NEPHAR 201
Analytical Chemistry II

Chapter 6
Chromatographic separations

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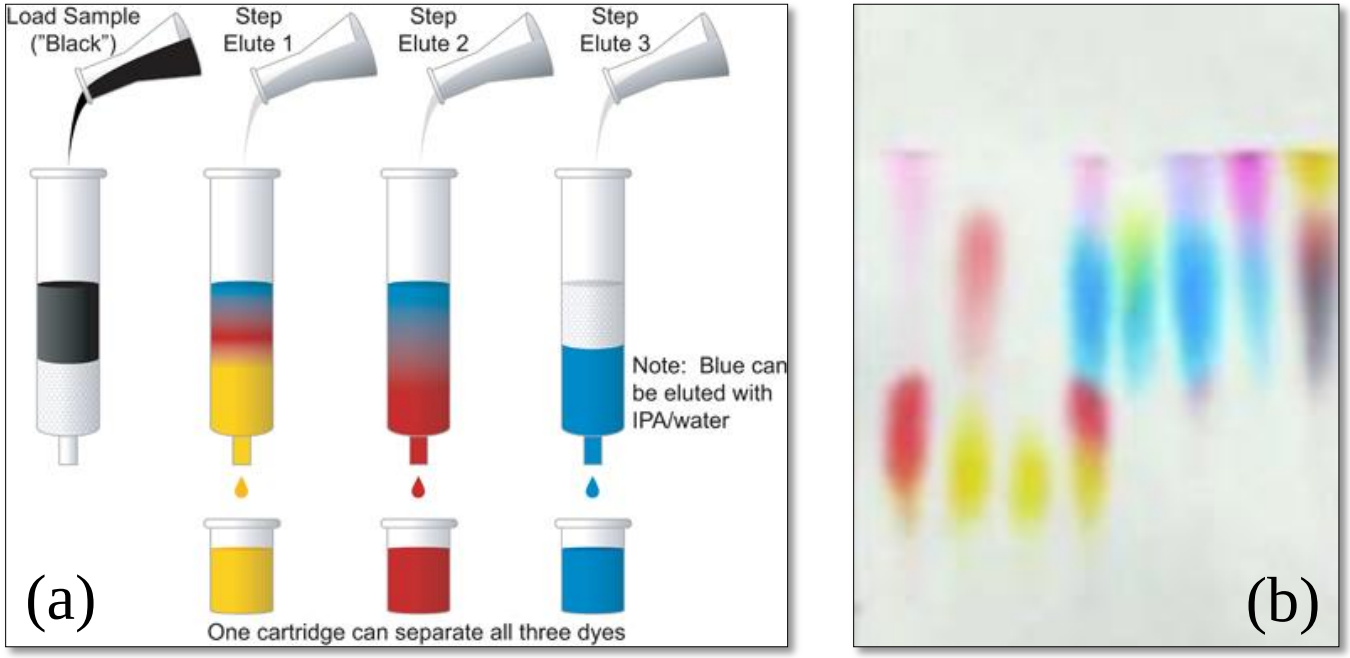
Week	Topic	Reference Material	Instructor
1 [14/09]	Introduction	Instructor's lecture notes	Alshana
2 [21/09]	An introduction to spectrometric methods	• Principles of Instrumental Analysis, Chapter 6, pages 116-142 • Enstrümantal Analiz- Bölüm 6, sayfa 132-163	Alshana
3 [28/09]	Components of optical instruments	• Principles of Instrumental Analysis, Chapter 7, pages 143-191 • Enstrümantal Analiz- Bölüm 7, sayfa 164-214	Alshana
4 [05/10]	Atomic absorption and emission spectrometry	• Principles of Instrumental Analysis, Chapter 9, pages 206-229, Chapter 10, pages 230-252 • Enstrümantal Analiz- Bölüm 9, sayfa 230-253, Bölüm 10 sayfa 254-280	Alshana
5 [12/10]	Ultraviolet/Visible molecular absorption spectrometry	• Principles of Instrumental Analysis, Chapter 13, pages 300-328 • Enstrümantal Analiz- Bölüm 13, sayfa 336-366	Alshana
6 [19/10]	Infrared spectrometry	• Principles of Instrumental Analysis, Chapter 16, pages 380-403 • Enstrümantal Analiz- Bölüm 16, sayfa 430-454	Alshana
	Quiz 1 (12.5 %)		
7 [26/10]	Chromatographic separations	• Principles of Instrumental Analysis, Chapter 26, pages 674-700 • Enstrümantal Analiz- Bölüm 26, sayfa 762-787	Alshana
8 [02-07/11]	MIDTERM EXAM (25 %)		
9 [09/11]	High-performance liquid chromatography (1)	• Principles of Instrumental Analysis, Chapter 28, pages 725-767 • Enstrümantal Analiz- Bölüm 28, sayfa 816-855	Alshana
10 [16/11]	High-performance liquid chromatography (2)		Alshana
11 [23/11]	Gas, supercritical fluid and thin-layer chromatography	• Principles of Instrumental Analysis, Chapter 27, pages 701-724, Chapter 29 pages 768-777 • Enstrümantal Analiz- Bölüm 27, sayfa 788-815, Bölüm 29 sayfa 856-866, Bölüm 28 sayfa 848-851	Alshana
12 [30/11]	Capillary electrophoresis	• Principles of Instrumental Analysis, Chapter 30, pages 778-795 • Enstrümantal Analiz- Bölüm 30, sayfa 867-889	Alshana
13 [07/12]	Quiz 2 (12.5 %)		Alshana
	Extraction techniques	Instructor's lecture notes	
14 [14/12]	Revision	Instructor's lecture notes and from the above given materials	Alshana
15 [21-31/12]	FINAL EXAM (50 %)		

Omitted

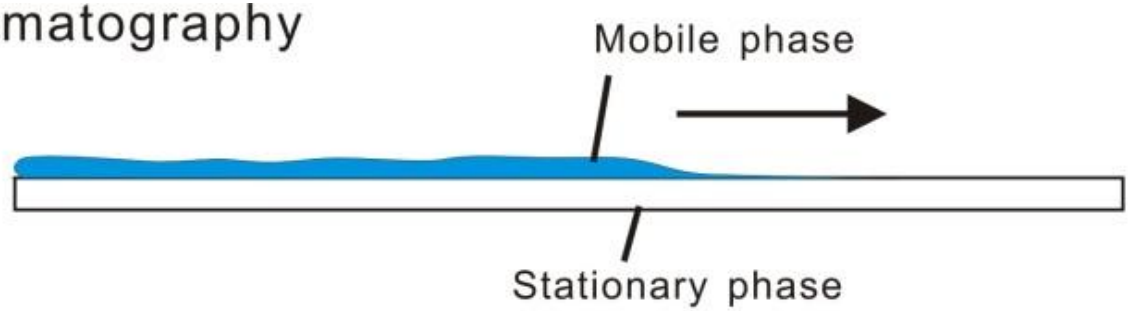
- **Chromatography**: is a method by which a mixture is separated by distributing its components between two phases (i.e., stationary phase and mobile phase).



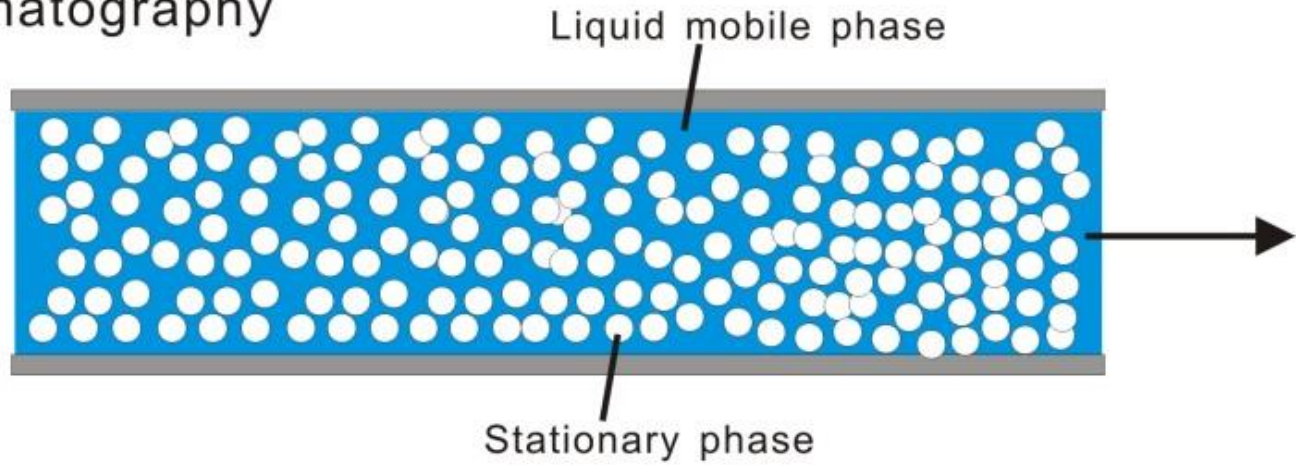
Examples of (a) column and (b) paper chromatography

- The **stationary phase** remains fixed in place while the **mobile phase** carries the components of the mixture through the medium being used.

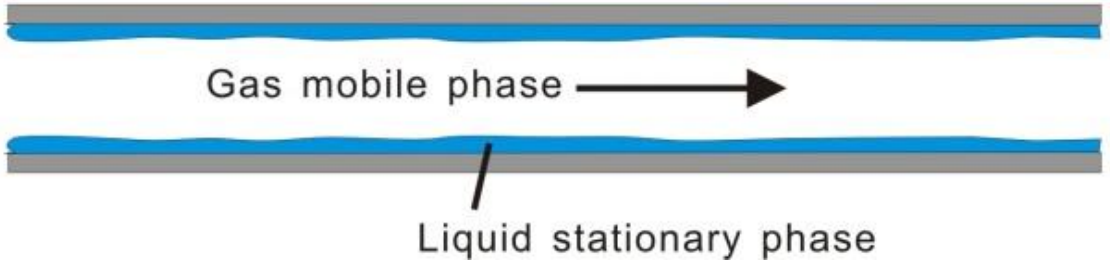
A. Planar chromatography



B. Liquid chromatography



C. Gas chromatography



- In all chromatographic separations, the sample is transported by a **mobile phase**, which may be **a gas, a liquid, or a supercritical fluid**. This mobile phase is then forced through a **stationary phase**, which is fixed in place **in a column** or **on a solid surface**.

- **The two phases are chosen so that the components of the sample distribute themselves between the mobile and stationary phase to varying degrees.**

Classification of Chromatographic Methods

Chromatographic methods can be categorized in **three** ways:

1. Based on interaction of solute (analyte) with the stationary phase,
2. Based on chromatographic bed shape,
3. Based on physical state of the mobile phase.

1. Based on interaction of solute (analyte) with the stationary phase:

a) Adsorption chromatography,

b) Partition chromatography,

c) Ion-exchange chromatography,

d) Size exclusion chromatography.

Classification of Chromatographic Methods

2. Based on chromatographic bed shape:

a) 2D [Thin-Layer Chromatography (TLC), Paper Chromatography (PC)],

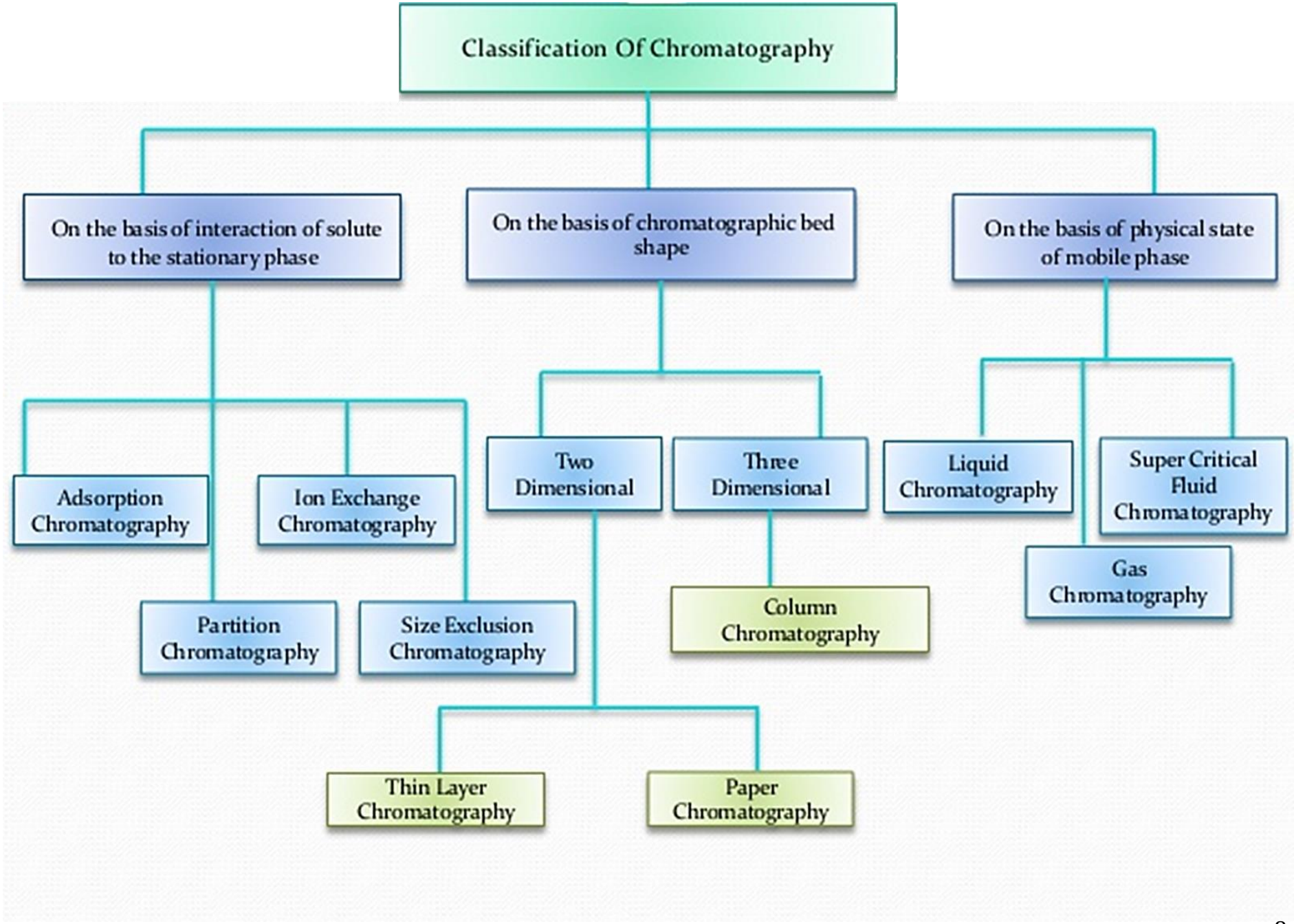
b) 3D (Column chromatography).

3. Based on physical state of the mobile phase:

a) Liquid Chromatography (LC),

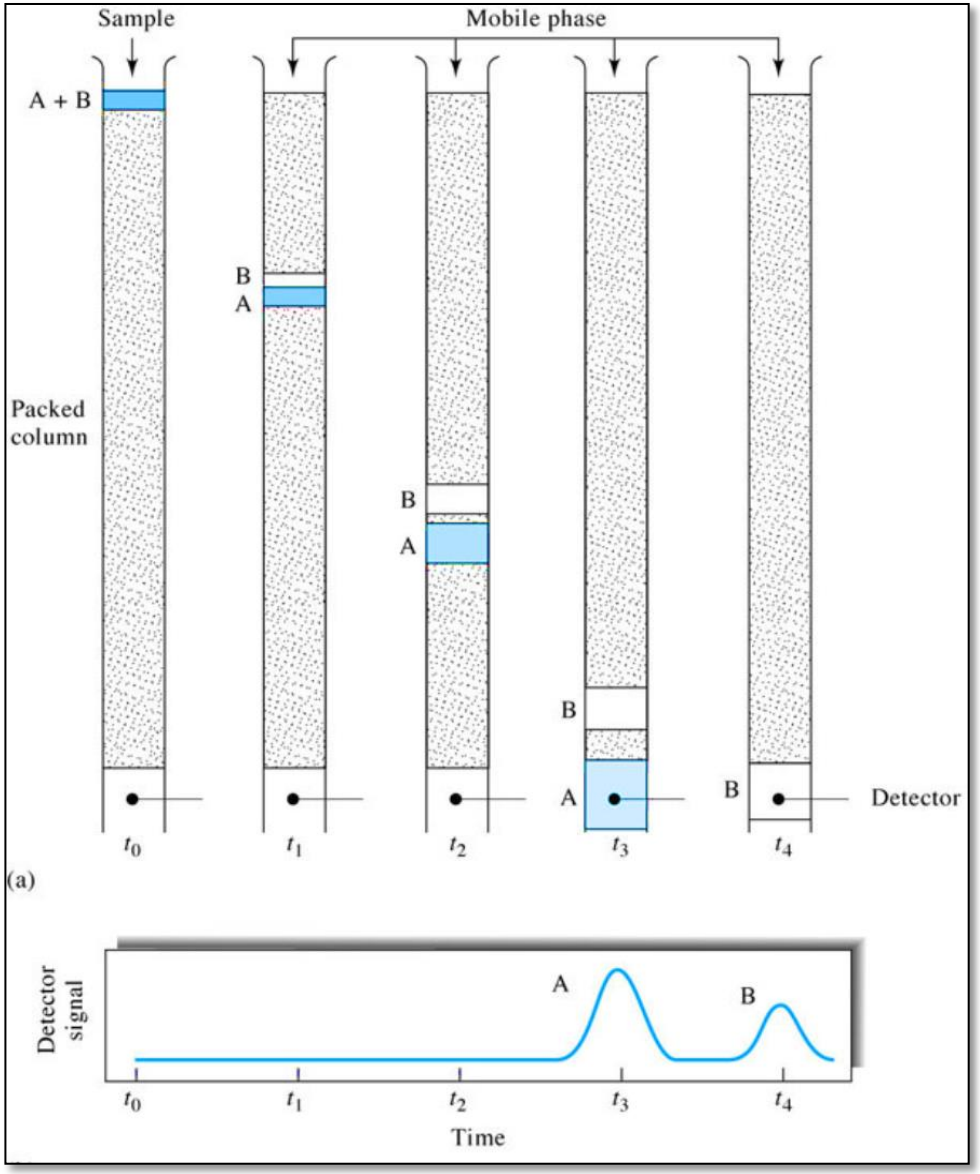
b) Gas Chromatography (GC),

c) Supercritical Fluid Chromatography (SFC).


Classification Of Chromatography
On the basis of interaction of solute to the stationary phase
On the basis of chromatographic bed shape
On the basis of physical state of mobile phase
Adsorption Chromatography
Ion Exchange Chromatography
Partition Chromatography
Size Exclusion Chromatography
Two Dimensional
Three Dimensional
Column Chromatography
Thin Layer Chromatography
Paper Chromatography
Liquid Chromatography
Super Critical Fluid Chromatography
Gas Chromatography

Elution in Columns

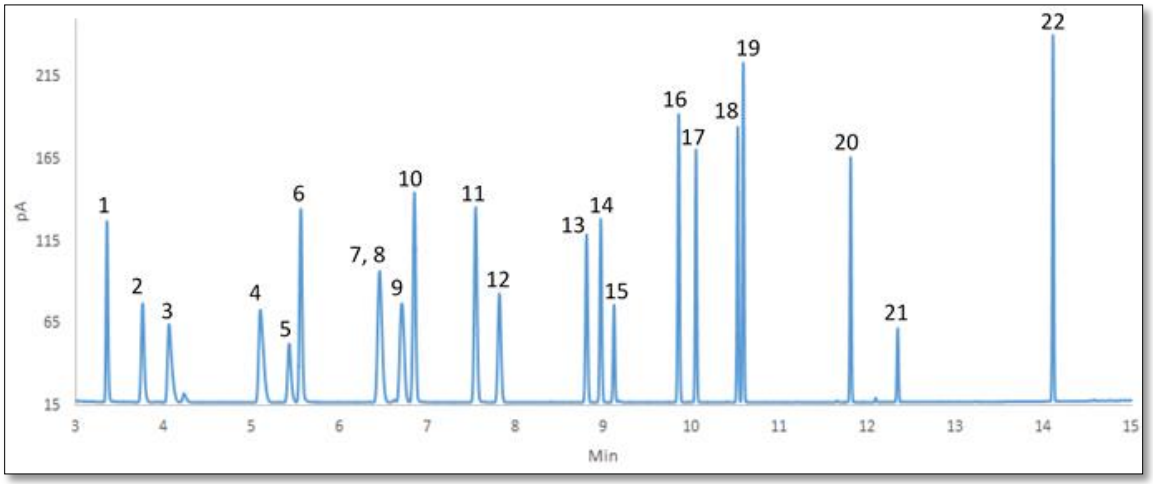
- **Elution** means pushing a species through a column by continuous addition of fresh solvent.
- The sample is introduced at the head of a column, whereupon the components of the sample distribute themselves between the two phases.
- Introduction of additional **mobile phase (the eluent)** forces the solvent containing a part of the sample down the column, where further partition between the mobile phase and fresh portions of the stationary phase occurs.



Chromatograms

- If a detector that responds to solute concentration is placed at the end of the column and its signal is plotted as function of time, a series of peaks is obtained. Such a plot, called a **chromatogram**, is useful for both qualitative and quantitative analysis.
- The **positions of peaks** on the time axis may serve to identify the components of the sample (**qualitative** analysis) .
- The **area under the peak** (or peak height) provides a **quantitative** measure of the amount of each component.

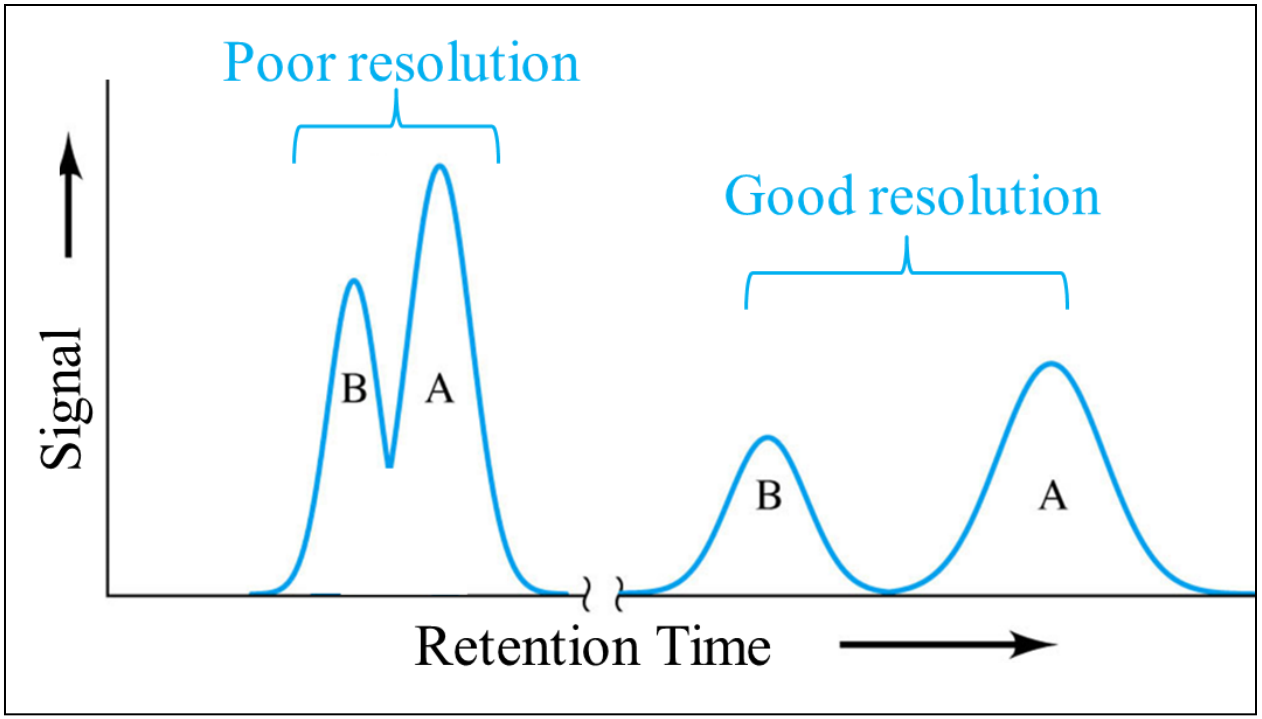
An example of a chromatogram



Peaks	Peaks
1. Ethanol	13. Methyl methacrylate
2. Allyl chloride	14. n-Propyl acetate
3. Acetone	15. 2-Chloroethanol
4. Acrylonitrile	16. 4-Methyl-2-pentanone
5. Vinyl acetate	17. Ethyl methacrylate
6. Allyl alcohol	18. n-Butyl acetate
7. Ethyl Acetate	19. 2-Hexanone
8. Methyl acrylate	20. 1-Bromo-4-fluorobenzene
9. 2-Butanone	21. Pentachloroethane
10. Propargyl alcohol	22. Nitrobenzene
11. Isobutyl alcohol	
12. Isopropyl acetate	

MIGRATION RATES OF SOLUTES (ANALYTES)

- 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.



Distribution Constants

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

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

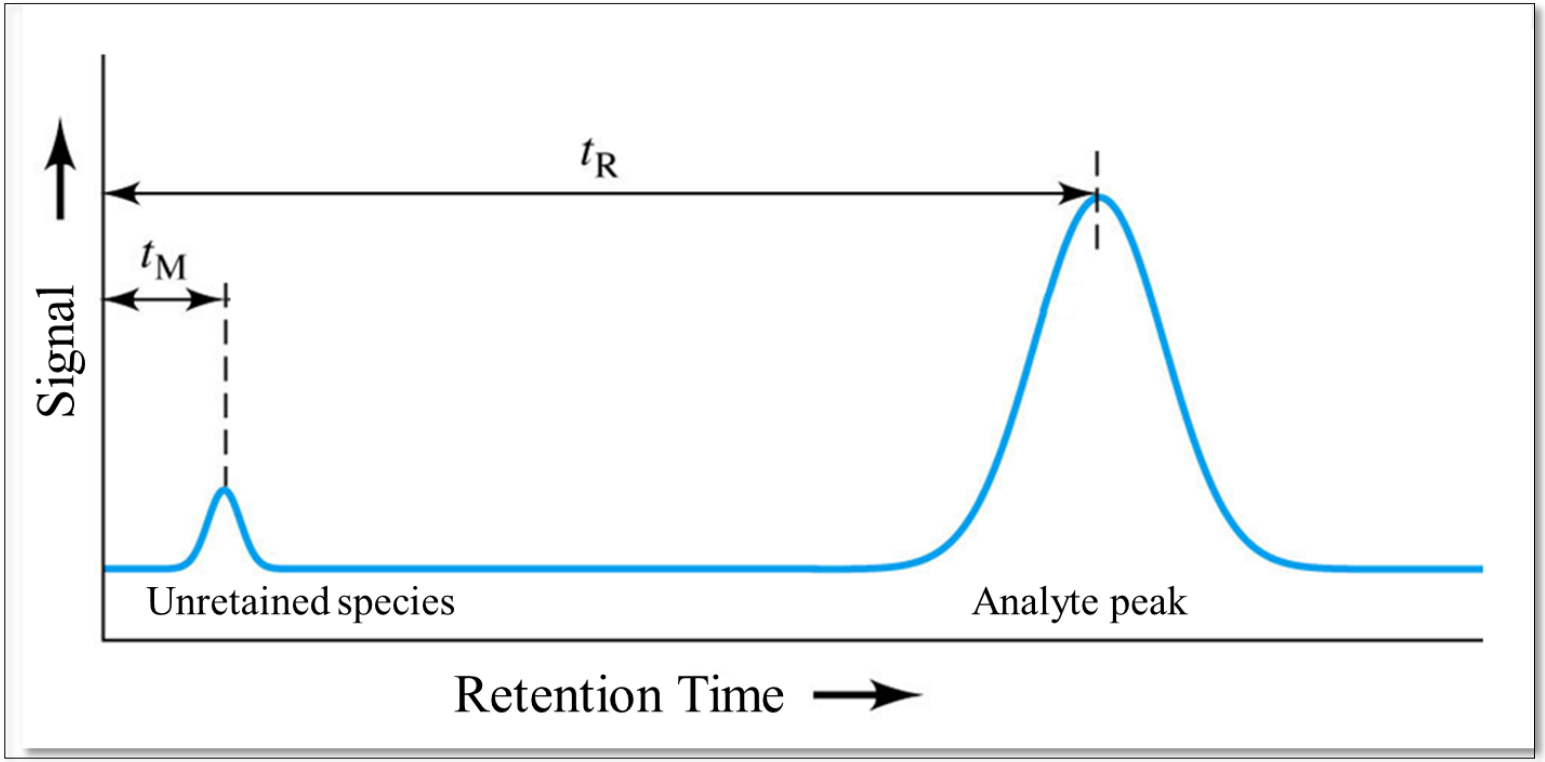
The equilibrium constant (***K***) for this reaction is called the distribution constant, the partition ratio, or the partition coefficient,

$$K = \frac{c_S}{c_M}$$

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. *K* is constant over a wide range of solute concentrations.

Retention Time

- **Retention time (t_R)**: is the time it takes after sample injection for the analyte to reach the detector.
- **Dead time (t_M):** is the time for an unretained species to reach the detector.

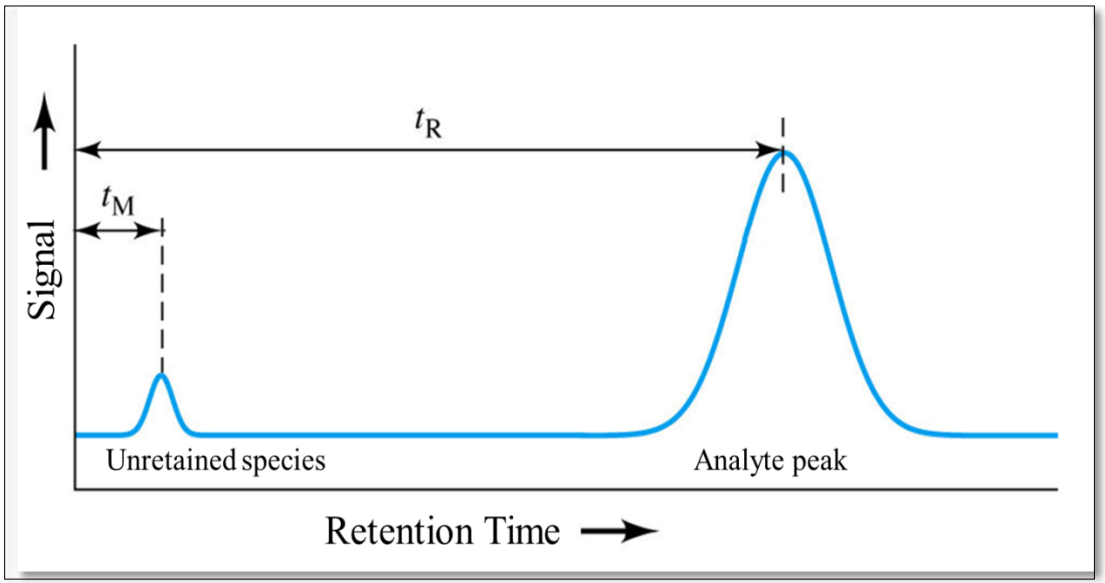


The Retention (Capacity) Factor

- **The retention factor**, or capacity factor, is an important parameter that is widely used to describe the migration rates of solutes on columns.
- For a solute A, the retention factor (k'_A) is defined as:

$$k'_A = \frac{t_R - t_M}{t_M}$$

- t_R and t_M can be readily obtained from the chromatogram.

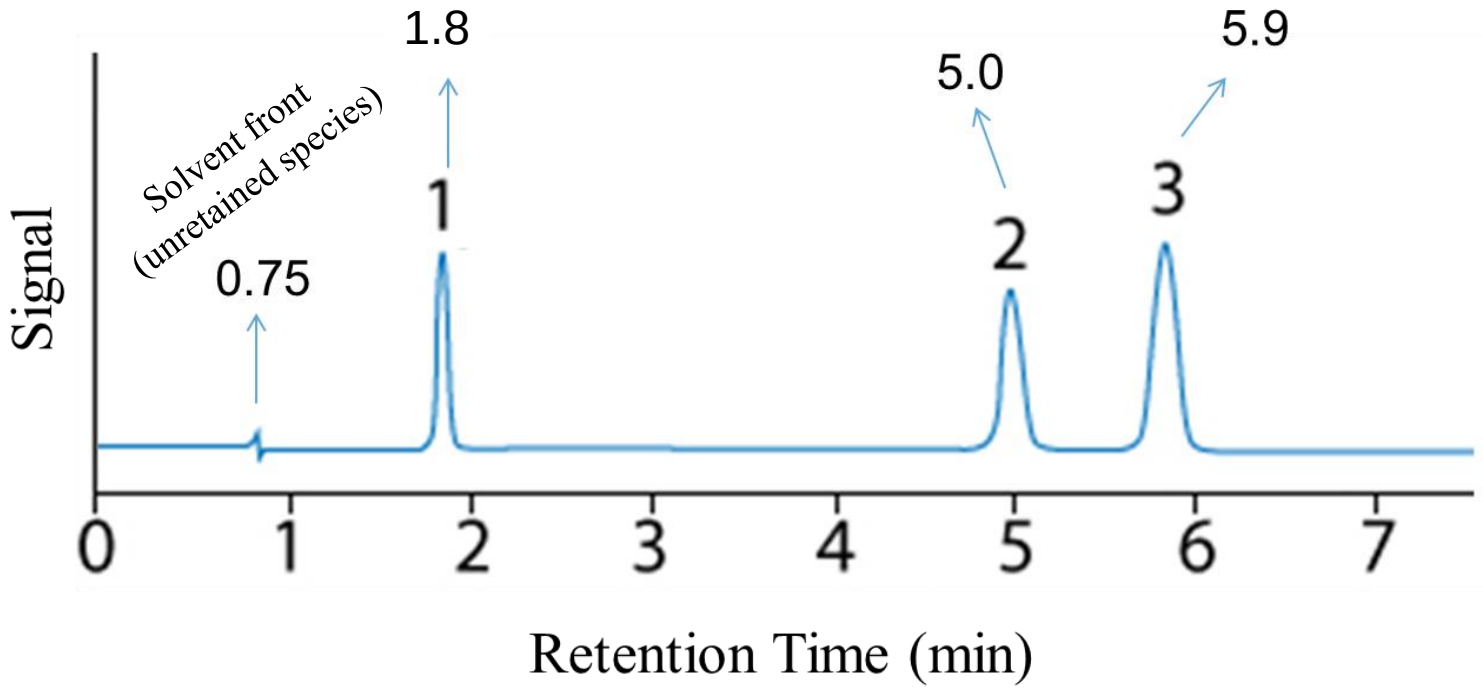


- When the retention factor for a solute is **less than 1.0**, elution occurs so **rapidly** that accurate determination of the retention times is difficult.
- When the retention factor is **larger than perhaps 20 to 30**, elution times become inordinately **long**.
- **Ideally**, separations are performed under conditions in which the retention factors for the solutes in a mixture lie in the range between **2 and 10**.

$$\mathbf{2 \leq k'_A \leq 10}$$

Calculating Retention Factor (k')

Calculate the retention factor (k') for the peaks 1, 2 and 3 in the chromatogram shown below.



Solution

$$k'_A = \frac{t_R - t_M}{t_M}$$

$$k'_1 = \frac{t_{R,1} - t_M}{t_M} = \frac{1.8 - 0.75}{0.75} = 1.40$$

$$k'_2 = \frac{t_{R,2} - t_M}{t_M} = \frac{5.0 - 0.75}{0.75} = 5.67$$

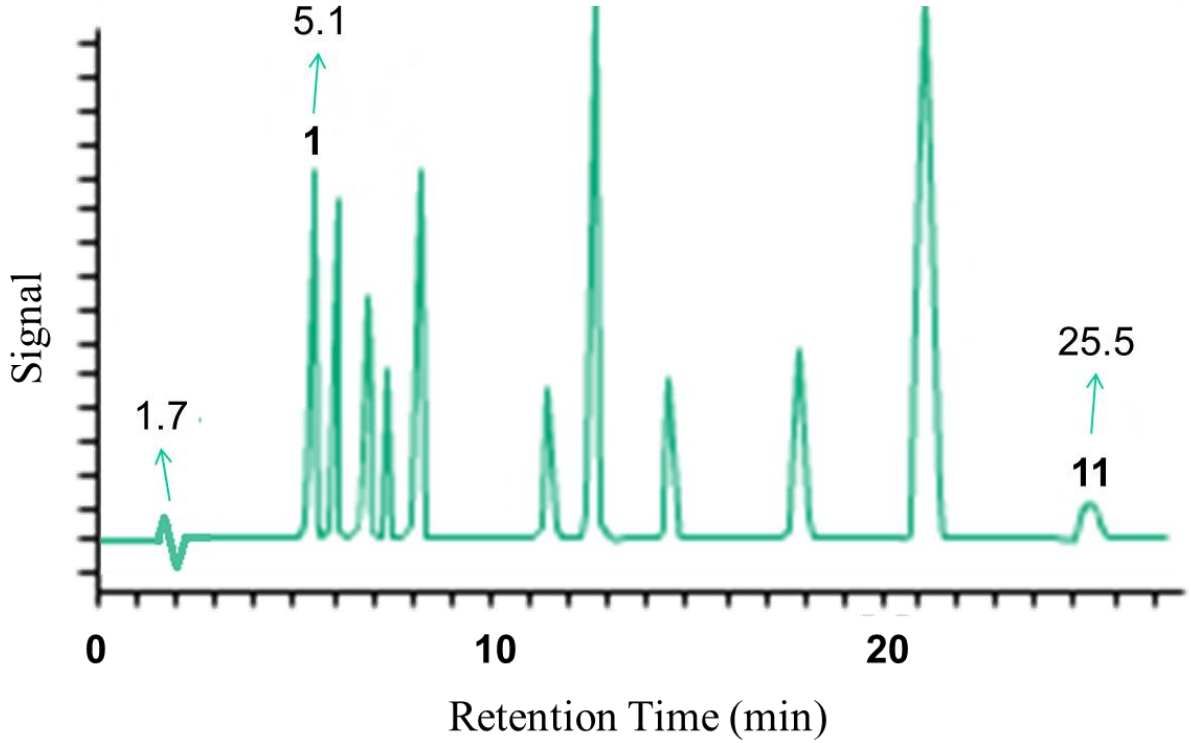
$$k'_3 = \frac{t_{R,3} - t_M}{t_M} = \frac{5.9 - 0.75}{0.75} = 6.87$$

- Conclusion

✓ Since all of *k*' values for 2 and 3 lie in the preferred range of 2-10, the peaks are suitable for quantitation. However, peak 1 is not.

Calculating Retention Factor (k')

Calculate the retention factor for the peaks 1 and 11 in the chromatogram shown below. Comment on the quality of those peaks for quantitation.



Solution

$$k'_A = \frac{t_R - t_M}{t_M}$$

$$k'_1 = \frac{t_{R,1} - t_M}{t_M} = \frac{5.1 - 1.7}{1.7} = 2.00$$

$$k'_{11} = \frac{t_{R,11} - t_M}{t_M} = \frac{25.5 - 1.7}{1.7} = 14.0$$

➢ Conclusion

✓ Since k'_1 is equal to 2.00, peak 1 is suitable for quantitation. However, since k'_{11} *is larger than* 10, peak 11 is not.

The selectivity Factor (α)

- **The selectivity factor (α)** of a column is defined as the degree of separation between successive peaks (generally called as **critical pair**).
- For the two species A and B, α is defined as:

$$\alpha = \frac{k'_B}{k'_A}$$

where k'_B and k'_A are the retention factors of B and A, respectively.

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

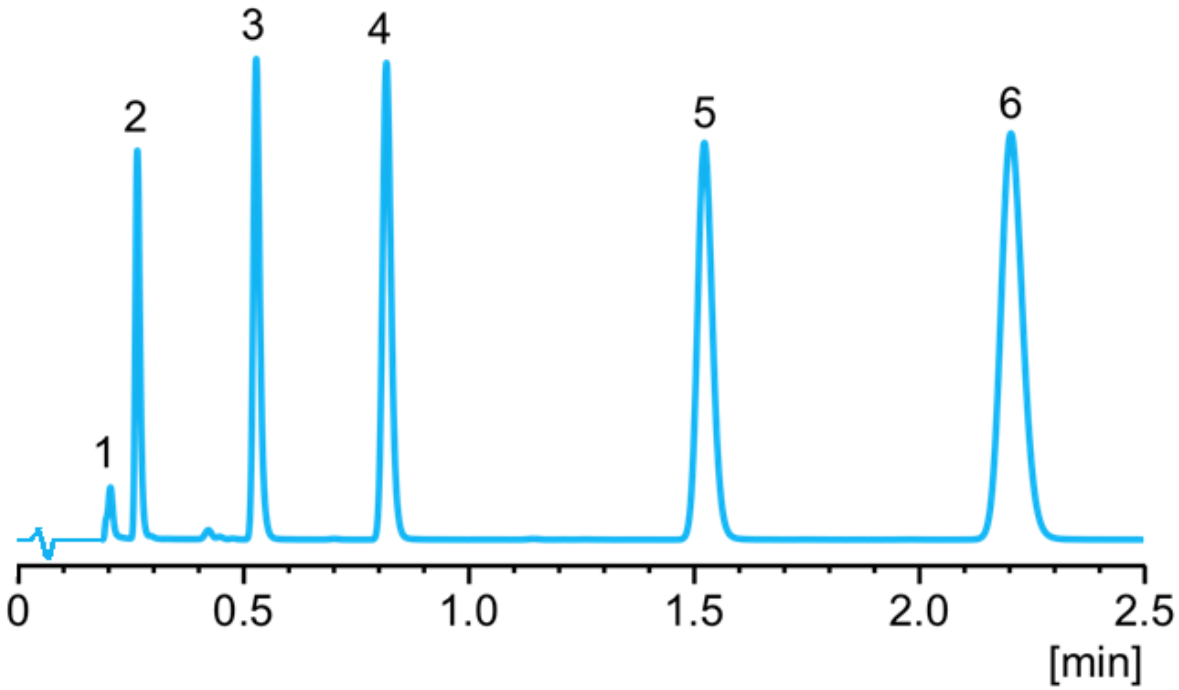
$$\propto = \frac{(t_R)_B - t_M}{(t_R)_A - t_M}$$

- The selectivity factor (α) is one of the most critical factors in chromatography.
- α should be large enough so that each peak is sufficiently resolved.
- Since A in the above equation is for the substance that is retained less and B is for the one that is retained more, α is always larger than 1.0

Calculating Selectivity Factor (α)

Calculate the selectivity factor (α) for the peak pairs of 1,2 and 3,4 and 5,6 in the chromatogram shown below.

Retention times (in min) from left to right are: 0.20, 0.25, 0.53, 0.83, 1.52 and 2.25. (The retention time for the unretained peak is 0.08 min)



Solution

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

1) For the peak pair 1,2:

$$\propto_{1,2} = \frac{(t_R)_2 - t_M}{(t_R)_1 - t_M} = \frac{0.25 - 0.08}{0.20 - 0.08} = 1.42\ (no\ unit)$$

2) For the peak pair 3,4:

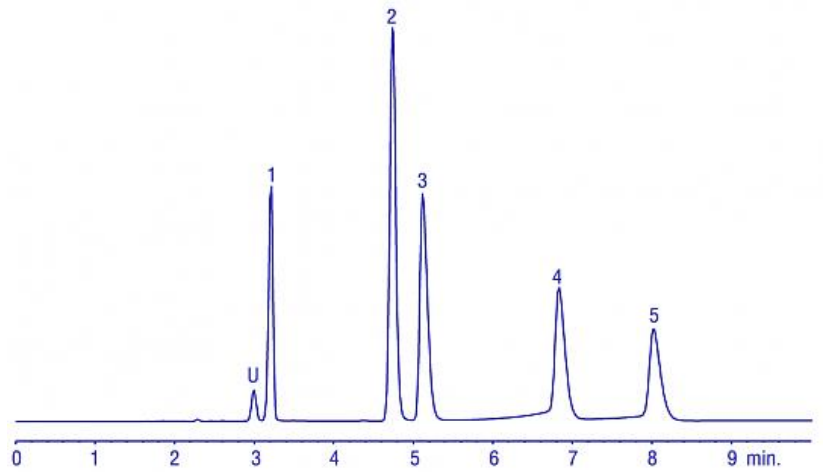
$$\propto_{3,4} = \frac{(t_R)_4 - t_M}{(t_R)_3 - t_M} = \frac{0.83 - 0.08}{0.53 - 0.08} = 1.67$$

3) For the peak pair 5,6:

$$\propto_{5,6} = \frac{(t_R)_6 - t_M}{(t_R)_5 - t_M} = \frac{2.25 - 0.08}{1.52 - 0.08} = 1.51$$

Analgesic acetaminophen and narcotic analgesics were separated using ultra-high performance liquid chromatography (UPLC) on an ultra C_{18} column and the following chromatogram was obtained.

Peak	Analyte	Retention time (min)
u	Unretained	3.0
1	Morphine sulfate	3.2
2	Acetaminophen	4.8
3	Codeine phosphate	5.2
4	Oxycodone HCl	6.9
5	Hydrocodone bitartrate	8.0



a) Calculate the retention factor (*k'*) for the five peaks in the chromatogram shown above.
b) Calculate the selectivity factor (α) for the peak pairs of 2,3 and 3,4 and 4,5.

Methods for Describing Column Efficiency

- Two related terms are widely used as quantitative measures of chromatographic column efficiency: (1) **plate height (*H*)** and (2) **theoretical plate number (*N*)**.
- The two are related by the equation:

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

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

- The efficiency of chromatographic columns increases as ***N*** becomes greater and as the ***H*** becomes smaller.

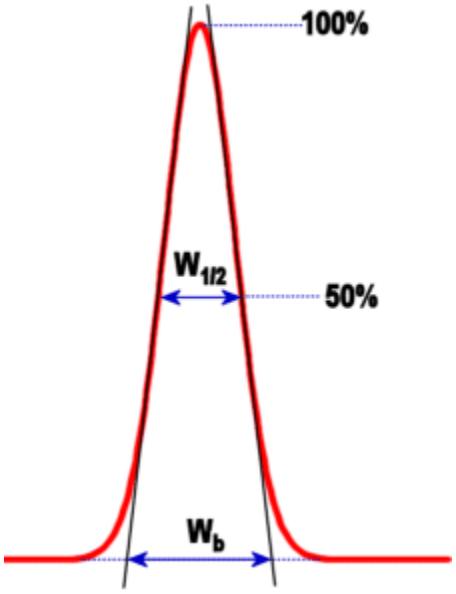
Theoretical plates

- ✓ A chromatographic column is made up of numerous discrete but continuous narrow layers called **theoretical plates**.
- ✓ At each plate, equilibration of the solute between the mobile and stationary phase is assumed to take place.
- ✓ Movement of the solute down the column is then treated as a stepwise transfer of equilibrated mobile phase from one plate to the next.

The Experimental Evaluation *H* and *N*

- N can be calculated from two time measurements t_R and peak width (W); to obtain H, the length of the column packing (L) must also be known.
- Another method for approximating N, is to determine $W_{1/2}$, the width of peak at half its maximum height. The theoretical plate number (N) is then given by:

$$N = 5.54 \left(\frac{t_R}{W_{1/2}}\right)^2$$



- N and H are widely used in the literature and by instrument manufactures as measures of column performance.

Column Resolution

- The resolution R_s of a column provides a quantitative measure of its ability to separate two analytes. Column resolution is defined as:

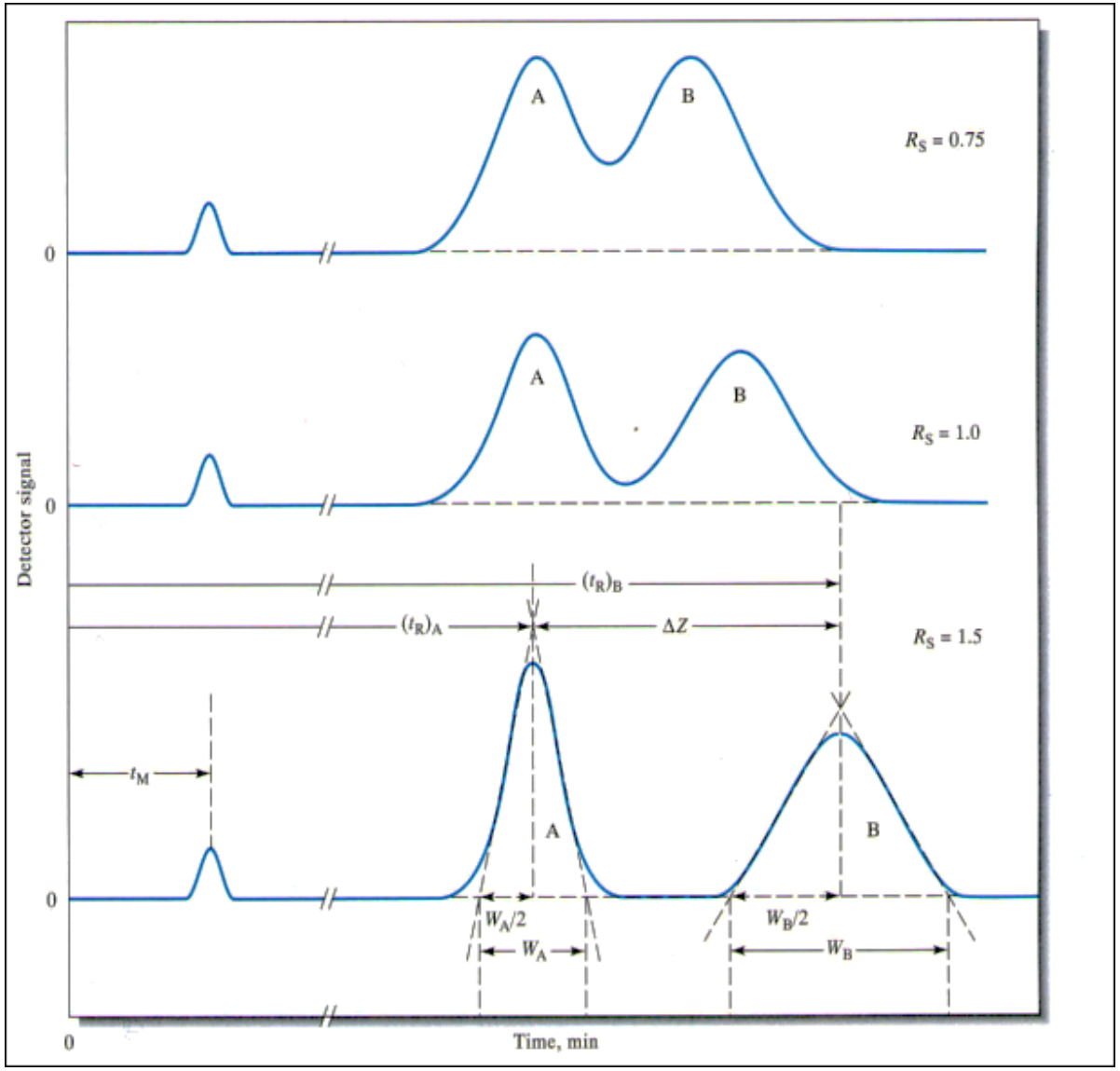
$$R_s = \frac{\Delta Z}{W_A/2 + W_B/2} = \frac{2\Delta Z}{W_A + W_B} = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B}$$

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

$$R_s = \frac{\Delta Z}{W_A/2 + W_B/2}$$

$$R_s = \frac{2\Delta Z}{W_A + W_B}$$

$$R_s = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B}$$



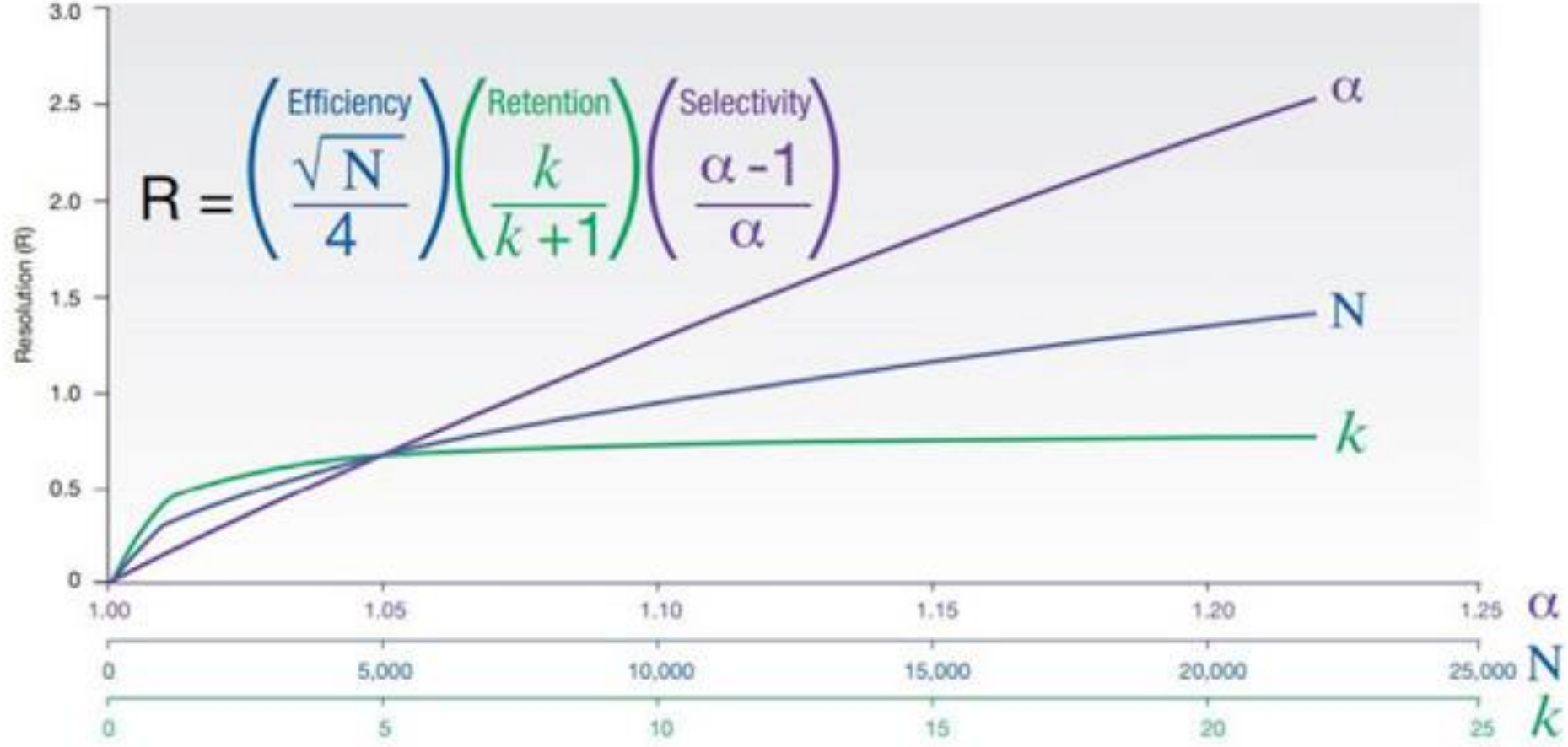
The Effect of Retention and Selectivity Factors on Resolution

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

$$R_S = \left(\frac{\sqrt{N}}{4}\right)\left(\frac{k'}{k'+1}\right)\left(\frac{\propto -1}{\propto}\right)$$

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

The Impact of Selectivity on Resolution



APPLICATIONS OF CHROMATOGRAPHY

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

Qualitative Analysis

- A chromatogram provides only a single piece of **qualitative information** about each species in a sample, namely, its **retention time**. 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.

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.

Analyses Based on Peak Height

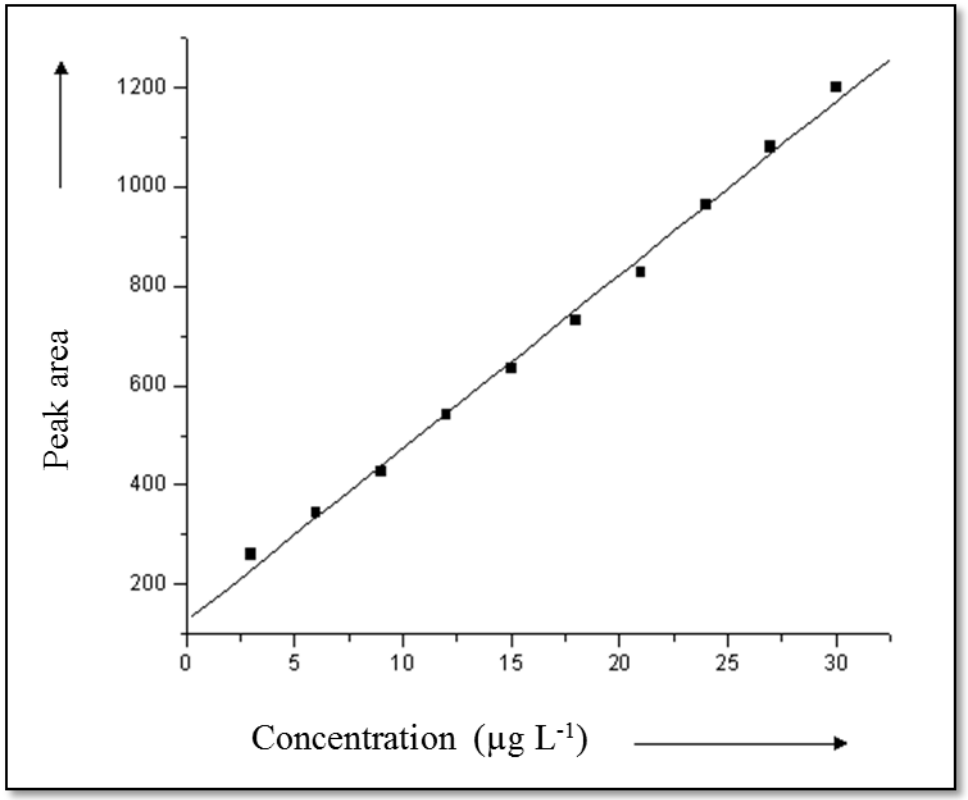
- The height of a chromatographic peak is obtained by connecting the base lines on either side of the peak by a straight line and measuring the perpendicular distance from this line to the peak.
- This measurement can ordinarily be made with reasonably high precision.
- Accurate results are obtained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

Analyses Based on Peak Areas

- Peak areas are a more satisfactory analytical variable than peak heights.
- On the other hand, peak heights are more easily measured and, for narrow peaks, more accurately determined. Most modern chromatographic instruments are equipped with digital electronic integrators that permit precise estimation of peak areas.

• Calibration and Standards

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



An example of a calibration curve