BASIC TERMS AND CONCEPTS

1. Retention Time (t_R), Void Time (t_M), Peak Height (h), and Peak Width (w_b):

The time between the sample injection and the peak maximum is called the retention time (t_R).The retention time of an unretained component or the first baseline disturbance by the sample solvent is called the void time (t_M) or hold-up time.

t_M is the total time spent by any component in the mobile phase. The adjusted retention time, t_R' is equal to ($t_R - t_M$), i.e., the time the solute resides in the stationary phase. Thus, $t_R = t_R' + t_M$ or the retention time is the total time the solute spends in the stationary phase (t_R') and in the mobile phase (t_M).

The peak width is measured at the base (w_b) or at the peak half-height ($w_{1/2}$).

2. Retention Volume (V_R), Void Volume (V_M), and Peak Volume:

The retention volume (V_R) is the volume of mobile phase needed to elute the analyte at given flow rate (F).

$$\text{retention volume, } V_R = t_R F$$

void volume, $V_M = t_M F$

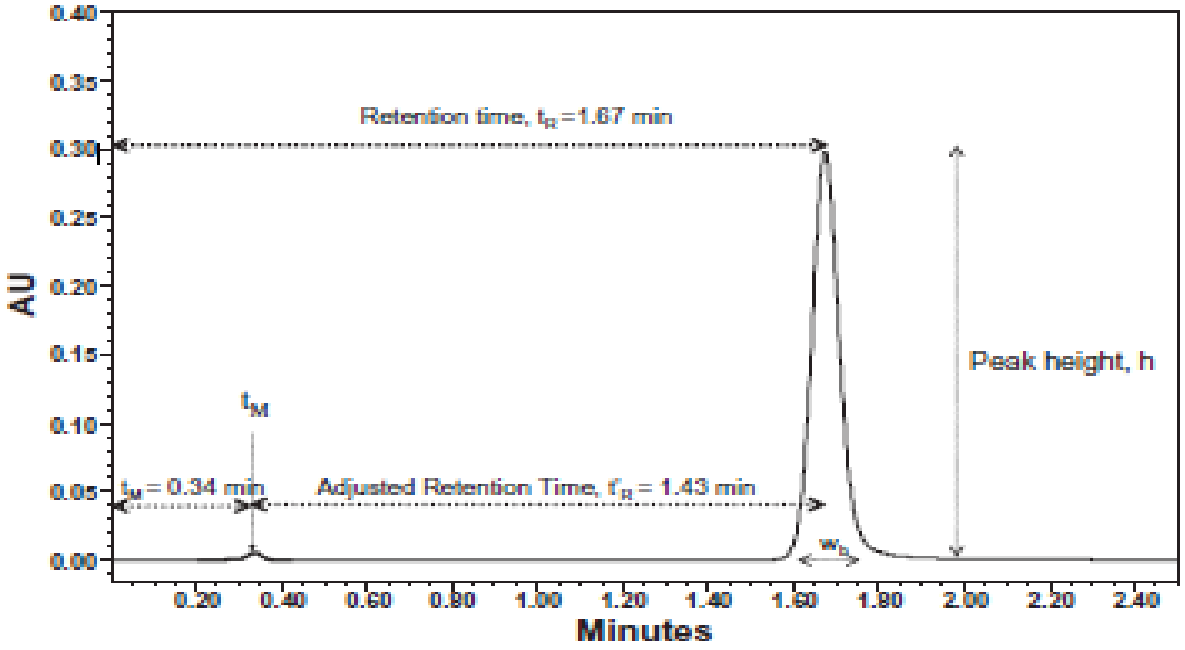


Figure 2.1. A chromatogram showing retention time (t_R), void time (t_M), peak width (w_b), and peak height (h).

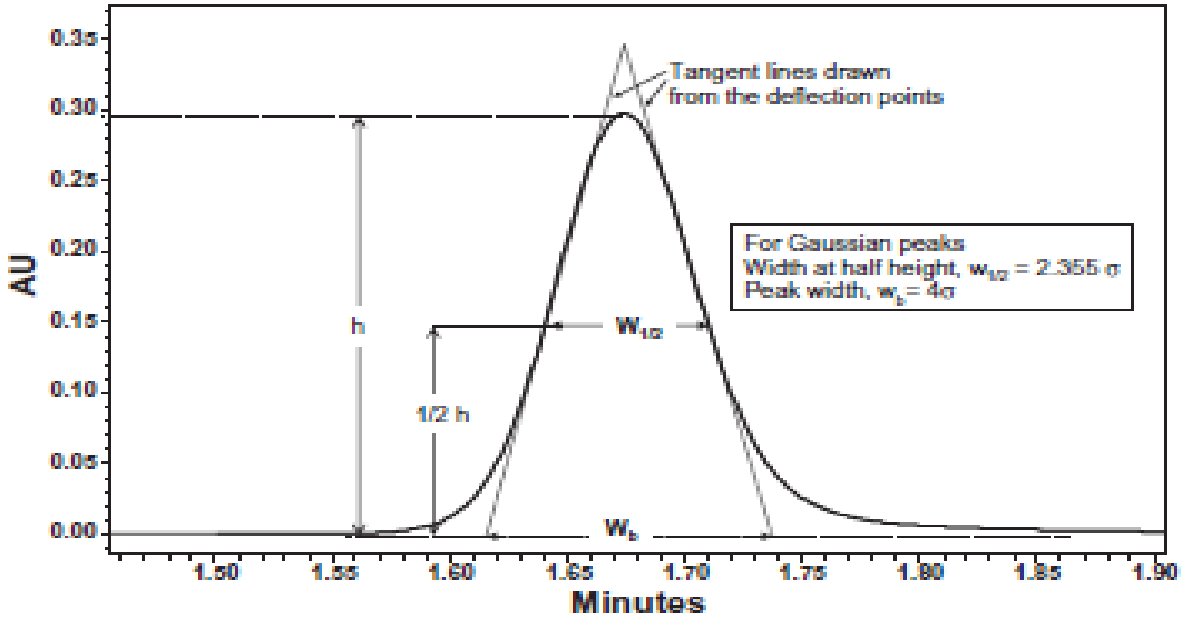


Figure 2.2. Diagram illustrating how peak width (w_b) and peak width at half height ($w_{1/2}$) are measured.

The void volume (V_M) is the total volume of the liquid mobile phase contained in the column (also called hold-up volume). It is the volume of the empty column (V_c) minus the volume of the solid packing.

Note that V_M is the sum of the intraparticle volume (V_0) and the interstitial volumes (V_e) inside the pores of the solid support. For most columns, the void volume can be estimated by the equation:

$$V_M = 0.65\ V_c = 0.65\ \pi\ r^2\ L,$$

where r is the inner radius of the column and L is the length of the column.

V_M can also be estimated from t_M in the chromatogram, since $V_M = t_M\ F$

The peak volume, also called bandwidth, is the volume of mobile phase containing the eluted peak:

$$\text{Peak volume} = w_b\ F.$$

Peak volume is proportional to V_M, and therefore smaller columns produce smaller peak volumes.

3. Retention Factor (k):

The retention factor (k) is the degree of retention of the sample component in the column. k is defined as the time the solute resides in the stationary phase (t_R') relative to the time

it resides in the mobile phase (t_M), k, was often referred to as k′ or capacity factor in many references.

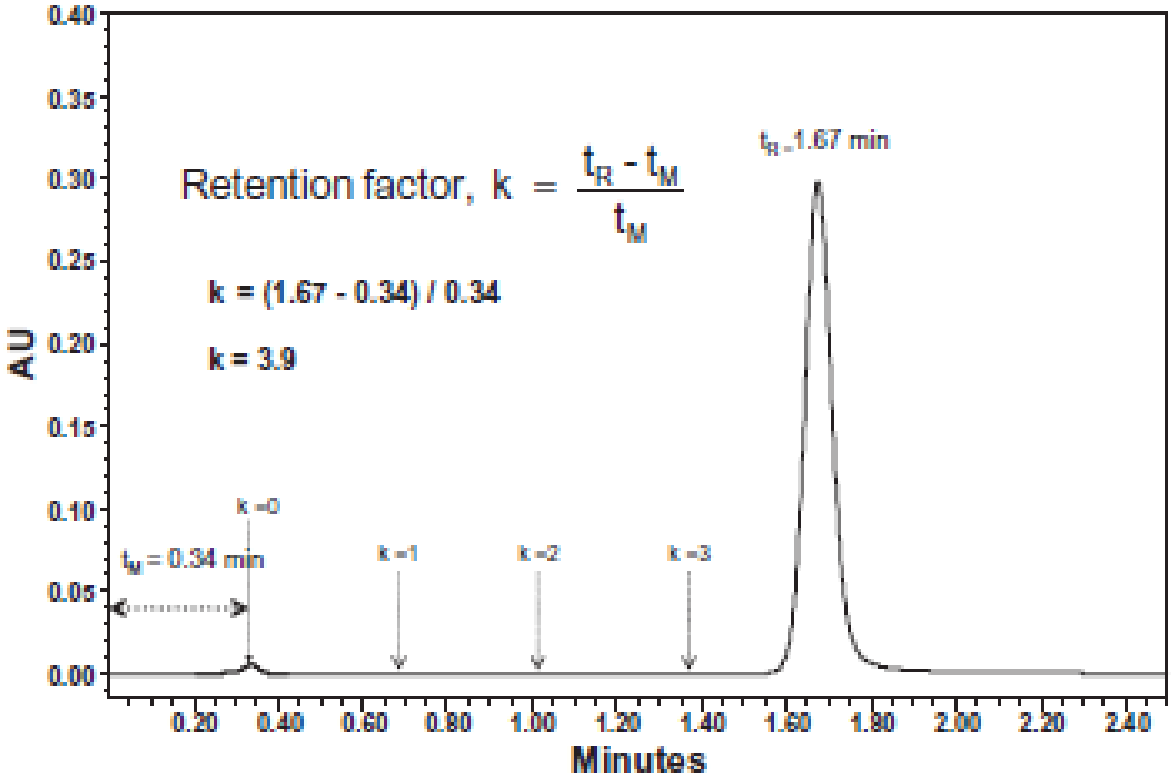


Figure 2.3. A chromatogram showing the calculation for retention factor (k), which is equal to t_R'/t_0. k is an important parameter defining the retention of the analyte. Desirable k values for isocratic analyses are 1 to 20.

Retention factor, $k = \frac{tR'}{tM} = \frac{tR - tM}{tM}$

$t_R = t_M + t_M k = t_M (1+ k)$.

The retention time is proportional to k.

By multiplying both sides by the flow rate, F, a similar equation for V_R is obtained:

Retention volume, $V_R = F\ t_R = F\ t_M\ (1+ k)$ *or* $V_R = V_M\ (1+ k)$

A peak with k = 0 is a component that is unretained by the stationary phase and elutes with the solvent front. k > 20 indicates that the component is highly retained. In most analyses, analytes elute with k between 1 and 20 so that they

have sufficient opportunity to interact with the stationary phase. Analytes eluting with k > 20 are difficult to detect

due to excessive band broadening.

Chromatography is a thermodynamically based method of separation, where each component in the sample is distributed between the mobile phase and the stationary phase.

$X_m \leftrightarrow X_s$ Partition coefficient $K = [X_s] / X_m$

where $[X_m]$ and $[X_s]$ are the concentrations of analyte X in the mobile phase and stationary phase, respectively. The distribution of analyte X is governed by the partition coefficient, K. Also,

$$\text{Retention factor, } k = \frac{\textit{Moles of X in stationary phase}}{\textit{Moles of X in mobile phase}}$$

$$= \frac{[Xs]}{[Xm]}\ \frac{Vs}{VM} = K\ \frac{Vs}{VM}$$

where V_s is the volume of the stationary phase and V_m is the volume of the mobile phase in the column or the void volume. k is controlled by the strength of the mobile phase, the nature of stationary phase, and the temperature at which the separation is performed.

4. **Separation Factor (α):**

It is a measure of relative retention k_2/k_1 of two sample components. Selectivity must be >1.0 for peak separation. Selectivity is dependent on many factors that affect K such as the nature of the stationary phase, the mobile phase composition, and properties of the solutes.

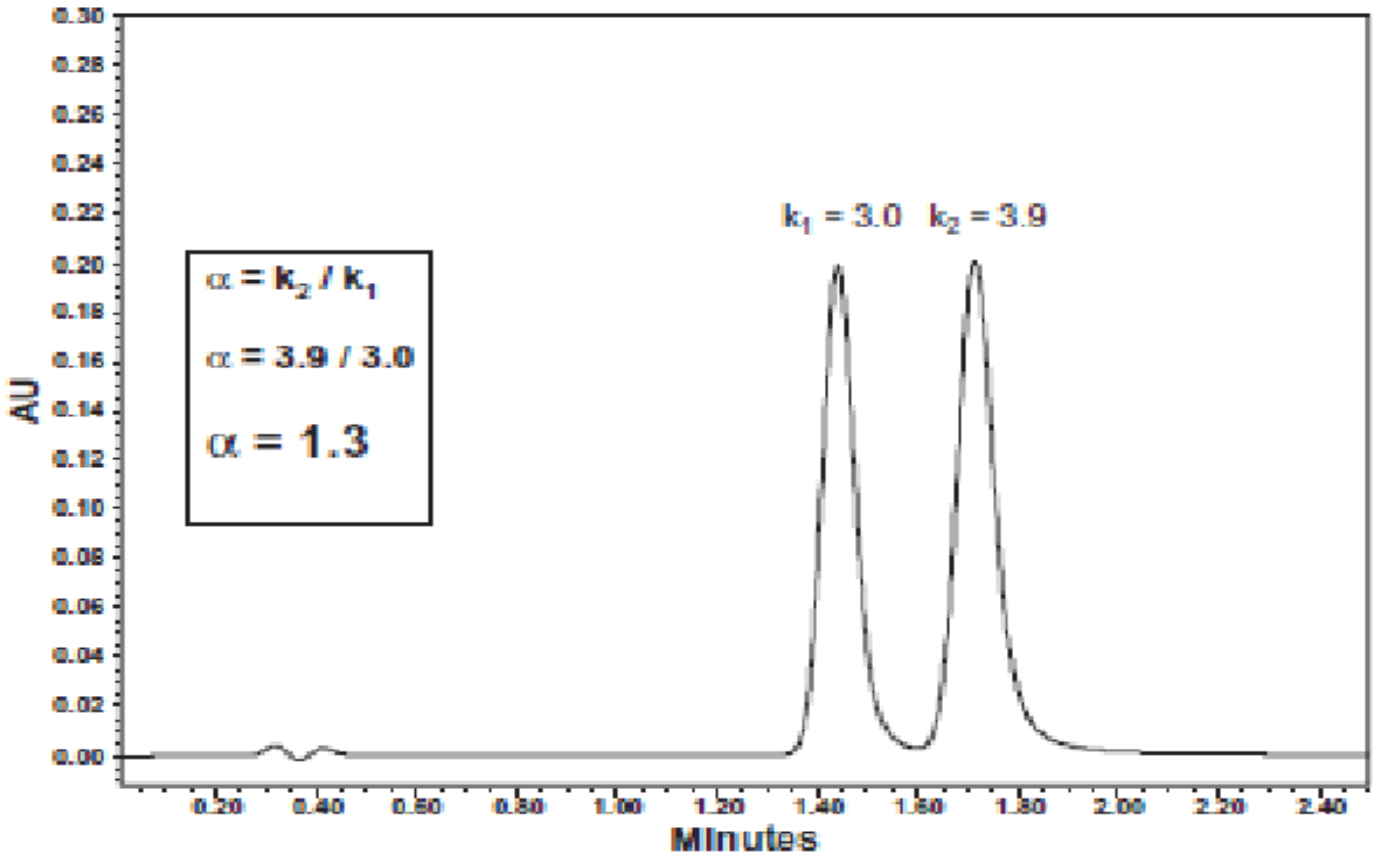


Figure 2.4. A chromatogram of two peaks with a selectivity factor (α) of 1.3.

5. Column Efficiency and Plate Number (N):

An efficient column produces sharp peaks and can separate many sample components in a relatively short time. As seen in most chromatograms, peaks tend to be Gaussian in shape and broaden with time, where w_b becomes wider with longer t_R. The number of theoretical plates or plate number (N) is a measure of the efficiency of the column. N is defined as the

square of the ratio of the retention time divided by the standard deviation of the peak (σ). Since w_b is equal to 4σ for a Gaussian peak,

$$\text{Number of theoretical plates, } N = \left(\frac{t_R}{\sigma}\right)^2 = \left(\frac{4t_R}{w_b}\right)^2 = 16\left(\frac{t_R}{w_b}\right)^2.$$

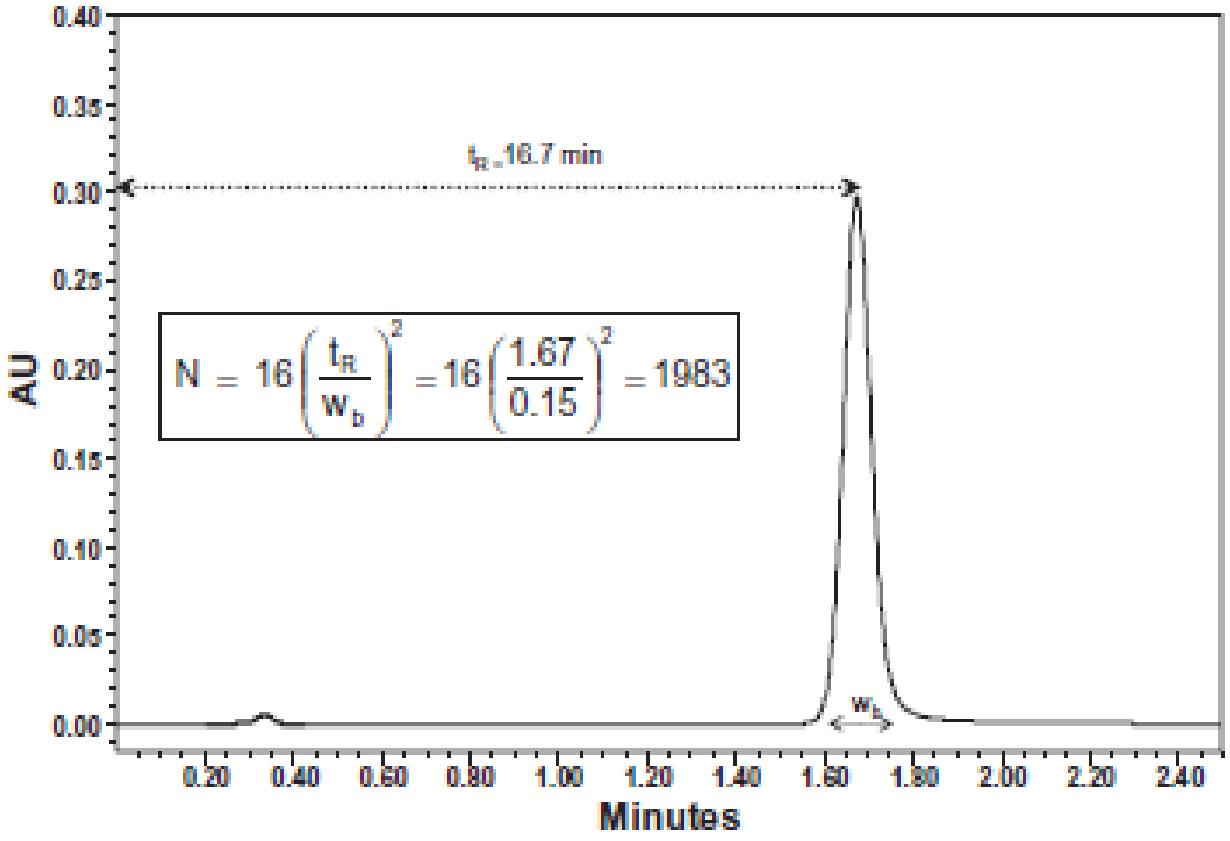


Figure 2.5. A chromatogram showing a peak from a column with N = 1,983.

Since it is more difficult to measure σ or w_b, a relationship using ($w_{1/2}$) is used to calculate N.

$$N = \left(\frac{t_R}{\sigma}\right)^2 = \left(\frac{2.355\, t_R}{w_{1/2}}\right)^2 = 5.546\left(\frac{t_R}{w_{1/2}}\right)^2.$$

6. Peak Volume:

Peak volume is the volume of mobile phase or eluate containing the eluting peak. Peak volume is proportional to k and V_M. This relationship between peak volume and these factors can be derived by rearranging:

$$N = 16\left(\frac{t_R}{w_b}\right)^2 \quad \frac{N}{16} = \left(\frac{t_R}{w_b}\right)^2 \quad \frac{\sqrt{N}}{4} = \left(\frac{t_R}{w_b}\right),$$

then multiplying both numerator and denominator by F,

$$\frac{\sqrt{N}}{4} = \left(\frac{F\, t_R}{F\, w_b}\right) = \left(\frac{V_R}{F\, w_b}\right) = \left(\frac{V_R}{Peak\ Volume}\right).$$

$$Peak\ volume = \frac{4V_R}{\sqrt{N}} = \frac{4\, V_M(1+k)}{\sqrt{N}}.$$

Since for a given column and a set of operating conditions N is approximately constant and (1 + k) is roughly equal to k in most cases where k is much greater than 1, peak volume is proportional to k and V_M. This relationship is important because of the increasing use of smaller-diameter columns (column i.d.< 3 mm) with smaller V_M.

7. Height Equivalent to a Theoretical Plate or Plate Height (HETP or H):

The height equivalent to a theoretical plate (HETP or H) is equal to the length of the column (L) divided by the plate number (N):

$$\text{HETP, } H = L / N$$

In HPLC, the main factor controlling H is the particle diameter of the packing (d_p). For a well-packed column, H is roughly equal to 2.5 d_p.

A typical 15-cm-long column packed with 5-μm materials should have N = L / H = 150,000μm / (2.5 × 5μm) = 12,000 plates.

Similarly, a 15-cm column packed with 3-μm material should have N = L / H = 150,000μm / 7.5 μm = 20,000 plates. Thus, columns packed with smaller particles are usually more efficient and have a higher plate number.

8. Resolution (R_s):

The goal of most HPLC analyses is the separation of one or more analytes in the sample from all other components present. Resolution (R_s) is a measure of the degree of separation of two adjacent analytes. R_s is defined as the difference in retention times of the two peaks divided by the average peak width. Since peak widths of adjacent peaks tend to be similar, the average peak width is approximated by one of the w_b's:

$$\text{Resolution, } R_s = \frac{t_{R2} - t_{R1}}{\left(\frac{w_{b1} + w_{b2}}{2}\right)} = \frac{\Delta t_R}{w_b}.$$

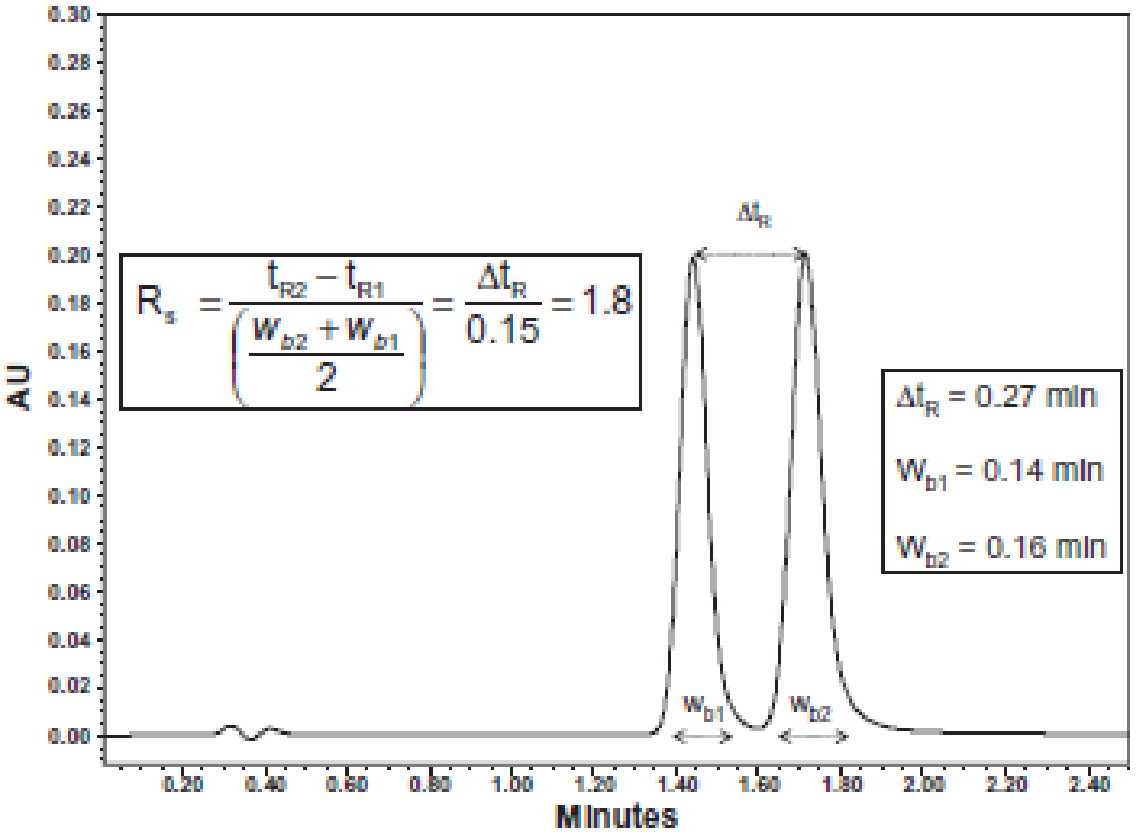


Figure 2.6. A chromatogram of two peaks with a resolution (R_s) of 1.8.

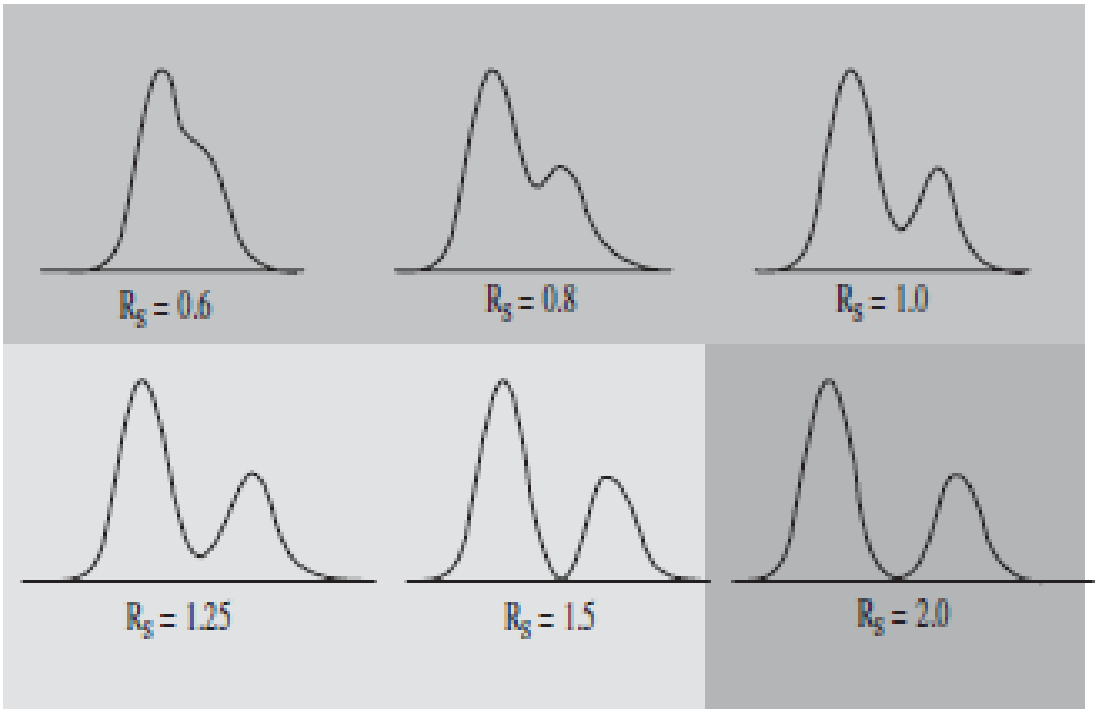


Figure 2.7. Diagrams showing two closely eluting peaks at various resolution values from 0.6 to 2.0. Figure reprinted with permission from Academy Savant.

R_s = 0 indicates complete co-elution or no separation. R_s = 0.6 indicates a slight partial separation. R_s = 1 indicates a partial separation and it is the minimum separation required for quantitation. R_s = 1.5 indicates baseline separation.

Ideally, the goal of most HPLC methods is to achieve baseline separation (R_s = 1.5–2.0).

9. Peak Symmetry: Asymmetry Factor (A_s) and Tailing Factor (T_f):

Under ideal conditions, chromatographic peaks should have Gaussian peak shapes with perfect symmetry. In reality, most peaks are not perfectly symmetrical and can be either fronting or tailing. The asymmetry factor (A_s) is

used to measure the degree of peak symmetry and is defined at peak width of 10% of peak height ($W_{0.1}$).

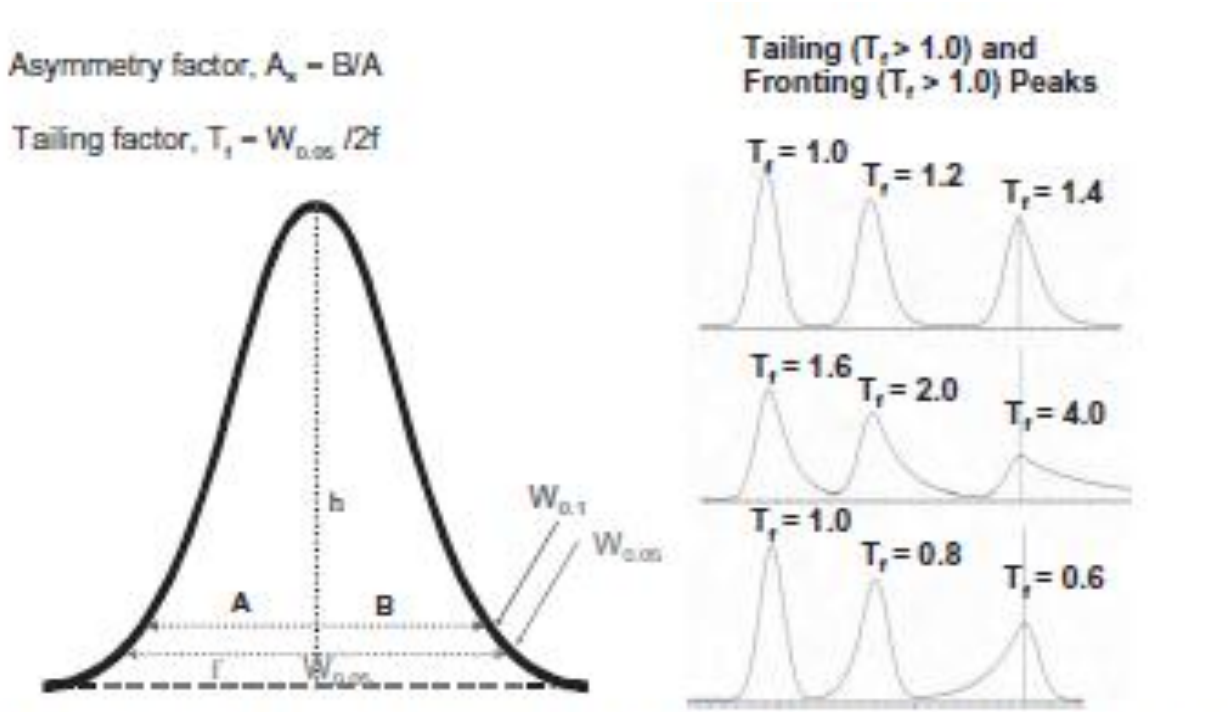


Figure 2.8. A diagram showing the calculation of peak asymmetry (A_s) and tailing factor (T_f) from peak width at 5% height ($W_{0.05}$) according to the USP. Inset diagrams show fronting and tailing peaks.

$$\text{Asymmetry factor, } A_s = B / A$$

Tailing factor (T_f) is calculated using the peak width at 5% peak height ($W_{0.05}$):

$$\text{Tailing factor } T_f = W_{0.05} / 2f$$

T_f = 1.0 indicates a perfectly symmetrical peak. T_f > 2 indicates a tailing peak that is typically not acceptable due to difficulty in integrating the peak area precisely. For most peaks ($0.5 < T_f < 2.0$), the values of A_s and T_f are similar. For tailing peaks, A_s is larger than T_f.

Peak tailing is typically caused by adsorption. Peak fronting is typically caused by column overloading or chemical reaction of the analyte during chromatography. Many basic analytes (amines) display some peak tailing due to the polar interaction with residual acidic silanol groups in silica-based columns.

MOBILE PHASE:

The mobile phase is the solvent that moves the solute (analyte) through the column. In HPLC, the mobile phase interacts with both the solute and the stationary phase and has a powerful influence on solute retention and separation.

General Requirements:

Solvents used as HPLC mobile phases should have these characteristics:

• High solubility for the sample components.

• Noncorrosive to HPLC system components.

• High purity, low cost, UV transparency.

• Low viscosity, low toxicity, and nonflammability.

Solvent Strength and Selectivity:

Solvent strength refers to the ability of a solvent to elute solutes from a column. Solvent strengths under normal phase conditions are often characterized by Hildebrand’s elution

strength scale (E^o). Solvent strength is related to its polarity. Nonpolar hexane is a weak solvent in normal phase chromatography whereas water is a strong solvent. The opposite is true in RPLC since the stationary phase is hydrophobic. Here water is a weak solvent and organic solvents are strong and in reversed order of the Hildebrand scale of THF > ACN > MeOH >> water. Water is a weak solvent because it is a poor solvent for nonstrongly H-bonding organics.

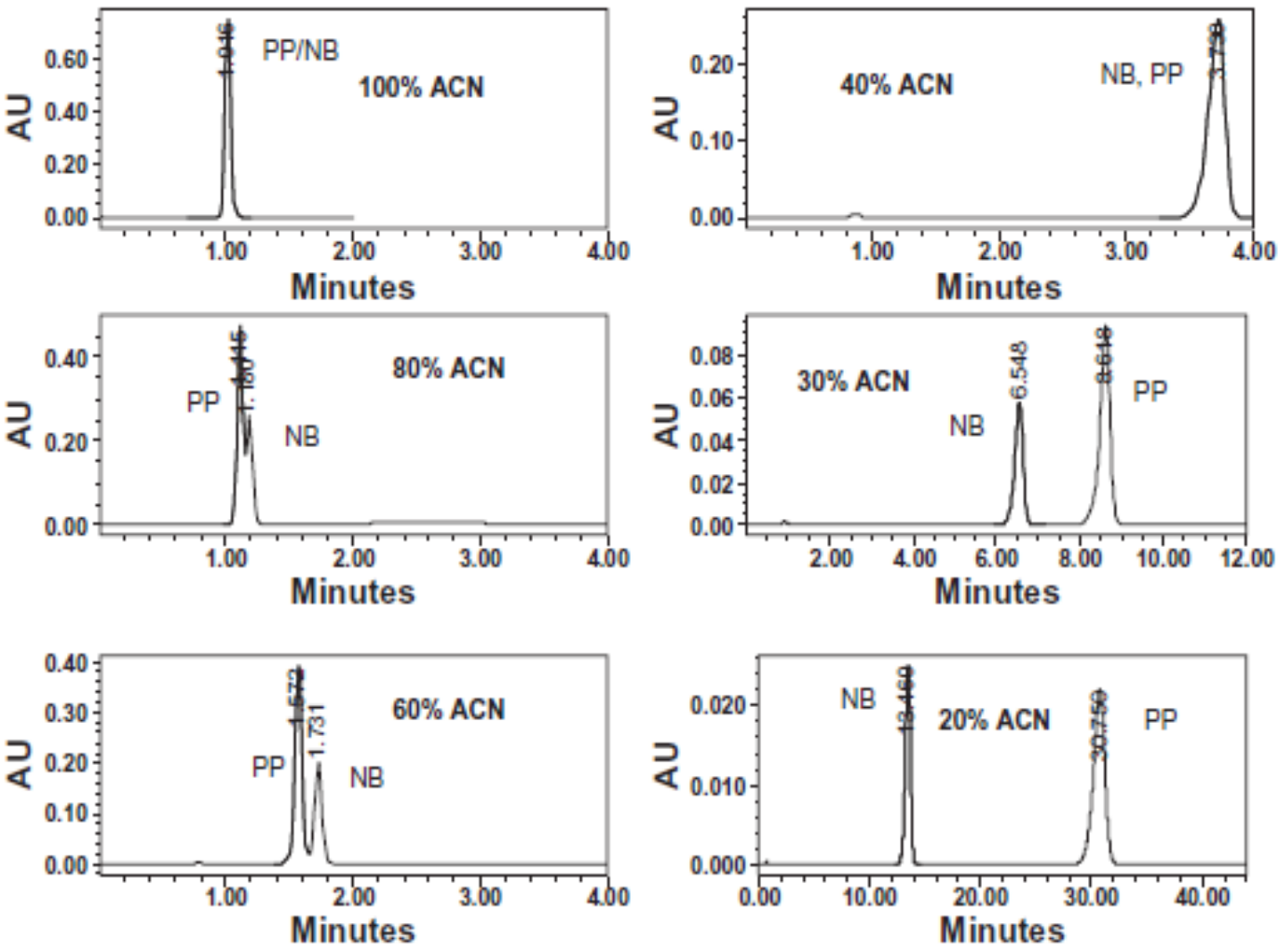


Figure 2.10. Six RPLC chromatograms illustrating the effect of mobile phase solvent strength on solute retention and resolution. LC conditions were: column: Waters Symmetry C18, 3 μm, 75 × 4.6 mm, 1 mL/min, 40°C, Detection at 258 nm. Mobile phase is mixture of acetonitrile (ACN) and water. Solutes were nitrobenzene (NB) and propylparaben (PP).

This Figure shows a series of six chromatograms to illustrate the effect of solvent strength in RPLC. Here, the two components (nitrobenzene and propylparaben) are eluted with mobile phases of decreasing solvent strength (i.e., decreasing concentration of acetonitrile (ACN)). At 100% ACN, both components are not retained by the column and elute with a k close to zero. At 60% ACN, the peaks are slightly retained (k close to 1) and are partially separated. The

two components merge back together at 40% ACN. At 30% ACN, the two components are well separated. At 20% ACN, propylparaben is highly retained with a k of 31.

The following observations can be made:

• Both t_R and k increase exponentially with decreasing percentage of organic solvents (or solvent strength) in the mobile phase.

• α and R_s generally increase with decreasing solvent strength.

• ACN is a stronger solvent than MeOH and can typically elute solutes faster in RPLC at similar concentration.

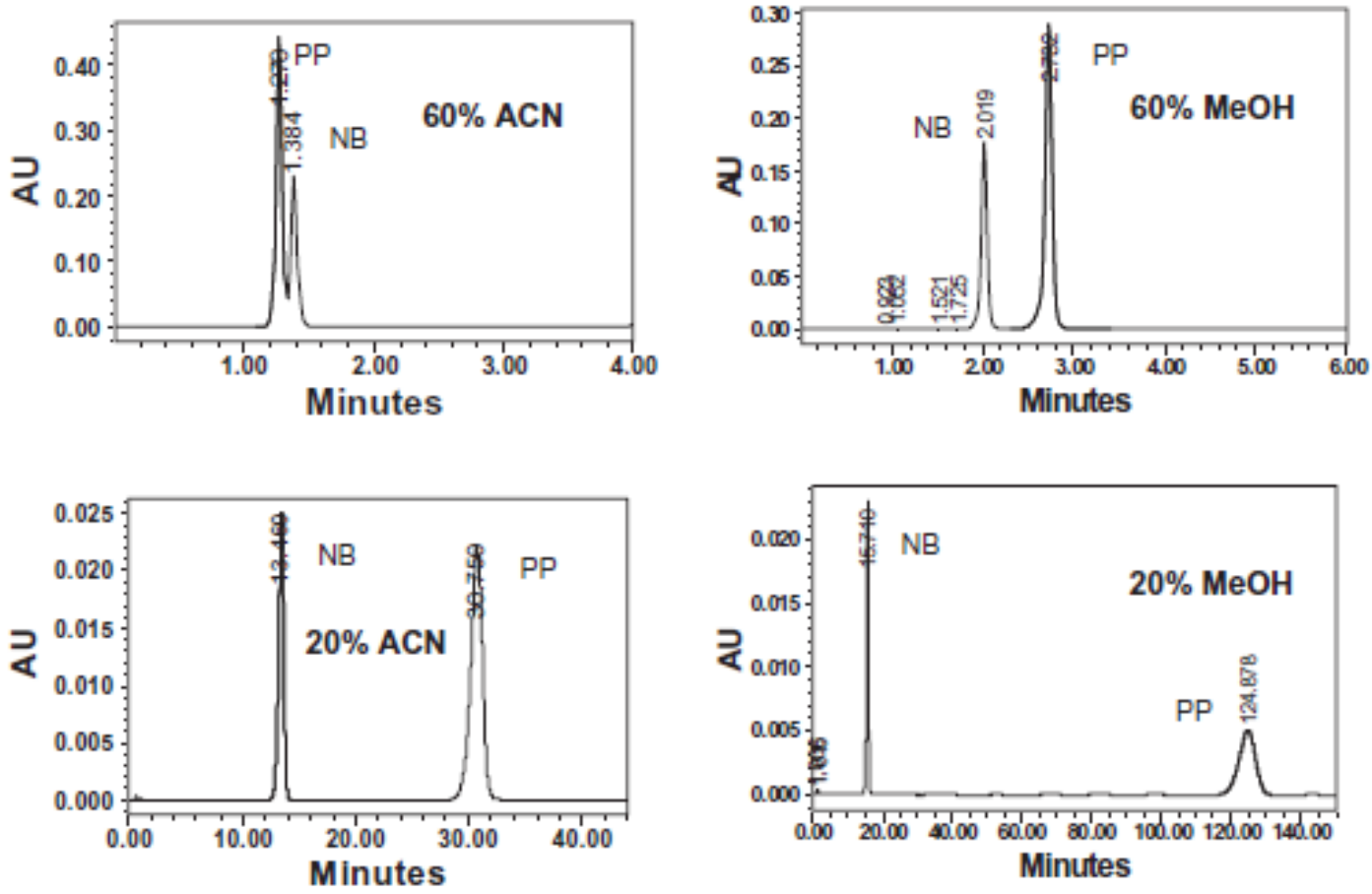


Figure 2.12. Four RPLC chromatograms illustrating the effect of mobile phase strength and selectivity of acetonitrile (ACN) and methanol (MeOH). See Figure 2.10 for LC conditions.

Buffers:

The pH of the aqueous component in the mobile phase has an important effect on the retention of ionizable (acidic or basic) analytes. In RPLC, the ionized form of the solute does not partition well into the hydrophobic stationary phase and has significantly lower k than the neutral form. The k of the analyte is plotted against the pH of the mobile phase (with percentage organic solvent in the mobile phase unchanged).

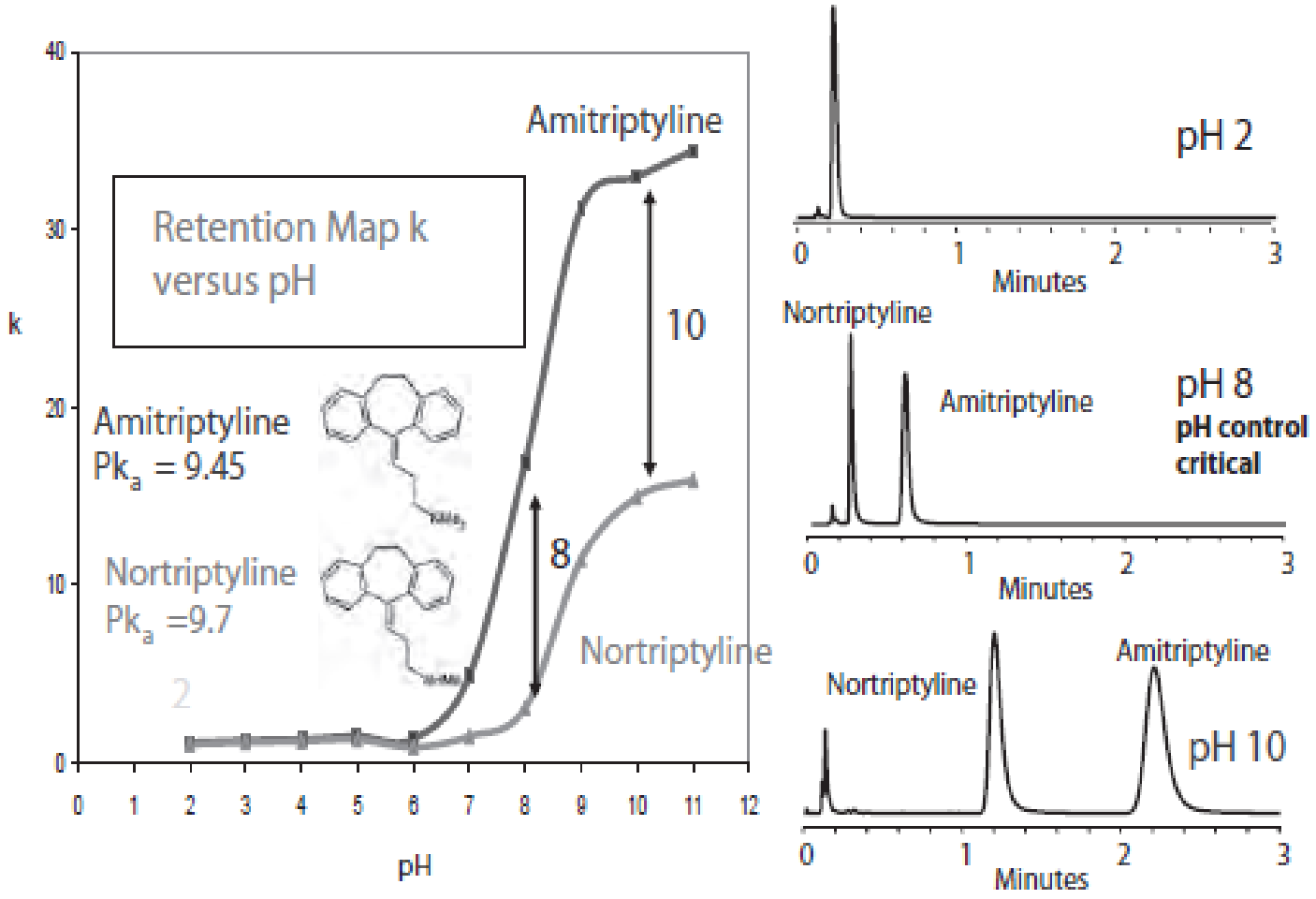


Figure 2.13. Retention map and chromatograms of two basic antidepressants using mobile phases at various pH with percentage organic modifier being kept constant. The diagram illustrates the importance of pH in the separation of basic analytes. Figure reprinted with permission from Waters Corporation.

At pH 2.0, both ionized solutes are not retained and elute as a single peak. At pH 8, the solutes are partially ionized and separate well. At pH 10, both un-ionized solutes are highly retained and resolved. Buffers are required to control the pH of the mobile phase.

Acidic Mobile Phases:

In RPLC, acidic pH of 2.5–3 is used for many applications. The low pH suppresses the ionization of weakly acidic analytes, leading to higher retention. Most silica-based bonded phases are not stable below pH 2 due to acid catalyzed hydrolytic cleavage of the bonded groups. Common acids used for mobile phase preparations are phosphoric acid, trifluoroacetic acid (TFA), formic acid, and acetic acid. However, basic analytes are ionized at low pH and might not be retained.

Ion-Pairing Additives:

Ion-pairing reagents are detergent-like molecules added to the mobile phase to provide retention of acidic or basic analytes. Long-chain alkyl sulfonates (C5 to C12) combine with basic solutes under acidic pH conditions to form neutral "ion-pairs" that are retained in RPLC. Retention is proportional to the length of the hydrophobic chain of the ion-pairing agent and its concentration. TFA has some ion-pairing capability and is useful in RPLC of proteins and peptides.

For acidic analytes, ion-pairing reagents such as tetraalkylammonium salts are used.

High pH Mobile Phases:

Prior to 1990, the use of a high-pH mobile phase was not feasible with silicabase columns due to the dissolution of the silica support at pH > 8. The development of improved bonding chemistries and hybrid particles now extends the useful pH range from 2 to 10 or, in many cases, from 1 to 12.

This offers an important alternative approach for the separation of basic analytes and, in particular, for impurity testing of water-soluble basic drugs. The previous Figure illustrates the basis of this approach in the separation of two closely related basic drugs, amitriptyline and nortriptyline. At low pH, both analytes are ionized and coelute with the solvent front. At pH close to the pK_a of the analytes, the partially ionized solutes are well separated with a large selectivity (α) value. At high pH, the non-ionized solutes are well retained and resolved.

Other Operating Parameters:

Flow Rate (F) and Column Temperature (T):

Typical flow rates for analytical columns (4.6 mm i.d.) are 0.5–2 mL/min. Operating at higher flow rates (F) increases column back pressure (ΔP) but reduces retention time and analysis time:

$$\Delta P = 1000 \frac{F\eta L}{\pi r^2 d_p^2}$$

where L = column length, η = mobile phase viscosity, r = column radius, and d_p = packing particle diameter.

If the mobile phase composition remains constant throughout the analysis, flow rate has no impact on k or α, since flow has the same effect on t_R of each solute. Flow also has significant effect on N. Operating flow rate should be proportional to the square of the column inner diameter. For instance, reducing the column diameter from 4mm to 2mm, the operating flow rate should be reduced from 1 mL/min to 0.25 mL/min to maintain the same linear flow rate, resulting in significant reduction of solvent usage for small-diameter columns.

Higher column temperatures (T) lower the viscosity of the mobile phase and usually have significant effects on retention (k), efficiency (N), and selectivity (α).

THE RESOLUTION EQUATION:

The degree of separation or resolution (R_s) between two solutes is dependent on both thermodynamic factors (retention, k, and selectivity, α) and kinetic factors (peak width and column efficiency, N). Resolution is controlled by three factors (retention, selectivity, and efficiency) as expressed quantitatively in the resolution equation:

The Resolution Equation $R_S = \left(\frac{k}{k+1}\right)\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{\sqrt{N}}{4}\right)$

To maximize R_s, k should be large, k values >10 will drive the retention term of k/(k + 1) to approach unity. No separation is possible if k = 0, since R_s must equal zero if k is zero in the resolution equation.

Selectivity (α) is typically between 1.01 and 1.50 for closely eluting solutes. When α = 1, R_s will equal to zero and co-elution of the analytes occur.

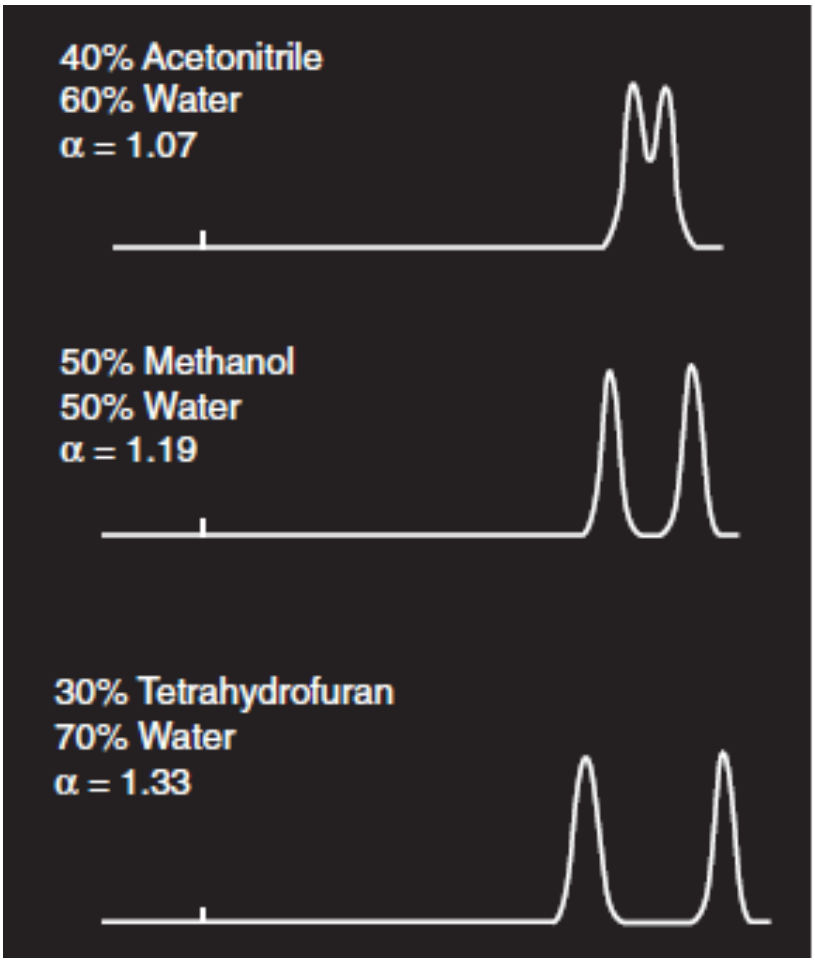
Selectivity is maximized by optimizing column and mobile phase conditions during method development.

Small change of selectivity can have a major effect on resolution as resolution is proportional to (α − 1).

Finally, the plate number of the column (N) should be maximized by using a longer column or a more efficient column. But increasing N is not an efficient way to achieve resolution since R_s is proportion to $\sqrt{N}$. Doubling N by doubling the column length increases analysis time by 2 but only increases resolution by $\sqrt{2}$ or by 41%. In contrast, increasing α from 1.05 to 1.10 will almost double the resolution.

Efficiency Retention Selectivity

$$R = \frac{\sqrt{N}}{4} \quad \frac{k}{k+1} \quad \frac{\alpha-1}{\alpha}$$



This Figure illustrates how two analytes can be separated more effectively by changing the organic solvent in the mobile phase from acetonitrile to methanol or tetrahydrofuran through increasing the selectivity (α) of the separation.

THE VAN DEEMTER EQUATION:

The van Deemter equation was developed in the 1950s to explain band broadening in chromatography by correlating HETP or plate height with linear flow velocity (V). Van Deemter curve (HETP vs. V), is a composite curve from three relatively independent terms, which are in turn controlled by particle size (d_p) of the packing, and diffusion coefficients (D_m) of the solute. The dip, or minimum point on the van Deemter curve, marks the minimum plate height (H_{min}) and the optimum velocity, which is the flow velocity at maximum column efficiency or H_{min}.

The van Deemter Equation $HETP = A + B/V + CV.$

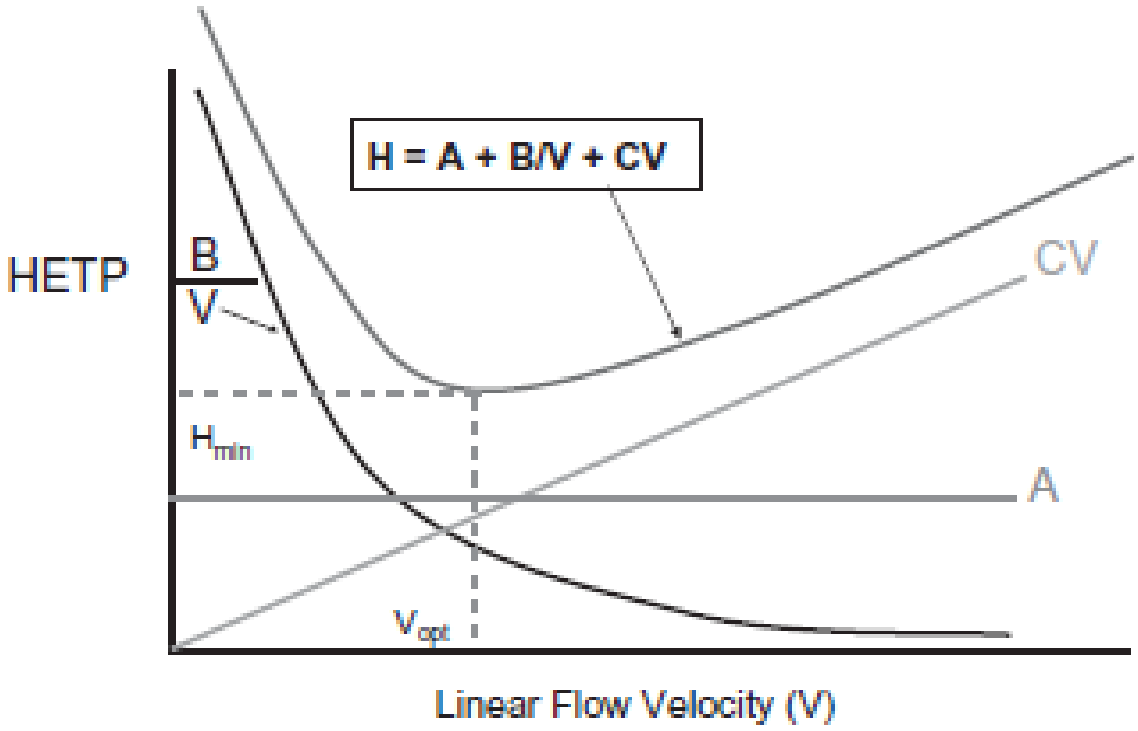


Figure 2.18. Van Deemter curve showing the relationship of HETP (H) vs. average linear velocity. The van Deemter curve has a classical shape and is a composite plot of A, B/V, and CV terms (plotted below to show their contributions). H_{min} = minimum plate height, V_{opt} = optimum velocity.

• The A term represents "eddy diffusion or multi-path effect" experienced by the various solute molecules as they transverse the packed bed. A term is proportional to d_p and is smaller in well-packed columns.

• The B term represents "longitudinal diffusion" of the solute band in the mobile phase and is proportional to D_m of the solute. Note that contribution from the B term is only important at very low flow rate.

• The C term represents "resistance to mass transfer" due to time lags caused by the slower diffusion of the solute band in and out of the stationary phase. The C term is proportional to

(d_p^2/D_m) and becomes important at high flow rates, especially for columns packed with larger particles.

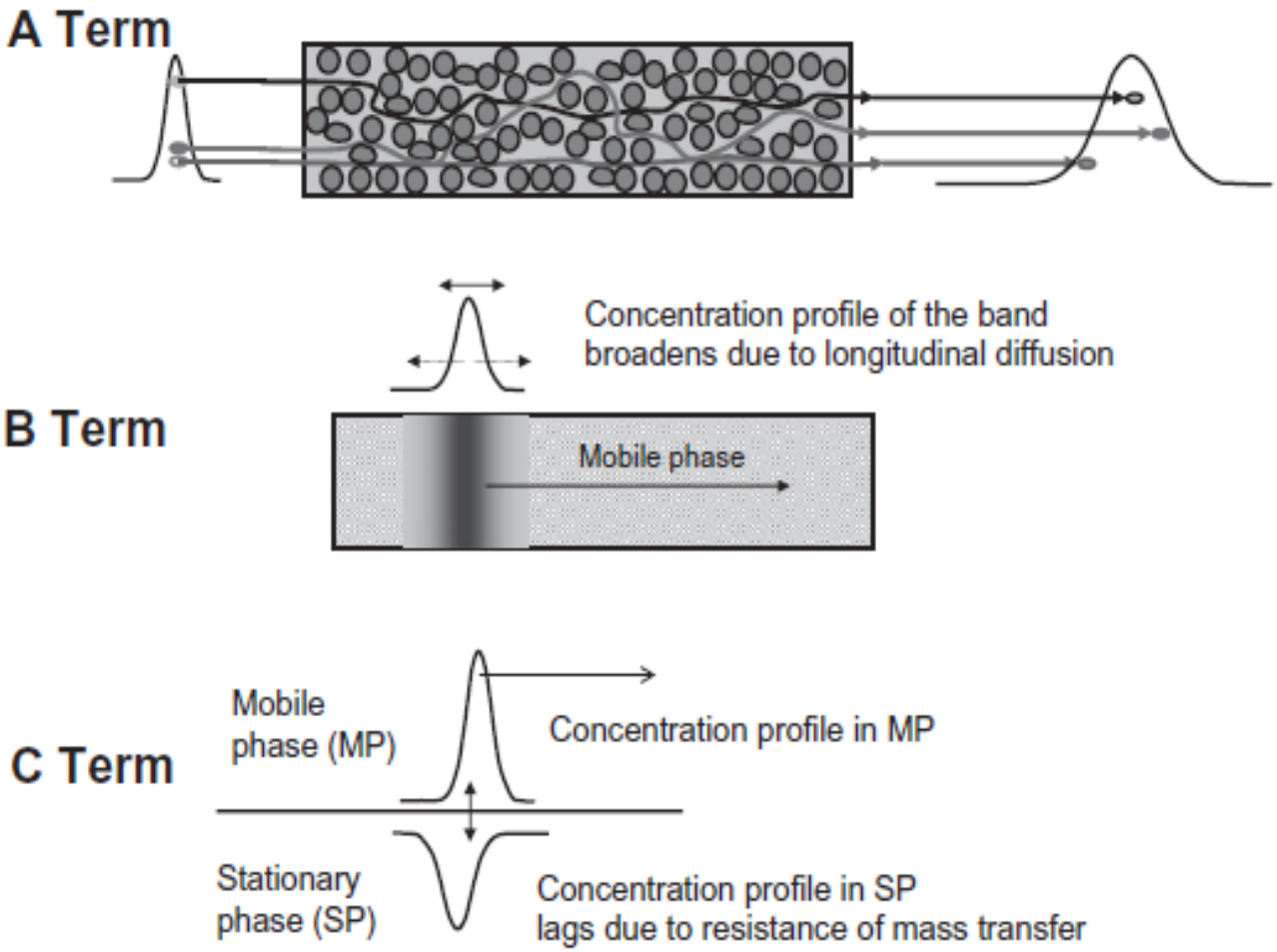


Figure 2.19. Diagrams illustrating the mechanism of the various van Deemter terms contributing to chromatographic band broadening. These diagrams are adapted from ideas from various Web resources.

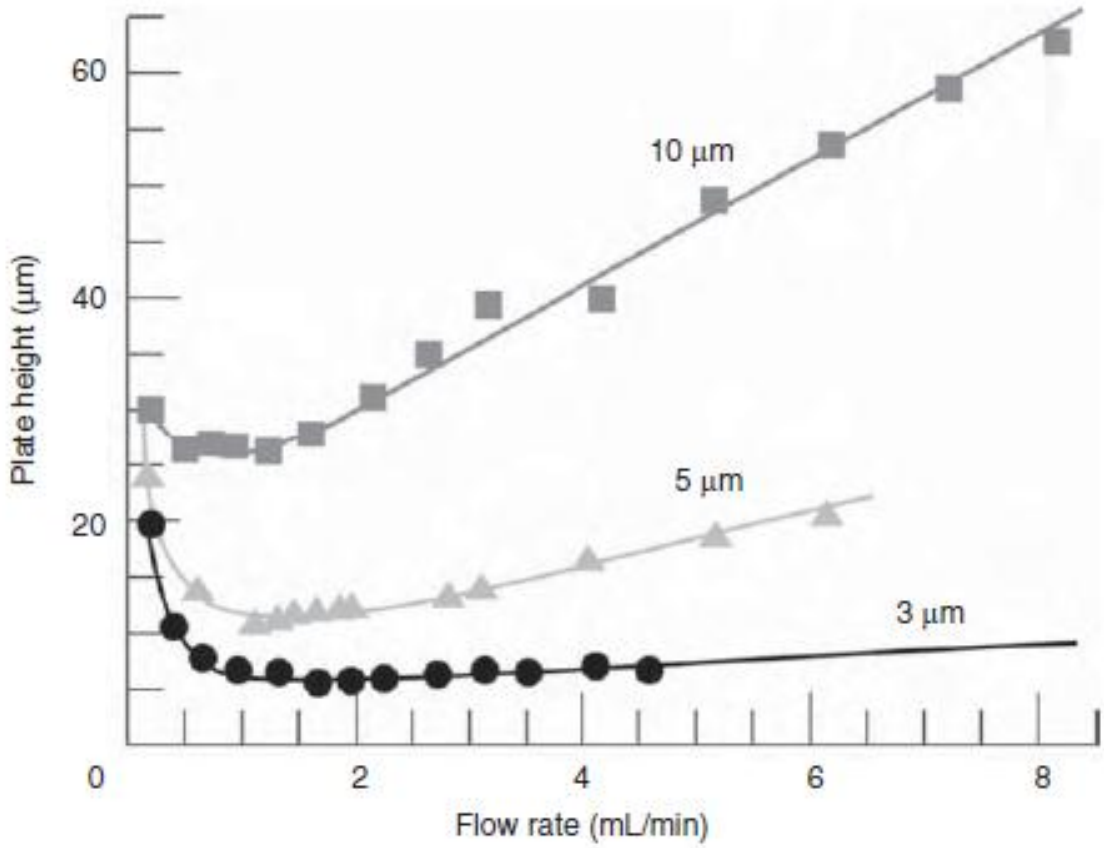


Figure 2.20. Experimental van Deemter curves of three columns packed with 10-, 5-, and 3-μm particles. Diagram reprinted with permission from reference 17.

This Figure shows the effect of d_p on van Deemter curves for columns packed with 10-, 5-, and 3-μm particles.

The following observations can be made.

• Small d_p yields smaller H_{min}. Note that H is approximated by 2.5 d_p.

• Small-particle columns suffer less efficiency loss at high flow rates since the van Deemter curved at high flow is

dominated by contribution from the C term, which is in turn proportional to d_p^2.

For these reasons, smaller-particle columns (i.e., 3μm) are becoming popular in modern HPLC because of their higher efficiency.

ISOCRATIC VS. GRADIENT ANALYSIS:

Most HPLC separations are performed under isocratic conditions in which the same mobile phase is used throughout the elution of the entire sample.

Although isocratic analysis is good for simple mixtures, gradient analysis, in which the strength of the mobile phase is increased with time during sample elution, is preferred for more complex samples containing analytes of diverse polarities.

Advantages of gradient analysis are:

- Better suited for complex samples and applications that require quantitation of all peaks or multiple analytes of diverse polarities.
- Better resolution of early and late eluting peaks.
- Better sensitivity of late eluting peaks.
- Higher peak capacity (fit more peaks in the chromatogram)

Disadvantages are:

- More complex HPLC instrument (i.e., binary pump) is required.
- Longer assay times.

There are several additional parameters in gradient analysis not present in isocratic HPLC that need to be optimized. These are initial and final mobile phase composition, gradient time or duration (t_G), flow (F), and etc…

Optimization of all these parameters can be accomplished by software simulation programs.

1. **Peak Capacity (n)**:

In isocratic analysis, peaks are broadened with elution time, but in gradient HPLC, peaks have similar peak widths since they are eluted with an increasingly stronger mobile phase. Peak capacity (n) is the maximum number of peaks that can fit in a chromatogram with resolution value of one. (n) is a useful concept for comparing column performance under different gradient conditions.

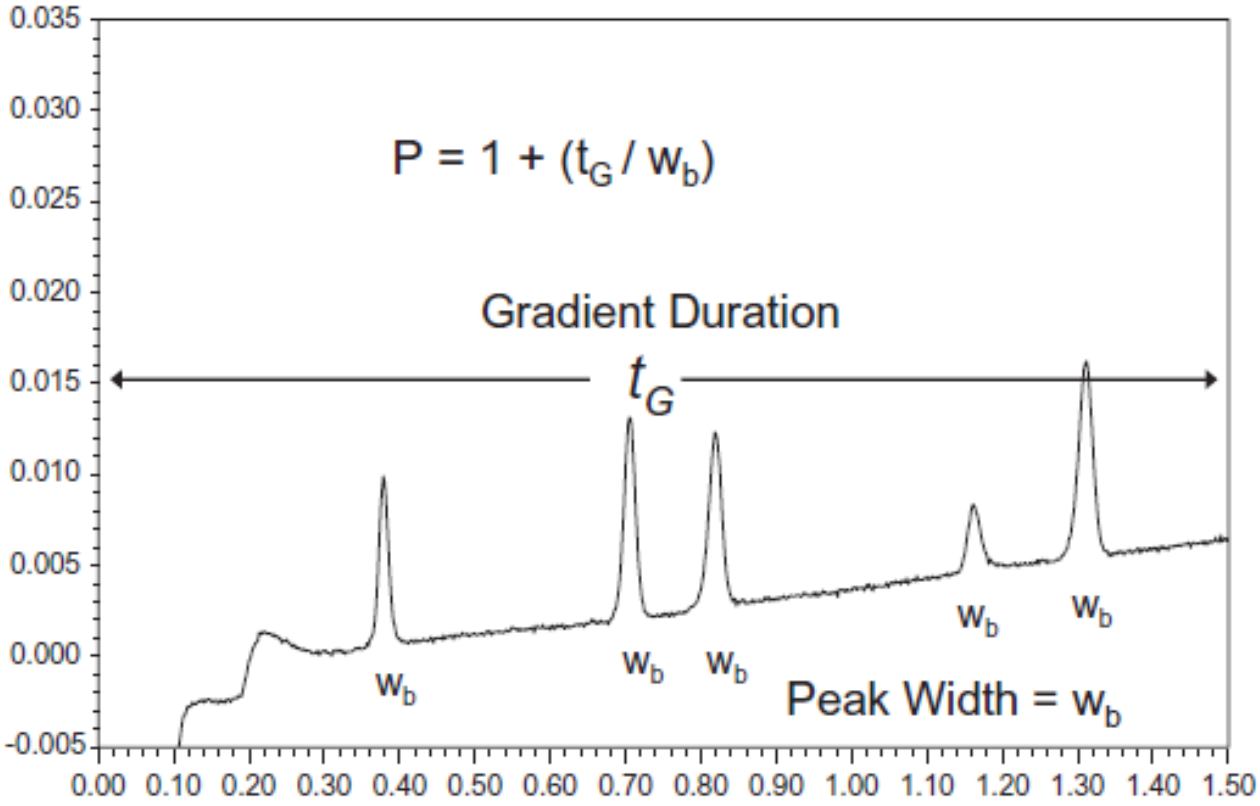


Figure 2.21. Chromatogram illustrating the concept of peak capacity (n), which is the maximum number of peaks that can be accommodated in a chromatogram with a resolution of one.

This Figure shows that peak capacities can be approximated by t_G/w_b. Higher peak capacities of 100–200 are possible in gradient analysis versus typical peak capacities of 50–100 for isocratic analysis. This is a result of narrower peak widths achieved in gradient HPLC.

$$\text{Peak Capacity, } n = t_G/w_b$$

2. Key Gradient Parameters (Initial and Final Solvent Strength, Gradient Time (t_G), and Flow Rate):

Gradient methods are more difficult to develop because the separation is controlled by several additional parameters, such as starting and ending solvent strength, flow rate (F), and gradient time (t_G). The concept of retention factor k is also more complex in gradient analysis and is best represented by an average k or k*,

$$k^* = t_G F / 1.15\ S\ \Delta\phi\ V_M$$

where k* = average k under gradient conditions, $\Delta\varphi$ = change in volume fraction of strong solvent in RPLC, S is a constant that is close to 5 for small molecules, F = flow rate (mL/min), t_G = gradient time (min), and V_M = column void volume (mL). In gradient analysis, F has a dramatic influence on retention (k*). Operating at higher F increases k*, since a greater volume of a lower-strength mobile phase is pumped through the column. This is equivalent to operating at a lower F and longer t_G. Shorter analysis time can be achieved by reducing t_G, increasing F, and using a smaller column (lower V_M).

3. The 0.25 Δt_G Rule: When Is Isocratic Analysis More Appropriate?

A rule called the "0.25 Δt_G rule" is useful for checking whether a sample run under gradient conditions can be more effectively handled by isocratic analysis. In this approach, the

sample is first analyzed under RPLC using a broad gradient (i.e., 5–95% organic solvent). If all analyte peaks elute within a time span of 0.25 Δt_G, then isocratic analysis is preferred. If the elution span is above 0.4 Δt_G, gradient is necessary. Between 0.25 Δt_G and 0.4 Δt_G, gradient is most likely preferred. The following Figure shows an example illustrating this useful rule.

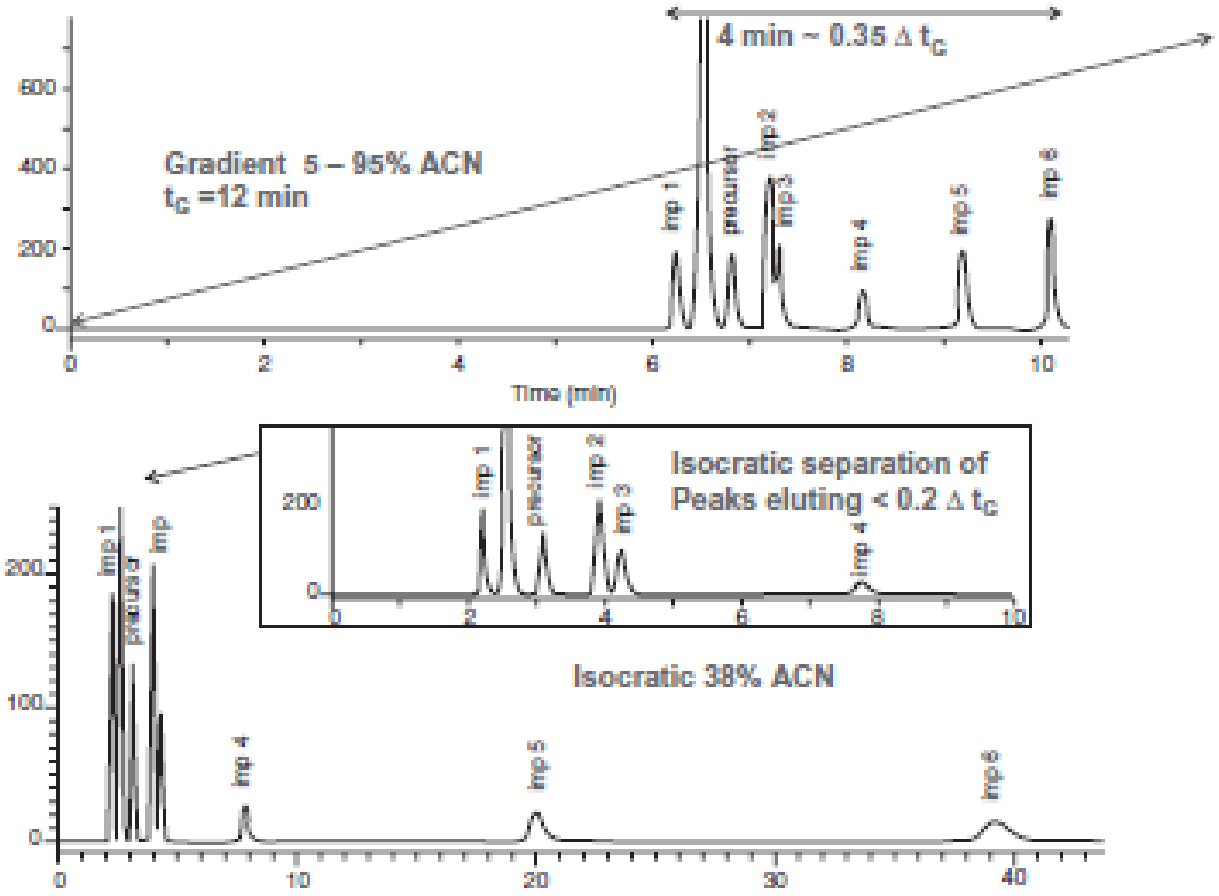


Figure 2.23. An example illustrating the 0.25 Δt_G rule used to indicate when a gradient separation can be more effectively carried out under isocratic conditions. The top chromatogram is obtained under gradient condition where all eight peaks elute in 4 minutes or 0.35 Δt_G. The bottom chromatogram shows the separation of these eight components under isocratic conditions. If all peaks elute <0.25 Δt_G in the gradient chromatogram, isocratic separation is preferred (as shown in the inset where the first six peaks are well-separated under isocratic conditions of 38% ACN. If all peaks eluting between 0.25 and 0.4 Δt_G, then both isocratic and gradient can be used. If all peaks elute in a time span >0.4 Δt_G, gradient separation is necessary.

SAMPLE CAPACITY:

Sample capacity or loading capacity of the column is the maximum amount of solute in milligrams per gram of packing material that can be injected without reducing column efficiency (e.g., by 10%). Since the amount of packing material is proportional to the column volume or size, sample capacity is proportional to the square of column diameter and the column length.

It is therefore important in preparative work to maximize the yield of purified material in each sample run by increasing the column size.

GLOSSARY OF HPLC TERMS:

Asymmetry (As)—Factor describing the shape of a chromatographic peak

Band Broadening—The process of increasing peak width and dilution of the solute as it travels through the column.

Column—A tube that contains packed solid material containing the stationary phase. Typical HPLC columns are stainless steel tubes packed with silica-based bonded phases.

Efficiency or Plate Number (N)—A measure of column performance. N is calculated from retention times and peak widths.

Mobile Phase—A solvent that carries the sample through the column. Typical mobile phases in RPLC are mixtures of water with acetonitrile or methanol.

Plate Height (H)—Height equivalent to a theoretical plate as defined by dividing the column length by N. H_{min} is equal to 2.5 d_p for a well-packed column.

Peak Capacity (n)—The maximum number of peaks that can be resolved in a chromatogram with a resolution of one.

Peak Width (w_b)—The width at the base of a chromatographic band during elution from the column. Higher-efficiency columns typically yield smaller-peak widths.

Resolution (R_s)—The degree of separation between two sample components as defined by the difference of their retention times divided by their average peak width. *Retention*—The tendency of a solute to be retained or retarded by the stationary phase in the column.

Retention Factor (k)—A measure of solute retention obtained by dividing the adjusted retention time by the void time. Also known as capacity factor or k′. *Sample Capacity*—The maximum mass of sample that can be loaded on a column without decreasing column efficiency.

Separation Factor or Selectivity (α)—The ratio of retention factors (k) of two adjacent peaks.

Stationary Phase—The immobile phase responsible for retaining the sample component in the column. In RPLC, this

is typically the layer of hydrophobic groups bonded on silica solid support materials.

Void Volume (V_M)—The total volume of liquid held up in the column. It can be approximated as 65% of the volume of the empty column.