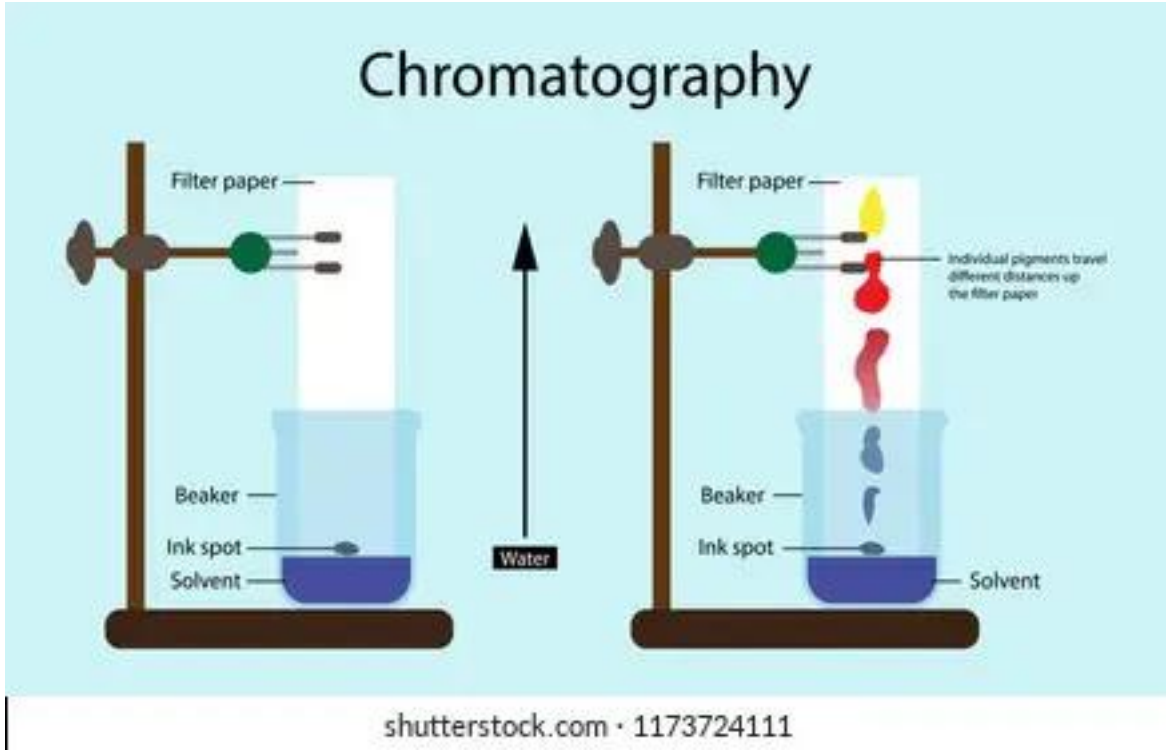
MODERN PHYSICAL AND CHEMICAL METHODS OF ANALYSIS



CHROMATOGRAPHY

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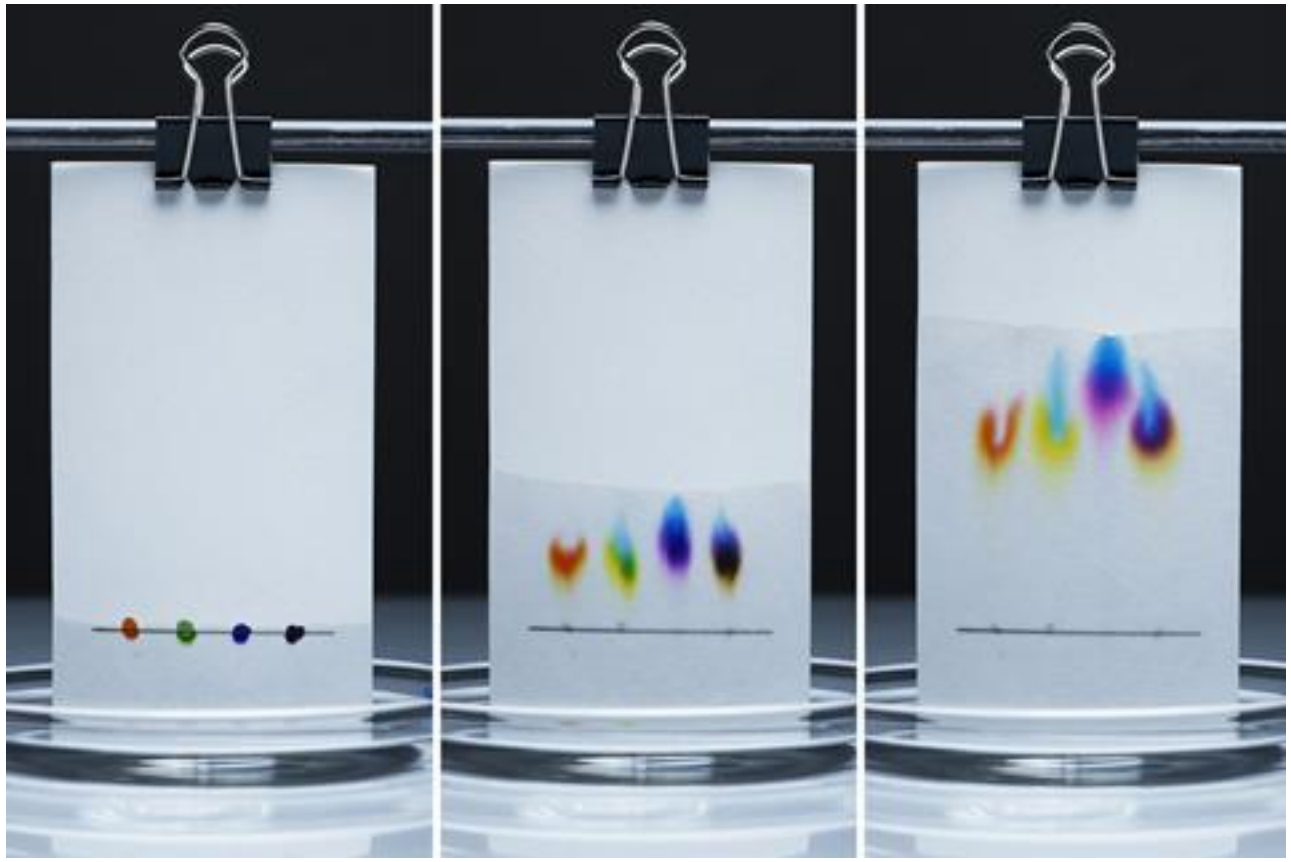
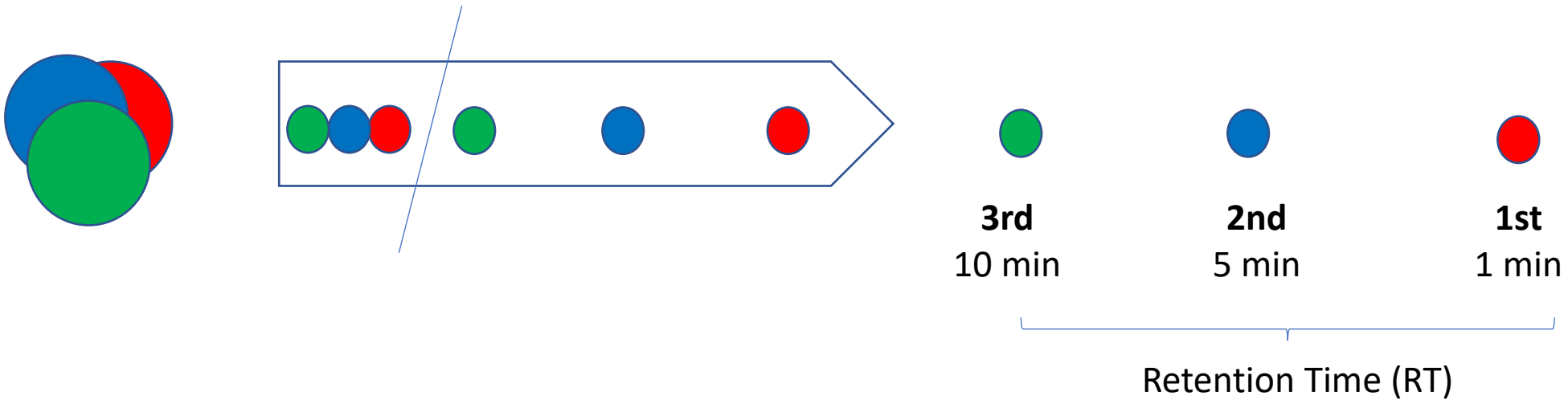
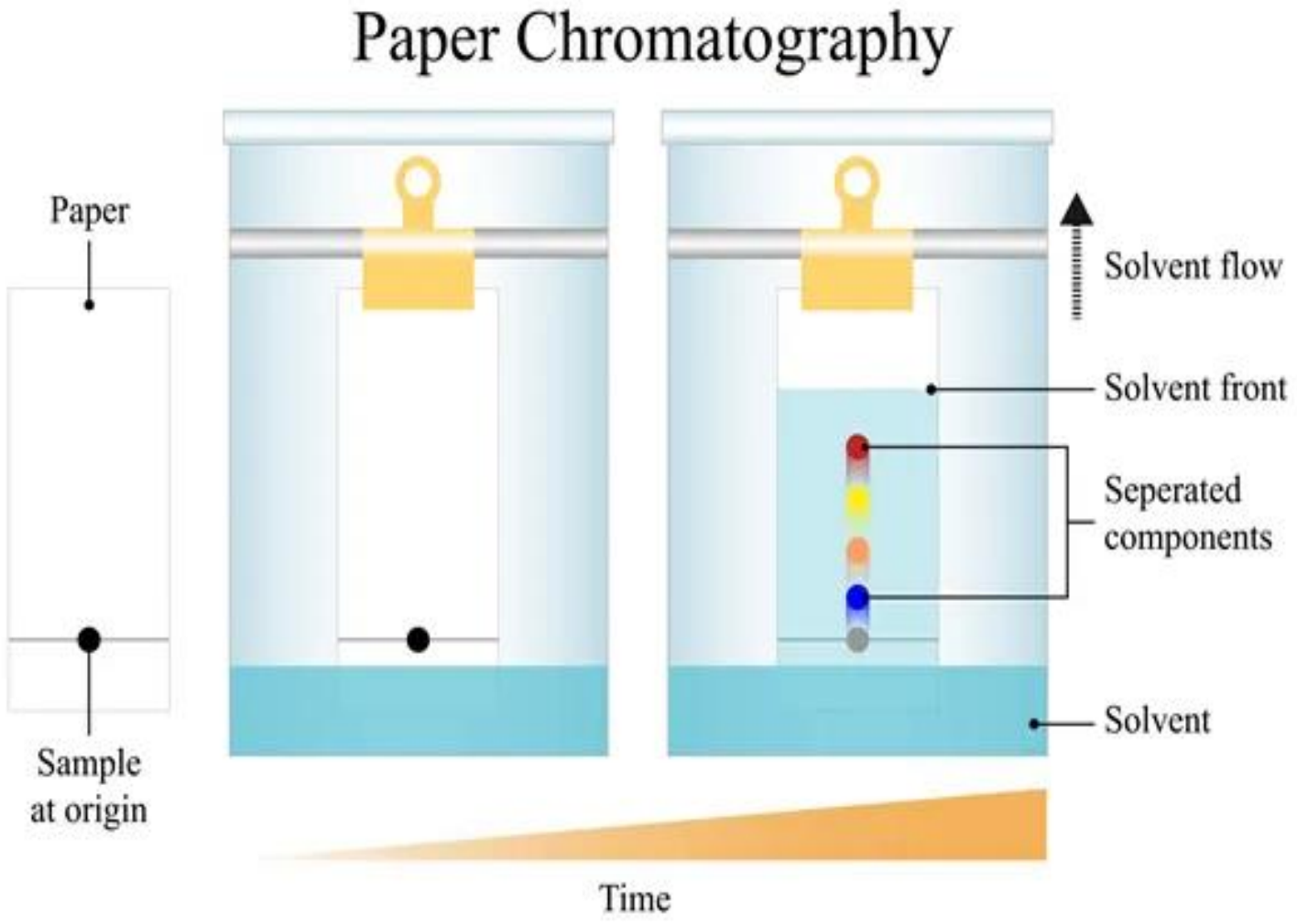


Chromatography

Chromatography is based on the differential interaction of different compounds within a sample, while they are being carried out throughout a system (the carrier is called “mobile phase”), with another phase (called stationary phase).



Key concepts in chromatography:



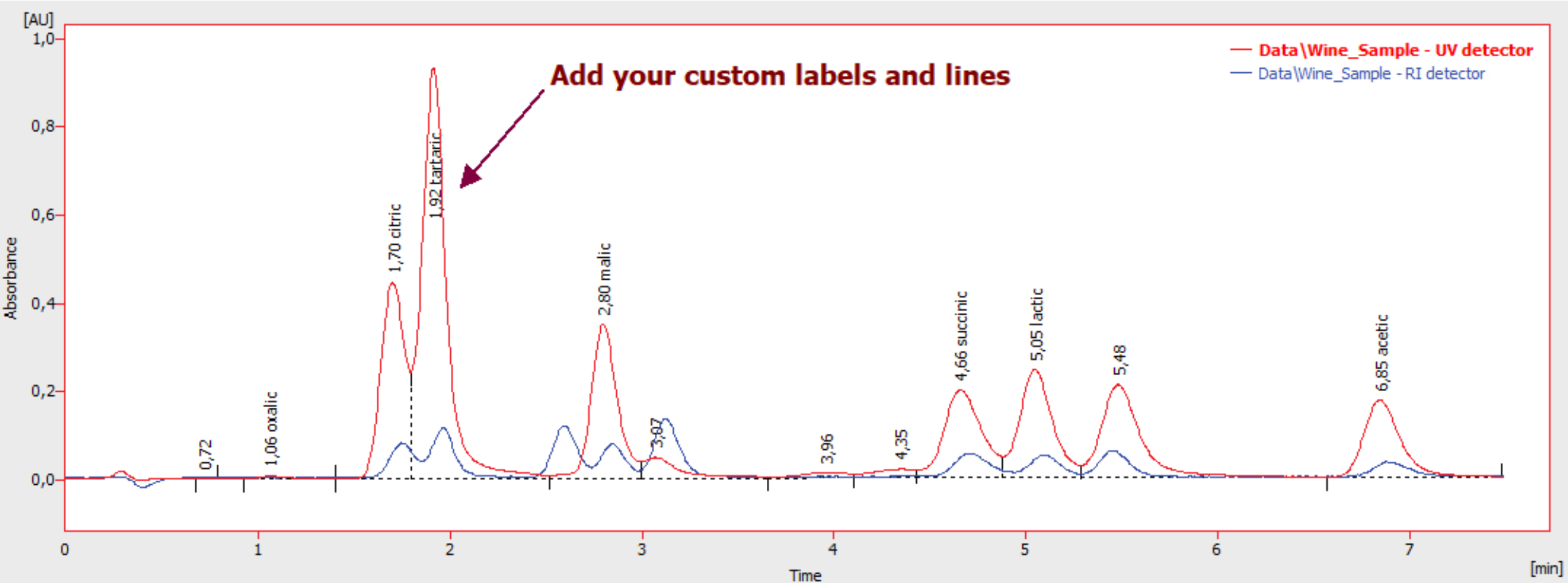
Sample: A sample is a portion of material selected in some manner to represent a larger body of material.

Analyte: Analyte is the chemical substance that is determined in the analytical procedure. Analytes are contained within a sample.

Mobile phase: The fluid (liquid, gas, or supercritical fluid) that flows through a chromatography system, moving the materials to be separated at different rates over the stationary phase.

Stationary phase: It is a solid or liquid which that does not move with the sample. It acts as a constraint on many of the components in a mixture, slowing them down to move slower than the mobile phase.

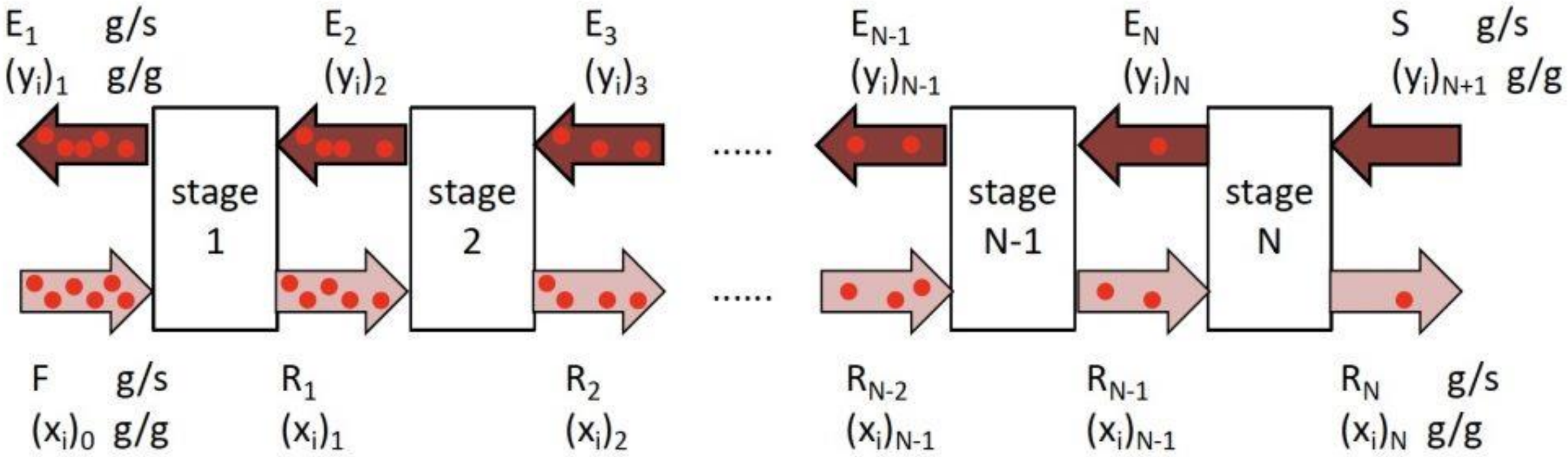
Chromatogram



Retention time (RT) is the time elapsed between sample introduction (beginning of the chromatogram) and the maximum signal of the given analyte at the detector. It is a measure of the time taken for an analyte to pass through a chromatography system.

Development of chromatography:

Countercurrent extraction

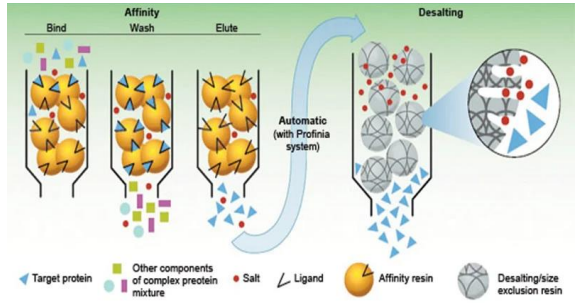


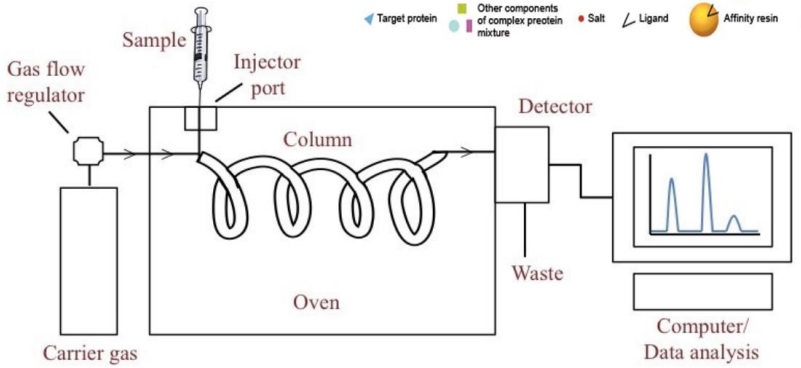
Countercurrent extraction refers to a serial extraction process. It separates two or more solutes with different partition coefficients from each other by a series of partitions between two immiscible liquid phases extraction. It is the same basis as in partition chromatography and each of the extraction steps would be the equivalent of a theoretical plate in chromatography.

Classification of chromatography:

Chromatography technique can be explained / **classified** into three fundamental ways:

- Based on the shape of chromatographic bed.
  - Planar chromatography
  - Column chromatography
- Based on the physical nature of the stationary and mobile phases.
  - Gas chromatography (GC)
  - Liquid chromatography (LC, HPLC, UHPLC)
  - Supercritical fluid chromatography (SFC)
- Based on the mechanism of the separation.
  - Ion- exchange
  - Affinity
  - Size exclusion





Method	*Mobile/phase*	*Stationary phase*	*Retention varies with*
Gas-liquid chromatography	Gas	Liquid	Molecular size/polarity
Gas-solid chromatography	Gas	Solid	Molecular size/polarity
Supercritical fluid chromatography	Supercritical fluid	Solid	Molecular size/polarity
Reversed-phase chromatography	Polar liquid	Nonpolar liquid or solid	Molecular size/polarity
Normal-phase chromatography	Less polar liquid	More polar liquid or solid	Molecular size/polarity
Ion-exchange chromatography	Polar liquid-Ionic solid	Ionic solid	Molecular charge
Size-exclusion chromatography	Liquid	Solid	Molecular size
Hydrophobic interaction chromatography	Polar liquid	Nonpolar liquid or solid	Molecular size/polarity
Affinity chromatography	Water	Binding sites	Specific structure

Different types of chromatography

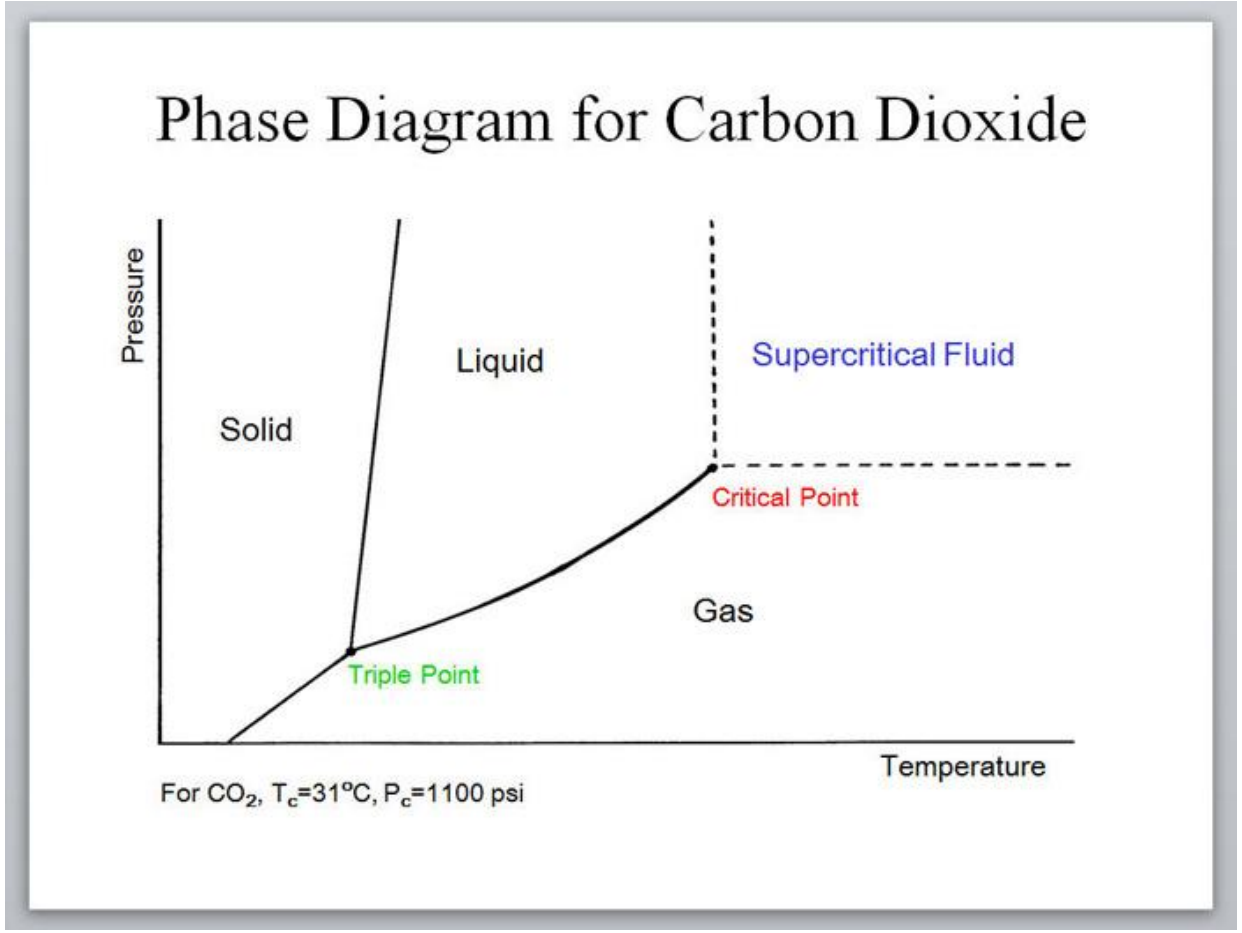
~~(Will be deeply covered later on)~~

Chromatography technique can be explained / **classified** into three fundamental ways:

- Based on the shape of chromatographic bed.
  - ~~Planar chromatography (TLC and paper chromatpography)~~
  - ~~Column chromatography~~
- Based on the physical nature of the stationary and mobile phases.
  - ~~Gas chromatography (GC)~~
  - ~~Liquid chromatography (LC, HPLC, UHPLC)~~
  - **Supercritical fluid chromatography (SFC)**
- Based on the mechanism of the separation.
  - **Ion- exchange**
  - **Affinity**
  - **Size exclusion**
  - **Hydrophobic interaction**

Supercritical fluid chromatography (SFC):

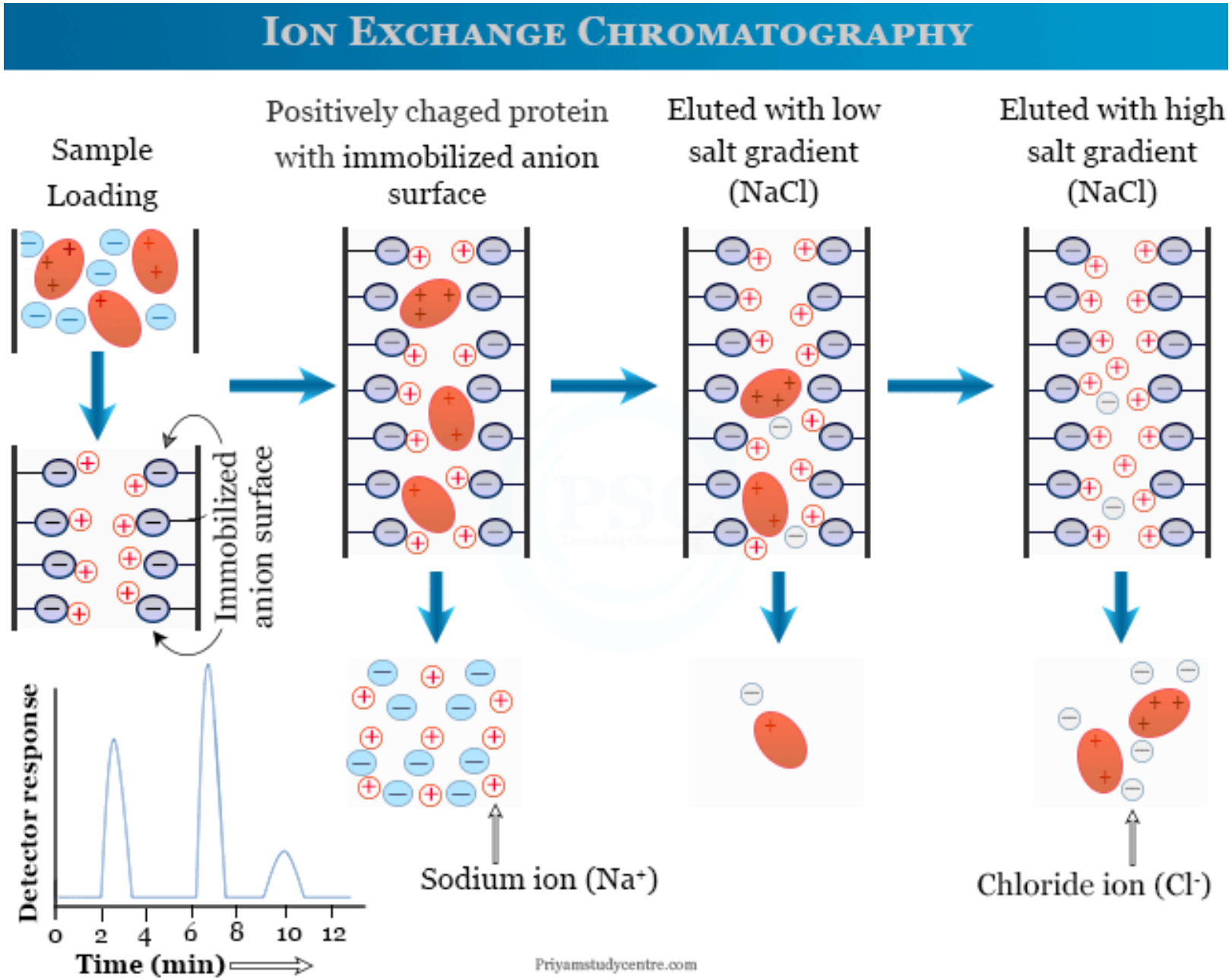
Supercritical fluid chromatography (SFC) is a form of normal phase chromatography that uses a **supercritical fluid** such as carbon dioxide as the mobile phase. It is used for the analysis and purification of low to moderate molecular weight, **thermally labile molecules** and can also be used for the separation of chiral compounds. Principles are similar to those of high performance liquid chromatography (HPLC), however SFC typically utilizes carbon dioxide as the mobile phase; therefore the entire chromatographic flow path must be pressurized. Because the supercritical phase represents a state in which liquid and gas properties converge, supercritical fluid chromatography is sometimes called convergence chromatography.



Ion exchange chromatography:

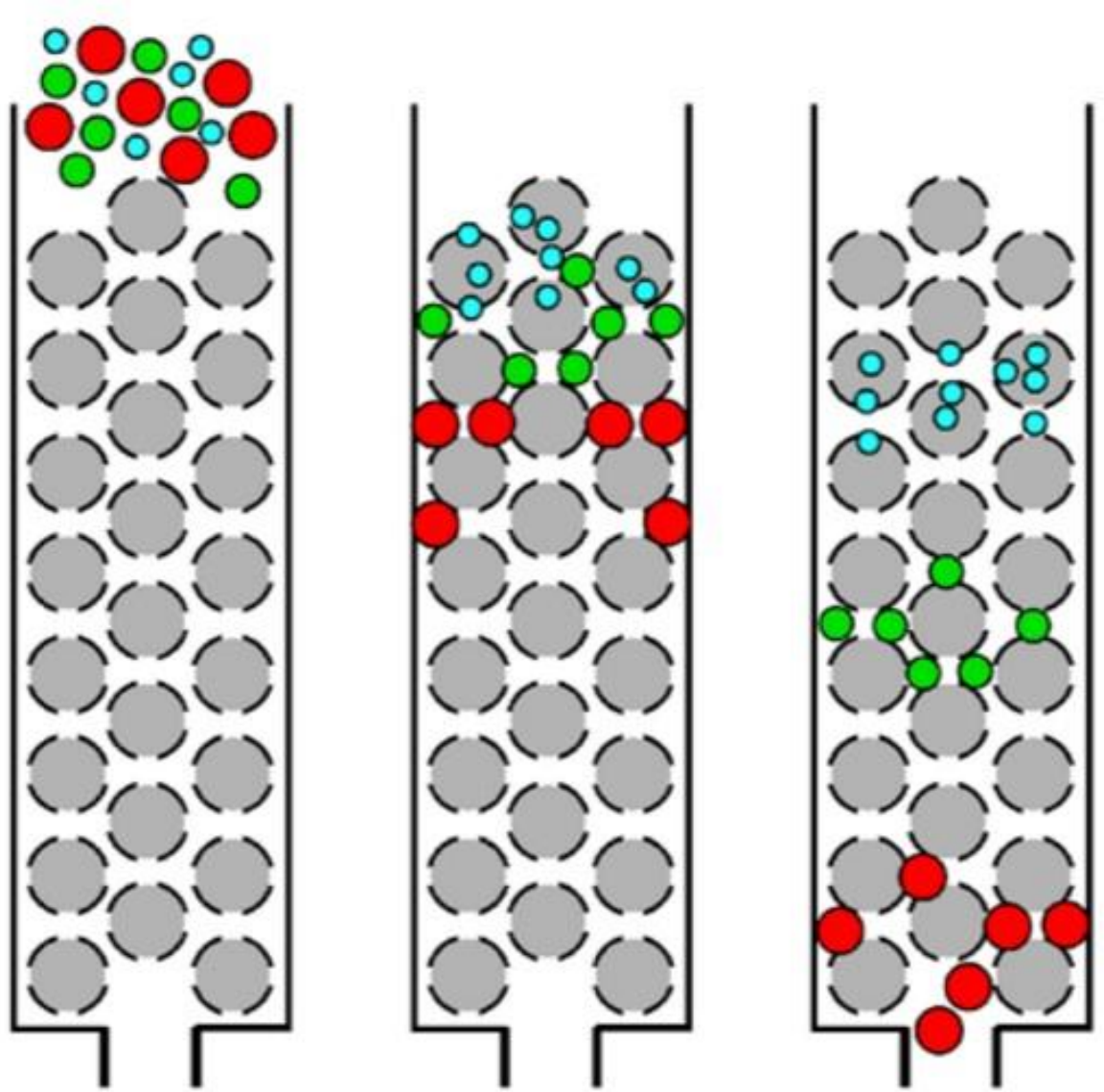
Ion exchange chromatography separates ions and polar molecules based on their **affinity to the ion exchanger**. It works on almost any kind of charged molecule, including large proteins, small nucleotides, and amino acids.

Moiety: In organic chemistry, the term moiety is used to denote a portion of a molecule. In Ion exchange chromatpography, stationary phase consist of molecules with a charged moiety.



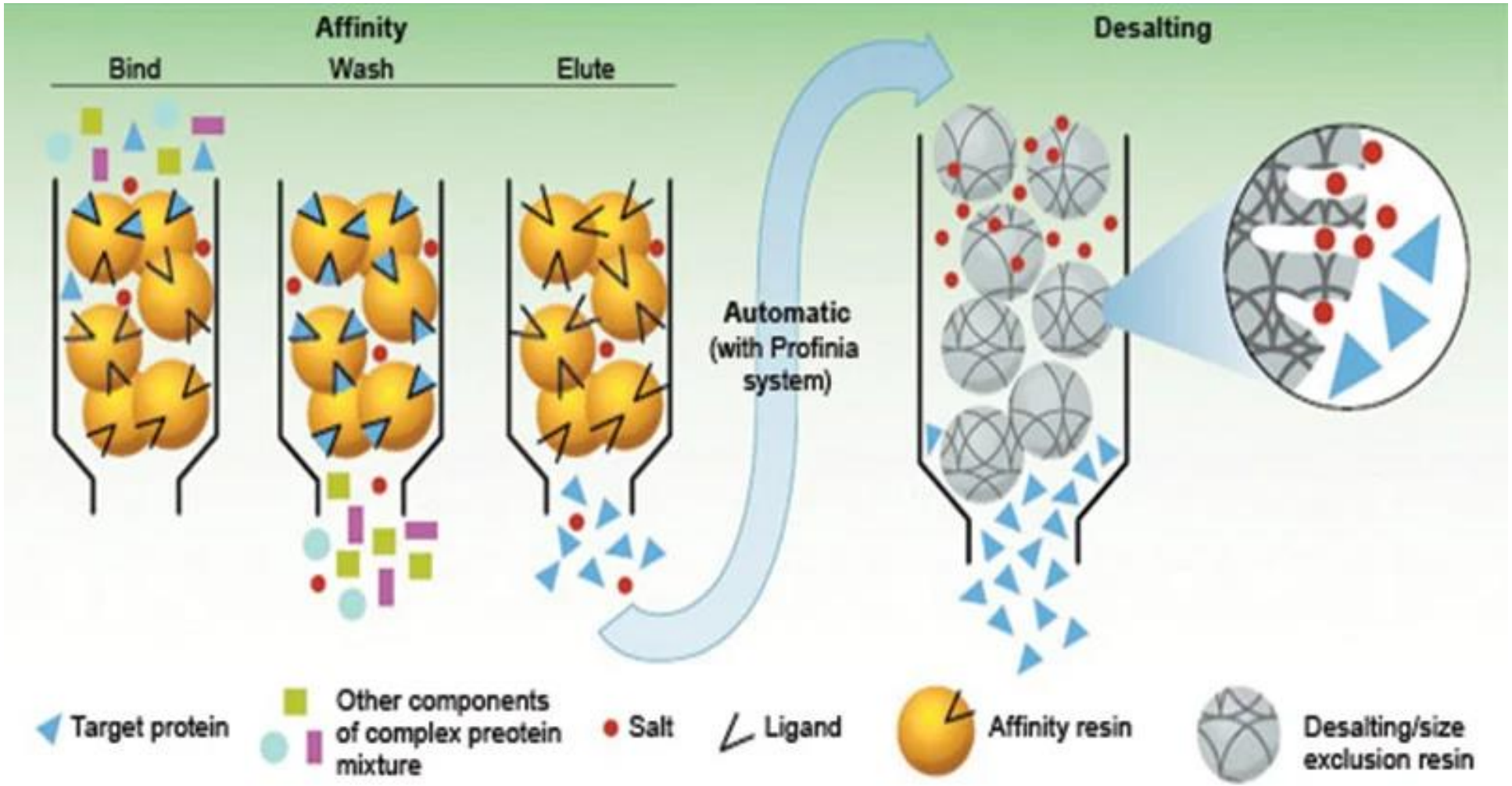
Size exclusion chromatography (SEC):

Size exclusion chromatography (SEC) separates molecules based on their **size** by filtration through a gel. The gel consists of spherical beads containing pores of a specific size distribution. Separation occurs when molecules of different sizes are included or excluded from the pores within the matrix. Small molecules diffuse into the pores and their flow through the column is retarded according to their size, while large molecules do not enter the pores and are eluted in the column's void volume. Consequently, **molecules separate based on their size** as they pass through the column and are eluted in order of decreasing molecular weight (MW).



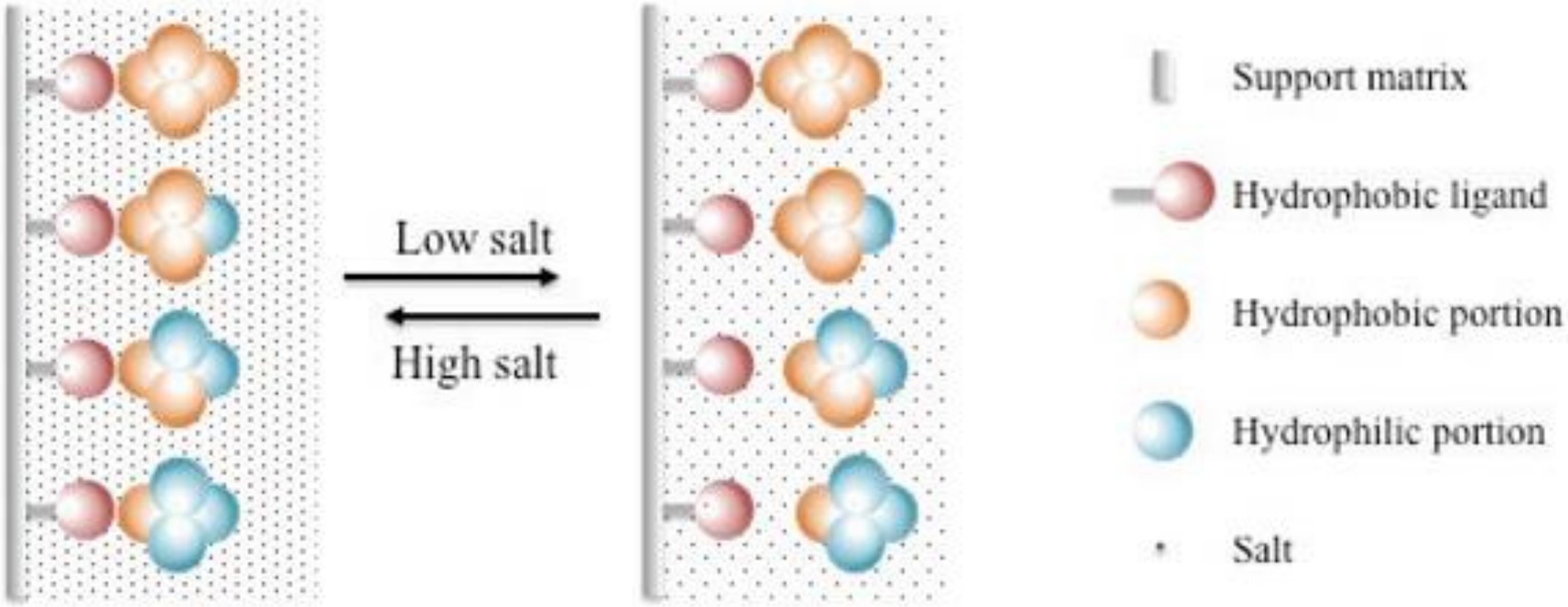
Affinity chromatography:

Affinity chromatography is a separation method based on a **specific binding interaction** between an immobilized ligand and its binding partner. Examples include antibody/antigen, enzyme/substrate, and enzyme/inhibitor interactions.



Hydrophobic interaction chromatography (HIC):

In Hydrophobic interaction chromatography (HIC) biomolecules adsorb to a weak hydrophobic surface at high salt concentration. Elution of adsorbed molecules is achieved by decreasing the salt concentration of the mobile phase over time.

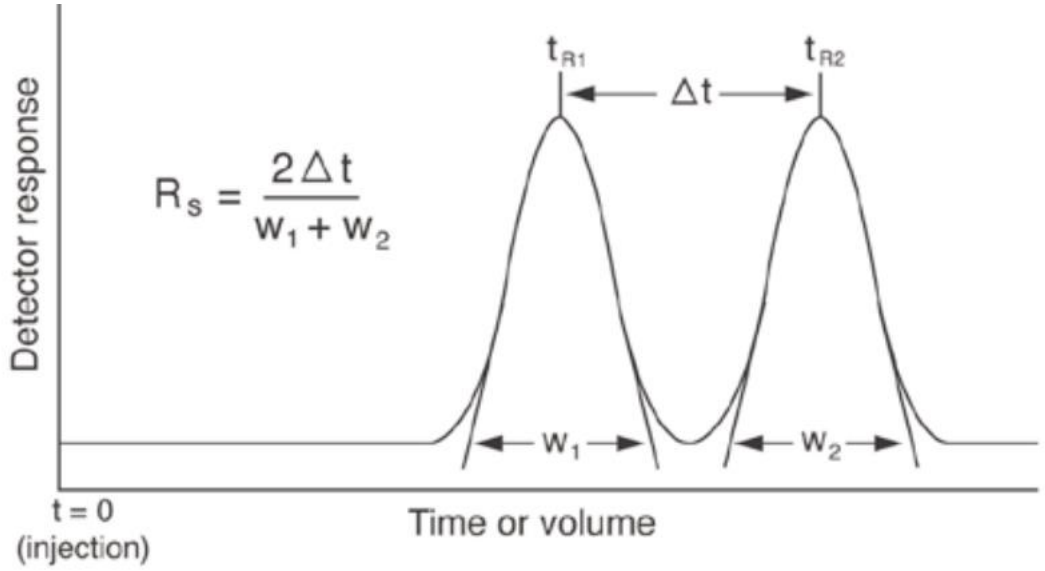


Other key concepts in chromatography:

An **eluotropic series** is listing of various compounds in order of eluting power for a given adsorbent. The "eluting power" of a solvent is largely a measure of how well the solvent can "pull" an analyte off the adsorbent to which it is attached.

A **theoretical plate** in many separation processes is a hypothetical zone or stage in which two phases, such as the liquid and vapor phases of a substance, establish an equilibrium with each other.

Resolution is a measure of the separation of two peaks of different retention time t in a chromatogram

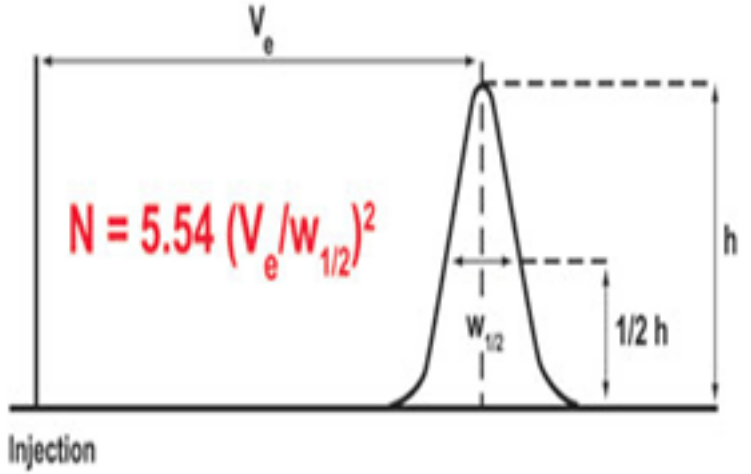


where:

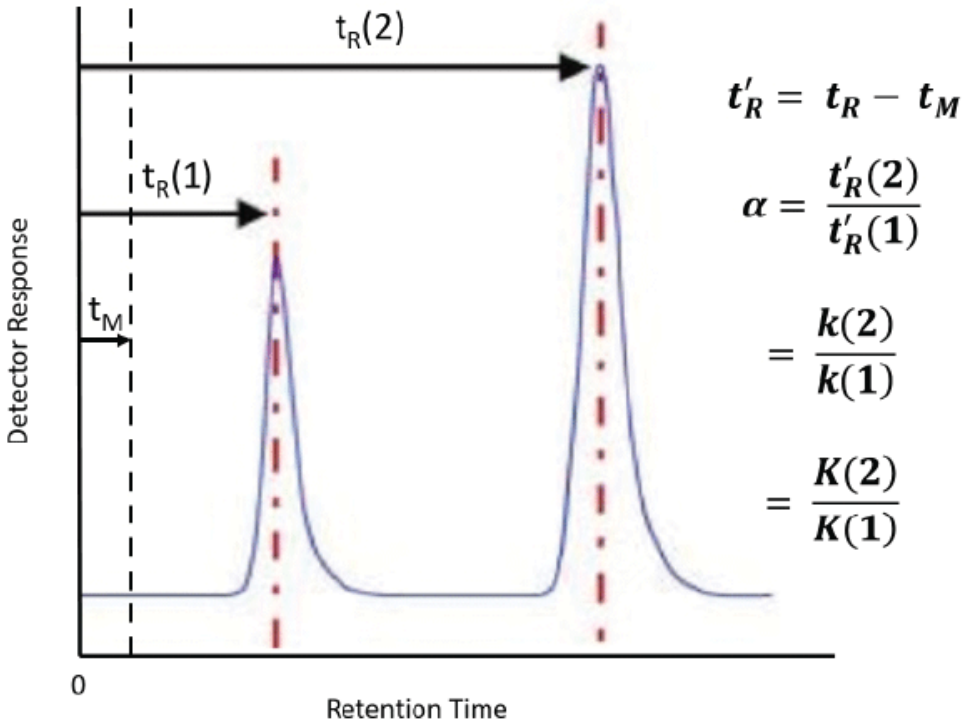
R_s = resolution
Δt = difference between retention times of peaks 1 and 2
w_2 = width of peak 2 at baseline
w_1 = width of peak 1 at baseline

Other key concepts in chromatography:

For chromatography columns, **efficiency** refers to the quality of separation. Given that each peak represents a component in the sample, you're looking for how many peaks can be separated. High resolution results in narrow peaks taking up less space, meaning that more peaks can be separated



Column efficiency, indicated as the number of theoretical plates per column, is calculated as $N = 5.54\ (t_R / w_{0.5})^2$ where t_R is the retention time of the analyte of interest and $w_{0.5}$ the width of the peak at half height



$$t'_R = t_R - t_M$$

$$\alpha = \frac{t'_R(2)}{t'_R(1)}$$

$$= \frac{k(2)}{k(1)}$$

$$= \frac{K(2)}{K(1)}$$

The **selectivity (or separation) factor (α)** is the ability of the chromatographic system to 'chemically' distinguish between sample components. It is usually measured as a ratio of the retention (capacity) factors (k) of the two peaks in question and can be visualized as the distance between the apices of the two peaks.

Validation of analytical data

Validation of analytical data. Central Tendency

Mean/Average can be defined as the sum of all the numbers divided by the total number of values.

where:

$\bar{x}$ = mean

$$\bar{x} = \frac{x_1 + x_2 + x_3 + \ldots + x_n}{n} = \frac{\Sigma x}{n}$$

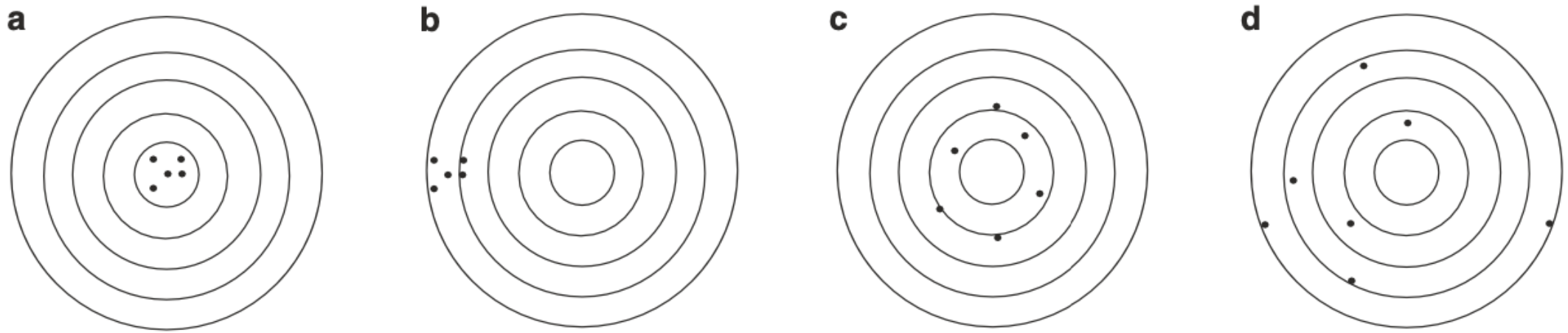
x_1, x_2, etc. = individually measured values (x_i)

n = number of measurements

$$\bar{x} = \frac{64.53 + 64.45 + 65.10 + 64.78}{4} = 64.72\%$$

Median: the midpoint or middle number within a group of numbers

Validation of analytical data. Precission and accuracy



Precision: This parameter is a measure of how reproducible or how close replicate measurements become.

Accuracy: How close a particular measure is to the true or correct value

a. Precise and accurate
b. Precise and non accurate
c. Accurate and non precise
d. Non precise and non accurate

Validation of analytical data. Standard deviation; indicators of dispersion

Standard deviation: a measure of the amount of variation or dispersion of a set of values

$$\sigma = \sqrt{\frac{\Sigma(x_i - \mu)^2}{n}}$$

where:

σ = standard deviation
xi = individual sample values
μ = true mean
n = total population of samples

However, true mean is generrally not known, thus:

SD (Standard deviation) is calculated as:

$$SD = \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{n}}$$

If n>30

$$SD = \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{n-1}}$$

If n<30

Validation of analytical data. Indicators of dispersion

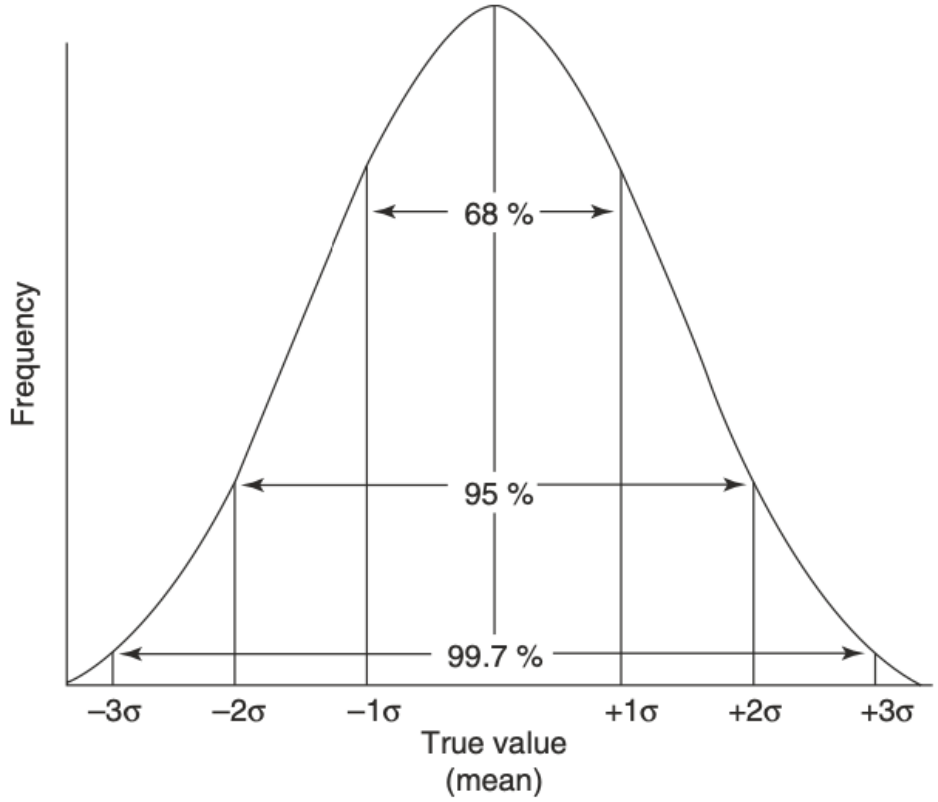
The co-efficient of variation (CV) is a statistical measure of the relative dispersion of data points in a data series around the mean. It represents the ratio of the standard deviation to the mean.

$$\%\,\text{Coefficient of variation}\,(\%\text{CV}) = \frac{\text{SD}}{\bar{x}} \times 100$$

Example

$$\%\,\text{CV} = \frac{0.293}{64.72} \times 100 = 0.453\%$$

Example of normal distribution



Validation of analytical data. Example

	Ret Time	O3	O2	O1	Promedio	Desvest	CV	
2	4.137	295059079	277504502	445823766	339462449	92528850.1	3.66872006	N,N-Dimethylglycine
3	5.017	2315560	2055277	3046185	2472340.67	513721.519	4.81260873	1H-Indole-3-acetamide
4	5.135	6881953	6208980	9148757	7413230	1540215.45	4.81311234	Butane
5	5.371	7907657	6837196	10749916	8498256.33	2022115.26	4.20265674	Acetic acid
6	9.708	24195093	18120441	16317997	19544510.3	4127122.54	4.73562637	Trimethyl phosphate
7	9.82	128289476	115737882	181405039	141810799	34859195.5	4.06810304	Glycerol
8	10.519	3128954	4508963	3484703	3707540	716483.396	5.17463492	Butanedioic acid
9	12.557	5177189		6435548	5806368.5	889794.182	6.52551862	Methylsuccinic acid
10	13.611	8059000	7316440	10366869	8580769.67	1590742.58	5.39419122	3,4-Dihydroxybutanoic acid
11	15.614	26975890	16646617	13207013	18943173.3	7165969.7	2.64349057	L-5-oxoproline
12	18.291	3044469	2955835	3658212	3219505.33	382507.06	8.41685205	Dodecanoic acid
13	21.291	6493304	5553756	7402634	6483231.33	924480.156	7.01283991	Alpha-Glycerophosphoric acid
14	22.247	41981439	38422239	51925294	44109657.3	6998579.04	6.30265903	D-(-)-Tagatofuranose
15	22.418	100206466	89539687	131086996	106944383	21577635.8	4.95626046	D-Psicofuranose
16	23.785	211440842	189461975	266376743	222426520	39616716.5	5.61446126	1,3,5-Triazine
17	23.989	135008895	119276139	69037992	107774342	34456617.8	3.12782707	Maltose
18	25.15	7481099	7490852	9855339	8275763.33	1367961.35	6.04970554	Cyclohexadecane, 1,2-diethyl-
19	25.582	5954604	6118611	7363904	6479039.67	770690.107	8.4068027	N,N-Diethyl-butyrothioamide
20	25.833	58044726	53960463	81580186	64528458.3	14907761.9	4.32851416	Glucopyranose
21	25.998	9604862	8745714	9583295	9311290.33	489922.163	19.0056524	Hexanamide, N-hexanoyl-N-allyl-

Validation of analytical data. Indicators of dispersion

A confidence interval is the mean of your estimate plus and minus the variation in that estimate. This is the range of values you expect your estimate to fall between if you redo your test, within a certain level of confidence. Confidence, in statistics, is another way to describe probability

$$\text{Confidence interval (CI)} = \bar{x} \pm Z \text{ value} \times \frac{\text{standard deviation (SD)}}{\sqrt{n}}$$

$$\text{CI (at 95\%)} = 64.72 \pm 1.96 \times \frac{0.2927}{\sqrt{25}}$$

$$= 64.72 \pm 0.115\%$$

If n>25

$$\text{CI} = \bar{x} \pm t \text{ value} \times \frac{\text{standard deviation (SD)}}{\sqrt{n}}$$

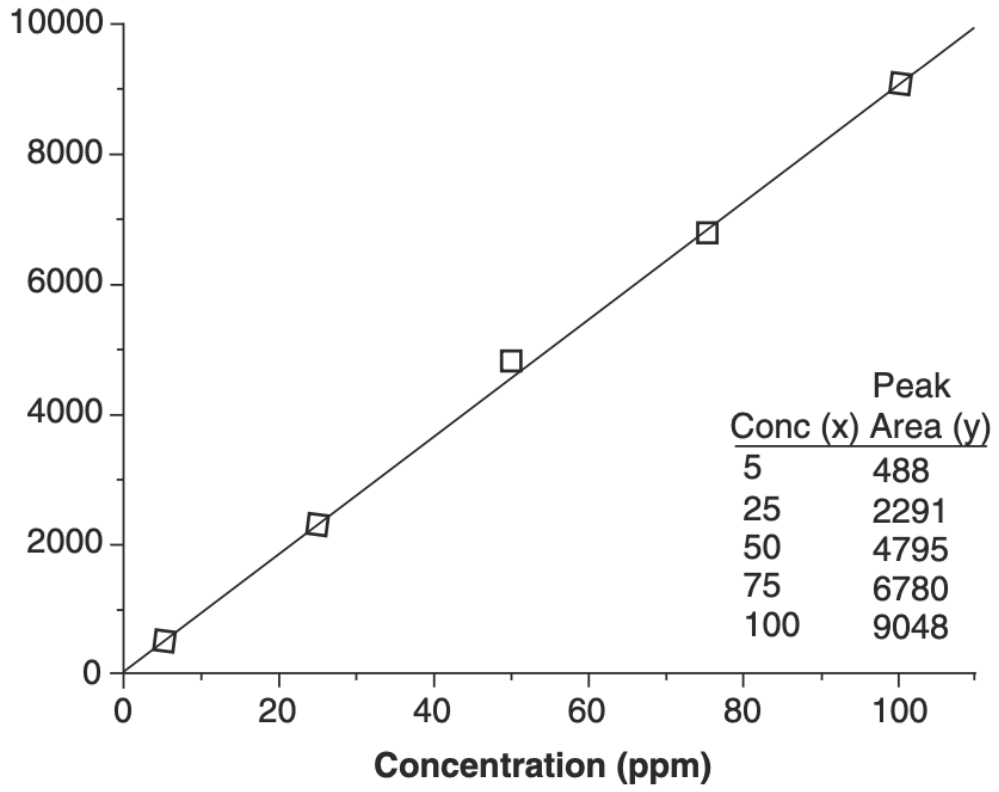
$$\text{CI (at 95\%)} = 64.72 \pm 3.18 \times \frac{0.2927}{\sqrt{4}}$$

$$= 64.72 \pm 0.465\%$$

If n<25

Quantitation of analytical data. Calibration curve

A calibration curve is a way to identify the concentration of an unknown substance. These curves use data points of known substances at varying concentrations, and researchers or developers can use these curves to find where an unknown substance plots.



Conc (x)	Peak Area (y)
5	488
25	2291
50	4795
75	6780
100	9048

$$\text{slope } a = \frac{\Sigma(x_i - \bar{x})(y_i - \bar{y})}{\Sigma(x_i - \bar{x})^2}$$

$$y - \text{intercept } b = \bar{y} - a\bar{x}$$

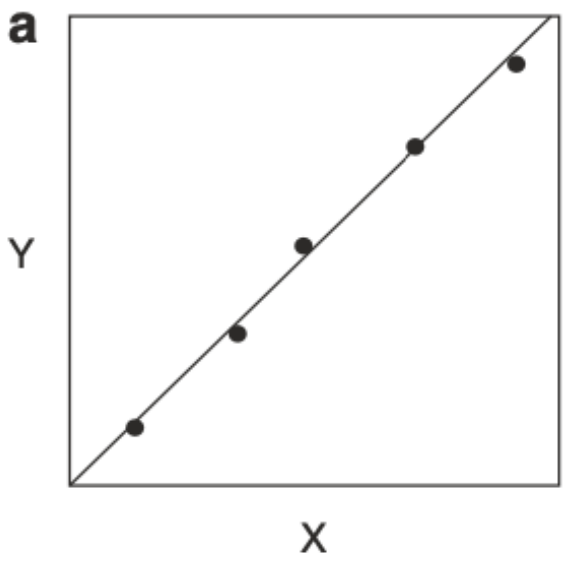
where:

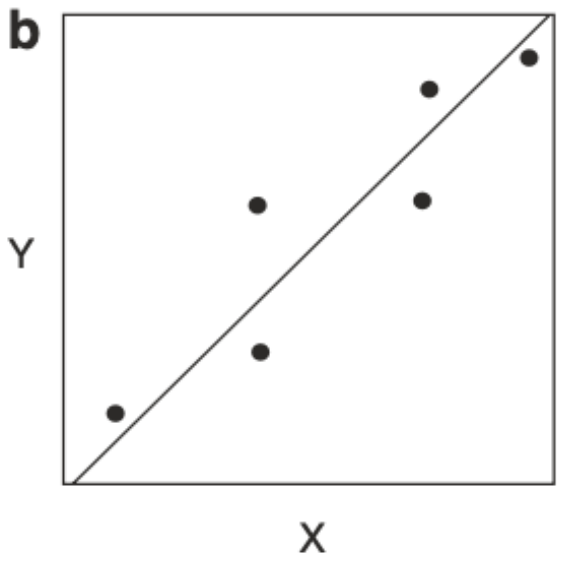
x_i and y_i = individual values

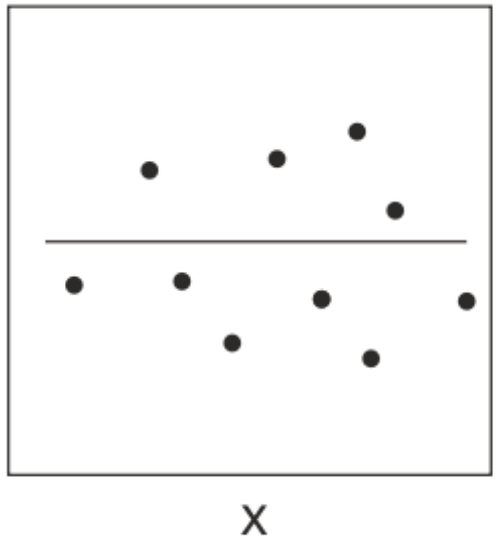
$\bar{x}$ and $\bar{y}$ = means of the individual values

$$y = ax + b \qquad x = \frac{y - b}{a}$$

Quantitation of analytical data. Calibration curve







High linearity (r^2) = 0.999

Not so high linearity (r^2) = 0.93

No linearity (r^2) = 0.2

correlation coefficient =

$$r = \frac{\Sigma(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\left[\Sigma(x_i - \bar{x})^2\right]\left[\Sigma(y_i - \bar{y})^2\right]}}$$

Coefficient of determination (r^2/ R^2): It provides a measure of how well observed outcomes are replicated by the mode

Quality of analytical data. LOQ and LOD

Limit of detection (LOD) –X_{LD}-: the lowest possible amount that we can detect with some degree of confidence (or statistical significance).

$$X_{LD} = X_{Blk} + (3 \times SD_{Blk})$$

where:

X_{LD} = minimum detectable concentration
X_{Blk} = signal of a blank
SD_{Blk} = standard deviation of the blank readings

$$X_{LQ} = X_{Blk} + (10 \times SD_{Blk})$$

Limit of quantification (LOQ) –X_{LQ}-: the lowest possible amount that we can detect with some degree of confidence (or statistical significance).

Quality of analytical data. Control charts