}{} \quad \underset{(B)}{} \quad \underset{(C)}{}$$

$$H = 2\lambda d_p + \frac{2\gamma D_m}{v} + \frac{\omega d_p^{\,2}}{D_m} v \qquad (1)$$

where d_p is the average particle diameter of packing, D_m the diffusivity of sample in the mobile phase, v the mobile phase velocity, and λ, γ, ω are constants of order unity, characteristic of the type of packing and bed structure. Equation (1) consists of three terms. The first (A) is the contribution of eddy diffusion, or diffusion due to solute molecules moving, not in a straight line, but around and under particles, and is generally considered to be independent of mobile phase

velocity. The second factor (B) causing band broadening is axial (or longitudinal) diffusion, the tendency of solute to diffuse away from band centre with time, and is inversely proportional to solvent velocity. The last term (C) is the resistance of sample to mass transfer and is proportional to mobile phase velocity. 'Mass transfer' is an expression of the time spent by solute molecules diffusing into or out of the support and/or bound to specific sites in adsorption chromatography. While the above equation is somewhat oversimplified, it

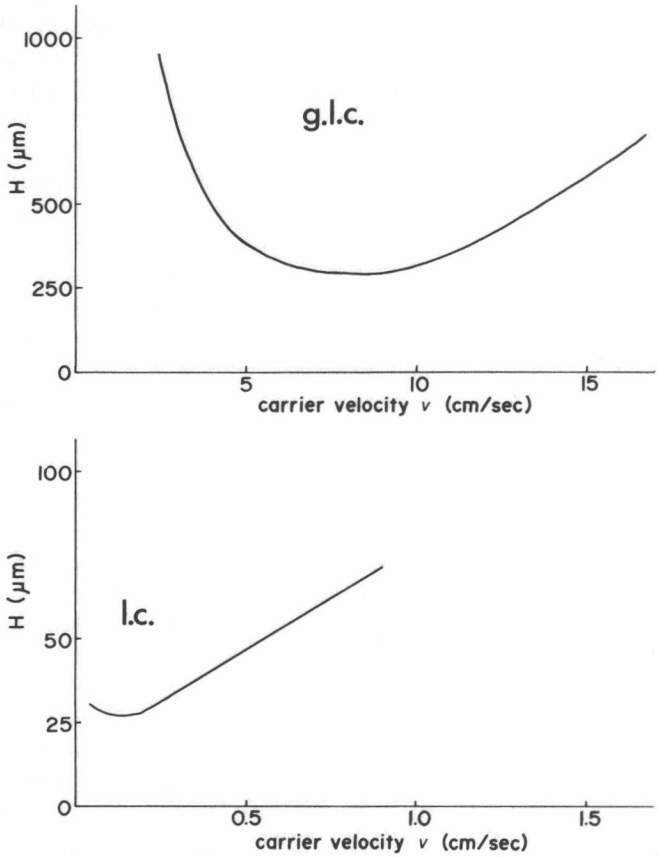

Figure 2 Approximation of curves obtained for the dependency of theoretical plate height (H) on carrier velocity (v) for g.l.c. (upper) and l.c. (lower). Note that the axes are 10-fold exaggerated below because of the lower values of H and v in l.c.

nevertheless provides valuable insight into the factors controlling band broadening, so that we may understand how to maximize efficiency.

The most important differences between the techniques of l.c. and g.l.c. are attributable to the difference in properties of liquids and gases. As pointed out by Giddings (1965), the most crucial distinction is that gases show almost no attraction for solute molecules, whereas liquid mobile phases can interact with solutes by several mechanisms. Thus, in l.c., *selectivity* is determined both by

mobile phase and stationary phase, while in g.l.c. only the stationary phase is of importance. Differences in column *efficiency* between l.c. and g.l.c. arise because diffusivity of solute is 10^4–10^5 times slower in liquids than gases, and viscosities of liquids are $\sim 10^2$ higher than those of gases. Returning to the van Deemter equation, we see that the enormously lower solute diffusivity in l.c. will make the axial transfer term (B) of very little consequence. Since this term is inversely proportional to solvent velocity, van Deemter-type plots for l.c. show less tendency for 'optimum' carrier velocities than in g.l.c., for only at inconveniently slow carrier velocities is an increase in H (decrease in N) noted (figure 2). The mass transfer term (C) is proportional to mobile phase velocity, but inversely proportional to solute diffusivity. To obtain high efficiency in l.c., mobile phase velocity must be lower than in g.l.c. to accommodate the lower diffusivity. The far lower sample diffusivities in l.c. diminish the importance of the B term, but increase the importance of the C term, when compared to g.l.c.

Decreasing the packing particle diameter will not only decrease band broadening due to eddy diffusion (A), but especially band broadening due to mass transfer (C) which is dependent on the square of particle diameter. Thus, there has been a steady trend towards smaller l.c. packings with the current technology represented by 'microparticulate' packings in the 5–10 μm range. While it is theoretically desirable to use small diameter packings in g.l.c., it is not generally feasible to use packings smaller than 125–150 μm (100–120 mesh), as otherwise column back pressure becomes excessive.

The high viscosity of liquids compared to gases ($\sim 100\times$) immediately suggests the need for proportionately higher column inlet pressures in l.c. This situation is mitigated by the lower carrier velocities used in l.c. (typically $\sim 0{\cdot}25$ cm/s versus ~ 10 cm/s in g.l.c.). On the other hand, the column permeability is inversely proportional to the *square* of particle diameter, so that the use of microparticulate packings frequently leads to back pressures of 30–200 atm ($\sim$ 500–3,000 psi) for a 25–30 cm column. Snyder (1971) has pointed out the desirability of using eluents with the lowest viscosity, because increased viscosity causes an almost directly proportional loss in N (with carrier velocity and column identity held constant). Again considering equation (1), the sample diffusivity (D_m) is higher in less viscous solvents, which decrease the C term (a major contributor to l.c. band spreading), but increase the less important B term.

The preceding considerations explain why a modern analytical l.c. column is shorter, packed with smaller particles, and eluted more slowly than a g.l.c. column. Since H for a properly packed 10 μm silica l.c. column may approach 0·02 mm ($= 2d_p$), a 25 cm analytical column can exhibit 10,000 plates. In contrast, an efficiently packed g.l.c. column will on occasion show $H = 0{\cdot}3$ mm, requiring a 300 cm column to produce 10,000 plates.

Retention

Retention in l.c. is measured by a parameter termed the capacity factor (k'), defined as the ratio of the amount of solute in the stationary phase (N_s) to the amount of solute in the mobile phase (N_m). Larger values of k' indicate more of

the solute to be in the stationary phase, and hence more strongly retained. Equation (2) shows that k' is also equal to the thermodynamic equilibrium distribution coefficient, K, times the ratio of the volume of stationary

$$k' = \frac{N_s}{N_m} = K\frac{V_s}{V_m} \tag{2}$$

phase (V_s) to the volume of mobile phase (V_m) in the column (more commonly termed the void volume, V_0). From other relationships, it can be shown that

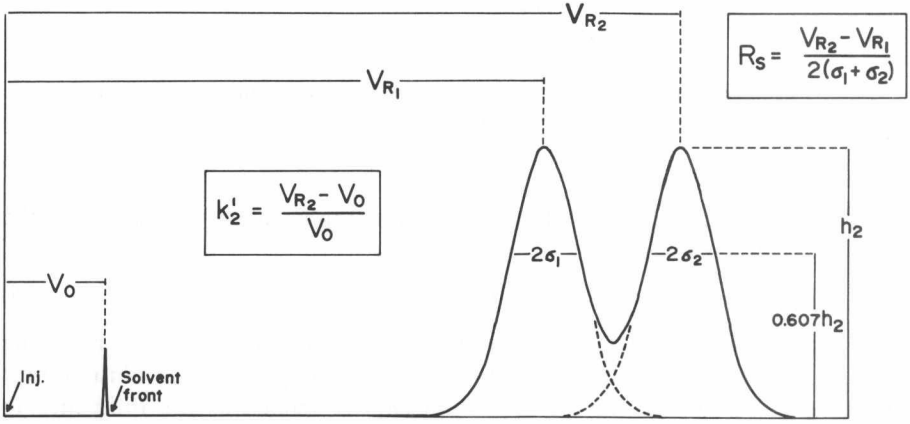

Figure 3 Parameters used for measuring (a) retention (capacity factor, k') and (b) resolution (R_s). Measurement of column void volume (V_0) is usually obtained with an unretained solute. While some texts recommend measurement of w for R_s determination, measurement of 2σ is easier for partially overlapping peaks

k' is related to the experimentally observed V_0 and the retention volume (V_R) of the solute peaks as shown in equation (3) and figure 3.

$$k' = \frac{V_R - V_0}{V_0} \tag{3}$$

It is occasionally of interest to compare R_f values observed on t.l.c. to k' values obtained for the same solutes on l.c. (Table 1). The relationship between k' and R_f is given by the expressions in equations (4).

$$k' = \frac{1 - R_f}{R_f}; \quad R_f = \frac{1}{k' + 1} \tag{4}$$

Strictly speaking, such a transformation is valid only if adsorbent of identical structure and activity is used for both the l.c. and t.l.c. experiments. These equations may nevertheless provide a means of extrapolating from existing t.l.c. data for choice of an approximate solvent system for l.c. analysis of an unfamiliar solute. For a systematic study of this problem, see Hara (1977). The recent introduction of alkyl-bonded (C_{18}) reversed-phase t.l.c. plates will be valuable for scouting solvent systems for reversed-phase l.c.

Table 1 Comparison of R_f and k'

R_f	k'
1	0
0·75	0·333
0·5	1·0
0·4	1·5
0·333*	2·0
0·2	4
0·1	9
0	∞

*Snyder (1968) has shown that an R_f of $\frac{1}{3}$ gives optimum resolution (valid for single bed development in t.l.c. only).

Resolution

The relative retention (α) of two chromatographic peaks is defined as the ratio of their k' values (equation 5).

$$\alpha = k'_2/k'_1 \tag{5}$$

When $\alpha = 1{\cdot}1$ or less, the resolution of peaks is fairly difficult on l.c., requiring 2,000–5,000 theoretical plates (depending on how complete the resolution must be). The quantitative value of resolution (R_s) is defined as the difference in retention volumes of two peaks divided by half the sum of their band widths (4σ) (equation 6).

$$R_s = \frac{V_{R2} - V_{R1}}{2\sigma_2 + 2\sigma_1} \simeq \frac{V_{R2} - V_{R1}}{4\sigma} \tag{6}$$

From equation (6) and other relationships, a general equation (7) can be derived which expresses resolution in terms of selectivity (*I*), capacity factor (*II*) and efficiency (*III*) (values of k' and N are measured for the slower eluting component).

$$\underset{(I)}{\left(\frac{\alpha-1}{\alpha}\right)}\ \underset{(II)}{\left(\frac{k'}{1+k'}\right)}\ \underset{(III)}{(\sqrt{N})}$$

$$R_s = \frac{1}{4}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k'}{1+k'}\right)(\sqrt{N}) \tag{7}$$

Equation (7) provides insight into the factors controlling resolution, and therefore which parameters should be altered to improve a separation.

The first step in developing a separation is to find a proper k' value (i.e. to optimize the solvent polarity). The retention term in equation (7) shows that as k' goes to zero, so does resolution. There is also little point in choosing excessively long k' values (> 10). While it is important to choose an efficient column, term (*III*) of equation (7) shows that resolution increases only with the square root of N. For a given column, N is maximized by choosing low viscosity solvents or heating the column to reduce the viscosity. Other approaches to increase N are to use a column with packing of smaller d_p (equation 1) or to increase

column length by adding series-connected columns. The latter approach may frequently be a last resort, since quadrupling column length is required to double resolution. For trace analyses, highly efficient columns are desired because they produce sharper peaks of higher amplitude, enhancing detectability.

The selectivity term (I) of equation (7) is characteristic of the column packing and solvent combination. Usually the most direct way of improving a difficult l.c. separation is to choose either a different solvent mixture or column, analogous to changing the stationary phase in g.l.c. The ability of various solvents to interact with solutes *via* different mechanisms—dispersion, dipole interaction, or hydrogen bonding (either proton donors or acceptors)—provides many possibilities for altering α.

MODES OF LIQUID CHROMATOGRAPHY

Classically, four modes of l.c. are recognized, based on the mechanism of separation. Liquid–solid chromatography (l.s.c.) is historically the oldest technique, with separations based on the selective adsorption of samples by a solid from the mobile phase. Commonly used adsorbents are silica, alumina, magnesium silicate, and occasionally others such as charcoal.

Liquid–liquid or partition chromatography (l.l.c.) is similar to l.s.c. except that the solid support is coated with a liquid stationary phase which is immiscible with the mobile phase. It is necessary to presaturate the mobile phase with stationary phase to retard the dissolution of the stationary phase from the column, and to control carefully the temperature of the system. Usually a precolumn is inserted between the pump and injector to assure that the eluent is presaturated with stationary phase.

In both l.s.c. and l.l.c, it is rare that a pure solvent is used as the mobile phase. Usually, mixed solvents must be used to adjust the solvent strength to the proper level. It may frequently be difficult to decide if a separation is purely adsorptive or purely partitioning; in many cases a mixture of processes may be occurring. This is especially true if the eluent contains appreciable amounts of polar components such as water, acids, glycols, or alcohols, which are strongly bound by supports such as silica or alumina.

Both l.l.c. and l.s.c. can be further subdivided into 'normal' and reversed-phase categories, according to the nomenclature of Howard and Martin (1950). 'Normal' phase consists of a polar stationary phase or adsorbent, and reversed-phase (r.p.) utilizes a non-polar stationary phase or support (silica coated with paraffin oil; charcoal) eluted with a polar solvent usually containing water. In r.p. chromatography, hydrophilic ('more polar') substances elute first, and stronger solvents (leading to faster elution) are less polar. Due to the reversal of polarity considerations, it is usually desirable to speak of solvent strength rather than polarity.

Currently the most popular sorbents for l.c. are modified silicas with functionality chemically bonded to surface silanol groups. The most frequently used of these sorbents are those with bonded alkyl functionality, usually C_{18},

employed in the reversed-phase mode. These supports pose a problem of nomenclature because there is considerable controversy whether they function by an adsorption (l.s.c.) or partition (l.l.c.) mechanism. In a recent review, Horvath and Melander (1977) have argued that the partition mechanism is unlikely, as the layer of bonded hydrocarbon is only a monolayer thick and subject to translational and rotational constraints. Colin and Guiochon (1977) reviewed in detail the various retention mechanism theories, and concurred with the opinion of Pryde (1974) that 'it seems a little irrelevant to argue whether the mechanism is by partition or adsorption for neither term is strictly applicable'. The latter suggested the term liquid–solid-partition chromatography, although bonded reversed-phase chromatography seems as descriptive. Recent data of Scott and Kucera (1977) show that the hydrocarbon chains of several r.p. sorbents (C_2, C_8, and C_{18}) associate with the organic component of the eluent, forming a monolayer which is *not* displaced by the sample. From this they conclude that the sample most likely interacts with the monolayer of solvent and not with the hydrocarbon chain.

Gel chromatography, also termed exclusion chromatography or gel-permeation chromatography (g.p.c.), is used to separate on the basis of molecular weight. Theoretical treatment of gel chromatography is different from that presented earlier in this review for l.l.c. and l.s.c. since there is no true 'retention' mechanism. The separation is controlled by the column packing, not by the solvent (in the absence of unwanted adsorptive interactions). The synthetic gel packings contain a large volume of pores which imbibe solvent and admit small molecules easily, medium-sized ones with more difficulty, and larger ones not at all. By controlling pore size, the molecular weight range for which a gel is effective is altered. A solute of molecular weight (or size) such that it is totally excluded from the pores elutes at the exclusion volume (V_0), whereas a small solute which is free to totally permeate the pores elutes at V_t. The quantity of solvent held within the pores (V_i) is equal to ($V_t - V_0$). As V_0 and V_i are frequently nearly the same, the number of peaks separable on a given gel is rather small. Nevertheless, the technique has been indispensable in polymer and protein chemistry for analytical and preparative purposes. For researchers in pesticide and natural products chemistry, g.p.c. can be the easiest way of separating low molecular weight substances of interest from triglyceride and other higher molecular weight components.

Ion-exchange chromatography has a multitude of applications in biochemistry and inorganic chemistry. In fact, Snyder and Kirkland (1974) assert that probably more separations are currently achieved by ion-exchange than any other l.c. mode. Supports are usually cross-linked polystyrene beads functionalized to contain cationic or anionic sites, although in other applications functionalized silicas or gel permeation materials such as Sephadex are used. The mobile phase is usually an aqueous buffer containing a counter ion with the same charge as the sample ion, and separation is achieved by the competition of these ions for the oppositely charged ionic group on the support. In addition to the ionic mechanism of separation, it is not uncommon to find a tendency towards

reversed-phase-type interactions between organic species in aqueous solutions and the polystyrene-based ion-exchange supports. Ion-exchange Sephadex gels used in biochemistry may show mixtures of ion-exchange and molecular exclusion mechanisms.

Recently the technique of ion-pair chromatography was developed by Schill and coworkers (reviewed by Gloor and Johnson, 1977, and by Schill *et al*, 1977) as an alternative to ion-exchange chromatography. Originally the technique consisted of coating an ionic organic compound onto a support such as silica or cellulose. Ionic organic species of opposite charge may then interact by forming ion-pairs with the reagent with varying degrees of both ionic affinity and lipophilicity. More recently the popular bonded-phase l.c. columns have been used for reversed-phase ion-pair chromatography, with ion-pairing reagents dissolved in the mobile phase. This modification has the advantage that both ionized and neutral components may be separated under the same conditions. The technique has also been termed soap chromatography, when alkyl sulphonates or sulphates are used as the ion-pairing reagent for separation of cationic organic species. Many factors can be altered to control retention, such as the type and size of counter ion, its concentration, mobile phase pH, and organic modifier type and concentration. Scott and Kucera (1977) have investigated the importance of wetting of the bonded-r.p. packings by the mobile phase on ion-pair chromatographic retention. With high water content where the *hydrophobic* column surface is non-wetted, the ionic reagent was found to be adsorbed to the column with concomitant increase in retention of counter-ionic species. When the hydrophobic packing was wetted (moderate to high organic modifier content), the ionic reagent was totally in the mobile phase, and counter-ionic species showed decreased retention. It is not yet clear if this will be true of all ion-pairing reagents.

Thus, in addition to the four classically recognized modes of l.c., two newer variants deserve separate classification due to their distinctiveness and utility: bonded-phase l.c. and ion-pair l.c. Bonded-r.p. l.c. has already proved to be extremely useful in pesticide metabolism studies, and ion-pair l.c. has great promise of further advancing this field in facilitating isolation of polar, ionic conjugates.

PACKINGS AND COLUMNS

An extremely comprehensive review of this subject was published recently (Majors, 1977), including specifications of over 110 types of l.c. packings and/or prepacked columns.

Types of Column Packings

Following the introduction of commercial liquid chromatographs in the late 1960s, porous layer beads (or pellicular adsorbents) were developed to provide more efficient columns. The porous layer beads consist of 30–40 μm glass