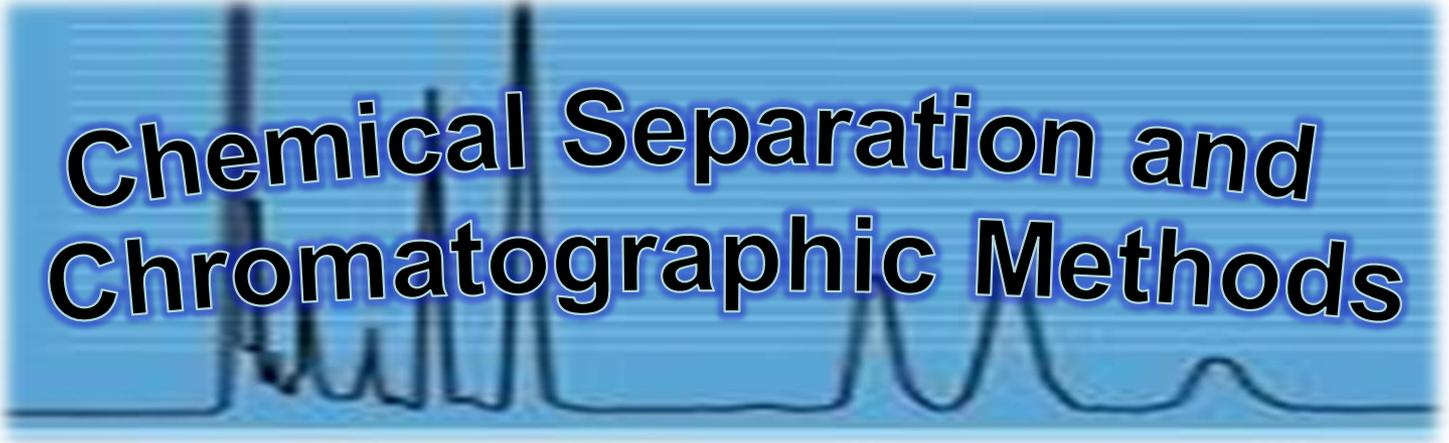# Chemical Separation and Chromatographic Methods

An Introduction to Chromatographic Separations

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Advanced Materials
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Definition

According to the IUPAC definition, 1993

"Chromatography is a physical method of separation in which the components to be separated are distributed between two phases; one of which is stationary (stationary phase) while the other moves in a definite direction (mobile phase)".

Chromatography derives its name from two Greek words as; (chroma) meaning "color" and (graphy) meaning "writing".

Chromatography is the collective term for a **family** of laboratory techniques for the separation of mixtures.

Chromatography is not restricted to analytical separations and identifications.

Other applications:
- Preparation of pure substances (purification),
- The study of the kinetics of reactions,
- Structural investigations on the molecular scale,
- Determination of physicochemical constants,
(including stability constants of complexes, enthalpy, entropy and free energy).

History

1906: Mikhail Tswett, invented adsorption chromatography. He separate various plant pigments such as chlorophylls and xanthophylls from leaves by passing solutions of these compounds through a glass column packed with finely divided chalk ($CaCO_3$) using petroleum ether as eluent.

Tswett

This work regarded as the first to describe the separation process, which led to born the technique of chromatography with the twentieth century. Tswett is credited as "**father of chromatography**" principally because he coined the term chromatography and scientifically described the process.

1931: Lederer and coworkers, separated lutein and zeaxanthine in carbon disulphide and the xanthophylls from egg yolk on a column of calcium carbonate powder.

1937: Paul Karrer, awarded the Nobel Prize in chemistry for his investigations on carotenoids, flavins and vitamins A and B2.

Karrer

1938: Richard Kuhn, awarded the Nobel Prize in chemistry for his work on carotenoids and vitamins.

Kuhn

1939: Leopold Ruzicka, awarded the Nobel Prize in chemistry for his work on polymethylenes and higher terpenes.

Ruzicka

1940-1949: Arne Wilhelm Tiselius, classify chromatographic process into three groups, namely frontal analysis, displacement chromatography and elution chromatography. He awarded the Nobel Prize in 1948 for his research on electrophoresis and adsorption analysis.

Tiselius

1952: Archer Martin and Richard Synge, awarded the Nobel Prize for developing a separation procedure for the isolation of acylated amino acids from protein hydrolysates by extraction of the aqueous phase with a chloroform organic phase.

This system successfully separated the acetylated amino acids according to their partition coefficients and marked the beginning of partition chromatography.

Martin   Synge

Perhaps more impressive is a list of twelve Nobel Prize awards that were based upon work in which chromatography played a vital role.

Chromatography is still continuously growing and its fields of application are widening.

Market size

The global chromatography market was worth USD 6.6 billion in 2011.

Expected to reach USD 8.9 billion in 2017.
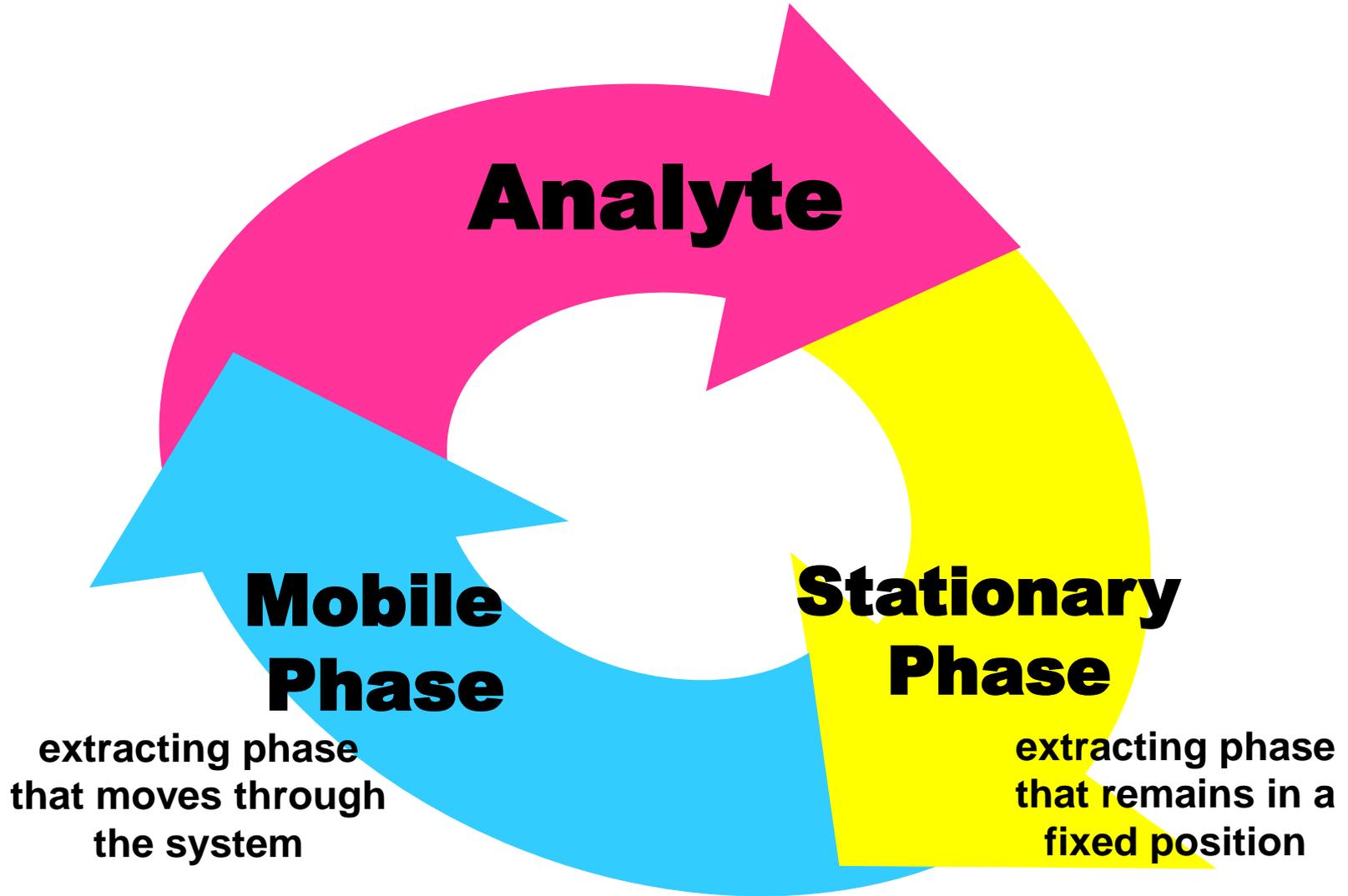
Annual growth rate about 5.5%.

HPLC is the largest product segment in the analytical instruments industry (about 50% of the total chromatography market).

General description of chromatography

Chromatography is based on a flow system containing two phases, **mobile** and **stationary**, and the **sample components** are separated according to differences in their distribution between the two phases.

In all chromatographic separations, the sample is dissolved in a mobile phase. This mobile phase is then forced through an immiscible stationary phase. The two phases are chosen so that the components of the sample distribute themselves between the mobile and stationary phase to varying degree. As continues moving of the mobile phase, the components in the mixture move through the system at different rates due to the difference in their interaction between the mobile and stationary phases.

Chromatography is competition between the mobile and stationary phase to separate the sample components

Analyte

Mobile Phase

extracting phase that moves through the system

Stationary Phase

extracting phase that remains in a fixed position

Classification of chromatographic methods

Chromatographic methods can be categorized in several ways:

(1) Based on the physical state of the mobile and stationary phases.

Mobile phase could be **gas**, **liquid** or **a supercritical fluid**.
Stationary phase could be **liquid** or **solid**.

(a) Homogeneous techniques:
Have both m.p. and s.p. same physical state (liquid); liquid-liquid chromatography.

(b) Heterogeneous techniques:
Employ different m.p. and s.p. states, e.g., liquid-solid, gas-solid, gas-solid chromatography.

(2) Based on the kind of equilibria involved in the transfer of solutes between phases, principle of separation used (separation mechanism).

(a) Partition chromatography:
Separation based on solubility.
Stationary phase is liquid.

(b) Adsorption chromatography:
Separation based on polarity.
Stationary phase is solid.

(c) Ion exchange chromatography:
Separation based on charge.

(d) Size exclusion chromatography:
Separation based on molecular size.

(3) Based on the shape of stationary phase, surface on which the separation to be performed or the way on which the mobile phase pass through the stationary phase.

(a) Planar or plane chromatography:

The stationary phase is placed on a plane surface (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.
- Paper chromatography
- Thin layer chromatography (TLC)
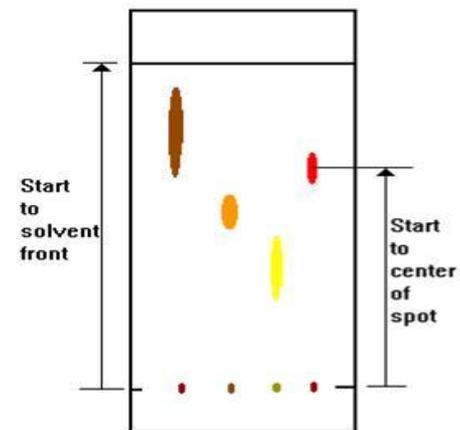
(b) Columnar or column chromatography:
The stationary phase is held in a narrow tube through which the mobile phase is forced under pressure or by gravity.
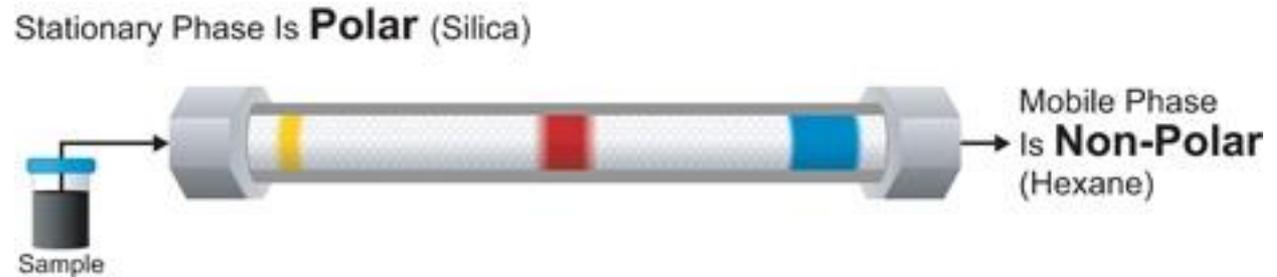- HPLC
- GC

## (4) Based on the chemical nature of stationary and mobile phases.

(a) Normal-phase chromatography:
Here the stationary phase is polar in nature and the mobile phase is in non-polar nature.

Stationary Phase Is **Polar** (Silica)

Mobile Phase
Is **Non-Polar**
(Hexane)

Sample

(b) Reverse-phase chromatography:
This is reverse to the above method. The stationary phase is non-polar in nature and the mobile phase is in polar nature.

Stationary Phase Is **Non-Polar** (C_{18})

Mobile Phase
Is **Polar**
(Aqueous)
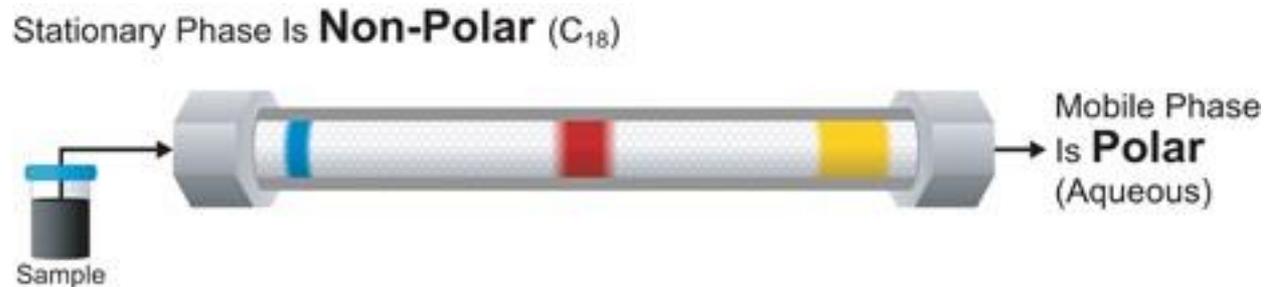
Sample

(5) Based on the purpose of chromatography experiment.

(a) Analytical chromatography:
Used for smaller amounts of materials.
- **Qualitative analysis:** What is in the sample?
- **Quantitative analysis:** How much is in the sample?

(b) Preparative chromatography:
Used for larger amounts of materials and to separate the components of a mixture for more advanced use (purification and sample preparation).

Classification of column chromatographic methods

General classification	Specific method	Stationary phase	Type of equilibrium
Liquid chromatography (LC) (m.p.: liquid)	Liquid-liquid, or partition	Liquid adsorbed or bonded on a solid	Partition between immiscible liquids or between liquid and bonded phase
	Liquid-solid, or adsorption	Solid	Adsorption
	Ion exchange	Ion-exchange resin	Ion exchange
	Size exclusion	Liquid in interstices of a polymeric solid	Partition/sieving
Gas chromatography (GC) (m.p.: gas)	Gas-liquid	Liquid adsorbed or bonded on a solid	Partition between gas and liquid or between liquid and bonded surface
	Gas-solid	Solid	Adsorption
Supercritical-fluid chromatography (SFC) (m.p.: supercritical fluid)		Organic species bonded to a solid surface	Partition between supercritical and bonded surface

Elution chromatography on columns

The sample is introduced at the head of a column, whereupon the components of the sample distribute themselves between the two phases. Introduction of additional mobile phase (continuously) 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.

Ideally, the resulting differences in rates cause the components in a mixture to separate into bands, or zones, located along the length of the column.

Like dissolve like (like attract like)
Non-polar stationary phases best for non-polar analytes
Polar stationary phases best for polar analytes
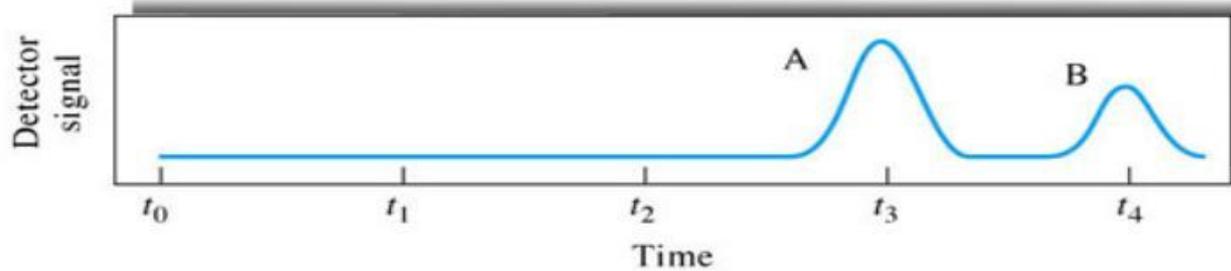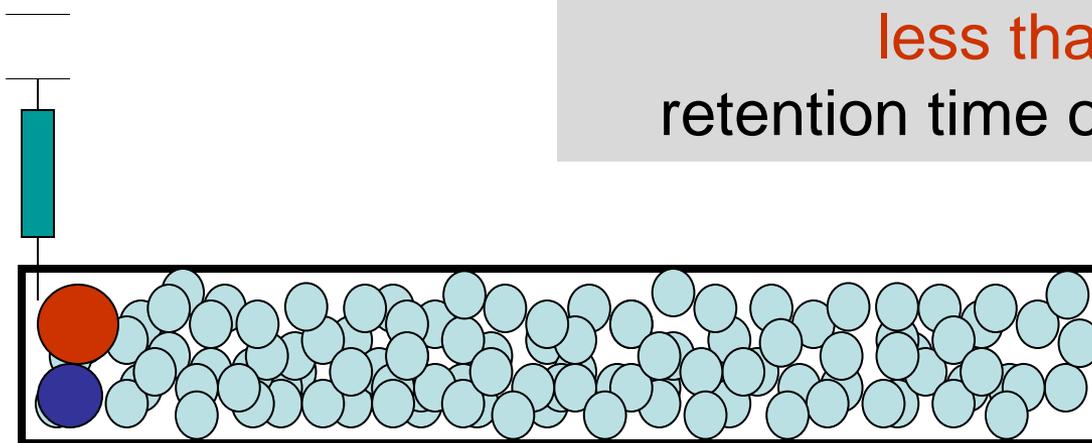
Separation of a mixture of components (A & B)
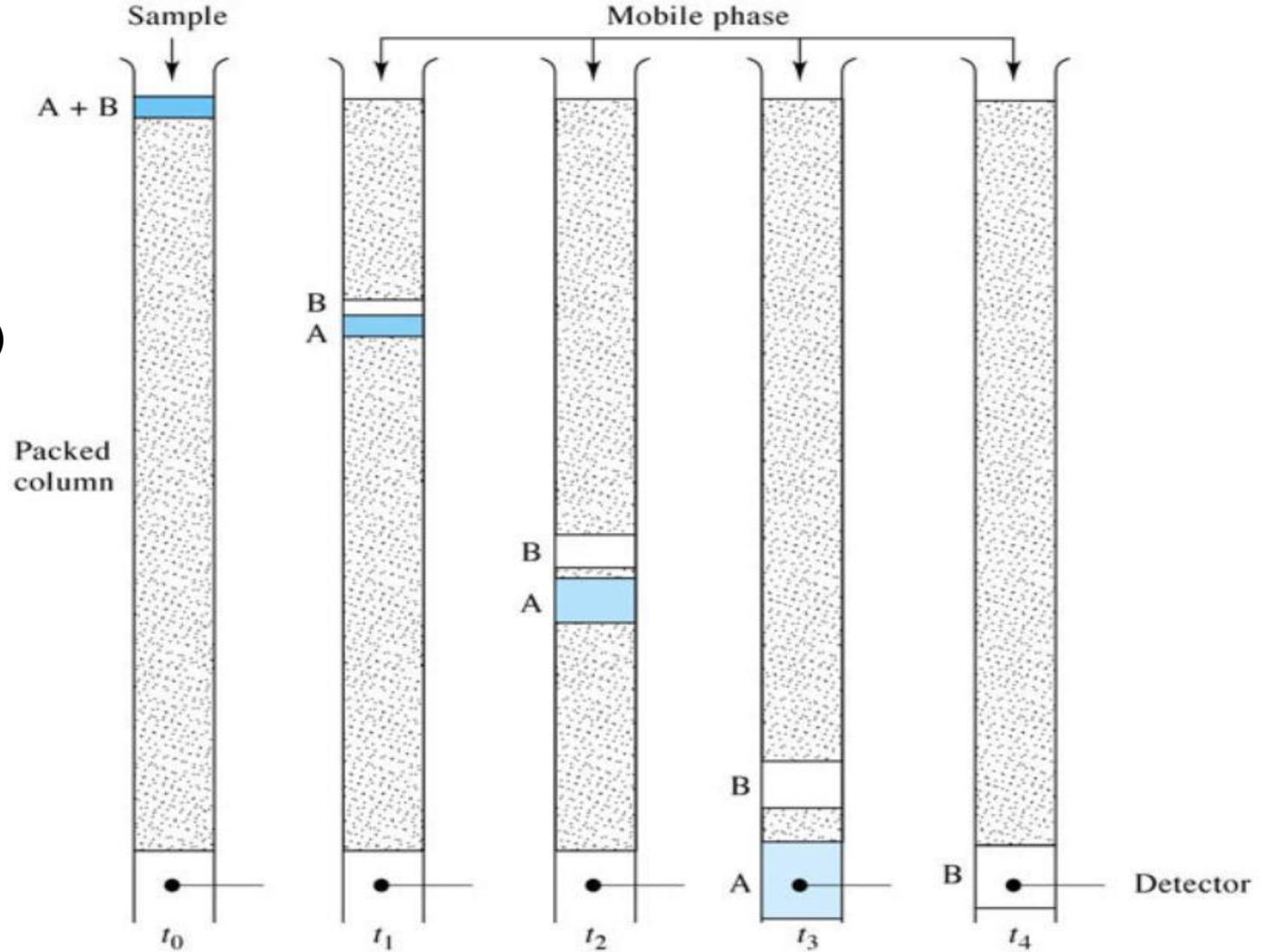
The output of the signal detector

Like dissolve like (like attract like)

Retention time of CCl_4 is
less than
retention time of CH_3OH

polar column

CCl_4 ●

CH_3OH ●
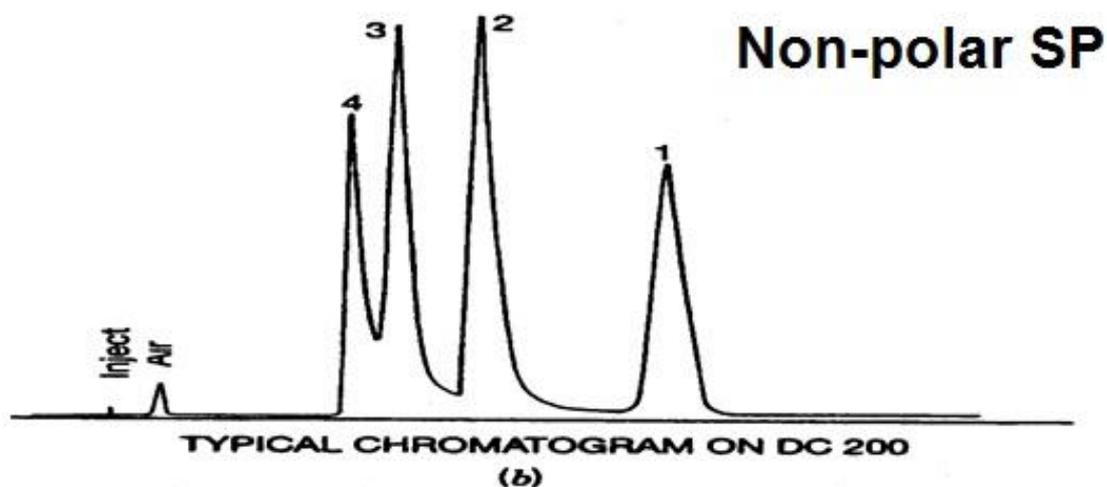
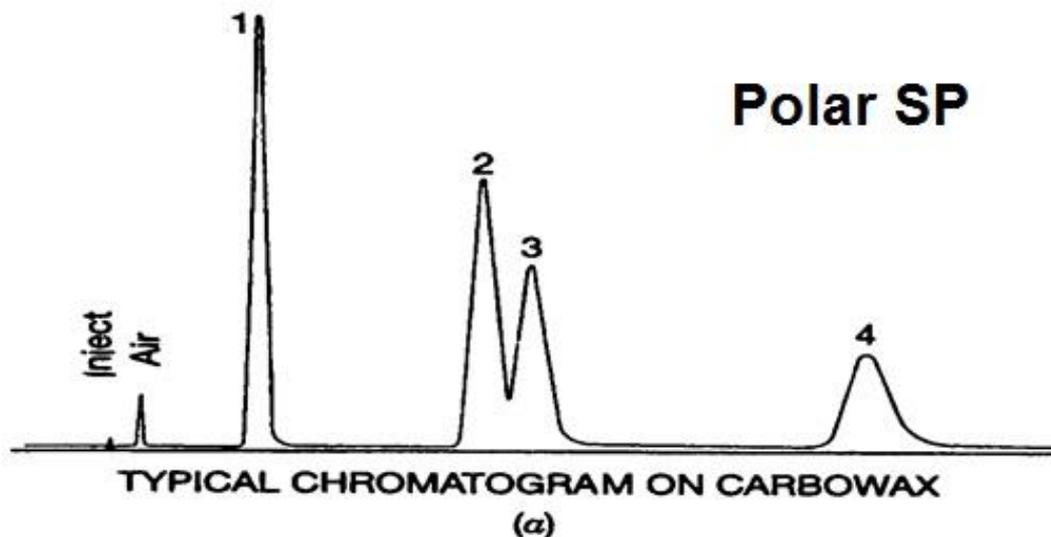SP ●

Methyl alcohol (CH_3OH) is polar and is attracted to SP and therefore travels slowly through column.

Carbon tetrachloride (CCl_4) is nonpolar and is less attracted to SP and therefore travels rapidly through column.

# Effect of stationary phase on retention times

(1) n-heptane
(2) tetrahydrofuran
(3) 2-butanone
(4) n-propanol

Polar SP

Inject Air

TYPICAL CHROMATOGRAM ON CARBOWAX

(a)

Non-polar SP

Inject Air

TYPICAL CHROMATOGRAM ON DC 200

(b)

Chromatograms

If a detector is placed at the end of the column and its signal is plotted as function of time, a series of peaks is obtained. Such a plot, called a **chromatogram**. The chromatogram can be used to provide information on separation process.
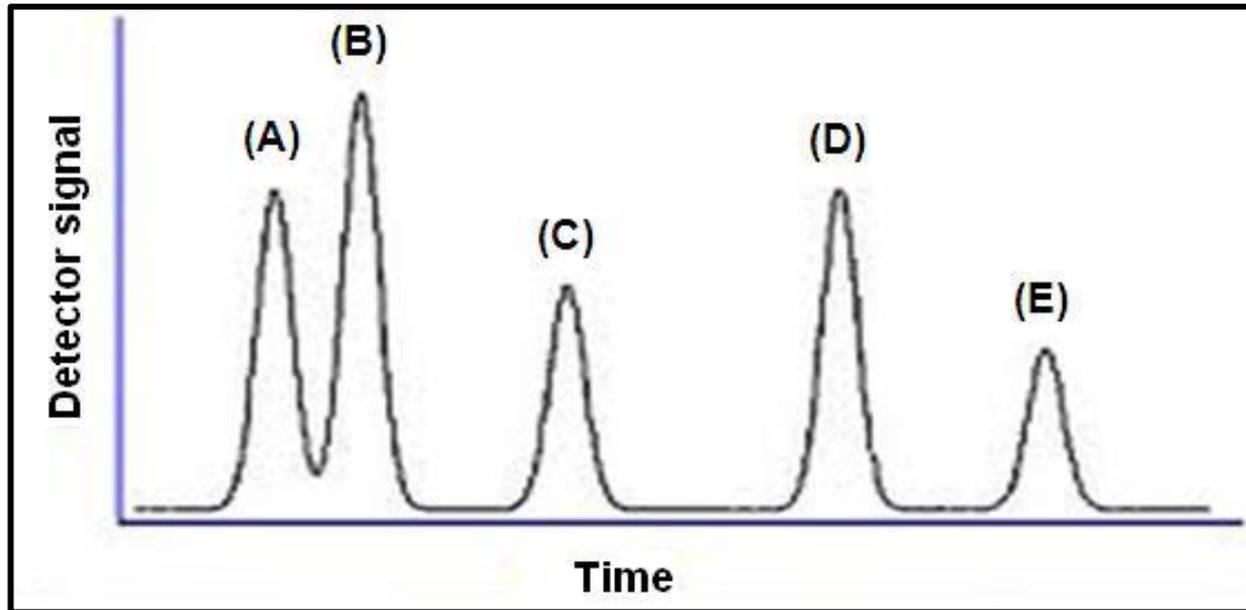

Typical chromatogram of detector response as a function of retention time.

A chromatogram is useful for both **qualitative** and **quantitative** analysis;
- Every peak represents one component.
- The positions of peaks on the time axis may serve to identify the components of the sample.
- The peaks heights or areas provide a quantitative measure of the amount of each component.
- Species E is more strongly retained; thus, E lags during the migration.

Peak shape …

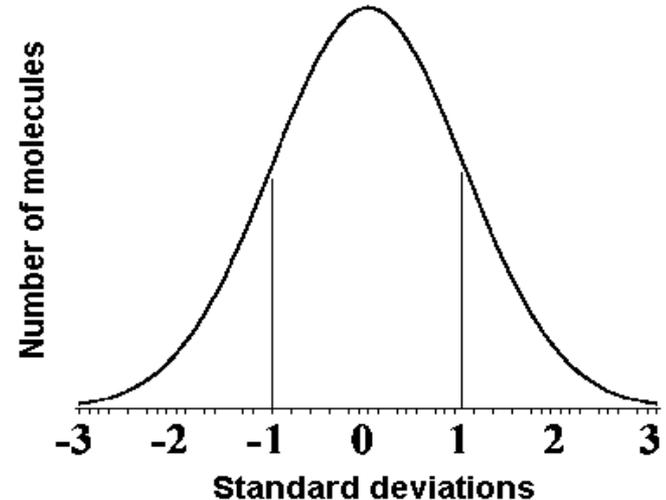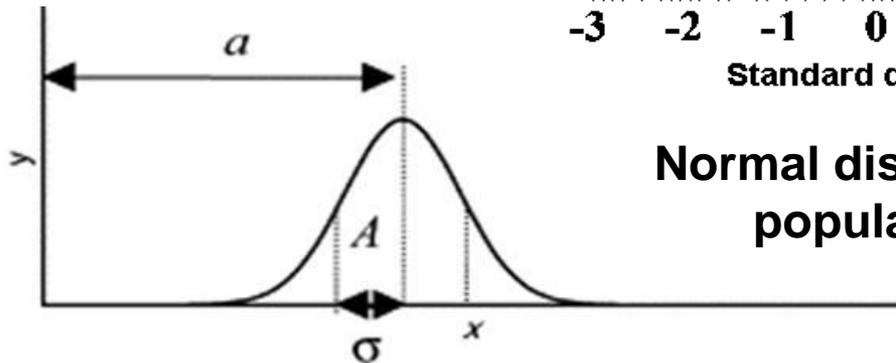The recorder will give **peak shape** exactly like the **mass distribution peak**.
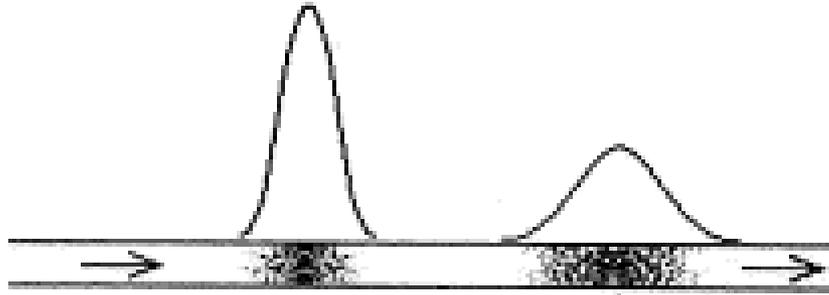
The mass distribution is symmetrical throughout but the sample zone is expanding while moving, the detector will record a steeper peak front and a prolonged peak tail. The zone breadth is directly related to residence time on the column and inversely related to the velocity at which the mobile phase flows.

Number of molecules (y-axis)

Standard deviations (x-axis: -3 -2 -1 0 1 2 3)

Normal distribution population

Basic Gaussian curve:

$(A, a, \sigma$ are independent)

$$y(x) = \frac{A}{\sqrt{2\pi}\sigma} e^{-[(x-a)/(\sqrt{2}\sigma)]^2}$$

Chromatographic properties and evaluation

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.

- **Partition coefficients**

- **Retention time**

- **Capacity factor**

- **Selectivity factor**

- **Resolution**

- **Efficiency**

Partition coefficients ...

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

For the analyte species, *A*

$$A_{mobile} \rightleftharpoons A_{stationary}$$
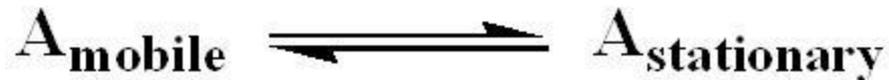
The equilibrium constant, *K* for this equilibrium is called the **distribution constant**, the **partition ratio**, or the **partition coefficient**,

$$K = c_S / c_M$$

Where, c_s is the molar concentration of the solute in the stationary phase and c_M is its molar concentration in the mobile phase.

Retention time ...

The time it takes after sample injection for the analyte peak to reach the detector. And is given the symbol t_R.

The time t_M for the unretained species to reach the detector is called the **dead time**. The rate of migration of the unretained species is the same as the average rate of motion of the mobile phase molecules.
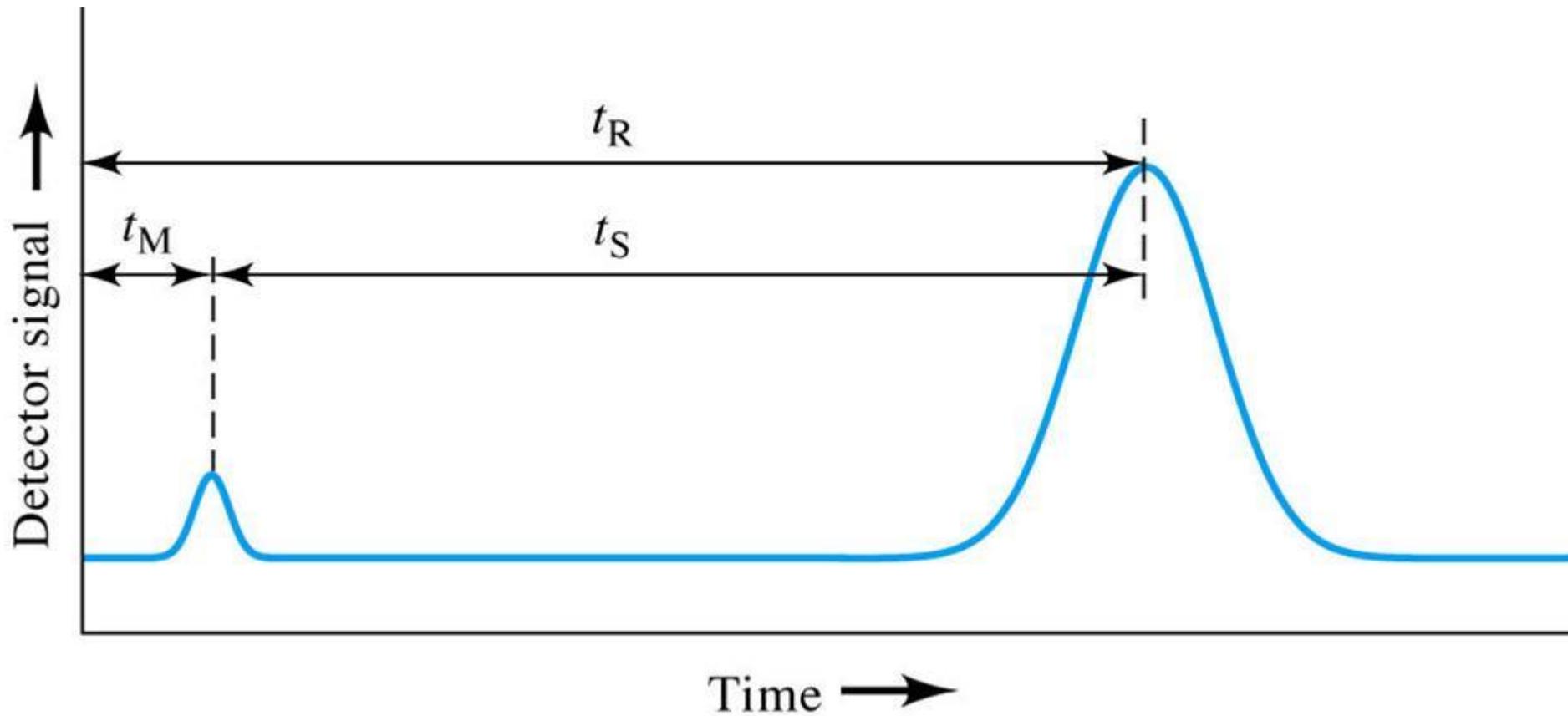
The average linear rate of solute migration $\bar{u}$ is:

$$\bar{u} = L / t_R$$

Where, L is the length of the column packing (column length). The average linear rate of movement u of the molecules of the mobile phase is:

$$u = L / t_M$$

Where, t_M is the time required for an average molecule of the mobile phase to pass through the column.

t_R: retention time; time elapsed from point of injection to maximum of peak = $t_M + t_S$

t_M: the time the component resides in the mobile phase

t_S: the time the analyte is retained on the stationary phase (also called **adjusted retention time**) = $t_R - t_M$

The capacity factor ...

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

For a solute **A**, the retention factor k'_A is defined as the ratio between the reduced and the unretained time:

$$k'_A = K_A V_S / V_M = (t_R - t_M)/t_M = (V_R - V_M)/V_M$$

$$\bar{u} = V_M / t_M$$

$$F = V_R / t_R$$

Where, V_R is the retention volume, V_M is the mobile phase volume (also called void volume).

When the retention factor for a solute is much less than unity, elution occurs so rapidly that accurate determination of the retention times is difficult. When the retention factor is larger than perhaps 20, elution times become inordinately long. Typically, a k value between 2 and 5 represents a good balance between analysis time and resolution.

The selectivity factor ...

The **selectivity factor** is the ability of the chromatographic system to discriminate different analytes.

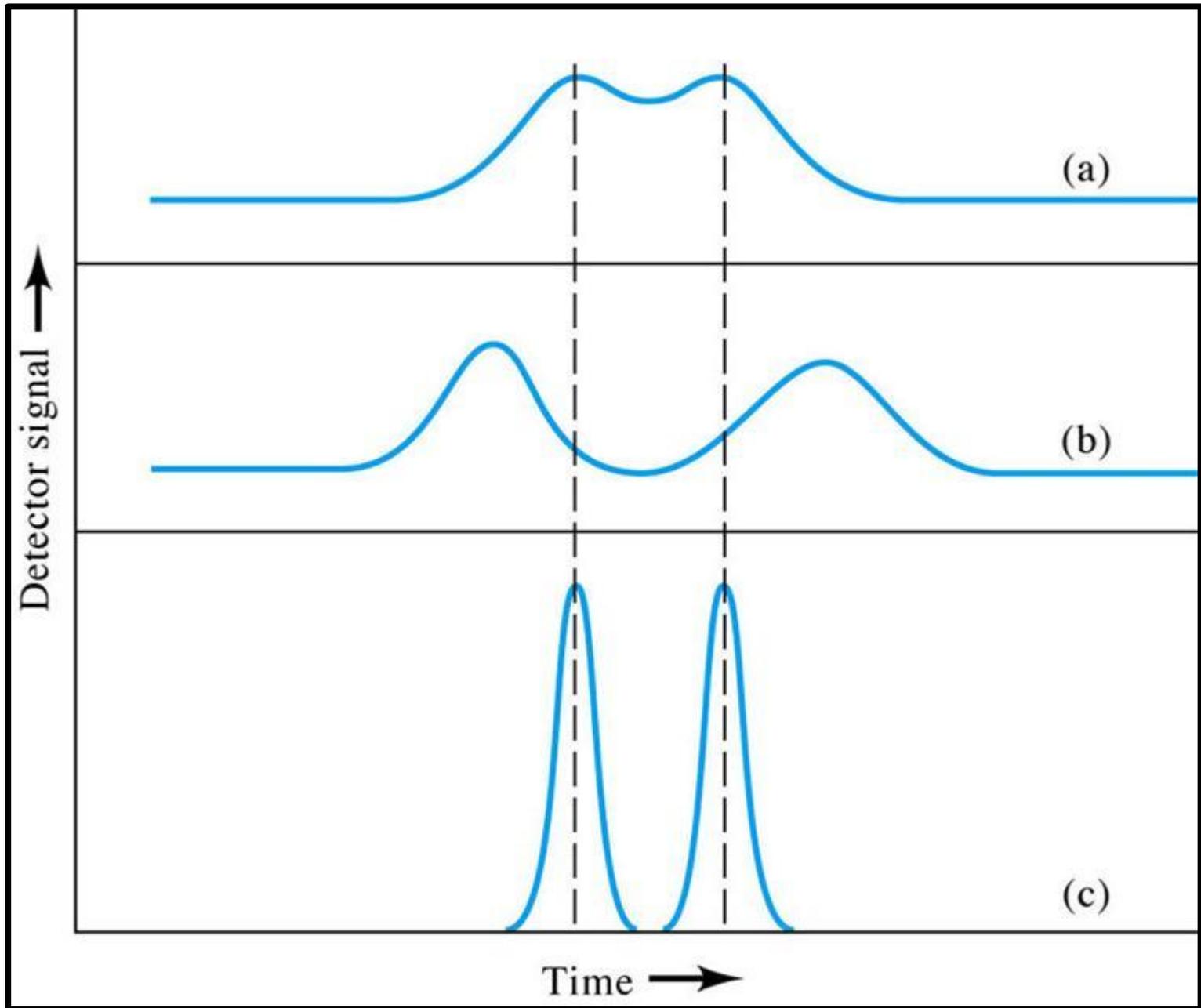The selectivity factor α of a column for the two species A and B is defined as:

$$\alpha = K_B/K_A = k'_B/k'_A = (t_{RB} - t_M) / (t_{RA} - t_M)$$

The distance between the peak maxima reflects the selectivity of the system.

The greater the distance, the higher the selectivity. α is always greater than unity.

Selectivity factor not take into account the **peak width**.

Selectivity vs. Resolution

Resolution ...

Resolution (R_s) is a term used to describe the degree of separation between neighboring solute bands or peaks (distance between the peaks). It is the ratio of the distance between two peaks to the average width of these peaks, and this descriptor encompasses both the efficiency and selectivity.

$$R_s = 2\,\frac{t_{R2} - t_{R1}}{w_1 + w_2} = 1.18\,\frac{t_{R2} - t_{R1}}{w_{(1/2)1} + w_{(1/2)2}}$$

The resolution is the ability of the column to resolve two analytes into separate peaks (or chromatographic zones). It is affected by the selectivity, efficiency and capacity of the column. The resolution equation describes those factors and indicates how they can be manipulated in order to improve the resolution between two peaks.

Typically, an R_s value greater than 1 is required for accurate quantification of two peaks. A value of 1, for two equally sized peaks, indicates an overlap of about 2% for one band over the other. A complete separation requires at least R_s > 1.5 units.

$R_s = 0.5$

$R_s = 1$

$R_s = 1.5$
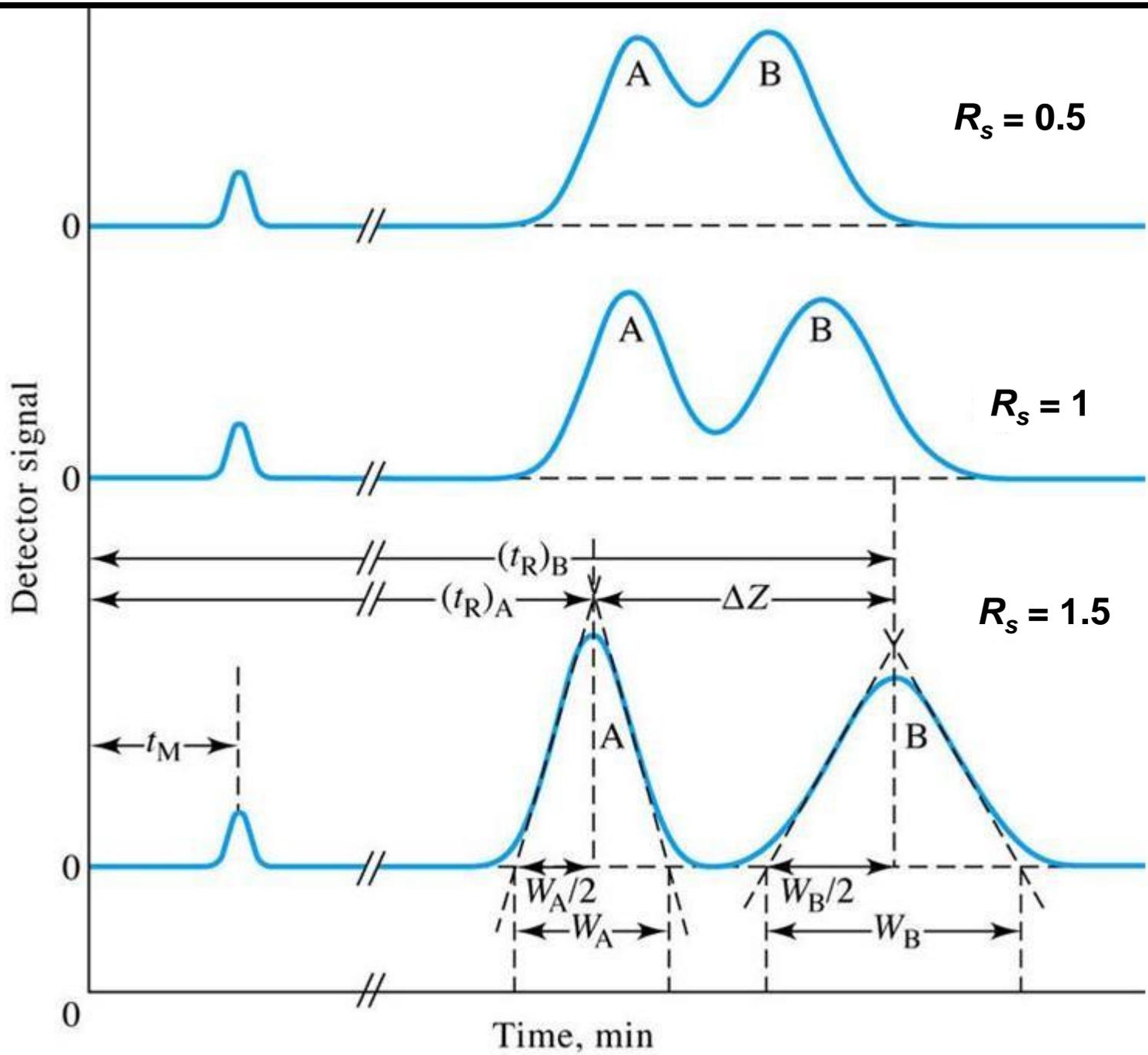
Column efficiency ...

The efficiency of a column is a number that describes **peak broadening** as a function of **retention**.

When a sample mixture is first applied to the head of a column, the width of the sample is very narrow zone. While this zone is moving through the column, it gets broadened. This phenomenon occurs because, as the sample mixture moves down the column, the various sample components interact with, and are retained to various degrees by the stationary phase. This interaction along with the path of the sample components through the packing material, causes the increase in band width (band broadening).

The amount of band broadening determines, to an extent, the degree to which two components can be separated; thus, band broadening should be kept to a minimum.

Band broadening (peak broadening)

Time ⟶

Detector signal

Time →
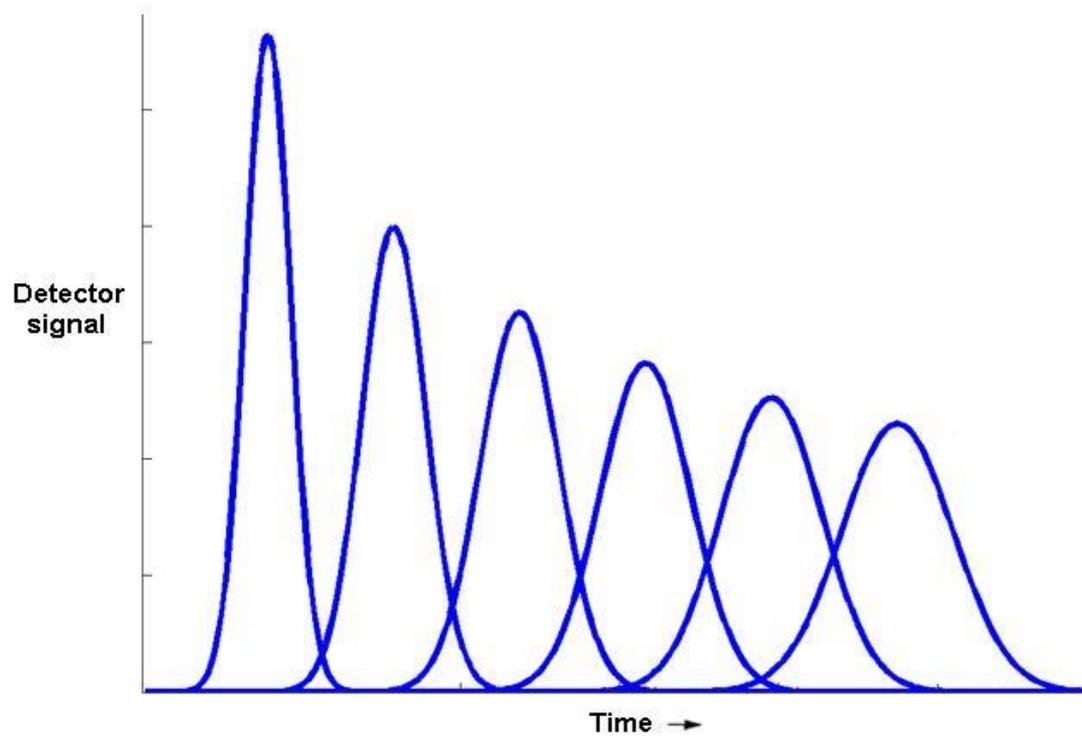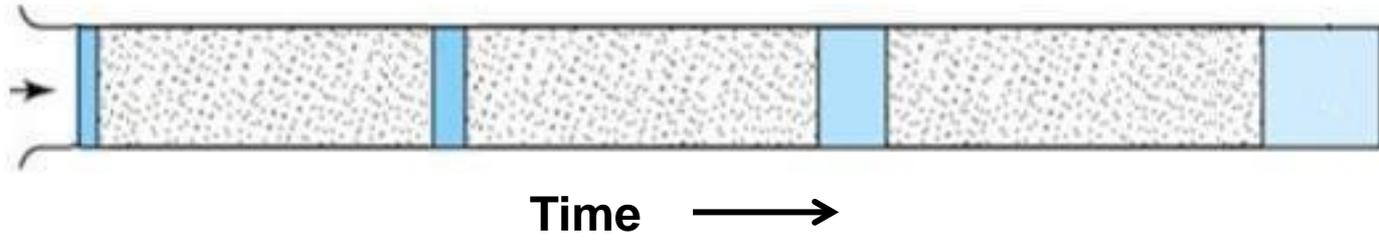
Two major theories have been developed to describe column efficiency, both of which are used in modern chromatography.

(1) The plate theory
- Proposed by Martin and Synge, 1941.
- Provides a simple and convenient way to measure column performance and efficiency.

(2) The rate theory
- Developed by van Deemter, Zuiderweg and Klinkenberg, 1956.
- Provides a means to measure the contributions to band broadening and thereby optimize the efficiency.

Plate theory ...

The major assumption in the plate theory is that there is an instantaneous equilibrium set up for the solute between the stationary and mobile phases.
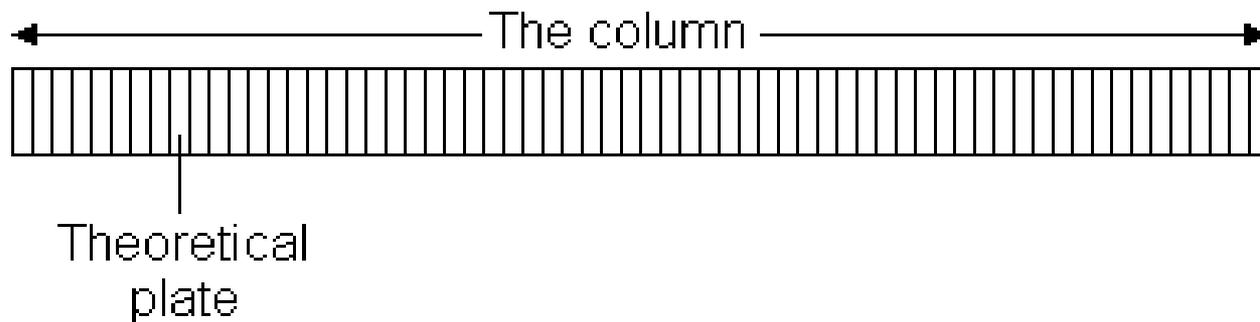
The column is considered to consist of a number of hypothetical thin sections or "**plates**". Each plate has a finite height (**height of effective theoretical plate**, HETP, *H*), and the solute spends a finite time in this plate. This time is considered to allow the solute to equilibrate between the stationary and mobile phases.

The smaller the plate height or the greater the number of theoretical plates (*N*), the more efficient the analyte exchange is between two phases and the greater is the efficiency of the column, which mean the better the separation. That is why column efficiency is measured by *N*.

The movement of a solute along the column is viewed as a stepwise transfer from one theoretical plate to the next. The thinner the theoretical plates, the greater the number that can be envisaged within a given length of column.

These terms are related as follows:

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

Where, **L** is the length of the column.

The number of the theoretical plates in the column is usually calculated using the following equation:

$$N = \left(\frac{t_R}{\sigma}\right)^2 = 16\left(\frac{t_R}{w}\right)^2 = 5.54\left(\frac{t_R}{w_{1/2}}\right)^2$$

Where, **σ** is the standard deviation as a measure for peak width **w** = 4σ, **$w_{1/2}$** = 2.354σ, **w** is the peak width measured in time units as the distance between the intersections of the tangents to the peak inflection points with the baseline and **$w_{1/2}$** is the peak width at half height.

In general, the *H* value is smaller for small stationary phase particle sizes (thin), low mobile phase flow rates, less viscous mobile phases, higher separation temperatures and smaller solute molecule sizes. The width of the chromatographic peak reflects the system band broadening and thus efficiency.

The efficiency can be varied by changing physical column parameters such as the length, diameter and construction material of the container of the column. It can also be varied by changing chemical parameters such as the size of the particles constituting the packing material or the mobile phase velocity.

The main criticisms of the plate theory are that it does not consider the effects of band broadening on separation, nor does it consider the influence of chromatographic variables such as particle size, stationary phase loading, eluent viscosity and flow rate on column performance.

Rate theory ...

The rate theory developed by van Deemter *et al.*, and later modified by others, considers the diffusional factors that contribute to band broadening in the column (column variance, σ^2_{col}) and avoids the assumption of an instantaneous equilibrium inherent in the plate theory. Van Deemter equation expresses the extent to which a component band spreads as it passes through the column in terms of physical constants and the velocity of the mobile phase. The most general form of van Deemter equation is:

$$H = 2\lambda d_{\mathrm{p}} + \frac{2\gamma D_{\mathrm{m}}}{u} + f(k)\frac{d_{\mathrm{p}}^2}{D_{\mathrm{m}}}u$$

Where, d_p is particle diameter (average), λ is a packing constant depends on the particle size distribution. The narrower the distribution, the smaller λ, γ is an obstruction factor for diffusion in a packed bed, D_m the diffusion coefficient in the mobile phase and $f(k)$ is a function of the retention factor (k).

$$H = 2\lambda d_{\mathrm{p}} + \frac{2\gamma D_{\mathrm{m}}}{u} + f(k)\frac{d_{\mathrm{p}}^2}{D_{\mathrm{m}}}u$$

$$H = A + \frac{B}{u} + C \cdot u$$
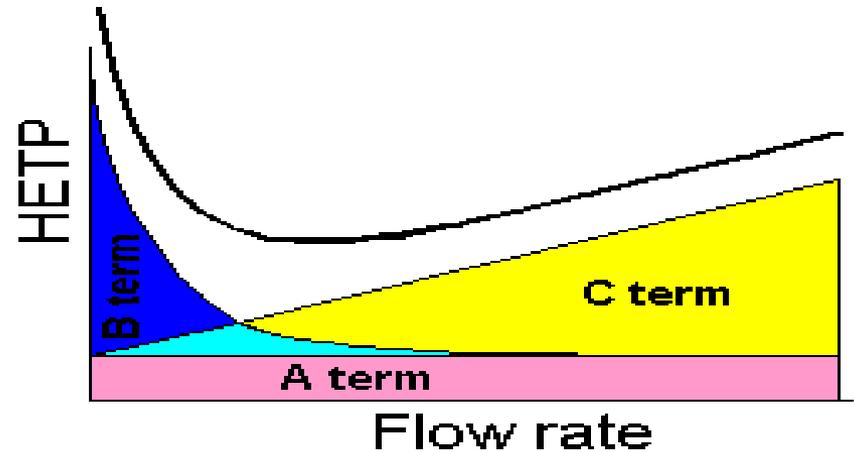
H = height equivalent to a theoretical plate, a measure of the resolving power and efficiency of the column (m).

A = Eddy diffusion parameter, related to channeling through a non-ideal packing or multipath effect (m).

B = Diffusion coefficient of the eluting particles in the longitudinal direction or ordinary molecular diffusion, resulting in dispersion ($m^2.s^{-1}$).

C = Contribution from non-equilibrium or resistance to mass transfer coefficient of the analyte between mobile and stationary phase (s).

u = Linear velocity of the mobile phase ($m.s^{-1}$).

The van Deemter equation is a **hyperbolic function** that predicts that there is an **optimum velocity** at which there will be the minimum variance per unit column length and, thence, a maximum efficiency.

(1) Eddy diffusion or multipath effect (A term)
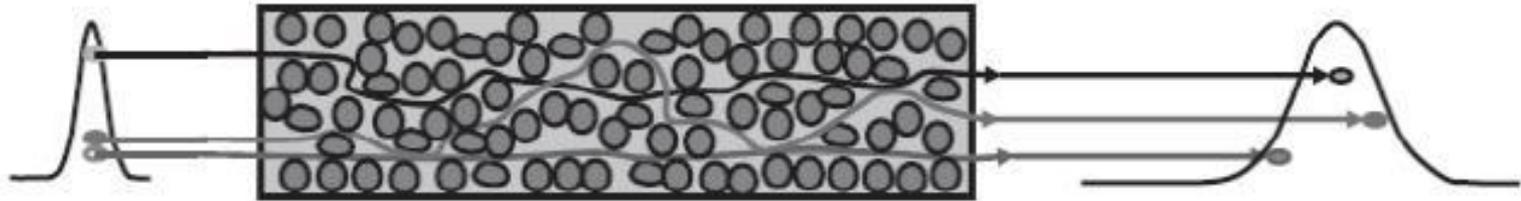
As a solute molecule passes through the column, it can follow a variety of different paths around the stationary phase particles. Each of the paths will be of a different length, so that as solute molecules of the same species follow different paths, they will arrive at the outlet of the column at different times.

This band broadening process is constant and dependent completely on the stationary phase and is independent of the flow rate of the mobile phase. A term is proportional to particle size and it can be minimized if the column is well packed with uniformly particles of constant size (uniformity bed distribution, uniform particle shape and size).

(2) Longitudinal diffusion (*B* term)
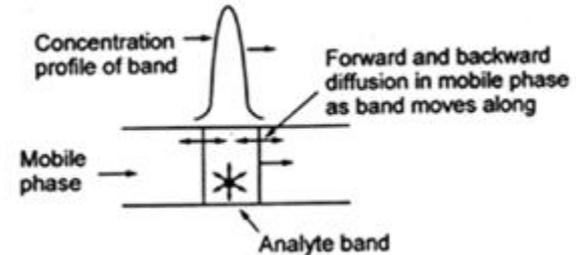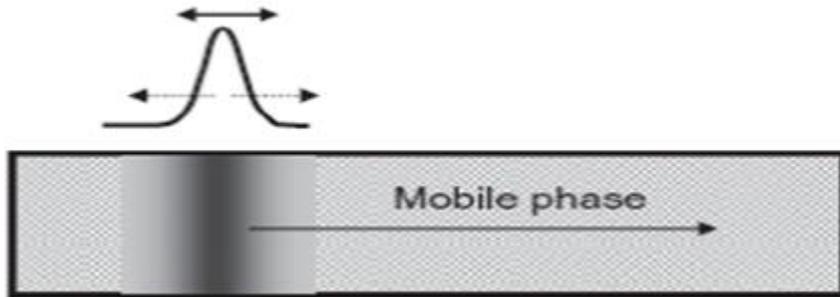
In chromatography, the sample mixture ideally travels through the column as tight zones of individual sample components separated by zones of mobile phase. Whenever a concentration gradient exists, however, diffusion of molecules will occur, from a region of high concentration (center) to a region of low concentration (edges). This form of diffusion is known as longitudinal diffusion and is represented by the *B* term of the proportional to van Deemter equation. *B* term is proportional to diffusion coefficient of the solute. It is related only to the mobile phase and is independent of the stationary phase.

Longitudinal diffusion occurs in all directions. As diffusion is a time dependent process, the longitudinal diffusion effect increases at low mobile phase flow rates. The contribution of longitudinal diffusion is inversely proportional to the mobile phase velocity. The diffusion from the center of the band to the edges has less time to occur.

(3) Resistance to mass transfer (C term)
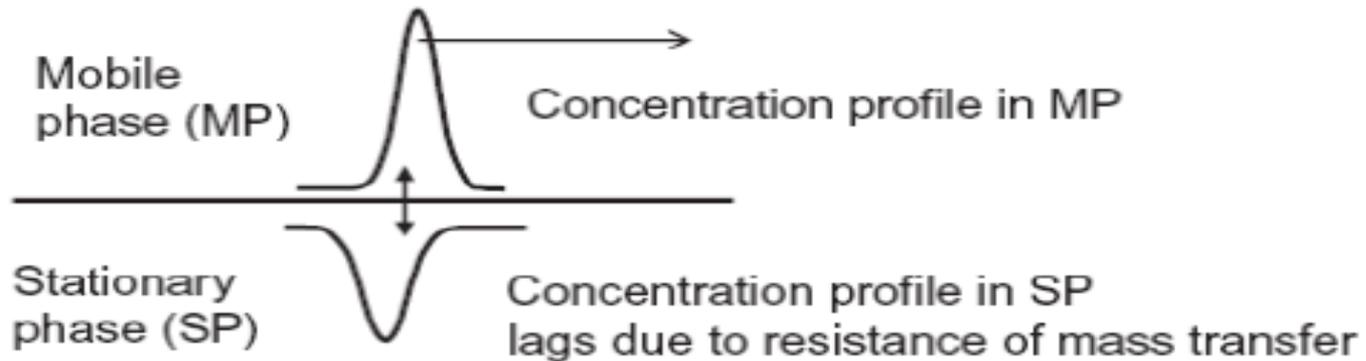
It take time for a solute to reach an equilibrium between the mobile and stationary phases. In the plate theory, it was assumed that the transfer of solute molecules between the mobile and the stationary phase was instantaneous. In the rate theory, it is accepted that there is a finite rate of mass transfer. In addition, molecules of the same species may spend different lengths of time in the stationary and mobile phases.

C is the time lags caused by the slower diffusion of the solute band in and out of the stationary phase. Thus, C term becomes important at high flow rates, due to a bigger lag between a retained molecule compared to a molecule moving with the flow. The faster the mobile phase moves, the less time there is for equilibrium to be approached. Mass transfer resistance can be reduced by using smaller particles and thinner stationary phase.

A van Deemter plot, is a graph of plate height versus the average linear velocity of the mobile phase. The data are determined experimentally using measured values for retention time, void volume or dead time, and peak width to determine *N* and hence *H* at various flow rates.

$$H = A + \frac{B}{u} + C \cdot u$$
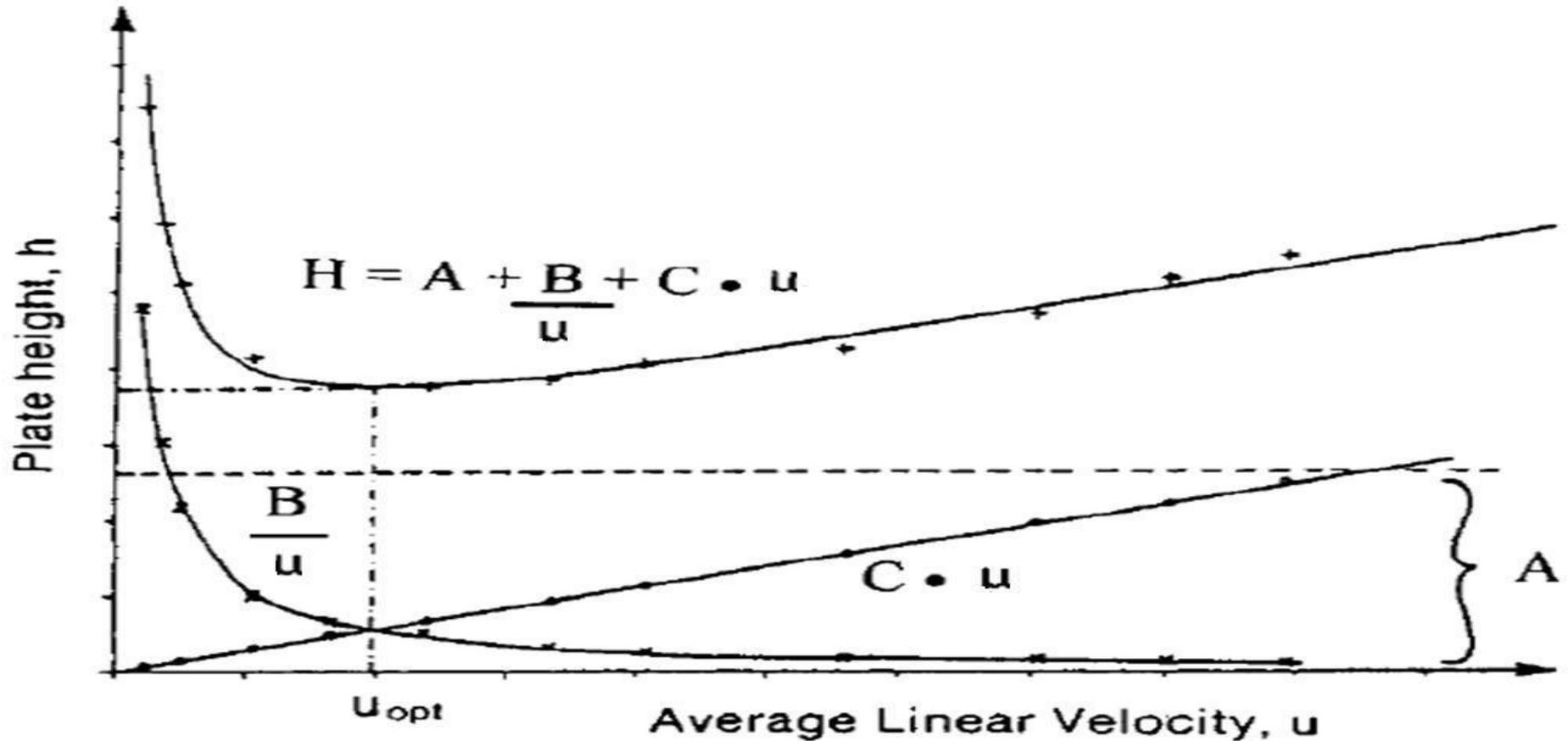
According to the plot, at flow rates below the optimum the overall efficiency is dependent on diffusion effects (*B* term). At higher flow rates, the efficiency decreases because the mass transfer, or *C* terms become more important. The minimum of the van Deemter curve represents the ideal flow velocity where maximum column efficiency is obtained. It is a compromise between the *B* and *C* terms. The *A* term is a constant, independent of flow rate.

The van Deemter equation can be modified and written in the form:

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

$$H = A + B/u + C_s u + C_M u$$

In conclusion, the plate theory assumes that an instantaneous equilibrium is set up for the solute between the stationary and mobile phases, and it does not consider the effects of diffusional effects on column performance.

The rate theory avoids the assumption of an instantaneous equilibrium and addresses the diffusional factors that contribute to band broadening in the column, namely, eddy diffusion, longitudinal diffusion and resistance to mass transfer in the stationary phase and the mobile phase. The experimental conditions required to obtain the most efficient system can be determined by constructing a van Deemter plot.
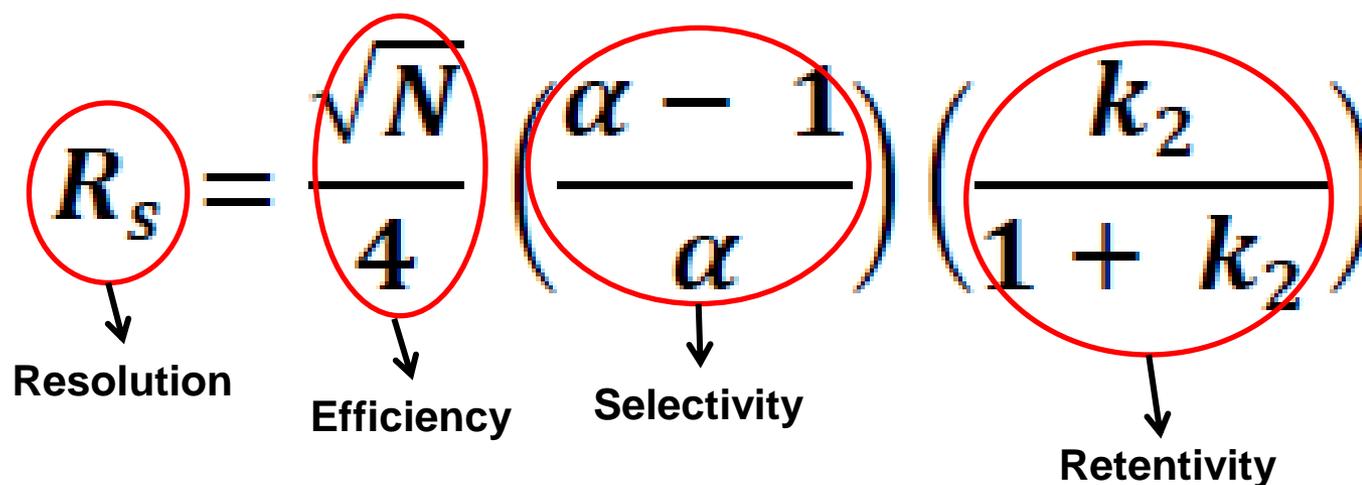
Effect of capacity and selectivity factors on resolution

The efficiency and resolution could also expressed in terms of capacity factor and selectivity by the **Purnell equation**:

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2}{1 + k_2}\right)$$

Effect of resolution on retention time

$$t_{R2} = \frac{16 R_s^2 H}{u}\left(\frac{\alpha}{\alpha - 1}\right)^2 \left(\frac{1 + k_2}{k_2}\right)^2$$

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k_2}{1 + k_2}\right)$$

Resolution

Efficiency

Selectivity

Retentivity

Peak symmetry ...

For practical reasons, the peak symmetry is usually measured at 10% of peak height, where **A** is the distance from peak front to peak maximum, and **B** is the distance from peak maximum to peak end.

$$symmetry = B / A$$

Ideally symmetry should be 1, i.e., **A** = **B**.
Values > 1 indicate peak tailing,
whilst values < 1 characterize peak fronting.

$A_S = B/A$

A | B

10% of peak height

$A_s < 1$ $A_s > 1$

Fronting   Tailing
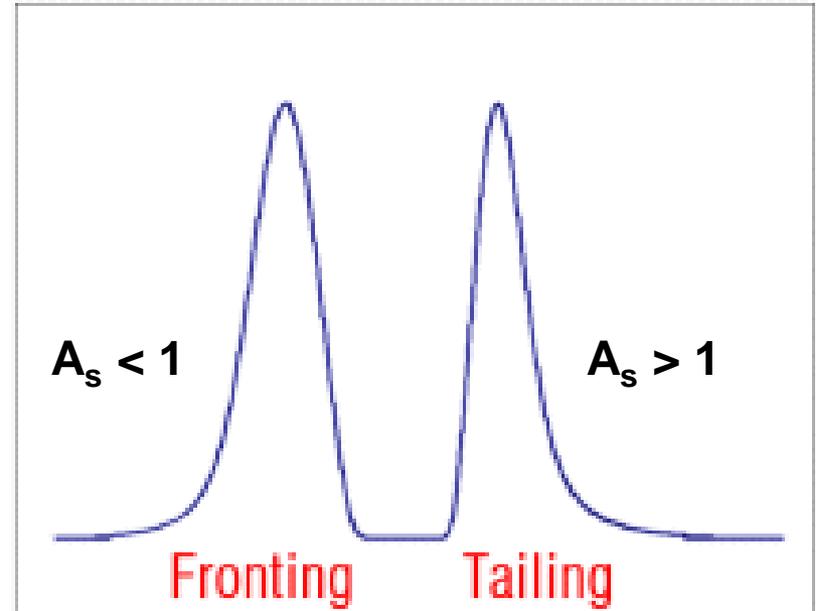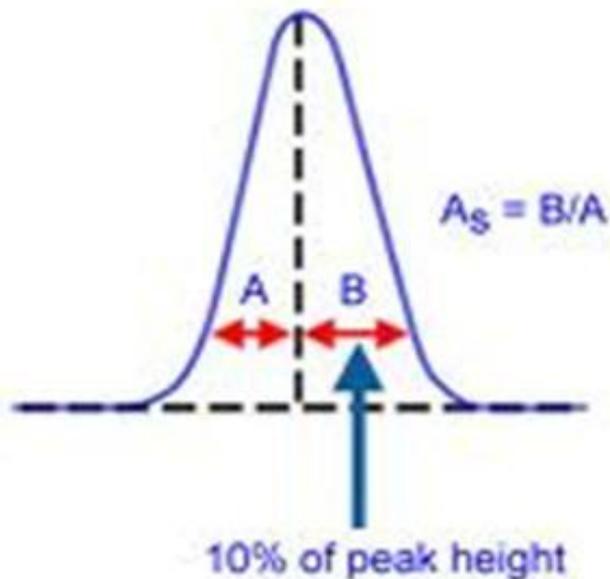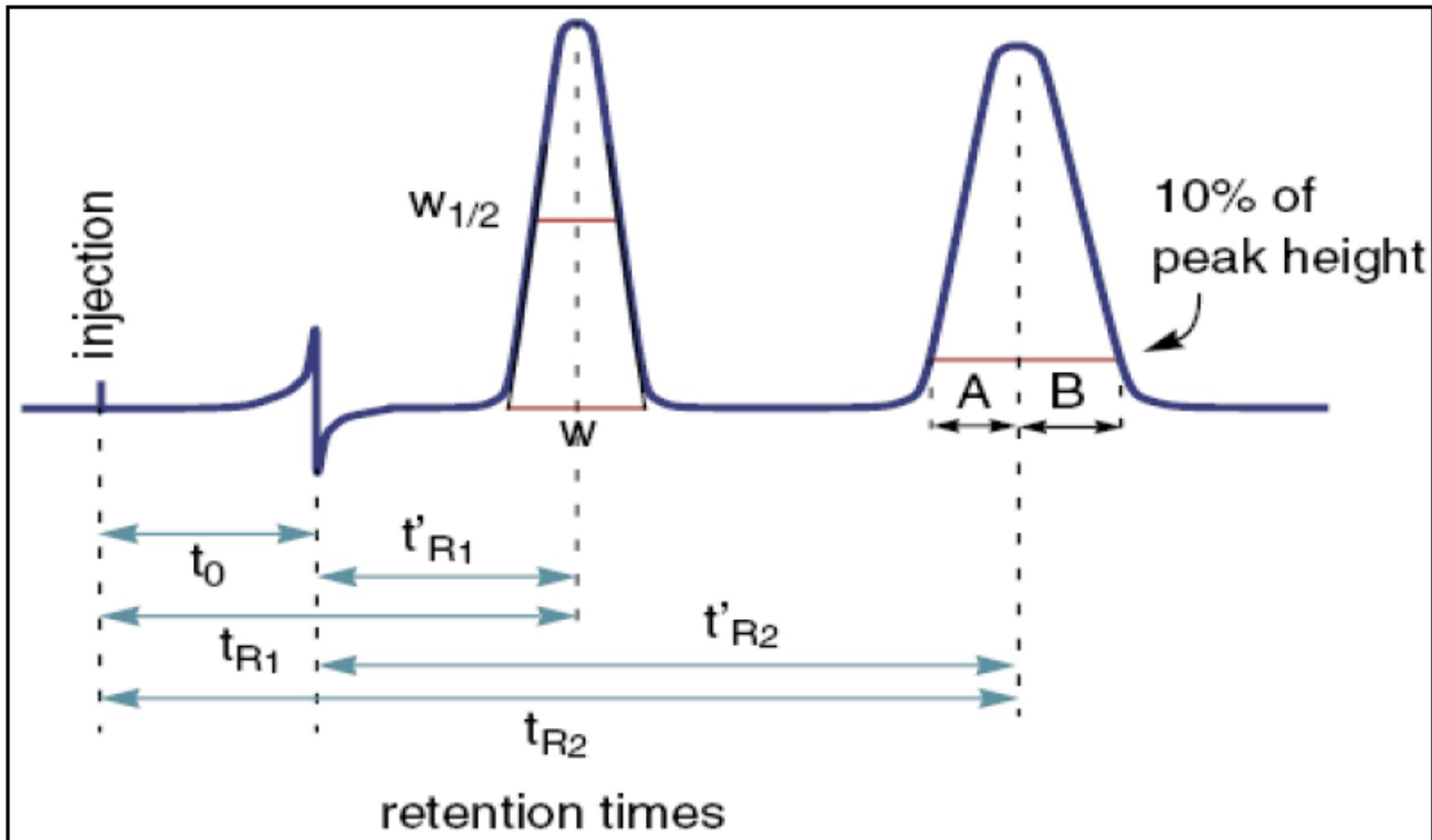
Typical chromatogram and its characteristic parameters.

Example:

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 B are 1.11 and 1.21 min, respectively.
Calculate: (a) column resolution, (b) average number of plates in the column, (c) plate height, (d) length of column required to achieve a resolution of 1.5, and (e) time required to elute substance B on the longer column.

(a) $R_s = 2(17.63 - 16.40) / (1.11 + 1.21) = 1.06$
(b) $N = 16 (16.40 / 1.11)^2 = 3493$ and $N = 16 (17.63 / 1.21)^2 = 3397$
   $N_{avg} = (3493 + 3397) / 2 = 3445$
(c) $H = L I N = 30.0 / 3445 = 8.7 \times 10^{-3}$ cm
(d) k' and α do not change greatly with increasing N and L. Thus,

$$\frac{(R_s)_1}{(R_s)_2} = \frac{\sqrt{N_1}}{\sqrt{N_2}} \quad == \quad \frac{1.06}{1.5} = \frac{\sqrt{3445}}{\sqrt{N_2}} \quad == \quad N_2 = 3445 \left(\frac{1.5}{1.06}\right)^2 = 6.9 \times 10^3$$
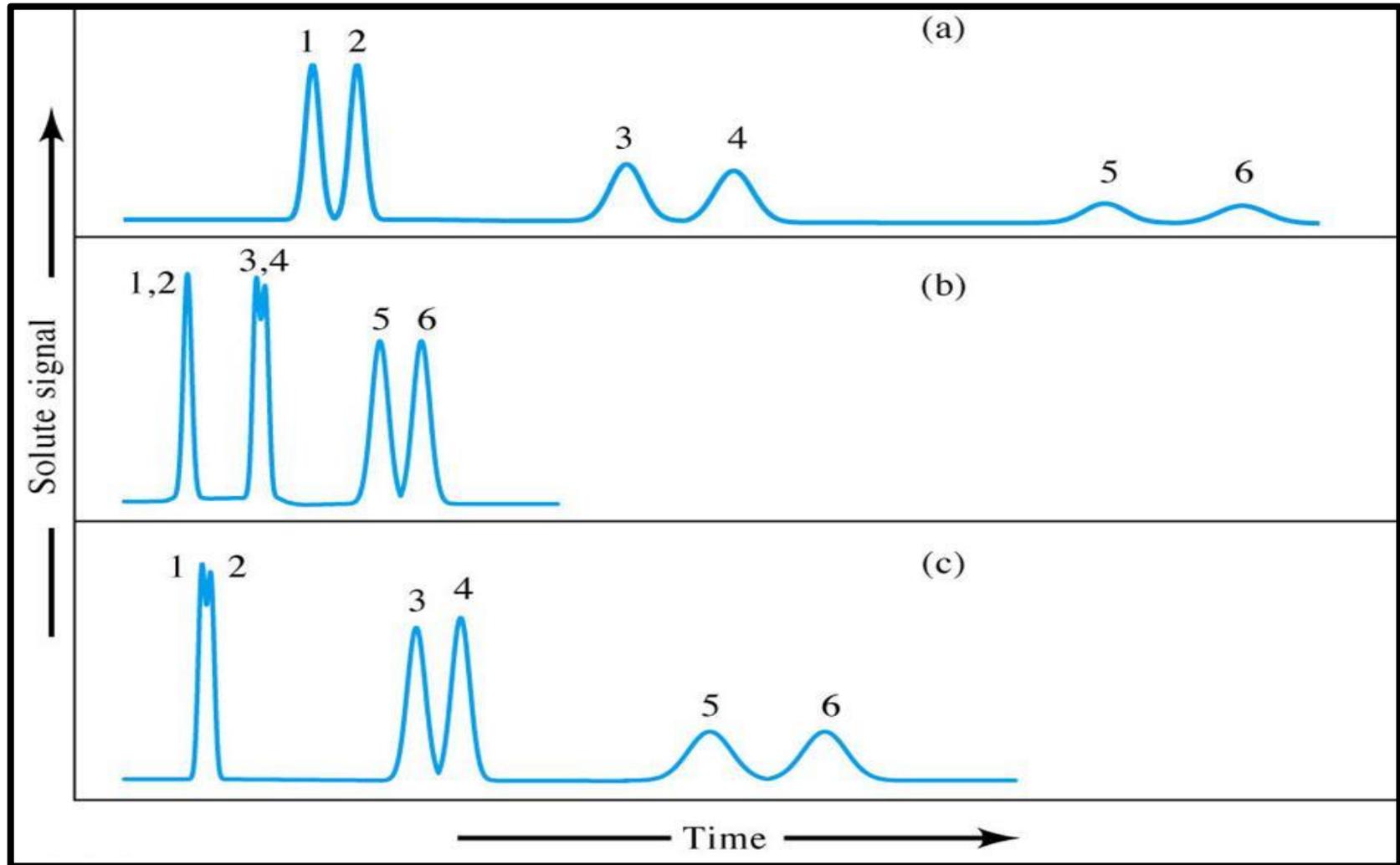
   $L = N H = 6.9 \times 10^3 \times 8.7 \times 10^{-3} = 60$ cm

(e) $$\frac{(t_R)_1}{(t_R)_2} = \frac{(R_s)_1^2}{(R_s)_2^2} = \frac{17.63}{(t_R)_2} = \frac{(1.06)^2}{(1.5)^2}$$

   $(t_R)_2 = 35$ min
   Thus, to obtain the improved resolution, the separation time must be doubled.

The general elution problem

What is desired in chromatography is the highest possible resolution in the shortest possible elapsed time.
The solution is to change the conditions to produce chromatogram like (a) for (1) and (2), then change the conditions to elute (5) and (6) as in chromatogram (b), then change the conditions again to elute (3) and (4) as in chromatogram (c).

The solution is to change the conditions during the run;
- For LC, gradient elution
- For GC, temperature programming

Isocratic *vs.* Gradient elution

Column dimensions

A modern chromatographic columns is **stainless steel**, **plastic tubes**, **glass** or **fused silica capillaries** filled with the stationary phase and arranged with end fittings designed to provide sealed connection with the eluent inlet and outlet lines and to retain packing material inside while allowing the mobile phase to pass through.

The length, diameter and construction material of the column affect the lifetime, efficiency and speed of separation. The size and nature of the packing material affect resolution.

Different HPLC columns length and diameter dimensions.
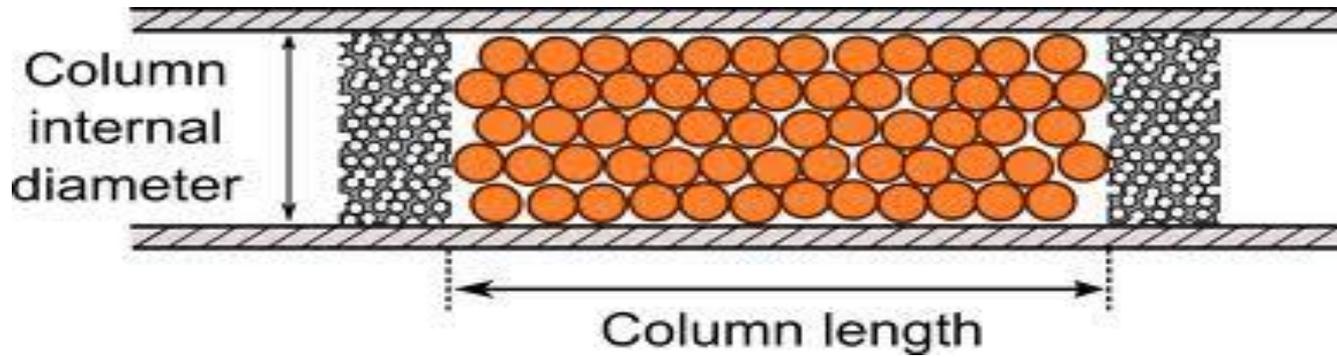
Internal diameter of column

The i.d. of a column is a critical aspect that determines quantity of analyte that can be loaded onto the column, the peak dilution and the flow rate. The larger the i.d., the greater is the loading capacity and the higher is the flow rate. However, peak dilution increases with i.d., and therefore mass sensitivity decreases. In contrast, smaller columns i.d. influences sensitivity and reduce the solvents, samples and reagents consumption at the expense of loading capacity.

Column length

Column length affects both the efficiency and the speed of the separation. Longer columns have higher plate numbers and yield better resolution but with longer analysis times. Note that column pressure drop is also proportional to the column length. However, the column efficiency tends to increase with length. In general, short columns are used for simple separations, while long columns are used for complex mixtures.

Column diameter

Names and definitions for HPLC techniques, relation between columns i.d., flow rate and sensitivity gain

| HPLC technique	Column i.d.	Flow rate	Injection volume	Relative gain in sensitivity
Preparative	> 10mm	> 20mL/min	-----	-----
Semi-preparative	5.0–10mm	5.0–20mL/min	-----	-----
Conventional	3.2–4.6mm	0.5–2.0mL/min	100µL	1
Narrowbore	1.5–3.2mm	100–500µL/min	19µL	5
Microbore	0.5–1.5mm	10–100µL/min	4.7µL	21
Micro capillary	150–500µm	1–10µL/min	490nL	235
Nano capillary	10–150µm	10–1000nL/min	12nL	3800
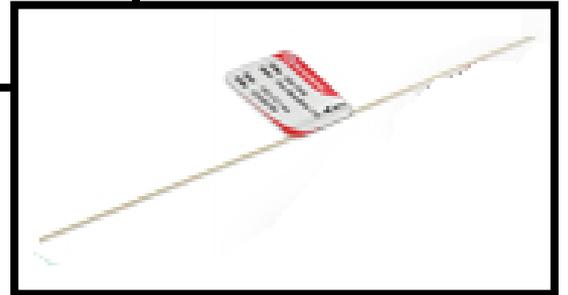
Particle diameter
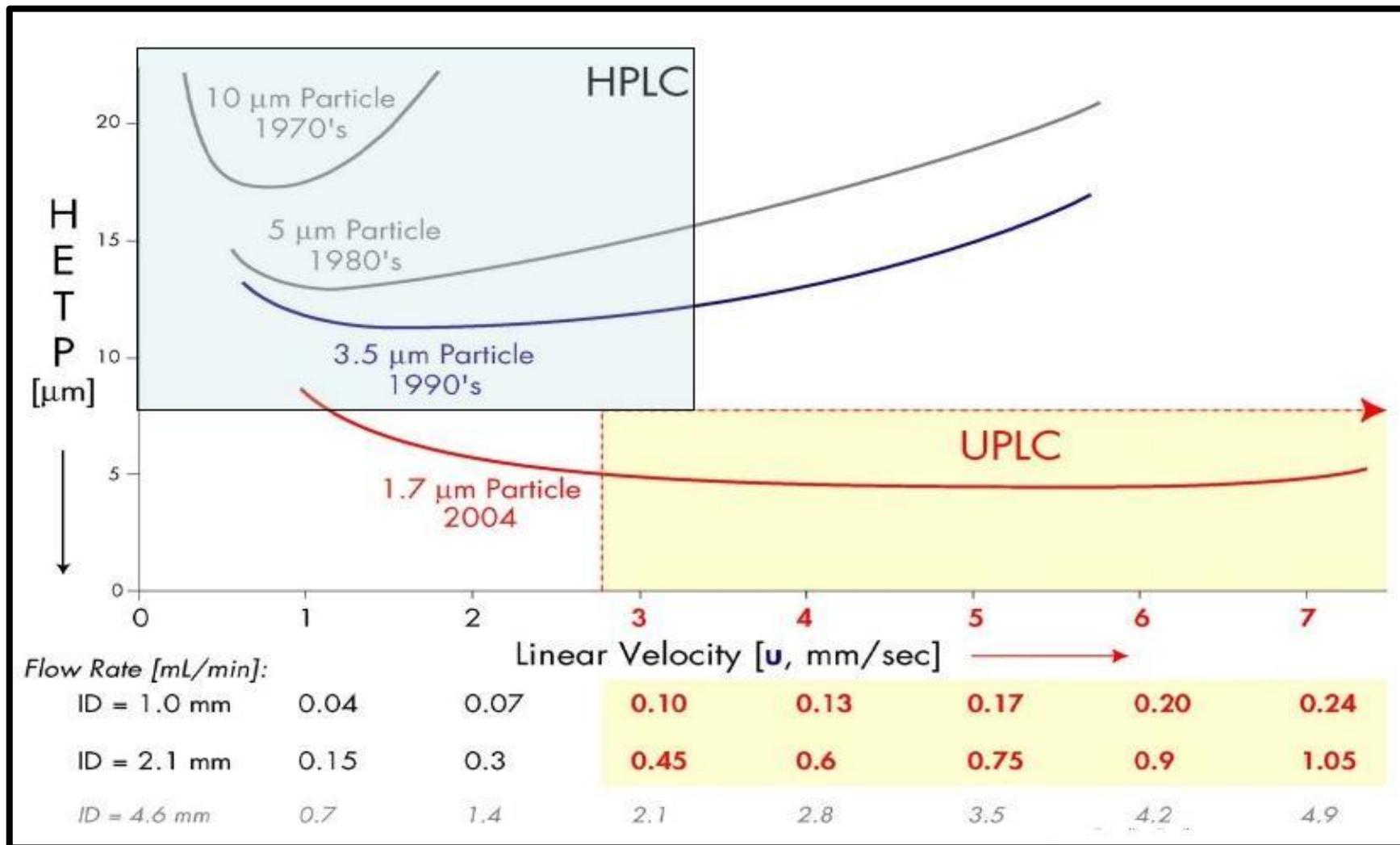
van Deemter plot, illustrating the evolution of particle sizes.

A chromatographic separation is optimized by varying experimental conditions until the components of a mixture are separated cleanly with a minimum expenditure of time.

Important variables affect method efficiency:

(1) Particle diameters
(2) Column diameters
(3) Column length
(4) Column type (stationary phase)
(5) Mobile phase type
(6) Mobile phase composition
(7) Gradient and temperature program
(8) Flow rate
(9) Pressure
(10) Detection method

Selection of an **appropriate** separation **method**

Stationary Phase

Detection Method

Mobile Phase
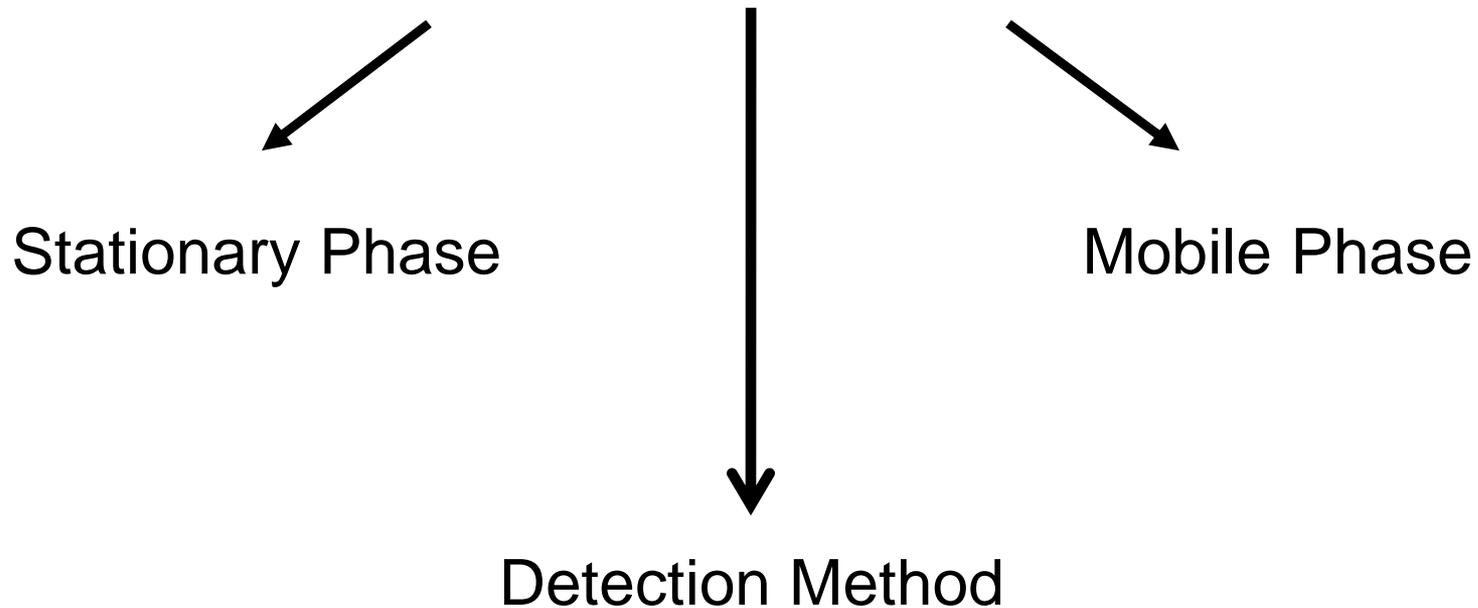
The goal in chromatography is the **highest possible resolution in the shortest possible elapsed time**.

Unfortunately, these goals tend to be incompatible and cannot both be optimized under the same conditions, consequently, a compromise between the two is usually necessary.

A chromatographic methods are a compromised techniques
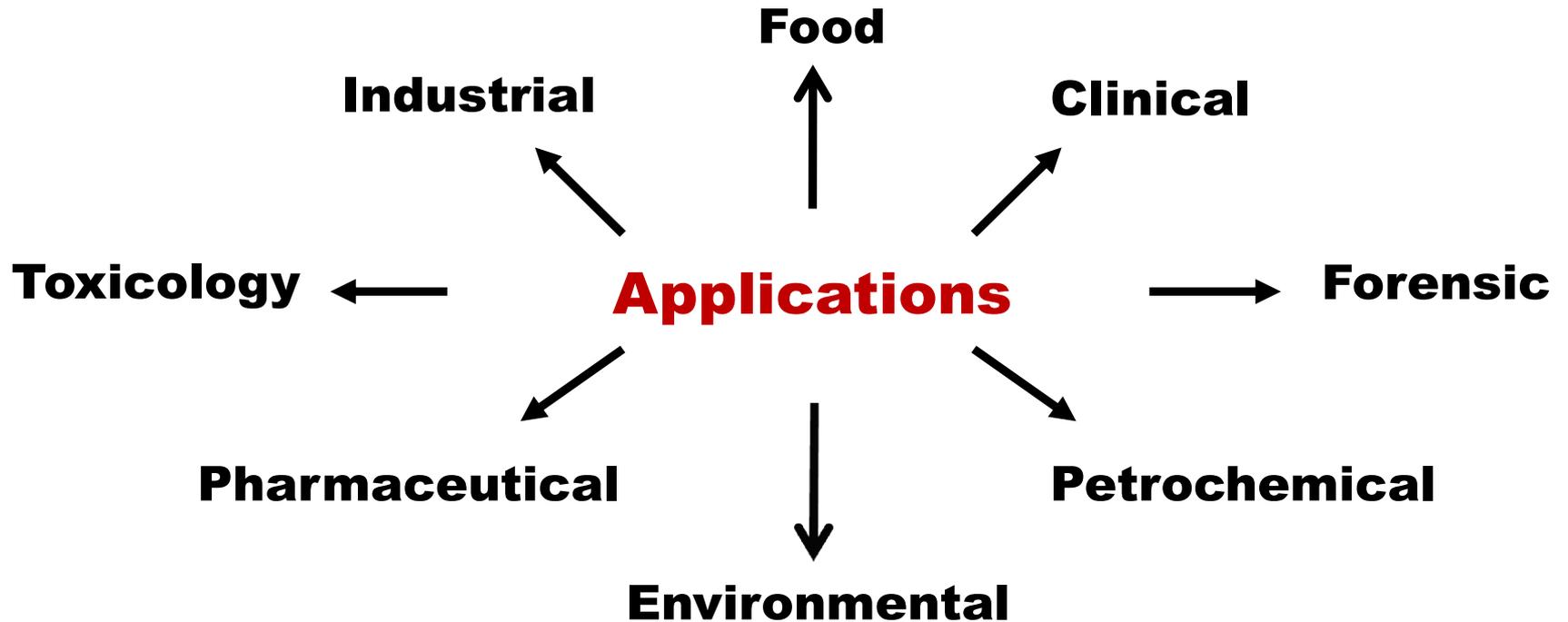
Applications of chromatography

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.

Modern **Chromatographic methods** have many applications including:

Separation

Identification

Purification
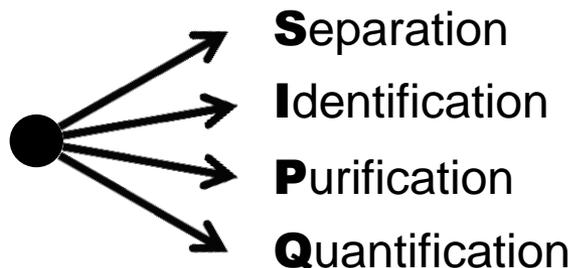
Quantification

Application areas

Qualitative analysis

- Qualitative analysis is the identification of the mixture constituents separated by chromatography.

- It is generally based on comparison of reference standards to the unknown chromatogram peaks.

- Components having the same retention time (or retention parameters) are assumed to be the same.

- Other qualitative criteria can be taken into account to confirm this identification: UV-spectra (Diode array) or mass spectra.

A chromatogram provides only a single piece of qualitative information about each species in a sample, namely, its retention time or its position on the stationary phase after a certain elution period.

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. Positive spectroscopic identification would be impossible without a preliminary chromatographic separation on a complex sample.
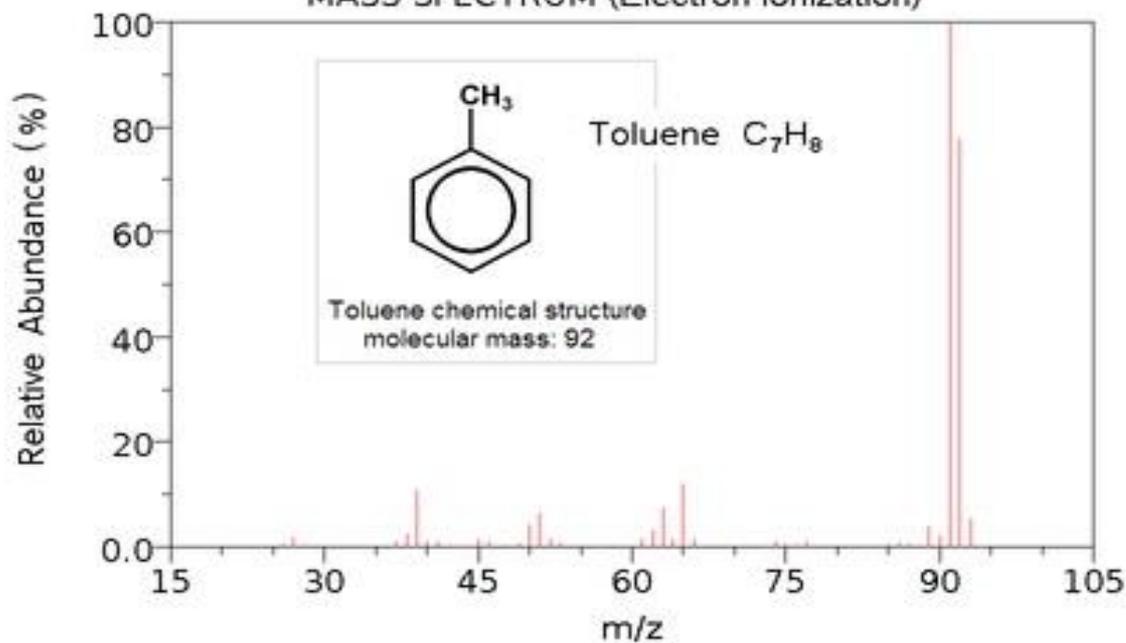
VIS-spectra in DMSO


UV-spectra (diode array detector, DAD)
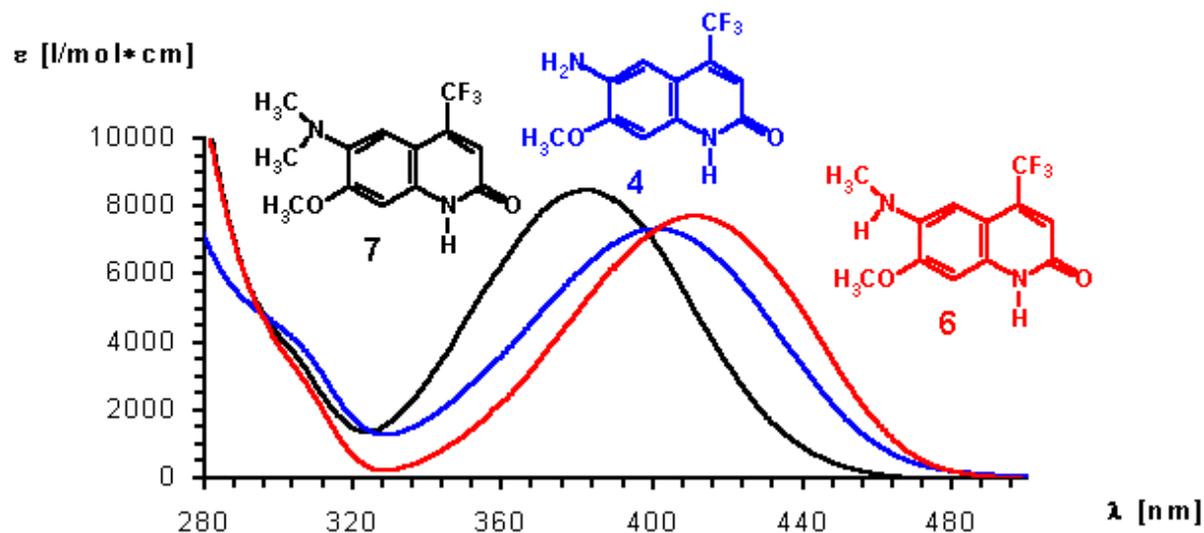
Mass spectra (mass spectrometric detector, MS)

Quantitative analysis

Chromatography can provide useful quantitative information about the separated species. 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. If conditions are properly controlled, these parameters vary linearly with concentration.

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.
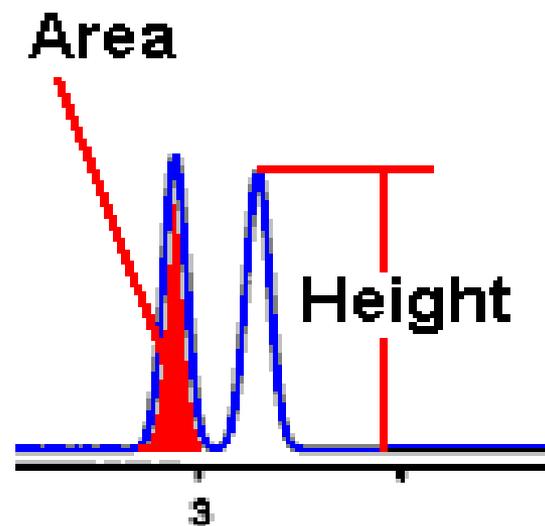
Quantitative analysis is the determination the concentration of each constituent in the separated mixture.

The detector basically responds to the concentration of the compound band as it passes through the flow cell

The more concentrated is the constituent, the stronger its signal; this is seen as a greater height response from the baseline