Liquid Chromatography for the Analyst

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The object of an LC analysis is to establish the probable identity, and determine the precise amount, of each of the pertinent components present in the sample. The pertinent components may include all the substances present in the mixture or only those of specific interest. The identity of the peak is determined from its position on the chromatogram, that is, the time required for it to be eluted, whereas, the quantity of a component present is determined from the peak height or peak area. It must be emphasized that a single LC analysis on a *hitherto unknown sample* can not unambiguously confirm the presence of a particular compound on the basis of retention data alone. Retention data, whether it is corrected retention volume, capacity factor or the separation ratio of the solute to that of a standard, can only indicate the probability of substance identity. Retention data from a second analysis, using a different phase system, increases the confidence level but absolute verification requires confirmation by another analytical technique. This might include infrared spectrometry, mass spectrometry or nuclear magnetic resonance spectroscopy. Such evidence would be essential for litigation purposes.

There are in-line LC/spectroscopic systems available, but in most cases it is easier to carry out a semi-preparative separation, collect the material and carry out the spectroscopic examination off-line. However, for routine quality control analyses, where the *sample*

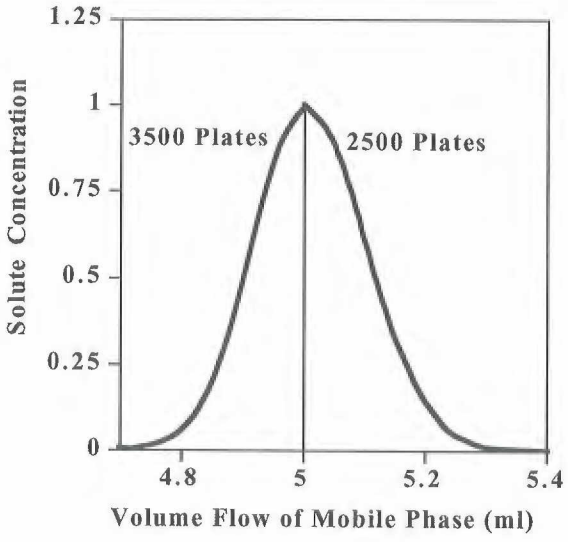
characteristics are already well established, retention data can be quite sufficient for identification purpose.

Qualitative Analysis

In LC both quantitative and qualitative accuracy depends heavily on the components of the sample being adequately resolved from one another. The subject of resolution has already been discussed, but it is necessary to consider those areas where uncertainty can still arise. Unfortunately, unless the analyst is aware of the pitfalls and how to deal with them, false assumptions of resolution can be made very easily.

Consider the liquid chromatography peak shown in figure 1.

Figure 1 **A Single LC Peak**



It is seen that the peak shown in figure 1 is asymmetric, which is typical of many peaks eluted from LC columns. This particular peak is not grossly asymmetric, the front half of the peak having an efficiency equivalent to 3500 theoretical plates and the latter half an efficiency of

2500 theoretical plates. This situation frequently occurs in LC and can be caused by a number of different effects. The two major causes of peak asymmetry have already been touched upon and can arise from a difference in peak dispersion occurring in the front half of the peak relative to the rear half or to the solute distribution coefficient being different for the two halves of the peak.

Re-iterating the HETP equation (10) given on page 104,

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

where the symbols have the meanings previously defined.

Now, it was also shown on page 145 that for a given column, solute, mobile phase and flow rate, equation (1) can be reduced to an alternative abbreviated form which is given as follows,

$$H = a + bD_m + \frac{c_1 d_p^2}{D_m} + \frac{c_2 d_f^2}{D_S} \qquad (2)$$

where $a = 2\lambda d_p$, $b = \frac{2\gamma}{u}$, $c_1 = f_1(k')u$ and $c_2 = f_2(k')u$.

On page 6, it was shown that in the front half of the peak, there will be a net transfer of solute from the mobile phase to the stationary phase and thus the resistance to mass transfer in the mobile phase will dominate. At the rear half of the peak there is a net transfer of solute from the stationary phase to the mobile phase and in this case the resistance to mass transfer in the stationary phase will dominate. Then if the resistance to mass transfer in the stationary phase is greater than that for the mobile phase, the rear part of the peak will be broader than the front half. In which case,

$$\frac{c_1 d_p^2}{D_m} < \frac{c_2 d_f^2}{D_S} \qquad (3)$$

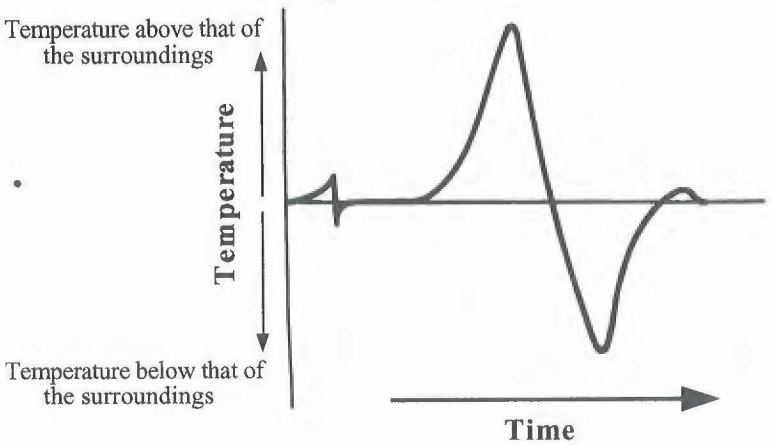
Consequently, the peak will exhibit the asymmetry shown in figure 1. It is seen that the relative values of the resistance to mass transfer terms is controlled by the functions, $\frac{c_1 d_p^2}{D_m}$ and $\frac{c_2 d_f^2}{D_S}$.

It would seem that, in practice, the inequality defined in (3) can frequently occur but the converse does not appear to be true. Thus, peak asymmetry (in part or whole) resulting from inequality in mass transfer between the two phases manifests itself in the form shown in figure 1.

Alternatively, peak asymmetry could arise from thermal effects. During the passage of a solute along the column the heats of adsorption and desorption that are evolved and adsorbed as the solute distributes itself between the phases. At the front of the peak, where the solute is being continually adsorbed, the heat of adsorption will be *evolved* and thus the front of the peak will be at a temperature above its surroundings. Conversely, at the rear of the peak, where there will be a net desorption of solute, heat will be *adsorbed* and the temperature or the rear of the peak will fall below its surroundings.

Figure 2

Temperature Profile of a Peak Passing Through a Heat of Adsorption Detector



In fact, this phenomenon has been used as the basis of a very sensitive detecting system. An example of the temperature profile of an adsorbent as a peak passes over it is shown in figure 2. Unfortunately, it was found almost impossible to produce a true simulation of the *concentration* profile of the peak from the *temperature* profile and interest in the detector declined.

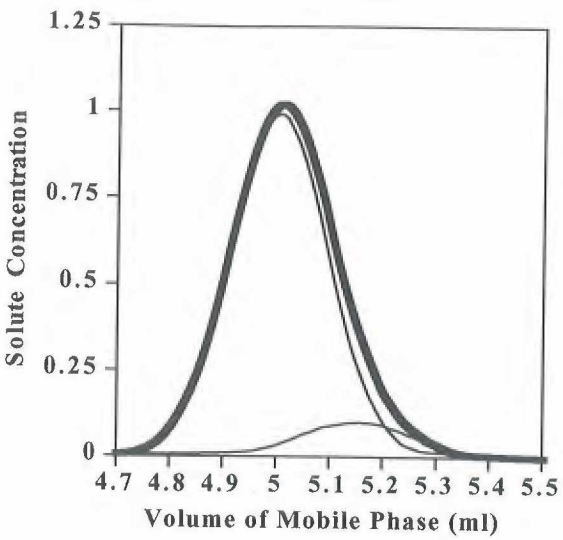
Nevertheless, it can be seen from figure 2 that the evolution and adsorption of heat will cause the front of the peak to travel through the column at an elevated temperature relative to the rear of the peak. Furthermore as the heat evolved will be proportional to the mass adsorbed, the largest temperature changes will occur at the highest concentrations in the peak. As a result, the distribution coefficient of the solute with respect to the stationary phase will be slightly smaller at the front of the peak and at the high concentrations, i.e. the peak maximum. Now, it has already been established that the velocity of a solute band through a column is inversely proportional to its distribution coefficient. Hence, the high concentrations of the peak-front will move slightly faster through the column than the lower concentrations of the peak. This will cause the peak maximum to move towards the front of the peak and thus produce peak asymmetry. The effect of the heat of adsorption on peak shape has been treated quantitatively for both LC (1) and GC (2).

In practice, it is probable that both of the effects discussed contribute to the overall peak asymmetry. Unfortunately, peak asymmetry varies in extent from the very obvious to the barely noticeable and because of this, peak asymmetry is often dismissed as the normal shape of a single solute peak. Such an assumption can cause serious errors in both qualitative and quantitative analysis.

Under circumstances where two solutes are incompletely resolved and one of the pair is present at a much lower concentration than the other, the profile of the pair often resembles a normal peak with slight asymmetry. Consider the combined elution profile of the two peaks shown in figure 3.

Figure 3

The Combined Elution Profile of Two Unresolved Peaks



It is seen that the peak profile shown in figure 1, resulting from an asymmetric single peak, is indistinguishable from the asymmetric peak shown in figure 3 that results from a 10% impurity eluting relatively close to the parent peak. Care must be taken not to interpret peak asymmetry as the normal elution profile when, in fact, it is an indication of incomplete resolution. If there is any uncertainty, the solvent should be changed and the peak in question carefully monitored. It does not matter if, in changing the solvent mixture, the resolution of other components is lost, as only the integrity of the peak in question is being examined. The composition of the mobile phase should be changed at least twice to be sure that only a single solute is present. If no second component can be detected from the peak profiles, then the original mobile phase can be used for the analysis and the integrity of the peak assumed.

This an excellent example of the value of the diode array detector. If the chromatogram shown in figure 3 was monitored at two different wavelengths, then a peak ratio curve would immediately disclose the presence of the second peak (see page 175) and it would no longer be necessary to resort to changes in mobile phase composition to establish the presence of the impurity.

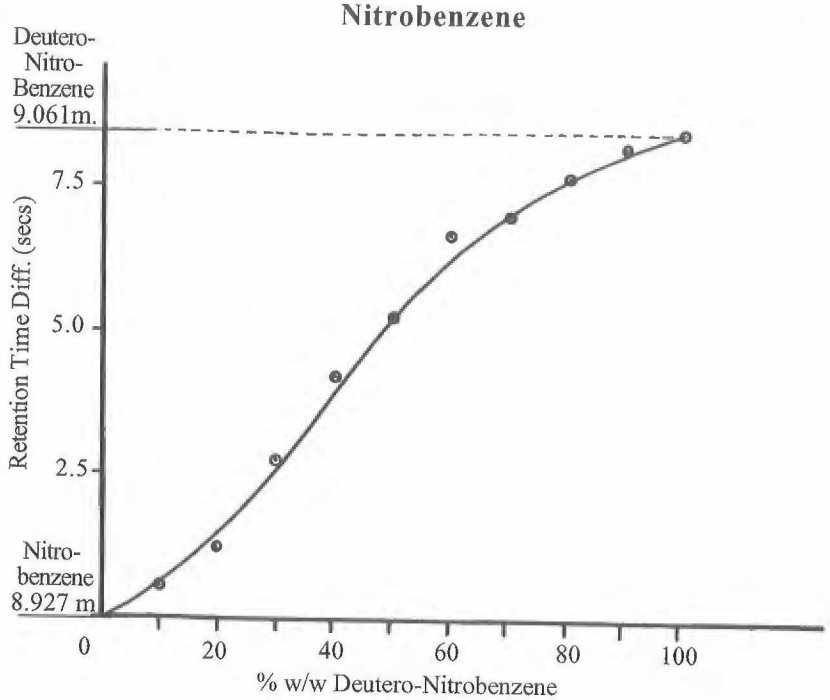
A second component, even at low concentration, will not only give an erroneous value for the amount of solute present but, on examining figure 3 it is seen that the retention time of the major peak is also significantly changed. This effect can be used for quantitative estimation of the unresolved mixture providing the chromatographic sytem can provide the necessary high precision.

Scott and Reese (3) measured the accuracy and precision that could be obtained from a well designed liquid chromatograph and their work will be referred to again later. In the course of their work they found that, providing the retention time of a mixed peak could be measured with sufficient accuracy and the retention times of the two pure components were known, then the proportion of the two components could be determined, even if only a single peak was apparent. The theory behind this work is a little complex (4), and it is not appropriate to discuss the details here. The basis of the method was to calculate the theoretical retention time from the combined elution curves for the two solutes over a range of mixtures. The curve relating retention time to composition was then used as a calibration curve to determine the composition of an unknown mixture from the retention time of the combined peak. The calibration curve that was calculated and the experimental points they obtained are shown in figure 4.

It is seen that the solutes used were nitrobenzene and deutero-nitrobenzene. These two solutes are so similar in physical and chemical characteristics that neither can be exclusively detected in a solvent without interference by the other. It is also seen that the retention times of the two solutes are 8.927 minutes and 9.061 minutes giving a difference of only 8.04 sec.

Figure 4

Graph of Retention Time Difference against Sample Composition for Mixtures of Nitrobenzene and Deutero-Nitrobenzene

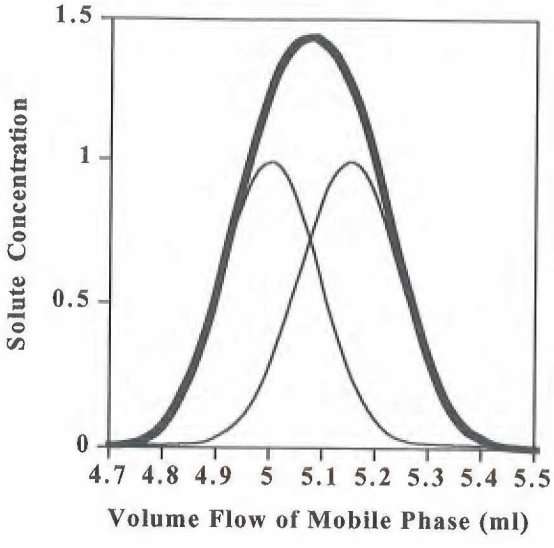


It follows that measurements must be made with a precision of about 0.2 second if quantitative results are to be of any value. It is seen from figure 4 that the experimental points lie very close to the line and a fairly accurate measurement of the distribution of the two isotopes can be obtained from retention time measurements. This method has very limited areas of application and is given here, more to demonstrate the effect of unresolved impurities on retention time, than to suggest it as an alternative to adequate chromatographic resolution. In some cases, however, particularly in the analysis of isotopes, it may be the only practical way to obtain a quantitative evaluation of the mixture by a liquid chromatographic method.

Finally, it must be emphasized that the uncertainty arising from the slight asymmetry shown by the presence of a 10% impurity in figure 3 does not depend on the magnitude of the impurity. In figure 5 the elution curve resulting from a 50% mixture of two closely eluting components is shown.

Figure 5

The Elution Curve of a Binary Mixture Containing Equal Quantities of Each Solute



It is seen that the profile of the combined peaks is perfectly symmetrical and displays no hint that there are two solutes present. Obviously an absorption ratio curve from a diode array detector would quickly disclose the presence of the two components, as would an appropriate changes in mobile phase composition. However, there would be a further clue for the analyst to follow that would give warning of the "duplicity" of the peak. The double peak would be very broad and be inconsistent with the change in peak width of the other solute peaks with retention time. The peak width of a solute increases regularly with retention time but, unfortunately, the relationship is not smooth. There are good reasons for this, but they

are not pertinent to this discussion. However, a peak such as that shown in figure 5 would be excessively broad and obviously out of order with the peak widths of neighboring solutes. This should alert the analyst to the possibility of an unresolved pair of solutes. The analyst should always be alert to the width of each peak in relationship to its position on the chromatogram, as this is the first indication of the presence of a composite peak.

The Effect of Temperature on Retention Volume Measurement

There are two major factors that influence retention volume measurement and they are temperature and solvent composition. In order to measure retention volume with adequate precision it is necessary to know the relationship between retention time and temperature so that the control limits of the column temperature can be specified.

The effect of temperature on retention time was investigated by Scott and Reese (3), who measured the retention volume of the solutes o-dinitro-benzene, 2-ethoxy naphthalene and p-chlorophenatole over a range of temperatures. The chromatographic conditions used are as follows,

Chromatographic Conditions

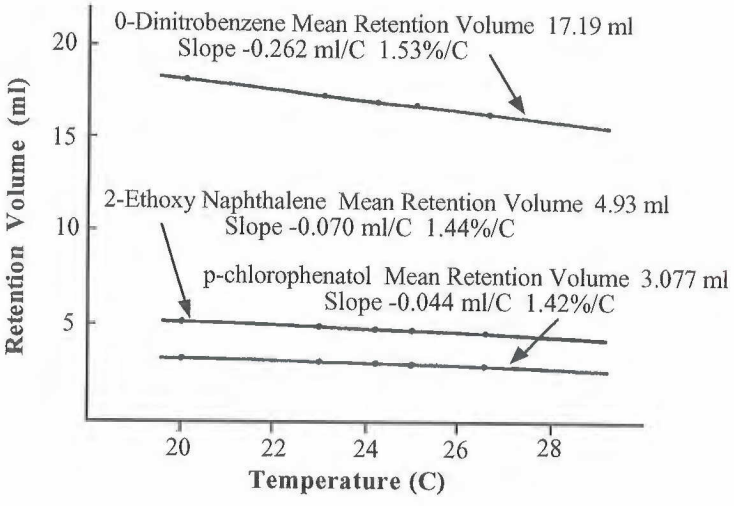
Column	Silerex 1
Column Length	25 cm
Column Diameter	4.6 mm
Column Packing	Silica Gel (particle size 10μm)
Mobile Phase	44% butyl chloride and 56% n-heptane
Flow Rate	1 ml/min.
Detector	UV adsorption at 254 nm
Sample Volume	1 μl

Scott and Reese chose to monitor retention volume as opposed to retention time, as retention volume is always the primary dependent variable in LC. Retention time is not a primary measurement because it must also include the reproducibility of the flow-rate delivered by

the pump. The retention volume was measured absolutely by means of a burette connected to the end of the column. The column was situated in a water bath that was controlled to +/- 0.05°C. Precautions were taken to ensure the mobile phase attained the column temperature before entering the column. The results obtained are shown in figure 6A.

Figure 6A

Graphs of Retention Volume against Temperature for Three Solutes



It is seen that in order to measure retention volumes with a precision of 0.1%, the temperature control must be +/- 0.04°C. This level of temperature control on a thermostat bath is not difficult to achieve but it is extremely difficult, if not impossible, to return to a specific temperature to within +/- 0.04°C after prior change. To achieve a precision of retention volume measurement of 1%, the temperature control must be +/- 0.4°C. This is far more practical as most column oven temperature can be set to a given temperature to within +/- 0.25°C. Although the data was obtained for three specific solutes, the results can be taken as reasonably representative for all solutes and phase systems. In most practical analyses, the precision limits of retention volume measurement will be about 1% but this will not include the reproducibility of the flow rate given by the pump. As

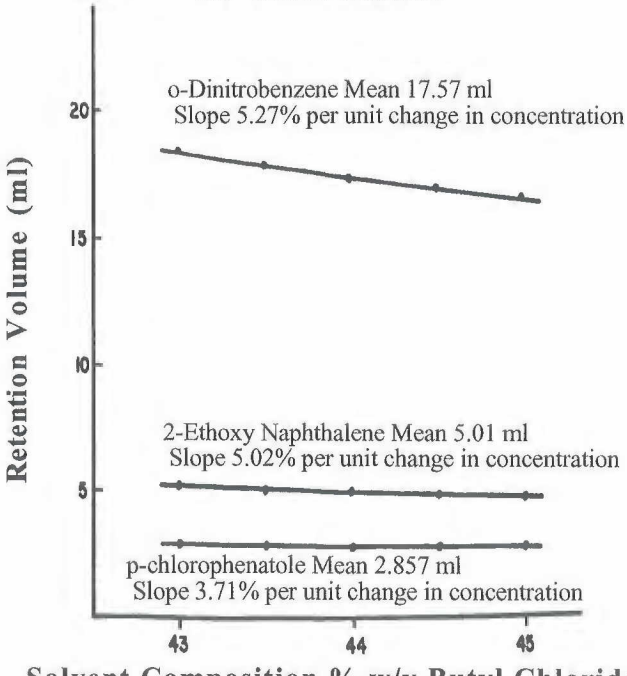
retention *times* are usually measured in analytical LC, the precision of measurement may be significantly greater than +/-1%. It follows that if the identity of the peak must be confirmed, and retention data is being used for the purpose, then it is essential that the column is carefully thermostatted.

The Effect of Solvent Composition on Retention Volume Measurement

Scott and Reese (3) also measured the change in retention volume with solvent composition using the same LC apparatus as that used for investigating the effect of temperature. The column was thermostatted at 24.7°C and the results that were obtained are shown in figure 6B.

Figure 6B

Graphs of Retention Volume against Solvent Composition for Three Solutes



It is seen that small changes in solute composition can have a profound effect on the retention volume, but it must be borne in mind that the magnitude of the effect will vary somewhat, between different solutes and different phase systems. The results shown in figure 6B indicate that if a retention volume is to be measured with a precision of 0.1%, the solvent composition must be maintained constant to within 0.02% w/v. Normally, the solvent composition can be kept constant within those limits providing a closed solvent system is used to prevent evaporation. However, it would be extremely difficult, if not impossible to make up another solvent mixture of the same composition within those limits of precision. This would be particularly difficult if either of the solvents were volatile. If the precision required for retention volume measurement was 1%, the solvent composition would have to be maintained constant with a precision of +/- 0.2 % w/v which should be quite practical. Furthermore, it would be fairly straightforward to make up replacement solvents to the same concentration within the same precision limits. For a routine analysis, however, it might well be advantageous to prepare the mobile phase in large volumes and store it in an appropriate manner. Scott and Reese (3) examined the repeatability of retention time measurements by two procedures, one employing computer processed data and the other by manual measurement of the distance in centimeters from the injection point to the peak maximum on a potentiometric recorder chart. They carried out twelve replicate analyses and the resulting statistical analysis is shown in table 1.

Table 1

The Precision of Retention Data Measurement Made Manually and by Computer

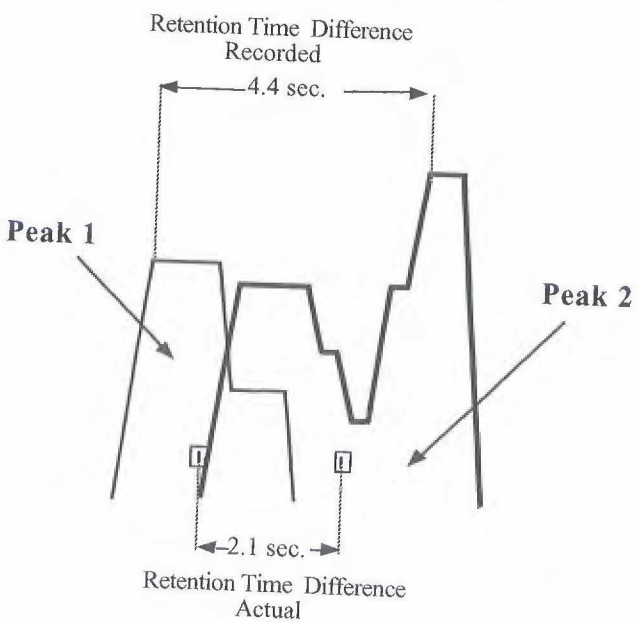
Parameter	Peak 1	Peak 2	Peak 3	Peak 4
Capacity factor	0.22	0.94	1.50	5.21
Mean (cm)	10.17	16.0	20.59	51.14
S.D.(% Mean)	0.85	0.245	0.19	0.15
Mean (min.)	3.97	6.27	8.11	20.14
S.D.(% Mean)	0.31	0.20	0.17	0.33

It is a little surprising to see from table 1 that there is only a minor difference between the precision of the two methods of measurement. This might suggest that the difference between replicate values was not arising from the methods of measurement but was caused by variations elsewhere in the chromatographic system. In order to gain some insight into the source of the variations in retention data, Scott and Reese (3) reconstructed the tips of the two peaks that gave the smallest and largest value of the twelve set of replicates for peak 4. The two peaks are shown in figure 7.

Figure 7

Tips of Peaks Having Extreme Retention Values Reconstructed by the Computer

Peak Crests (between 99.9 and 100% of the peak heights)



By taking the mean positions of the peaks, it is seen that they are only 2.1 sec apart. However, as a result of a noise spike on the front of the

first peak and a similar noise spike on the back of the second peak, the computer interpreted each noise spike as the peak maxima, which resulted in a retention difference of 4.4 sec. It is clear that a significant part of the scatter of retention data can be due to detector noise, either arising from the detector itself or from changes in operating conditions of the chromatograph. This once more emphasizes the importance of using a chromatograph that can maintain stable operating conditions in conjunction with a detector having as little long term noise as possible. The results also indicate that unless the maximum sensitivity is essential for the analysis, the sample size should be adjusted so that the detector can be operated well below its maximum sensitivity to reduce the consequence of the noise.

Quantitative Analysis

Quantitative estimates of the mass of a particular solute present in a sample are obtained from either peak height or peak area measurements. The values obtained are then compared with the peak height or area of a reference solute present in the sample at a known concentration or mass. In this chapter quantitative analysis by LC will be discussed but the procedures described should not be considered as entirely appropriate for other types of chromatographic analysis. Those interested in general quantitative chromatographic analysis including GC and TLC are referred to the book by Katz (4).

Peak Height Measurements

Most detectors are concentration sensitive devices and thus the peak height will be proportional to the maximum concentration in the peak, which, in turn, will be proportional to the total area of the peak. The total area of the peak is proportional to the total mass of solute contained in the peak providing it is not excessively tailing. As the peak height is inversely related to the peak width, then, if peak heights are to be used for analytical purposes, all parameters that can affect the peak width must be held constant. This means that the capacity factor of the solute (k') must remain constant and, consequently, the solvent

composition is held stable. The temperature must also be held constant and an highly repeatable method of sample injection must be used. If computer data acquisition and processing are employed, then a direct printout of the peak heights is obtained and, with most systems, the calculated analysis is also presented. If the peak heights are to be measured manually, which even today is carried out in the majority of LC analyses, then the base line is produced beneath the peak and the height between the extended base line and the peak maximum measured. In general, the measurements should be made estimating to the nearest 0.1 mm.

Peak Area Measurements

The area of a peak is the integration of the peak height (concentration) with respect to time (volume flow of mobile phase) and thus is proportional to the total mass of solute eluted. Measurement of peak area accommodates peak asymmetry and even peak tailing without compromising the simple relationship between peak area and mass. Consequently, peak area measurements give more accurate results under conditions where the chromatography is not perfect and the peak profiles not truly Gaussian or Poisson.

Unfortunately, neither the computer nor the potentiometric recorder measures the primary variable, *volume of mobile phase*, but does measure the secondary variable, *time*. This places stringent demands on the LC pump as the necessary accurate and proportional relationship between time and volume flow depends on a constant flow rate. Thus, peak area measurements should never be made unless a good quality pump is used to control the mobile phase flow rate. Furthermore, the pump must be a constant flow pump and *not* a constant pressure pump.

Peak areas can be measured manually in a number of ways, the simplest being the product of the peak height and the peak width at 0.6065 of the peak height. This does not give the true peak area but providing the peak is Poisson, Gaussian or close to Gaussian it will

always give accurately the same proportion of the peak area. Other methods involve the use of a planimeter (an instrument that provides a numerical value for the area contained within a perimeter traced out by a stylus), and the measurement of the product of the peak height and the peak width at the base. The first method is extremely tedious and the second somewhat inaccurate. The most accurate manual method of measuring peak area is to cut the peak out and weigh it. A copy of the chromatogram should be taken and the peaks cut out of the copy. This procedure is also a little tedious (not as tedious as the use of a planimeter) but does provide very accurate values for the peak area. It is particularly effective for skewed or malformed peaks where other methods of manual peak area measurement (with the exception of the planimeter) fail dismally and give very inaccurate results. The recommended method is to use the product of the peak height and the peak width at 0.6065 of the peak height but this does require adequate resolution of the components of the mixture.

Procedures for Quantitative Analysis

There are two basic methods used in quantitative analysis; one uses a reference standard with which the peak areas (peak heights) of the other solutes in the sample are compared; the other is a normalization procedure where the area (height) of any one peak is expressed as a percentage of the total area (heights) of all the peaks. There are certain circumstances where each method is advantageous, and providing they are used carefully and appropriately all give approximately the same accuracy and precision.

Quantitative Analysis Using Reference Standards

Reference standards can be used in two ways; a weighed amount of the standard can be added directly to the sample and the area of the peaks of interest compared with that of the standard; alternatively, a weighed amount of the standard can be made up in a known volume of solvent, a sample placed on the column and chromatographed under exactly the same conditions as the original sample. The peak area

(height) of the reference solute in the reference chromatogram is compared to the peak areas (heights) of the solutes of interest in the sample chromatogram. The details of the two methods will now be described separately.

The Internal Standard Method

The use of an internal standard probably gives the most accurate quantitative results. However, the procedure depends upon finding an appropriate substance that will elute in a position on the chromatogram where it will not interfere or merge with any of the natural components of the mixture. If the sample contains numerous components, this may be difficult. Having identified a reference standard, the response factors for each component of interest in the mixture to be analyzed must be determined. A synthetic mixture is made up containing known concentrations of each of the components of interest and the standard. If there are (n) components, and the (r) component is present at concentration (C_r) and the standard at a concentration (C_{st}).

Then,

$$\frac{C_r}{C_{st}} = \frac{A_r}{A_{st}} \alpha_r$$

where (A_r) is the area of the peak for component (r),
(A_{st}) is the area of the peak for the standard,
and (α_r) is the response factor for component (r).

Thus, the response factor (α_r) for the component (r) is given by

$$\alpha_r = \frac{C_r A_{st}}{C_{st} A_r}$$

If peak heights are used instead of peak areas then

$$\frac{C_r}{C_{st}} = \frac{H_r}{H_{st}}\alpha_r$$

where (H_r) is the height of the peak for component (r),
(H_{st}) is the height of the peak for the standard,
and (α_r) is the response factor for component (r).

Thus, the response factor (α_r) for the component (r) is then given by

$$\alpha_r = \frac{C_r H_{st}}{C_{st} H_r}$$

A weighed amount of standard is now added to the sample so that its concentration is now $(C_{st(s)})$ and the sample chromatographed. If the concentration of any component (p) is (C_p) and the peak areas of the component (p) and that of the standard are (A_p) and $(A_{st(s)})$ respectively then

$$C_p = \frac{A_p C_{st(s)}}{A_{st(s)}}\alpha_p$$

Again if peak heights are employed instead of peak areas,

$$C_p = \frac{H_p C_{st(s)}}{H_{st(s)}}\alpha_p$$

where (H_p) is the peak height of component (p) in the sample,
and $(H_{st(s)})$ is the peak height of the standard in the sample.

Thus, the concentration of any (or all) of the components present in the mixture can be determined providing they all adequately separated from one another. If the same type of mixture is being analyzed, the operating conditions are maintained constant and there is not an extreme change in the composition of the samples, the response factors usually need be determined only once a day.

The External Standard Method

The external standard method requires that the standard is chromatographed separately from the sample and thus, the chromatographic conditions must be maintained extremely constant. The great advantage of the external standard method is that the reference standard (or standards) can be identical to the solute (or solutes) of interest in the sample. Thus, a synthetic mixture can be made up in which the concentration of the components is closely similar to those of the sample.

Thus, if the (p)th solute in the mixture is at a concentration of ($C_{p(s)}$) in the sample and ($C_{p(st)}$) in the standard solution, then

$$C_{p(s)} = \frac{A_{p(s)}}{A_{p(st)}} C_{p(st)}$$

where ($A_{p(s)}$) is the area of the peak for solute (p) in the sample chromatogram,
($A_{p(st)}$) is the area of the peak for solute (p) in the reference chromatogram,
and ($C_{p(st)}$) is the concentration of the standard in the reference solution.

If peak heights are employed, then

$$C_{p(s)} = \frac{H_{p(s)}}{H_{p(st)}} C_{p(st)}$$

where ($H_{p(s)}$) is the area of the peak for solute (p) in the sample chromatogram,
($H_{p(st)}$) is the area of the peak for solute (p) in the reference chromatogram,
and ($C_{p(st)}$) is the concentration of the standard in the reference solution.

Theoretically, providing the chromatographic conditions are kept constant, then, the reference chromatogram need only be run once a day. However, it is advisable to run the reference chromatogram at least every two hours and many analysts run a reference chromatogram immediately after each sample.

The Normalization Method

The normalization method is the easiest and most straightforward to use but, unfortunately, it is also the least likely to be appropriate for most LC analyses. To be applicable, the detector must have the same response to all the components of the sample. An exceptional example, where the normalization procedure is frequently used, is in the analysis of polymers by exclusion chromatography using the refractive index detector. The refractive index of a specific polymer is a constant for all polymers of that type having more than 6 monomer units. Under these conditions normalization is the obvious quantitative method to use.

The percentage x(p)% of any specific polymer (p) in a given polymer mixture can be expressed by

$$x_p = \frac{A_p}{A_1 + A_2 + A_3 + \ldots + A_n} 100$$

where (A_p) is the peak area of polymer (p)

or •

$$x_p = \frac{A_p}{\sum_{p=1}^{p=n} A_p} 100$$

If peak heights are used, the percentage x(p)% of any specific polymer (p) in a given polymer mixture can be expressed by similar equations,

$$x_p = \frac{H_p}{H_1 + H_2 + H_3 + \ldots + A_n} 100$$

where (H_p) is the peak area of polymer (p)

or

$$x_p = \frac{H_p}{\sum_{p=1}^{p=n} H_p} 100$$

The Relative Precision of Peak Height and Peak Area Measurements

In their work on the precision of contemporary liquid chromatographic measurements, Scott and Reese (3) also evaluated the precision that could be expected from a computer measuring peak heights and peak areas. They again used twelve replicate samples and the results they obtained are shown in table 2.

Table 2

Precision Obtained from the Measurements of Peak Heights and Peak Areas by Means of a Computer

Parameter	**Peak 1**	**Peak 2**	**Peak 3**
Capacity Ratio (k')	0.94	1.50	5.21
Peak Heights			
Mean	1785	15191	75140
Stand.Dev. (S.D.)	63	319	1183
S.D.(% of Mean)	3.5	2.1	1.6
Peak Areas			
Mean	282929	3344011	41042092
Stand.Dev. (S.D.)	17301	64503	583521
S.D.(% of Mean)	6.11	1.93	1.42

It is seen that there is not a great difference between the use of peak heights or peak areas for quantitative analysis, except possibly for very early peaks, where the results seem to indicate that peak height measurements might be more precise. However, it again must be emphasized that the measurements made by Scott and Reese were *overall* precision measurements that will include all variations in the chromatographic system. The difference between the two methods of measurement may well be significant, but the absolute values for precision will not, by any means, be solely dependent on the method of peak measurement.

Peak De-Convolution

In some data acquisition and processing systems, software is included that mathematically analyze convoluted (unresolved) peaks, identify the individual peaks that make up the composite envelope and then determine the area of the individual peaks.

It must be stressed at this point that clever algorithms are no substitute for good chromatography.

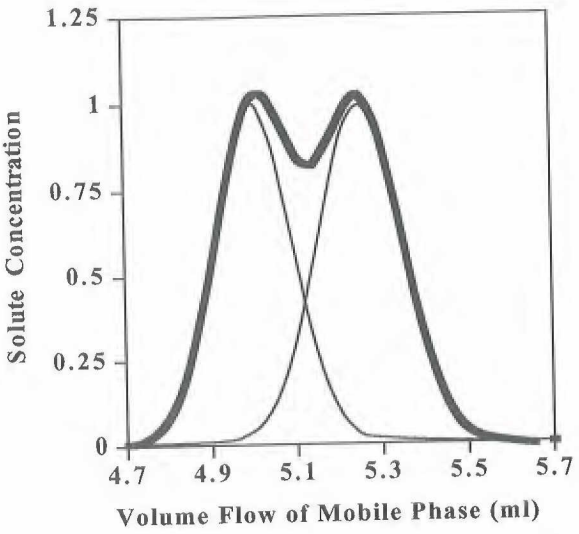
Sometimes it is not possible to improve the resolution of a complex mixture beyond a certain level and, under these circumstances, the use of some de-convolution technique may be the only solution. The algorithms in the software must contain certain tentative assumptions in order to analyze the peak envelope. Firstly, a particular mathematical function must be assumed that describes the peaks. The function used is usually Gaussian and, in most cases, no account is taken of the possibility of asymmetric peaks. Furthermore it is also assumed that *all* the peaks can be described by the *same* function (i.e. the efficiency of all the peaks are the same) which, as has already been discussed, is also not generally true. Nevertheless, providing the composite peak is not too complex, de-convolution can be reasonably successful.

If resolution is partial and the components are present in equal quantities, then the de-convolution approach might be appropriate. An

example of the convolution of two partially resolved peaks of equal size is shown in figure 8.

Figure 8

The De-Convolution of Two Partially Resolved Peaks Representing Solutes Present in Equal Quantities

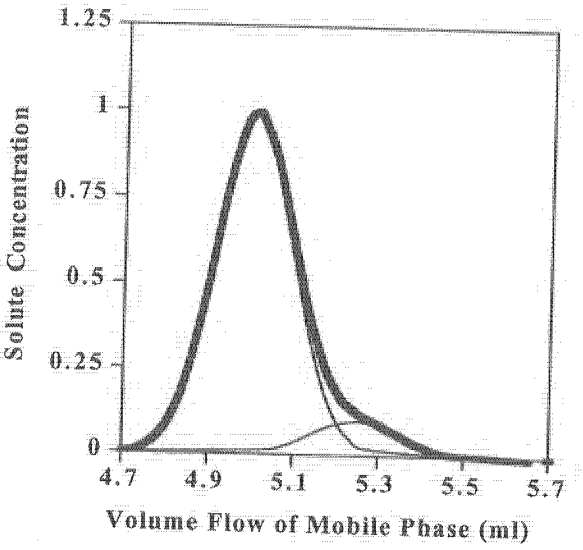


It is seen that two Gaussian shaped peaks can be easily extracted from the composite envelope and the software could also supply values for either the heights of the de-convoluted peaks or their area. It should be noted, however, that the two peaks are clearly discernible as unresolved components in the original chromatogram. As a consequence, the de-convoluting software can easily identify the approximate positions of the peak maxima and can also obtain a crude value for the peak widths. Such information allows the software to arrive at a valid analysis quickly and with reasonable accuracy. Nevertheless, it should also be pointed out, that in the example shown in figure 8 most data processing software would construct a perpendicular from the valley to the base line, thus bisecting the combined peak envelope. The area of each half would then be taken as the respective area of each peak.

The software will also function well if the same partial resolution is obtained but for peaks of unequal size. An example of the analysis of such a composite peak is shown in figure 9.

Figure 9

The De-Convolution of Two Partially Resolved Peaks Representing Solutes Present in Unequal Quantities



It seen that the de-convolution is likely to be successful as the position of the peak maximum, and the peak width, of the major component is easily identifiable. This would mean that the software could accurately determine the constants in the Gaussian equation that would describe the profile of the major component. The profile of the major component would then be subtracted from the total composite peak leaving the small peak as difference value. This description oversimplifies the calculation processes which will include a number of iteration steps to arrive at the closest fit for the two peaks.

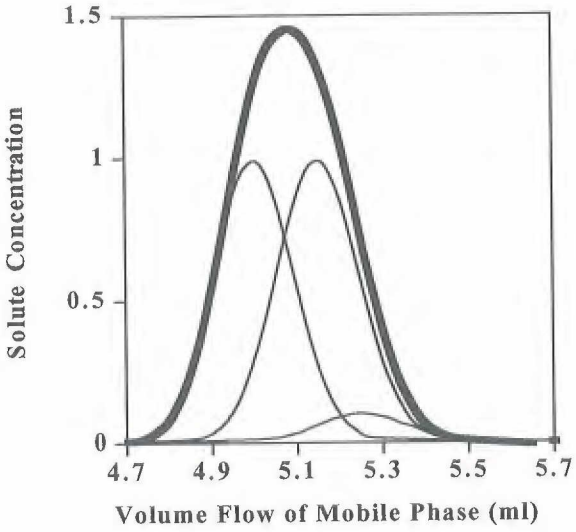
However, in the examples taken the solutes were at least partly resolved and, unfortunately, as the resolution becomes less and less

and the need for an accurate de-convolution technique becomes even greater, the value of the software presently available appears to become minimal.

A typical example at the other extreme where a de-convolution technique would be useless is given in figure 10.

Figure 10

The De-Convolution of Three Completely Unresolved Peaks



It is seen that however sophisticated the software might be, it would be virtually impossible to de-convolute the peak into the three components. The peaks shown in the diagram are discernible because the peaks themselves were assumed and the composite envelope calculated. The envelope, however, would provide no basic data; there is no hint of an approximate position for any peak maximum and absolutely no indication of the peak width of any of the components. The use of the diode array detector, monitoring at different wavelengths, might help by identifying uniquely one or more of the

components. If the software could import extra data of this type then de-convolution might be possible. The use of de-convoluting software has limited application and must be used with considerable caution. Every effort should be made to separate all the components of a mixture by chromatography and only employ peak de-convolution as a last resort.

Reporting Analytical Results

Modern data processing software varies widely in both the data that is provided and the manner in which it is presented. Most systems give a choice of peak height, or peak area measurements, or both, which provide a routine for determining the response factors for each solute from the calibrating chromatogram. Retention times are taken as the time at which the peak maximum is reached and if the dead volume peak is defined, then the capacity ratios of the components will also be calculated. From the capacity factors a simple ratio algorithm can provide the retention ratio of each component to any previously defined solute peak. Some data systems provide peak-valley identification and also peak-valley-to-baseline construction. A peak valley is the minimum between two incompletely resolved peaks. Peak skimming routines are also common which are used when a small peak is eluted on a badly tailing major peak. The tail of the peak is constructed (actually extended) under the small peak and the area of the small peak taken as that peak area above the extended tail.

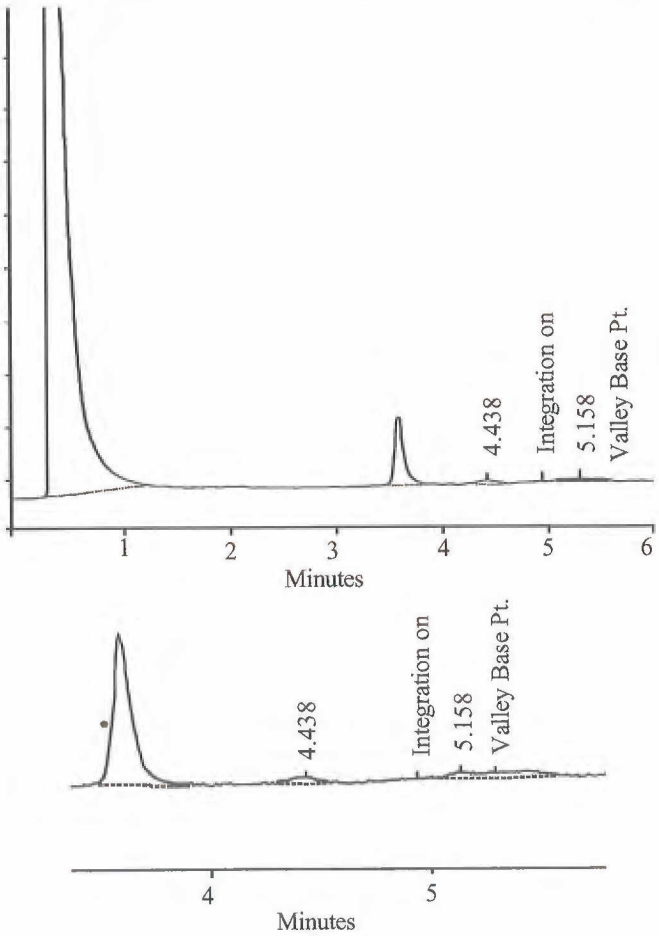
The information provided on the print-out also varies considerably from one manufacturer to another. Some provide all the chromatographic information, including solvent composition, the gradient program if one is used, column type, dimensions and operating temperature together with the mobile phase flow rate. In some cases the type of detector detector operating conditions and the sensitivity (or attenuation) setting is reported. In general, all the above information is normally reported with the analysis and so it is very convenient to have the information included in the computer print-out.

Figure 11 Computer Print-Out of an LC Analysis

Sample Volume 1µl Dilution 1 to 1

External Standard Area 10000 External Standard Concentration 1000 fmole

Retention Time	Peak Area	Peak Height	Concentration fmol
0.363	33318956	7439.579	-
3.590	206343	34.884	-
4.430	16853	1.541	-
5.150	27775	1.208	27775



As one might expect, computer data processing is an expensive adjunct to a liquid chromatograph and the more sophisticated reporting systems can be very expensive. An example of a computer print-out of

a trace analysis is shown in figure 11. The example that is taken is not as neat and explicit as those usually shown in the literature but more typical of those that the analyst is likely to meet in day-to-day analyses. The sample is of biological origin and the material of interest is present in trace quantities. The area of the chromatogram depicting the separation of the substance being determined is shown enlarged at the base of the diagram. This facility for enlarging a chosen portion of the chromatogram, for detailed inspection, is also a common feature of many data processing systems. It is seen that the substance of interest, that eluted at a retention time of 5.158 min., is incompletely resolved from its neighbor which is eluted later. It is also seen that the software marks where integration is initiated and where it ends at the valley-base line. The analyst can, therefore, check the limits of the integration and thus, be assured that only the area of the peak of interest was being calculated.

Although computer systems have become relatively inexpensive and the software for processing chromatography data is fairly straightforward to write, the data acquisition and processing systems (albeit highly desirable) are still a significant added cost to the overall chromatograph. As a result, even today, manual measurements made directly on the chromatogram are still used in a large proportion of all LC analyses. Results obtained by manual measurements suffer from little loss, if any, in accuracy and precision but are certainly time consuming. If LC techniques are being used for quality control analyses, where a large number of samples are chromatographed every day, then some form of data acquisition and processing facilities are essential.

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