Chapter 26
An Introduction to Chromatographic Separations

- Chromatography, a powerful separation method that finds applications in all branches of science,
- invented and named by the Russian botanist Mikhail Tswett.
- He employed the technique to separate various plant pigments such as chlorophylls and xanthophylls by passing solutions of these compounds through a glass column packed with finely divided calcium carbonate,
- The separated species appeared as colored bands on the column, which accounts for the name he chose for the method (Greek chroma meaning "color“ and graphein meaning "writing"),
- The applications of chromatography have grown explosively in the last half century, due not only to the development of several new types of chromatographic techniques but also to the growing need by scientists for better methods for characterizing complex mixtures.
- The tremendous impact of these methods on science is attested by the 1952 Nobel Prize in Chemistry that was awarded to A. J P, Martin and R. LM, Synge for their discoveries in the field, Many of the Nobel Prizes awarded since that time have been based on work in which chromatography played a vital role.

General Description of Chromatograpy

- Chromatography permit the scientist to separate closely related components of complex mixtures.
- In all chromatographic separations the sample is transported in a mobile phase, which may be a gas, a liquid, or a supercritical fluid.
- This mobile phase is then forced through an immiscible stationary phase, which is fixed in place in a column or on a solid surface.
- The two phases are chosen so that the components of the sample distribute themselves between the mobile and stationary phase to varying degrees.
- some components in sample interact more strongly with stationary phase and are more strongly retained

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- Those components strongly *retained by the sta*tionary phase move only slowly with the flow of mobile phase.
- In contrast, components that are weakly held by the stationary phase travel rapidly.
- As a consequence of these differences in migration rates, sample components separate into discrete *bands, or zones,* that can be analyzed qualitatively and quantitatively.

26 A-1 Classification of Chromatographic Methods

Chromatographic methods can be categorized in two ways.

1- The first classification is based upon the *physical means by which the stationary and mobile phases* are brought into contact.

- In column chromatography, the stationary phase is held in a narrow tube through which the mobile phase is forced under pressure or gravity
- In planar chromatography, the stationary phase is supported on a flat plate or in the interstices of a paper; here, the mobile phase moves through the stationary phase by capillary action or under the influence of gravity.

26 A-1 Classification of Chromatographic Method

2- A second and more fundamental classification of chromatographic methods is one based upon the *types of mobile and stationary phases and the kinds of equilibria* involved in the transfer of solutes between phases.

Three general categories of chromatography:

(1) liquid chromatography,

(2) gas chromatography, and

(3) supercritical-fluid chromatography.

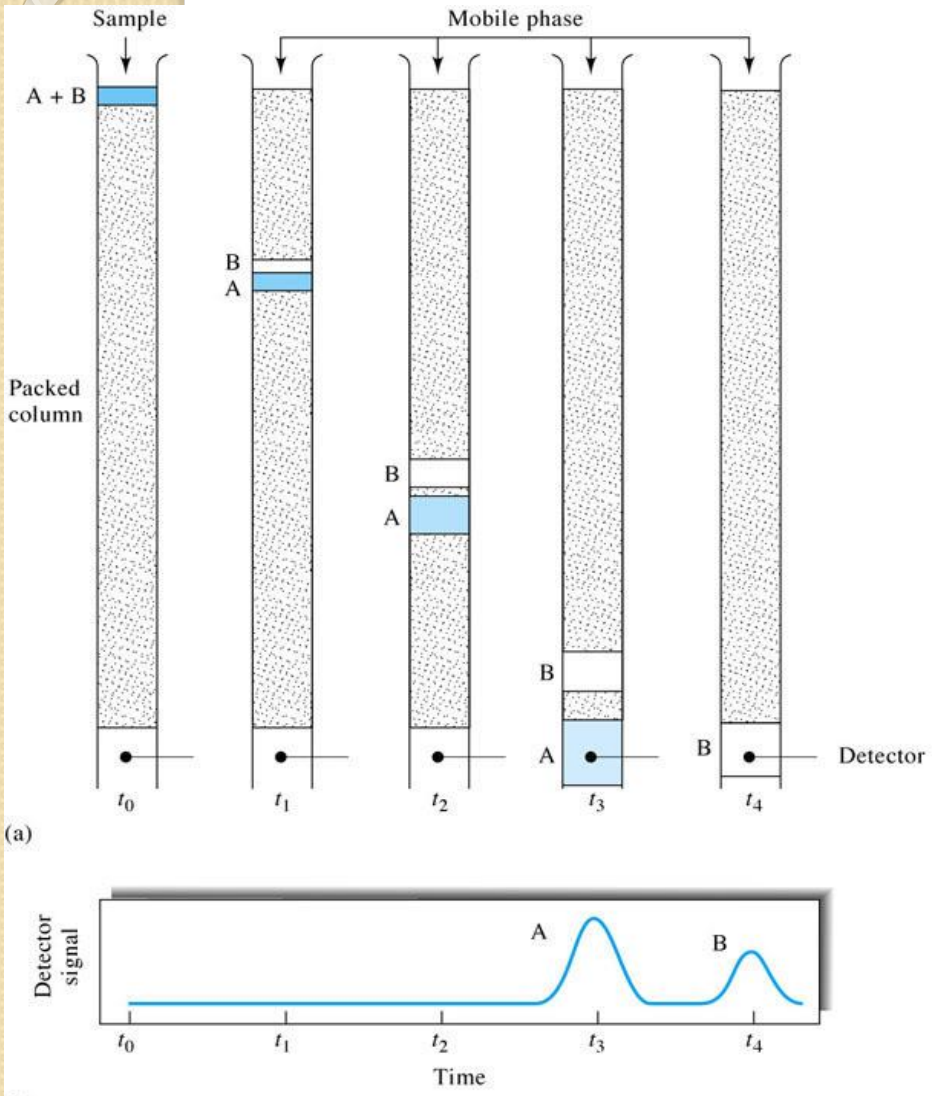
The mobile phases in the three techniques are liquids, gases, and supercritical fluids respectively.

TABLE 26-1 Classification of Column Chromatographic Methods

General Classification	Specific Method	Stationary Phase	Type of Equilibrium
1. Gas chromatography (GC)	a. Gas-liquid chromatography (GLC)	Liquid adsorbed or bonded to a solid surface	Partition between gas and liquid
	b. Gas-solid	Solid	Adsorption
2. Liquid chromatography (LC)	a. Liquid-liquid, or partition	Liquid adsorbed or bonded to a solid surface	Partition between immiscible liquids
	b. Liquid-solid, or adsorption	Solid	Adsorption
	c. Ion exchange	Ion-exchange resin	Ion exchange
	d. Size exclusion	Liquid in interstices of a polymeric solid	Partition/sieving
	e. Affinity	Group specific liquid bonded to a solid surface	Partition between surface liquid and mobile liquid
3. Supercritical fluid chromatography (SFC; mobile phase: supercritical fluid)		Organic species bonded to a solid surface	Partition between supercritical fluid and bonded surface

26 A-2 Elution in Column Chromatography

How 2 components, A and B are resolved on a column by elution chromatography?

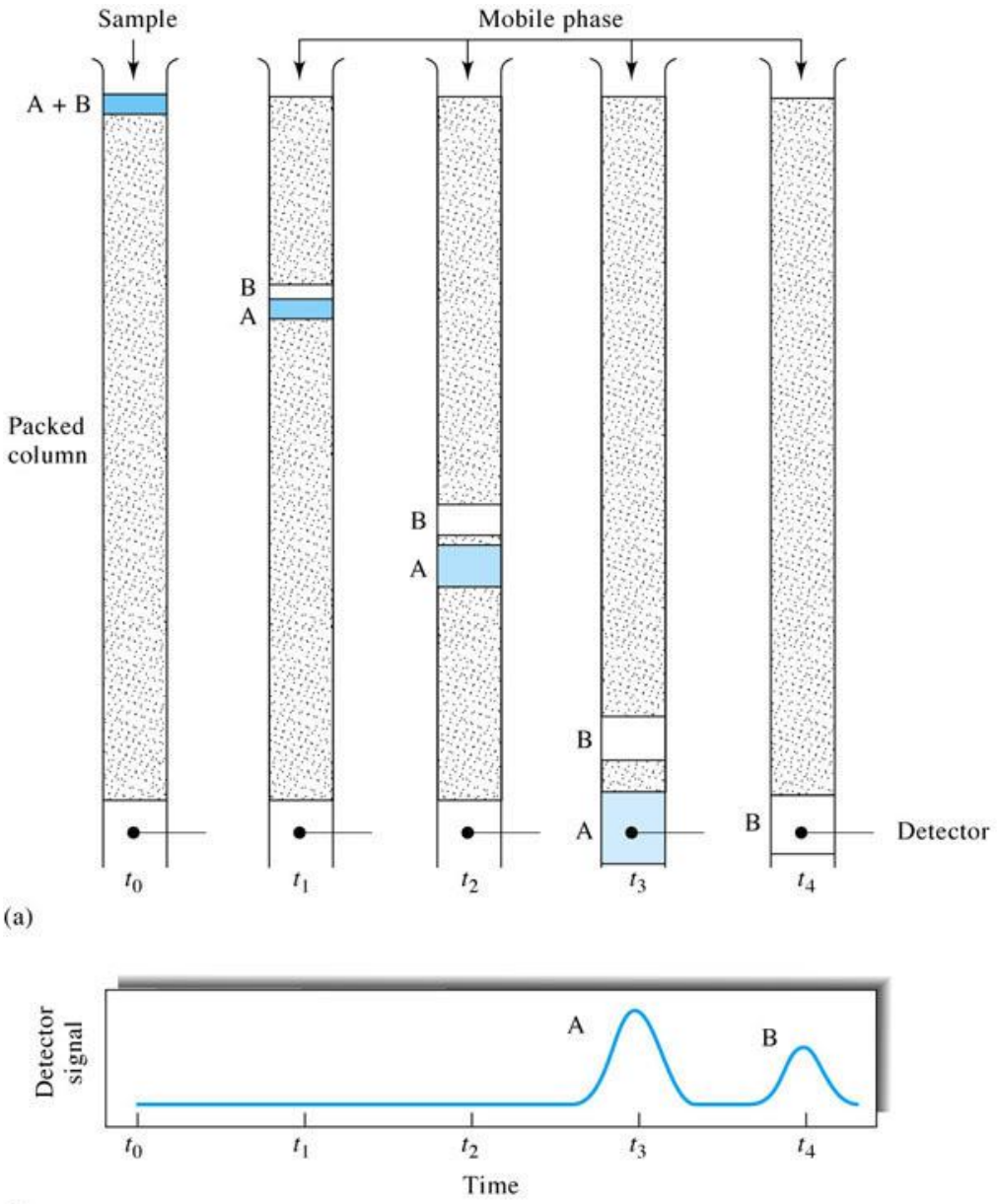


- The column: narrow-bore tubing packed with a finely divided inert solid that holds the stationary phase on its surface.
- The sample (A+B) is introduced at the head of a column, at time t_0 ,whereupon the components of the sample distribute themselves between the two phases
- Elution involves washing a species through a column by continuous addition of fresh solvent.
- additional mobile phase (the eluent) forces the solvent containing a part of the sample down the column, where further partition between the mobile phase and fresh portions of the stationary phase occurs.

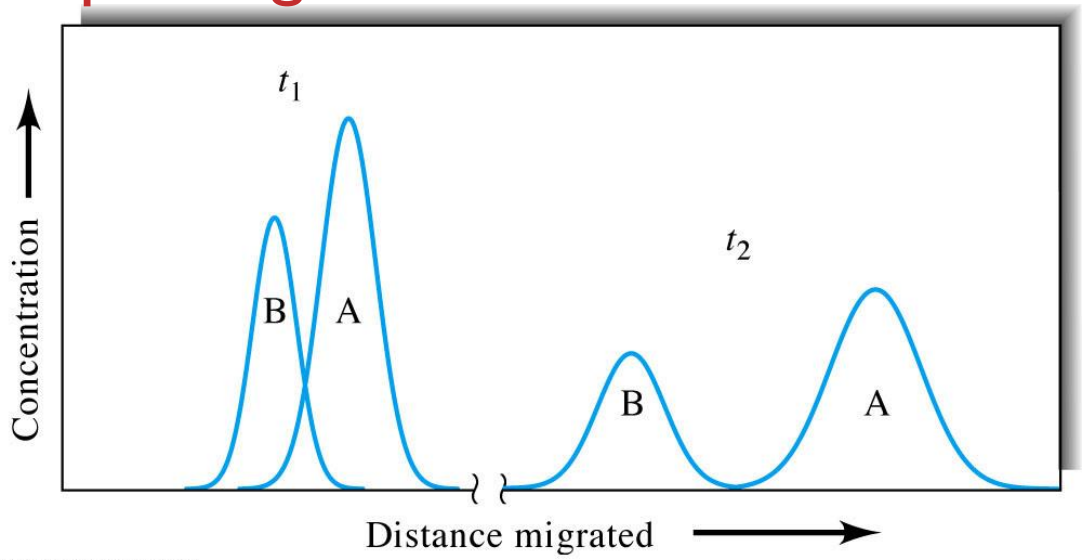
➢If a detector, that responds to solute concentration, is placed at the end of the column and its signal is plotted as function of time (or of volume of the added mobile phase), a series of peaks is obtained. Such a plot, called a *chromatogram*, is useful for both qualitative and quantitative analysis.

➢ The positions of peaks on the time axis may serve to identify the components of the sample(qualitatively)

➢the areas under the peaks provide a quantitative measure of the amount of each component.



Improving Column Performance



- Figure above shows the concentration profiles for the bands containing solutes A and B in Figure 26-1 at time t_1 and a later time t_2.
- Since B is more strongly retained by the stationary phase than is A, B lags during the migration and elutes last.
- movement down the column increases the distance between the two bands. At the same time, however, the broadening of both zones takes place, which lowers the efficiency of the column as a separating device.
- While band broadening is inevitable, conditions can ordinarily be found where it occurs more slowly than band separation. Thus, a clean separation of species is often possible provided the column is sufficiently long.

26B Migration Rates Of Solutes

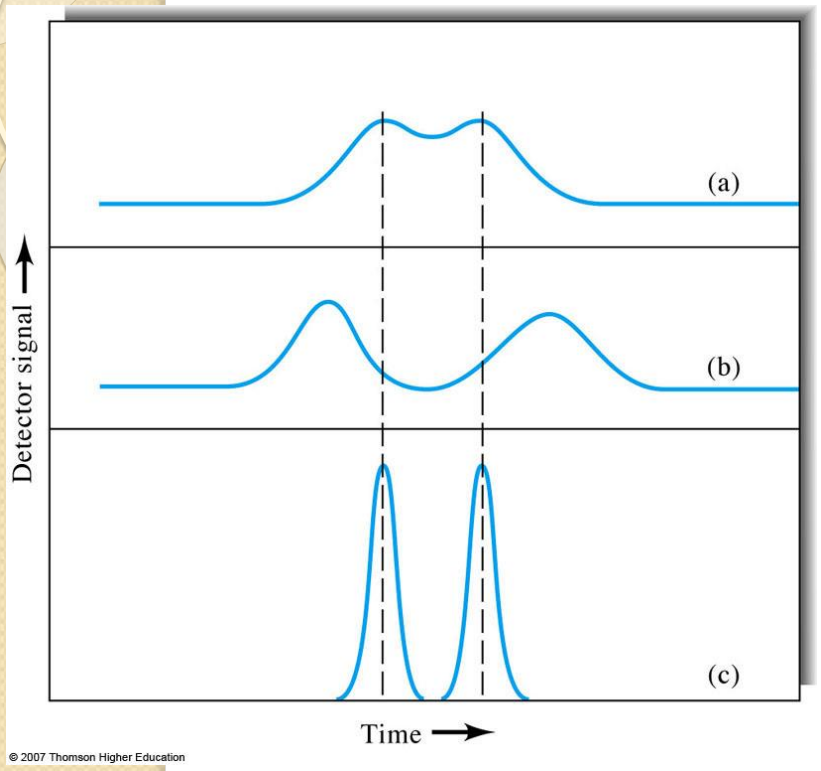


FIGURE26-3 Two-component chromatogram illustrating two methods for improving separation:
(a) original chromatogram with overlapping peaks,
(b) improvement brought about by an Increase in band separation,
(c) improvement brought about by a decrease in the widths.

- The effectiveness of a chromatographic column in separating two solutes depends in part upon the relative rates at which the two species are eluted.
- These rates are determined by the magnitude of the equilibrium constants for the reactions by which the solutes distribute themselves between the mobile and stationary phases.
- By changing experimental conditions, non-separated bands can be separated
  - adjust migration rates for A and B (increase band separation)
  - adjust zone broadening (decrease band spread)

26B-1 Distribution Constants:

- The distribution equilibria in chromatography involves the transfer of an analyte between the mobile and stationary phases.

$$A_{mobile} \rightleftharpoons A_{stationary}$$

The equilibrium constant, K_c for this reaction is called the distribution constant, the partition ratio, or the partition coefficient,

$$K_c = \frac{c_S}{c_M} = \frac{n_S/V_S}{n_M/V_M}$$

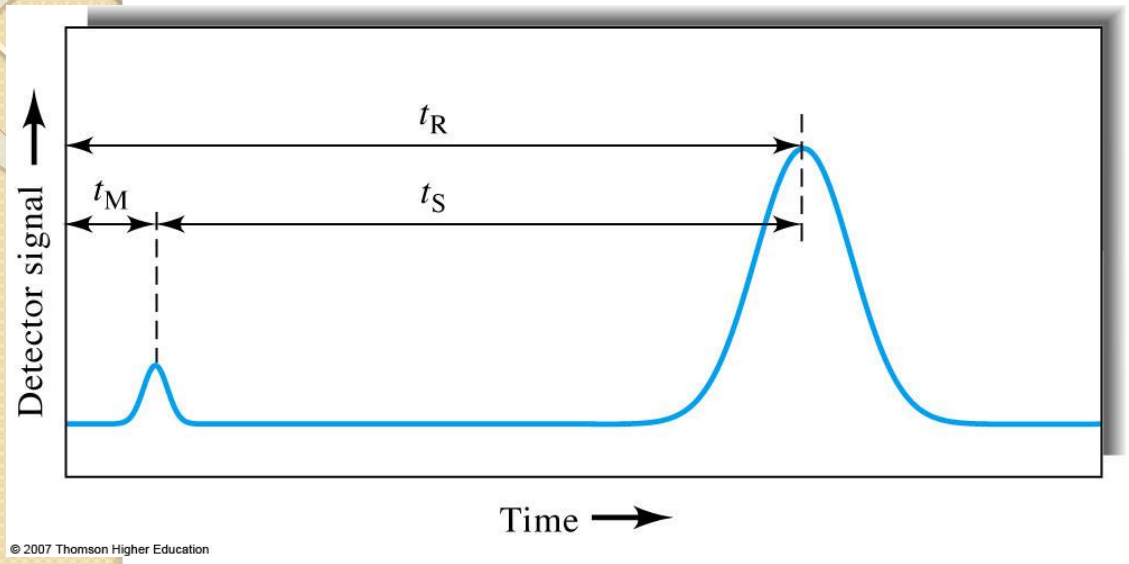
Where; c_s and c_M are the molar conc. of the solute;
n_S and n_M are the number of moles of analyte;
V_S *and* V_M *are the volumes of the* stationary and mobile phases

- K is constant *over a wide range* of solute concentrations.
- By appropriate choice of the mobile phase, the stationary phase or both the distribution constant can be manipulated within limits. By adjusting the volume of a phase, we can alter the molar ratio in the two phases.

Linear Chromatography: symmetric Gaussian-type peaks and retention times independent of the amount of analyte injected.

26B-2Retention Time:

K_c is not readily measured. Instead, we can measure a quantity called the *retention time* that is a function of K_c



- retention time, t_R, The time it takes after sample injection for the analyte peak to reach the detector
- dead time, t_M or void time, the time for the unretained species to reach the detector

- The analyte has been retained because it spends a time t_S in the stationary phase. The retention time is then $t_R = t_S + t_M$

- *The average linear rate of solute migration through the column* $\bar{v} = L/t_R$
  where, L is the length of the column packing.
- *The average linear rate of movement of the M.P molecules*, $u = L/t_M$
  where t_M, the dead time.

26B-3 Volumetric Flow Rate and Linear Flow Velocity

the mobile-phase flow is usually characterized by the volumetric flow rate, F (cm^3/min) at the column outlet.

For an open tubular column

- *F is related to the linear velocity at the column outlet, u_0*
- $F = u_0 A = u_0 \pi r^2$ *A: cross sectional area of the tube*

For a packed column

- *the entire column volume is not available to the liquid*
- $F = u_0 \pi r^2 \varepsilon$ ε = fraction of the total column volume available to the liquid (column porosity).

26B-4 The Relationship between Retention Time and Distribution Constant

• To relate the rate of migration of a solute to its distribution constant, we express the rate as a fraction of the velocity of the mobile phase:

$\bar{v}$ = u x (*fraction of time solute spends in* mobile phase)

This fraction, however, equals the average number of moles of solute in the mobile phase at any instant divided by the total number of moles of solute in the column

$\bar{v}$ = u x (*moles of solute A in* mobile phase / total moles of solute A)

$$\bar{v} = u \cdot \frac{C_M V_M}{C_M V_M + C_S V_S} = u \cdot \frac{1}{1 + C_S V_S / C_M V_M}$$

$$K_c = C_S / C_M \qquad \bar{v} = u \cdot \frac{1}{1 + K_c (V_S / V_M)}$$

26B-5 The Rate of Solute Migration: The Retention Factor

- The retention factor, k, or capacity factor, is an important parameter that is widely used to *compare the migration rates of solutes on columns*.

k, does not depend on column geometry or volumetric flow rate

For a given combination of solute, MP, SP , any column of any geometry operated at any mobile phase flow rate will give the same retention factor

For a solute A, the retention factor k_A is defined as

$$k_A = K_A V_S / V_M$$

where K_A is the distribution constant for the solute A.

$$\bar{v} = u \cdot \frac{1}{1 + k_A}$$

To show how k_A *can be derived from a chromatogram,*

$$\frac{L}{t_R} = \frac{L}{t_M} \times \frac{1}{1 + k_A}$$

$$k_A = (t_R - t_M)/t_M$$

When k_A is < 1.0, separation is poor, When k'_A is >30, separation is slow, When k_A is 2-10, separation is optimum

Relative Migration Rates: The selectivity Factor

How do we compare elution of two components A and B?

The selectivity factor, α, of a column for the two species A and B is defined as

$$\alpha = K_B/K_A$$

where K_B is the distribution constant for species B

K_A is the distribution constant for species A.

selectivity factor

$$\alpha = k_B/k_A$$

and retention factors

where k_B and k_A are the retention factors.

An expression for the determination of α from an experimental chromatogram:

$$\alpha = \frac{(t_R)_B - t_M}{(t_R)_A - t_M}$$

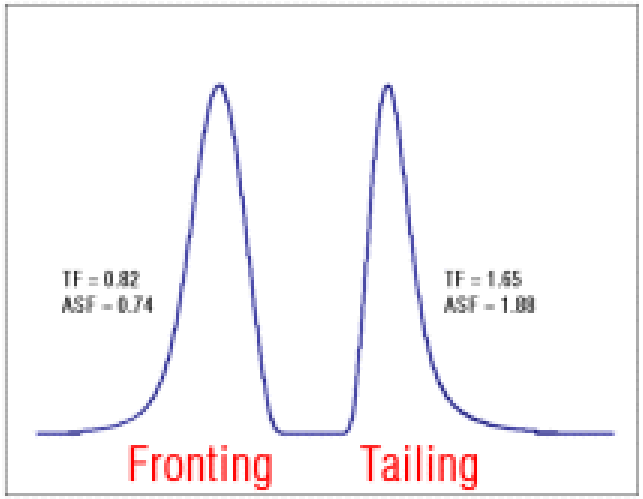
larger α = better separation

26C Band Broadening And Column Efficiency

The reasons that band becomes broader as they move down a column

26C-1 Rate Theory

- Describes the shapes and breadths of elution bands in quantitative terms based on a *random-walk* mechanism for the migration of molecules through a column.
- Individual molecule undergoes "random walk"
- Add up to give Gaussian peak (like random e
- Many thousands of adsorption/desorption pr
- Average time for each step with some +ve o
- Breadth of band increases down column bec
- Zone broadening is affected by separation eff broadening
- Distortions, nonideal peaks exhibit *tailing or fronting*



- cause of tailing and fronting is a distribution constant that varies with concentration. Fronting also arises when the amount of sample introduced onto a column is too large. Distortions of this kind are undesirable because they lead to poorer separations and less reproducible elution times.

26C-2A Quantitative Description of Column Efficiency

chromatographic column efficiency:

(I) plate height, H and

(II) plate count, or number of theoretical plates, N.

The two are related by the equation $N = L/H$

where; L is the length (usually in centimeters) of the column packing.

N is the plate count and H is the plate height

➢The efficiency of chromatographic columns increases as the plate count *N becomes greater* and as the plate height *H becomes smaller.*

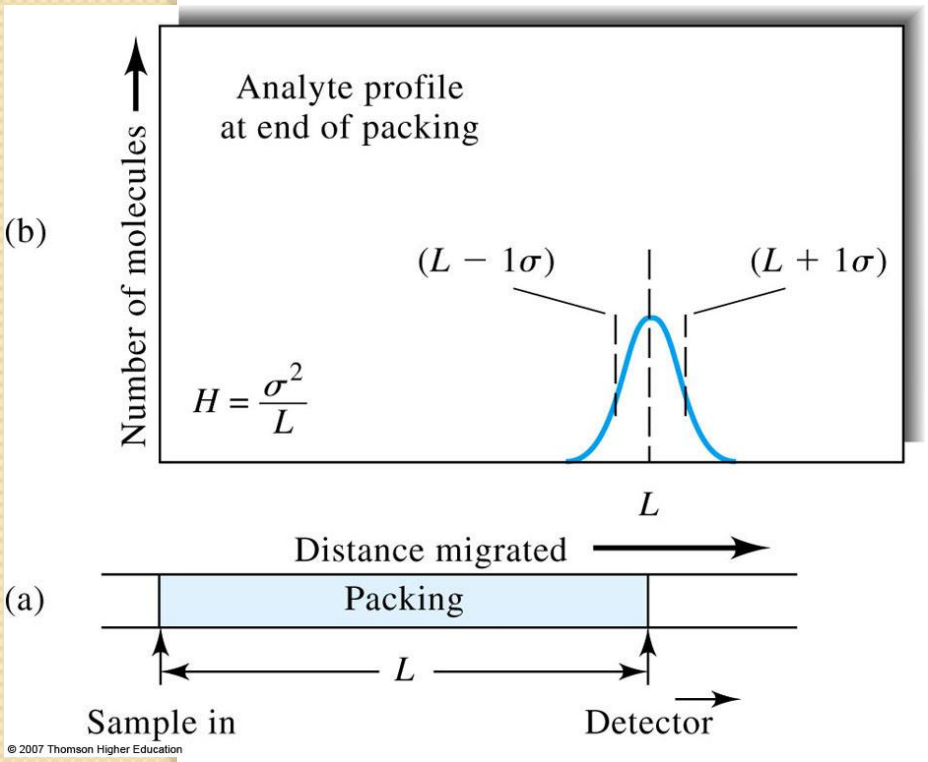
- a few 100 to several 100.000 plate numbers and
- plate heights ranging from a 1/10 to 1/1000 of a centimeter or smaller are common.

Plates are only theoretical -

- a pioneering theoretical study of Martin and Synge in which they treated a chromatographic column as if it were similar to a distillation column made up of numerous discrete but continuous narrow layers called *theorerical plates*.
- *At each plate,* equilibration of the solute between the mobile and stationary phases was assumed to take place.
- Movement of the solute down the column was then treated as a stepwise transfer of equilibrated mobile phase from one plate to the next.
- The theory was ultimately abandoned in favor of the *rate theory, however,* because it fails to account for peak broadening in a mechanistic way.

Definition of Plate Height:

✓ the breadth of a Gaussian curve is described by its standard deviation σ *or its variance* σ^2



The plate height H is given by

$$H = \sigma^2/L$$

- L carries units of centimeters and
- σ^2 units of centimeters squared;
- thus H represents linear distance in centimeters.
- *The plate height can be thought of as the length of column that contains a fraction of analyte that lies between L- σ and L.* Because the area under a normal error curve bounded by σ is about 68% of the total area, the plate height, as defined, contains approximately 34 % of the analyte.

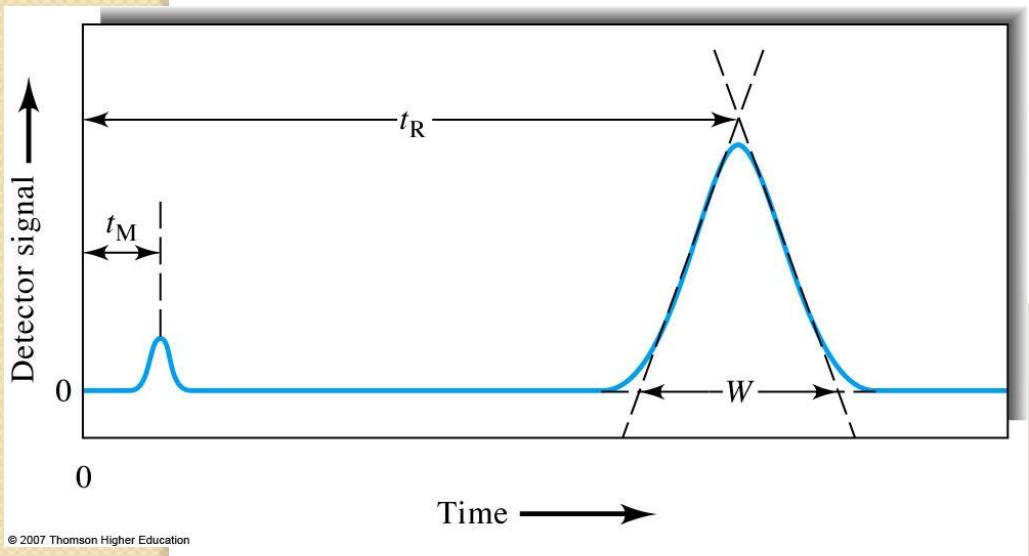
The Experimental Evaluation of H and N

- The plate count N and the plate height H are widely used in the literature and by instrument manufactures as measures of column performance.
- H can be calculated from an experimental chromotogram.

▪ Tangents at the inflection points on the two sides of the chromatographic peak are extended to form a triangle with the baseline of the chromatogram. The area of this triangle can be shown to be approximately 96% of the total area under the peak if the peak is Gaussian.

▪ In Section aIB-I of Appendix 1 it is shown that about 96% of the area under a Gaussian peak is included within plus or minus two standard deviations *($\pm 2\sigma$) of its maximum.*

▪ Thus, the intercepts shown in Figure 26-7 occur at approximately plus or minus two standard deviations of the solute peak *($\pm 2\tau$) from the maximum, and* $W = 4\tau$, where *W is the magnitude of the base of the triangle*. Substituting this relationship into Equation 26-18 and rearranging yields



$$\sigma = L W / 4\, t_R$$

$$H = L W^2 / 16\, t_R^2$$

$$N = L/H \qquad N = 16\,(t_R/W)^2$$

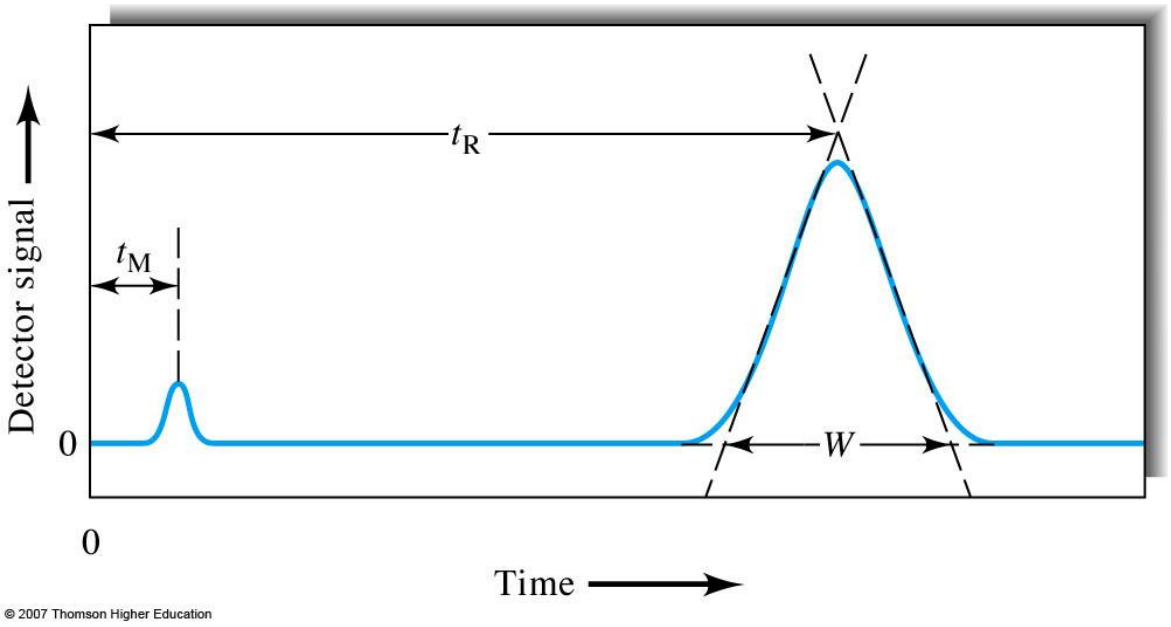
Thus, N can be calculated from two time measurements, t_R and W; to obtain H, the length of the column packing L must also be known.

Note that these calculations are only approximate and assume Gaussian peak shapes.

The Experimental Evaluation of H and N

Another method for approximating N, is to determine $W_{1/2}$, the width of peak at half its maximum height. The plate count is then given by

$$N = 5.54(t_R/W_{1/2})^2$$



26C-3 Kinetic Variables Affecting Column Efficiency

- Band broadening reflects a loss of column efficiency.
- The slower the rate of mass-transfer processes occurring while a solute migrates through a column the broader the band at the column exit.
- Some of the variables that affect mass-transfer rates are controllable and can be exploited to improve separations.

TABLE 26-2 Variables That Influence Column Efficiency

Variable	Symbol	Usual Units
Linear velocity of mobile phase	u	cm s^{-1}
Diffusion coefficient in mobile phase*	D_M**	cm^2 s^{-1}
Diffusion coefficient in stationary phase*	D_S	cm^2 s^{-1}
Retention factor (Equation 26-12)	k	unitless
Diameter of packing particles	d_p	cm
Thickness of liquid coating on stationary phase	d_f	cm

The Effect of Mobile-Phase Flow Rate

- The extent of band broadening depends upon the length of time the mobile phase is in contact with the stationary phase, which in turn depends upon the flow rate of the mobile phase.
- Efficiency studies have generally been carried out by determining H as a function of mobile-phase velocity u.
- Higher mobile phase velocity, less time on column, less zone broadening
- Both Figure show a minimum H *(or a maximum in efficiency) at low linear* flow rates, the minimum for LC usually occurs at flow rates well below those for GC. GC separations are much more shorter, faster than LC one.
- Often these flow rates are so low that the minimum *H is not observed for LC* under normal operating conditions.

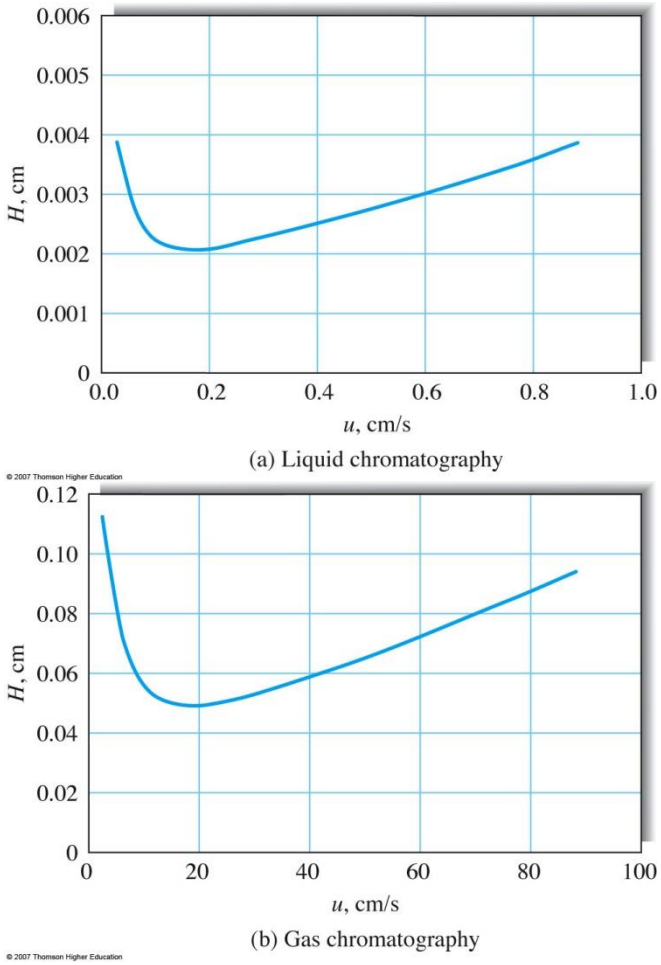


FIGURE26-8 Effect of mobile-phase flow rate on plate height for
(a) LC and (b)GC.
Note very different flow rate and plate height scales.

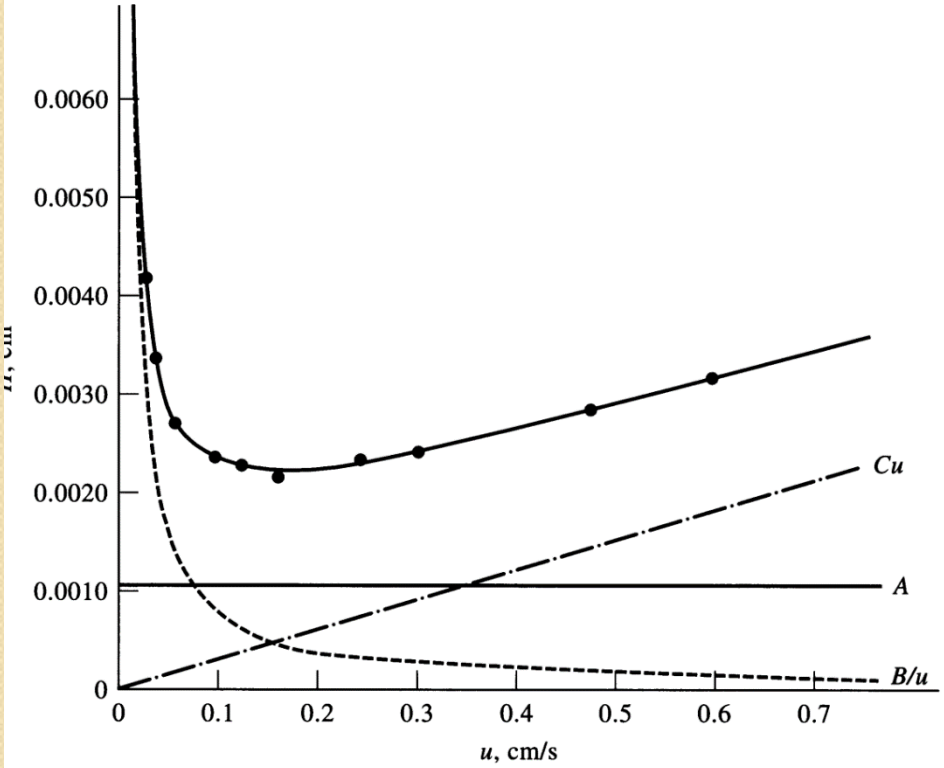
Theory of Band Broadening

The efficiency of chromatographic columns can be approximated by the expression ,Van Deemter equation.

$$H = A + B/u + Cu$$

$$= A + B/u + (C_s + C_M)u$$

H: the plate height (cm),
u: the linear velocity of the mobile phase (cm/s)
A; *multiple flow paths* coefficient (eddy diffusion)
B; *longitudinal diffusion* coefficient *and*
C; *mass transfer* between phases, respectively.



The C coefficient can be broken into two coefficients, one related to the stationary phase (C_s) and one related to the mobile phase (C_M). The van Deemter equation contains terms linearly and inversely proportional to, as well as independent of, the mobile phase velocity.

TABLE 26-3 Processes That Contribute to Band Broadening

Process	Term in Equation 26-23	Relationship to Column* and Analyte Properties
Multiple flow paths	A	$A = 2\lambda d_p$
Longitudinal diffusion	B/u	$\frac{B}{u} = \frac{2\gamma D_M}{u}$
Mass transfer to and from stationary phase	$C_S u$	$C_S u = \frac{f(k) d_f^2}{D_S} u$
Mass transfer in mobile phase	$C_M u$	$C_M u = \frac{f'(k) d_p^2}{D_M} u$

* *u, Ds, D_M, d_f, d_p and k are as defined in Table 26-2.*

f(k) and f'(k) are functions of k, retention factors.

λand γ are constants that depend on quality of the packing.

B is the coefficient of longitudinal diffusion.

C_s and C_M are coefficients of mass transfer in stationary and mobile phase, respectively.

The Multipath Term(A)

- *Molecules move through different paths.* Zone broadening arises in part from the multitude of pathways by which a molecule (or ion) can find its way through a packed column.

- The length of these pathways may differ significantly; *Larger difference in pathlengths for larger particles* , thus, the residence time in the column for molecules of the same species is also variable. ,

- *At low flow rates, diffusion allows particles to switch between paths quickly and reduces variation in transit time*

- Solute molecules then reach the end of the column over a time interval, which leads to a broadened band. This effect which is called *eddy diffusion*, is directly proportional to the diameter of the particles making up the column packing.

TABLE 26-3 Processes That Contribute to Band Broadening

Process	Term in Equation 26-23	Relationship to Column* and Analyte Properties
Multiple flow paths	A	$A = 2\lambda d_p$

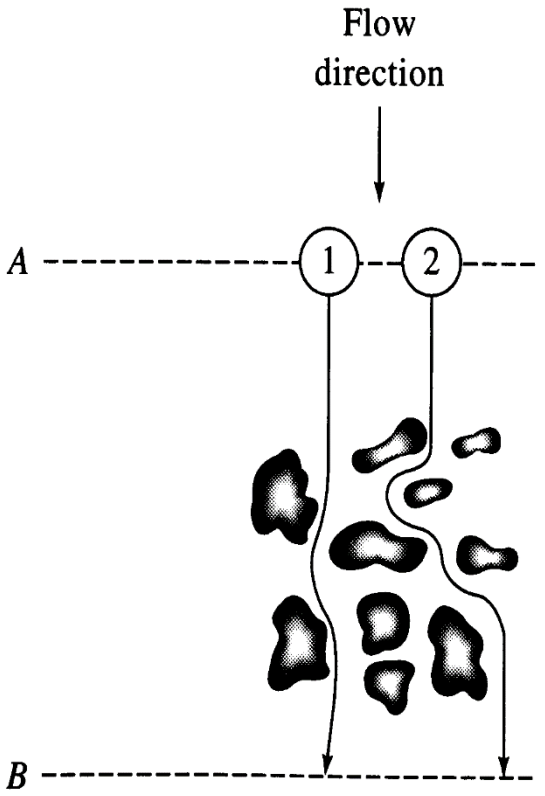


FIGURE26-9 Typical pathways of two molecules during elutionmolecule 2 will arrive at B later than molecule 1.

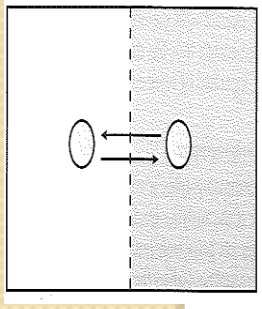
The Longitudinal Diffusion Term (B/u)

- Longitudinal diffusion in column chromatography is a band broadening process in which solutes diffuse from the concentrated center of a zone to the more dilute regions ahead of and behind the zone center.
- The longitudinal diffusion term is directly proportional to the mobile-phase diffusion coefficient D_M.
- The contribution of longitudinal diffusion is seen to be inversely proportional to the mobile phase velocity. High flow, less time for diffusion.
- Longitudinal diffusion is a common source of band broadening in GC because gaseous molecules diffuse at relatively high rates. The phenomenon is of little significance in LC where diffusion rates are much smaller.

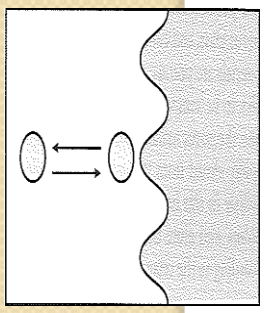
Mass-transfer Coefficients (C_s and C_M)

- The need for the two mass-transfer coefficients C_s and C_M arises because the equilibrium between the mobile and the stationary phase is established so slowly that a chromatographic column always operates under non-equilibrium conditions.
- C_S is rate for adsorption onto stationary phase
- C_M is rate for analyte to desorb from stationary phase
- Effect proportional to flow rate - at high flow rates less time to approach equilibrium
- Slower rate of mass transfer results an increase in plate height.

The Stationary-Phase Mass-Transfer Term $C_s u$,



- When the stationary phase is an immobilized liquid, the mass-transfer coefficient is directly proportional to the square of the thickness of the film on the support particles d_t^2 *and inversely proportional to the diffusion* coefficient D_s *of the solute in the film.*
  - ✓ *These effects* can be understood by realizing that both reduce the average frequency at which analyte molecules reach the liquid-liquid interface where transfer to the mobile phase can occur. That is, *with thick films, molecules must on the average travel farther to reach the surface, and with smaller diffusion coefficients, they travel slower. The consequence is a slower rate of mass transfer and an increase in plate height.*



- When the stationary phase is a solid surface, the mass-transfer coefficient C_s is directly proportional to the time required for a species to *be adsorbed or desorbed*, which in turn is inversely proportional to the first-order rate constant for the processes.

The Mobile-Phase Mass-Transfer Term $C_M u$,

- The mass-transfer processes that occur in the mobile phase are sufficiently complex that we do not yet have a complete quantitative description. On the other hand, we have a good qualitative understanding of the variables affecting zone broadening from this cause, and this understanding has led to vast improvements in all types of chromatographic columns.
- The mobile-phase mass-transfer coefficient C_M is known to be inversely proportional to the diffusion coefficient of the analyte in the mobile phase. D_M.
- For packed columns, C_M is proportional to the square of the particle diameter of the packing material, d_p^2.
- *For open* tubular columns, C_M is proportional to the square of the column diameter, d_c^2.
- The contribution of mobile-phase mass transfer to plate height is the product of the mass-transfer coefficient C_M (which is a function of solvent velocity) and the velocity of the solvent itself. Thus, the net contribution of $C_M u$ to plate height is not linear in *u (see the* curve labeled $C_M u$ in Figure 26-10) but bears a complex dependency on solvent velocity.

Effect of Mobile Phase Velocity on Terms in Van Deemter Equation

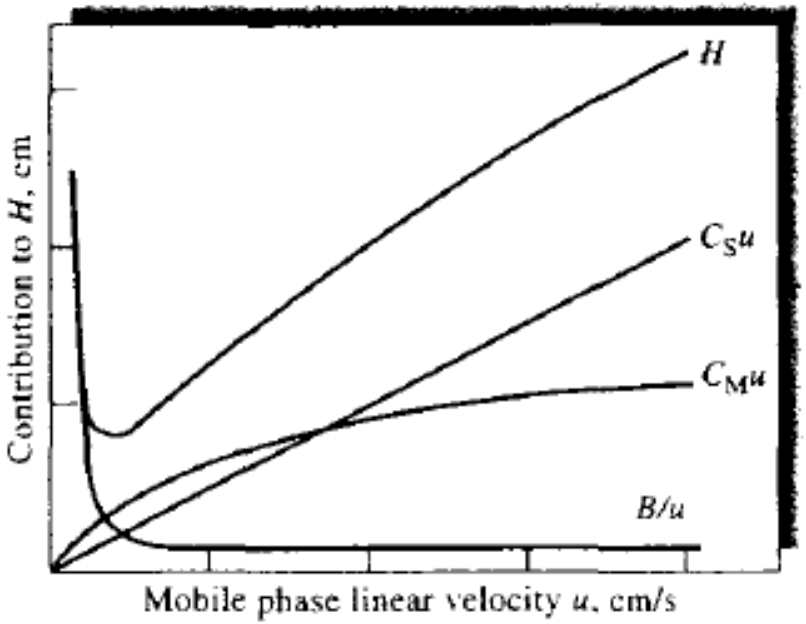


FIGURE 26-10 Contribution of various mass-transfer terms to plate height. $C_S u$ arises from the rate of mass transfer to and from the stationary phase, $C_M u$ comes from a limitation in the rate of mass transfer in the mobile phase, and B/u is associated with longitudinal diffusion.

The top curve is the summation of these various effects. Note that there is an optimal flow rate at which the plate height is a minimum and the separation efficiency is a maximum.

Methods for Reducing Zone Broadening

- Two important controllable variables that affect column efficiency

1) the diameter of the particles making up the packing for (packed columns) and
2) the diameter of the column (for open tubular columns).

- To take advantage of the effect of column diameter, narrower and narrower columns have been used in recent years.
- With gaseous mobile phases, the rate of longitudinal diffusion can be reduced appreciable by lowering the temperature and thus the diffusion coefficient D_M. The consequence is significantly smaller plate heights at low temperatures.
- With liquid stationary phases. the thickness of the layer of adsorbed liquid should be minimized because C_s in Equation 26-23 is proportional to the square of this variable.

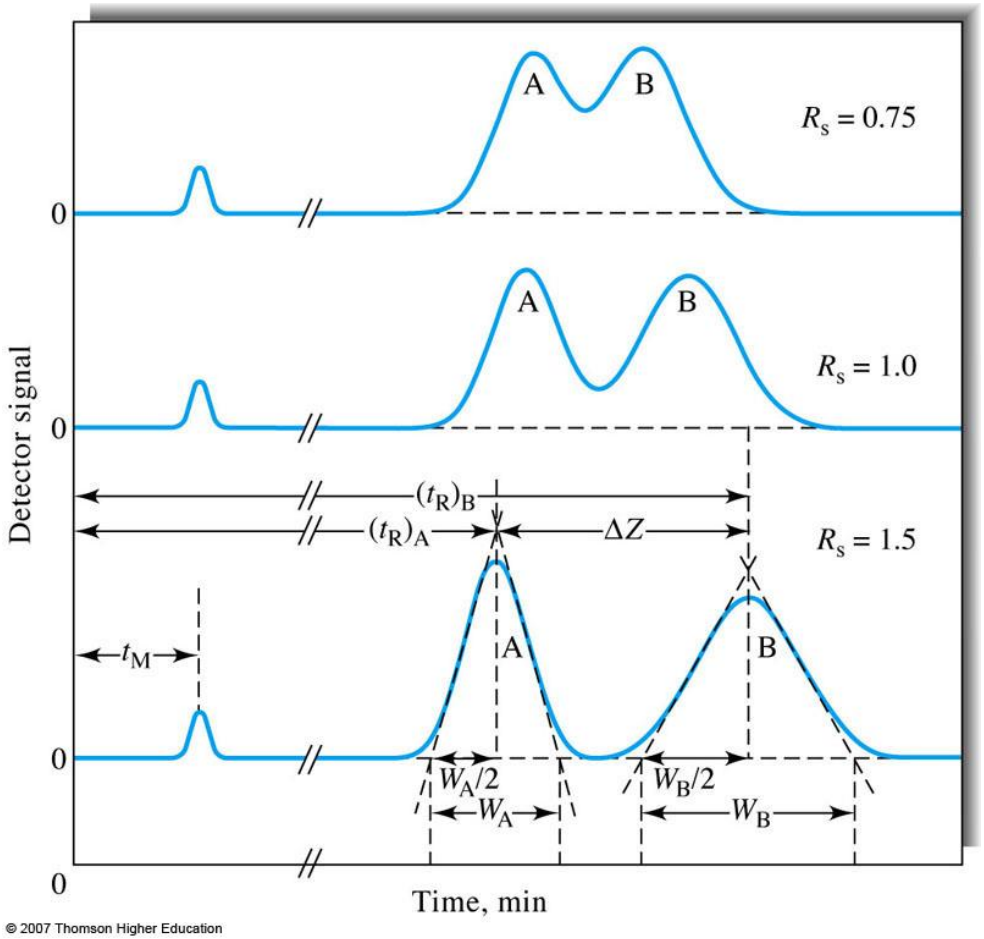
26D- Optimization Of Column Performance

- A chromatographic separation is optimized by varying experimental conditions until the components of a mixture are separated cleanly in a minimum amount of time.
- Optimization experiments are aimed at either
  - (1) reducing zone broadening or
  - (2) altering relative migration rates of the components.
- zone broadening is increased by those kinetic variables that increase the plate height of a column.
- Migration rates, on the other hand, are varied by changing those variables that affect retention and selectivity factors

26D-1 Column Resolution

- The resolution R_s of a column provides a quantitative measure of its ability to separate two analytes, and tells us how far apart two bands are relative to their widths.

$$R_S = \frac{\Delta Z}{W_A/2 + W_B/2} = \frac{2\Delta Z}{W_A + W_B} = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B}$$



- a resolution of 1.5 gives an essentially complete separation of the two components, whereas a resolution of 0.75 does not.
- At a resolution of 1.0, zone A contains about 4% B and zone B contains a similar amount of A.
-At a resolution for 1.5, the overlap is about 0.3% . The resolution for a given stationary phase can be improved by lengthening the column, thus increasing the number of plates.

The Effect of Retention and Selectivity Factors on Resolution

Relationship between the resolution of a column and the retention factors $k`_A$ and $k`_B$ for two solutes, the selectivity factor , and the number of plates

Assumptions: $W_A \cong W_B \cong W$

$K_A \cong K_B$

$k_A \cong k_B \cong k$

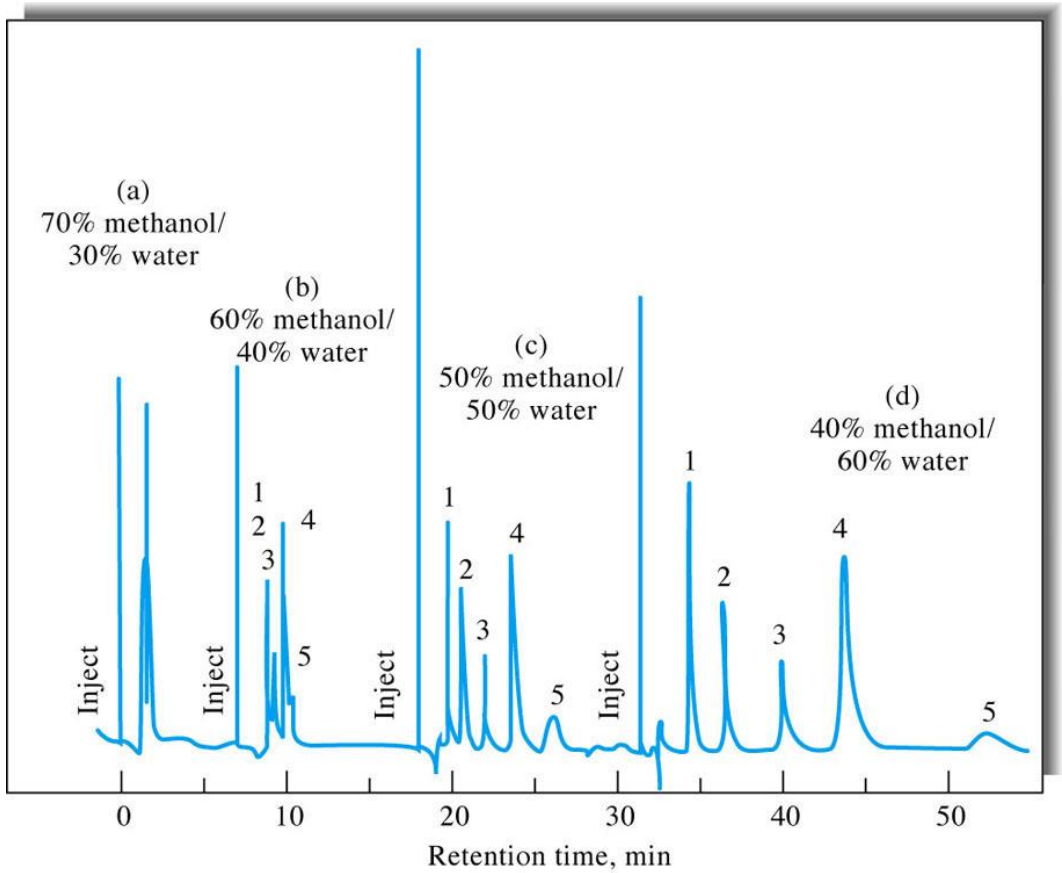
$$N = 16R_s^2\left(\frac{1}{\alpha - 1}\right)^2\left(\frac{1+k'}{k'}\right)^2$$

Where k' is the average of k'_A and k'_B

EXAMPLE 26-1 Substances A and B have retention times of 16.40 and 17.63 min. respectively, on a 30.0-cm column. An unretained species passes through the column in 1.30 min. The peak widths (at base) for A and Bare 1.11 and 1.21 min. respectively. Calculate
(a) the column resolution (b) the average number of plates in the column,
(c) the plate height, (d) the length of column required to achieve a resolution of 1.5.
(e) the time required to elute substance B on the column that gives an *R, value of 1.5,* and
(f) the plate height required for a resolution of 1.5 on the original 30-cm column and in the original time.

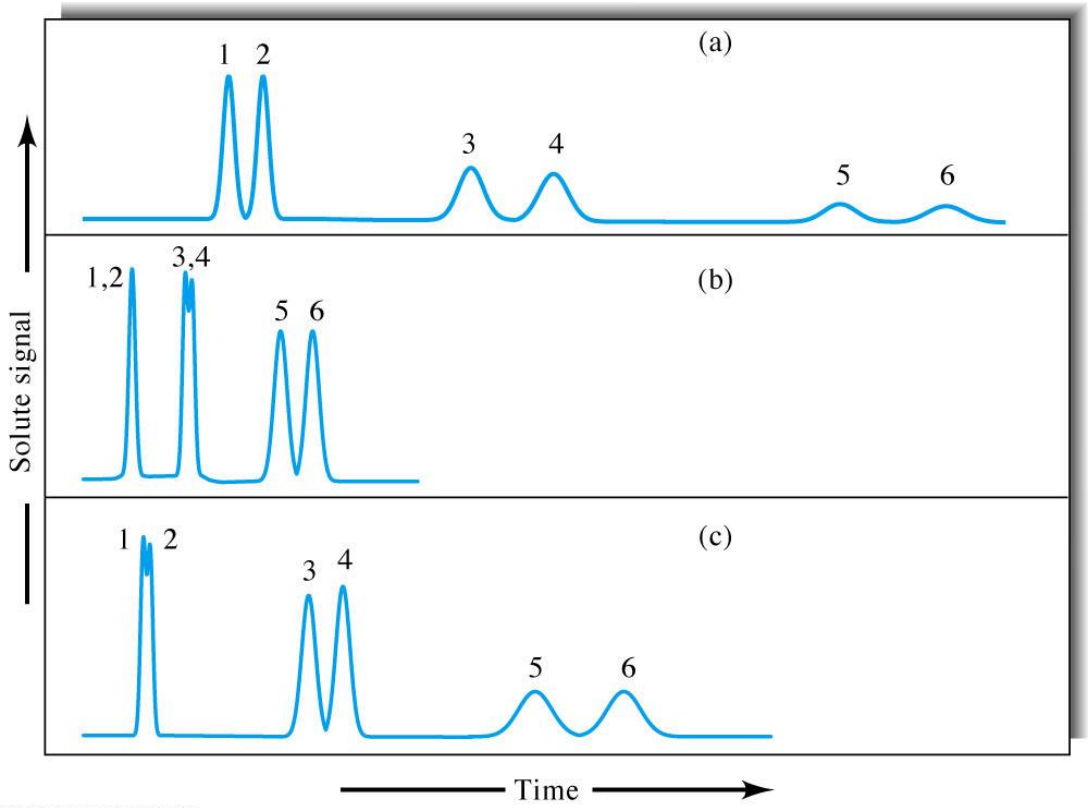
Resolution (and zone broadening) depends on;

- u (linear flow rate) - low flow favors increased resolution (van Deemter plot)
- H (plate height) (or N number of plates) - use smaller particles, lengthen column, viscosity of mobile phase (diffusion)
- α (selectivity factor) - vary temperature, composition of column/mobile phase
- k_A (capacity factor) - vary temperature, composition of column/mobile phase



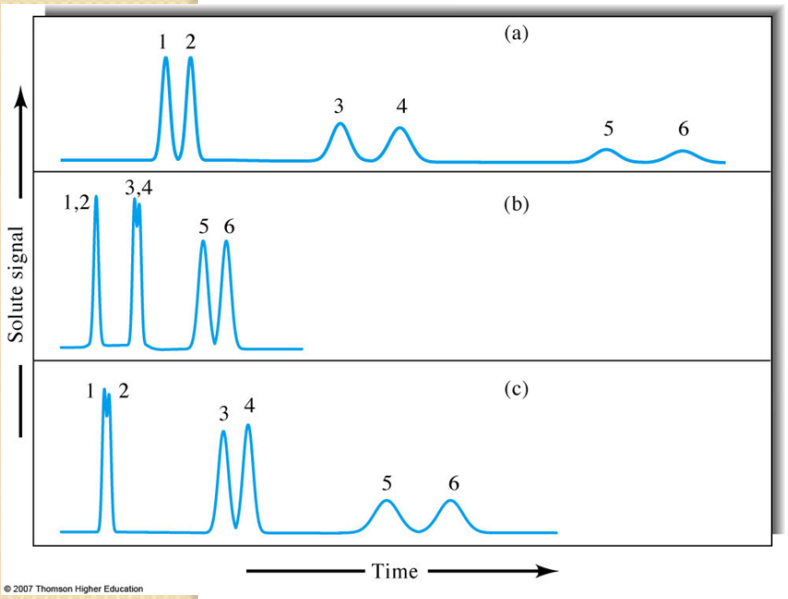
General Elution Problem:

- For multiple components, conditions are rarely optimum for all components.
- The phenomenon illustrated in Figure 26-15 is encountered often enough to be given a name, the general elution problem.



A common solution to this general elution problem

- to change conditions that determine the values of *k during* the separation.
- These changes can be performed in a stepwise manner or continuously.
- For LC, variations in *k can be achieved by varying* the composition of the mobile phase during elution, called *gradient elution or solvent programming.*
- *Elution with constant mobile-phase* composition is called *isocratic elution.*
- *For GC, the* temperature can be changed in a known fashion to bring about changes in *k. This temperature programming* mode can help achieve optimal conditions for many separations.



- Conditions at the beginning of the separation could be those producing chromatogram (a).
- Immediately after the elution of components 1 and 2, conditions could be changed to those that are optimal for separating components 3 and 4, as in chromatogram (c).
- With the appearance of peaks for these components, the elution could be completed under the conditions used for producing chromatogram (b).

Often, such a procedure leads to satisfactory separation of all the components of a mixture in minimal time.

TABLE 26-4 Important Chromatographic Quantities and Relationships

Name	Symbol of Experimental Quantity	Determined From
Migration time, unretained species	t_M	Chromatogram (Figure 26-7)
Retention time, species A and B	$(t_R)_A$, $(t_R)_B$	Chromatogram (Figures 26-7 and 26-12)
Adjusted retention time for A	$(t'_R)_A$	$(t'_R)_A = (t_R)_A - t_M$
Peak widths for A and B	W_A, W_B	Chromatogram (Figures 26-7 and 26-12)
Length of column packing	L	Direct measurement
Volumetric flow rate	F	Direct measurement
Linear flow velocity	u	F and column dimensions (Equations 26-6 and 26-7)
Stationary-phase volume	V_S	Packing preparation data
Concentration of analyte in mobile and stationary phases	c_M, c_S	Analysis and preparation data

TABLE 26-5 Important Derived Quantities and Relationships

Name	Calculation of Derived Quantities	Relationship to Other Quantities
Linear mobile-phase velocity	$u = \frac{L}{t_M}$	
Volume of mobile phase	$V_M = t_M F$	
Retention factor	$k = \frac{t_R - t_M}{t_M}$	$k = \frac{KV_S}{V_M}$
Distribution constant	$K = \frac{kV_M}{V_S}$	$K = \frac{c_S}{c_M}$
Selectivity factor	$\alpha = \frac{(t_R)_B - t_M}{(t_R)_A - t_M}$	$\alpha = \frac{k_B}{k_A} = \frac{K_B}{K_A}$
Resolution	$R_s = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B}$	$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_B}{1 + k_B}\right)$
Number of plates	$N = 16\left(\frac{t_R}{W}\right)^2$	$N = 16R_s^2\left(\frac{\alpha}{\alpha - 1}\right)^2\left(\frac{1 + k_B}{k_B}\right)^2$
Plate height	$H = \frac{L}{N}$	
Retention time	$(t_R)_B = \frac{16R_s^2 H}{u}\left(\frac{\alpha}{\alpha - 1}\right)^2 \frac{(1 + k_B)^3}{(k_B)^2}$	

APPLICATIONS OF CHROMATOGRAPHY

Chromatography has grown to be the premiere method for separating closely related chemical species. In addition, it can be employed for qualitative identification and quantitative determination of separated species.

Qualitative Analysis

- A chromatogram provides only a single piece of qualitative information about each species in a sample, namely, its retention time, t_R.
- It is a widely used tool for recognizing the presence or absence of components of mixtures containing a limited number of possible species whose identities are known.
- the amount of information revealed by chromatography is small compared with the amount provided by a single IR, nuclear magnetic resonance, or mass spectrum. Furthermore, spectral wavelength or frequency data can be determined with much higher precision than can their chromatographic counterpart t_R.
- However, positive spectroscopic identification would be impossible without a preliminary chromatographic separation on a complex sample.
- It is important to note that, although chromatograms may not lead to positive identification of species present in a sample, they often provide sure evidence of the absence of certain compounds.
- Thus, if the sample does not produce a peak at the same retention time as a standard run under identical conditions, it can be assumed that the compound in question is either absent or is present at a concentration level below the detection limit of the procedure

Quantitative Analysis

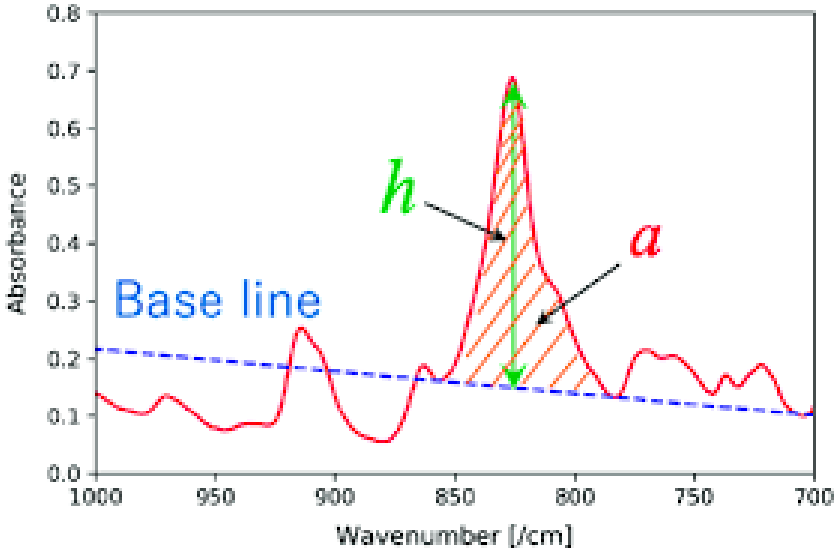
* Chromatography can provide useful quantitative information about the separated species.

- Chromatography owes its rapid growth during the past four decades in part to its *speed, simplicity, relatively low cost, and wide applicability* as a tool for separations.
- Quantitative column chromatography is based upon a comparison of either the height or the area of the analyte peak with that of one or more standards.

For planar *chromatography, the area covered by the separated species serves as the analytical* parameter. If conditions are properly controlled, these parameters vary linearly with concentration.

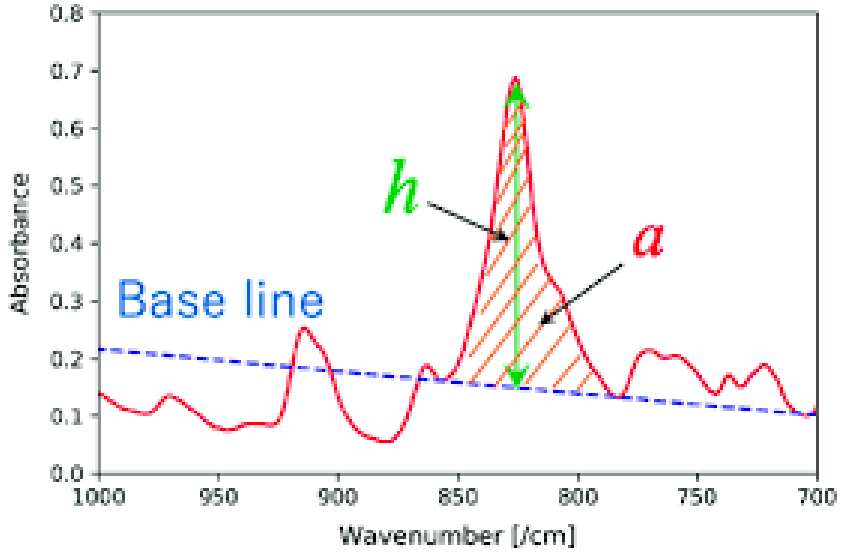
Analyses Based on Peak Height

The height of a chromatographic peak is obtained by connecting the baselines on either side of the peak by a straight line and measuring the perpendicular distance from this line to the peak. This measurement can ordinarily be made with reasonably high precision. Accurate results are obtained with peak heights only if variations in column conditions do not alter the peak widths during the period required to obtain chromatograms for samples and standards. The variables that must be controlled closely are column temperature, eluent flow rate, and rate of sample infection.



Analyses Based on Peak Areas

Peak areas are a more satisfactory analytical variable than peak heights. On the other hand, peak heights are more easily measured and, for narrow peaks, more accurately determined. Most modern chromatographic instruments are equipped with digital electronic integrators that permit precise estimation of peak areas. If such equipment is not available, manual estimate must be made. A simple method, which works well for symmetric peaks of reasonable widths, is to multiply the height of the peak by its width at one half the peak height.



26-22 The relative areas for the five gas chromatographic peaks obtained in the separation of five steroids are given next. Also shown are the relative responses of the detector to the five compounds. Calculate the percentage of each component in the mixture.

Compound	Peak Area, Relative	Detector Response, Relative
Dehydroepiandrosterone	27.6	0.70
Estradiol	32.4	0.72
Estrone	47.1	0.75
Testosterone	40.6	0.73
Estriol	27.3	0.78

26-22.

	A	B	C	D	E
1	**Problem 26-22**				
2	Compound	Peak area	Response factor, *F*	Area X *F*	% Compound
3	Dehydroepiandrosterone	27.6	0.7	19.32	14.99
4	Estradiol	32.4	0.72	23.33	18.10
5	Estrone	47.1	0.75	35.33	27.40
6	Testosterone	40.6	0.73	29.64	22.99
7	Estriol	27.3	0.78	21.29	16.52
8			Sum	128.91	100.00
9	**Spreadsheed Documentation**				
10	D3=B3*C3				
11	D8=SUM(D3:D7)				
12	E3=D3/D8*100				
13	E8=SUM(E3:E7)				

Thus, dehydroepiandrosterone = 14.99%; Estradiol = 18.10%; Estrone = 27.40%;

Testosterone = 22.99%; Estriol = 16.52%

Calibration and Standards

The most straightforward method for quantitative chromatographic analyses involves the preparation of a series of standard solutions that approximate the composition of the unknown. Chromatograms for the standards are then obtained and peak heights or areas are plotted as a function of concentration. A plot of the data should yield a straight line passing through the origin.

The Internal Standard Method

The highest precision for quantitative chromatography is obtained by use of internal standards because the uncertainties introduced by sample injection are avoided. In this procedure, a carefully measured quantity of an internal standard substance is introduced into each standard and sample, and the ratio of analyte to internal standard peak areas (or heights) serves as the analytical parameter. For this method to be successful, it is necessary that the internal standard peak be well separated from the peaks of all other components of the sample.