Analytical chemistry

Lecture №30

Introduction to Chromatographic methods of analysis

• **Chromatography** is a technique in which the components of a mixture are separated based on differences in the rates at which they are carried through a fixed or **stationary phase** by a gaseous or liquid **mobile phase.**

• The **stationary phase** in chromatography is a phase that is fixed in place either in a column or on a planar surface.

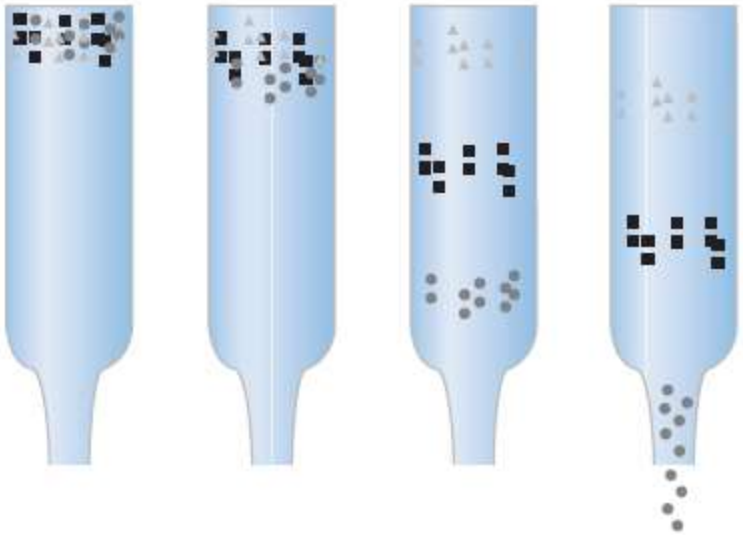
• The **mobile phase** in chromatography is a phase that moves over or through the stationary phase carrying with it the analyte mixture. The mobile phase may be a gas, a liquid, or a supercritical fluid.

Russian botanist, Michael Tswett (1872–1919), inventor of chromatography.

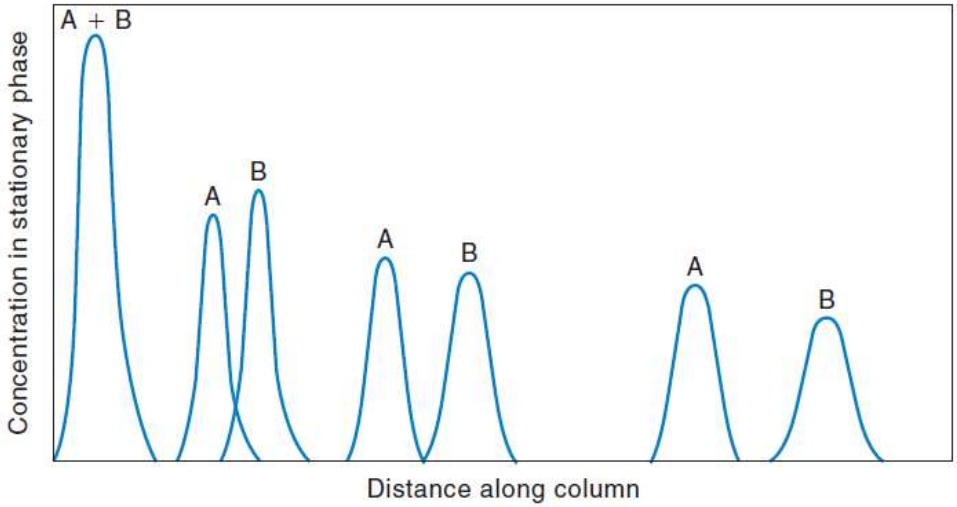
In 1901, the Russian botanist, Mikhail Tswett, invented adsorption chromatography during his research on plant pigments. He separated different colored chlorophyll and carotenoid pigments of leaves by passing an extract of the leaves through a column of calcium carbonate, alumina, and sucrose, eluting them with petroleum ether/ethanol mixtures. He coined the term **chromatography** in a 1906 publication, from the Greek words *chroma meaning "color" and graphos meaning "to write." Tswett's original* experiments went virtually unnoticed in the literature for decades, but eventually others adopted it. Today there are several different types of chromatography. Chromatography is taken now to refer generally to the separation of components in a sample by distribution of the components between two phases—one that is stationary and one that moves, usually (but not necessarily) in a column.

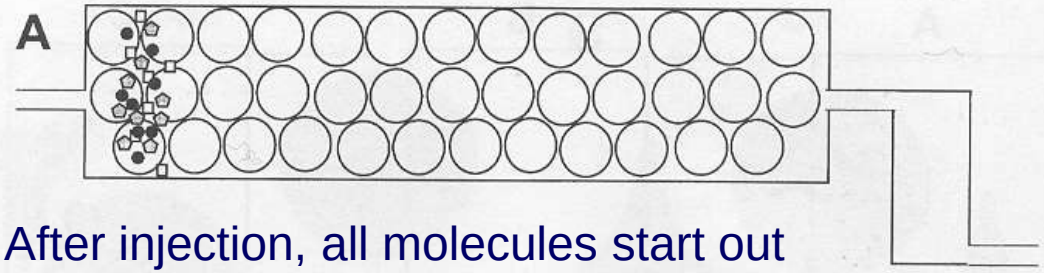
Principles of Chromatographic Separations

While the mechanisms of retention for various types of chromatography differ, they are all based on the dynamic distribution of an analyte between a fixed stationary phase and a flowing mobile phase. Each analyte will have a certain affinity for each phase.

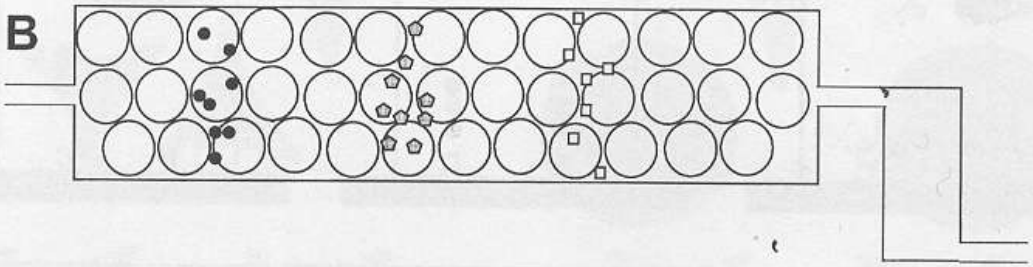


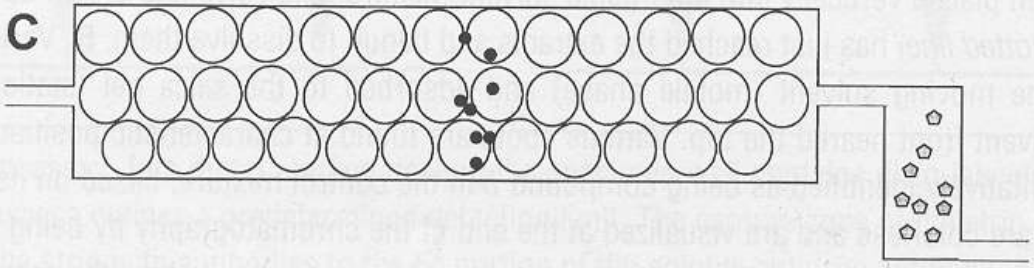
A small volume of sample is placed at the top of the column, which is filled with particles constituting the stationary phase and the solvent. Rather than an equilibrium-based "plate view" of chromatography, many hold that a "rate view" of chromatography to be more rigorous: in this view, the partition ratio is simply the ratio of the time a solute spends in the stationary phase to that it spends in the mobile phase. More solvent, functioning as mobile phase, is added to the top of the column and percolates through the column. The individual components interact with the stationary phase to different degrees.

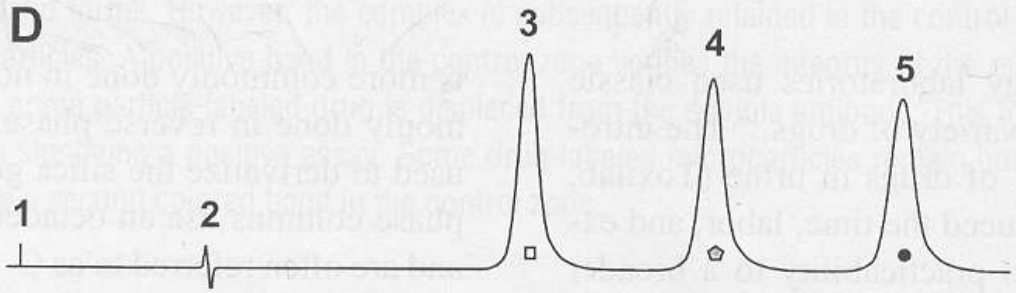




After injection, all molecules start out overlapping.







Separation of individual sample components is always based on their *relative affinity for the mobile versus the stationary phases.* Because some molecules have higher affinity for the stationary phase, they will pass through the column *slowerthan others and, therefore, will be separated from each other (we say they have been "retained" by the column).*

Due to varying relative affinity for the stationary versus the mobile phases, individual molecules begin to separate.

As the different molecules elute off of the column, they are detected as resolved "peaks".

Peak “area” generally correlates with the amount of drug loaded onto a column and, thus, the original drug concentration.

However, there can be sample-to-sample variation due to extraction efficiency, loading volumes, detection efficiency, etc.

Again, the *internal standardis utilized to correct for variations. Similar to the relative retention time, a relative peak intensityis defined and related to drug concentration.*

Variations in the peak area are not always similar for all molecules (e.g. some molecules may have a lower extraction efficiency due to proteinuriaand others may not; it is important that the internal standard shares the same *susceptibility to interferences or variations in efficiency as the analyte).*

Therefore, the internal standard is chosen to be chemically similar to the analyteof interest to best correct for variations.

However, adequate similarity is *noteasy to predict or establish.*

Extensive validation is ultimately necessary to satisfy the rigor of your assay. Need to test all anticipated interferences and also un-expected variabilitiesusing “real” patient samples, either with a large correlation study, with a gold standard method and/or with addition/recovery studies.

A known amount of an internal standard is added to every sample (including controls and calibrators) before any other preparative step.
All samples are brought through the identical preparative steps, separated by a chromatographic method and quantitatively detected.
The relative peak intensities are measured for a series of calibrators with a fixed amount of internal standard and varying amounts of a known analyte. These relative peak intensities are fit to an equation, generally linear, to define a *calibration curve.*
Similarly, the relative peak intensities of the unknown samples are calculated and related to the *calibration curve to quantify the concentration of the analyte (drug) in the original clinical sample.*

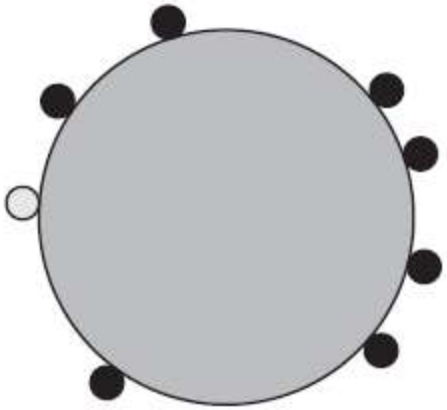
Classification of Chromatographic Methods

• Two common approaches are used to bring the mobile phase and stationary phase into contact. In **column chromatography,** the stationary phase is placed in a narrow column through which the mobile phase moves under the influence of gravity or pressure. The stationary phase is either a solid or a thin, liquid film coating on a solid particulate packing material or the column's walls. In **planar chromatography** the stationary phase coats a flat glass, metal, or plastic plate and is placed in a developing chamber. A reservoir containing the mobile phase is placed in contact with the stationary phase, and the mobile phase moves by capillary action.

• Chromatographic methods fall into three categories based on the nature of the mobile phase: liquid, gas, and supercritical fluid.

Gas chromatography separates gaseous substances on partitioning in a stationary phase from a gas phase. ***Liquid chromatography*** includes techniques such as *size exclusion* (separation based on molecular size), *ion exchange* (separation based on charge), and *high-performance liquid chromatography* (HPLC—separation based on partitioning from a liquid phase), *thin-layer chromatography* (TLC)

• The mechanism by which solutes separate provides a third means for characterizing a separation



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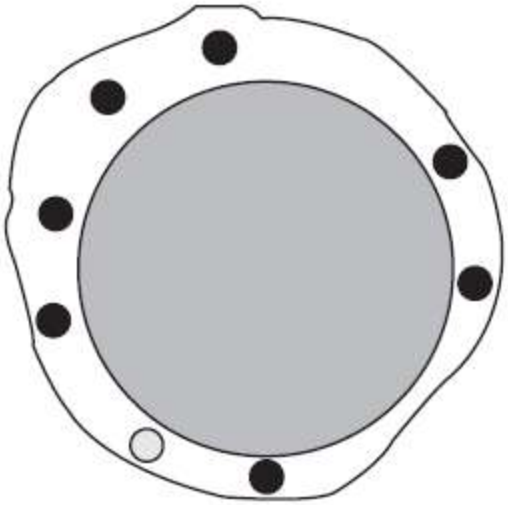

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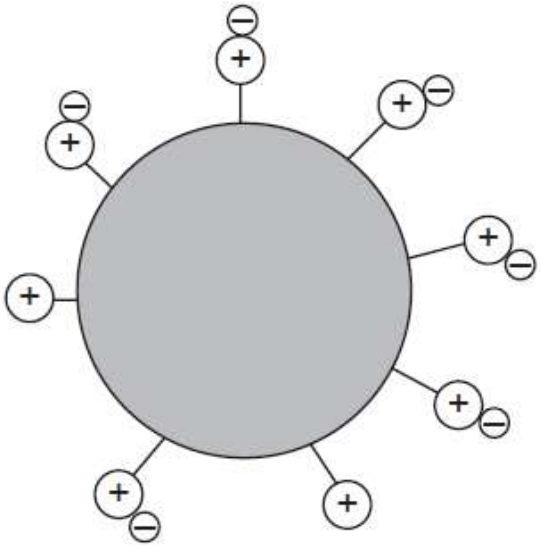

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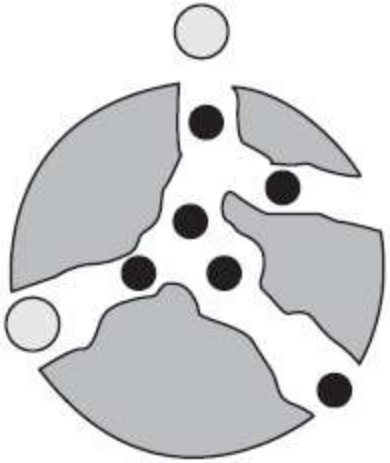

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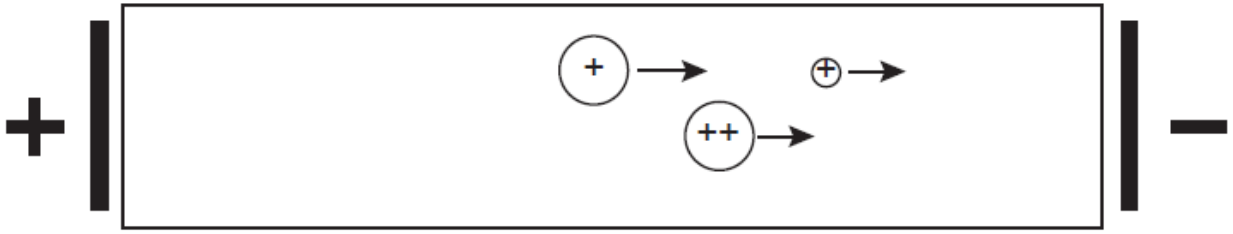


(e)

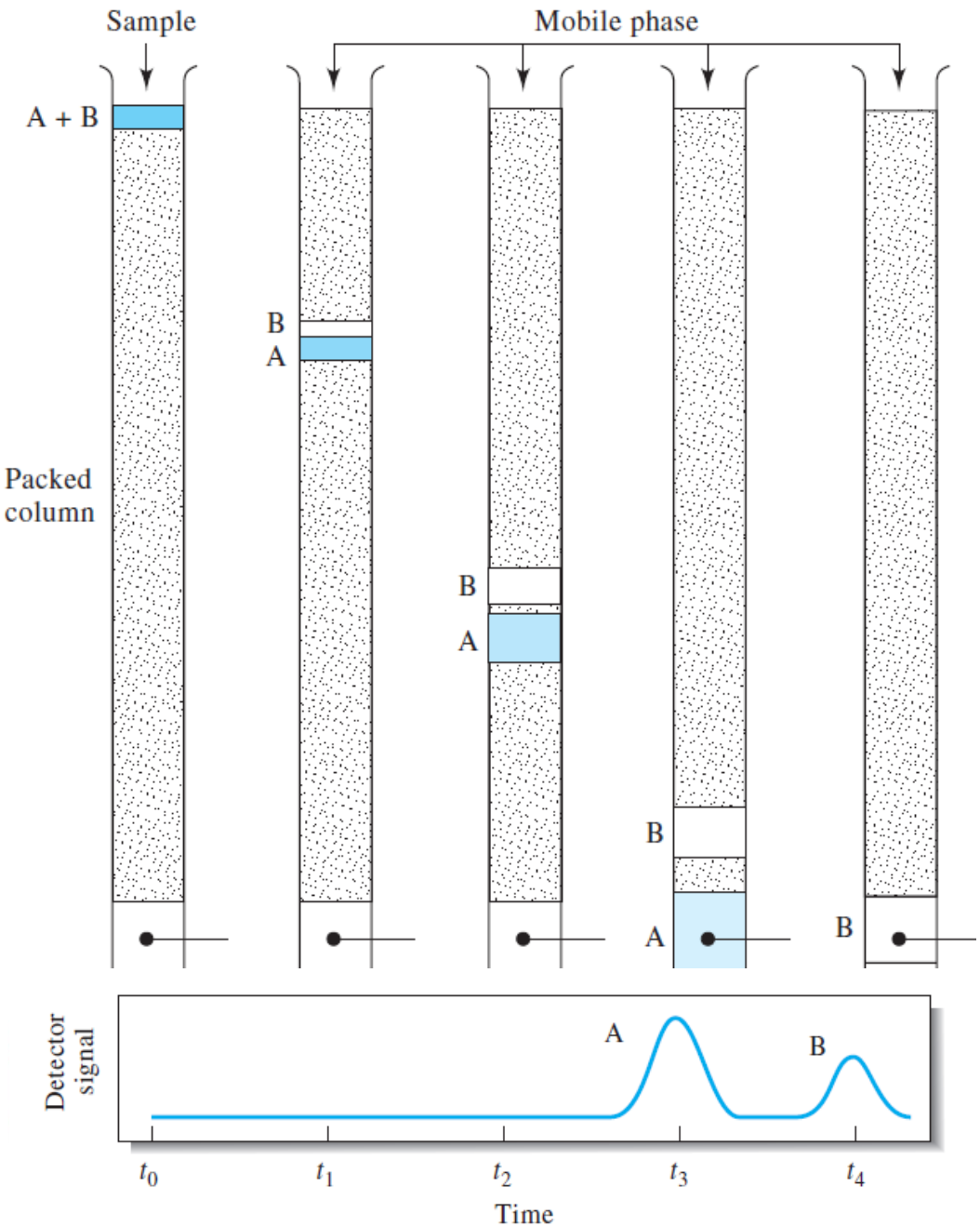
(a) adsorption chromatography, (b) partition chromatography, (c) ion-exchange chromatography, (d) size exclusion chromatography, and (e) electrophoresis. For the separations in (a), (b), and (d) the solute represented by the solid circle (•) is the more strongly retained.

In ***adsorption chromatography***, solutes separate based on their ability to adsorb to a solid stationary phase. In ***partition chromatography***, a thin liquid film coating a solid support serves as the stationary phase. Separation is based on a difference in the equilibrium partitioning of solutes between the liquid stationary phase and the mobile phase. Stationary phases consisting of a solid support with covalently attached anionic (e.g., $–SO_3^-$)or cationic (e.g., $–N(CH_3)_3^+$) functional groups are used in ***ion-exchange chromatography***. Ionic solutes are attracted to the stationary phase by electrostatic forces. Porous gels are used as stationary phases in ***size-exclusion chromatography***, in which separation is due to differences in the size of the solutes. Large solutes are unable to penetrate into the porous stationary phase and so quickly pass through the column. Smaller solutes enter into the porous stationary phase, increasing the time spent on the column. Not all separation methods require a stationary phase. In an ***electrophoretic separation***, for example, charged solutes migrate under the influence of an applied potential field. Differences in the mobility of the ions account for their separation.

Classification of Column Chromatographic Methods

General Classification	Specific Method	Stationary Phase	Type of Equilibrium
1. Gas chromatography (GC)	a. Gas-liquid (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

General Theory of Column Chromatography

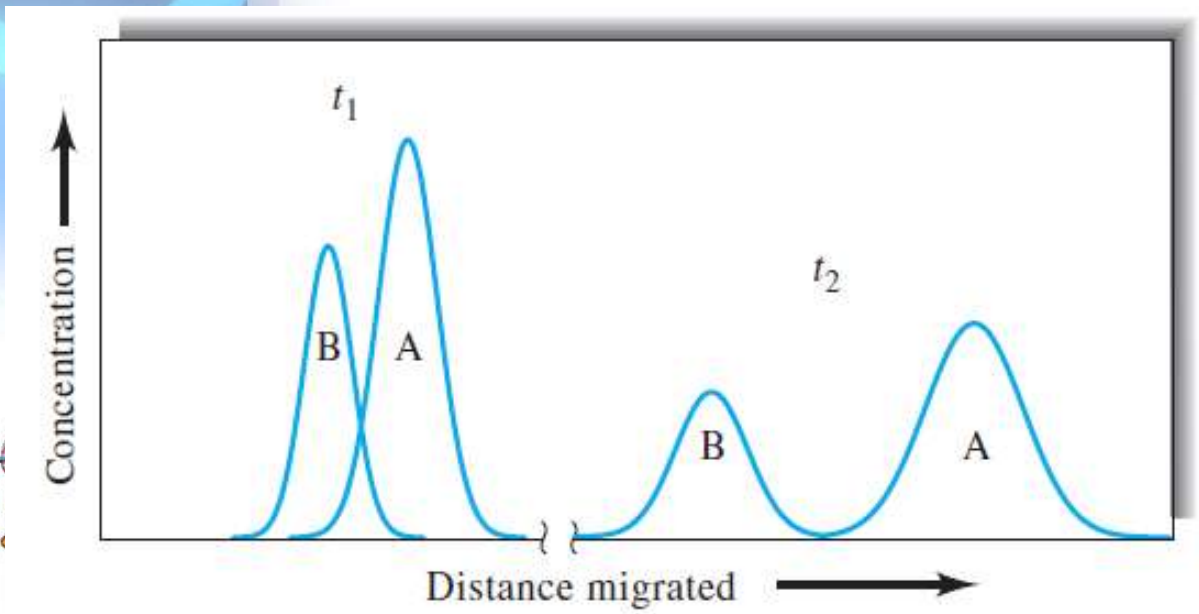


components A and B of a sample are resolved on a packed column by **elution.** The column consists of narrow-bore tubing that is packed with a finely divided inert solid that holds the stationary phase on its surface. The mobile phase occupies the open spaces between the particles of the packing. Initially, a solution of the sample containing a mixture of A and B in the mobile phase is introduced at the head of the column as a narrow plug at time t_0. *The two components distribute themselves between the mobile phase and* the stationary phase. Elution then occurs by forcing the sample components through the column by continuously adding fresh mobile phase. With the first introduction of fresh mobile phase, the **eluent,** the portion of the sample contained in the mobile phase moves down the column, where further partitioning between the mobile phase and the stationary phase occurs (time t_1). Further additions of solvent carry solute molecules down the column in a continuous series of transfers between the two phases.

• **Elution** is a process in which solutes are washed through a stationary phase by the movement of a mobile phase. The mobile phase that exits the column is termed the **eluate.**

An **eluent** is a solvent used to carry the components of a mixture through a stationary phase.

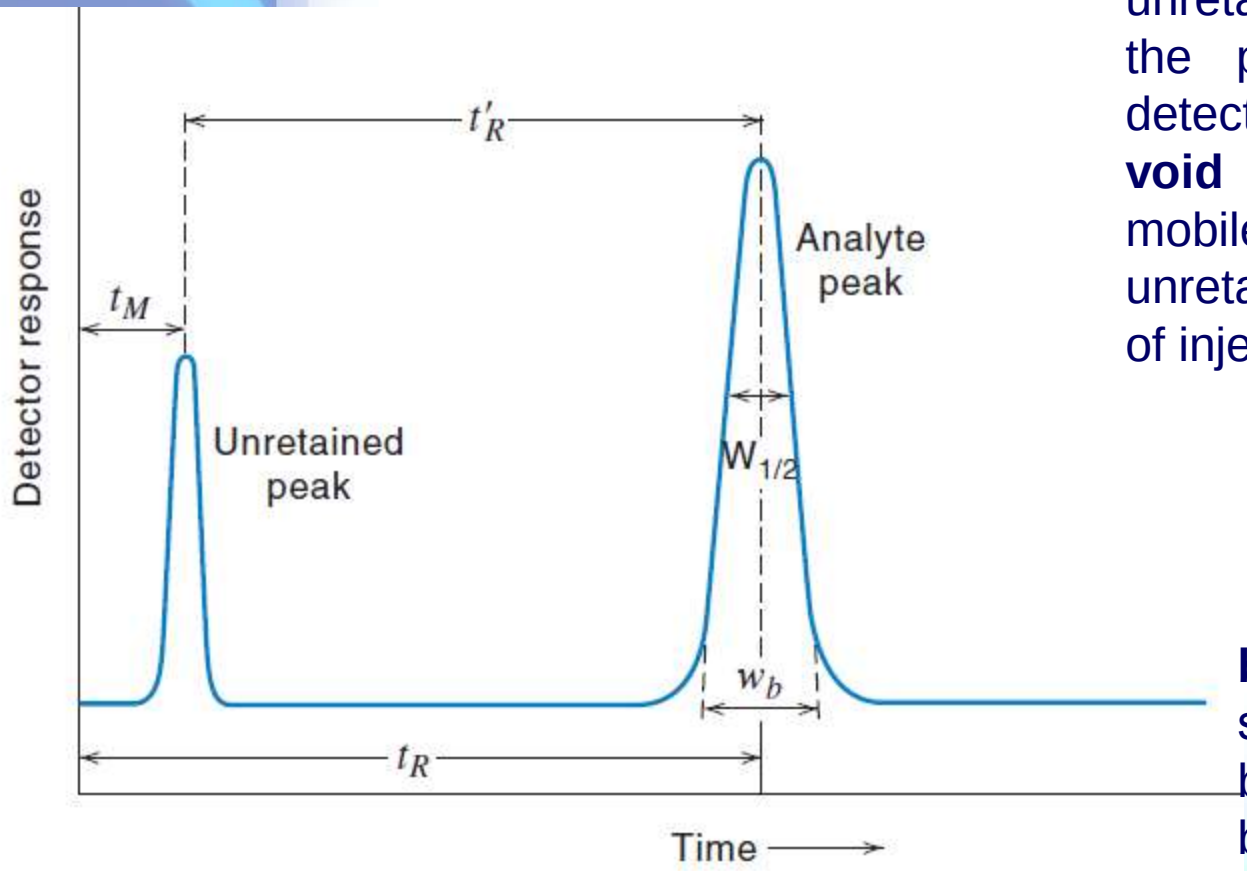
If a detector that responds to solute concentration is placed at the end of the column during elution and its signal is plotted as a function of time (or of volume of added mobile phase), a series of peaks is obtained. Such a plot called a **chromatogram**, is useful for both qualitative and quantitative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



Chromatogram A plot of the detector's signal as function of elution time or volume.

The **retention time, t_r,** is the elapsed time from the introduction of the solute to the peak maximum. The retention time also can be measured indirectly as the volume of mobile phase eluting between the solute's introduction and the appearance of the solute's peak maximum. This is known as the **retention volume, V_r.** Dividing the retention volume by the ***mobile phase's flow rate, u,*** gives the retention time.

t_R' is the adjusted retention time



void time-The time required for unretained solutes to move from the point of injection to the detector (***t_m***).

void volume-The volume of mobile phase needed to move an unretained solute from the point of injection to the detector.

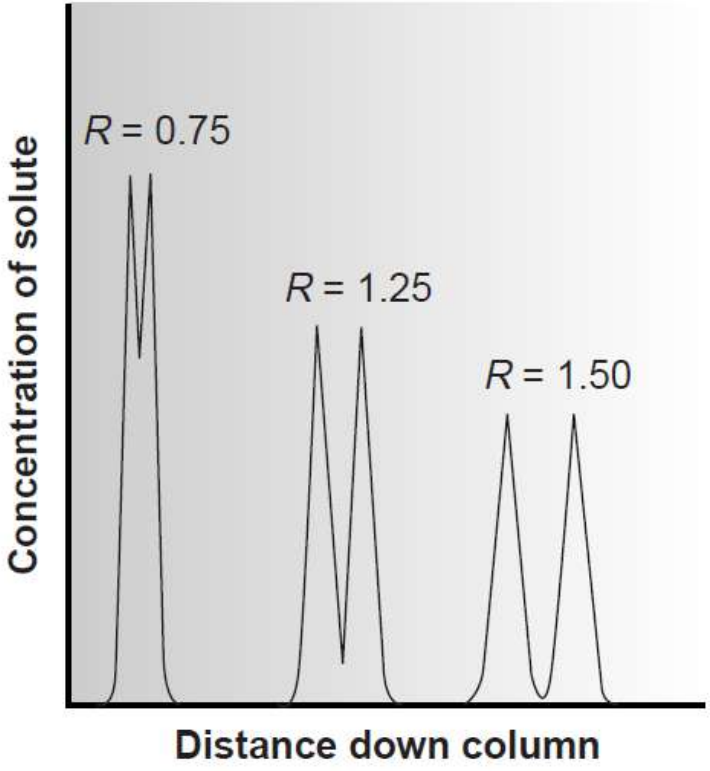
$$t'_R = t_R - t_M$$

baseline width-the width of a solute's chromatographic band measured at the baseline (*w).*

Chromatographic Resolution

Resolution is a quantitative measure of the degree of separation between two chromatographic peaks, A and B.

The degree of separation between two chromatographic peaks improves with an increase in *R. For two peaks of equal size, a resolution of* 1.5 corresponds to an overlap in area of only 0.13%. Because resolution is a quantitative measure of a separation's success, it provides a useful way to determine if a change in experimental conditions leads to a better separation.



$$R = \frac{t_{r,B} - t_{r,A}}{0.5(w_B + w_A)} = \frac{2\Delta t_r}{w_B + w_A}$$

retention factor, k - it is the ratio of the time the solute spends in the stationary phase to the time it spends in the mobile phase:

$$k = \frac{t'_R}{t_M}$$

The retention factor is an important experimental parameter that is widely used to compare the migration rates of solutes on columns.

A retention factor much less than unity means that the solute emerges from the column at a time near that of the void time. When retention factors are larger than perhaps 20 to 30, elution times become inordinately long. Ideally, separations are performed under conditions in which the retention factors for the solutes of interest in a mixture lie in the range between 1 and 5.

Capacity Factor

The distribution of a solute, S, between the mobile phase and stationary phase can be represented by an equilibrium reaction

$S_m \rightleftarrows S_s$

and its associated partition coefficient, *K_D, and distribution ratio, D,*

$$K_D = \frac{[S_s]}{[S_m]} \qquad D = \frac{[S_s]_{tot}}{[S_m]_{tot}}$$

where the subscripts m and s refer to the mobile phase and stationary phase, respectively. As long as the solute is not involved in any additional equilibria in either the mobile phase or stationary phase, the equilibrium partition coefficient and the distribution ratio will be the same.

$$f_m = \frac{(\text{moles S})_m}{(\text{moles S})_{tot}} = \frac{V_m}{V_m + DV_s}$$

$$k' = D\frac{V_s}{V_m}$$

fraction of solute in the mobile phase, *f_m,*

capacity factor
A measure of how strongly a solute is retained by the stationary phase (*k'*).

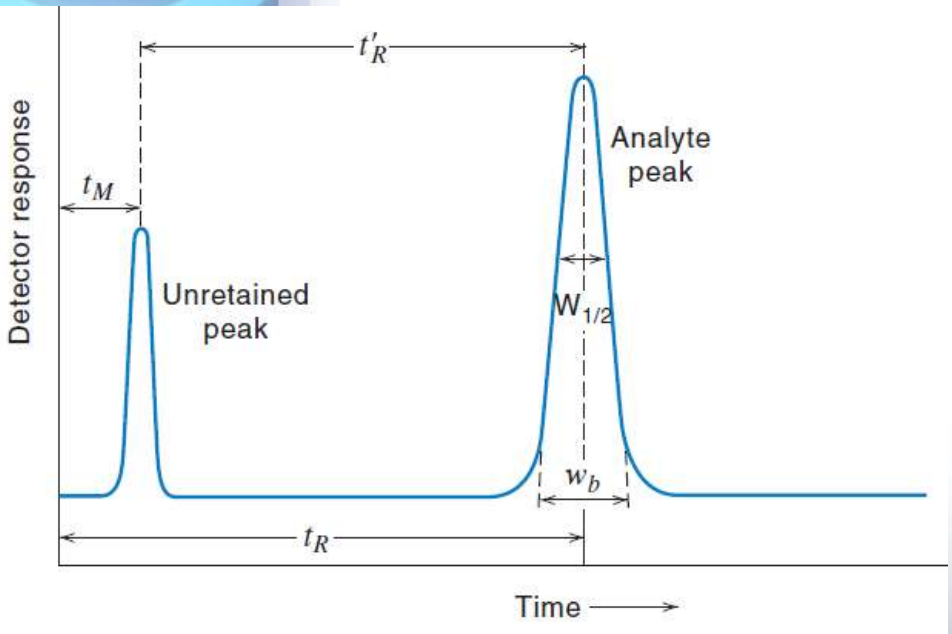
Theory of Column Efficiency in Chromatography

The separation efficiency of a column can be expressed in terms of the number of theoretical plates in the column. A theoretical plate is a concept derived from distillation theory, whereby each theoretical plate in chromatography can be thought of as representing a single equilibrium step, such as in our Excel simulations. They are a measure of the efficiency or resolving power of a column; the more the number of plates, the more efficient is the column. The **plate height, *H,*** is the length of a column, *L, divided by the number of theoretical plates, N:*

$$H = \frac{L}{N}$$

$$N = \left(\frac{t_R}{\sigma}\right)^2$$

$$N = 5.545\left(\frac{t_R}{w_{1/2}}\right)^2$$



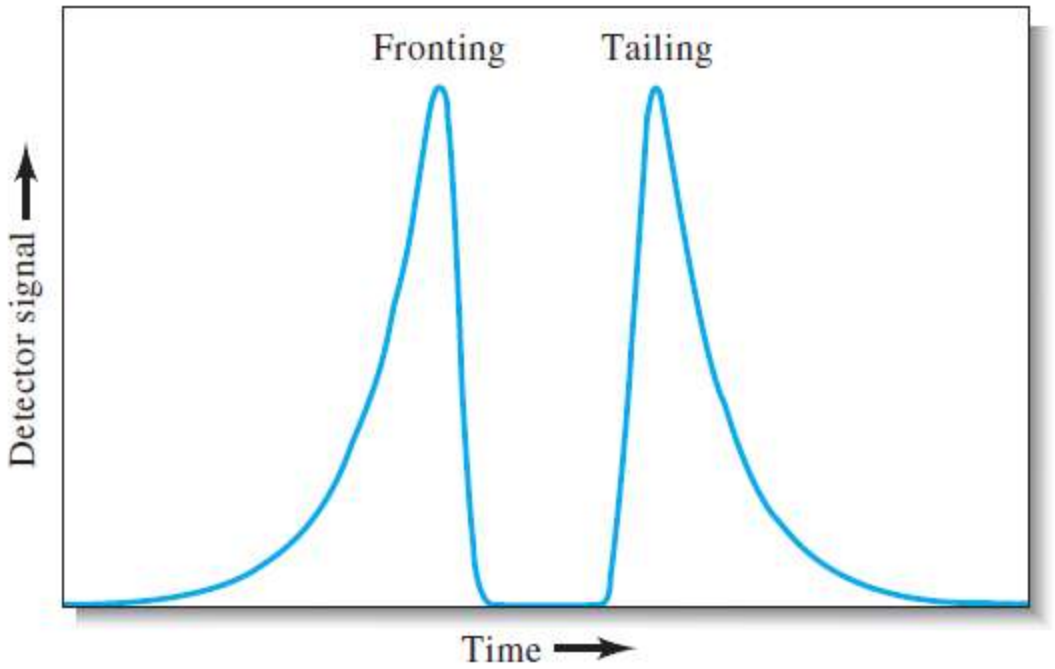
$$N = 16\left(\frac{t_R}{w_b}\right)^2$$

Martin and Synge first proposed the '**plate theory'** in 1941, whereby they merely compared the GC separation to fractional distillation. Thus, the 'theoretical' plate is the portion of the column wherein the solute is in complete equilibrium with the mobile and the stationary phase. *H is the so-called height equivalent of a theoretical plate (plate height) or* HETP value. The quantity *H (equal to L/N) measures the efficiency of a given* column (operated under a specific set of operating conditions) per unit length of column. Small *H values mean more efficient columns and large N values. A* central goal in LC practice is the attainment of small *H values for maximum N* and highest column efficiencies. Therefore, large *N values and improved separation are usually favored by long* columns packed with small particles, by nonviscous mobile phases flowing relatively slowly through the column, and by higher separation temperatures.

As the '*Plate Theory' has two serious limitation, viz., first: it does not speak of the separating power of* a definite length of column, and second: it does not suggest means of improving the performance of the column; the 'Rate Theory' has been introduced which endeavours to include the vital fact that-'the mobile-phase flows continuously, besides the solute molecules are constantly being transported and partitioned in a gas chromatographic column'.

Nonideal Behavior

The treatment of chromatography outlined assumes that a solute elutes as a symmetrical band. This ideal behavior occurs when the solute's partition coefficient, K_D, *is constant for all concentrations* of solute. In some situations, chromatographic peaks show nonideal behavior, leading to asymmetrical peaks. The chromatographic Peak is an example of "**fronting"** and is most often the result of overloading the column with sample. An example of "**tailing,"** occurs when some sites on the stationary phase retain the solutes more strongly than other sites.

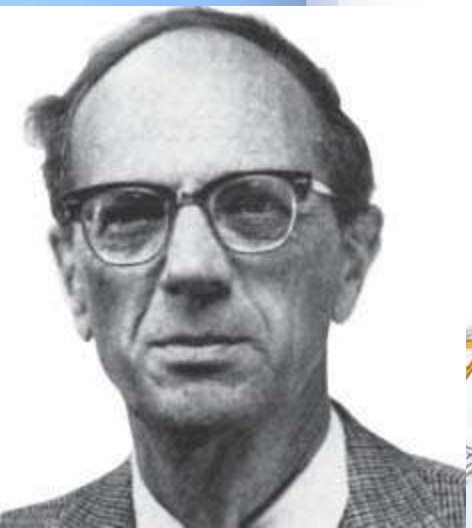


RATE THEORY OF CHROMATOGRAPHY-THE VAN DEEMTER EQUATION

The plate theory of chromatography and equilibrium models cannot explain the dynamics of separation in a quantitative fashion. How might the separation efficiency change, for example, if we double the mobile phase flow? The van Deemter equation is the best known and most used to explain and determine conditions for efficient separations.

J. J. van Deemter, the man who, with his colleagues at Royal Dutch Shell, developed the famous equation describing the efficiency of chromatography columns that bears his name.

$$H = A + \frac{B}{\overline{u}} + C\overline{u}$$



where the constants *A, B, and C are* coefficients of multiple path effects, longitudinal diffusion, and mass transfer, respectively. Today, we consider the van Deemter equation to be appropriate only for packed columns at high flow velocities.

RATE THEORY OF CHROMATOGRAPHY-THE VAN DEEMTER EQUATION

$$H = A + \frac{B}{\overline{u}} + C\overline{u}$$

•u –average linear mobile phase velocity

•A –represents the contribution to zone broadening by eddy diffusion

•B –represents the contribution of longitudinal diffusion

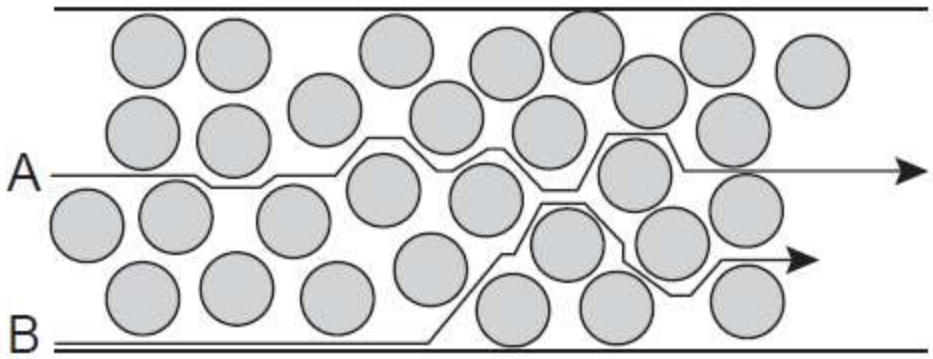
•C –represents the contribution of resistance to mass transfer in both the stationary and mobile phases.

•Eddy Diffusion –(A term) –results from the inhomogeneity of flow velocities and path lengths around the packing particles (individual flow paths for packed columns are of different lengths).

•Small uniformly packed particles in columns are the most efficient and A is very small.

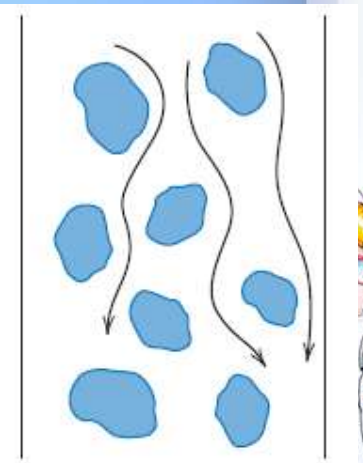
•A is zero for open tubular columns.

$$H = \frac{B}{u} + C_S u + C_M$$



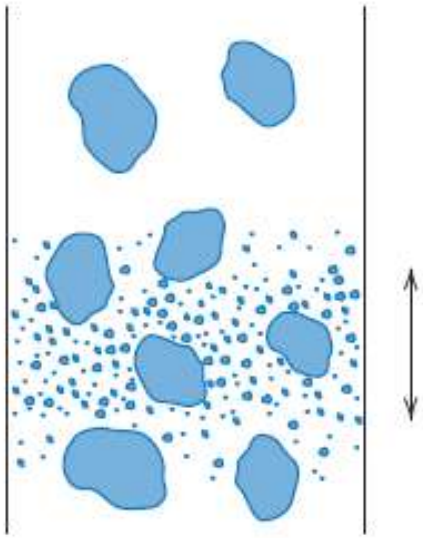
Multiple Paths

Solute molecules passing through a chromatographic column travel separate paths that may differ in length. Because of these differences in path length, solute molecules injected simultaneously elute at different times. The principal factor contributing to this variation in path length is a nonhomogeneous packing of the stationary phase in the column. Differences in particle size and packing consistency cause solute molecules to travel paths of different length. Some solute molecules follow relatively straight paths through the column, but others follow a longer, more tortuous path.



Eddy diffusion.

eddy diffusion *and is due to the variety of tortuous (variable-length)* pathways available between the particles in the column and is independent of the gas- or mobile-phase velocity. It is minimized by using small and uniform particles, packing them well. However, gas chromatography is used at modest pressures, and very fine tightly packed supports are not used.



Molecular diffusion.

The Longitudinal Diffusion Term, B/u.

Diffusion is a process in which species migrate from a more concentrated part of a medium to a more dilute region. The rate of migration is proportional to the concentration difference between the regions and to the **diffusion coefficient** D_M of the species. The latter, which is a measure of the mobility of a substance in a given medium, is a constant for a given species equal to the velocity of migration under a unit concentration gradient.

In chromatography, longitudinal diffusion results in the migration of a solute from the concentrated center of a band to the more dilute regions on either side (that is, toward and opposed to the direction of flow). Longitudinal diffusion is a common source of band broadening in gas chromatography where the rate at which molecules diffuse is high. The phenomenon is of little significance in liquid chromatography where diffusion rates are much smaller.

Longitudinal diffusion along the axis of the column results in zone broadening.

•ß = 2gDm.

•Obstructive factor, g–is unity for coated capillary columns. Longitudinal diffusion is hindered by packing.

•Dm–solute diffusion coefficient in the mobile phase.

•Diffusion rate depends on temperature and pressure of the mobile phase.

•Dm decreases with decreasing temperature and increasing pressure.

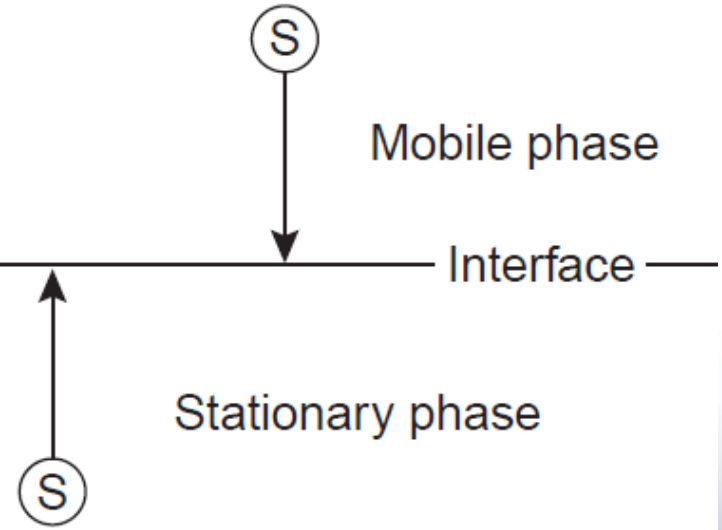
•LC has a much lower β than GC since diffusion rates are much larger in gases.

•So gases of higher MW's are favored as mobile phases since diffusion is lower.

•As mobile phase velocity increases β becomes less.

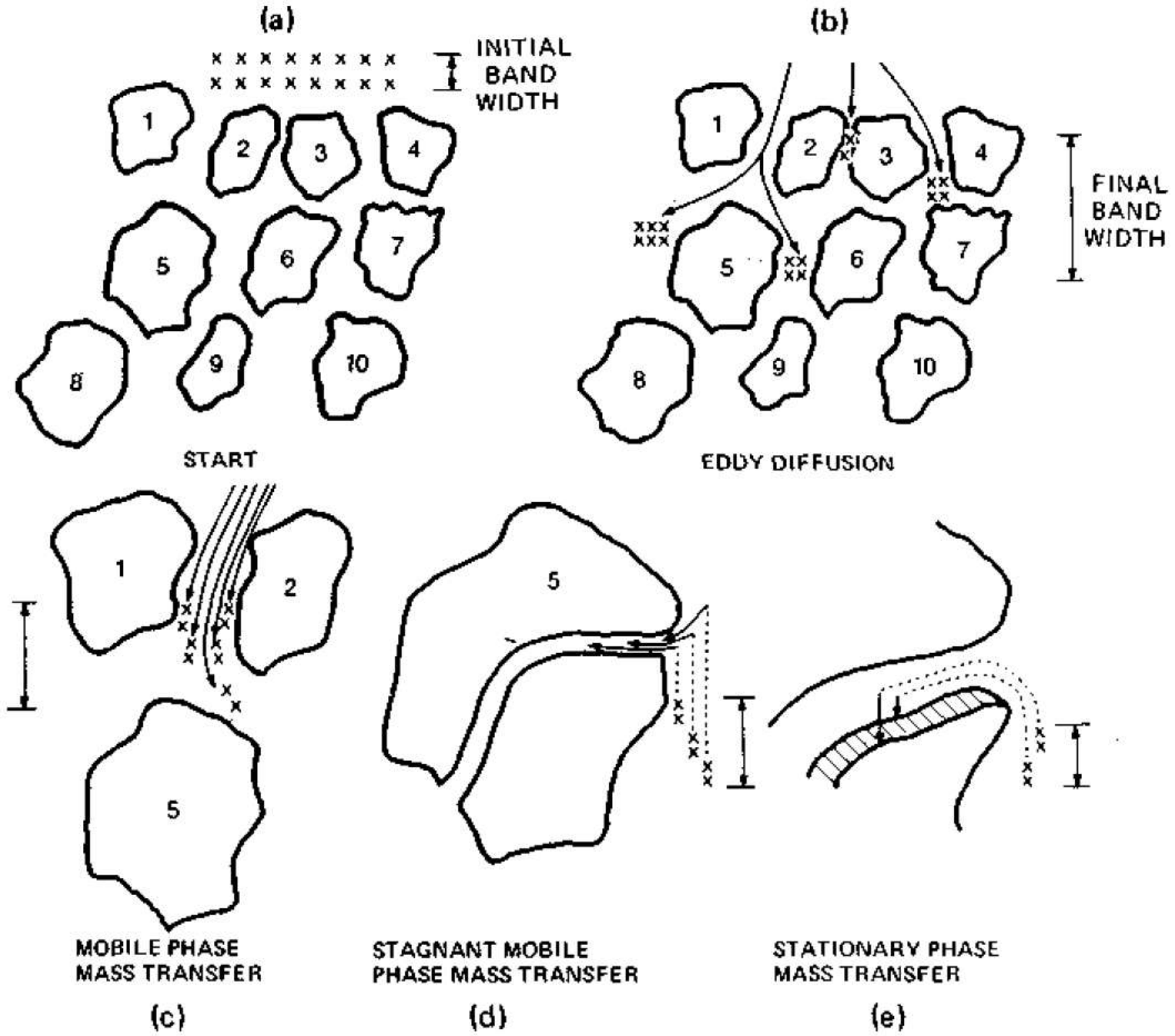
Mass Transfer

The final two contributions to band broadening result from the finite time required for a solute molecule to diffuse through the stationary phase and mobile phase. A chromatographic separation occurs because solutes move between the stationary and mobile phases. For a solute to move from one phase to the other, it must first diffuse to the interface between the two phases—a process called **mass transfer.** A contribution to band broadening occurs whenever the solute's movement to the interface is not fast enough to maintain a true equilibrium distribution of solute between the two phases. Thus, solute molecules in the mobile phase move farther down the column than expected before passing into the stationary phase. Solute molecules in the stationary phase, on the other hand, take longer than expected to cross into the mobile phase.

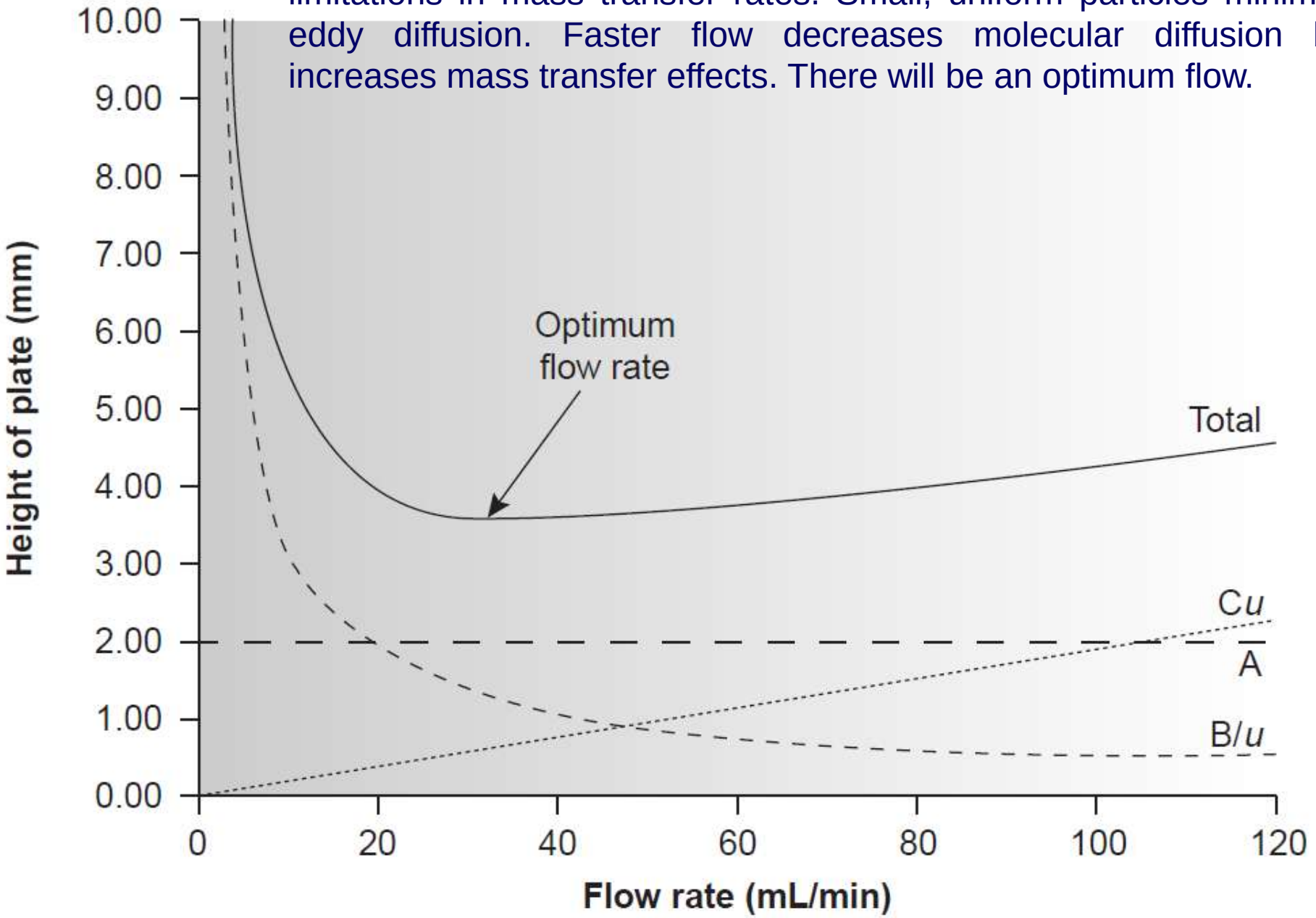


Mass Transfer –(C term) –most important term in GC, LC, and SFC.

- For a stationary phase –Csis small for solid phases (since transfer of analyte on and off a surface is rapid), but is a factor for liquid stationary phases.
- C for liquids depends on thickness of the film, the diffusion coefficient of the analyte in the stationary phase and geometric nature of the packing.
- For a mobile phase –Cm–depends on the capacity factor of the analyte and the particle diameter of the stationary phase (packed) or internal diameter of the column (open tubular). Efficiency increases as particle size or column diameter decreases.


(a)
INITIAL BAND WIDTH
1
2
3
4
5
6
7
8
9
10
START
(b)
FINAL BAND WIDTH
EDDY DIFFUSION
MOBILE PHASE MASS TRANSFER
(c)
STAGNANT MOBILE PHASE MASS TRANSFER
(d)
STATIONARY PHASE MASS TRANSFER
(e)

Peaks are broadened by eddy diffusion, molecular diffusion, and limitations in mass transfer rates. Small, uniform particles minimize eddy diffusion. Faster flow decreases molecular diffusion but increases mass transfer effects. There will be an optimum flow.



Summary of Methods for Reducing Band Broadening.

For packed columns, one variable that affects column efficiency is the diameter of the particles making up the packing. For capillary columns, the diameter of the column itself is an important variable.

With gaseous mobile phases, the rate of longitudinal diffusion can be reduced appreciably by lowering the temperature and thus the diffusion coefficient.

The result is significantly smaller plate heights at lower temperatures.

This effect is usually not noticeable in liquid chromatography because diffusion is slow enough that the longitudinal diffusion term has little effect on overall plate height.

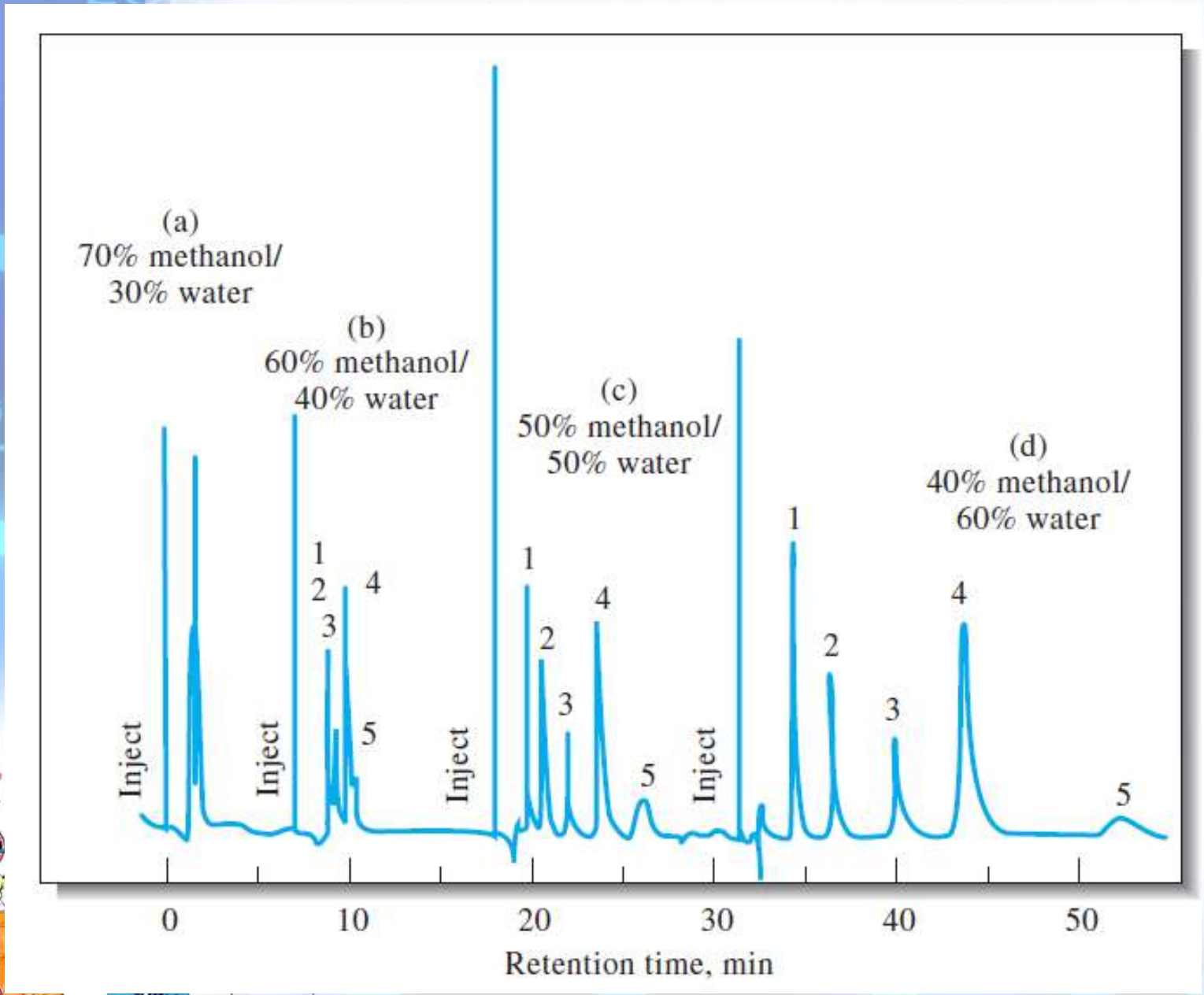
The resolution of a column improves as the square root of the number of plates increases. However, that increasing the number of plates is expensive in terms of time unless the increase is achieved by reducing the plate height and not by increasing column length.

Methods for minimizing plate height, include reducing the particle size of the packing material, the diameter of the column, and the thickness of the liquid film. Optimizing the flow rate of the mobile phase is also helpful.

capillary column

A narrow bored column that usually does not contain a particulate packing material.

Effect of solvent variation on chromatograms.



Optimization of Chromatographic Performance

Resolution Equation

$$R = \tfrac{1}{4}\sqrt{N} \times (k/(k+1)) \times (\alpha-1)$$

Efficiency Retention Factor Selectivity

- The first part relates to the kinetic effects that lead to band broadening … $(N)^{1/2}$ or H/u.
- The second and third terms are related to the thermodynamics of the separation.

Second term which contains k, depends on the properties of both the solute and the column.

Third term which contains, a, the selectivity term, depends on the properties of the mobile and stationary phases.

- The parameters N (or H), k, α, can be adjusted.

α and k can be varied by varying the temperature or composition of the mobile phase. Or a different column packing can be used.

- N can be changed by altering the length of the column.
- H can be changed by altering the flow of the mobile phase, the particle size of the packing, the viscosity of the mobile phase, and thickness of the liquid stationary phase.

For liquid chromatography, variations in *k are brought about by varying the* composition of the mobile phase during elution. Such a procedure is called **gradient elution or solvent programming.** Elution under conditions of constant mobile-phase composition is called **isocratic elution.** For gas chromatography, 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.

Applications of Chromatography

Chromatography is a powerful and versatile tool for separating closely related chemical species. In addition, it can be used for the qualitative identification and quantitative determination of separated species.

Objectives

•Qualitative Applications

Confirm presence or absence of compounds in samples. Screening unknowns.

•Quantitative Applications

Establish the amount of individual components in a sample by comparing with standards used for quality control.

•Preparative Applications

Purifying samples.

Important Chromatographic Quantities and Relationships

Name	Symbol of Experimental Quantity
Migration time, unretained species	t_M
Retention time, species A and B	$(t_R)_A$, $(t_R)_B$
Adjusted retention time for A	$(t'_R)_A$
Peak widths for A and B	W_A, W_B
Length of column packing	L
Volumetric flow rate	F
Linear flow velocity	u
Stationary-phase volume	V_S
Concentration of analyte in mobile and stationary phases	c_M, c_S

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	$k = \frac{t_R - t_M}{t_M}$	$k = \frac{K V_S}{V_M}$
Distribution constant	$K = \frac{k V_M}{V_S}$	$K = \frac{c_S}{c_M}$
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	$(t_R)_B = \frac{16R_s^2 H}{u}\left(\frac{\alpha}{\alpha - 1}\right)^2 \frac{(1 + k_B)^3}{(k_B)^2}$