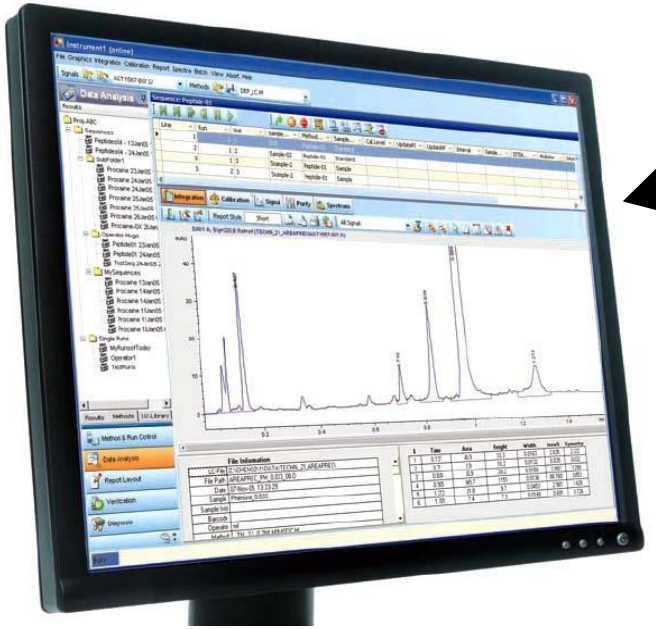
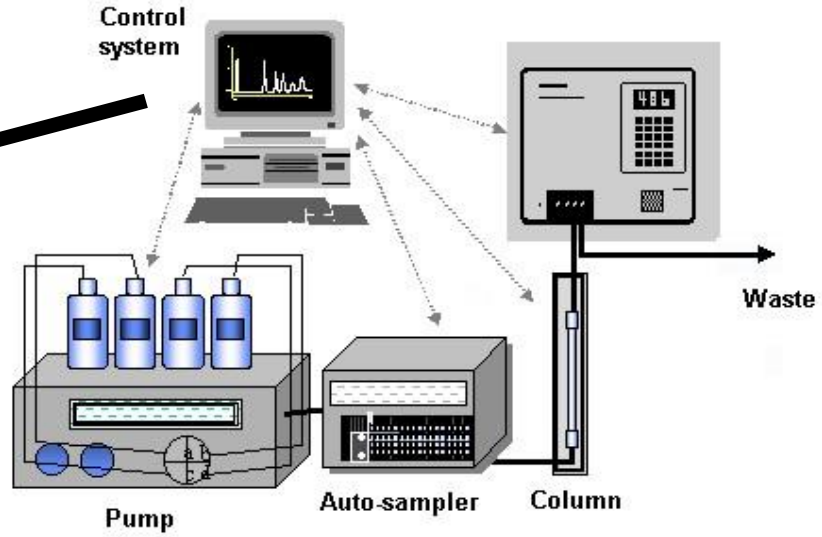
Chemical Analysis by HPLC-UV Detector Device

مقدمة

التحليل الكيميائي بواسطةجهاز HPLC-UV

Dr. Wedad Alonzi





OUTLINE

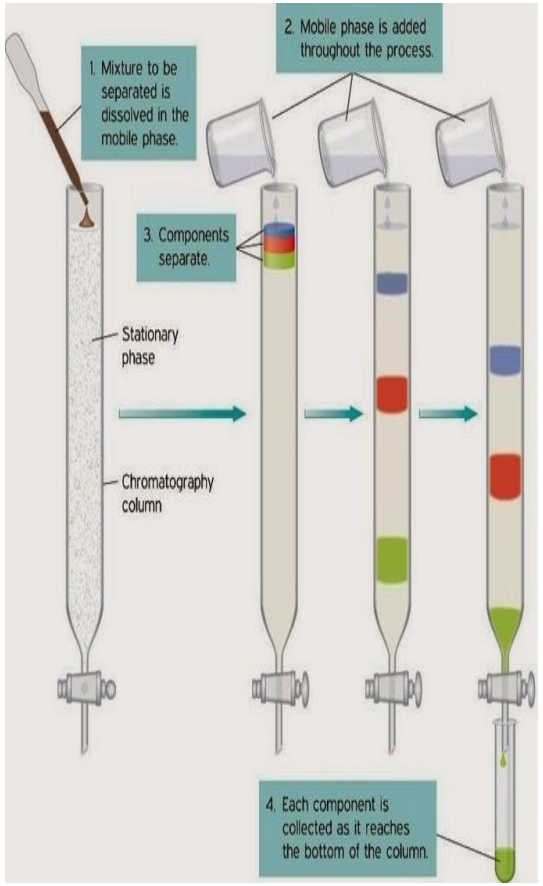
- INTRODUCTION
- TYPES OF CHROMATOGRAPHY
- RP LIQUID CHROMATOGRAPHY
- INSTRUMENTATION
- CHROMATOGRAM
- METHOD DEVELPOMENT LAYOUT
- ADVANTAGES OF HPLC

What is chromatography:

"Chromatography is a physical method of separation in which the components to be separated are distributed between two phases; one of which is:

stationary (stationary phase)

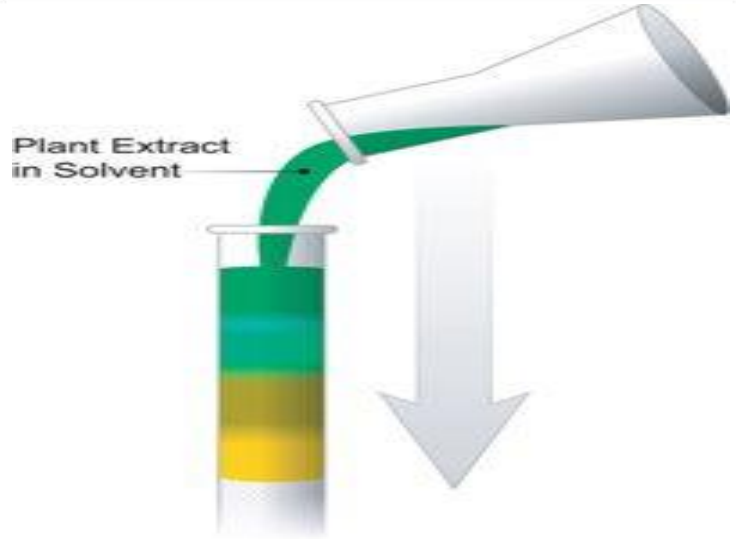
while the other moves in a definite direction (mobile phase)".



Chromatography; What does it mean?

To write with colors literally translated from its Greek roots ***chroma*** and ***graphein*** , chromatography was first developed by the Russian botanist Mikhail Tswett in 1903 as he produced a color ful separation of plant pigments through a column of calcium carbonate.

Mikhail Tswett the Russian botanist who separated six pigments from a leaf pigment extraction.

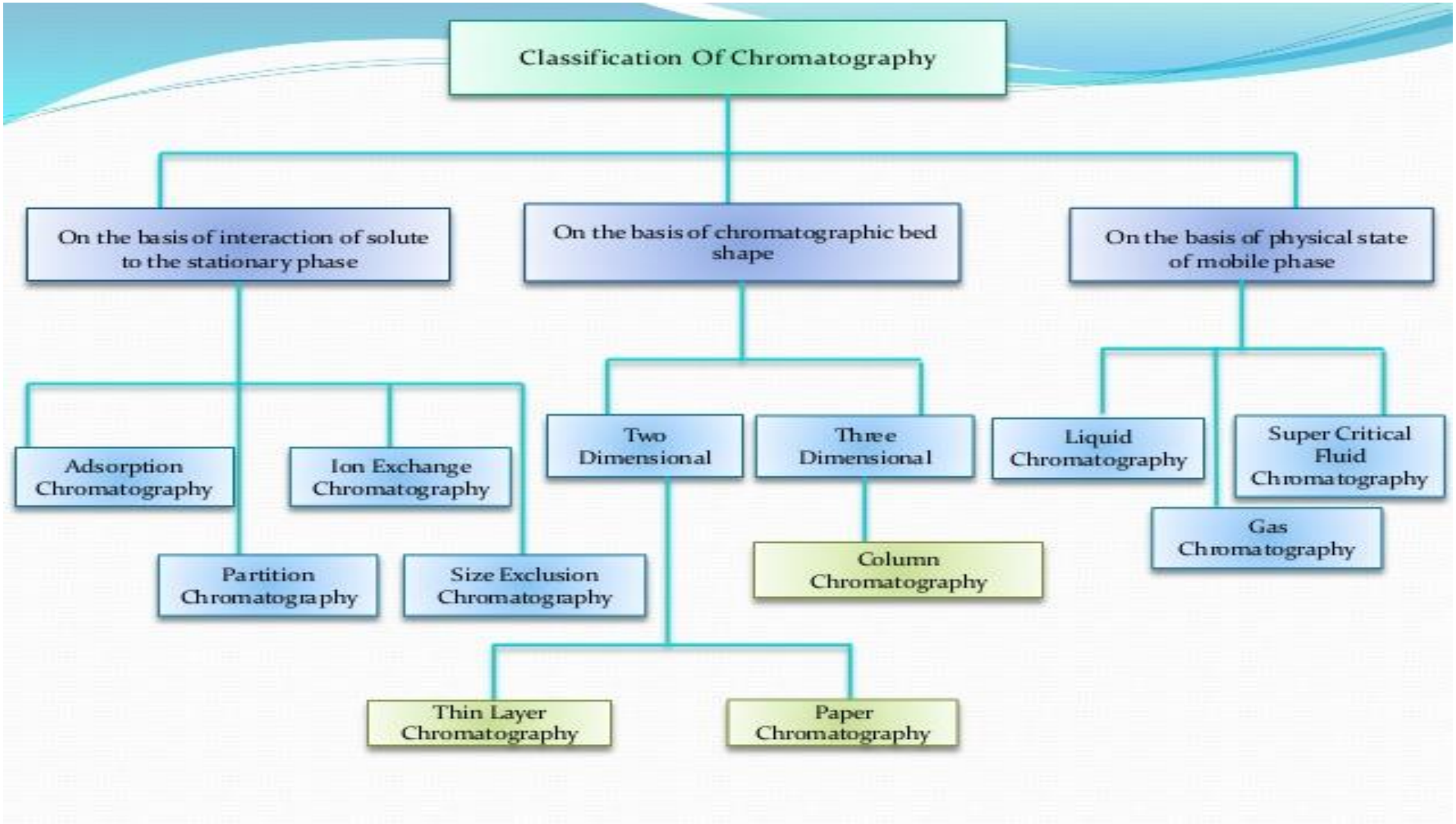


History of Chromatography

n the 1940s, two scientists named Archer John Porter Martin and Richard Laurence Millington Synge made an important breakthrough in the field of chromatography. They developed a new approach to chromatography, which used two liquid phases instead of one. This helped separate compounds with different partition coefficients

12 Nobel prizes were awarded between 1937 and 1972 alone for work in which chromatography played a vital role

During the 1970s, new breakthroughs in chromatography technology helped refine this popular technique even more. It was at this time that pumps were developed to help push the liquid phase and compounds through the stationary phase. As a result, the compounds were able to pass through and be separated more quickly. It was called high performance—or in some cases high pressure—liquid chromatography.

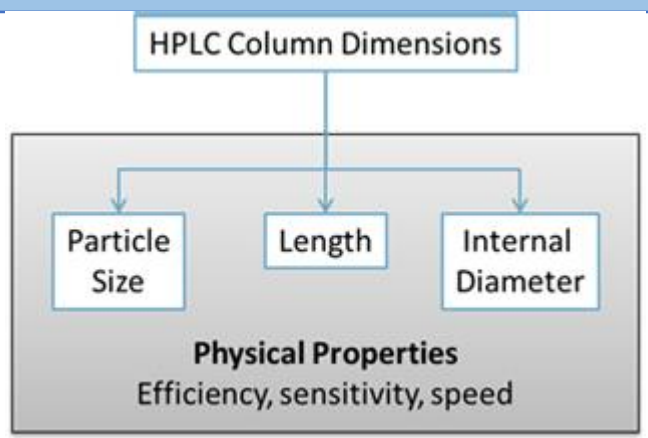

Classification Of Chromatography
On the basis of interaction of solute to the stationary phase
On the basis of chromatographic bed shape
On the basis of physical state of mobile phase
Adsorption Chromatography
Ion Exchange Chromatography
Partition Chromatography
Size Exclusion Chromatography
Two Dimensional
Three Dimensional
Column Chromatography
Thin Layer Chromatography
Paper Chromatography
Liquid Chromatography
Super Critical Fluid Chromatography
Gas Chromatography

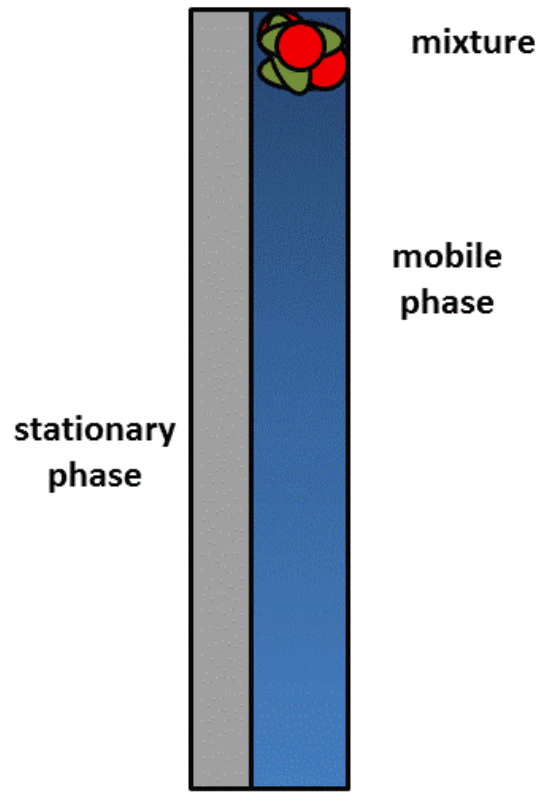
H: High

P: Performance , pressure

L: Liquid

C: Chromatography




mixture
mobile phase
stationary phase

HPLC is really the automation of traditional liquid chromatography under conditions which provide for enhanced separations

during shorter periods of time,

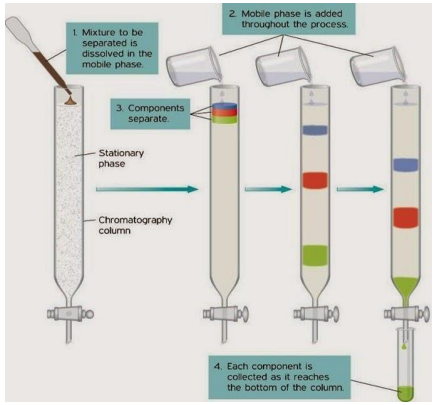
utilizing very small particles,

small column diameters, and

very high fluid pressures

What is HPLC?

HPLC is a technique for separation, identification and quantification of components in a mixture. It is especially suitable for compounds which are not easily volatalised, thermally unstable and have high molecular weights.

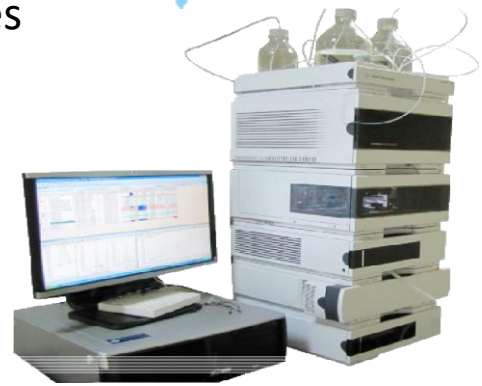


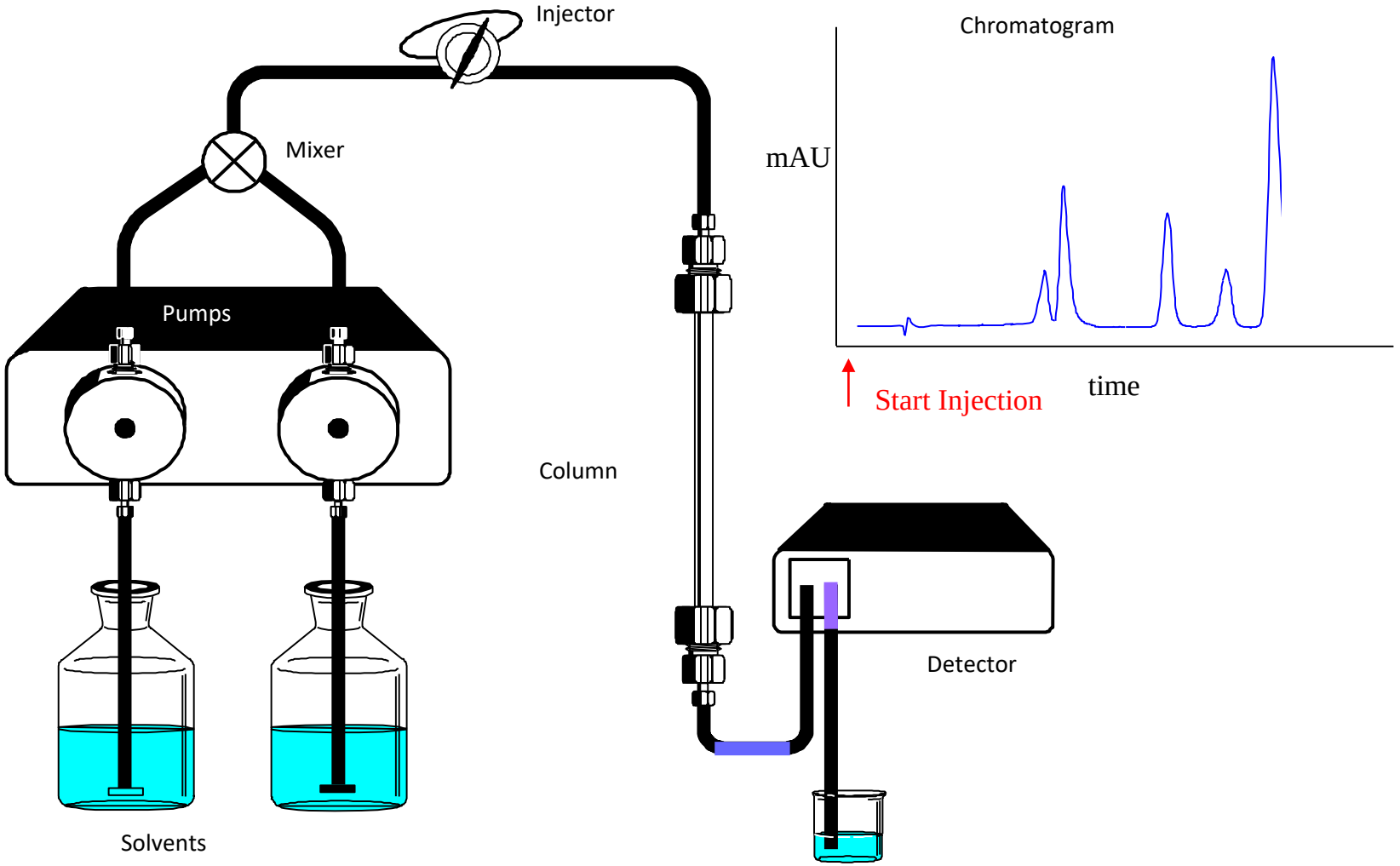
HPLC is a type of liquid chromatography where the sample is forced through a column that is packed with a stationary phase composed of irregularly or spherically shaped particles, a porous monolithic layer , or a porous membrane by a liquid (mobile phase) at high pressure.

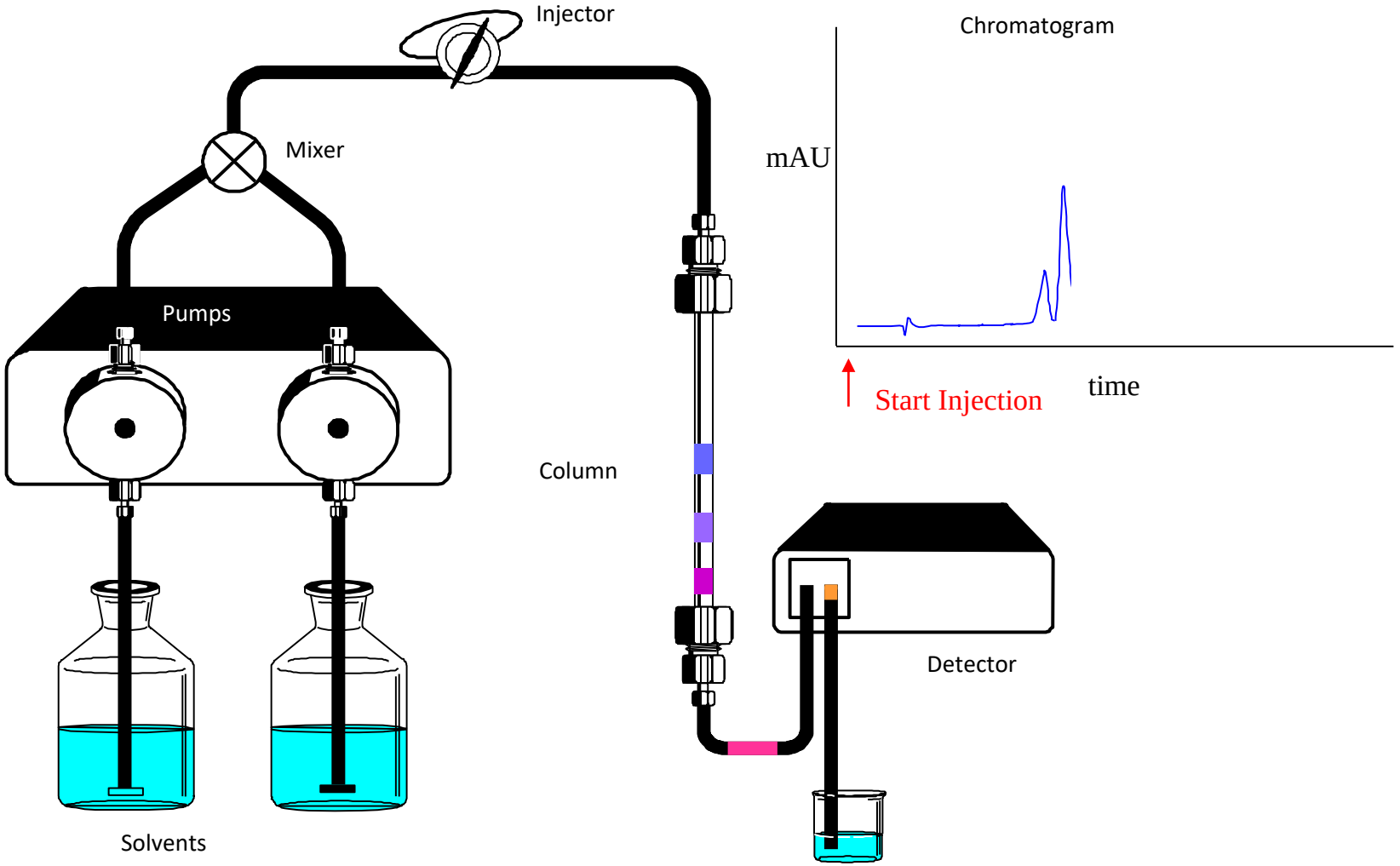
To understand the principle of HPLC , we must first look at the principle behind liquid chromatography Liquid chromatography is a separation technique that involves: •the placement (injection) of a small volume of liquid sample •into a tube packed with porous particles (stationary phase) •where individual components of the sample are transported along the packed tube (column) by a liquid moved by gravity.

The main principle of separation is adsorption . •When a mixture of components are introduced into the column . various chemical and/or physical interactions take place between the sample molecules and the particles of the column packing .

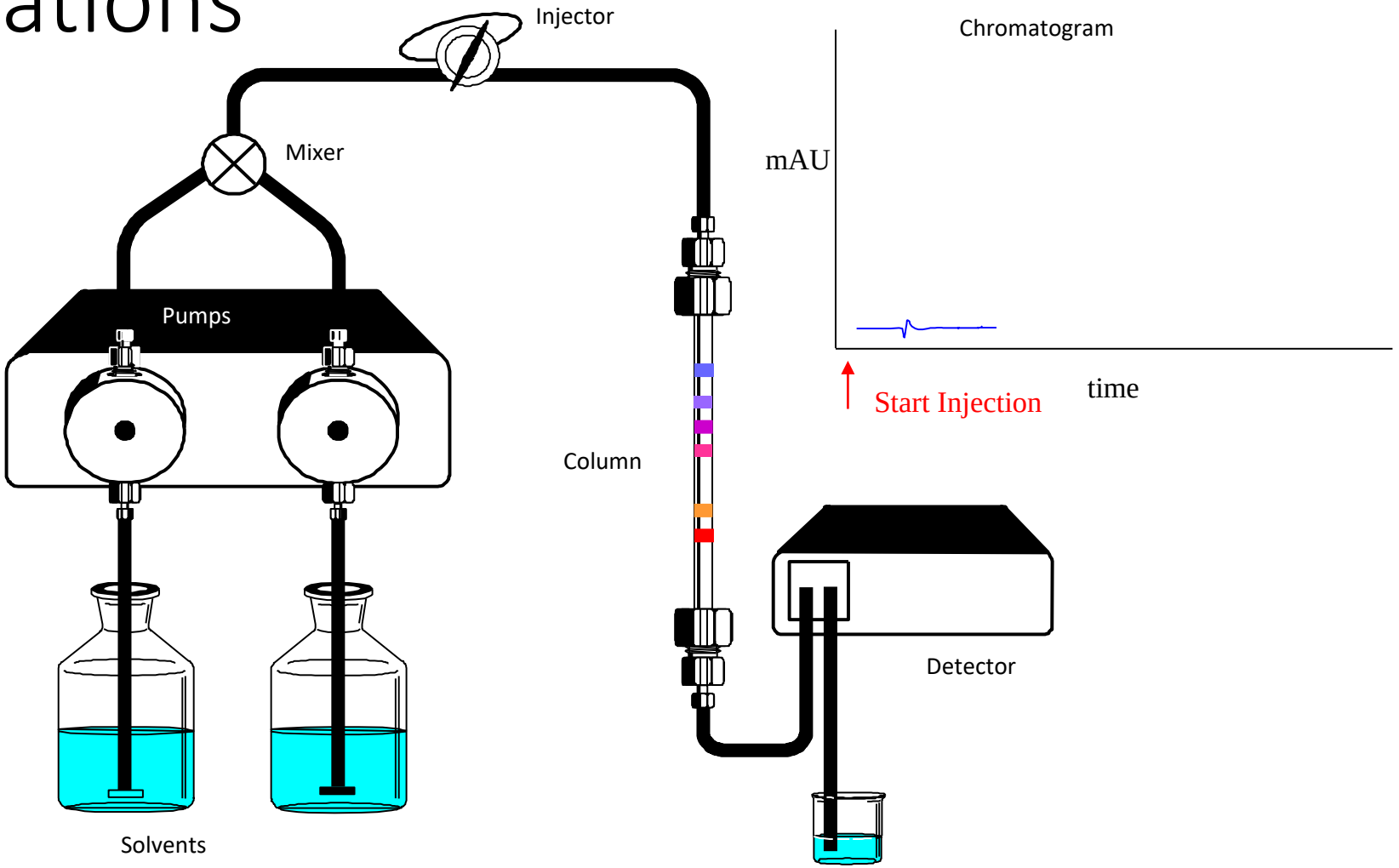
EVOLUTION

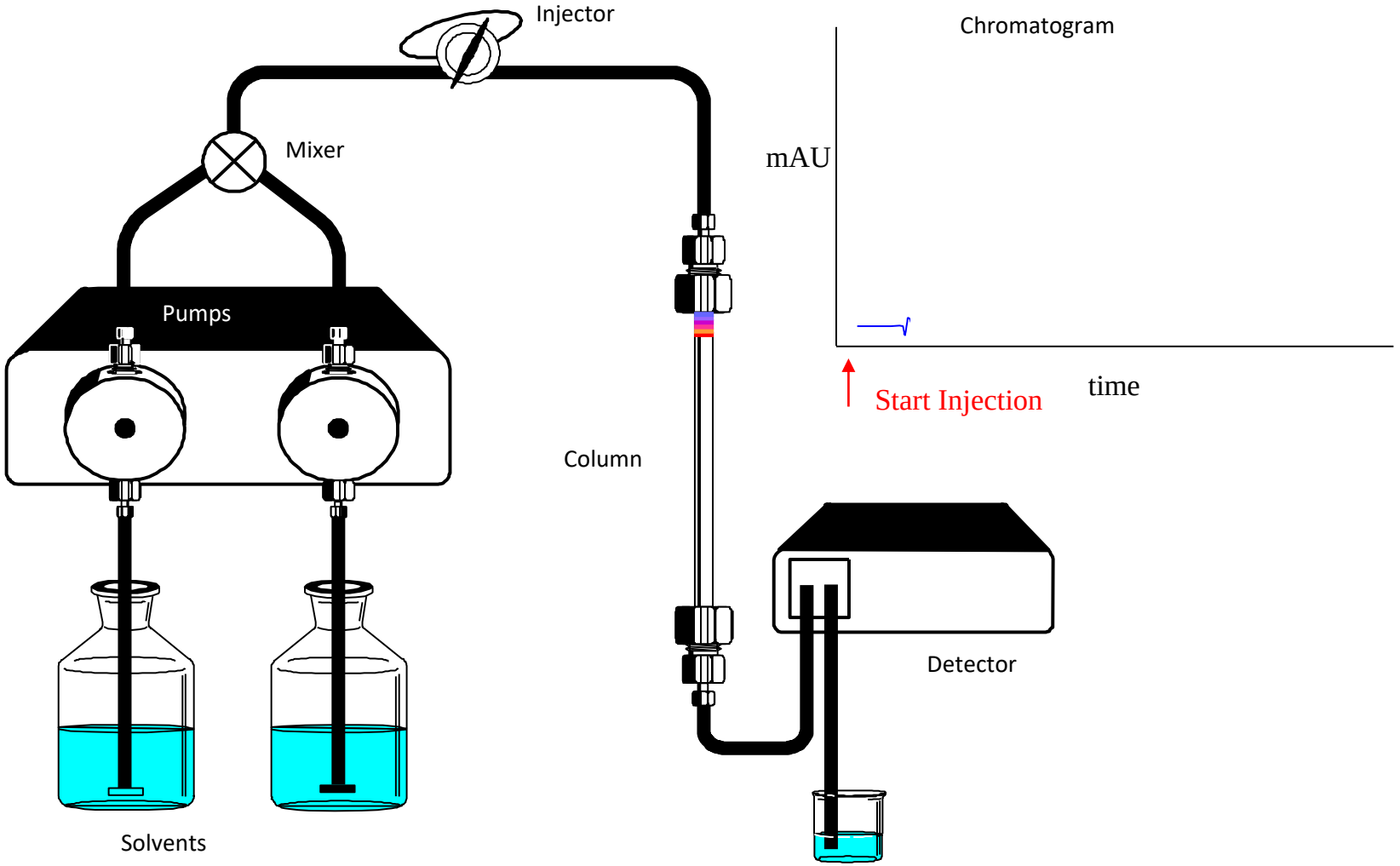


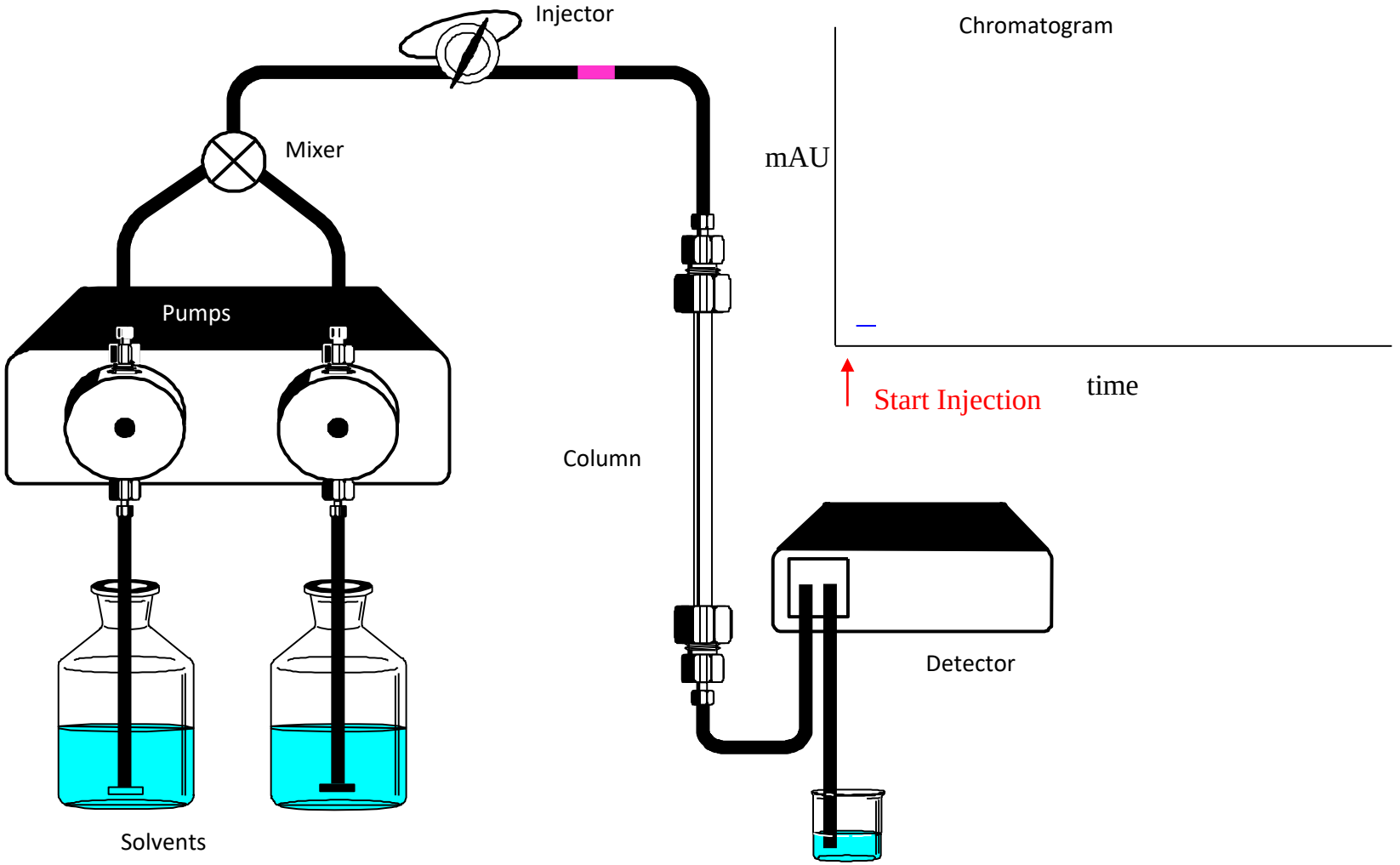

Injector
Mixer
Pumps
Column
Solvents
Detector
Chromatogram
mAU
time
Start Injection


Injector
Chromatogram
Mixer
mAU
Pumps
Column
Start Injection
time
Detector
Solvents

Separations



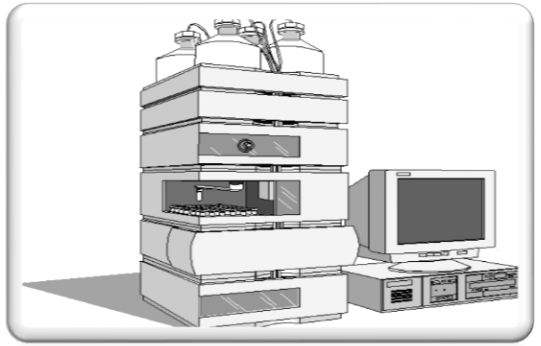

Injector
Chromatogram
Mixer
mAU
Pumps
Column
Start Injection
time
Detector
Solvents

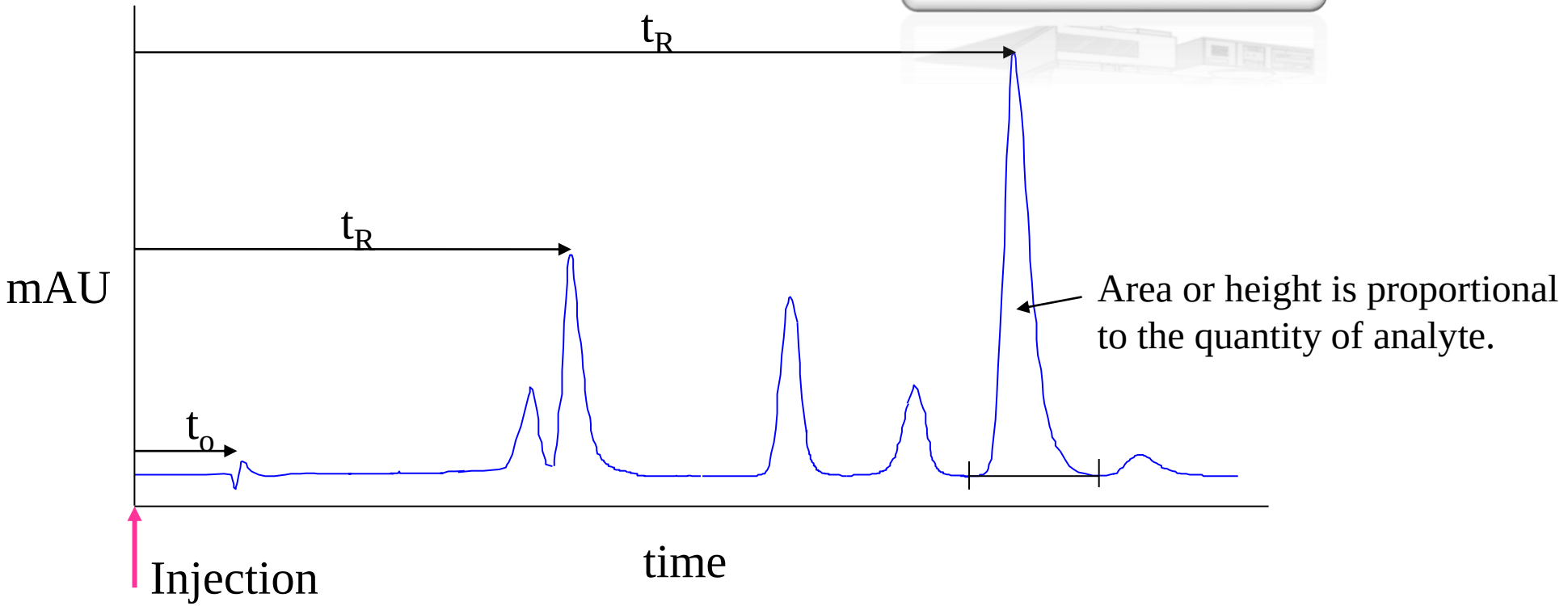

Injector
Chromatogram
Mixer
mAU
Pumps
Column
Start Injection
time
Detector
Solvents

The Chromatogram

t_o - elution time of unretained peak

t_R- retention time - determines sample identity





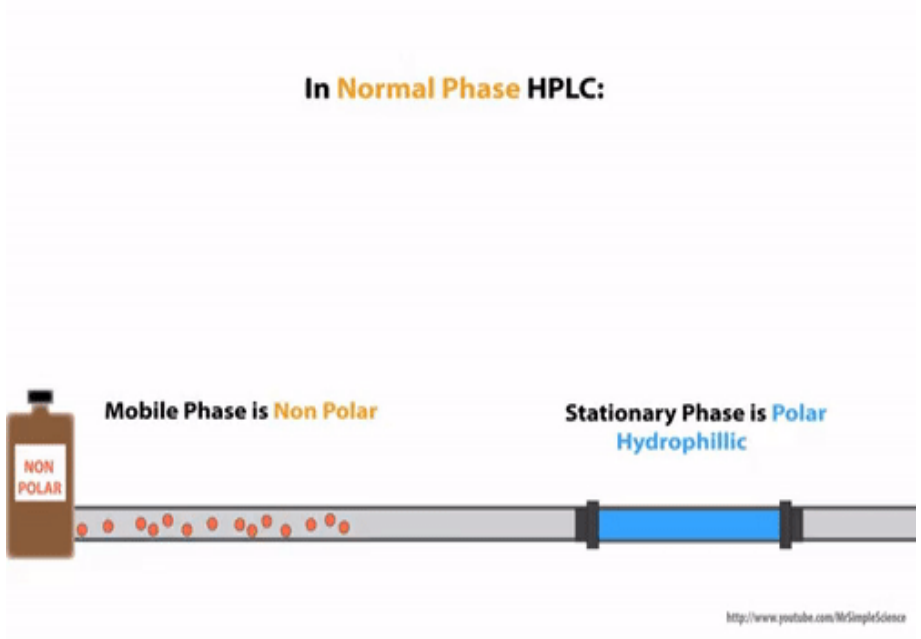
The HPLC column stationary phase is where the separation occurs and is the most important part of the system. Different types of analysis are classified based on the type of stationary phase and mechanism behind the separation in the column.

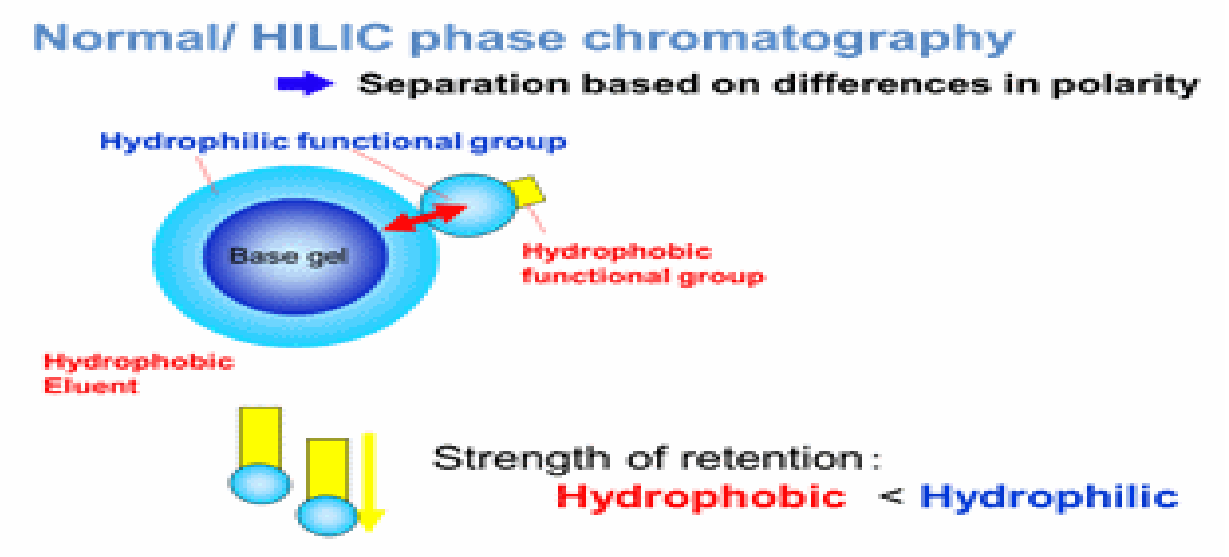
Modes of HPLC

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

Normal phase

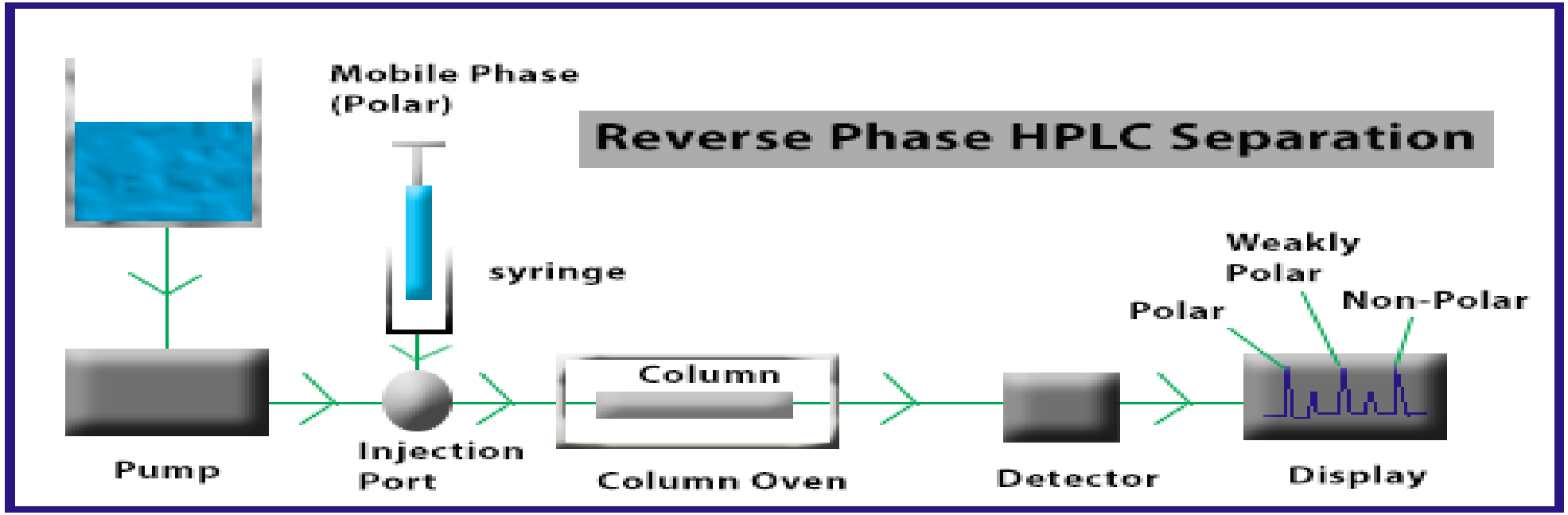
In this column type, the retention is governed by the interaction of the polar parts of the stationary phase and solute. For retention to occur in normal phase, the packing must be more polar than the mobile phase with respect to the sample

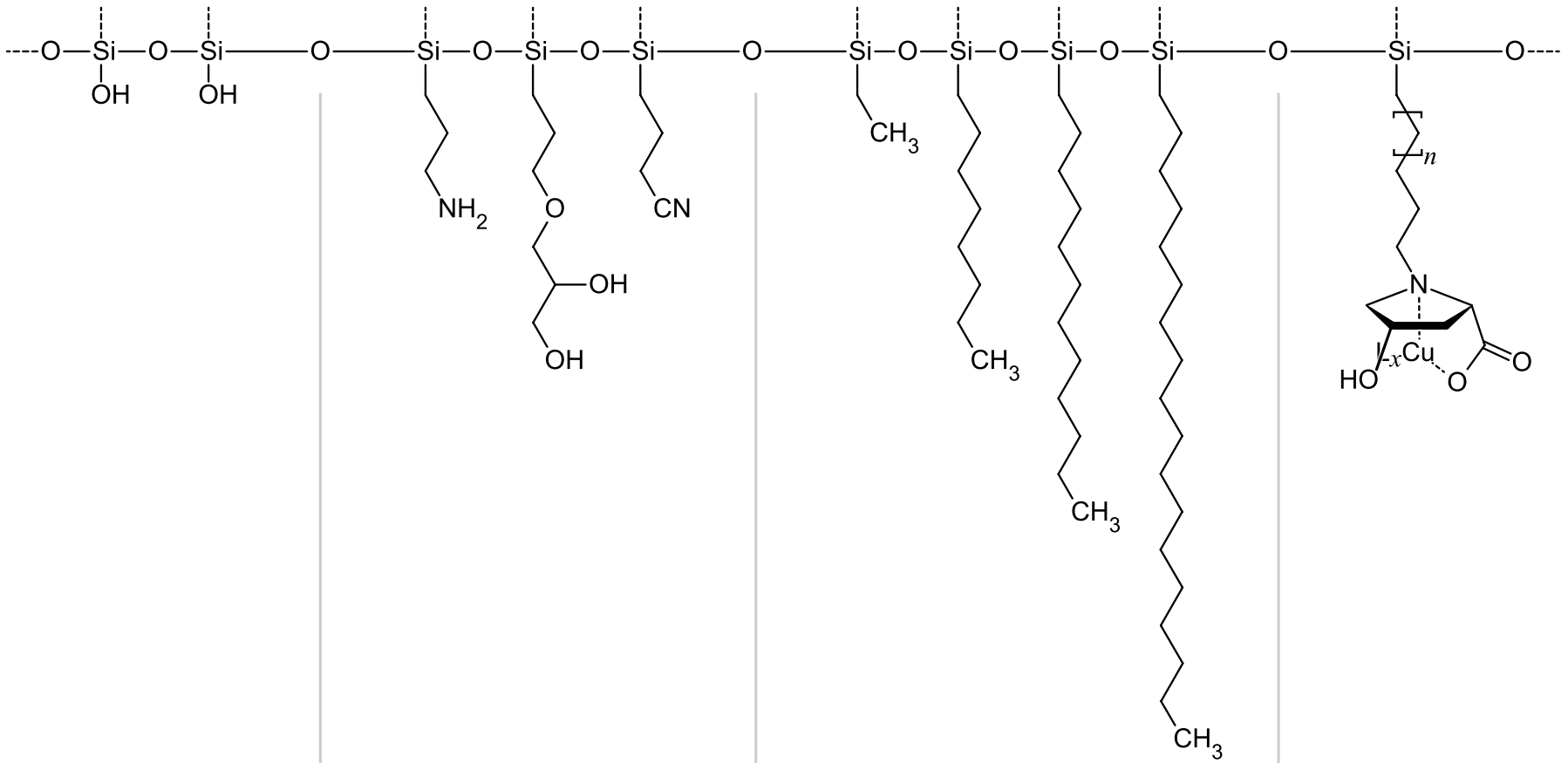




Reverse phase

- In this column the packing material is relatively nonpolar and the solvent is polar with respect to the sample. Retention is the result of the interaction of the nonpolar components of the solutes and the nonpolar stationary phase. Typical stationary phases are nonpolar hydrocarbons, waxy liquids, or bonded hydrocarbons (such as C18, C8, etc.) and the solvents are polar aqueous-organic mixtures such as methanol-water or acetonitrile-water.





polare Phase | mittelpolare Phasen | unpolare Phasen | chirale Phase

Bonded Phases

- C-2 Ethyl Silyl $-Si-CH_2-CH_3$
- C-8 Octyl Silyl $-Si-(CH_2)_7-CH_3$
- C-18 Octadecyl Silyl $-Si-(CH_2)_{17}-CH_3$
- CN Cyanopropyl Silyl $-Si-(CH_2)_3-CN$

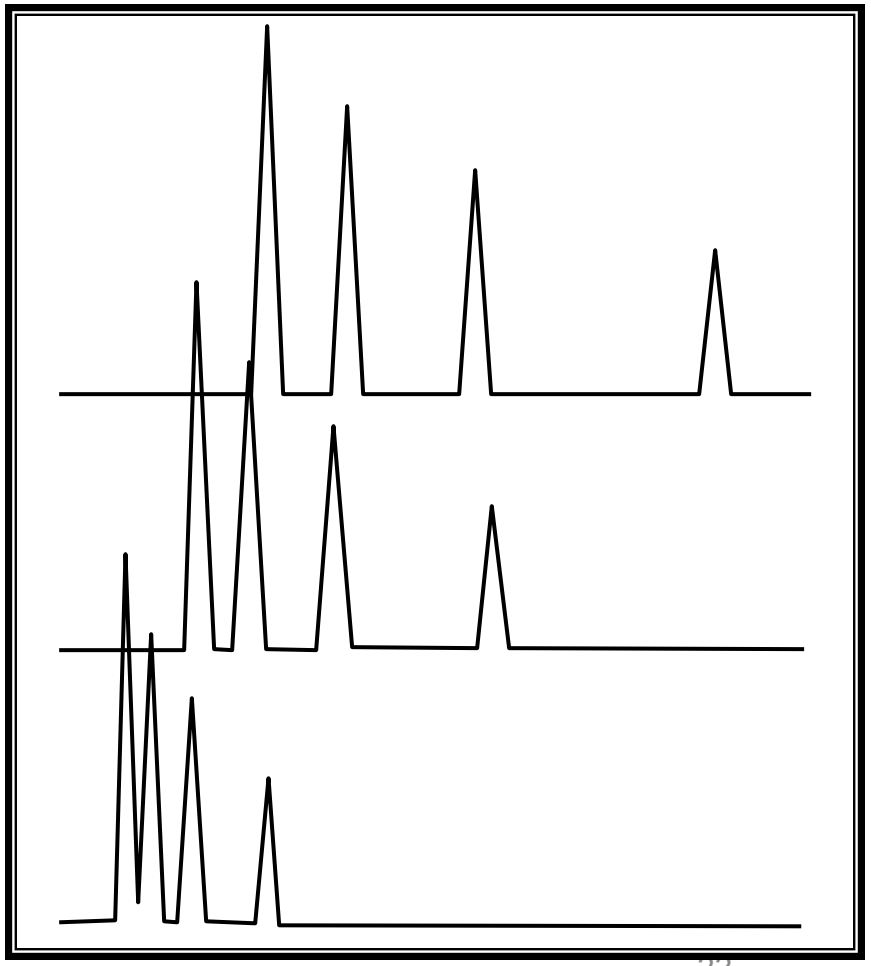
Summery of the modes of separation

- **Other Aspects**

Type of compounds	Mode	Stationary Phase	Mobile Phase
Neutrals, weak acids, weak bases	Reversed-phase	C8, C18, cyano, amino	Water, organics
Ionics, acids, bases	Ion pair	C8, C18	Water/organic ion-pair reagent
Compounds not soluble water	Normal phase	Amino, cyano, diol, silica	Organics
Ionics, inorganic compounds	Ion exchange	Anion or Cation exchange resin	Aqueous/Buffer
High molecular weight compounds	Size exclusion	Polystyrene, silica	Gel filtration: aqueous Gel permeation: organic

- Unretained compounds like uracil or potassium nitrate are used to determine dead volume (t_0) for a reversed-phase column. A non-polar compound like 1,3,5-tri-*tert*.-butylbenzene (TTBB) is used for the same purpose in normal-phase chromatography (i.e., silica).

Relationship between Polarity of Eluent and Retention Time in Reversed Phase Mode



Eluent: Methanol / Water

60/40

70/30

80/20

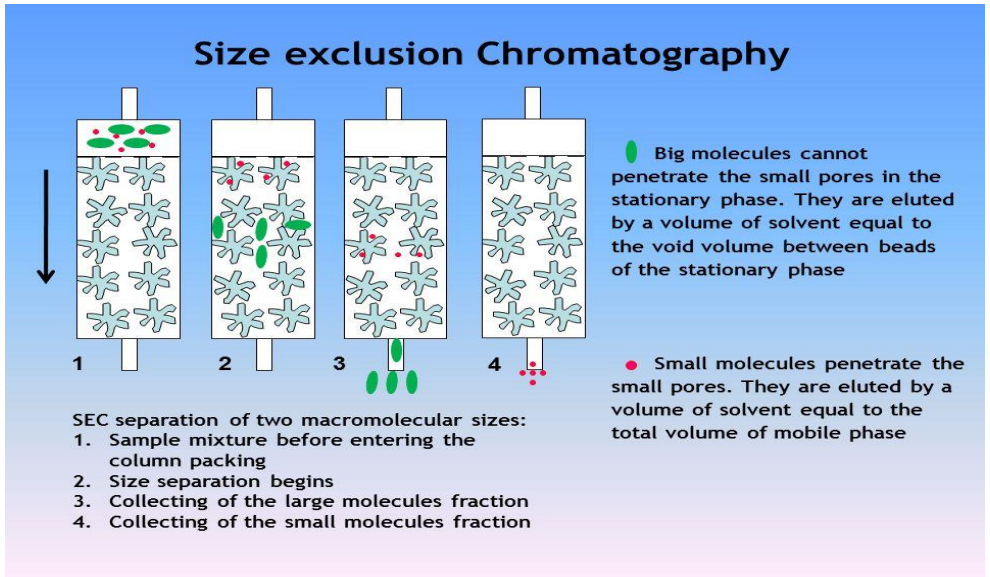
Size exclusion SEC

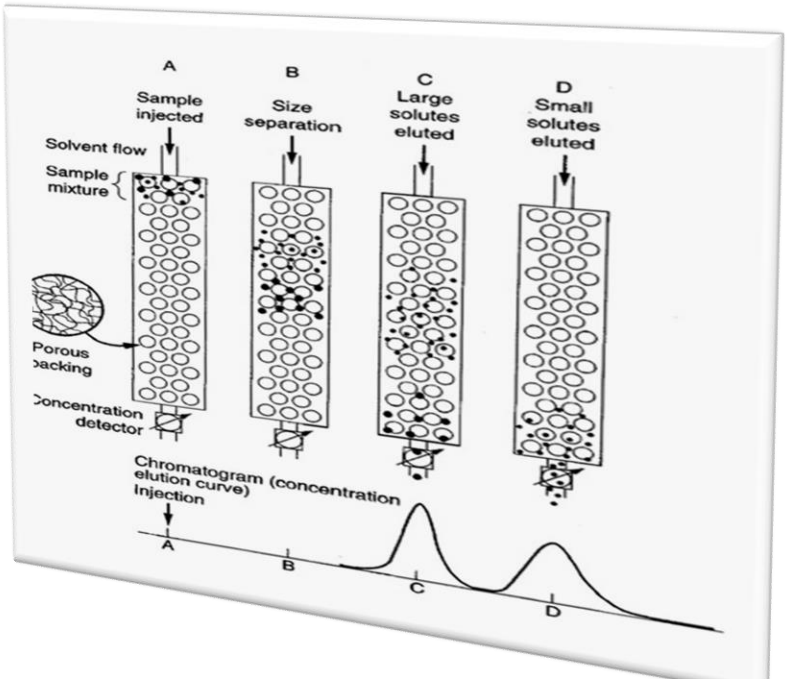
Packings are porous polymeric (resins) or silica based materials Two names used for the same process:

1)Gel filtration chrom. = aqueous solvent

2)Gel permeation chromatography = nonaqueous mobile phase

Column packing works like a molecular filter allowing small molecules access to every pore, retarding their progress –large molecules pass thru more quickly





Ion exchange Chromatography:

Synthetic organic resins are normally employed for separation or water soluble ionizable compounds. Anion exchangers have positive centres on surface and are used to separate compounds having sulfonate, phosphate or carboxylate groups.

Cation exchangers have negative canters on the surface and are used to separate basic substances such as amines.

Cross-linked styrene divinylbenzene is typical base material with charged groups linked to phenyl rings. Charges on packing material attract oppositely charged molecules from mobile phase and release them in inverse order of the attraction forces.
Separation of components can be controlled by
control of pH of mobile phase,
temperature,
ionic composition and addition of modifiers.

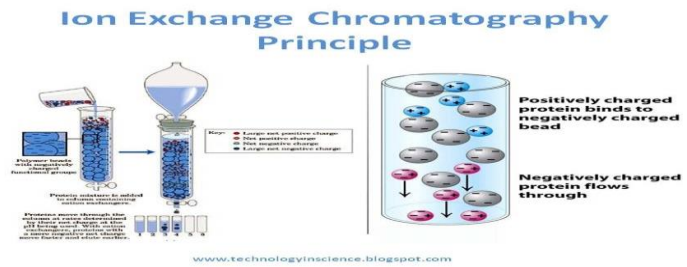
Resin Type	Cation Exchanger	Anion Exchanger
Net charge of molecule of interest	+	−
Charge of resin	−	+
Running conditions	0.5–1.5 pH units below the pI of the molecule of interest	0.5–1.5 pH units above the pI of the molecule of interest

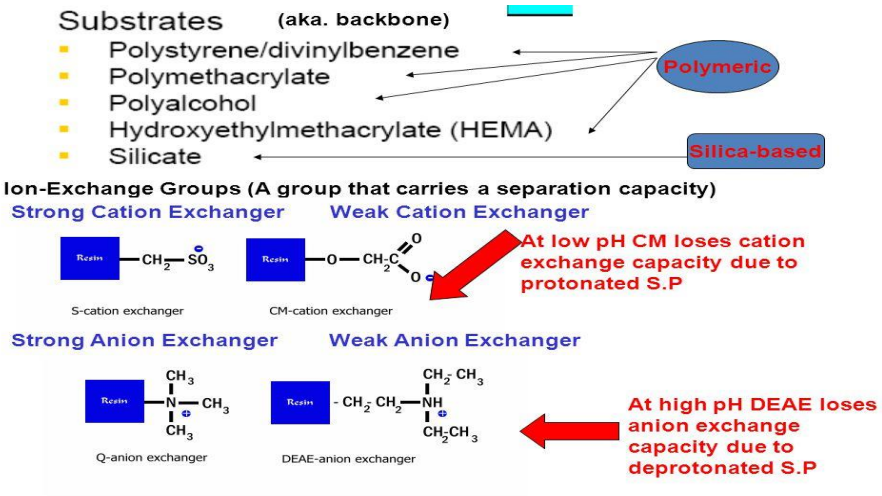
Can write reactions in general format

$xRSO_3^- H^+ + M^{x+} \rightleftharpoons (RSO_3^-)_x M^{x+} + xH^+$

solid solution solid solution

Where R = polymer support (styrene divinylbenzene)





HPLC Applications

Chemical

polystyrenes
dyes
phthalates

Bioscience

proteins
peptides
nucleotides

Pharmaceuticals

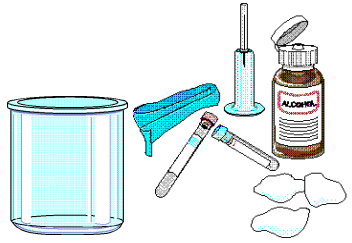
tetracyclines
corticosteroids
antidepressants
barbiturates

Consumer Products

lipids
antioxidants
sugars

Environmental

polyaromatic hydrocarbons
Inorganic ions
herbicides



Clinical

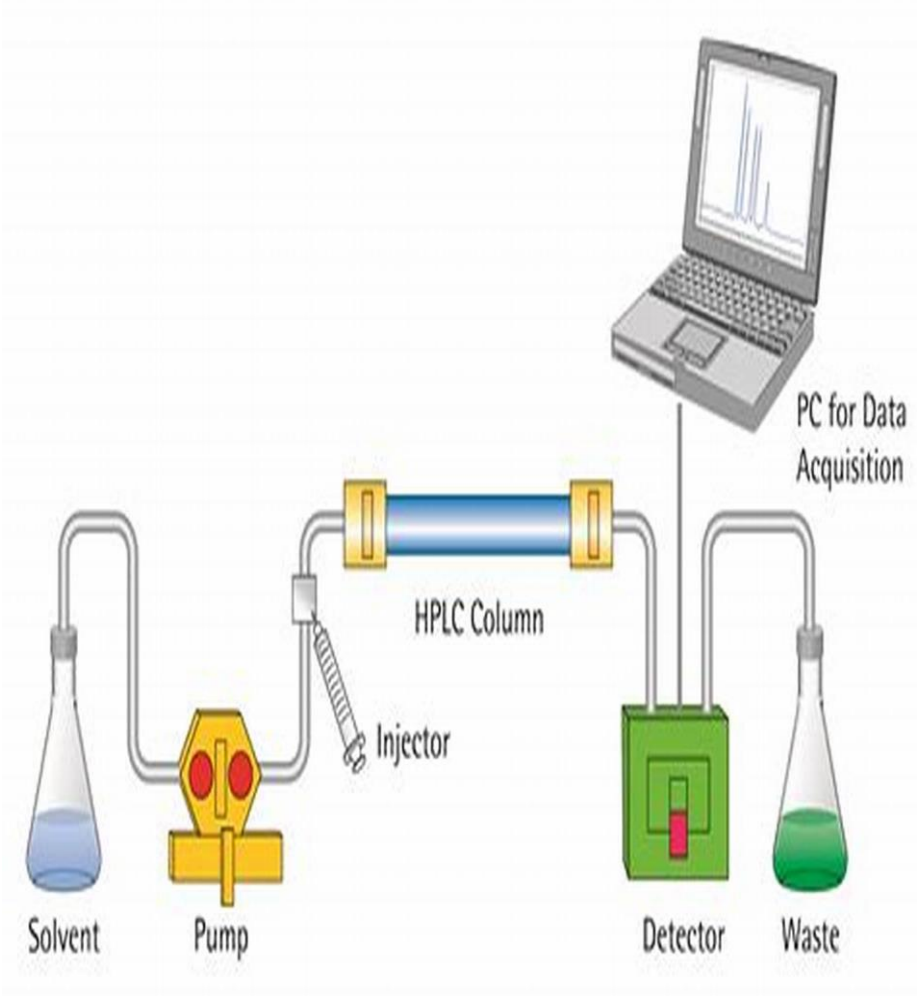
amino acids
vitamins
homocysteine

Advantages of High Performance Liquid Chromatography

- High separation capacity, enabling the batch analysis of multiple components
- Superior quantitative capability and reproducibility
- Moderate analytical conditions
- Unlike GC, the sample does not need to be vaporized.
- Generally high sensitivity
- Low sample consumption
- Easy preparative separation and purification of samples

Components of HPLC

1. Solvent Reservoir
2. Pumps
3. Sample Injection System
4. Columns
5. Detectors
6. Data Processing
7. Waste

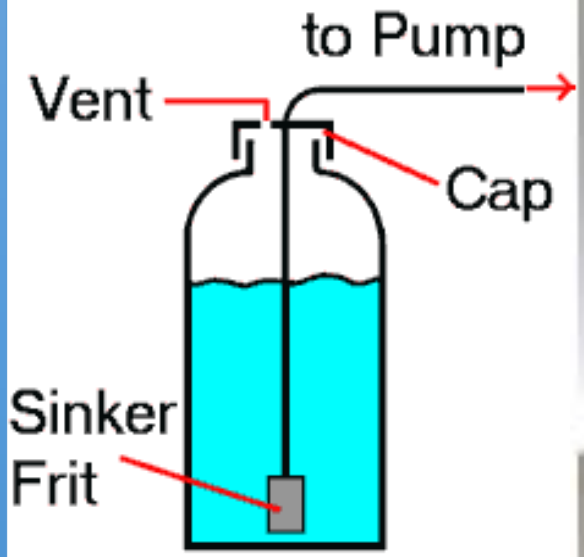

PC for Data
Acquisition
HPLC Column
Injector
Solvent
Pump
Detector
Waste

HPLC Hardware:

Detectors and Their Ranges of Application

Mobile Phase Reservoirs

These are inert containers for mobile phase storage and transport. Generally transparent glass bottles are used so as to facilitate visual inspection of mobile phase level inside the container. Stainless steel particulate filters are provided inside for removal of particulate impurities in the mobile phase if any.



Elution modes

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

Gradient elution

- A separation in which the mobile phase composition is changed during the 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

Gradient vs. Isocratic Conditions


Pump

Isocratic

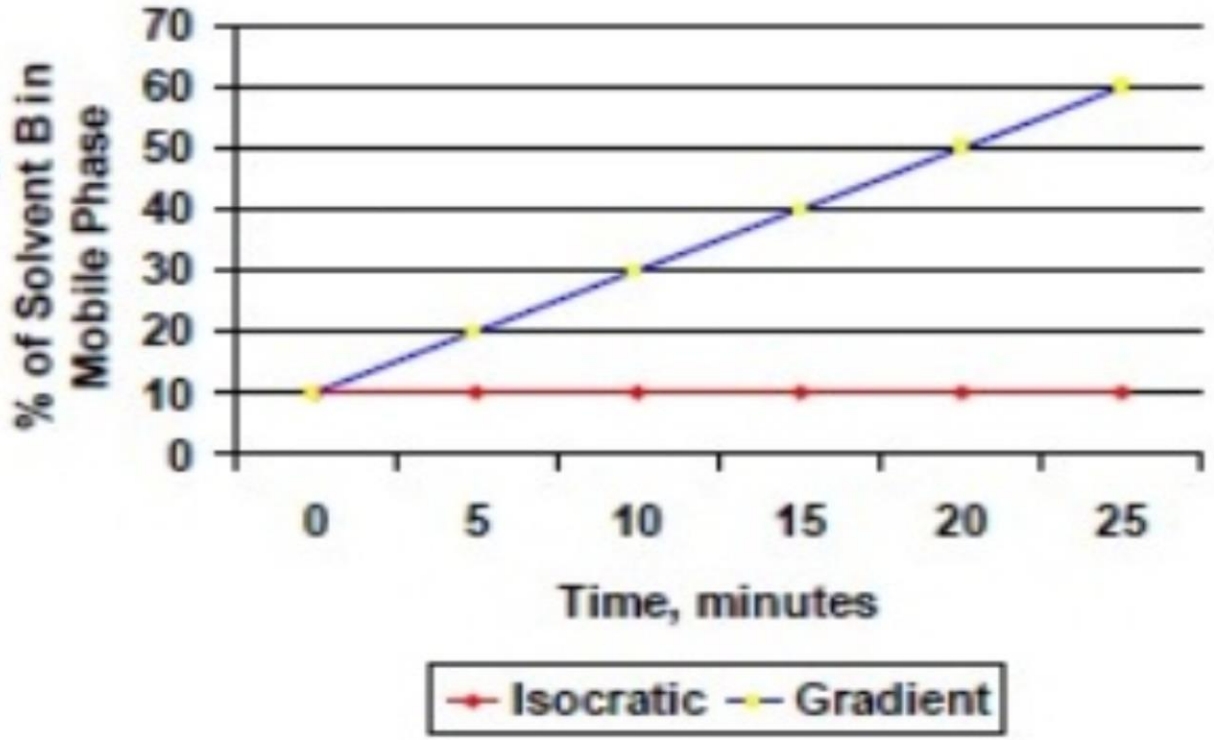
mobile phase solvent composition remains constant with time

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

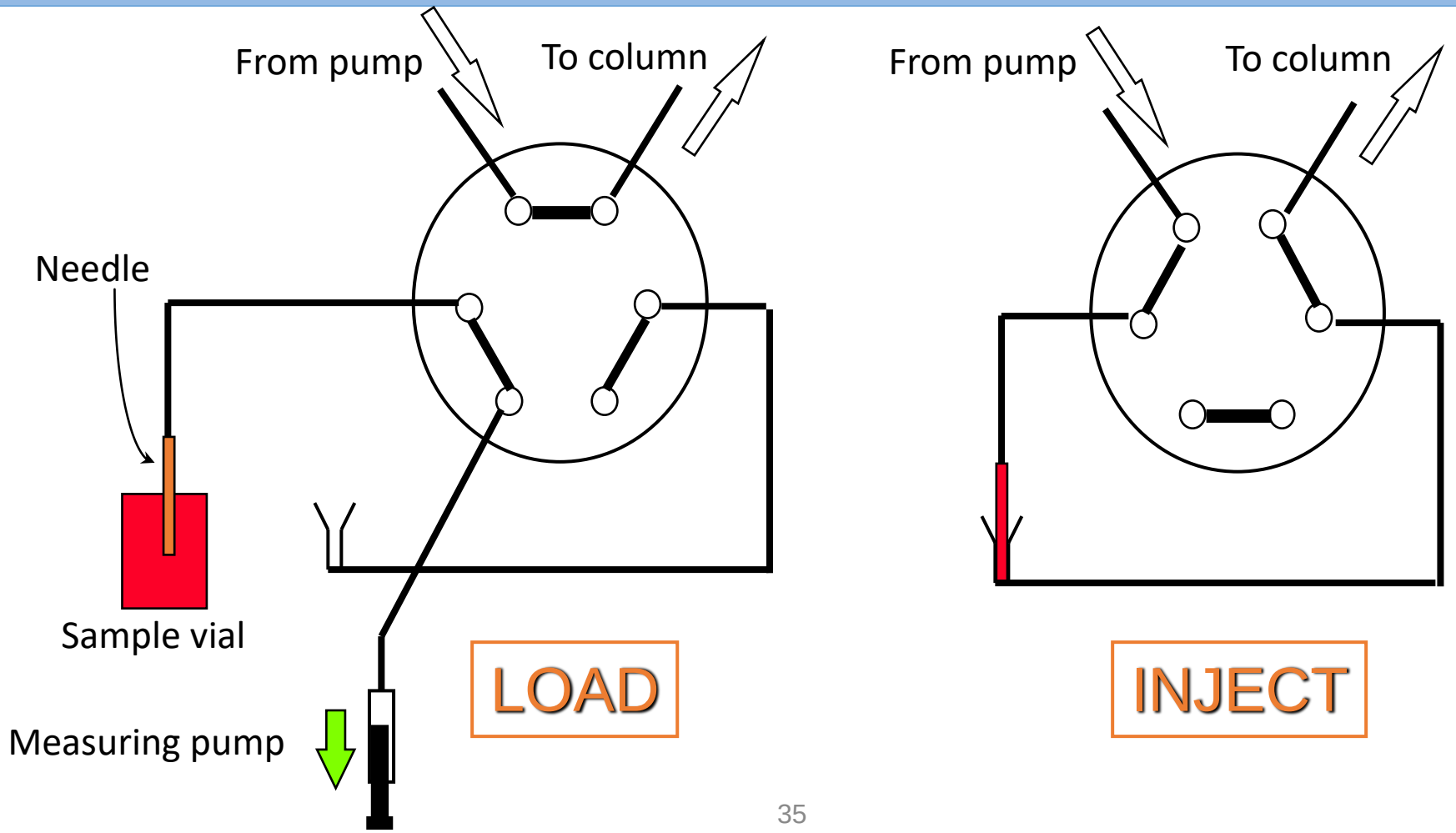
Gradient

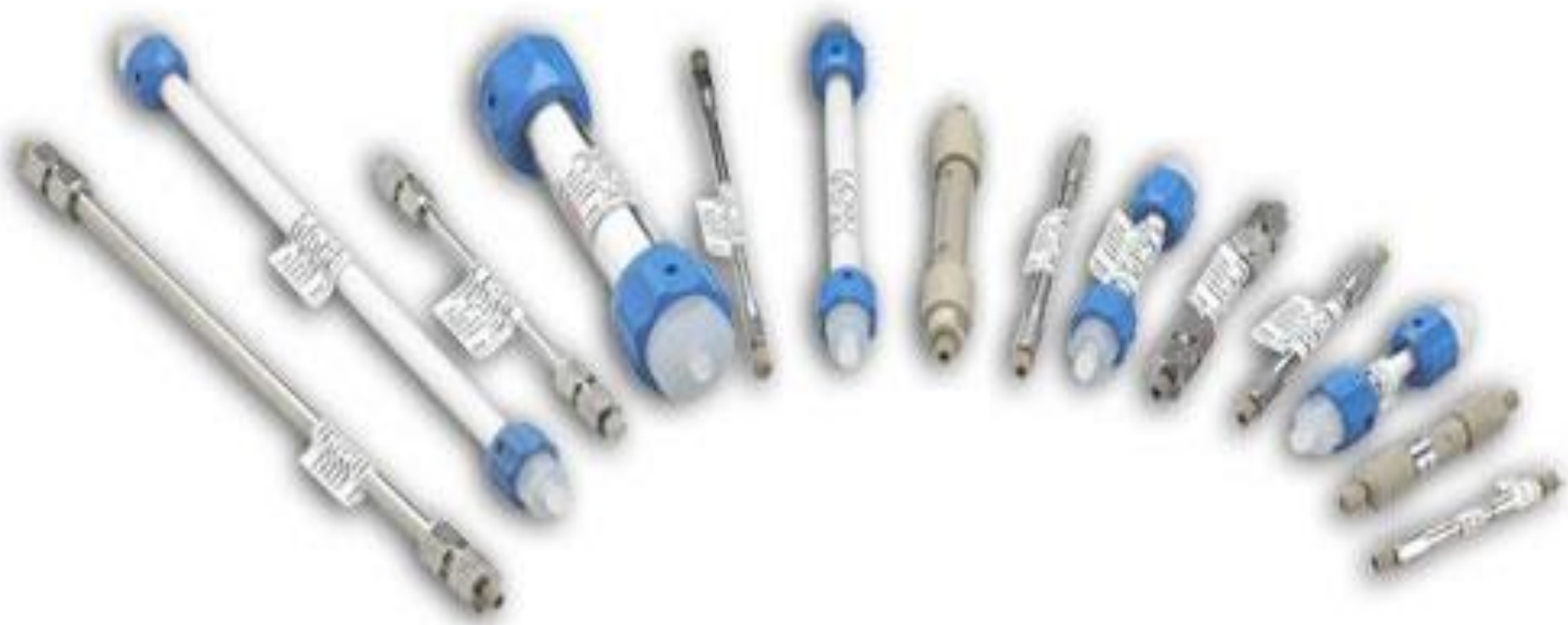
mobile phase solvent ("B") composition increases with time

- Best for the analysis of complex samples
- Often used in method development for unknown mixtures
- Linear gradients are most popular (for example, the "gradient" shown at right)



Injector: Autosampler





Detection Condition Requirements

- Sensitivity
  - The detector must have the appropriate level of sensitivity.
- Selectivity
  - The detector must be able to detect the target substance without, if possible, detecting other substances.
- Adaptability to separation conditions
- Operability, etc.

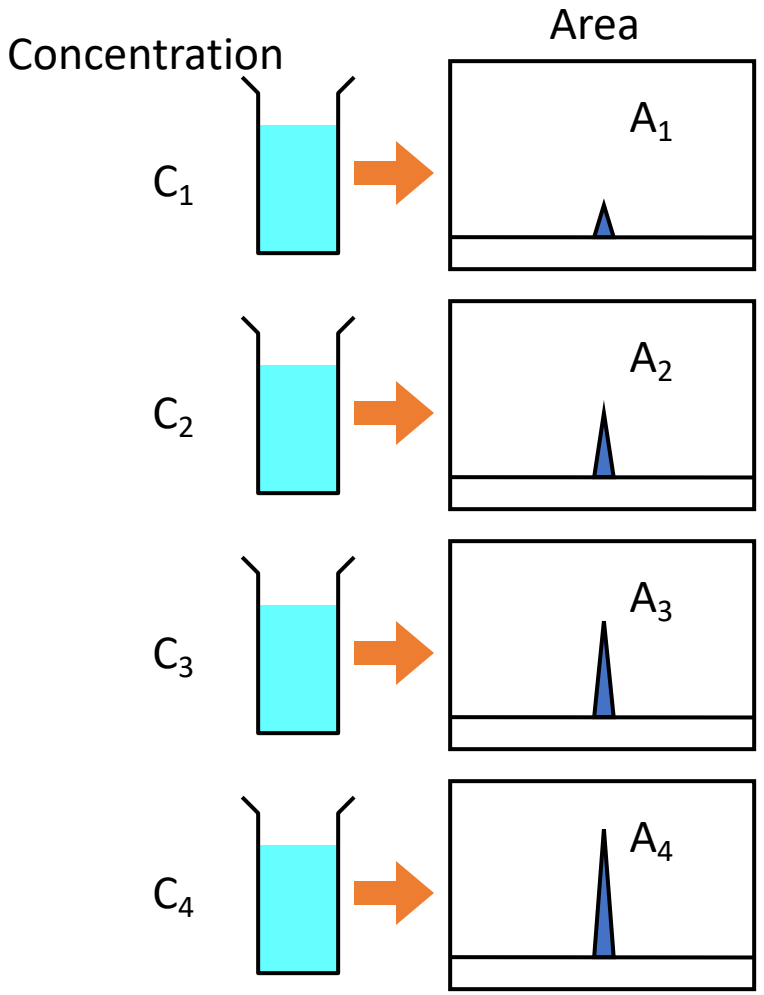
Qualitative Analysis

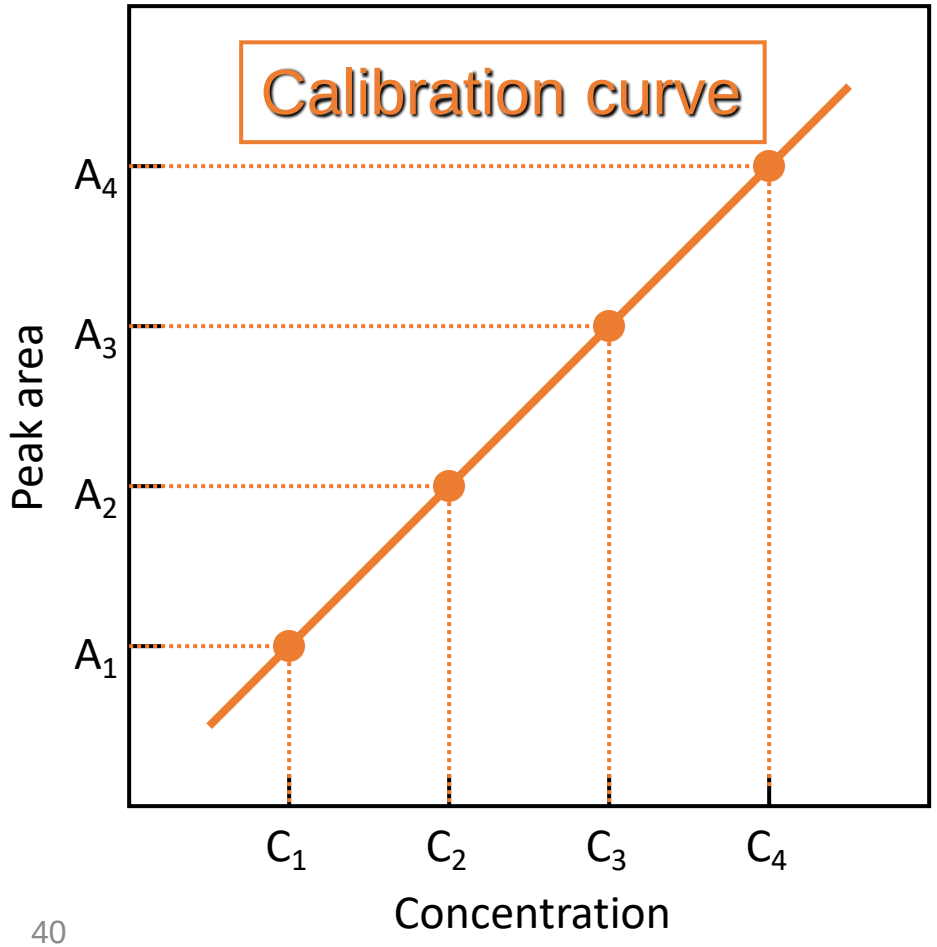
- Identification based on retention time
- Acquisition of spectra with detector
  - UV spectra
  - MS spectra
- Transfer to other analytical instruments after preparative separation

Quantitative Analysis

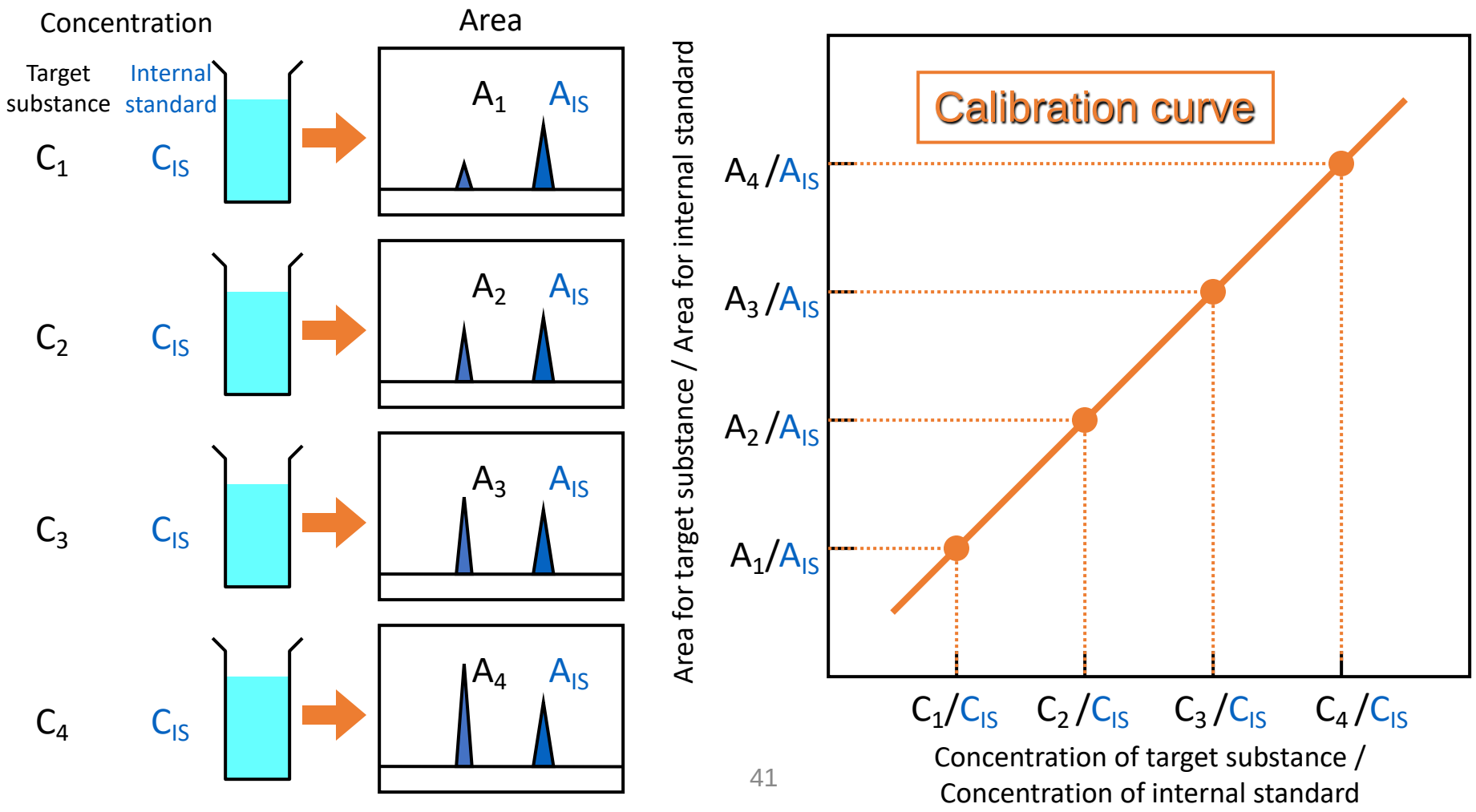
Absolute Calibration Curve Method and Internal Standard Method

Calibration Curve for Absolute Calibration Curve Method





Calibration Curve for Internal Standard Method



Selection Criteria for Internal Standard

- It must have similar chemical properties to the target substance.
- Its peak must appear relatively near that of the target substance.
- It must not already be contained in the actual samples.
- Its peak must be completely separated from those of other sample components.
- It must be chemically stable.

Substances That Must Not Be Injected into the Column

- Insoluble substances (e.g., microscopic particles and precipitation)
- Substances that are precipitated in the eluent
- Substances that irreversibly adsorb to the packing material
- Substances that dissolve, or chemically react, with the packing material