High Performance Liquid Chromatography

Dr. Joohee Pradhan

OUTLINE

- Definition of HPLC
- HPLC principle
- Major components of HPLC and their function
- Application of HPLC
- Factors affecting function of HPLC
- Advantages & disadvantages of HPLC

What is HPLC?

- Originally referred to as ***High-Pressure Liquid Chromatography***
- Now more commonly called ***High Performance Liquid Chromatography***
- 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.*

Introduction

- HPLC is a form of liquid chromatography used to separate compounds that are dissolved in solution. HPLC instruments consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector.

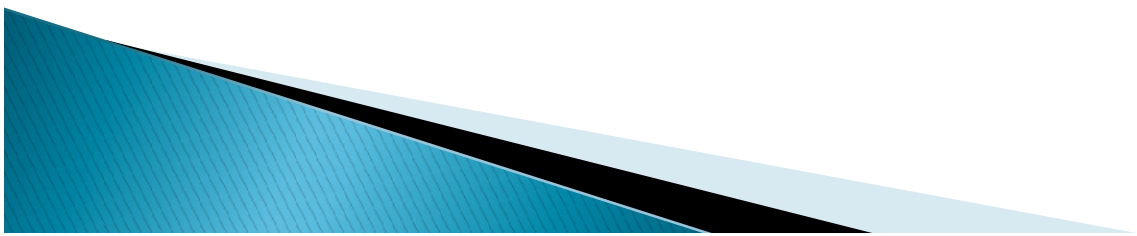
- Compounds are separated by injecting a sample mixture onto the column. The different component in the mixture pass through the column at differentiates due to differences in their partition behavior between the mobile phase and the stationary phase. The mobile phase must be degassed to eliminate the formation of air bubbles.

Points to remember……

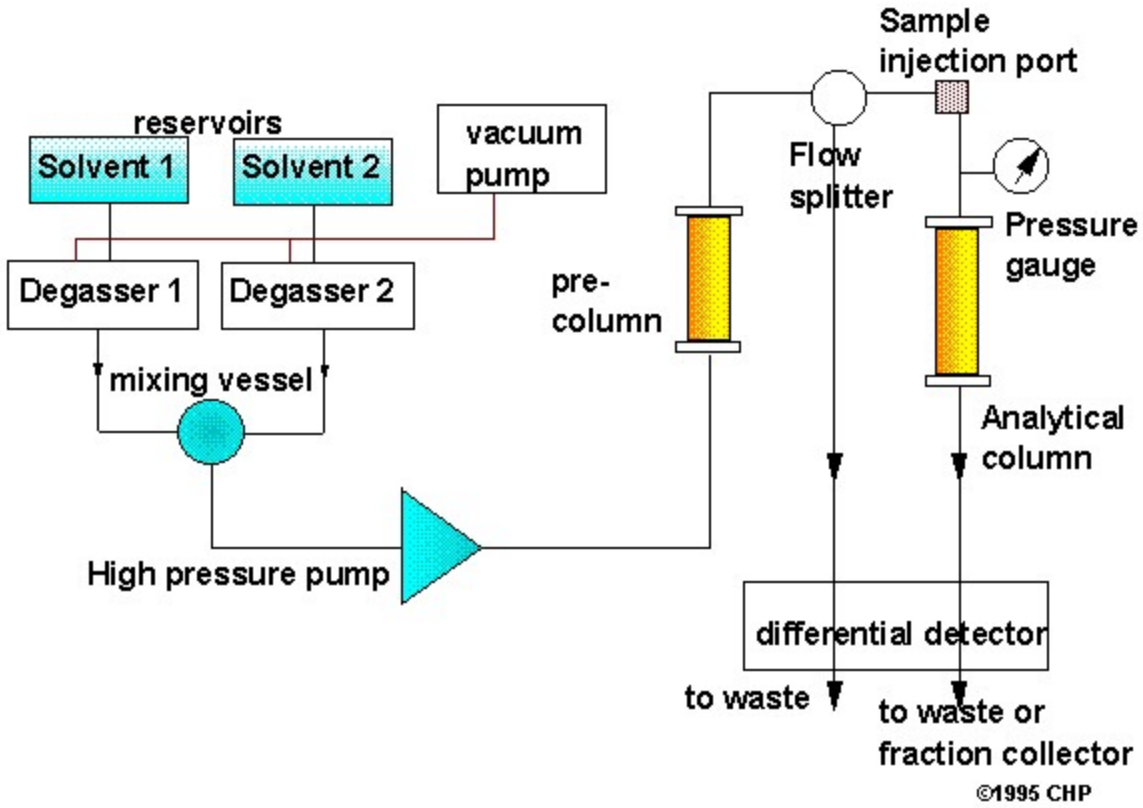
- Called High Performance Liquid Chromatography because: As many as 50,000 Theoretical Plates/ m
- Called High Pressure Liquid Chromatography because: as high as 1000 to 4000 psi
- Particle dimensions: 30μ or less
- Column diameter: 1-3 mm
- 100 times faster separation than conventional column chromatography

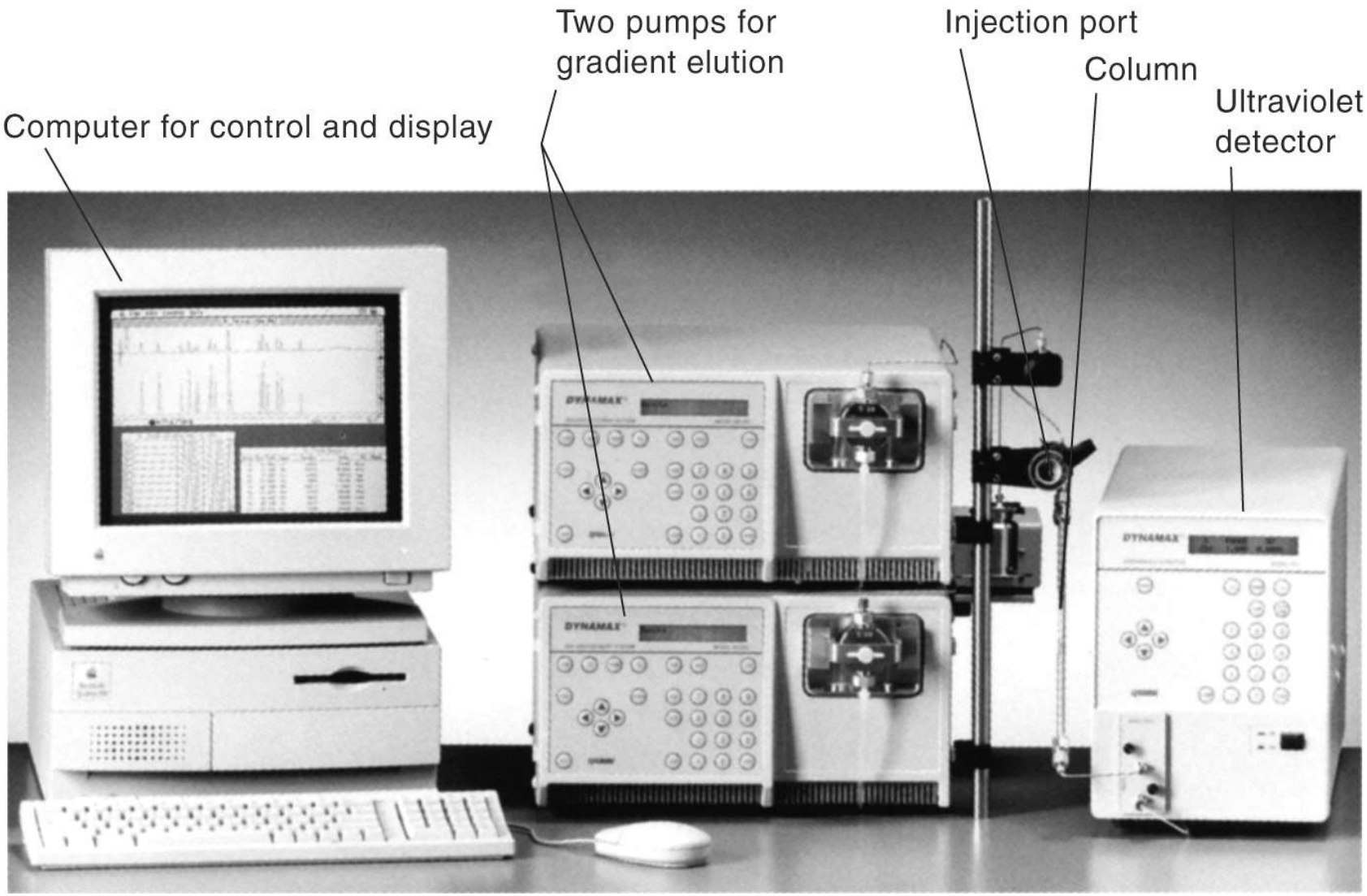
FOUR TYPES OF LIQUID CHROMATOGRAPHY

- Partition chromatography
- Adsorption, or liquid-solid chromatography
- Ion exchange chromatography
- Size exclusion, or gel, chromatography



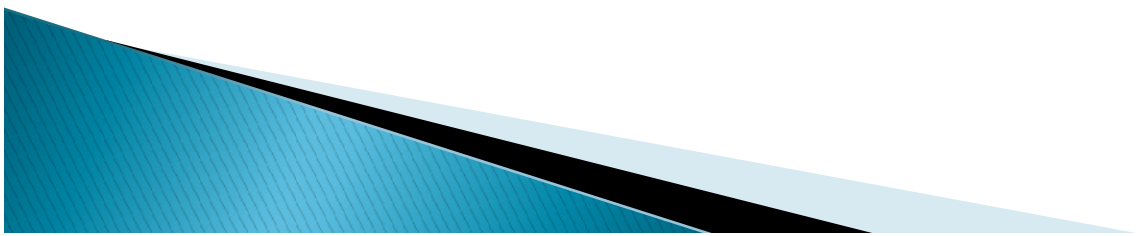
HPLC system


Sample
injection port
reservoirs
Solvent 1
Solvent 2
vacuum
pump
Flow
splitter
Pressure
gauge
Degasser 1
Degasser 2
pre-
column
mixing vessel
Analytical
column
High pressure pump
differential detector
to waste
to waste or
fraction collector
©1995 CHP


Computer for control and display
Two pumps for gradient elution
Injection port
Column
Ultraviolet detector

Stationary Phases

- Polar ("Normal" Phase):
  - Silica, alumina
- Non-Polar ("Reversed Phase")
  - ODS Silica gel
  - C18, C8



The Mobile Phase

- Normal chromatography

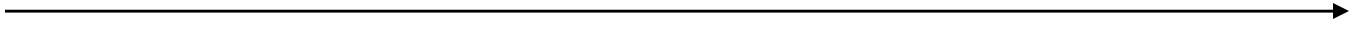
  Hexane ; dichloromethane; isopropanol; methanol

  Increasing strength

- Reverse phase chromatography

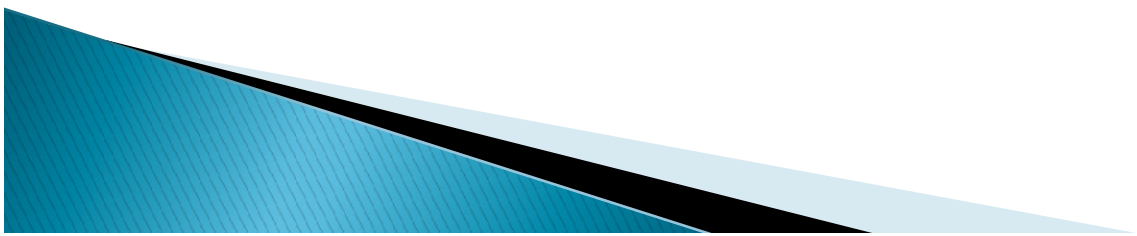
  water ; methanol; acetonitrile; tetrahydrofuran (THF)

  Increasing strength

Components of HPLC

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



Solvent Reservoir

- Mobile phase
  - isocratic elution - single solvent separation teachnique
  - gradient elution - 2 or more solvents, varied during separation
- To carry sample into the column

Pumps

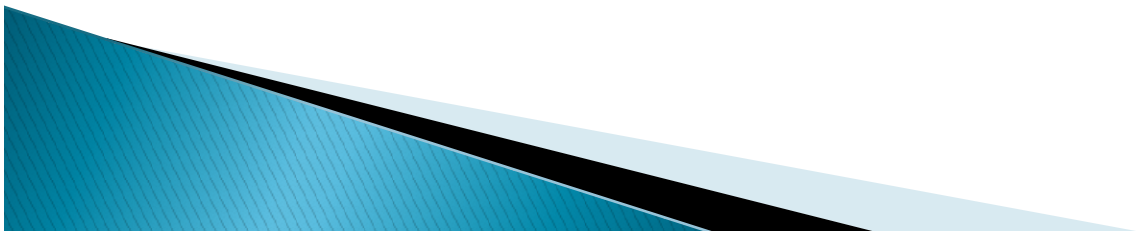
- To produce an appropriate pressure to push solvent into the sample.
- A pump capable of pumping solvent up to a pressure of 4000 psi and at flows of up to 10 ml/min

Sample Injection System

- sample valve
- Syringe/injector

- Syringe :
  - manual
  - Autoinjector

- A fixed-volume loop of between 1 – 200 μl (20 μl is often used as standard)



Columns

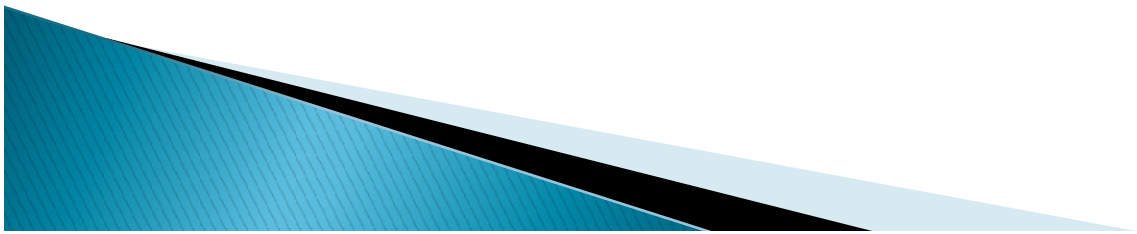
- straight, 15 to 150 cm in length; 2 to 3 mm i.d.
- packing - silica gel, alumina, Celite

HPLC columns

- The column is one of the most important components of the HPLC chromatograph because the separation of the sample components is achieved when those components pass through the column. The High performance liquid chromatography apparatus is made out of stainless steel tubes with a diameter of 3 to 5mm and a length ranging from 10 to 30cm.
- Normally, columns are filled with silica gel because its particle shape, surface properties, and pore structure help to get a good separation. Silica is wetted by nearly every potential mobile phase, is inert to most compounds and has a high surface activity which can be modified easily with water and other agents. Silica can be used to separate a wide variety of chemical compounds, and its chromatographic behavior is generally predictable and reproducible.

Several column types (can be classified as)

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

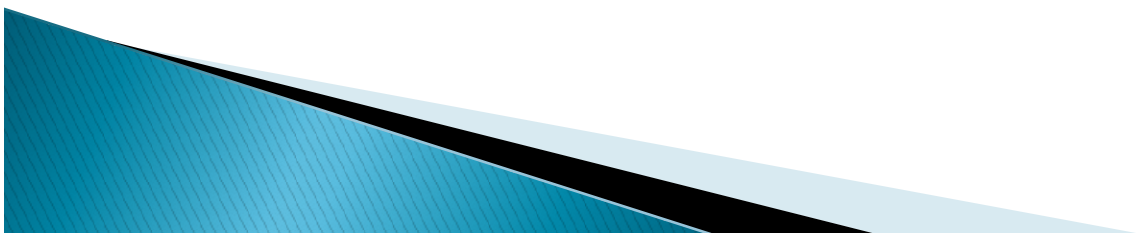


Normal phase

- 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.

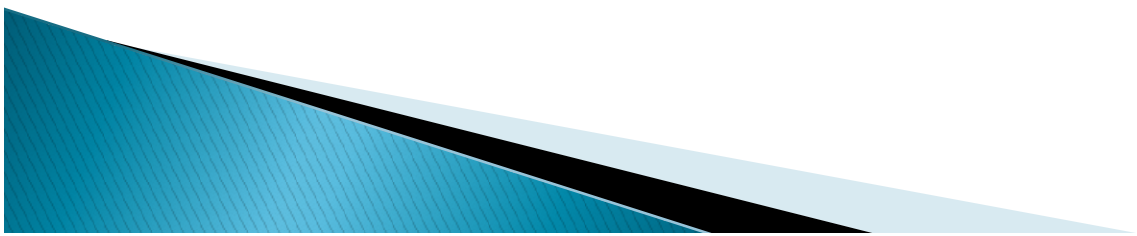


Size exclusion

- In size exclusion the HPLC column is consisted of substances which have controlled pore sizes and is able to be filtered in an ordinarily phase according to its molecular size. Small molecules penetrate into the pores within the packing while larger molecules only partially penetrate the pores. The large molecules elute before the smaller molecules.

Ion exchange

- In this column type the sample components are separated based upon attractive ionic forces between molecules carrying charged groups of opposite charge to those charges on the stationary phase. Separations are made between a polar mobile liquid, usually water containing salts or small amounts of alcohols, and a stationary phase containing either acidic or basic fixed sites.



HPLC Detectors

most common HPLC detectors:

- Refractive index
- UV/Vis
  - Fixed wavelength
  - Variable wavelength
  - Diode array
- Fluorescence

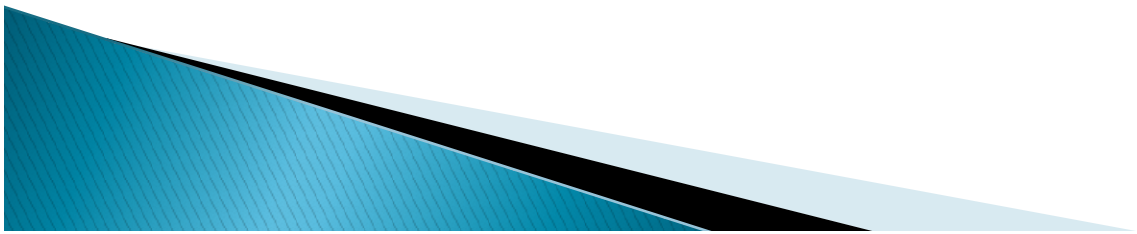
less common, but important

- Conductivity
- Mass-spectrometric (LC/MS)
- Evaporative light scattering

Good detectors…

Regardless of the principle of operation, an ideal LC detector should have the following properties:

- Low drift and noise level (particularly crucial in trace analysis).
- High sensitivity.
- Fast response.
- Wide linear dynamic range (this simplifies quantitation).
- Low dead volume (minimal peak broadening).
- Cell design which eliminates remixing of the separated bands.
- Insensitivity to changes in type of solvent, flow rate, and temperature.
- Operational simplicity and reliability.
- It should be tuneable so that detection can be optimized for different compounds.
- It should be non-destructive.



THE REFRACTIVE INDEX DETECTOR

Principles

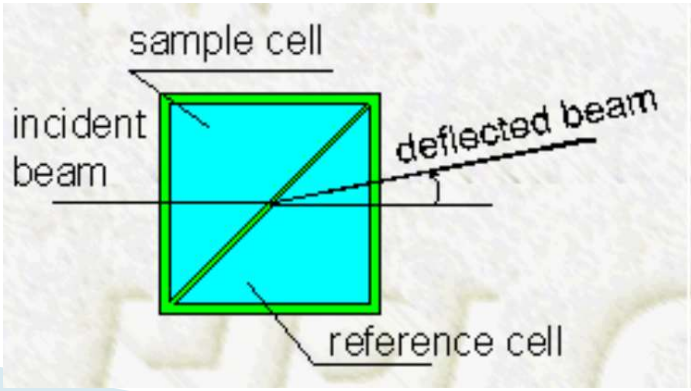
- The refractive index (RI) detector is the only universal detector in HPLC.
- Involves measuring of the change in refractive index of the column effluent passing through the flow-cell.
- The greater the RI difference between sample and mobile phase, the larger the imbalance will become.
- Thus, the sensitivity will be higher for the higher difference in RI between sample and mobile phase.

THE REFRACTIVE INDEX DETECTOR

- In complex mixtures, sample components may cover a wide range of refractive index values and some may closely match that of the mobile phase, becoming invisible to the detector.
- RI detector is a pure differential instrument, and any changes in the eluent composition require the rebalansing of the detector.
- This factor is severely limiting RI detector application in the analyses requiring the gradient elution, where mobile phase composition is changed during the analysis to effect the separation.
- RI detectors require the use of a two-path cell where the sample-containing side is constantly compared with the non-sample-containing reference side.

Deflection detectors (RI)

- This detector based on the deflection principle of refractometry, where the deflection of a light beam is changed when the composition in the sample flow-cell changes in relation to the reference side (as eluting sample moves through the system). When no sample is present in the cell, the light passing through both sides is focused on the photodetector (usually photoresistor). As sample elutes through one side, the changing angle of refraction moves the beam. This results in a change in the photon current falling on the detector which unbalances it. The extent of unbalance (which can be related to the sample concentration) is recorded on a strip chart recorder.



The advantages of this type of detector are:

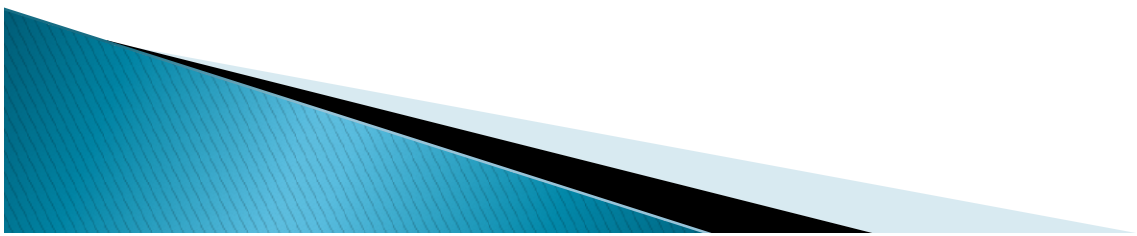
- (1) universal response;
- (2) low sensitivity to dirt and air bubbles in the cells; and
- (3) the ability to cover the entire refractive index range from 1.000 to 1.750 RI with a single, easily balanced cell.

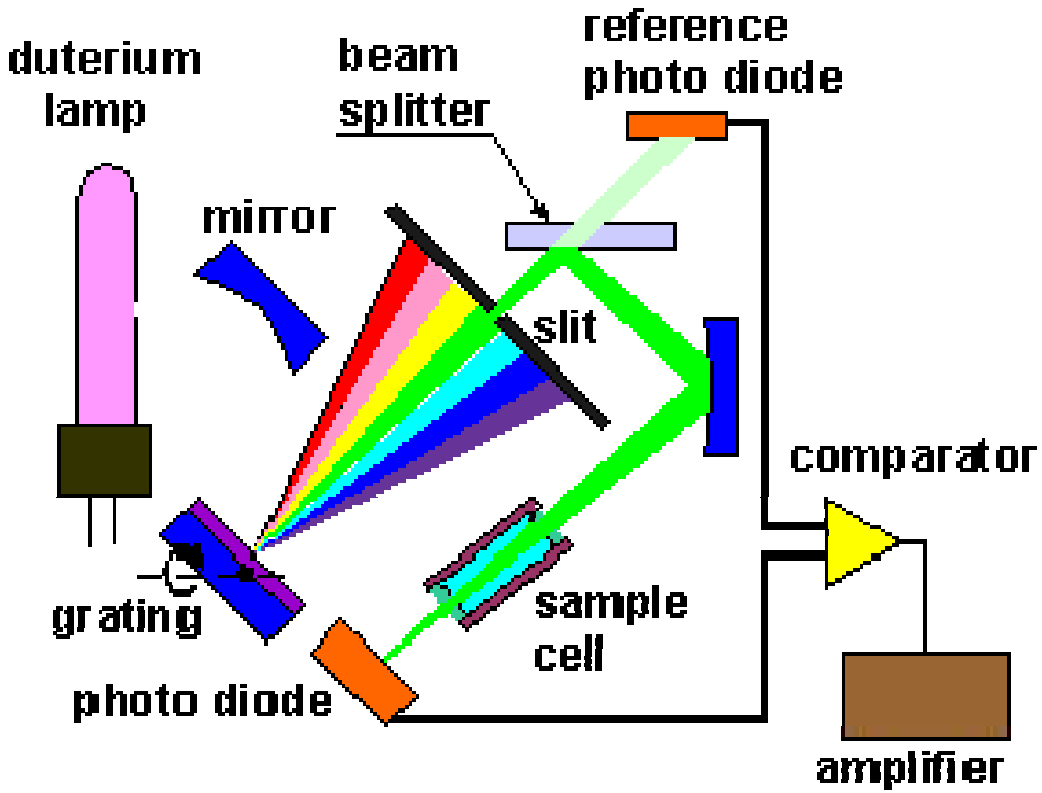
The **disadvantages** are the relatively low sensitivity and a general disability to easily remove and clean or replace the cell when filming or clogging occurs.

Variable-wavelength UV detectors

- Detectors which allow the **selection of the operating wavelength** called variable wavelength detectors and they are particularly useful in three cases:
- offer best sensitivity for any absorptive component by selecting an appropriate wavelength;
- individual sample components have high absorptivity at different wavelengths and thus, operation at a single wavelength would reduce the system's sensitivity;

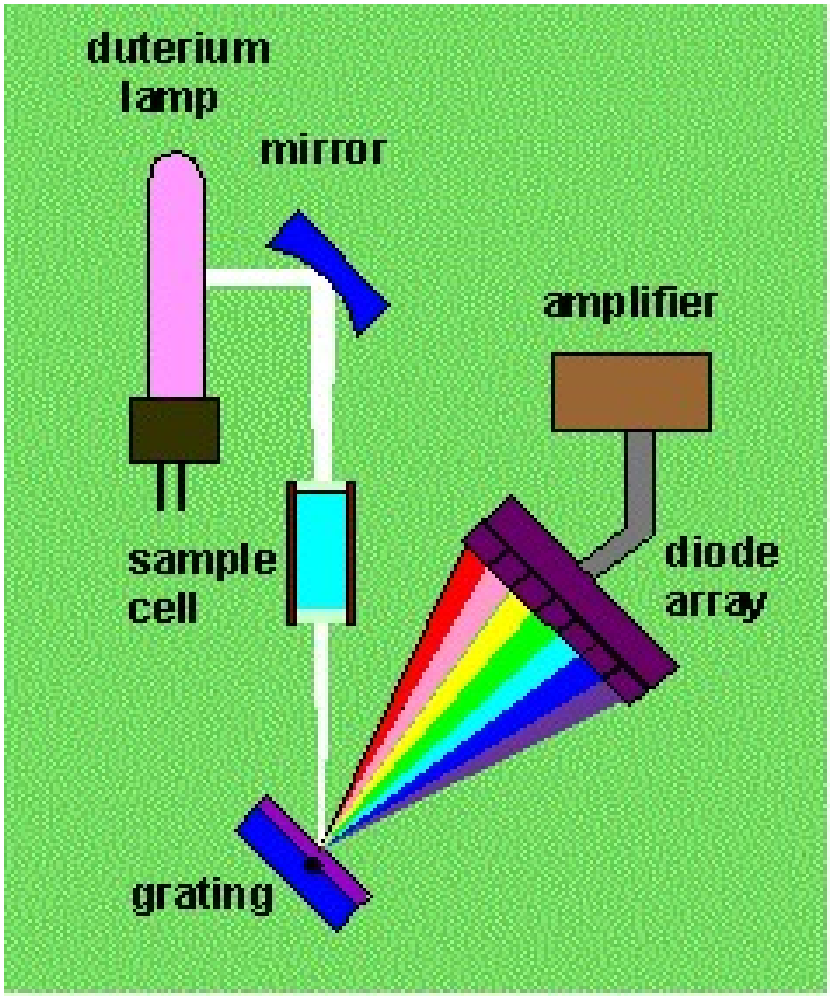
- Depending on the sophistication of the detector, wavelength change is done manually or programmed on a time basis into the memory of the system.




duterium
lamp
beam
splitter
reference
photo diode
mirror
slit
comparator
grating
sample
cell
photo diode
amplifier

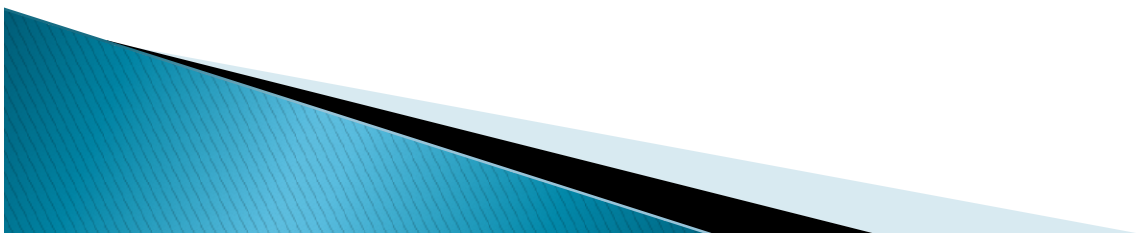
Diode-array detectors

- variable wavelength UV detectors can perform spectroscopic scanning. Helps in identification beyond simple identification by retention time.
- can use best wavelength(s) for actual analysis.
- The second major advantage is related to the problem of peak purity. Often, the peak shape in itself does not reveal that it actually corresponds to two (or even more) components. Absorbance rationing at several wavelengths can help decide whether the peak represents a single compound or, is in fact, a composite peak.

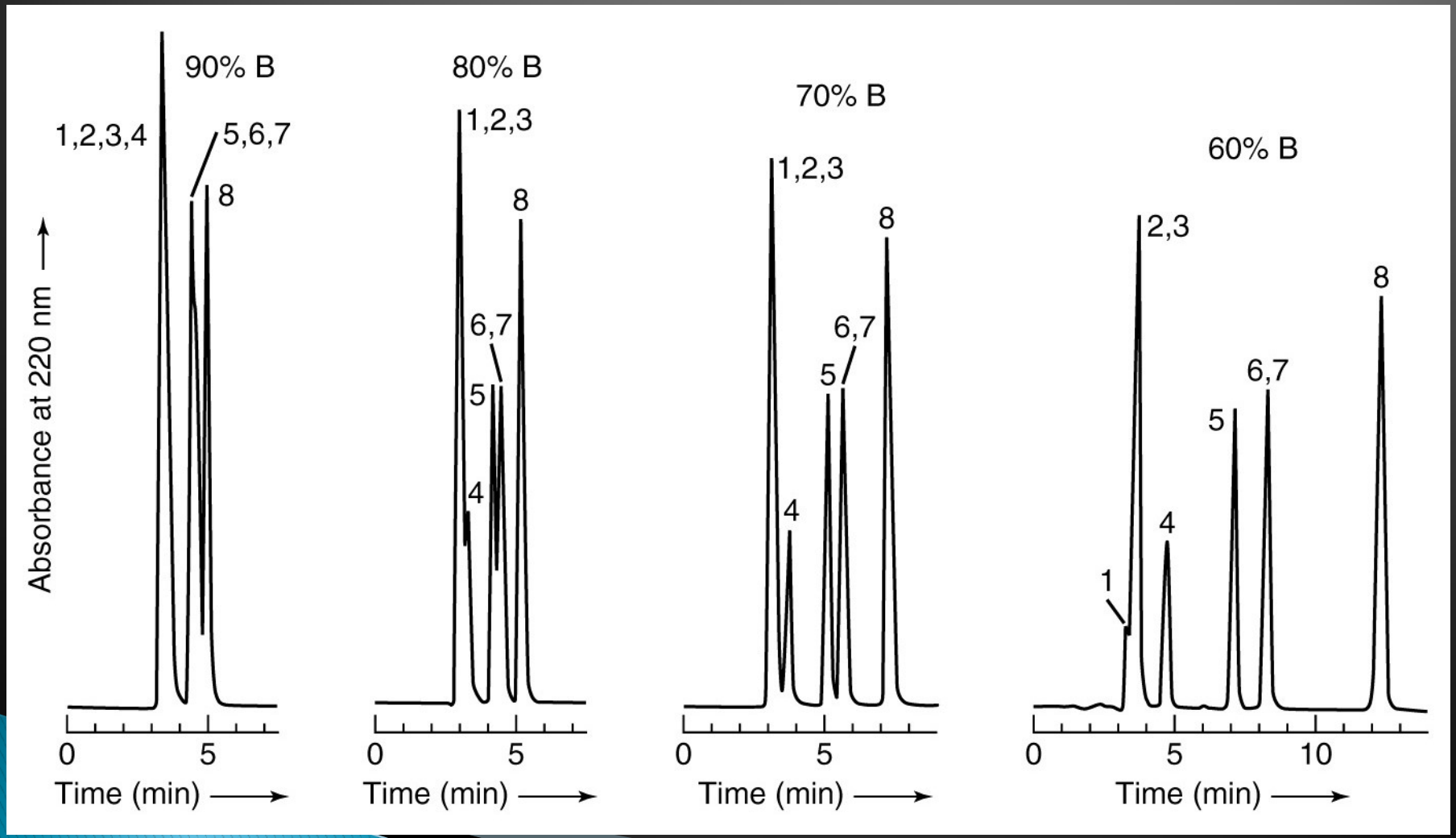

duterium
lamp
mirror
amplifier
sample
cell
diode
array
grating

Data Processing

- Using specific sowtare that is connected to HPLC machine
- Receive the information from HPLC machine and present it as a graph
- The graph describes about qualitative data (Retention time) and quantitative data (area under curve)

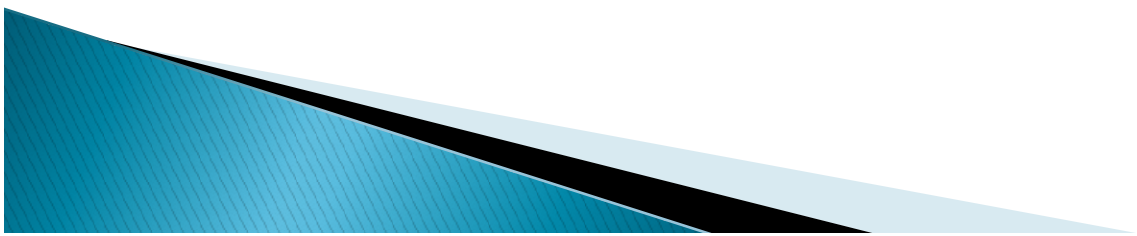


HPLC CHROMATOGRAM



Retention Time

- The **retention time** of a solute is taken as the elapsed time between the time of injection of a solute and the time of elution of the peak maximum of that solute.



Application of HPLC

1. Pharmaceuticals industry
   - To control the drug stability
   - Quantity of drug determination from pharmaceutical dosage forms, ex. Paracetamol determination in panadol tablet
   - Quantity of drug determination from biological fluids, ex: blood glucose level

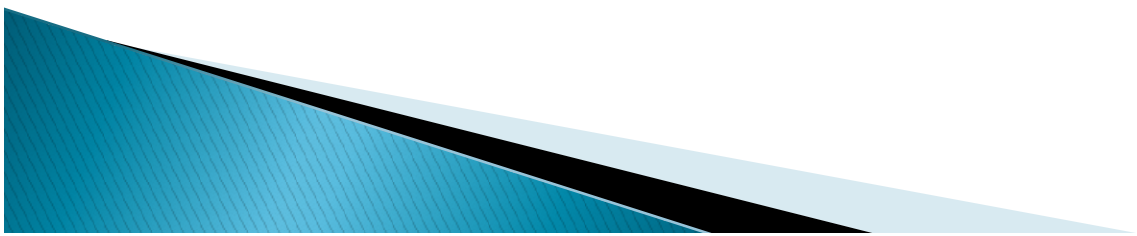
2. Analysis of natural contamination
   - Phenol & Mercury from sea water

3. Forensic test
   - Determination of steroid in blood, urine & sweat.
   - Detection of psychotropic drug in plasma

Application of HPLC

4. Clinical test
   - Monitoring of hepatic chirosis patient through aquaporin 2 in the urine.

5. Food and essence manufacture
   - sweetener analysis in the fruit juice
   - preservative analysis in sausage.

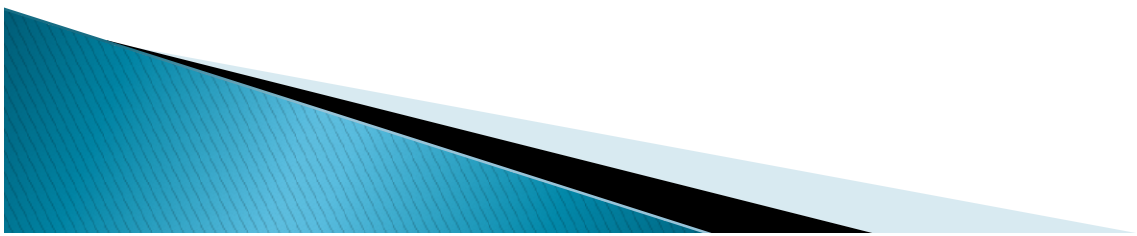


The factors which influence the HPLC performance

1. Internal diameter of column
   - the smaller in diameter, the higher in sensitivity
2. Pump pressure
   - the higher in pressure, the higher in separation
3. Sample size
4. The polarity sample, solvent and column
5. Temperature
   - the higher in temperature, the lesser the time of separation

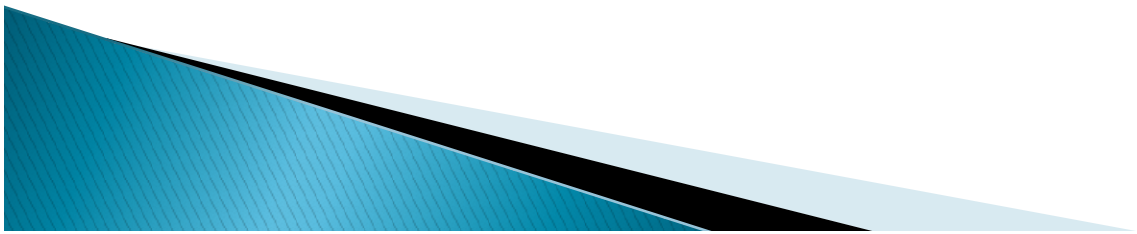
advantages

1. Needs a small sample with a high accuracy and precision
2. Non-destructed sample during operation compared to GC.



Disadvantages

- Need a skill to run the instruments
- Solvents consuming



Thank You