CHAPTER 6. CHROMATOGRAPHY

6.1 INTRODUCTION

In nature one rarely has a sample with only one component, in fact most samples are very complex mixtures, *e.g.*, sea water or blood. Even so, in some cases the separation of a sample into its components may not be necessary for analysis. The techniques of atomic absorption and ICP atomic emission rarely require separations before analysis. However, most analytical methods usually require some type of separation prior to the analysis of a complex sample. Thus the last topic in this text will be a discussion of one of the most powerful separation techniques, chromatography.

Most separations are performed using systems with two phases, *e.g.*, a solid and a liquid. In many separation techniques the component of interest is concentrated in one phase while the other sample components are concentrated in the other phase. The separation is then carried out by simply physically separating the phases. In the case of a liquid and a solid, the solid is filtered off.

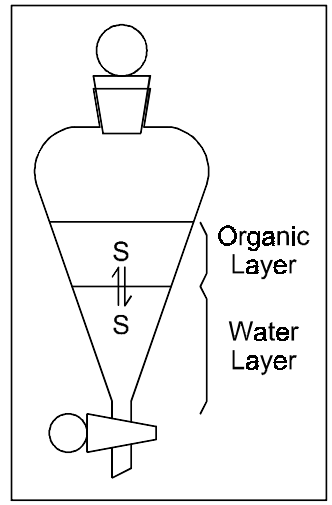


Figure 6-1

As an example and also to introduce the concept of partition, the case of two immiscible liquids, one organic (*e.g.* hexane), the other water, will be examined. These are placed in a separatory funnel as shown in Figure 6-1. If a sample, S, which is soluble to some extent in both liquids, is dissolved in one or the other of the liquids and the funnel is shaken, S will distribute itself between the two solvents.

$$S_w \rightarrow S_o$$

where the subscript "w" represents the water phase and "o" the organic phase. At equilibrium:

$$K = \frac{[S]_o}{[S]_w} \tag{6-1}$$

where the equilibrium constant, K, is called the *partition coefficient* or *partition ratio*. As K increases, the affinity of the sample for the organic phase increases. Thus to separate two substances, one substance should have a large K (prefer the organic phase) and the other should have a small K (prefer the water phase).

For example, assume that the volumes of the two phases are equal, or $V_o = V_w$. Then:

$$K = \frac{[S]_o}{[S]_w} = \frac{[S]_o V_w}{[S]_w V_w} = \frac{[S]_o V_o}{[S]_w V_w} = \frac{mol(S)_o}{mol(S)_w} \tag{6-2}$$

$$K = \frac{mol(S)_{total} - mol(S)_w}{mol(S)_w} = \frac{mol(S)_t}{mol(S)_w} - 1 \tag{6-3}$$

This can be rearranged to

$$\frac{mol(S)_w}{mol(S)_t} = \frac{1}{K+1} \tag{6-4}$$

Thus, in a separation of two substances, A and B, if A has $K_A = 10$, $molA_w/molA_t = 0.091$ (from Eqn. (6-4)), or 9.1% of this material remains in the water while 90.9% will be found in the organic phase. If B has $K_B = 0.10$, then $molB_w/molB_t = 0.909$ and 90.9% of this material will remain in the water

while 9.1% will be in the organic phase. This separation is certainly not outstanding, and yet requires that one partition coefficient be 100 times greater than the other. With partition coefficients a factor of 100 apart, the two substances will not be very similar to each other. It would be most desirable to be able to separate materials with partition coefficients much closer to each other than this.

If the same two phases discussed above are used, but arranged differently (not in a separatory funnel), significant improvements can be made in the ability to separate substances. Imagine a long tube of inert material (*e.g.* glass) which is coated with the immiscible organic liquid. The second phase, water, is placed in the centre of the tube as shown in Figure 6-2.

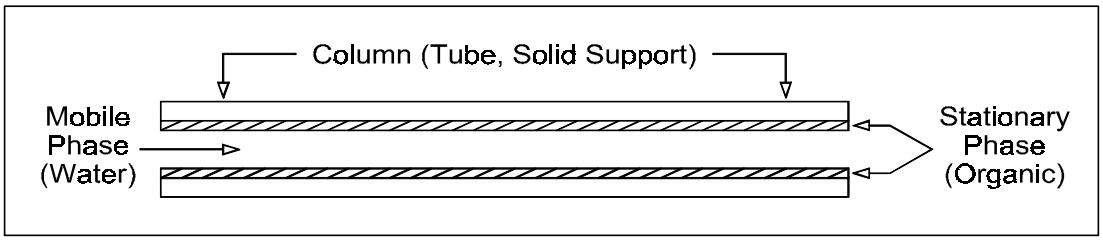


Figure 6-2

Now place a solute, S, in the tube at some location. This will distribute itself between the water and organic phases just as if it were in a separatory funnel and will form a small band of solute molecules. For reasons discussed below, the concentration of solute will be higher in the centre of the band and will go to zero at the band edges. A small segment of a column containing such a sample band is shown in Figure 6-3.

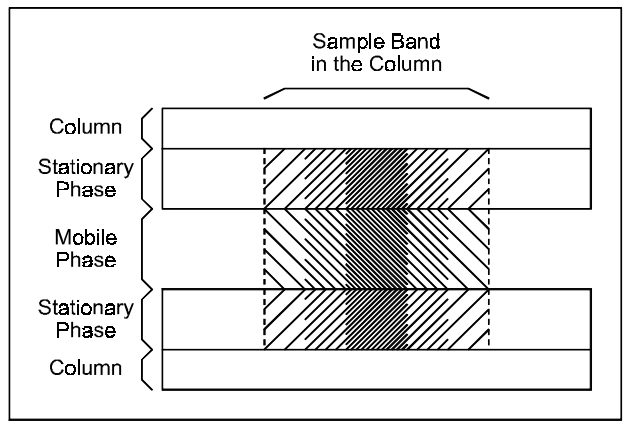


Figure 6-3

If the material in the centre of the tube (water in this example) is moved through the tube, what will happen to the substance dissolved in the water? The band of solute in the water will move down the column with the water and get ahead of the band in the organic phase (which does not move) as shown in Figure 6-4. As soon as this happens the molecules of S at the leading edge of the band in the water find themselves in a non-equilibrium situation in which their concentration is too high in comparison with that of the molecules in the stationary phase. Thus some molecules will move from the water into the organic phase as shown to try to reestablish equilibrium. Likewise the molecules at the trailing edge of the band in the organic phase also find themselves in a non-equilibrium condition, their concentration also being too high, so molecules of S move back into the water

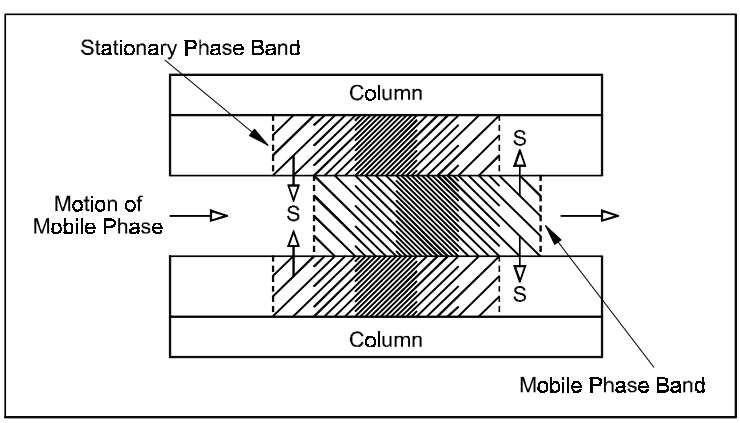


Figure 6-4

phase (as shown). This causes the band of molecules in the organic phase to move down the tube also; however, this band will always trail somewhat behind that in the water phase. The total band of solute will not move as rapidly as the water because the solute is continually exchanging between the two phases and while in the organic phase the solute undergoes no net motion in the direction in which the water phase moves.

In this example water was mobile and the organic liquid was stationary, but this does not have to be the case. In general the tube which holds the stationary phase and encloses the entire system is called a *column*, the liquid coated on the column is the *stationary phase*, and the liquid which moves through the column is the *mobile phase* or *eluent*. The solutes partition between the stationary and mobile phases according to:

$$S_m \rightarrow S_s$$

Now the partition coefficient is defined as

$$K = \frac{C_s}{C_m} \qquad (6\text{-}5)$$

where the subscript "m" refers to the mobile and "s" to the stationary phase, and *C* stands for the molar concentration of solute in a particular phase. It is customary always to place the concentration in the stationary phase in the numerator of the equilibrium constant.

At this point a brief introduction to the types of chromatography in use should be presented. The stationary phase can either be a solid or a liquid held in place by an inert solid support and the mobile phase can be either a liquid or a gas. If the stationary phase is a solid, its surface must be active and the materials to be separated must be attracted to and adsorb on that surface. Often the type of chromatography in use is classified by the phases employed, providing four varieties: gas-liquid chromatography (GLC), gas-solid chromatography (GSC), liquid-liquid chromatography (LLC), and liquid-solid chromatography (LSC). The example presented above is LLC. More will be said about the types of chromatography later.

It should also be mentioned that there are two common types of chromatographic columns in use. The open tubular column is the type described above and will be used in other examples below. This is done because it is perhaps easier to understand the processes involved when using an open tubular column as an example. Open tubular columns must be very small (in diameter) in order to keep the volumes of the two phases close to each other. For this reason open tubular columns are often called capillary columns.

The other type of column in common use is the packed column. Such a column still has an inert outer tube (*e.g.* glass or stainless steel), but the stationary phase is not coated on the tube. Instead it is coated on small particles, usually spherical and of approximately uniform size, that are inert, These particles are packed into the tube and form a "bed", as pictured in Figure 6-5. If the stationary phase is a solid, these particles themselves are the stationary phase. The mobile phase passes through this bed and solutes partition between the mobile phase and the stationary phase. The mechanism of separation is the same on both packed and open tubular columns. The great advantage of packed columns is that they can be made very large (in diameter)

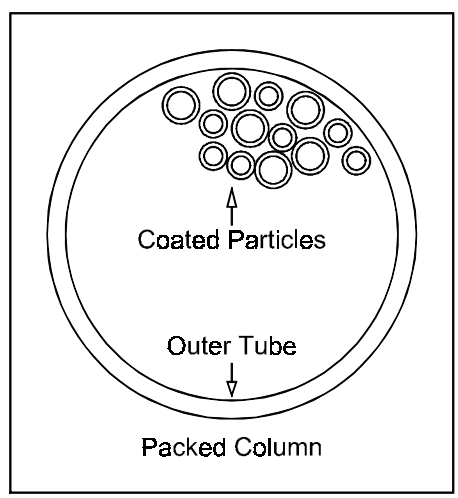


Figure 6-5

and thus can separate large samples. Capillary columns usually separate more efficiently, but can only accommodate very small samples.

6.2 THEORY OF CHROMATOGRAPHY

Since the speed at which a band of solute moves through the column depends on the amount of time that the solute spends in the stationary phase (if it spends no time at all in the stationary phase, it will move with the velocity of the mobile phase), the velocity of band motion will depend on the value of K. Thus it might be possible to separate two substances if their K values are different. For the following discussion refer to Figure 6-6. Two substances, A and B, are introduced together at the top of a column (shown as the left column). As they are moved through the column with time (centre column), A gets ahead of B since it has a slightly higher velocity. When they approach the bottom of the column (right column), A has completely separated from B and the two can be collected as pure materials. To predict whether separation is possible, a relationship between the velocity of the sample bands and the parameters of the system needs to be developed.

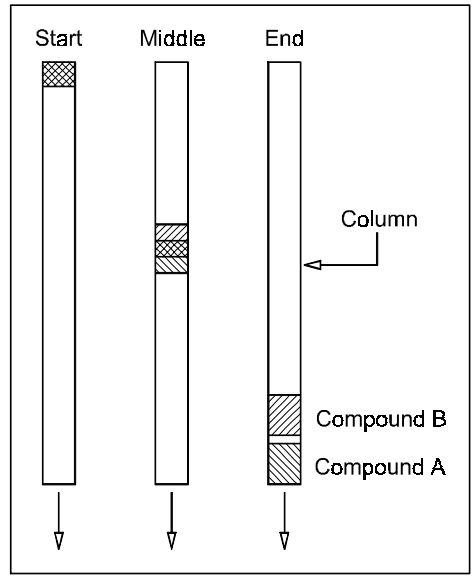


Figure 6-6

The first chromatographic separations were done with coloured materials using packed, glass columns, allowing the separations to be observed visually. This gave rise to the name "chromatography", which comes from the Greek meaning to write with colour.

As stated above the stationary phase in chromatography can be either a liquid or a solid. If the stationary phase is a liquid, the term often used to describe this type of chromatography is *partition chromatography*. *Adsorption chromatography* is the term used to describe chromatography in which the stationary phase is a solid, since the mechanism of attachment of the sample components to the stationary phase is adsorption. In developing the theory there are small differences between partition and adsorption chromatography.

6.2.1 BAND VELOCITY

Partition chromatography.

Since sample molecules move down the column only when they are in the mobile phase, it can be stated that sample velocity, v, will equal the mobile phase velocity, v_m, multiplied by the fraction of time spent in the mobile phase. Now the fraction of time spent in the mobile phase is also equal to the fraction of molecules (or mols), f_m, of substance in the mobile phase. Thus

$$v = v_m f_m \tag{6-6}$$

The fraction of solute in the mobile phase is determined as follows:

$$f_m = \frac{mol_m}{mol_t} = \frac{V_m C_m}{V_s C_s + V_m C_m} \tag{6-7}$$

where V represents the volume of a particular phase. Dividing numerator and denominator by C_m and making use of Eqn. (6-5) leads to:

$$f_m = \frac{V_m}{V_m + KV_s} \tag{6-8}$$

Thus by substituting Eqn. (6-8) into (6-6) the velocity of the sample band is given by

$$v = \frac{v_m V_m}{V_m + KV_s} \tag{6-9}$$

However, the sample velocity is not the most useful parameter in practical circumstances. Two parameters which are both more useful and more easily measured are the eluent *flow rate* (usually measured in mL/s or mL/min), F, and the sample *retention time*, t_r, measured in seconds or minutes. The retention time is the time between placing the sample on the column and the emergence of the maximum of the sample band at the end of the column. Very simply

$$t_r = L/v \tag{6-10}$$

where L is the column length (in cm). Thus substituting (6-9) into (6-10)

$$t_r = \frac{L(V_m + KV_s)}{v_m V_m} \tag{6-11}$$

The eluent velocity, v_m, is not easily measured, but is related to the mobile phase area, A_m, (in cm^2) and the flow rate by

$$v_m = F/A_m \tag{6-12}$$

The volume of the mobile phase in the column is simply

$$V_m = A_m L \tag{6-13}$$

Substituting (6-12) and (6-13) into (6-11) leads to

$$t_r = \frac{A_m L(V_m + KV_s)}{FV_m} = \frac{V_m + KV_s}{F} \tag{6-14}$$

One additional parameter, the *retention volume*, V_r, will now be defined. This is the volume of eluent necessary to bring the maximum of the sample band through the column.

$$V_r = t_r F = (V_m + KV_s) \tag{6-15}$$

The flow rate, F, is easily measured experimentally, V_s is usually known from the method of preparing the column, V_m, the volume of mobile phase in the column (sometimes called the column dead space volume), may also be known from column preparation but can easily be determined by measuring the retention volume of a substance which has no affinity for the stationary phase. For a material which is not retained by the stationary phase, K = 0.

$$V_r = (V_m + (0)V_s) = V_m \qquad \text{if } K = 0 \tag{6-16}$$

Eqn. (6-15) provides the relationship between the band velocity of a substance (given in the more useful terms of retention volume or retention time), the value of K, and the column parameters V_m and V_s.

Calculate t_r and V_r for a compound with a K of 10.0 when chromatographed on a column 30.0 cm in length with a mobile phase flow rate of 0.100 mL/min. A non-retained sample elutes from the column in 1.86 min. 0.0500 mL of stationary phase was used to prepare the column.

V_s = 0.0500 cm^3, F = 0.100 cm^3/min

$V_m = Ft_r$(non-retained) = 0.100cm^3/min(1.86min) = 0.186 cm^3.

V_r = (0.186 + (10)(0.0500))cm^3 = 0.686 mL

$t_r = V_r/F$ = 0.686cm^3/0.100cm^3/min = 6.86 min.

A second, slightly different treatment for band velocity is often employed. The *capacity factor*, *k'*, a new constant, is defined as the ratio of the time spent in the stationary phase to that spent in the mobile phase. Thus as *k'* increases, band velocity decreases and retention time and retention volume increase. The ratio of time in the stationary phase to that in the mobile phase is equal to the ratio of mols in the stationary phase to mols in the mobile phase, or

$$k' = \frac{\text{mol}_s}{\text{mol}_m} = \frac{C_s V_s}{C_m V_m} = \frac{\text{K} V_s}{V_m} \tag{6-17}$$

or

$$\text{K} = \frac{k' V_m}{V_s} \tag{6-18}$$

which, when substituted into Eqn. (6-14) above, gives

$$t_r = \frac{V_m + \text{K} V_s}{F} = \frac{V_m + V_s k' V_m / V_s}{F} = \frac{V_m (1 + k')}{F} \tag{6-19}$$

Now V_m/F equals the time for eluent (or a non-retained material) to travel through the column. If this is represented by t_m, then

$$t_r = t_m (1 + k') \tag{6-20}$$

and

$$V_r = F t_r = F t_m (1 + k') = V_m (1 + k') \tag{6-21}$$

As an example of this approach calculate the capacity factor for a material that elutes from a column in 6.85 min. A non-retained substance elutes in 45 s.

6.85 min = (45 s/60 s/min)(1 + *k'*)

k' = 6.85 min/0.75 min - 1 = 8.1

In comparing the two approaches, note that both K and *k'* are dimensionless constants, but *k' is not an equilibrium constant*. K depends only on the materials involved, that is the solute and the mobile and stationary phases, and is independent of how the column is constructed. Thus it will be the same from column to column as long as the same materials are used in each column. *k'* depends not only on the substances used, but also on the method of column preparation, and thus is a constant only for a

given column. On the other hand *k'* has the advantage of being easily determined experimentally without exact knowledge of the method of column preparation. Note in the example above that *k'* was determined without reference to any of the materials needed to prepare the column. K could not be calculated in this way.

Adsorption chromatography.

In partition chromatography the solutes dissolve in the stationary phase, forming conventional solutions. In adsorption chromatography, the solutes adsorb on the surface of the stationary phase. Nevertheless, the distribution of the sample components between mobile and stationary phases in adsorption chromatography is still written in the same fashion:

$$S_m \rightarrow S_s$$

and

$$K = \frac{C_s}{C_m} \tag{6-5}$$

where C_m is the molar concentration of S in the mobile phase. However, C_s is a rather unusual concentration (one that the reader might not have seen before). It is a surface concentration defined as

$$C_s = \frac{\text{mol S}}{\text{unit area}} = \frac{\text{mol}_s}{A_s} \tag{6-22}$$

since the S molecules are not dissolved in the solid phase, but rather adsorbed on its surface. In Eqn. (6-22) A_s is the surface area of the stationary phase. The units of C_s are usually either mol/m^2 or mol/cm^2. K is no longer a dimensionless constant, but rather has the units of length.

The relationship for the fraction of S in the mobile phase, f_m, now becomes

$$f_m = \frac{V_m C_m}{V_m C_m + A_s C_s} \tag{6-23}$$

This leads (in exactly the same fashion as was done for partition chromatography above) to the following expressions for t_r and V_r.

$$t_r = (V_m + KA_s)/F \tag{6-24}$$

$$V_r = (V_m + KA_s) \tag{6-25}$$

To use these equations, one must know the surface area of the stationary phase in the column. This is not usually known, but can be measured, if desired.

The definition of the capacity factor becomes

$$k' = \frac{C_s A_s}{C_m V_m} = \frac{KA_s}{V_m} \tag{6-26}$$

for adsorption chromatography. However, all of the other equations involving the capacity factor are the same for both partition and adsorption chromatography. *k'* remains a dimensionless constant in both cases. Thus, for example, the retention time is given by

$$t_r = t_m(1 + k') \tag{6-27}$$

regardless of whether one is employing partition or adsorption chromatography. This is one advantage of the treatment using the capacity factor; all equations are the same regardless of the chromatography used.

A chromatographer packs a 30.0 cm × 4 mm id column with 7.54 g of silica gel stationary phase which is reported to have a surface area of 100 m^2/g. She collects 10.0 mL of eluent from the column in 12.34 min. A non-retained substance gives a retention time of 56 s and a sample gives a time of 6.87 min. Calculate both K and *k'* for this sample.

A_s = (7.54 g)(100 m^2/g) = 754 m^2 = 7.54x10^6 cm^2

t_m = (56 s)/(60 s/min) = 0.933 min

t_r = 6.87 min

F = (10.0 mL)/(12.34 min) = 0.8104 mL/min

V_m = (0.933 min)(0.8104 mL/min) = 0.7563 mL

$t_r = (V_m + KA_s)/F$ or $K = (t_rF - V_m)/A_s$

K = {(6.87 min)(0.8104 cm^3/min) - 0.7563 cm^3}/7.54x10^6 cm^2

K = (4.811 cm^3)/7.54x10^6 cm^2 = 6.38x10^{-7} cm

$t_r = t_m(1 + k')$ or $k' = (t_r - t_m)/t_m$

k' = (6.87 min - 0.933 min)/0.933 min = 6.36

6.2.2 BAND SHAPE

At this point the actual shape of a band of solute must be considered as it proceeds down and off the column. Most instruments that perform chromatographic experiments, called *chromatographs*, use a detector at the end of the column. This is a device, such as a spectrophotometer, that senses the concentration of the sample molecules in the eluent. The detector then provides a signal which is proportional to sample concentration to a recording device such as a strip chart recorder or computer which displays sample concentration as a function of time. This arrangement is diagrammed in Figure 6-7. A typical recording from a chromatograph, called a *chromatogram* is shown in Figure 6-8.

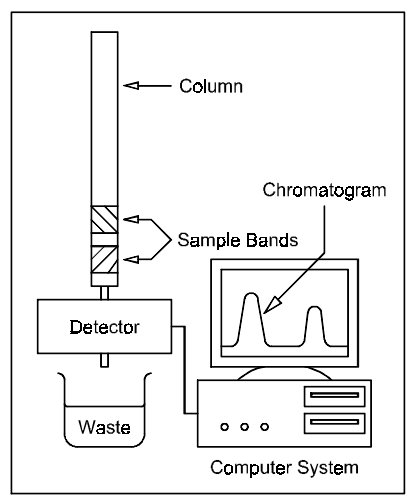


Figure 6-7

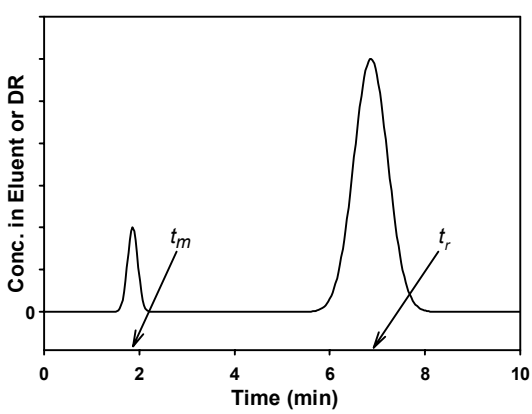


Figure 6-8

This chromatogram would result from the experiment described in the first example given in the section on partition chromatography above. The small peak at t = 1.86 min is for the non-retained substance (K = 0) and can be used to determine the values of t_m and V_m. The second peak at t = 6.86 min is for the sample (K = 10.0).

The problem of separating one compound from another will now be reexamined. For example, assume that a sample contains the material used in the first example above (K_1 = 10.0) and a second compound with K_2 = 12.0. If this sample is passed through the same column (V_s = 0.0500 cm^3, V_m = 0.186 cm^3, F = 0.100 mL/min), what will be the retention time for the second substance? Using Eqn. (6-14)

$$t_r = (0.186 + 12.0(0.0500))\text{cm}^3/0.100\text{cm}^3/\text{min} = 7.86 \text{ min.}$$

Thus the band maximum of compound 1 exits the column at 6.86 min and that for compound 2 at 7.86 min. Does this mean that the two substances are satisfactorily separated? *Not necessarily!* The separation depends not only on the difference in retention times, but also on the widths of the peaks as they exit the column. Compare the two chromatograms shown in Figure 6-9. The retention times of the peaks in both chromatograms are the same (6.86 and 7.86 min). So are the areas under the peaks, indicating that the amount of material in both chromatograms is the same. However, the widths of the peaks in the upper chromatogram are three times the widths in the second, while the heights are three times less. In the upper chromatogram the individual peaks, 1 and 2, overlap considerably. The detector response, which is the sum of both of the individual peaks, looks itself like a single peak and might be mistaken for one. Note that the detector will only record the total response, not the individual peaks in this case. On the other hand the separation in the lower chromatogram is quite satisfactory with the detector following the individual compounds with very little overlap.

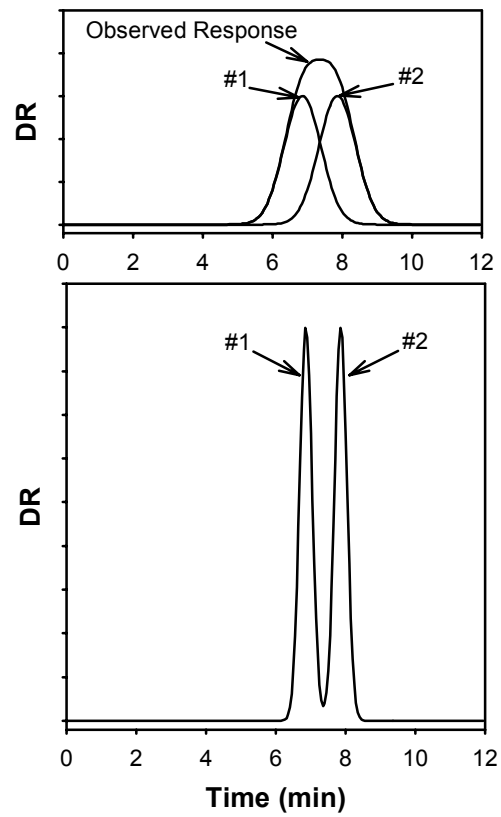


Figure 6-9

It is obvious that if peak width can be kept narrow, very similar compounds can be separated (K_1 = 10, K_2 = 12). It is also obvious that peak width is as important in achieving good separations as is difference in retention time. The theory that has been developed so far does not address peak width. Thus a different theory which includes peak width must be developed. In other words, the reasons why a band broadens as it moves through a column will have to be investigated.

6.2.3 CHROMATOGRAPHIC BANDWIDTH - PLATE THEORY

Plate theory was the first theory developed to explain the shape of chromatographic peaks and was modelled on the theory of distillation columns. Distillation columns were divided into regions called plates in which a fractional distillation equilibrium was considered to exist. In chromatographic plate theory the column was divided into small regions, again called plates, in which equilibrium was considered to exist. Thus a column could be thought of as a series of very small separatory funnels linked together. Equilibrium would exist in each funnel, and each funnel would be a plate. This is not a particularly accurate description of a chromatographic column, but it was the best available for some time.

None of the derivations involved in plate theory will be presented, just the important results. As one might guess, the greater the number of plates in a column, the narrower the bands and the better the

separations. The larger the number of plates, the smaller the length of the column taken up by a plate. Thus one of the very important parameters that came from plate theory is the concept of the length of a plate, or the *height equivalent of a theoretical plate*, HETP. This is very often used in chromatography to describe the quality of a column. If N is the number of plates in the column, then

$$N = L/\text{HETP} \tag{6-28a}$$

or

$$\text{HETP} = L/N \tag{6-28b}$$

where L is the column length. Note that HETP has the units of length, since N has no units.

Plate theory also provided an expression for the shape of a chromatographic band or peak. As shown in the figures above, an *ideal* peak is symmetrical and bell shaped with a shape referred to as "Gaussian". This means that the peak can be described by a particular equation, the Gaussian equation:

$$\text{Detector Response} = \text{DR} = h\exp\left[\frac{-(t-t_r)^2}{2s^2}\right] \tag{6-29}$$

where h is the peak height (height at band maximum), t is time, t_r is the retention time, and s is a measure of the peak width in time units. Note that Detector Response = h when $t = t_r$, and that response decreases both before and after t_r. This equation is very similar to that used in statistics to describe the distribution of values in a population, the normal error equation (see section 1.1.3 and Eqn. (1-8)). In this regard, "t_r" acts as the mean and "s" as the standard deviation (it is still a measure of the width of the peak). Band shape in chromatography and the distribution of values in a population follow the same curve for much the same reasons. In chromatography the molecules of each substance have an associated average velocity (referred to as "v" above). More molecules travel at this velocity than any other. However, some molecules travel at velocities either higher or lower than the average and exit the column before or after the band maximum. The farther the velocity is from the average, the fewer the number of molecules that travel at that velocity. This is one factor that gives rise to the bell-shape of the peaks. The same arguments can be made about the distances travelled by the molecules in the column. Since the concept of standard deviation is so closely associated with Gaussian curves, it is also used in chromatographic theory to represent a measure of the peak width. In this text the standard deviation of a chromatographic peak will be referred to in this regard. If this is bothersome, simply recall that it is the peak width that is under discussion.

Often Gaussian peaks are plotted in a slightly different way to aid in understanding the various terms. Instead of plotting Detector Response *versus* time (as in Figure 6-8), in Figure 6-10 it is plotted *vs* $(t-t_r)/s$, a dimensionless term which is zero at t_r and which increases or decreases in units of the standard deviation. Recall from Section 1.1.3 that for a Gaussian curve 68.3% of the area of the peak lies between +1s and -1s, 95.5% lies between +2s and -2s, and 99.7% lies between +3s and -3s. When $(t-t_r)/s = \pm 1$ (at $\pm 1s$ on either side of t_r), DR (Detector Response) = $he^{-1/2}$ = 0.607h, at $\pm 2s$ DR = 0.135h, and at $\pm 3s$ DR = 0.011h. Thus the peak is about 6s wide at its base.

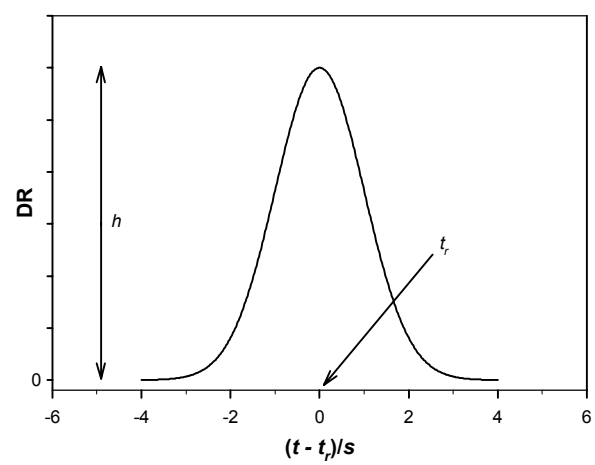


Figure 6-10

Often the peak width is measured at one-half of its maximum height, h. When DR = 0.5h, the following applies.

$$\frac{\text{DR}}{h} = 0.500 = \exp\left[\frac{-(t-t_r)^2}{2s^2}\right] \tag{6-30}$$

Taking the logarithm of both sides of Eqn. (6-30) provides

$$0.639 = \frac{(t-t_r)^2}{2s^2} \tag{6-31}$$

or

$$\frac{t-t_r}{s} = \sqrt{1.386} = \pm 1.177 \tag{6-32}$$

Thus the width of an ideal peak, $w_{1/2}$, at half of its height, $h_{1/2}$, is $2(1.177s)$.

$$w_{1/2} = 2(1.177s) = 2.35s \tag{6-33}$$

Plate theory not only predicts that bandwidths go down as N increases, but more specifically that

$$\sigma = \sqrt{N} \tag{6-34}$$

where σ is the bandwidth or standard deviation in terms of the number of plates. In this text the symbol, σ, will always be used for this particular bandwidth or standard deviation, which has no units.

Exactly what is a bandwidth in terms of the number of plates? Assume that a column has 100 plates. Plate theory states that $\sigma = 100^{1/2} = 10$. This means that for a chromatographic band centred at the end of the column (note that half of such a band will have already left the column), 34.1% of the band will be in the last 10 plates, 47.7% will occupy the last 20 plates, and 49.8% will occupy the last 30 plates. Each plate occupies a certain distance in the column (HETP) and thus the band is a certain distance across in the column. The number of plates in a column thus predicts the bandwidth of the peak, both in terms of plates and in terms of width or distance in the column.

It may seem strange that plate theory predicts that as N increases, σ increases, yet at the same time it predicts that as N increases, bandwidth decreases. What is decreasing is not bandwidth in terms of plates, it is bandwidth, s_d, in terms of the width or length of a band in the column. The symbol, s_d, will be used to indicate bandwidth in terms of distance or length and s_t to indicate bandwidth in terms of time (as observed on a chromatogram, see the figures above).

An example should help in understanding all this. Assume that two columns are to be compared, both 30 cm in length. Column #1 has 100 plates, column #2 has 10000.

For column #1: HETP = L/N = 30cm/100 = 0.30 cm.

For column #2: HETP = 30cm/10000 = 0.0030 cm.

When the band maximum reaches the end of column #1 (the place where one normally observes it), it will have traversed 100 plates and therefore $\sigma = 100^{1/2} = 10$, or $s_d = 10(0.30\text{ cm}) = 3$ cm. The half of the band still remaining on the column will extend back 3σ (30 plates) or $3s_d = 9.0$ cm. This is diagrammed in Figure 6-11.

When a band maximum reaches the end of column #2, it will have traversed 10000 plates, and therefore $\sigma = 10000^{1/2} = 100$. So σ for #2 is larger than σ for #1.

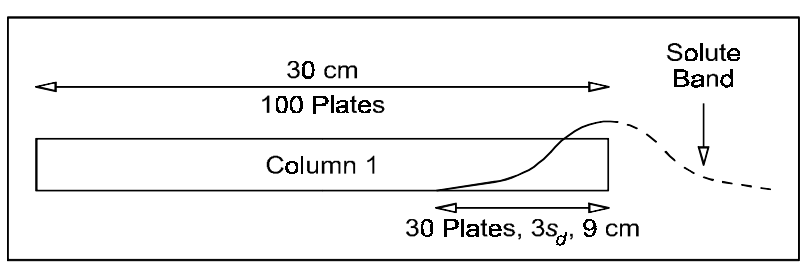


Figure 6-11

However, s_d for #2 = 100(0.0030cm) = 0.30 cm, and this is smaller than s_d for #1. The half of the band still remaining on column #2 extends back only 0.90 cm. Thus the physical bandwidth on column #2 is smaller than that on #1. This situation is diagrammed in Figure 6-12. The actual distance that a band occupies in a column decreases (by $N^{1/2}$) as N increases.

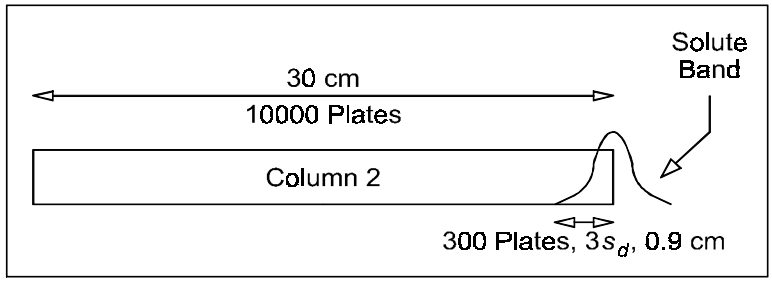


Figure 6-12

If all this emphasis on plates seems a bit esoteric, do not let the jargon bother you. Simply look upon N or HETP as a "figure of merit" for chromatographic columns. As N increases or as HETP decreases, the physical bandwidths decrease and separations get better. Bandwidth is a function of the square root of either N or HETP.

Since N (or HETP) is such an important parameter, a method must be devised to determine its value. This would be easily done if one could look into a column and measure the actual width of a band. From Eqn. (6-34) and the relationship between σ and s_d,

$$s_d = \sigma(\text{HETP}) = \sqrt{N}(\text{HETP}) \tag{6-35}$$

Substituting (6-33) in (6-35) gives

$$\frac{w_{1/2}}{2.35} = \sqrt{N}(\text{HEPT}) \tag{6-36}$$

Rearranging and substituting (6-28b) into (6-36) gives

$$N = \left(\frac{w_{1/2}}{2.35(\text{HETP})}\right)^2 = \frac{(w_{1/2})^2 N^2}{5.55L^2} \tag{6-37}$$

Solving for N provides the result

$$N = \frac{5.55L^2}{(w_{1/2})^2} \tag{6-38}$$

Thus N could be easily calculated from a knowledge of the column length and the half-peak bandwidth as shown in Figure 6-13. Unfortunately, most bands cannot be directly observed on columns. However, if one has a chromatogram (a plot of Detector Response *vs* time, as shown in Figure 6-14), one can perform the calculation even more easily than if one could observe a band directly on the column. The retention time of a substance is directly proportional to the column length and the width of the band in time is directly proportional to the width of the band in distance on the column with the same proportionality constant (that constant is simply the band velocity, v).

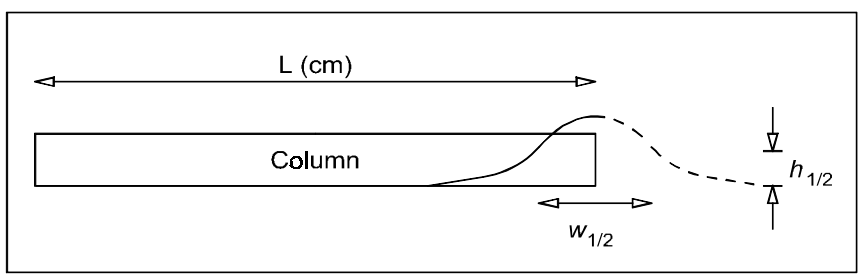


Figure 6-13

$$L = vt_r \qquad (6\text{-}39)$$

and

$$(w_{1/2})_d = v(w_{1/2})_t \qquad (6\text{-}40)$$

Substituting (6-39) and (6-40) into (6-38) gives

$$N = \frac{5.55(vt_r)^2}{[v(w_{1/2})_t]^2} = \frac{5.55t_r^{\ 2}}{(w_{1/2})_t^{\ 2}} \qquad (6\text{-}41)$$

providing a simple relationship between N, t_r, and $w_{1/2}$. There are other, similar methods for measuring N.

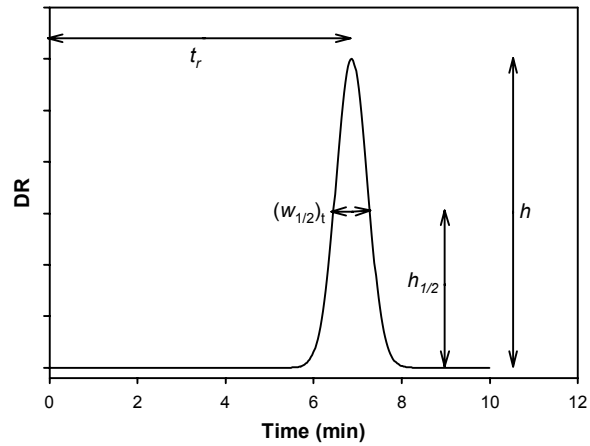


Figure 6-14

A 2.00 m packed CG column provides the following data for a hydrocarbon peak: t_r = 9.65 min and the times when the detector response was ½ the peak height = 9.40 and 9.89 min. What are the values of N and HETP for this column?

$(w_{1/2})_t$ = 9.89 - 9.40 min = 0.49 min

N = 5.55(9.65 min)^2/(0.49 min)^2 = 2.15x10^3

HETP = 2.00 m/2.15x10^3 = 9.3x10^{-4} m = 0.93 mm

The parameter, N, provides a method of comparing columns in terms of their efficiency of separation. Unfortunately plate theory does not provide a model that explains the factors that influence HETP. Thus it gives no clue as to how to design a column to provide better separations. A better theory is still needed.

6.2.4 RATE THEORY

One way to study the causes of band broadening is to vary different parameters and to observe the effect on HETP or N. N (or HETP) is easily measured as demonstrated above. The parameter that is most often varied is the velocity of the mobile phase, v_m, (or F). The behaviour shown in Figure 6-15 is observed if HETP is measured and plotted as a function of v_m.

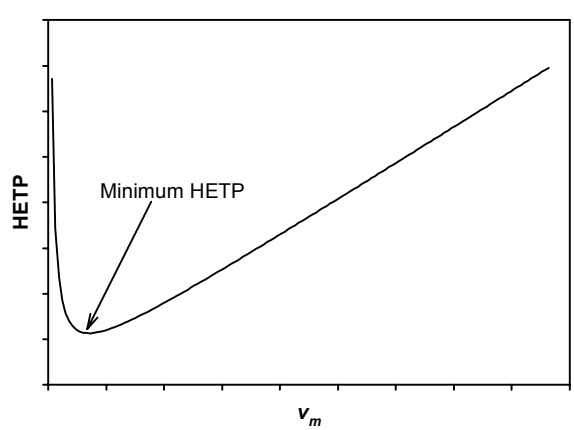


Figure 6-15

It was pointed out by VanDeempter that this sort of behaviour could be fit by a function of the following form:

$$\text{HETP} = A + B/v_m + Cv_m \qquad (6\text{-}42)$$

where A, B, and C are constants.

This equation is often referred to as the VanDeempter equation for this reason. It is strictly an empirical equation - in other words it seems to fit the data well, but it does not come *directly* from any

simple, all encompassing theory of chromatography. At low v_m the Cv_m term goes to zero and the B/v_m term controls HETP. Likewise at high v_m, the B/v_m term goes to zero and HETP is controlled by the Cv_m term. Often the A term is quite small and may not be particularly important in influencing the overall behaviour.

An attempt must now be made to explain how this type of behaviour could arise. Keep in mind that an increase in HETP means a decrease in N, the number of plates, and an increase in bandwidth. As HETP increases, separation efficiency decreases. Thus there is an optimum flow rate for the best separations, as shown in Figure 6-15.

The discussion is begun by first examining the B/v_m term and asking - why should the bands broaden as the mobile phase velocity becomes very low? The answer is that the band will do this because of the "natural desire" of the molecules in the high concentration band centre to diffuse toward lower concentrations. This is referred to as *longitudinal diffusion*, which is depicted in Figure 6-16. Thus the band will naturally become more diffuse (broader) with time. At very low flow rates (low v_m) all bands move through the column slowly and all have enough time to broaden under the influence of this type of diffusion. As v_m increases, all bands move through the column more rapidly and have less time to broaden due to longitudinal diffusion.

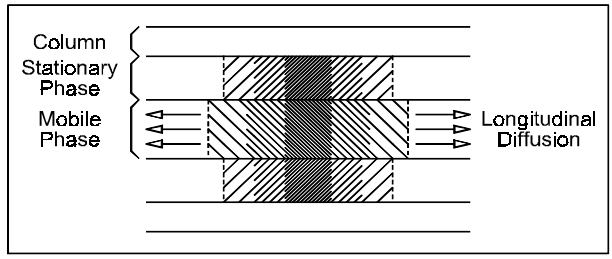


Figure 6-16

Can anything be done about this? Unfortunately the answer is no. All one can do is keep the flow rate high enough so that this is not a significant problem.

Next consider the A term. What effects could give rise to a contribution to bandwidth which is independent of flow rate? First of all, bands must start with a finite size - you cannot make the initial bandwidth infinitely narrow. This will contribute to the A term. There is a finite spread in molecular velocity (as explained above some molecules will move faster than the average, some slower) which will cause the band to broaden a fixed amount, independent of flow rate. Finally for packed columns, if the column is not well packed, channels may develop in the bed (obviously not a problem with capillary columns) that cause the molecules to travel vastly different path lengths. Anytime path lengths of differing sizes are available to the sample, molecules will traverse the column in different times (some molecules will travel longer distances and as a result take more time than others) and the bands will broaden. If the column is well packed and if the initial sample size is small (the initial bandwidth is small), the A term is usually also very small and is sometimes neglected in discussing band broadening.

Finally consider the Cv_m term. The factors giving rise to this term must depend directly on flow rate. Any slow step in the mechanism of column operation that affects the approach to equilibrium contributes to this term. If the flow rate is low, these slow steps have enough time to take place, equilibrium is achieved, and the separate sample bands in the two phases, which combine to make up the total band, stay together. When the mobile phase velocity increases, these steps do not have enough time to occur and the band in the mobile phase tends to get ahead of that in the stationary phase.

If equilibrium were instantly achieved between the mobile and stationary phases, the bands in these two phases would be in exactly the same place in the column. If equilibrium is not achieved, then the band in the mobile phase moves ahead of that in the stationary phase. The more slowly equilibrium is approached, the farther ahead the band in the mobile phase appears with respect to that in the stationary phase. The total sample bandwidth includes both of these individual bands. This is illustrated in Figure 6-17.

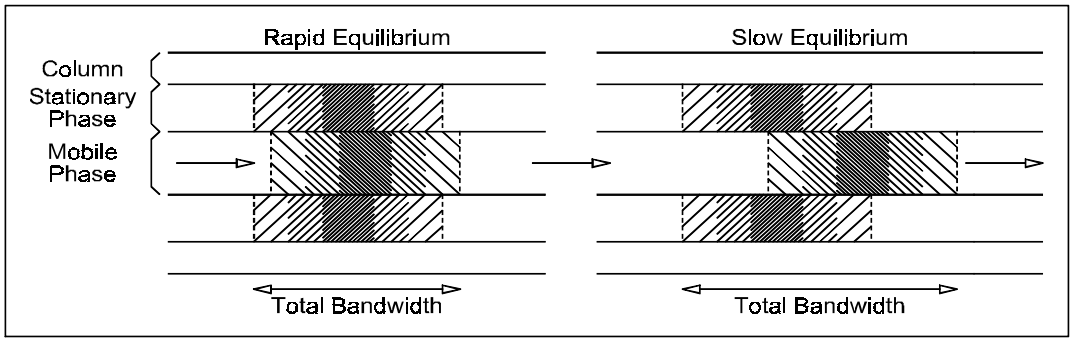


Figure 6-17

What factors might tend to slow down the approach to equilibrium? The first is *transverse diffusion* in the *mobile phase*. It takes time for solute molecules in the mobile phase to get to the phase boundary so that they can partition into the stationary phase, as shown in Figure 6-18. During this time they are also moving down the column, away from the band in the stationary phase. The solution to this problem is to make the distances travelled in the mobile phase as short as possible. In a packed column this requires the use of very small particles, in a capillary column, the use of a very small diameter tube.

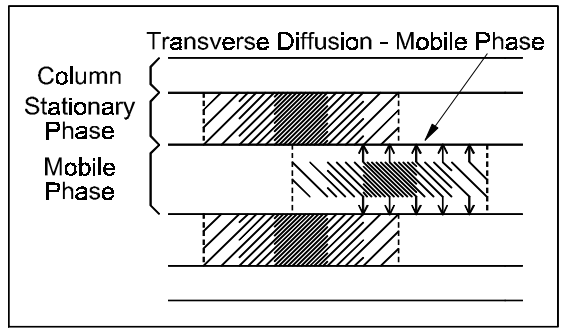


Figure 6-18

The second factor is transverse diffusion in the *stationary phase* (this does not apply when the stationary phase is a solid surface). This is the same effect as above, except the phase is different. During the time it takes sample molecules to diffuse up to the phase boundary to re-enter the mobile phase the band in the mobile phase is moving down the column, as illustrated in Figure 6-19. The solution in this case is to use a "light liquid loading", in other words keep the layer of stationary phase thin so that molecules do not have to travel very far to get to the mobile phase. This means that sample size must be kept small in order not to overload the stationary phase. When overloading occurs, band shape degrades markedly.

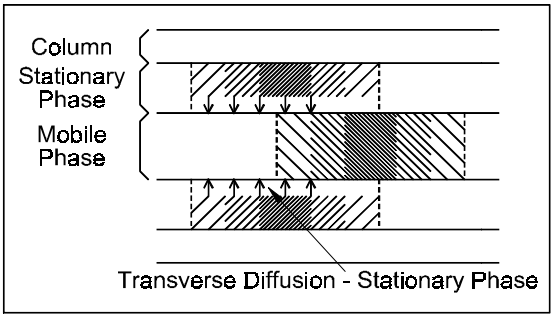


Figure 6-19

A third factor is adsorption on the "inert" solid support (the column itself or the particles used in packed columns) when the stationary phase is a liquid (GLC and LLC) or simply adsorption when the stationary phase is a solid (GSC or LSC). Adsorption equilibrium is often slow. Thus it will take time to desorb any solute that adsorbs on the solid surface and during this time the mobile phase band gets farther ahead of that in the stationary phase, which is diagrammed in Figure 6-20.

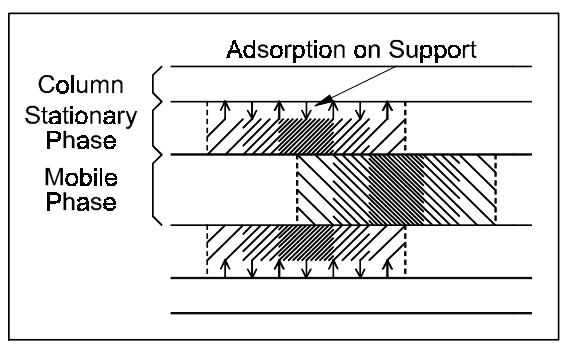


Figure 6-20

For GLC or LLC the solution is to use a truly inert support, one that does not adsorb solute molecules, or to treat the solid support to make it more inert. For GSC or LSC the solution is to design solids with uniform surfaces at which the adsorption equilibrium is fast.

A fourth factor could be slow partition equilibrium at the mobile phase - stationary phase boundary in GLC or LLC. This is not usually a problem. If it should arise one could raise the temperature or change one or both of the phases.

One can summarize the above to obtain a set of guidelines for obtaining the best separations:

1) Operate at minimum HETP (optimum v_m). This will mean running more slowly than you might like. High speed and good separation are often mutually exclusive.

2) Use a small sample size (small initial bandwidth).

3) Place the sample on your column evenly (again small initial bandwidth).

4) Pack your column evenly - avoid channels (for packed columns).

5) Use a small diameter solid support (for packed columns).

6) Use a solid support of uniform diameter (keeps path lengths even for packed columns).

7) For liquid stationary phases, use a small amount of liquid (requires small sample size).

8) For liquid stationary phases, use a truly inert solid support.

9) For solid stationary phases, use a solid with a uniform surface.

6.2.5 RESOLUTION

From the discussions above it is obvious that the ability to separate two compounds will depend on both the difference in retention times (or volumes) and the bandwidths of the two compounds. One might ask - is there a quantitative way to measure the "goodness" of separation? One method often used is to determine the *resolution*. Resolution, R, is simply defined as:

$$R = \text{(peak separation)/(peak width)}$$

$$R = \frac{(t_r)_B - (t_r)_A}{t_w} \tag{6-43}$$

where A and B represent the two materials being separated and t_w is the peak width in time. t_w is the width of the peak at the baseline, not the half-width. For reasons which will become obvious later, t_w is taken as $4s_t$ (not $6s_t$). This treatment assumes that $(t_w)_A = (t_w)_B$ - one can look upon t_w as an average of the two. Usually any two materials which one is worried about resolving will have bandwidths very close to one another and the above approximation is valid. (More rigorous but more complex treatments which do not make this assumption are available.) Thus

$$R = \frac{(t_r)_B - (t_r)_A}{4s_t} \tag{6-44}$$

where s_t is the average band standard deviation in time units. Note that R could have been just as easily defined as a volume or distance ratio.

If $R = 0.5$, the peaks will be separated by $2s$, if the two peaks contain equal amounts of material (peak areas are equal, assuming equal detector response), and if they are split one from the other

(separated) at the valley between them (in this case the peaks are too close to each other and there is no observed valley), each split will contain 84.1% of the major component and 15.9% of the minor component. Table 6-1 lists these split values for several values of resolution.

Table 6-1

Resolution	$\Delta t_r(s_t)$	Split(%)
0.50	2.0	84.1 to 15.9
0.75	3.0	93.3 to 6.7
1.00	4.0	97.7 to 2.3
1.50	6.0	99.9 to 0.1
2.00	8.0	99.99 to 0.01

Figure 6-21 shows graphically how changes in resolution affect the shape of the chromatogram for the conditions described above. The upper curves represent the actual chromatograms observed and the lower curves represent the individual peaks that overlap to produce the actual chromatogram. It is observed from Table 6-1 that any separation in which R is above 1.0 is good and that any separation in which R is above 1.5 is quantitative. Figure 6-21 shows that if R is 1.5, the separation is almost "baseline", if it is 1.0, the separation is not complete, but it is obvious that two compounds are present, and if it is 0.5, only one peak is actually observed, but the peak shape is somewhat distorted, indicating that the peak is made up of more than one component.

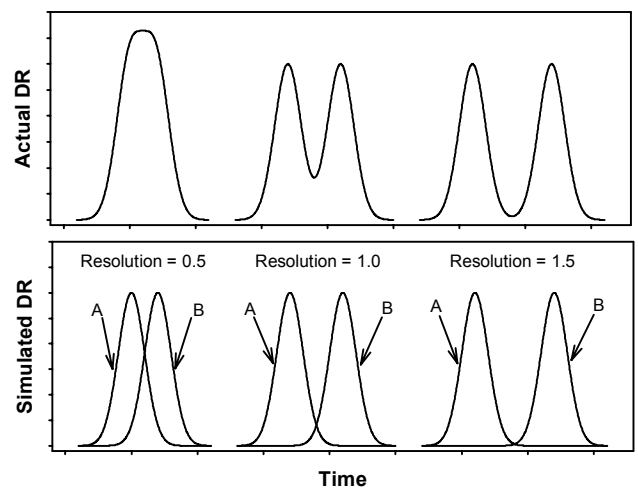


Figure 6-21

The factors that influence resolution need to be investigated in more detail. This can be done by substituting Eqn. (6-14) for $(t_r)_B$ and $(t_r)_A$ into Eqn. (6-44).

$$R = \frac{\left(\frac{(V_m + K_B V_s)}{F}\right) - \left(\frac{(V_m + K_A V_s)}{F}\right)}{4s_t} = \frac{V_s(K_B - K_A)}{4Fs_t} = \frac{V_s \Delta K}{4Fs_t} \qquad (6\text{-}45)$$

The standard deviation (in time) is related to s_d by

$$s_t = s_d / v \qquad (6\text{-}46)$$

where v is the sample band velocity, and s_d is related to column parameters as follows:

$$s_d = \sigma(\text{HETP}) = \left(\sqrt{\frac{L}{\text{HETP}}}\right)\text{HETP} = \sqrt{L(\text{HETP})} \qquad (6\text{-}47)$$

Substituting (6-47) , (6-39), and (6-14) into (6-46) leads to

$$s_t = \frac{\sqrt{L(\text{HEPT})}}{v} = \frac{t_r\sqrt{L(\text{HEPT})}}{L} = \frac{V_m + \text{K}V_s}{F}\sqrt{\frac{\text{HEPT}}{L}} \tag{6-48}$$

In Eqns. (6-46) to (6-48) the values of s_t, s_d, v, t_r, and K should be considered averages of the values for compounds A and B. Substituting (6-48) and (6-28a) into (6-45) produces

$$R = \left(\frac{F}{V_m + \text{K}_{\text{avg}}V_s}\right)\sqrt{\frac{L}{\text{HEPT}}}\left(\frac{V_s\Delta\text{K}}{4F}\right) = \frac{V_s\Delta\text{K}\sqrt{N}}{4(V_m + \text{K}_{\text{avg}}V_s)} \tag{6-49}$$

The treatment using the capacity factor instead of the distribution ratio is obtained by substituting Eqn. (6-18) into (6-49).

$$R = \frac{V_s(V_m/V_s)\Delta k'\sqrt{N}}{4\{V_m + V_s(V_m/V_s)k'_{\text{avg}}\}} = \frac{\Delta k'\sqrt{N}}{4(1 + k'_{\text{avg}})} \tag{6-50}$$

As expected, Eqns. (6-49) and (6-50) predict that R will be directly proportional to $N^{1/2}$ and to ΔK (or $\Delta k'$). Since resolution depends on $N^{1/2}$ and since N can always be increased by increasing the length of the column, better resolution can always be obtained by increasing the column length. However, the square root dependence means that to increase resolution by a factor of two the column must be lengthened by a factor of four. Thus in practice using longer columns to provide better resolution is usually avoided.

Resolution depends on the actual values of K (or k') and the method of column preparation in a bit more complex manner. Recall that retention time increases as either K or k' increases. If the retention time is large (the compounds emerge late in the chromatogram), $\text{K}V_s >> V_m$ ($k' >> 1$) then

$$R \cong \frac{\Delta\text{K}\sqrt{N}}{4\text{K}_{\text{avg}}} = \frac{\Delta k'\sqrt{N}}{4k'_{\text{avg}}} \tag{6-51}$$

In this case R depends on the *relative* difference in the distribution ratios and is independent of the amount of stationary or mobile phase used in preparing the column. If the retention times are small, in the limit the denominator becomes constant (independent of K or k'). If $V_m >> \text{K}V_s$ or $1 >> k'$,

$$R \cong \frac{V_s\Delta\text{K}\sqrt{N}}{4V_m} = \frac{\Delta k'\sqrt{N}}{4} \tag{6-52}$$

Here resolution does depend on the amounts of the different phases used in making the column. Assuming a constant value of ΔK, it will take fewer plates in a column to separate two substances as retention time decreases.

This treatment is useful for estimating the number of plates needed to separate two materials to any desired resolution.

Consider two compounds. If A has k' = 10.00 and B has k' = 10.50, calculate the retention times for both compounds and the number of plates needed to separate them with a resolution of 1.25 (a value considered to give a satisfactory separation). Do the same calculations if A has k' = 2.00 and B has k' = 2.50. In both cases $\Delta k'$ is 0.50. The column used in both cases has t_m = 1.50 min.

For the compounds with longer retention times:

$(t_r)_A$ = (1.50 min)(1 + 10.00) = 16.5 min

$(t_r)_B$ = (1.50 min)(1 + 10.50) = 17.25 min

k'_{avg} = (10.00 + 10.50)/2 = 10.25

$\Delta k'$ = 10.50 - 10.00 = 0.50

$R = 1.25 = 0.50(N)^{1/2}/4(1 + 10.25)$

$N = \{1.25(4)(11.25)/0.50\}^2$ = 12,700 = 13000 plates

For the compounds with the shorter retention times:

$(t_r)_A$ = (1.50 min)(1 + 2.00) = 4.50 min

$(t_r)_B$ = (1.50 min)(1 + 2.50) = 5.25 min

$R = 1.25 = 0.50(N)^{1/2}/4(1 + 2.25)$

$N = \{1.25(4)(3.25)/0.50\}^2$ = 1060 = 1100 plates

6.2.6 PEAK SHAPE

It was stated in Section 6.2.3 above that plate theory predicts a symmetrical, "Gaussian" shaped peak. In the ideal case this is what is observed. An ideal chromatographic system is one in which the partition coefficient, K, is truly independent of concentration. A common method of determining how K depends on concentration is to plot the concentration in the stationary phase, C_s, *vs* the concentration in the mobile phase, C_m, for the system. Such a plot is often referred to as an *isotherm* since it is done at constant temperature. The partition coefficient, K, is simply the slope of this plot. For an ideal system this plot is a straight line. An isotherm for an ideal chromatographic system and the resulting chromatographic peak shape are shown in Figures 6-22 and 6-23 respectively.

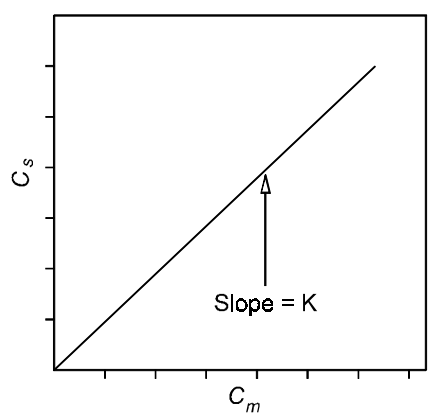


Figure 6-22

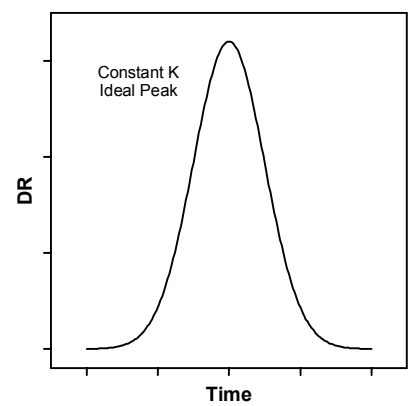


Figure 6-23

The common deviation from ideality is the case where K decreases as the overall concentration increases, resulting in the isotherm shown in Figure 6-24. This is often observed in systems where adsorption occurs, either on the solid support when the stationary phase is a liquid or on the active surface itself when the stationary phase is a solid. The reason for this is that the surface sites for adsorption are not uniform in their attraction for the sample molecules. The high energy sites fill up first (at low concentrations). Since these sites are strongly attractive, K will be large. When the high energy sites are full, lower

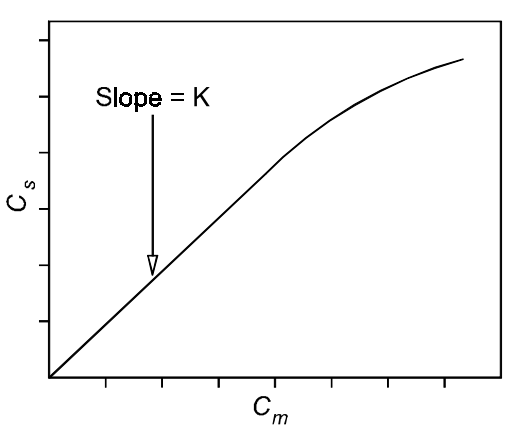


Figure 6-24

energy sites begin to fill (at higher concentrations). Since these sites are less attractive, K decreases. If all sites should be filled (surface saturation) the isotherm curve will completely flatten out.

The resulting chromatographic peak shape is shown in Figure 6-25. Since K decreases as concentration increases, K will be smaller at the band maximum than at the band edges and the band maximum will move more rapidly than the band edges. Thus the band maximum will catch up to the leading edge of the band and leave the trailing edge behind. This results in a peak with a very sharp leading edge and a long trailing edge. This behaviour is often referred to as "tailing". Since this is commonly observed with adsorption processes, which are part of the separation mechanism when the stationary phase is a solid (GSC or LSC), these types of chromatography are usually less ideal than chromatography which uses a liquid stationary phase (GLC or LLC).

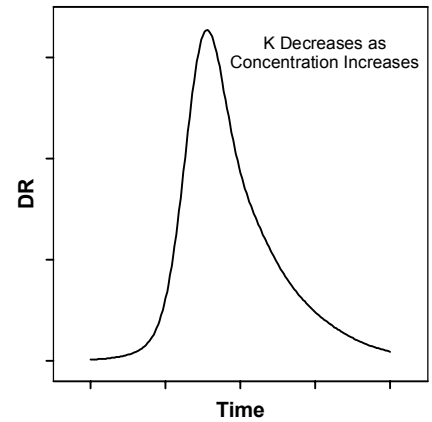


Figure 6-25

Obviously the other case of non-ideality would involve an increase in K with overall increasing concentration. The isotherm for this case is shown in Figure 6-26. This is sometimes observed when a chromatographic column using a liquid as stationary phase is overloaded. When this occurs, the sample actually begins to make up a significant fraction of the stationary phase and the properties of the stationary phase begin to change. Since the attraction of sample molecules for one another is often greater than the attraction of sample molecules for molecules of the stationary phase, as the concentration of sample in the stationary phase increases, its attraction for that phase also increases, resulting in an increase in K.

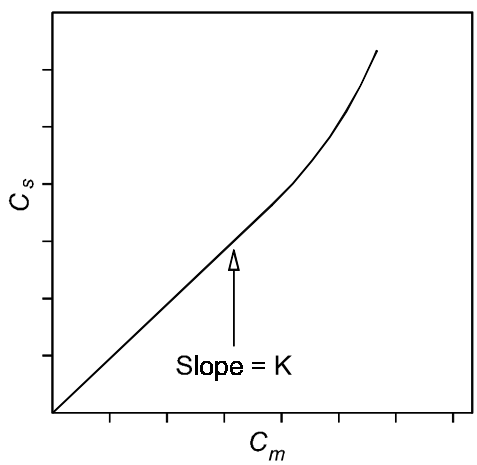


Figure 6-26

The chromatographic peak shape resulting from this behaviour is shown in Figure 6-27. In this case the band centre or maximum moves more slowly than the band edges. Thus the leading edge moves ahead of the band maximum while the trailing edge catches up to the maximum. This results in a peak with a very long leading edge and a very sharp trailing edge.

Unfortunately in both non-ideal cases, the actual peak width is larger than in the ideal case. Columns in which non-ideality occurs will have fewer plates than ideal columns, all other factors being equal. Polar compounds, which are more highly attracted to most stationary phases, tend to show less ideal behaviour than non-polar compounds. Non-ideality should be avoided if at all possible.

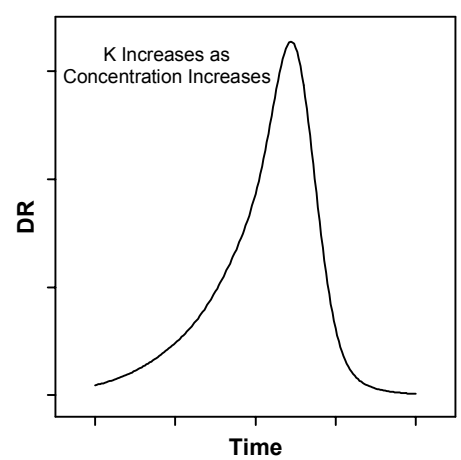


Figure 6-27

6.3 TYPES OF CHROMATOGRAPHY

So far the discussion of chromatography has been fairly general, mentioning only mobile and stationary phases, and assuming that sample molecules dissolve to some extent in both phases. It is now time to get more specific about the details of columns, phases, and separation mechanisms. As mentioned earlier there are two types of mobile phases - gases and liquids, and also two types of stationary phases - liquids and solids. Thus one classification method uses the types of phases involved in the separation. The major distinction between various chromatographic methods is made according to the mobile phase. Thus one speaks of *gas chromatography* or *liquid chromatography*.

6.3.1 GAS CHROMATOGRAPHY

In gas chromatography the mobile phase is always a gas, usually H_2, He, or N_2. The stationary phase can be either a liquid or a solid. However, solids are seldom used in practice (although there are certain applications where a solid must be used, *e.g.* analysis of gas mixtures) and the term "gas chromatography" is invariably taken to mean "gas-liquid chromatography". Only GLC will be discussed in this text. In this type of chromatography the stationary phase is always a liquid, usually very high boiling, which is either coated on the solid support or chemically bonded to it. The best way to proceed may be to describe the instrument, called a *gas chromatograph*. A block diagram of a gas chromatograph is shown in Figure 6-28.

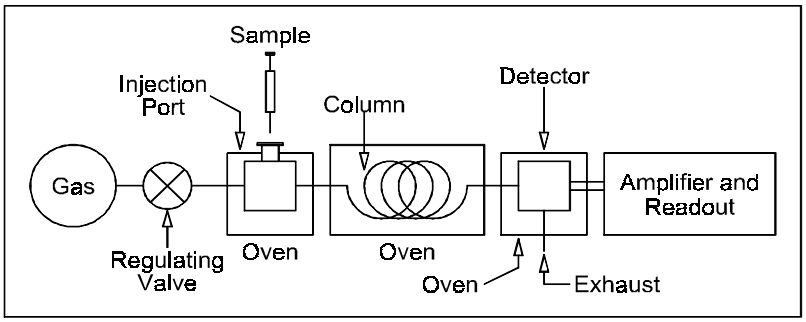


Figure 6-28

The mobile phase, called the *carrier gas*, is stored in a large metal cylinder under high pressure. The carrier gas should be light and inert. Thus He, H_2, and N_2 are usually used in this regard. Helium is the most inert and probably the best all-around choice, but also the most expensive. Hydrogen is the lightest, which has certain advantages, but is the least inert. A regulating valve is used to reduce the tank pressure to about 1-2 atm above ambient for use in the column.

The sample is usually measured in a glass syringe of about 1 to 10 μL capacity. Thus samples are not large and some sampling error results when trying to accurately deliver such small sample sizes. To place a sample on the column, the syringe needle pierces a rubber membrane called a septum and the sample is rapidly injected into a small, hot chamber called an injection port. The sample then volatilizes rapidly and is carried onto the column in the mobile phase. The injection port oven is usually maintained at a higher temperature than the column. The injection port must not be so hot as to pyrolyze the sample and must not react with the sample. Sample injection is somewhat of an art in gas chromatography.

The column has its own oven, the temperature of which can be changed quite rapidly. During an experiment the temperatures of the injection port and detector ovens are held constant, but the column oven, and thus the column, often change temperature. The need for this will be explained later. As mentioned previously, two types of columns are used, packed and capillary. Packed columns are typically 1 to 3 m in length and 3 to 6 mm in internal diameter with 1000 to 10000 plates. The inert support upon which the stationary phase is coated is usually the skeletons of one-celled plants called diatoms. This material is made of silica (SiO_2), is hollow, and has a diameter of about 0.15 mm. Capillary columns are small silica tubes, 0.1 to 0.5 mm in internal diameter and 10 to 100 m in length with 10000 to 250,000

plates. The stationary phase is usually bonded to their walls. These have become very popular in recent years and provide the best separations of all types of chromatography (they have the largest number of plates). Capillary columns require very small sample sizes and special injection ports.

Several types of detectors are in use, too many to describe each one individually. In order to keep the sample molecules in the gas phase, all detectors also are mounted in ovens, which are usually maintained at a higher temperature than the column. The most popular detector is the *flame ionization detector*, which is diagrammed in Figure 6-29. In this detector some or all of the effluent from the column is mixed with hydrogen gas and burned in an H_2/air flame. Molecules that contain C-H bonds produce ions and electrons in such a flame. These ions and electrons are collected by polarized plates placed on either side of the flame. The small current from these ions is amplified by the amplifier and displayed by some type of recording device or computer. This detector does not respond well to materials which contain no C-H bonds. Thus it is useful mainly for organic molecules, but since very few other substances are volatile enough to get through a gas chromatograph, this detector is almost a universal gas chromatographic detector.

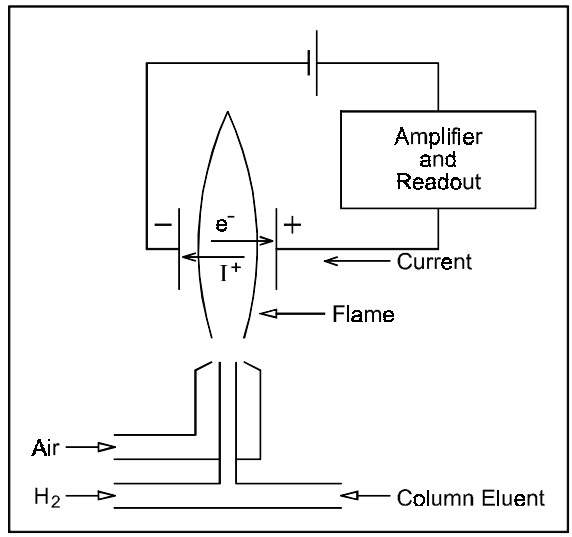


Figure 6-29

The carrier gas in gas chromatography is ideal, and therefore does not interact with the sample molecules. Thus the type of carrier gas used has very little effect on the separations. The factors which are most important in determining the preference of the sample for either the mobile or the stationary phase are the temperature and the polarity of the liquid (stationary) phase. The higher the temperature, the greater the vapour pressure of the sample components, and the greater the affinity for the mobile phase. Thus gas chromatography separates mainly based on sample boiling point (or vapour pressure). Components with higher boiling points (lower vapour pressures) will move more slowly through the column, and thus will have higher retention times.

If two sample components have the same vapour pressures, separations can usually be achieved if the molecules have different polarities. A non-polar molecule will prefer to dissolve in a non-polar stationary phase, likewise a polar molecule prefers polar stationary phases (like prefers like). Thus if two components have the same vapour pressure and are chromatographed on a column with a polar stationary phase, the more polar of the components will have the longer retention time and *vice versa*. Many different liquid phases of various polarities are available. These are selected, often by trial and error, to fit the separation at hand. They must be stable and non-volatile at the temperatures used. This places an upper limit of about 250 - 300°C on the use of gas chromatography. Above this temperature most liquid phases either begin to decompose or to bleed from the column.

What will occur if a sample contains a mixture of components with a wide boiling range? If the column temperature is held constant (termed *isothermal* operation) those components with low boiling points will come through the column almost immediately, all bunched together, those with intermediate boiling points will separate well, and those with high boiling points, while still separating well, will take a very long time to come through the column and will exit as very broad, small peaks which may not even be recognized as peaks. No single temperature can be found to separate all components of such a sample well and quickly. Figure 6-30 demonstrates this behaviour.

At first glance this might seem contradictory. Since N should be a constant for a column, all sample components should have the same σ and s_d. This is indeed the case (for similar sample components). However, the longer the retention time, the lower the sample velocity, v, and the longer it will take a component to come from the column. Thus s_t increases as t_r increases, even though s_d does not. Put a different way, $s_t = s_d/v$, and s_t, which is related to the peak width on the chromatogram, increases as v decreases while s_d remains constant. Thus for peaks of constant area, as shown in Figure 6-30, the peak height decreases and the peak width increases as t_r increases. The first peaks in a chromatogram are typically tall and narrow, and the last peaks are short and wide.

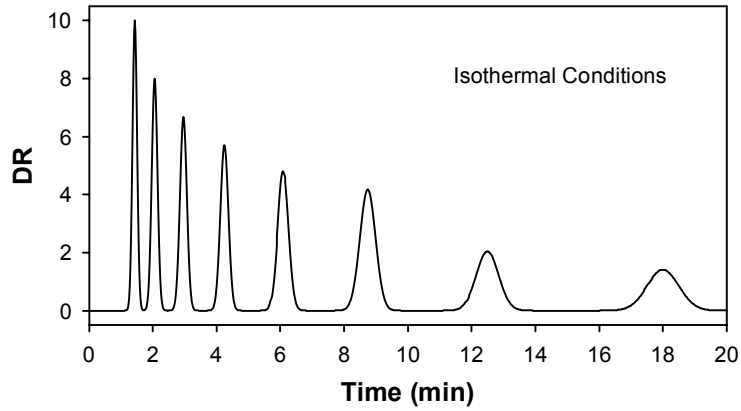


Figure 6-30

The solution to this dilemma is to change the column temperature during the experiment. This is called *temperature programming*. The temperature is initially held low so that the volatile sample components come through slowly enough to separate well. Then the temperature is raised so that the higher boiling components will come through more quickly while still separating well. Thus all peaks are fairly tall and narrow and the experiment can be done more rapidly. This is the reason that the column oven must be able to change temperature rapidly. Figure 6-31 shows a chromatogram of the same sample as shown in Figure 6-30 (run isothermally), but now run under temperature programming conditions. Note the difference in time scales in these two figures.

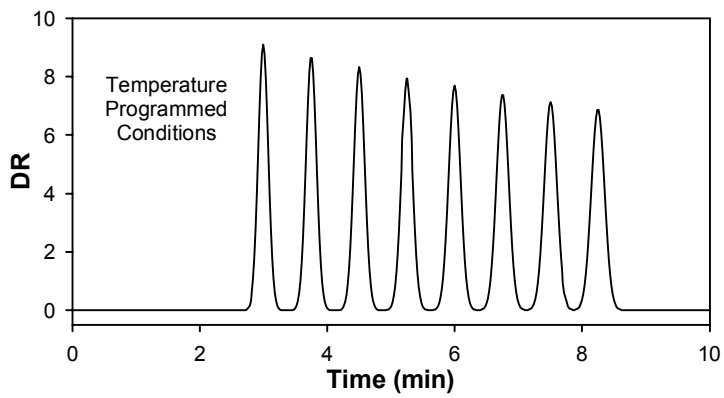


Figure 6-31

Normally in temperature programming the temperature is increased linearly at a rate of about 3 to 20°C/min. Sometimes the temperature is held constant for a short period of time before starting the program and often it is also held constant at the end of a run. More sophisticated chromatographs allow periods during the run when the temperature can be held constant. Note that the partition coefficient, K, and the capacity factor, *k'*, are both a function of temperature and will change during a temperature programmed run. Thus all equations derived above which assume that these parameters are constant are invalid for temperature programmed experiments.

Gas chromatography is probably the most *ideal* type of chromatography. It has available to it many sensitive detectors, the theory is well developed, most samples are well-behaved, and columns with large numbers of plates are readily available. Unfortunately sample components must be volatile at temperatures of 300°C or less (a sample component must have a minimum vapour pressure of about 2 mm Hg) and thermally stable at temperatures where they are volatile. This excludes many organic compounds, most compounds of biochemical interest (unless they are broken into smaller molecules

and/or derivatized), and almost all inorganic materials. Thus gas chromatography is best used for simpler organic molecules. For example, it finds wide use in the petroleum and chemical manufacturing industries.

6.3.2 LIQUID CHROMATOGRAPHY

Obviously liquid chromatography uses a liquid as the mobile phase. Today most analytical liquid chromatography is referred to as *HPLC*, which means either high pressure liquid chromatography or high performance liquid chromatography. This leads to an interesting bit of history regarding chromatography. The first type of chromatography was liquid chromatography. The columns used were large, open, had few plates, and were used mainly in preparatory work. This was the situation for many years before gas chromatography was developed. Rate theory was evolved by gas chromatographers, high-plate columns were developed, and liquid chromatography fell into disuse. Then liquid chromatographers read gas chromatographic theory and decided to make more ideal solid supports - more inert, more uniform, and much smaller in particle size. When this happened the number of plates in their columns increased dramatically (thus the "high performance"), but since the particle size was so small, the pressures needed to drive the mobile phase through the columns also increased dramatically (thus the "high pressure"). Liquid chromatography now approaches gas chromatography in its separation power and research in liquid chromatography has undergone a renaissance. Gas chromatography has certainly not fallen into disuse, but it is no longer at the forefront of chromatographic technology.

As was done with gas chromatography, the discussion will start with the instrument. In many respects the instrument is very similar to a gas chromatograph; however, there are significant differences. A block diagram of a liquid chromatograph is shown in Figure 6-32. The mobile phase or eluent is usually placed in a simple container such as a flask or bottle. This phase can be water, an organic solvent, or mixtures of different solvents. In fact mixtures are the most common eluents.

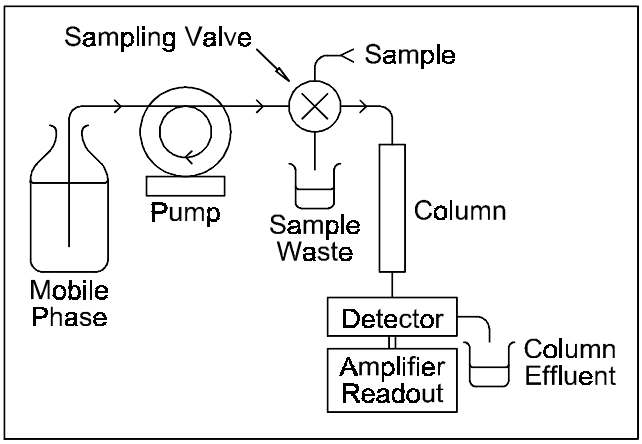


Figure 6-32

The pressures required for modern HPLC work usually range from about 100 atm to about 500 atm. Thus the next component in line after the solvent reservoir is a pump capable of providing these high pressures with a constant flow. In many of the more versatile liquid chromatographs two or more solvent reservoirs are provided and the pump is actually capable of mixing two or more solvents in any ratio. This ratio can be changed during an experiment.

Due to the high pressures involved, one cannot inject a sample onto the column directly with a syringe, as is done in gas chromatography. Instead a sampling valve is used. This valve contains a sample loop which has a fixed volume. The valve shown in Figure 6-33 is a six port valve, that is, it has six connections. It is shown in the sample loop filling position. In this position the high pressure pump is connected directly to the column and the sample loop is available for filling with sample. Once the loop is full the rotor is rotated 60° clockwise (with respect to the stator), which places the loop between the pump and the column. All the contents of the loop are thus forced onto the column. The valve can remain in this position as long as desired during the chromatogram, but the rotor must be rotated 60° counterclockwise to refill the loop.

The advantage of this arrangement is that a constant amount of sample can be placed on the column with each injection (unless air is somehow placed in the sample loop) without great difficulty. On the other hand, great care must be taken with syringe injection in gas chromatography to get constant sample amount. However, one cannot vary the sample size easily with an injection valve; one must change the sample loop to do so. With syringe injection, sample size variation is simply a matter of injecting different volumes.

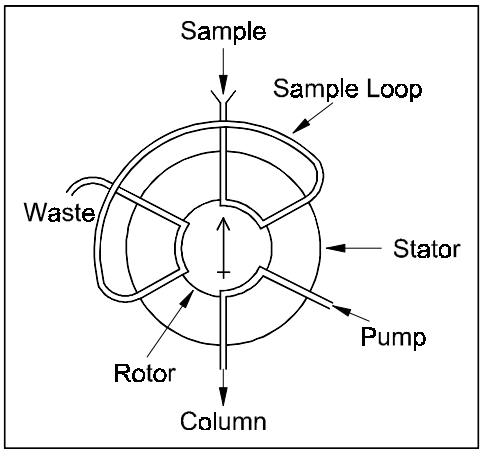


Figure 6-33

The columns themselves are usually stainless steel tubes packed with very small particles. The tube length is between 5 and 25 cm, the inner diameter is usually 1, 2, or 4 mm, and the particle size varies between 10 and 3 μm. The plate height (HETP) in liquid chromatography is smaller than in gas chromatography, but the columns are also much shorter because it would take tremendous pressures to drive solvents through long columns. Liquid chromatographic columns typically have between 2000 and 20000 plates.

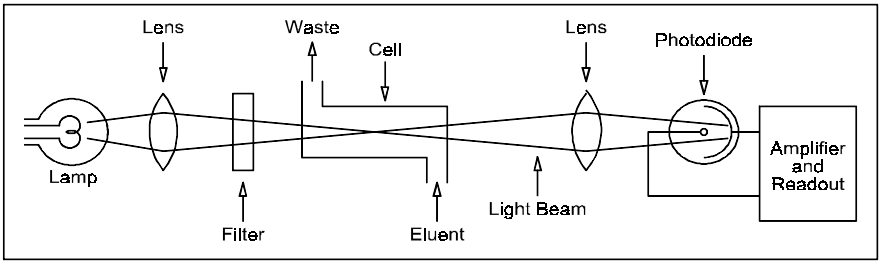


Figure 6-34

The detectors available for use in liquid chromatography are far different from those used in gas chromatography. In general LC detectors are less sensitive and less general than GC detectors. Spectrophotometers (see Figure 6-34) and fluorometers are two types of detectors commonly used in liquid chromatography. In these detectors a flow cell connected directly to the end of the column replaces the normal spectrophotometric cuvette. It is often the same length (1 cm). Obviously to use such detectors the sample must absorb light or fluoresce and the eluent must not. Not all samples absorb light and even fewer fluoresce, while some solvents do absorb light, especially in the ultraviolet. Thus these detectors are not general; that is, they will not be sensitive to all the components in a sample. There is no sensitive, general detector for liquid chromatography. However, any device capable of performing rapid analysis on a flowing stream can be used as an LC detector.

The amplifier and recording device are responsible for amplifying the detector signal and displaying it, just as with gas chromatography.

The actual mechanism of separation in liquid chromatography is somewhat different than in gas chromatography. In GC the mobile phase is ideal, it does not interact with the sample molecules, and thus the particular gas used is not important. Volatility differences between molecules are very important in determining whether good separations are achieved. In liquid chromatography the mobile phase is not ideal, it interacts strongly with the sample molecules, and thus it plays a very important role in the separations. On the other hand temperature is relatively unimportant, which is the reason many liquid chromatographs do not have ovens and are not thermostated. In liquid chromatography polarity, including sample, eluent, and stationary phase polarity, are the most important factors in determining whether good separations can be achieved.

In adsorption chromatography the "solid support" is not coated with anything. Its surface acts as the second phase for the separation, the place where the sample molecules reside when not in the mobile phase. The most common solid used in adsorption chromatography is silica (SiO_2), but alumina (Al_2O_3) is also often used. These can be obtained with various types of surfaces, depending on pretreatment. In general the mobile phases used in adsorption chromatography are relatively non-polar organic solvents such as hydrocarbons and chlorinated hydrocarbons. The more polar a sample molecule, the more it will prefer the silica surface to the liquid phase. Thus as the polarities of the sample molecules increase, so do the retention times, and separations are based on polarity. If it is desired to move polar samples more rapidly through a column, then the mobile phase is made more polar itself, either by changing the solvent completely (*e.g.* from hexane to methylene chloride or acetone) or mixing a more polar solvent with the original non-polar solvent.

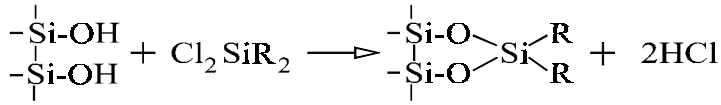
In partition chromatography the solid support is coated with the stationary phase. With most modern HPLC stationary phases, the liquid phase is actually chemically bonded to the solid support. This prevents the stationary phase from slowly leaching from the column. If one uses a non-bonded stationary phase, then one should saturate the mobile phase with molecules of the stationary phase before passing it through the column in order to slow down the leaching.

The most common solid support for bonded phases is silica. At first it might seem that bonding a liquid phase to a silica surface would be difficult, but it is easier than one might guess. The silica surface contains many -SiOH groups (silanol groups) that are relatively active. When these are reacted with a chloroorganosilane the following reaction occurs:

$$\text{-Si-OH} + \text{ClSiR}_3 \rightarrow \text{-Si-O-SiR}_3 + \text{HCl}$$

The "R" groups connected to the second silicon atom on the right make up the liquid phase. These are commonly either 8 or 18 carbon atoms in length and are hydrocarbons. When one refers to a "C18 bonded phase" one is speaking about the above type of stationary phase where the R groups are 18 carbons in length.

Variations in the stationary phase are achieved in several ways. One can change the length of the R group, *e.g.* from 4 to 8 to 18 carbons. One can add polar substituents to the R group such as -OH and $-NH_2$. Finally one can pack the surface with different amounts of stationary phase. The reaction shown above will attempt to provide three R groups per surface -SiOH group. The reaction below reduces this to one R group per -OH.



Although variety can be obtained with bonded phases as explained above, these phases are typically rather non-polar and the eluents used with them are usually polar, often involving solvents such as water, methanol, or acetonitrile (CH_3CN). On these types of columns the non-polar sample molecules prefer the stationary phase; the lower the sample polarity, the greater the retention time. To bring a non-polar sample through the column faster, one uses a less polar eluent or mixes a less polar component into the eluent. In terms of polarity, this behaviour is the reverse of that in adsorption chromatography (which was the first type of liquid chromatography employed). For this reason adsorption chromatography or any partition chromatography in which polar samples are more tightly held by the stationary phase and in which eluent polarity is increased during an experiment is called *normal phase chromatography*.

Partition chromatography in which non-polar compounds are more tightly held by the stationary phase and in which eluent polarity is decreased during an experiment is called *reversed phase chromatography*.

If one has a sample of many components which have a variety of polarities, it will be difficult to select an eluent with a polarity that will separate all the components well. Some components will come through too rapidly and will not separate, some will separate well, and some may not elute from the column. This is analogous to a sample in gas chromatography with a wide variety of volatilities. To separate such a sample in LC one must change the eluent polarity during the experiment. This is done by using two or more solvents of differing polarity and continuously changing the proportion of these in the mixture. This is termed *solvent programming* and is the reason why the more sophisticated liquid chromatographs have pumps with this mixing capability. Solvent programming is the LC equivalent of temperature programming in GC. An LC experiment in which the solvent composition is constant is termed *isocratic*.

The exact type of system that works best with a particular sample must often be decided by trial and error. Obviously, if one has a sample that can be separated by gas chromatography, one would use GC since it is simpler and more "ideal". Thus liquid chromatography finds use in separating temperature sensitive substances and non-volatile substances, which include many of the materials with which scientists deal. Many biological and biochemical separations use liquid chromatography as do most inorganic separations.

6.3.3 OTHER TYPES OF CHROMATOGRAPHY

There are several types of chromatography which do not easily fit into the above classifications. Three types, all forms of liquid chromatography will be discussed below.

Gel permeation chromatography

Gel permeation or *size exclusion* chromatography uses a porous solid phase that is neither coated (as in LLC) nor does it adsorb any of the sample molecules (ideally). This solid phase can be either an organic polymer or an inorganic structure, but in either case it must have small pores of a uniform size. It is produced in the form of small, uniform particles and is packed into columns in the usual manner. The behaviour of sample molecules toward the stationary phase will depend on the size of the sample molecules. If the sample molecule is too large to enter any of the pores, it is not retained at all, and it moves with the velocity of the mobile phase (in other words $t_r = t_m$). Another way to look at this is to say that since it has no affinity for the stationary phase, K = 0, and thus, using Eqn. (6-16) from Section 6.2.1

$$V_r = V_m \tag{6-16}$$

and thus

$$t_r = t_m \tag{6-53}$$

If the sample molecule is so small that it can enter all the pores, then all of the volume of the stationary phase is available to it (it can go anywhere it likes in the porous stationary phase) and K = 1. This states that such sample molecules have equal affinity for the mobile and stationary phases. In this case the molecules will spend time in the stationary phase and will travel more slowly than the large molecules that could not enter the stationary phase. Using Eqns, (6-14) and (6-15)

$$t_r = (V_m + V_s)/F \tag{6-54}$$

and

$$V_r = V_m + V_s \tag{6-55}$$

Eqns. (6-54) and (6-55) represent the maximum values of t_r and V_r that any material can have, assuming no interaction with the solid walls of the pores. If the sample molecules are intermediate in size, then some but not all of the pores will be available to them. Thus they will spend some time in the stationary phase, but not as much time as the small molecules. They will move more rapidly than the small molecules, but not as rapidly as the very large molecules. In this case $0 < K < 1$ and t_r and V_r will be intermediate between the two cases discussed above.

Typically V_s is between 4 and 10 times the value of V_m for gel permeation columns. This means that the maximum retention time on such columns is also roughly between 4 and 10 times t_m (the minimum retention time). Thus chromatograms for such columns take very little time and the number of plates in these columns is small. Nevertheless, since this type of chromatography separates on the basis of size rather than polarity or volatility, it is very useful.

Stationary phases are available with different pore size ranges. The behaviour of three such phases is shown in Figure 6-35. Each pore size separates a range of molecular weight materials well. For example, Figure 6-35 indicates that a stationary phase with an average pose size of 10 nm will separate molecules that range in molecular weight from about 100 to about 10,000. Compounds with molecular weights above and below this optimum range are separated poorly or not at all. Thus you choose the stationary phase pore size to fit your sample, generally the average pore size should fit the molecule of median size in your sample. This type of chromatography is particularly useful to biochemists and polymer chemists, both of which work with large molecules, and molecules with a large range of sizes.

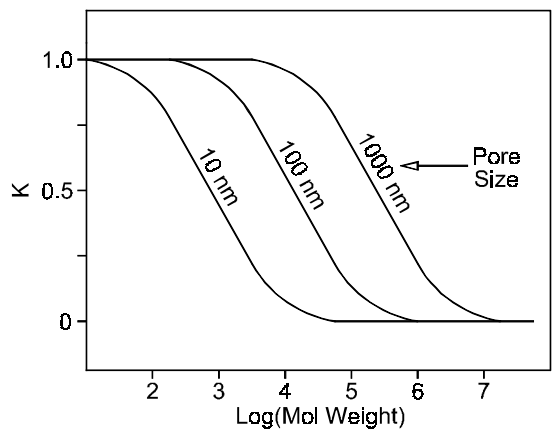


Figure 6-35

Often in GPC a linear relationship exists between log(molecular weight) (logMW) and t_r (see Figure 6-35). A standard with MW of 35,000 Da has a retention time of 7.52 min and a standard of 62,000 Da has t_r of 4.21 min. What is the approximate MW of a material with a retention time of 5.13 min?

For a linear relationship $\log MW = a + bt_r$

$b = \text{slope} = \{\log(MW)_1 - \log(MW)_2\}/(t_{r1} - t_{r2})$

$b = (\log 35000 - \log 62000)/(7.53 - 4.21)\ \text{min} = -0.0748\ \text{min}^{-1}$

$a = \log MW - bt_r = \log 35000 + (0.0748\ \text{min}^{-1})(7.52\ \text{min}) = 5.11$

$\log MW_u = 5.11 - (0.0748\ \text{min}^{-1})(5.13\ \text{min}) = 4.72$

MW = 53000 Da

Ion exchange chromatography.

So far most of the discussion has concerned itself with types of chromatography that would be most useful to organic or biochemists. There is one type that is particularly useful to inorganic chemists,

ion exchange chromatography. In this type of chromatography the stationary phase is usually an organic polymer which is somewhat porous, but more importantly, contains groups that can ionize.

One of the most common polymers used for the stationary phase is polystyrene, made from styrene (also named vinylbenzene or phenylethene) as shown in Figure 6-36. This results in molecules which are long strings or chains. Such molecules do not form a rigid, yet porous, matrix, the type that is desired. To achieve a rigid, yet porous, matrix, a small amount of *p*-divinylbenzene (shown in Figure 6-37) is added to the mixture before polymerization occurs. This molecule acts to link the separate long chains together and is termed a *crosslinking* agent. The crosslinked structure is depicted in Figure 6-38. The larger the amount of such a reagent in the polymerization mixture, the smaller the pore size and the more rigid the matrix.

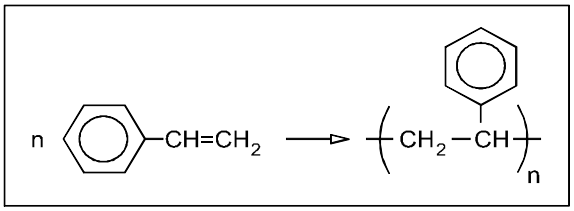


Figure 6-36

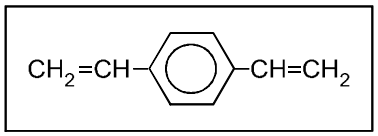


Figure 6-37

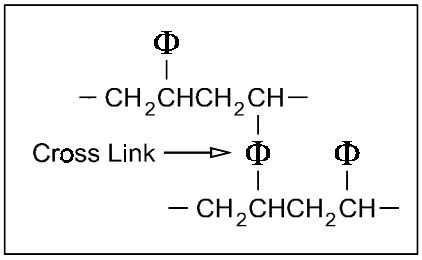


Figure 6-38

The type of material that we have described so far can be used in gel permeation chromatography as described above. However, to act as an ion exchange medium, ionizable groups must be added to the matrix. One way to do this is to sulfonate the benzene rings in the polymer, which results in the structure shown in Figure 6-39. The sulfonate group is permanently attached to the polymer backbone, but the hydrogen ion associated with it can be replaced by any other cation, *e.g.* sodium or calcium.

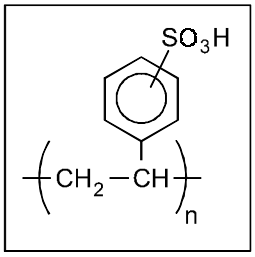


Figure 6-39

The polymer backbone is usually represented simply as "R", so the material which has been used in this example would be RSO_3H or $RSO_3^-H^+$. This is called a *strong acid cation exchanger* because the proton can be exchanged at any pH (it acts like a strong acid) and because it exchanges cations. If the sulfonate group were replaced with a carboxylate group, giving RCOOH, a weak acid cation exchanger would result. This material exchanges cations only above a pH of about 5. Anion exchangers result when the amine group or the quaternary ammonium group is substituted for the sulfonate group. $RNH_3^+OH^-$ is a weak base anion exchange resin which exchanges anions below pH of about 9 and $RN(CH_3)_3^+OH^-$ is a strong base anion exchange resin which can exchange anions at any pH.

Small beads of one of these ion exchange polymers are packed into a column in the usual fashion and mobile phase is passed through the column. The mobile phase is almost always an aqueous solution, also containing added ionic materials. Before placing a sample on the column, all of the ionizable groups on the resin are coupled with the same ion on purpose. The strong acid cation exchange resin will continue to be used as the example. For this type of material, the hydrogen ion usually is employed. The resin is converted to the hydrogen form by passing a solution of concentrated acid through the column. The result is that each RSO_3^- group has associated with it a hydrogen ion.

Now imagine that a sample is placed on the column, and that the sample contains two or more types of ions, other than H^+, which are to be separated. These might be Na^+ and K^+. What happens? The sodium ion and the potassium ion can exchange with the hydrogen ion.

$$RSO_3^-H^+ + Na^+ \rightarrow RSO_3^-Na^+ + H^+$$

$$RSO_3^-H^+ + K^+ \rightarrow RSO_3^-K^+ + H^+$$

Thus a sodium or potassium ion from the solution replaces a hydrogen ion on the resin and the hydrogen ion goes into solution. While the sodium and potassium ions are on the resin associated with the RSO_3^- groups, they do not move. At some later time another hydrogen ion from solution will exchange with the sodium or potassium ions, allowing the latter to go back into solution. Thus the bands of these ions move down the column, continuously exchanging with hydrogen ion. If one of these ions is more attracted to the resin than the other, it will move more slowly, and separation will be achieved.

Anion exchange chromatography works in exactly the same way, except that anions are continuously moving back and forth between the mobile and stationary phases. These again can separate on the basis of differences in affinity between ions for the stationary phase.

Note that the mobile phase or eluent *must* contain the ion that is associated with the resin in the beginning, H^+ in the example above. If it did not, the Na^+ and K^+ would go onto the stationary phase, but would never come off, since there would be no ions in solution with which to exchange.

$$RSO_3^-Na^+ + H^+ \rightarrow RSO_3^-H^+ + Na^+$$

Ion exchange chromatography is very useful in separating inorganic materials, both cations and anions. However, one cannot normally separate both the anions and cations in a sample in one experiment. Ion exchange chromatography, for example, is very useful for identifying the ionic components in acid rain and other polluted natural water samples. However, this type of chromatography also works well with organic ions. There are types of amino acid analyzers which use ion exchange columns to separate the amino acids in a sample one from another. Ion exchange resins are also used in water purification. "Deionized" water results when ordinary water is passed through a mixture of a cation exchange resin in the hydrogen form and an anion resin in the hydroxide form. All cations are exchanged for H^+ and all anions for OH^-. These two ions react with each other to produce water. Thus no ions remain in the water (except for the residual hydrogen and hydroxide ions). This is a better way of removing ions from water than distillation.

Thin layer chromatography.

Thin layer chromatography (TLC) is not a different form of chromatography, like ion exchange chromatography, it is simply adsorption chromatography (LSC) done in a different manner. Rather than packing the stationary phase, usually SiO_2, into a column, it is spread in a thin layer on a glass plate or piece of plastic sheet (thus the name TLC). Often a binding agent, *e.g.* calcium sulfate, is added to the stationary phase to make it adhere better to the plate.

Samples are placed on the plate near the bottom in a small spot and the spotted plate is placed upright in a tank which contains a small amount of eluent (mobile phase). The level of the eluent in the tank must be below the position of the spot on the plate. The eluent moves up the plate by capillary action, dissolves the sample and carries the sample up the plate with it. The normal chromatographic processes occur as the sample and eluent move along the plate and the sample components separate. The plate is removed from the tank before the eluent reaches the top.

The plate is then dried (the eluent evaporates) and developed, if necessary. Development usually involves treating the plate with a substance that makes the sample components coloured. The resulting chromatogram is a series of spots, which represent the sample components, spread along the plate. Since the sample components never leave the plate, retention times are not measured. Instead the distance from the original sample spot to the solvent front when the experiment was ended, d_f, and the distance

from the original sample spot to the final sample component spot, d_s, are measured. The *retardation factor*, R_f, is the ratio of these two distances.

$$R_f = d_s/d_f \quad (R_f \leq 1) \tag{6-56}$$

Since sample components move different distances along the plate, they have different R_fs, and qualitative identification is made on the basis of R_f.

TLC gives the same types of separations as LSC with the same types of samples and eluents. Its great advantages are simplicity and low cost. It requires no expensive instrumentation and more than one sample can be run at the same time. Its ability to do quantitative analysis is limited, but it is excellent as a survey technique, for optimizing parameters for a separation to be done later by LSC, or as a test for sample purity.

You are attempting to devise a more direct synthesis for an organic compound of interest. You run thin layer chromatography on a pure sample of the compound you wish to prepare and obtain the following results: distance of original spot to solvent front = 18.3 cm, distance of original spot to final sample spot = 12.5 cm. You subject the final reaction mixture of your new method to TLC (run in the same way) and obtain the following: distance of original spot to solvent front = 16.6 cm, to sample spot #1 = 8.5 cm, to spot #2 = 11.3 cm, and to spot #3 = 13.1 cm. Have you succeeded in your synthesis?

The R_f for the pure known compound is calculated as follows:

R_f = 12.5/18.3 = 0.683

The R_fs for the compounds in the reaction mixture are:

R_{f1} = 8.5/16.6 = 0.512

R_{f2} = 11.3/16.6 = 0.681

R_{f3} = 13.1/16.6 = 0.789

Compound #2 in the reaction mixture has the same R_f as the target compound. Therefore you are probably successful; however, more definitive proof must be obtained before you can be sure.

6.4 USES OF CHROMATOGRAPHY

6.4.1 SEPARATIONS

Chromatography was introduced as a separation technique, and it always is used to perform separations. However, it may be used to do analysis as well as separations. The first use to be considered will be to perform separations only - in this regard the term "*preparative chromatography*" is often used. When chromatography is used simply to separate the components of a mixture for preparative purposes, a detector is not necessary (but may be very useful) and if a detector is employed, it should be non-destructive or should sample only a small fraction of the column effluent.

A device called a fraction collector is normally used to split the effluent from the column into small fractions. Most fraction collectors consist of a movable stage containing a series of tubes which can individually be positioned for connection to the column. The position of the stage is controlled by a timer and positioning motors so that each tube spends an exactly controlled amount of time attached

to the column. Thus each tube in the fraction collector will contain a fixed fraction of the eluent that moves through the column. If the chemist sets the system up correctly, the sample components will appear separated in different tubes in the fraction collector. After the experiment the chemist can then select any of the tubes for whatever purpose is desired.

Packed columns are almost universally used in preparative chromatography because these can separate much larger samples. Since large packed columns usually do not contain as many plates as small packed columns or capillary columns, the separations are usually not as good in preparative chromatography as in *analytical* chromatography.

6.4.2 QUALITATIVE ANALYSIS

When chromatography is used for analytical purposes, either for qualitative or quantitative analysis, a detector is almost always used and thus a chromatogram is available. A certain amount of qualitative information is available from this chromatogram. On a given column, any specific sample component will always have the same retention time, and thus retention time can be used to identify sample components. What is usually done is to pass standards (known substances) through the chromatograph and measure the various t_r values. Such standards must be run on each new column because t_r will change from column to column, even though the same materials are used for all columns. Column characteristics also change somewhat with time, so it is wise to remeasure the standards from time to time on the same column. Then the sample is run and if a peak in the sample occurs at the same t_r as one of the standard peaks, that component in the unknown is assumed to be the same as the standard.

This is not foolproof and cannot be taken as absolute proof of identity. Whereas it is true that a given component will always have the same retention time from run to run (provided that the column has not aged to any extent), it may also happen that another substance in the sample will have the same retention time. Thus to positively identify a substance, information additional to the retention time will be necessary. However, often in routine analysis when a series of well characterized samples is being analyzed, retention time alone is considered sufficient to identify the presence of a specific component.

Sometimes a sample whose characteristics are less well established is analyzed and component peaks need to be identified. If no standards are available which correspond to the suspected unknowns, can anything be done? One can still go to the literature to see if another chemist has analyzed a similar sample. However, one cannot directly use t_r or V_r values from the literature for identification, even if your column is packed with the same materials as that in the literature. The reason is that your column will almost certainly have a different V_m and V_s than the one in the literature. *k'* for your column will also be different from that of the literature column. However, if you use the same conditions (same stationary and mobile phases, same temperature or solvent composition) as those used in the literature, the value of K, the partition coefficient, will be the same for both systems. Is there any way this can be used?

Proceed as follows:

$$V_r = V_m + KV_s \qquad (6\text{-}15)$$

or

$$V_r - V_m = KV_s \qquad (6\text{-}57)$$

Now assume that you add a material to your sample which is not originally in the sample, but which must also have been in the samples described in the literature. This is essentially an internal standard. Its peak must not interfere with any of the other peaks in your sample. One uses the internal standard peak to calculate a parameter called the *relative retention ratio* (*RRR*) as follows:

$$RRR = \frac{(V_r - V_m)_{unk}}{(V_r - V_m)_{std}} = \frac{K_{unk}V_s}{K_{std}V_s} = \frac{K_{unk}}{K_{std}} \quad (6\text{-}58)$$

Since the Ks are the same for both columns, the relative retention ratios will also be the same, regardless of how the column is packed (provided the same materials are used). The values of relative retention ratios should be the same from lab to lab. Thus, if the *RRR* for your peak matches one from the literature, this can be taken as tentative identification, keeping in mind that more than one compound can have the same *RRR*. Retention times can be used to calculate *RRR* values as well as retention volumes.

$$RRR = \frac{(V_r - V_m)_{unk}}{(V_r - V_m)_{std}} = \frac{(t_r - t_m)_{unk}}{(t_r - t_m)_{std}} \quad (6\text{-}59)$$

Since the use of *RRR*s assumes that the Ks are constant, this method of qualitative analysis cannot be used with temperature programming in gas chromatography or solvent programming in liquid chromatography.

You are in the middle of a series of analyses for chlorinated hydrocarbons using GC and you must replace the column in your chromatograph with one made by a different manufacturer. The new column is made from the same materials as the old column. You do not have time to run all the standards, so you run chlorobenzene through the new column and calculate the retention times for all the other compounds from the chlorobenzene data. On the old column the retention times are as follows: "air" peak (non-retained peak) - 0.57 min, chlorobenzene - 5.23 min, *m*-dichlorobenzene - 8.46 min. For the new column the retention times are: "air" peak - 0.82 min, chlorobenzene - 4.96 min. What should the retention time of *m*-dichlorobenzene be on the new column?

Let chlorobenzene be the standard and *m*-dichlorobenzene be the unknown. For the old column:

RRR = (8.46 min - 0.57 min)/(5.23 min - 0.57 min) = 1.693

For the new column:

1.693 = (t_r - 0.82 min)/(4.96 min - 0.82 min)

t_r = 1.693(4.96 min - 0.82 min) + 0.82 min = 7.83 min

6.4.3 QUANTITATIVE ANALYSIS

The chromatogram also contains quantitative information. If the detector response is linear in concentration (and it usually is) then the peak area is directly proportional to concentration.

$$A = kC \quad (6\text{-}60)$$

where A is peak area, C is sample concentration and k is the proportionality constant. Thus the measurement of concentration becomes a problem in the measurement of the area of a peak. This can be done in several ways. Figure 6-40 diagrams and "explains" the terms used in the discussion of the methods of area measurement to follow.

Peak height approximation.

Assume that peak height, h, is proportional to area. Thus

$$A = k_1 h \qquad (6\text{-}61)$$

and

$$h = k_2 C \qquad (6\text{-}62)$$

Since h is easily measured, this should be a simple way to do quantitative analysis if the assumption holds. Why might h not be proportional to area? Peak height depends on sampling technique - the narrower the starting band of the sample, the larger all peak heights will be, all other parameters being constant. Thus peak height will not necessarily always be proportional to area.

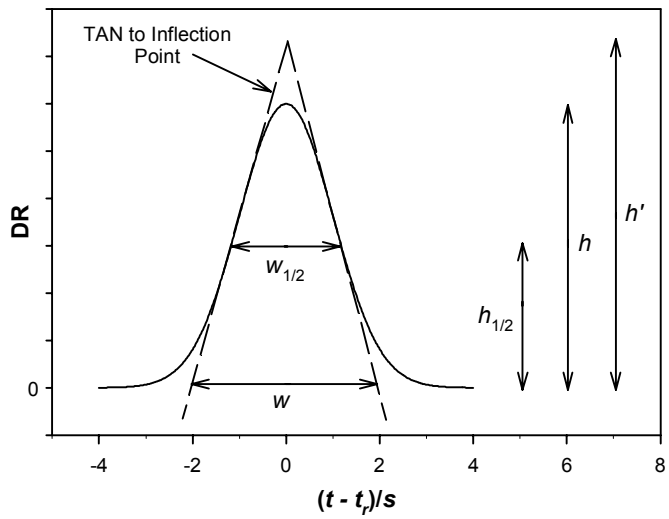


Figure 6-40

It is easiest to use this approximation for the first peaks in a chromatogram, those that come through the column most rapidly. Why? The longer the retention time of a peak, the greater the width of a peak (t_w or s_t). For this reason, peak height is most easily and accurately measured on the tall, narrow peaks at the start of a chromatogram. Refer to the discussion of temperature programming in gas chromatography (Figure 6-30) for an illustration of this effect.

Geometric area approximation.

Approximate the area using the peak height and width taken from the chromatogram. There are two common ways of doing this. (A) measure h and $w_{1/2}$ and assume that

$$A = hw_{1/2} = k_3 C \qquad (6\text{-}63)$$

The area calculated this way will always be 93.9% of the actual area of an ideal peak. (B) Draw tangents to the inflection points on the peak (at $\pm 1s$). These will cross the base line at $\pm 2s$ and will cross each other at h' above the actual peak (actually at $1.21h$). This forms a triangle, the base of which is w ($4s$) and the height of which is h'. The area of this triangle, which approximates the area of the peak, is $h'w/2$. Thus one assumes that

$$A = h'w/2 = k_4 C \qquad (6\text{-}64)$$

The area calculated in this way will always be 96.8% of the actual area of an ideal peak.

Cut and weigh.

Trace or photocopy the chromatogram, cut out the peak on the tracing, and weight the cutout peak. Here one assumes that

$$A = k_5(\text{mass}) \qquad (6\text{-}65)$$

and thus

$$\text{mass} = k_6 C \qquad (6\text{-}66)$$

Use an integrator.

An integrator is either a mechanical or an electronic device that measures the actual area under the curve and records it. There are several ways in which these devices work, which will not be mentioned. One word of caution, they measure the total area under a curve, which would include any drift in the baseline, not just the area due to the component of interest.

In general an integrator provides the best area estimate, followed by geometric approximation (especially for the later peaks in a chromatogram), and then by cut and weigh and peak height. Once one has established a method for measuring peak area, then one runs a standard to determine the proportionality constant between area and concentration, or better, one prepares a calibration curve using several standards, and then runs the sample. Using the calibration curve or the constant, one then determines the unknown concentration in the usual fashion. If area is actually directly proportional to concentration, then the method of standard addition can also be used.

Recall that in liquid chromatography a sampling valve with a fixed sampling loop is employed for sample introduction. When used properly, the same volume of sample is placed on the column with every injection. To prepare a calibration curve, standards with different concentrations of the unknown must be injected and the areas measured. However, in gas chromatography, a very small syringe, 1 to 10 μL, is used and the calibration curve can be prepared by injecting different volumes of the same standard and plotting area *vs* amount (usually in mols) injected.

Continuing with your analysis of chlorinated compounds (see the example above), a standard solution containing 0.0100 mol/L of chlorobenzene when chromatographed gave a peak height of 8.41 cm and a width (at half-height) of 33 s from a 5.0 μL injection. An unknown produced a peak height of 5.36 cm and a half-width of 31 s from a 2.0 μL injection. What is the concentration of chlorobenzene in the unknown?

Since the volumes injected are not equal, you must work with mols, since more fundamentally area is proportional to mols (only to concentration when the injected volumes are equal).

A_1 = (8.41 cm)(33 s) = 276 cm·s = K(mols)

K = 2.76×10^2 cm·s/(0.01 mol/L)(5.0×10^{-6} L) = 5.55×10^9 cm·s/mol

A_2 = (5.36 cm)(31 s) = 166 cm·s

mol_u = 166 cm·s/5.55×10^9 cm·s/mol = 2.99×10^{-8} mol

C_u = 2.99×10^{-8} mol/2.0×10^{-6} L = 0.015 mol/L

Internal standards.

One problem was mentioned above with regard to sampling with a syringe - the difficulty of reproducing or accurately measuring injection volumes with such a small syringe. Such injection is normally precise to no better than 10%. If more precision is needed, one can use an internal standard as described in Chapter 3 on the introduction to instrumental methods. Recall that the internal standard is a substance which is not in the sample, which is similar in its behaviour to the unknowns, which will not have the same retention time as any of the unknowns, and which is added to all standard solutions and to all samples in the same concentration.

One chromatographs all the samples (both standards and unknowns), usually injecting all solutions in the same volume, measures the areas of the analyte and the internal standard peaks in all experiments, and calculates the ratio of the areas of analyte to internal standard, A_{unk}/A_{std}, for each component and each experiment. The calibration curve, as shown in Figure 6-41, is prepared by plotting the area ratio *vs* the analyte concentration for the standards. The ratio depends on the accuracy with which you prepared the standard solutions, not on the volume injected. If the injection volume should increase by 10%, both areas, the internal standard and the analyte, would also increase by 10%, but the ratio would remain constant. Thus variations in injected volume do not affect the quantitative result.

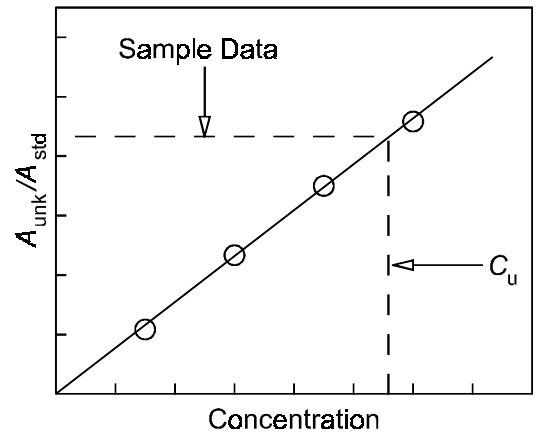


Figure 6-41

The final results are obtained from the area ratios of the unknowns and the calibration curve in the usual manner.

You are using GC for the analysis of fats (lipids). One of the main components of saturated fats is palmitic acid (hexadecanoic acid, $C_{15}H_{31}COOH$). Large carboxylic acids are difficult to chromatograph using GC (their volatility is low), but their esters are not. Thus the acids are often esterified prior to analysis by GC. 2.0 μL of a standard solution of 0.0100 M methyl palmitate and 0.0100 M methyl undecanoate (internal standard) produced areas of 386 and 291 units respectively on an electronic integrator. 1.00 mL of 0.100 M methyl undecanoate is added to 9.00 mL of a sample and this is analyzed in the same way, giving areas of 186 and 268 units for the unknown and the internal standard respectively. What is the concentration of methyl palmitate in the unknown?

Assume $A_u/A_s = KC_u/C_s$ or $K = A_uC_s/A_sC_u$

K = 386(0.0100 mol/L)/291(0.0100 mol/L) = 1.326

$C_u = A_uC_s/A_sK$ = 186(0.100 mol/L)(1.00 mL/10.00 mL)/268(1.326)

C_u = 0.00523 mol/L

However, the unknown was diluted with the standard. Thus

Original C_u = (0.00523 mol/L)(10.00 mL/9.00 mL) = 0.00581 mol/L

6.5 PROBLEMS

Sections 6.1 and 6.2: Basic Chromatographic Theory

6-1) 5.00 g of CCl_4 (d = 1.60 g/mL), the stationary phase, are loaded onto a KelF support (a polymer of C, Cl, and F), slurried with water, the mobile phase, and packed into a column. A solute which does not dissolve in CCl_4 (*e.g.* NaCl) has a V_r of 18.3 mL. A high molecular weight alcohol has a V_r of 52.1 mL on the same column. Calculate the partition coefficient for the alcohol. Ans: 10.8.

6-2) Determine V_r for the column of Problem 6-1 for alcohols with K = 8.61 and 12.82. Ans: 45.2 and 58.4 mL.

6-3) A 6.4 mm o.d. by 25.0 cm LC column is packed with 2.48 g of a 10 μ particle size silica gel (for adsorption chromatography) with a surface area of 300 m^2/g. The peak for a non-retained material emerges from the column after 95 s and the sample peak of interest after 343 s. Calculate K for the sample if the flow rate is 1.00 mL/min. Ans: 5.56×10^{-7} cm.

6-4) Assume that 20.0 μL of a 1.00×10^{-5} M solution of a material with an area per molecule of 100 $Å^2$ (1 Å = 0.1 nm) were injected onto the column of Problem 6-3. What is the minimum width (in cm) of the band that this material would occupy if it all adsorbed on the stationary phase (none remains in the mobile phase)? Ans: 4.05×10^{-6} cm.

6-5) A typical GC capillary column (open column) has a bore (inner diameter) of 0.250 mm and a length of 50.0 m. The column walls are coated to a thickness of 0.0100 mm. Calculate V_r for such a column for a compound with a K of 50.0. Ans: 20.9 mL.

6-6) The following are typical values in gas chromatography: F = 30.0 mL/min, t_m = 30 s, t_r = 300 s, wt% of the stationary phase with respect to the total column packing = 5.0%, weight of the column packing = 2.00 g, density of the liquid stationary phase = 0.90 g/mL. Calculate typical values for K and k'. Ans: 1.22×10^3, 9.00.

6-7) A 0.125 inch o.d. (outside diameter) GC column is packed as follows: 10.00g of inert support are mixed with a solution containing 1.000 g of stationary phase and the solvent is evaporated. The stationary phase density is 0.917 g/mL. As much coated support as possible is packed into the column; that remaining unpacked weighed 8.58 g. When operated at a flow rate of 20.0 mL/min of He the "air" peak (non-retained) emerged at 26.5 s and a sample peak at 138.1 s. What are the values of K and k' for the sample? Ans: 155 and 4.21.

6-8) The following data were taken in a GC experiment: t_r of air (non-retained) = 33 s, t_r of benzene = 275 s, t_r of toluene = 342 s. From the literature it is known that the partition coefficient of benzene for the stationary phase in your column and at your column temperature is 650. Calculate K for toluene. Ans: 830.

Section 6.2: Plate and Rate Theory

6-9) The column of Problem 6-1 would have to have 558 plates to separate the alcohols of Problem 6-2 with baseline resolution. If the plate height were 0.80 mm, how long a column would be necessary for such a separation? Ans: 45 cm.

6-10) A packed GC column with 4000 plates is a very good column. What is the half peak width for a peak with a retention time of 432 s on such a column? Ans: 16.1 s.

6-11) The bandwidth in chromatography could be defined as the width (distance) between the two inflection points on the peak (the points at which the slope changes sign). Assuming an ideal peak (Gaussian), show mathematically what this bandwidth would be in terms of s. At what height would this bandwidth be measured? Ans: $2s$, DR = $0.607h$.

6-12) Typical values for the column length, the number of plates, the flow rate of eluent, and the mobile phase volume are shown below for LC, packed column GC, and capillary column GC.

	LC	Packed GC	Capillary GC
L	10 cm	2 m	50 m
N	10,000	5,000	100,000
F	1 mL/min	20 mL/min	2 mL/min
V_m	0.400 mL	12.00 mL	2.00 mL

Compare these types of chromatography by calculating HETP, the halfwidth (in time) of a peak for a component with $k' = 10.0$, and the k' for a component that is separated by two halfwidths from the peak for $k' = 10$ (this separation is quantitative). Ans: 0.0010 cm, 0.040 cm, 0.050 cm, 6.22 s, 13.2 s, 4.92 s, 10.52, 10.73, and 10.16.

6-13) The flow rate for the column in Problem 6-5 is 2.00 mL/min and the halfwidth of the peak is 6.22 s. How many plates does the column contain? What is HETP? Ans: 5.65×10^4, 0.0884 cm.

6-14) The following data were taken on a 3.00 metre GC column using nitrogen gas as the carrier (mobile phase) and dibutyl phthalate as the stationary phase:

# of plates	gas velocity	# of plates	gas velocity
364	1.0 cm/sec	1875	20.0 cm/sec
850	2.5 "	1550	30.0 "
1460	5.0 "	1250	40.0 "
2000	10.0 "	1050	50.0 "
2030	15.0 "		

Plot the above data and determine (approximately) the coefficients (A, B, and C) of the Van Deempter equation. Assuming that V_m is 20.0 mL, determine the optimum flow rate. If 3.45 mL of the stationary liquid phase were used, determine the retention volume and retention time (under optimum conditions) for a substance with a K value of 50.0. Ans: A = 0.0202 cm, B = 0.799 cm^2/sec, C = 0.00498 sec., F = 50.7 mL/min, V_r = 192.5 mL, and t_r = 3.80 min.

6-15) The Van Deempter equation is usually written in terms of the velocity of the mobile phase, but it can also be written in terms of the flow rate of the mobile phase since flow rate is directly proportional to velocity. Thus one could write HETP = $A + B/F + CF$. A 3.00 m packed column is prepared using a solid support loaded with 1.89 mL of stationary phase. At a flow rate of 10.0 mL/min a non-retained substance exhibits a t_r of 2.83 min. A series of experiments determines that A = 0.0554 cm, B = 0.1682 cm·mL/min, and C = 0.00481 cm·min/mL. Determine the best flow rate, the minimum value for HETP and the maximum number of plates for this column. For a compound with a K of 30.0, determine the retention times for flow rates of 5.00, 10.0, and 20.0 mL/min. Ans: 5.91 mL/min, 0.112 cm, 2670, 17.0 min, 8.50 min, 4.25 min.

Sections 6.3 and 6.4: Chromatographic Analysis

6-16) Many commercial chemicals are not pure compounds but isomeric mixtures. Polychlorinated biphenyls, reputed by some to be very dangerous substances, are good examples. There are six isomers

alone of the dichlorobiphenyls (DCBs) (where the chlorines are on different rings). A commercial testing laboratory reports the following data:

Compound	V_r	Compound	V_r
"air"	4.3 mL	2,2'-dichlorobiphenyl	52.4 mL
2-bromobiphenyl	48.2	3,4'-DCB	53.2
2,3'-DCB	49.6	3,3'-DCB	53.9
2,4'-DCB	50.3	4,4'-DCB	54.6

and you observe the following values run on an unknown containing 2-bromobiphenyl as internal standard:

"air"	9.5 mL	Unknown #2	45.5 mL
2-bromobiphenyl	42.4	Unknown #3	47.1
Unknown #1	44.0		

Assuming that the temperature and column materials are the same for the two data sets, determine which components are in the sample. Ans: 2,2'-, 2,4'-, and 4,4'-DCB.

6-17) A mixture containing pentane (internal standard) and all the isomers of hexane was chromatographed (GLC) giving the following retention volumes: "air", 11.5 mL; pentane, 23.3 mL; 2,2-dimethylbutane, 37.2 mL; 2,3-dimethylbutane, 45.6 mL; 2-methylpentane, 47.1 mL; 3-methylpentane, 49.8 mL; and hexane 54.0 mL. Irv Gratch, an infamous undergraduate student, then used the chromatograph and from that moment on no further peaks were obtained from the instrument. A new column was packed (same materials as the first) and an unknown hexane sample (with pentane added) was run at the same temperature as the above mixture with the following results (V_r): "air" 5.7 mL; pentane, 27.2 mL; Unk #1, 67.8 mL; Unk #2, 75.6 mL; Unk #3, 83.1 mL. What isomers of hexane are in the sample? Ans: 2,3-DMB, 3-MP, and H.

6-18) An electronic integrator provides the following data for a set of standards and an unknown analyzed by GLC:

Sample	Reading(C_6H_{12})	Reading(C_6H_6)
0.00100 mol C_6H_{12} + 0.000100 mol C_6H_6	937	83
0.00100 mol C_6H_{12} + 0.000250 mol C_6H_6	1042	231
0.00100 mol C_6H_{12} + 0.000500 mol C_6H_6	868	384
0.00100 mol C_6H_{12} + 0.001000 mol C_6H_6	945	836
0.00100 mol C_6H_{12} + 1.00 mL of Unknown	998	291

The cyclohexane is used as an internal standard. All samples are diluted to 10.0 mL with a suitable solvent. Show whether the instrument response is linear in concentration. Compute the concentration of benzene in the original sample. Ans: 0.329 mol/L.

6-19) An internal standard is to be used for quantitative analysis in LC. 0.50 mL of 1.00×10^{-3} M internal standard is added to 4.50 mL of sample, giving mixture one. A 20 µL injection of mixture one produces areas of 35.4 (arbitrary units) for the internal standard and 15.5 for the unknown. 1.00 mL of the internal standard is added to 9.00 mL of a 1.00×10^{-2} M solution of the unknown to form mixture two. 0.20 mL of mixture two are added to 1.00 mL of mixture one to form mixture three. A 20 µL injection

of mixture three produces areas of 31.9 for the internal standard and 42.6 for the unknown. What is the concentration of unknown in the original sample? Ans: 7.52×10^{-4} M.

6-20) The composition of various oils used in cooking is of great concern since saturated oils have been linked to heart disease. These oils are the triesters of glycerol ($CH_2OHCHOHCH_2OH$) and long chain fatty acids. One method of analysis is to saponify the oil (the triglycerides), remove the glycerol, prepare the methyl esters of the acids, and analyze the methyl esters by GC. The fatty acids usually contain 16 or 18 carbons and are saturated or contain one or two double bonds. The chromatogram of Figure A-1 was taken from the literature and shows the following peaks: A - $C_{15}H_{29}COOCH_3$, B - $C_{15}H_{31}COOCH_3$, C - $C_{17}H_{31}COOCH_3$, D - $C_{17}H_{33}COOCH_3$, E - $C_{17}H_{35}COOCH_3$, and F - $C_{17}H_{35}COOC_2H_5$, added as internal standard. The chromatogram in Figure A-2 was taken on a column made from the same materials and run at the same temperature in an analysis laboratory. What compounds are contained in the sample from the second chromatogram? Ans: A, D.

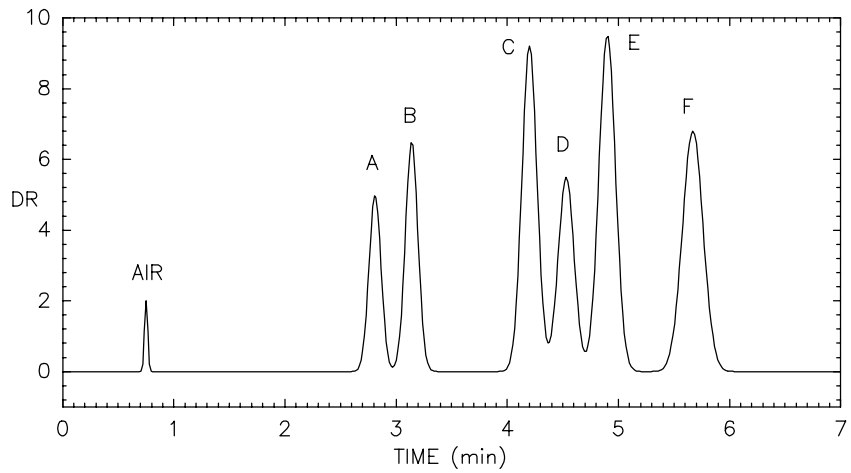


Figure A-1

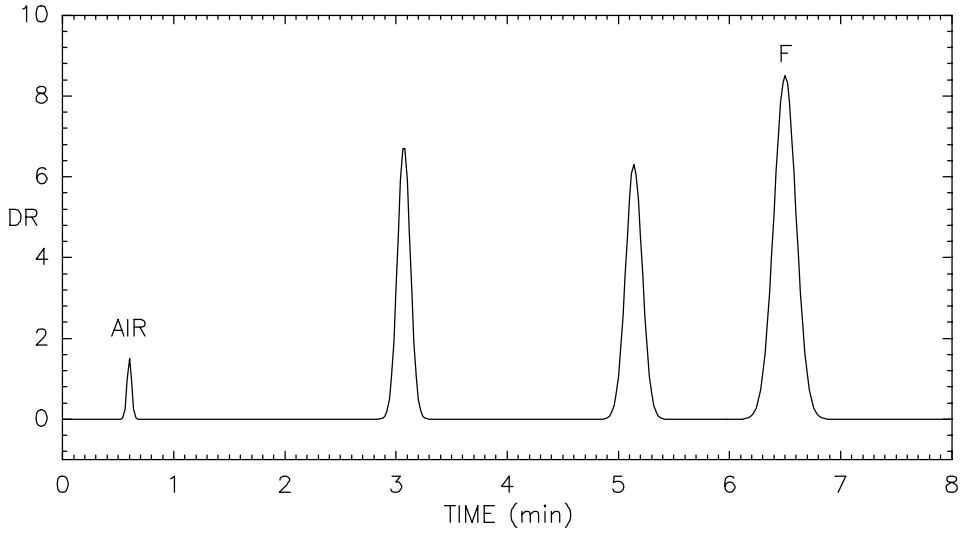


Figure A-2

6-21) To analyze caffeine in urine you prepare a 1.00×10^{-4} M solution of caffeine in methanol, inject 20.0 µL of this into a liquid chromatograph which uses a spectrophotometric detector operating at 254 nm and obtain the chromatogram shown in Figure A-3. You then obtain a urine sample, remove some of the sample components by extraction (not changing the sample volume) and inject 20.0 µL of this sample into the same column, obtaining the chromatogram shown in Figure A-4. Assuming that area is directly proportional to concentration, determine the concentration of caffeine in the urine sample. Ans: 2.90×10^{-5} M.

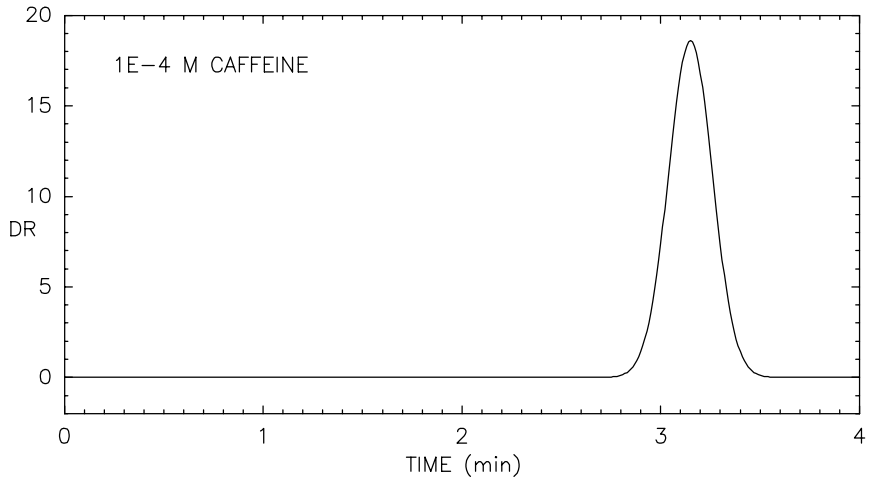


Figure A-3

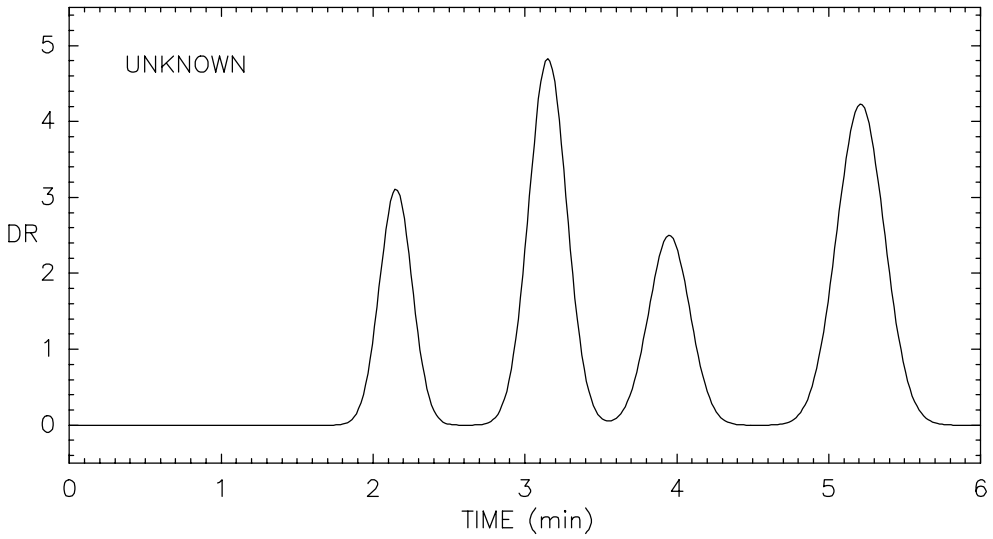


Figure A-4