CHROMATOGRAPHY

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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

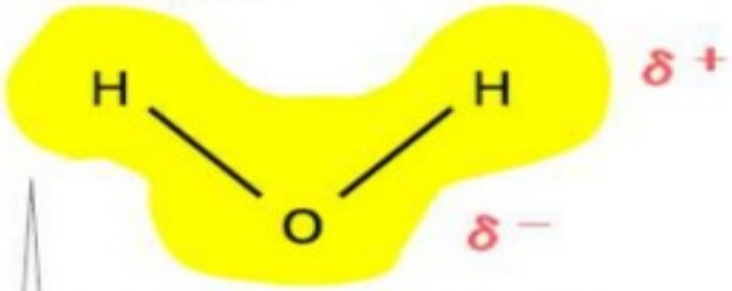
LC mode	Packing materials	Mobile phase	Interaction
Normal phase chromatography	Silica gel	n-Hexane/IPE	Adsorption
Reversed phase chromatography	Silica-C18(ODS)	MeOH/Water	Hydrophobic
Size exclusion chromatography	Porous polymer	THF	Gel permeation
Ion exchange chromatography	Ion exchange gel	Buffer sol.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

HPLC

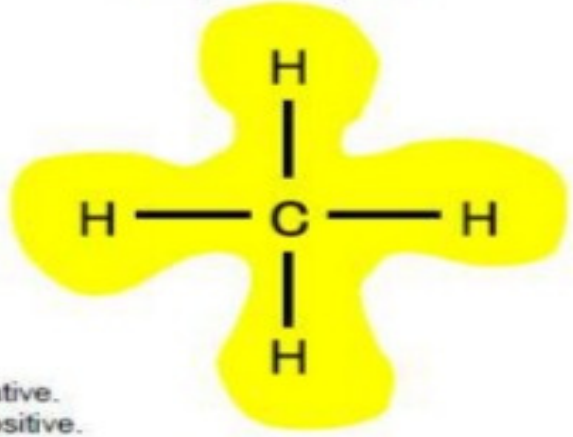
3. Separation mode

Polar compounds

Polar compound

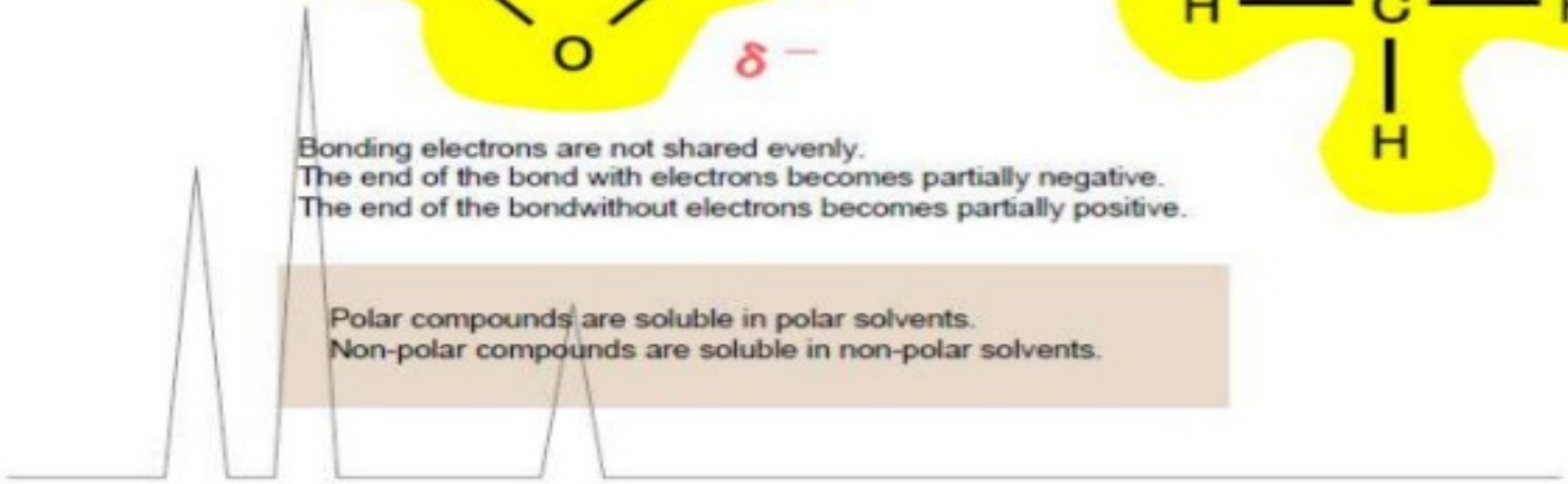


Non-polar compound



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.



HPLC

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

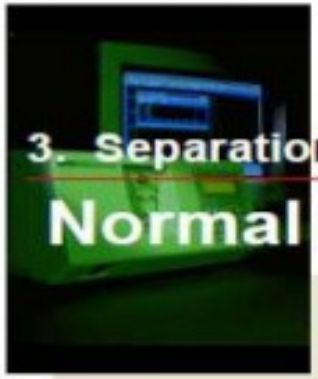
- Mechanism:

✓ 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.

HPLC

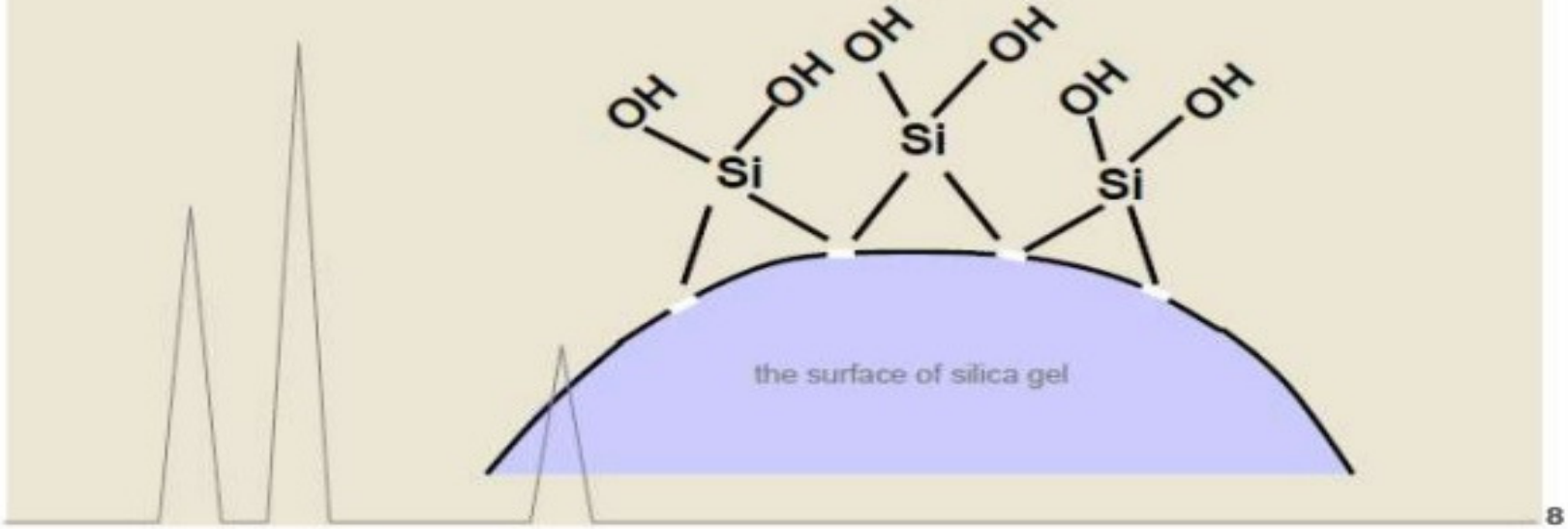


3. Separation mode

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.



8

HPLC

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

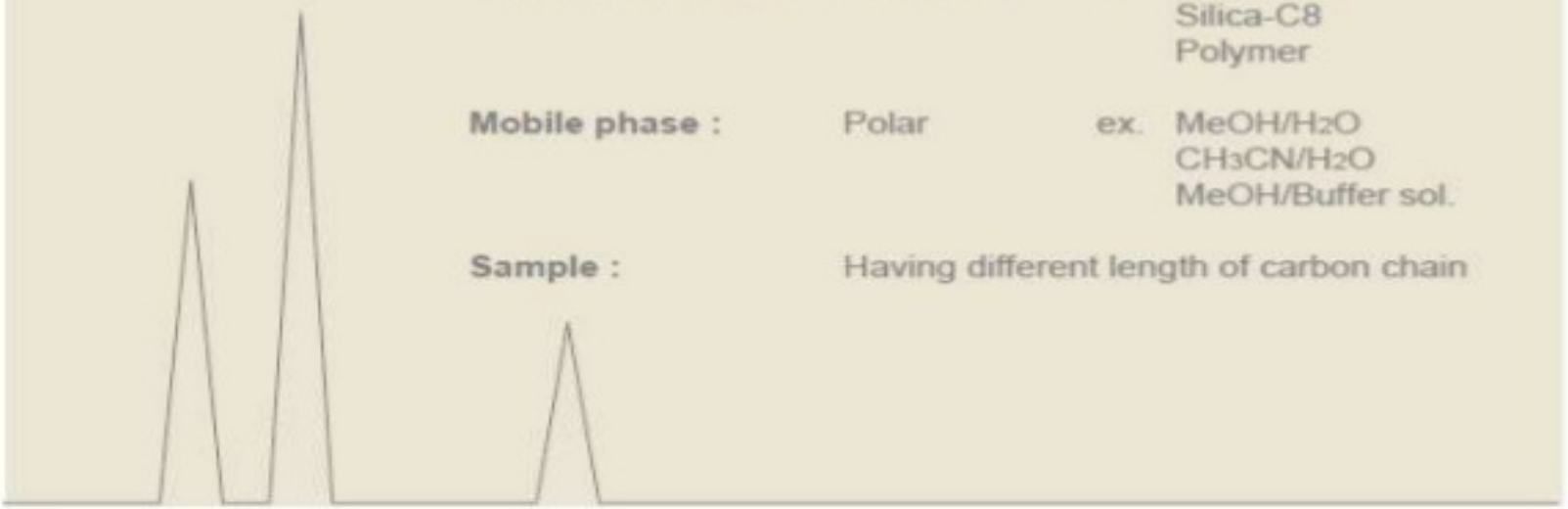
✓ Non-Polar compounds travels slower & eluted slowly due to higher affinity to st.phase

HPLC

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		



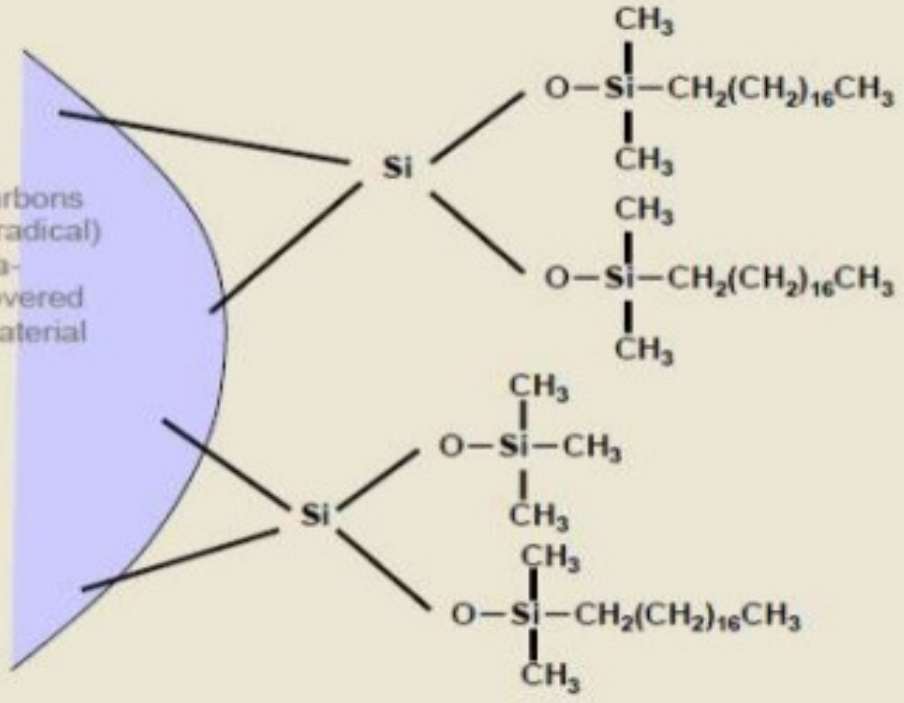
HPLC

3. Separation mode

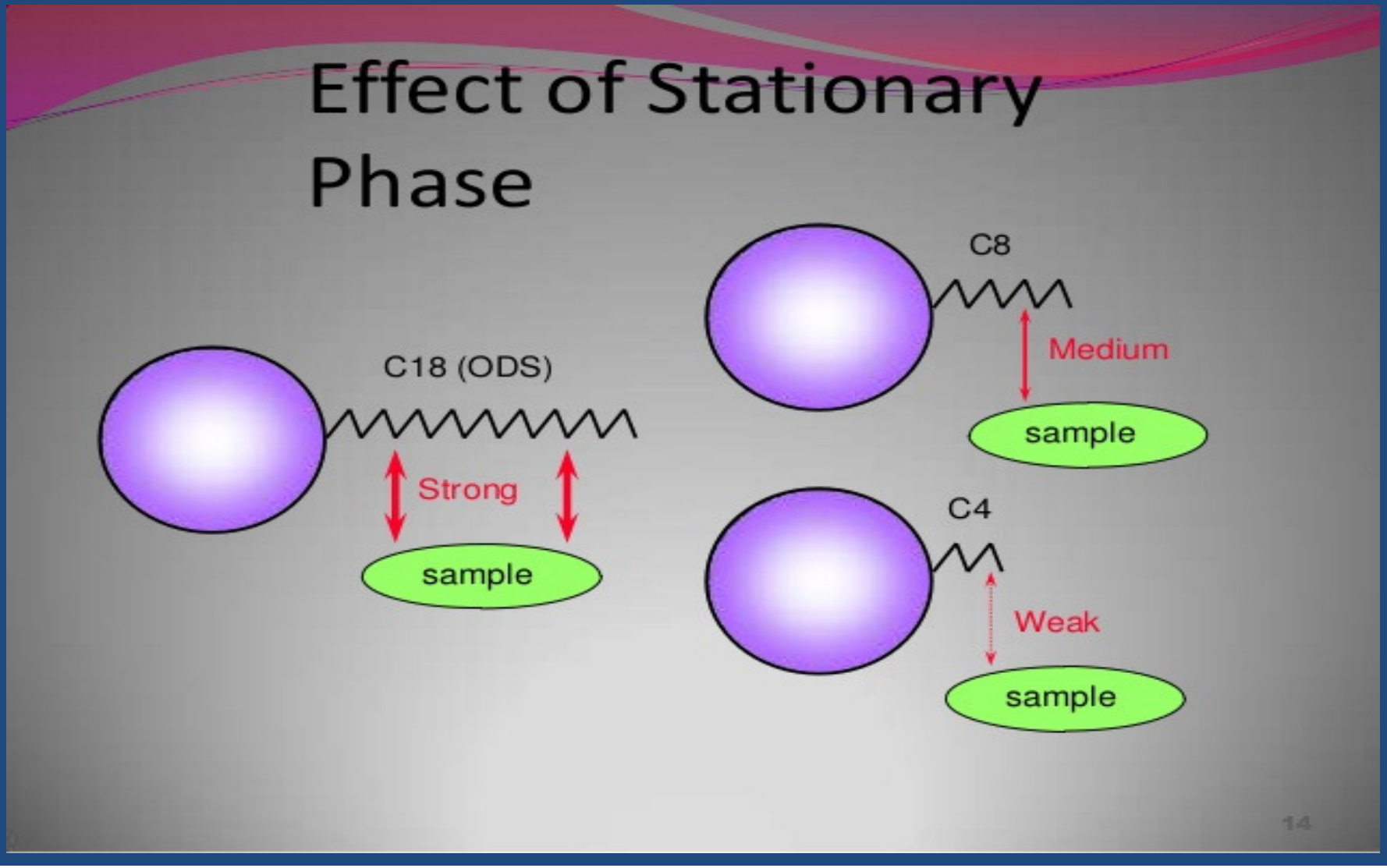
Reversed Phase Chromatography

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.



HPLC


Effect of Stationary Phase
C8
Medium
sample
C18 (ODS)
Strong
sample
C4
Weak
sample
14

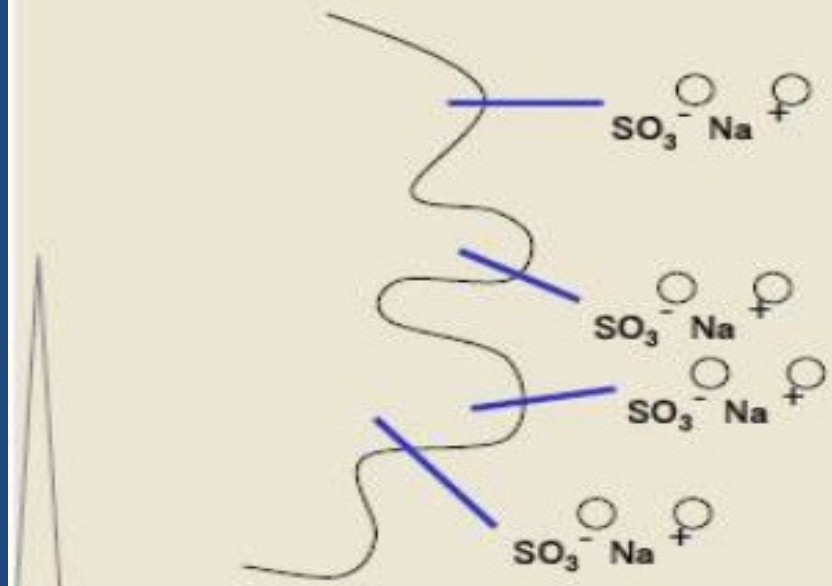
HPLC

ION-EXCHANGE CHROMATOGRAPHY:

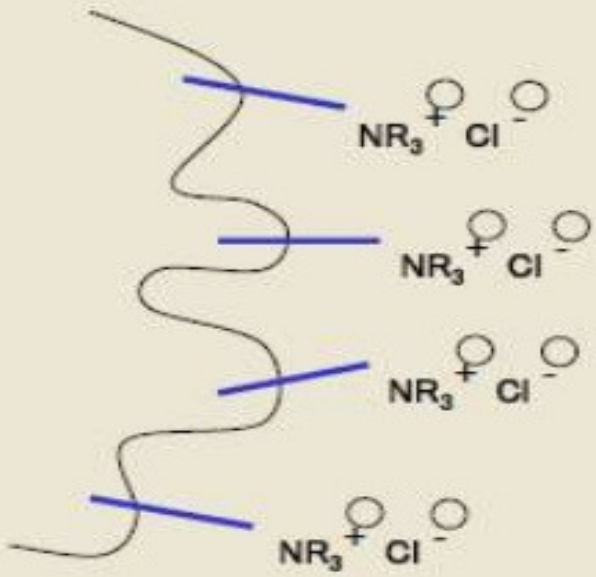
- It is the process by which similar charged ions such as cations, anions can be separated.
- By using the suitable ion exchange resin it can be separated.
- It exchanges the ions according to their relative affinities.
- The exchange takes place in a reversible manner between the ions of the solution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

HPLC

Ion-exchange Gel



Cation exchange gel



Anion exchange gel

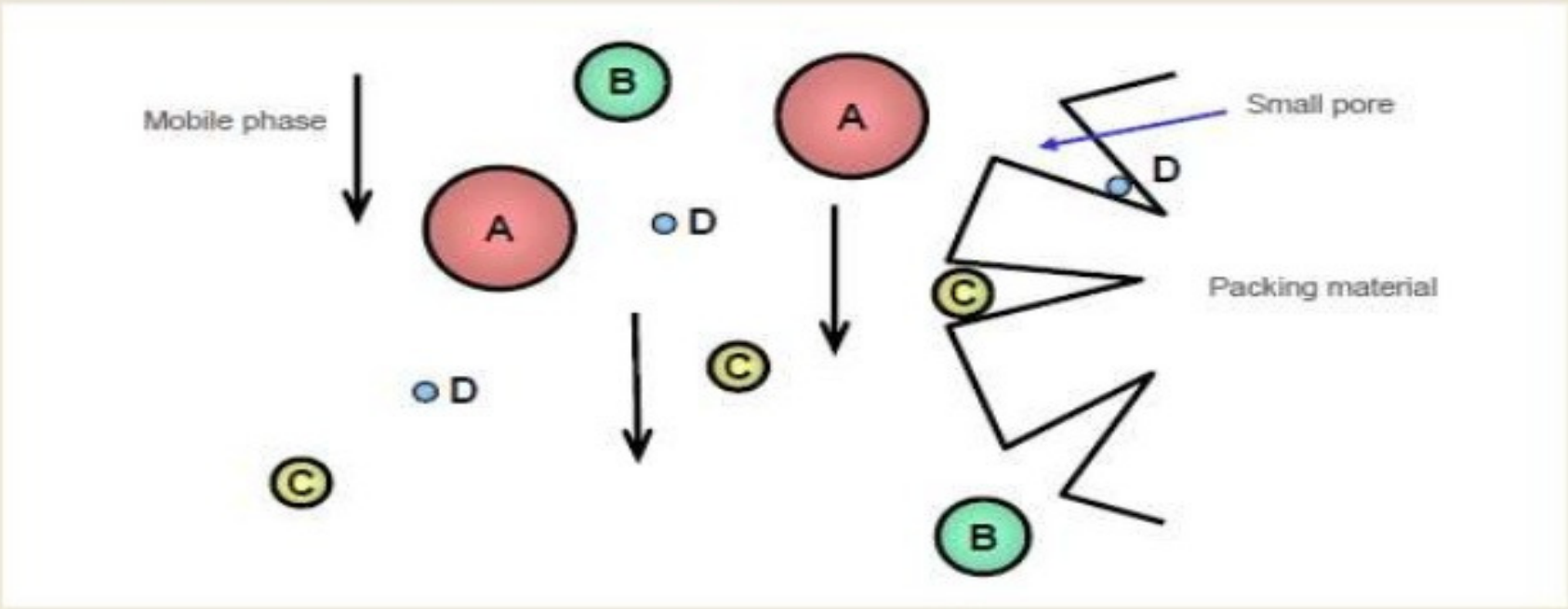
HPLC

MOLECULAR EXCLUSION CHROMATOGRAPHY:

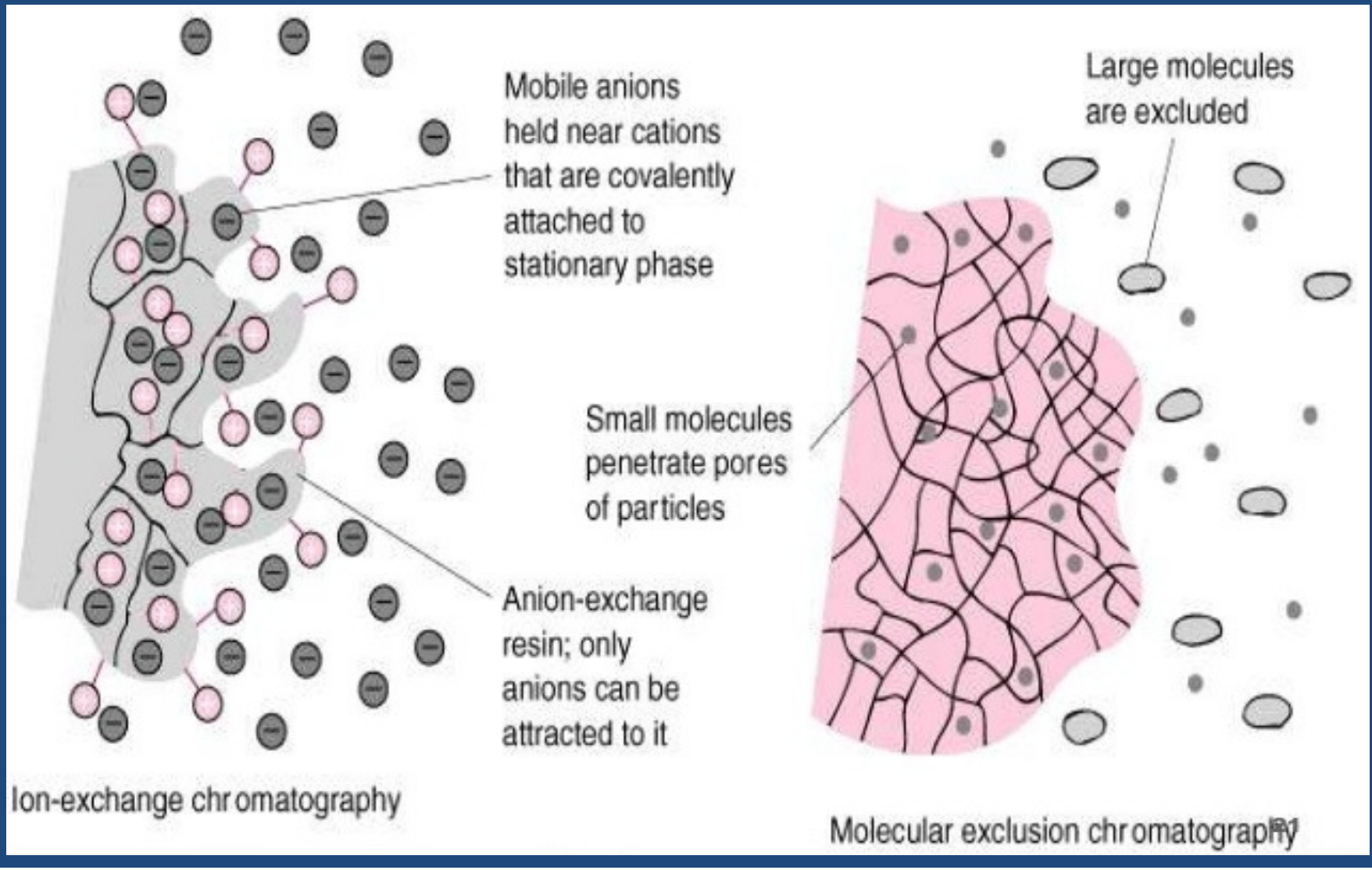
- A mixture of components with different molecular sizes are separated by using gels.
- The gels used acts as molecular sieve & hence a mixture of substances with different molecular sizes are separated.
- Soft gels like dextran, agarose or poly acrylamide are used.
- Semi-rigid gels like polystyrene,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

HPLC

SEC Separation mechanism

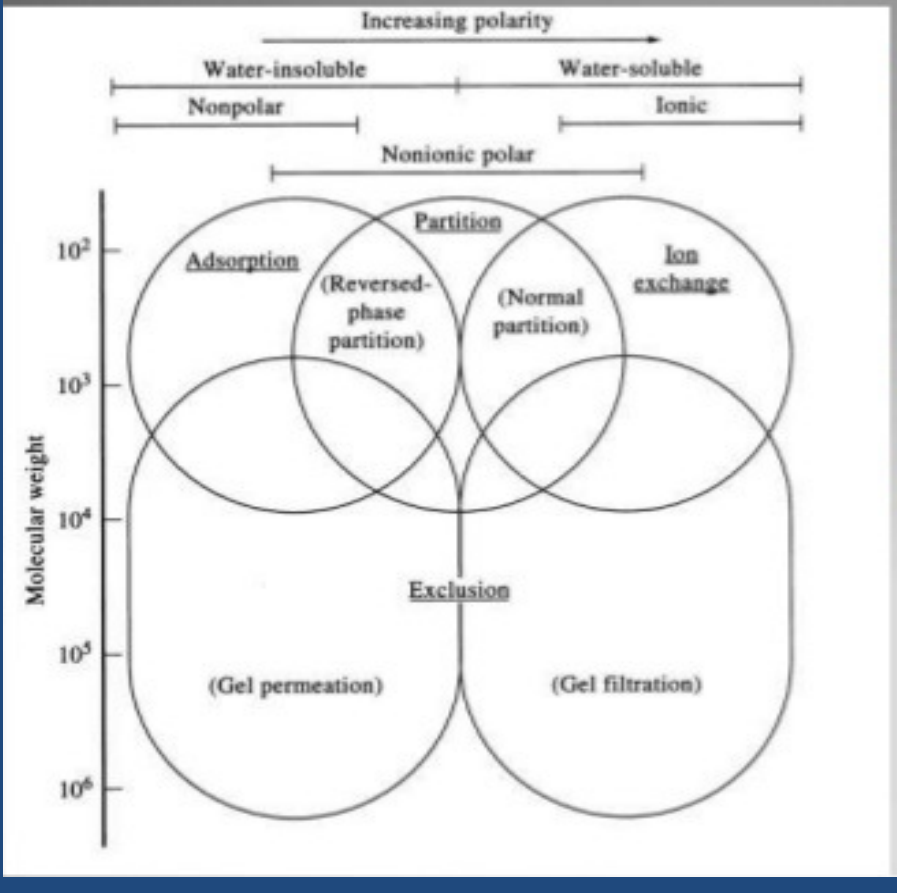


HPLC


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

HPLC

TYPES OF HPLC DEPENDS ON:



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

HPLC

III. BASED ON ELUTION TECHNIQUE

1.Isocratic elution

•A separation in which the mobile phase composition remains constant throughout the procedure is termed **isocratic elution**

•In isocratic elution, peak width increases with retention time linearly with the number of theoretical plates. This leads to the disadvantage that late-eluting peaks get very flat and broad.

- Best for simple separations
- Often used in quality control applications that support and are in close proximity to a manufacturing process

HPLC

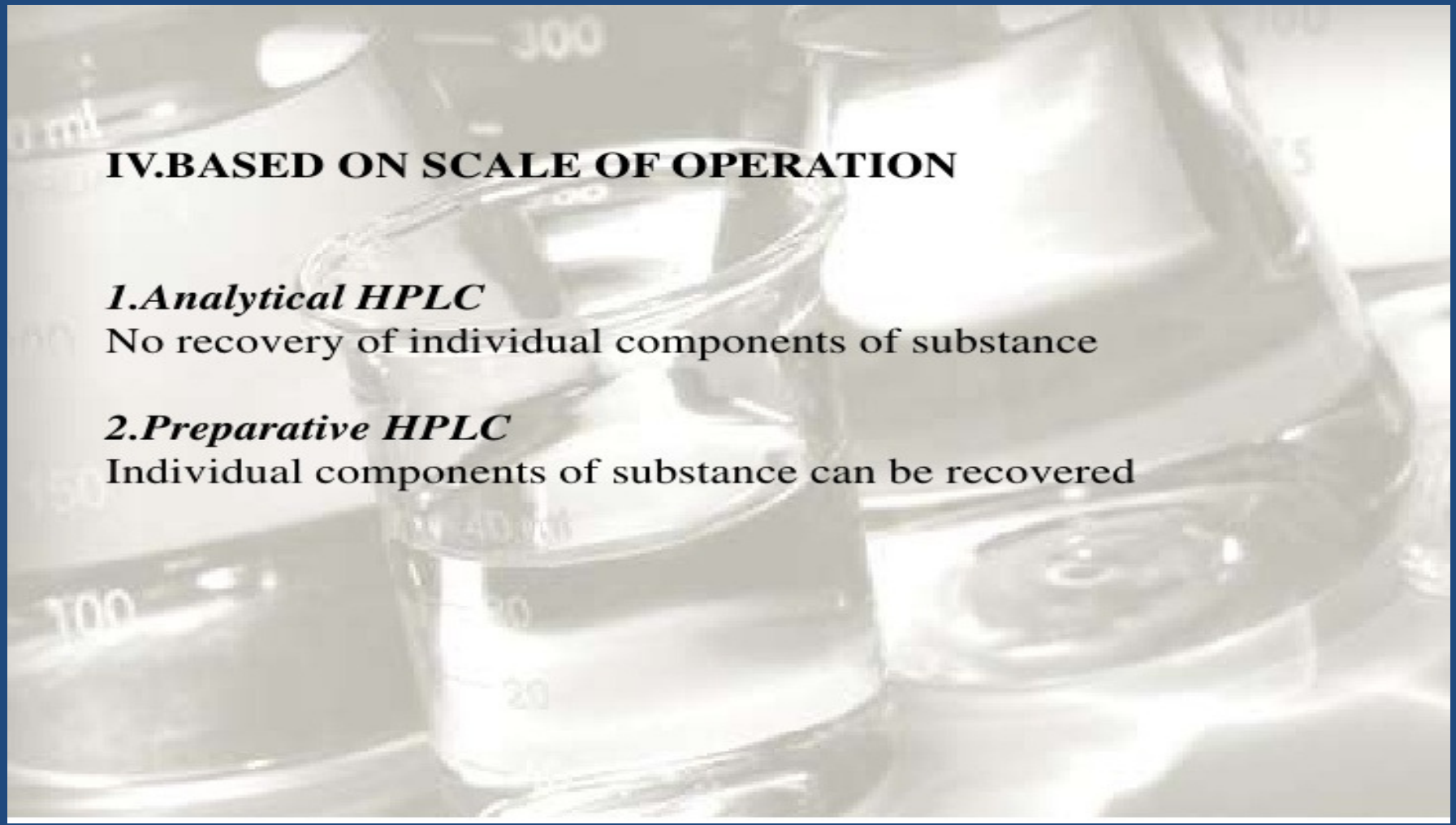
2. Gradient elution

•A separation in which the mobile phase composition is changed during the separation process is described as a **gradient elution**

•Gradient elution decreases the retention of the later-eluting components so that they elute faster, giving narrower peaks . This also improves the peak shape and the peak height

- Best for the analysis of complex samples
- Often used in method development for unknown mixtures
- Linear gradients are most popular

HPLC


IV.BASED ON SCALE OF OPERATION
1.Analytical HPLC
No recovery of individual components of substance
2.Preparative HPLC
Individual components of substance can be recovered

HPLC

V.BASED ON TYPE OF ANALYSIS

1.Qualitative analysis

Analysis of a substance in order to ascertain the nature of its chemical constituents

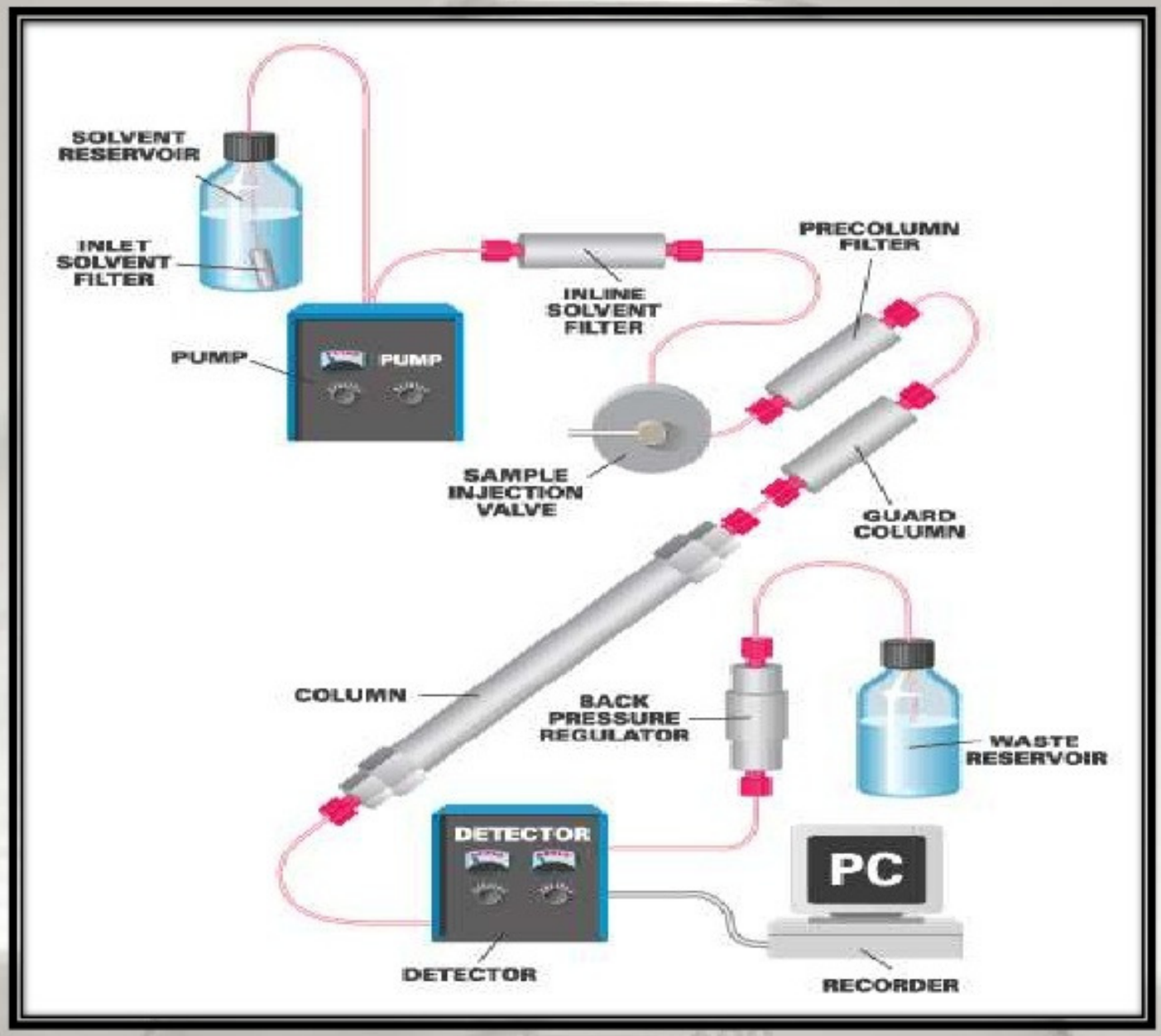
We can separate individual components but cannot assess the quantity in this analysis

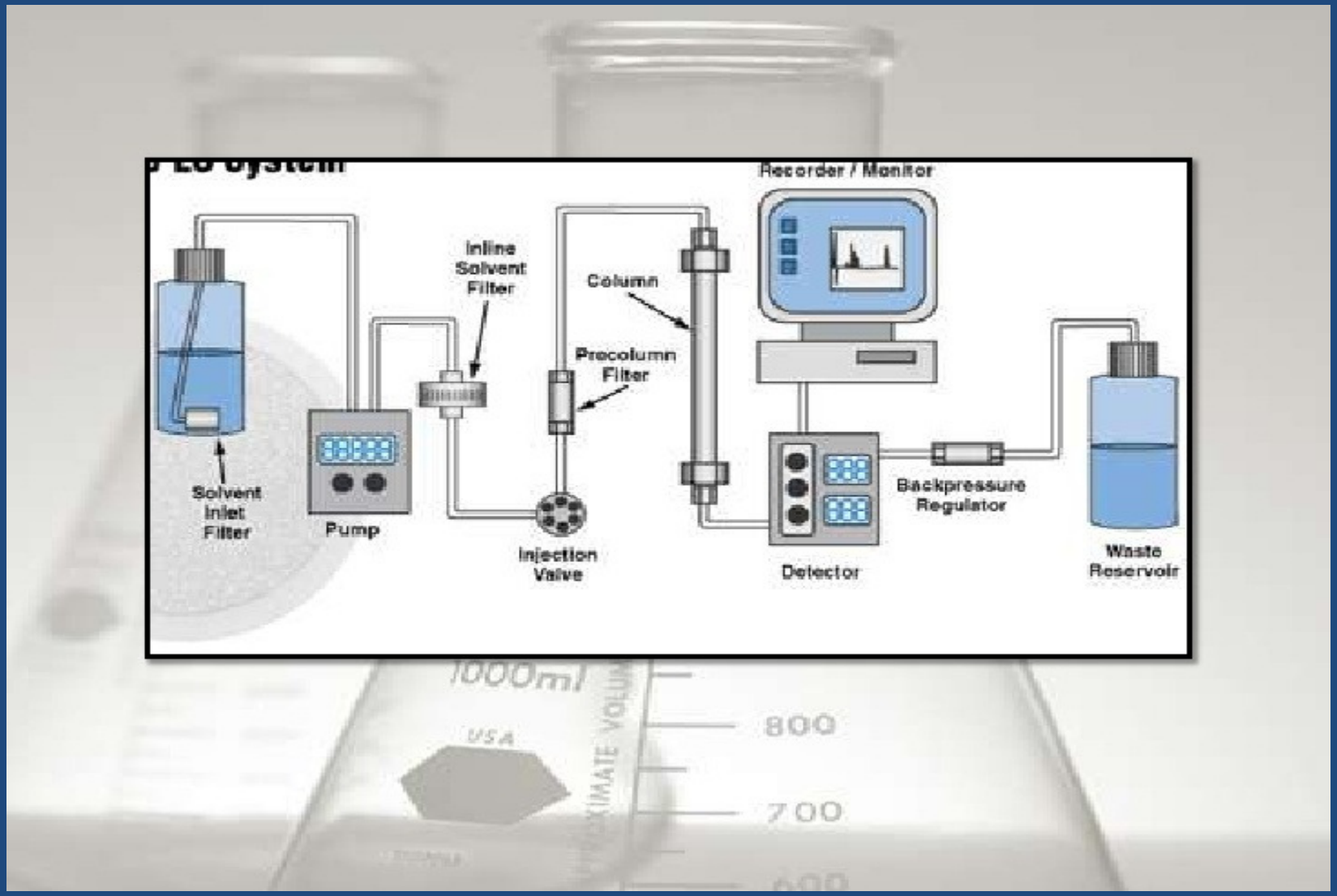
2.Quantitaive analysis

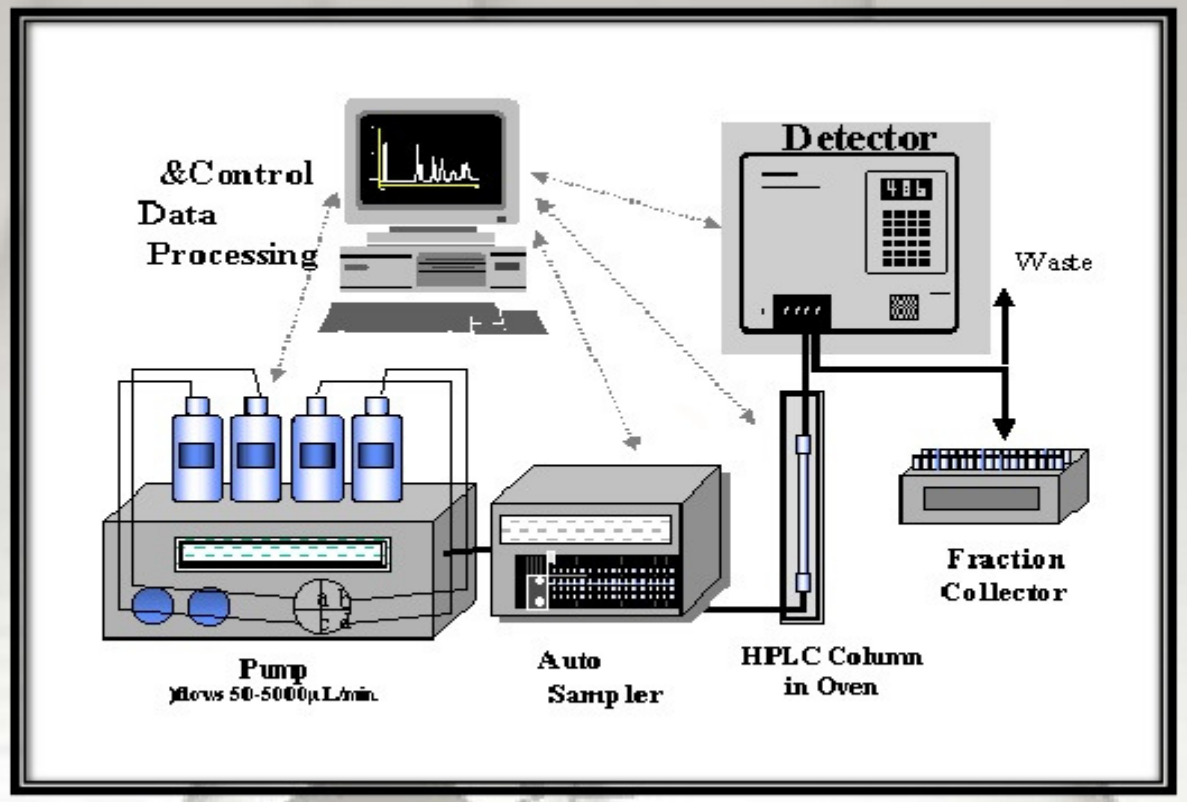
Determining the amounts and proportions of its chemical constituents .

Quantity of the impurity and individual components can be assessed

INSTRUMENTATION


SOLVENT RESERVOIR
INLET SOLVENT FILTER
PUMP
PUMP
INLINE SOLVENT FILTER
PRECOLUMN FILTER
SAMPLE INJECTION VALVE
GUARD COLUMN
COLUMN
BACK PRESSURE REGULATOR
WASTE RESERVOIR
DETECTOR
DETECTOR
PC
RECORDER


Recorder / Monitor
Inline
Solvent
Filter
Column
Precolumn
Filter
Solvent
Inlet
Filter
Pump
Injection
Valve
Detector
Backpressure
Regulator
Waste
Reservoir


&Control
Data
Processing
Detector
Waste
Fraction
Collector
Pump
flows 50-5000µL/min.
Auto
Sampler
HPLC Column
in Oven

HPLC

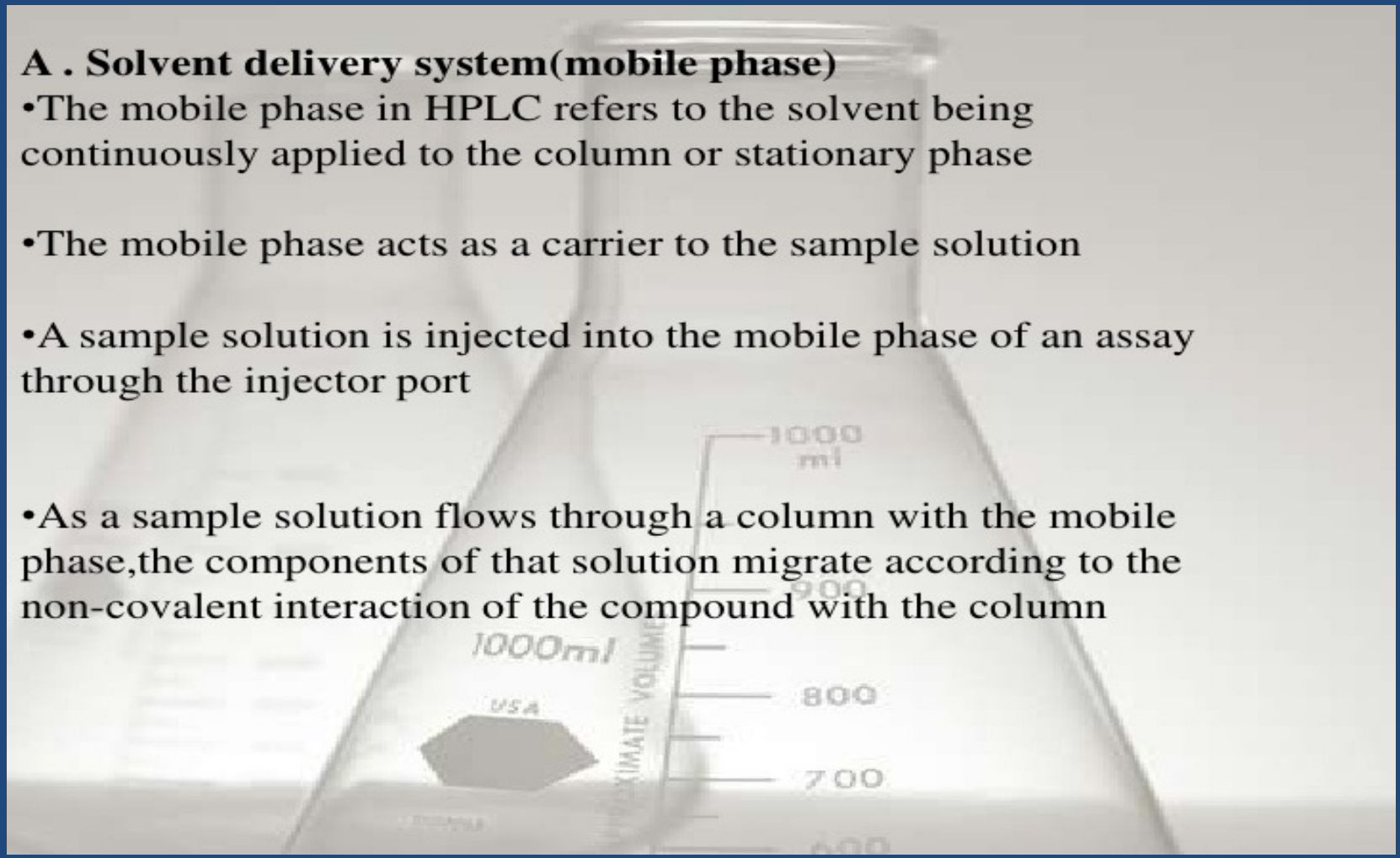
A . Solvent delivery system(mobile phase)

•The mobile phase in HPLC refers to the solvent being continuously applied to the column or stationary phase

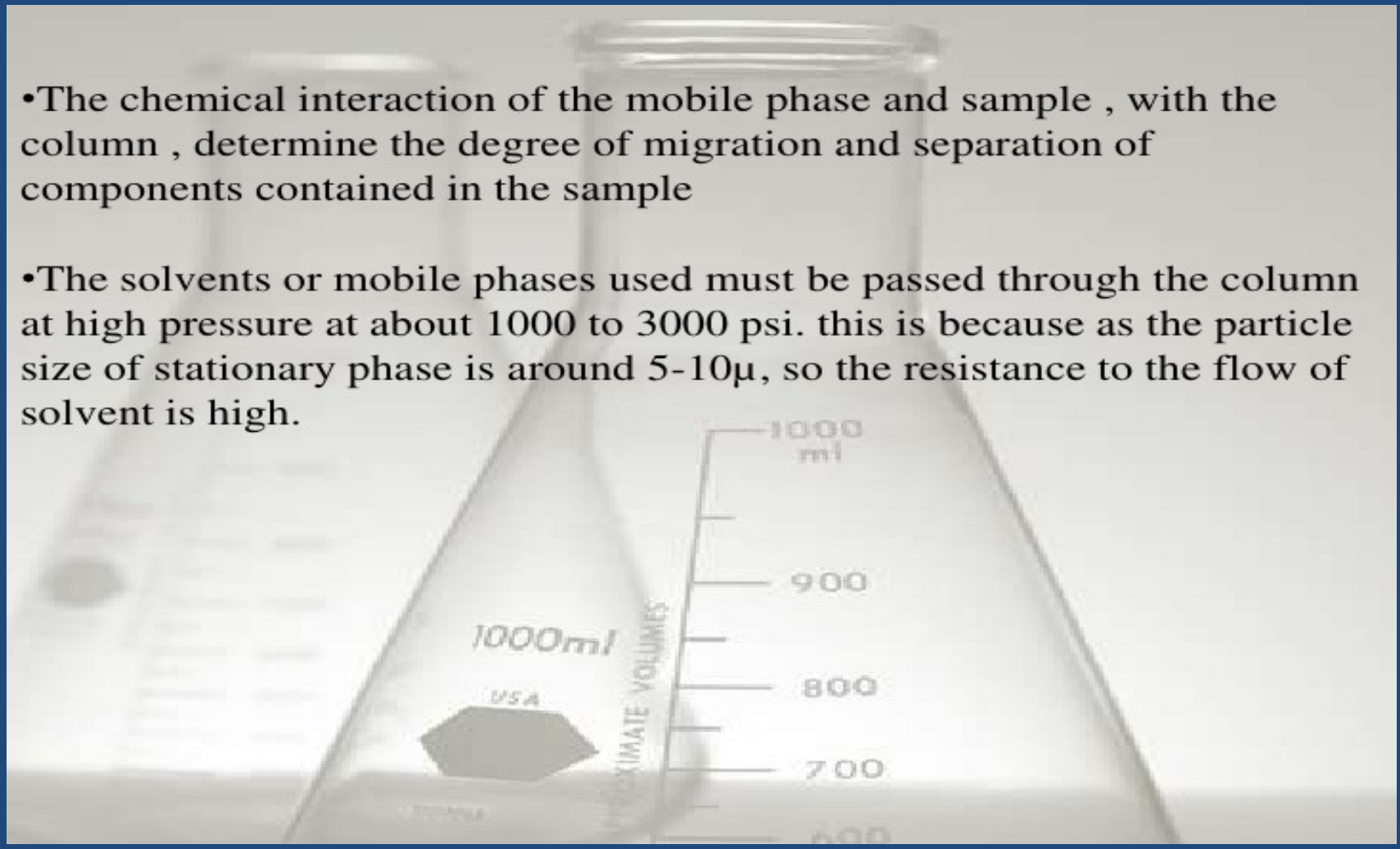
•The mobile phase acts as a carrier to the sample solution

•A sample solution is injected into the mobile phase of an assay through the injector port

•As a sample solution flows through a column with the mobile phase,the components of that solution migrate according to the non-covalent interaction of the compound with the column



HPLC



•The chemical interaction of the mobile phase and sample , with the column , determine the degree of migration and separation of components contained in the sample

•The solvents or mobile phases used must be passed through the column at high pressure at about 1000 to 3000 psi. this is because as the particle size of stationary phase is around 5-10µ, so the resistance to the flow of solvent is high.

B.Pumps

•The role of the pump is to force a liquid (called the mobile phase) through the liquid chromatograph at a specific flow rate, expressed in milliliters per min (mL/min).

•Normal flow rates in HPLC are in the 1-to 2-mL/min range.

•Typical pumps can reach pressures in the range of 6000-9000 psi (400- to 600-bar).

•During the chromatographic experiment, a pump can deliver a constant mobile phase composition (isocratic) or an increasing mobile phase composition (gradient).

Types of HPLC pumps

There are several types of pumps used for HPLC analysis, most commonly used are reciprocating piston pump,syringe pump and constant pressure pump

1.Reciprocating piston pumps:

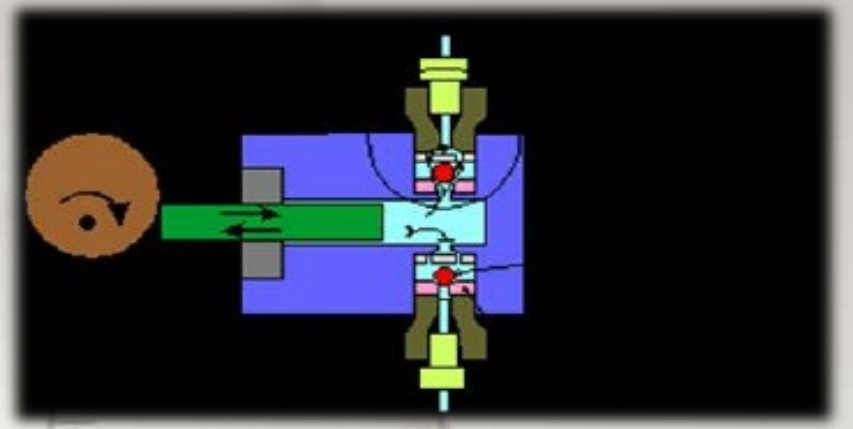
•Consists of a small motor driven piston which moves rapidly back and forth in a hydraulic chamber that may vary from 35-400µL in volume

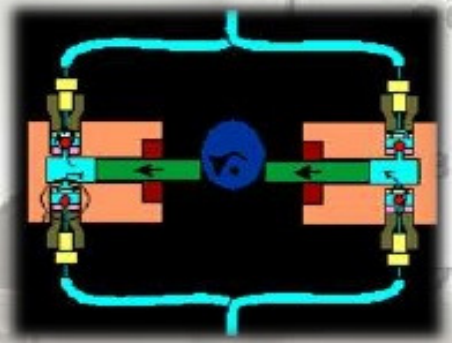
•On the back stroke , the separation column valve is closed , and the piston pulls in solvent from the mobile phase reservoir

•On the forward stroke,the pump pushes solvent out of the column from the reservoir

•A wide range of flow rates can be attained by altering the piston stroke volume during each cycle , or by altering the stroke frequency.

•Dual and triple head pump consists of identical piston chamber units which operate at 180 or 120 degrees out of phase(this system is significantly smoother because one pump is filling while the other is in the delivery cycle.

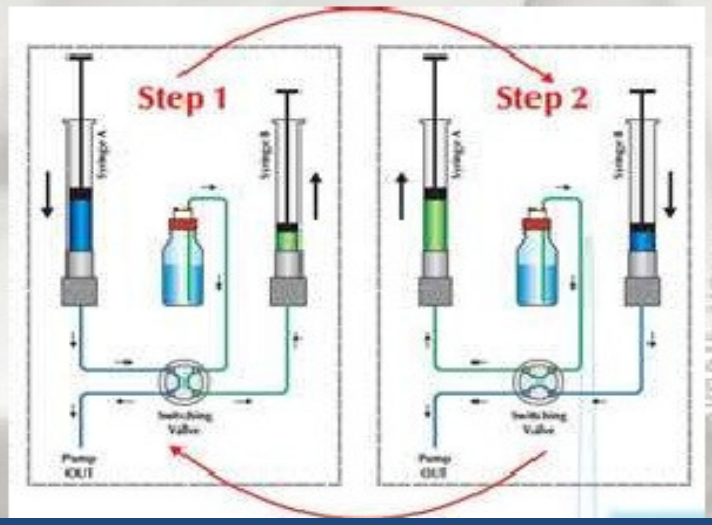




2.Syringe type pump

•These are most suitable for small bore columns because this pump delivers only a finite volume of mobile phase before it has to be refilled. These pumps have a volume between 250 to 500mL

•The pump operates by a motorized lead screw that delivers mobile phase to the column at a constant rate .The rate of solvent delivery is controlled by changing the voltage on the motor.



3.Constant pressure pump

•In these types of pumps , the mobile phase is driven through the column with the use of pressure from the gas cylinder

•.A low-pressure gas source is needed to generate high liquid pressures

•The valving arrangement allows the rapid refill of the solvent chamber whose capacity is about 70mL

•This provides continuous phase flow rates

C. Injector:

•The injector serves to introduce the liquid sample into the flow stream of the mobile phase for analysis.

•It is equipped with six port valves so that a sample can be injected into the flow path at continuous pressure

•For a manual injector, the knob is manually operated to deliver the sample to the column

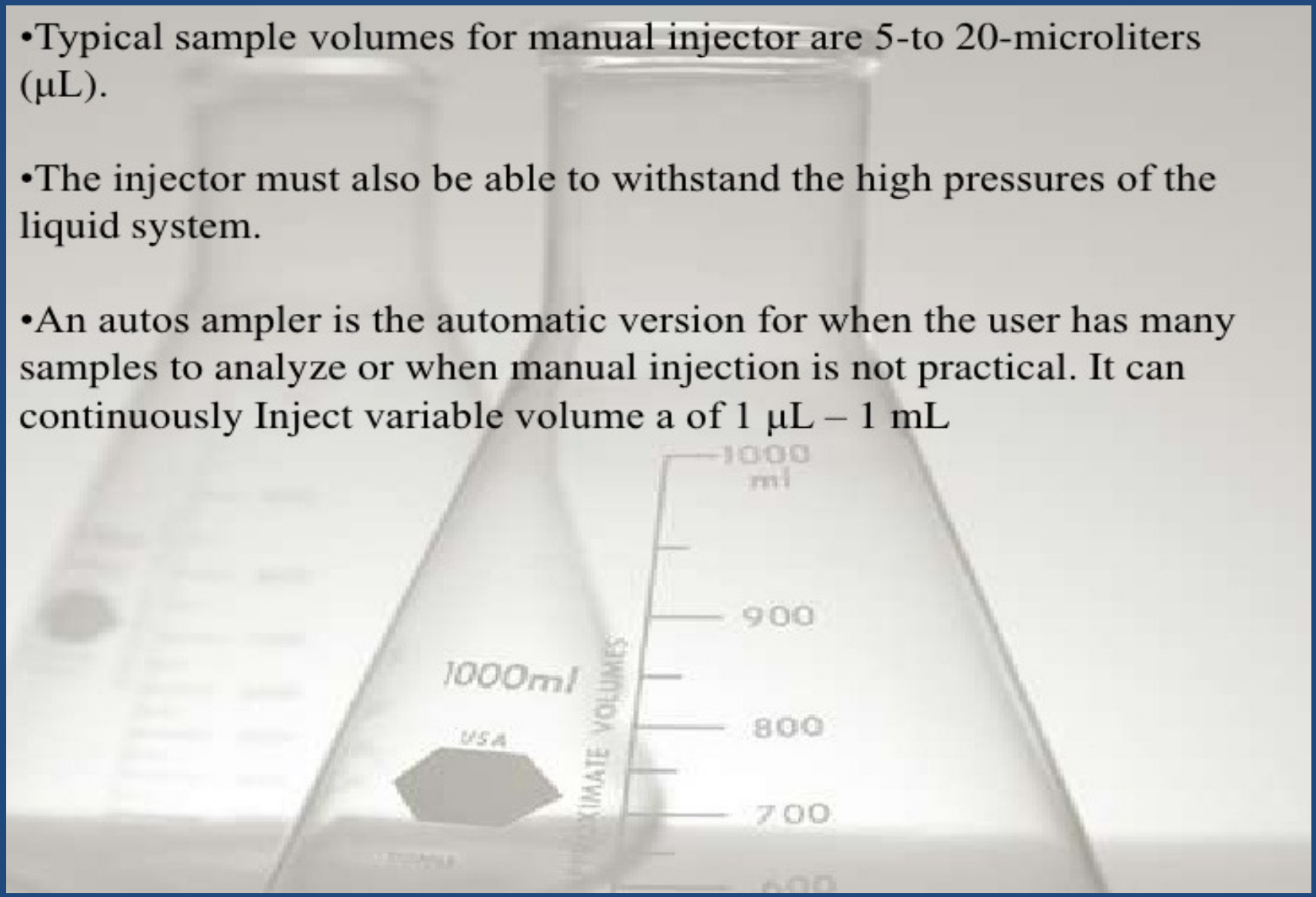
•The knob is set to LOAD position for sample injection using a syringe , the sample is injected into the sample loop , which is separated from the flow path

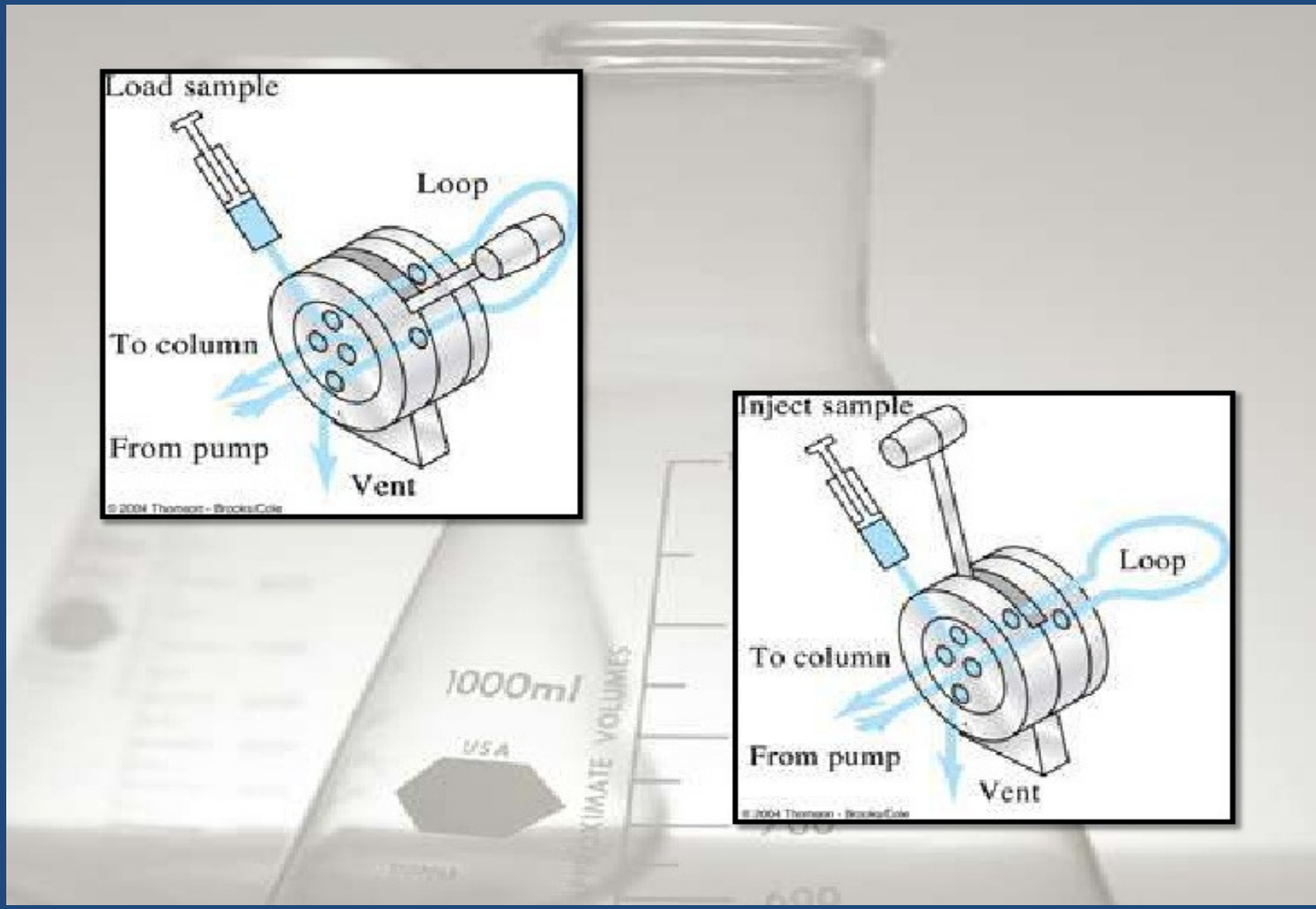
•The knob is turned to INJECT position and the eluent travels through the loop from the pump and delivers the sample to the column

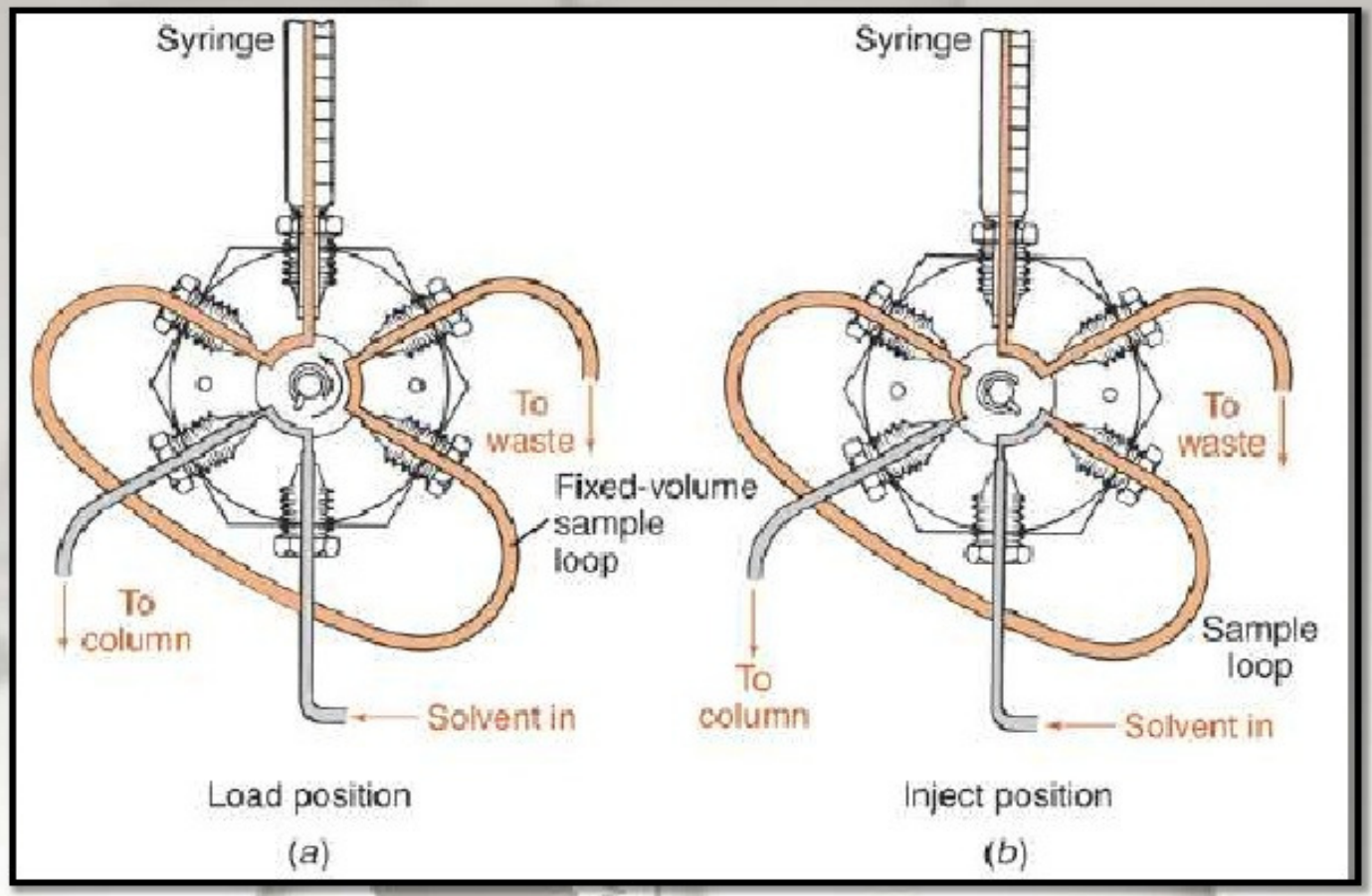
•Typical sample volumes for manual injector are 5-to 20-microliters (μL).

•The injector must also be able to withstand the high pressures of the liquid system.

•An autos ampler is the automatic version for when the user has many samples to analyze or when manual injection is not practical. It can continuously Inject variable volume a of 1 μL – 1 mL




Load sample
Loop
To column
From pump
Vent
Inject sample
Loop
To column
From pump
Vent


Syringe
To waste
Fixed-volume sample loop
To column
Solvent in
Load position
(a)
Syringe
To waste
Sample loop
To column
Solvent in
Inject position
(b)

D.Column

•Considered the "heart of the chromatograph" the column's stationary phase separates the sample components of interest using various physical and chemical parameters.

•It is usually made of stainless steel to withstand high pressure caused by the pump to move the mobile phase through the column packing other material include PEEK and glass

•The small particles inside the column are called the "packing" what cause the high back pressure at normal flow rates.

•Column packing is usually silica gel because of its particle shape , surface properties , and pore structure give us a good separation

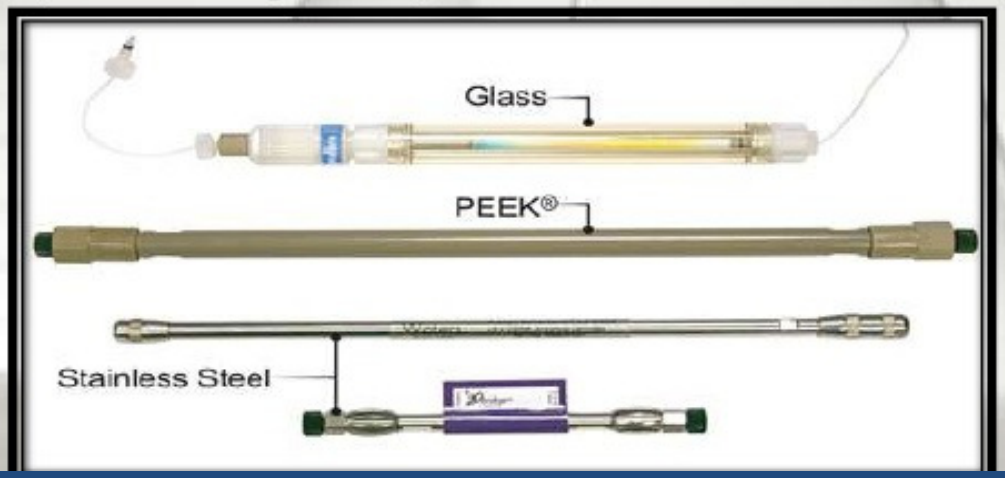
•Other material used include alumina, a polystyrene-divinyl benzene synthetic or an ion-exchange resin

– Pellicular particle: original, Spherical, nonporous beads, proteins and large biomolecules separation (dp: 5 μm)

– Porous particle: common used, dp: 3 ~ 10 μm. Narrow size distribution, porous microparticle coated with thin organic film

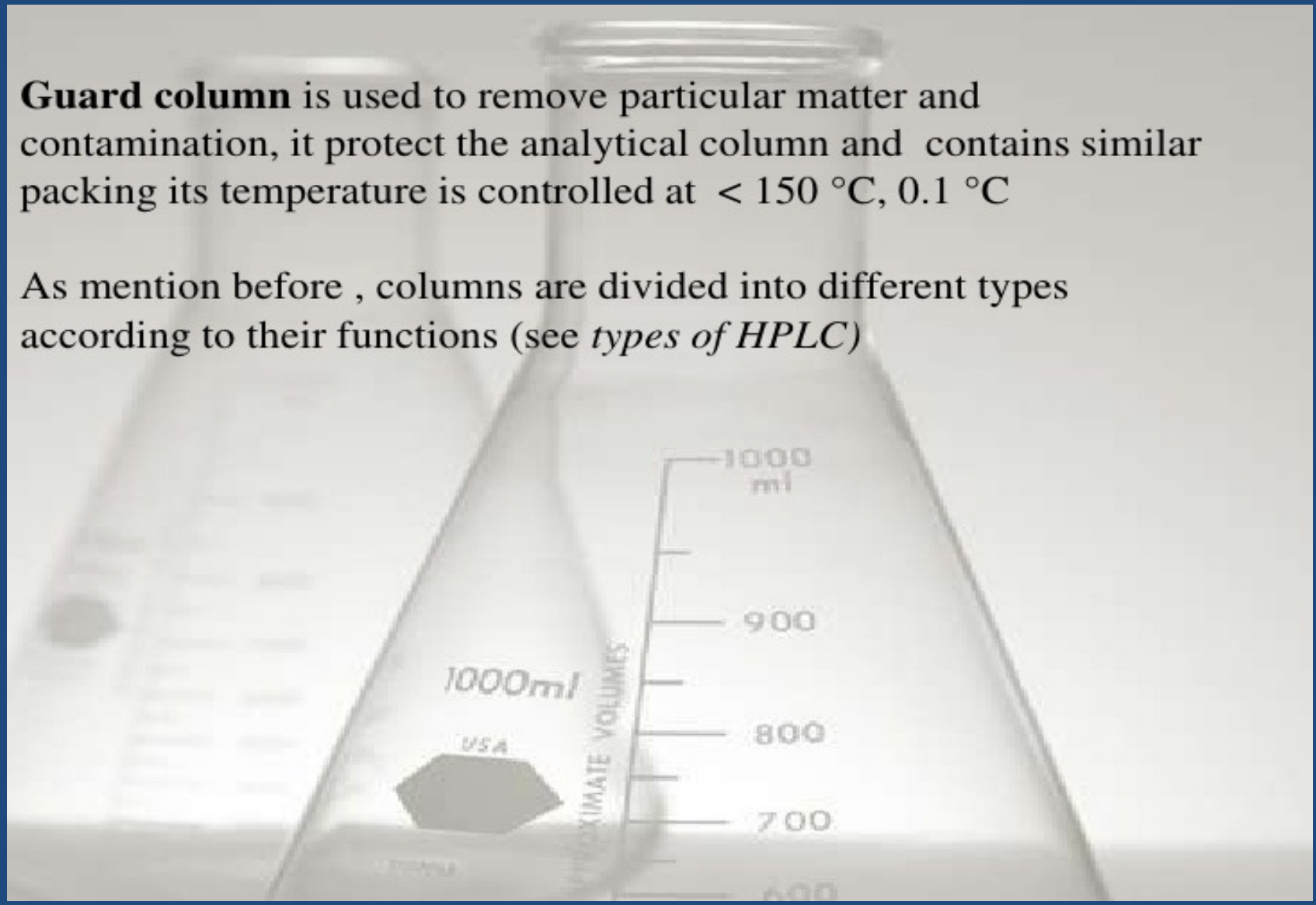
•The dimensions of the analytical column are usually

-straight, Length(5 ~ 25 cm), diameter of column(3 ~ 5 mm), diameter of particle(35 μm). Number (40 k ~ 70 k plates/m)



Guard column is used to remove particular matter and contamination, it protect the analytical column and contains similar packing its temperature is controlled at < 150 °C, 0.1 °C

As mention before , columns are divided into different types according to their functions (see *types of HPLC)*



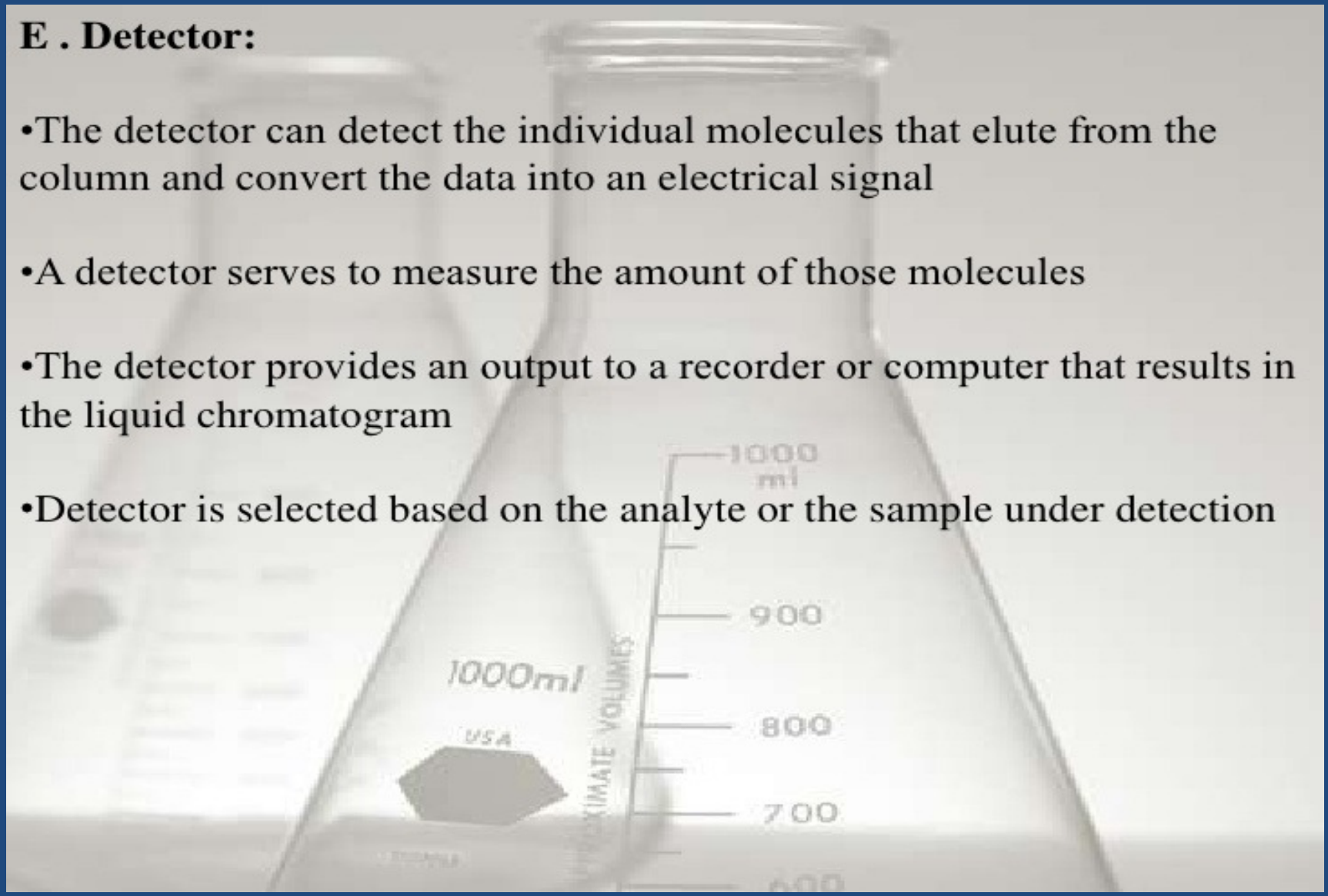
E . Detector:

•The detector can detect the individual molecules that elute from the column and convert the data into an electrical signal

•A detector serves to measure the amount of those molecules

•The detector provides an output to a recorder or computer that results in the liquid chromatogram

•Detector is selected based on the analyte or the sample under detection



Commonly used detectors in HPLC

Ultraviolet (UV)

•This type of detector responds to substances that absorb light.

•The UV detector is mainly to separate and identify the principal active components of a mixture.

•UV detectors are the most versatile, having the best sensitivity and linearity.

•UV detectors cannot be used for testing substances that are low in chromophores (colorless or virtually colorless) as they cannot absorb light at low range.

•They are cost-effective and popular and are widely used in industry

Fluorescence

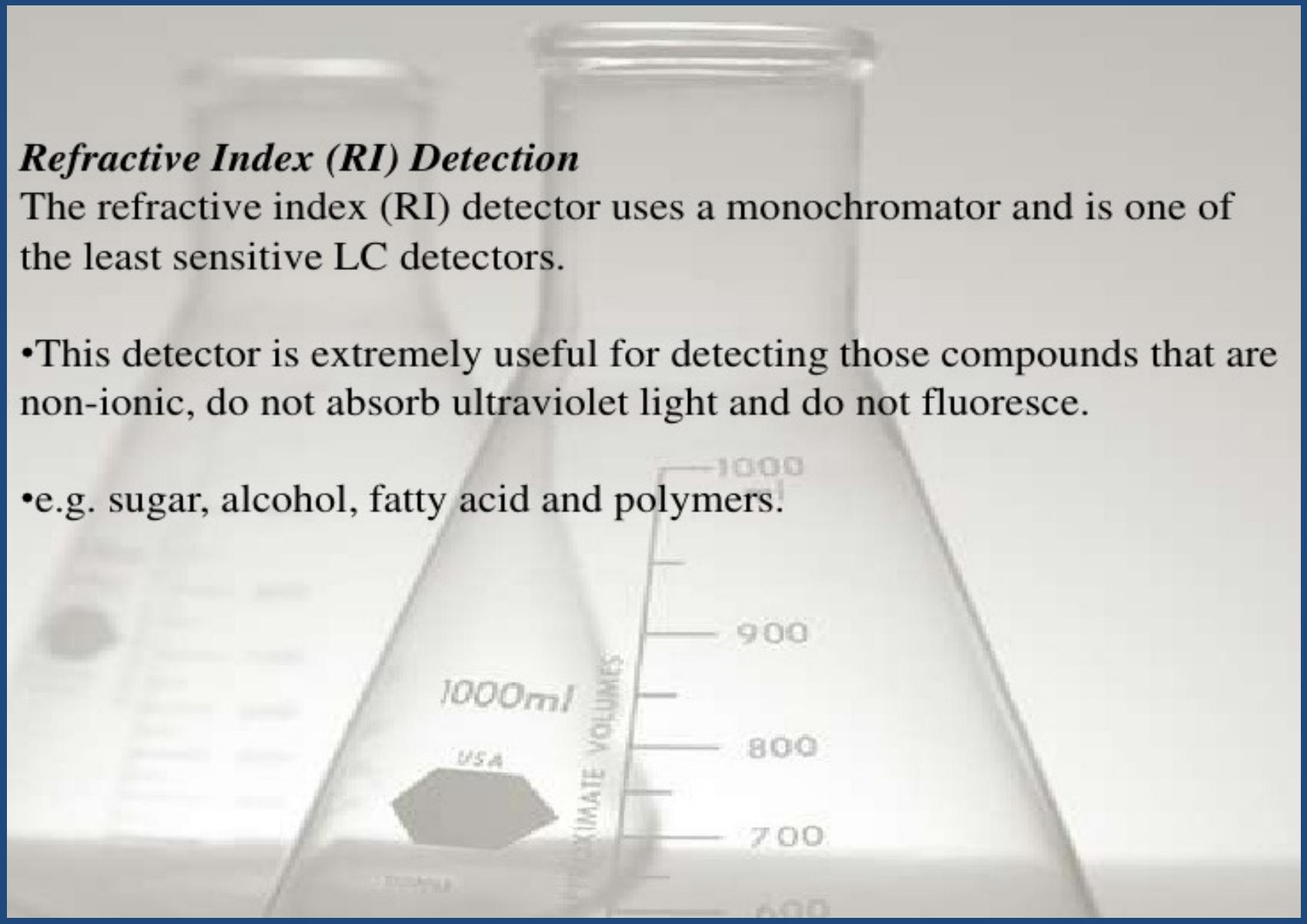
•This is a specific detector that senses only those substances that emit light. This detector is popular for trace analysis in environmental science.

•As it is very sensitive, its response is only linear over a relatively limited concentration range. As there are not many elements that fluoresce , samples must be syntesized to make them detectable.

Mass Spectrometry

•The mass spectrometry detector coupled with HPLC is called HPLC-MS. HPLC-MS is the most powerful detector,widely used in pharmaceutical laboratories and research and development.

•The principal benefit of HPLC-MS is that it is capable of analyzing and providing molecular identity of a wide range of components.



Refractive Index (RI) Detection

The refractive index (RI) detector uses a monochromator and is one of the least sensitive LC detectors.

•This detector is extremely useful for detecting those compounds that are non-ionic, do not absorb ultraviolet light and do not fluoresce.

•e.g. sugar, alcohol, fatty acid and polymers.

F . Data processing unit (Computer)

•Frequently called the data system, the computer not only controls all the modules of the HPLC instrument but it takes the signal from the detector and uses it to determine the time of elution (retention time) of the sample components (qualitative analysis) and the amount of sample (quantitative analysis).

•The concentration of each detected component is calculated from the area or height of the corresponding peak and reported.