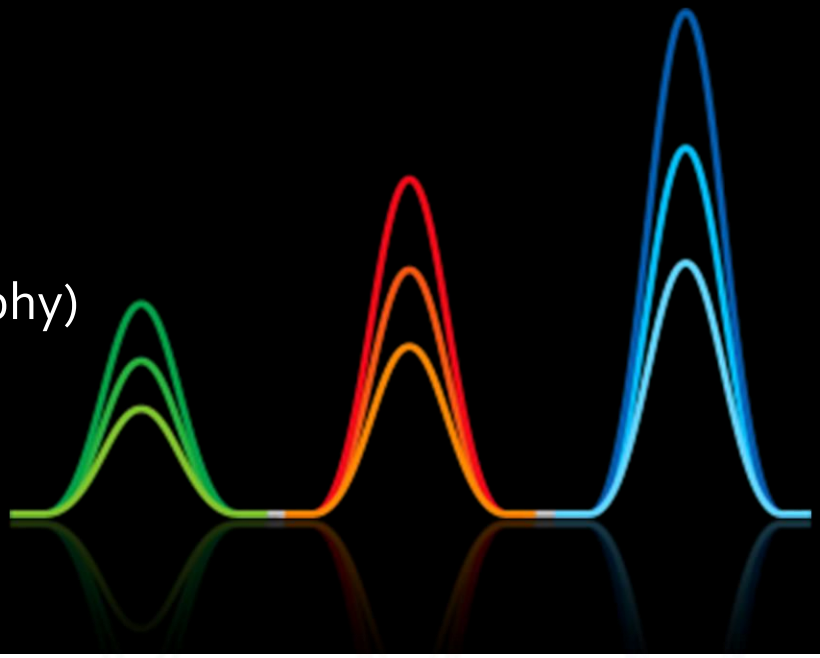
Instrumental Chemical Analysis

Chromatography

(General aspects of chromatography)



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Chromatography

- Tswett (1906) stated that " chromatography is a method in which the components of a mixture are *separated* on **adsorbent** column in a flowing system".
- **Chromatography** is a physical method of separation that *distributes* components (in certain matrix) between **two phases**, one is stationary (stationary phase), the other is moving in a definite direction (the mobile phase) .
- Chromatography covers a wide variety of techniques which are applied in all over chemistry in research and development, manufacturing, diagnostics, quality assurance etc. The common in these analytical methods is that the components of the sample are *partitioning* between a stationary and a mobile phase.
- The components are **separated from each other because of the difference in the strength of interactions for each component with the stationary phase and as a result the times spent on the stationary phase are different**.

Chromatography

Chromatographic terms

- The **analyte** is the substance to be separated during chromatography. It is also normally what is needed from the mixture.
- **Elution** is the process of passing the mobile phase through the column.
- **Eluent** (**mobile phase)** is the phase that moves in a definite direction. It may be a liquid, a gas, or a supercritical fluid. The mobile phase moves through the chromatography column (the stationary phase) where the sample interacts with the stationary phase and is separated.
- **Eluate** is the solution that moved out from the chromatography column. It contains the sample components separated/analyzed and the eluent.
- The **stationary phase** is the substance fixed in place for the chromatography procedure.
- The **detector** refers to the instrument used for qualitative or/and quantitative detection of analytes after separation (eluate).
- **Chromatogram** is the graph for the detector response (which is related to the analyte concentration) versus elution time or volume.

Chromatography

Chromatographic techniques can be grouped several ways:

A. Based on sample nature:
   1. Paper or TLC (1D or 2D)
   2. Column chromatography
      a. Liquid chromatography
      b. Gas chromatography

B. Based on stationary phase nature:
   1. Normal phase mode
   2. Reverse phase mode

C. Based on stationary phase – analyte interaction type:
   1. Adsorption chromatography
   2. Ion exchange chromatography
   3. Partition chromatography
   4. Size exclusion

D. Based on elution technique:
   1. Isocratic separation
   2. Gradient separation
   3. Programmed temperature

E. Based on the scale of operation
   1. Analytical
   2. Preparative

F. Based on the purpose
   1. Screening
   2. Qualitative analysis
   3. Quantitative analysis
   4. Purification

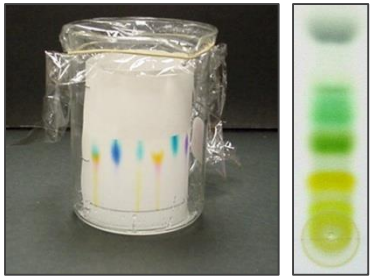
G. Based on sample loading method
   1. Manual injection with sampling loops
   2. Autosampler system
   3. On-line (pre- and post column) derivatization

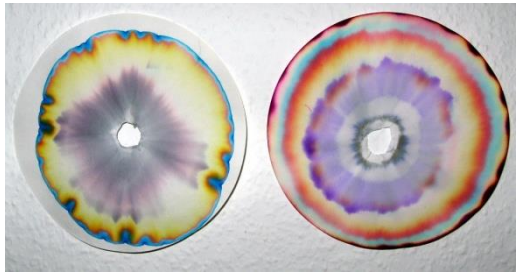
H. Based on pressure applied
   1. Low pressure (flash chromatography)
   2. High pressure (HPLC)
   3. Ultra high pressure (UPLC)

I. Based on detection technique
   1. Electrochemical (IC and Voltammetry)
   2. Spectrometry (UV/Vis, DAD, Flu, MS,RI,… etc)
   3. Others (thermal, e capture, flame ionization)

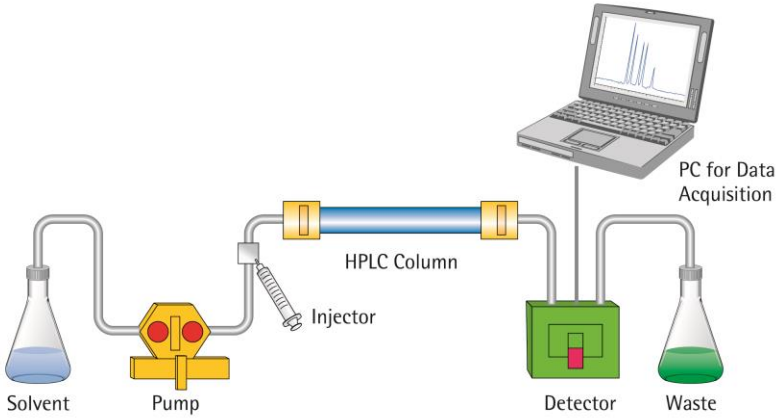
Chromatography

- **Planar chromatography** - flat stationary phase, mobile phase moves through capillary action or gravity





- **Column chromatography** - tube of stationary phase, mobile phase moves by pressure or gravity



Chromatography

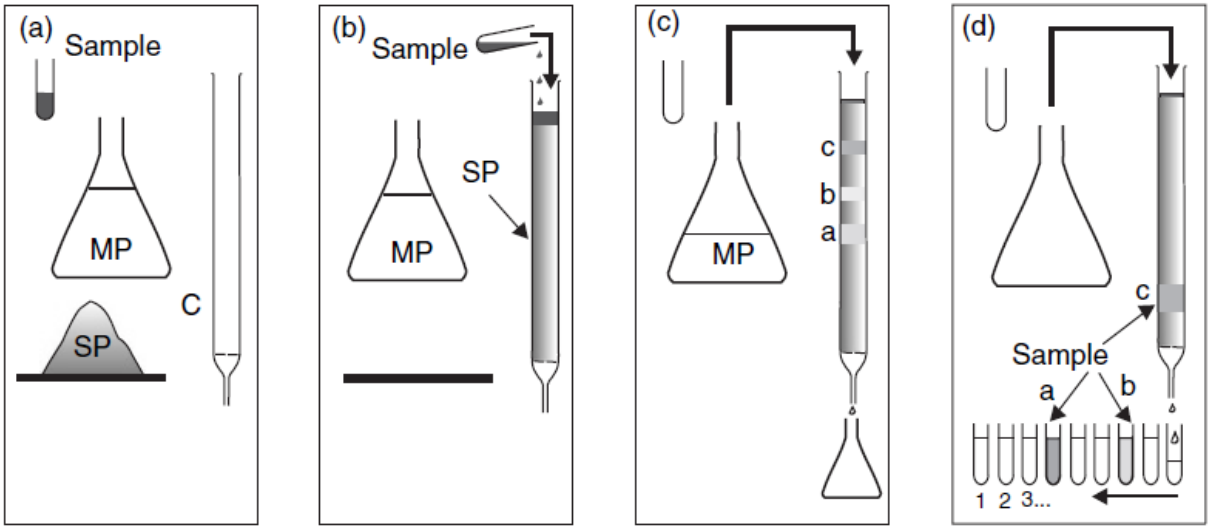


Figure 1.1 *A basic experiment in chromatography.* (a) The necessary ingredients (C, column; SP, stationary phase; MP, mobile phase; and S, sample); (b) introduction of the sample; (c) start of elution; (d) recovery of the products following separation.

- To identify compounds in a mixture, we need to measure the immigration times and compare it with standard reference materials. To do that, an optical device (or other detecting device/method) was placed at the column exit, which indicated the variation of the composition of the eluting phase with time.

Chromatography

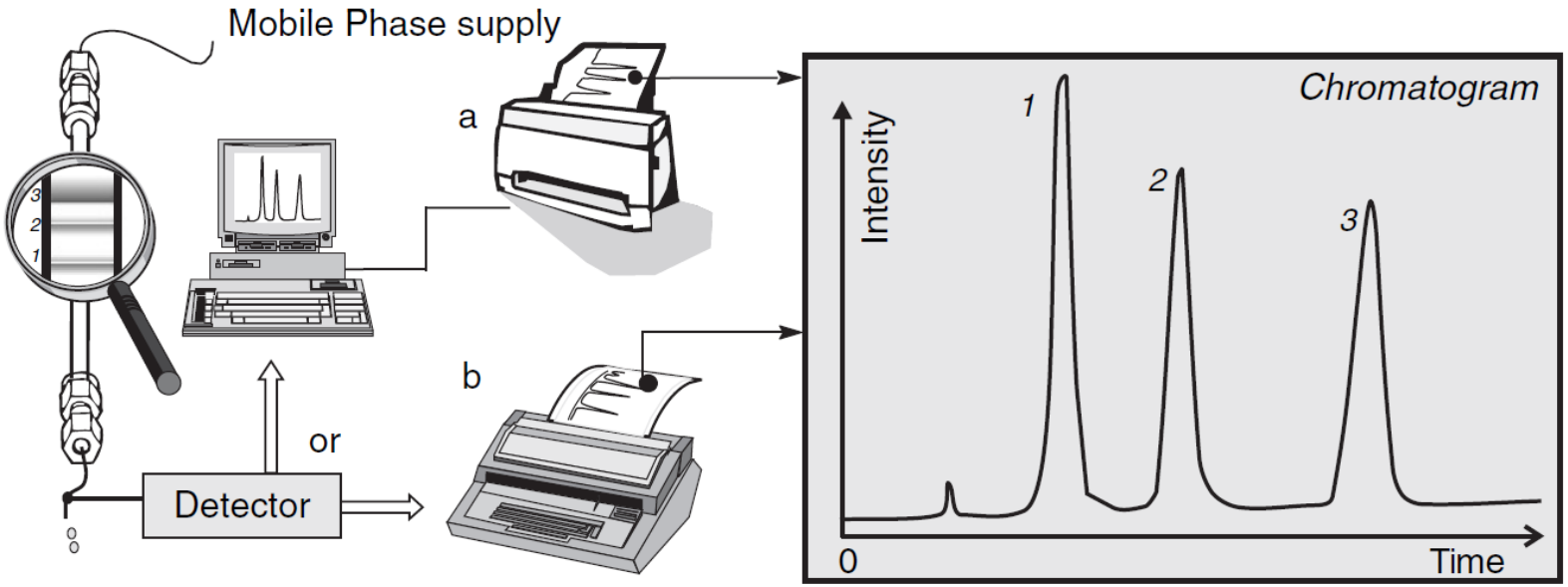
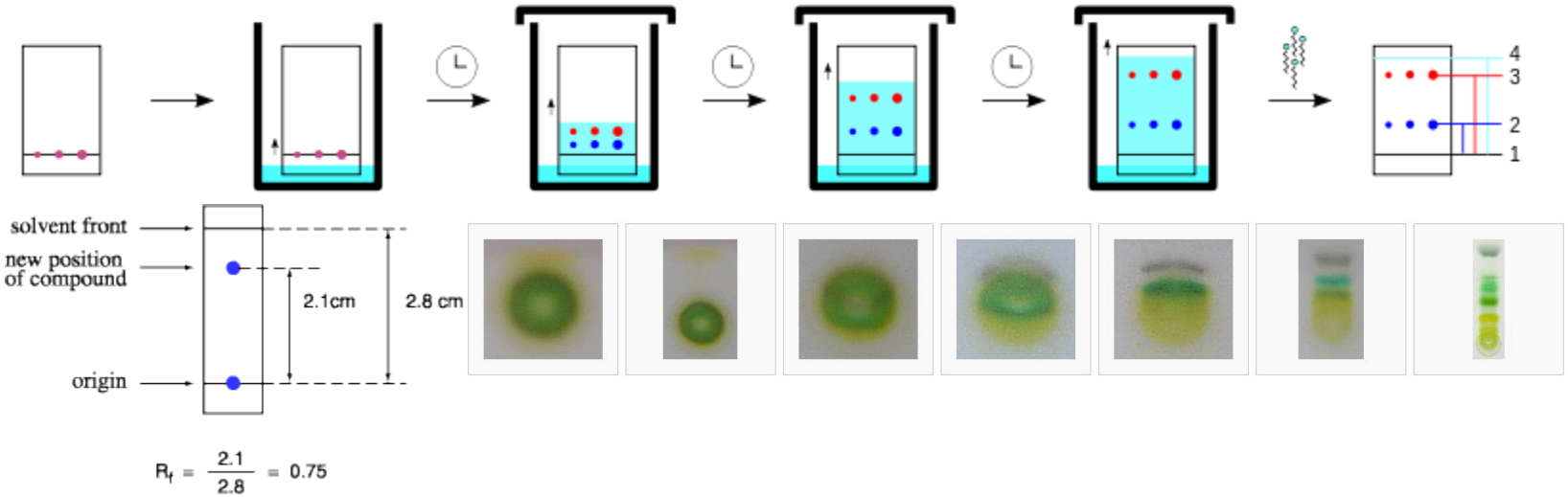


Figure 1.2 *The principle of analysis by chromatography*. The chromatogram, the essential graph of every chromatographic analysis, describes the passage of components. It is obtained from variations, as a function of time, of an electrical signal emitted by the detector. It is often reconstructed from values that are digitized and stored to a microcomputer for reproduction in a suitable format for the printer. (a). For a long time the chromatogram was obtained by a simple chart recorder or an integrator (b). Right, a chromatogram illustrating the separation of a mixture of at least three principal components. Note that the order of appearance of the compounds corresponds to the relative position of each constituent on the column.

Paper and Thin Layer Chromatography

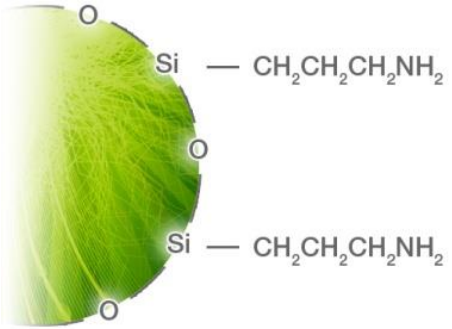
- Thin layer chromatography (TLC) is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide, or **cellulose**. This layer of adsorbent is known as the stationary phase.

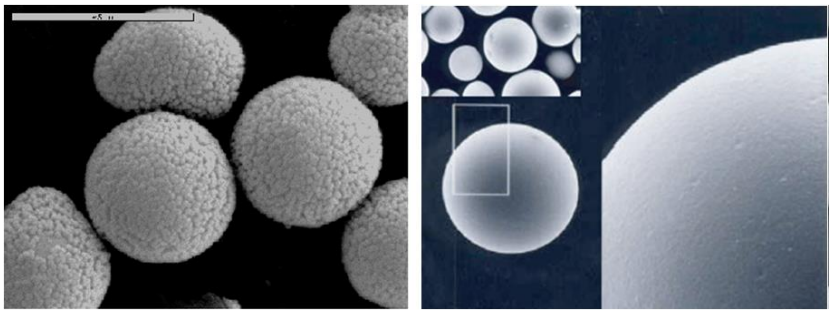


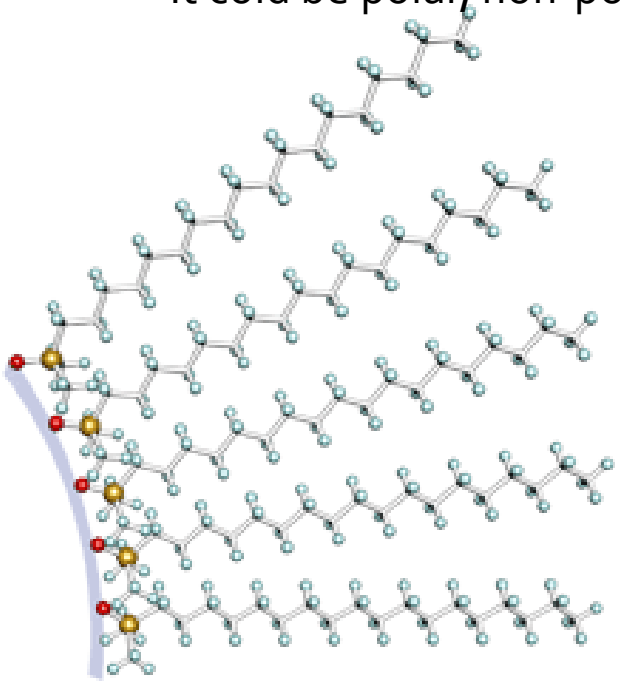
- The order of mobile phase strength/weakness depends on the coating (stationary phase) of the TLC plate. For silica gel coated TLC plates, the eluent strength increases in the following order: Perfluoroalkane (weakest), Hexane≈Pentane, Carbon tetrachloride, Toluene, Benzene, Diethyl ether, Dichloromethane, Acetone, Ethylacetate, Acetonitrile, 1-Propanol, Ethanol, Methanol, Acetic acid, Water, (strongest). For C18 coated plates the order is reverse.
- Strong mobile phase: a solvent that quickly elutes solutes from the column.

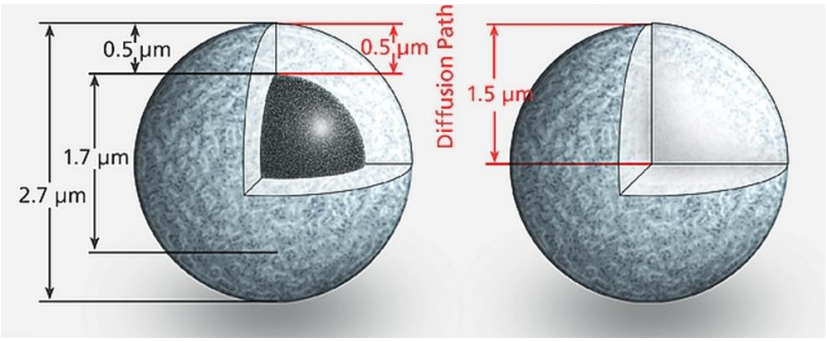
Stationary Phase

- It may be a solid, a gel or a liquid.
- If a liquid, it should be distributed on a solid support. The liquid may be chemically bonded covalently to the solid (bonded phase) or immobilized onto it (immobilized phase).
- It could be packed or coated.
- It cold be polar, non-polar or inert.

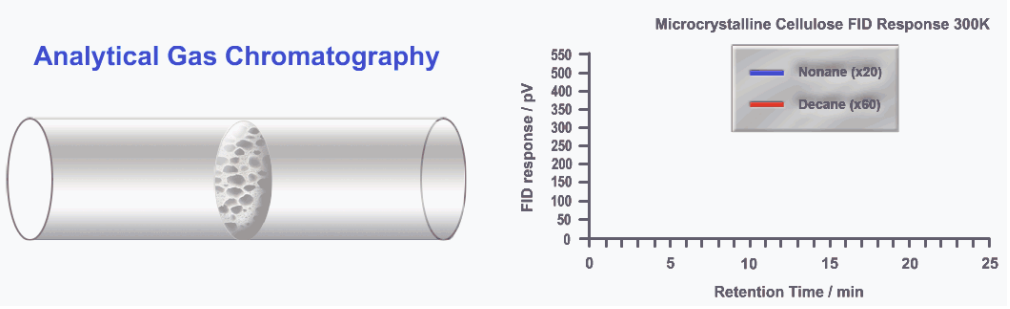








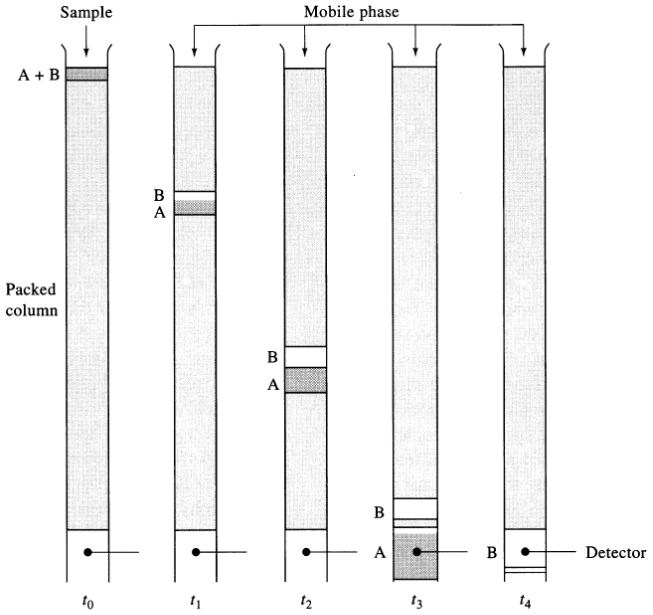
Column Elution Chromatography

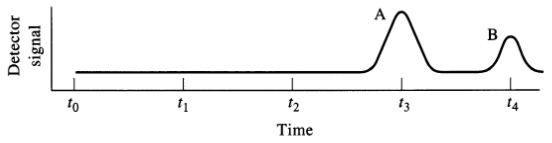


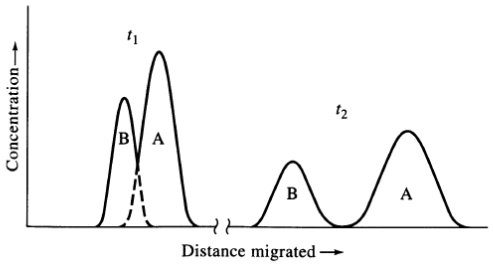
- Elution mechanism depends on the fact that the solute will establish an equilibrium state between the stationary and mobile phases. The distribution of the solute into the two phases is described by the **distribution coefficient K,**

$$K = \frac{[X]_s}{[X]_m}$$

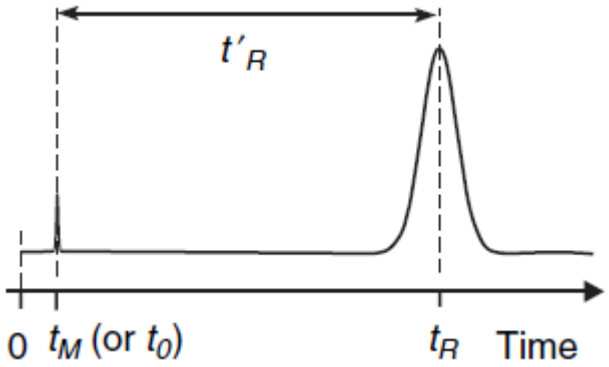
- Solutes with large K will be retained strongly by the stationary phase (slower movement on the column).
- K is defined for a given chemical compound in a specific chromatographic system.







Chromatogram

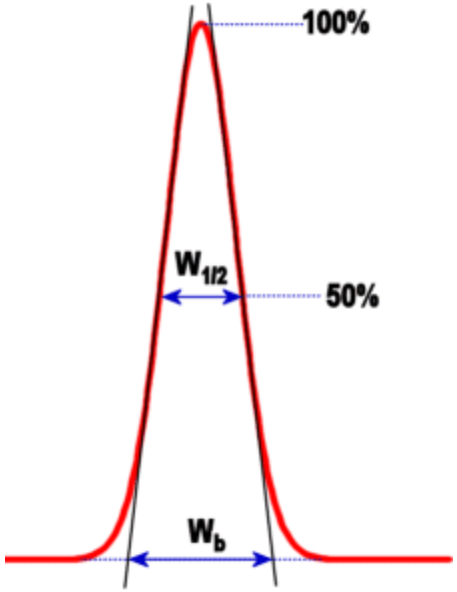


$F = \text{flow rate}$

$t_M = \text{retention time of mobile phase}$

$t_R = \text{retention time of analyte (solute)}$

$L = \text{length of the column}$



Baseline width (W_b). This is measured by drawing tangents to the front and back slopes of the peak. The baseline width is the distance between the intersections of these tangents with the baseline.

The **width-at-half-height ($W_{½}$)** is the distance from the front slope of the peak to the back slope of the peak measured at 50% of the maximum peak height.

If the peak has the ideal "Gaussian" shape, then **W_b = 1.7 $W_{½}$**, but for most "real" peaks, baseline width is somewhat larger than this.

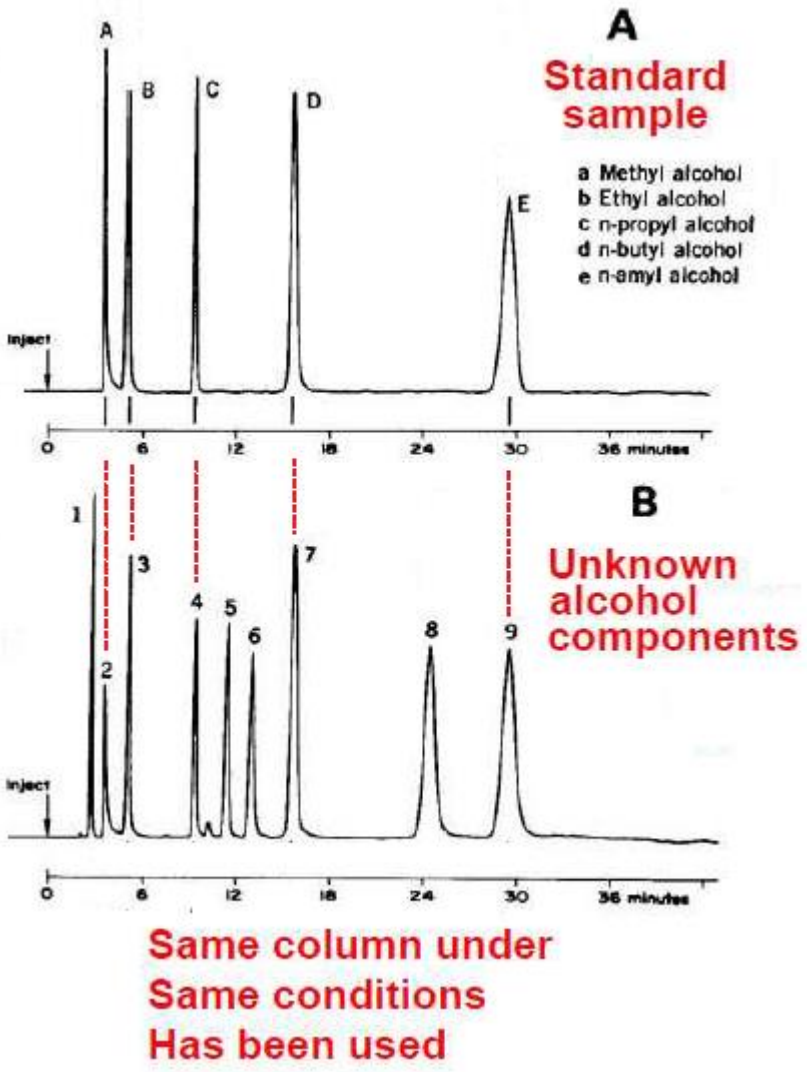
Chromatogram

- Retention time (t_R) is defined by the time taken between the moment of sample injection into the chromatograph and the analyte peak maximum recorded on the chromatogram.
- The hold-up time (t_M), called, void time or the dead time sometimes denoted by (t_o). It is the time required for the mobile phase to pass through the column.
  - (t_M) also is retention time for material that does not interact with the stationary phase at all and hence move totally with mobile phase.
- Retention volume (or elution volume) is the volume of mobile phase needed to elute the analyte, $V_R = t_R \times F$.
- Hold-up volume (or dead volume) $V_M = t_M \times F$.
- For all analytes in a sample if $t_R = t_M$ then no separation (analytes move as quick as mobile phase).
- The difference between the retention time and the hold-up time is designated by the *adjusted retention time* of the compound, (t'_R).

Chromatogram

For qualitative analysis

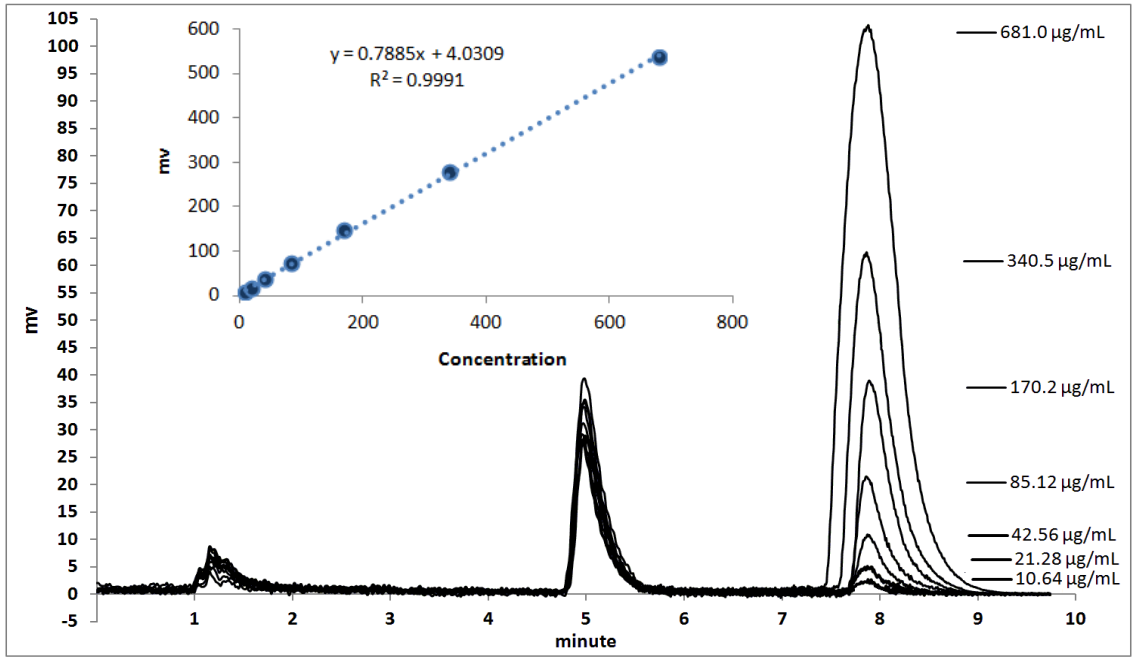
- Retention time is used for identification purposes but needs another qualitative test for confirmation.
- By running a standard reference material (SRM) at a given operation conditions, the t_R for the analyte of interest can be designated.
- Then running an unknown sample where the analyte is expected to be present (at identical operational conditions as the SRM)
  - If there is no peak at t_R of the SRM then we are sure that the unknown does not contain the analyte
  - If there is a peak at the t_R then we have to use another test to confirm.



Chromatogram

For quantitative analysis

- The detector should have a linear response with the analyte concentration.
- Then the area under the peak (from the chromatogram) can be related to the concentration of the analyte.
- Again a SRM should be used to prepare a calibration curve from which the analyte with unknown concentration can be determined.



Chromatogram

Important Chromatographic Quantities and Relationships

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

Chromatography Theories

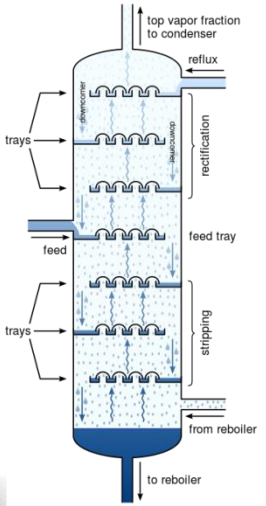
"Like light rays in the spectrum, the different components of a pigment mixture, obeying a law, are resolved on the calcium carbonate column and then can be qualitatively and quantitatively determined. I call such a preparation a chromatogram and the corresponding method the chromatographic method."

Mikhail Semenovich Tswett, Russian botanist (1872–1919)

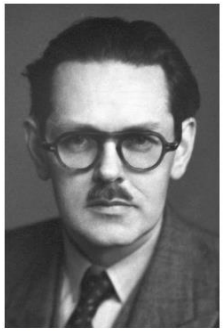
Chromatography Theoies

Plate Theory, 1941

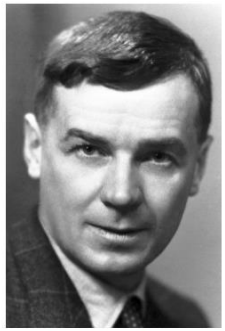
Chromatography column assumed to be similar to a distillation column. Separation occurs across a series of theoretical plates. Higher number of theoretical plates improves column performance.



- The Nobel Prize in Chemistry 1952 was awarded jointly to Archer John Porter Martin and Richard Laurence Millington Synge "for their invention of partition chromatography."



Archer John Porter Martin

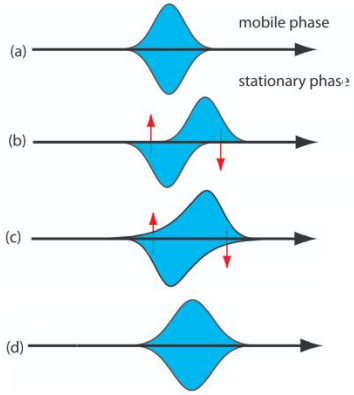


Richard Laurence Millington Synge

Chromatography Theories

Rate Theory, 1956

Plate theory does not explain band spreading and peak broadening. Does not take into account packing material properties, mobile phase flow rate and column geometry. Rate theory takes into account various factors that cause chromatographic peak broadening and reduction of separation efficiency.



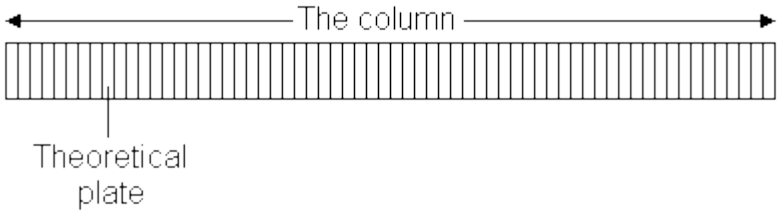
Longitudinal diffusion and resistance to mass transfer as causes of nonideality in chromatography

J J VAN DEEMTER*, F. J. ZUIDERWEG* and A. KLINKENBERG**

Chemical Engineering Science, 1956, Vol 5, pp 271 to 289

Plate theory

- Consider a column of length **L** is sliced horizontally into **N** number of small plate-like discs of same height **H** (also called The equivalent height of a theoretical plate HETP). For each plate the concentration of the solute in the mobile phase is in equilibrium with the concentration of this solute in the stationary phase.

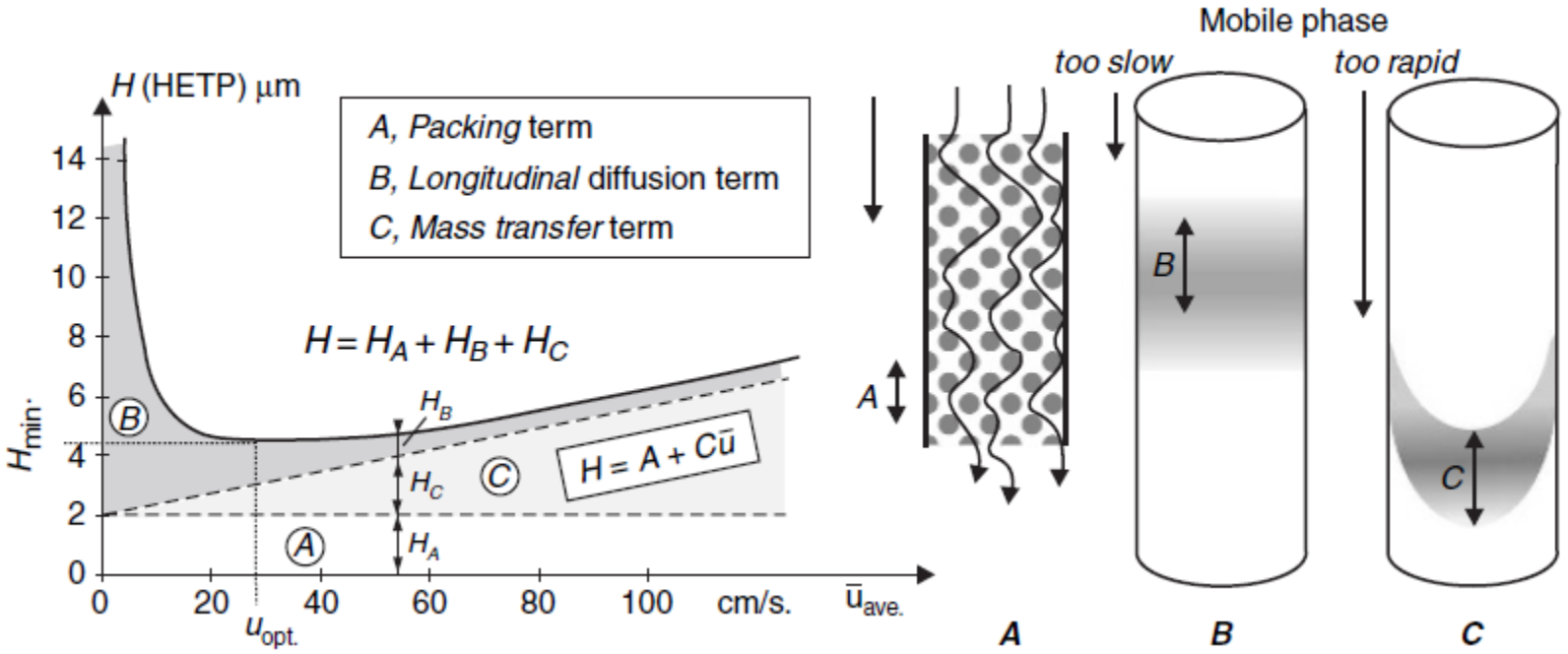


- Each analyte is considered to be moving through the column in a sequence of distinct steps, although the process of chromatography is a continuous phenomenon.
- Each step correspond to a new equilibrium and the solute move through the column by a distance of one plate through a process of (e.g. adsorption/desorption cycle).
- All chromatographic separation are based on differences in the extent to which solutes are distributed between mobile phase and stationary phase.

Rate theory (*Van Deemter Equation*)

$$H = A + B/\mu + C \cdot \mu$$

- A: Eddy Diffusion, Multi-path Effect
- B/μ: Longitudinal Diffusion
- C · μ: Mass Transfer (Kinetics)



Rate theory (*Van Deemter Equation*)

Processes That Contribute to Band Broadening

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

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	k	unitless
Diameter of packing particles	d_p	cm
Thickness of liquid coating on stationary phase	d_f	cm

λ is particle shape (with regard to the packing)

γ, f and f' are constants

The Chromatographic Parameters

Partition Coefficient , K

- Nernst partition coefficient (K) (also called distribution coefficient K_d) is a fundamental parameter in chromatography which relate the concentrations of each compound within the two phases :

$$C_M \Leftrightarrow C_S$$

$$K = \frac{C_S}{C_M} = \frac{\text{Molar concentration of the solute in the stationary phase}}{\text{Molar concentration of the solute in the mobile phase}}$$

- Partition coefficient indicates the amount of time that a compound spends in stationary phase as the opposed to the amount of time spends solvated by the mobile phase. This relationship determines the amount of time it will take for the compound to travel the length of the column. The more time spent in the stationary phase, the more time the compound will take to travel the length of the column.
- Because of the band broadening phenomenon the concentration of a particular analyte is varying depending on its place in the column. However <u>their ratio C_S/C_M remains constant</u>.

See: https://www.youtube.com/watch?v=naUKY6yoUu4

The Chromatographic Parameters

Retention (or Separation) factor, k

- Express the ability of the column to retain different compounds
- Used to compare the **migration rates** of solutes in columns.
- When a compound is injected onto a column, its total mass m_T is divided in two quantities: m_M, the mass in the mobile phase and m_S, the mass in the stationary phase.
- During solute's migration down the column:
  - m_S and m_M remain constant
  - their ratio, the retention factor (***k***) is constant and independent of m_T

$$k = \frac{m_S}{m_M} = K\frac{V_S}{V_M}$$

- ***k*** is useful for comparing results obtained on different systems since it is independent on column length and flow-rate. However, it depends on the experimental conditions.
- ***k*** (could be galculated from the chromatogram) is related to *K* (i.e. thermodynamic information).

The Chromatographic Parameters

Retention (or Separation) factor, k

- it expresses how much longer a sample component is retarded by the stationary phase than it would take to travel through the column with the velocity of the mobile phase.

- Values of retention factor ***k***:
  - If ***k*** less than 1: solute emerge from the column close to mobile phase
  - If ***k*** is high (e.g. 30): elution time is too long
  - Optimum value of ***k*** is (1-5)
- So optimizing the separation for the analyte (A) of interest is done by optimizing k_A
  - In gas chromatography by change temperature
  - In liquid chromatography by changing mobile phase composition

The Chromatographic Parameters

Retention (or Separation) factor, k

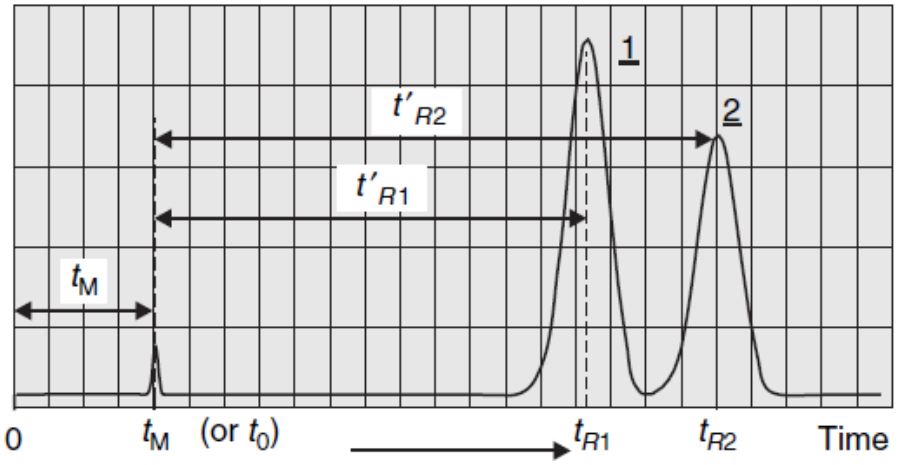
- Retention factor can be given from chromatogram

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

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

$$V_R = V_M(1+k)$$

$$V_R = V_M + KV_S$$



here,

$k_1 = \frac{t'_{R1}}{t_M}$ $k_1 = 3.07$

$k_2 = \frac{t'_{R2}}{t_M}$ $k_2 = 3.92$

$\alpha = \frac{t'_{R2}}{t'_{R1}}$ $\alpha = 1.27$

Retention factor does not take into account the peak width!!!!

The Chromatographic Parameters

Column efficiency, N and H

- Column efficiency expressed in term of number of theoretical plates **N** or plate height **H**
  - The greater the **N** the more efficient column
  - Column efficiency is **N = L/H**
  - Improve column efficiency by **increase the length of the column and/or reduce the height of the theoretical plate**
- Theoretical column efficiency is best given from the chromatogram by this equation

$$N = 16 \frac{t_R^2}{w^2} \quad \text{or} \quad N = 5.54 \frac{t_R^2}{w_{1/2^2}}$$

  - w: width of the peak at the base
  - $w_{1/2}$: width at half height of the peak

- The efficiency of a column is a function of several parameters. These include the size of the column packing particles, the uniformity of the packing, the flow of eluent, and the rapidity with which equilibrium is established between the two phases.

The Chromatographic Parameters

Selectivity (or separation) factor, α

- Used to measure the ability of the chromatographic system to distinguish between sample components.

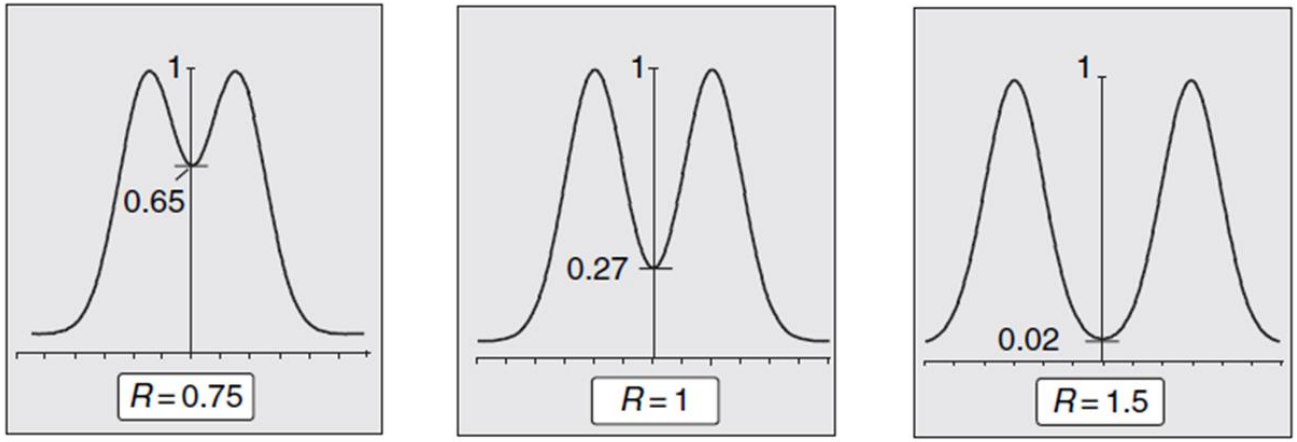
$$\alpha = \frac{k_2}{k_1} = \frac{K_2}{K_1}$$

- By definition **α** is greater than unity (species 1 elutes faster than species 2).
- **α** is related to **retention factor k** and **partition coeff. K**
- As the selectivity of a separation is dependent upon the chemistry of the analyte, mobile, and stationary phases all of these factors may be altered in order to change or optimize the selectivity of an HPLC separation.
- **α** optimized by manipulating:
  - Temperature
  - Composition of mobile phase (pH, organic %, ionic strength, additives)
  - Composition of stationary phase.
- Selectivity factor does not take into account the peak width. Therefore **α** alone is not enough to determine whether the separation is really possible.

The Chromatographic Parameters

Resolution factor between two peaks, Rs or R

- Quantitative measure of a column ability to separate two compounds
  - Take into account retention time and peak width
  - Minimum ***R*** = 1.5 is required to obtain complete separation

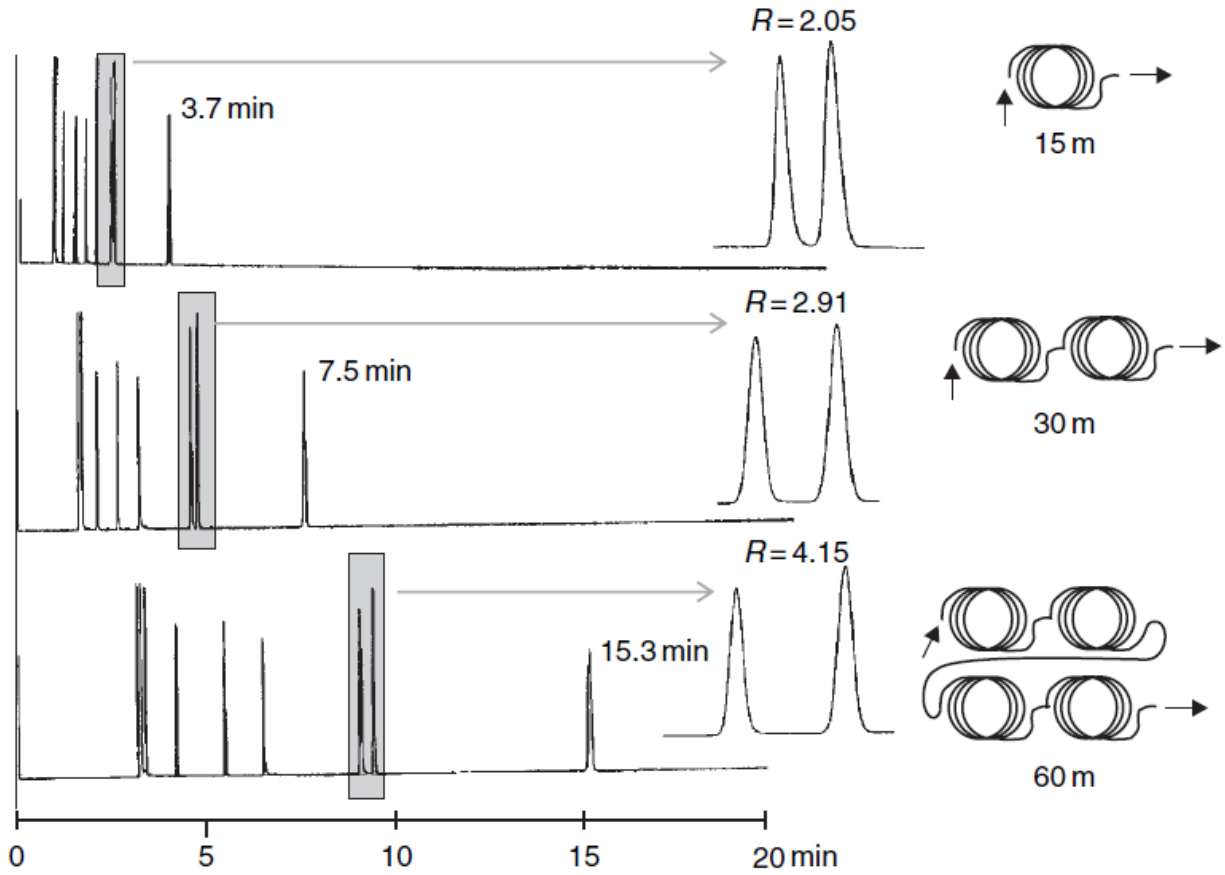


$$R = 2\frac{t_{R(2)} - t_{R(1)}}{w_1 + w_2}$$

$$R = \frac{1}{4}\sqrt{N} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k_2}{1 + k_2}$$

The Chromatographic Parameters

Resolution factor between two peaks, Rs or R



Q. How are the R, H, k, K, t_R, t_m, t_R', and α will be affected by doubling the column length?

The Chromatographic Parameters

Summary

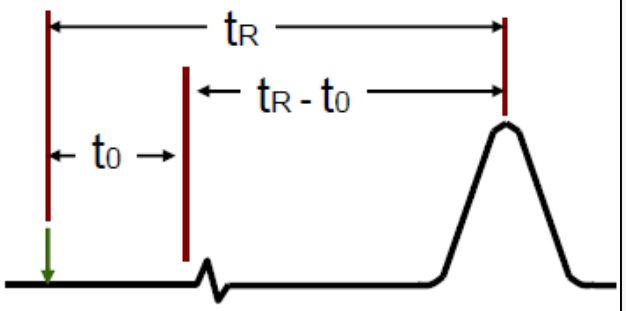
Important Derived Quantities and Relationships

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

The Chromatographic Parameters

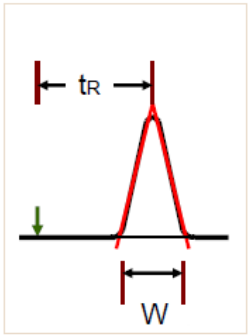
Summary

k' : capacity factor

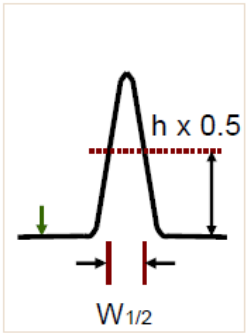


$$k' = \frac{t_R - t_0}{t_0}$$

The number of theoretical plates N



$N = 16 (t_R / W)^2$

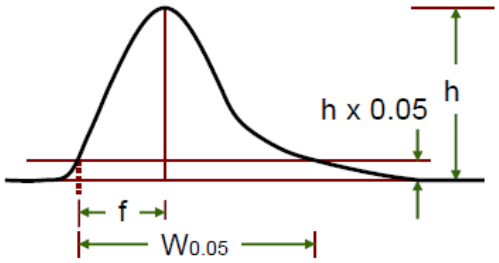


$N = 5.545 (t_R / W_{0.5})^2$

The height of the theoretical plate H

H = L / N L : Column length

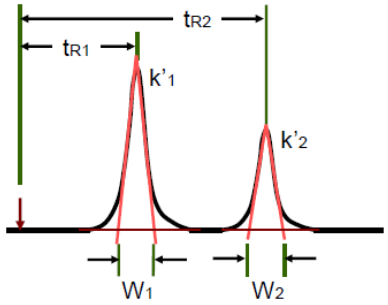
S : Symmetry factor (T : Tailing factor)



$$S = \frac{W_{0.05}}{2f}$$

S = 1 : The peak is completely symmetric.
S > 1 : Tailing
S < 1 : Leading

Degree of separation



Resolution : $R_s = 2 \times \frac{t_{R2} - t_{R1}}{W_1 + W_2}$

Separation factor : $\alpha = \frac{k'_2}{k'_1}$