CHEMISTRY 5570

Advanced Analytical Chemistry

Lecture 12

Chromatography

IUPAC Definition of Chromatography

A method, used primarily for separation of the components of a sample in which the components are distributed between two phases, one of which is stationary while the other moves. The stationary phase may be a solid, liquid supported on a solid, or a gel. The stationary phase may be packed in a column, spread as a layer, or distributed as a film. The mobile phase may be gaseous or liquid.

[Notice that the definition neglects supercritical fluid chromatography (SFC)].

Chromatography

General Principles: Classification Methods

- **Classify by Mobile Phase**
  - **Three areas:**
    - **Liquid Chromatography (LC)**
    - **Gas Chromatography (GC)**
    - **Supercritical Fluid Chromatography (SFC)**

Further classify by specifying stationary phase, i.e. gas-solid, gas-liquid, etc.

For LC:

A nonpolar mobile phase with polar stationary phase is called normal-phase chromatography.

A polar mobile phase with nonpolar stationary phase is called reversed-phase chromatography.

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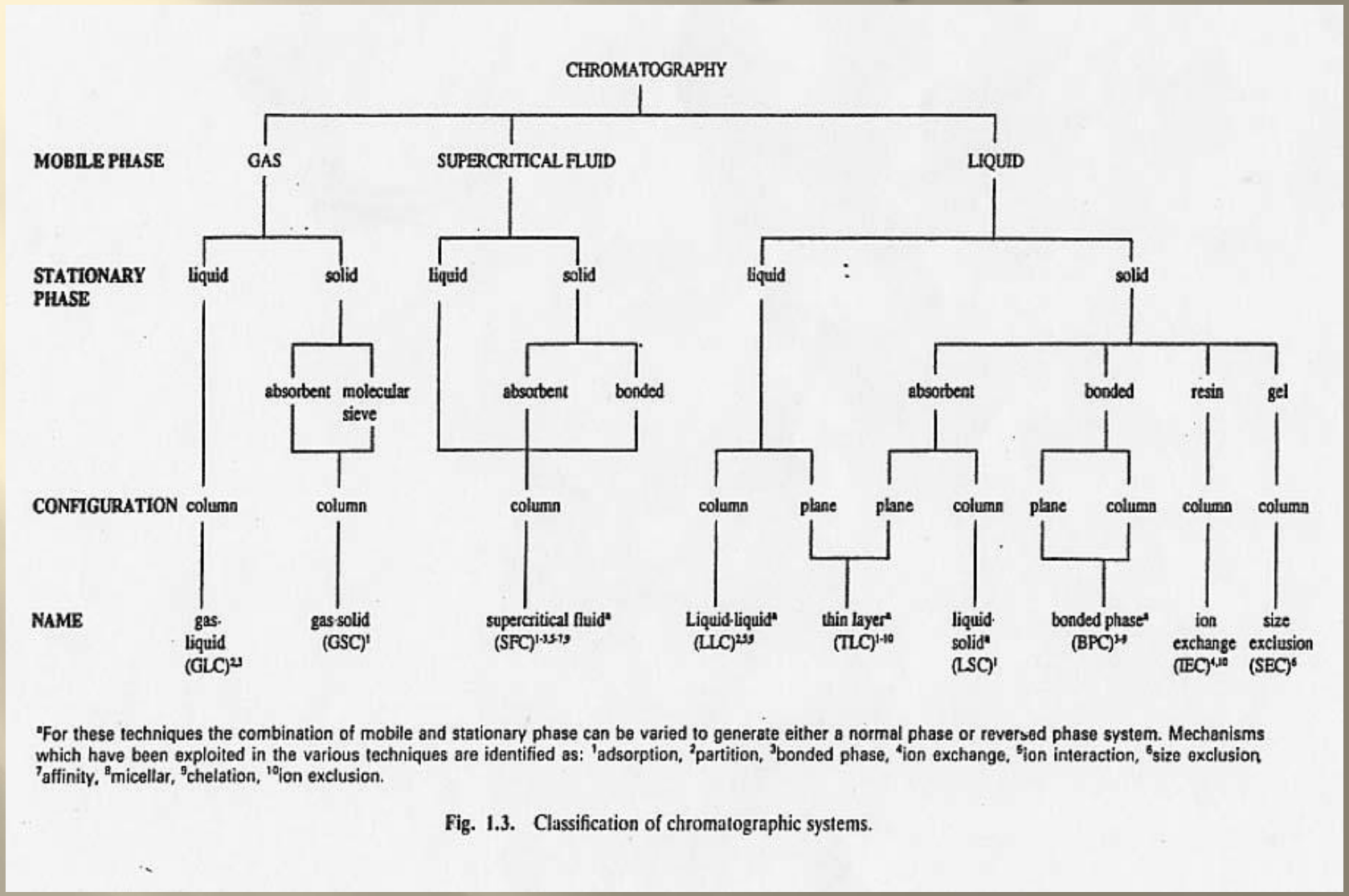

[a]For these techniques the combination of mobile and stationary phase can be varied to generate either a normal phase or reversed phase system. Mechanisms which have been exploited in the various techniques are identified as: [1]adsorption, [2]partition, [3]bonded phase, [4]ion exchange, [5]ion interaction, [6]size exclusion, [7]affinity, [8]micellar, [9]chelation, [10]ion exclusion.

Fig. 1.3. Classification of chromatographic systems.

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Separation Mechanism

The nature of interaction between sample components and the two phases form a further basis of classification.

Reflects the forces involved which are usually weak such as van der Waals or hydrogen bonding, ionic interactions, etc…

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Separation Mechanism

Adsorption – solute and mobile phase molecules compete for active sites on the surface of the solid stationary phase (the adsorbent).

Partition – partitioning of a solute between two immiscible liquids but one liquid is held stationary on a solid support. A solute in contact with two immiscible liquids will distribute itself between them according to its distribution coefficient, K.

Ion-exchange

Size exclusion – physical sieving process (gel permeation is a size exclusion process).

Affinity – stationary phase is a bioactive liquid bonded to solid support. Antibody-antigen interactions, chemical, chiral, etc.

Micellar or pseudophase – modifier added to mobile phase – surfactants.

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Qualitative Analysis

In general, chromatography is a blind method.

It indicates the presence of a substance but not what it is.

Qualitative data can also be obtained even with non-discriminating detectors.

While the detectors used for GC and LC are not the same, quantitative methods are identical.

Each detector will produce a response/unit concentration.

This is substance dependent so standards must always be used.

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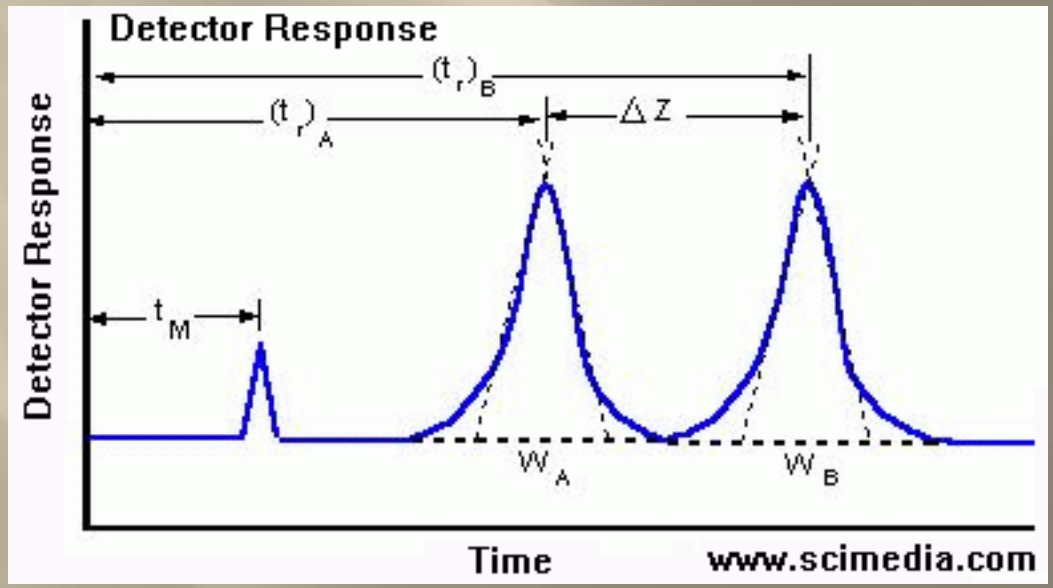
Peak Measurements

Each method of quantitation assumes you have one or more reasonably resolved peaks.

Need to identify baseline as well as beginning and end of peak.



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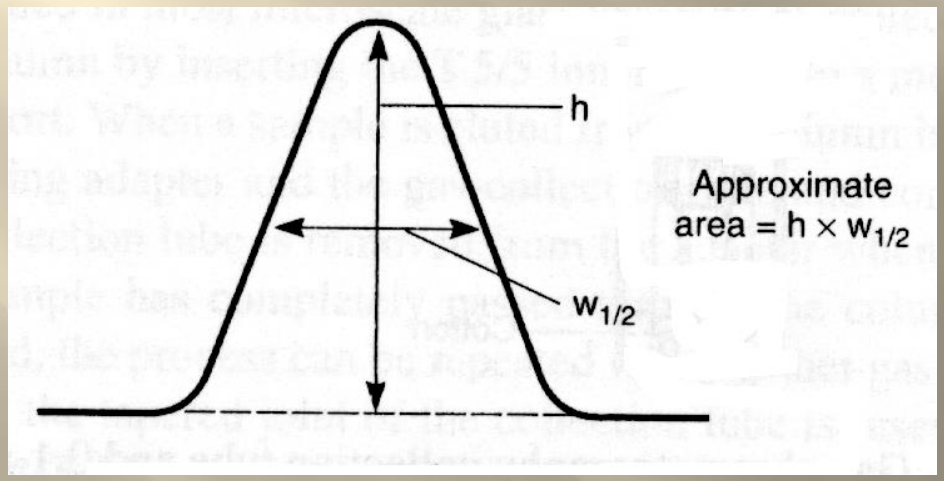
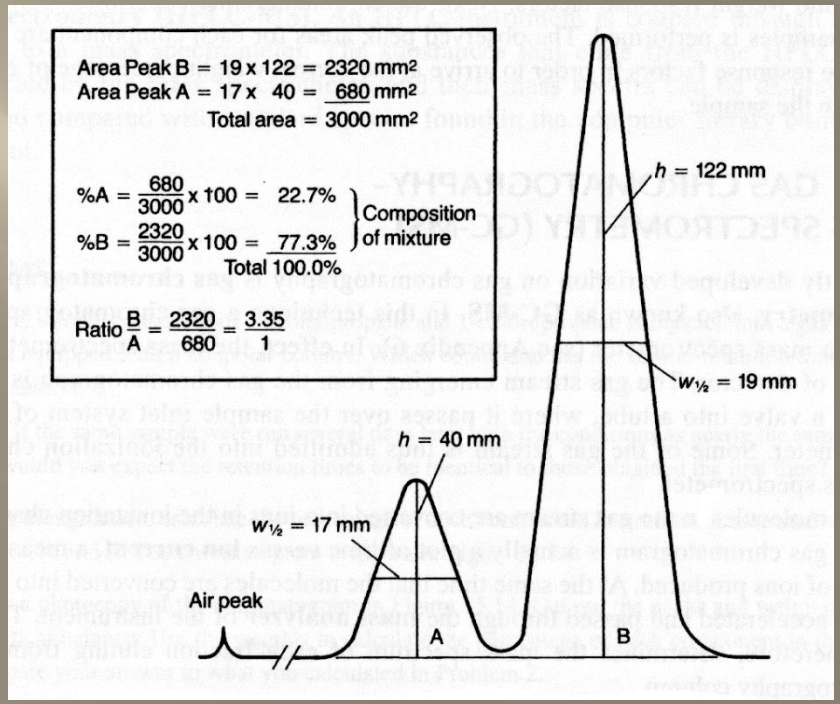
Peak Height

In some cases, can assume peak height is proportional to concentration. (typically only used with capillary columns)

Advantage: simple and rapid
Disadvantage: height is more variable than area





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Peak Area

This is the major approach for establishing a relationship between peaks and concentration.

$$\text{area} \propto \text{concentration}$$

Area is determined from a large number of measurements and detectors usually have very large dynamic ranges.

This results in a reliable method of measurement.

Once you have the peak areas – a relationship between concentration and area must be established.

Several approaches can be used – depending on the chromatographic experiment.

Methods:

External standard method

Internal standard method

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External Standard Method

Requirements for proper use:

Standard solution contains all eluents to be quantified.

Standard eluents should be of similar concentration as unknowns.

The standard and sample matrix should be similar as possible.

Analysis conditions must be identical – stable instrumental conditions, same sample size, etc...

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External Standard Method

Assume response is linear over entire concentration range or measure it. Use same injection volume for both unknown and standard.

$$[Analyte] = \frac{Area_{Analyte}}{Area_{Std}} [\text{Std}]$$

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External Standard Method

Example:
Determination of X in $MeCl_2$.
Prepare a standard of X: 20 mg in 100 mL $MeCl_2$ (0.200 ug/uL).
Use same injection volume for both of 5 uL.

Measure the areas produced by both the analyte and standard.

$Area_{Std}$ = 2000 units
$Area_{Unk}$ = 3830 units

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External Standard Method

Now determine the [X] in unknown.

$$[Analyte] = \frac{Area_{Analyte}}{Area_{Std}} [\text{Std}]$$

$$[Analyte] = \frac{3830}{2000} \text{ x } 0.200 \text{ ug/uL} = 0.384 \text{ ug/uL}$$

Can convert to whatever units needed.

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Internal Standard Method

Overall, the most reliable approach.

Basis

A known substance is added at a constant concentration to all standards and samples – internal standard.

Since the internal standard is always present at a constant amount, it can be used to account for variations such as injection volume during an analysis.

Requirements for an internal standard:

-Must be present at a constant concentration in all samples and standards.

-Must be stable and measurable under the analysis conditions.

-Must not interfere with the analysis or co-elute with sample components.

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Internal Standard Method

Three common approaches are used:

Classical method – weighed portions of the standard and sample are combined.

Stock solution – a known volume of the sample is "spiked" with a known volume of the standard.

Calibration plot – a series of standards are run and a curve plotted based on corrected peak areas.

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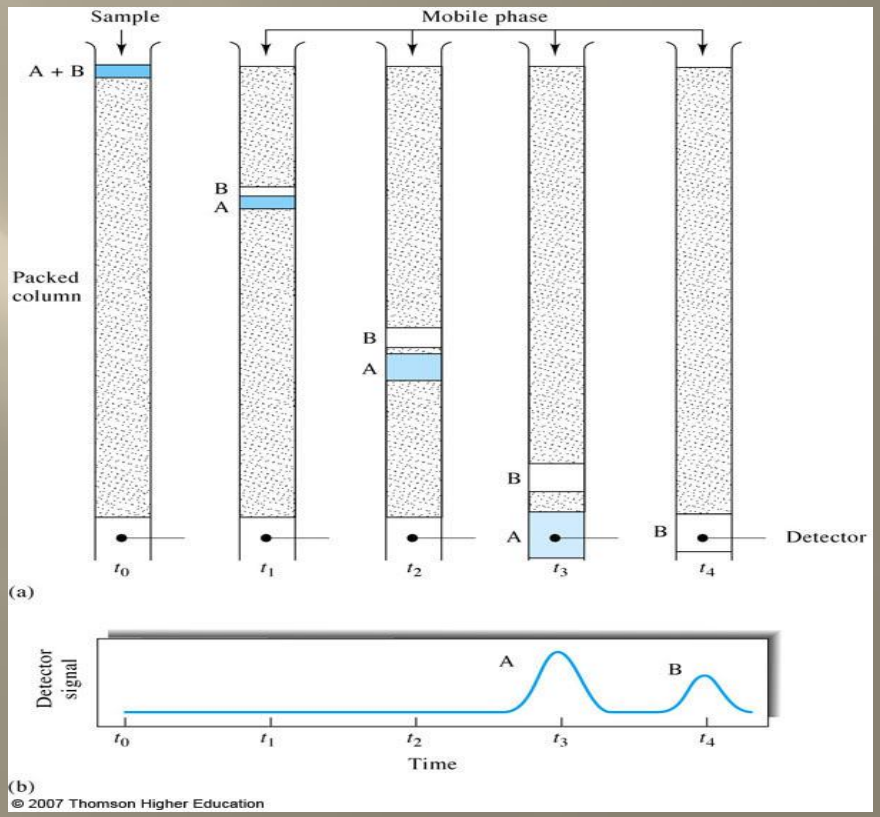
Elution

A solute partitions between two phases (equilibrium).

Separation is based on relative retention.

Making the column longer will increase the degree of separation.



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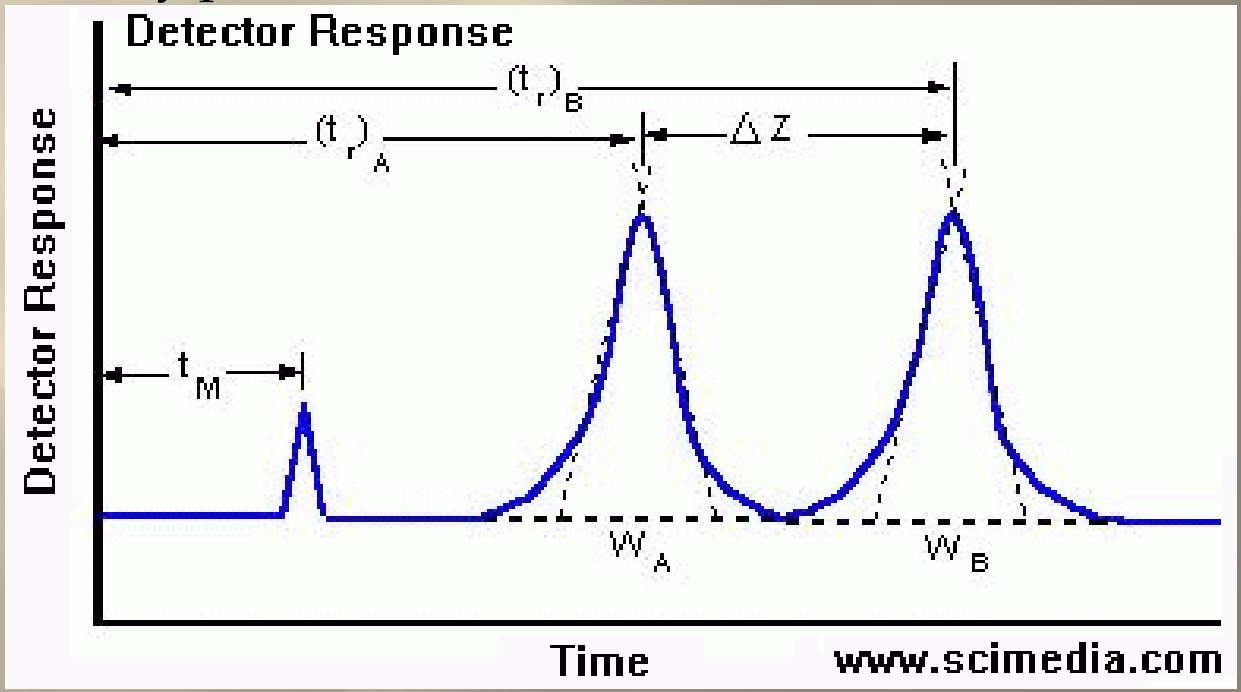
Elution

Reflects the distribution of a solute between the mobile and stationary phases.



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Notice the retention time an analyte spends in the system is composed of two components: tr′ and tm

tm - is the time it takes a solute to pass through the space occupied by the mobile phase (dead space, column void volume).

tm is the same for all analytes in a given system – the solutes migrate with the same velocity of the mobile phase.

tm represents no separation process.

The adjusted retention time, tr′- represents the time the analyte spends retained by the stationary phase.

tr′ represents the separation process or interaction of analyte with stationary phase.

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Partition Coefficient, Distribution Coefficient

K – concentration of the analyte in the stationary and mobile phase

$$K = \frac{C_s}{C_m}$$

Cs – concentration of the analyte in stationary phase

Cm – concentration of the analyte in mobile phase

K = 1 when the analyte is distributed equally

K is assumed to be independent of concentration but can be altered by such factors as temperature.

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Capacity Factor, Partition Ratio, k´ (Capacity Ratio)

k´ – solute partition ratio is an important parameter routinely used in HPLC but not as much in GC.

k´ – the ratio of the total amount of a solute in the stationary phase to the amount in the mobile phase at equilibrium.

$$k' = \frac{m_s}{m_m}$$

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Capacity Factor, Partition Ratio, k´ (Capacity Ratio)

This probability can be related to time, which is easier to measure.

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

In practice, accurate measure of column void volume is difficult in HPLC (difficult to find marker compounds), but for GC the practice is simple and accurate.

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Column Efficiency

One goal of chromatography is to achieve sharp symmetrical peaks, thus optimizing analyte separation and improving detection. The sharpness of the peak represents the efficiency of the chromatographic column (actually the entire system).

Two general approaches have been developed to measure column efficiency – plate theory and rate theory.

Plate theory

Proposed by Martin and Synge in 1941

Rate theory

Proposed by van Deemter in 1956

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Plate Theory

Models a chromatographic column as a series of narrow, discrete sections called theoretical plates.

Assume that at each plate equilibrium of the analyte is established between mobile and stationary phase.

Movement of analyte and mobile phase is viewed as a series of transfers from one plate to the next. Efficiency of a column increases as the number of equilibrations (i.e. theoretical plates) increases.

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Plate Theory

According to plate theory, a column is mathematically equivalent to a distillation plate column.

The total length is divided into segments each representing one equilibrium stage (or theoretical plate).

Equilibrium is established at each stage.

When a peak is produced – it can be used to determine the number of theoretical plates in a column.

Knowing the width and retention time of the peak (assuming Gaussian distribution), the number of plates can be determined.

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Plate Theory

Efficiency of a column increases as the number of equilibrations (i.e. theoretical plates) increases.

n - # of theoretical plates or plate number

n_{theor} – measures the column efficiency

$$n = 5.54\left(\frac{t_r}{w_{1/2}}\right)^2$$ (assumes a Gaussian peak)

$$n = 16\left(\frac{t_r}{w_b}\right)^2$$ n is a dimensionless quantity

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Plate Theory

Efficiency of a column increases as the number of equilibrations (i.e. theoretical plates) increases.

Example: Can have analytes that have same elution time but different number of plates.

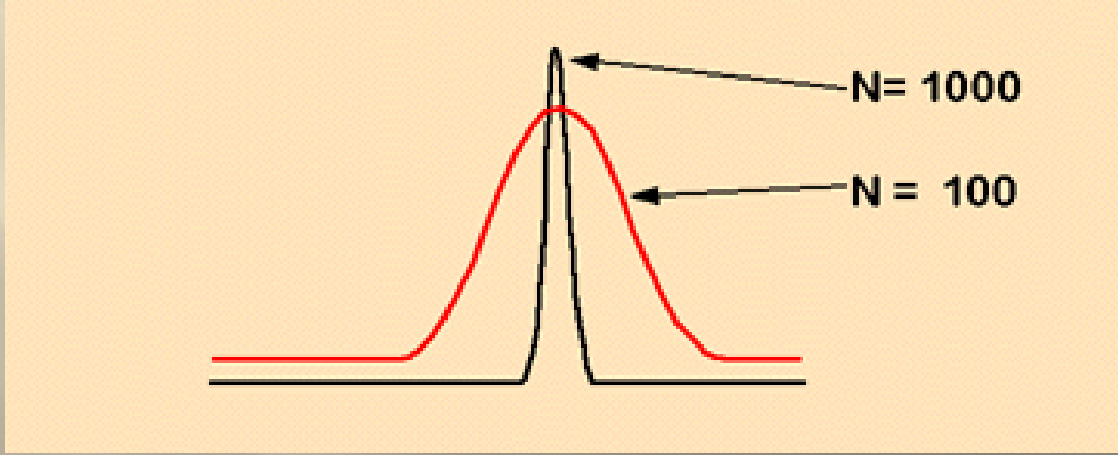

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Plate Theory

N or Neff or neff – Effective plate number or number of effective theoretical plates. Used especially if void volume is large or for early eluting peaks.

$$N = 5.54\left(\frac{t_r - t_m}{w_{1/2}}\right)^2 = 5.54\left(\frac{t_r'}{w_{1/2}}\right)^2$$

$$N = 16\left(\frac{(t_r - t_m)^2}{w_b}\right) = 16\left(\frac{t_r'}{w_b}\right)^2$$

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Plate Theory

If the peak is asymmetrical then calculation of N is more complex.

$$N = \frac{41.71(t_r / w_{0.1})^2}{A_s + 1.25}$$ (for asymmetrical peak)

As – asymmetry factor

$w_{0.1}$ – peak width at 10% of peak height

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Plate Theory

Plate number and effective plate number depend on the length of the column, so other parameters have been developed.

h or hetp or h_{etp} – the height equivalent to a theoretical plate or plate height

$$h = \frac{L}{n}$$

L – length of the column

H or HETP – the effective plate height or height equivalent to an effective theoretical plate.

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

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Plate Theory

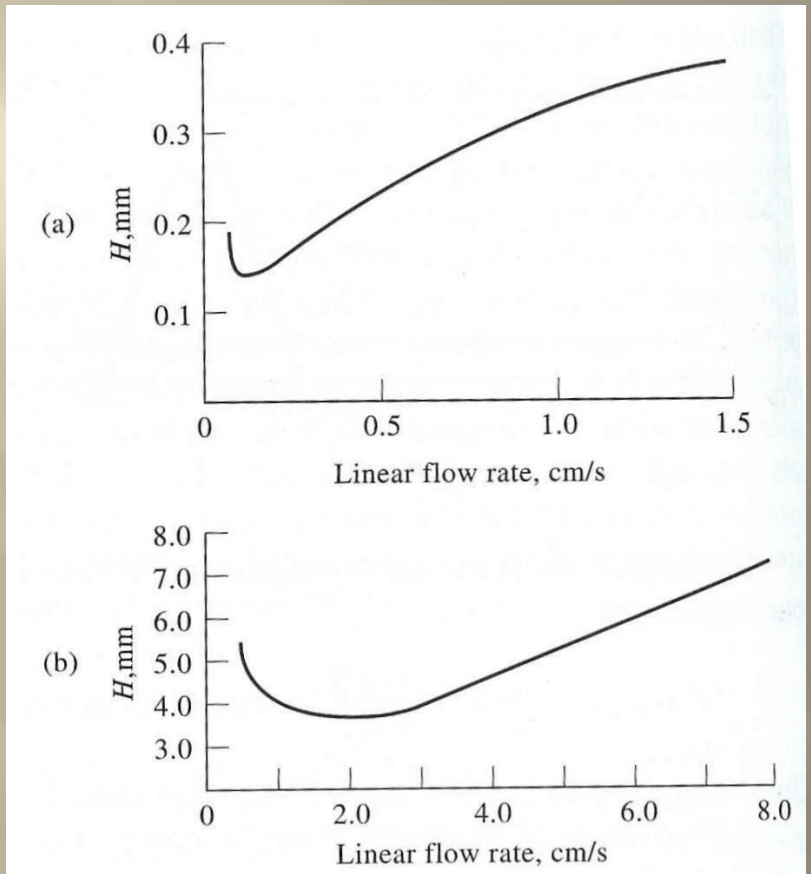

Figure 26-7 Effect of mobile-phase flow rate on plate height for (a) liquid chromatography and (b) gas chromatography.

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Rate Theory

Rate theory can be used to predict effects on column performance, i.e.

- Phase properties
- Phase thickness
- Solute diffusivities
- Support size
- Support porosity
- Partition coefficients
- Phase velocity
- Flow rates

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Rate Theory

Focuses on the contributions of various kinetic factors to zone or band broadening.

Column Dispensivity, H, is assumed to be the sum of the individual contributions of the kinetic factors.

Van Deemter set up the equation as:

$H = A + B/u + Cu$ (van Deemter equation)

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Rate Theory

$H = A + B/u + Cu$ (van Deemter equation)

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.

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Rate Theory

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.

$A = 2\lambda dp$ (λ – correction factor for the irregularity of the packing)

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Rate Theory

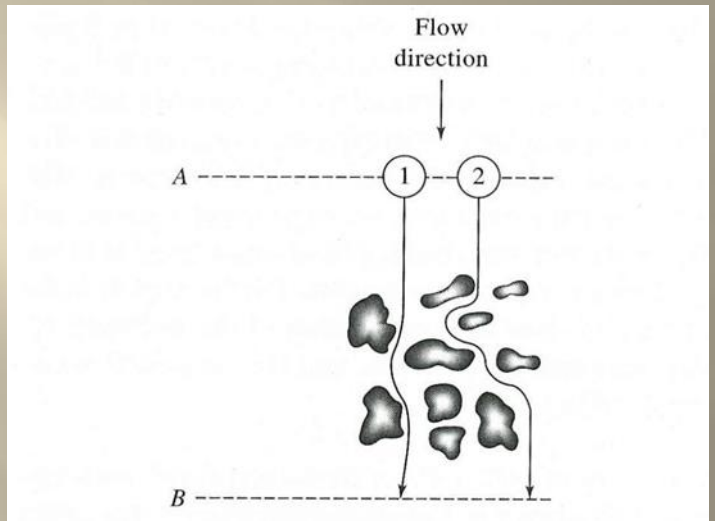
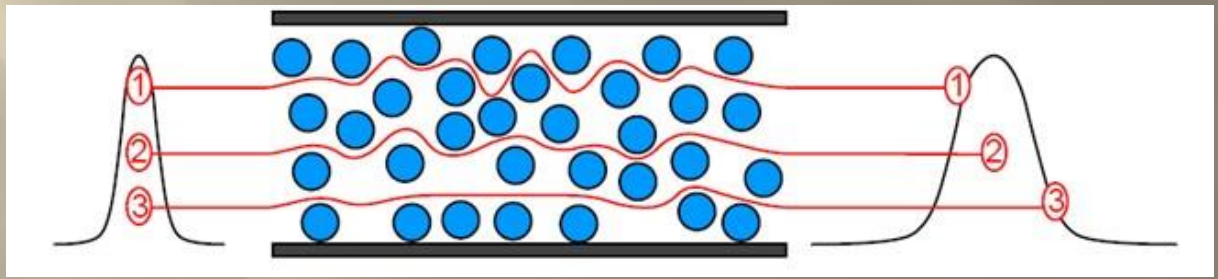


Figure 26-8 Typical pathways of two molecules during elution. Note that distance traveled by molecule 2 is greater than that traveled by molecule 1. Thus, molecule 2 would arrive at *B* later than molecule 1.



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Rate Theory

Longitudinal (molecular) Diffusion – (B term) – arises from the random molecular motion of analyte molecules in the mobile phase. Longitudinal diffusion along the axis of the column results in zone broadening.

$ß = 2\gamma D_m$.

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

D_m – solute diffusion coefficient in the mobile phase.

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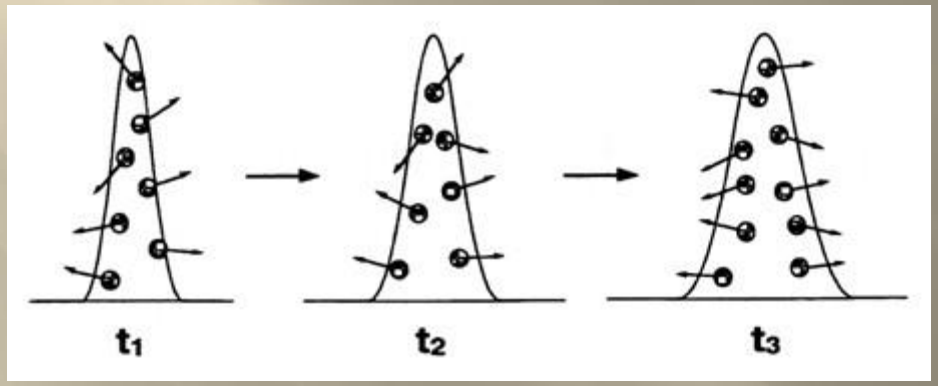
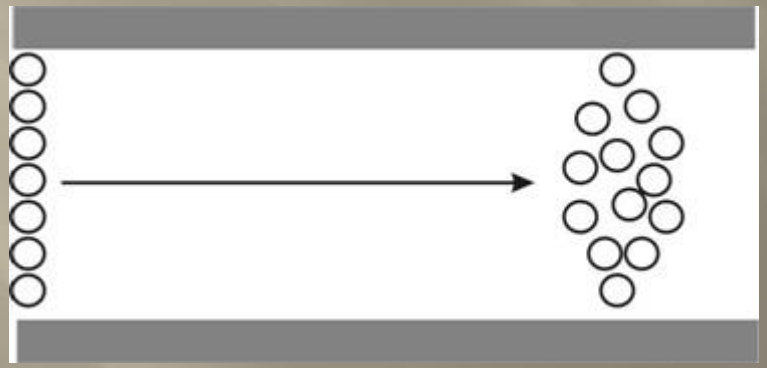
Rate Theory

Longitudinal (molecular) Diffusion – (B term)





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Rate Theory

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

D_m 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.

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Rate Theory

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

For a stationary phase – Cs is 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.

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Rate Theory

$$H = A + B/u + Cu = A + B/u + (Cs + Cm)\,u$$

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

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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	$C_M u$	$C_M u = \frac{f'(k) d_p^2}{D_M} u$

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Rate Theory

Goal is to find H_{min} from van Deemter plot

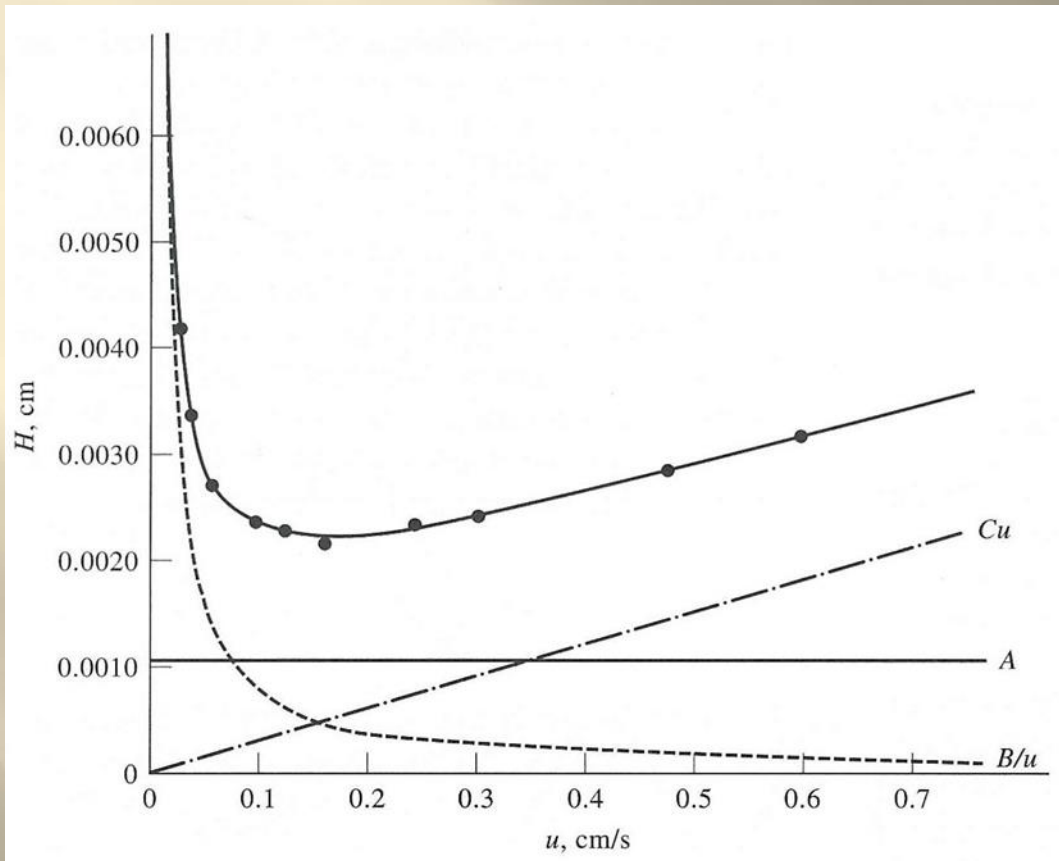

Figure 26-9 A van Deemter plot for a packed liquid chromatographic column. The points on the upper curve are experimental. The contributions of the various rate terms are shown by the lower curves: *A*, multipath effect; *B/u*, longitudinal diffusion; *Cu*, mass transfer for both phases. *(From E. Katz, K. L. Ogan, and R. P. W. Scott,* J. Chromatogr., ***1983,*** 270, *51. With permission.)*

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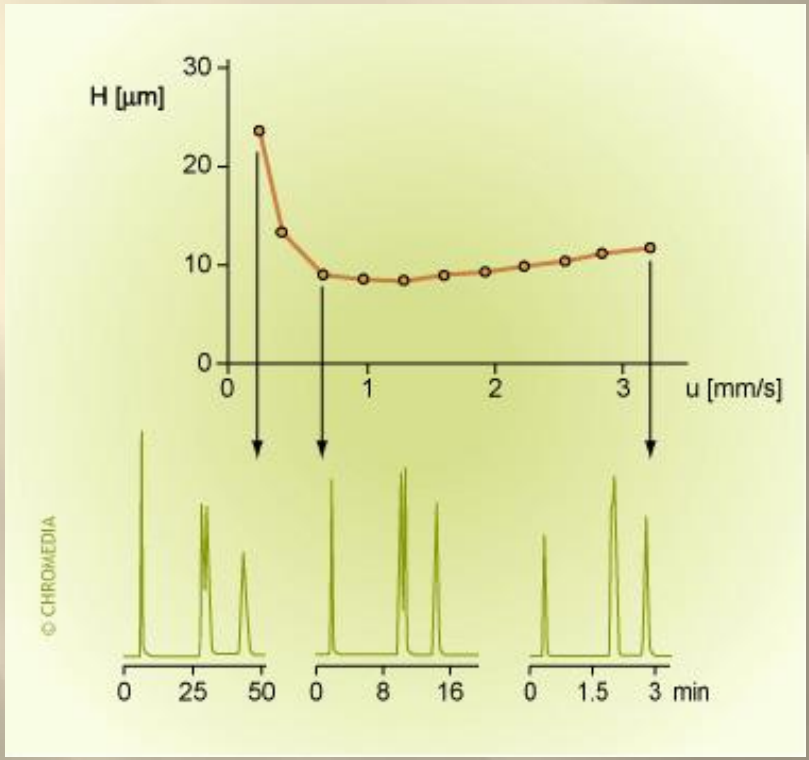
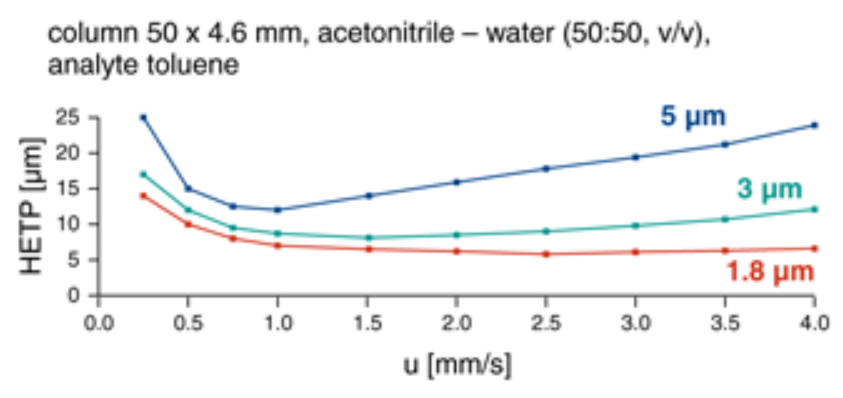
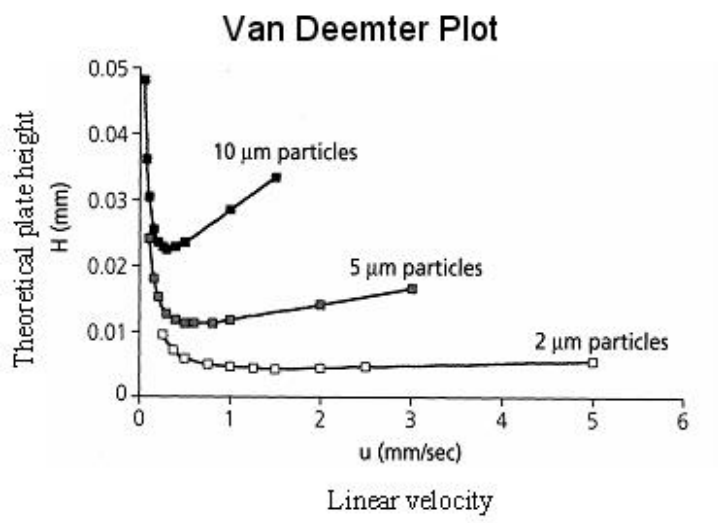
van Deemter plots - examples







Chromatography

Resolution

Knowing how well a column retains a solute is not enough – also need to deal with multiple eluents.

Need to measure Resolution, Rs. – or how well two neighboring peaks are separated from each other.

For symmetrical peaks – a good working equation:

$$R_s = \frac{(t_{r2} - t_{r1})}{\frac{1}{2}(w_{b1} + w_{b2})} \qquad R_s = 1.18\frac{(t_{r2} - t_{r1})}{w_{1/2(1)} + w_{1/2(2)}}$$

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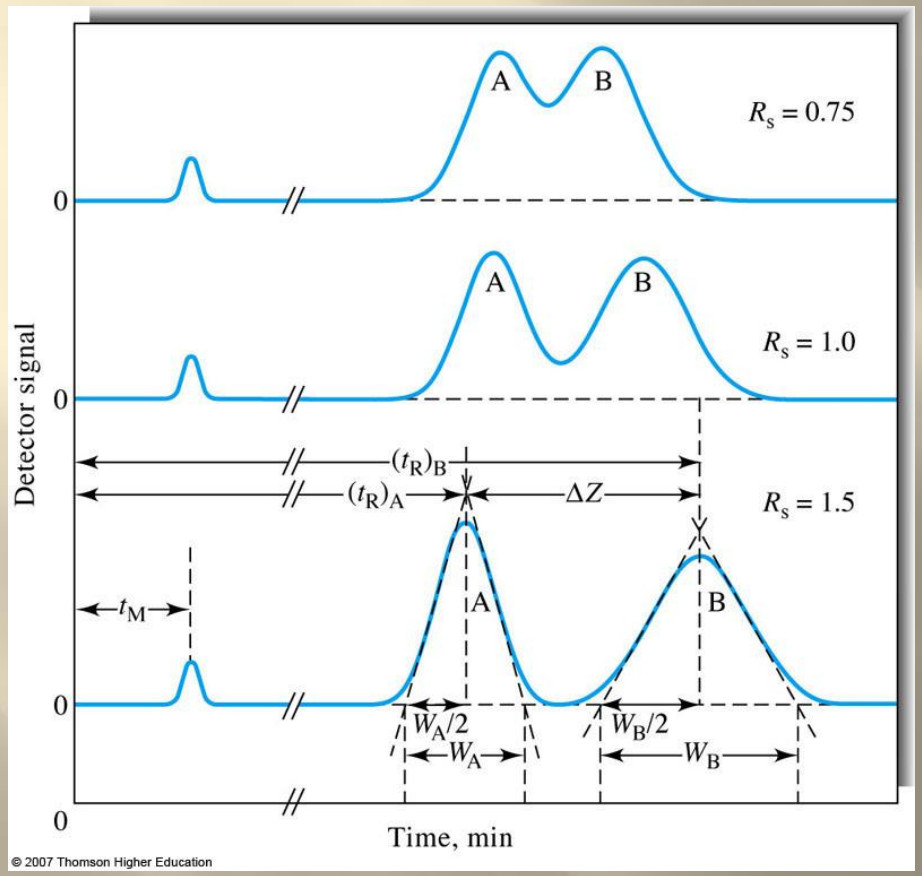
Resolution



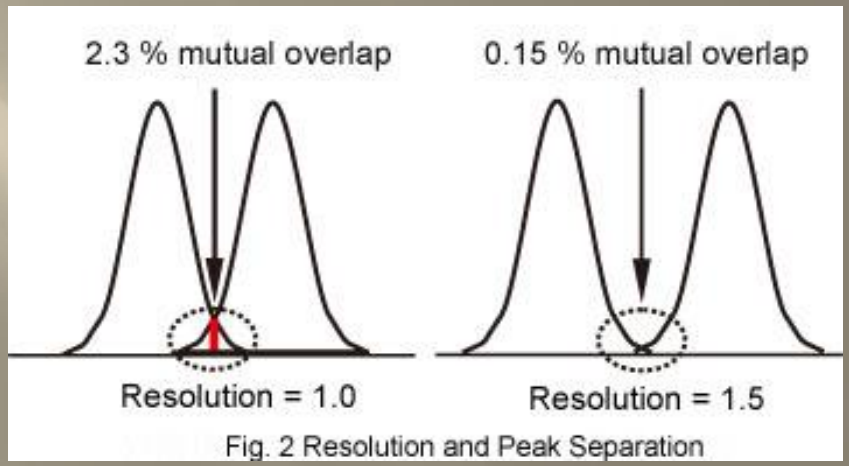


Fig. 2 Resolution and Peak Separation

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Resolution

The Fundamental Resolution Equation

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

Efficiency Retention Factor Selectivity

α = Selectivity (influenced by mobile and stationary phase)

$= k_2/k_1$

N = Column Efficiency (influenced by length and particle size)

k = Capacity Factor (retention) (influenced by stationary and mobile phase). $= (k_1+k_2)/2$ (average)

Best for LC.

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Resolution

There are other forms of the resolution equation, however, these make certain assumptions.

When the k of both compounds is almost the same, one can use just the "k" value. The same is possible for N. If the difference in k is very small, also the alpha will be small and the resolution equation could be simplified.

If the difference in k between the 2 compounds increases, the alpha also increases rapidly and. The "alpha-term" approaches "1", meaning R value will not increase at higher alpha's, which is not correct.

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Resolution

The Fundamental 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.

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Resolution

The Fundamental Resolution Equation

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

Efficiency Retention Factor Selectivity

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.

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Resolution

The Fundamental Resolution Equation

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

Efficiency Retention Factor Selectivity

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.

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Resolution

The Fundamental Resolution Equation

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

Efficiency Retention Factor Selectivity

Ideally, we want sufficient resolution
(Rs of 1.5 or greater for analyte/solute of interest peaks)

We also want the separation performed in a minimum amount of time.

Other parameters may also be of importance:

-sufficient quantity if performing "prep" scale separation

-sufficient sensitivity for detection (covered more with instrumentation and quantitation)

-ability to identify unknowns (e.g. with MS detection)

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Resolution Optimization

Trade-offs in reducing H

In packed columns, going to small particle sizes results in greater back-pressure at higher flow rates.

In GC, small column and film diameters means less capacity and can require longer analysis times.

Trade-offs in lengthening column (N = L/H)

Longer experimental times due to more column length and can lead to peak broadening at later eluting peaks.

Increasing column length is not usually the most desired way to improve resolution (because required time increases and signal to noise ratio decreases).

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Resolution Optimization

Alternatively, k values can be increased (use lower T in GC or weaker solvents in HPLC); or α values can be increased (use different solvents in HPLC or column with better selectivity) but effect on RS is more complicated.

How to improve resolution?

Increase N (increase column length, use more efficient column)

Increase a (use more selective column or mobile phase)

Increase k values (increase retention)

Which way works best?

Increase in k is easiest (but best if k is initially small)

Increase in a is best, but often hardest

Often, changes in k lead to small, but unpredictable, changes in α also

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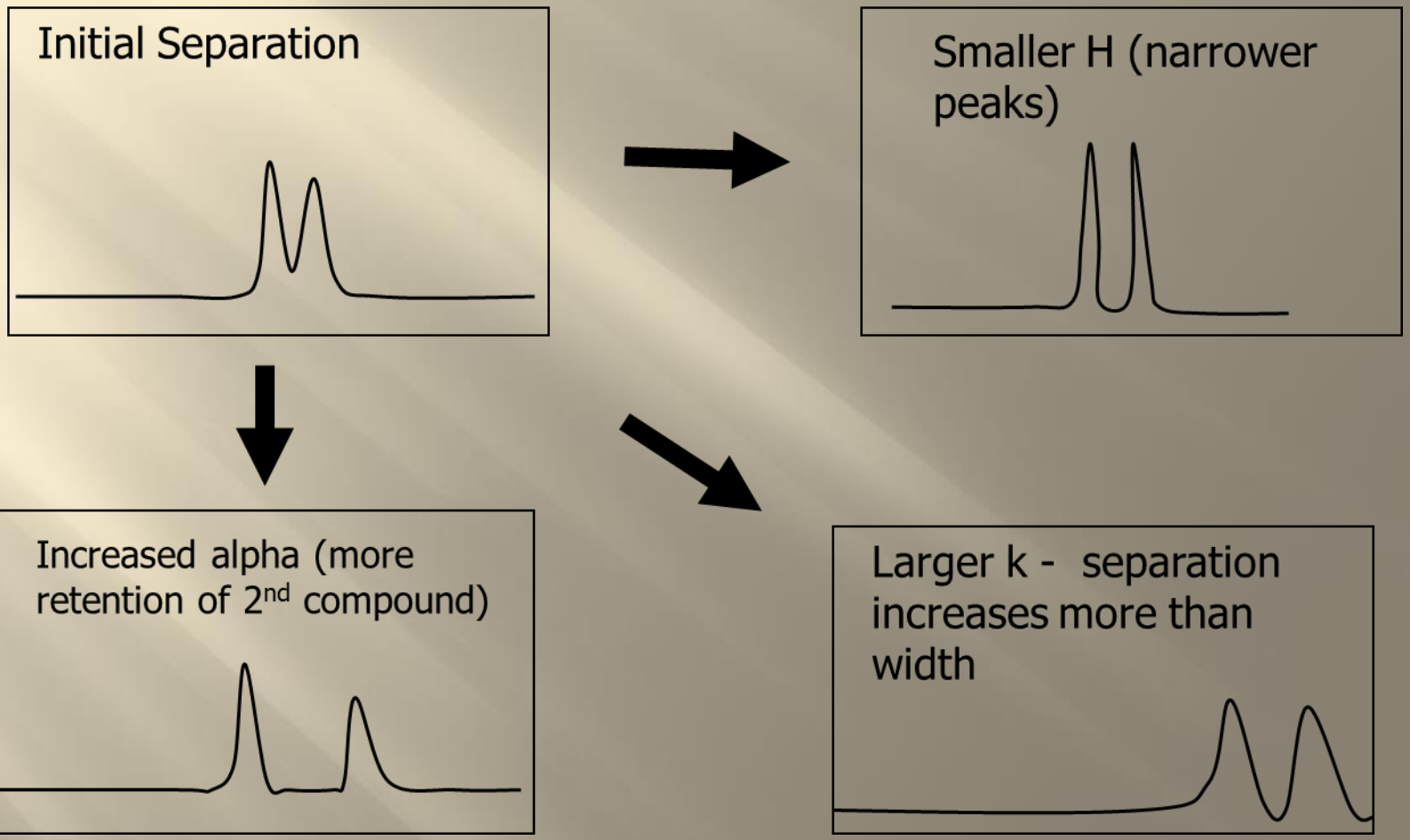
Resolution Optimization



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Resolution

Peak capacity and resolution equations work for packed columns but not so well for capillary columns, since assume that the peak width is proportional to retention volume measured from point of injection.

The peaks produced on a capillary column are narrower than predicted by theory.

For this reason packed columns come close to pure plate theory.

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Resolution

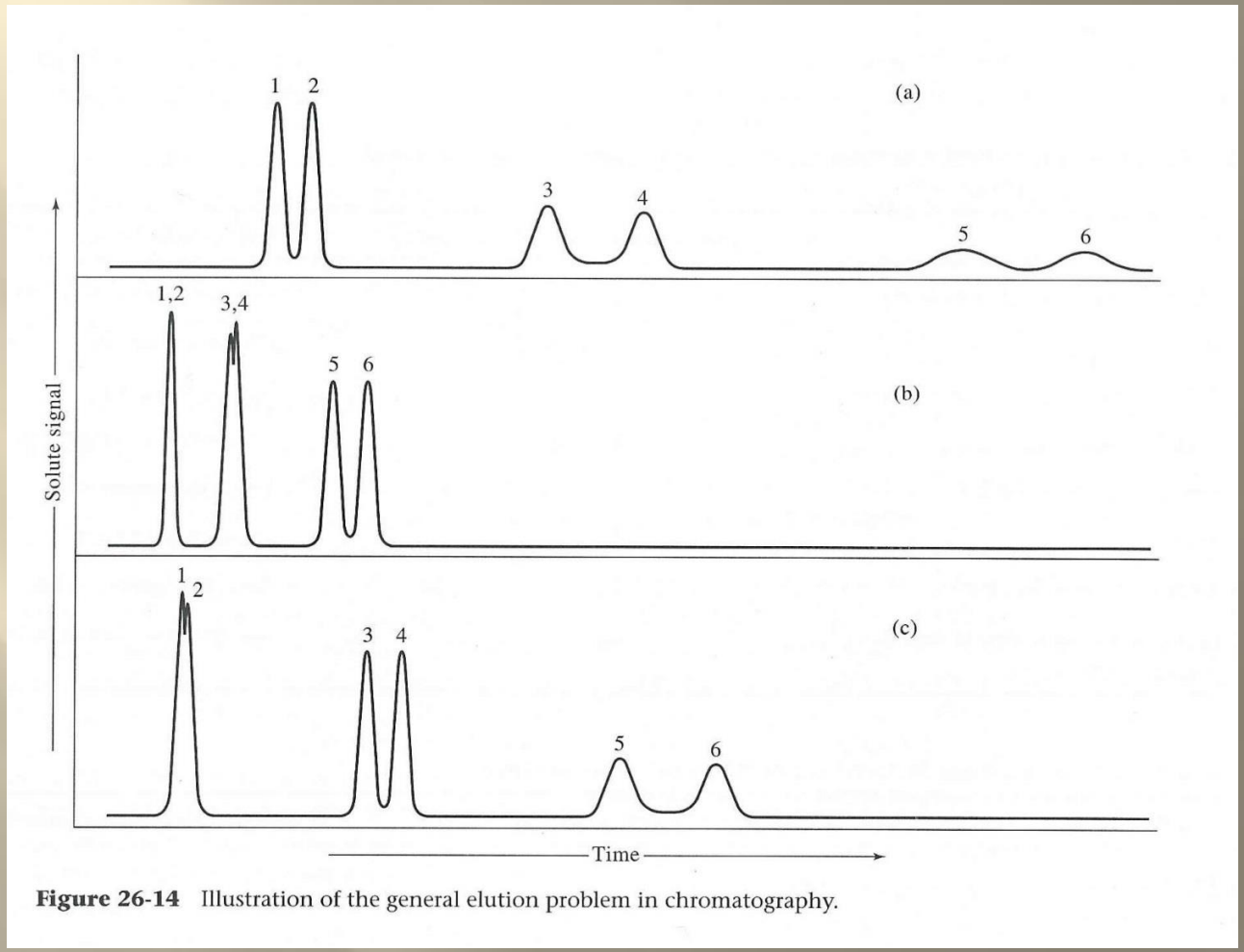
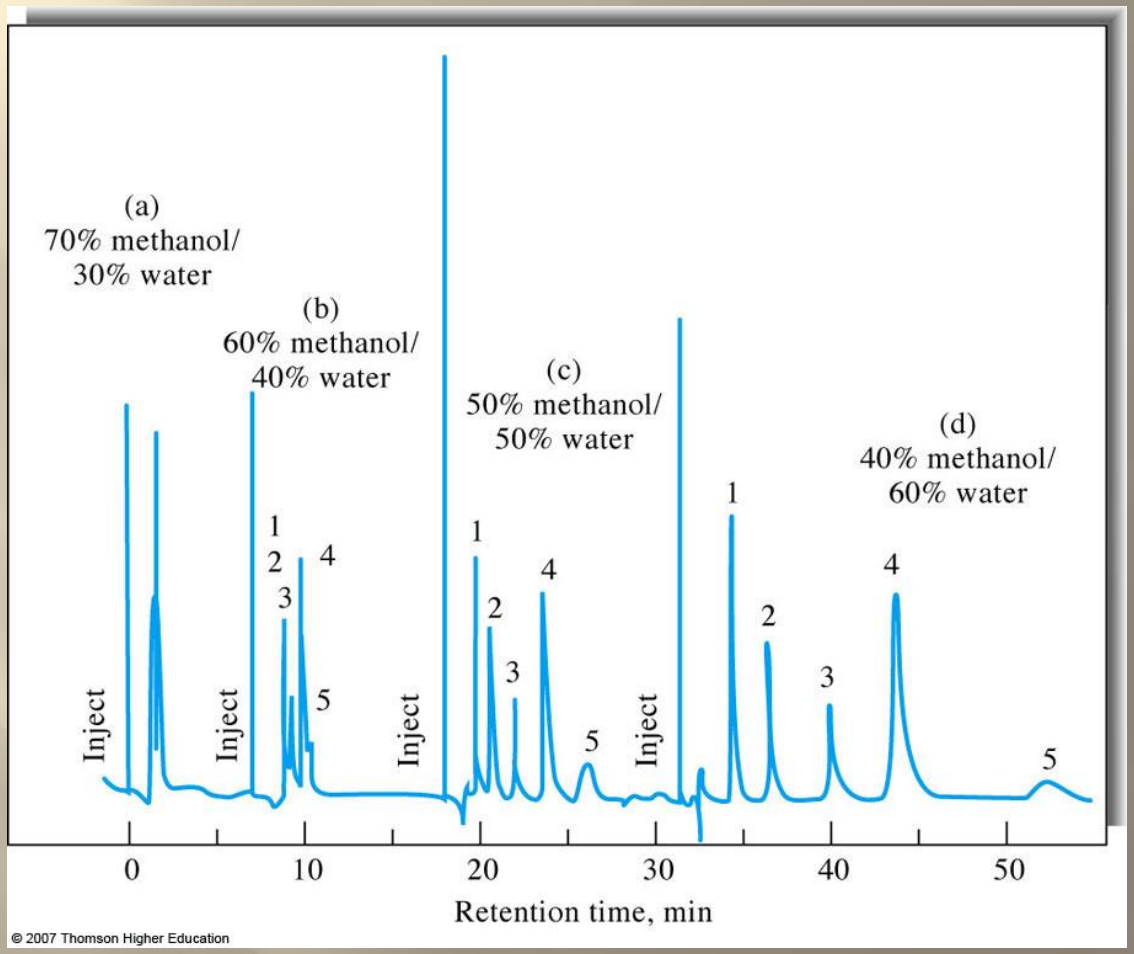


Figure 26-14 Illustration of the general elution problem in chromatography.

Chromatography

Resolution



Chromatography

Parameters

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	$(t_R)_B = \frac{16R_s^2 H}{u}\left(\frac{\alpha}{\alpha - 1}\right)^2 \frac{(1 + k_B)^3}{(k_B)^2}$	

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Assignment

- Outline of research paper Due 10-12-20

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