HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

BY
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CHROMATOGRAPHY :

- Chromatography is defined as the method of separating a mixture of components into individual components.
- This techniques is based on the differences in the rate at which the components of a mixture move through a porous medium(stationary phase) under the influence of some solvent or gas(mobile phase).

WHY USE HPLC?

- Simultaneous analysis
- High resolution
- High sensitivity
- Good repeatability
- Moderate analysis condition
- Easy to fractionate and purify
- Not destructive

HPLC

- HPLC- It was originally referred to as High Pressure Liquid Chromatography since high pressure is applied using a pumping system to the column.
- This pressure works by forcing the mobile phase through, at much higher rate increasing the resolution power.
- Due to its high efficiency and performance High Pressure Liquid Chromatography is referred to as High Performance Liquid Chromatography.

TYPES OF LIQUID CHROMATOGRAPHY

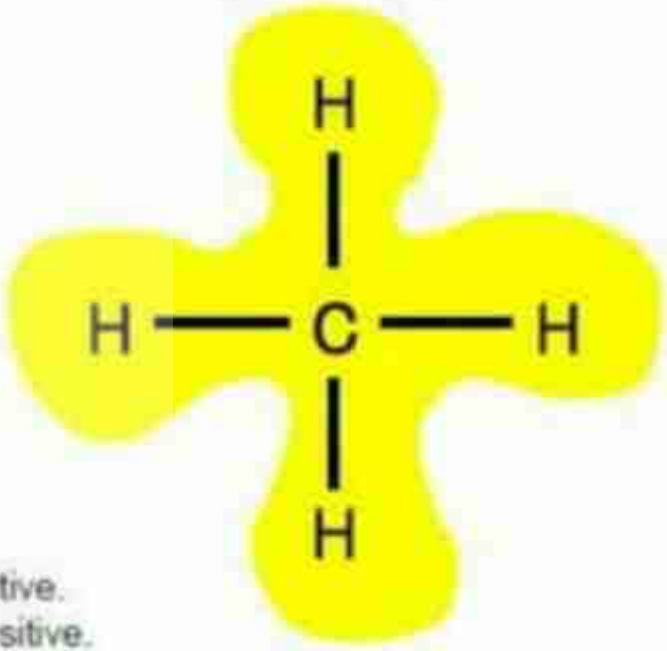
LC mode	Packing materials	Mobile phase	Interaction
Normal phase chromatography	Silica gel	n-Hexane/IPE	Adsorption
Reversed phase chromatography	Silica-C18(ODS)	MeOH/Water	Hydrophobic
Size exclusion chromatography	Porous polymer	THF	Gel permeation
Ion exchange chromatography	Ion exchange gel	Buffer sol.	Ion exchange
Affinity chromatography	Packings with ligand	Buffer sol.	Affinity

3. Separation mode

Polar compounds

Polar compound

Non-polar compound



Bonding electrons are not shared evenly.
The end of the bond with electrons becomes partially negative.
The end of the bondwithout electrons becomes partially positive.

Polar compounds are soluble in polar solvents.
Non-polar compounds are soluble in non-polar solvents.

1.NORMAL PHASE CHROMATOGRAPHY:

- Stationary Phase – Polar nature.

Eg: SiO_2,Al_2O_3

- Mobile Phase – Non-Polar nature.

Eg:heptane,hexane,cyclohexane,$CHCl_3$,CH_3OH

- Mechanism:

- ✓ Polar compounds travels slower & eluted slowly due to higher affinity to st.phase
- ✓ Non-polar compounds travels faster & eluted 1st due to lower affinity to st.phase.
- This technique is not widely used in pharmaceutical separations.

3. Separation mode

Normal Phase Chromatography

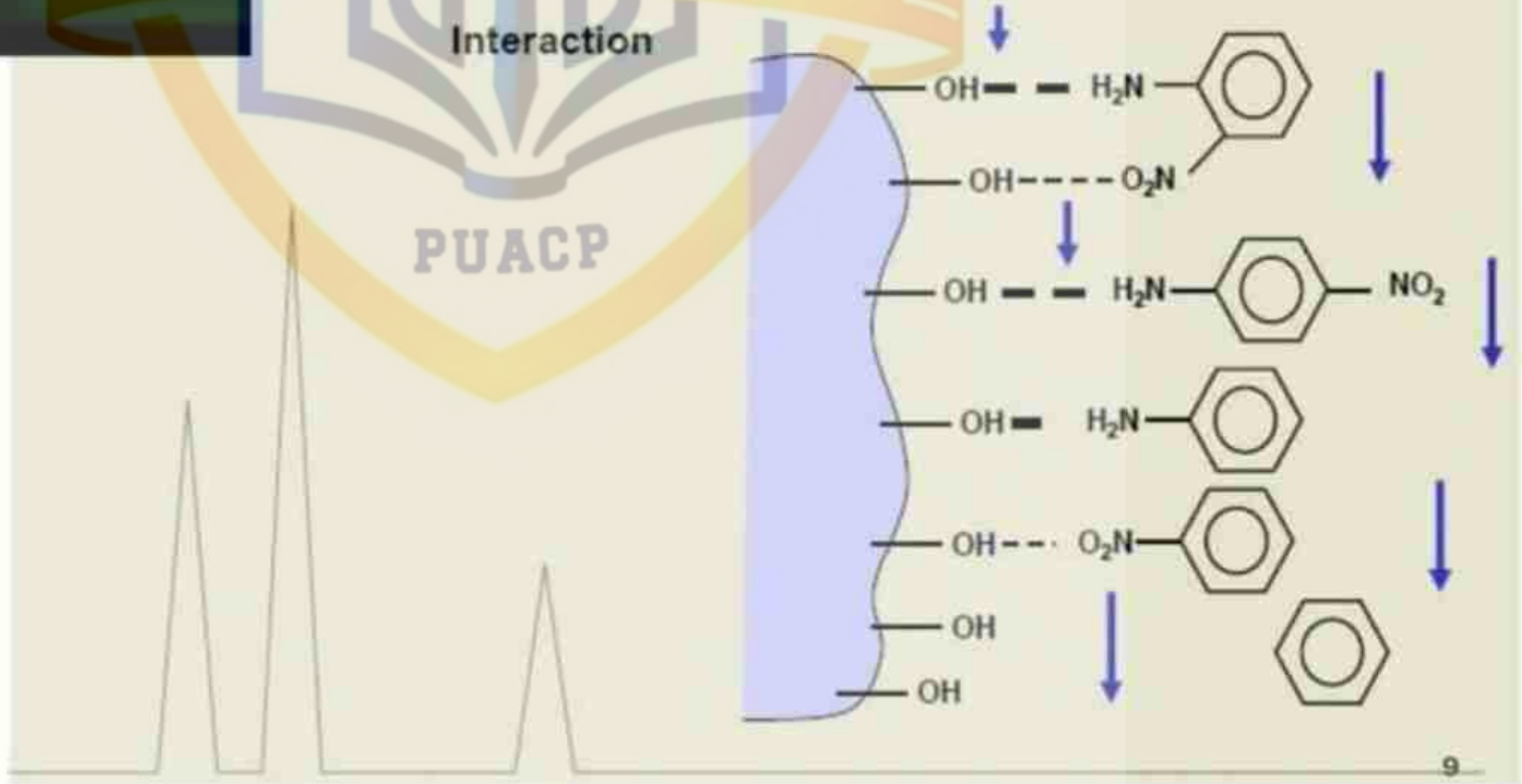
Packing material

The most popular packing material is silica gel.

It is believed that silanol radicals (-Si-OH) on the surface of silica gel act as the active site and the sample is separated.

3. Separation mode

Normal Phase Chromatography



3. Separation mode

Normal Phase Chromatography

Mobile phase solvents

n-Hexane	(n-Hex)
iso-Octane	(iso-Oct)
Chloroform	($CHCl_3$)
Dichloromethane	(CH_2Cl_2)
Ethylacetate	(AcOEt)
Isopropylalchol	(IPA)
Tetrahydrofran	(THF)
Dioxane	
Acetonitrile	(CH_3CN)
Ethanol	(EtOH)
Methanol	(MeOH)
Amines	
Acids	

Low

Polarity

High

2.REVERSE PHASE CHROMATOGRAPHY:

- Stationary Phase – Non-Polar nature.

  Eg: n-octadecyl, n-octyl, ethyl, phenyl diol, hydrophobic polymers.

- Mobile Phase – Polar nature.

  Eg: methanol or acetonitrile/water or buffer sometimes with additives of THF or dioxane.

- Mechanism:

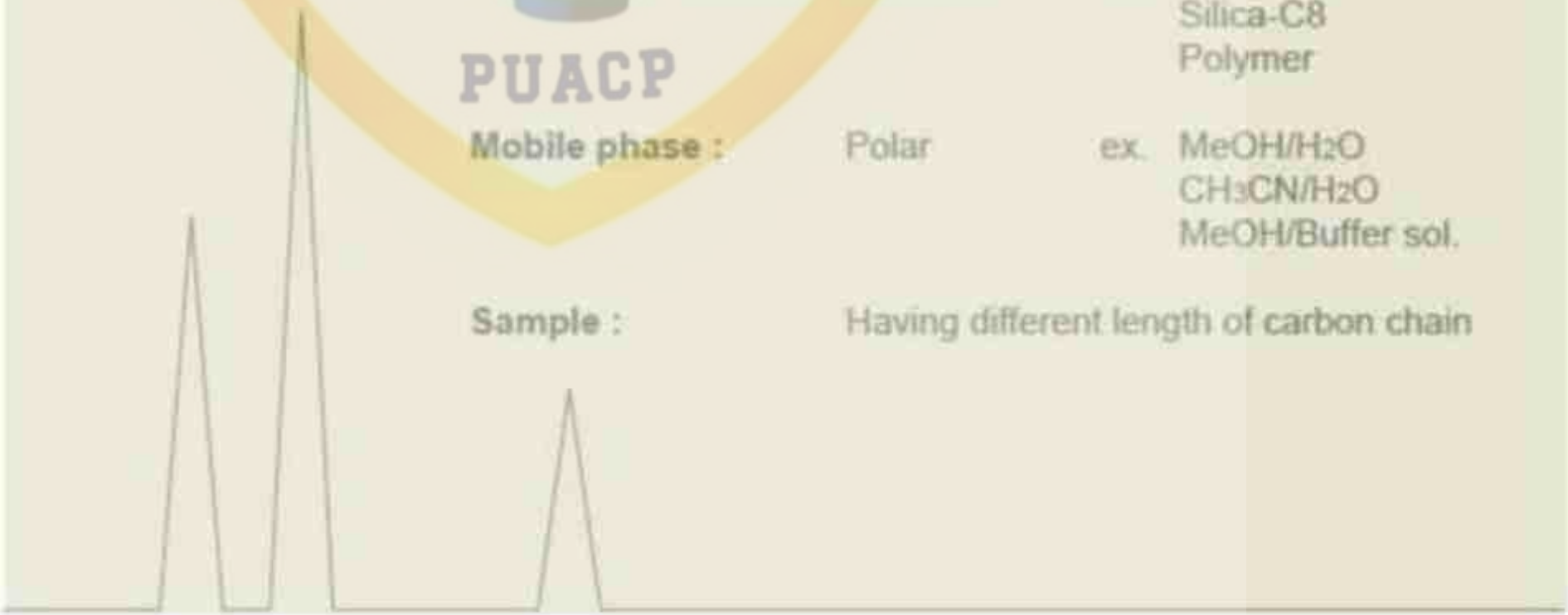
✓ Polar compounds travels faster & eluted 1st due to lesser affinity to st.phase

✓ Non-Polar compounds travels slower & eluted slowly due to higher affinity to st.phase

3. Separation mode

Reversed Phase Chromatography

Interaction :	Hydrophobic		
Packing materials :	Non-polar	ex.	Silica-C18 Silica-C8 Polymer
Mobile phase :	Polar	ex.	MeOH/H_2O CH_3CN/H_2O MeOH/Buffer sol.
Sample :	Having different length of carbon chain		

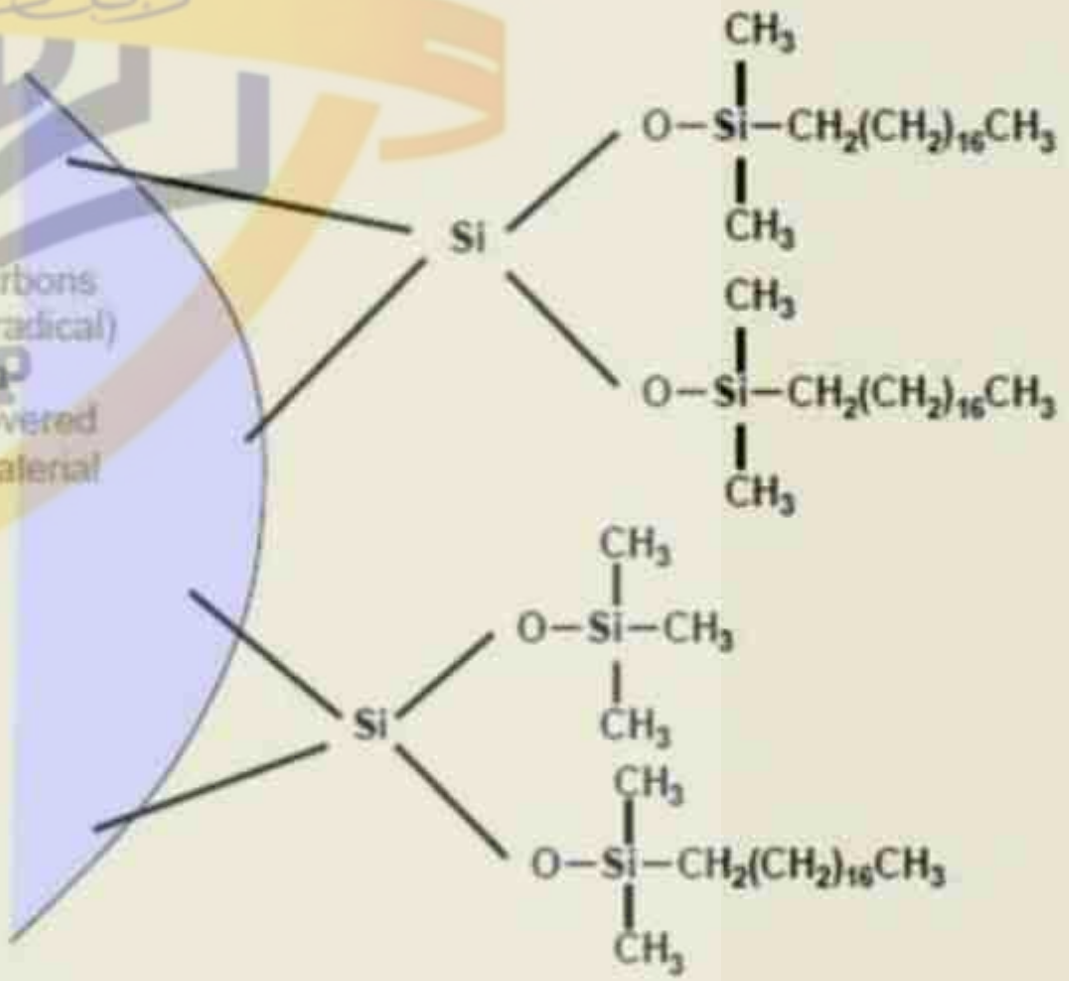


3. Separation mode

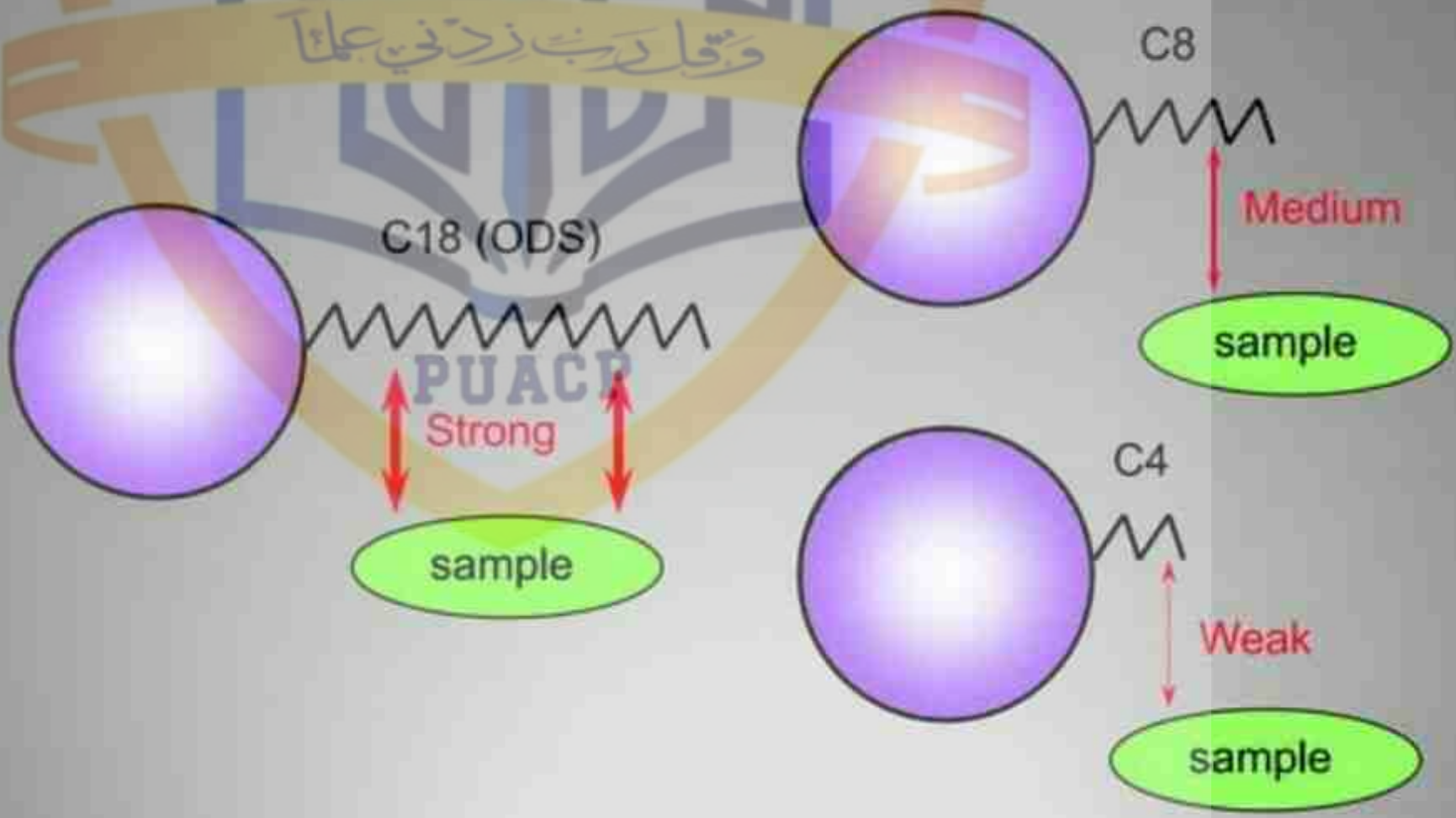
Reversed Phase Chromatography

Silica-C18 Packing materials

Commonly used packing materials are hydrocarbons having 18 carbon atoms (called the Octadecyl radical) which are chemically bonded to silica gel (Silica-ODS).Since the surface of the Silica-ODS is covered with hydrocarbon, the polarity of the packing material itself is very low.



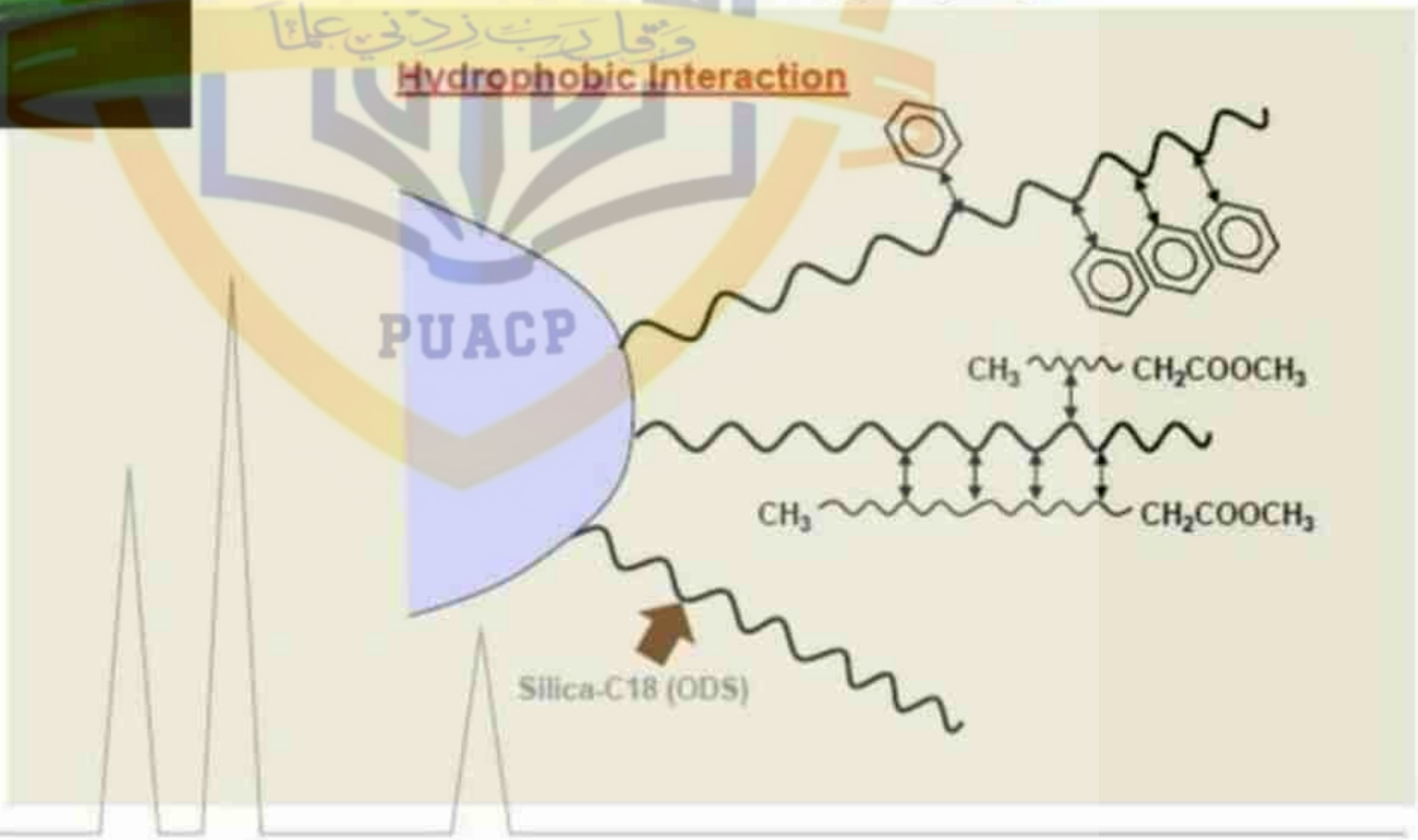
Effect of Stationary Phase



3. Separation mode

Reversed Phase Chromatography

Hydrophobic Interaction



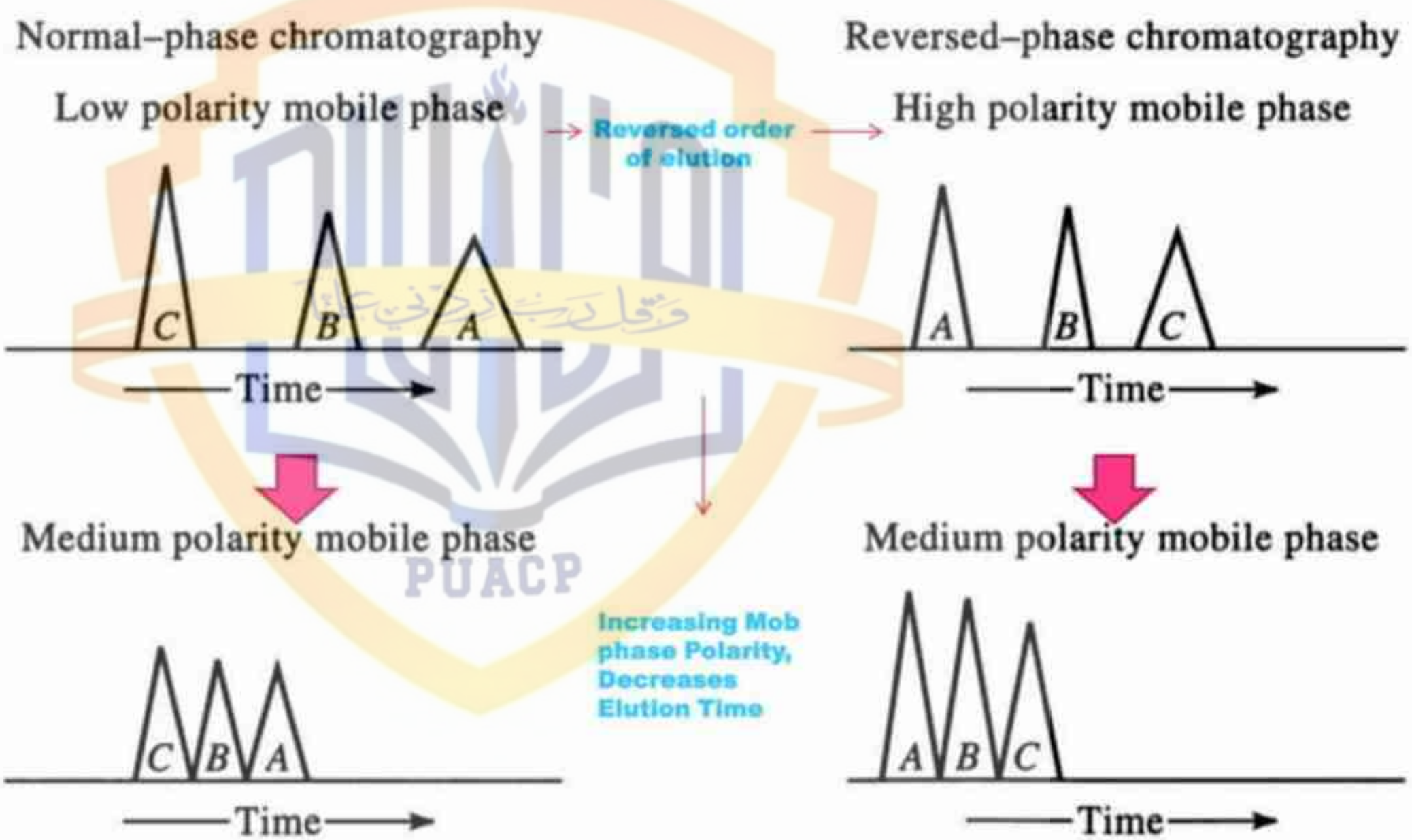
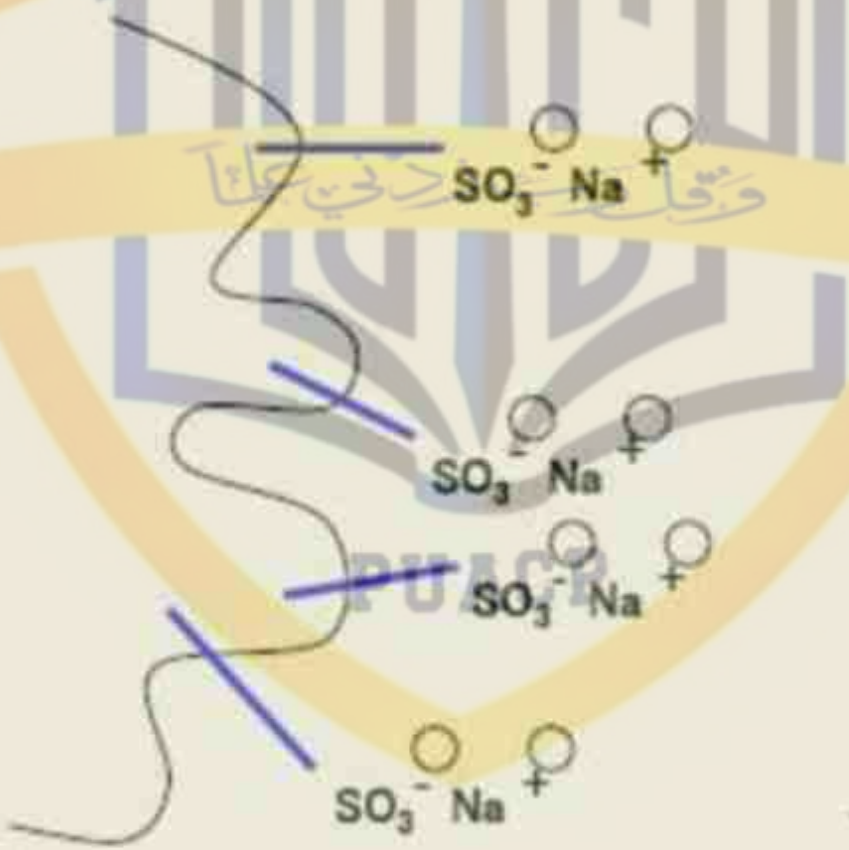


Figure 28-14 The relationship between polarity and elution times for normal-phase and reversed-phase chromatography.

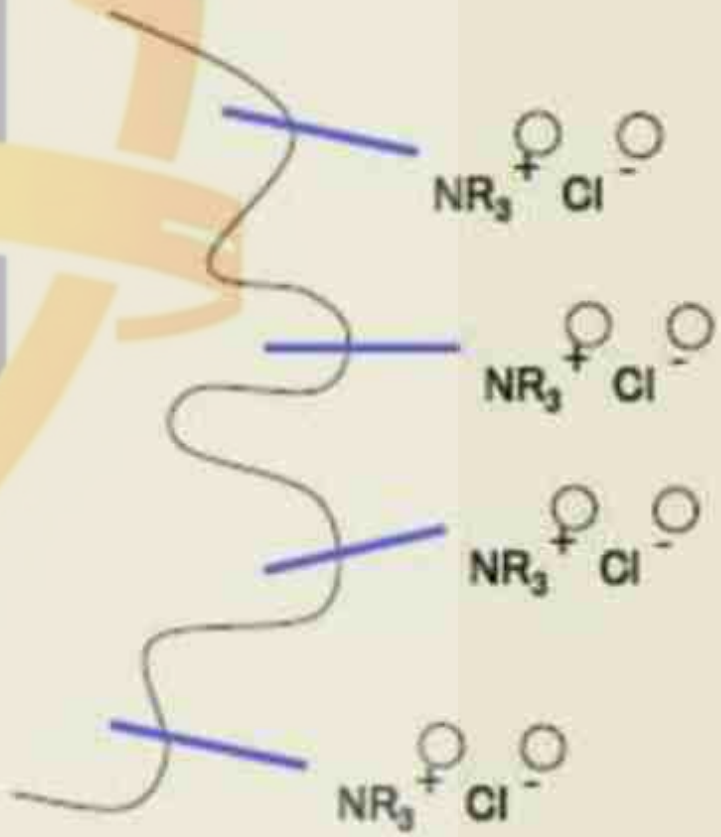
5.ION-EXCHANGE CHROMATOGRAPHY:

- It is the process by which similar charged ions such as cations, anions can be separated.
- By using the suitable ion exchange resin it can be separated.
- It exchanges the ions according to their relative affinities.
- The exchange takes place in a reversible manner between the ions of the solution and the ions present in the ion exchange resin .

Ion-exchange Gel



Cation exchange gel

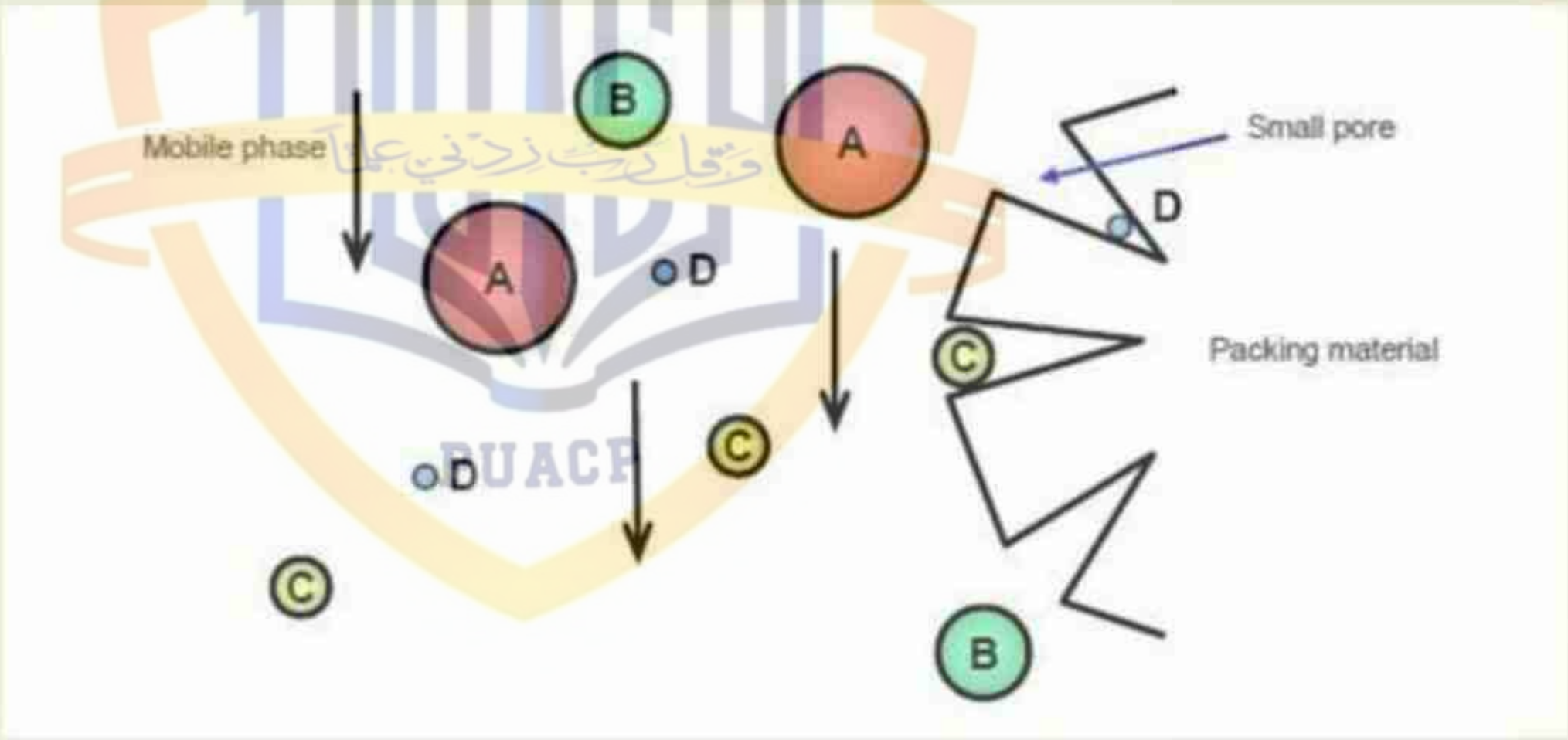


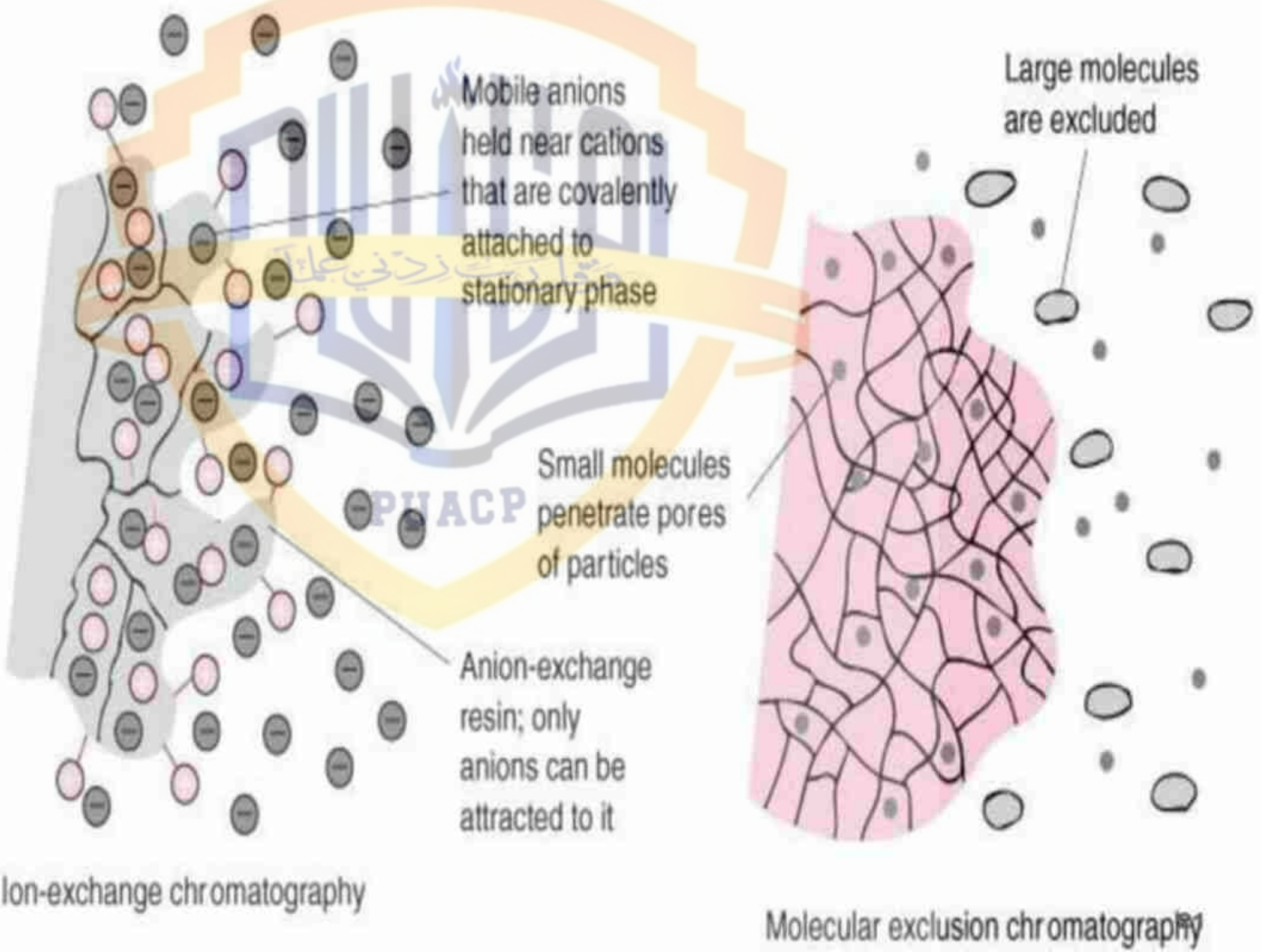
Anion exchange gel

6.MOLECULAR EXCLUSION CHROMATOGRAPHY:

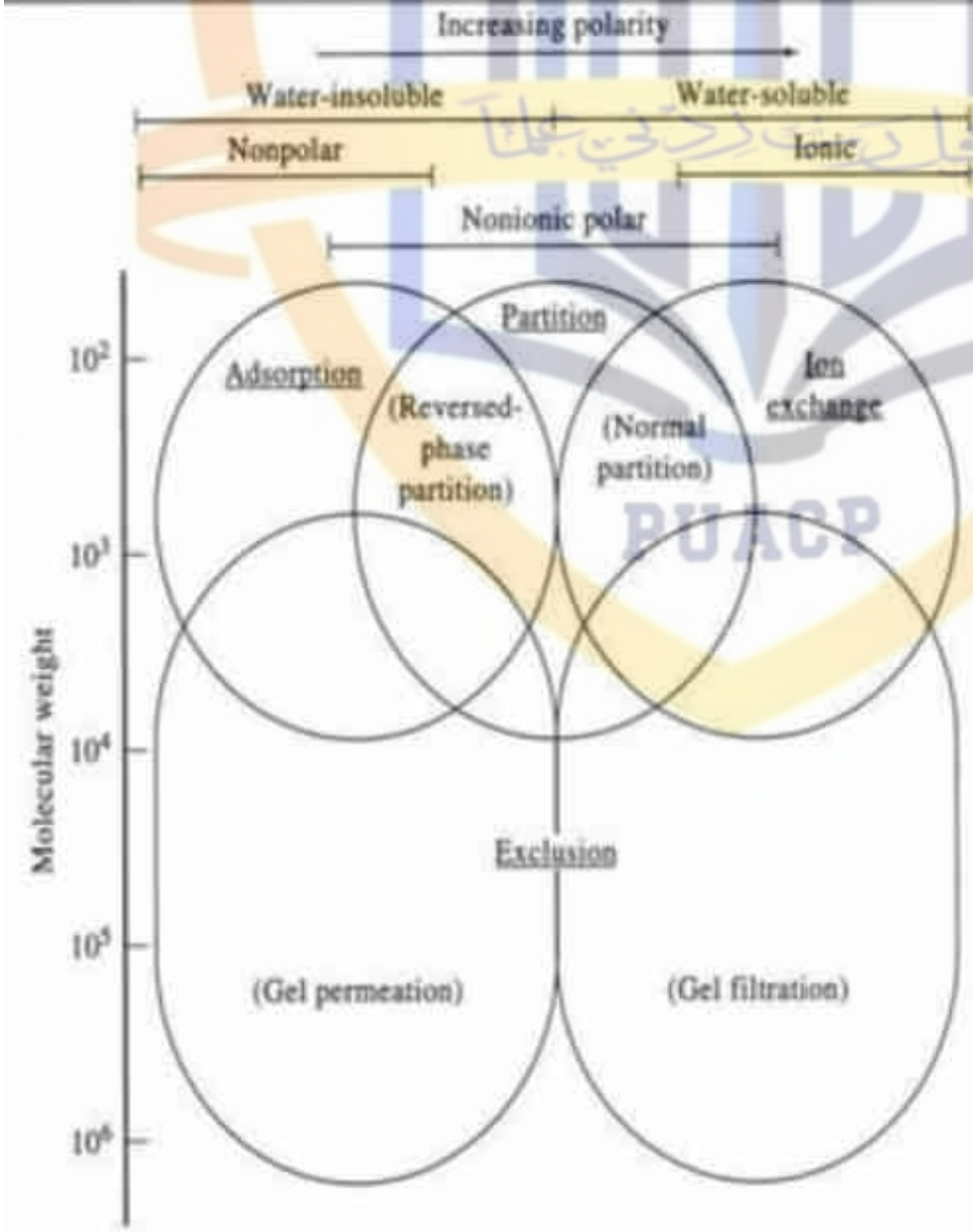
- A mixture of components with different molecular sizes are separated by using gels.
- The gels used acts as molecular sieve & hence a mixture of substances with different molecular sizes are separated.
- Soft gels like dextran, agarose or poly acrylamide are used.
- Semi-rigid gels like polystyrene, alkyl dextran in non aqueous medium are also used.

SEC Separation mechanism




Mobile anions held near cations that are covalently attached to stationary phase
Small molecules penetrate pores of particles
Anion-exchange resin; only anions can be attracted to it
Large molecules are excluded
Ion-exchange chromatography
Molecular exclusion chromatography

TYPES OF HPLC DEPENDS ON:



- Molecular weight of solute
- Water solubility of solute
- Polarity of solute
- Ionic and non-ionic character of solute

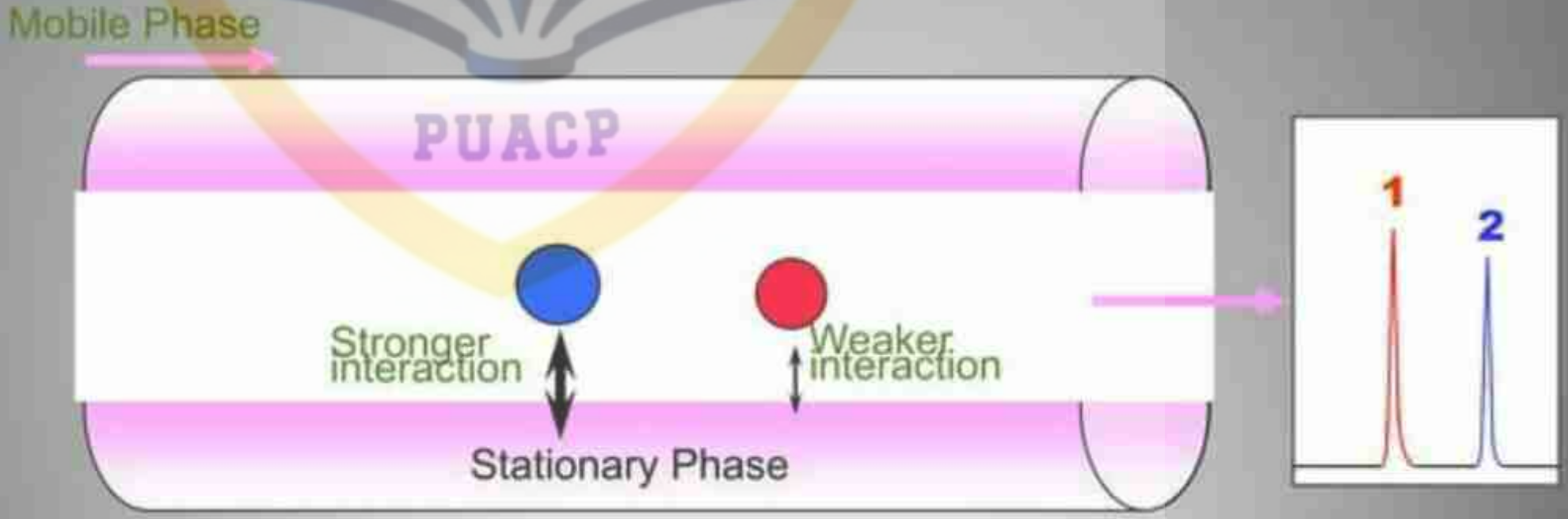

وقل رب زدني علما
PRINCIPLE
PUACP

ADSORPTION CHROMATOGRAPHY:

- The principle of separation is adsorption .
- Separation of compounds takes place based on the difference in the affinity of the compunds towards stationary phase as in the normal and reverse phase.
- The lesser the affinity of the sample particles towards the stationary phase the faster the time of elution of the sample.

Separation Mechanism

Due to different interaction between stationary phase and different sample, the molecules move at different rate, therefore separation can be done.



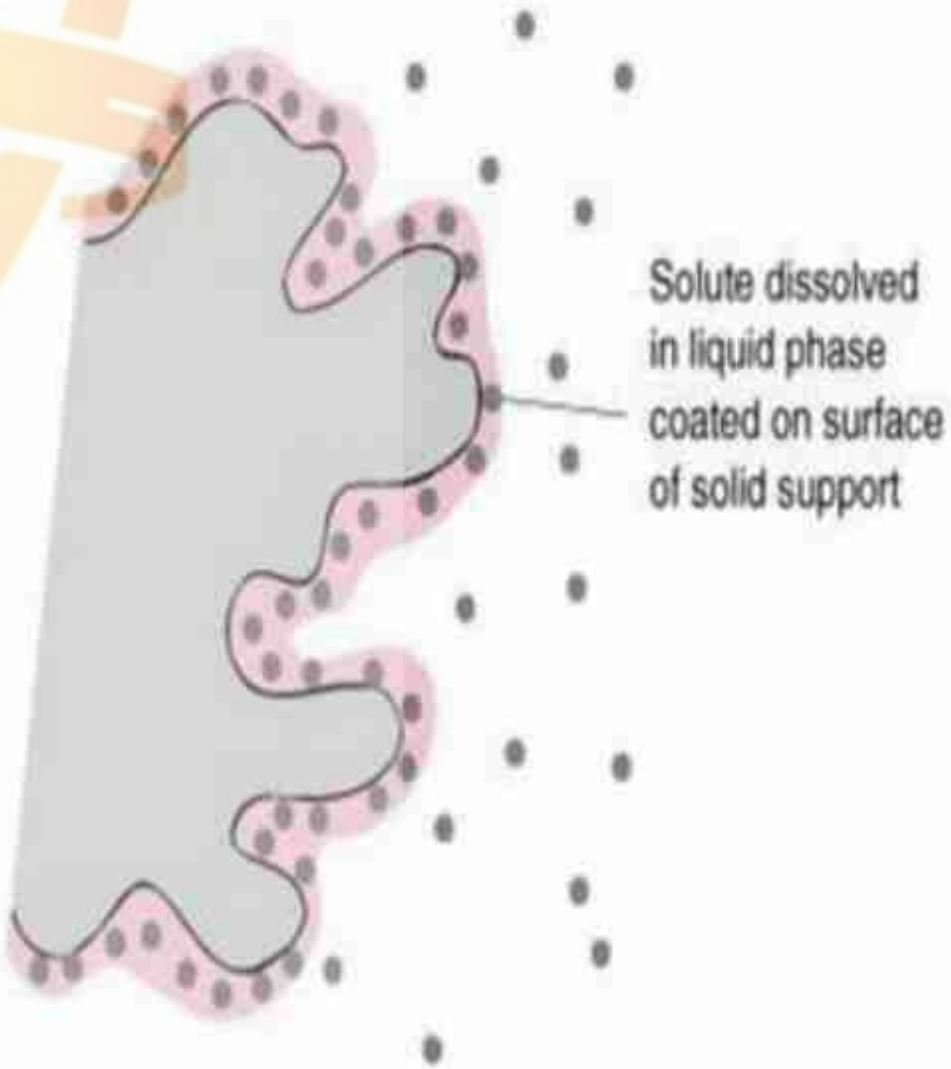
PARTITION CHROMATOGRAPHY:

- In this the stationary phase is a liquid which is coated on the solid support on the column.
- The mobile phase is also a liquid.
- When solute along with the mobile phase is passed over the stationary phase it gets dissolved to the surface of the liquid coated to the solid support.
- The compounds which have more partition co-efficient are eluted slowly when compared to the compounds with low partition co-efficient.

ADSORPTION CHRT:

Adsorption chr omatography

PARTITION CHRT:

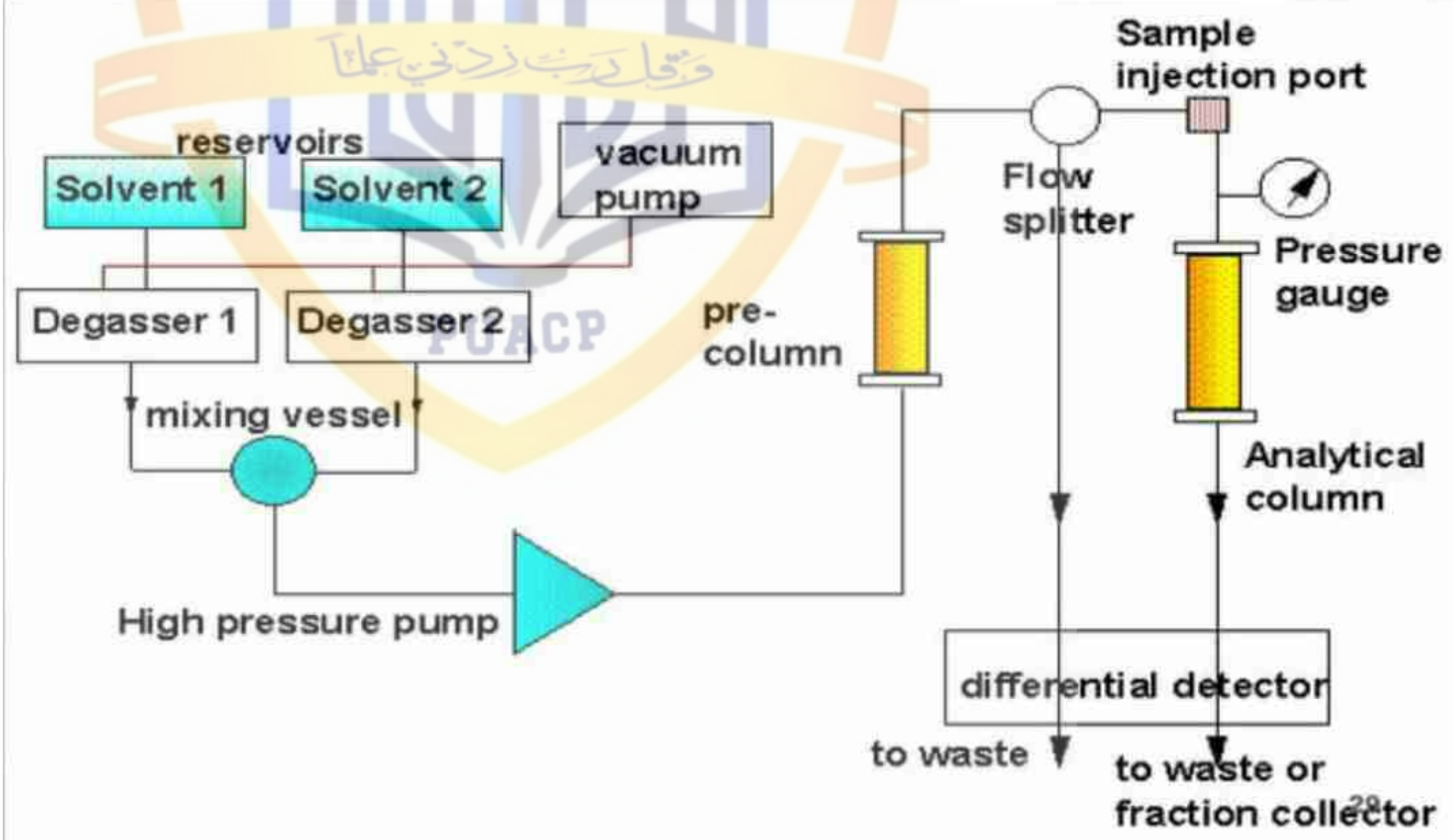


Partition chromatography

INSTRUMENTATION OF HPLC

- Solvent storage bottle
- Gradient controller and mixing unit
- De-gassing of solvents
- Pump
- Pressure gauge
- Pre-column
- Sample introduction system
- Column
- Detector
- Recorder

FLOW DIAGRAM OF HPLC INSTRUMENT

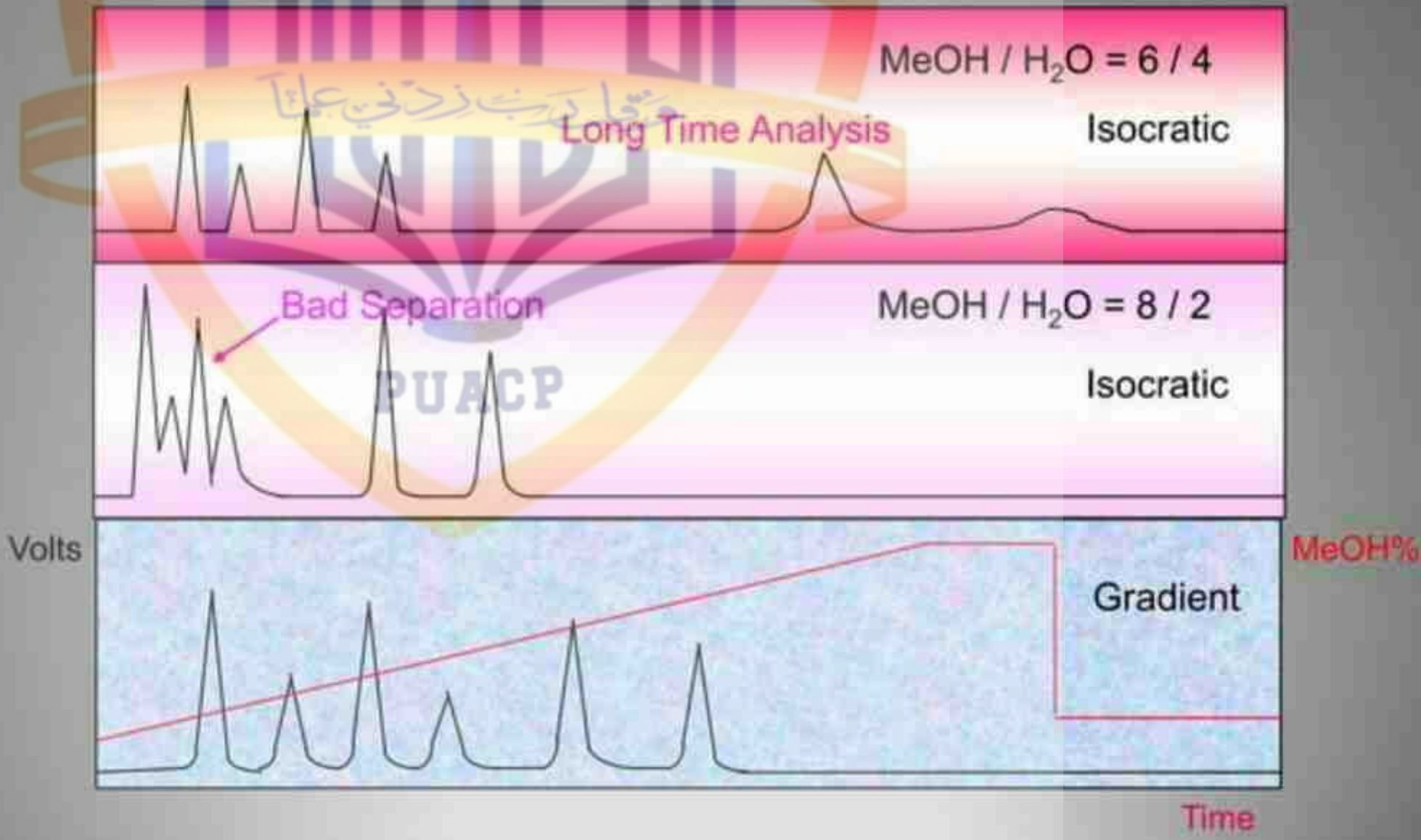


Outline of LC-2010

❖GRADIENT CONTROLLER

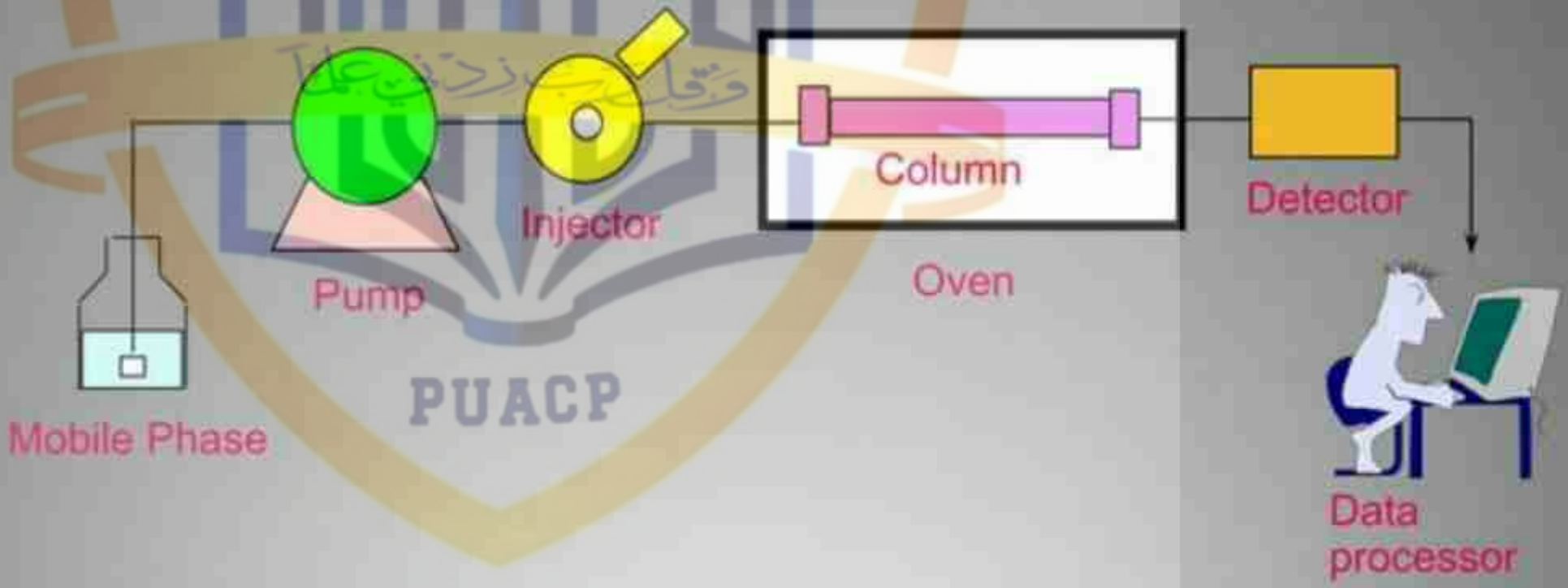
- Isocratic solvents- mobile phase is prepared by using pure solvent or mixture of solvents which has same eluting power or polarity.
- Gradient solvents- in this the polarity of the solvent is gradually increased & hence the solvent composition has to be changed.
- Hence this gradient controller is used when two or more solvent pumps are used for such separations.

Elution Modes



(Column : ODS)

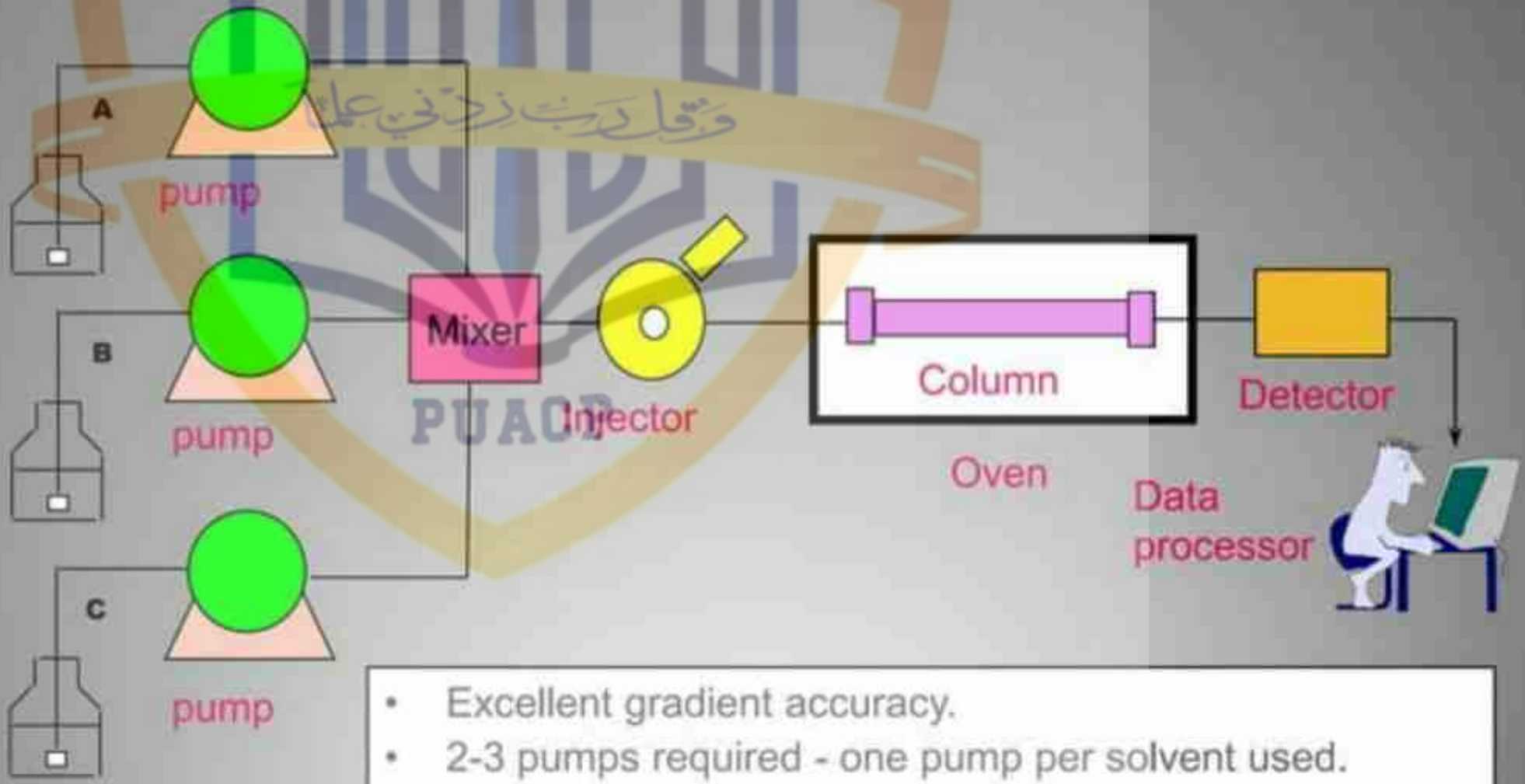
Isocratic System



Simple system with one pump and one solvent reservoir.

If more than one solvent is used, solvents should be premixed.

High-pressure Gradient System



- Excellent gradient accuracy.
- 2-3 pumps required - one pump per solvent used.
- On-line degassing may not be critical.

❖MIXING UNITS:

Mixing units

- Low pressure-mixing chamber which uses Helium for de-gassing
- High pressure-mixing chamber does not require Helium for de-gassing

Mixing of solvents done by

- Static mixer-which is packed with beads
- Dynamic mixer-with magnetic stirrer & operates under high pressure

❖DEGASSING OF SOLVENTS:

- Several gases are soluble in organic solvents, when high pressure is pumped, the formation of gas bubbles increases which interferes with the separation process, steady baseline & shape of the peak.
- Hence de-gassing is very important and it can be done by various ways.

(I) VACUUM FILTRATION:

- ✓ De-gassing is accomplanished by applying a partial vacuum to the solvent container.
- ✓ But it is not always reliable & complete.

(II) HELIUM PURGING:

- ✓ Done by passing Helium through the solvent.
- ✓ This is very effective but Helium is expensive.

(III) ULTRASONICATION:

- ✓ Done by using ultrasonicator which converts ultra high frequency to mechanical vibrations.

❖PUMP:

- The solvents or mobile phase must be passed through a column at high pressures at up to 6000 psi(lb/in^2) or 414 bar.
- As the particle size of stationary phase is smaller (5 to 10μ) the resistance to the flow of solvent will be high.
- That is, smaller the particle size of the stationary phase the greater is the resistance to the flow of solvents.
- Hence high pressure is recommended.

➢REQUIREMENTS FOR PUMPS:

- Generation of pressure of about 5000 psi.
- Pulse free output & all materials in the pump should be chemically resistant to solvents.
- Flow rates ranging from 0.1 to 10 mL/min
- Pumps should be capable of taking the solvent from a single reservoir or more than one reservoir containing different solvents simultaneously.

Types of pumps used in HPLC

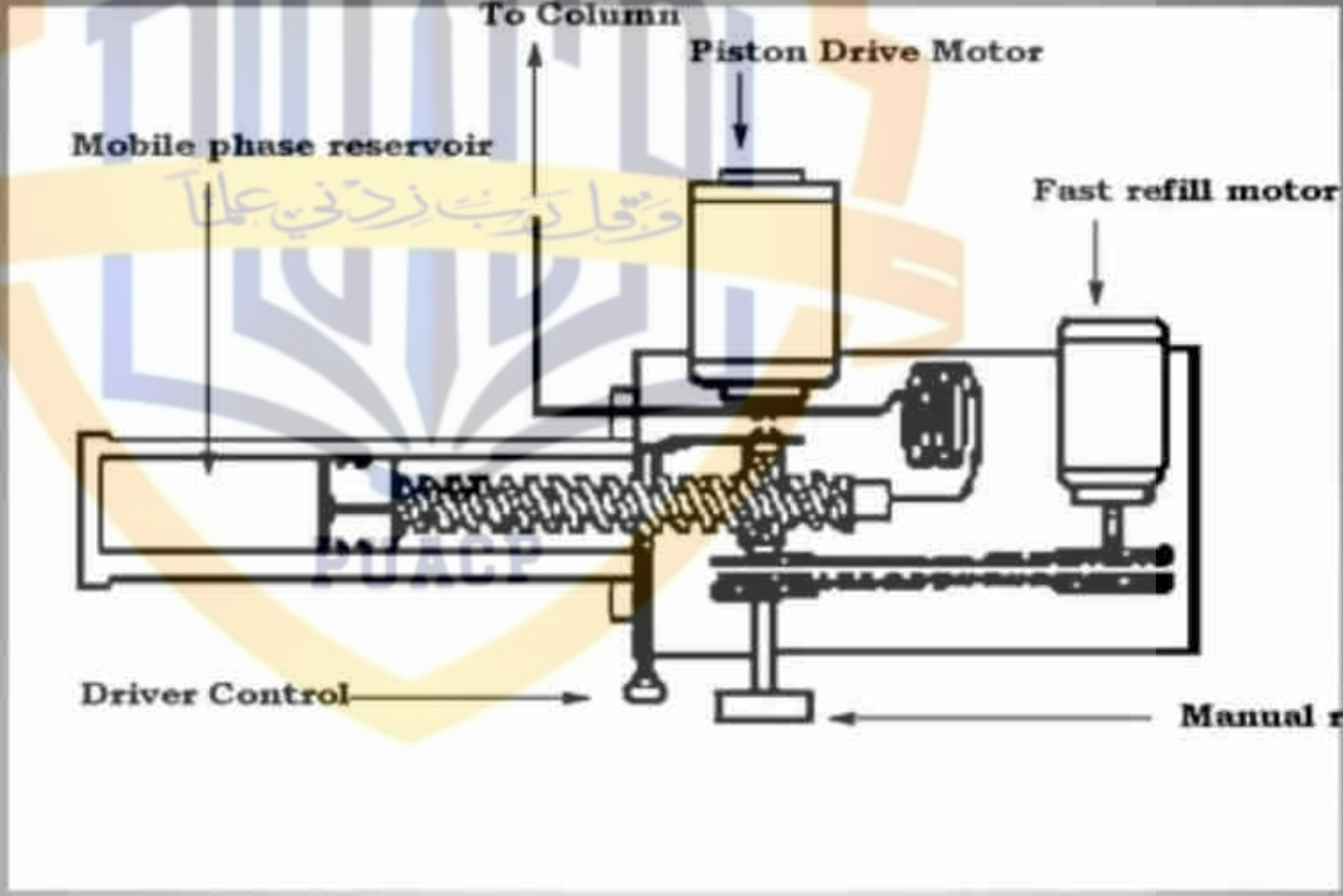
- DISPLACEMENT PUMPS
- RECIPROCATING PUMPS
- PNEUMATIC PUMPS

PUMP A

PUMP B

➢DISPLACEMENT PUMPS

- It consists of large, syringe like chambers equipped with a plunger activated by a screw driven mechanism powered by a stepping motor.
- So it is also called as Screw Driven Syringe Type Pump.
- Advantages:- It produces a flow that tends to be independent of viscosity & back pressure.
- Disadvantages:- It has a limited solvent capacity(~250) & considerably inconvenient when solvents must be changed.



DISPLACEMENT PUMP

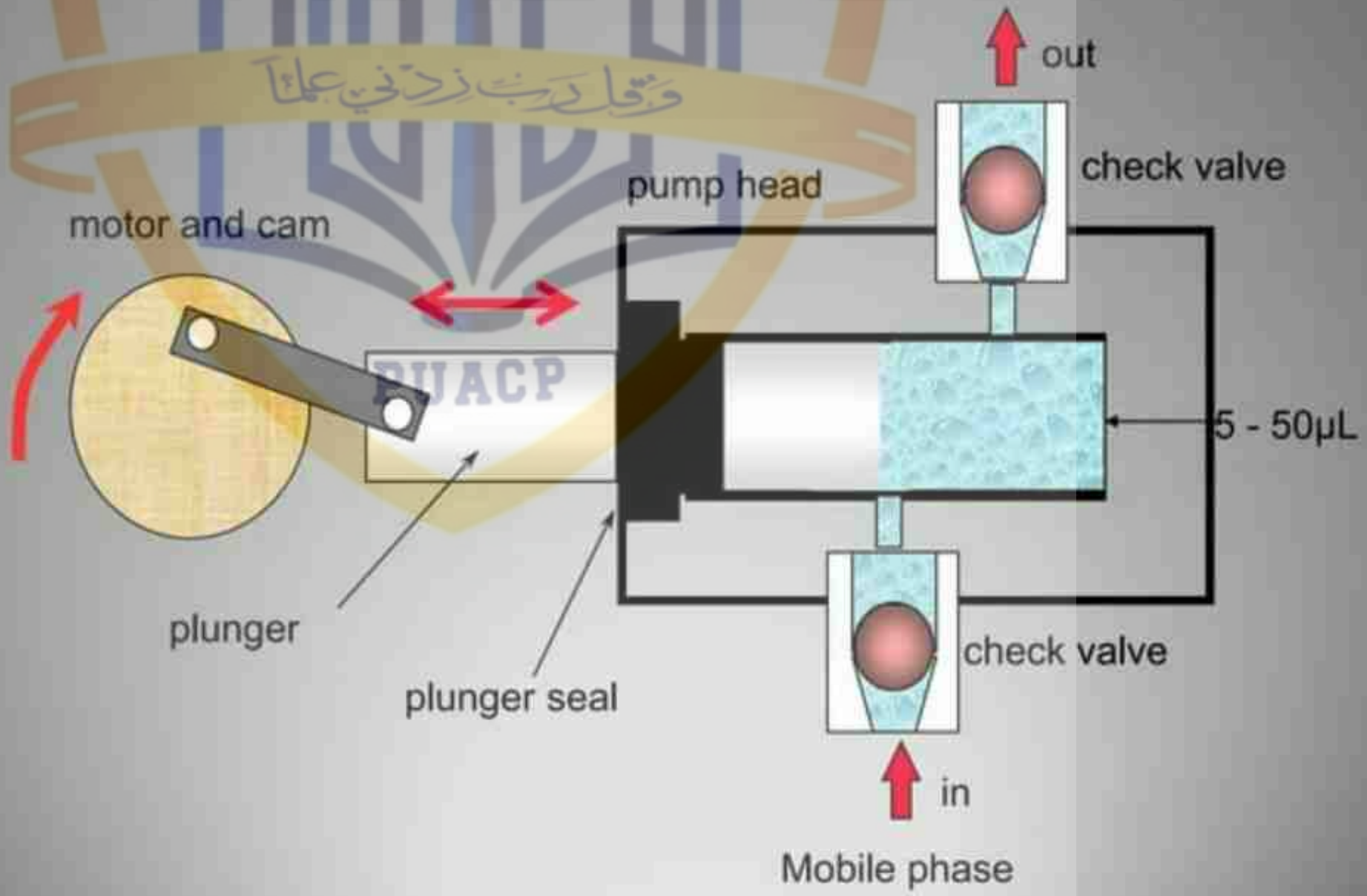
➢RECIPROCATING PUMPS:

- This pump transmits alternative pressure to the solvent via a flexible diaphragm ,which in turn is hydraulically pumped by a reciprocating pump.
- DISADVANTAGES:-

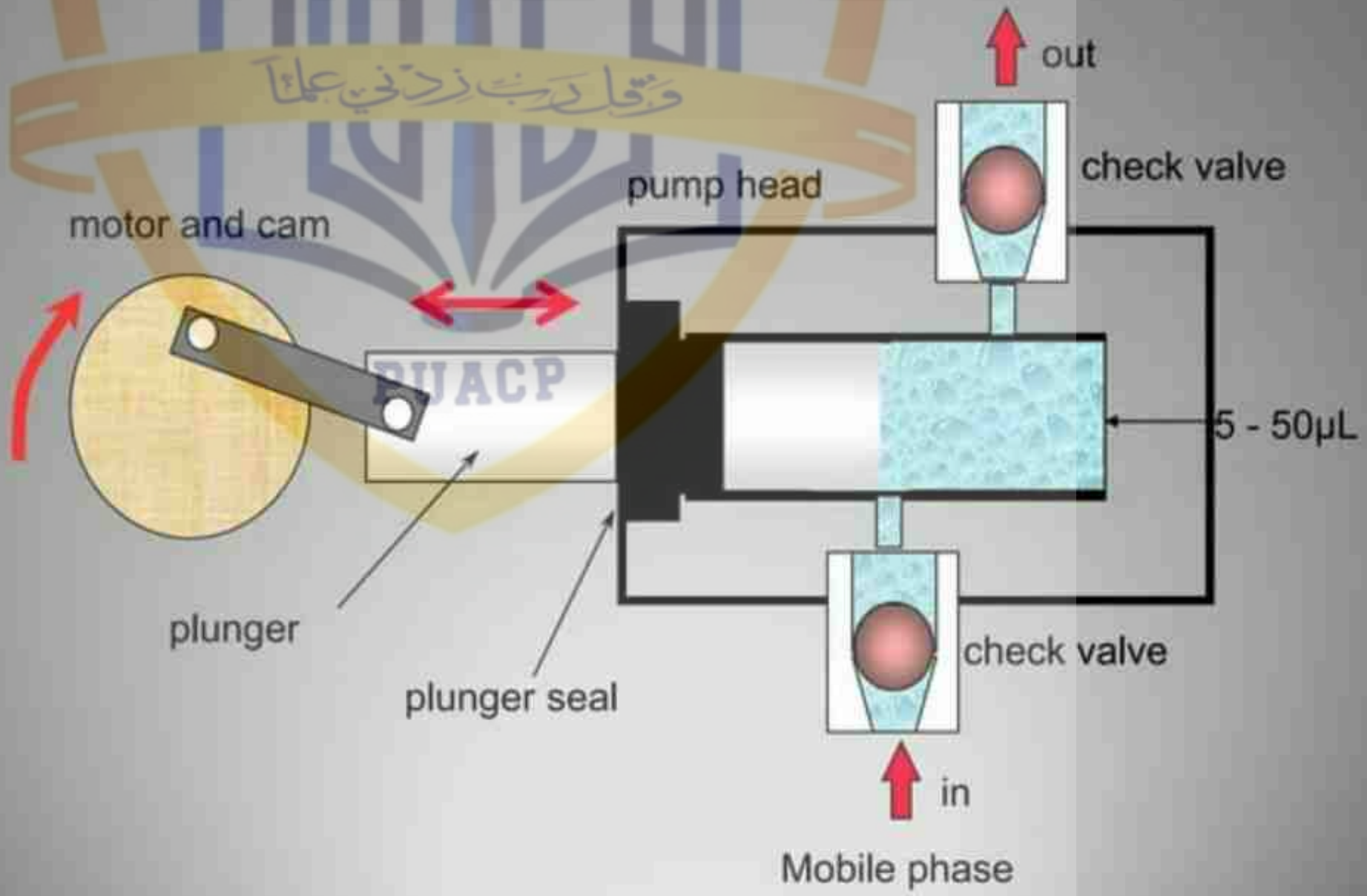
  Produces a pulsed flow which is damped because pulses appear as baseline noise on the chromatograph.

  This can be overcome by use of dual pump heads or elliptical cams to minimize such pulsations.

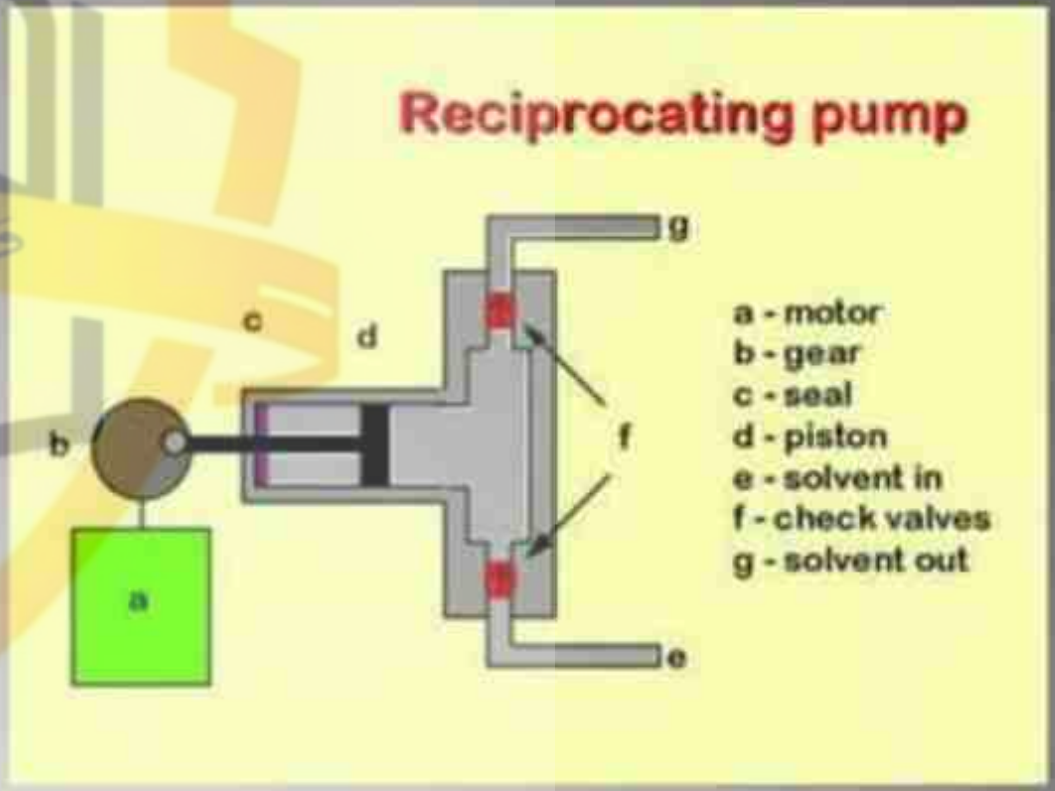
Plunger Reciprocating Pump



Plunger Reciprocating Pump



- Solvent is pumped back and forth by a motor driven piston
- Two ball check valves which open & close which controls the flow
- The piston is in direct contact with the solvent
- Small internal volume 35-400µL
- High output pressure up to 10,000 psi
- Ready adaptability to gradient elution and constant flow rate

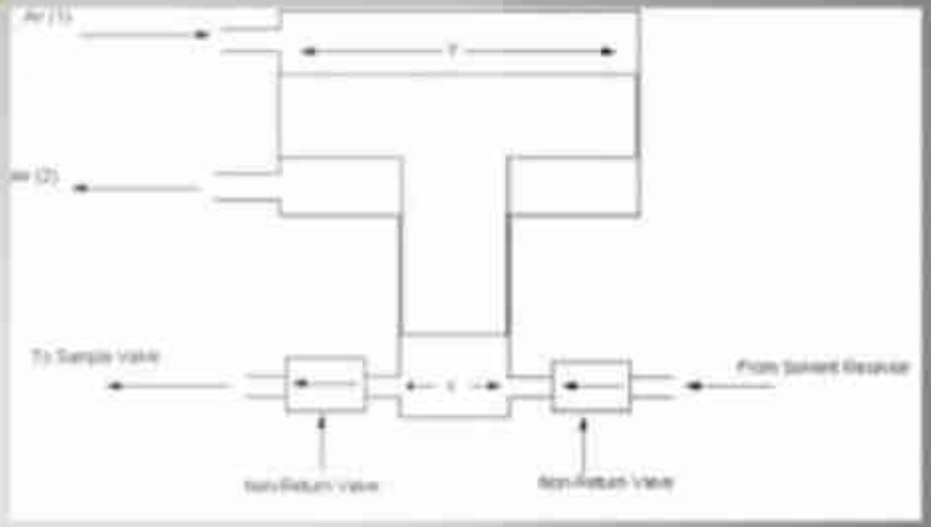


ADVANTAGES:

- Have small internal volume of 35-400µL
- Higher output pressures up to 10,000 psi.
- Adaptability to gradient elution.
- Large solvent capacities & constant flow rates.
- Largely independent of column back pressure & solvent viscosity.

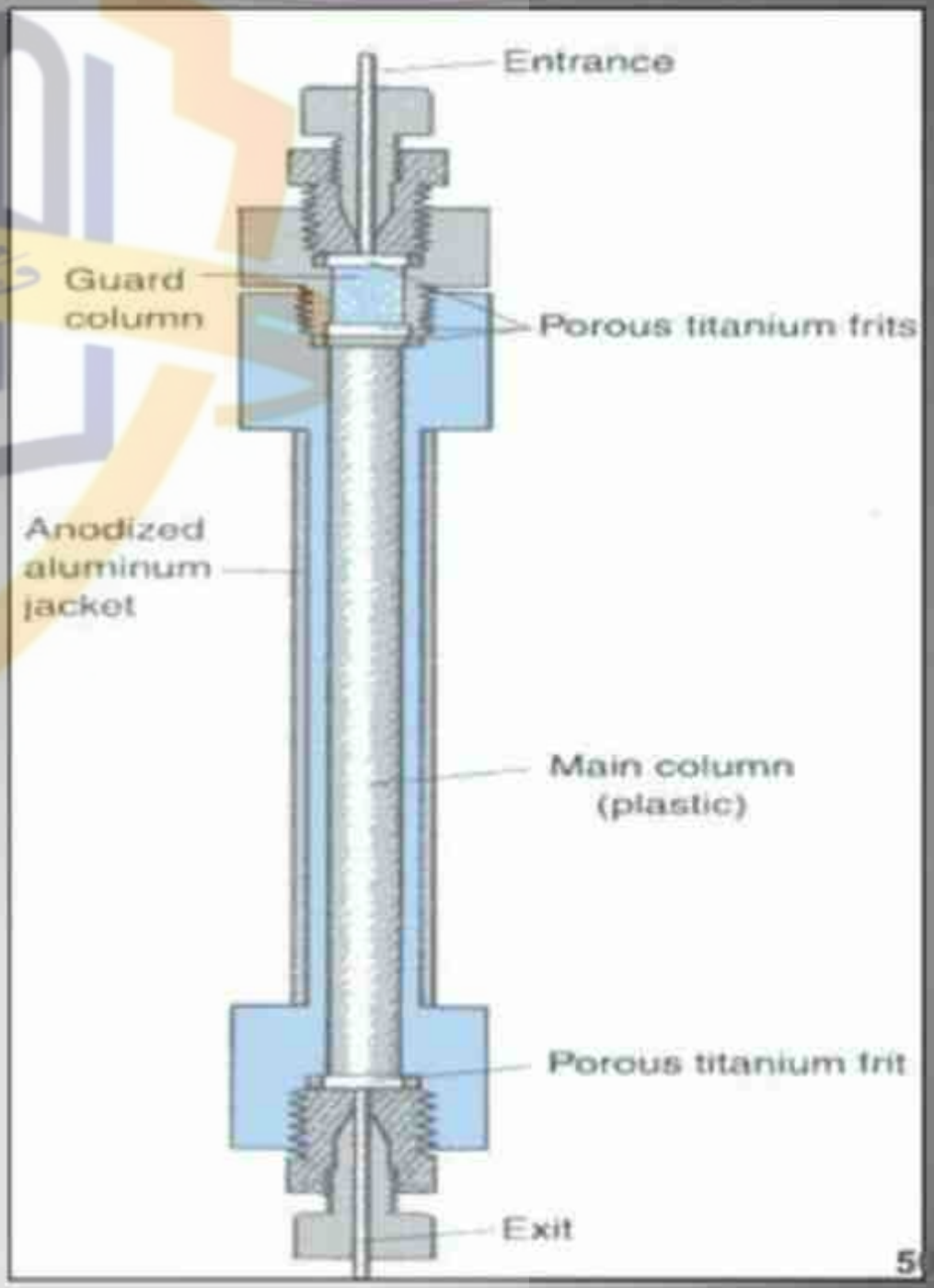
➢ PNEUMATIC PUMPS:

- In this pumps, the mobile phase is driven through the column with the use of pressure produced from a gas cylinder.
- It has limited capacity of solvent



❖ COLUMN:

- There are various columns that can be used in HPLC method.
- They are as follows:
  - Guard Column
  - Derivatizing Column
  - Capillary Column
  - Fast column
  - Analytical Column
  - Preparatory Column



➢GUARD COLUMN:

- Guard columns are placed anterior to the separating column.
- This protects and prolongs the life & usefulness of the separating column.
- They are dependable columns designed to filter or remove:-

✓ particles that clog the separating column,

✓ compounds and ions that could ultimately cause 'baseline drift', decreased resolution, decreased sensitivity and create false peaks.

- Compounds that may cause precipitation upon contact with the stationary or mobile phase.
- Compounds that may co-elute and cause extraneous peaks & interfere with the detection and quantification.
- These columns must be changed on a regular basis in order to optimize their protectiveness.

➢DERIVATIZING COLUMN

- Derivatization involves a chemical reaction between an analyte and a reagent to change the chemical and physical properties of an analyte.
- The four main uses of derivatization in HPLC are:
  - ✓ Improve detectability,
  - ✓ Change the molecular structure or polarity of analyte for better chromatography,
  - ✓ Change the matrix for better separation,
  - ✓ Stabilize a sensitive analyte.

- Pre or post primary column derivatization can be done.
- Derivatization techniques includes –acetylation, silylation, acid hydrolysis.
- DISADVANTAGES: It becomes a complex procedure and so it acts as a source of error to analysis and increases the total analysis time.
- ADVANTAGES: Although derivatization has drawbacks, it may still be required to solve a specific separation or detection problem.

➢CAPILLARY COLUMNS:

- HPLC led to smaller analytical columns called as micro-columns, capillary columns which have diameter less than a millimeter.
- Sample used – is in nanolitre volumes, decreased flow rate, decreased solvent volume usage which leads to cost effectiveness.
- Disadvantage:- since it is miniatured flow rate is difficult to produce & gradient elution is not efficient.

- MICROBORE and SMALLBORE columns are also used for analytical and small volumes assay.
- Diameter of small-bore columns is 1-2mm.
- The instrument must also be modified to accommodate these smaller capacity columns.

- FAST COLUMNS:
- This column also have the same internal diameter but much shorter length than most other columns & packed with particles of 3µm in diameter.
- Increased sensitivity, decreased analysis time, decreased mobile phase usage & increased reproducibility.

ANALYTICAL COLUMN:

- This is the most important part of HPLC which decides the efficiency of separation
- Length- 5 to 25 cm ,Internal Diameter 3 to 5mm.
- Particle size of packing material is 3 to 5µm.
- LC columns achieve separation by different intermolecular forces b/w the solute & the stationary phase and those b/w the solute & mobile phase.

➢PREPARATORY COLUMN:

- Length – 10 to 15 cm, Int. diameter – 4.6mm
- Packed with particles having 5µm as diameter.
- Columns of this time generate 10,000 plates per column.
- It consists of back pressure regulator and fraction collector.
- This back pressure regulator is placed posterior to the HPLC detector.

❖SAMPLE INJECTOR SYSTEM:

- Several injector devices are available either for manual or auto injection of the sample.

(i) Septum Injector

(ii)Stop Flow Injector

(iii)Rheodyne Injector

Rheodyne Manual injector

(I)SEPTUM INJECTOR:

- These are used for injecting the sample through a rubber septum.
- This kind of injectors cannot be commonly used , since the septum has to withstand high pressures.

(II)STOP FLOW(ON LINE):

- In this type the flow of mobile phase is stopped for a while & the sample is injected through a valve.

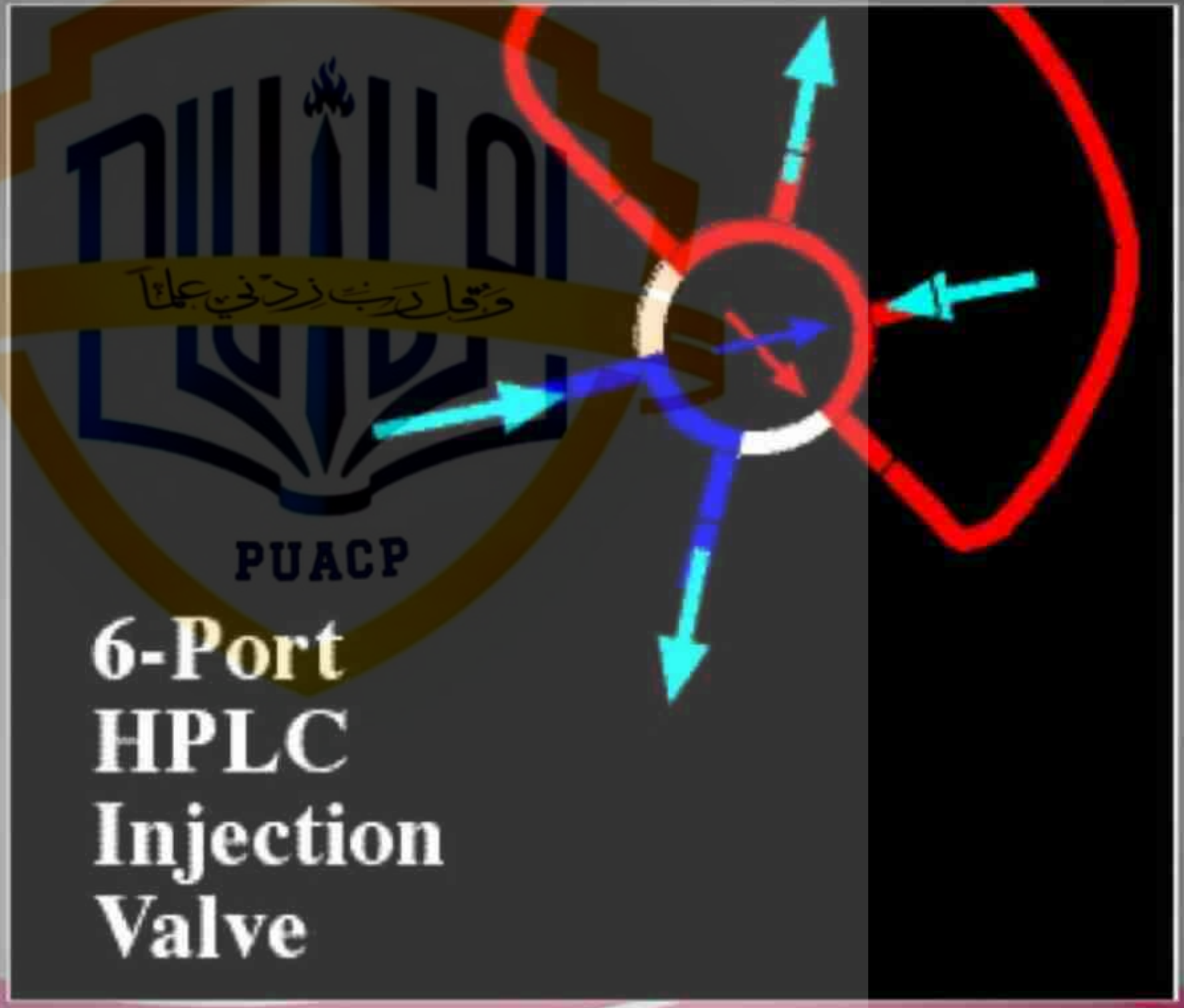
(III)RHEODYNE INJECTOR:

- It is the most popular injector and is widely used.
- This has a fixed volume of loop, for holding sample until its injected into the column, like 20µL, 50µL or more.
- Through an injector the sample is introduced into the column.
- The injector is positioned just before the inlet of the column.

SELECTION VALVE:
By using the selection valve we can select whether the purpose is for analytical purpose or preparative purpose.

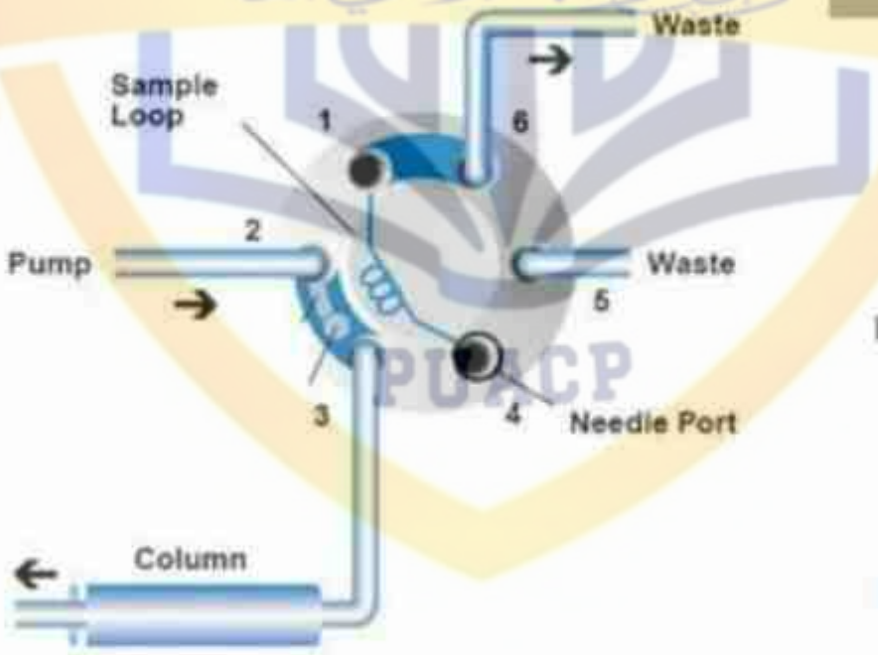
LOAD POSITION:
In this position the sample is loaded into the sample loop .

INJECT POSITION: In this position the loaded sample is injected into the column by the forcful flow of the solvent into the sample loop by which the sample is introduced into the column.

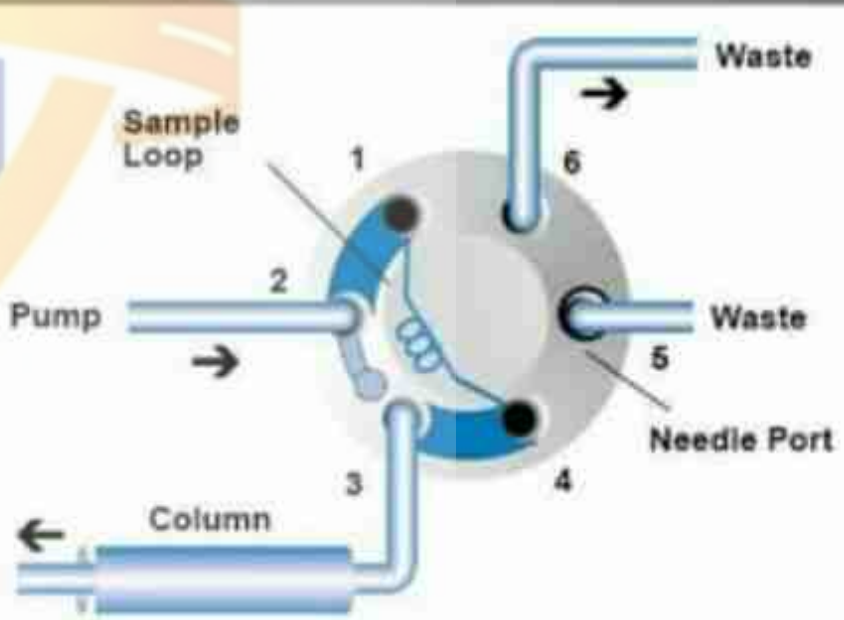

وقل رب زدني علما
PUACP
6-Port
HPLC
Injection
Valve


وقل رب زدني علما
PUACP

6 Port Valve System



Position 1 (LOAD)



Position 2 (INJECT)

Typical sample loop volume is 5-200 µl.

HPLC Auto Injectors

Inside of SIL-20AC

➢COLUMN TEMPERATURE CONTROLLER:

- For obtaining better and reproducible chromatograms constant column temperature should be maintained.
- Some are equipped with heaters that control temperatures to a few tenths of a degree from near ambient.
- Columns may also be fitted with water jackets fed from a constant temperature bath to give precise temperature control.
- For some applications, close control of column temperature is not necessary & columns are operated at R.T

TYPES OF DETECTORS

General – respond to mob phase bulk properties which vary in the presence of solutes. (e.g. refractive index)

Specific – respond to some specific property of the solute (not possessed by the mob phase (e.g. UV absorption)

"Hyphenated" detector – LC-MS

DETECTORS:

❖ DETECTORS:

- Absorbance (UV/Vis or PDA)
- Refractive index (detects the change in turbidity)
- Fluorescence (if the analyte is fluorescent)
- Electrochemical (measures current flowing through a pair of electrodes, on which a potential difference is imposed, due to oxidation or reduction of solute)
- Conductivity (for ions)
- Light scattering
- Mass spectrometry (HPLC-MS)

Selection of Detectors

Detectors	Type of compounds can be detected
UV-Vis & PDA	Compounds with chromophores, such as aromatic rings or multiple alternating double bonds.
RF	Fluorescent compounds, usually with fused rings or highly conjugated planar system.
CDD	Charged compounds, such as inorganic ions and organic acid.
ECD	For easily oxidized compounds like quinones or amines.
RID & ELSD	For compounds that do not show characteristics usable by the other detectors, eg. polymers, sccharides.

ABSORBANCE DETECTORS:

- The UV/Vis source usually comes from a monochromator so the wavelength can be selected, or scanned.
- If wavelength scanning is desired, the flow is stopped long enough for the scan to take place.
- Fixed wavelength-measures at single wavelength usually 254nm.
- Variable wavelength-measures at single wavelength at a time but can detect over a wide range of wavelengths simultaneously.

Refractive Index Detector

Refraction

Photodiode

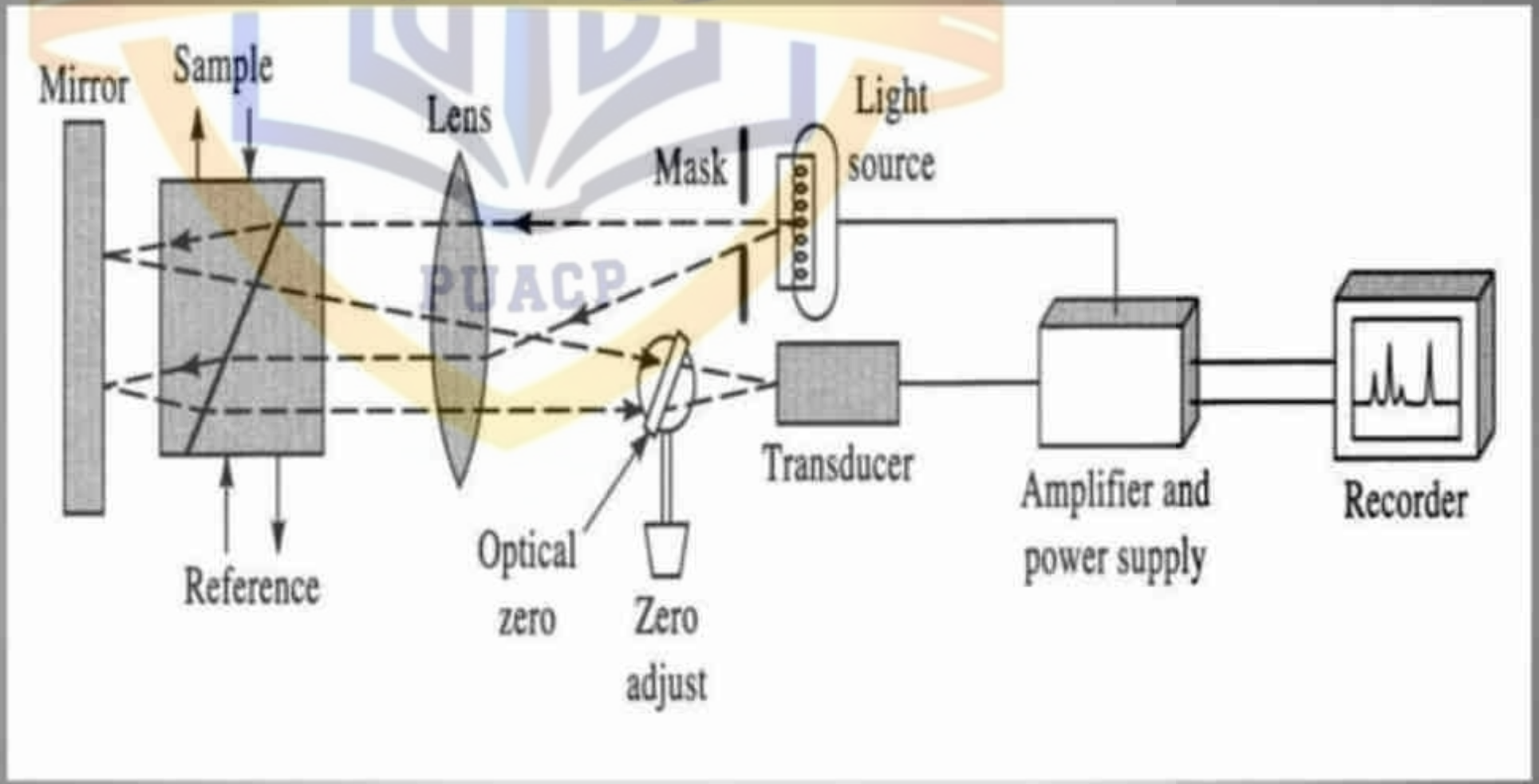
Reference

Sample

W Lamp

REFRACTIVE INDEX (RI) DETECTOR:

- Nearly universal but poor detection limit.



- Detection occurs when the light is bent due to samples eluting from the columns, and this is read as a disparity b/w the two channels.
- It is not much used for analytical applications because of low sensitivity & specificity.
- When a solute is in the sample compartment, refractive index changes will shift the light beam from the detector.

➢FLUORIMETRIC DETECTORS:

- It is based on the fluorescent radiation emitted by some compounds.
- The excitation source passes through the flow cell to a photodetector while a monochromator measures the emission wavelengths.
- More sensitive and specific.
- The disadvantge is that most compounds are not fluorescent in nature.

Fluorescence of Compounds

Fluorescence is a type of luminescence in which the light energy is released in the form of a photon in nanoseconds to microseconds

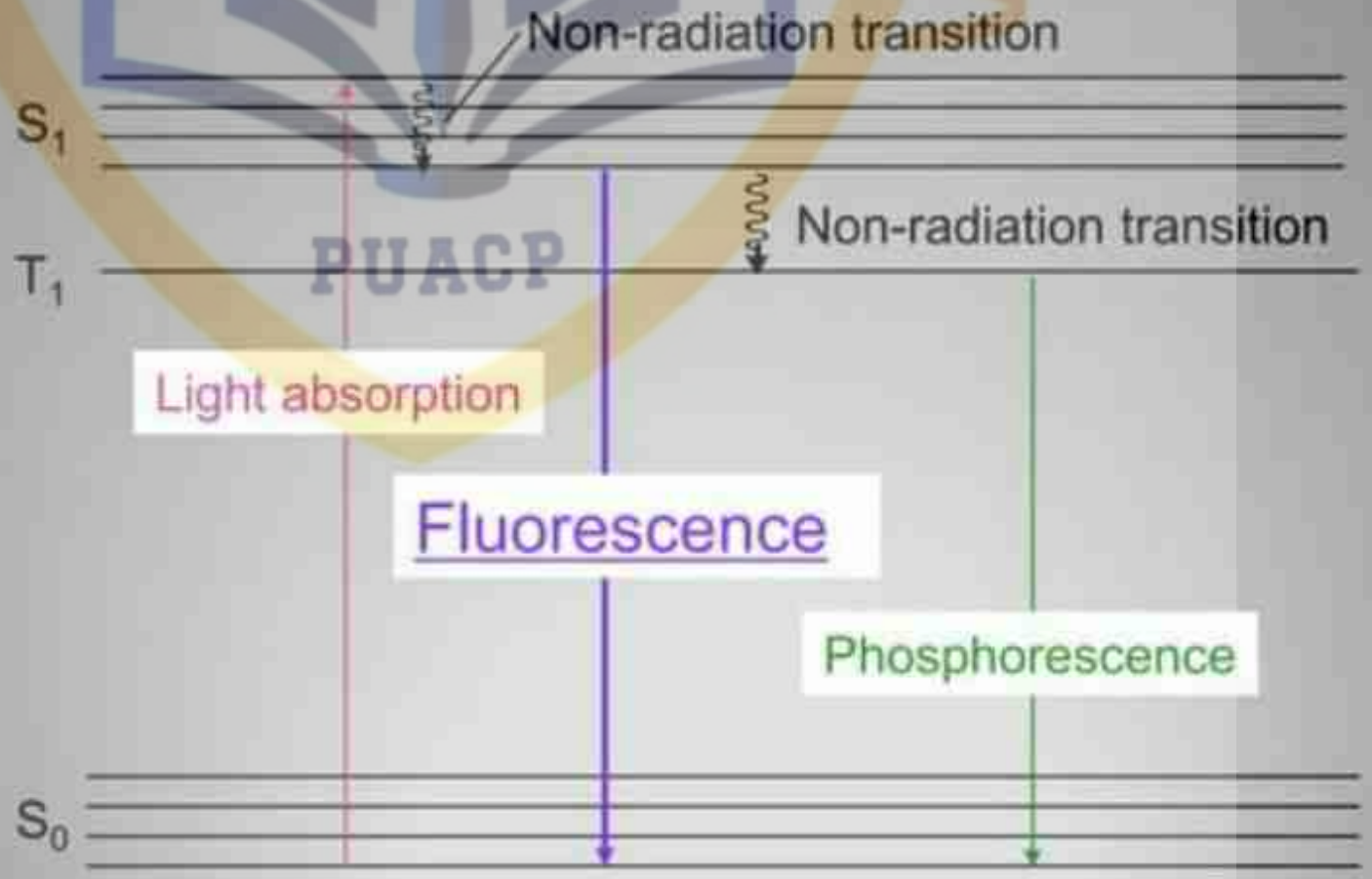
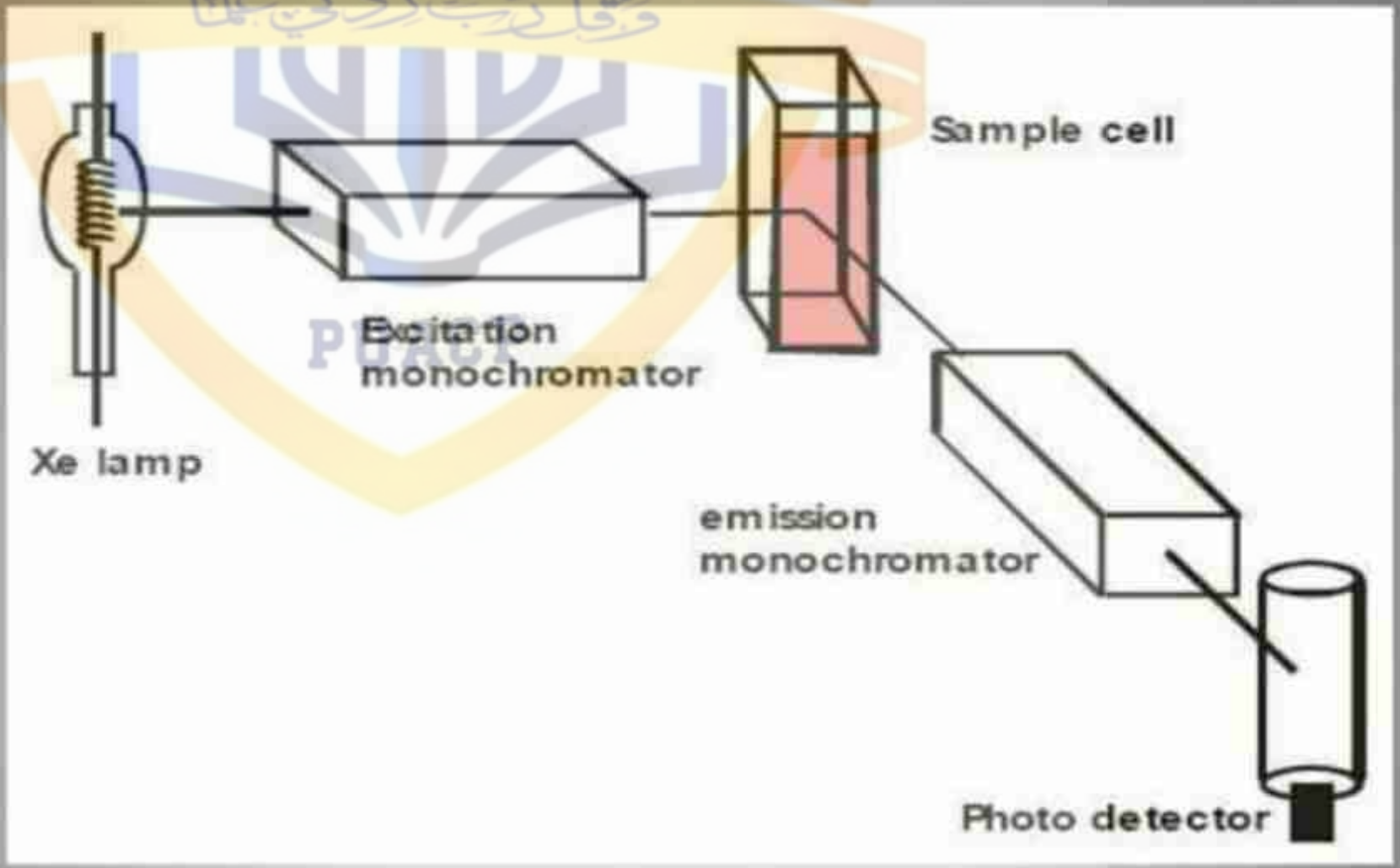


DIAGRAM OF FLUORESCENCE DETECTORS



Fluorescence Detector

Advantage

- Sensitivity is higher than UV-Vis detector
- Selectivity is high because relatively few compounds fluorescence
- Compatible with gradient elution

Disadvantage

- Difficult to predict fluorescence
- Greatly affected by environment
  - Solvent
  - pH
  - Temperature
  - Viscosity
  - Ionic strength
  - Dissolved gas

➢ AMPEROMETRIC DETECTOR:

- Amperometric detectors works based on the reducing and oxidizing property of the sample when a potential is applied.
- The diffusion current recorded is directly proportional to the concentration of the compound recorded.
- DISADVANTAGE: This detector is applicable only when the functional groups present in the sample can be either oxidized or reduced.
- ADVANTAGE: Highly sensitive detector.

AMPEROMETRIC DETECTOR:

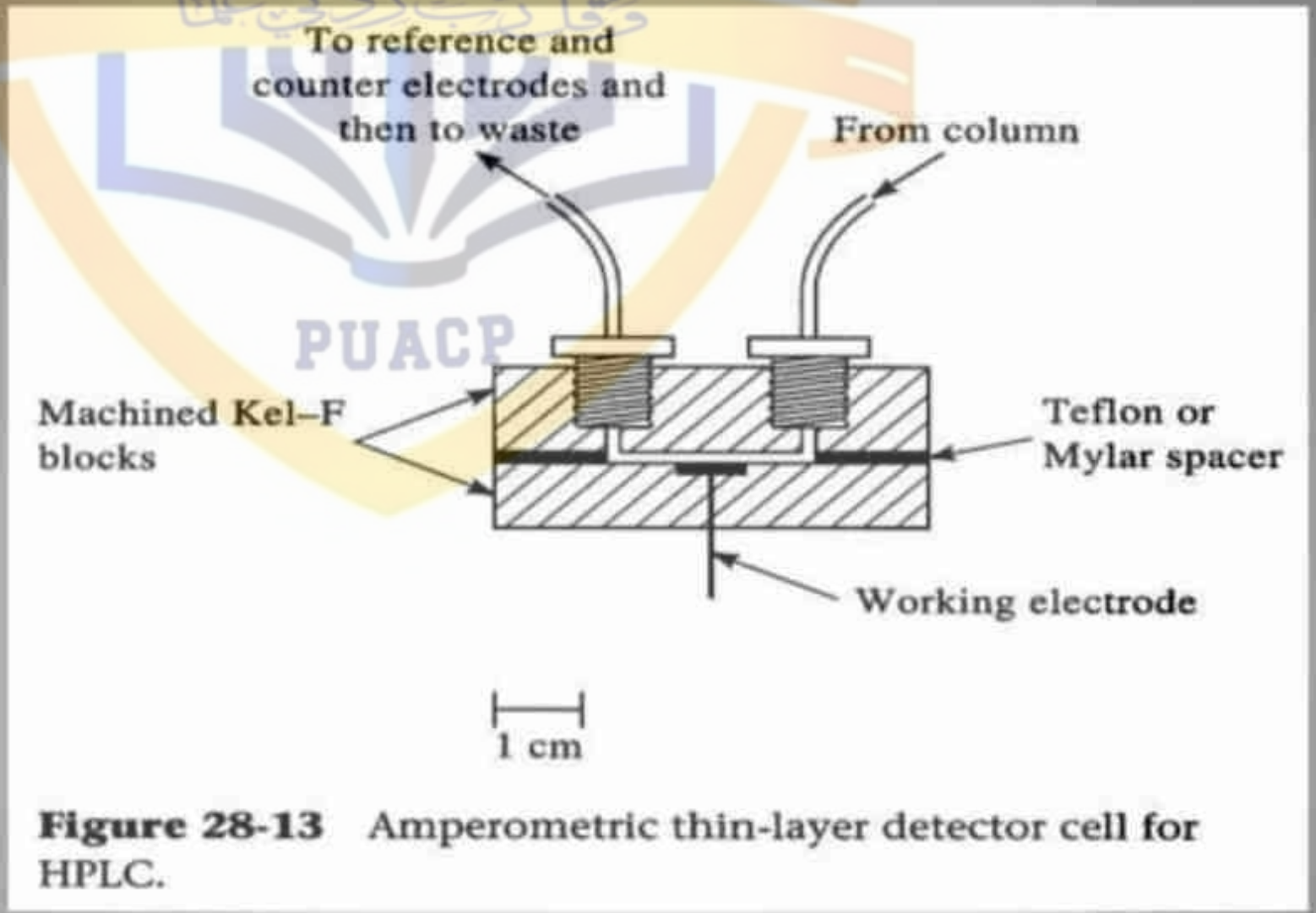
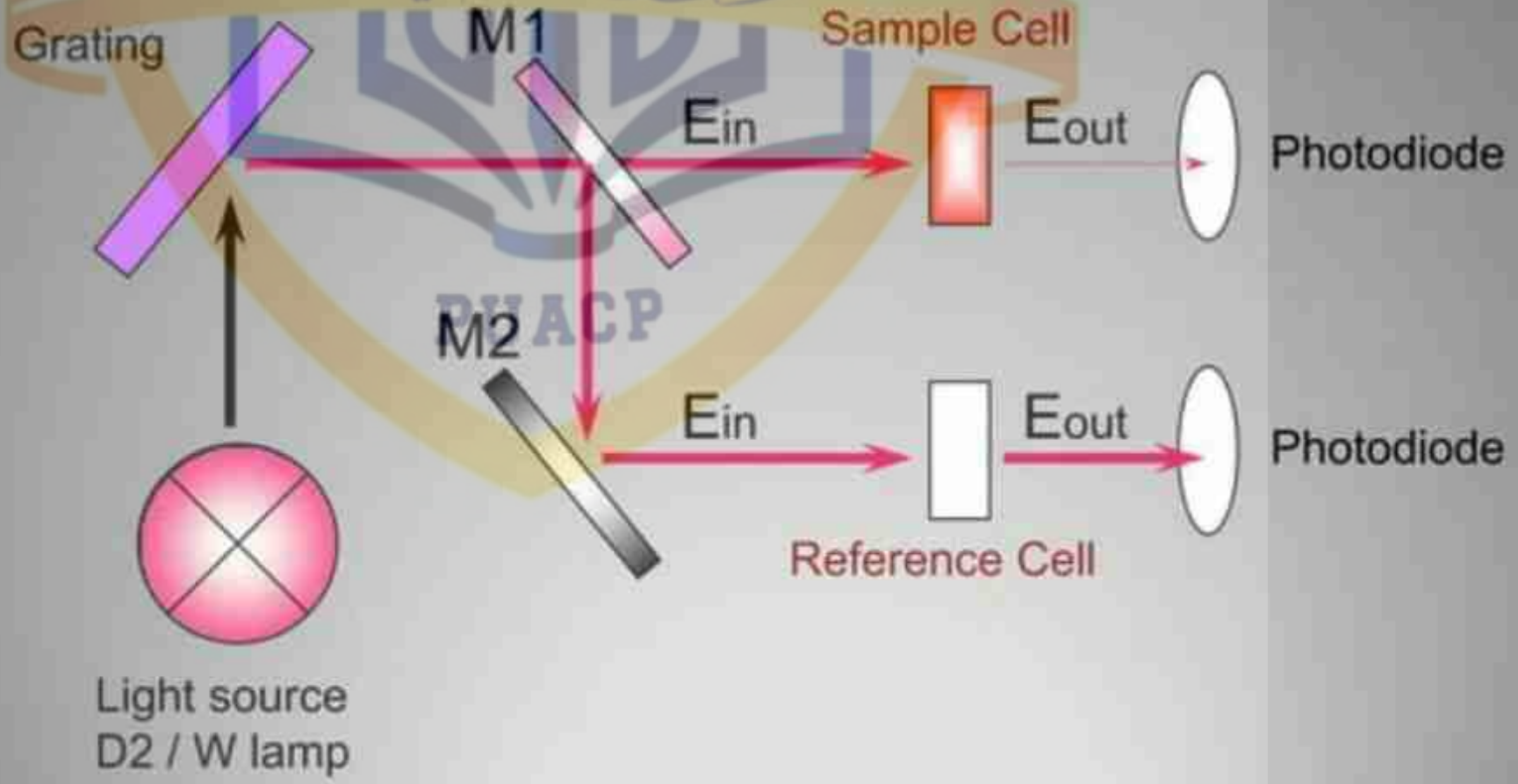


Figure 28-13 Amperometric thin-layer detector cell for HPLC.

Instrumentation of UV-Vis Detector



Ultraviolet / Visible Detector

Advantage:

- Sensitivity is high
- Relative robust to temperature and flow rate change
- Compatible with gradient elution

Disadvantage:

- Only compounds with UV or visible absorption could be detected.

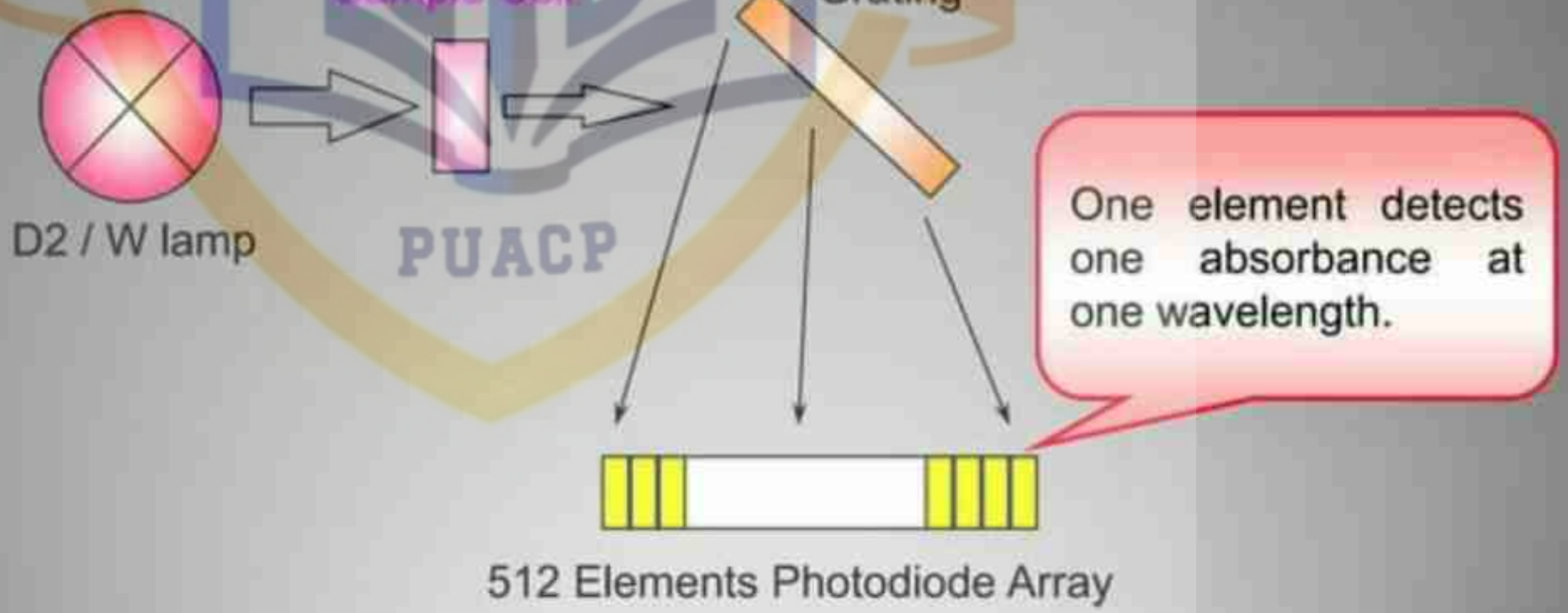
Additional Functions

- Dual Wavelength mode
- Wavelength Time Program mode
- Wavelength Scan mode

PHOTODIODE ARRAY DETECTORS:

- This is a recent detector which is similar to UV detector which operates from 190-600nm.
- Radiations of all wavelength fall on the detector simultaneously.
- The resulting spectra is a three dimensional plot of Response Vs Time Vs Wavelength.
- ADVANTAGE: The wavelength need not be selected but detector detects the responses of all compounds.

➢PHOTODIODE ARRAY DETECTORS:

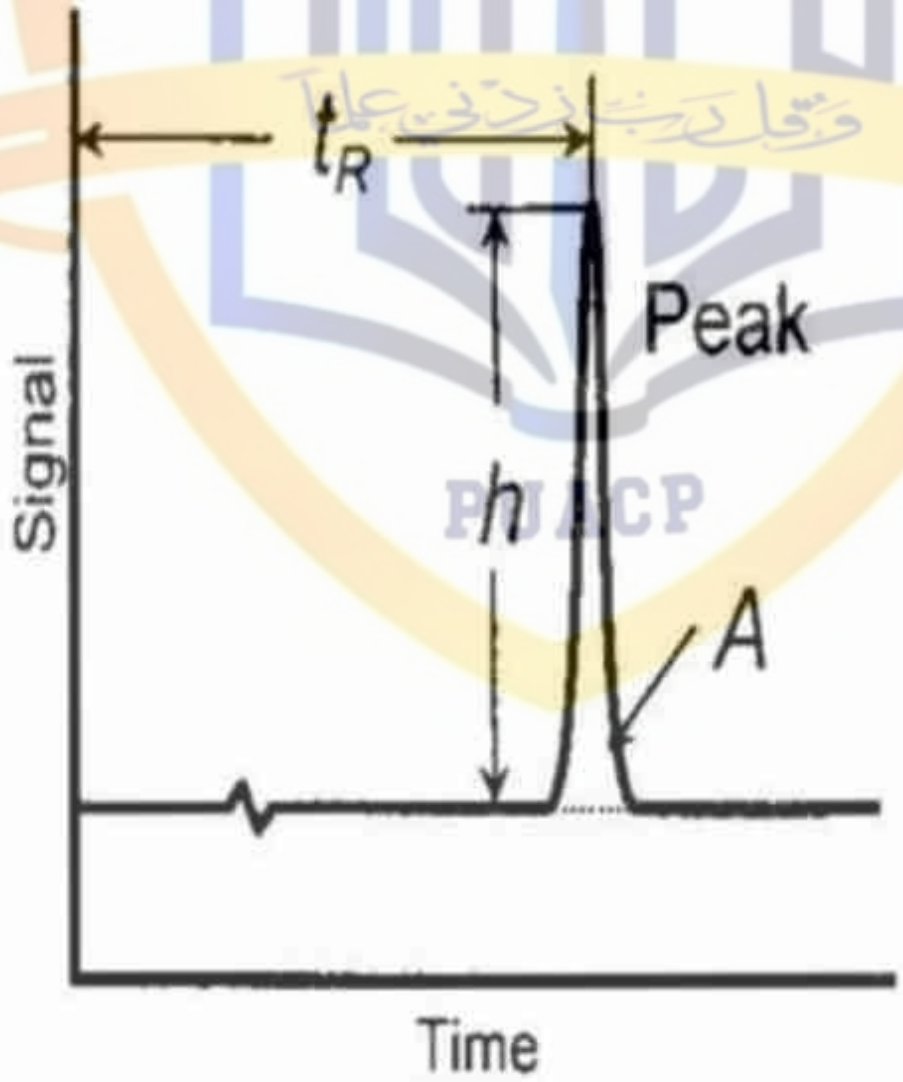


❖RECORDERS AND INTEGRATORS:

- Recorders are used to record responses obtained from the detectors after amplification,if necessary.
- They record the baseline & all the peaks obtained with respect tot ime.
- Retention time can be found out from this recordings,but area under curve cannot be determined.

❖INTEGRATORS:

- These are improved versions of recorders with some data processing capabilities.
- They can record the individual peaks with retention time,height,width of peaks,peak area,percentage area,etc.
- Integrators provides more information on peaks than recorders.
- In recent days computers and printers are used for recording and processing the obtained data & for controlling several operations.

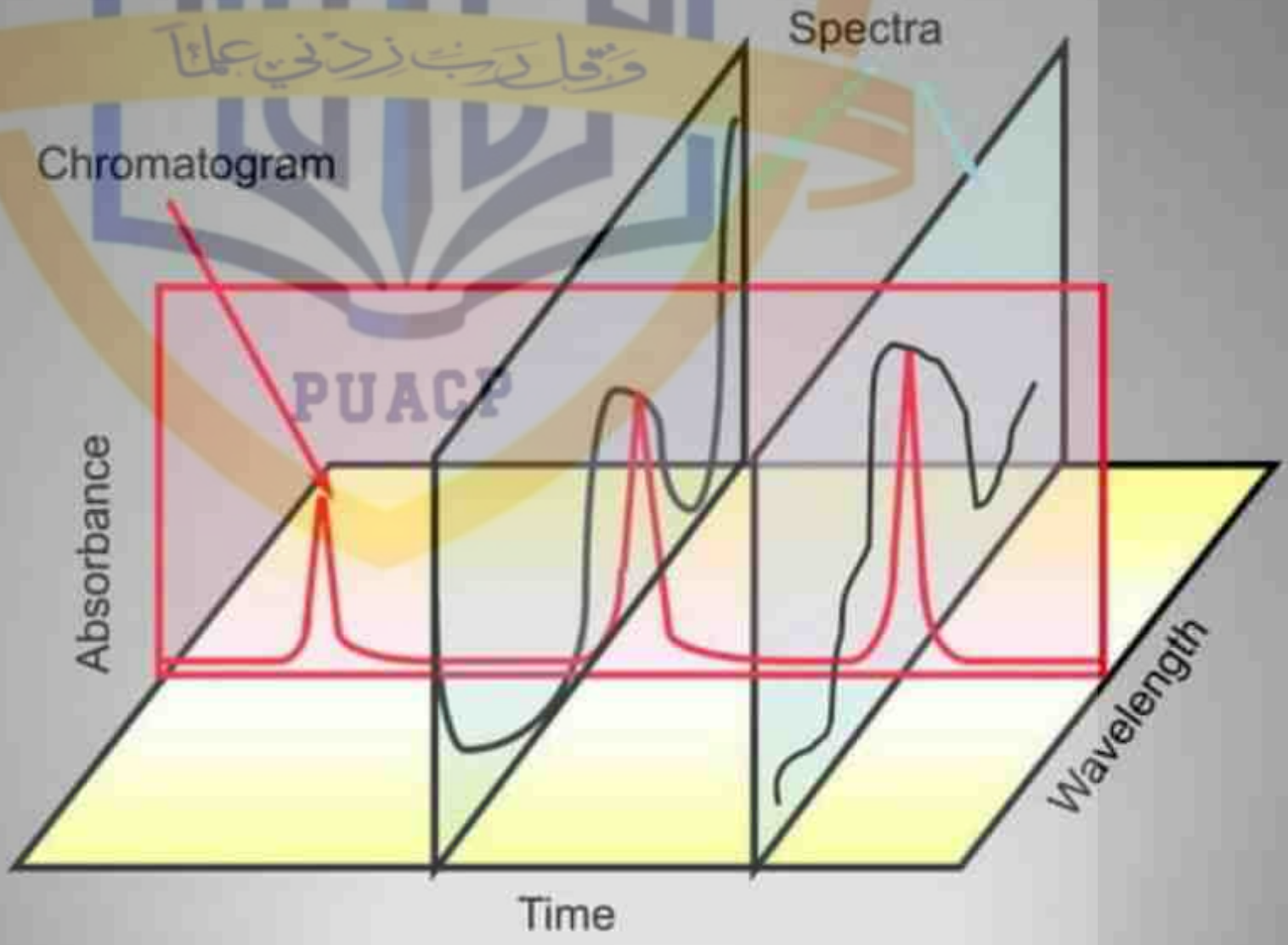


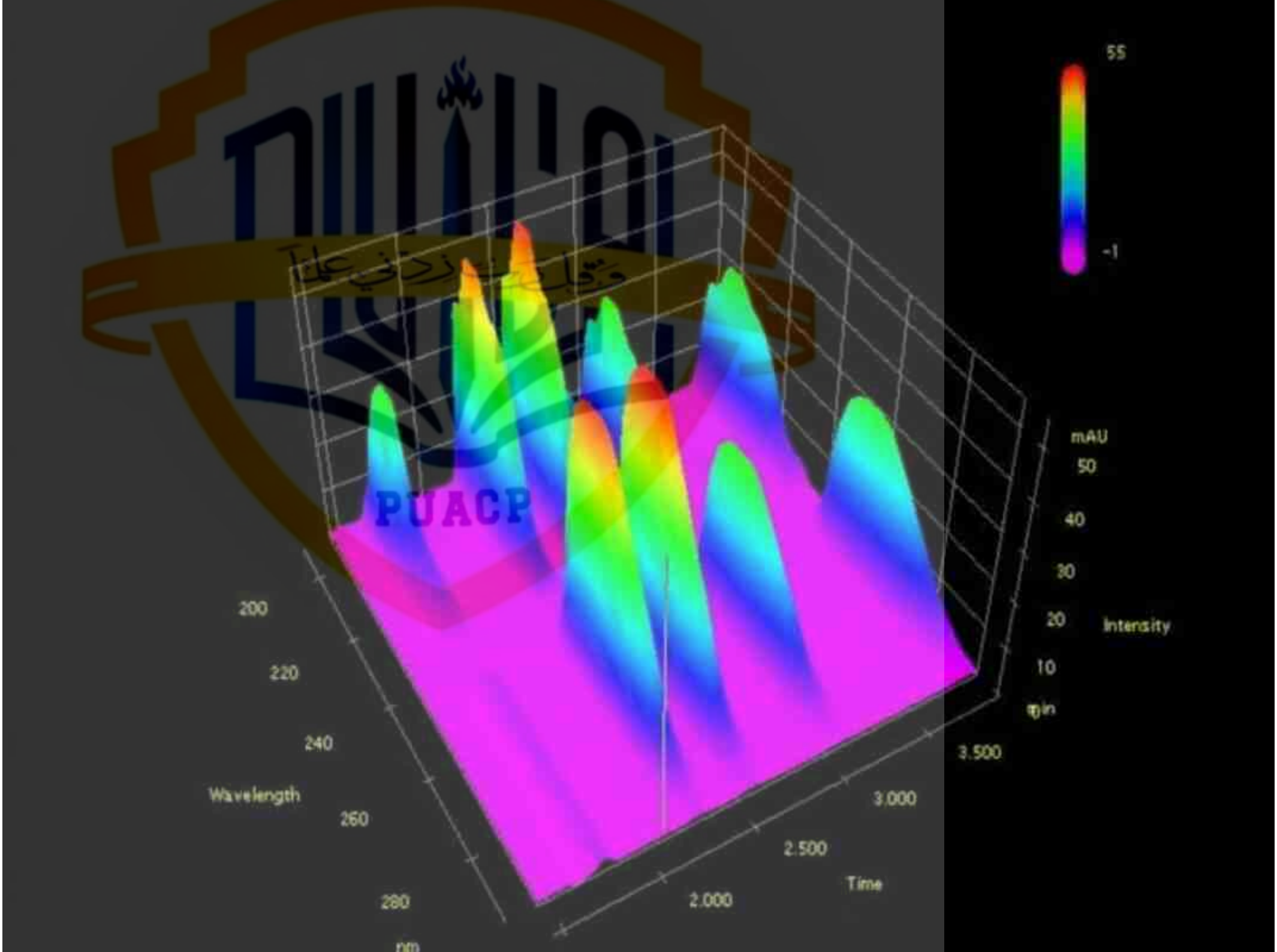
t_R : Retention time

A : Area

h : Height

Photodiode Array Detector (3-D Data)




55
-1
mAU
50
40
30
20
10
Intensity
Wavelength
200
220
240
260
280
nm
2.000
2.500
3.000
3.500
Time
min

PARAMETERS:

- Retention time(R_t)
- Retention volume(Vr)
- Separation factor(S)
- Resolution
- Theoritical plates
- Column efficiency
- Assymetry factor

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THANK U