CHAPTER 1

Introduction, Chromatography Theory, and Instrument Calibration

1.1 Introduction

Analytical chemists have few tools as powerful as chromatography to measure distinct analytes in complex samples. The power of chromatography comes from its ability to separate a mixture of compounds, or "analytes", and determine their respective identity (chemical structure) and concentration. Chromatography can be divided into three basic types that include gas, liquid, and supercritical fluid chromatography. Liquid chromatography can further be divided into ion exchange, separations based on size, and even extended to gel-based electrophoretic techniques. This book will provide a basic introduction to different types of liquid and gas chromatography. The relationship between each type of chromatography is illustrated in Figure 1.1.

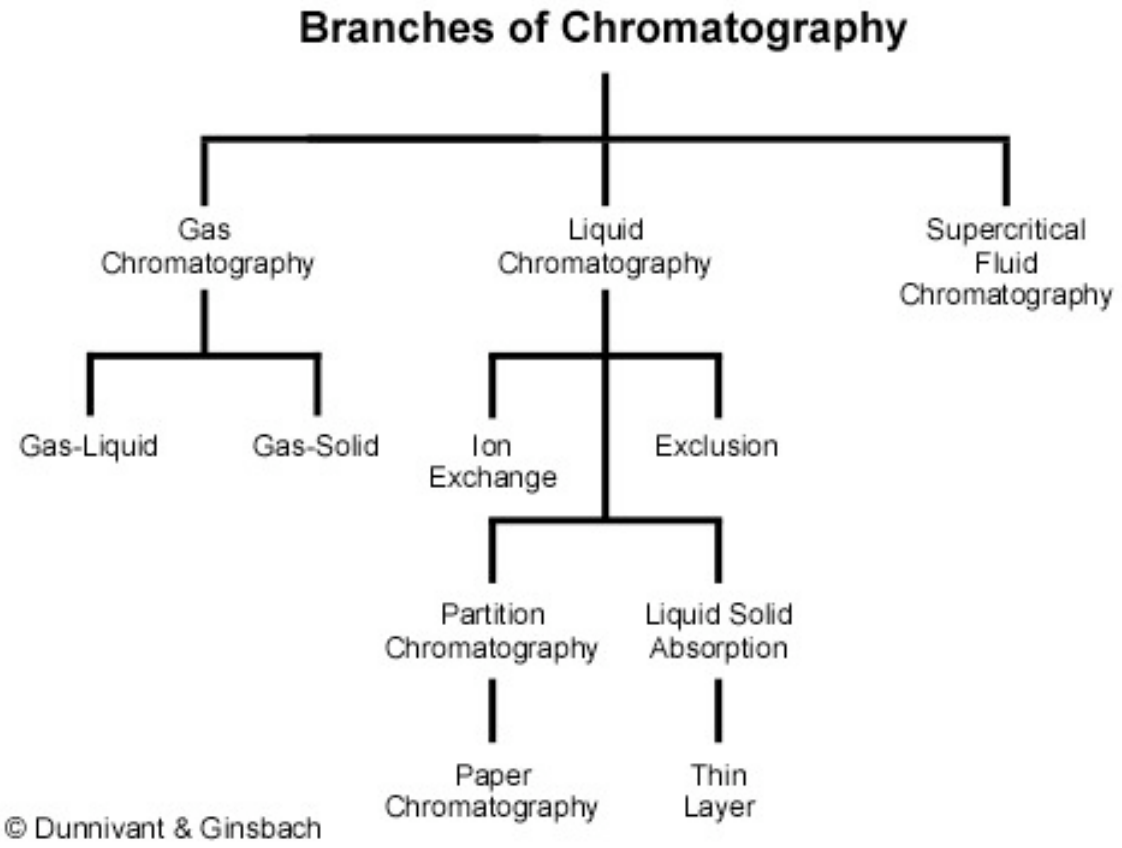


Figure 1.1. Categories of Chromatography and Their Relationship to Each Other.

In general, each type of chromatography is comprised of two distinct steps: chromatography (or separation of individual compounds in distinct elution bands) and identification (detection of each elution band). Gas chromatography is the process of taking a sample and injecting it into the instrument, turning the solvent and analytes into gaseous form, and separating the mixture of compounds into individual peaks (and preferably individual compounds). Liquid chromatography completes the same process except the separations occur in a liquid phase. Individual band or peaks exit the column and identification occurs by a relatively universal detector. One particularly common detector for both gas and liquid chromatography is mass spectrometry (MS) which transforms each analyte from a chemically neutral species into a positive cation, usually breaking various bonds in the process. Detecting the mass of the individual pieces (referred to as fragments) allows for conclusive identification of the chemical structure of the analyte. Principles of gas chromatography (GC) will be covered in Chapter 2, liquid chromatography (LC) in Chapter 3, capillary electrophoresis (CE) in Chapter 4 and mass spectrometry (MS) in Chapter 5.

In mass spectrometry, the combination of compound separation and ion fragment identification (the subject of Chapter 6) yields an extremely powerful analysis that is said to be confirmatory. Confirmatory analysis means the analyst is absolutely sure of the identity of the analyte. In contrast, many other individual techniques and detectors are only suggestive, meaning the analyst thinks they know the identity of an analyte. This is especially true with most universal GC and LC detectors since these detectors respond similarly to many compounds. The only identifying factor in these chromatographic systems is their elution time from the column. In order to obtain confirmatory analysis the sample would need to analyzed by at least two or more techniques (for example, different separation columns) that yield the same results. Mass spectrometry and nuclear magnetic resonance (NMR) are two confirmatory techniques in chemistry.

At this point, it is important to understand the different applications GC-MS and LC-MS offer for two different types of chemists, analytical and synthetic organic chemists. Organic chemists attempt to create a desired chemical structure by transforming functional groups and intentionally breaking or creating bonds; in their resulting identification procedures they already have a relatively good idea of the chemical structure. To characterize the resulting product the chemist will use Infrared Spectroscopy (IR) to observe functional groups, Mass Spectrometry (MS) to obtain the compound's molecular weight, and Nuclear Magnetic Resonance (NMR) spectroscopy to determine the molecular structure. Information from all three techniques is used to conclusively identify the synthesized product.

Analytical chemists are forced to approach identification in a different way, because they have no *a priori* knowledge of the chemical structure and because the analyte is usually present at low concentrations where IR and NMR are inaccurate. Often, analysis is performed to look for a desired compound by

comparing the sample analysis to that of a known (reference) compound. The reference is used to identify the unknown compound by matching retention time (in chromatography) and ion fragmentation pattern (in mass spectrometry). With today's computer mass spectral libraries that contain ion fractionation patterns for numerous chemicals, the analyst has the option of not using a reference standard. This is especially valuable if a reference compound is not available or is expensive. In some cases, especially with low analyte concentration, this approach may only result in a tentative identification.

This book will focus on GC-MS and LC-MS applications from an analytical chemistry perspective even though many synthetic chemists will also find much of this information useful for their applications.

1.2 Chromatographic Theory

All chromatographic systems have a mobile phase that transports the analytes through the column and a stationary phase coated onto the column or on the resin beads in the column. The stationary phase loosely interacts with each analyte based on its chemical structure, resulting in the separation of each analyte as a function of time spent in the separation column. The less analytes interact with the stationary phase, the faster they are transported through the system. The reverse is true for less mobile analytes that have stronger interactions. Thus, the many analytes in a sample are identified by retention time in the system for a given set of conditions. In GC, these conditions include the gas (mobile phase) pressure, flow rate, linear velocity, and temperature of the separation column. In HPLC, the mobile phase (liquid) pressure, flow rate, linear velocity, and the polarity of the mobile phase all affect a compounds' retention time. An illustration of retention time is shown in Figure 1.2. The equation at the top of the figure will be discussed later during our mathematic development of chromatography theory.

$$R_S = \frac{2\left(t_{R'B} - t_{R'A}\right)}{W_A + W_B}$$

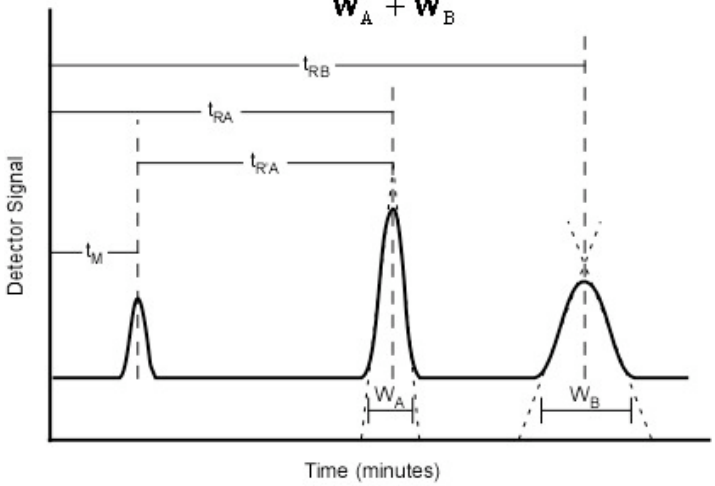
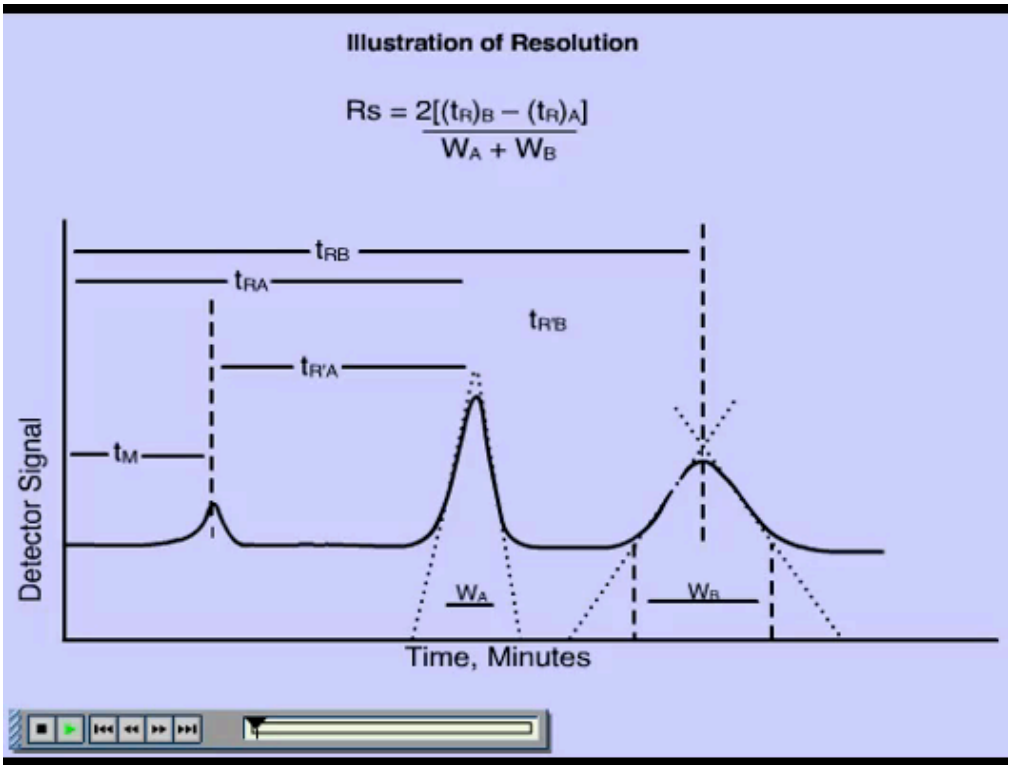


Figure 1.2. Identification of Analytes by Retention Time.

In the above figure, the minimum time that a non-retained chemical species will remain in the system is t_M. All compounds will reside in the injector, column, and detector for at least this long. Any affinity for the stationary phase results in the compound being retained in the column causing it to elute from the column at a time greater than t_M. This is represented by the two larger peaks that appear to the right in Figure 1.2, with retention times t_{RA} and t_{RB}. Compound B has more affinity for the stationary phase than compound A because it exited the column last. A net retention ($t_{R'A}$ and $t_{R'B}$) time can be calculated by subtracting the retention time of the mobile phase(t_M) from the peaks retention time (t_{RA} and t_{RB}).

Figure 1.2 also illustrates how peak shape is related to retention time. The widening of peak B is caused by longitudinal diffusion (diffusion of the analyte as the peak moves down the length of the column). This relationship is usually the reason why integration by area, and not height, is utilized. However, compounds eluting at similar retention times will have near identical peak shapes and widths.

A summary of these concepts and data handling techniques is shown in Animation 1.1. Click on the figure to start the animation.



Animation 1.1. Baseline Resolution.

Chromatographic columns adhere by the old adage “like dissolves like” to achieve the separation of a complex mixture of chemicals. Columns are coated with a variety of stationary phases or chemical coatings on the column wall in capillary columns or on the inert column packing in packed columns. When selecting a column’s stationary phase, it is important to select a phase possessing similar intermolecular bonding forces to those characteristic of the analyte. For example, for the separation of a series of alcohols, the stationary should be able to undergo hydrogen bonding with the alcohols. When attempting to separate a mixture of non-polar chemicals such as aliphatic or aromatic hydrocarbons, the column phase should be non-polar (interacting with the analyte via van der Waals forces). Selection of a similar phase with similar intermolecular forces will allow more interaction between the separation column and structurally similar analytes and increase their retention time in the column. This results in a better separation of structurally similar analytes. Specific stationary phases for GC and HPLC will be discussed later in Chapter 2 and 3, respectively.

Derivation of Governing Equations: The development of chromatography theory is a long established science and almost all instrumental texts give nearly exactly the same set of symbols, equations, and derivations. The derivation below follows the same trends that can be found in early texts such as Karger et al. (1973) and Willard et al. (1981), as well as the most recent text by Skoog et al. (2007). *The reader should keep two points in mind as they read the following discussion. First, the derived equations establish a relatively simple mathematical basis for the interactions of an analyte between the mobile phase (gas or liquid) and the stationary phase (the coating on a column wall or resin bead). Second, while each equation serves a purpose individually, the relatively long derivation that follows has the ultimate goal of yielding an equation that describes a way to optimize the chromatographic conditions in order to yield maximum separation of a complex mixture of analytes.*

To begin, we need to develop several equations relating the movement of a solute through a system to properties of the column, properties of the solute(s) of interest, and mobile phase flow rates. These equations will allow us to predict (1) how long the analyte (the solute) will be in the system (retention time), (2) how well multiple analytes will be separated, (3) what system parameters can be changed to enhance separation of similar analytes. The first parameters to be mathematically defined are flow rate (F) and retention time (t_m). Note that “F” has units of cubic volume per time. Retention behavior reflects the distribution of a solute between the mobile and stationary phases. We can easily calculate the volume of stationary phase. In order to calculate the mobile phase flow rate needed to move a solute through the system we must first calculate the flow rate.

$$F = (\pi r_c^2)\ \varepsilon\ (L/t_m)$$

$$F = \pi\ (d_c/2)^2\ \varepsilon\ (L/t_m)$$

$$F = \left(\frac{\pi\, d_c}{4}\right) \varepsilon\ (L/t_m)$$

$$\text{where } \left(\frac{\pi\, d_c}{4}\right) = \text{cross sectional area of column}$$

$$\varepsilon = \text{porosity of column packing}$$

$$(L/t_m) = \text{average linear velocity of mobile phase}$$

In the equations above, r_c is the internal column radius, d_c is the internal column diameter, L is the total length of the column, t_m is the retention time of a non-retained analyte (one which does not have any interaction with the stationary phase). Porosity (e) for solid spheres (the ratio of the volume of empty pore space to total particle volume) ranges from 0.34 to 0.45, for porous materials ranges from 0.70 to 0.90, and for capillary columns is 1.00. The average linear velocity is represented by u-bar.

The most common parameter measured or reported in chromatography is the retention time of particular analytes. For a non-retained analyte, we can use the retention time (t_M) to calculate the volume of mobile phase that was needed to carry the analyte through the system. This quantity is designated as V_m,

$$\underset{mL}{V_M} = \underset{min.}{t_M}\ \underset{mL/min}{F} \qquad \text{Eqn 1.1}$$

and is called the dead volume. For a retained solute, we calculate the volume of mobile phase needed to move the analyte through the system by

$$\underset{mL}{V_R} = \underset{min.}{t_R}\ \underset{mL/min}{F} \qquad \text{Eqn 1.2}$$

where t_R is the retention time of the analyte.

In actual practice, the analyst does not calculate the volume of the column, but measures the flow rate and the retention time of non-retained and retained analytes. When this is done, note that the retention time not only is the transport time through the detector, but also includes the time spent in the injector! Therefore

$$V_M = V_{column} + V_{injector} + V_{detector}$$

The net volume of mobile phase (V'_R) required to move a retained analyte through the system is

$$V_R' = V_R - V_M \qquad \text{Eqn 1.3}$$

where V_R is the volume for the retained analyte and V_M is the volume for a nonretained (mobile) analyte.

This can be expanded to

$$t_R' F = t_R F - t_M F$$

and dividing by F, yields

$$t_R' = t_R - t_M \qquad \text{Eqn 1.4}$$

Equation 1.4 is important since it gives the net time required to move a retained analyte through the system (Illustrated in Figure 1.2, above)

Note, for gas chromatography (as opposed to liquid chromatography), the analyst has to be concerned with the compressibility of the gas (mobile phase), which is done by using a compressibility factor, j

$$j = \frac{3\left[\left(\frac{P_i}{P_o}\right)^2 - 1\right]}{2\left[\left(\frac{P_i}{P_o}\right)^2 - 1\right]}$$

where P_i is the gas pressure at the inlet of the column and P_o is the gas pressure at the outlet. The net retention volume (V_N) is

$$V_n = j V_R' \qquad \text{Eqn 1.5}$$

The next concept that must be developed is the partition coefficient (K) which describes the spatial distribution of the analyte molecules between the mobile and stationary phases. When an analyte enters the column, it immediately distributes itself between the stationary and mobile phases. To understand this process, the reader needs to look at an instant in time without any flow of the mobile phase. In this “snap-shot of time” one can calculate the concentration of the analyte in each phase. The ratio of these concentrations is called the equilibrium partition coefficient,

$$K = C_s / C_M \qquad \text{Eqn 1.6}$$

where C_s is the analyte concentration in the solid phase and C_M is the solute concentration in the mobile phase. If the chromatography system is used over

analyte concentration ranges where the “K” relationship holds true, then this coefficient governs the distribution of analyte anywhere in the system. For example, a K equal to 1.00 means that the analyte is equally distributed between the mobile and stationary phases. The analyte is actually spread over a zone of the column (discussed later) and the magnitude of K determines the migration rate (and t_R) for each analyte (since K describes the interaction with the stationary phase).

Equation 1.3 ($V'_R = V_R - V_M$) relates the mobile phase volume of a non-retained analyte to the volume required to move a retained analyte through the column. K can also be used to describe this difference. As an analyte peak exits the end of the column, half of the analyte is in the mobile phase and half is in the stationary phase. Thus, by definition

$$V_R\, C_M = V_M\, C_M + V_S\, C_S \qquad \textit{Eqn 1.7}$$

Rearranging and dividing by C_M yields

$$V_R = V_M + K\, V_S \quad \textit{or} \quad V_R - V_M = K\, V_S \qquad \textit{Eqn 1.8}$$

Now three ways to quantify the net movement of a retained analyte in the column have been derived, Equations 1.2, 1.4, and 1.8.

Now we need to develop the solute partition coefficient ratio, k’ (also knows as the capacity factor), which relates the equilibrium distribution coefficient (K) of an analyte within the column to the thermodynamic properties of the column (and to temperature in GC and mobile phase composition in LC, discussed later). For the entire column, we calculate the ratio of total analyte mass in the stationary phase ($C_S V_S$) as compared to the total mass in the mobile phase ($C_M V_M$), or

$$k' = \frac{C_s V_s}{C_m V_m} = K \frac{V_s}{V_m} \qquad \textit{Eqn 1.9}$$

where V_S/V_M is sometimes referred to as b, the volumetric phase ratio.

Stated in more practical terms, k’ is the additional time (or volume) a analyte band takes to elute as compared to an unretained analyte divided by the elution time (or volume) of an unretained band, or

rearranged, gives

$$k' = \frac{t_r - t_m}{t_m} = \frac{V_s - V_m}{V_m} \qquad \text{Eqn 1.10}$$

$$t_r = t_m (1 + k') = \frac{L}{u} (1 + k') \qquad \text{Eqn 1.11}$$

where μ is the linear gas velocity and the parameters in Equation 1.10 were defined earlier. So, the retention time of an analyte is related to the partition ratio (k'). Optimal k' values range from ~1 to ~5 in traditional packed column chromatography, but the analysts can use higher values in capillary column chromatography.

Multiple Analytes: The previous discussions and derivations were concerned with only one analyte and its migration through a chromatographic system. Now we need to describe the relative migration rates of analytes in the column; this is referred to as the selectivity factor, α. Notice Figure 1.2 above had two analytes in the sample and the goal of chromatography is to separate chemically similar compounds. This is possible when their distribution coefficients (Ks) are different. We define the selectivity factor as

$$\alpha = \frac{K_B}{K_A} = \frac{k'_B}{k'_A} \qquad \text{Eqn 1.12}$$

where subscripts A and B represent the values for two different analytes and solute B is more strongly retained. By this definition, α is always greater than 1. Also, if one works through the math, you will note that

$$\alpha = \frac{V'_B}{V'_A} = \frac{t_{R,B} - t_m}{t_{R,A} - t_m} = \frac{t'_{R,B}}{t'_{R,A}} \qquad \text{Eqn 1.13}$$

The relative retention time, α, depends on two conditions: (1) the nature of the stationary phase, and (2) the column temperature in GC or the solvent gradient in LC. With respect to these, the analyst should always first try to select a stationary phase that has significantly different K values for the analytes. If the compounds still give similar retention times, you can adjust the column temperature ramp in GC or the solvent gradient in LC; this is the general elution problem that will be discussed later.

Appropriate values of α should range from 1.05 to 2.0 but capillary column systems may have greater values.

Now, we finally reach one of our goals of these derivations, an equation that combines the system conditions to define analyte separation in terms of column properties such as column efficiency (H) and the number of separation units (plates, N) in the column (both of these terms will be defined later). As analyte peaks are transported through a column, an individual molecule will undergo many thousands of transfers between each phase. As a result, packets of analytes and the resulting chromatographic peaks will broaden due to physical processes discussed later. This broadening may interfere with "resolution" (the complete separation of adjacent peaks) if their K (or k') values are close (this will result in an a value close to 1.0). Thus, the analyst needs a way to quantify a column's ability to separate these adjacent peaks.

First, we will start off with an individual peak and develop a concept called the theoretical plate height, H, which is related to the width of a solute peak at the detector. Referring to Figure 1.2, one can see that chromatographic peaks are Gaussian in shape, can be described by

$$H = \frac{\sigma^2}{L} \qquad \text{Eqn 1.14}$$

where H is the theoretical plate height (related to the width of a peak as it travels through the column), σ is one standard deviation of the bell-shaped peak, and L is the column length. Equation 1.14 is a basic statistical way of using standard deviation to mathematically describe a bell-shaped peak. One standard deviation on each side of the peak contains ~68% of the peak area and it is useful to define the band broadening in terms of the variance, σ^2 (the square of the standard deviation, σ). Chromatographers use two standard deviations that are measured in time units (t) based on the base-line width of the peak, such that

$$\tau = \frac{\sigma}{L/t_R} \qquad \text{Eqn 1.15}$$

Here, L is given in cm and t_R in seconds. Note in Figure 1.2, that the triangulation techniques for determining the base width in time units (t) results in 96% if the area or ±2 standard deviations, or

$$W = 4\tau = 4\frac{\sigma}{L/t_R} \quad or \quad \sigma = \frac{WL}{4t_R} \qquad \text{Eqn 1.16}$$

Substitution of Equation 1.16 into Equation 1.14, yields

$$H = \frac{LW^2}{16t^2_R} \qquad \text{Eqn 1.17}$$

H is always given in units of distance and is a measure of the efficiency of the column and the dispersion of a solute in the column. Thus, the lower the H value the better the column in terms of separations (one wants the analyte peak to be as compact as possible with respect to time or distance in the column). Column efficiency is often stated as the number of theoretical plates in a column of known length, or

$$N = \frac{L}{H} = 16\left(\frac{t_R}{W}\right)^2 \qquad \text{Eqn 1.18}$$

This concept of H, theoretical plates comes from the petroleum distillation industry as explained in Animation 1.2 below. Click on the Figure to play the animation.

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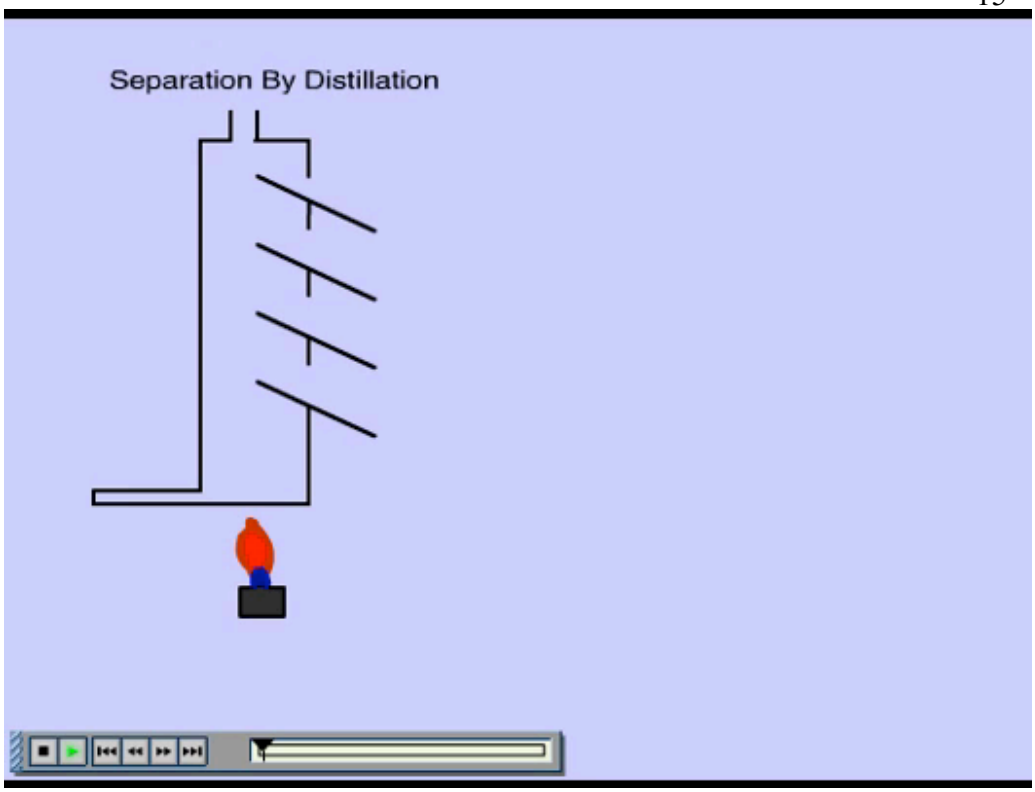
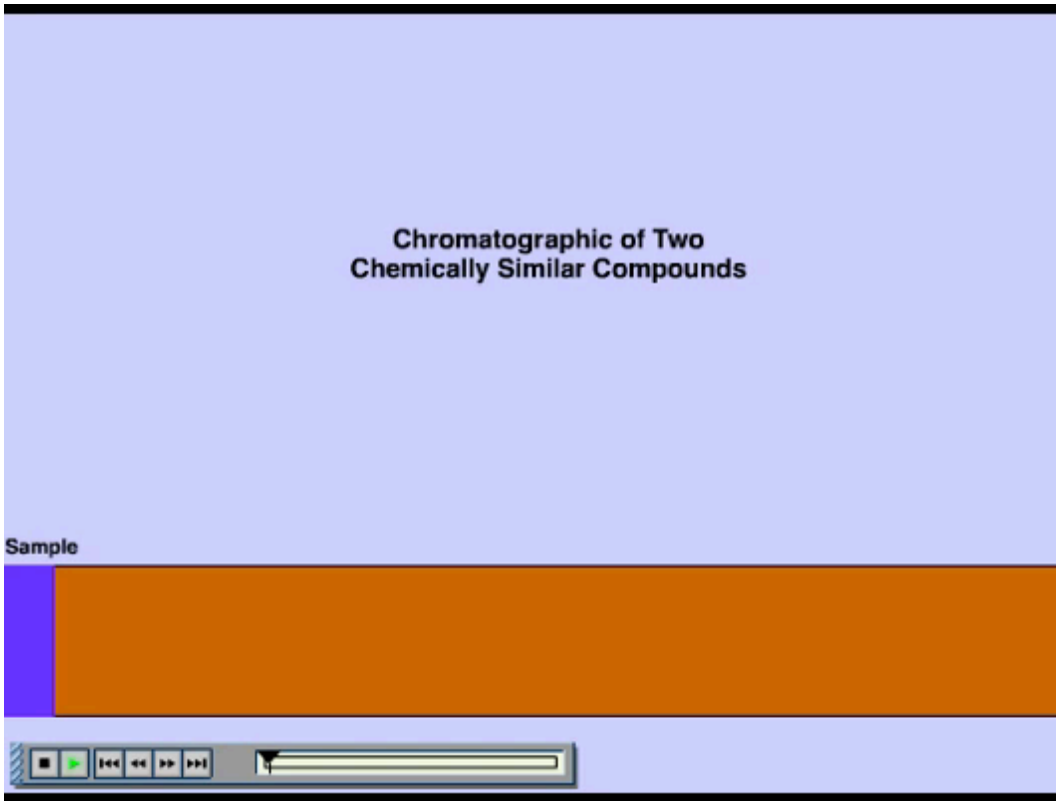


Animation 1.2 Origin of H and the Theoretical Plate Height Unit

To summarize Animation 1.2 with respect to gas and liquid chromatography, a theoretical plate is the distance in a column needed to achieve baseline separation; the number of theoretical plates is a way of quantifying how well a column will perform.

We now have the basic set of equations for describing analyte movement in chromatography but it still needs to be expanded to more practical applications where two or more analytes are separated. Such an example is illustrated in Animation 1.3 for a packed column.



Animation 1.3 Separation of Two Analytes by Column Chromatography.

Separation of two chemically-similar analytes is characterized mathematically by resolution (R_s), the difference in retention times of these analytes. This equation, shown earlier in Figure 1.2, is

$$R_s = \frac{2(t_{R'B} - t_{R'A})}{W_A + W_B} \qquad \text{Eqn 1.19}$$

where $t_{R'B}$ is the corrected retention time of peak B, $t_{R'A}$ is the corrected retention time of peak A, W_A is the peak width of peak A in time units and W_B is the width of peak B. Since $W_A = W_B = W$, Equation 1.19 reduces to

$$Rs = \frac{t_{R'B} - t_{R'A}}{W} \qquad \text{Eqn 1.20}$$

Equation 1.18 expressed W in terms of N and t_R, and substitution of Equation 1.17 into Equation 1.18, yields

$$R_s = \left(\frac{t_{R'B} - t_{R'A}}{t_{R'B}}\right)\frac{\sqrt{N}}{4} \qquad \textit{Eqn 1.21}$$

Recall from Equation 1.10, that

$$k' = \frac{t_R - t_m}{t_m}$$

substitution into Equation 1.21 and upon rearrangement, yields

$$R_s = \left(\frac{k'_B - k'_A}{1 + k'_B}\right)\frac{\sqrt{N}}{4} \qquad \textit{Eqn 1.22}$$

Recall that we are trying to develop an equation that relates resolution to respective peak separations and although k' values do this, it is more useful to express the equation in terms of α, where $\alpha = k'_B/k'_A$. Substitution of α into Equation 1.22, with rearrangement, yields

$$R_s = \frac{\alpha - 1}{\alpha}\,\frac{k'_B}{1 + k'_B}\,\frac{\sqrt{N}}{4} \qquad \textit{Eqn 1.23}$$

or the analyst can determine the number of plates required for a given separation:

$$N = 16\,R_s^2\left(\frac{\alpha - 1}{\alpha}\right)^2\left(\frac{k'_B}{1 + k'_B}\right)^2 \qquad \textit{Eqn 1.24}$$

Thus, the number of plates present in a column can be determined by direct inspection of a chromatogram, where R_s is determined from Equation 1.20, k'_A and k'_B are determined using Equation 1.10, and α is determined using Equation 1.12.

$$R_s = \frac{t_{R,B} - t_{R,A}}{W} \qquad \textit{Eqn 1.20}$$

$$k' = \frac{t_r - t_m}{t_m} = \frac{V_s - V_m}{V_m} \qquad \textit{Eqn 1.10}$$

$$\alpha = \frac{K_B}{K_A} = \frac{k'_B}{k'_A} \qquad \textit{Eqn 1.12}$$

Another use of Equation 1.23 is that it can be used to explain improved separation with temperature programming of the column in GC and gradient programming in HPLC. Recall that poorer separation will result as peaks broaden as they stay for extended times in the column and several factors contribute to this process. N can be changed by changing the length of the column to increase resolution but this will further increase band broadening. H can be decreased by altering the mobile phase flow rate, the particle size of the packing, the mobile phase viscosity (and thus the diffusion coefficients), and the thickness of the stationary phase film.

To better understand the application of the equations derived above a useful exercise is to calculate all of the column quantification parameters for a specific analysis. The chromatogram below (Figure 1.3) was obtained from a capillary column GC with a flame ionization detector. Separations of hydrocarbons commonly found in auto petroleum were made on a 30-meter long, 0.52-mm diameter DB-1 capillary column. Table 1.1 contains the output from a typical integrator.

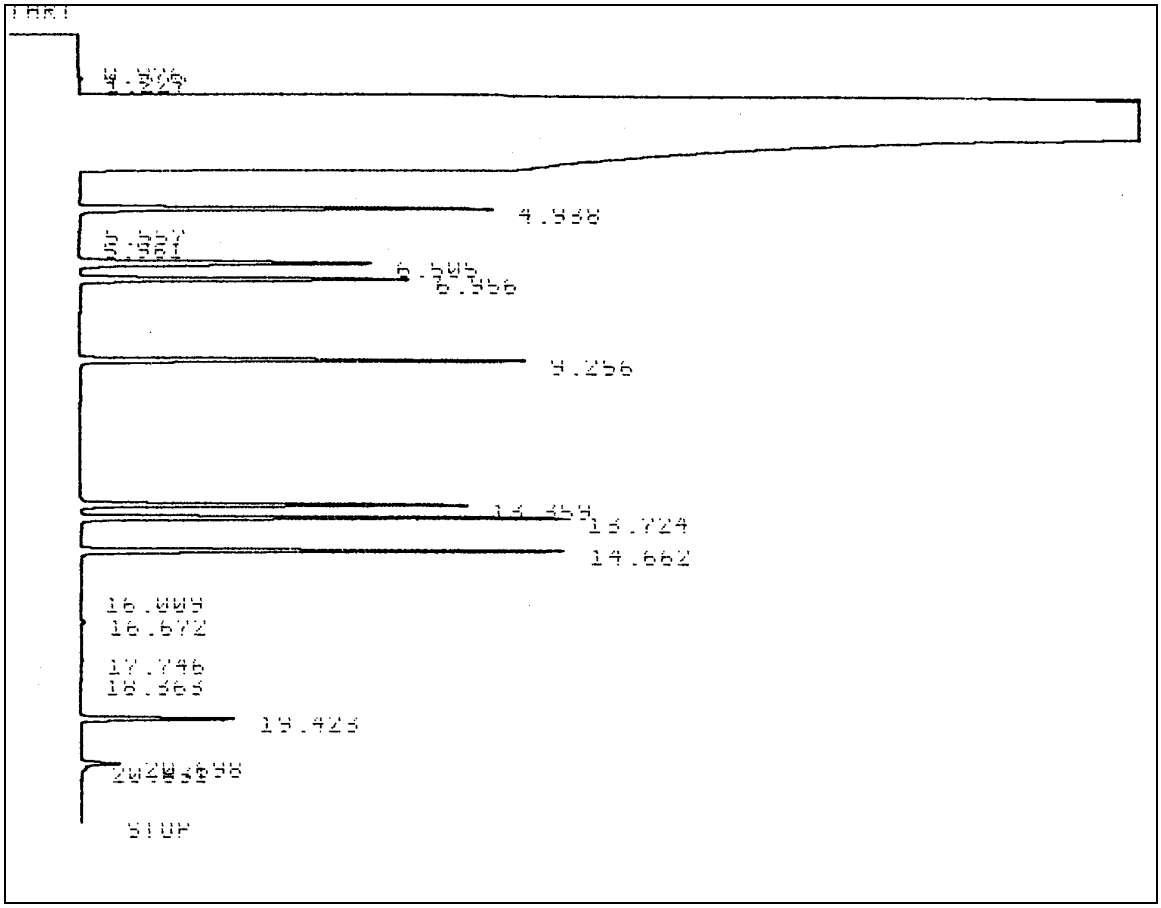


Figure 1.3 Integrator Output for the Separation of Hydrocarbons by Capillary Column GC-FID.

Table 1.1 Integrator Output for the Chromatogram shown in Figure 1.3.

Analyte	Retention Time (min)	Area	Peak Width at the Base (in units of minutes)
Solvent (t_M)	1.782	NA	NA
Benzene	4.938	598833	0.099
Iso-octane	6.505	523678	0.122
n-Heptane	6.956	482864	0.100
Toluene	9.256	598289	0.092
Ethyl Benzene	13.359	510009	0.090
o-Xylene	13.724	618229	0.087
m-Xylene	14.662	623621	0.088

Example 1.1
Calculate k', α, R_s, H, and N for any two adjacent compounds in Table 1.1.

Solution:

Using peaks eluting at 13.724 and 13.359 minutes the following values were obtained.

$$k' = \frac{t_R - t_M}{t_M} = \frac{13.359 - 1.782}{1.782} = 6.50$$

$$\alpha = \frac{t_{RB} - t_M}{t_{RA} - t_M} = \frac{13.724 - 1.782}{13.359 - 1.782} = 1.03$$

$$H = \frac{L\,W^2}{16\,t_R^2} = \frac{(30\text{ m})\,(0.090)^2}{16\,(13.359)^2} = 8.51 \times 10^{-5}\text{ m or } 85.1\ \mu\text{m}$$

$$N = \frac{L}{H} = \frac{30\text{ m}}{8.51 \times 10^{-5}\text{ m}} = 352{,}519\text{ plates}$$

$$R = \left[\frac{t_{RB} - t_{RA}}{t_{RB}}\right]\frac{\sqrt{N}}{4} = \frac{13.724 - 13.359}{13.724}\,\frac{\sqrt{352{,}519}}{4} = 4.12$$

Problem 1.1
Figure 1.4 and Table 1.2 contain data from an HPLC analysis of four s-Triazines (common herbicides). Calculate k', α, R_s, H, and N for any two adjacent compounds. Compare and contrast the results for the resin packed HPLC column to those of the capillary column in the GC example given above.

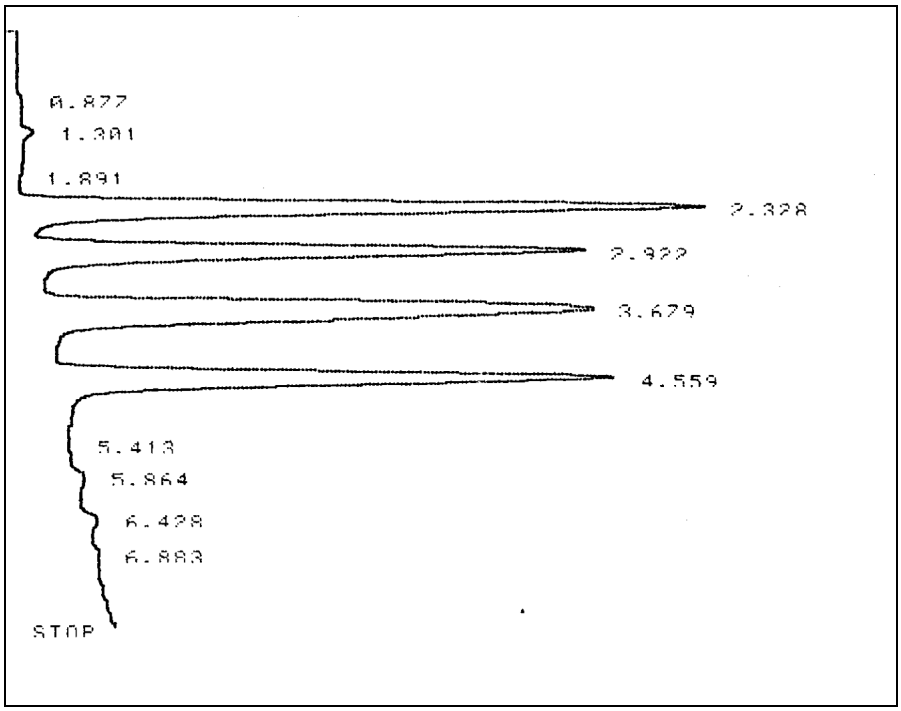


Figure 1.4 HPLC Chromatogram of Four Triazines. The analytical column was an 10.0 cm C-18 stainless steel column with 2 μm resin beads.

Table 1.2 Integrator Output for the HPLC Chromatogram shown in Figure 1.4.

Analyte	Retention Time (min)	Area	Peak Width at the Base (in units of minutes)
Solvent (t_M)	1.301	NA	NA
Peak 1	2.328	1753345	0.191
Peak 2	2.922	1521755	0.206
Peak 3	3.679	1505381	0.206
Peak 4	4.559	1476639	0.198

1.3 Optimization of Chromatographic Conditions

Now we will review and summarize this lengthy derivation and these complicated concepts. Optimization of the conditions of the chromatography system (mobile phase flow rate, stationary phase selection, and column temperature or solvent gradient) are performed to achieve base-line resolution for the most difficult separation in the entire analysis (two adjacent peaks). This process results in symmetrically-shaped peaks that the computer can integrate to obtain a peak (analyte) area or peak height. A series of known reference standards are used to generate a linear calibration line (correlating peak area or

height to analyte concentration) for each compound. This line, in turn, is used to estimate the concentration of analyte in unknown samples based on peak area or height.

An instrument's resolution can be altered by changing the theoretical plate height and the number of theoretical plates in a column. The plate height, as explained in the animation below, is the distance a compound must travel in a column needed to separation two similar analytes. The number of theoretical plates in a column is a normalized measure of how well a column will separate similar analytes.

Now it is necessary to extend the concept of theoretical plate height (H) a bit further to understand its use in chromatography. Since gas and liquid chromatography are dynamic systems (mobile flow through the column), it is necessary to relate a fixed length of the column (the theoretical plate height) to flow rate in the column. Flow rate is measured in terms of linear velocity, or how many centimeters a mobile analyte or carrier gas will travel in a given time (cm/s). The optimization of the relationship between H and linear velocity (μ), referred to as a van Deemter plot, is illustrated in Figure 1.5 for gas chromatography.

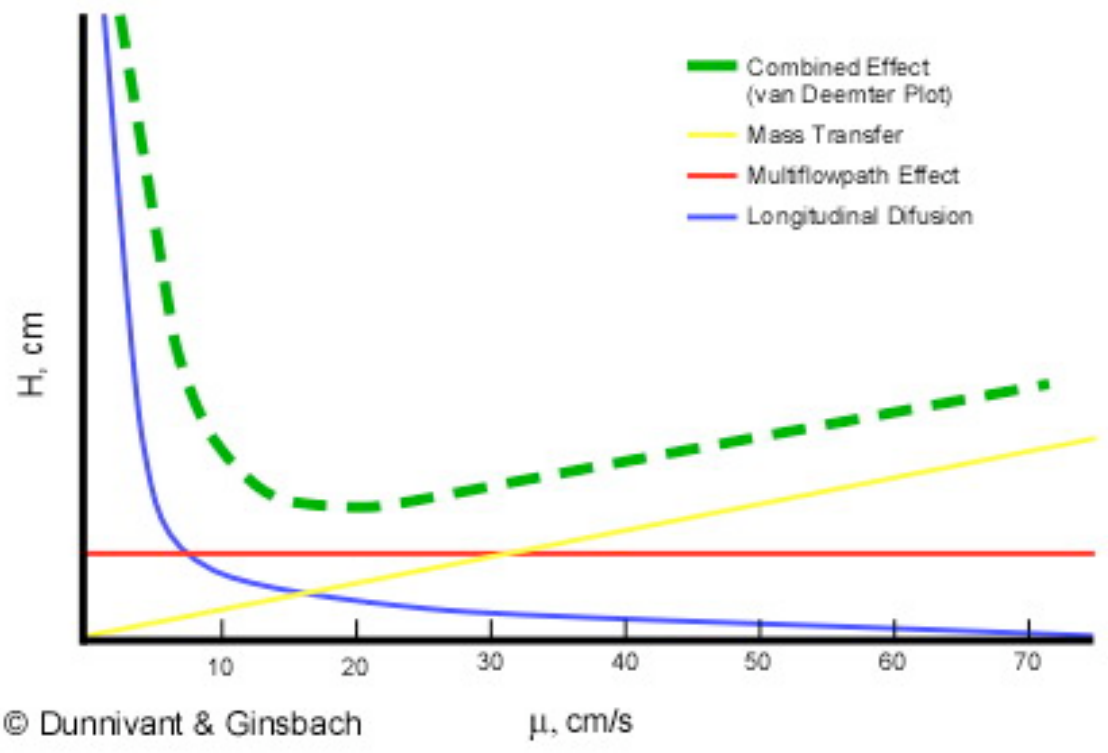


Figure 1.5. A Theoretical van Deemter Plot for a Capillary Column showing the Relationship between Theoretical Plate Height and Linear Velocity.

It is desirable to have the smallest plate height possible, so the maximum number of plates can be "contained" in a column of a given length. Three factors contribute to the effective plate height, H, in the separation column. The first is the longitudinal diffusion, B (represented by the blue line in Figure 1.5) of the analytes that is directly related to the time an analyte spends in the column. When the linear velocity (μ) is high, the analyte will only spend a short time in the column and the resulting plate height will be small. As linear velocity slows, more longitudinal diffusion will cause more peak broadening resulting in less resolution. The second factor is the multi-flow path affect represented by the red line in Figure 1.5. This was a factor in packed columns but has been effectively eliminated when open tubular columns (capillary columns) became the industry standard. Third are the limitations of mass transfer between and within the gas and stationary phases, C_u (the yellow line in Figure 3) defined by

Mass Transfer to and from the Stationary Phase $$C_s u = \frac{f(k)\ d_f^2}{D_s}\ \mu$$

Mass Transfer in the Mobile Phase $$C_M u = \frac{f'(k)\ d_p^2}{D_M}\ \mu$$

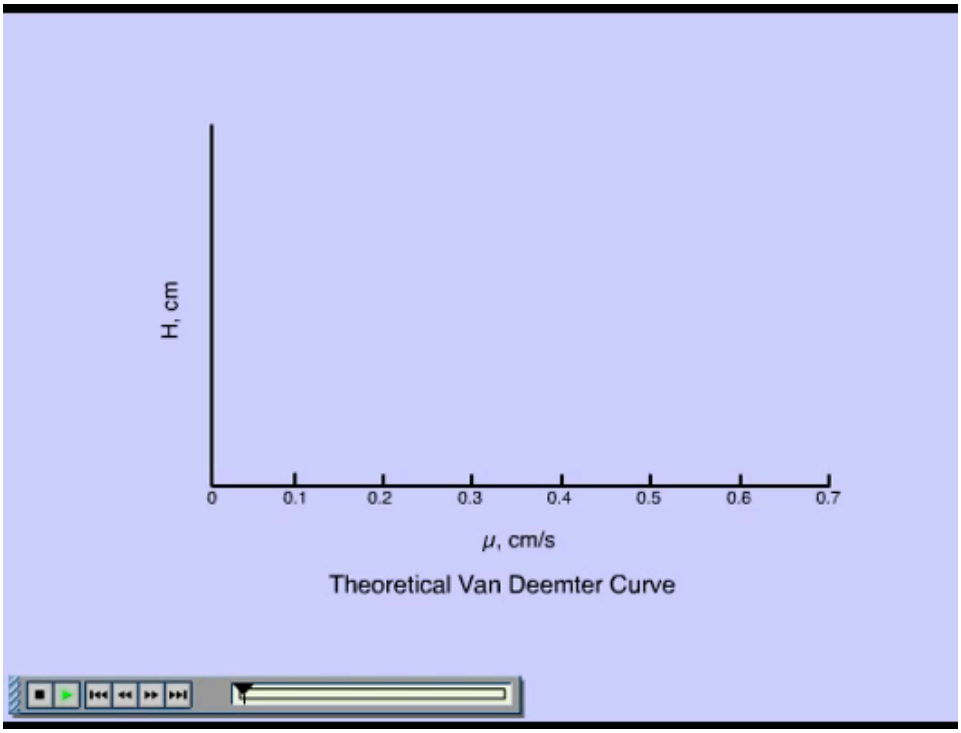
where μ is the mobile phase linear velocity, D_s and D_m are diffusion coefficients in the stationary and mobile phases respectively, d_f and d_p are the diameter of the packing particles and the thickness of liquid coating on the stationary phase particles respectively, k is the unitless retention or capacity factor, and f(k) and f'(k) are mathematical functions of k.

If the linear velocity of the mobile phase is too high, the entire "packet" of a given analyte will not have time to completely transfer between the mobile and stationary phase or have time to completely move throughout a given phase (phases are coated on the column walls and therefore have a finite thickness). This lack of complete equilibrium of the analyte molecules will result in peak broadening for each peak or skewing of the Gaussian shape. This, in turn, will increase H and decrease resolution.

The green line in Figure 1.5 represents the van Deemter curve, the combined result of the three individual phenomena. Since the optimum operating conditions has the smallest plate height; the flow rate of the GC should be set to the minimum of the van Deemter curve. For gas chromatography this occurs around a linear velocity of 15 to 20 cm/s. However, in older systems, as the oven and column were temperature programmed, the velocity of the gas changed

which in turn changed the mobile phase flow rate and the linear velocity. This has been overcome in modern systems with mass flow regulators, instead of pressure regulators, that hold the linear velocity constant.

These concepts are reviewed in Animation 1.4. Click the figure to start the animation.

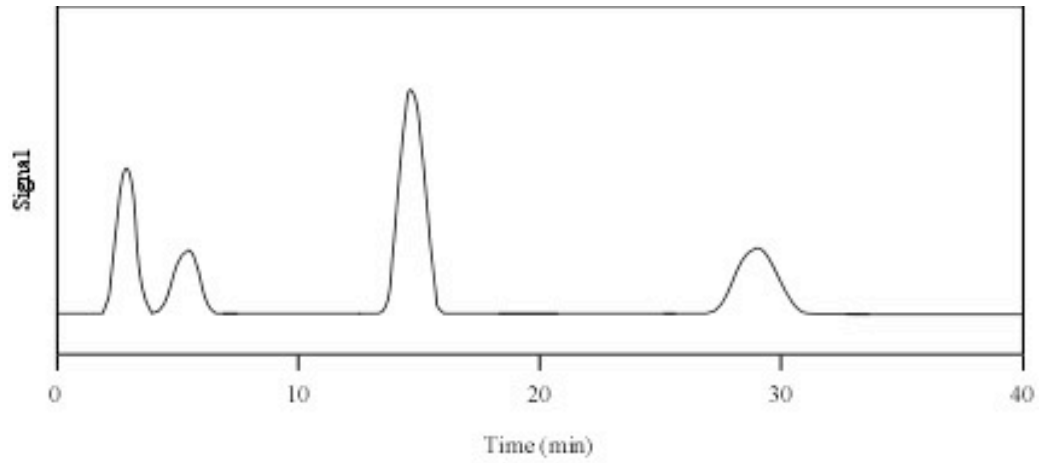


Animation 1.4 Construction of a van Deemter Curve for an HPLC System

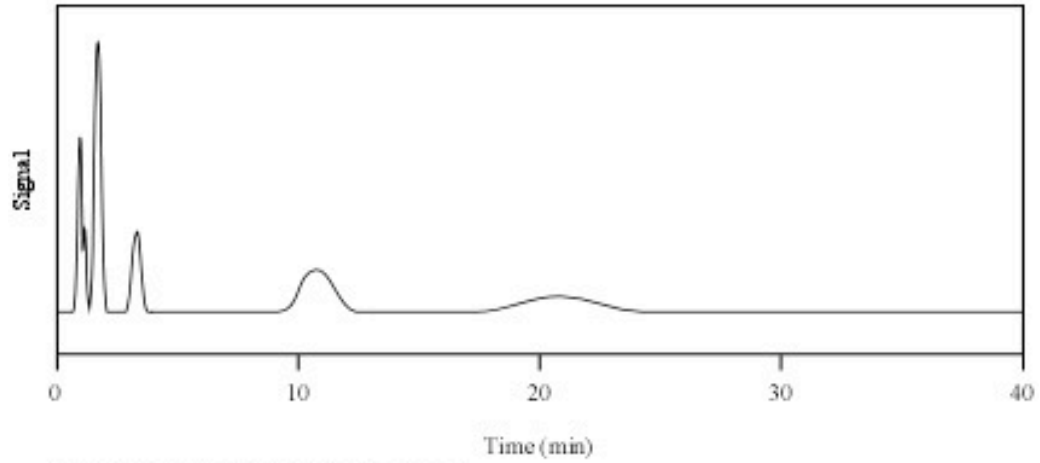
Now that the theoretical basis for understanding chromatographic separation has been established, it is necessary to extend these ideas one step further. Remember, the power of chromatography is the separation of complex mixtures of chemicals; not just for two chemicals as illustrated previously. In most cases separating mixtures of many compounds is required. This requires that the resolution, R_s, be constantly optimized by maintaining H at its minimum value in the van Deemter curve.

This optimization is accomplished by systematically altering the column temperature in GC or the solvent composition in HPLC. Analytes in the separation column spend their time either “dissolved” in the stationary phase or vaporized in the mobile phase. When analytes are in the stationary phase they are not moving through the system and are present in a narrow band in the length of the column or resin coating. As the oven temperature is increased, each unique analyte has a point where it enters the mobile phase and starts to move down the column. In GC, analytes with low boiling points will move down

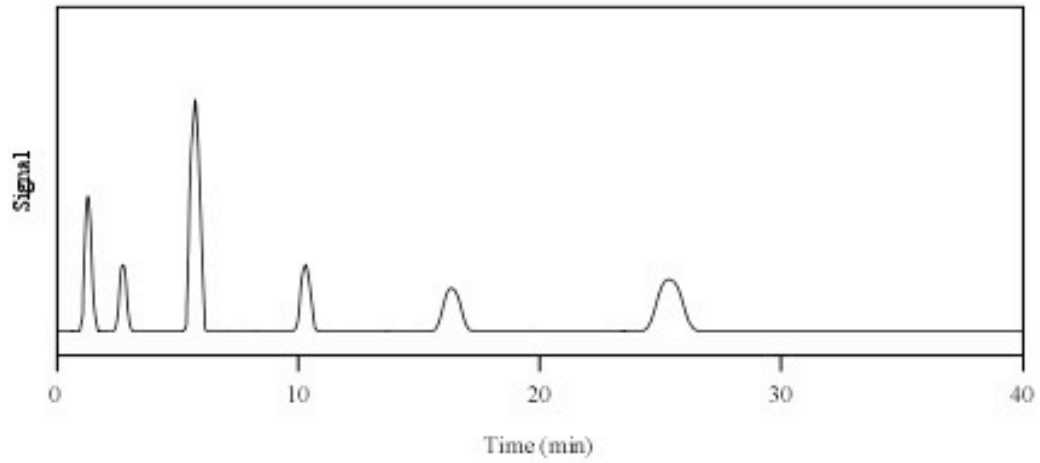
the column at lower temperatures, exit the system, and be quantified. As the temperature is slowly increased, more and more analytes (with higher boiling points) likewise exit the system. In reverse-phase HPLC, analytes with more polarity will travel fastest and less polar analytes will begin to move as the polarity of the mobile phase is decreased. Thus, the true power of GC separation is achieved by increasing the oven/column temperature (referred to as ramping) while in LC the separation power is in gradient programming (composition of the mobile phase). This is “the general elution problem” that is solved by optimizing the mobile phase, linear velocity, and the type of stationary phase. As noted, temperature programming is used to achieve separation of large numbers of analytes in GC. An example of the effects of temperature programming on resolution is illustrated in Figure 1.6. In Figure 1.6a, a low isothermal temperature is used to separate a mixture of six analytes with limited success as some peaks contain more than one analyte. A higher isothermal temperature, shown in Figure 1.6b, is more successful for analytes with higher boiling points but causes a loss of resolution for peaks that were resolved at the lower isothermal temperature. The temperature program used to produce Figure 1.6c achieves adequate separation and good peak shape for a complex solution.



a) Isothermal temperature at a 40 C



b) Isothermal temperature at 200 C



c) Temperature program from 40 C to 200 C

Figure 1.6. Temperature Programming: The Solution to the General Elution Problem for GC Applications.

1.4 Calibration of an Instrument/Detector

We now have a basis for understanding separation science with respect to chromatography. All chromatography systems rely on these principles. But how does the analyst relate instrument output to analyte concentration in a sample? Instruments yield signals (also referred to as responses) that are specific to the type of detector being used. Most GC detectors result in electrical currents while most LC detectors yield absorbance values. MS units can be attached to both GC and LC systems and yield counts of ions per time. But before actual samples are analyzed each instrument detector must be calibrated. Two common forms of calibration are internal and external calibration.

Detector response yields two useful means of quantification in chromatography: peak area and peak height. In the "old days" these measurements were made manually; a strip chart recording was obtained by passing a strip of paper consisting of uniform weight past a pen that moved relative to the detector signal. The shape of the peak was drawn on the paper and the peak height was measured with a ruler or the peak area was measured either by triangulation or by actually weighing a cutout of the paper containing the peak! Fortunately for us, these archaic methods are no longer required. The major disadvantage of these techniques is that the range of detector responses was limited by the height of the paper. Today, peak area and height measurements are calculated by electronic integrators or computers, and most systems are automated such that peak area/height are directly correlated between standards and samples. Most systems use peak area to generate calibration lines, which are usually linear relationships between the detector response and the concentration or mass of analyte injected into the instrument. Such a plot is shown for an external calibration method in Figure 1.7.

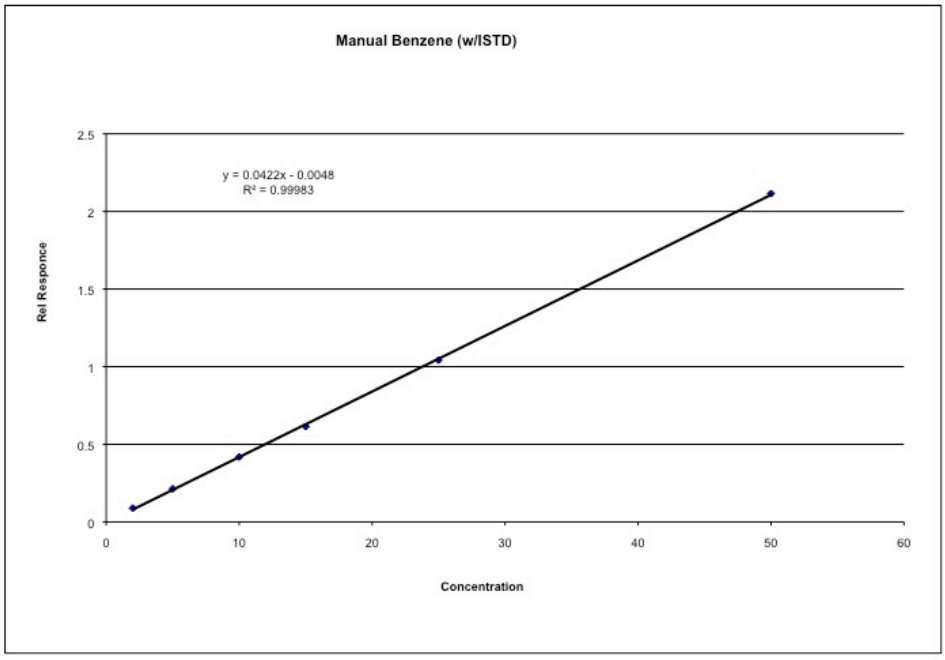
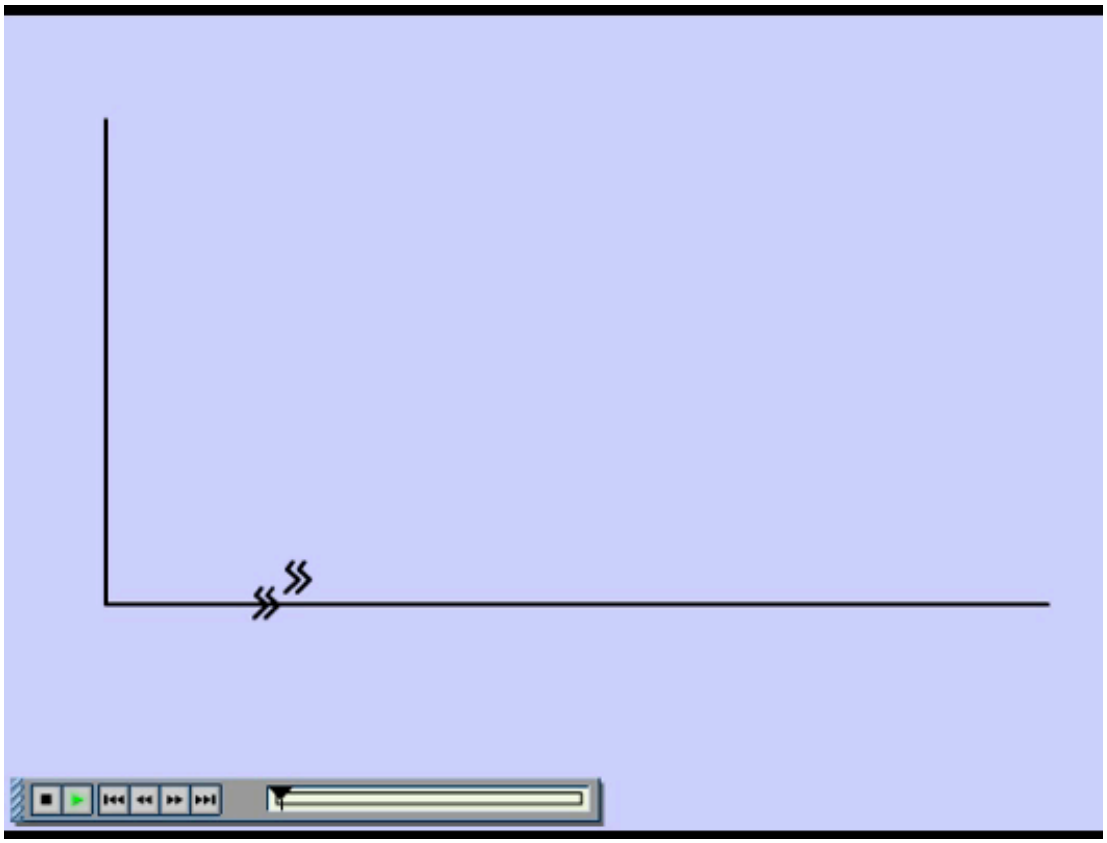


Figure 1.7 External Calibration of Benzene on a Capillary Column GC.

A summary of integration concepts is illustrated in Animation 1.5. Click on the figure to begin the animation.



Animation 1.5 Integration of Chromatographic Peaks.

After an instrument has been calibrated, a sample extract is analyzed under the same conditions as the standards. The calculated area for the sample is then analyzed by a linear regression of the standard line and a mass or concentration of the analyte in the sample is calculated. Usually a dilution factor adjustment is made and the concentration of analyte in the original sample is then calculated.

A special type of additional calibration is used in capillary column gas chromatography because of analyte losses during sample injection and due to the possibility of inconsistent injections when manual injections are preformed. This method is referred to as an “internal standard” where every sample and standard injected into the instrument contains an identical concentration of a compound with similar chemical structure to the analyte but one that has a unique retention time in the column. The instrument is set to measure a constant concentration (and therefore measured area) of the internal standard and adjusts all injections to that constant value. For example, if a sample is found to only contain 90 percent of the internal standard, then it is assumed that 10 percent of the injection was lost and all analyte concentrations are increased by 10 percent. Similarly adjusts can be made of over injecting a sample.

The next chapters of this book will focus on the components of GC, LC, CE and MS with an additional chapter on interpretation of MS fragmentation patterns. Both GC and LC rely on the chromatography theory discussed in this chapter, but CE requires a different derivation that will discussed in Chapter 4. All instruments rely on some form of calibration if quantitative results are required.

1.5 Questions

1. List the three basic types of chromatography. What are the subcategories of each type?

2. What does confirmatory analysis mean with respect to chromatography? What two ways can it be accomplished?

3. What are mobile and stationary phases in chromatography?

4. With respect to GC and LC, what types of gradients are used to improve analyte separation?

5. Hand draw a chromatography with a solvent peak and two analytes. Label components of the diagrams with respect to retention time.

6. Explain the old adage “like dissolves like” with respect to chromatography.

7. What possible intermolecular forces can be involved in “like dissolves like?”

8. How are time, gas/liquid volume, and flow rate related in GC and LC?

9. Why is retention time so important in chromatography?

10. Explain the concept of capacity factor, k’. Is k’ a factor for one analyte or many analytes?

11. How is the capacity factor related to retention time of an analyte?

12. What are the acceptable range for k’ for traditional chromatography?

13. How are the capacity factor and the selectivity factor mathematically related?

14. What is the value/purpose of using the selectivity factor?

15. What are acceptable values for selectivity factors?

16. Explain the theoretical plate height, H. What is the origin of H?

17. How is the total number of plates in a column related to the length of the column and H?

18. Why is proper column packing so important in packed-column GC and LC?

19. Explain resolution with respect to chromatography. What is the mathematical relationship between resolution and retention time and peak width?

20. Explain each component in the governing equation for resolution, equation 1.23.

21. How is equation 1.23 used to improve separation of chemically similar analytes in GC and LC?

22. Using the data in Table 1.1 and Example 1.1, calculate k’, α, R_s, H, and N for iso-octane and n-heptane.

23. Why do capillary columns provide higher resolution as compared to packed columns?

24. Draw a van Deemter curve for a GC analysis and explain each factor that contributes to H.

25. Use figures and words to explain the “General Elution Problem.” How is this solved in GC and LC?

26. Why is instrument calibration so important in chromatography?

27. What is the difference in internal and external calibration?

1.6 References

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