TECHNIQUES IN LIQUID CHROMATOGRAPHY

Edited by

C. F. SIMPSON

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Preface

During the course of the Residential School in High Performance Liquid Chromatography held at the University of Sussex in July 1980 under the auspices of the Royal Society of Chemistry, discussions were held between the writer and the lecturers participating in the School about the desirability of producing a book, based upon the lectures presented but considerably expanded. The response was extremely favourable, and this book is the result of the efforts of the authors, who are recognized as being leaders in the field, to present the state of the art in liquid chromatography. Further, the authors have also attempted to indicate the way in which the technique is likely to develop over the course of the next few years.

It has been my privilege to edit the various contributions and seek to ensure consistent terminology and use of symbols throughout. Any errors which may be present are my responsibility.

I would like to thank the authors contributing to this volume for presenting me with first-class manuscripts, and the instrument manufacturers and suppliers who generously allowed the participants in the School to have 'hands on' experience with modern equipment. A large number of the experiments performed are included in this book to provide the reader with the methodology employed, with typical results.

It is my hope that the information contained in this book will be useful to both beginners and to people who have considerable experience in the techniques presented. To this end, the contents present both theory and practice. The theory attempts to show how chromatography 'works', and the thinking which has gone into producing what is now accepted as being the most likely mechanism of separations. From the practical viewpoint, the everyday problems which can be experienced are discussed and advice is given on how they may be eliminated.

My only regret is that readers will not have the opportunity to discuss at first hand their own problems, as the participants in the School were able to do.

Chelsea College 1982 COLIN SIMPSON

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1 An Introduction to Liquid Chromatography and Some Fundamental Relationships

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1.1 INTRODUCTION

Chromatography is a method of separation which depends for its efficiency upon the equilibrium distribution of the solute molecules between two phases, one stationary over which the second flows. Those solutes preferentially distributed in the mobile phase will pass rapidly through the system while those preferentially distributed in the stationary phase will pass more slowly through the system. The stationary phase is contained within a suitable tube called the column and the mobile phase is pumped (or allowed to flow under gravity) through the column. The sample is placed at the column head using a suitable sample introduction system, and after passage through the column the (hopefully) separated components are detected using an appropriate detector whose output is displayed on a strip chart recorder.

A block diagram illustrating the various parts of the chromatograph is shown in Fig. 1.1 while Fig. 1.2 shows a typical detector response together with the various parameters which may be directly measured from the chromatogram.

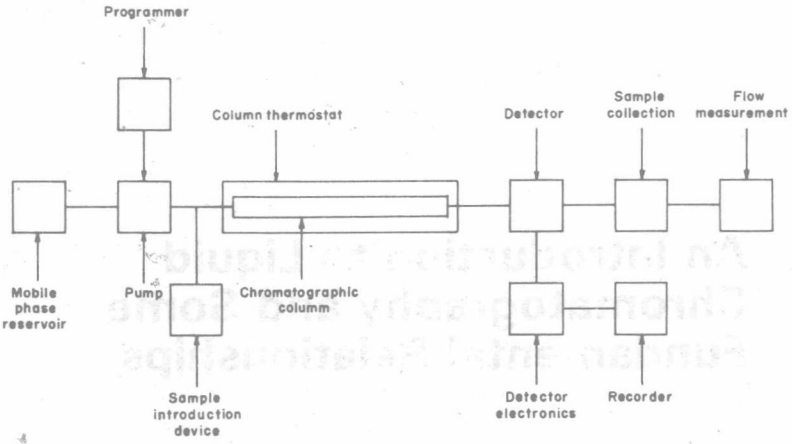

Figure 1.1. Block diagram of liquid chromatograph.

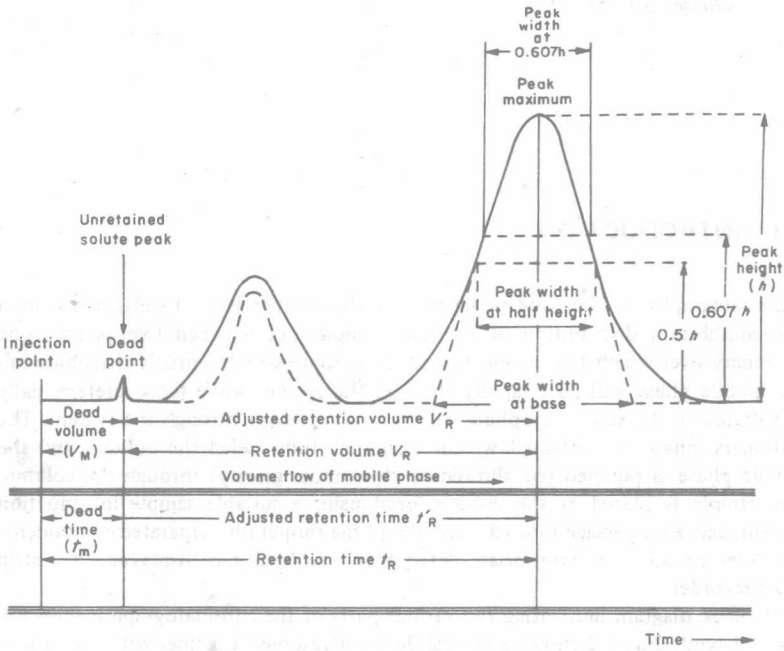

Figure 1.2. The parameters of a chromatogram.

1.2 BASIC THEORY

This definition of the chromatographic method includes the term 'equilibrium distribution' and it would be useful to consider briefly exactly what is meant by this term and how it controls separation. A system is in equilibrium when its entropy is at a maximum; indeed, separation may be considered to be the opposite of mixing, a process favoured by the second law of thermodynamics. This implies that for any separation problem, work has got to be done on the system to achieve the desired end. Thus, if we consider a solute distributed between two phases, A and B, then at equilibrium:

$$(\Delta G)_{TP} = 0$$

or

$$\mu_A = \mu_B$$

where μ is the chemical potential of the solute in phases A and B. Since

$$\mu = \mu^0 + RT \ln a$$

where a is the activity of the distributed substance in a given phase and μ^0 is its standard chemical potential, i.e. $\mu = \mu^0$ at $a = 1$, then

$$\mu_A^0 + RT \ln a_A = \mu_B^0 + RT \ln a_B \tag{1.1}$$

Activities are not always known (indeed, rarely so in chromatography), but in chromatographic systems we are normally dealing with solutions which tend towards infinite dilution. So we can replace activity, at least to a first approximation, by the concentration, C. Making this substitution, and rearranging Eqn (1.1) gives:

$$\ln (C_A/C_B) = -(\mu_A^0 - \mu_B^0) \tag{1.2}$$

or

$$C_A/C_B = K = \exp(-\Delta\mu^0/RT) \tag{1.3}$$

K is termed the distribution (partition) ratio and should be constant at constant temperature since $\Delta\mu^0$ is a constant. Thus, a plot of C_A against C_B (the distribution isotherm) should be linear and of slope K. This is a condition which we always want to achieve in chromatography, because this leads to symmetrical elution profiles of the solute bands (Fig. 1.2).

However, there are occasions when the distribution isotherm is not linear over the entire concentration range (or even over a relatively limited range) and then the elution profiles will be asymmetric and show either leading or trailing edges. This is illustrated in Fig. 1.3 which shows the effect of non-linear isotherms on peak shape and retention volume of eluted peaks. When the isotherm is linear a symmetrical elution peak is obtained and the retention time of a peak of this nature is constant. The situation changes when the isotherm is convex. The peak shows

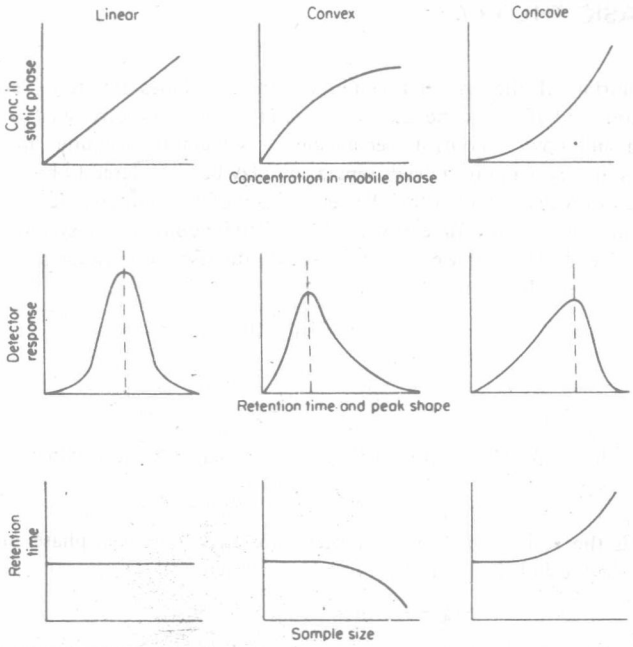

Figure 1.3. Three basic types of distribution isotherm showing the effect on peak shape and retention time.

marked tailing, indicating that adsorptive effects are occurring and the retention time of those components exhibiting this effect decreases with increasing sample size. In these circumstances it is necessary to change the distributive system to obtain symmetrical peaks. When the isotherm is concave, the peak shape exhibits a leading edge followed by a rapid fall off in sample concentration (within the peak). Increasing sample size increases the retention time of these components, and symmetrical peak shapes will usually be obtained by decreasing the sample load.

So far we have not stipulated the nature of the mobile and stationary phases, but consideration of the definition of chromatography given at the start of this discussion precludes gas–gas and solid–solid systems. Thus we are left with liquid–liquid and liquid–solid phases, the subject of this book, and gas–liquid and gas–solid which are outside its scope.

On the basis of what has been written, it would be instructive to consider a typical experiment similar to those undertaken by Tswett,[1] the originator of the technique, to see what conclusions may be drawn.

A glass column of about 1 cm^2 cross-section and 25–30 cm long is filled about three-quarters full with a suitable adsorbent. The sample is dissolved in a suitable solvent, which may also be the mobile phase, and applied to the head of the column

as an even band. Pure solvent is allowed to percolate through the column under gravity. Under the influence of the mobile phase, the initial band starts to migrate down the column and its components start to disengage. Those components held only weakly by the stationary phase pass rapidly through the chromatographic bed; those more strongly held move slowly, until a complete separation of the components of the initial band is achieved.

It will be clear from the above description that the solute bands migrate through the chromatographic bed at different velocities and this differential migration rate is a function of the equilibrium distribution of the components between the stationary and mobile phases. A further qualitative observation emerges from this experiment; that is, during the migration of the components of the initial sharp sample band applied to the head of the column these component bands progressively broaden. In the ideal case, upon development of the initial sharp band a series of sharp bands representing the individual components would be observed (see Fig. 1.4). Regrettably, this ideal situation is not obtained in practice; due to various diffusional effects which occur within the column, the original band starts to broaden and, as indicated in Fig. 1.2, the bands assume a normal distribution (providing adsorptive effects etc. are absent); that is, non-ideal development occurs.

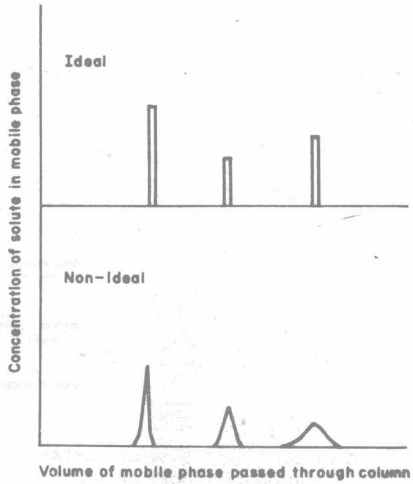

Figure 1.4. Elution development of a three-component mixture.

Under circumstances where the distribution isotherm is linear (linear non-ideal chromatography), the variance, σ^2, of the concentration profile (that is, the extent by which band broadening occurs) is proportional to its migration distance, z, i.e.

$$\sigma^2 \propto z \qquad (1.4)$$

The proportionality factor, σ^2/z, is termed the 'height equivalent to the theoretical plate' (HETP) or plate height, a term used by Martin and Synge[2] to describe the 'goodness' of a column. It is a function of many contributing factors depending on how the column has been packed, the general operating conditions, and so on (see later).

A sample band which interacts with the stationary phase migrates down the chromatographic bed at some fraction of the velocity of the mobile phase. This fraction is called R_f, the retardation factor, which is obtained from:

$$R_f = \frac{\text{rate of movement of the sample band}}{\text{rate of movement of the solvent front}} \tag{1.5}$$

R_f is the principal parameter of dry column and thin-layer chromatography.

If at some time during the migration of the component bands through the column the mobile phase flow is stopped, the individual components in the column will rapidly come into equilibrium between the stationary and mobile phases. If we consider one component only, the situation which occurs is shown in Fig. 1.5. The concentration profiles of the component in both the stationary and (stopped) mobile phases are exactly mirrored, apart from a possible difference in magnitude. In this state the concentration ratio in the two phases is equal to the distribution ratio, where:

$$K = C_s/C_m \tag{1.6}$$

C_s and C_m are the concentrations of the component in the stationary and mobile phases, respectively.

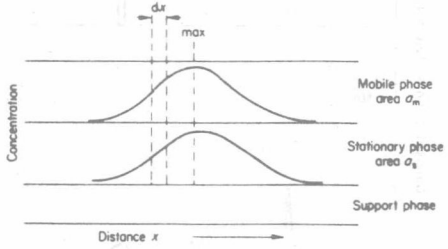

Figure 1.5. Equilibrium distribution of a solute between the stationary and 'stationary' mobile phase.

As we have seen, it is a condition for symmetrically shaped bands that the distribution ratio varies linearly with respect to the component concentration in each phase. If this condition is not met then asymmetric peaks occur and the retention time (volume) will vary with sample concentration (Fig. 1.3). Consider a small

section of these bands of length dx (Fig. 1.5); the ratio of concentrations of the component in the two phases in this section is termed the column capacity ratio, k'. This quantity is related to the distribution coefficient by:

$$k' = q_s/q_m = C_s A_s\, dx/C_m A_m\, dx = C_s A_s/C_m A_m$$
$$= C_s V_s/C_s V_m = K(V_s/V_m) \tag{1.7}$$

That is, the column capacity ratio is the product of the distribution coefficient and the ratio of stationary phase to mobile phase in the total column. A_s, A_m; V_s, V_m; q_s, q_m are the mean cross-sectional areas, the volumes and quantities of solute in the stationary phase and mobile phases respectively.

k' is a most important quantity in liquid chromatography. It relates the equilibrium distribution of the sample within the column to the thermodynamic properties of the column. Clearly, it is very difficult to know exactly the respective volumes of the stationary and mobile phases contained within the column (or surface areas if we are considering adsorptive systems, when an equivalent argument to that given above holds). Hence, we need some practical method whereby this quantity may be readily measured.

Consider the state of the system at equilibrium; all equilibria are dynamic, and there will be a continuous interchange of solute molecules between the stationary and 'stopped' mobile phases. If it were possible to observe one molecule of solute and follow its path over a period of time, it would be seen that the fraction of time the molecule spent in, say, the stationary phase would approach the instantaneous fraction of the molecules which are present in that phase at a given time, and over an indefinite period of time these two fractions would be equal within statistical limits. Thus we can write from Eqn (1.7):

average fraction of time spent by a molecule in the stationary phase

$$= \frac{q_s}{q_s + q_m} = \frac{k'}{1 + k'} \tag{1.8}$$

and similarly

average fraction of time spent by a molecule in the mobile phase

$$= \frac{q_m}{q_s + q_m} = \frac{1}{1 + k'} \tag{1.9}$$

Obviously the sum of these two fractions is unity, since the molecule must be in either the stationary or the mobile phase. (*Note*: it is assumed that the molecule does not interact with the walls of the column.)

If the mobile phase flow is now restarted so that it moves through the column at a constant linear velocity of U cm s^{-1}, then, when a solute molecule is present in the mobile phase it too will pass through the column at this velocity, but when it is present in the stationary phase, its velocity will be zero. Thus the average downstream velocity of a solute molecule depends upon the mobile phase velocity, U, and the fraction of time that the molecule spends in the mobile phase. Thus the

band velocity, U_{band}, is

$$U_{band} = U \cdot \frac{1}{1+k'} = U \cdot \frac{1}{1+K(V_s/V_m)} \tag{1.10}$$

Now R_f has been defined as the rate of movement of this sample band relative to the rate of movement of the mobile phase front (Eqn (1.5)); hence:

$$R_f = U_{band}/U = 1/(1+k') \tag{1.11}$$

or

$$k' = (1-R_f)/R_f \tag{1.12}$$

Therefore, it will be seen that R_f is a function of the equilibrium distribution of the sample within the column, and since elution chromatography is simply an extension of dry column chromatography, elution times may be related to this quantity.

Thus, from Fig. 1.2 for the first solute we can write:

$$U_{band}/U = t_m/t_{R_1} \tag{1.13}$$

t_m is the elution time of an unretained (unadsorbed) solute, and hence is equal to the time taken for the mobile phase front to pass completely through the column, and, similarly, t_{R_1} is the time taken for a solute which interacts with the stationary phase to pass through the column. From Eqn (1.10):

$$U_{band}/U = 1/(1+k')$$

hence

$$t_m/t_{R_1} = 1/(1+k')$$

or

$$k' = (t_{R_1} - t_m)/t_m \tag{1.14}$$

i.e. the column capacity ratio k' is the additional time a solute band takes to elute from the column compared to the time it takes for an unretained band to elute from the column. This difference in elution times is divided by the 'dead' time to give a dimensionless number for the column capacity factor and these quantities may readily be obtained from the chromatogram, providing the dead time (column dead volume) can be measured (see later).

The term k' has been related to the distribution coefficient by Eqn (1.7), and since K is a constant under conditions of linear non-ideal chromatography it follows that R_f is also a constant. Thus, if R_f lies between 0 and 1 the solute migrates through the column with a migration velocity U_{band}, and clearly, therefore, U_{band} must be the product of the mobile phase velocity U and the fraction of time the solute spends in the mobile phase R_f, i.e.:

$$U_{band} = UR_f \tag{1.15}$$

When $R_f = 0$, the band does not migrate at all ($U_{band} = 0$) and when $R_f = 1$, the band migrates at the same velocity as the mobile phase, ($U_{band} = U$). Now $U_{band} = L/t_{R_1}$ where L is the column length, t_{R_1} is the retention time of the band (see Fig. 1.2), and $U = L/t_m$, where t_m is the dead time. Thus from Eqn (1.15) and

$$t_{R_1} = t_m/R_f \tag{1.16}$$

and bearing in mind that $t_R = V_R/F_c$ and $t_m = V_m/F_c$, we get from Eqn (1.11):

$$V_R = V_m(1 + k') \tag{1.17}$$

or from Eqn (1.7)

$$V_R = V_m + KV_s \tag{1.18}$$

Equation (1.18) is a fundamental equation in chromatography. It relates the retention volume of a component to the column dead volume and the product of the distribution coefficient and the volume of stationary phase present within the column. This equation is correct for partition columns but for adsorption columns V_s would be replaced by A_s (the surface area of the adsorbent present in the column) or possibly, but not so usefully, by W_A (the weight of adsorbent present in the column).

Since V_m plays no part in the separation process and is, moreover, a constant in any given chromatographic analysis, the adjusted retention volume V'_R (sometimes called the net retention volume, V_N) is used:

$$V'_R = V_R - V_m = KV_s \quad (\text{or } KA_s) \tag{1.19}$$

1.3 BAND BROADENING: PLATE HEIGHT AND PLATE NUMBER

It has already been noted (Eqn (1.4); Fig. 1.2) that when chromatographic development takes place it does so with a concomitant broadening of the solute band that was originally placed at the head of the column as a narrow band. Clearly this broadening is detrimental to separation and considerable effort has been expended to explain how these band broadening processes occur and how the column must be designed to minimize these effects. A detailed study of the design of columns is given in Chapter 2. Here consideration will be given to how the 'goodness' of a column may be measured, that is, the extent to which band broadening occurs.

Martin and Synge[2] first described these effects, using the 'Plate Theory' of chromatography, in their historic paper on partition chromatography for which they received the Nobel Prize in 1952. They envisaged a chromatographic column as consisting of a large number of equal-volume elements, in each of which the concentration of the solute between the two phases present corresponded to the equilibrium value. Thus, in each plate:

$$C_s = KC_m \tag{1.20}$$

(see Eqn (1.3)). C_s and C_m are the concentrations of the solute in the stationary and mobile phases, respectively, and K is the distribution coefficient. Equation (1.20) is just an alternative way of writing the distribution law, and in the analysis which follows, attributed to Scott,[3] it is assumed that a linear relationship holds. The analysis is given in full here because of the novel approach.

Differentiating Eqn (1.20) gives:

$$dC_s = K \times dC_m \tag{1.21}$$

Consider the situation in three consecutive plates, p − 1, p and p + 1 contained in a column of n plates (illustrated in Fig. 1.6).

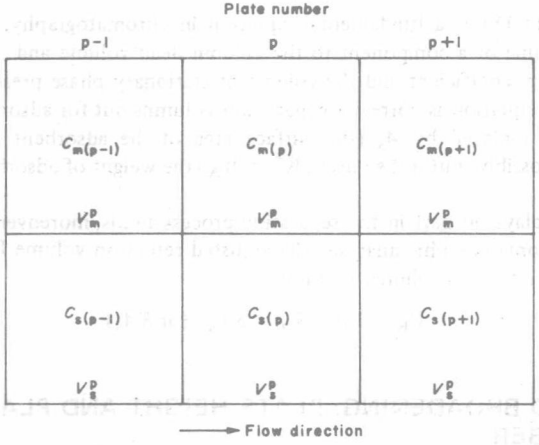

Figure 1.6. Representation of three consecutive plates in a chromatographic column.

V_m^p and V_s^p are the respective volumes of mobile and stationary phase present in each of the column elements, $C_{m(p-1)}$, $C_{s(p-1)}$, $C_{m(p)}$, $C_{s(p)}$ and $C_{m(p+1)}$, $C_{s(p+1)}$ are the concentrations of the solute present in the appropriate plate in the mobile and stationary phases respectively. Let a volume of mobile phase dV pass from plate p − 1 to plate p. At the same time an equal volume of mobile phase will be displaced from plate p to plate p + 1. In this operation there will be a change in the mass of the solute in plate p equal to the numerical difference between the mass of solute entering plate p from plate p − 1 and the mass of solute leaving plate p and entering plate p + 1. Hence we can write a mass balance for this process as:

$$dm = (C_{m(p-1)} - C_{m(p)})\, dV \tag{1.22}$$

where dm is the difference in mass effected on the transfer.

Now if equilibrium is maintained and it is assumed that re-equilibration is rapid, the change in mass, dm, will distribute itself in plate p between the two phases