HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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CHROMATOGRAPHY :

- Chromatography: method of separating a mixture of components into individual components.
- based on the differences in the rate at which the components of a mixture move through a porous medium(stationary phase) under the influence of some solvent or gas(mobile phase).

WHY USE HPLC?

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

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 HPLC 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

1.NORMAL PHASE CHROMATOGRAPHY:

- Stationary Phase – Polar , Eg: SiO_2,Al_2O_3
- Mobile Phase – Non-Polar
  Eg:heptane,hexane,cyclohexane,$CHCl_3$,CH_3OH
- Mechanism:

✓ Polar compounds travels slower & eluted slowly due to higher affinity to stationary phase

✓ Non-polar compounds travels faster & eluted 1st due to lower affinity to stationary phase.

- This technique is **not widely used** in pharmaceutical separations.

3. Separation mode

Normal Phase Chromatography

Mobile phase solvents

n-Hexane	(n-Hex)
iso-Octane	(iso-Oct)
Chloroform	($CHCl_3$)
Dichloromethane	(CH_2Cl_2)
Ethylacetate	(AcOEt)
Isopropylalchol	(IPA)
Tetrahydrofran	(THF)
Dioxane	
Acetonitrile	(CH_3CN)
Ethanol	(EtOH)
Methanol	(MeOH)
Amines	
Acids	

Low

Polarity

High

2.REVERSE PHASE CHROMATOGRAPHY:

- Stationary Phase – Non-Polar nature.

  Eg: n-octadecyl, n-octyl, ethyl, phenyl diol, hydrophobic polymers.

- Mobile Phase – Polar nature.

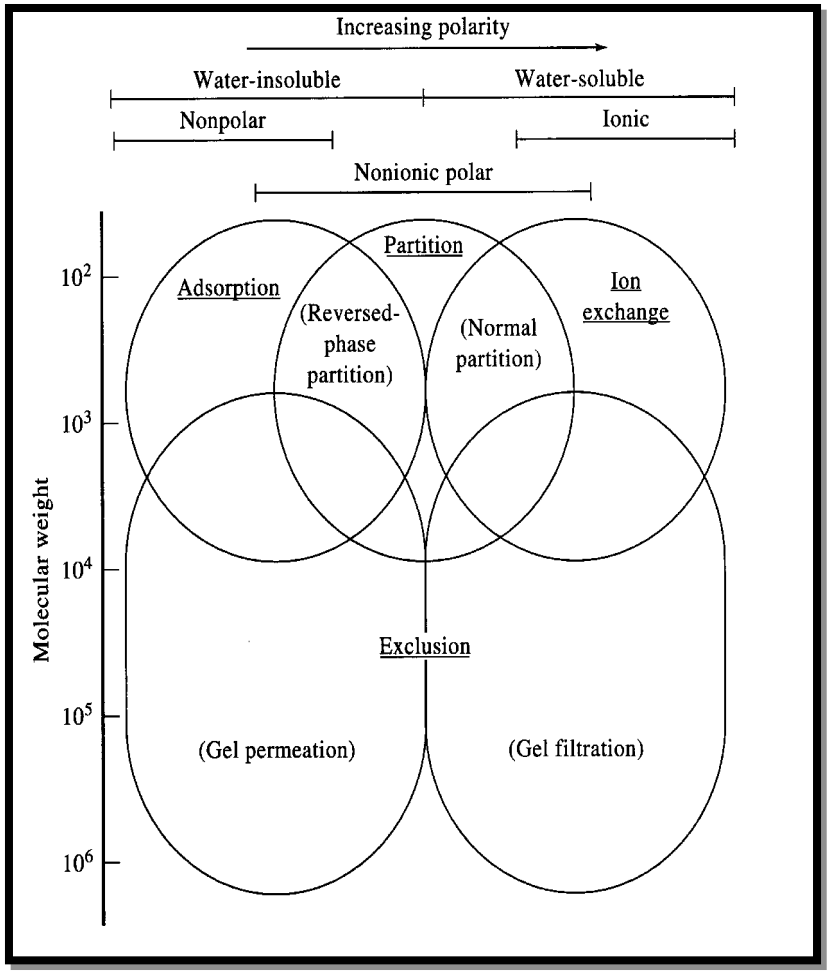
  Eg: methanol or acetonitrile/water or buffer sometimes with additives of THF or dioxane.

- 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

TYPES OF HPLC DEPENDS ON:



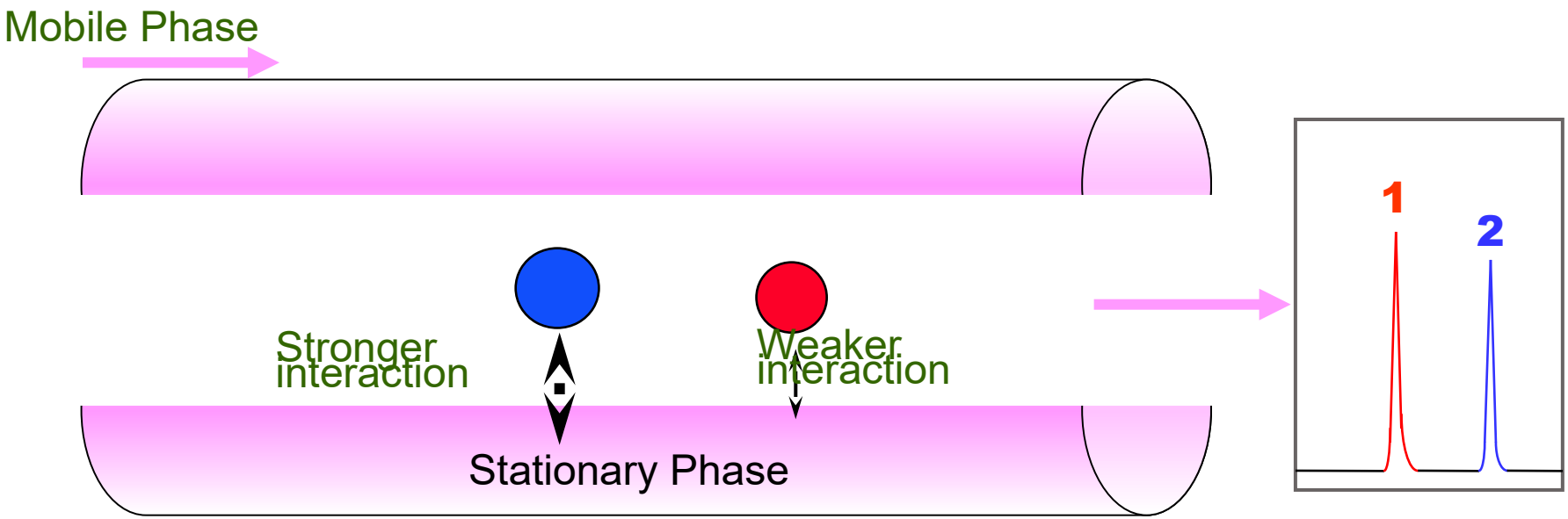
- Molecular weight of solute
- Water solubility of solute
- Polarity of solute
- Ionic and non-ionic character of solute

PRINCIPLE:

- The principle of separation is **adsorption** .
- based on the difference in the affinity of the compounds towards stationary phase as in the normal and reverse phase.
- The **lesser the affinity** of the sample particles towards the stationary phase the **faster the time of elution** of the sample.

Separation Mechanism

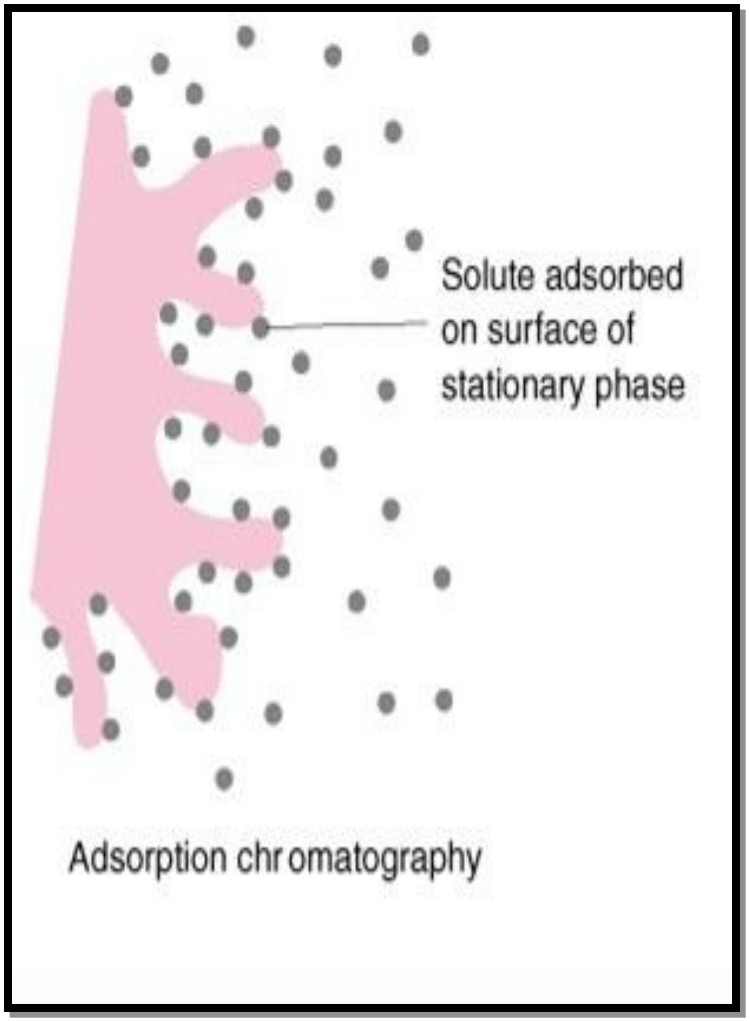
Due to different interaction between stationary phase and different sample, the molecules move at different rate, therefore separation can be done.



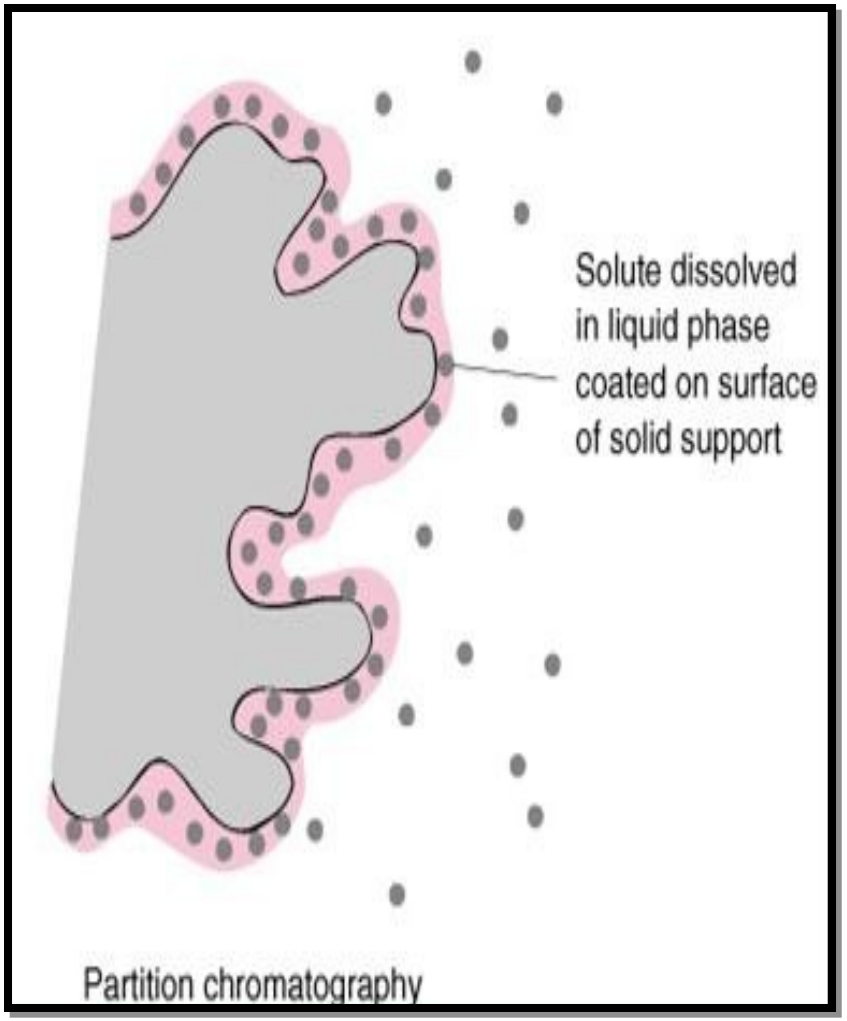
PARTITION CHROMATOGRAPHY:

- In this the **stationary phase is a liquid which is coated on the solid support on the column.**
- The mobile phase is also a liquid.
- When solute along with the mobile phase is passed over the stationary phase it gets dissolved to the surface of the liquid coated to the solid support.
- The compounds which have more partition co-efficient are eluted slowly when compared to the compounds with low partition co-efficient.

ADSORPTION CHROMATOGRAPHY:



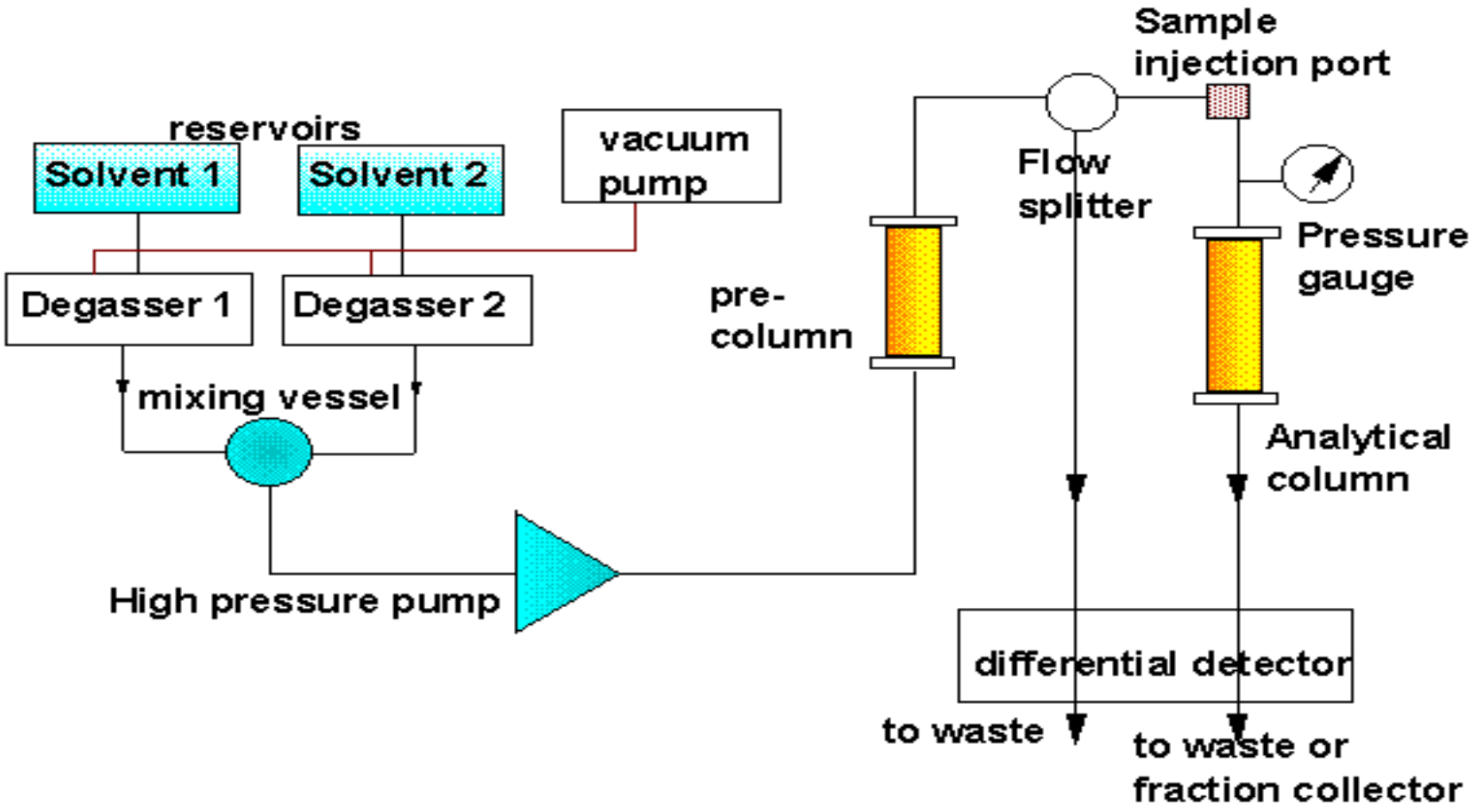
PARTITION CHROMATOGRAPHY:



INSTRUMENTATION OF HPLC

- Solvent storage bottle
- Gradient controller and mixing unit
- De-gassing of solvents
- Pump
- Pressure gauge
- Pre-column
- Sample introduction system
- Column
- Detector
- Recorder

FLOW DIAGRAM OF HPLC INSTRUMENT



Outline of LC-2010

Reservior Tray

Auto sampler

Column Oven

Degassing Unit

Pump Unit

Low pressure gradient device

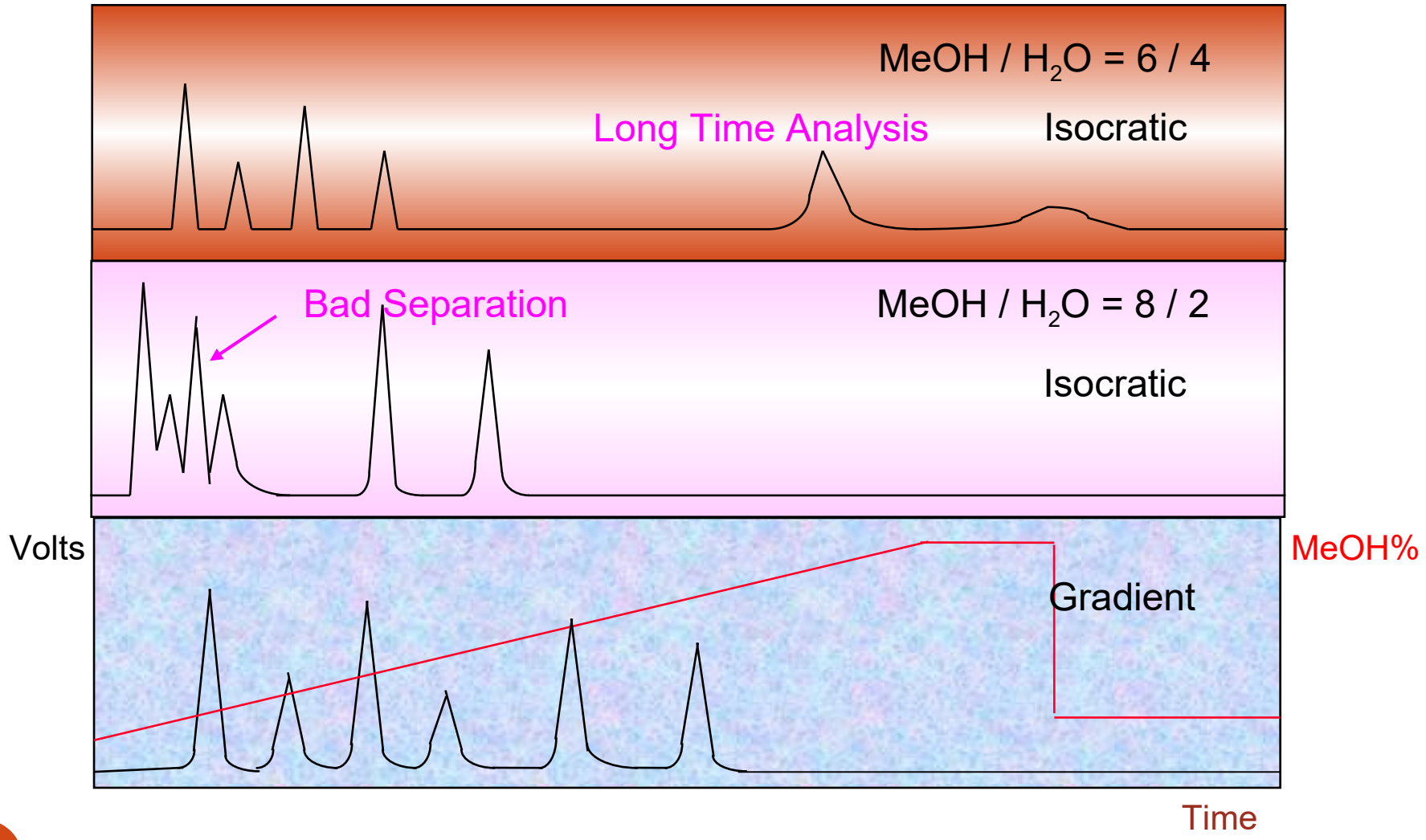
System Controller

UV detector

❖GRADIENT CONTROLLER

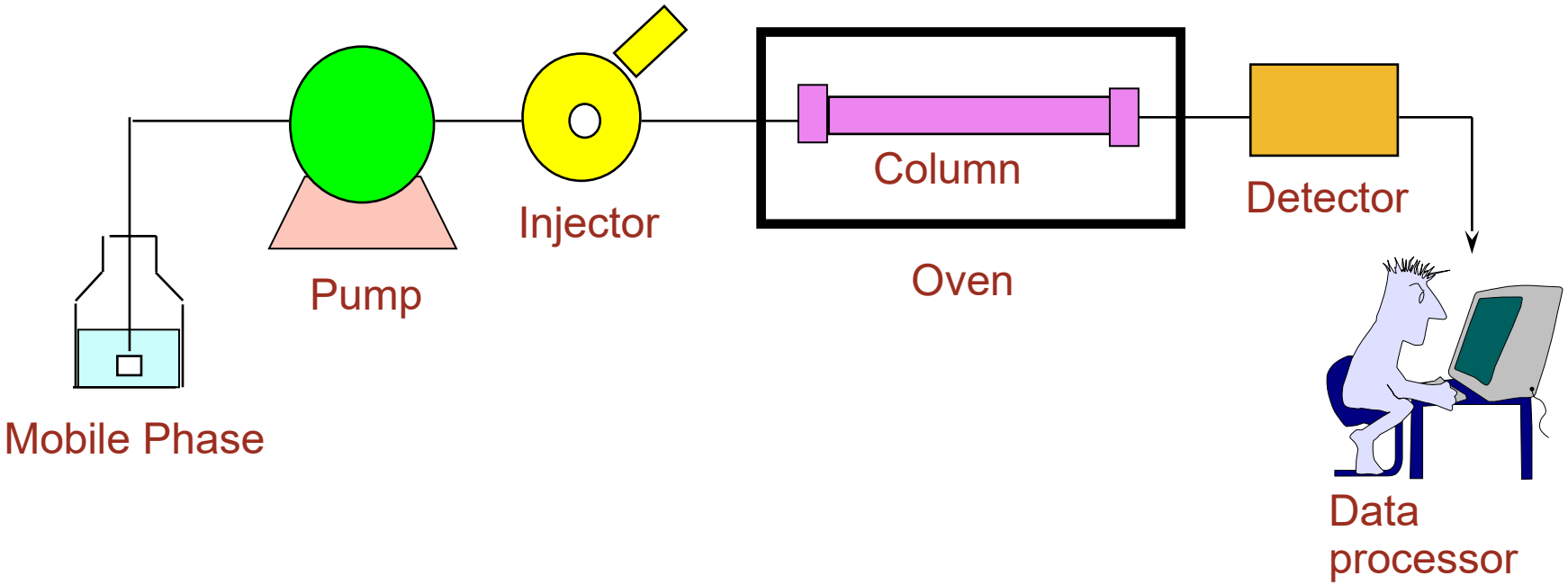
- **Isocratic solvents-** mobile phase is prepared by using **pure solvent or mixture of solvents** which has same eluting power or polarity.
- **Gradient solvents-** in this the polarity of the solvent is gradually increased & hence the solvent composition has to be changed.

Elution Modes



(Column : ODS)

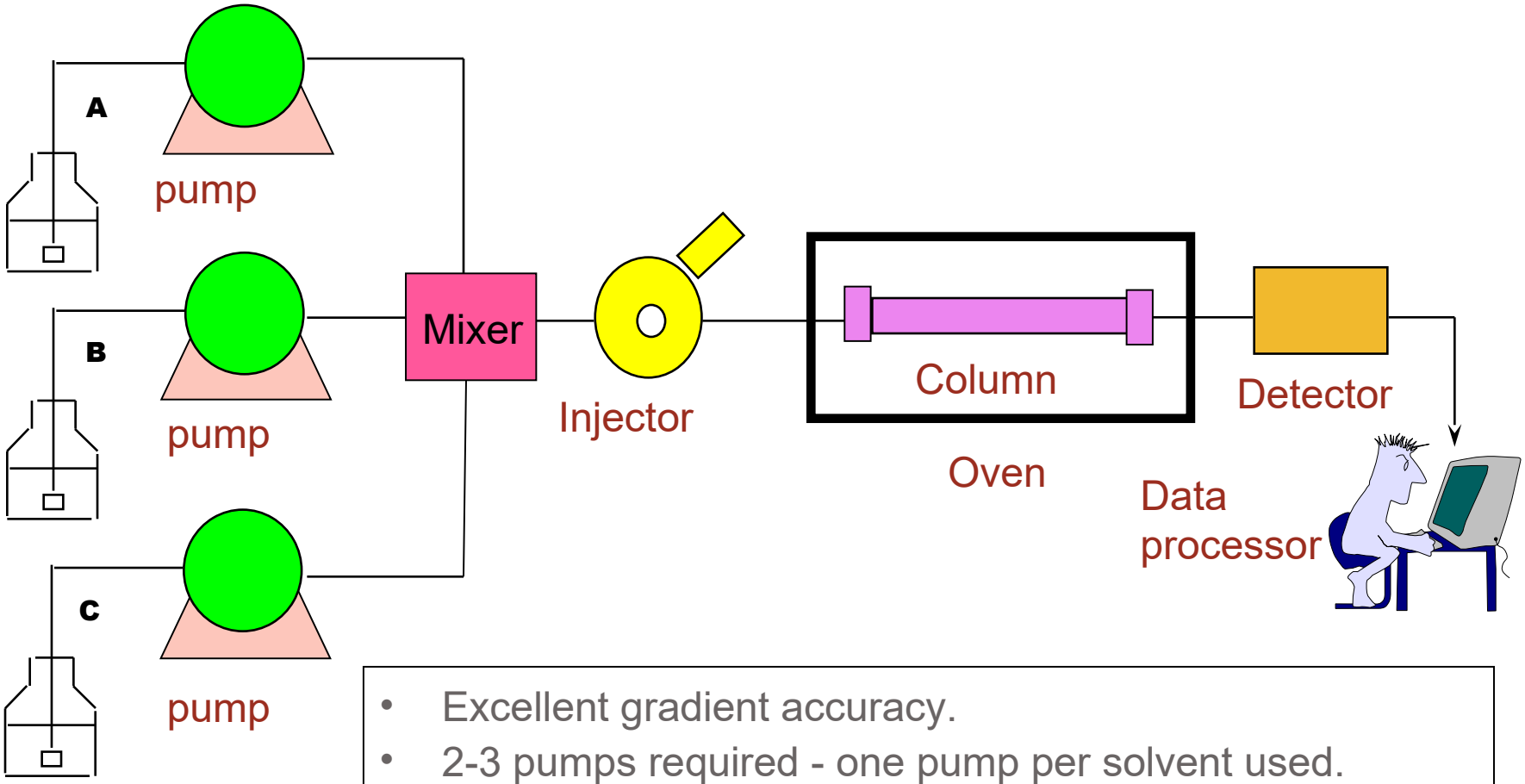
Isocratic System



Simple system with one pump and one solvent reservoir.

If more than one solvent is used, solvents should be premixed.

High-pressure Gradient System



- Excellent gradient accuracy.
- 2-3 pumps required - one pump per solvent used.
- On-line degassing may not be critical.

DEGASSING OF SOLVENTS:

- Several gases are soluble in organic solvents, when high pressure is pumped, the formation of gas bubbles increases which interferes with the separation process, steady baseline & shape of the peak.

Vacuum filtration: by applying a partial vacuum to the solvent container.

Helium Purging: Done by passing Helium through the solvent. This is very effective but Helium is expensive.

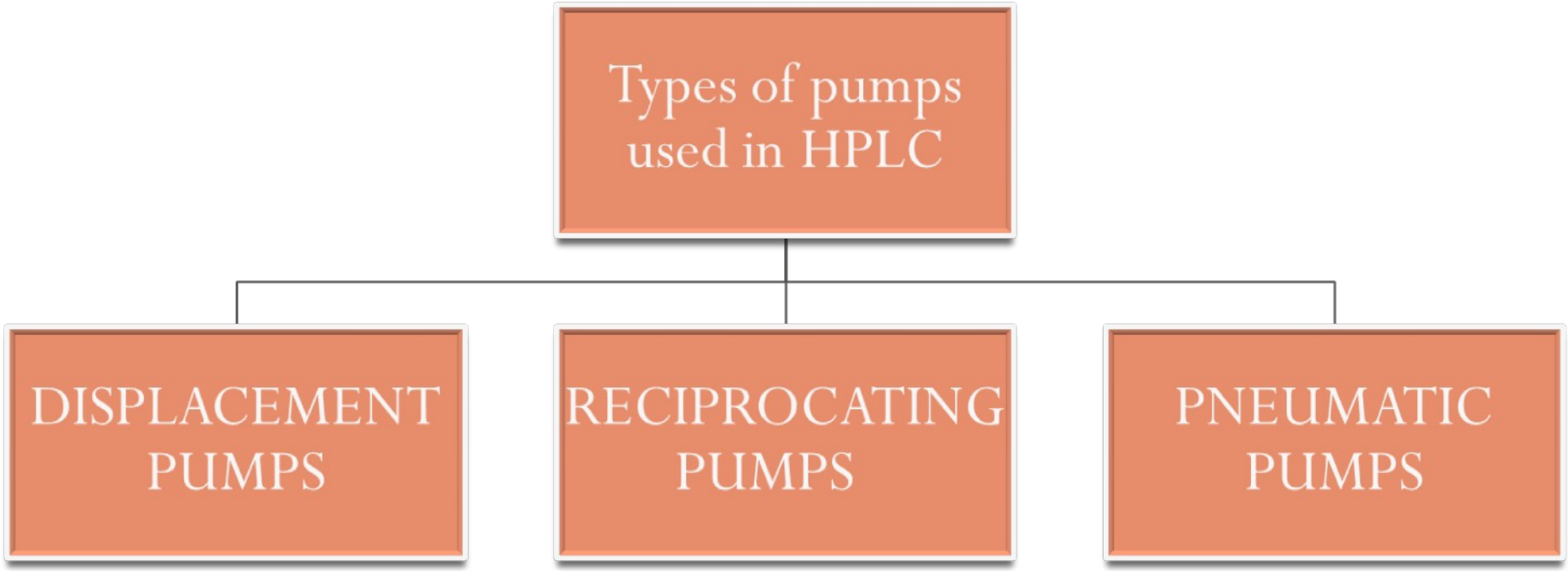
Ultrasonication: Done by using ultrasonication which converts ultra high frequency to mechanical vibrations.

❖PUMP:

- The solvents or mobile phase must be passed through a column at high pressures at up **to 6000 psi(lb/in²) or 414 bar.**
- As the particle size of stationary phase is smaller (5 to 10μ) the resistance to the flow of solvent will be high.
- That is, smaller the particle size of the stationary phase the greater is the resistance to the flow of solvents.
- Hence high pressure is recommended.

➢REQUIREMENTS FOR PUMPS:

- Generation of pressure of about **5000 psi.**
- all materials in the pump should be **chemically resistant** to solvents.
- Flow rates ranging from **0.1 to 10 mL/min**
- Pumps should be capable of taking the solvent from a **single reservoir or more than one reservoir** containing different solvents simultaneously.


Types of pumps used in HPLC
DISPLACEMENT PUMPS
RECIPROCATING PUMPS
PNEUMATIC PUMPS

PUMP A

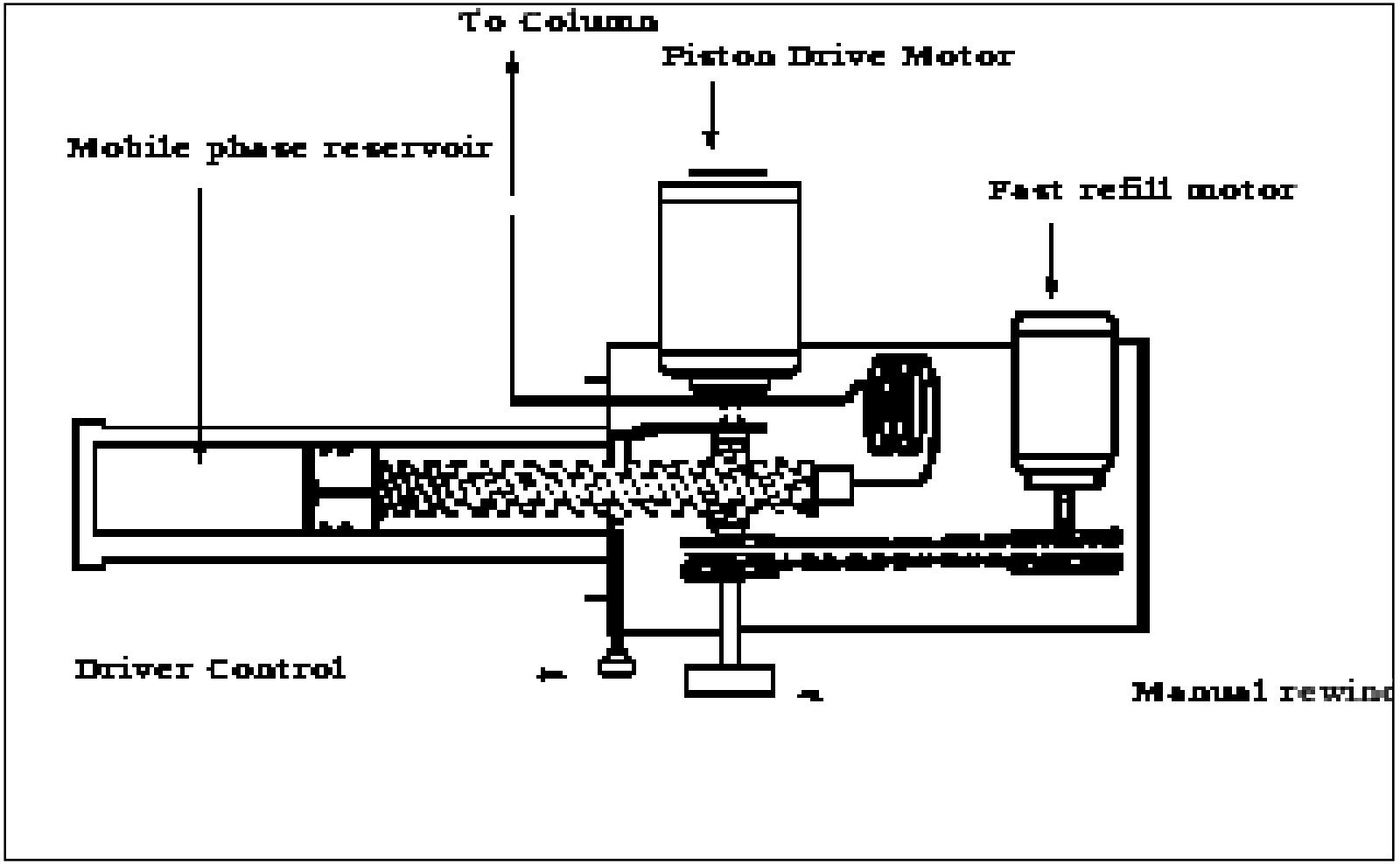
PUMP B


LC-8A
SHIMADZU
A
FLOW/PRESS
PRESSURE
P.MAX
P.MIN


SHIMADZU
B
FLOW/PRESS
PRESSURE
P.MAX
P.MIN

➢DISPLACEMENT PUMPS

- It consists of large, syringe like chambers equipped with a plunger activated by a screw driven mechanism powered by a stepping motor.
- Advantages:- It produces a flow that tends to be independent of viscosity & back pressure.
- Disadvantages:- It has a limited solvent capacity(~250) & considerably inconvenient when solvents must be changed.



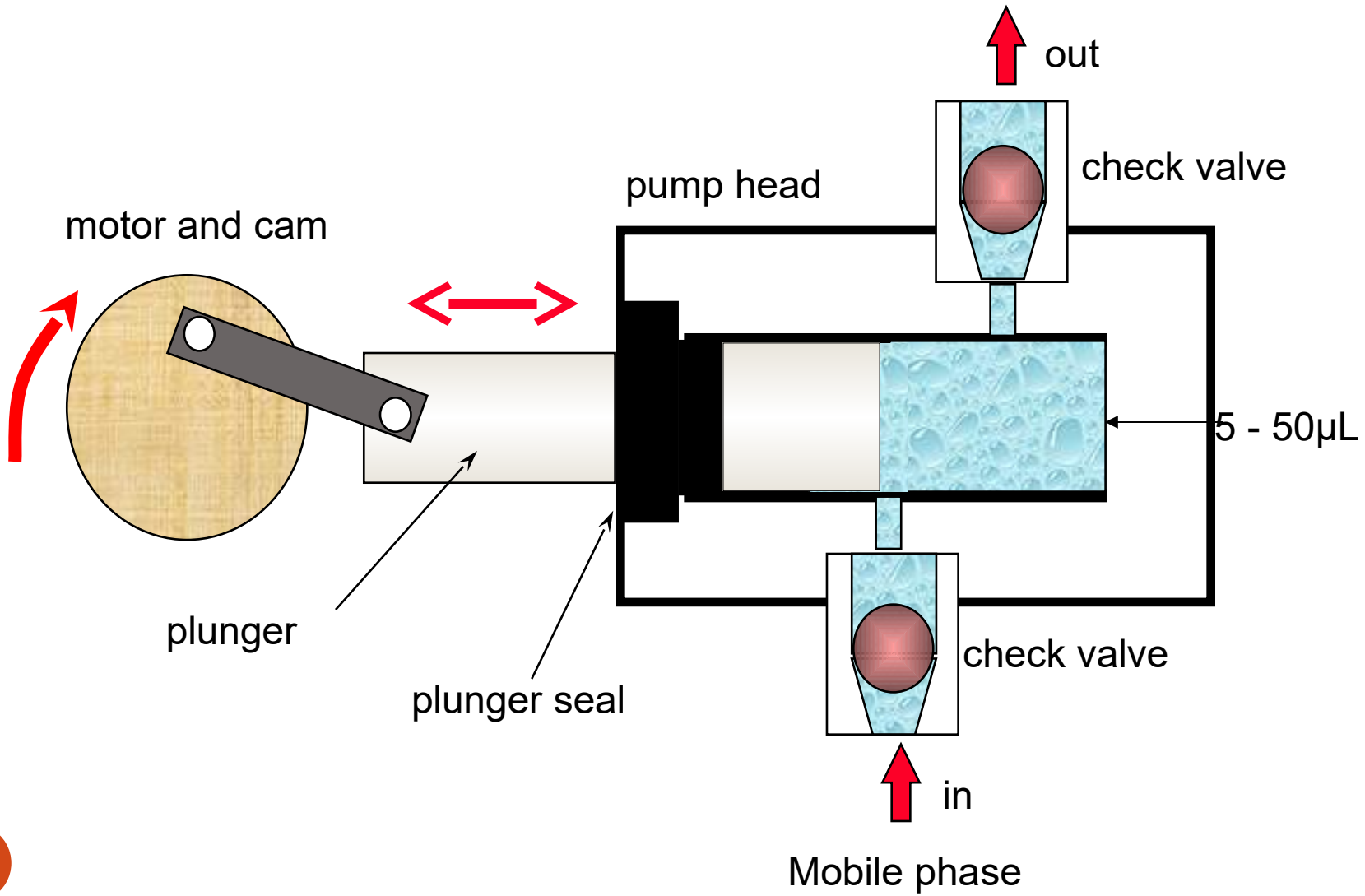
DISPLACEMENT PUMP

➢RECIPROCATING PUMPS:

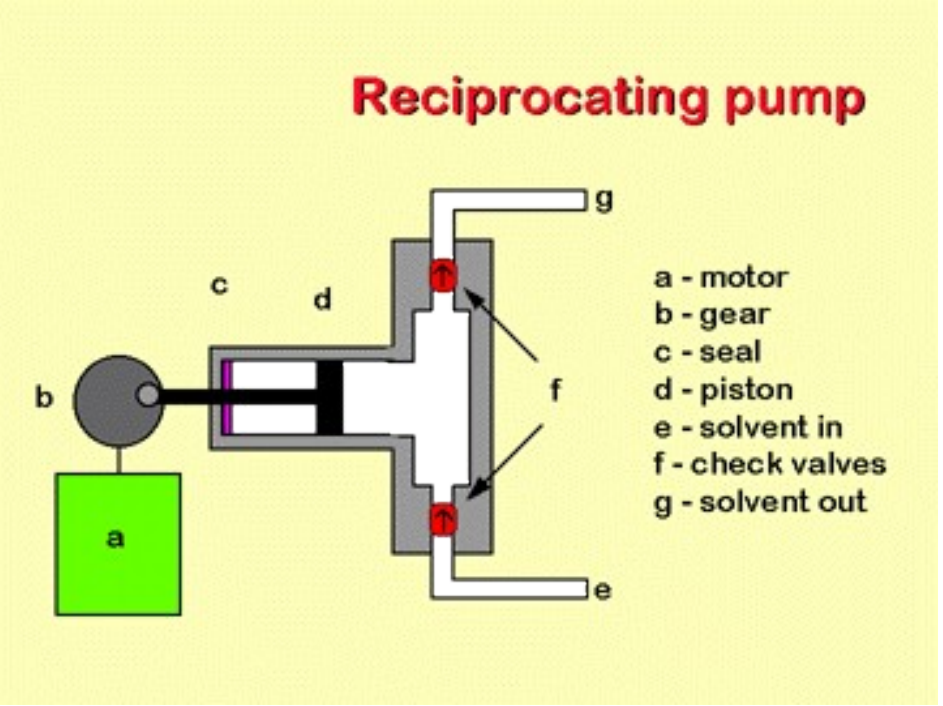
- This pump transmits alternative pressure to the solvent via a flexible diaphragm ,which in turn is hydraulically pumped by a reciprocating pump.
- **DISADVANTAGES**:-

  Produces a pulsed flow which is damped because pulses appear as baseline noise on the chromatograph

Plunger Reciprocating Pump



- motor driven piston :Solvent is pumped back and forth
- Two check valves (open & close): controls the flow

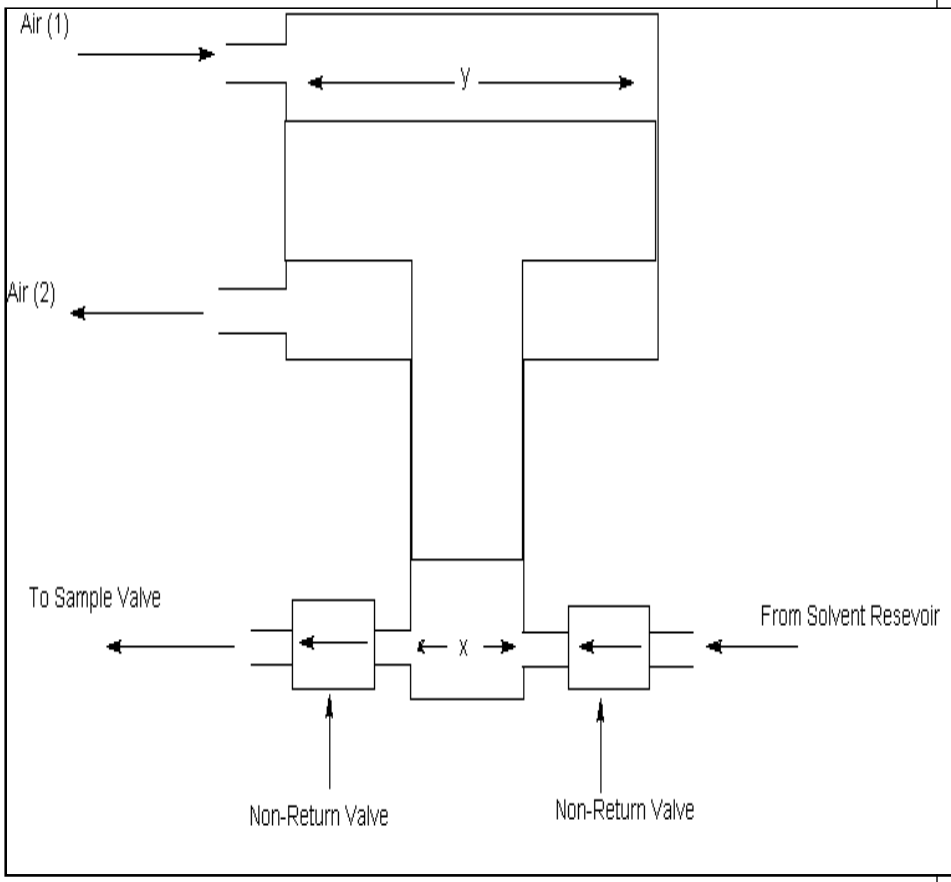


Advantages:

- Have small internal volume of 35-400µL
- Higher output pressures up to 10,000 psi.
- Adaptability to gradient elution.
- Large solvent capacities & constant flow rates.
- Largely independent of column back pressure & solvent viscosity.

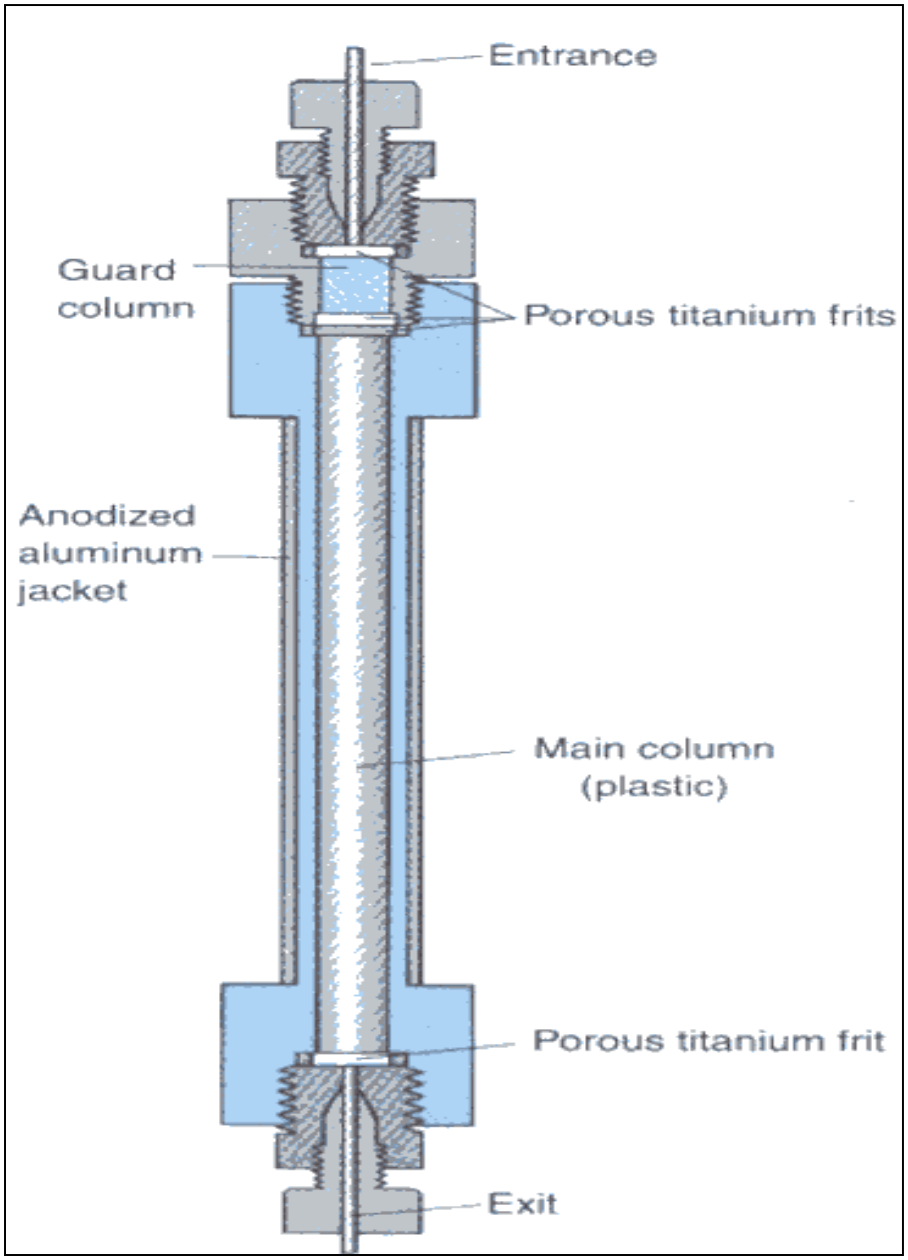
➢PNEUMATIC PUMPS:

- the mobile phase is driven through the column with the use of pressure produced from a gas cylinder.
- It has limited capacity of solvent
- Due to solvent viscosity back pressure may develop.



❖ COLUMN:

- Types:
- Guard Column
- Derivatizing Column
- Capillary Column
- Fast column
- Analytical Column
- Preparatory Column



➢GUARD COLUMN:

- Guard columns are placed anterior to the separating column.
- This **protects and prolongs the life & usefulness of the separating column.**
- They are dependable columns designed to filter or remove:-

✓ particles that clog the separating column,

✓ compounds and ions that causes decreased resolution, decreased sensitivity and create false peaks.

- ✓ Compounds that may cause precipitation upon contact with the stationary or mobile phase.
- ✓ Compounds that cause irrelevant peaks & interfere with the detection and quantification.
- ✓ These columns must be changed on a regular basis in order to optimize their protectiveness.

➢DERIVATIZING COLUMN

- Derivatization involves a chemical reaction between an analyte and a reagent to change the chemical and physical properties of an analyte.
- The four main uses of derivatization in HPLC are:

✓ Improve detectability,

✓ Change the molecular structure or polarity of analyte for better chromatography,

✓ Change the matrix for better separation,

✓ Stabilize a sensitive analyte.

- Pre or post primary column derivatization can be done.
- Derivatization techniques includes –acetylation, silylation, acid hydrolysis.
- **DISADVANTAGES**: It becomes a complex procedure and so it acts as a source of error to analysis and increases the total analysis time.
- **ADVANTAGES**: Although derivatization has drawbacks, it may still be required to solve a specific separation or detection problem.

➢CAPILLARY COLUMNS:

- HPLC led to smaller analytical columns called as micro-columns, capillary columns which have **diameter less than a millimeter.**
- Sample used – is in **nanolitre volumes**, decreased flow rate, decreased solvent volume usage which leads to cost effectiveness.
- Disadvantage:- since it is miniatured, flow rate is difficult to produce & gradient elution is not efficient.

- MICROBORE and SMALLBORE columns are also used for analytical and small volumes assay.
- Diameter of small-bore columns is 1-2mm.
- The instrument must also be modified to accommodate these smaller capacity columns.

FAST COLUMNS:

- This column also have the same internal diameter but much **shorter length** than other columns & packed with particles of 3µm in diameter.
- **Advantages:** Increased sensitivity, decreased analysis time, decreased mobile phase usage & increased reproducibility.

➢ANALYTICAL COLUMN:

- Length- 5 to 25 cm ,Internal Diameter 3 to 5mm.
- Particle size of packing material is 3 to 5µm.

➢PREPARATORY COLUMN:

- Length – 10 to 15 cm, Int. diameter – 4.6mm
- Packed with particles having 5µm as diameter.
- Columns of this time generate 10,000 plates per column.
- It consists of back pressure regulator and **fraction collector.**

❖SAMPLE INJECTOR SYSTEM:

- Several injector devices are available either for manual or auto injection of the sample.

(i) Septum Injector

(ii)Stop Flow Injector

(iii)Rheodyne Injector

Rheodyne Manual injector

(i)Septum Injector:

- used for injecting the sample through a rubber septum. cannot be commonly used , since the septum has to withstand high pressures.

(ii)Stop Flow(On Line):

- mobile phase is stopped for a while & the sample is injected through a valve.

(iii)Rheodyne injector:

- most popular injector and is widely used.
- This has a fixed volume of loop, for holding sample until its injected into the column, like 20µL, 50µL or more.
- Through an injector the sample is introduced into the column.
- The injector is positioned just before the inlet of the column.

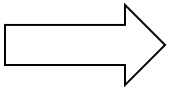
SELECTION VALVE:

By using the selection valve we can select whether the purpose is for analytical purpose or preparative purpose.

LOAD POSITION: In this position the sample is loaded into the sample loop .

INJECT POSITION: In this position the loaded sample is injected into the column by the forcful flow of the solvent into the sample loop by which the sample is introduced into the column.

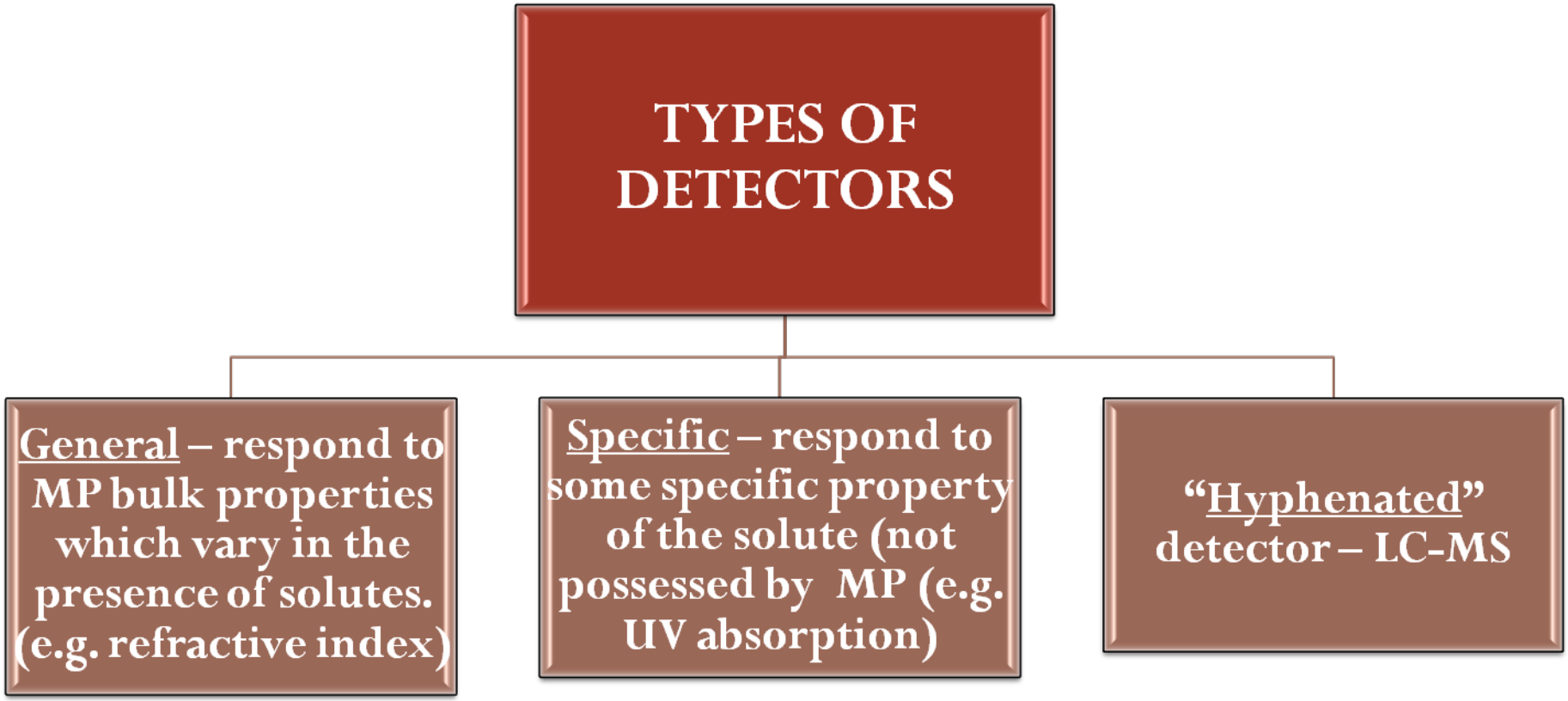
HPLC Auto Injectors



Inside of SIL-20AC

➢COLUMN TEMPERATURE CONTROLLER:

- For obtaining better and reproducible chromatograms constant column temperature should be maintained.
- Some are equipped with heater/ water to give precise temperature control.


TYPES OF DETECTORS
General – respond to MP bulk properties which vary in the presence of solutes. (e.g. refractive index)
Specific – respond to some specific property of the solute (not possessed by MP (e.g. UV absorption)
"Hyphenated" detector – LC-MS

DETECTORS:

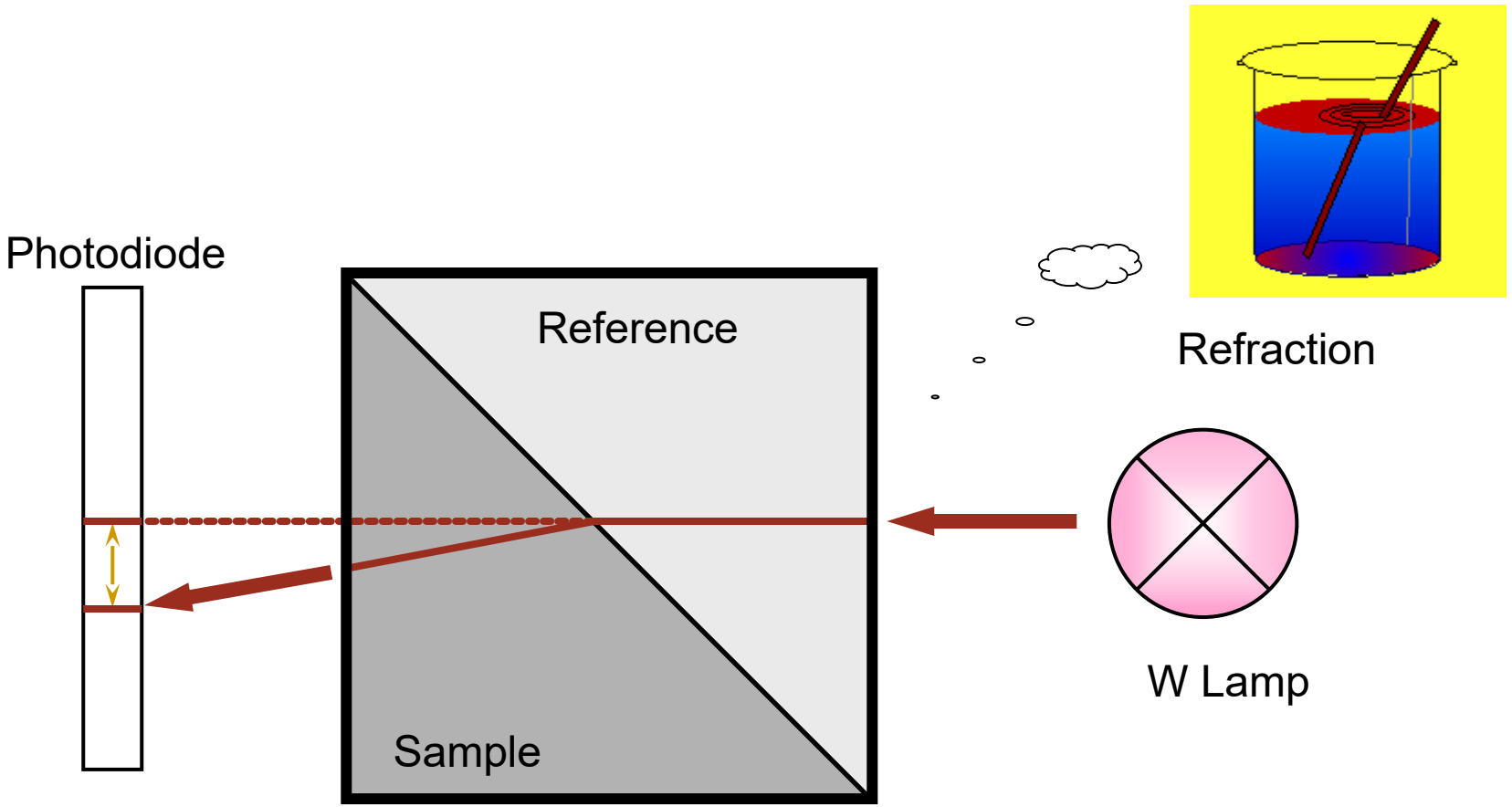
❖DETECTORS:

- Absorbance (UV/Vis)
- Refractive index (detects the change in turbidity)
- Fluorescence (if the analyte is fluorescent)
- Electrochemical (measures current flowing through a pair of electrodes, on which a potential difference is imposed, due to oxidation or reduction of solute)
- Conductivity (for ions)
- Light scattering
- Mass spectrometry (HPLC-MS)

❖ABSORBANCE DETECTORS:

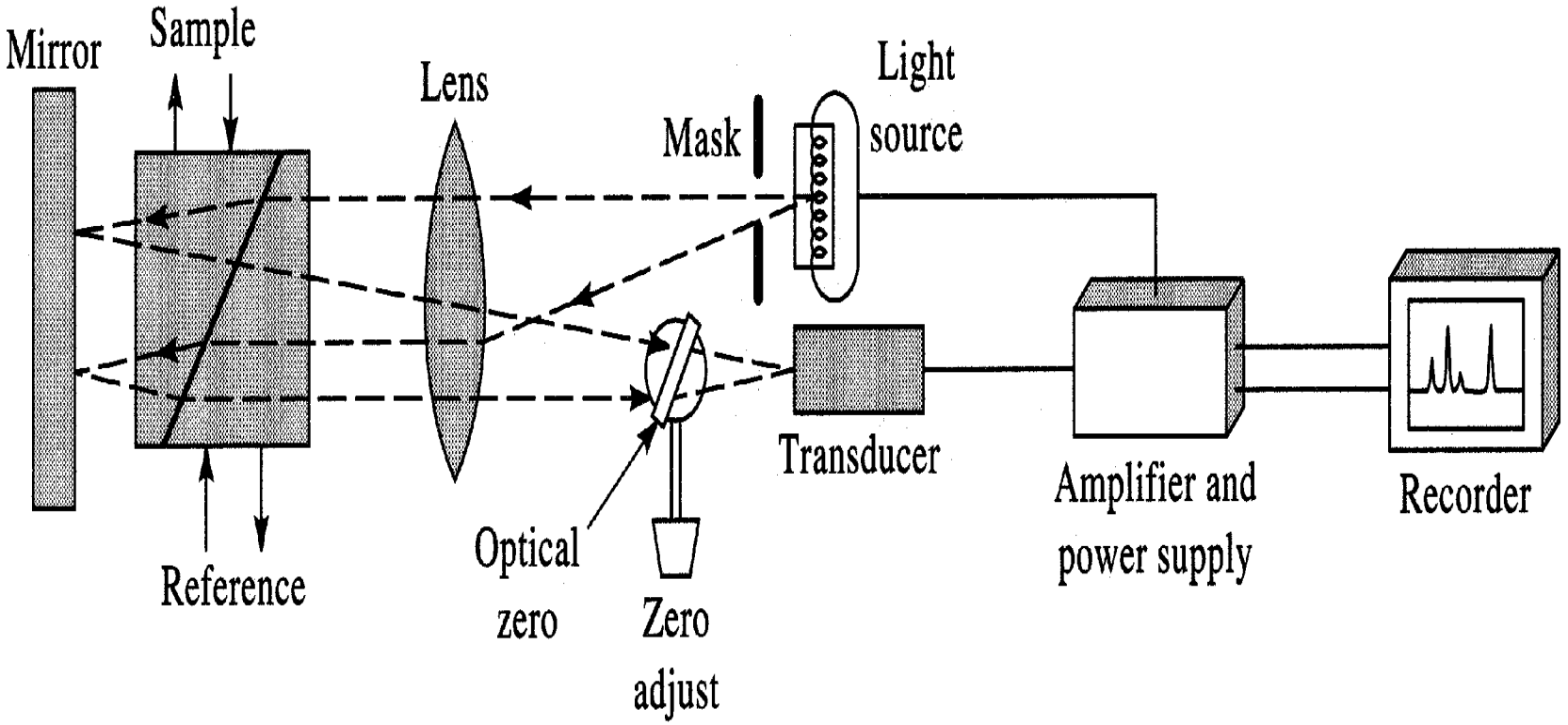
- The UV/Vis source usually comes from a monochromator so the wavelength can be selected, or scanned.
- Fixed wavelength-measures at single wavelength usually 254nm.
- Variable wavelength-measures at single wavelength at a time but can detect over a wide range of wavelengths simultaneously.

Refractive Index Detector



REFRACTIVE INDEX (RI) DETECTOR:

- Nearly universal but poor detection limit.



- Detection occurs when the light is bent due to samples eluting from the columns, and this is read as a disparity b/w the two channels.
- It is not much used for analytical applications because of low sensitivity & specificity.

➢FLUORIMETRIC DETECTORS:

- It is based on the fluorescent radiation emitted by some compounds.
- The excitation source passes through the flow cell to a photodetector while a monochromator measures the emission wavelengths.
- More sensitive and specific.
- The disadvantge is that most compounds are not fluorescent in nature.

Fluorescence of Compounds

Fluorescence is a type of luminescence in which the light energy is released in the form of a photon in nanoseconds to microseconds

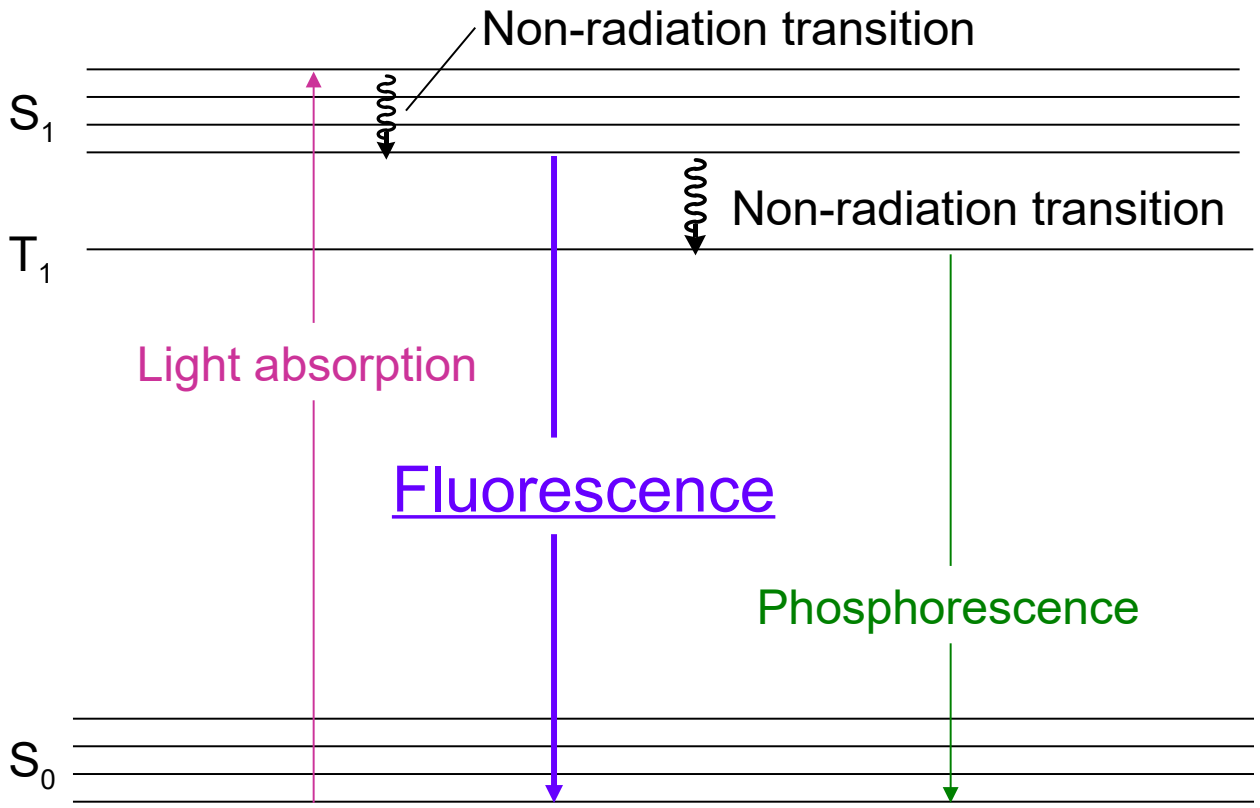
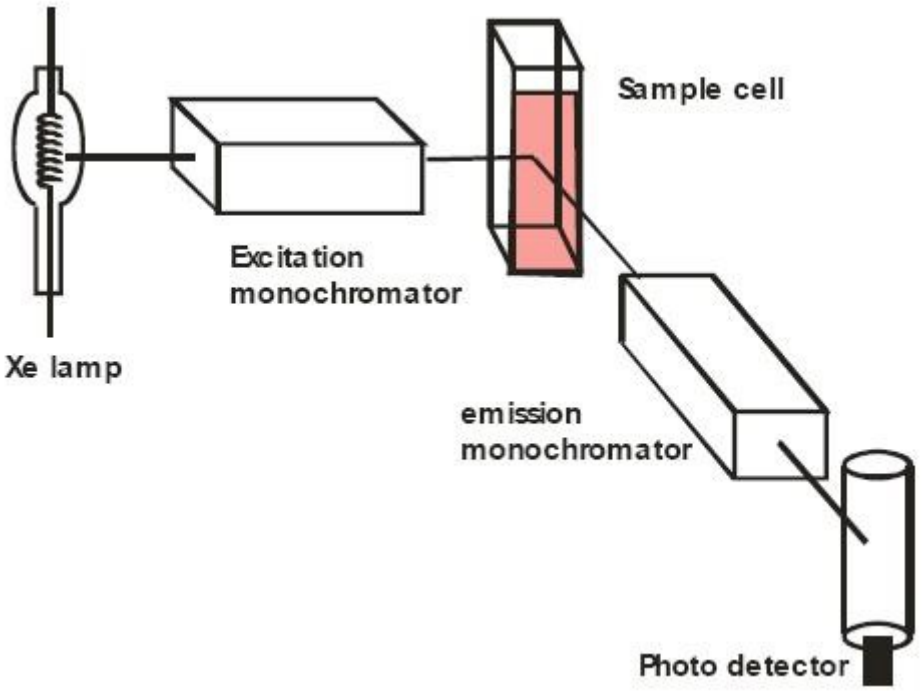


DIAGRAM OF FLUORESCENCE DETECTORS



Fluorescence Detector

Advantage

- Sensitivity is higher than UV-Vis detector
- Selectivity is high because relatively few compounds fluorescence
- Compatible with gradient elution

Disadvantage

- Difficult to predict fluorescence
- Greatly affected by environment
  - Solvent
  - pH
  - Temperature
  - Viscosity
  - Ionic strength
  - Dissolved gas

➢ AMPEROMETRIC DETECTOR:

- based on the reducing and oxidizing property of the sample when a potential is applied.
- The diffusion current recorded is directly proportional to the concentration of the compound recorded.
- DISADVANTAGE: This detector is applicable only when the functional groups present in the sample can be either oxidized or reduced.
- ADVANTAGE: Highly sensitive detector.

AMPEROMETRIC DETECTOR:

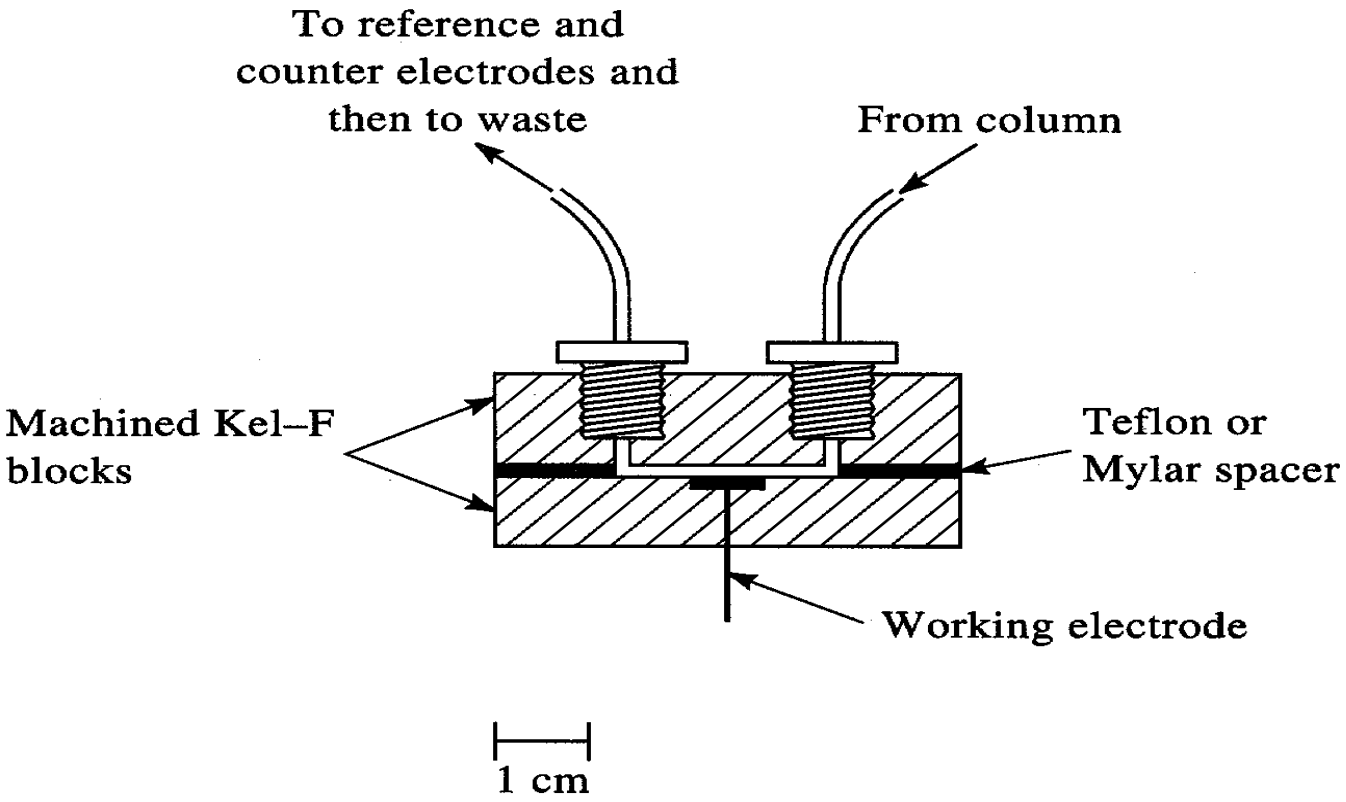
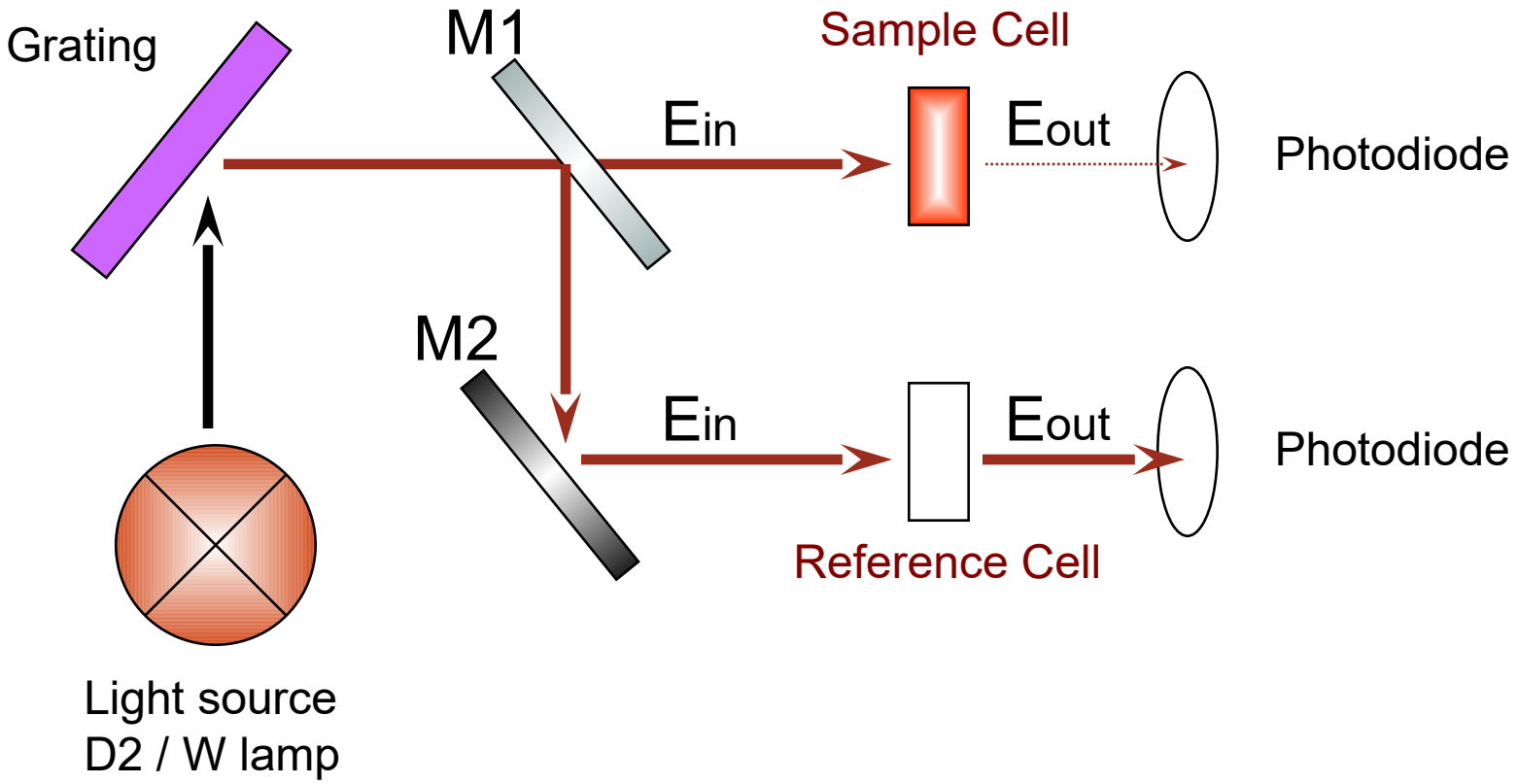


Figure 28-13 Amperometric thin-layer detector cell for HPLC.

Instrumentation of UV-Vis Detector



Ultraviolet / Visible Detector

Advantage:

- Sensitivity is high
- Relative robust to temperature and flow rate change
- Compatible with gradient elution

Disadvantage:

- Only compounds with UV or visible absorption could be detected.

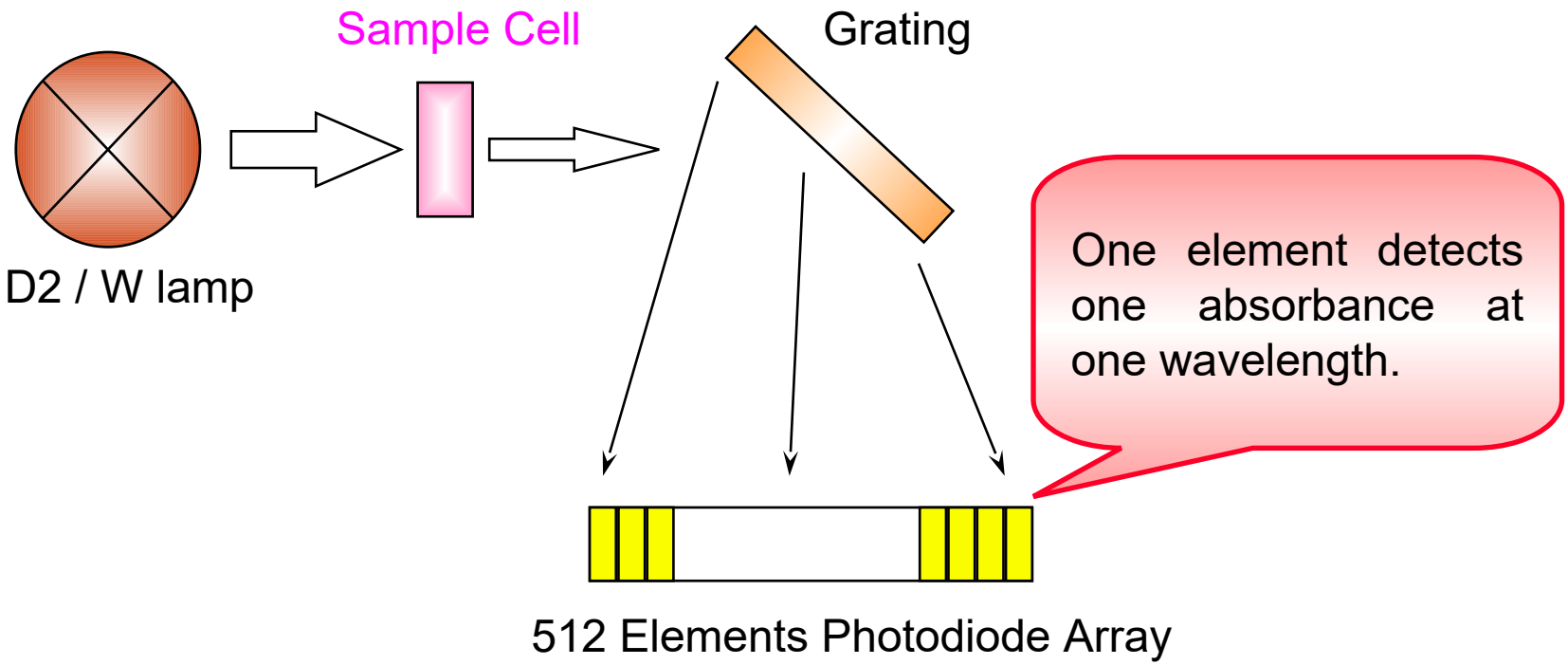
Additional Functions

- Dual Wavelength mode
- Wavelength Time Program mode
- Wavelength Scan mode

➢PHOTODIODE ARRAY DETECTORS :

- This is a recent detector which is similar to UV detector which operates from 190-600nm.
- Radiations of all wavelength fall on the detector simultaneously.
- The resulting spectra is a three dimensional plot of Response Vs Time Vs Wavelength.
- ADVANTAGE: The wavelength need not be selected but detector detects the responses of all compounds.

➢PHOTODIODE ARRAY DETECTORS:

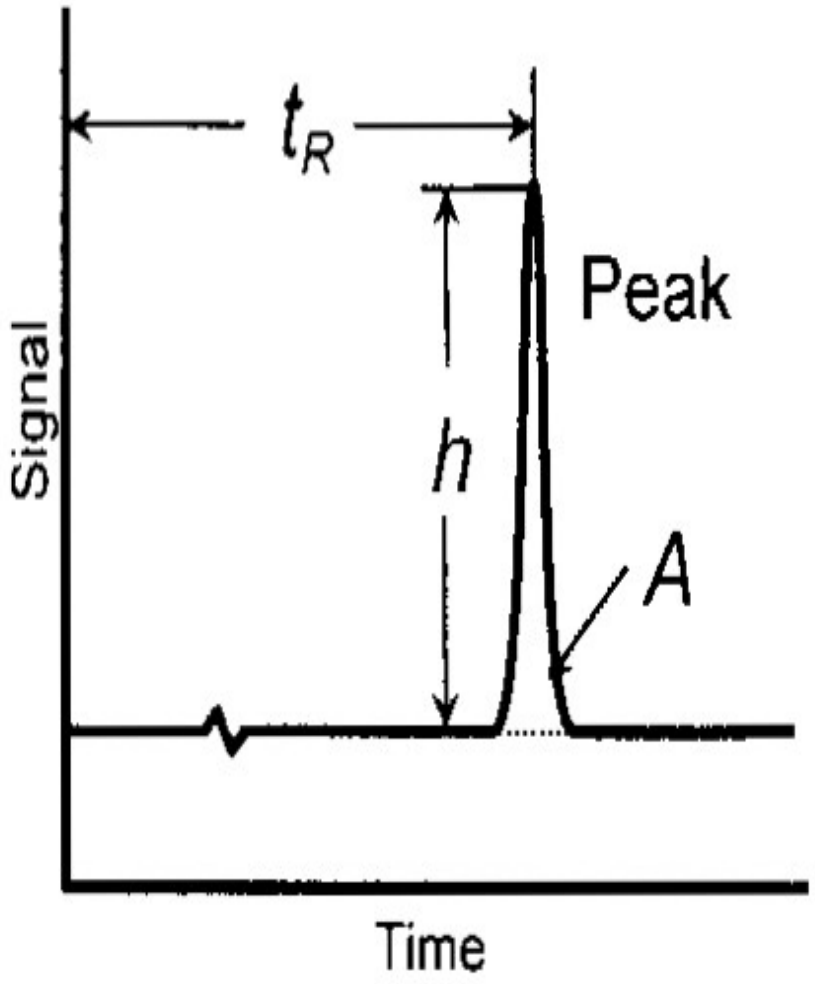


❖RECORDERS AND INTEGRATORS:

- Recorders are used to record responses obtained from the detectors after amplification,if necessary.
- They record the baseline & all the peaks obtained with respect tot ime.
- Retention time can be found out from this recordings,but area under curve cannot be determined.

❖INTEGRATORS:

- These are improved versions of recorders with some data processing capabilities.
- They can record the individual peaks with retention time,height,width of peaks,peak area,percentage area,etc.
- Integrators provides more information on peaks than recorders.
- In recent days computers and printers are used for recording and processing the obtained data & for controlling several operations.

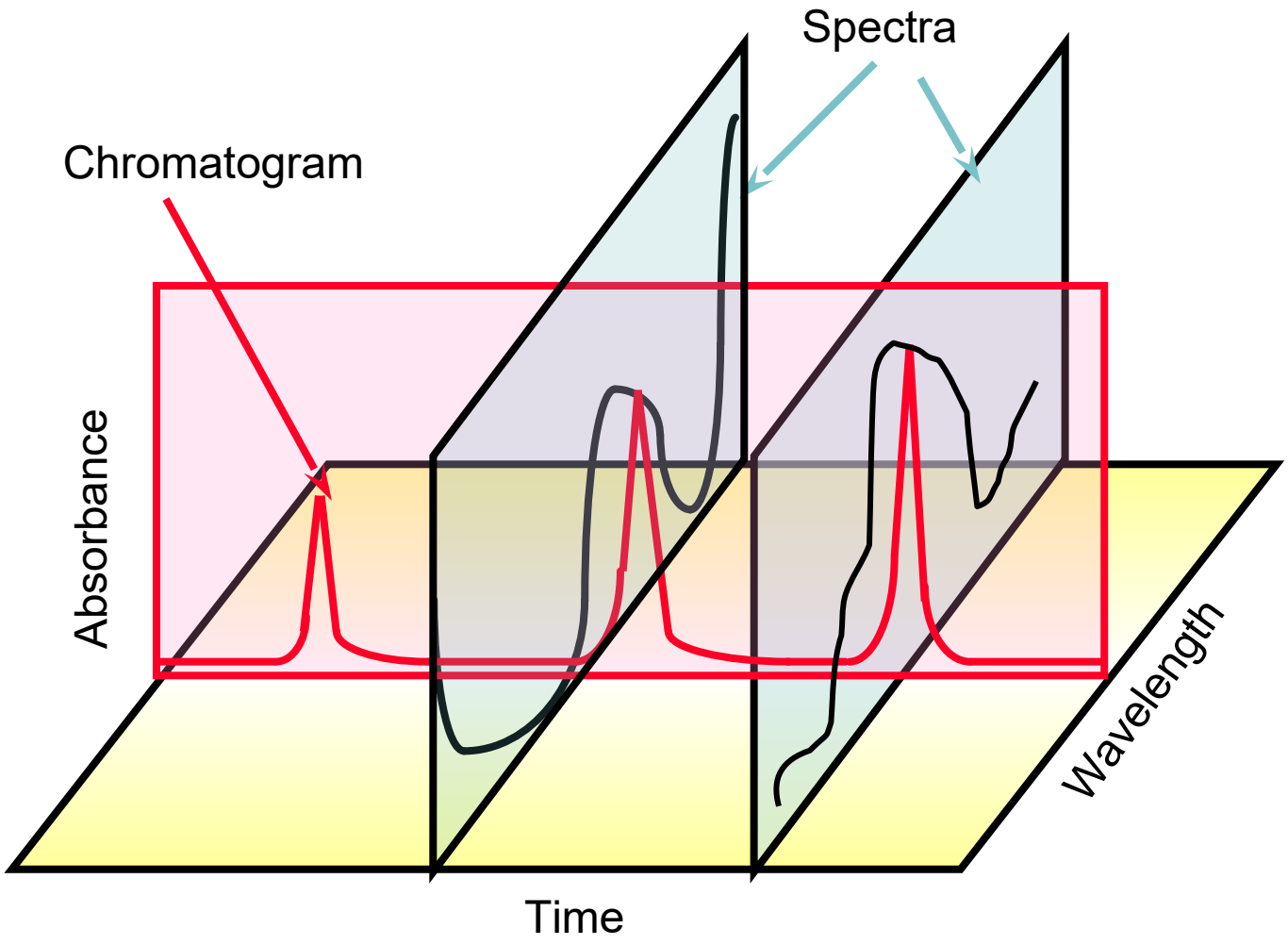


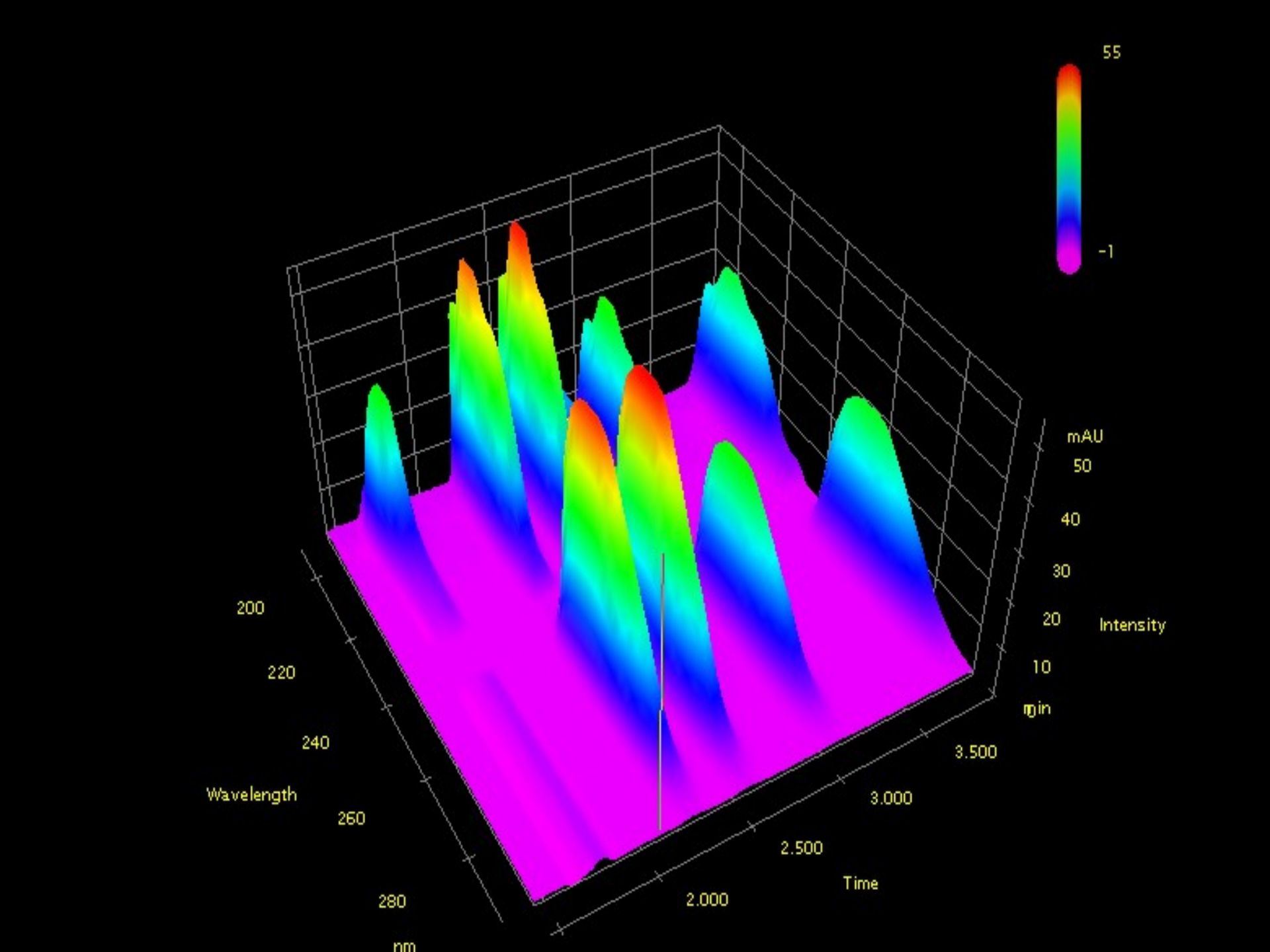
t_R : Retention time

A : Area

h : Height

Photodiode Array Detector (3-D Data)

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-1
mAU
50
40
30
20
10
Intensity
200
220
240
260
280
nm
Wavelength
2.000
2.500
3.000
3.500
min
Time

❖PARAMETERS:

- Retention time(R_t)
- Retention volume(Vr)
- Separation factor(S)
- Resolution
- Theoritical plates
- Column efficiency
- Assymetry factor

❑REFERENCE:

- Gurdeep R. Chatwal, Sham K. Anand, Instrumental Method Of Chemical Analysis, Himalaya Publishing House, 2003, p. 2.624 to 2.638
- P.D Sethi, Quantitative Analysis Of Pharmaceutical Preparations .
- Douglas A. Skoog, Instrumental Analysis, Brooks/Cole, 2007, p. 897 to 899
- Dr. S Ravi Shankar, Textbook Of Pharmaceutical Analysis, Rx Publications, 2005, p. 18-1 to 18-11
- Robert D. Braun, Introduction Instrumental Analysis, Pharma Book Syndicate, 2006, p. 860 to 863
- www.google.com

THANK YOU