بسم الله الرحمن الرحيم

Basic Principals Of Performance Liquid Chromatography (HPLC)

By: Dr. Reza Zadeh-dabbgh
Ph.D Analytical Chemistry
Shahid Chamran University
And
Minoo Barzkar
M.Sc Marine Chemistry
Khorramshahr Marine Science and Technology University

Ahvaz Jondishapur University of Medical Sciences
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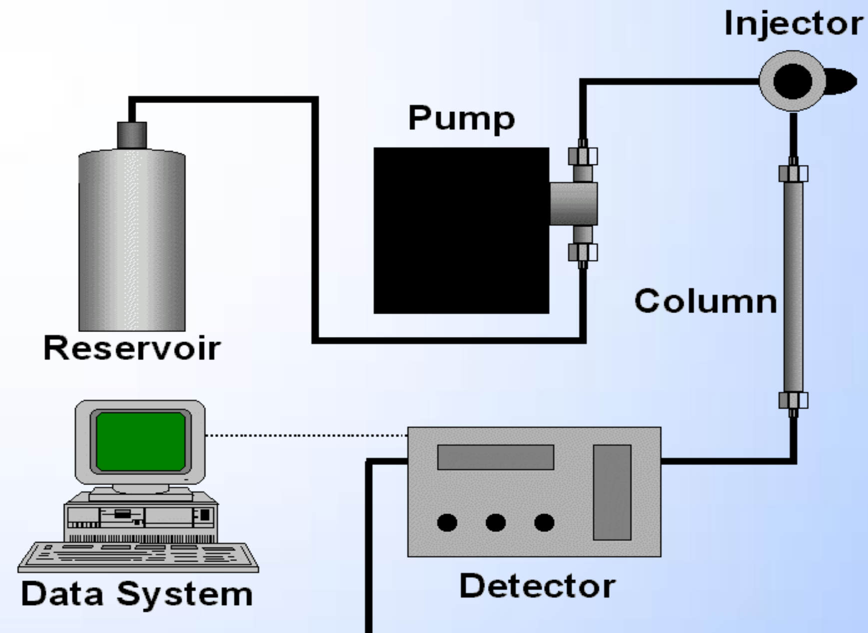

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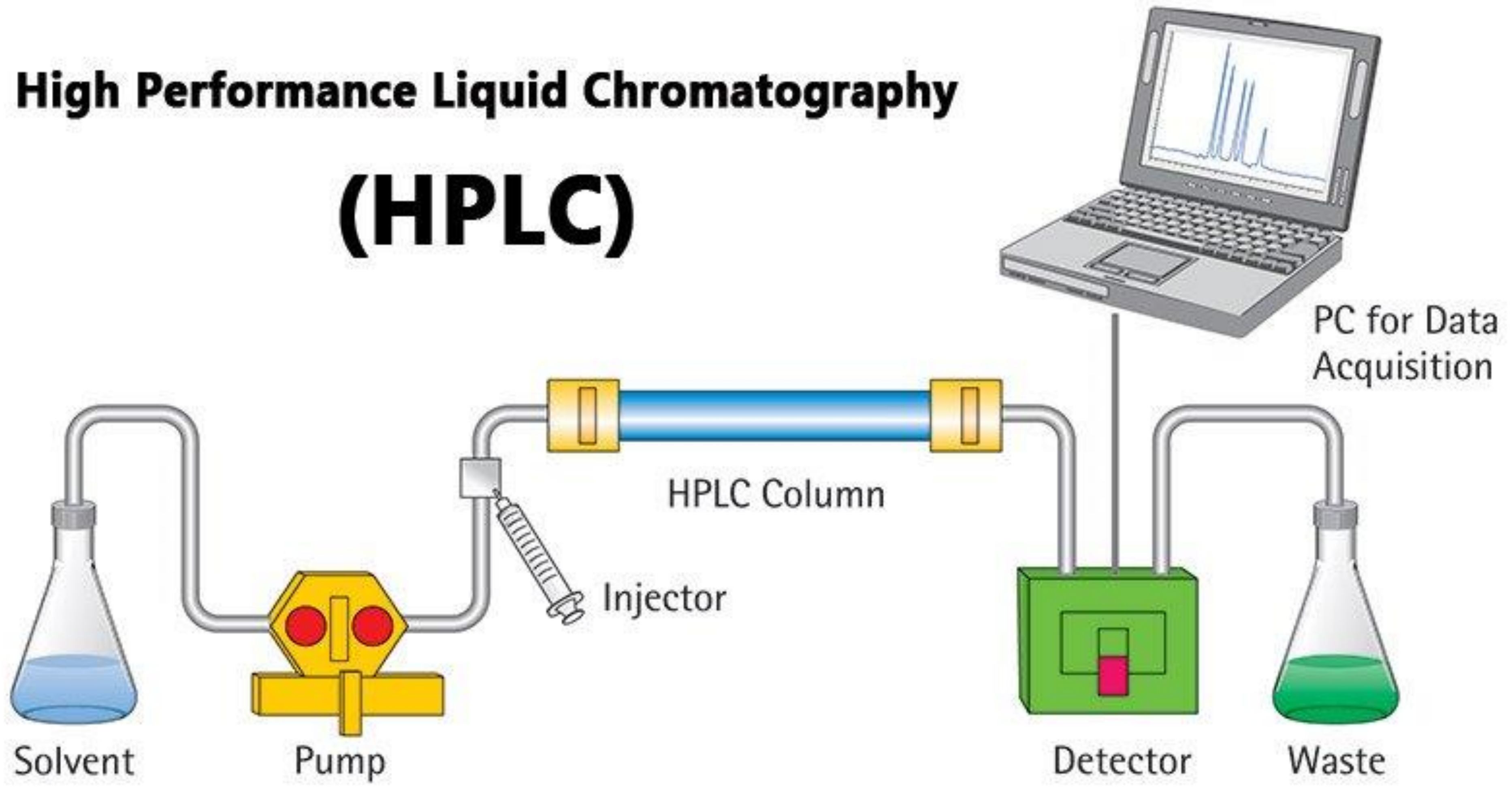
Table of Content

- How it Looks
- Introduction
- Principle of HPLC
- Instrumentation HPLC
- Types of HPLC
- Comparison of HPLC with different Chromatography
- Why We use HPLC
- Applications
- Advantages
- Limitation
- References

How it Looks

Introduction

- High performance liquid chromatography or commonly known as HPLC is an analytical technique used to separate, identify or quantify each component in a mixture.
- The mixture is separated using the basic principle of column chromatography and then identified and quantified by spectroscopy.
- In the 1960s the column chromatography LC with its low-pressure suitable glass columns was further developed to the HPLC with its high-pressure adapted metal columns.
- HPLC is thus basically a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres.

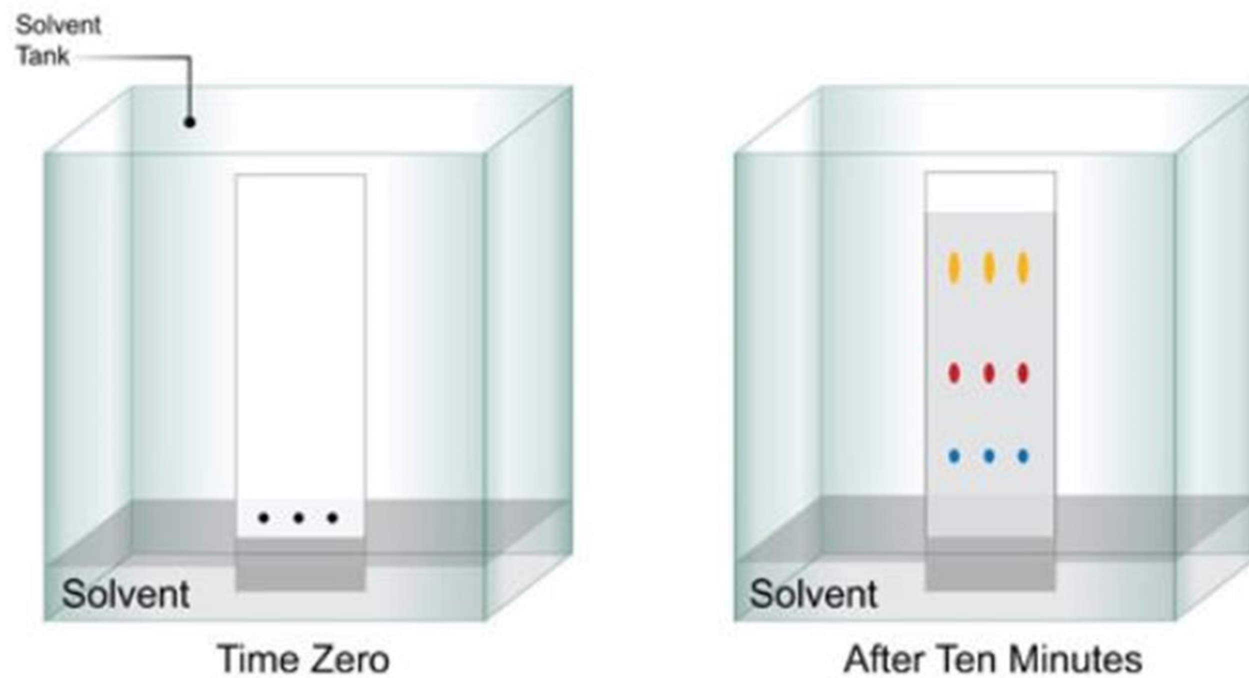

High Performance Liquid Chromatography
(HPLC)
PC for Data
Acquisition
HPLC Column
Injector
Solvent
Pump
Detector
Waste

Principle

- The purification takes place in a separation column between a stationary and a mobile phase.
- The stationary phase is a granular material with very small porous particles in a separation column.
- The mobile phase, on the other hand, is a solvent or solvent mixture which is forced at high pressure through the separation column.
- Via a valve with a connected sample loop, i.e. a small tube or a capillary made of stainless steel, the sample is injected into the mobile phase flow from the pump to the separation column using a syringe.

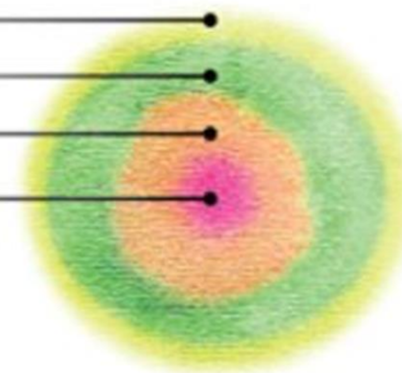
Cont...

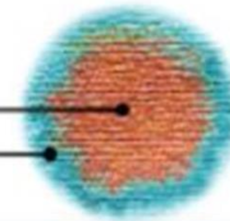
- Subsequently, the individual components of the sample migrate through the column at different rates because they are retained to a varying degree by interactions with the stationary phase.
- After leaving the column, the individual substances are detected by a suitable detector and passed on as a signal to the HPLC software on the computer.
- At the end of this operation/run, a chromatogram in the HPLC software on the computer is obtained.
- The chromatogram allows the identification and quantification of the different substances.


Thin Layer Chromatography (TLC)
Solvent Tank
Solvent
Time Zero
Solvent
After Ten Minutes

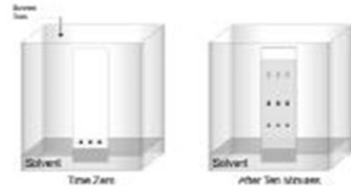
- Thin Layer Chromatography (TLC)

- **Paper Chromatography**

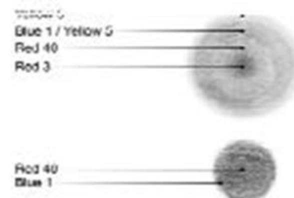




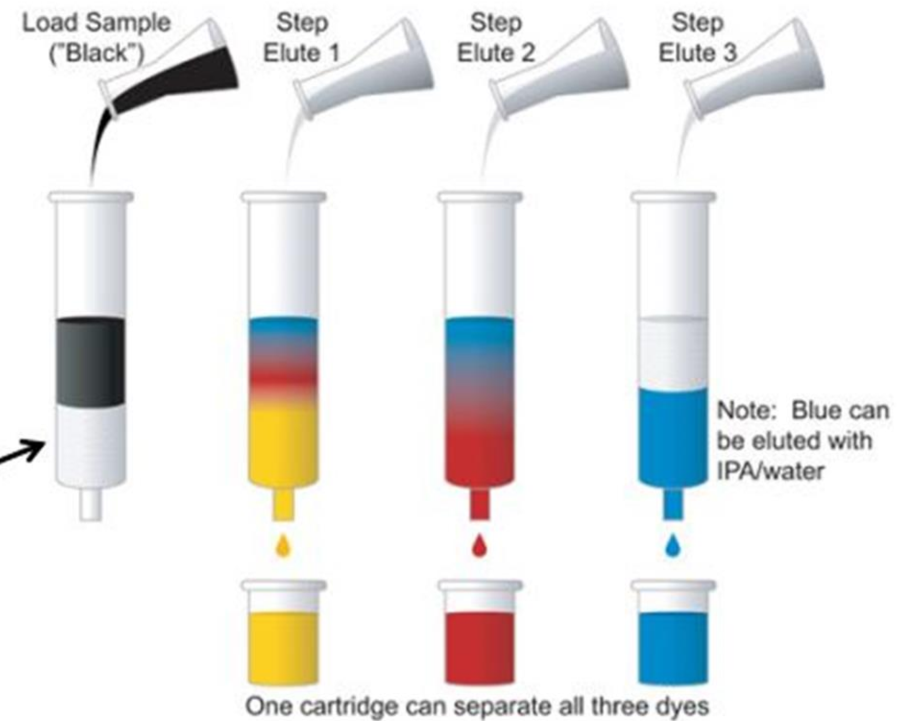
- Thin Layer Chromatography (TLC)



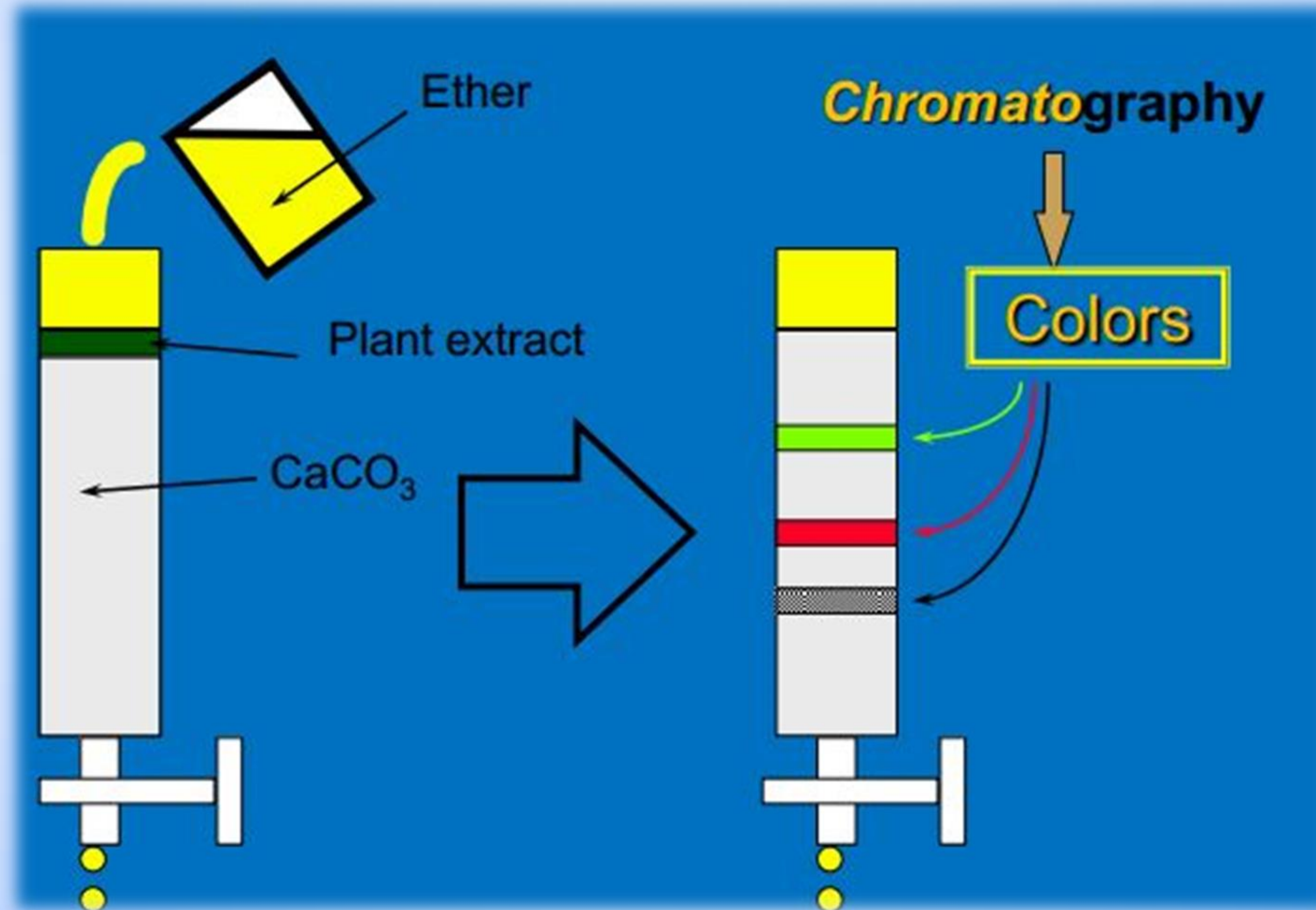
- Paper Chromatography



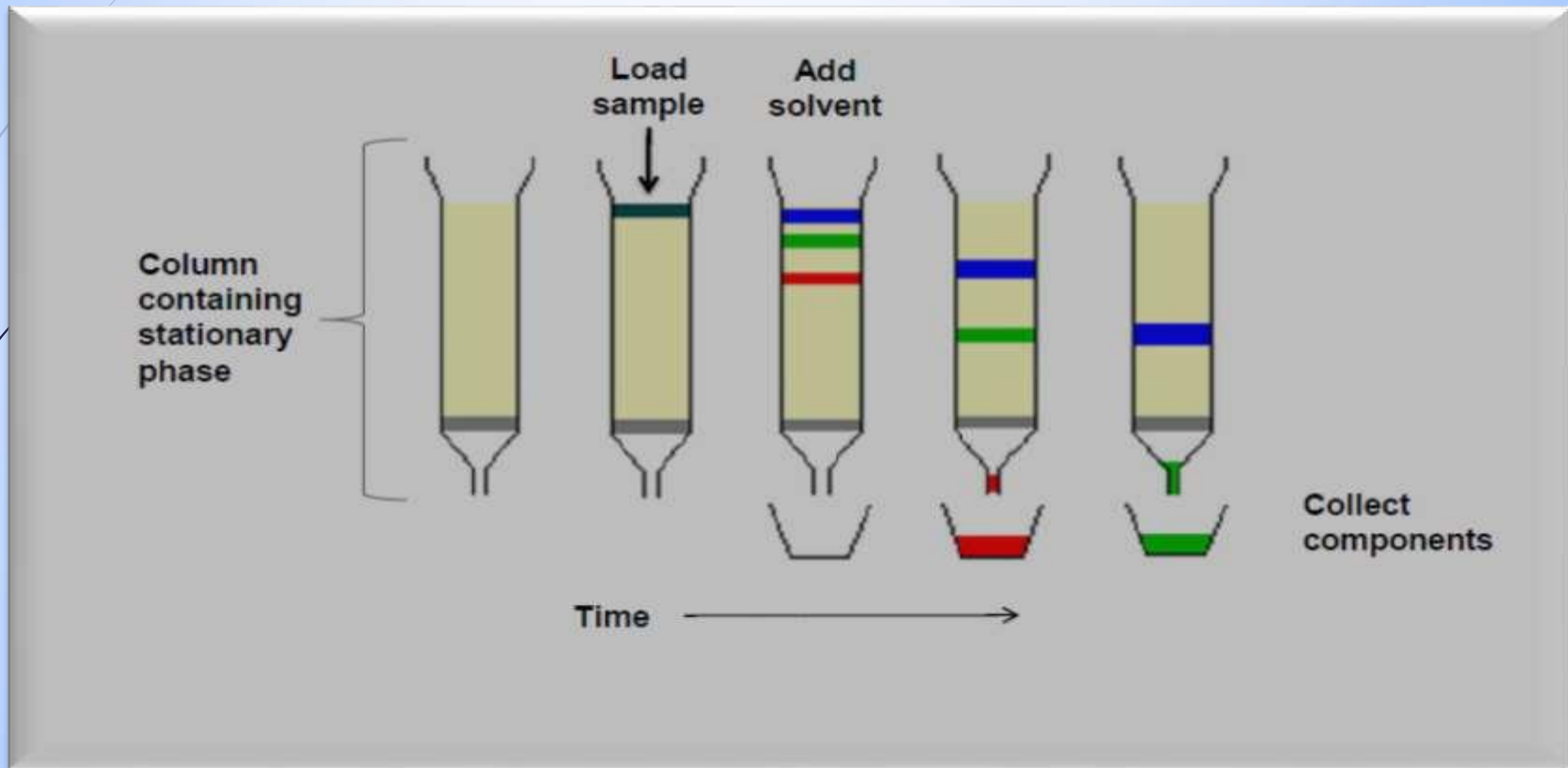
- **Column Chromatography**
  - **S**olid **P**hase **E**xtraction
  - HPLC
  - UPLC®
  - Flash Chromatography



Principle



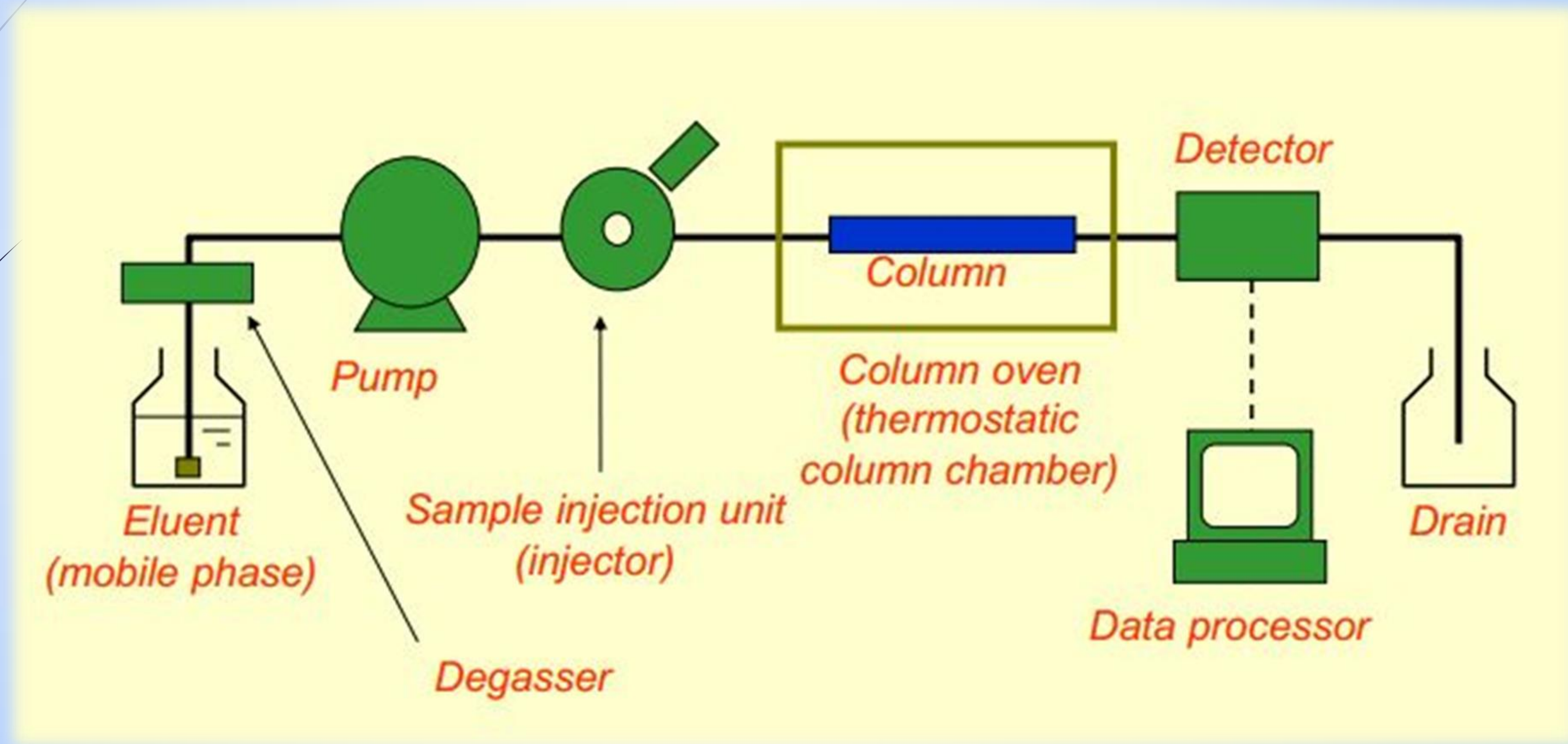
Diagrammatic View of HPLC Principle



Instrumentation of High-Performance Liquid Chromatography (HPLC)

- The Pump
- Injector
- Column
- Detector
- Recorder
- Degasser
- Column Header

Schematic of an HPLC Instrument

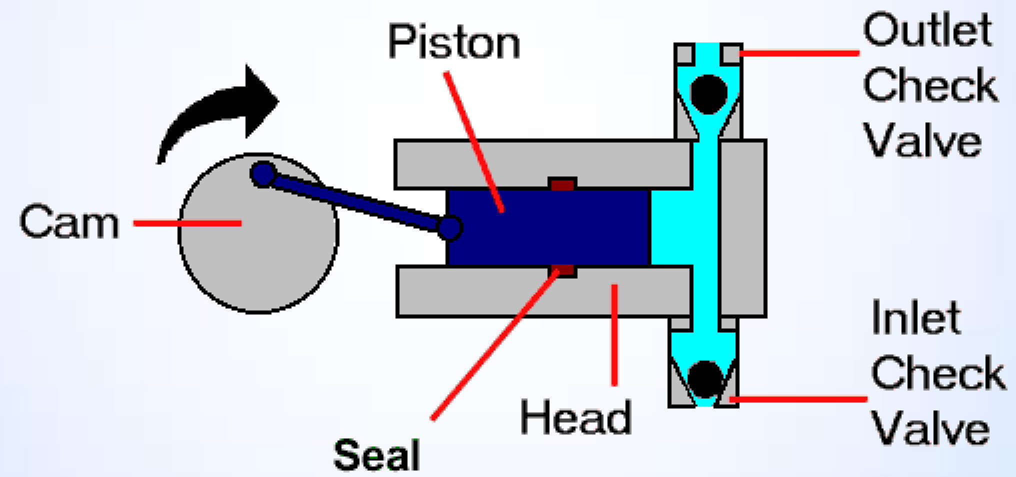


		Mobile phase		
		Gas	Liquid	Solid
Stationary phase	Gas			
	Liquid	Gas chromatography	Liquid chromatography	
	Solid			

The Pump

- The development of HPLC led to the development of the pump system.
- The pump is positioned in the most upper stream of the liquid chromatography system and generates a flow of eluent from the solvent reservoir into the system.
- High-pressure generation is a "standard" requirement of pumps besides which, it should also to be able to provide a consistent pressure at any condition and a controllable and reproducible flow rate.
- Most pumps used in current LC systems generate the flow by back-and-forth motion of a motor-driven piston (reciprocating pumps). Because of this piston motion, it produces "pulses".

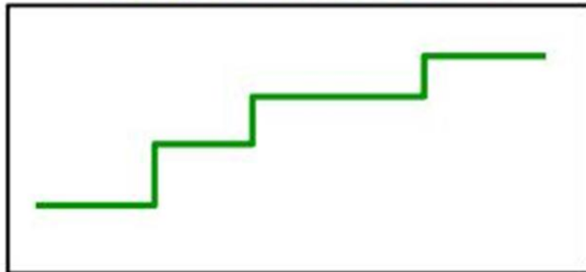
The Pump

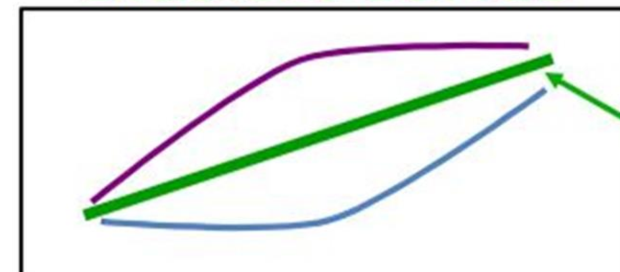


Eluent

- **Isocratic**
  - Constant eluent composition, same eluent: for example 50 % methanol
- **Gradient**
  - Varying eluent composition
    - HPGE (High Pressure Gradient): High gradient accuracy, complex system configuration (multiple pumps required)
    - LPGE (Low Pressure Gradient): Simple system configuration, degasser required

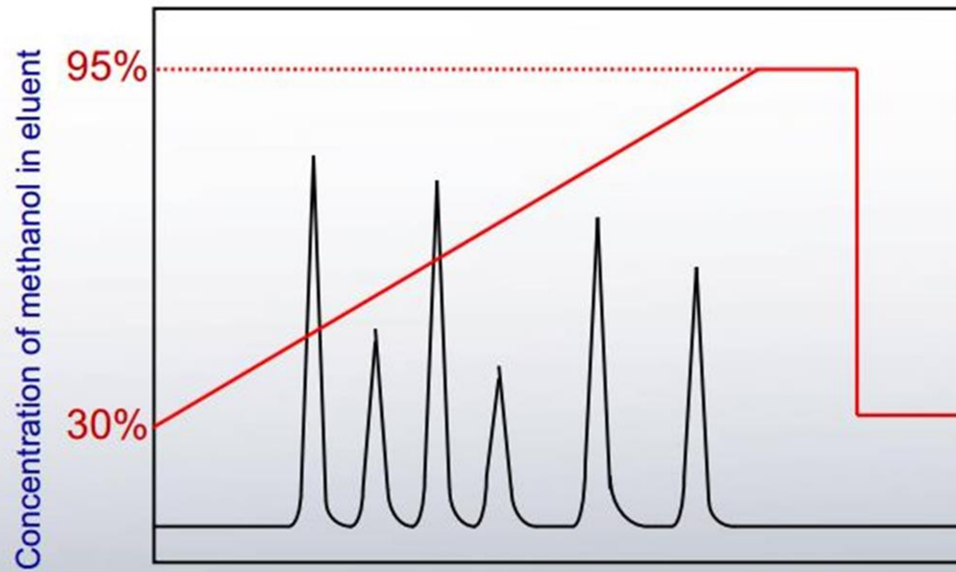
Isocratic
Same


Step-Gradient
Increasing Strength


Gradient Curves
Linear
Gradient
Increasing Strength

Eluent

- In gradient mode



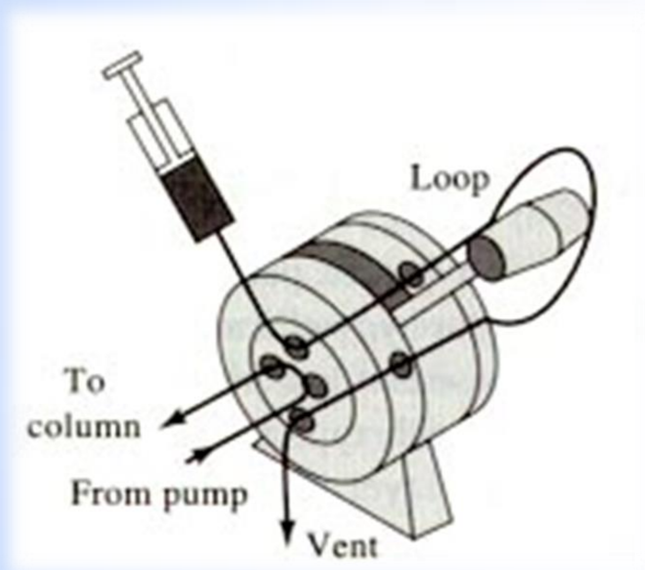
- In isocratic mode

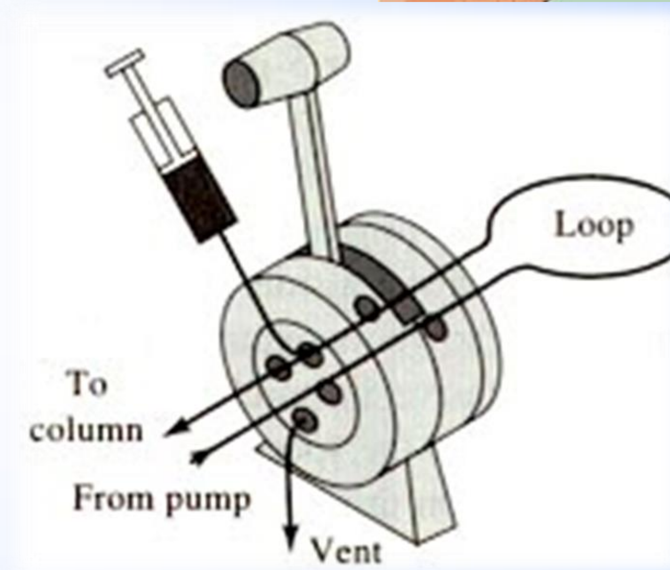
(Column: ODS type)

Injector

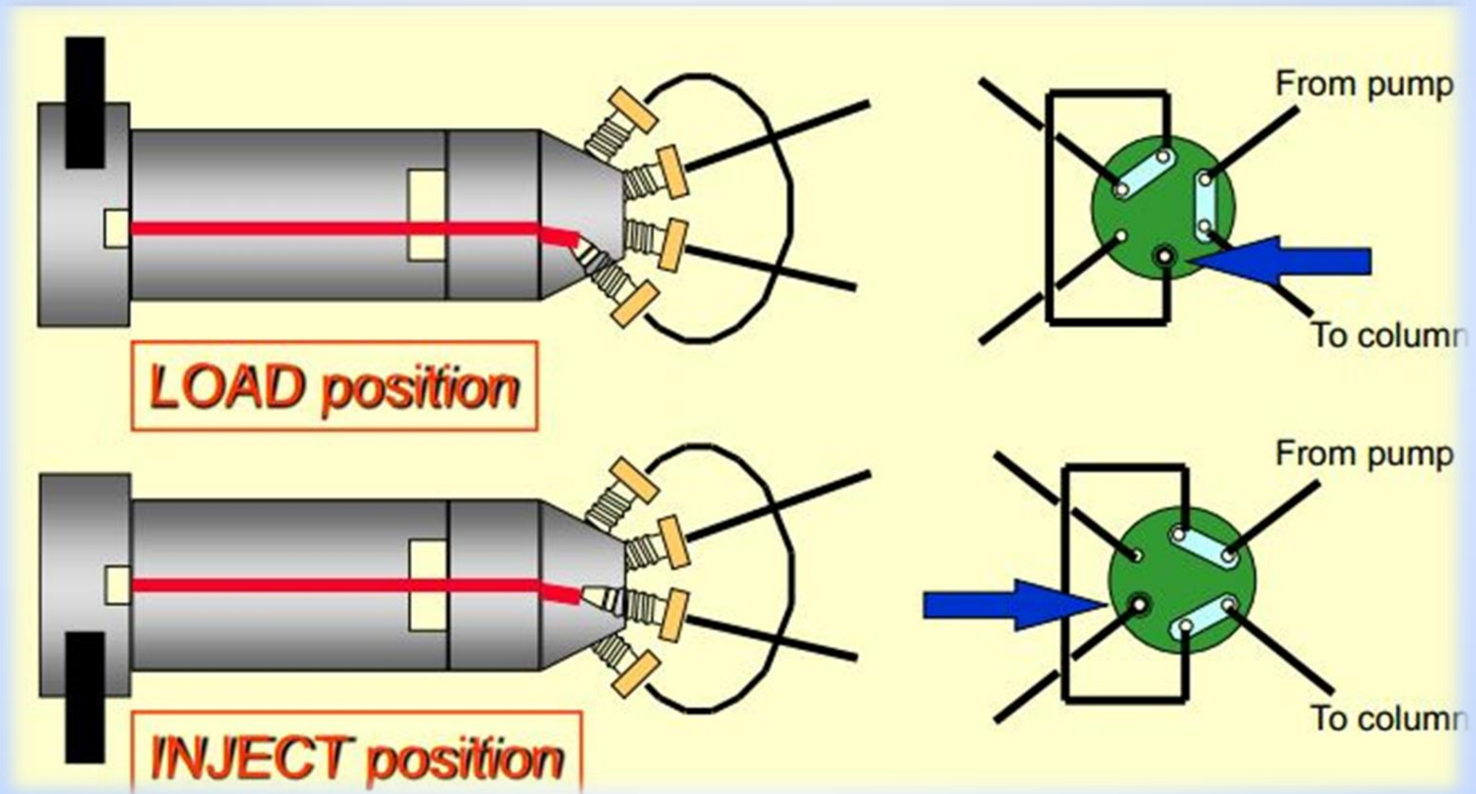
- An injector is placed next to the pump.
- The simplest method is to use a syringe, and the sample is introduced to the flow of eluent.
- The most widely used injection method is based on sampling loops.
- The use of the autosampler (auto-injector) system is also widely used that allows repeated injections in a set scheduled-timing.

Injector

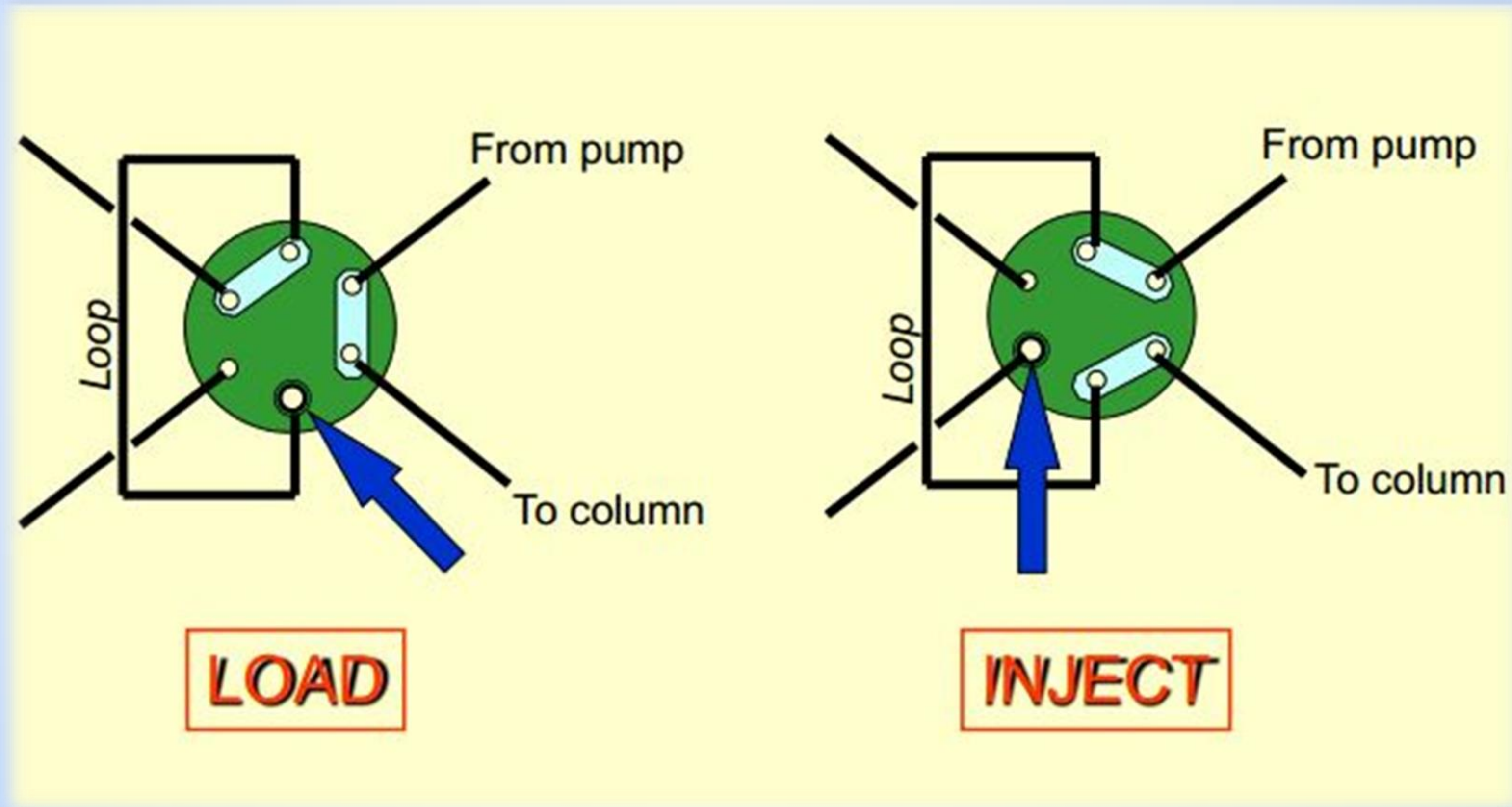




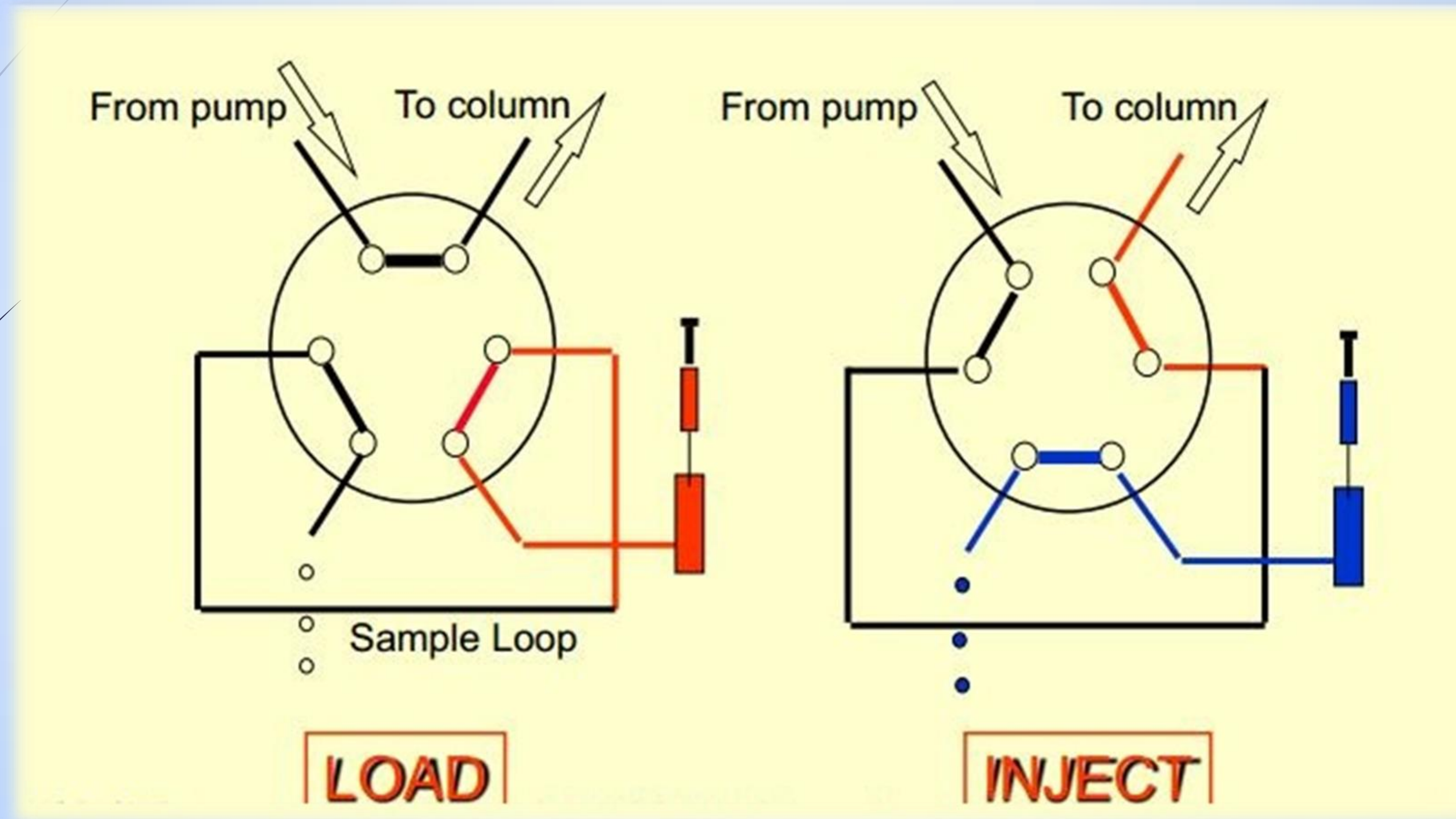
Injector



Injector



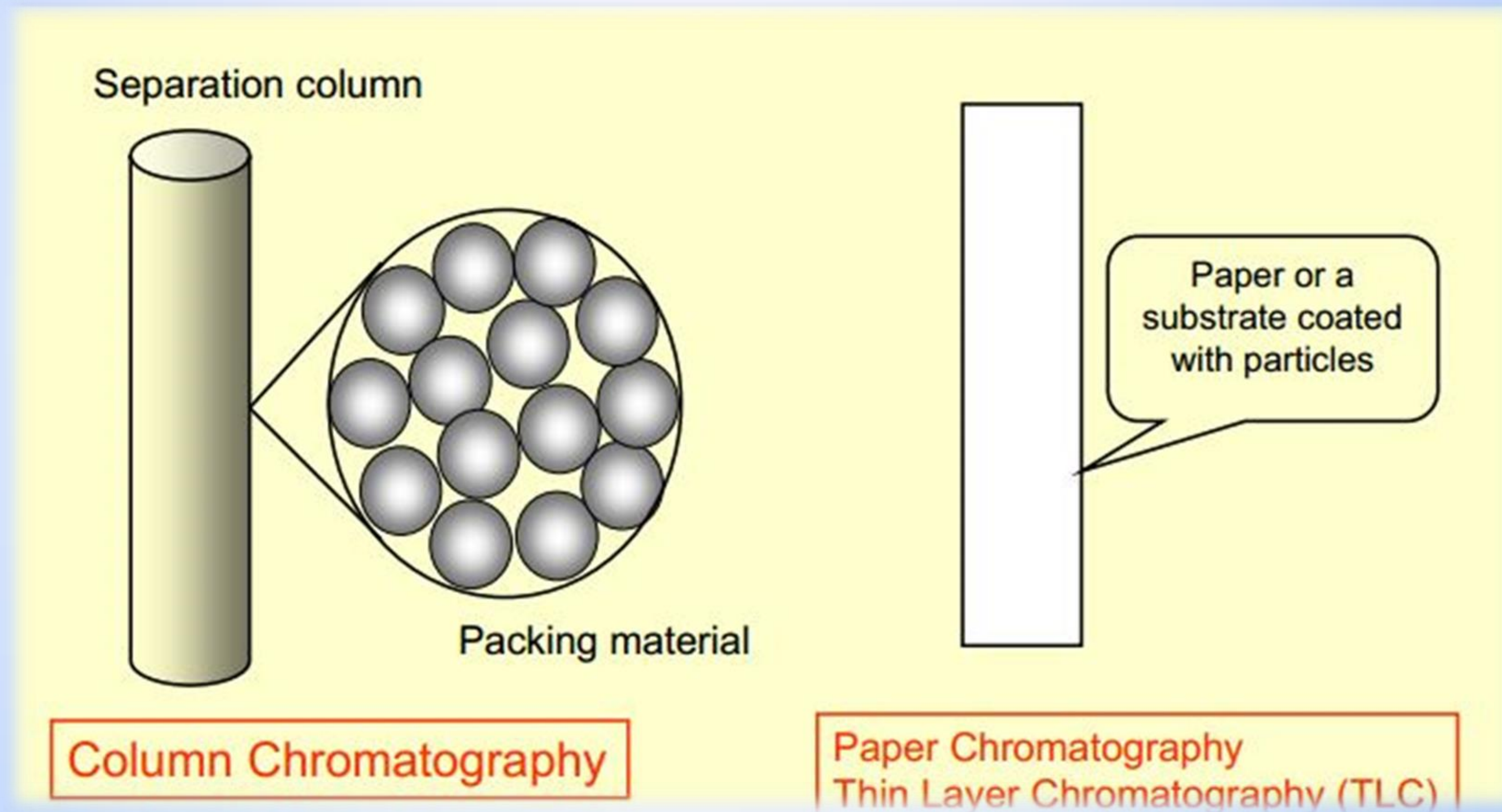
Injector



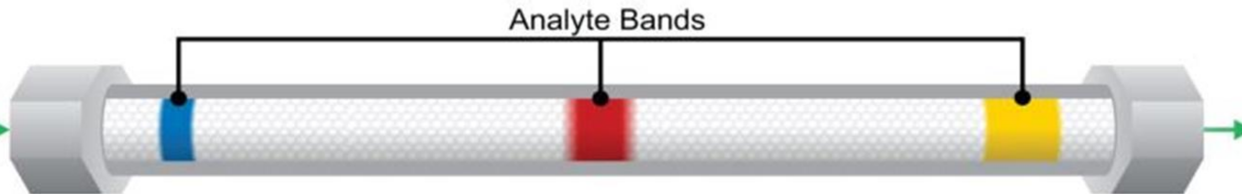
Column

- The separation is performed inside the column.
- The recent columns are often prepared in a stainless steel housing, instead of glass columns.
- The packing material generally used is silica or polymer gels compared to calcium carbonate.
  The eluent used for LC varies from acidic to basic solvents.
- Most column housing is made of stainless steel since stainless is tolerant towards a large variety of solvents.

Column



Column



Column Heaters

- Some separations of complex mixtures must be temperature optimised to achieve separations of overlapping peaks.
- Increased column temperature will also shorten retention times for a given column dimension.
- Selectivity of the column is also a function of temperature and elution orders of peaks may change and even reverse - some chiral and amino acid separations are very sensitive to column temperature effects.
- Eliminates retention time variation due to room temperature fluctuations.

Column Heater

The LC separation is often largely influenced by the column temperature.

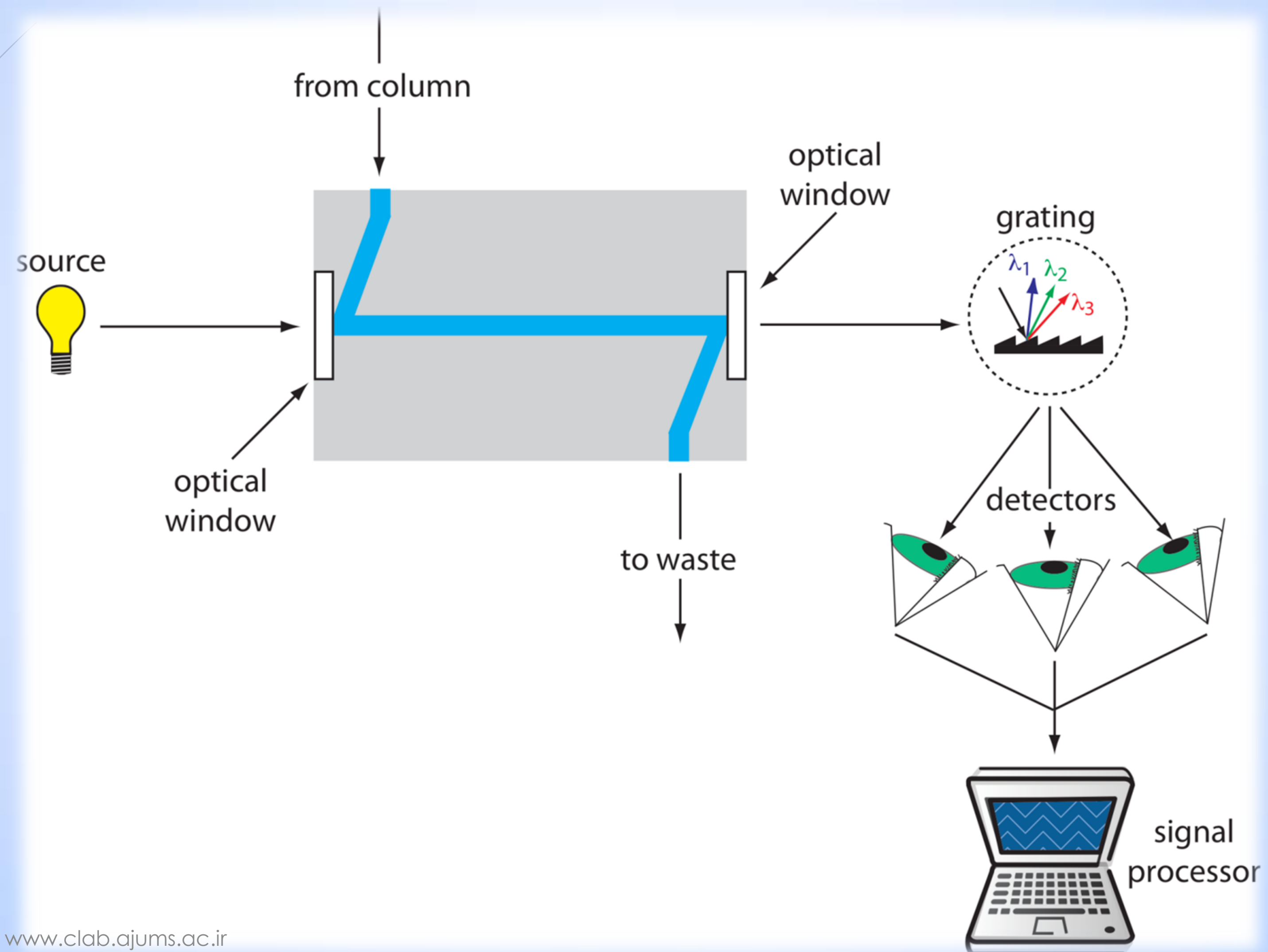
- In order to obtain repeatable results, it is important to keep consistent temperature conditions.
- Also for some analysis, such as sugar and organic acid, better resolutions can be obtained at elevated temperatures (50 to 80°C).
- Thus columns are generally kept inside the column oven (column heater).

Column Heaters

Detector

- Separation of analytes is performed inside the column, whereas a detector is used to observe the obtained separation.
- 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.
- This difference is monitored as a form of an electronic signal. There are different types of detectors available.

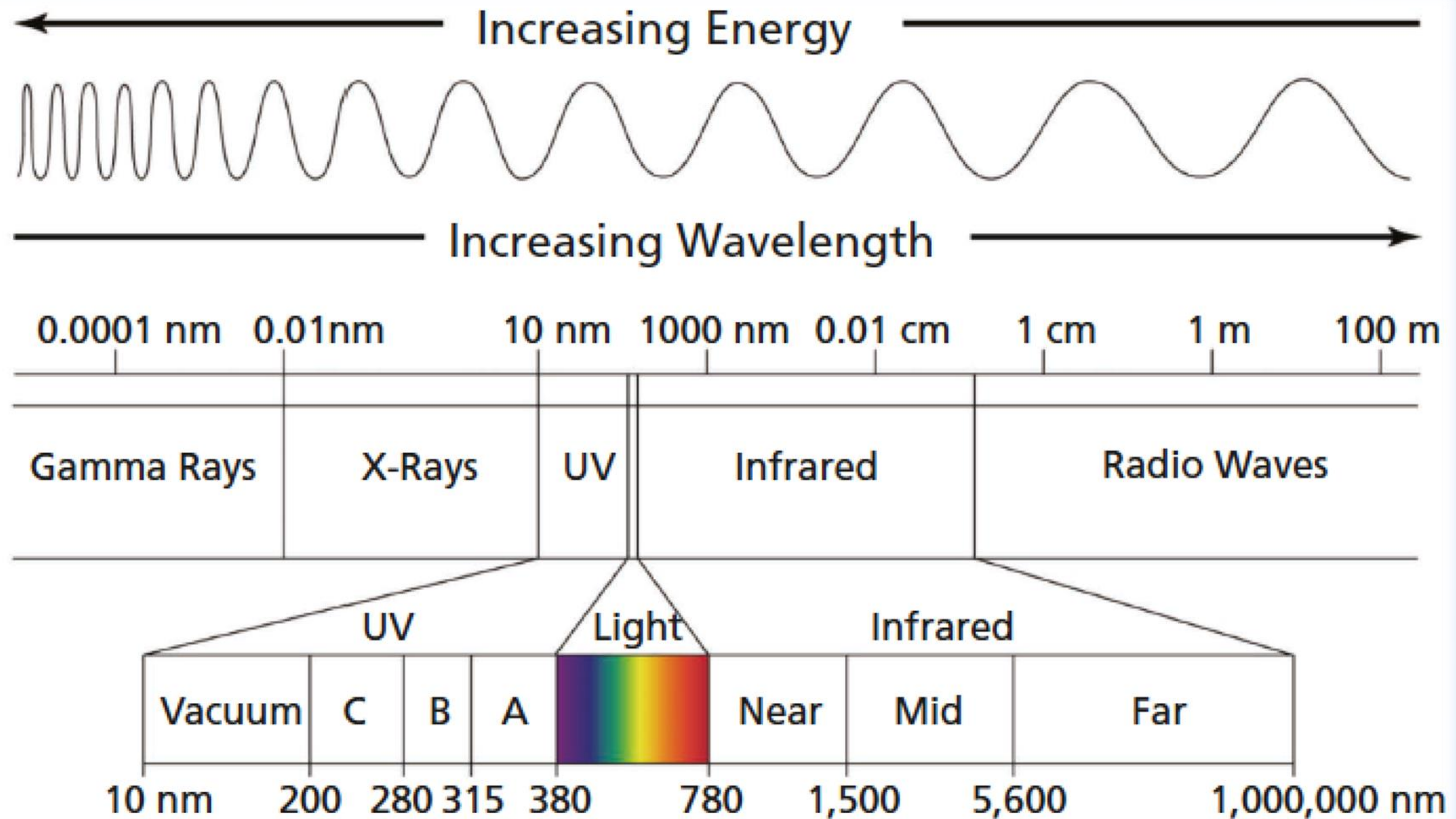
Detector



Types of Detectors

- ✓ UV-Vis absorbance detector
- ✓ Photodiode array-type UV-VIS absorbance detector (DAD)
- ✓ Fluorescence detector
- ✓ Refractive index detector
- ✓ Electrical conductivity detector
- ✓ Electrochemical detector
- ✓ Mass spectrometer

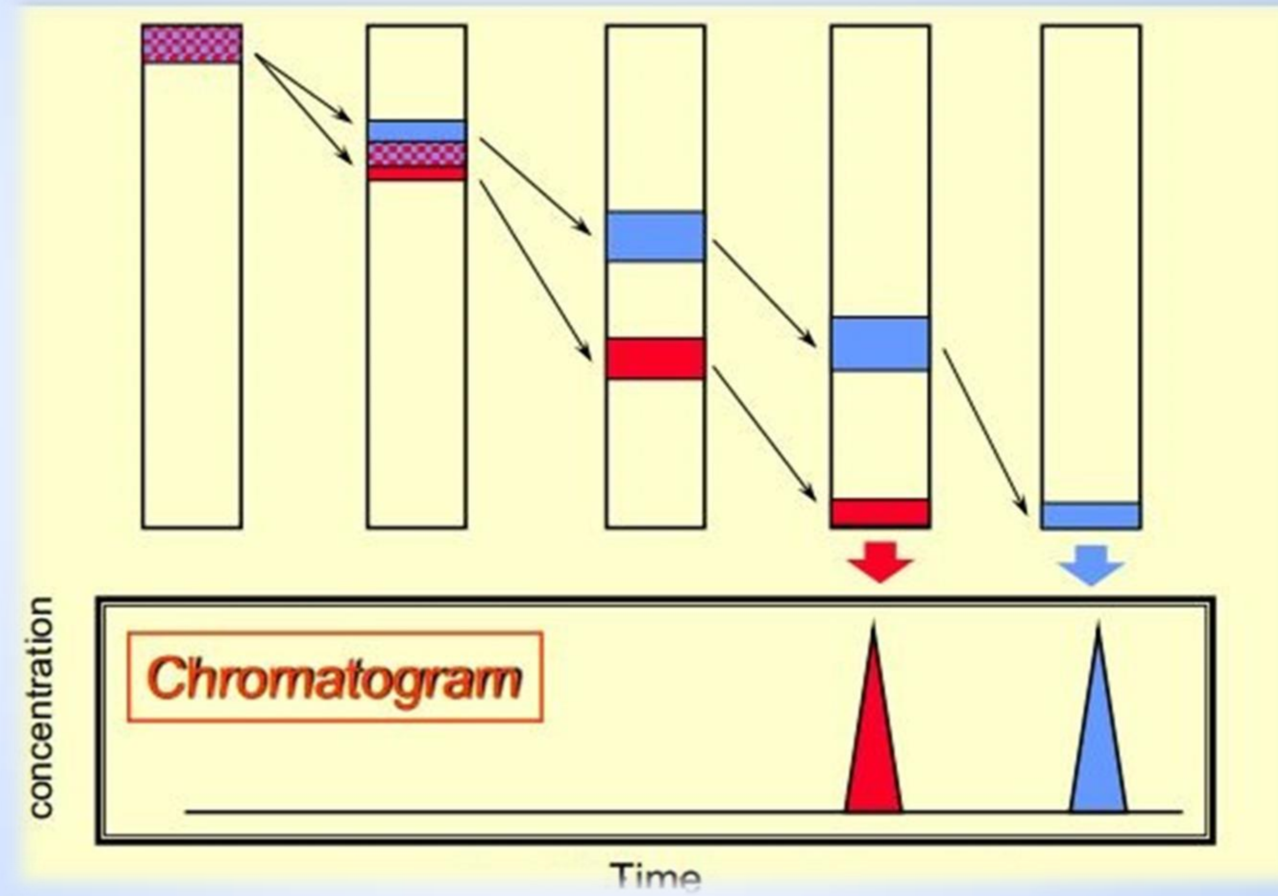
Detector

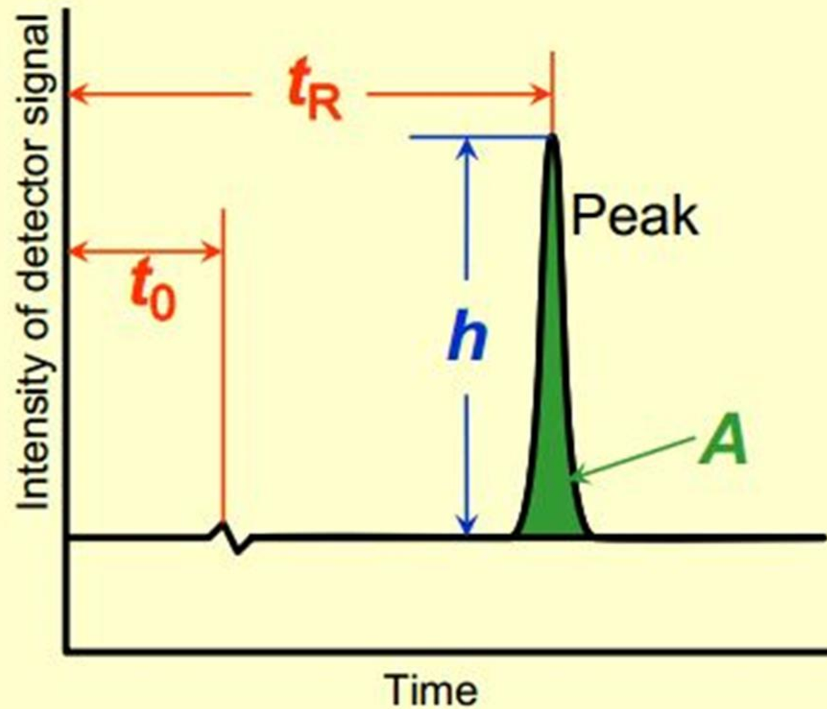


Recorder

- 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.
- In older days, the pen (paper)-chart recorder was popularly used. Nowadays, a computer-based data processor (integrator) is more common.
- There are various types of data processors; from a simple system consisting of the in-built printer and word processor while those with software that are specifically designed for an LC system which not only data acquisition but features like peak-fitting, baseline correction, automatic concentration calculation, molecular weight determination, etc.

Recorder





t_R : Retention time

t_0 : Non-retention time

A : Peak area

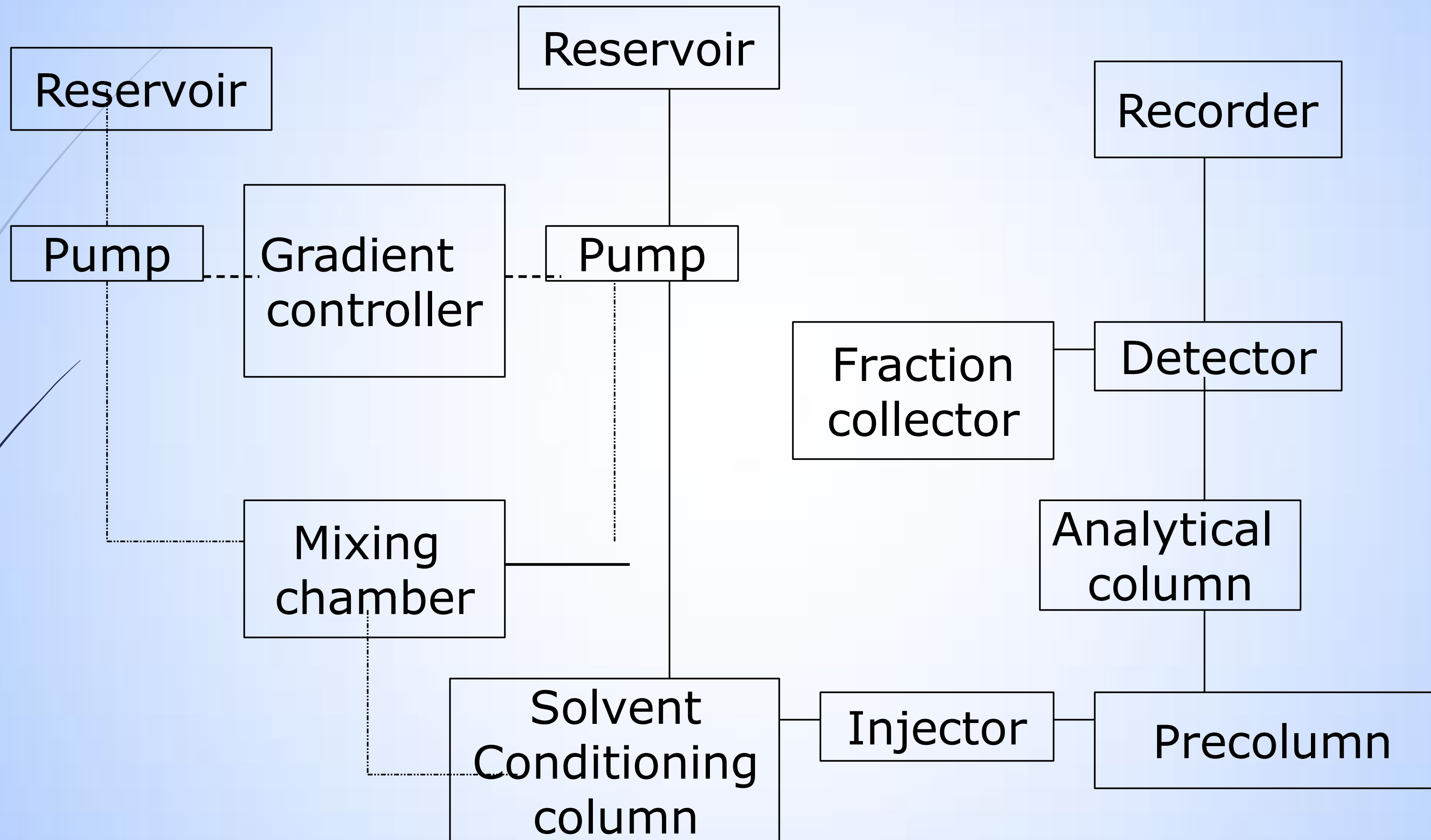
h : Peak height

Degasser

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.
- The numerous very small pores on the surface of the polymer tube allow the air to go through while preventing any liquid to go through the pore.

BLOCK DIAGRAM OF HPLC



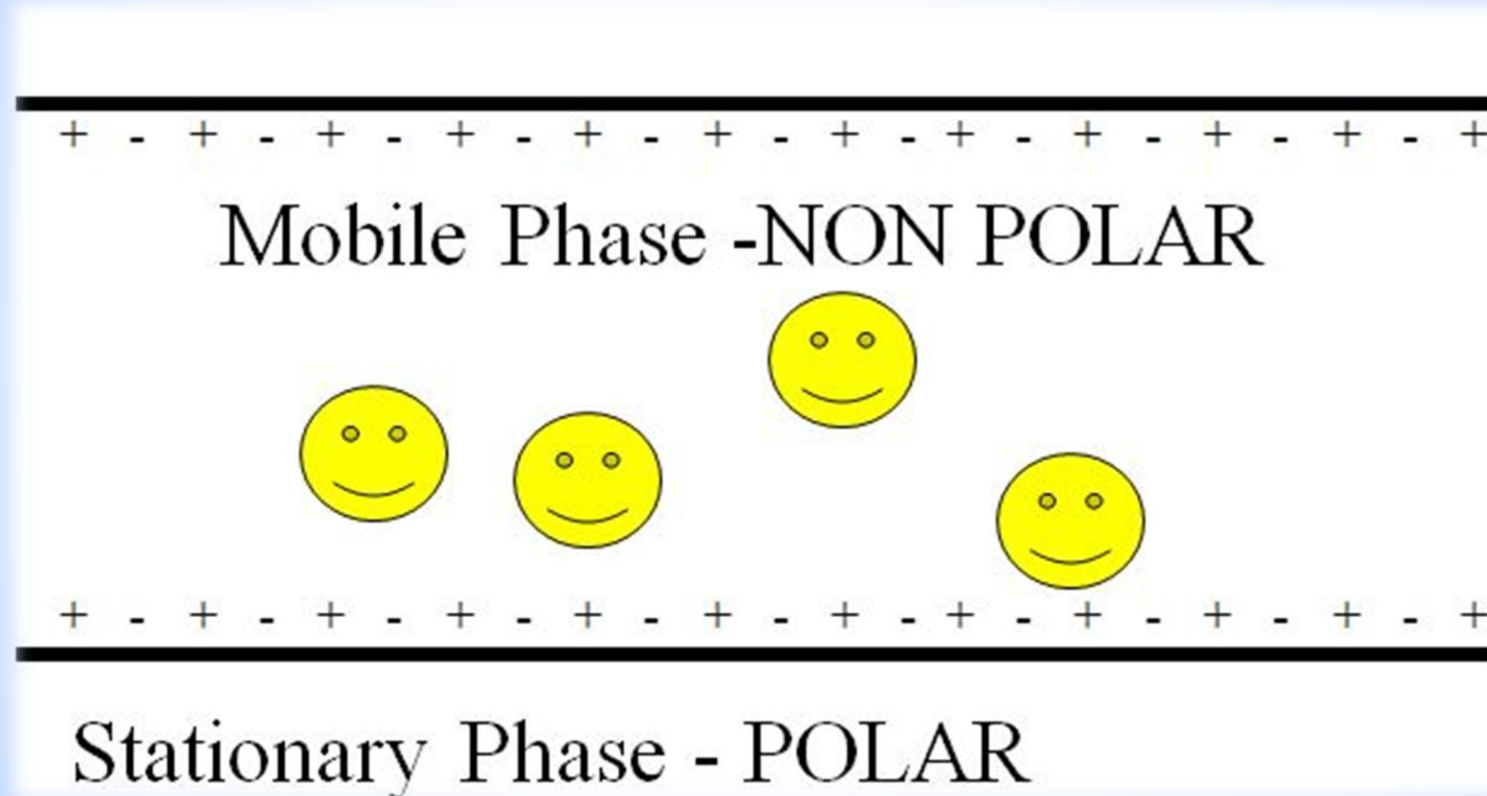
Types of High-Performance Liquid Chromatography (HPLC)

- Normal phase
- Reverse phase
- Ion exchange
- Size exclusion

Normal phase:

- This method separates analytes on the basis of polarity. NP-HPLC uses polar stationary phase and non-polar mobile phase.
- Therefore, the stationary phase is usually silica and typical mobile phases are hexane, methylene chloride, chloroform, diethyl ether, and mixtures of these.
- Polar samples are thus retained on the polar surface of the column packing longer than less polar materials.

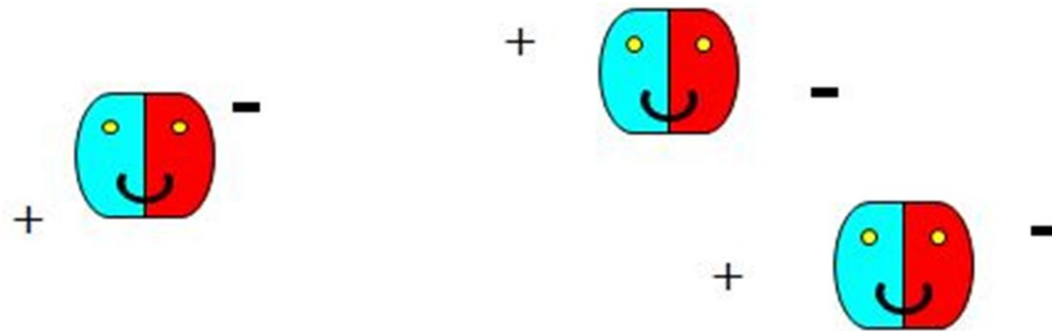
Normal phase



Reverse Phase HPLC

- The stationary phase is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile.
- It works on the principle of hydrophobic interactions hence the more nonpolar the material is, the longer it will be retained.

Reverse Phase HPLC



Reverse Phase HPLC

- Stationary phase: Low polarity
  - Octadecyl group-bonded silical gel (ODS)
- Mobile phase: High polarity
  - Water, methanol, acetonitrile
  - Salt is sometimes added.

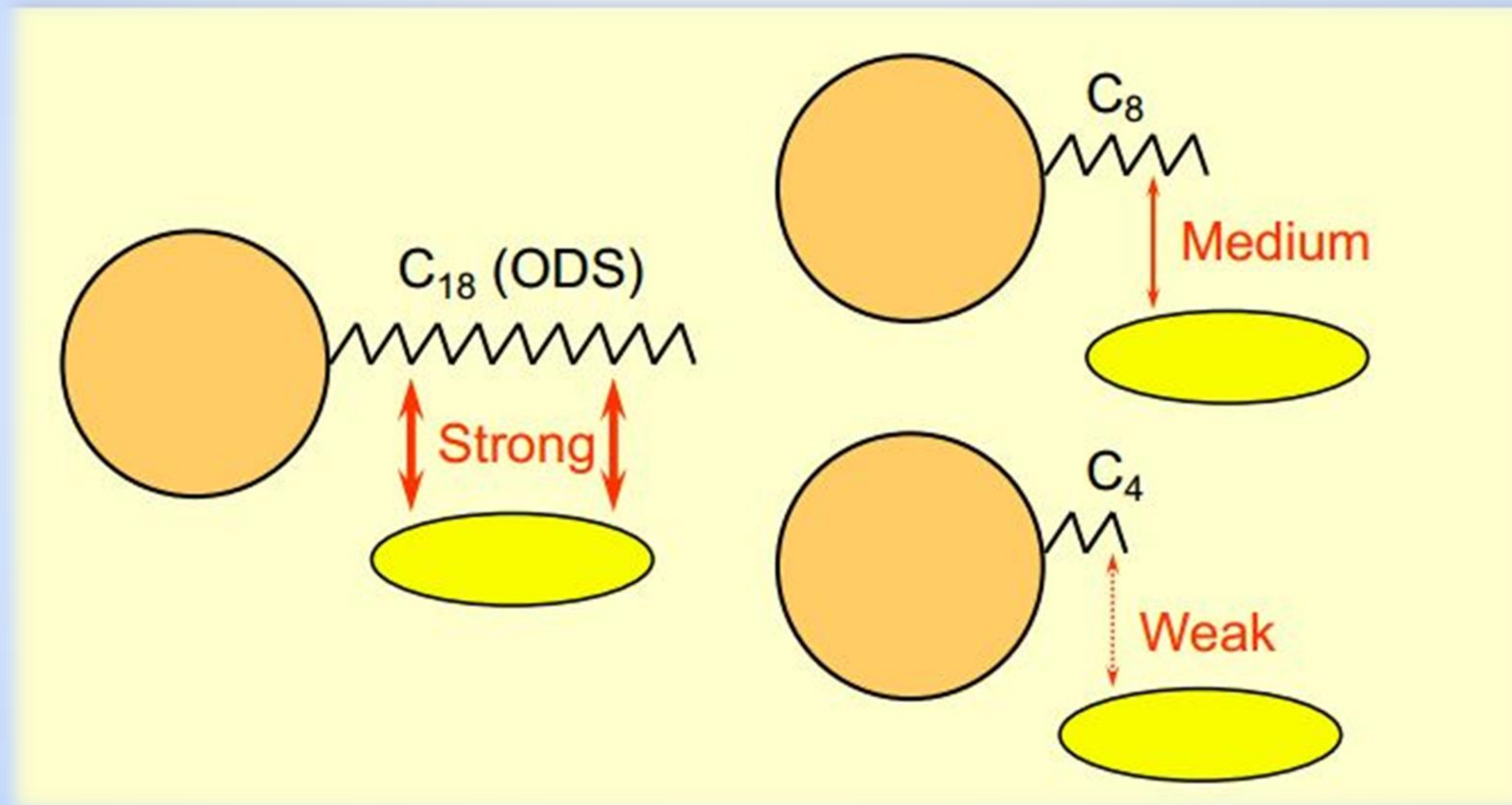
Separetion Column for Reversed Phase

- C_{18} (ODS) type
- C_8 (octyl) type
- C_4 (butyl) type
- Phenyl type
- TMS type
- Cyano type

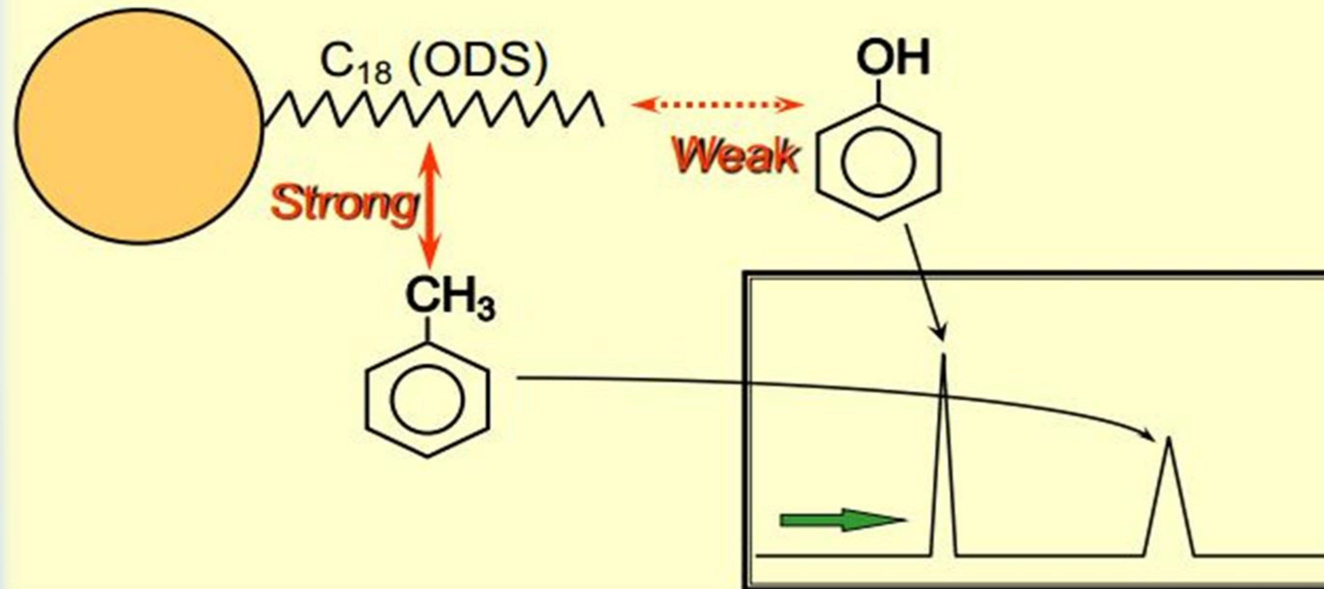
Si
-O-Si
CH_2 CH_2 CH_2 CH_2 CH_2 CH_2 CH_2 CH_2 CH_2
CH_2 CH_2 CH_2 CH_2 CH_2 CH_2 CH_2 CH_2 CH_3

C_{18} (ODS)

Separetion Column for Reversed Phase



Separetion Column for Reversed Phase



Normal phase and Reverse Phase

	Stationary phase	Mobile phase
Normal phase	High polarity (hydrophilic)	Low polarity (hydrophobic)
Reversed phase	Low polarity (hydrophobic)	High polarity (hydrophilic)

Ion-Exchange HPLC

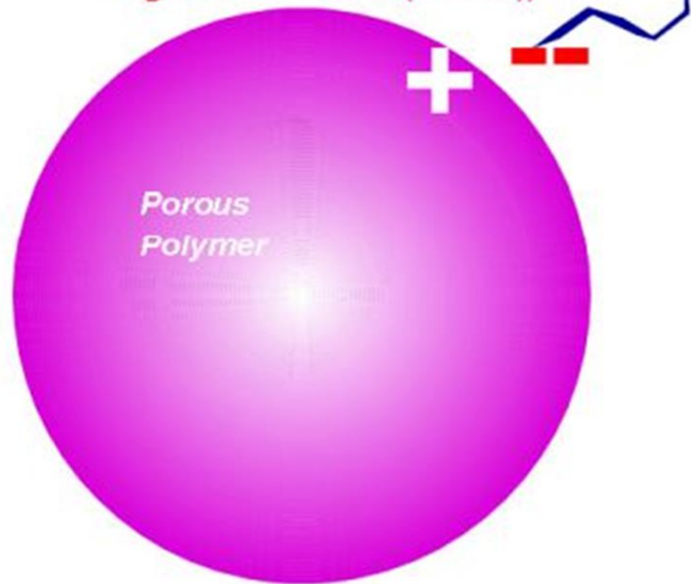
- The stationary phase has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples.
- The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time.

Ion-Exchange HPLC

Chromatography Mode
Ion Exchange

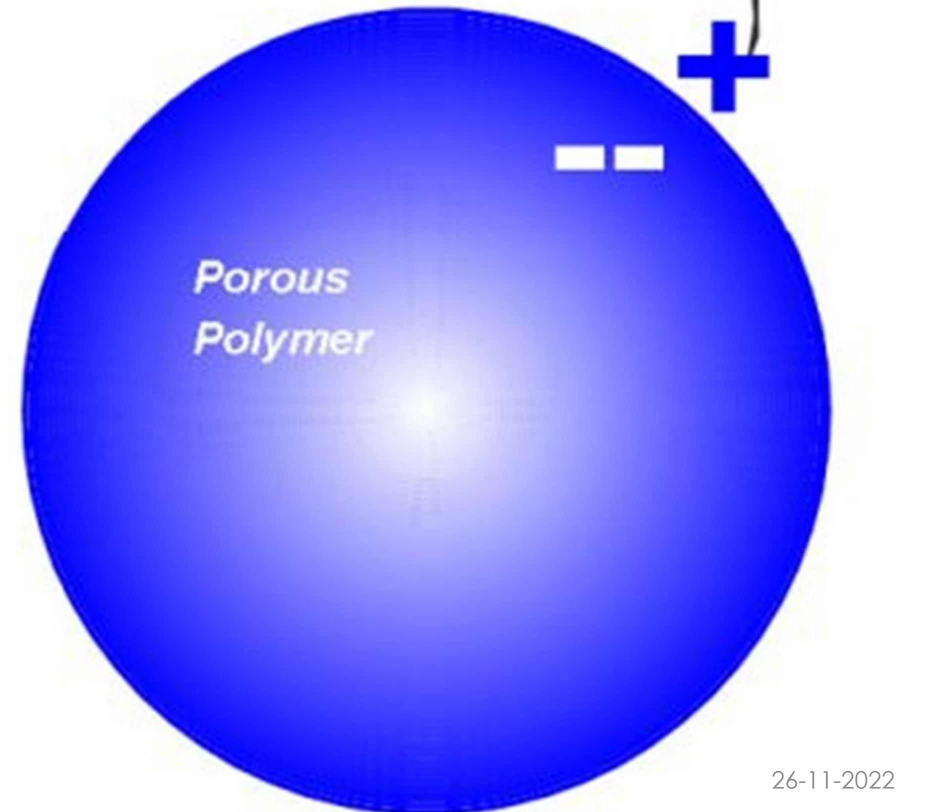
Anion Exchanger

(*Positive* surface attracts *Negative* molecules (anions))



Cation Exchanger

(Negative surface attracts *Positive* molecules (cations))

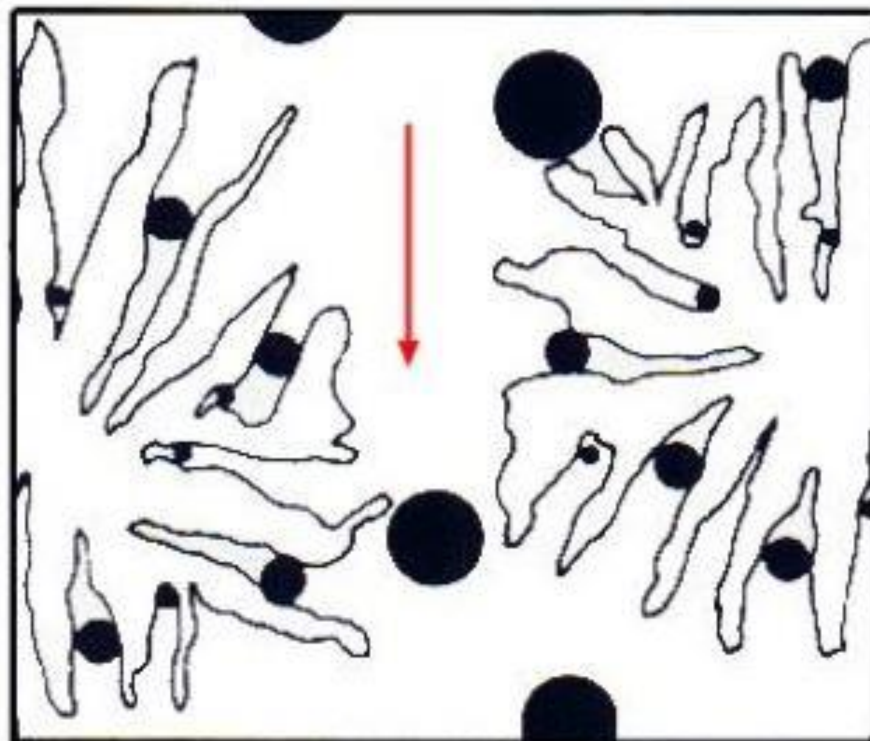


Size-exclusion HPLC

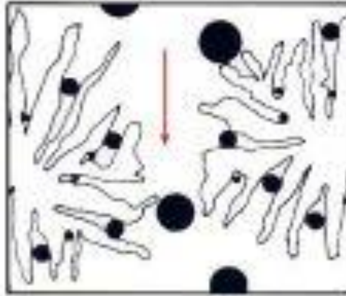
- The column is filled with material having precisely controlled pore sizes, and the particles are separated according to its their molecular size.
- Larger molecules are rapidly washed through the column; smaller molecules penetrate inside the porous of the packing particles and elute later.

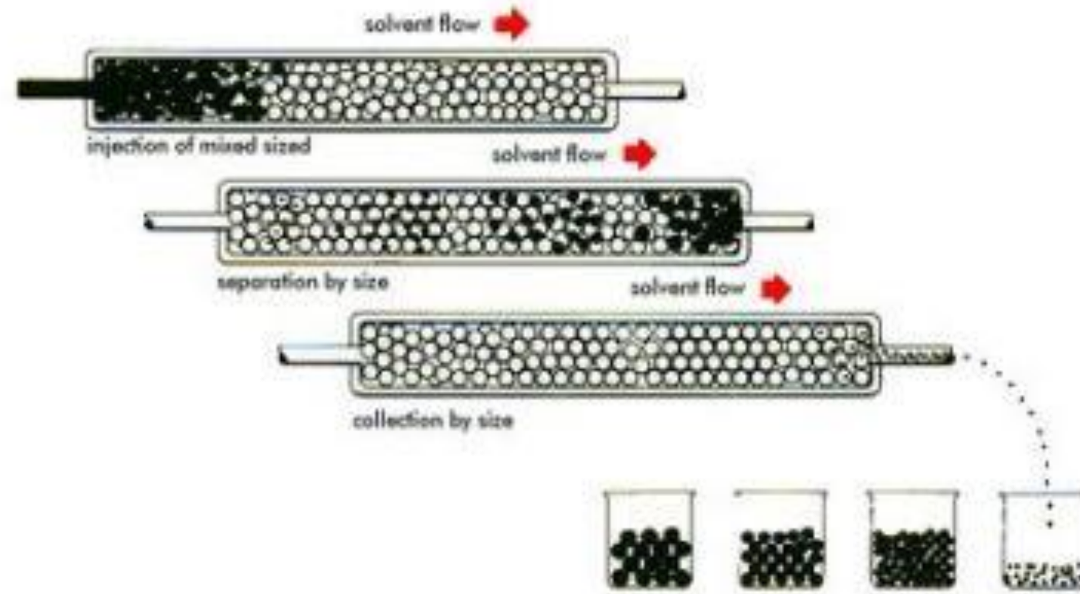
Size-exclusion HPLC

Cross sectional view of porous particle



Size-exclusion HPLC





Molecules of various sizes elute from the column at different rates. The column retains low molecular weight material (small black dots) longer than the high molecular weight material (large black dots). The time it takes for a specific fraction to elute is called its "retention time".

Comparision between HPTLC and HPLC

Sr.no.	HPTLC	HPLC
1	High Performance Thin Layer Chromatography	High Performance Liquid Chromatography
2	Planar Chromatography	Column Chromatography
3	Principle based on Adsorption Chromatography	Principle is based on Adsorption and Partition Chromatography
4	Simultaneous method for test as well as reference material	Not simultaneous method for test as well as reference material
5	It is simple, rapid, reproducible method	It is Tedious method
6	Sample preparation is simple	Sample preparation is complex
7	Limited Flexibility	Extreme Flexibility
8	Semiautomatic Technique	Automatic (Instrumental) Technique
9	Determination of Surface Area	Determination of Retention Time

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Comparision between GC and HPLC

SR.NO.	GC	HPLC
1	Gas Chromatography	High Performance Liquid Chromatography
2	Less resolution	High resolution
3	Limited Flexibility	Extreme Flexibility
4	Determination of Volatile compounds	Determination of Volatile and Non Volatile Compounds

COMPARISION BETWEEN HPLC AND UPLC

Parameters	HPLC Assay	UPLC Assay
Column	XTerra,C× 18,50 4.6mm	AQUITY UPLC BEH C18,50 ×2.1mm
Particle size	4µm particles	1.7µm particles
Flow rate	3.0ml per min	0.6ml per min
Injection volume	20µl	3µl partial loop fill or 5µl full loop fill
Total run time	10min	1.5min
Theoretical Plate count	2000	7500

61

Parameters	HPLC Assay	UPLC Assay
Lower limit of quantization	0.2µg/ml	0.054µl/ml
Total solvent consumption	Acetonitrile:10.5ml, water:21ml	Acetonitrile:0.53ml, water:0.66ml
Delay volume	720µl	110µl
Column temperature	° 30C	° 65C
Maximum back pressure	40-35Mpa less	103.5Mpa more
Resolution	Less	High
Method development cost	High	Low

WHY USE HPLC

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

Applications of HPLC

The information that can be obtained by HPLC includes resolution, identification and quantification of a compound. It also aids in chemical separation and purification. The other applications of HPLC include :

Pharmaceutical Applications

- *To control drug stability.*
- *Tablet dissolution study of pharmaceutical dosages form.*
- *Pharmaceutical quality control.*

Cont...

Environmental Applications

- *Detection of phenolic compounds in drinking water.*
- *Bio-monitoring of pollutants.*

Applications in Forensics

- *Quantification of drugs in biological samples.*
- *Identification of steroids in blood, urine etc.*
- *Forensic analysis of textile dyes.*
- *Determination of cocaine and other drugs of abuse in blood, urine etc.*

Cont....

Food and Flavour

- *Measurement of Quality of soft drinks and water.*
- *Sugar analysis in fruit juices.*
- *Analysis of polycyclic compounds in vegetables.*
- *Preservative analysis.*

Applications in Clinical Tests

- Urine analysis, antibiotics analysis in blood.
- Analysis of bilirubin, biliverdin in hepatic disorders.
- Detection of endogenous Neuropeptides in extracellular fluid of brain etc.

Fields in Which High Performance Liquid Chromatography Is Used

- Biogenic substances
  - Sugars, lipids, nucleic acids, amino acids, proteins, peptides, steroids, amines, etc.
- Medical products
  - Drugs, antibiotics, etc.
- Food products
  - Vitamins, food additives, sugars, organic acids, amino acids, polyphenols, biogenic amins
- Environmental samples
  - Inorganic ions
  - Hazardous organic substances, etc.
- Organic industrial products
  - Synthetic polymers, additives, surfactants, etc.

Advantages of HPLC

- Separations fast and efficient (high resolution power)
- Continuous monitoring of the column effluent
- It can be applied to the separation and analysis of very complex mixtures
- Accurate quantitative measurements.
- Repetitive and reproducible analysis using the same column.
- Adsorption, partition, ion exchange and exclusion column separations are excellently made.

Limitations

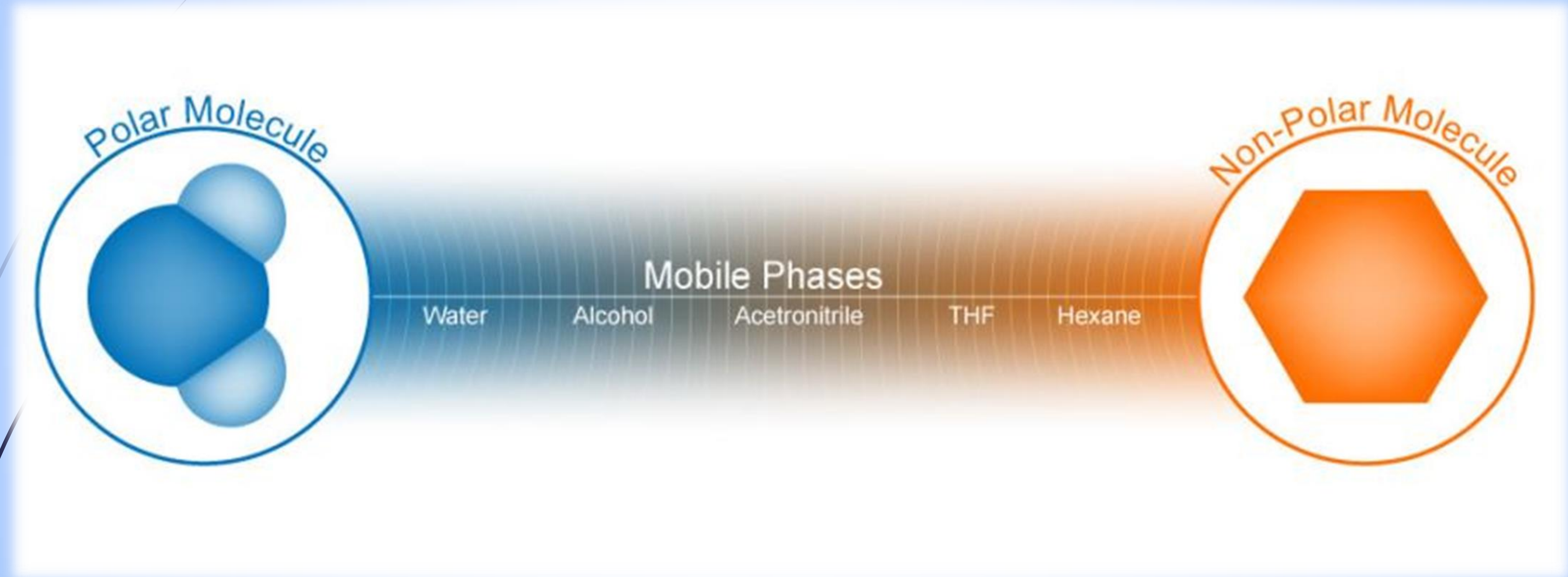
- **Cost:** Despite its advantages, HPLC can be costly, requiring large quantities of expensive organics.
- **Complexity**
- HPLC does have **low sensitivity** for certain compounds, and some cannot be detected as they are irreversibly adsorbed.
- Volatile substances are better separated by gas chromatography.

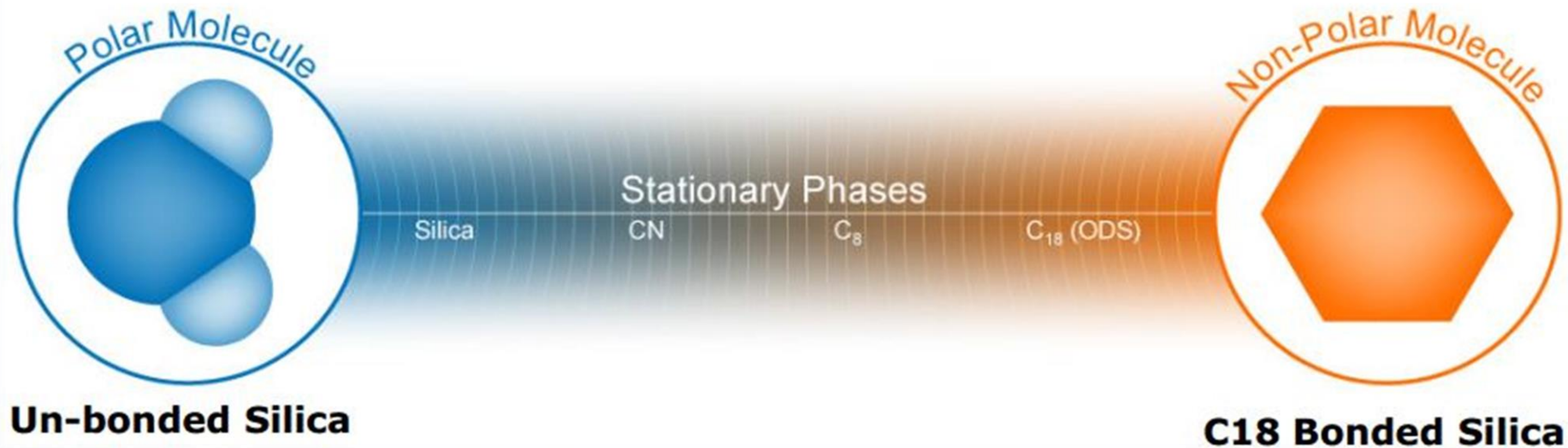
Report

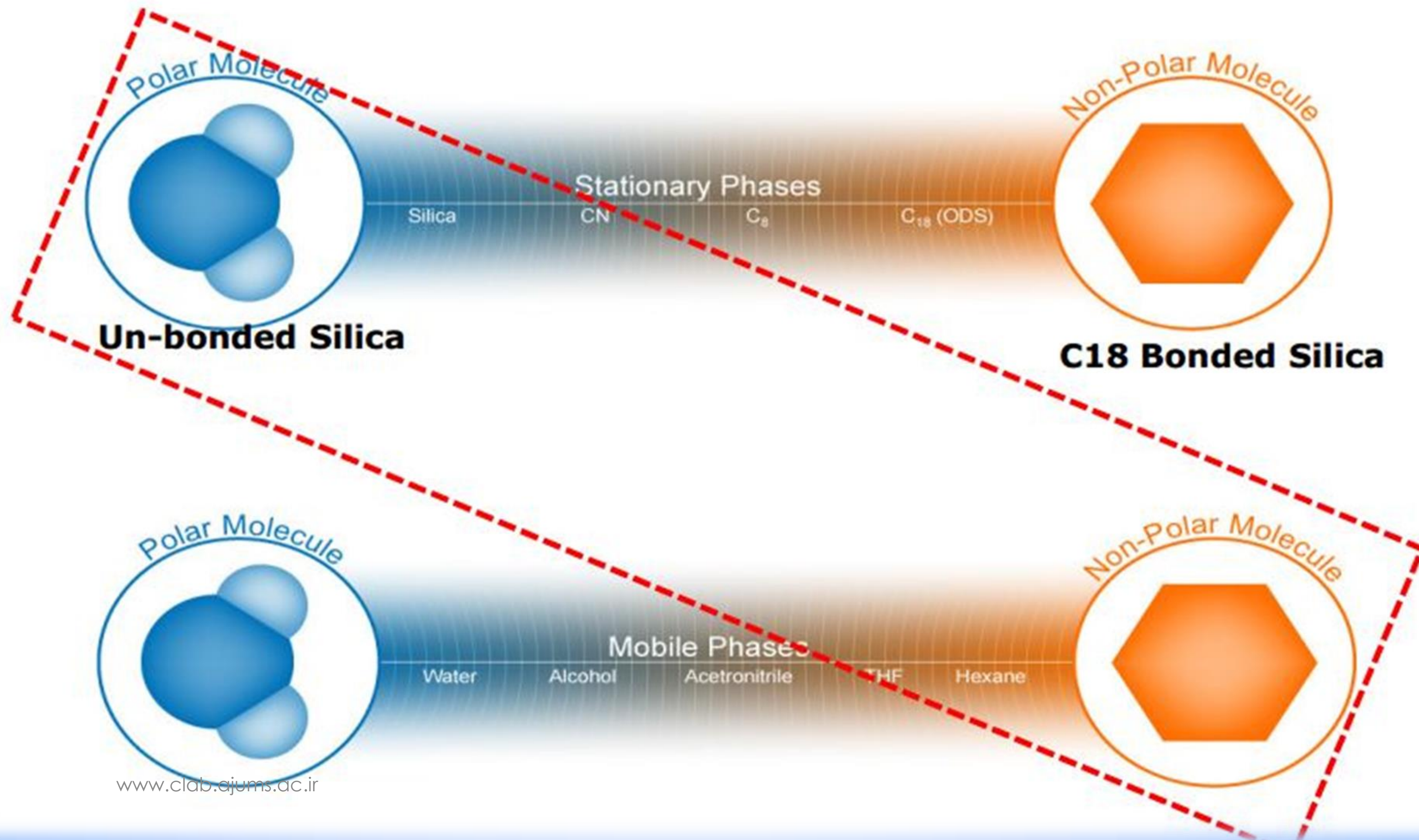
	Peak Name	RT (min)	Area (V *sec)	% Area	Height (V)	% Height	Amount	Units
1	benzoat	10.114	5111806	77.83	321740	79.52	80.000	ppm
2	sorbat	12.304	1456027	22.17	82881	20.48	20.000	ppm

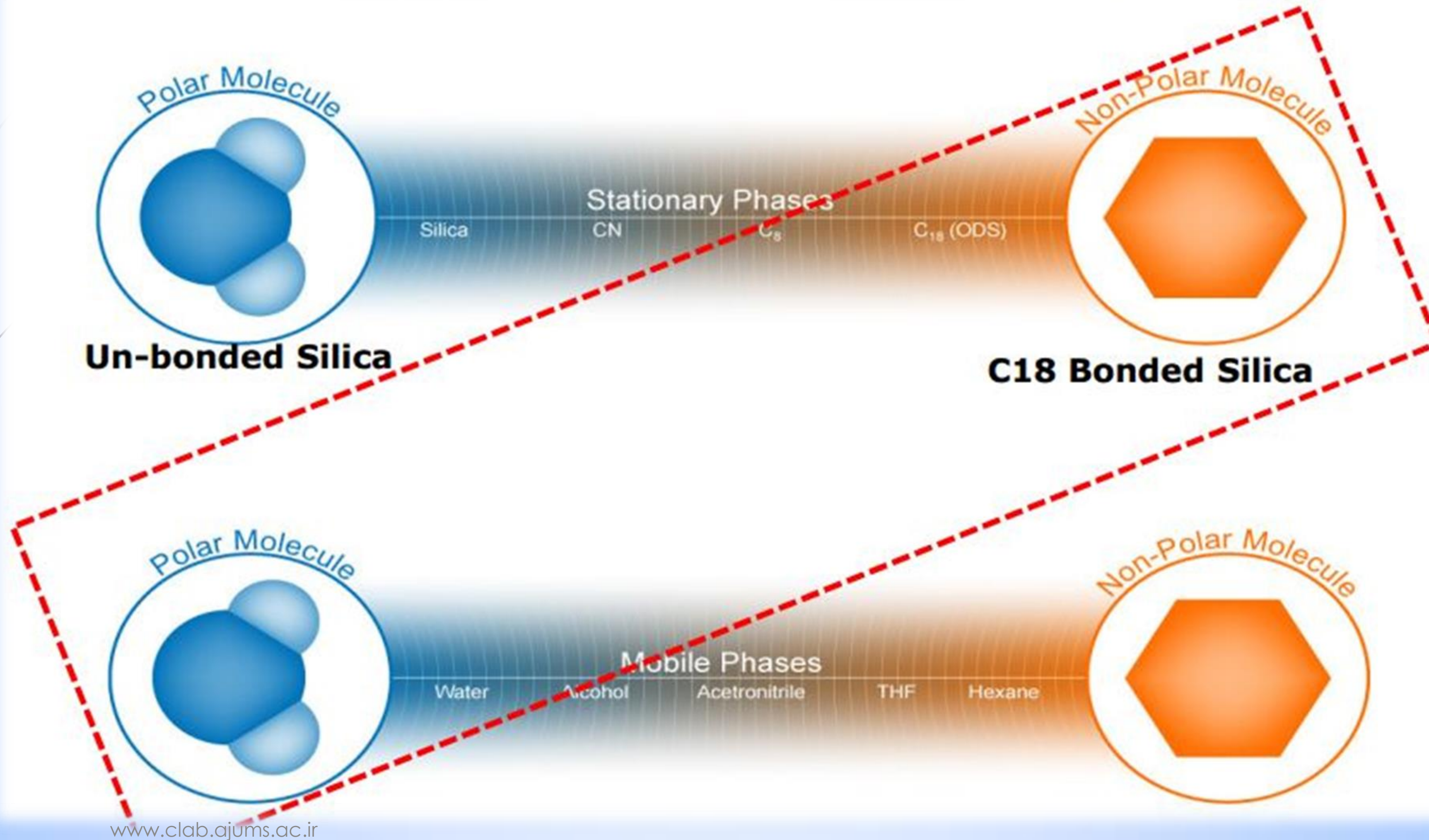
Part II

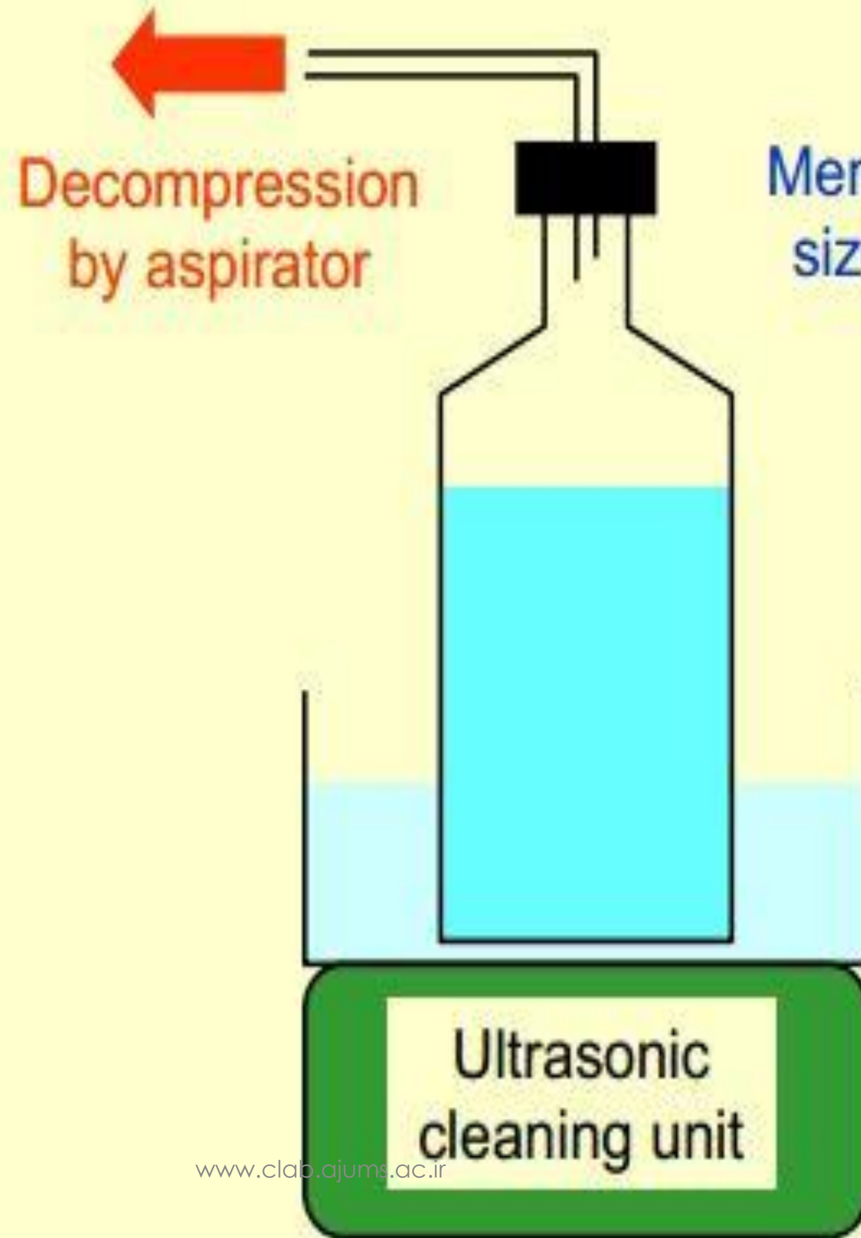
- Characteristic of Molecules based on their Structure and Electron Charge Distribution

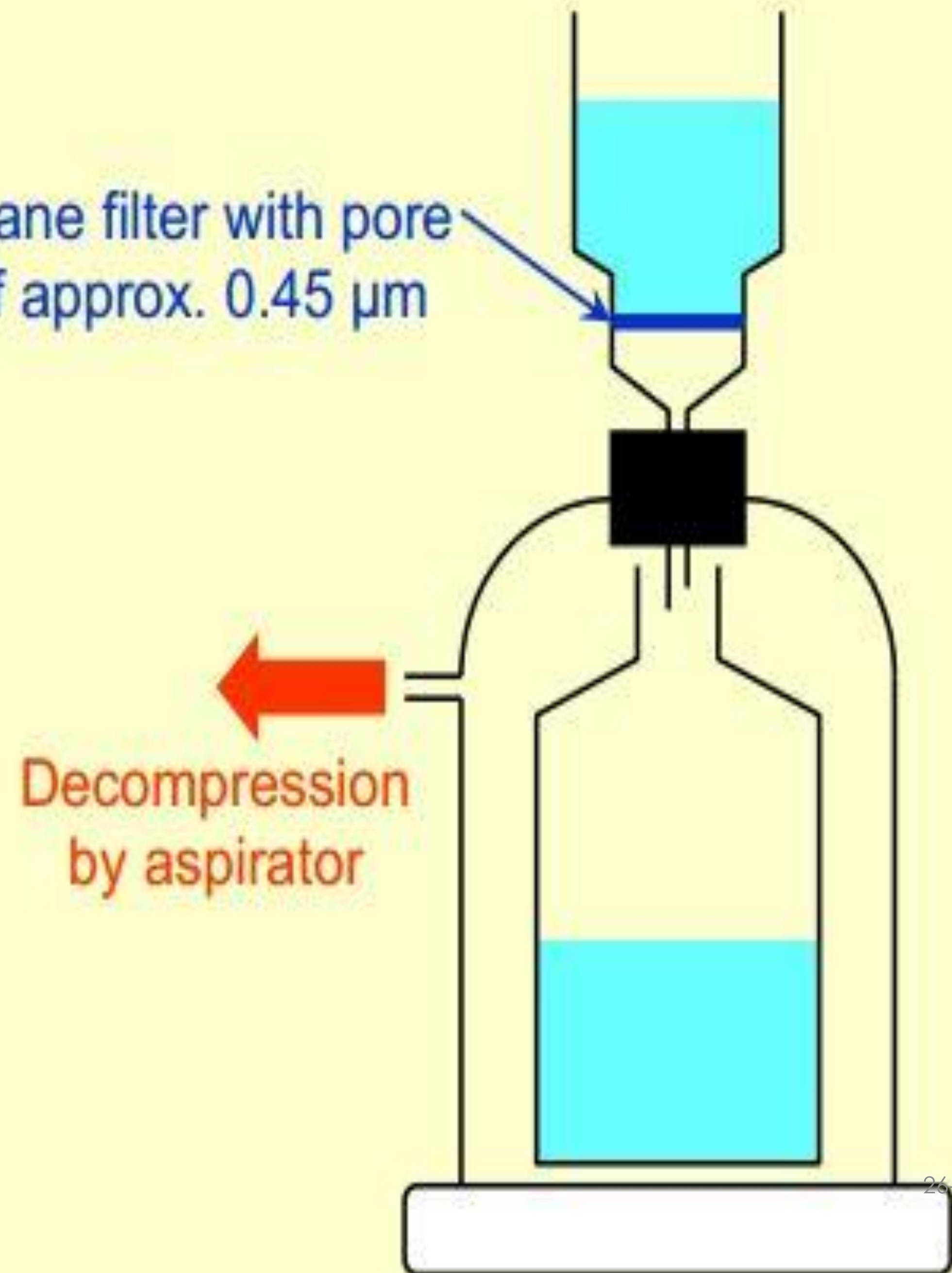

Polar Molecule
Mobile Phases
Water
Alcohol
Acetronitrile
THF
Hexane
Non-Polar Molecule

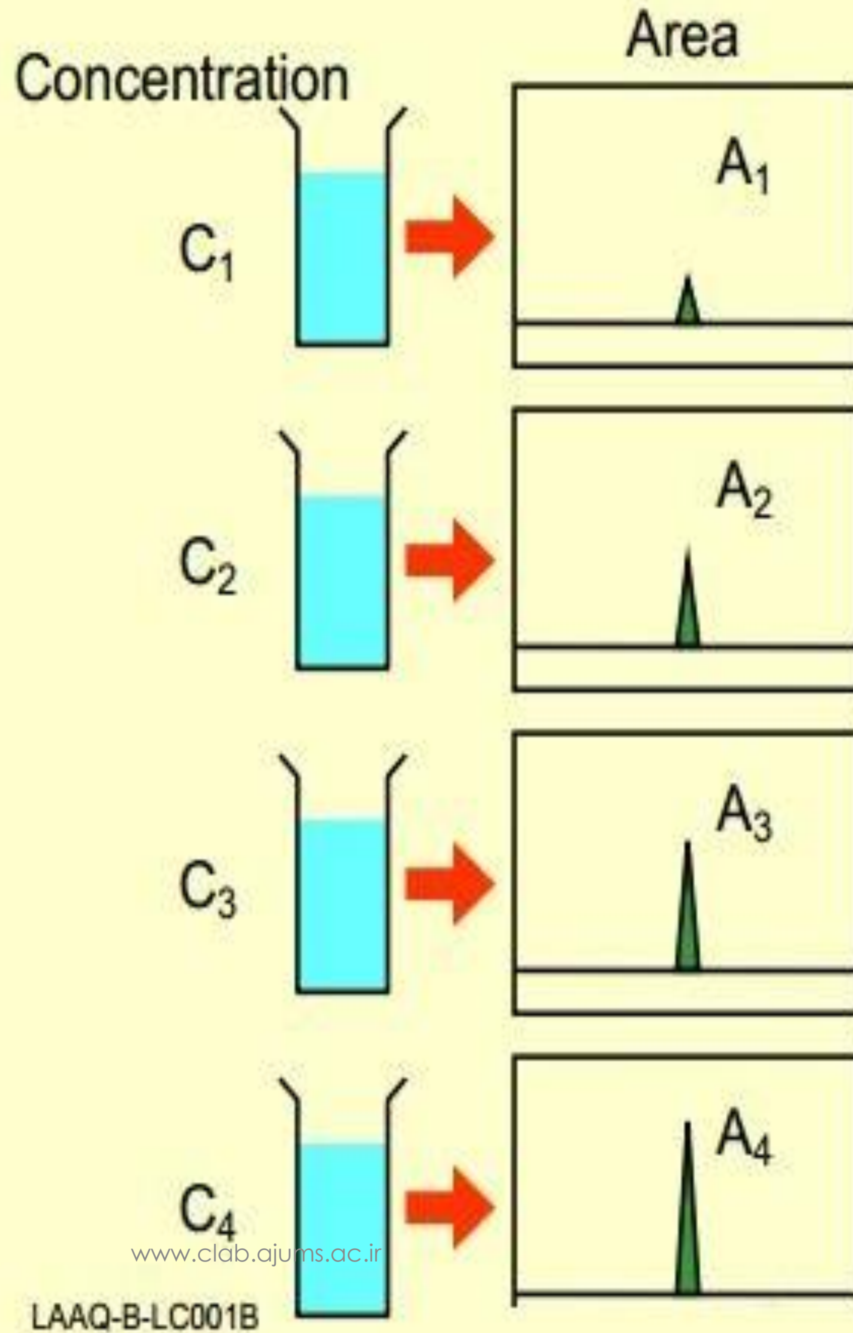

Polarity Scale-
Particle/Stationary Phase
Waters
THE SCIENCE OF WHAT'S POSSIBLE.
Polar Molecule
Non-Polar Molecule
Stationary Phases
Silica
CN
C8
C18 (ODS)
Un-bonded Silica
C18 Bonded Silica


Chromatography Mode
Normal Phase
Waters
THE SCIENCE OF WHAT'S POSSIBLE.
Polar Molecule
Non-Polar Molecule
Stationary Phases
Silica
CN
C8
C18 (ODS)
Un-bonded Silica
C18 Bonded Silica
Polar Molecule
Non-Polar Molecule
Mobile Phases
Water
Alcohol
Acetronitrile
THF
Hexane

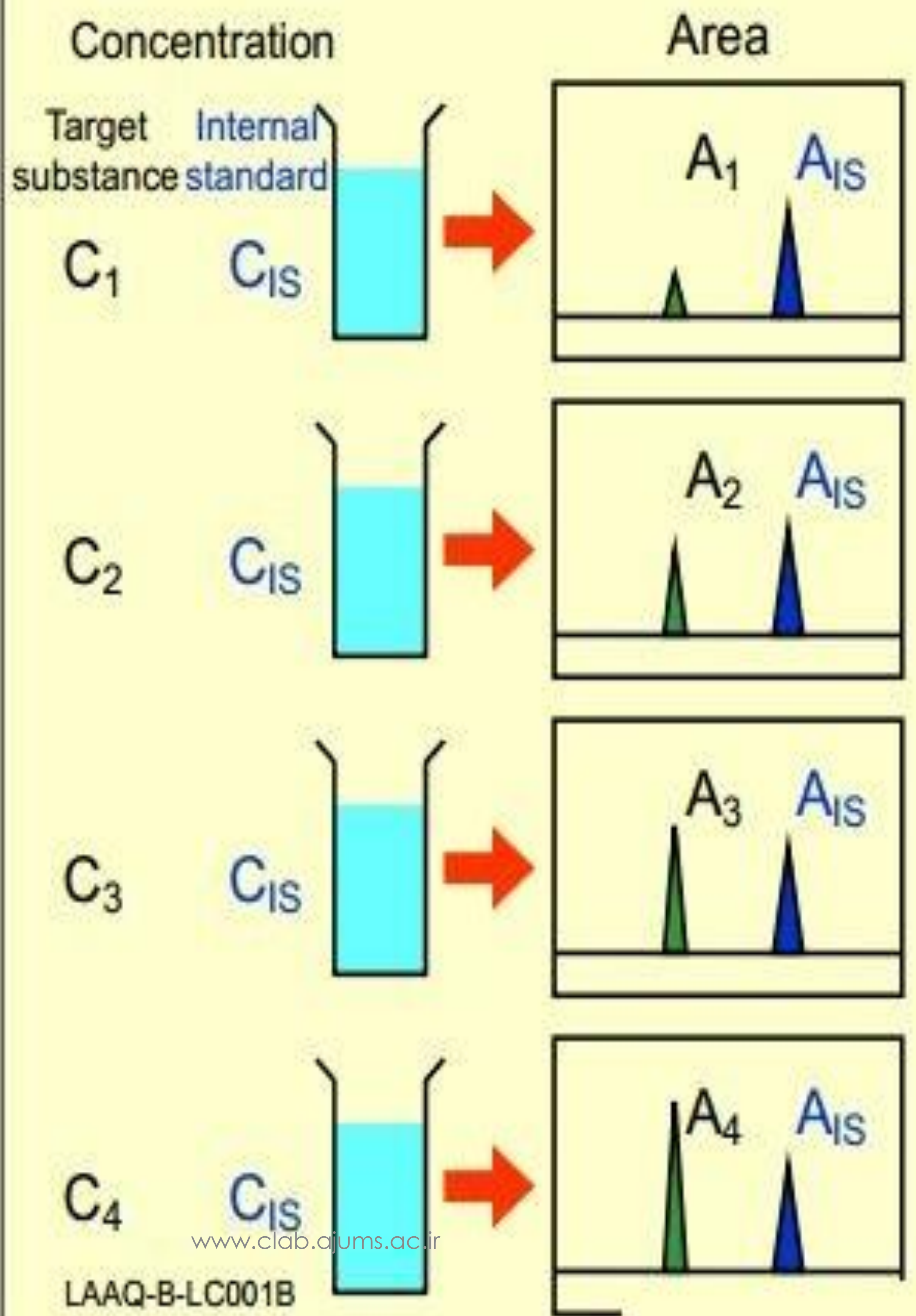

Chromatography Mode
Reversed - Phase
Waters
THE SCIENCE OF WHAT'S POSSIBLE.
Polar Molecule
Non-Polar Molecule
Stationary Phases
Silica
CN
C8
C18 (ODS)
Un-bonded Silica
C18 Bonded Silica
Polar Molecule
Non-Polar Molecule
Mobile Phases
Water
Alcohol
Acetronitrile
THF
Hexane


Decompression
by aspirator
Ultrasonic
cleaning unit


Membrane filter with pore
size of approx. 0.45 μm
Decompression
by aspirator



Concentration
Area
C_1
A_1
C_2
A_2
C_3
A_3
C_4
A_4
LAAQ-B-LC001B
Calibration curve
Peak area
A_4
A_3
A_2
A_1
C_1
C_2
C_3
C_4
Concentration


Concentration
Area
Target substance
Internal standard
C1 CIS
A1 AIS
C2 CIS
A2 AIS
C3 CIS
A3 AIS
C4 CIS
A4 AIS
LAAQ-B-LC001B

Area for target substance / Area for internal standard
Calibration curve
A4/AIS
A3/AIS
A2/AIS
A1/AIS
C1/CIS C2/CIS C3/CIS C4/CIS
Concentration of target substance /

Calculating Column Efficiency

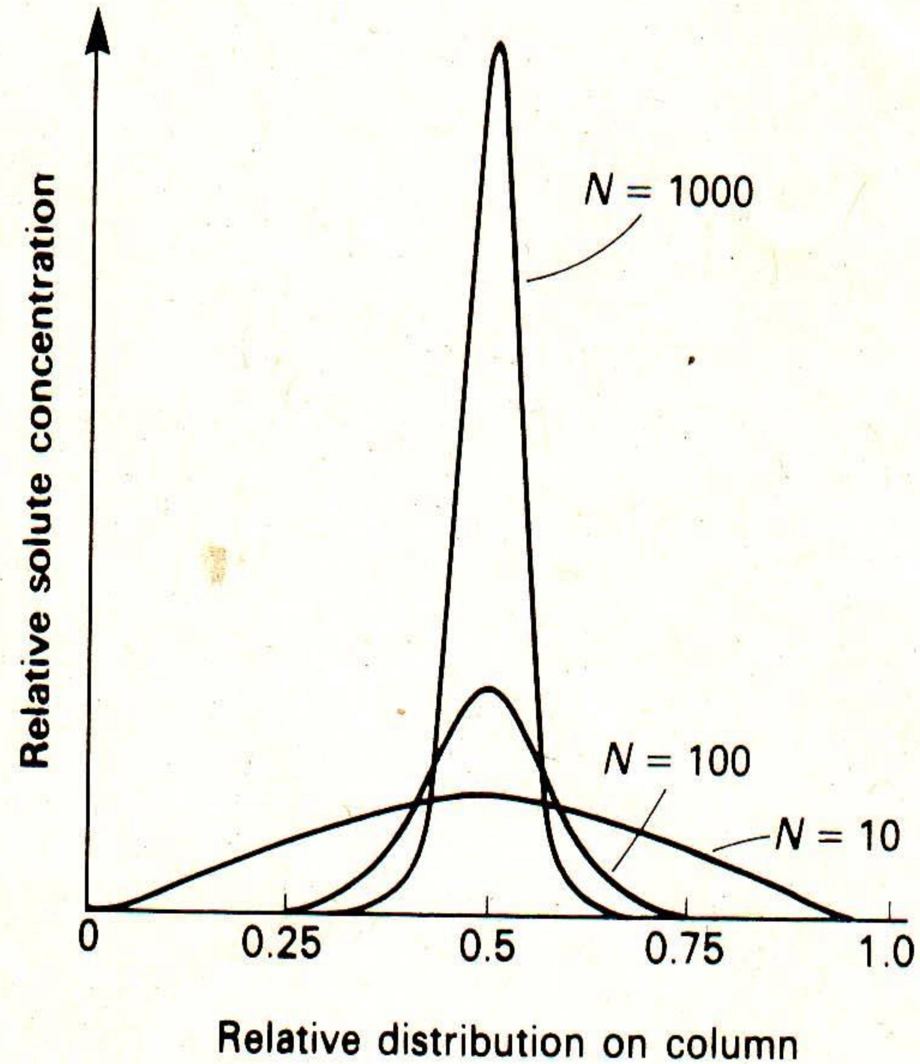
- Peak width at half height is used to calculate column efficiency.

- $N = 5.54 \, (T / W)^2$

Where N = theoretical plates

W = Width at half height T = Retention time

5.54 is a constant based on the normal distribution



Diagrammatic effect of the number of theoretical plates (N) on the shape of the solute.

Quantitative analysis

- Quantitation performed with peak area or height.
- Calibration curve created beforehand using a standard.
  - External standard method
  - Internal standard method
  - Standard addition method

External standard method

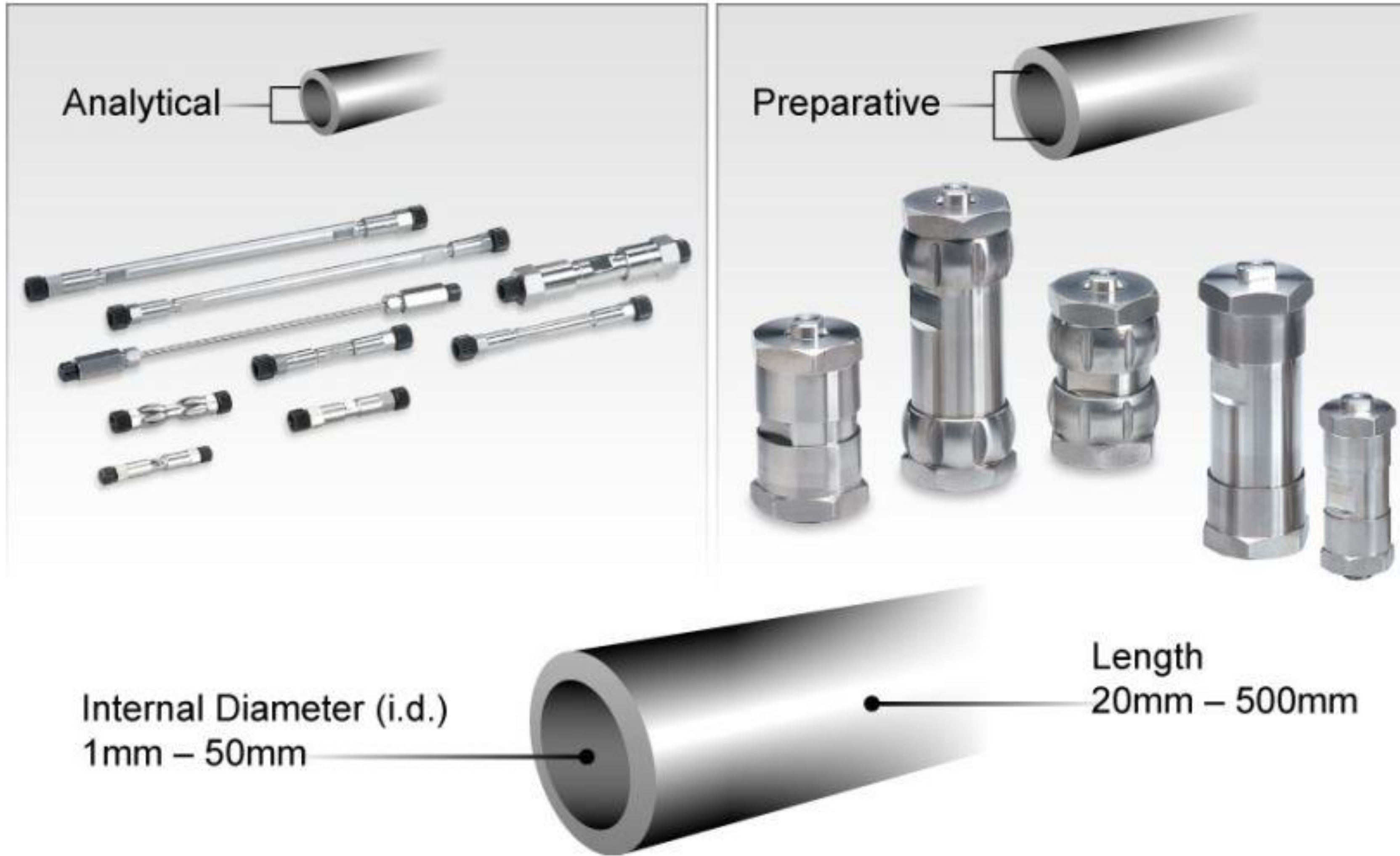
- The simplest method
- The accuracy of this method is dependent on the reproducibility of the injection volume.
- **Standard solutions of known concentrations of the compound of interest are prepared with one standard that is similar in concentration to the unknown.**
- A fixed amount of sample is injected.
- Peak height or area is then plotted versus the concentration for each compound. The plot should be linear and go through the origin.
- The concentration of the unknown is then determined according to the following formula:

$$\text{Conc.}_{\text{unknown}} = \left(\frac{\text{Area}_{\text{unknown}}}{\text{Area}_{\text{known}}} \right) \text{conc.}_{\text{known}}$$

Internal standard method

- The internal standard method tends to yield the most accurate and precise results
- **An equal amount of an internal standard, a component that is not present in the sample, is added to both the sample and standard solutions.**
- The internal standard selected should be chemically similar, to have similar retention time and derivatize similarly to the analyte, to be stable and does not interfere with any of the sample components.
- The internal standard should be added before any preparation of the sample so that extraction efficiency can be evaluated.
- Quantification is achieved by using ratios of peak height or area of the component to the internal standard.

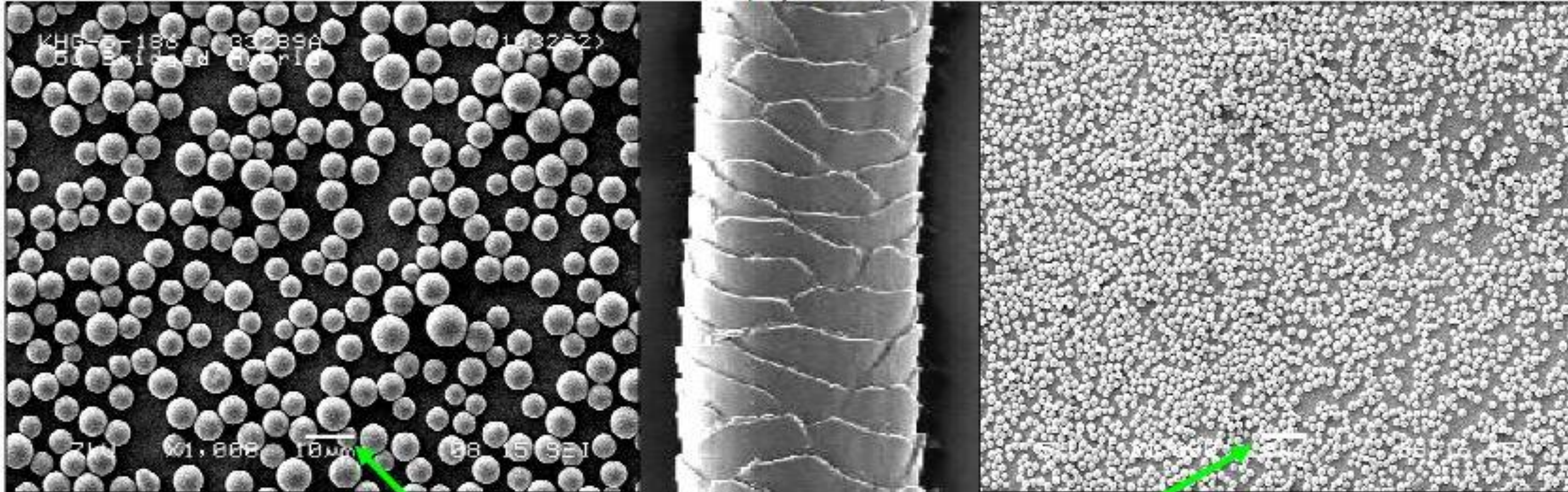
$$\text{Conc.}_{\text{unknown}} = \left(\frac{\text{Area}_{\text{Internal Std in known}}}{\text{Area}_{\text{Internal Std in unknown}}}\right) \times \left(\frac{\text{Area}_{\text{unknown}}}{\text{Area}_{\text{known}}}\right) \times \text{conc.}_{\text{known}}$$


Column Dimension
Waters
THE SCIENCE OF WHAT'S POSSIBLE.
Analytical
Preparative
Internal Diameter (i.d.)
1mm – 50mm
Length
20mm – 500mm

Waters Particle Technology

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

60 µm Human Hair
(very fine hair)



5 µm
Analytical Particles
(can fit 12 across hair)

1.7 µm
ACQUITY UPLC® Particles
(can fit 33 across hair)

Images are on the same scale (Bar = 10 µm)

Particle Size and Mechanical Separating Power*

Waters
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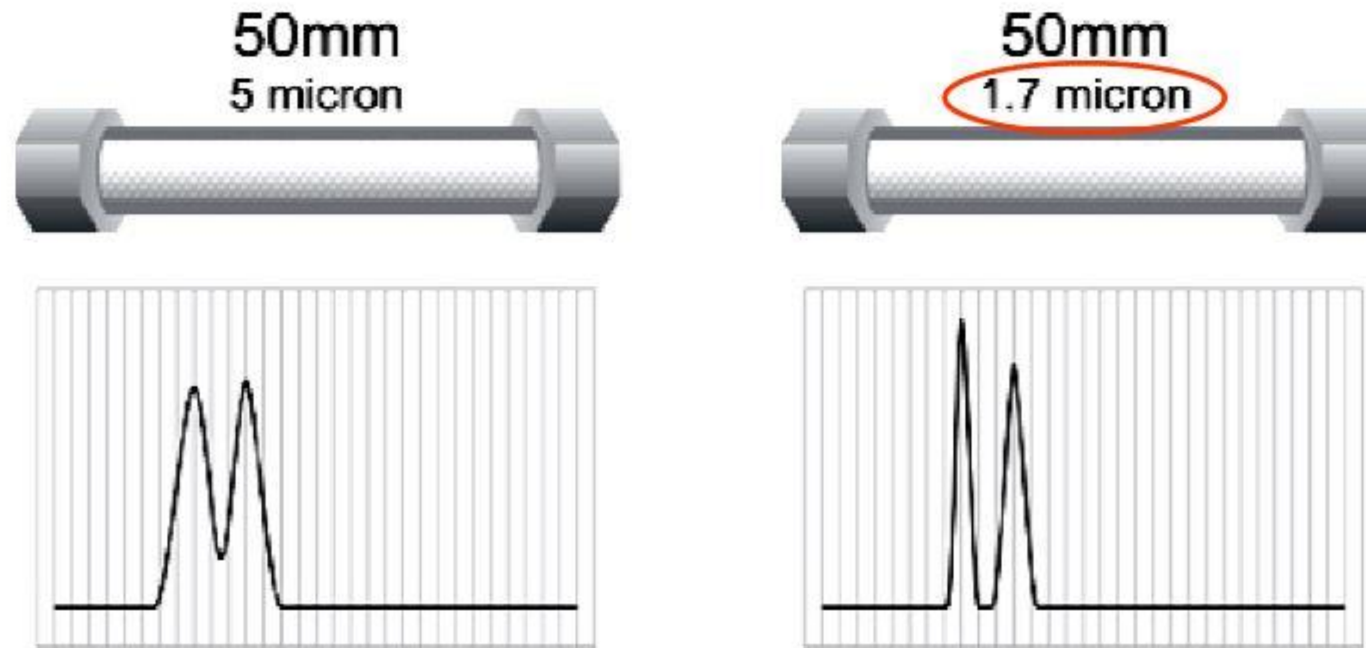
Columns contain the same packing material chemistry, are the same length with the same mobile phase. ***One column has particles which are a third the size.***



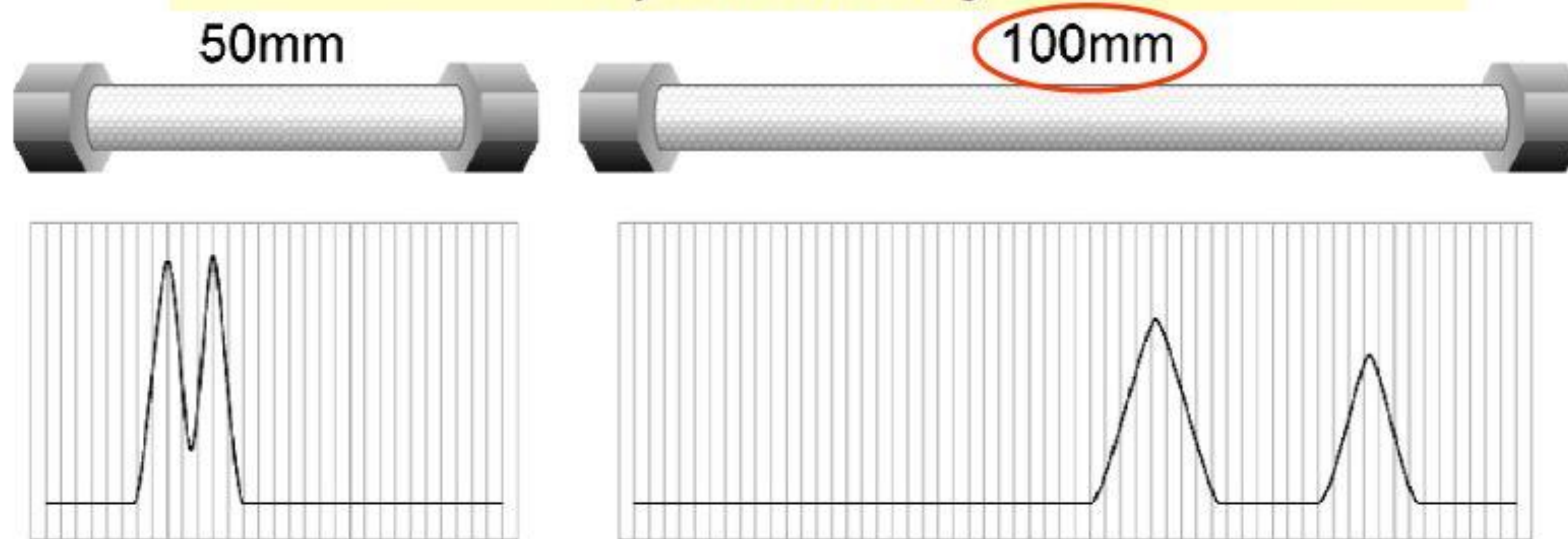
Smaller particle sizes provide for a better separation with the <u>same run time</u>. However, back pressure will increase.

* This is also called "Efficiency"

Column Length and Mechanical Separating Power*

Waters
THE SCIENCE OF WHAT'S POSSIBLE.

Columns contain the same packing material, same particle size and same mobile phase, *only one is twice as long*

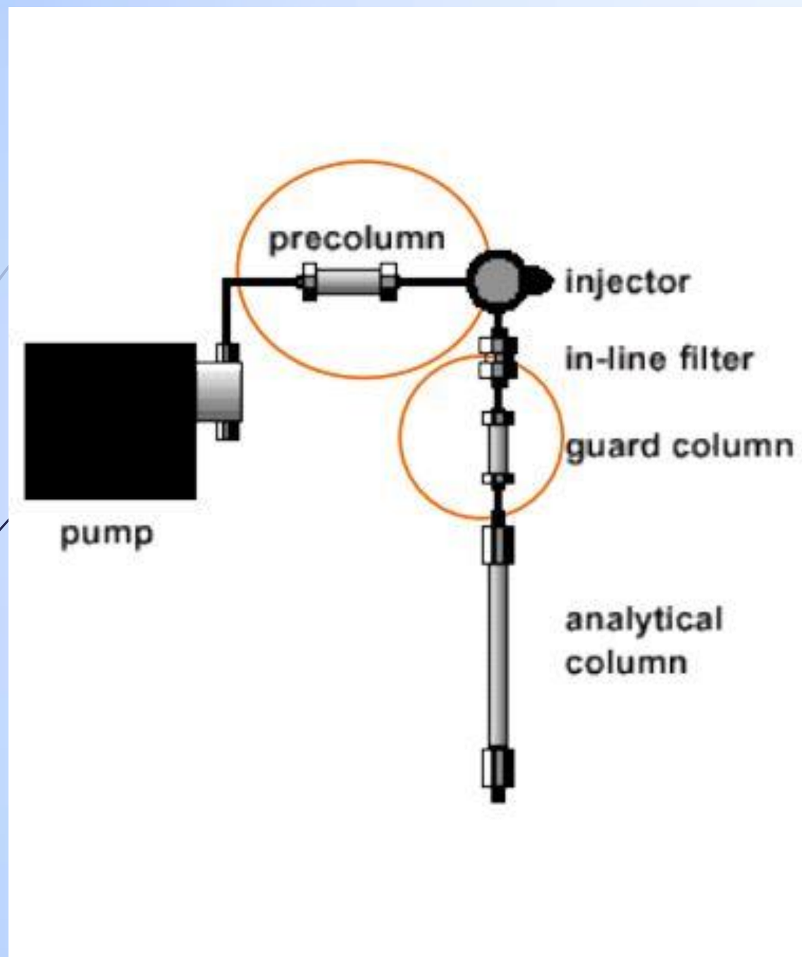


Additional column length does provide a better separation.

However, several "costs" are incurred: ***more time (2X) for the analysis, use more solvent, increased back pressure and the longer column costs more to buy.***

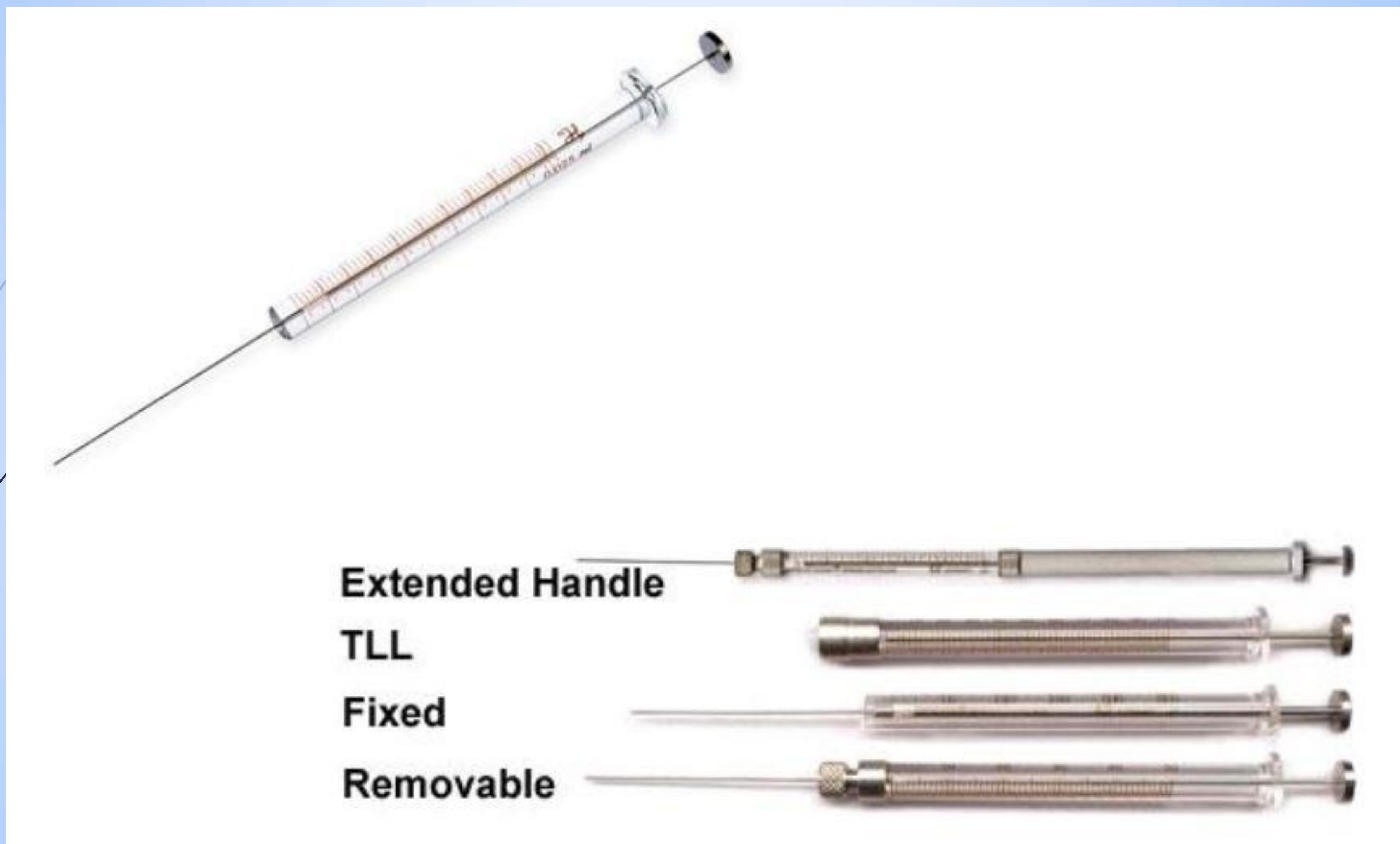
A better approach, would be to try a different particle chemistry/mobile phase combination or a smaller particle size that can create the separation in less time.

* This is also called "Efficiency"

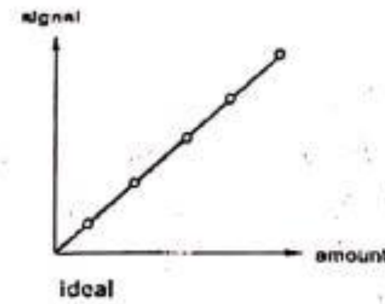

precolumn
injector
in-line filter
guard column
pump
analytical
column

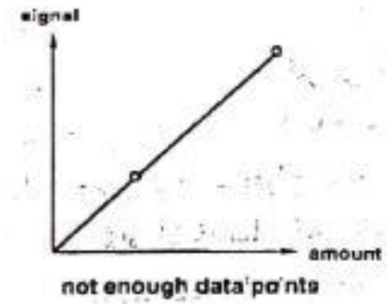
HPLC Guard

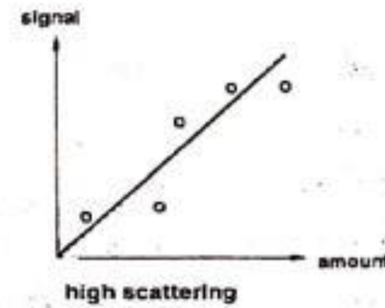
VANGUARD

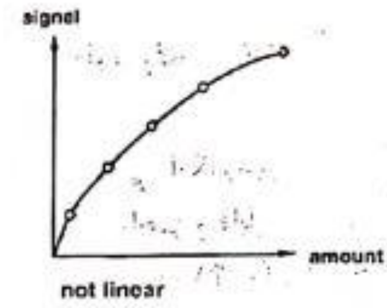

Extended Handle
TLL
Fixed
Removable

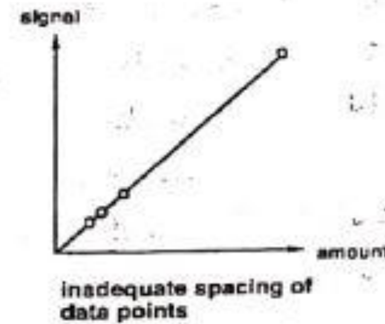
Calibration Curve

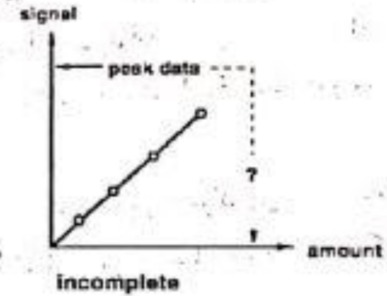


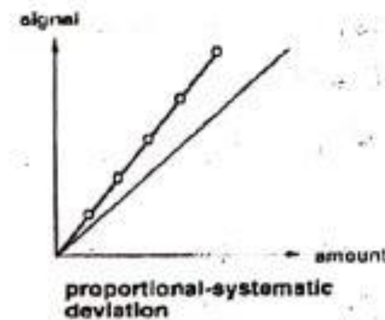


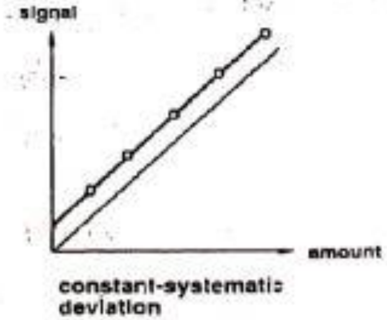




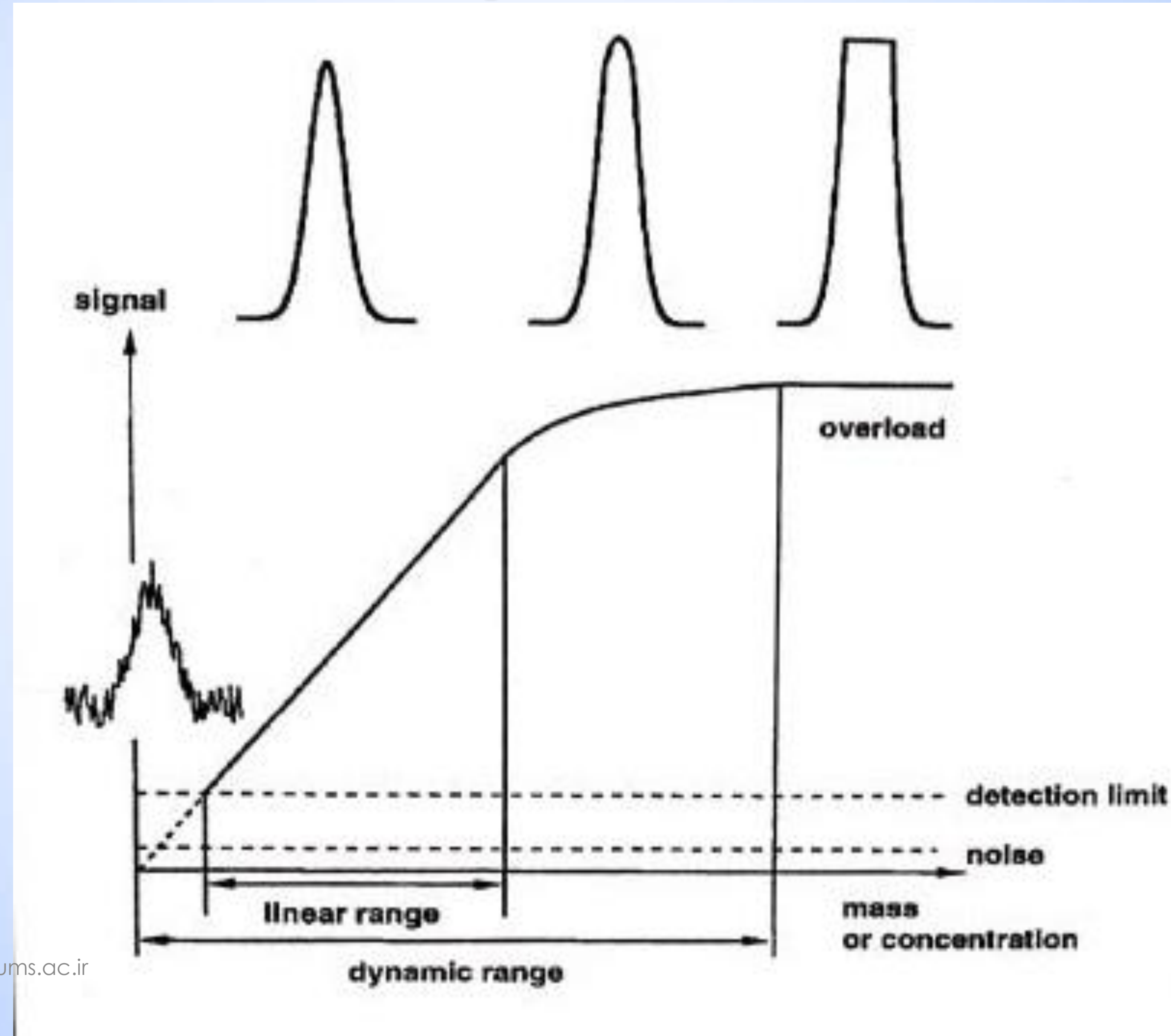




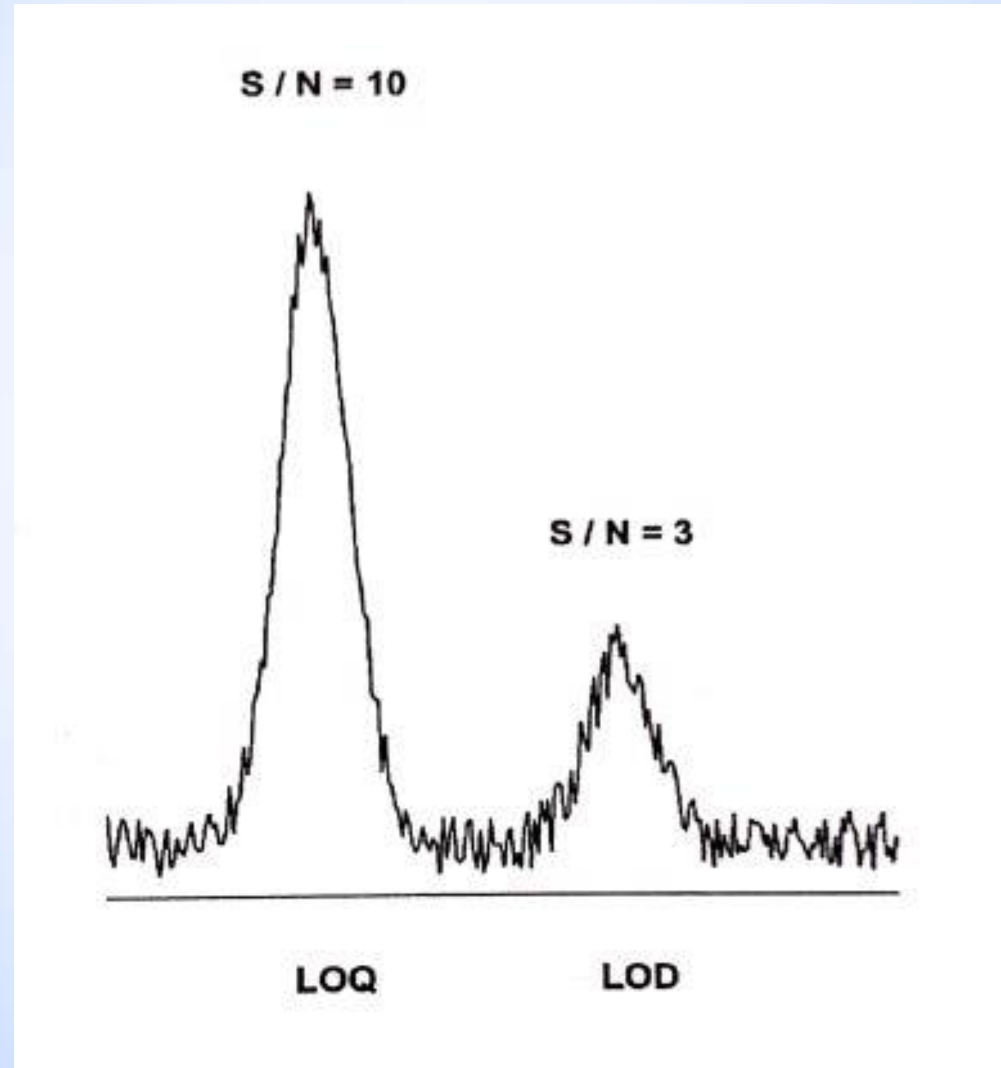




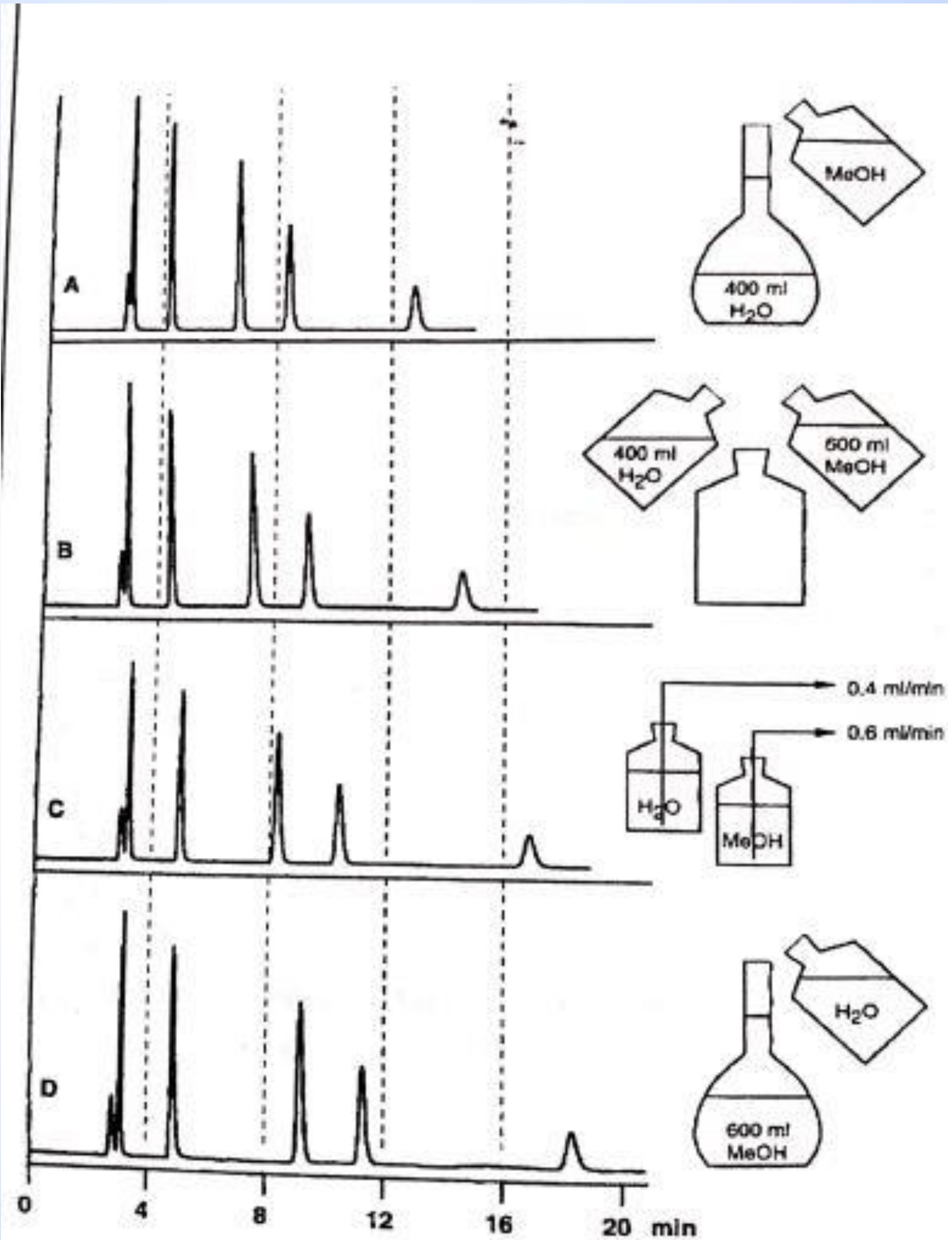
The curve and response of the detector



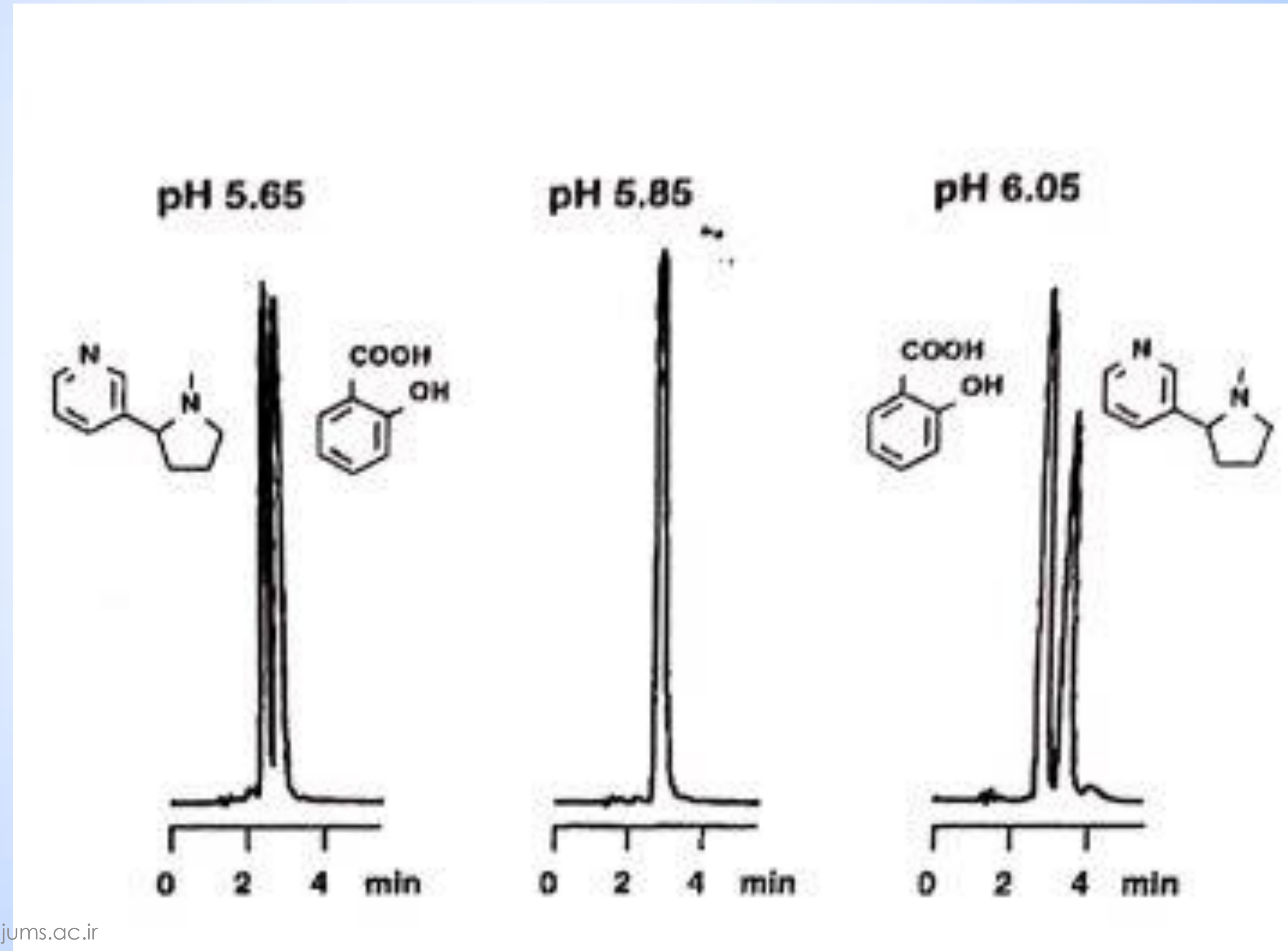
Baseline vibrations - Noise



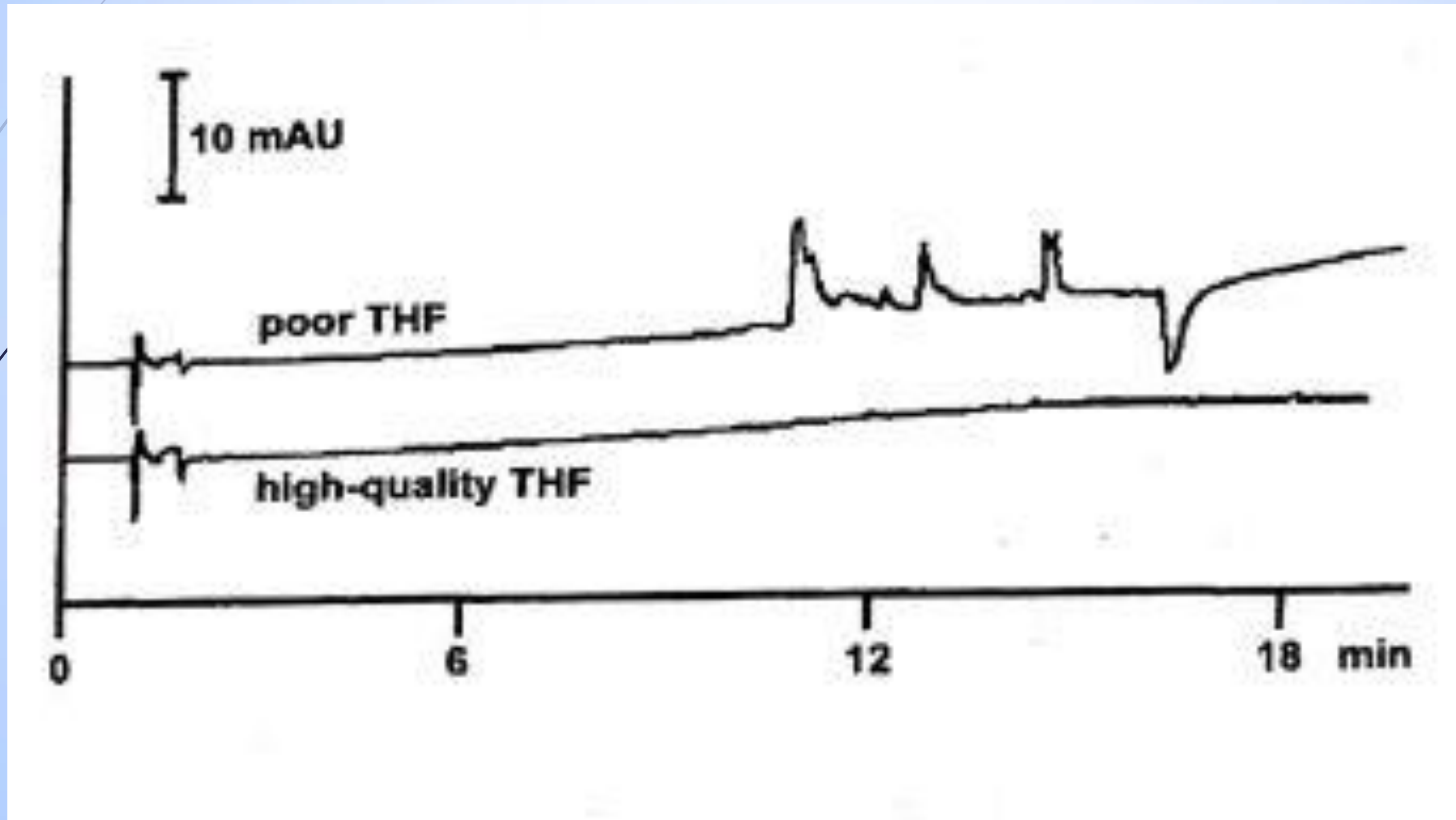
Mixing the mobile phase



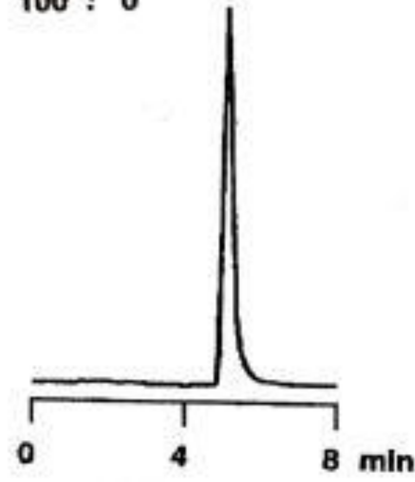
Mobile phase and pH

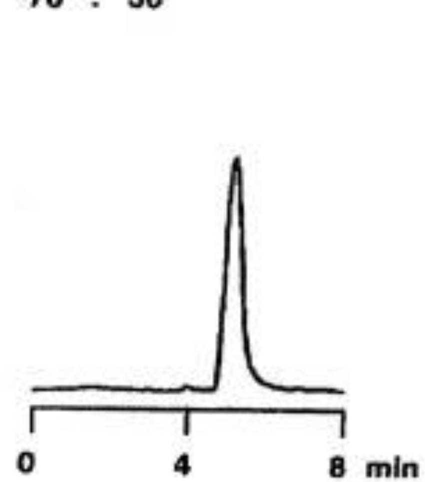


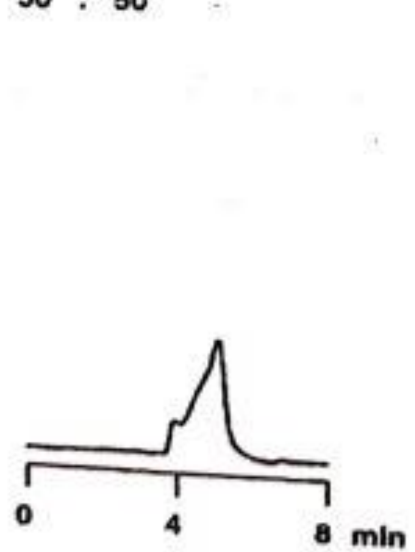
Solvent purity

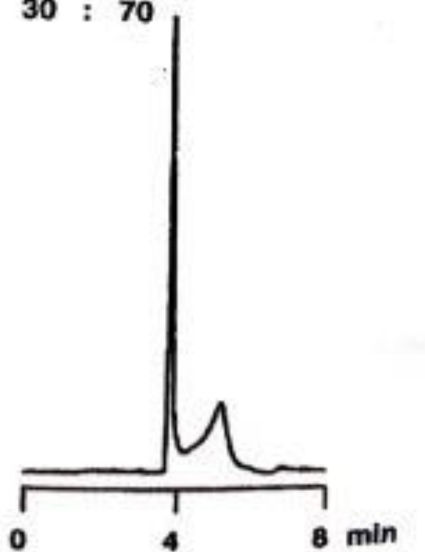


sample solvent:

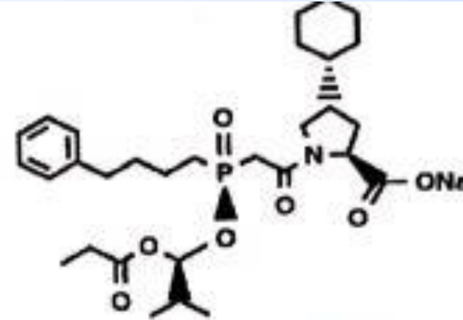


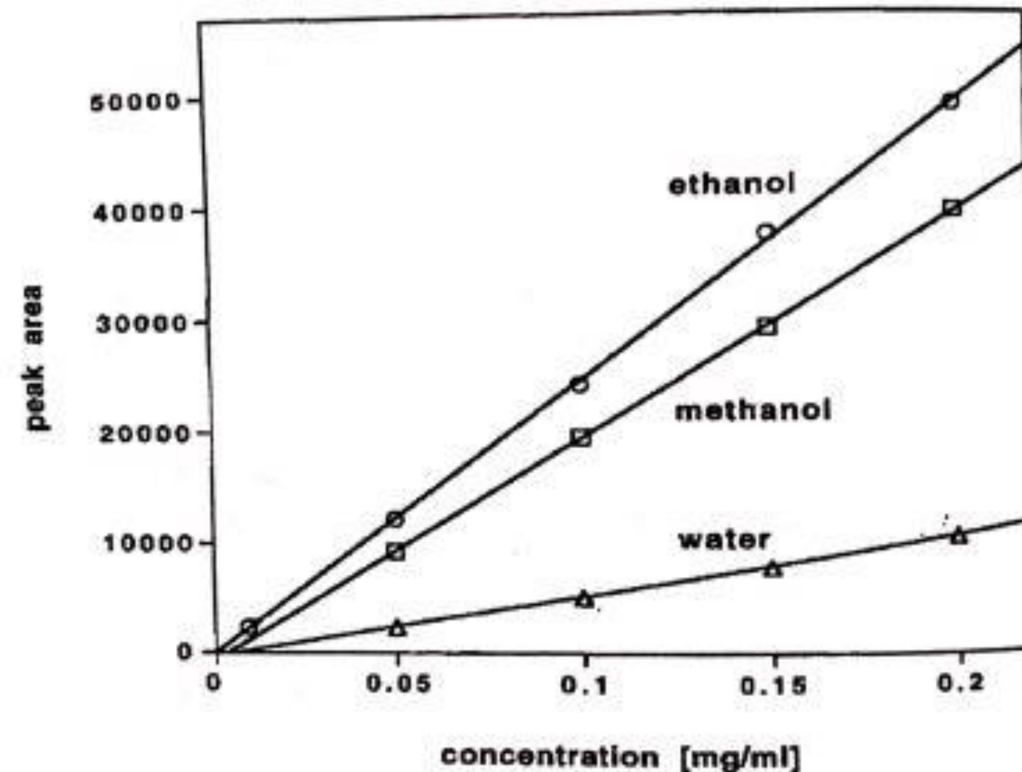




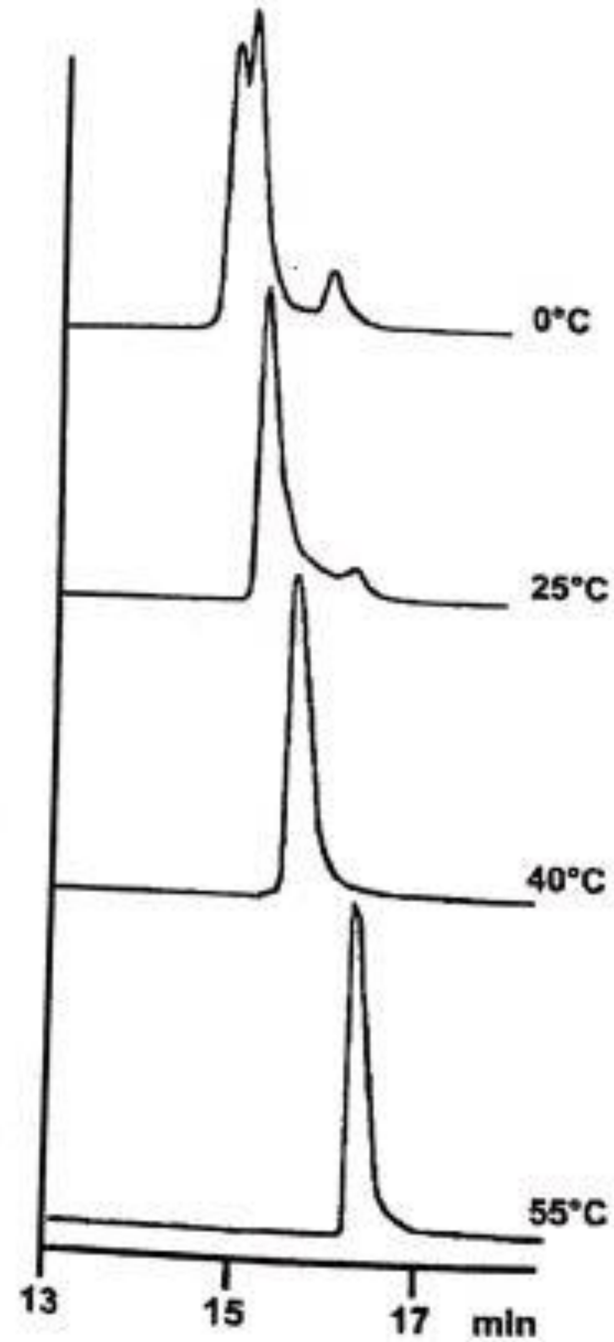


Sample solvent and calibration curve - sodium fusinopril





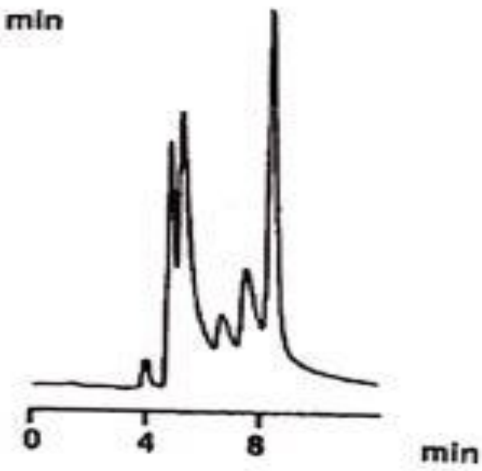
Temperature

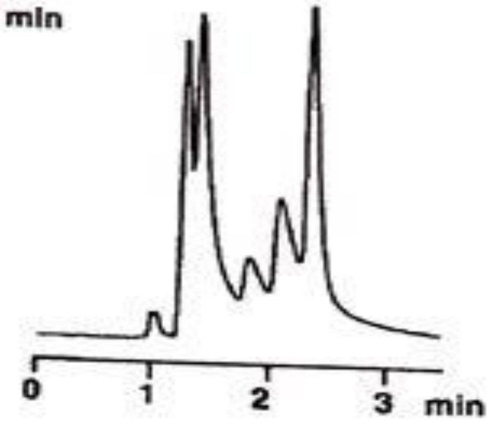


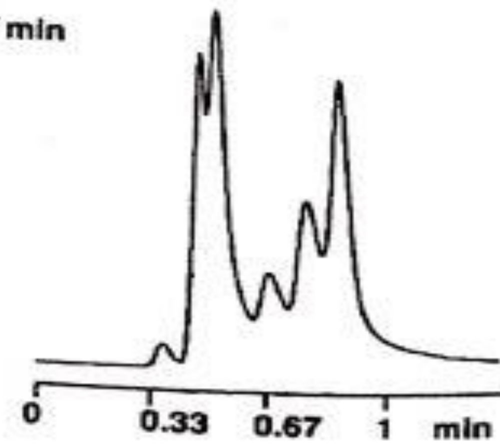
Temperature –

beta carotene

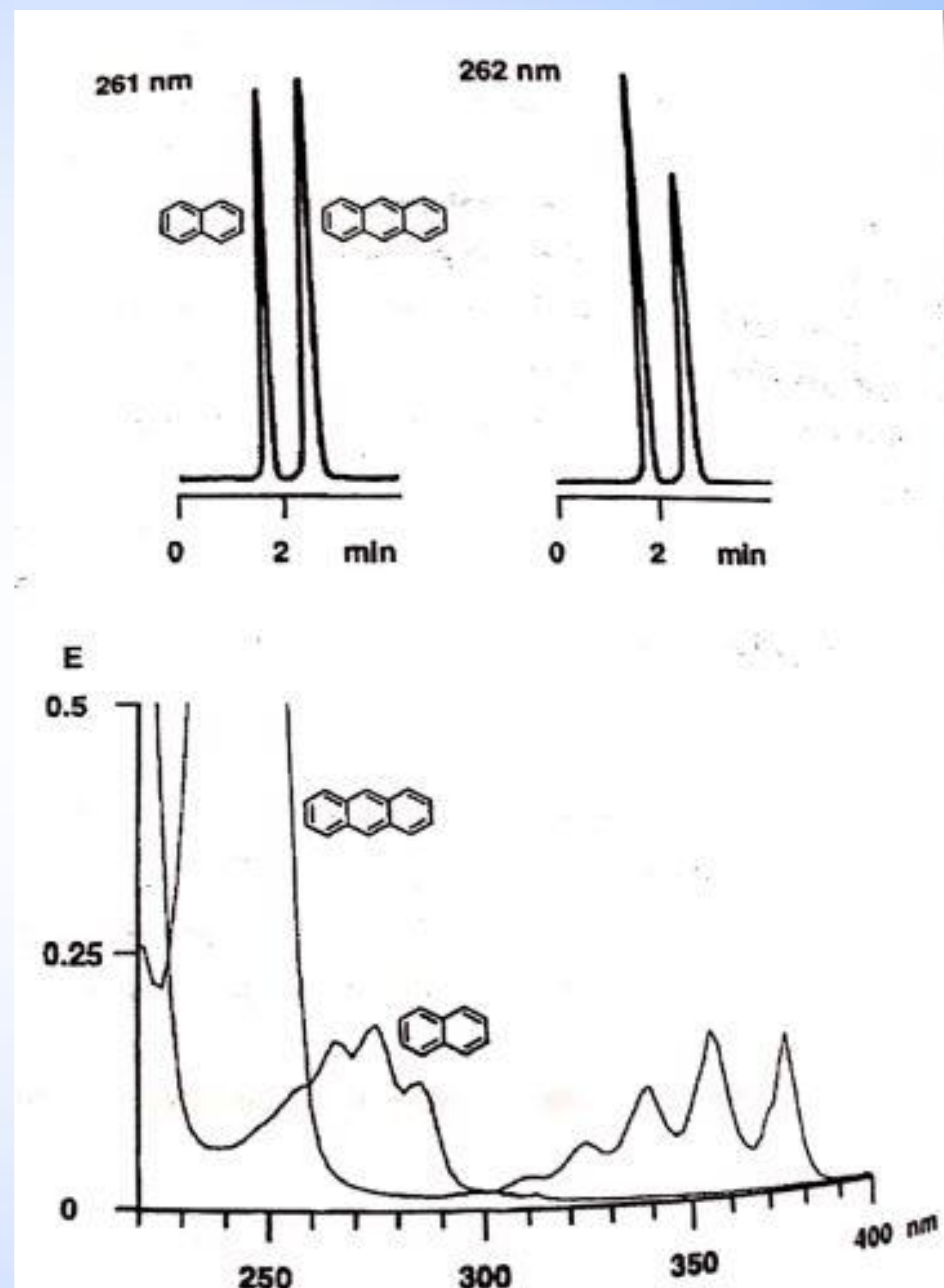
Flow rate



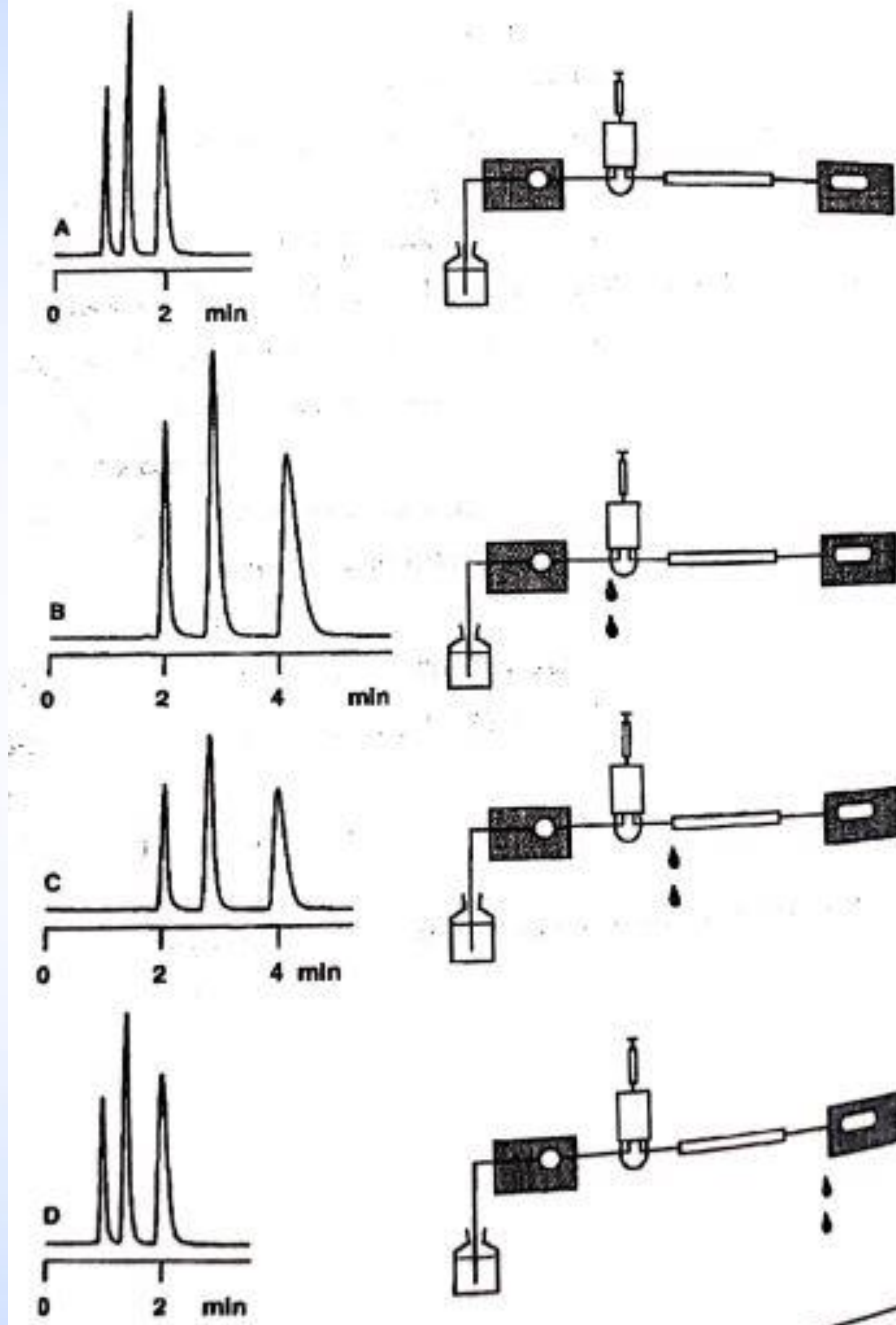




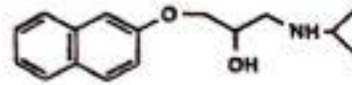
Wavelength

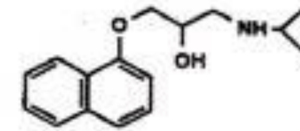


leakage

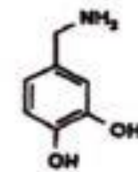


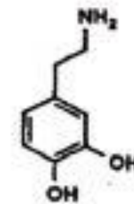
Internal standard

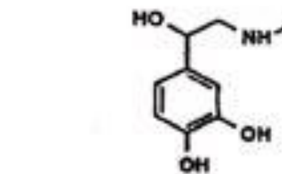




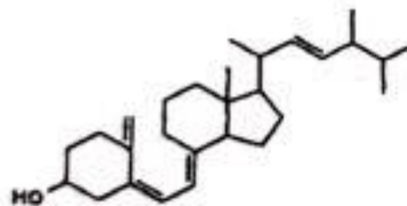
pronethalol for propranolol

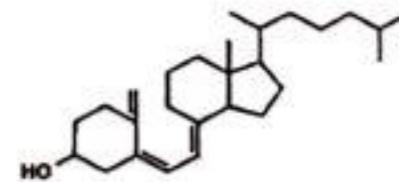






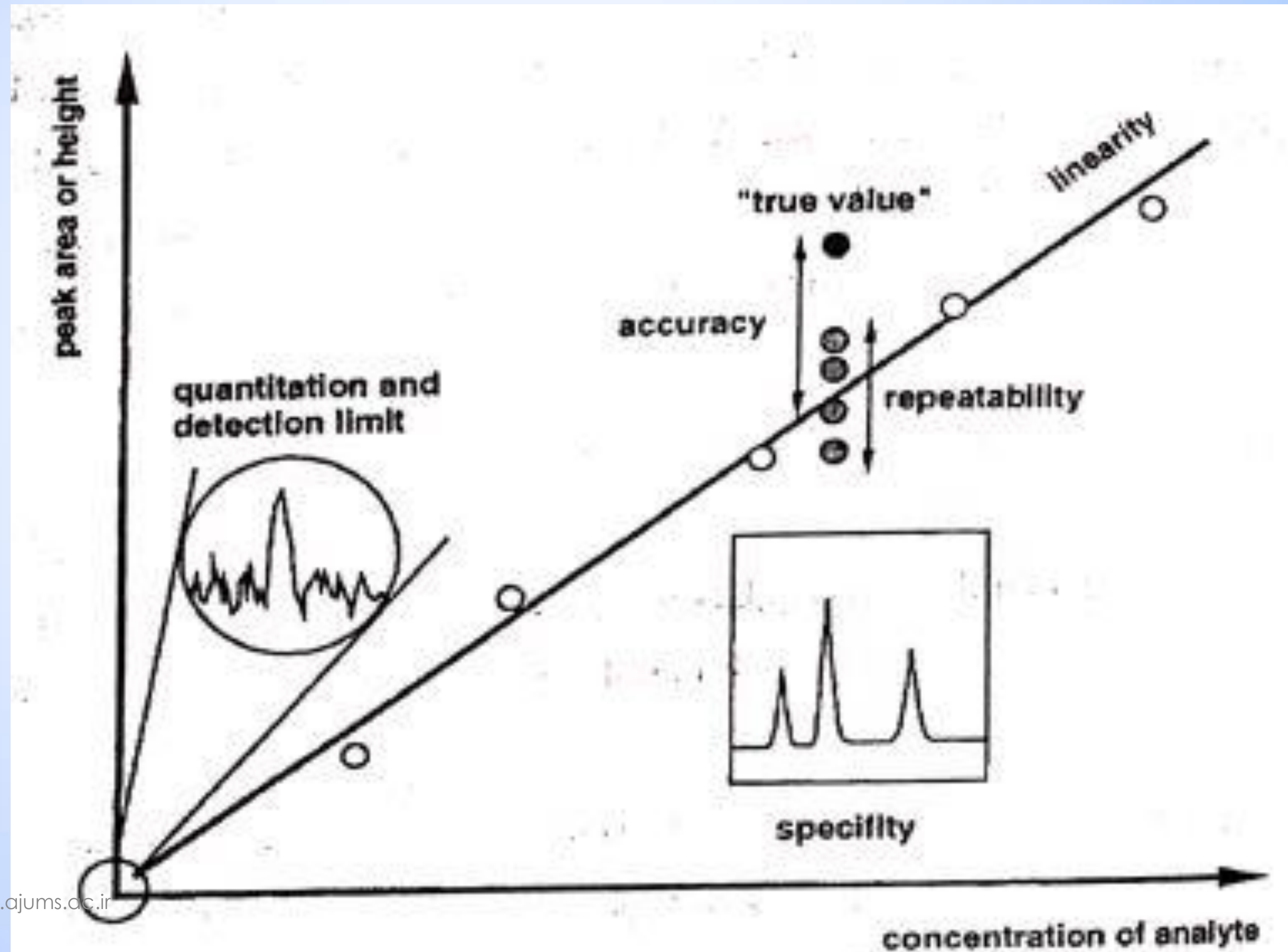
3,4-dihydroxy-benzylamine for dopamine and epinephrine



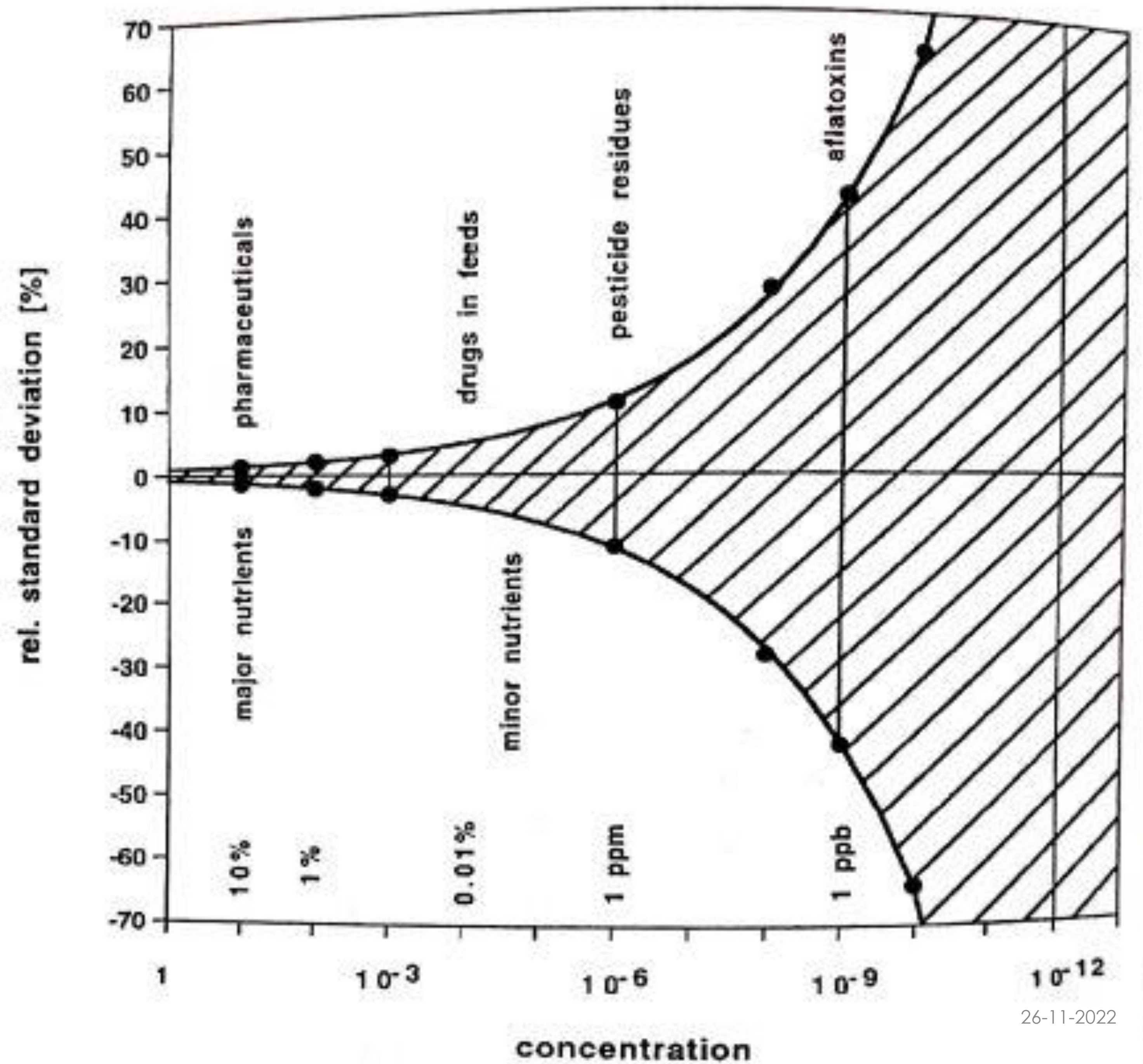


vitamin D_2 for vitamin D_3

validation



Horwitz



Thanks