Experiment 9

Chromatography (HPLC)

High-performance liquid chromatography

chromatography

Stationary phase ← الثابتة

Solid / liquid.

Mobile phase
(Solvent)
gas/ Liquid
اللي بتحرك

* بحركة ال (Mobile) خلال ال Stationary رح يتحرك

ال(analyte) وال analyte يعتمد بالحركة على ال attraction forces between it and analyte.

* المادة اللي إلها affinity أعلى لل Stationary رح يتأخر.

* المادة اللي إلها affinities أعلى لل (Mobile) رح تمشي أسرع.

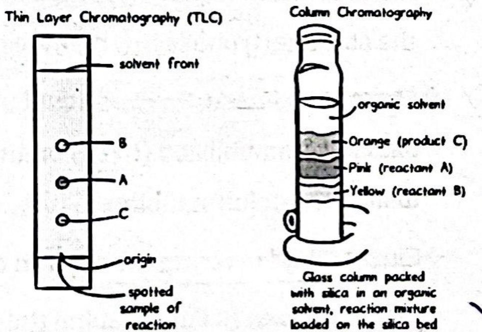
INTRODUCTION

* ال (chromatography) مُرتبط بشكل أساسي بال (Seperation).
بعد ما أفصلهم بقدر أحللهم بالطريقة اللي بدّي إيّاها.

Chromatography is an analytical technique **for separation** of a complex **mixture** of **chemical substances into its individual components,** so that the individual components can **be thoroughly analyzed.**

➤ **Its principle** relies on the **differential affinities** of **the individual substances for a gas or liquid mobile medium** and **for a solid or liquid stationary medium through which they pass.** So, the **components of the mixture will distribute unequally between the two immiscible phases.**

(analyte)



→ less polar

* ال (principle / اللي بقوم عليه ويعتمد عليه هو اختلاف ال affinities يعني اختلاف ال attraction لل (analyte) between two phases.

ال Silica ← polar

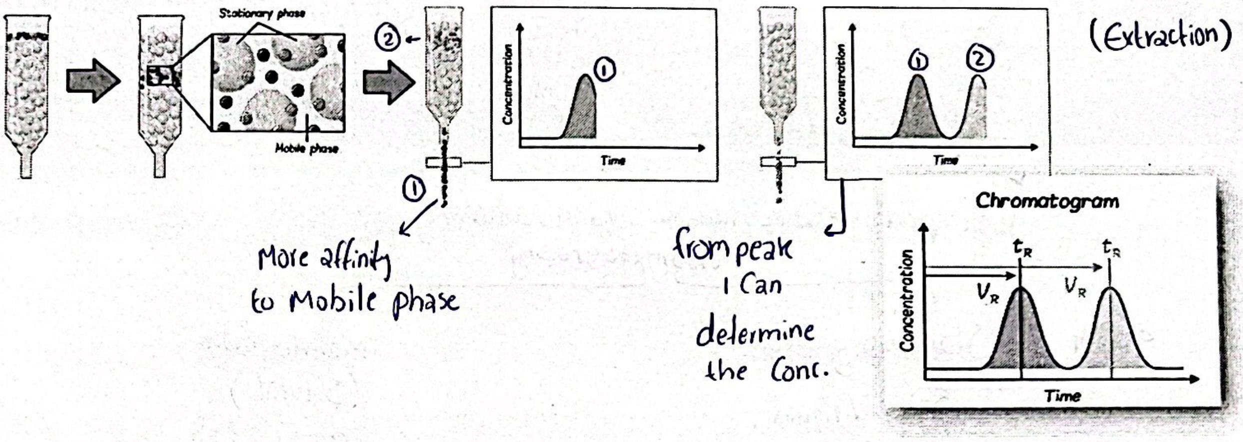
Purpose of Chromatography

بدّي أحدّد المكونات بال Mixture

① ➤ **Analytical:** determine chemical composition of a sample (qualitatively① and then quantitively)②.

② ➤ **Preparative:** purify and collect one or more components of a sample.

بدّي أفصّل المكونات بال Mixture
(Extraction)

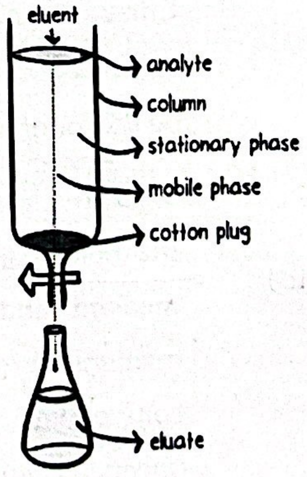


More affinity to Mobile phase

from peak I Can determine the Conc.

tR → identfiy the peaks.

TERMS RELATED TO CHROMATOGRAPHY



✓ ❖**Analyte:** The mixture of substances that are to be separated and analyzed during chromatography.

✓ ❖**Mobile Phase:** Liquid or gaseous solvent that flows over or through the stationary phase in a definite direction through the column.

✓ ❖**Stationary Phase:** Solid or liquid or a mixture of solid/liquid medium which is immobilized (fixed) on the support particles or on the inner wall of the column tubing.

❖**Eluent:** Fluid entering the column consist of mobile phase & analyte.

❖**Effluent (Eluate):** Fluid leaving the column.

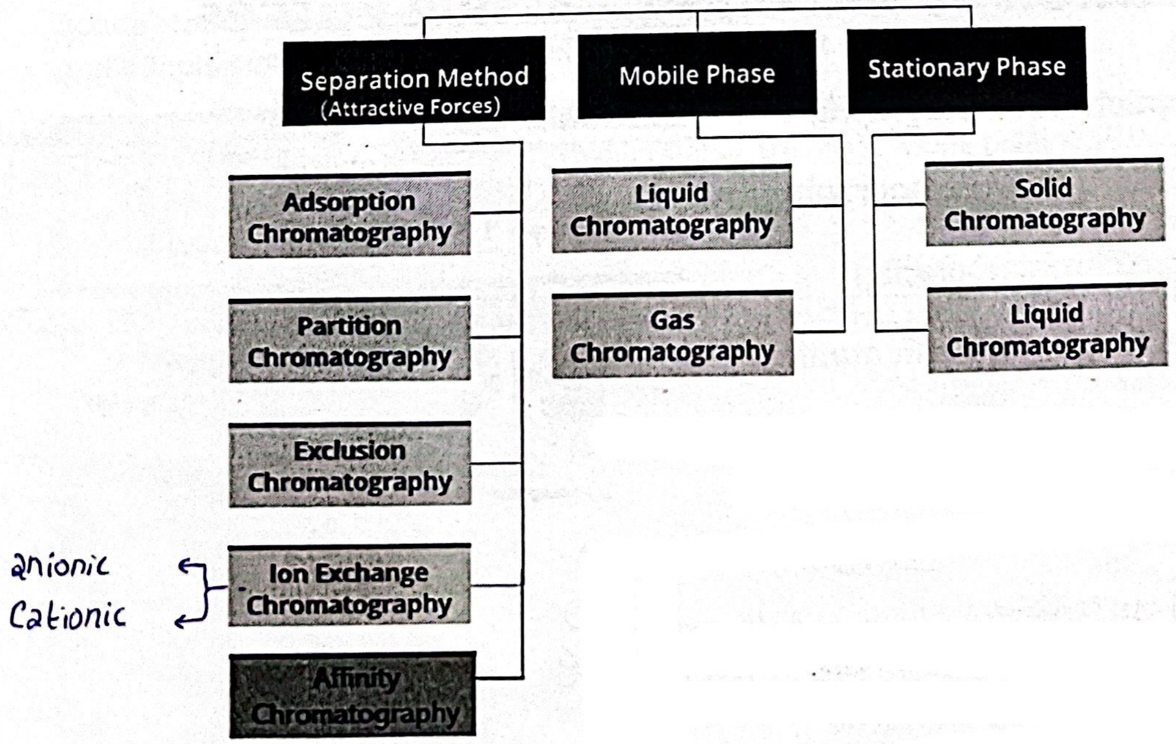
mobile phase → TLC
or mobile phase + analyte → Eluent

Applications for Chromatography

- **Pharmaceutical Company** – determine amount of each chemical found in new product
- **Hospital** – detect blood or alcohol levels in a patient's blood stream
- **Law Enforcement** – to compare a sample found at a crime scene to samples from suspects
- **Environmental Agency** – determine the level of pollutants in the water supply
- **Manufacturing Plant** – to purify a chemical needed to make a product

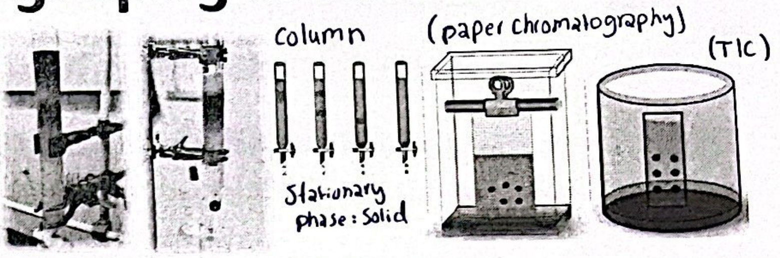
analyte ← Seperation ← الهدف مُشترك *

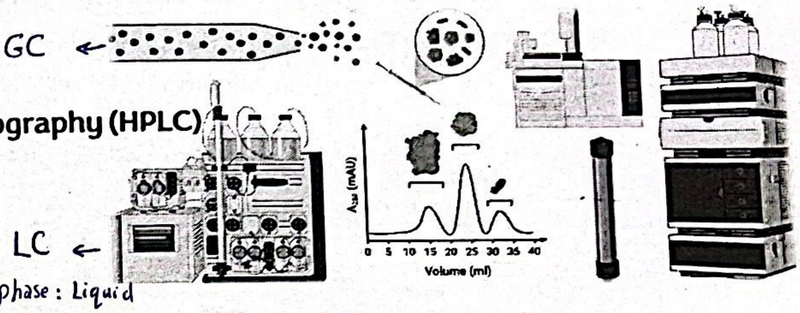
Classification of Chromatography



Types of Chromatography Methods

- Paper Chromatography
- Thin Layer Chromatography (TLC) ← Non volatile Mixture
- Gas Chromatography (GC)
- Column Chromatography ← Chemical Substance into individual Comp.
- Liquid Chromatography (LC)
- High Performance Liquid Chromatography (HPLC)





(Gas C) → seperete it depend on boiling point

mobile phase : Liquid

حركته بناءً على ال (P) مو الجاذبية .

* الفرق بين ال GC وال TLC :

↓ gas mobile and liquid Stationary phase

↙ Liquid Mobile and Solid Stationary phase

* الفرق بين ال paper وال (TLC)

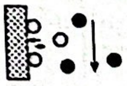
↓ Stationary phase : Silica

↓ Stationary phase : paper

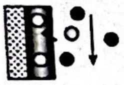
Classification based on Attractive Forces

1. Adsorption chromatography
2. Ion Exchange chromatography
3. Partition chromatography
4. Size Exclusion chromatography
5. Affinity chromatography

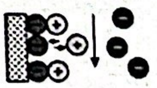
Adsorption chromatography
Separation based on adsorption of chemicals to the surface of a support



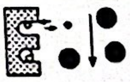
Partition chromatography
Separation based on partitioning of chemicals into a layer of the stationary phase



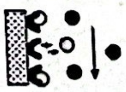
Ion-exchange chromatography
Separation of ions based on their binding to fixed charges on a support



Size-exclusion chromatography
Separation of chemicals based on their size and ability to enter a porous support



Affinity chromatography
Separation of chemicals based on their interactions with a biologically related binding agent



① الفصل بحتمد على bonds مُعينة

② الفصل بعتمد على ال (charge) المُختلفة رح تعمل (attraction) ، المُشابهة رح تتحرك مع ال mobile phase

③ الفصل بحمد على (differat Solubility of chemical Sub)

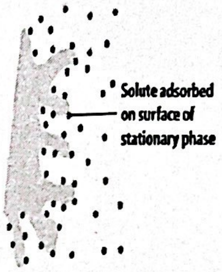
④ الفصل بتعتمد على ال (size) الصغيرة رح تعلق داخل بال Stationary ، الكبيرة رح تتحرك

⑤ بتعتمد على (biologic) ، نفس مبدأ ال (Enzyme) .

1. Adsorption Chromatography

of analyte

Separation based on their adsorption onto the surface of solid (stationary phase). Based on different non-covalent interactions (H-bond, ionic, hydrophobic, vander waal...).



- **Normal phase-separation: Polar analytes** → adsorbs strongly → Polar stationary phase (ex: Silica or Alumina) → Nonpolar mobile phase
- **Reverse phase-separation: Non-polar analytes**→ adsorbs strongly → Non-polar stationary phase (carbon chain bonded to silica) → Polar mobile phase

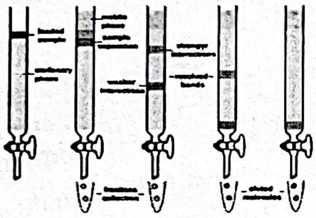
← More use in (TLC)

لازم الـ Stationary
يختلف عن Mobile
لازم immiscible
واحد nonpolar
والتاني polar

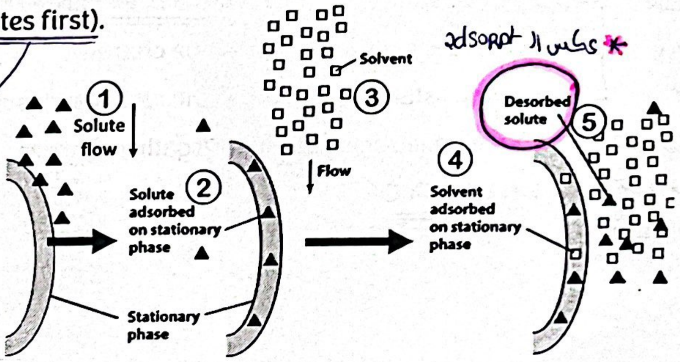
Degree of adsorption (less tightly bound → elutes first).

Ex: Column chromatography CC, TLC, HPLC

More tightly
رح يتأخر الـ elutes
إله ..

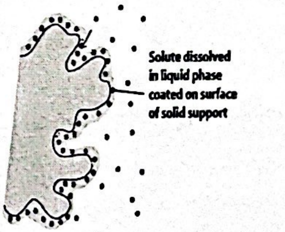


* عكس الـ adsorpt



* إللي بتنزل أسرع less tightly bonded

2. Partition Chromatography



Solute are separated based on their partition (K_d) between a liquid mobile phase and a liquid stationary phase coated on a solid support.

K_d→ ratio of concentration of solute in two phases (Solubility differences).

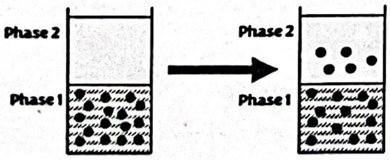
- **Normal** – analyte is nonpolar organic; stationary phase MORE polar than the mobile phase.
  Ex: TLC, Paper Chromatography

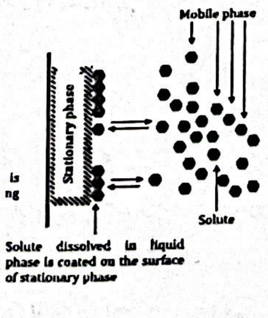
المختلف هو الـ analyte موجود بالـ Stationary وأنا بدّي بوصل وبروح للـ Mobile

* بدّي nonpolar

- **Reverse** – analyte is polar organic; stationary phase LESS polar than the mobile phase.
  Ex: HPLC

الـ analyte بدّي olar
نفس مبدأ الـ Normal



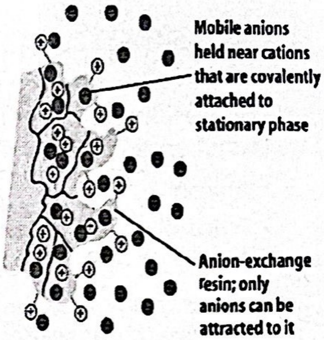


Solute dissolved in liquid phase is coated on the surface of stationary phase

* إذا بدي أفصل -Neg ستخدم (resin) مُحمل بشحنة +pos عشان يرتبط فيها وأجمعه (collection)

* الفصل بناءً على ال
① type of charge
② Strength of charge

3. Ion Exchange Chromatography



Use ionic stationary phase (Ionic exchanger)

- ions separated based on their tendency to displace counter ions adsorbed on stationary phase (Depends on charge, hydration, "solubility").
- Used for analysis of proteins, nucleic acid, amino acids and its base pair.

Stationary phase / Solid

- **Anionic stationary phases:** the resin carries positive charges (**attracts anions**) and allows the removal/separation of positive charges.

Mobile: Liquid or gas

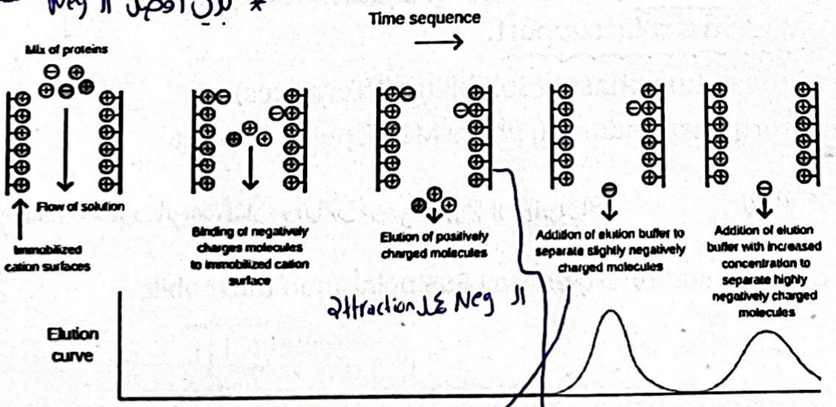
- **Cationic stationary phases:** the resin carries negative charges (**attracts cations**) and allows the removal/separation of negative charges.
- **Ex:** GC, HPLC

Covelent bond

عشان مايتوصل. باختلاف ال Cond.

Example of Anionic Exchanger:

* بدّي أفصل ال Neg ستخدم resin مُحمل بشحنة positive ⊕



ال Neg عل attraction

* مُلخّص *

Resin Type	Cationic Exchanger	Anionic Exchanger
Charge of the resin (stationary phase)	−	+
Net charge of molecule of interest	+	−

* buffer:

① ال .conc خفيفة عشان أكرق أوّلهم

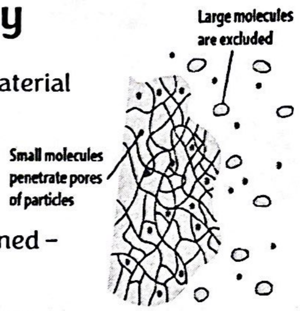
② ال bond ما تكون Covelent عشان أكرف أفضلهم عشان هيك بضيف ال (buffer)

buffer تاني ال .conc إله أعلى عشان أكرف أفضل.

4. Size (Molecular)Exclusion Chromatography

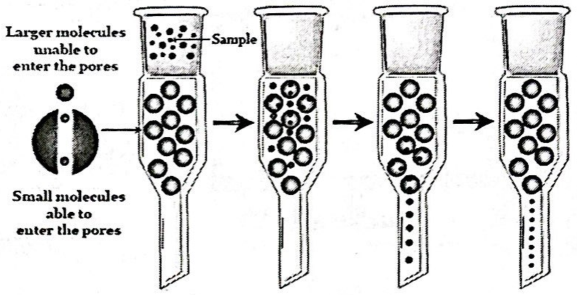
Separation is a result of "trapping" of molecules in the pores of the packing material

- Stationary phase is a porous matrix
- Very large molecules can't get into the pores – unretained
- Very small molecules get hung up into pores for a long time – most retained – longest retention time



Ex: GC, HPLC

* Stationary phase : Solid
* Mobile phase : Liquid /gas.

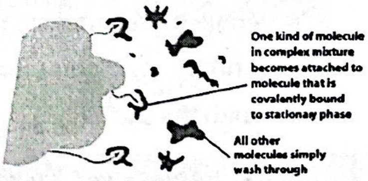


* كُل ما كانوا أصغر كُل ما علقوا أكثر ..

بال Solute في Mixture of Comp الهم affinity لـ (ligand) معين.

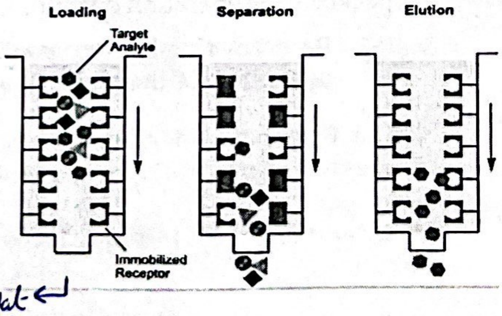
5. Affinity chromatography (Key-lock)

The mixture are separated when the element having an affinity towards the stationary phase binds to the stationary phase. In contrast, other components are eluted with the mobile phase.

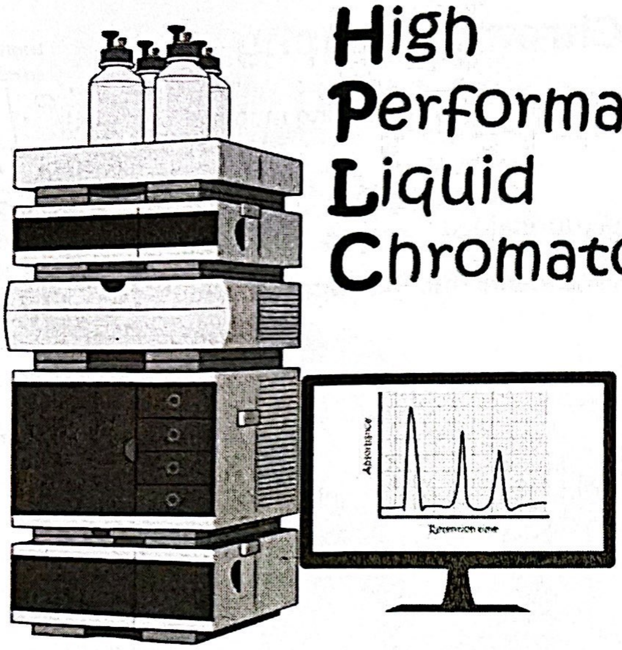


- The substrate/ ligand is bound to the stationary phase so that the reactive sites for the binding of components are exposed.
- Now, the mixture is passed through the mobile phase where the components with binding sites for the substrate bind to the substrate on the stationary phase while the rest of the components are eluted out with the mobile phase.
- The components attached to the stationary phase are then eluted by changing the pH, ionic strength, or other conditions.

* عشان يفصل

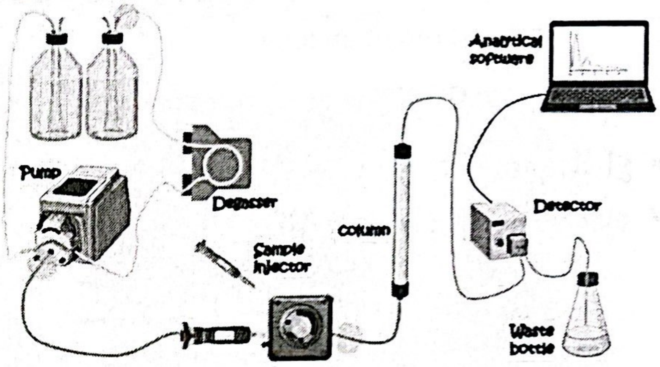


← non-covalent bond عن طريق أغير ال Cond.



High
Performance } High resolution power
Liquid } Mobile Phase is liquid
Chromatography

Stationary phase → Solid



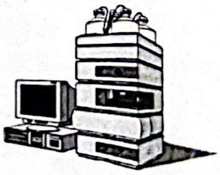
(Column) ↓ عن طريق الجاذبية

High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) is a modified form of column chromatography where the components of a mixture are separated based on their affinity with the stationary phase. It is an extension of conventional LC.

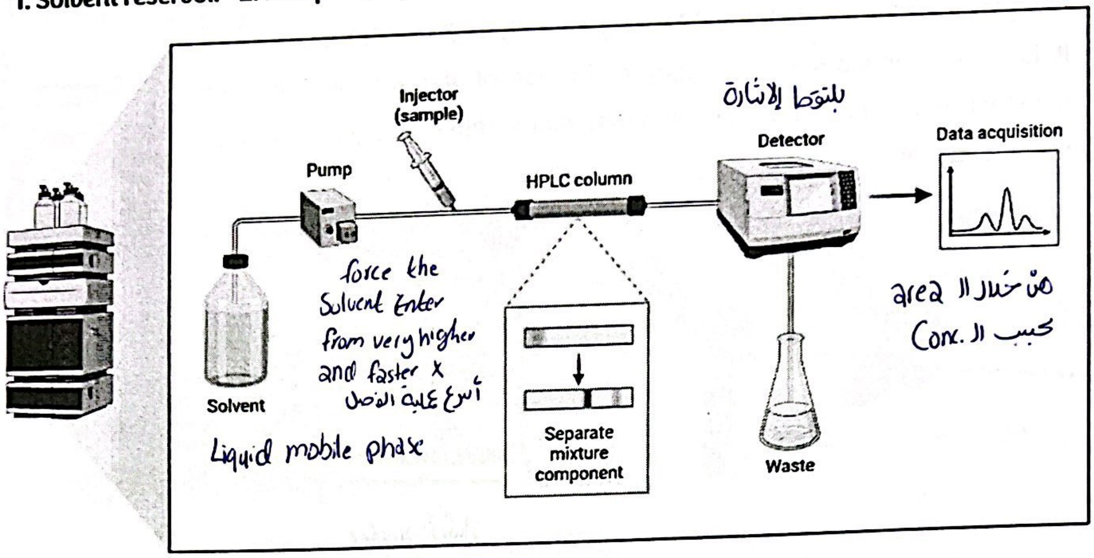
- ❖ Very powerful technique (High resolution & High Pressure). → from pump
- ❖ The stationary phase is a granular material with very small porous are tightly particles packed in a separation column. * أزيد الفصل .. ← عشان أزيد ال Surface area ← يزيد interaction

  Particles size is very small → larger surface area → higher interaction → Better separation of the molecules flowing through it.
- ❖ The Eluent (liquid mobile phase) is forced through the column with extremely high pressure (up to 400 atm or 6,000 psi) by a pump
- ❖ separation time is highly reduced to few minutes rather than hours and days.



HPLC Instrumentation Components

1. Solvent reservoir 2. Pump 3. Injector 4.Column 5.Detector 6.Recorder or Data system.



بلتقط الإشارة

force the Solvent Enter from very higher and faster x
أسرع عملية الفصل

Liquid mobile phase

من خلال ال area
نحسب ال Conc.

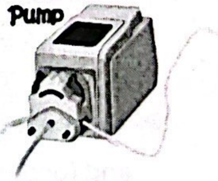
1. Solvent reservoir: Solvent reservoir is also known as mobile phase (liquid) reservoir. The high viscous solvent is discouraged to use as it takes much more time to travel through column, and high pressure is required for the viscous solvent. These leads to peak broadening, and hence better not to use such solvent.

ممكن بس أزيد ال P يكون كذي تكسير

- ➢ The choice of solvent depends on the nature of sample and the sensitivity of the detector.

2. Pump: The pump's function is to propel the liquid mobile phase through the column at a set flow rate, which is measured in (mL/min). Which forces the mobile phase at a much a higher velocity than the gravity-flow columns.

كُل ما زاد ال flow rate ↓ بزيد ال pressure



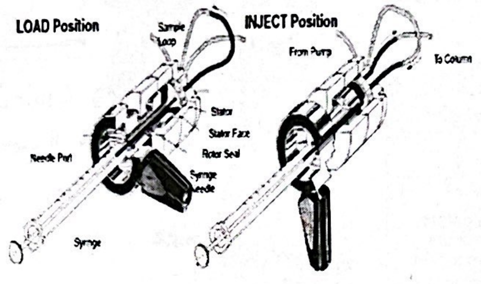
- ➢ A pump can deliver a constant mobile phase composition (isocratic) or a rising mobile phase composition (gradient) during the chromatographic experiment.

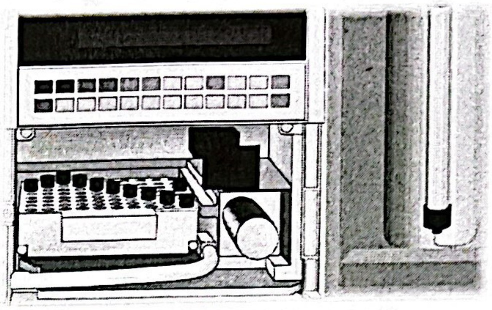
* two different Mobile phase

* mobile phase واحد تركيز

3. Injector: The injector is used to insert the liquid sample into the mobile phase's flow stream.

- ➤ Sample quantities range from 0.1 to 100 microliters (L) at high pressure.
- ➤ It is either done using a syringe or The use of the autosampler (auto-injector) system is also widely used that allows repeated injections in a set scheduled-timing.



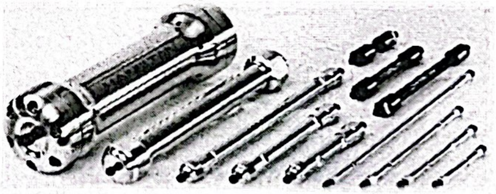


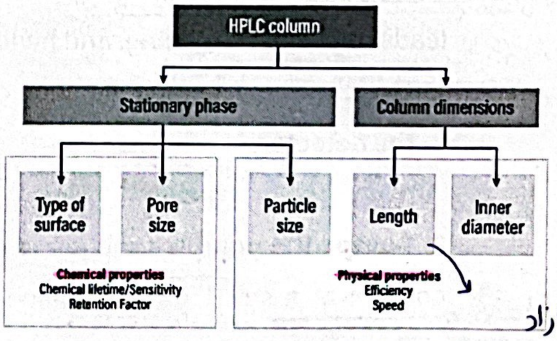
AutoSampler

عشان تتحمل الضغط.

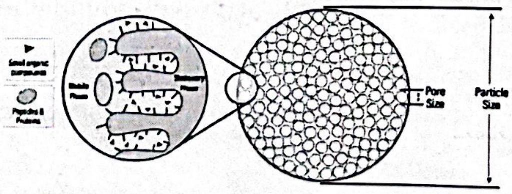
4. HPLC Column: It is known as the heart of the chromatograph. Mostly, stainless steel is used as materials for the construction of the tubing (High pressure).

- ➤ The column length generally varies from 5 cm to 30 cm, and its diameter ranges from 2-50 mm.
- ➤ Silica and alumina particle is used as packing materials. Particle size ranges from 3-5 µm. Pore size (pore size 80 to 120Å) are best for small molecules MW< 2000. For larger molecules with MW>2000, a popular pore size for proteins is 300Å.
- ➤ The mobile phase is aspirated from the solvent reservoir and forced through the system's column and detector by a pump.





كُل ما زاد طول الـ Column كُل ما كان أفضل ..



كُل ما أنا بدّي أفضل في الفصل تبعه سريع أو بطلع بسرعة ← أقصر ، less dia

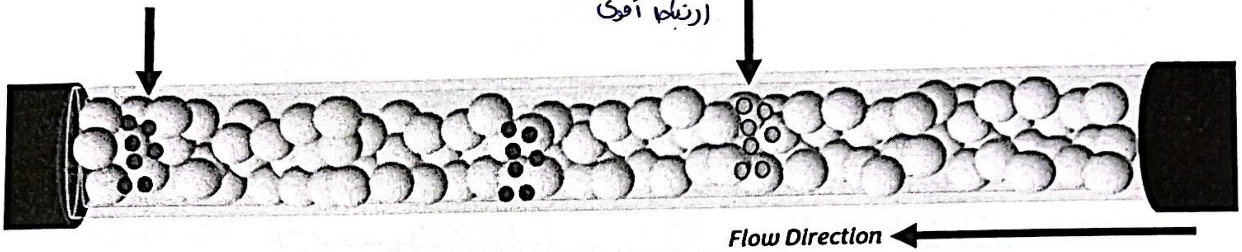
Normal Phase HPLC

The stationary phase is more polar than the mobile phase (e.g. toluene as the mobile phase, silica as the stationary phase).

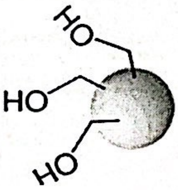
** Non-polar will be eluted first*

** Polar analyte moves the slowest (eluted last)*

ارتباط أقوى



Stationary phase has polar heads



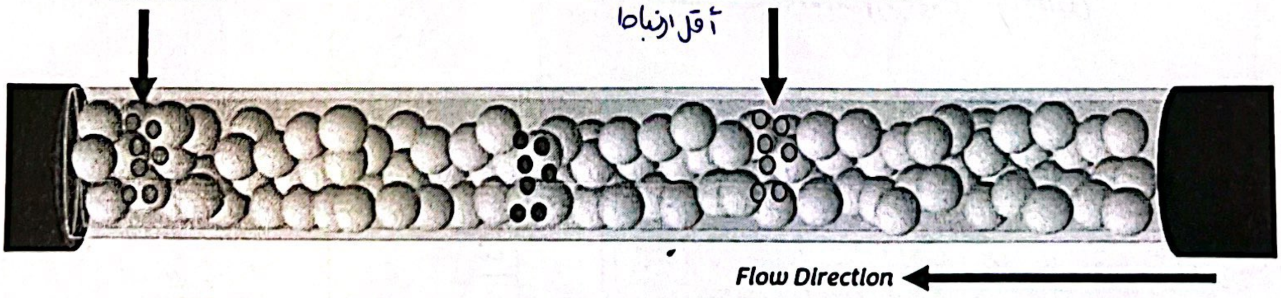
Reverse Phase HPLC

The stationary phase is less polar than the mobile phase (e.g., water-methanol mixture as the mobile phase, C18= octadecylsilyl (ODS) as the stationary phase).

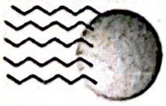
Polar analyte will be eluted first

Non-polar analyte moves the slowest (eluted last)

أقل ارتباطا



Stationary phase has Hydrophobic alkyls groups



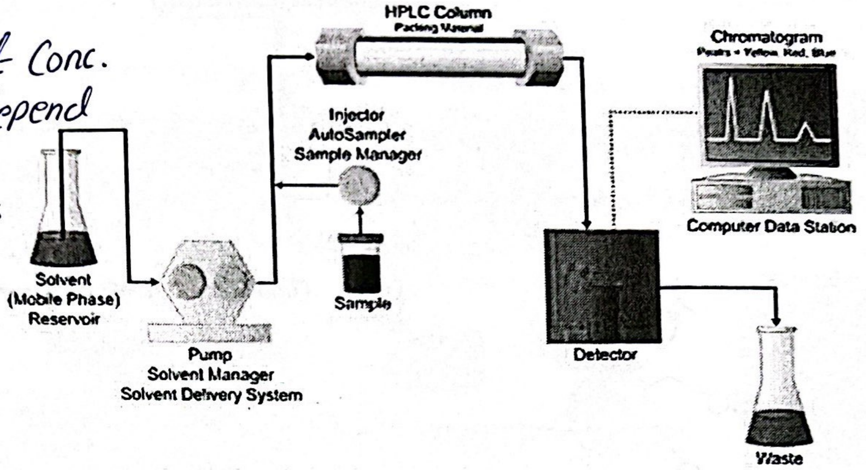
Elution Modes in HPLC:

* ما بحتاج Second

A. Isocratic

The mobile phase is either a pure solvent or a mixture *remains the same throughout the run.*

* Conctent Conc. does not depend on Column dimensions

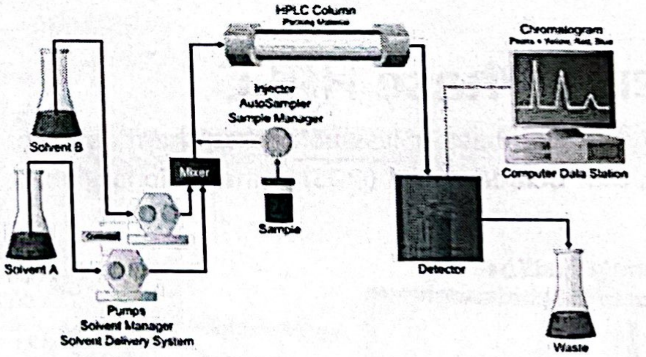


* أكثر من (pump)

A. Gradient

The mobile phase composition changes during the separation.

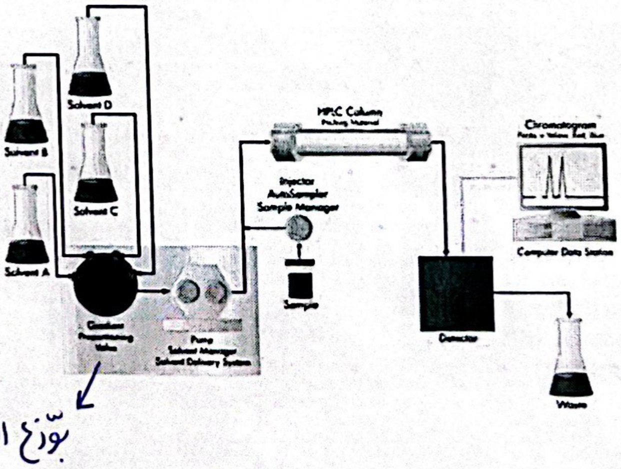
Multibele mobile phases during the Seperation



* متى بستخدمه ؟

لمّا ال Solute اللي بدّي أفصله عنده High affinity

ل Column / Stationary phase ، بدّي اسرع من استخراجه بستخدم Multi



بوزّع الكميات الداخلة بالطريقة الصحيحة عشان يصيرلهم ضخ

chromatography موجز من ال *

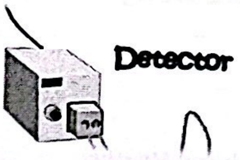
just Seperation

* بعد ما العينة صارلها Seperation ← detector

5. Detector: It is used to observe the obtained separated elute as it leaves the column. The composition of the eluent is consistent when no analyte is present. While the presence of analyte changes the composition of the eluent. What detector does is to measure these differences.

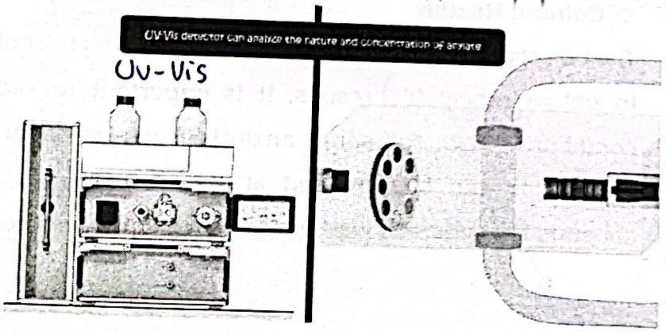
ال Qual وال Quan عبر أجهزة ثانية.

- ➢ This difference is monitored as a form of an electronic signal. This signal is proportional to the concentration of each separated component
- ➢ Crucial in trace elements (high sensitivity).
- ➢ Simplifies quantification * لأن بتعتمد ال absorbtion
- ➢ Insensitive to changes in type of solvent, flow rate and temp.
- ➢ There are different types of detectors available



The most widely used detection methods

- Spectrophotometers
- Fluorometers
- Electrochemical detectors
- Mass spectrometer
- Refractive index detector

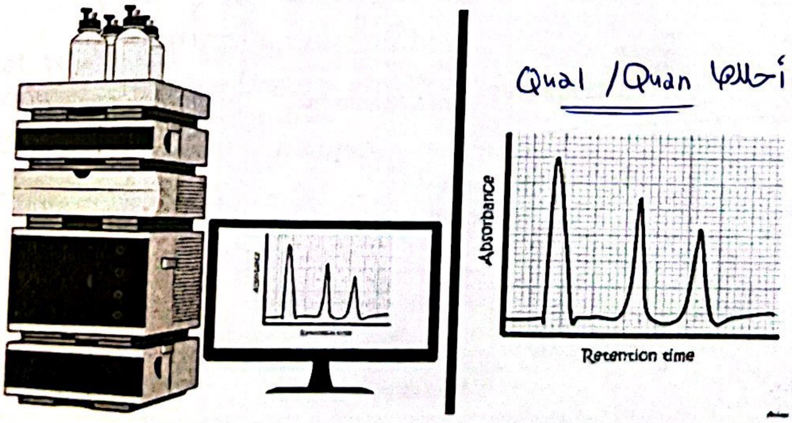


(Signal يجي ال)

6. Recorder: The change in eluent detected by a detector is in the form of an electronic signal, and thus it is still not visible to our eyes.

- ➢ A computer-based data processor (integrator) is used to process various types of data.
- ➢ It takes the signal from the detector and makes use of it to decide the time of elution (retention time) of the sample components (qualitative analysis) and the quantity of pattern (quantitative analysis).

* بقدر أحللها Qual /Quan



* Quan → peak area Under the Curve & Absorbane

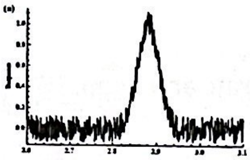
* Qual → retention time

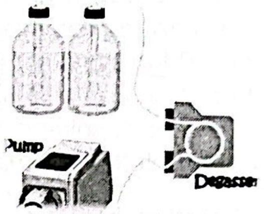
tR

❖ Degasser

→ * بحافظ على pump وبمنع تكون ال noise من وجود gases or bubbles

The eluent used for LC analysis may contain gases such as oxygen that are non-visible to our eyes. When gas is present in the eluent, this is detected as noise and causes an unstable baseline. Degasser uses special polymer membrane tubing to remove gases.



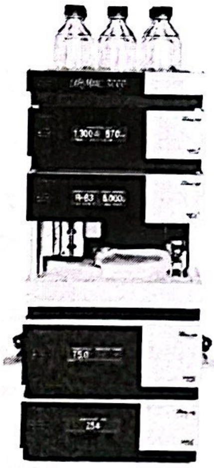


❖ Column Heater

The LC separation is often largely influenced by the column temperature. In order to obtain (repeatable) results, it is important to keep consistent temperature conditions. Also, for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperatures. (50 to 80°C). Thus, columns are generally kept inside the column oven (column heater).

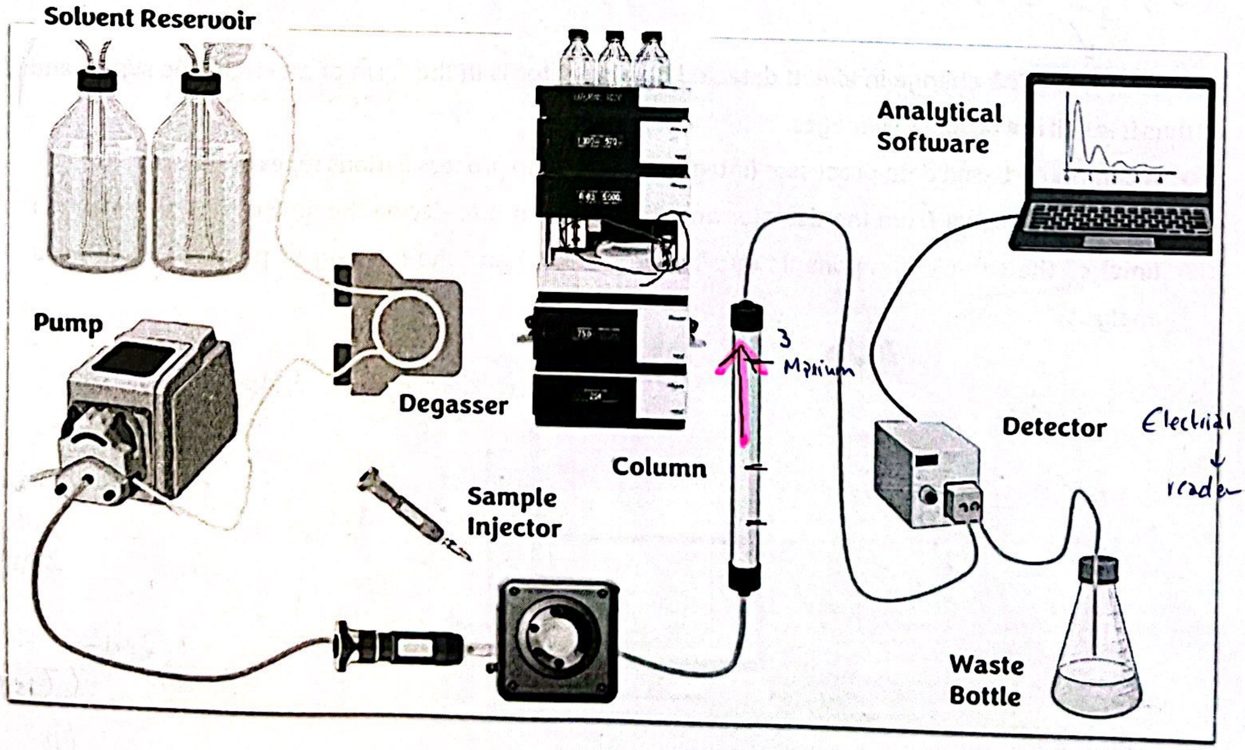
الفصل بين two peaks يكون واضح ويسهل التمييز بينهم

واضح Rt



← Column Heater

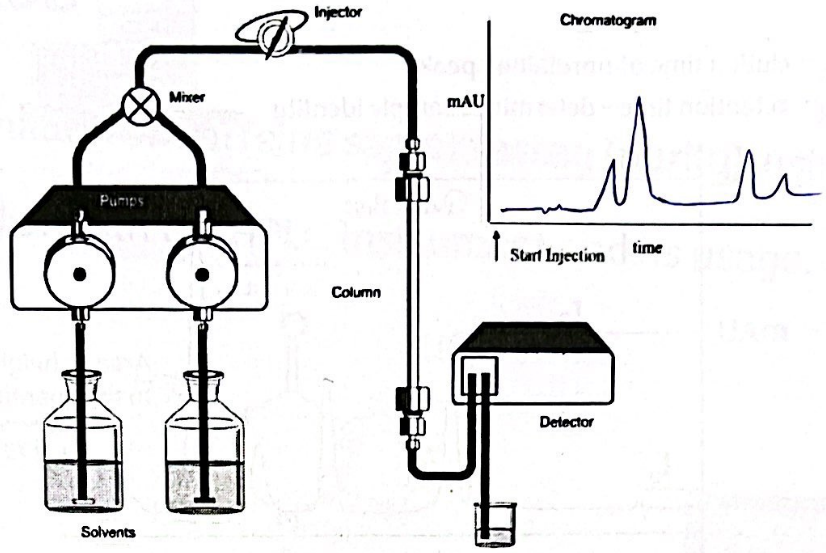
بحافظ عليها ضمن حرارة معينة خلال عملية التحليل



3 Maximum

Electrical signal ↓ reader

Separation in HPLC



* A more polar than B ← reversabile phase

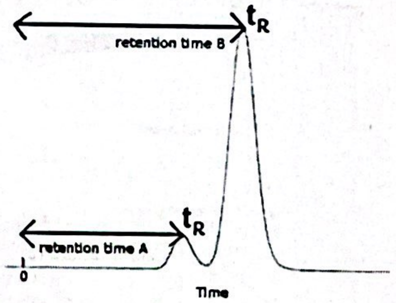
* B More polar than A ← normal phase

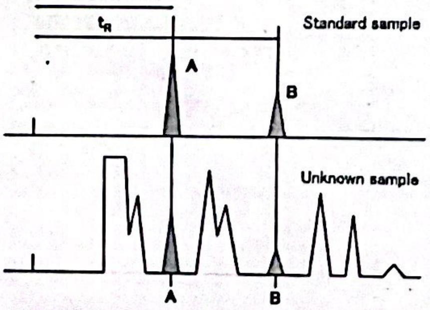
The Retention Time (t_R)

بعكس

+ الStability

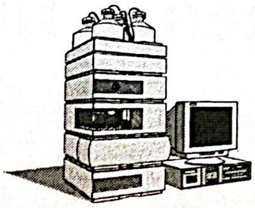
- The time taken for a particular compound to travel through the column to the detector
- From the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound.





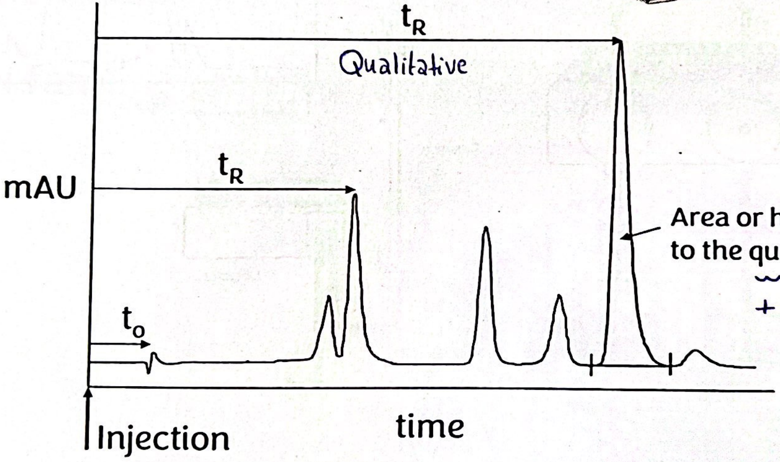
بقدر أقارن ال peaks اللي طلعتلي مع ال peaks لل Standard
عشان أميز نوعهم

The Chromatogram



t_o – elution time of unretained peak

t_R– retention time – determines sample identity



Area or height is proportional to the quantity of analyte.

+9421 → Conc.

كُلما زاد ال Conc.

يعني ال Area رح تكون أعلى وأكبر