Ministry of higher education and scientific research
AL-Mustaqbal University college
Department of medical physics

Analytical chemistry(practical)

Lecture 6

HPLC Technique

(High Performance Liquid Chromatography Technique)

E.Learning by :
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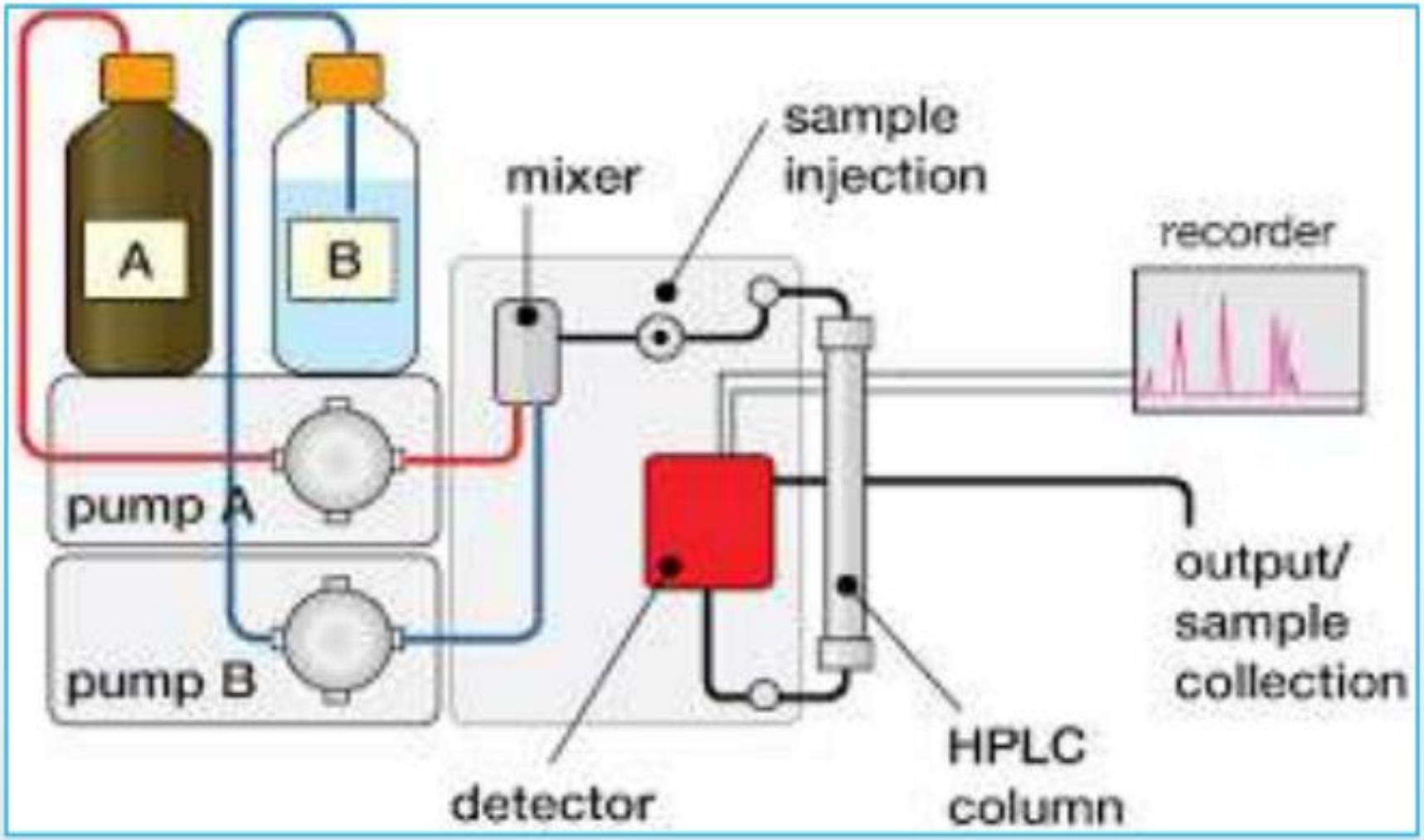
We discussed some of the main components of HPLC technique



How is a sample actually put into an HPLC system?

Sample Injection…

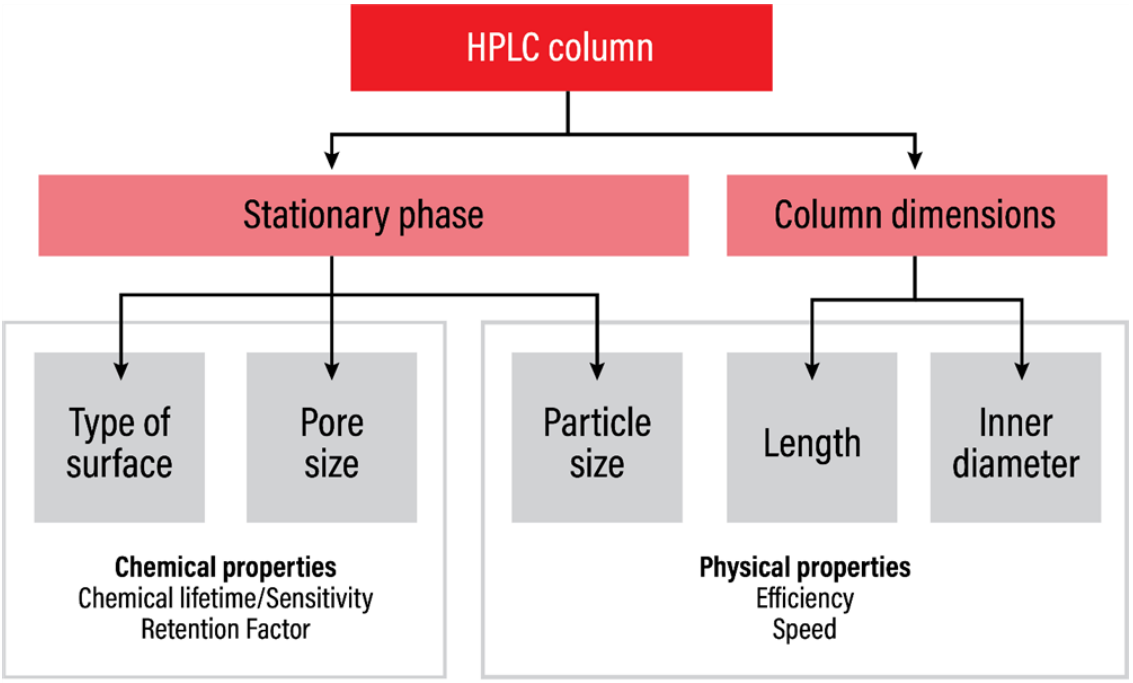
1- Manual Injector:
1.User manually loads sample into the injector using a syringe and
2.then turns the handle to inject sample into the flowing mobile phase… which transports the sample into the beginning (head) of the column, which is at high pressure.

2- Auto sampler:
1.User loads vials filled with sample solution into the auto sampler tray (100 samples)
2.and the autosampler automatically
a.measures the appropriate sample volume,
b.injects the sample,c.then flushes the injector to be ready for the next sample, etc., until all sample vials are processed

HPLC Columns Types

Within the Column is where separation occurs

Key Point –Proper choice of column is critical for success in HPLC.



Columns Types of columns in HPLC:

1)Analytical : [internal diameter (i.d.) 1.0 -4.6-mm; lengths 15 –250 mm]

2)Preparative: (i.d. > 4.6 mm; lengths 50 –250 mm)

3)Capillary: (i.d. 0.1 -1.0 mm; various lengths)

4)Nano: (i.d. < 0.1 mm, or sometimes stated as < 100 μm)

Materials of construction for the tubing:

1)Stainless steel :(the most popular; gives high pressure capabilities)

2)Glass :(mostly for biomolecules)

***3)PEEK polymer* :**(biocompatible and chemically inert to most solvents

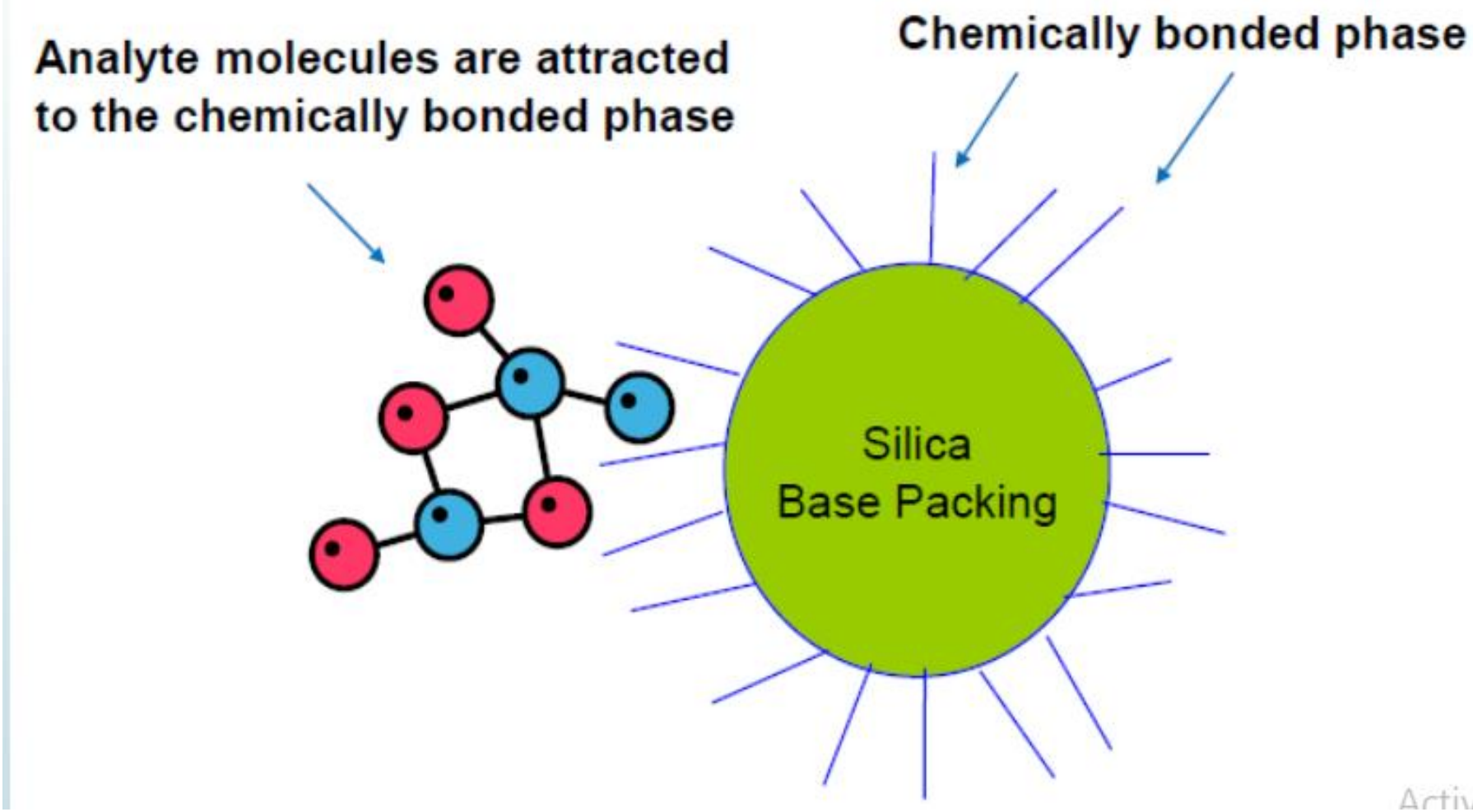
HPLC Columns Packing Materials

- ✓ Columns are packed with small diameter porous particles.

–The most popular sizes are: 5-µm, 3.5-µm and 1.8-µm

- ✓ Columns are packed using high-pressure to ensure that they are stable during use–most users purchase pre-packed columns to use in their liquid chromatographs.
- ✓ These porous particles in the column usually have a hemically bonded phase on their surface which interacts with the sample components to separate them from one another– for example, C18 is a popular bonded phase
- ✓ The process of retention of the sample components (often called analytes) is determined by the choice of column packing and the selection of the mobile phase to push the analytes through the packed column.

Mechanism of an HPLC Separation



Separation Modes of HPLC

Remember:
The correct selection of the column packing and the mobile phase are the most important factors in successful HPLC.

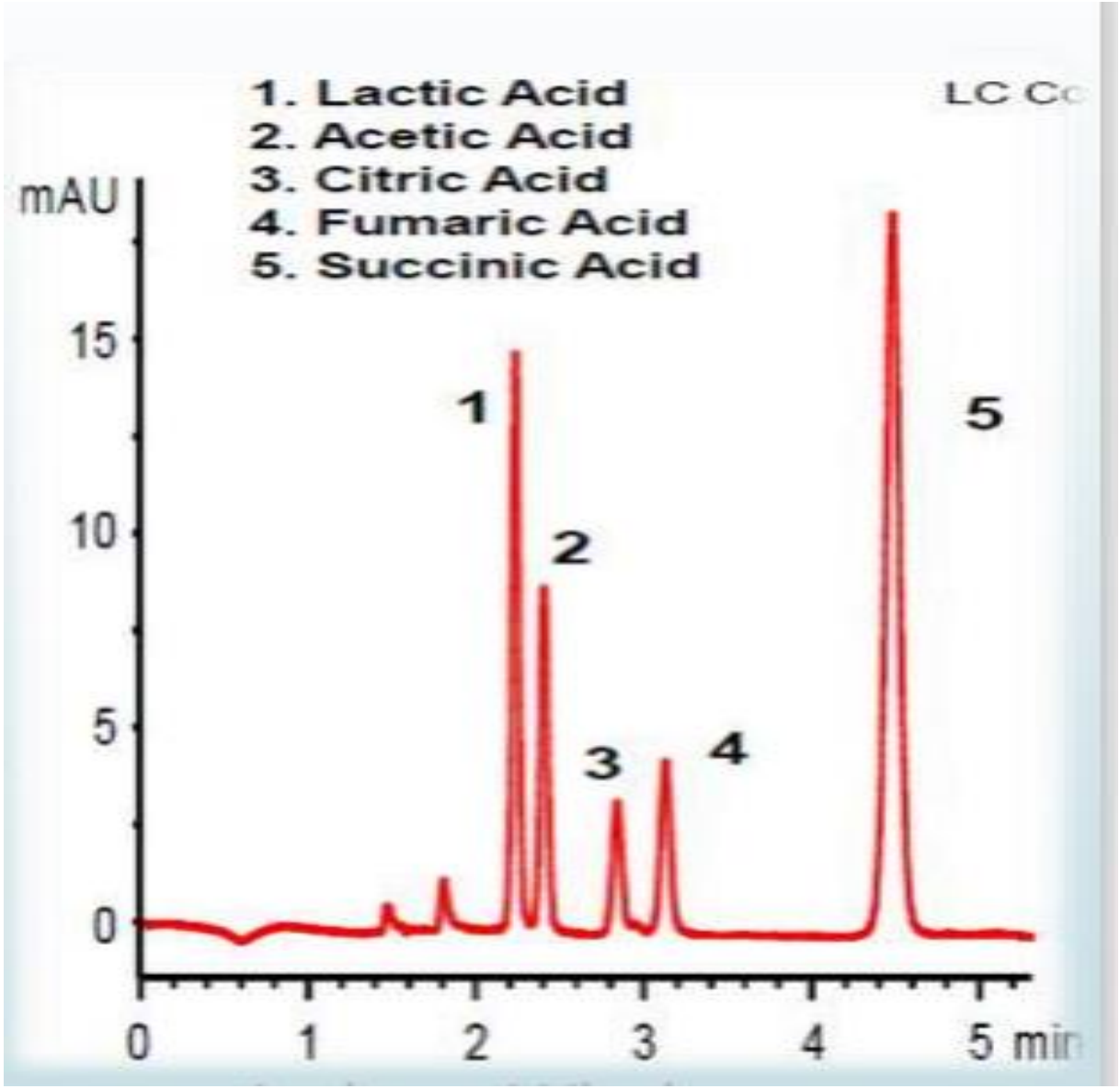
There are four major separation modes that are used to separate most compounds:

1.Reversed-phasechromatography
2.Normal-phase and adsorption chromatography
3.Ion exchange chromatography
4.Size exclusion chromatography

….Let’s briefly look at each mode of HPLC

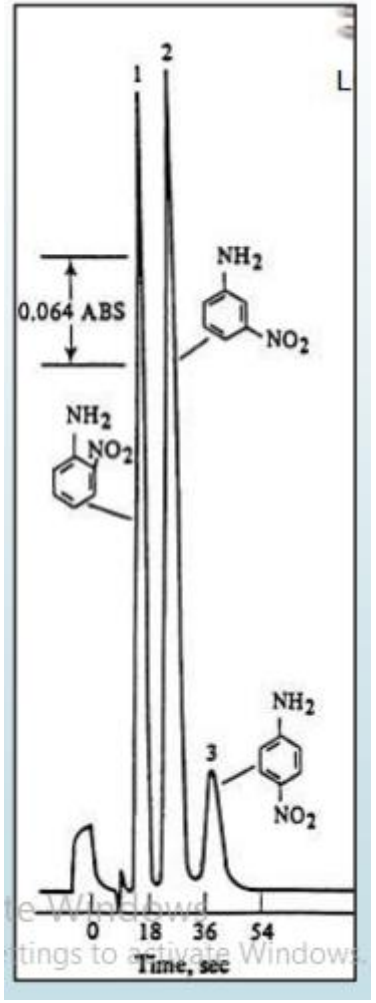
1.Reversed-Phase Chromatography(RPC):

- ✓ The column packing is non-polar (e.g. C18, C8, C3, phenyl, etc.) and the mobile phase is water (buffer) + water-miscible organic solvent (e.g. methanol, acetonitrile)
- ✓ RPC is, by far, the most popular mode …
- ✓ over 90% of chromatographers use this mode .
- ✓ The technique can be used for non-polar, polar, ionizable and ionic molecules …making RPC very versatile .
- ✓ For samples containing a wide range of compounds, gradient elution isoften used …
- ✓ One begins with a predominantly water-based mobile phase and then adds organic solvent as a function of time.
- ✓ The organic solvent increases the solvent strength and elutes compounds that are very strongly retained on the RPC packing ..


1. Lactic Acid
2. Acetic Acid
3. Citric Acid
4. Fumaric Acid
5. Succinic Acid
LC Cc
mAU
15
10
5
0
1
2
3
4
5
0
1
2
3
4
5 min

2. Normal Phase or Adsorption Chromatography

- ✓ In this mode, the column packing is polar (e.g. silica gel, cyanopropyl-bonded, amino-bonded, etc.) and the mobile phase is non-polar (e.g. hexane, isooctane, methylene chloride, ethyl acetate)
- ✓ Normal phase separations are performed less than 10% of the time.

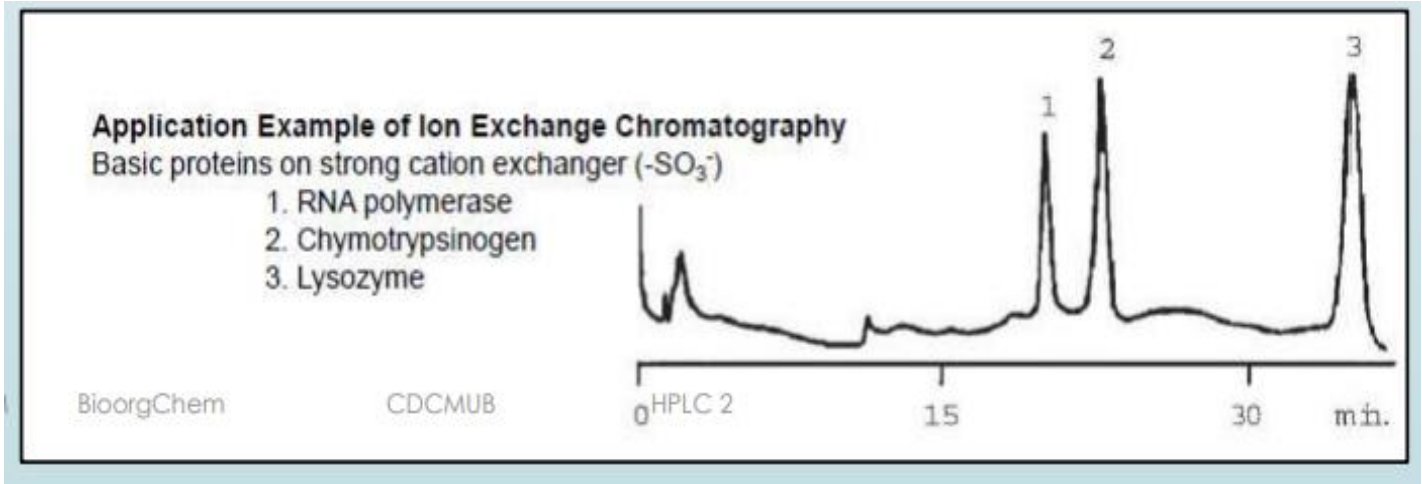


The technique is useful for:

- water-sensitive compounds.
- geometric isomers.
- cis-trans isomers.
- class separations.
- and chiral compounds.

3. Ion Exchange Chromatography

- ✓ In ion exchange, the column packing contains ionic groups(e.g. sulfonic,tetraalkylammonium) and the mobile phase is an aqueous buffer (e.g.phosphate, formate, etc.).
- ✓ Ion exchange is used by about 20%of the liquid hromatographers
- ✓ The technique is well suited for:
- ✓ the separation of inorganic and organic anions and cations in aqueous solution.
- ✓ Ionic dyes, amino acids, and proteins can be separated by ion exchange because such compounds are salt in brine water



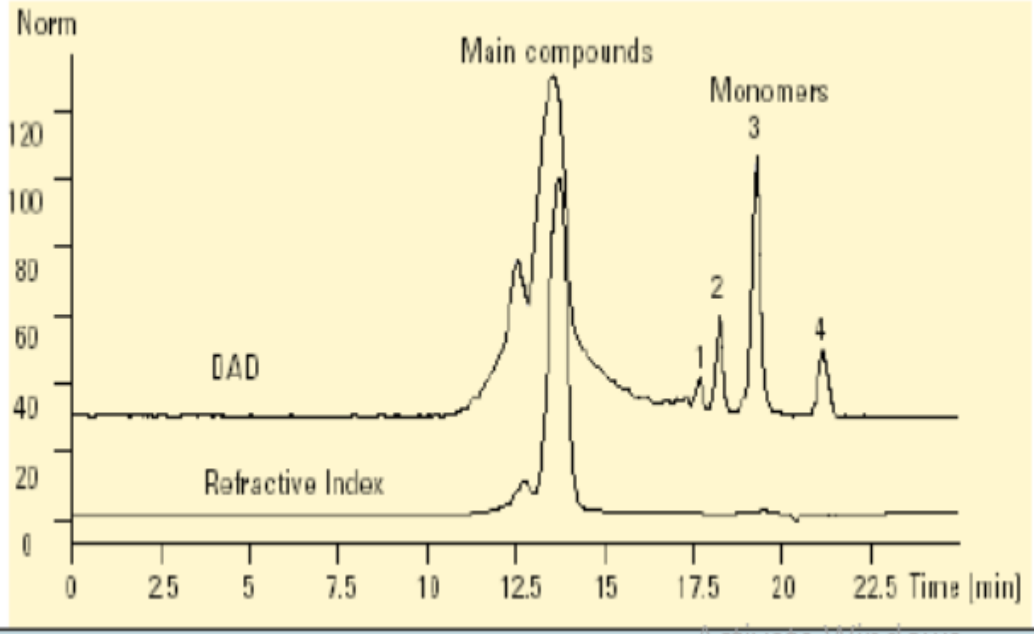
4. Size Exclusion Chromatography (SEC)

- ✓ In SEC, there is no interaction between the sample compounds and the column packing material. Instead, molecules diffuse into pores of a porous medium.
- ✓ Depending on their size relative to the pore size, molecules are separated.
- ✓ Molecules larger than the pore opening do not diffuse into the particles, while molecules smaller than the pore opening enter the particle and are separated.
- ✓ Large molecules elute first. Smaller molecules elute later.. Remember **Gel Filtration …**
- ✓ The SEC technique is used by 10-15% of chromatographers, mainly for polymer characterization and for proteins.

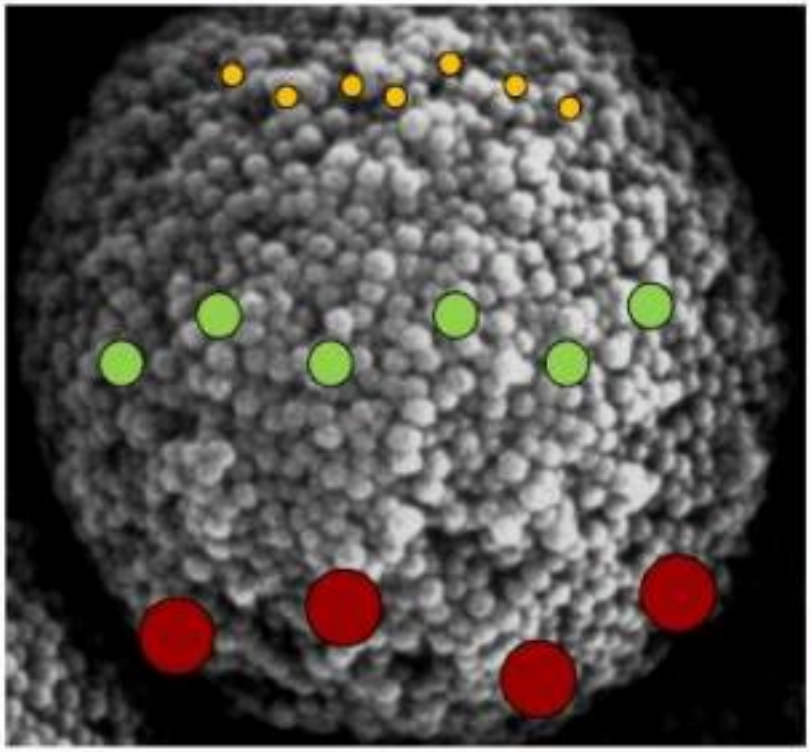
There are two modes:

- ✓ •non-aqueous SEC[sometimes termed Gel Permeation Chromatography (GPC)]
- ✓ •aqueous SEC[sometimes referred to an Gel Filtration Chromatography (GFC)].

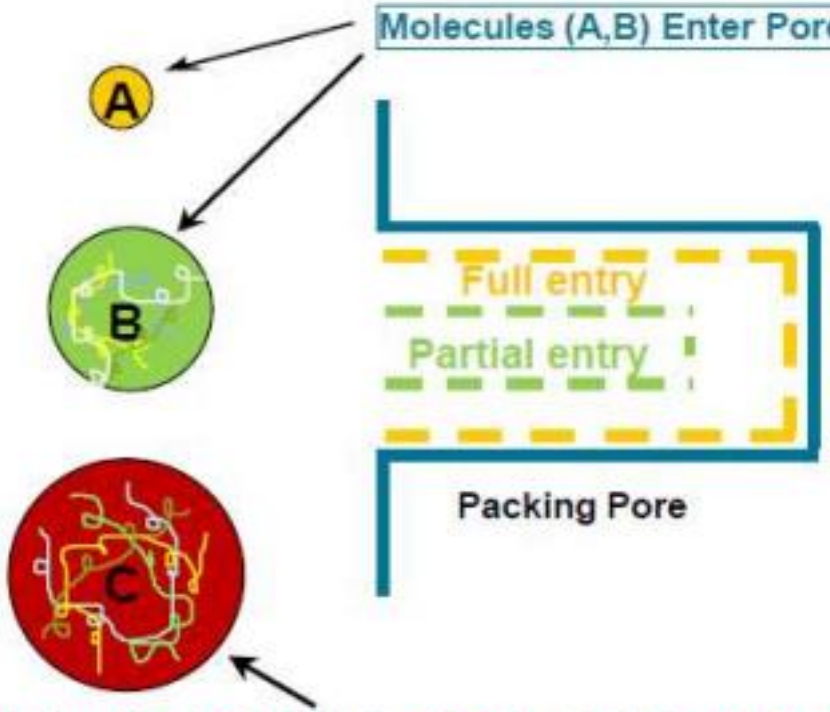
Gel Permeation Chromatogram of Polybutadiene polymer on non-aqueous SEC (GPC) column;
The monomers elute after the polymer;
column: PLgel mixed-D gel



Mechanism of SEC

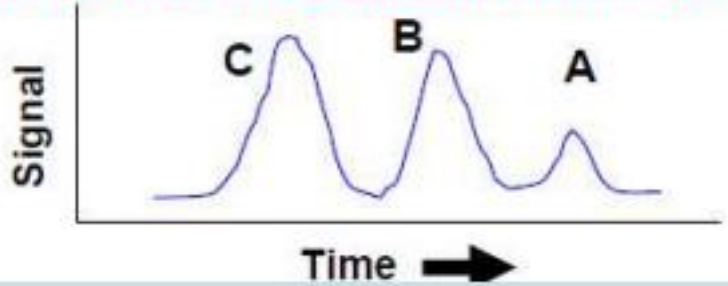


LC Columns



Molecules must freely enter and exit pores to be separated. Largest molecules elute first, followed by intermediate size molecules and finally the smallest molecules elute last.

Molecule (C) Will be Excluded from Pores



Temperature Control in HPLC

Why is it needed?

Reproducibility

•Retention in HPLC is temperature-dependent

•If temperature varies, then it is difficult to assign "peaks" to specific compounds in the chromatogram and the peak areas/heights may vary.

Solubility

• Certain chemical compounds may have low solubility in the HPLC mobile phase

• If they are injected into the flow stream they may precipitate or other difficulties may arise.

Stability

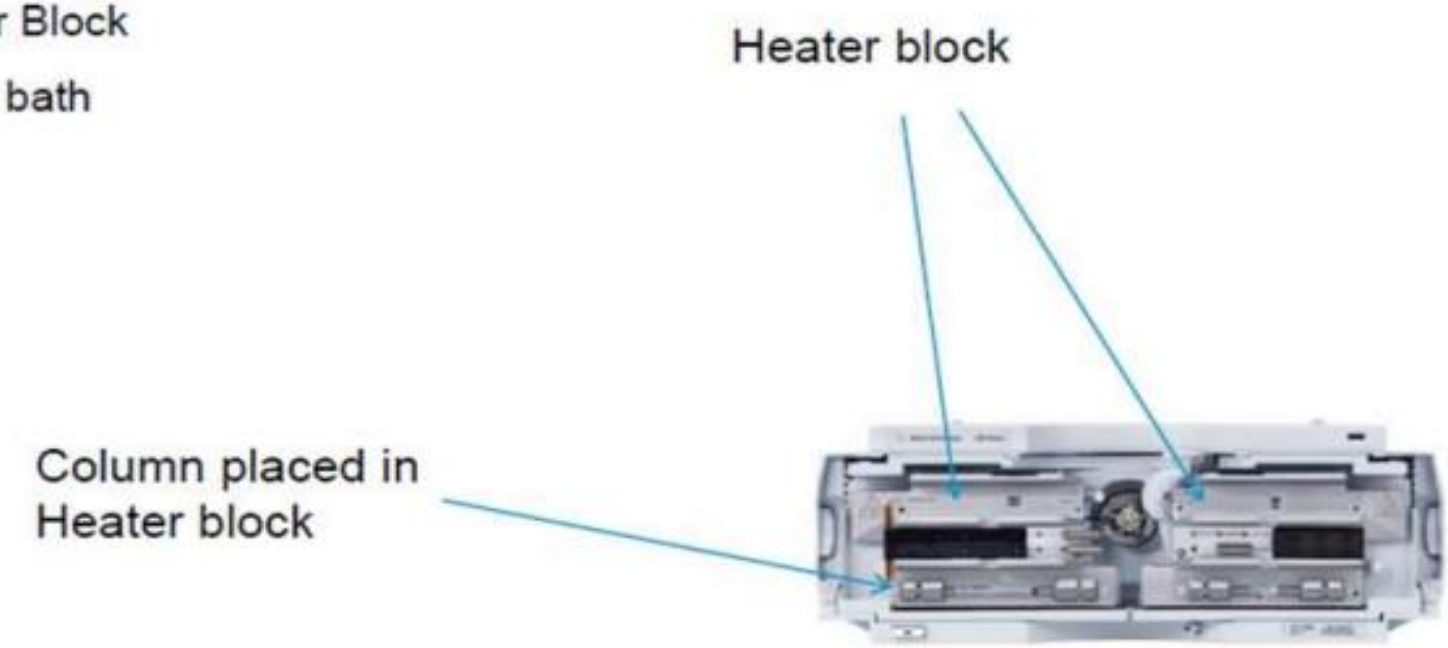
• Certain chemical compounds, especially biological compounds such as enzymes or proteins, may not be stable at room temperature or higher .

• The temperature needs to be much lower down to 4°C.

How is Temperature Control Achieved?

Three (3) ways the temperature of a column could be controlled, use:

1. Oven
2. Heater Block
3. Water bath



Agilent 1200 Series Column Compartment
(temperature range: 10 above ambient to 100°C)

Detection in HPLC

There are many detection principles used to detect the compounds eluting from an HPLC column..

The most common are:

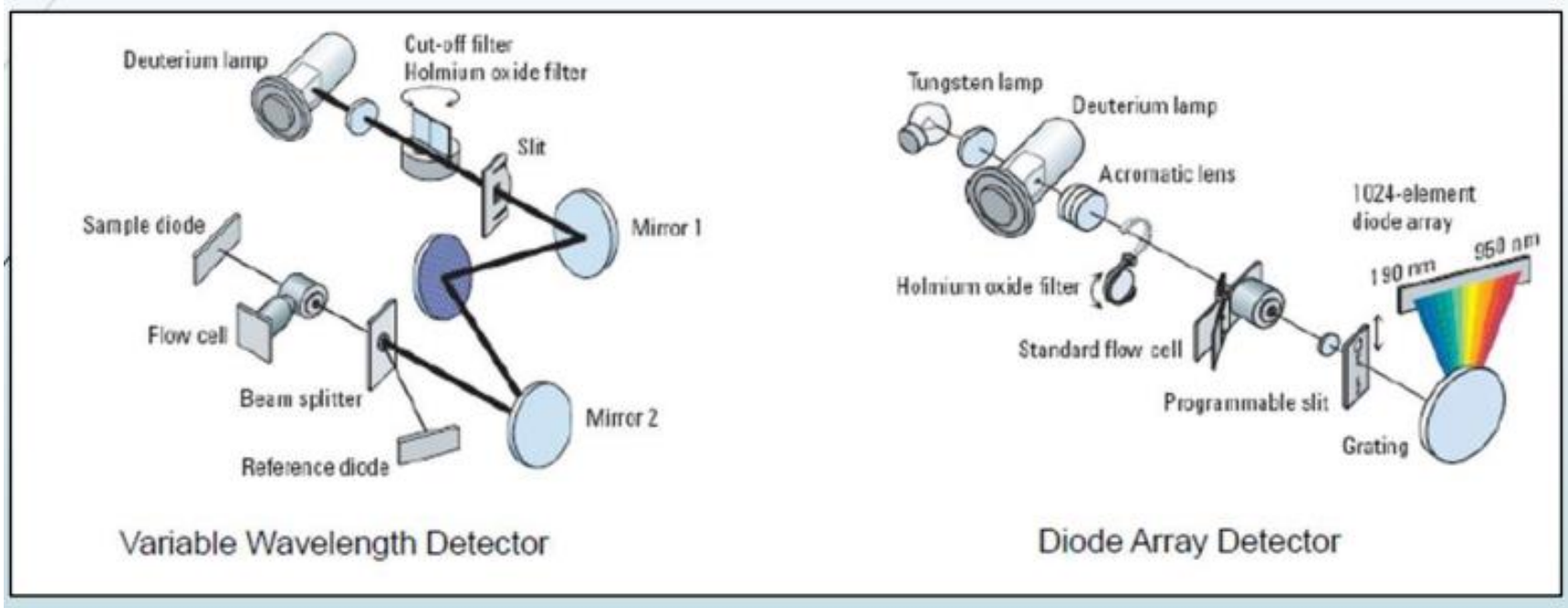
1. Spectroscopic Detection.
2. Refractive Index Detection.
3. Fluorescence Detection.

1-Spectroscopic Detection

Ultraviolet (UV) Absorption

- An ultraviolet light beam is directed through a flow cell and a sensor measures the light passing through the cell.
- If a compound elutes from the column that absorbs this light energy, it will change the amount of light energy falling on the sensor.
- The resulting change in this electrical signal is amplified and directed to a recorder or data system.
- A UV spectrum is sometimes also obtained which may aid in the identification of a compound or series of compounds.

1.a.Spectroscopic Detection (UV-visible Detector)

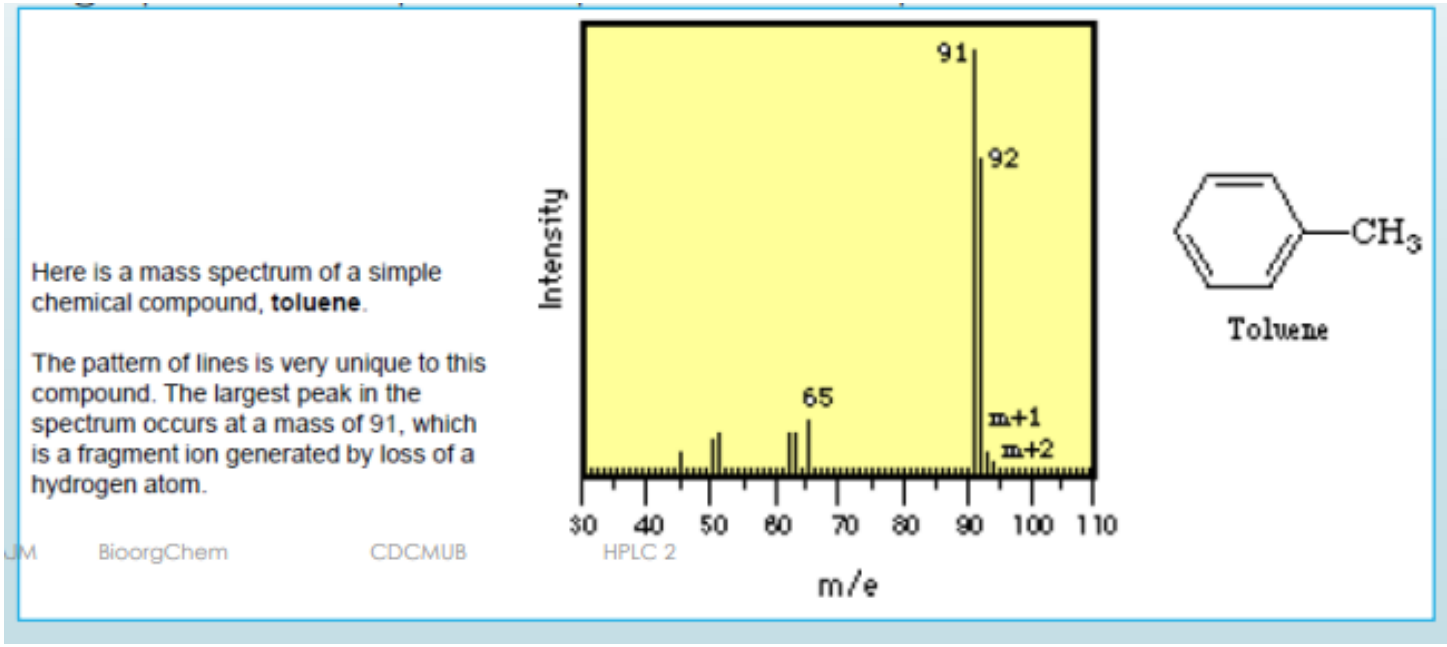


Spectroscopic Detection

1.b. Mass Spectroscopy (MS)

An MS detector senses a compound eluting from the HPLC column first by ionizing it then by measuring it's mass and/or fragmenting the molecule into smaller pieces that are unique to the compound.

The MS detector can sometimes identify the compound directly since its mass spectrum is like a fingerprint and is quite unique to that compound



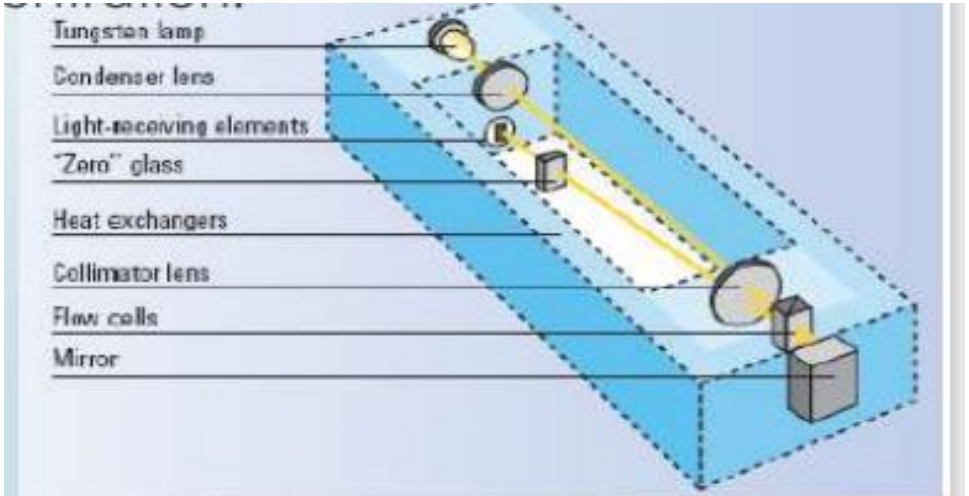
2.Refractive Index (RI) Detection

The ability of a compound or solvent to deflect light provides a way to detect it.

The RI is a measure of molecule's ability to deflect light in a flowing mobile phase in a flow cell relative to a static mobile phase contained in a reference flow cell.

The amount of deflection is proportional to concentration.

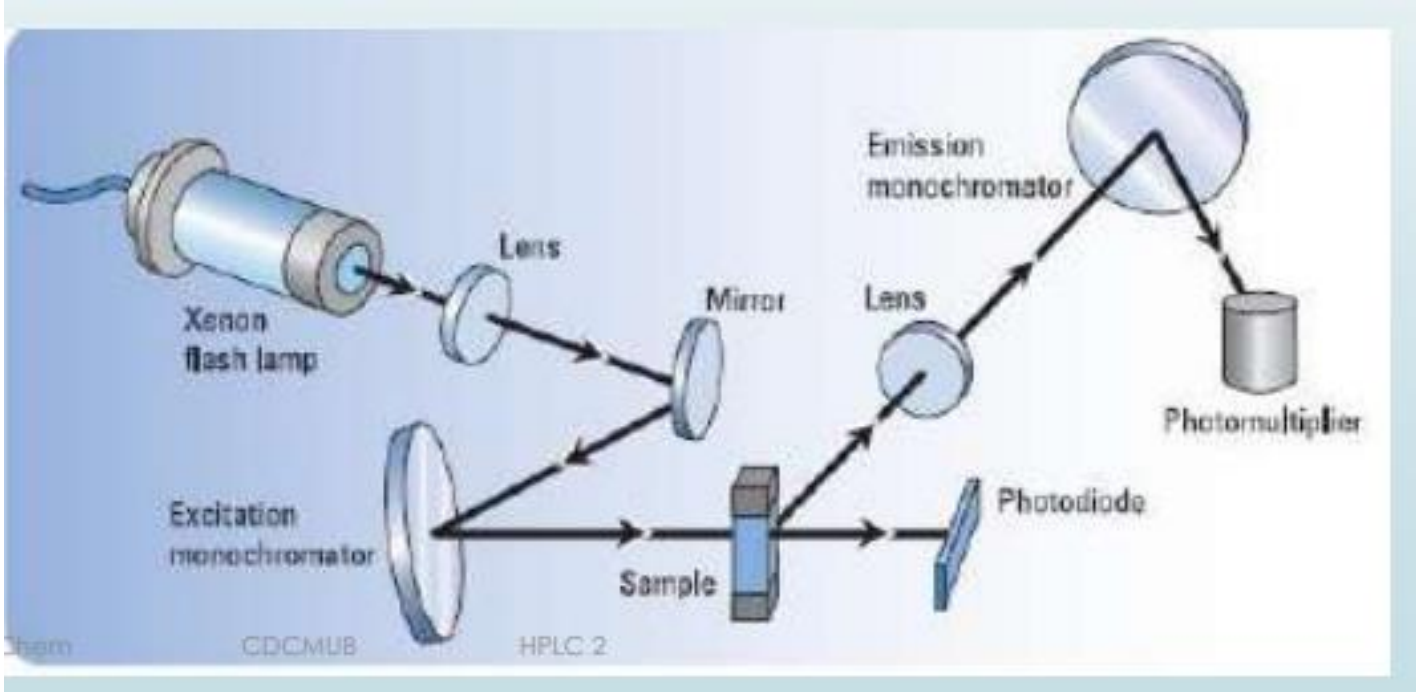
The RI detector is considered to be a universal detector but it is not very sensitive



Schematic of a Deflection Type of RI Detector

3. Fluorescence Detection

- Compared to UV-Vis detectors fluorescence detectors offer a higher sensitivity and selectivity that allows to quantify and identify compounds and impurities in complex matrices at extremely low concentration levels(trace level analysis).
- Fluorescence detectors sense only those substances that fluoresce




THANK YOU :)