High performance liquid chromatography [HPLC]

Chromatography

- It is define as, it is analytical method in which separation of active constituent in complex mixture, and the mixture was distributed in two phases i.e. stationary phase and mobile phase is known as chromatography.
- It is technique is **used for separation, purification, Identification and extraction of compound.**
- It is method it can consist of **two phases stationary phase and mobile phase.**
- **Stationary phase** is constant phase or column packaging material.
- **Mobile phase** is moveable phase.
- The **basic principle of chromatography is based on Adsorption and partition chromatography.**
- **Adsorption chromatography** - The affinity of molecules towards stationary phase is known as Adsorption chromatography.
- **Partition chromatography** - The molecule can moves in two phases of liquid is known as partition chromatography.
- It is important for **qualitative and quantitative analysis.**

TYPES OF CHROMATOGRAPHY

❖**Based on modes of chromatography**

1. Normal phase mode
2. Reverse phase mode

❖**Based on principle of separation**

1. Adsorption chromatography
2. Ion exchange chromatography
3. Partition chromatography
4. Size exclusion

❖**Based on elution technique**

1. Isocratic separation
2. Gradient separation

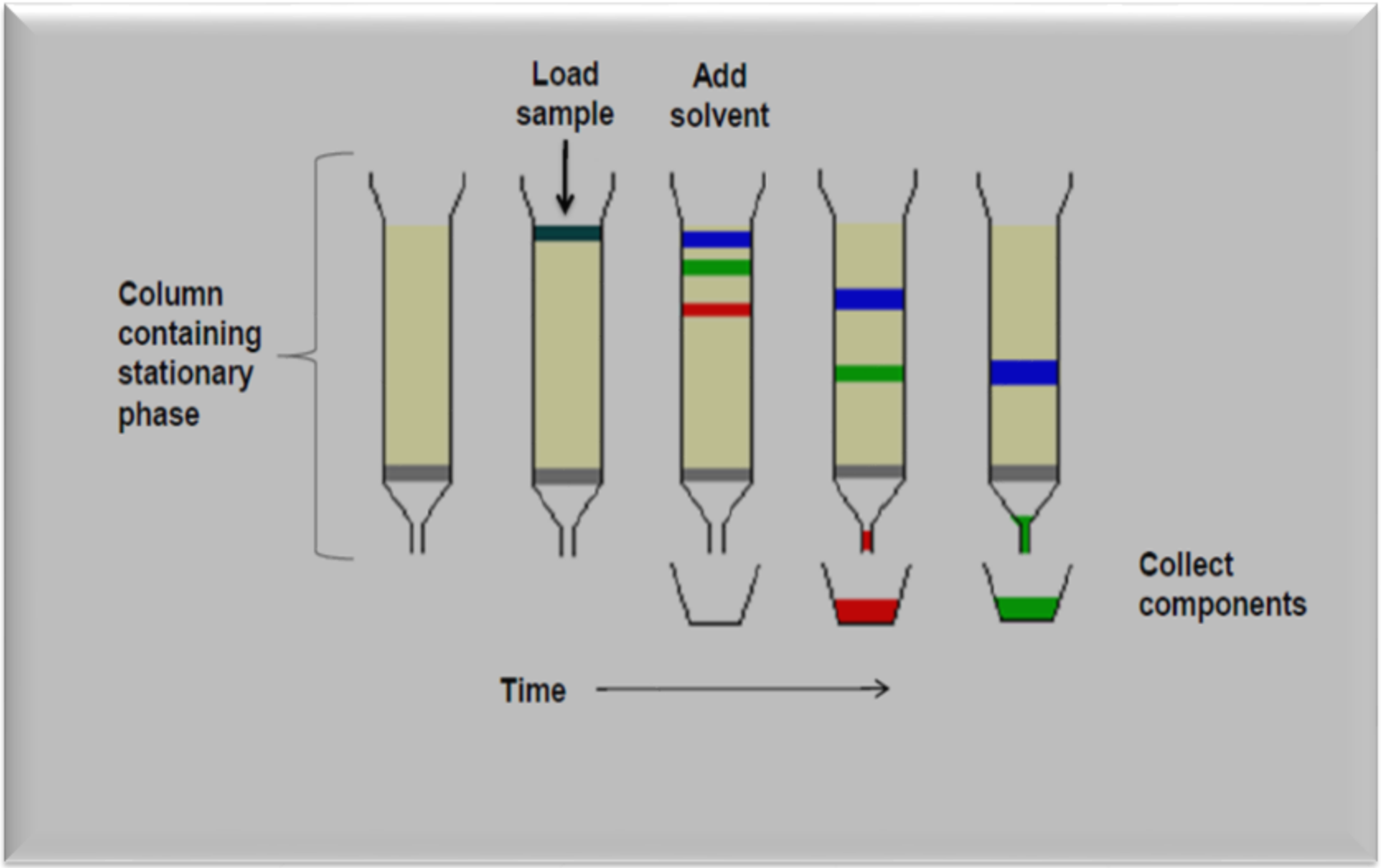
❖**Based on the scale of operation**

1. Analytical HPLC
2. Preparative HPLC

❖**Based on the type of analysis**

1. Qualitative analysis
2. Quantitative analysis

Principles of 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

- HPLC is a High **Performance** liquid Chromatography.
- High **Pressure** Liquid Chromatography.
- High **Priced** Liquid Chromatography.
- It is **column** chromatography.
- It is **Liquid** Chromatography.
- It is modified from of gas chromatography, it is **applicable for both Volatile as well as Non volatile compound.**
- It can mainly divided by two types **1. Normal phase HPLC 2. Reversed Phase HPLC.**
- It is having a **high resolution and separation capacity**.
- It is used as **qualitative as well as quantitative analysis.**
- High performance liquid chromatography (HPLC) is a chromatographic technique used to separate a mixture of compounds in analytical chemistry and biochemistry with the purpose of **identifying, quantifying or purifying the individual components of the mixture.**


Agilent Technologies
Quaternary

Principle

- High Performance Liquid Chromatography [HPLC] is principle is based on **adsorption as well as partition chromatography** is depending on the nature of stationary phase, if stationary phase is solid principle is based on adsorption chromatography and if stationary phase is liquid principle is based on partition chromatography.

- It is important for determination of **volatile and non volatile compounds**.

- It is important for determination **qualitative and quantitative analysis**.

- It is important for determination of **Retention Time** (the time is required , after sample injection maximum angle peak reaches to detector)

Advantages

- It is simple, rapid , reproducible.
- High sensitivity.
- High performance.
- Rapid process and hence time saving.
- It is having a high resolution and separation capacity.
- Accuracy and Precision.
- Stationary phase was chemically innert.
- Wide varities of stationary phase.
- Mobile phase was chemically innert.
- Less requirement of mobile phase in developing chamber.
- Early recovery of separated component.
- Easy visualization of separated components.
- It is having Good reproducibility and repeatability.
- It is analytical technique is important for validation of product, quality control studies of product.
- It is important for qualitative and quantitative analysis.
- It is used for both analytical and preparative purpose.

Types of HPLC Separations

- **Normal Phase**: Separation of polar analytes by partitioning onto a polar, bonded stationary phase.

- **Reversed Phase**: Separation of non-polar analytes by partitioning onto a non-polar, bonded stationary phase.

- **Adsorption**: In Between Normal and Reversed. Separation of moderately polar analytes using adsorption onto a pure stationary phase (e.g. alumina or silica)

- **Ion Chromatography**: Separation of organic and inorganic ions by their partitioning onto ionic stationary phases bonded to a solid support.

- **Size Exclusion Chromatography**: Separation of large molecules based in the paths they take through a "maze" of tunnels in the stationary phase.

HPLC Methods

Parameter Group	Method	Compounds
SDW05.23000's	EPA 555	Cl-PhenoxyAcids
WPP05.06000's	EPA 605	Benzidines
WPP05.13000's	EPA 610	PAHs
SHW06.26000's	SW-846 8316	Acrylics
SHW06.28000's	SW-846 8330's	Explosives
SHW07.06000's	SW-846 8325	Benzidines and N- Pesticides

Modes of High Performance Liquid Chromatography

Types of Compounds	Mode	Stationary Phase	Mobile Phase
Neutrals Weak Acids Weak Bases	Reversed Phase	C18, C8, C4 cyano, amino	Water/Organic Modifiers
Ionics, Bases, Acids	Ion Pair	C-18, C-8	Water/Organic Ion-Pair Reagent
Compounds not soluble in water	Normal Phase	Silica, Amino, Cyano, Diol	Organics
Ionics Inorganic Ions	Ion Exchange	Anion or Cation Exchange Resin	Aqueous/Buffer Counter Ion
High Molecular Weight Compounds Polymers	Size Exclusion	Polystyrene Silica	Gel Filtration-Aqueous Gel Permeation-Organic

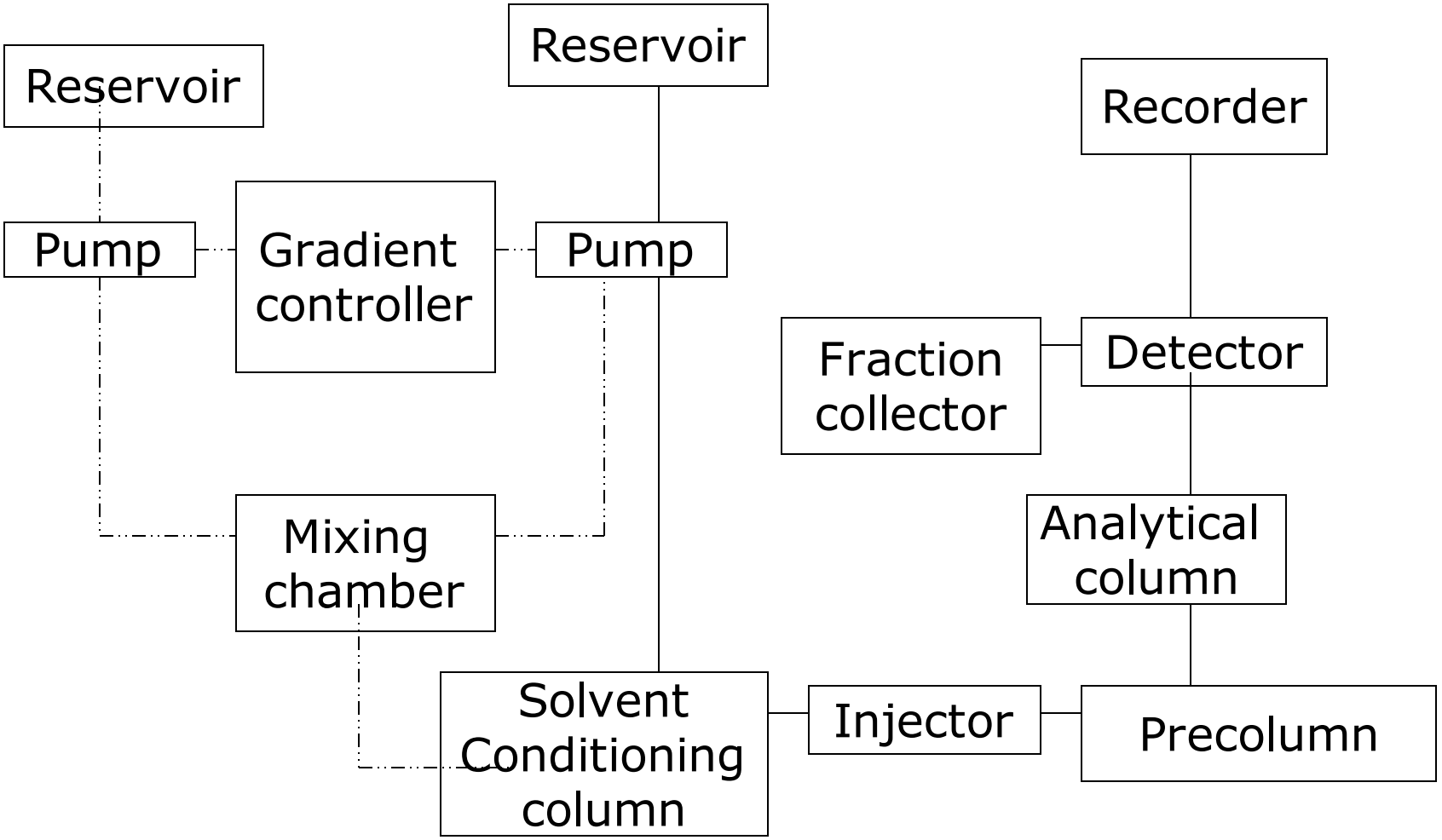
HPLC Contain

- **Stationary Phase -** It is non polar and The stationary solid surface is coated with a 2nd liquid (the Stationary Phase) which is immiscible with mobile phase.
- **Mobile Phase** - A polar mobile phase(ACN, MeOH, WATER + BUFFER).

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 $-Si-(CH_2)_3-CN$

Block diagram of hplc

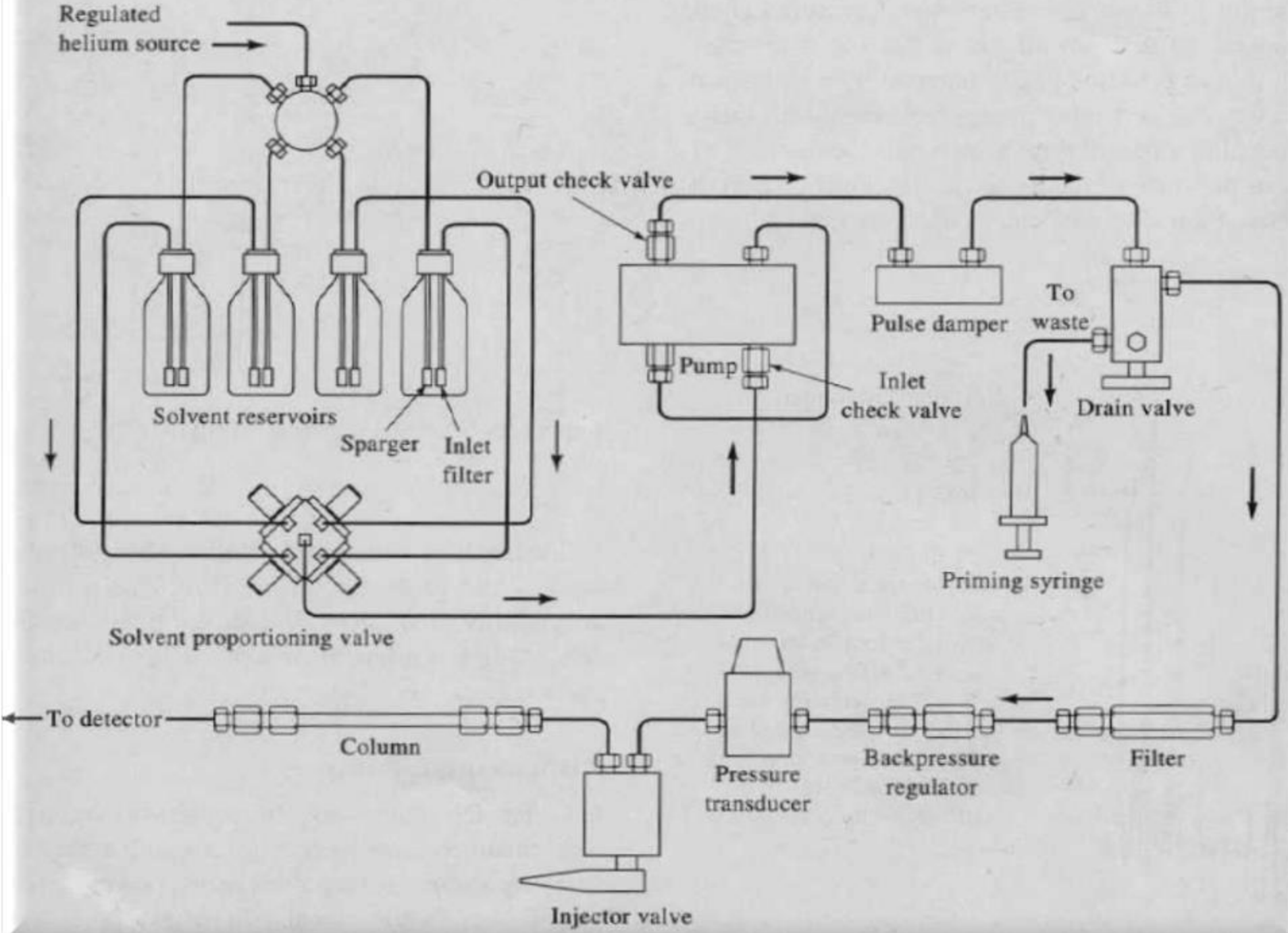


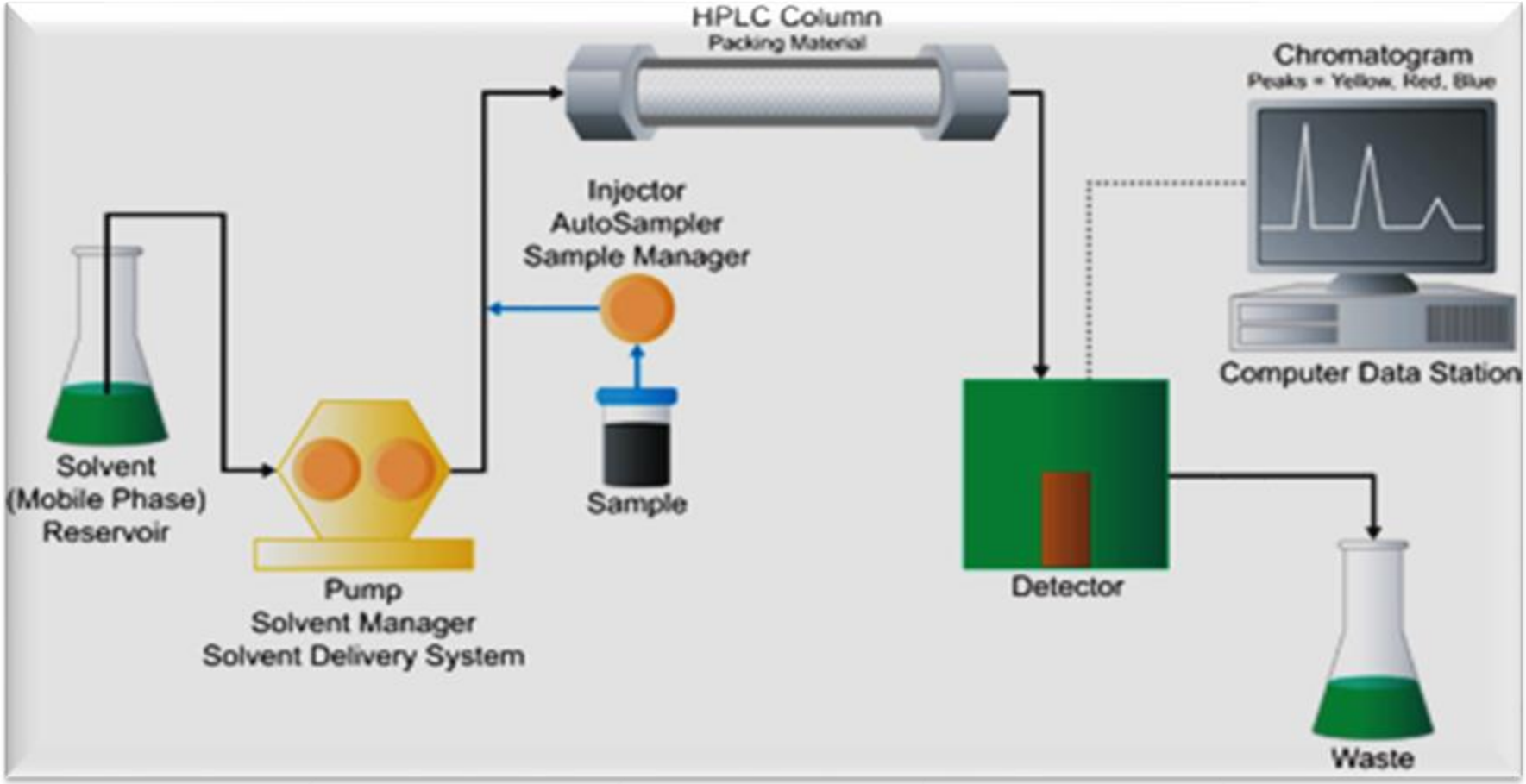
WHY USE HPLC

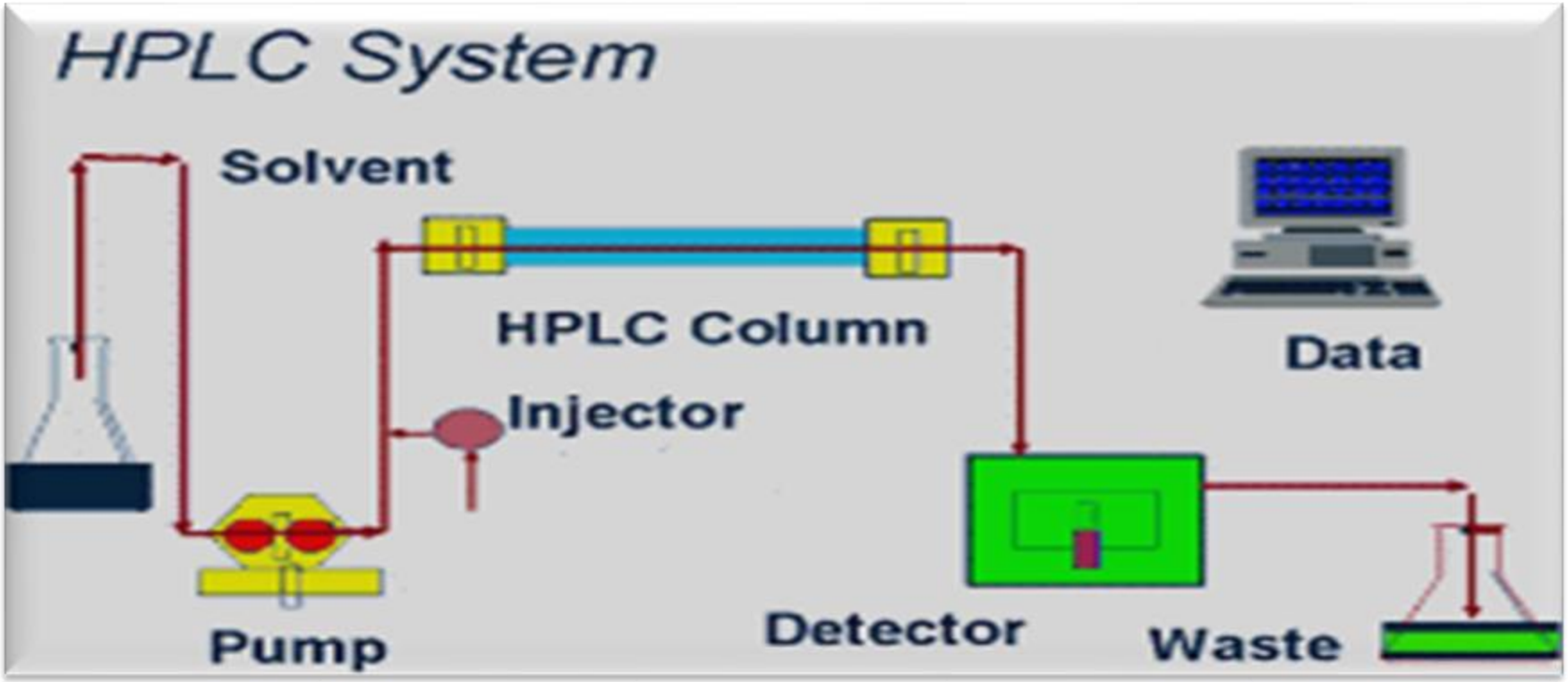
- Simultaneous analysis
- High resolution
- High sensitivity
- Good repeatability
- Moderate analysis condition
- Easy to fractionate and purify
- Not destructive

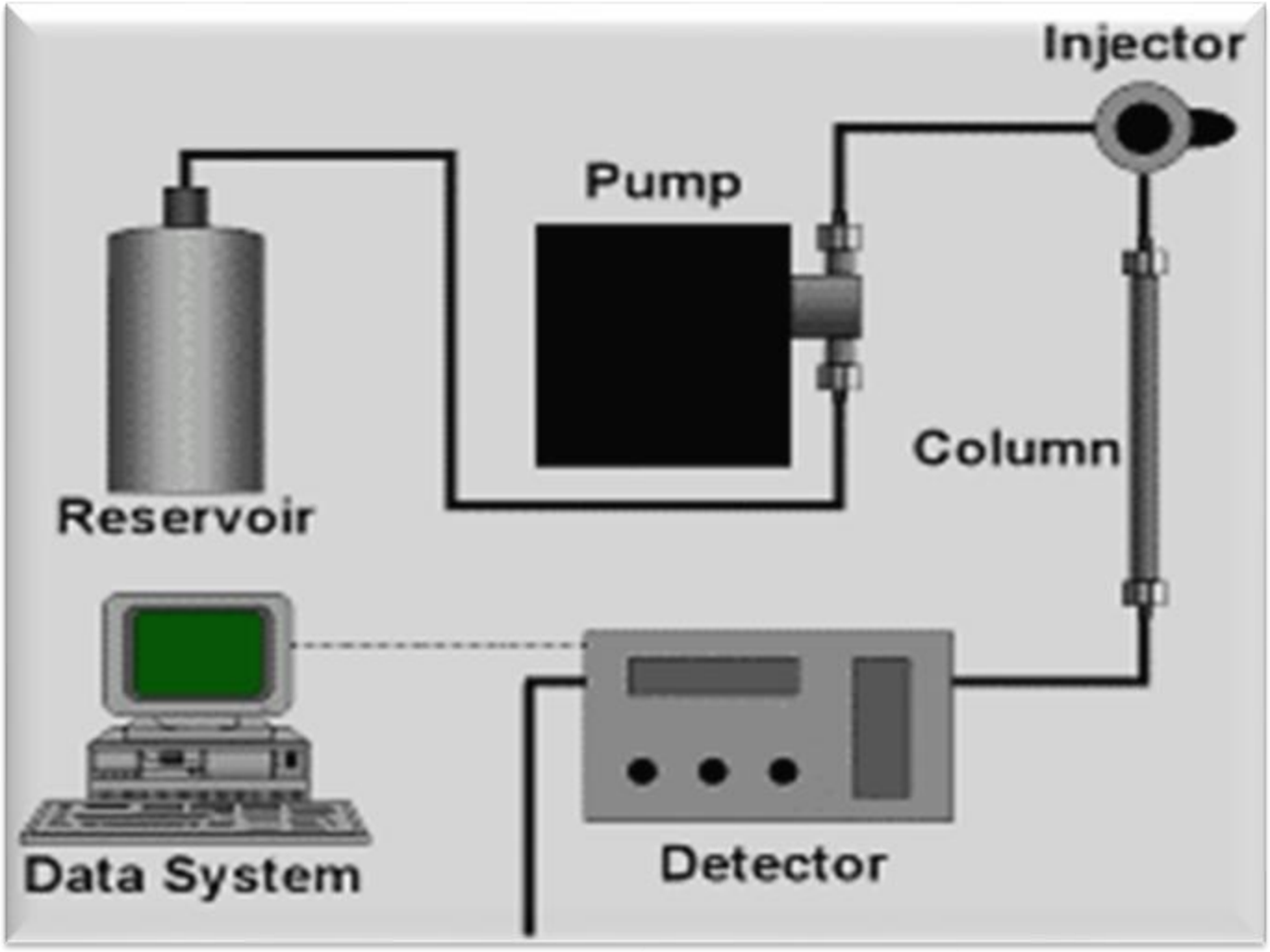
INSTRUMENTATION OF HPLC

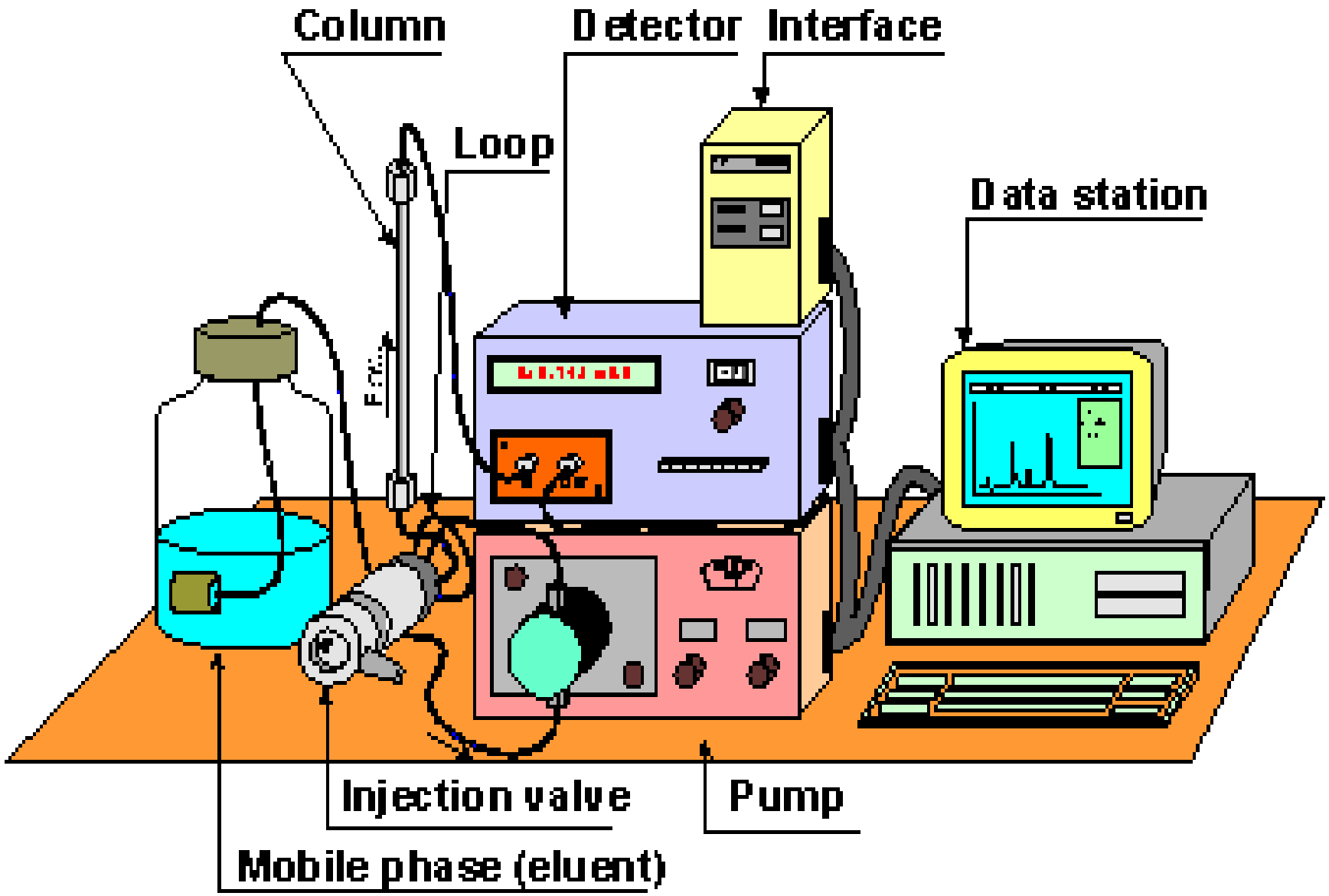
- Solvent storage bottle
- Gradient controller and mixing unit
- De-gassing of solvents
- Pump
- Pressure gauge
- Pre-column
- Sample introduction system
- Column
- Detector
- Recorder


Regulated
helium source
Output check valve
Pulse damper
To
waste
Pump
Inlet
check valve
Drain valve
Solvent reservoirs
Sparger
Inlet
filter
Priming syringe
Solvent proportioning valve
To detector
Column
Pressure
transducer
Backpressure
regulator
Filter
Injector valve


HPLC Column
Packing Material
Chromatogram
Peaks = Yellow, Red, Blue
Injector
AutoSampler
Sample Manager
Computer Data Station
Solvent
(Mobile Phase)
Reservoir
Sample
Pump
Solvent Manager
Solvent Delivery System
Detector
Waste


HPLC System
Solvent
HPLC Column
Injector
Data
Pump
Detector
Waste


Injector
Pump
Column
Reservoir
Data System
Detector


Column
Detector
Interface
Loop
Data station
Injection valve
Pump
Mobile phase (eluent)

OUTLINE OF LC-2010

Reservior Tray

Auto sampler

Column Oven

Degassing Unit

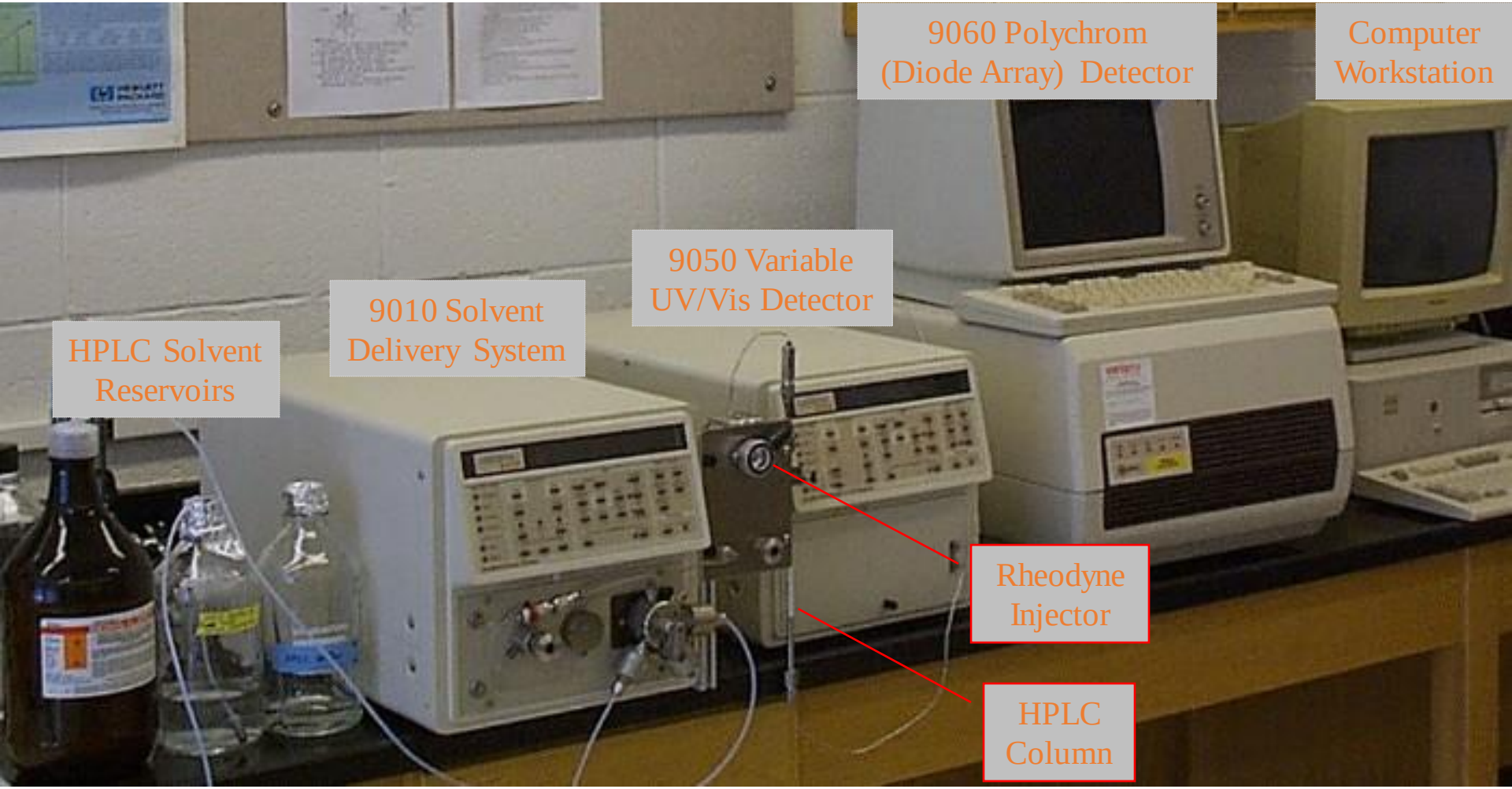
Pump Unit

Low pressure gradient device

System Controller

UV detector

HPLC System


9060 Polychrom
(Diode Array) Detector
Computer
Workstation
9050 Variable
UV/Vis Detector
9010 Solvent
Delivery System
HPLC Solvent
Reservoirs
Rheodyne
Injector
HPLC
Column

Solvent/ mobile phase reservoirs

- Glass or stainless-steel containers capable of holding up to 1 liter mobile phase (pure organic solvents or aqueous solutions of salts and buffers)
- Inert to a variety of aqueous and non aqueous mobile phases.
- Stainless steel should be avoided for use with solvents containing halide ions.

Degassing & filtration of mobile phase

- In many cases, aqueous solvents & some organic solvents are degassed prior to use
- Degassing is done to prevent formation of gas bubbles in the pump or detector (Mobile phases are degassed by stirring of the mobile phase under vacuum, sonication or sparing with helium gas)
- The mobile phase are filtered to remove particulate matter that may clog the system

Tubing

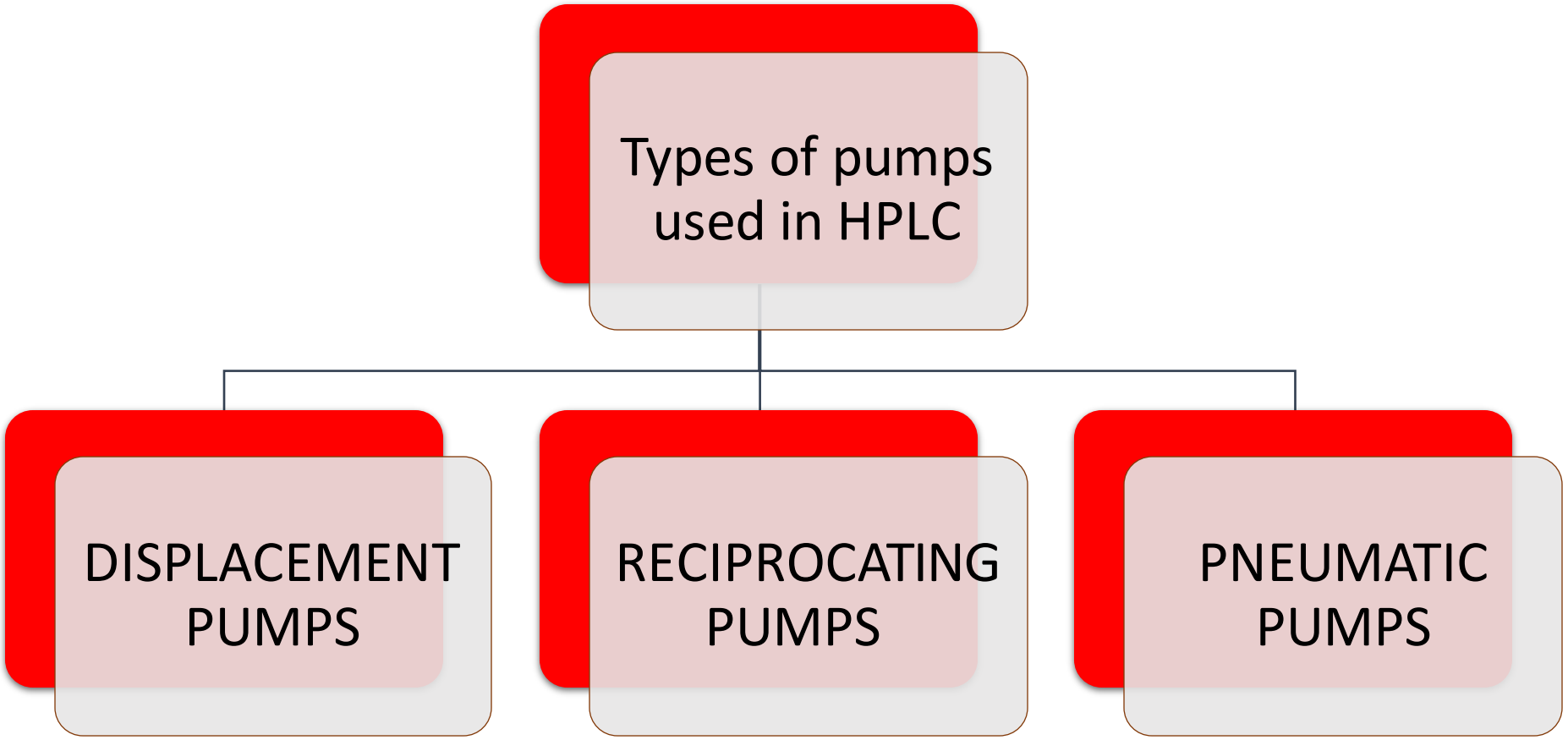
- Should be inert,
- have ability to withstand pressure
- able to carry sufficient volume

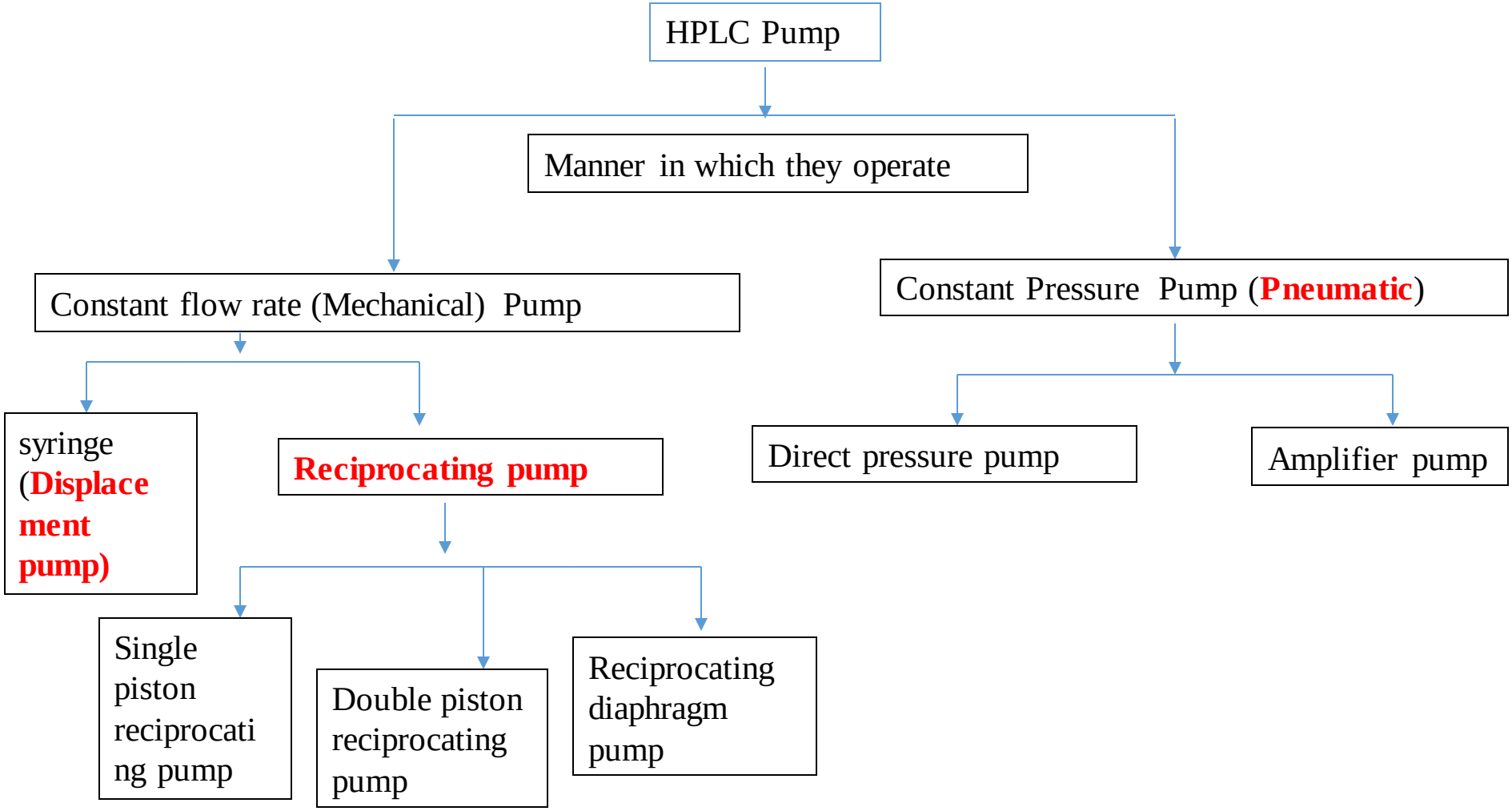
PUMP

- The solvents or mobile phase must be passed through a column at high pressures at up to 6000 psi(lb/in^2) 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

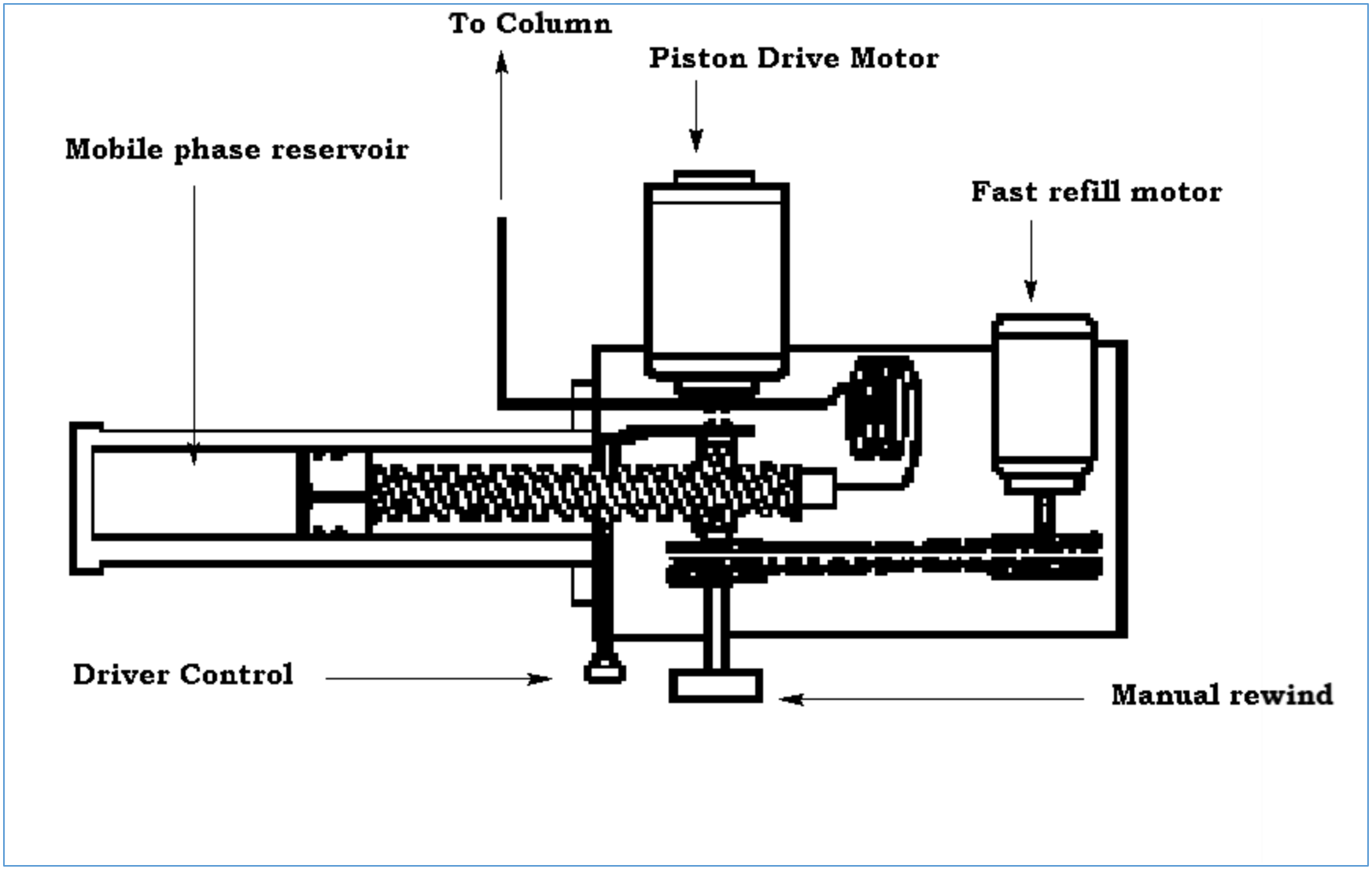
- Generation of pressure of about 6000 psi.
- Pulse free output & all materials in the pump should be chemically resistant to solvents.
- Flow rates ranging from 0.1 to 10 mL/min
- Pumps should be capable of taking the solvent from a single reservoir or more than one reservoir containing different solvents simultaneously.


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


HPLC Pump
Manner in which they operate
Constant flow rate (Mechanical) Pump
Constant Pressure Pump (Pneumatic)
syringe (Displacement pump)
Reciprocating pump
Direct pressure pump
Amplifier pump
Single piston reciprocating pump
Double piston reciprocating pump
Reciprocating diaphragm pump

DISPLACEMENT PUMPS

- It consists of large, syringe like chambers equipped with a plunger activated by a screw driven mechanism powered by a stepping motor.
- So it is also called as Screw Driven Syringe Type Pump.
- 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.

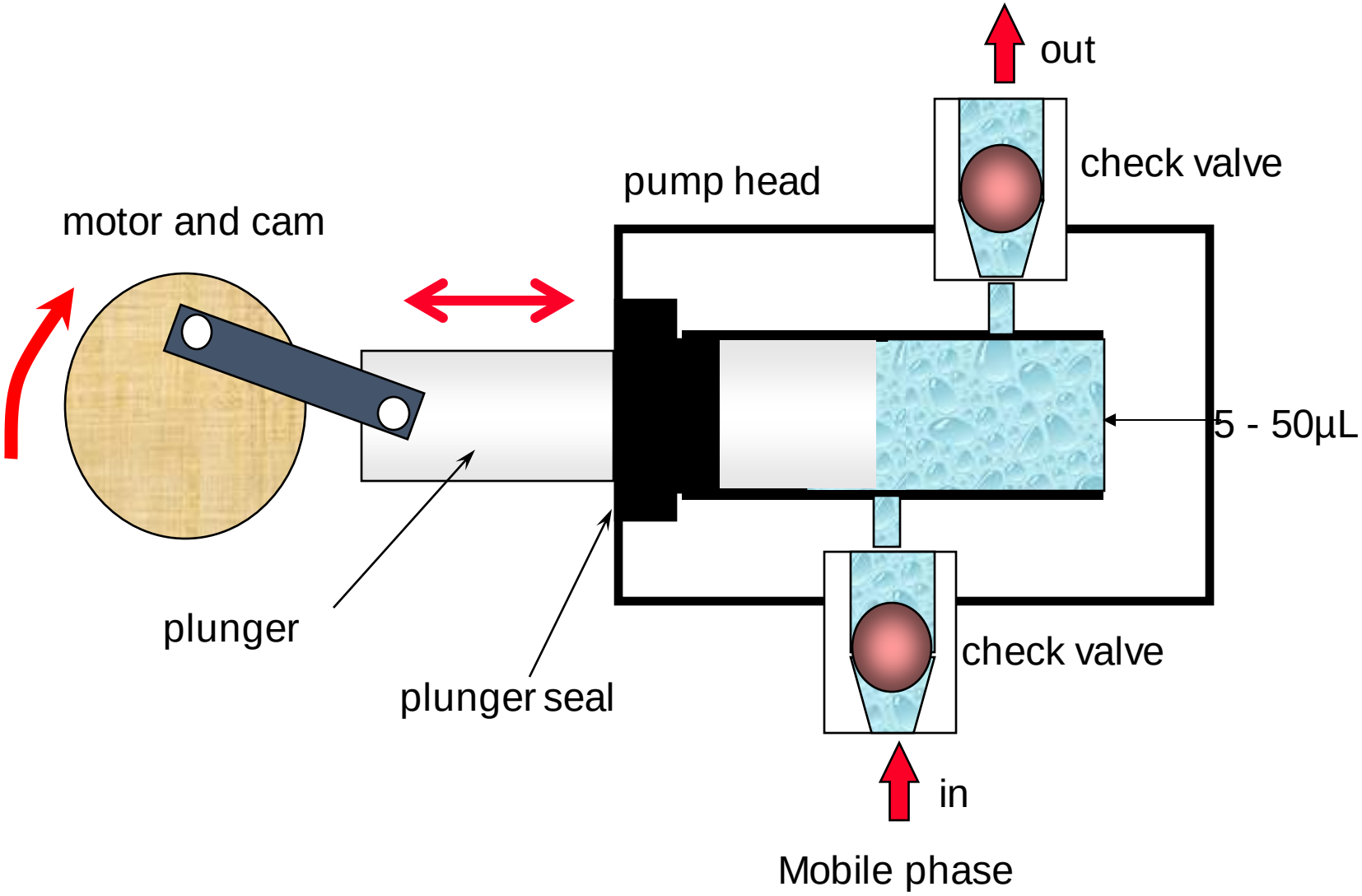

To Column
Piston Drive Motor
Mobile phase reservoir
Fast refill motor
Driver Control
Manual rewind

RECIPROCATING PUMPS

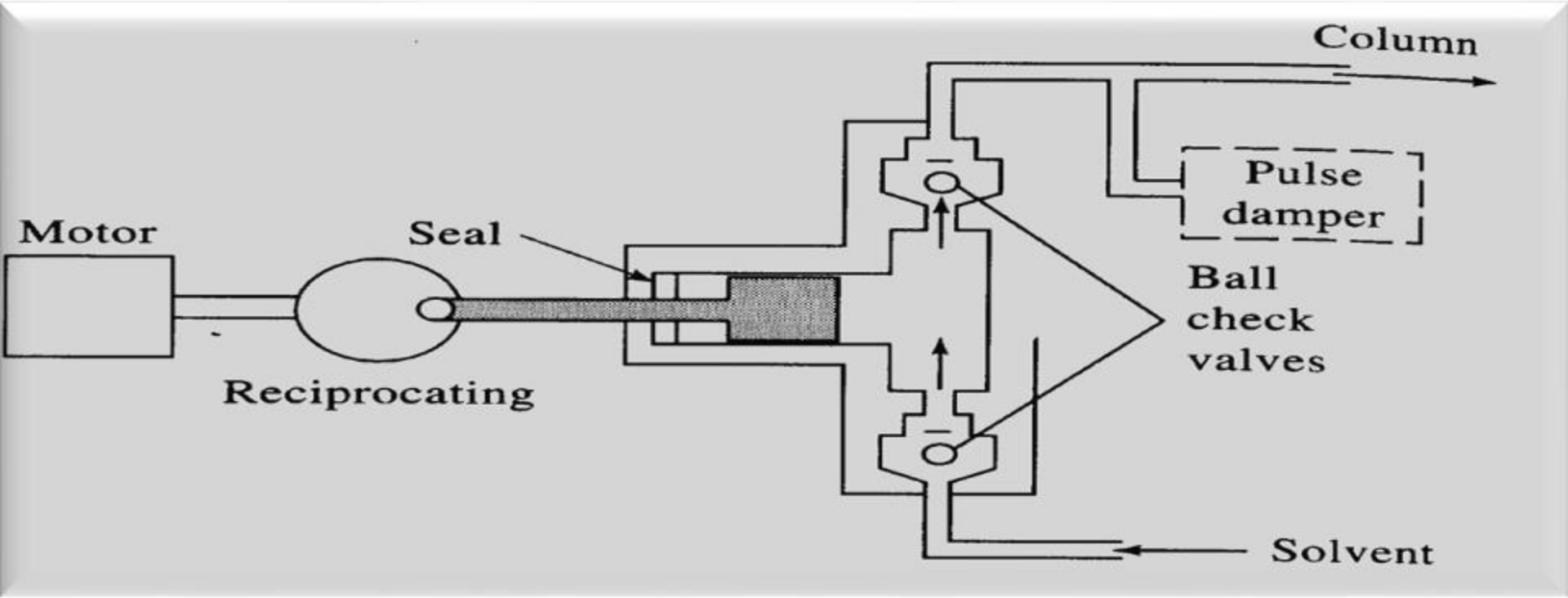
- This pump transmits alternative pressure to the solvent via a flexible diaphragm ,which in turn is hydraulically pumped by a reciprocating pump.
- Disadvantages

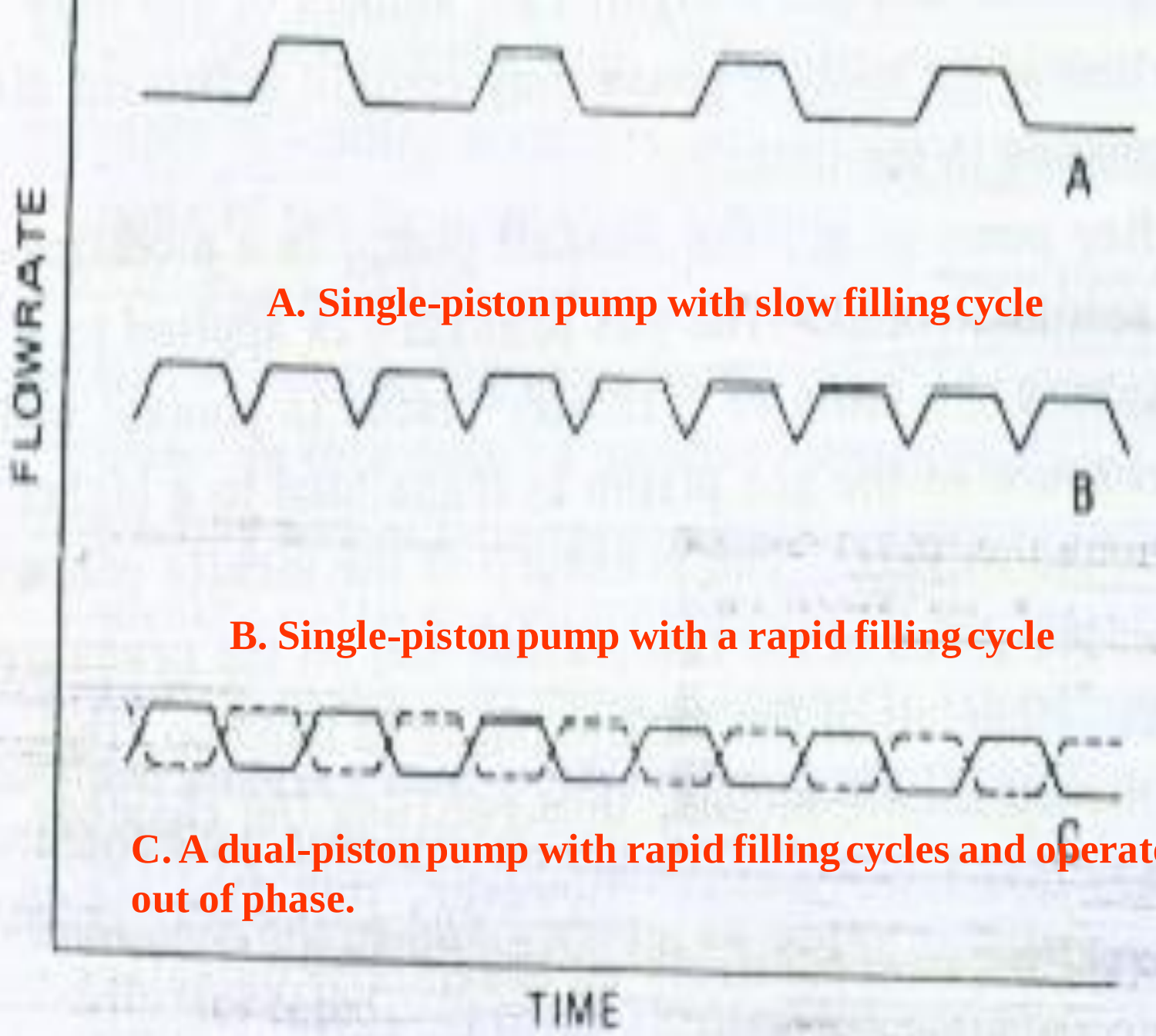
  Produces a pulsed flow which is damped because pulses appear as baseline noise on the chromatograph.

  This can be overcome by use of dual pump heads or elliptical cams to minimize such pulsations.


out
check valve
pump head
motor and cam
5 - 50µL
plunger
plunger seal
check valve
in
Mobile phase

- Solvent is pumped back and forth by a motor driven piston
- Two ball check valves which open & close which controls the flow
- The piston is in direct contact with the solvent
- Small internal volume 35-400μL
- High output pressure up to 10,000 psi
- Ready adaptability to gradient elution and constant flow rate





A. Single-piston pump with slow filling cycle

B. Single-piston pump with a rapid filling cycle

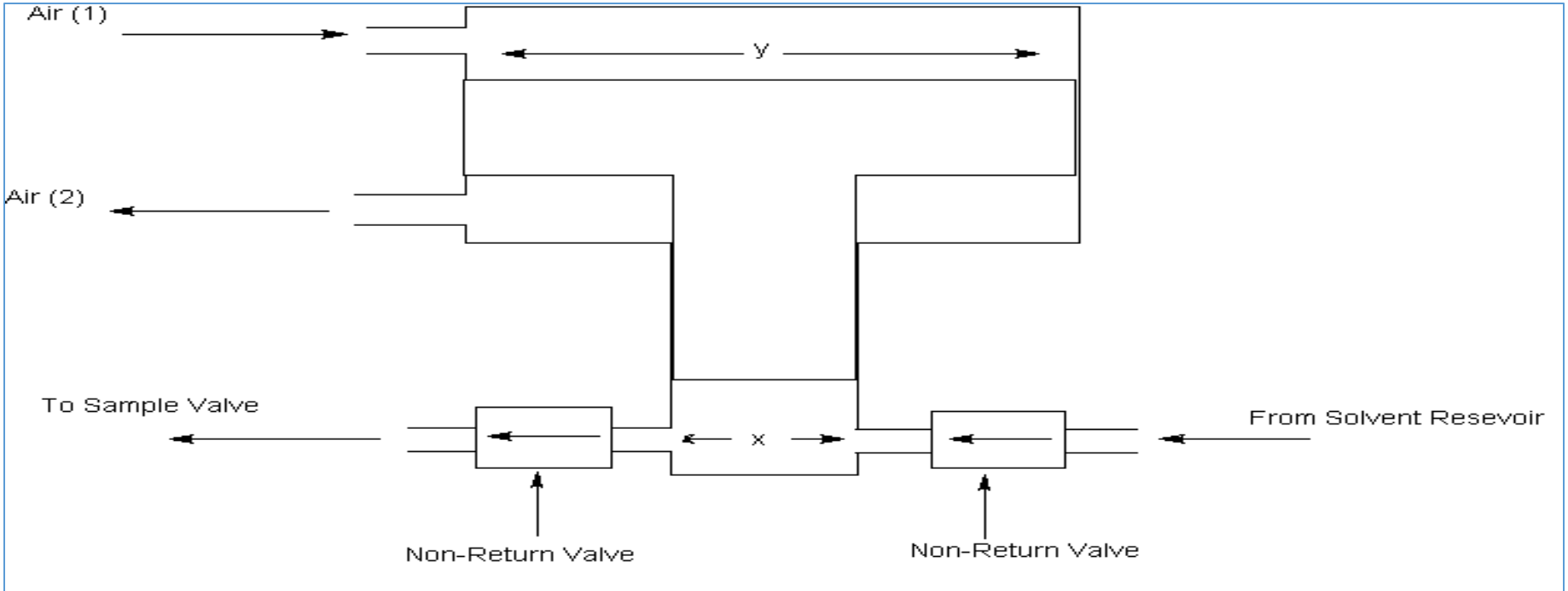
C. A dual-piston pump with rapid filling cycles and operate 180^0 out of phase.

Schematic representation of solvent flow from

- 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

- In this 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.



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

i. Septum Injector

- These are used for injecting the sample through a rubber septum.
- This kind of injectors cannot be commonly used , since the septum has to withstand high pressures.

ii. Stop Flow

- In this type the flow of mobile phase is stopped for a while & the sample is injected through a valve.

III. RHEODYNE INJECTOR

- It is the 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.

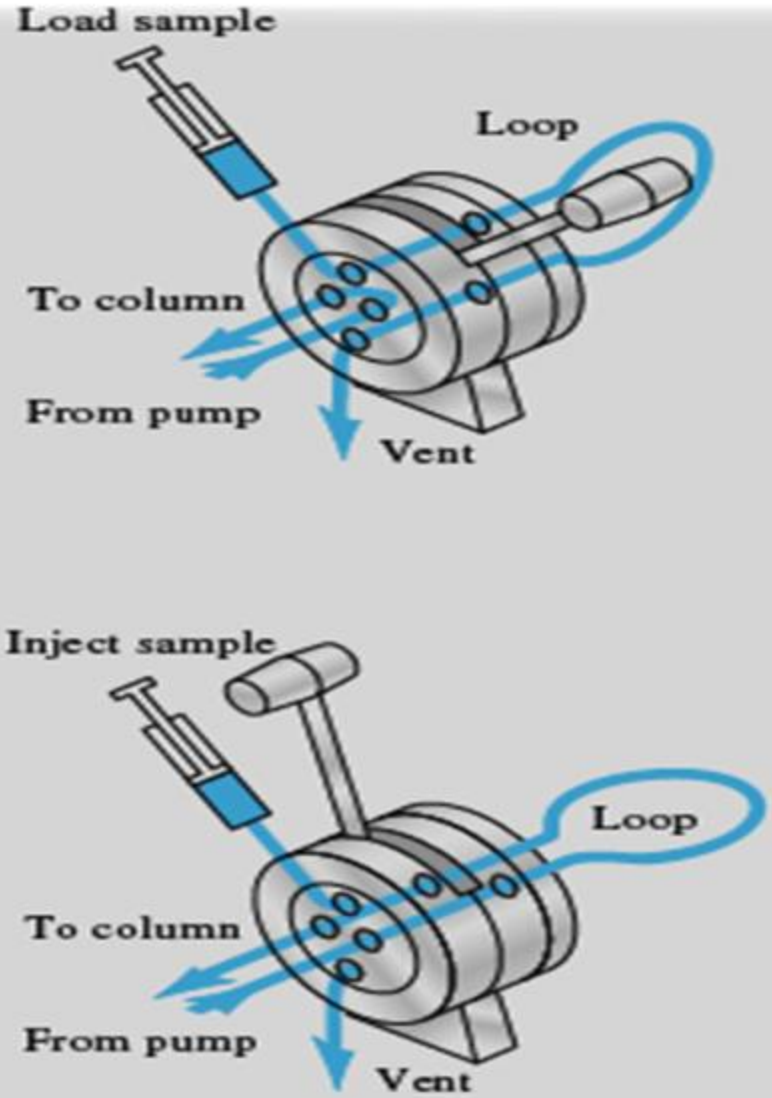


FIGURE 28-6 A sampling loop for LC. With the valve handle as shown on the left, the loop is filled from the syringe, and the mobile phase flows from pump to column. When the valve is placed in the position on the right, the loop is inserted between the pump and the column so that the mobile phase sweeps the sample onto the column. (Courtesy of Beckman-Coulter, Inc.)

SELECTION VALVE

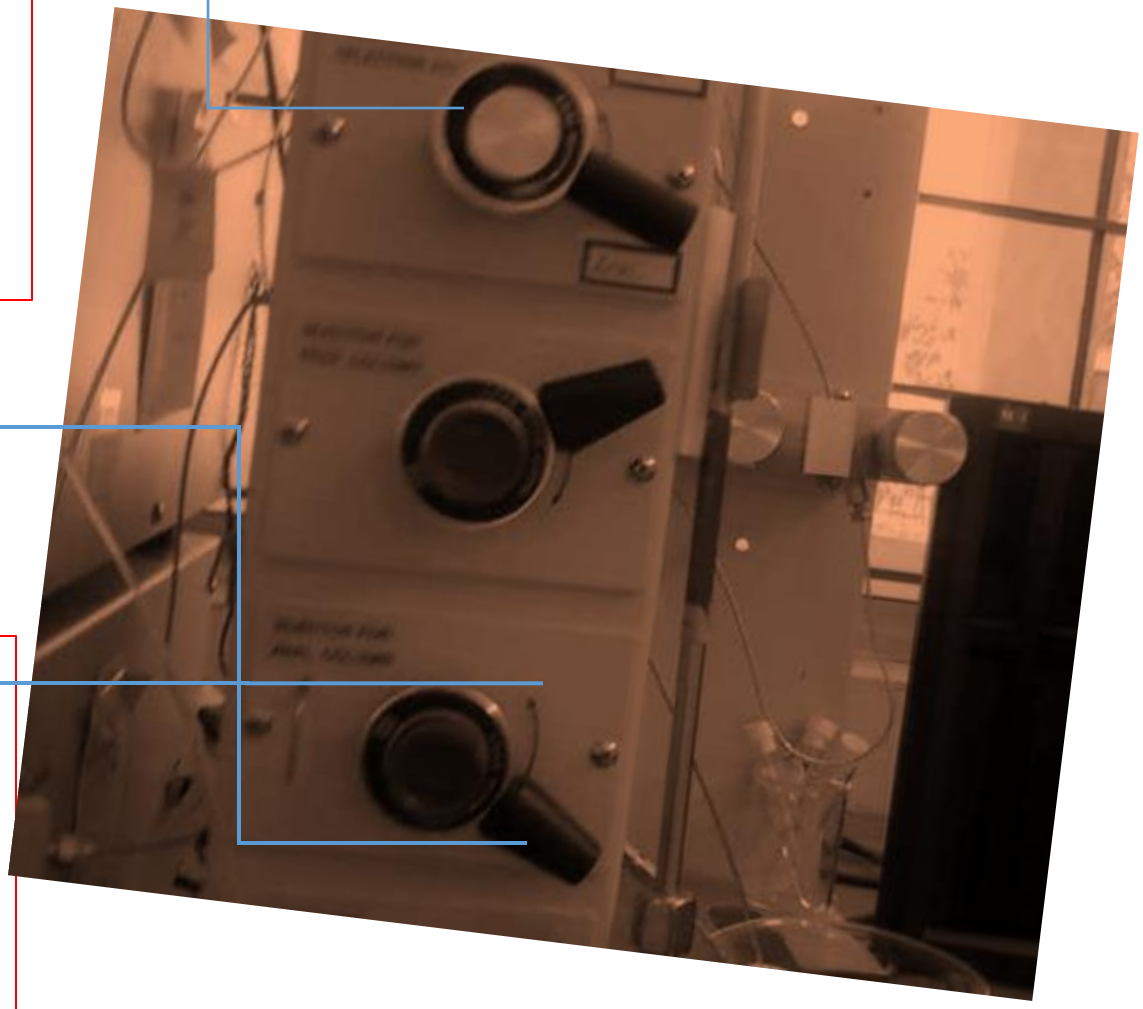
BY USING THE SELECTION VALVE WE CAN SELECT WHETHER THE PURPOSE IS FOR ANALYTICAL PURPOSE OR PREPARATIVE PURPOSE.

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 forceful flow of the solvent into the sample loop by which the sample is introduced into the column.



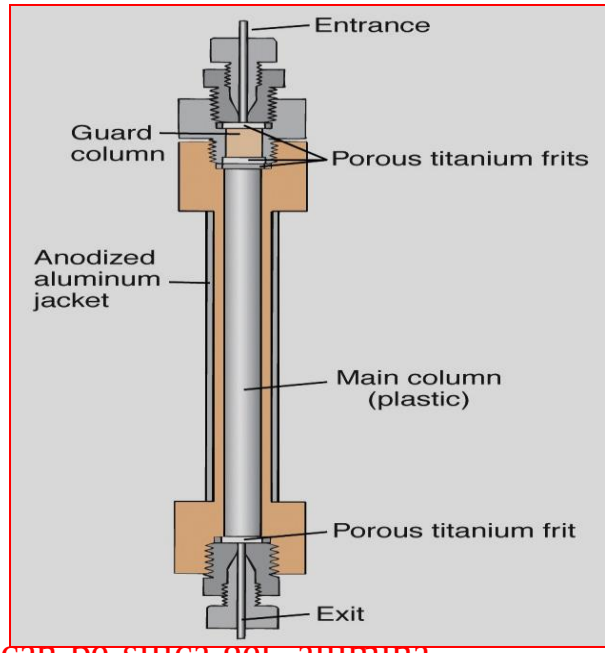
HPLC Auto Injectors

Inside of SIL-20AC

column

1. Precolumn

1.1 It contains a packing chemically identical to that in analytical column.
1.2 Mainly used to remove the impurities from the solvent and thus prevents contamination of the analytical column, it can protect analytical column.
1.3 It is also called as guard column or protective column.
1.4 it is having large particle size.
1.5 It is having short length of 2 to 10 cm, so does not affect separation.



2. Analytical column

2.1 The success or failure of analysis depends upon choice of column.

2.2 Actual separation is carried out here.

2.3 Stainless –steel tube

2.4 size – length -25 to 100 cm

2.5 Internal diameter – 2 to 4.6 mm

2.6 Column is filled with small particles 5 – 10 micron. The solid support can be silica gel, alumina.

2.7 The separation is result of different components adhering to or diffusion into the packing particles when the mobile phase is forced through column.

2.8 C8 and C18 columns are considered as examples of reversed phase liquid chromatography (RP).

2.9 The stationary phase here is seen as a thin film of non-polar liquid phase that has been designed to be chemically similar to an inert material (Silica gel particles).

2.10 The non-polar layer is chemically linked to the silica particles surface by reaction with the polar silanol groups on the stationary phase surface and so rendering them less polar or non-polar.

2.11 The difference between the two columns will be in the length of the carbon-chain attached to the silica surface.

2.12 Accordingly C8 hplc columns have packing material composed of silica particles attached to C8 carbon units.

2.13 C18 will, of course, have packing materials coated with C18 hydrophobic units.

2.14 Categorically both are reversed phase but C18 columns will definitely be more "hydrophobic rather than the C8 columns.

Detectors

- Absorbance (**UV/Vis** and **PDA**)
- **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)

Selection of Detectors

Detectors	Type of compounds can be detected
UV-Vis & PDA	Compounds with chromophores, such as aromatic rings or multiple alternating double bonds.
RF	Fluorescent compounds, usually with fused rings or highly conjugated planar system.
CDD	Charged compounds, such as inorganic ions and organic acid.
ECD	For easily oxidized compounds like quinones or amines.
RID & ELSD	For compounds that do not show characteristics usable by the other detectors, e.g. polymers, saccharides.

TYPES OF HPLC DETECTORS

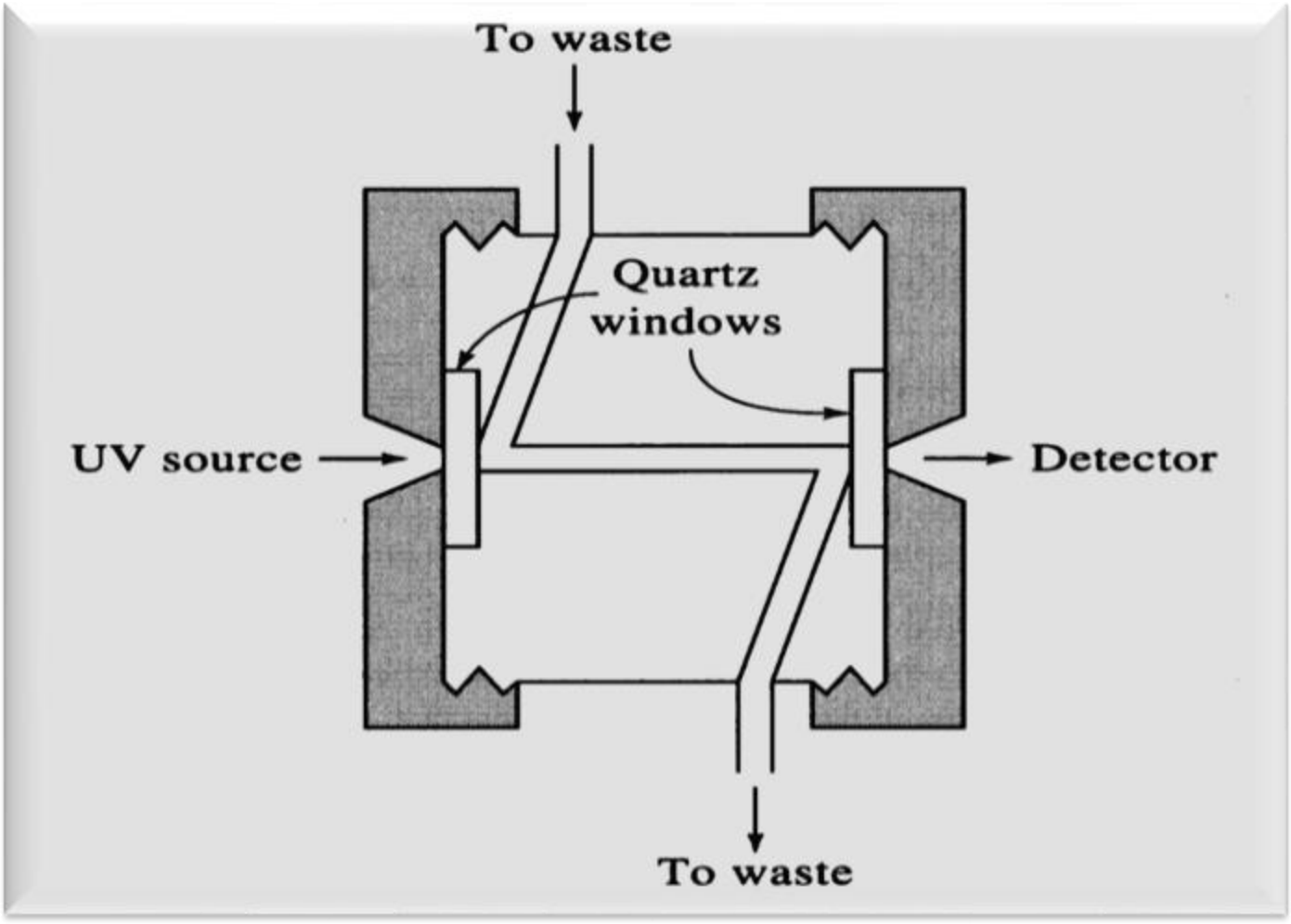
Name	Advantage	Disadvantage
UV-Vis	Works w/all molecules	Non-specific; complex samples; absorption wavelength
DAD	Works for all wavelengths	High LOD
Fluorescence	Very specific; low LOD	Not everything fluoresces
IR	Works w/all molecules	Many solvents IR active
Refractive Index	Works w/nearly all molecules	Temperature sensitive; high LOD
Scattering	Uniform response; 5ng/25mL LOD	Non-specific; interference from solvent
Electrochemical	Commercially available	Non-specific; high LOD
Mass Spec	Low LOD; analyte identification	Ability to ionize analyte

Ideal Detector Properties

- High Sensitivity
- Universality or predictable specificity
- Large linear response range
- Low dead volume
- Non-Destructive
- Insensitive to temperature & mobile phase
- Continuous operation
- Reliable and easy to use
- No single detector fits all these criteria

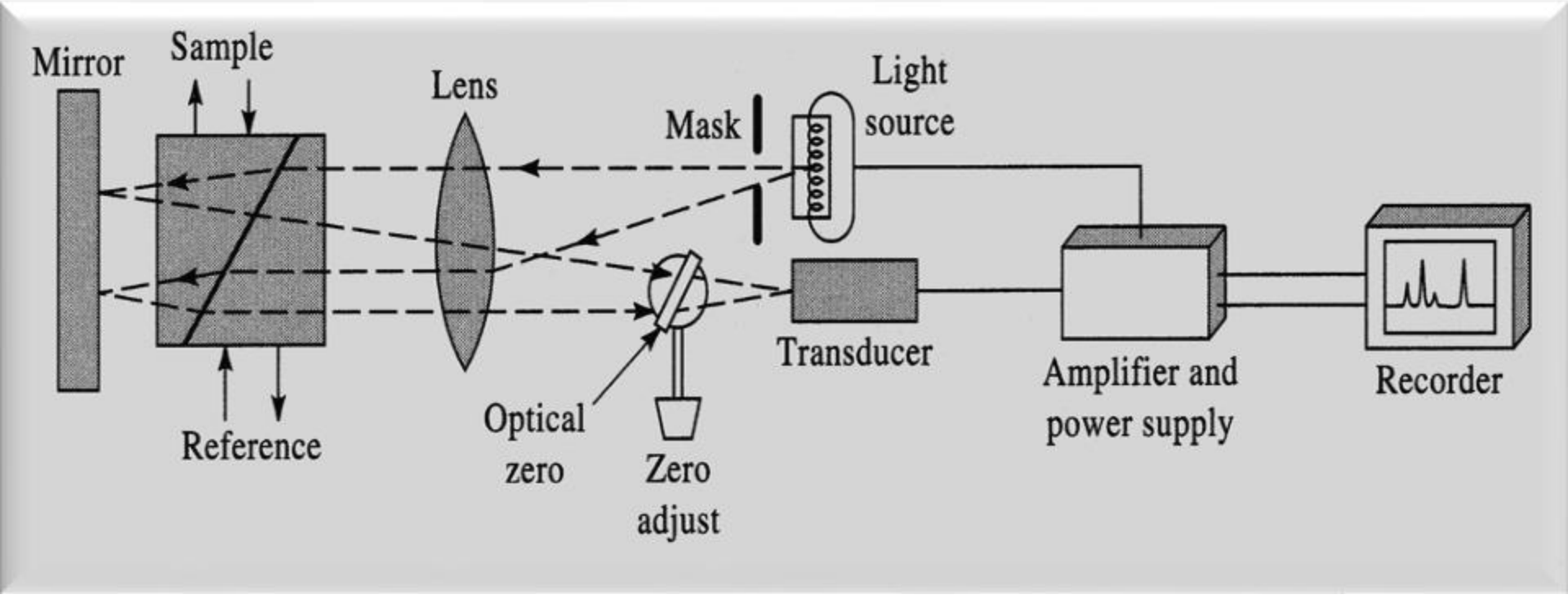
UV-visible detector

- UV visible detector is widely used as it detects large number of compounds because most drugs have appropriate structural characteristics for light absorption.
- These are useful for aromatic compounds and other type of unsaturated systems.
- These are classified as fixed or variable wavelength detectors.
- Fixed wavelength detectors employ filter as a source to provide appropriate wavelength.
- Most common fixed wavelength detectors are based on 254 nm.
- Variable wavelength detectors are employ a spectrophotometer to provide dispersion of light and selection of any wavelength in UV visible regions.
- Diffraction gratings are frequently used for wavelength dispersion.


To waste
Quartz windows
UV source
Detector
To waste

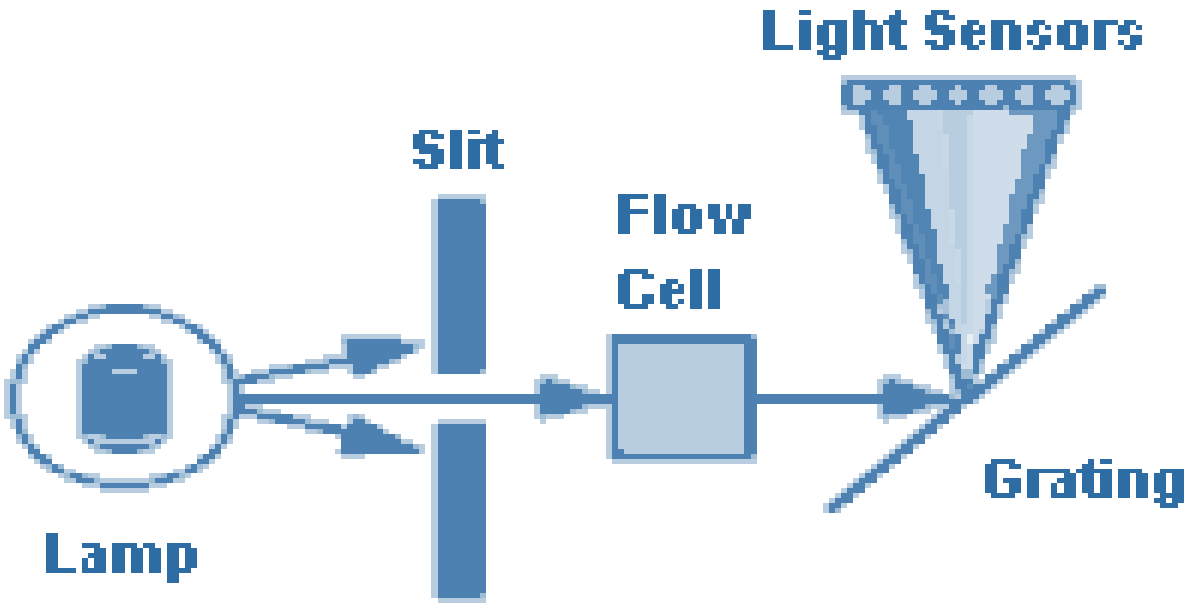
Refractive Index (RI) detector

- 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.
- When a solute is in the sample compartment, refractive index changes will shift the light beam from the detector.



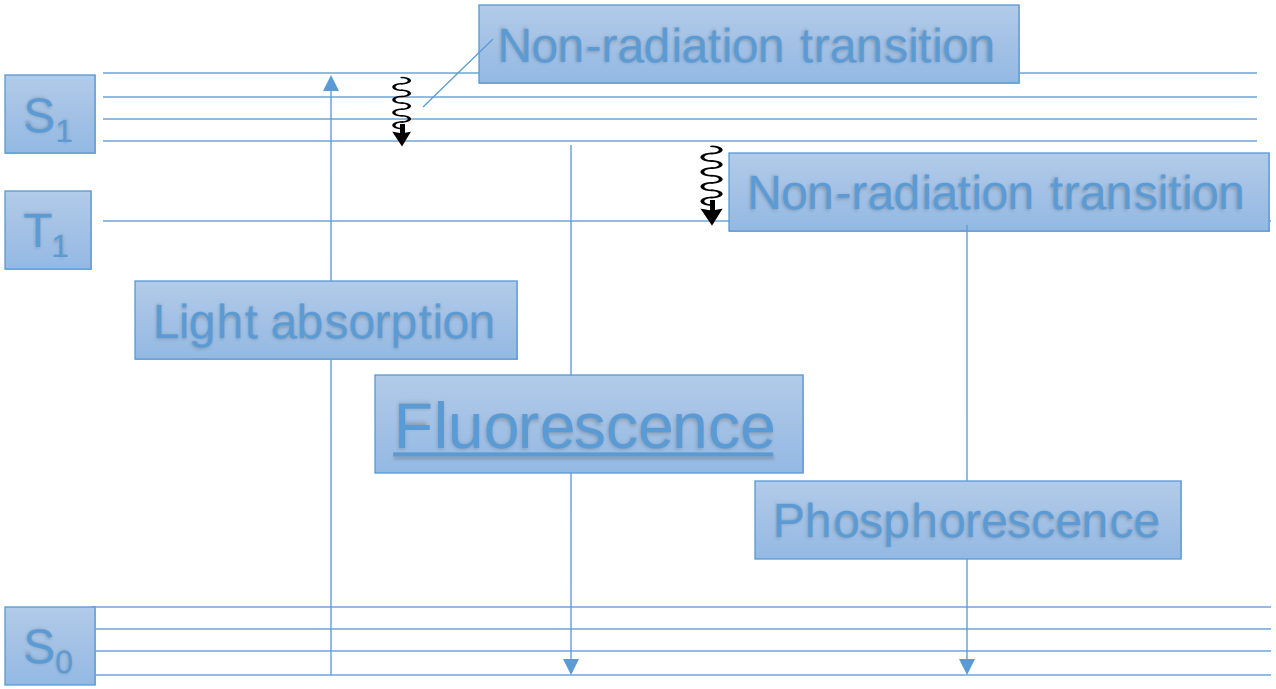
photodiode array (PDA)

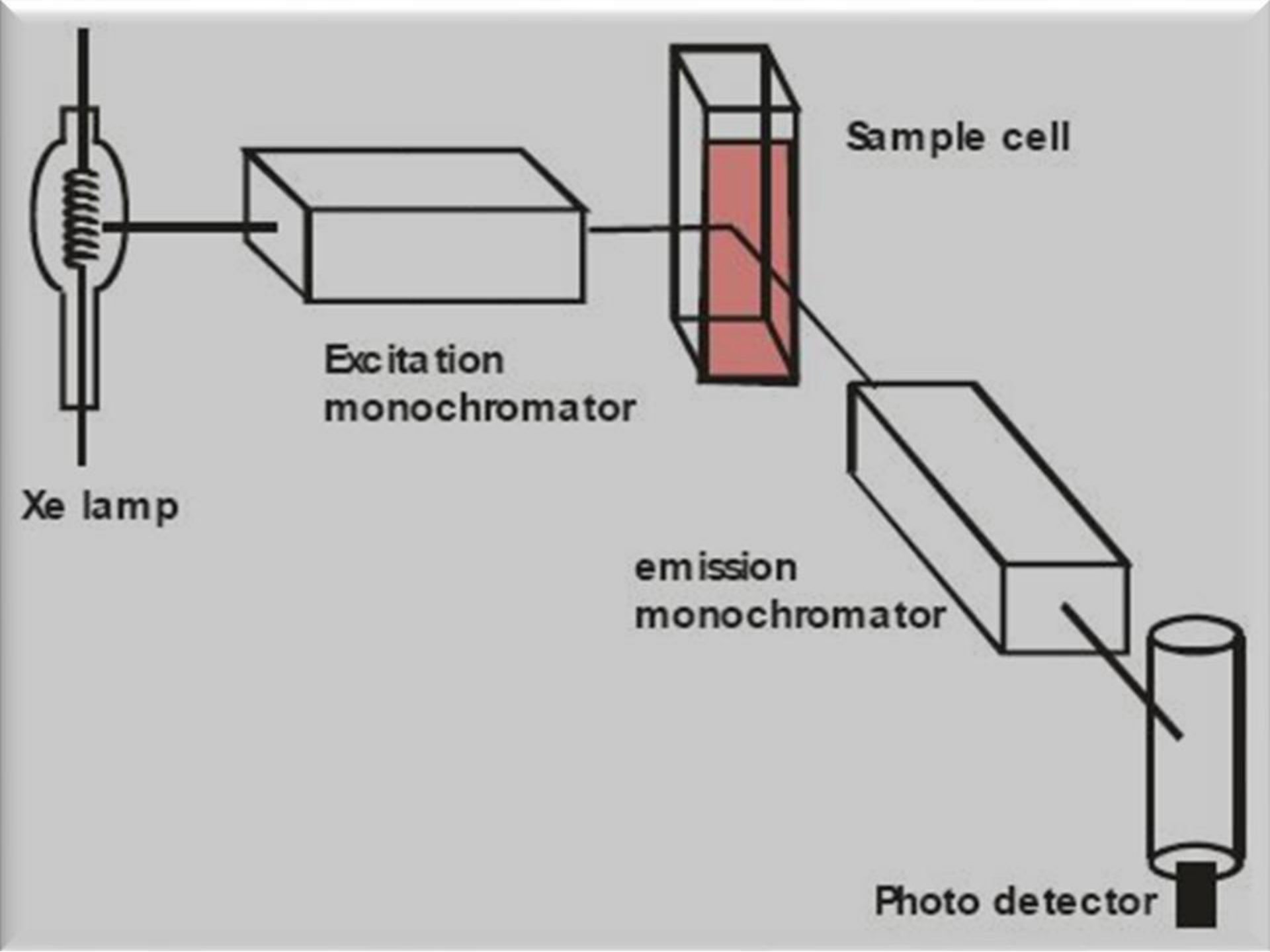
- A photodiode array (PDA) is a linear array of discrete photodiodes on an integrated circuit (IC) chip.
- Allows a range of wavelengths to be detected simultaneously. In this regard it can be thought of as an electronic version of photographic film. Array detectors are especially useful for recording the full Uv- vis is a absorption spectra of samples that are rapidly passing through a sample flow cell, such as in an HPLC detector.
- PDAs work on the same principle as simple. Photovoltaic detector
- similar to UV detector, non destructive 190-600 nm for quantization & identification Spectra is 3D, Response vs time vs WL.

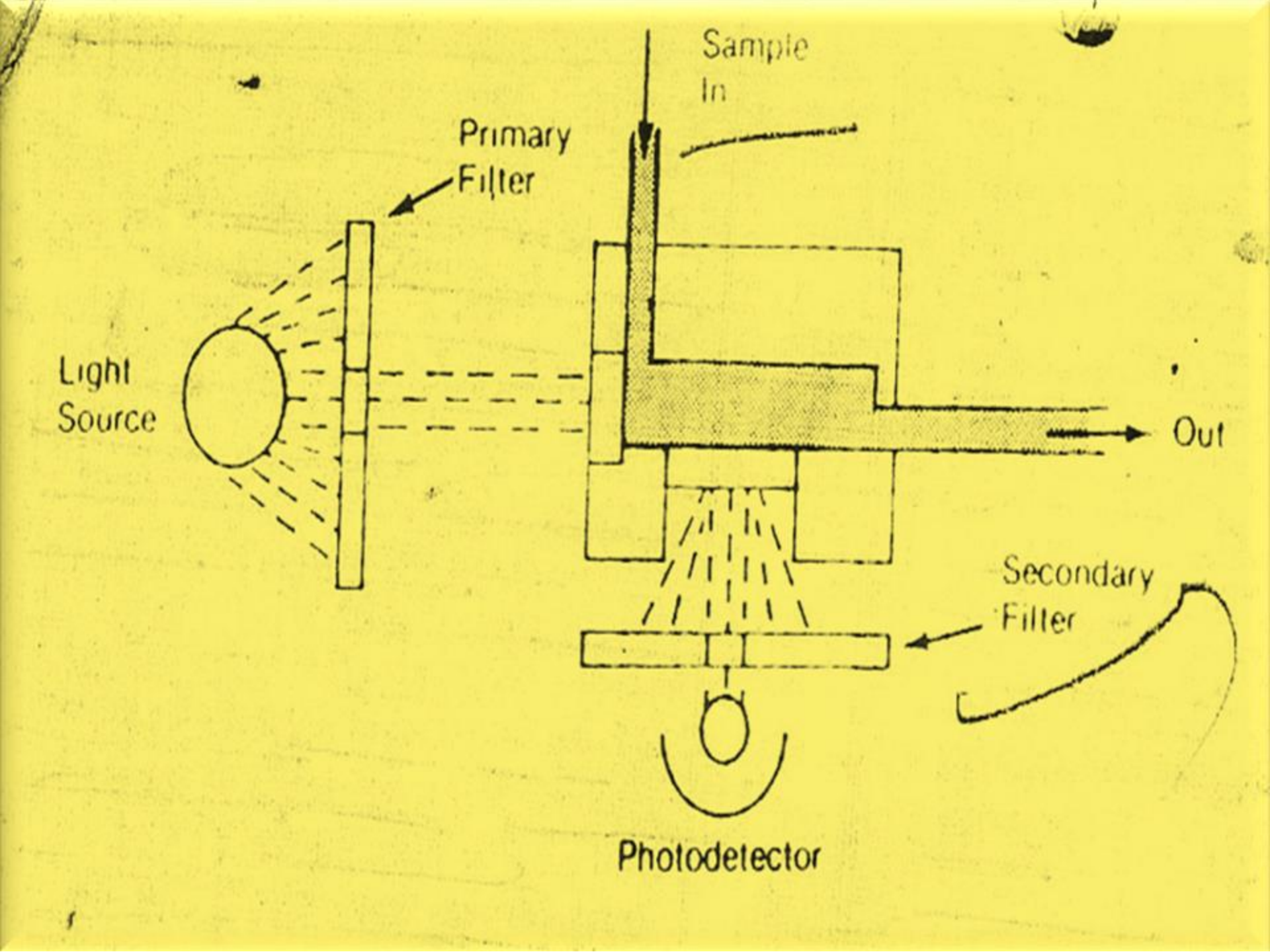


FLUORIMETRIC DETECTORS

- It is based on the fluorescent radiation emitted by some compounds.
- The excitation source passes through the flow cell to a photo detector while a monochromatic measures the emission wavelengths.
- More sensitive and specific.
- The disadvantage is that most compounds are not fluorescent in nature.
- Fluorescence is a type of luminescence in which the light energy is released in the form of a photon in nanoseconds to microseconds.




Sample cell
Excitation
monochromator
Xe lamp
emission
monochromator
Photo detector


Sample
In
Primary
Filter
Light
Source
Out
Secondary
Filter
Photodetector

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 time.
- Retention time can be found out from this recordings, but area under curve cannot be determined.
- The Integrators 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.

Comparision between hptlc and hplc

Sr.no.	HPTLC	HPLC
1	High Performance Thin Layer Chromatography	High Performance Liquid Chromatography
2	Planar Chromatography	Column Chromatography
3	Principle based on Adsorption Chromatography	Principle is based on Adsorption and Partition Chromatography
4	Simultaneous method for test as well as reference material	Not simultaneous method for test as well as reference material
5	It is simple, rapid, reproducible method	It is Tedious method
6	Sample preparation is simple	Sample preparation is complex
7	Limited Flexibility	Extreme Flexibility
8	Semiautomatic Technique	Automatic (Instrumental) Technique
9	Determination of Surface Area	Determination of Retention Time

Comparision between GC and hplc

SR.NO.	GC	HPLC
1	Gas Chromatography	High Performance Liquid Chromatography
2	Less resolution	High resolution
3	Limited Flexibility	Extreme Flexibility
4	Determination of Volatile compounds	Determination of Volatile and Non Volatile Compounds

Comparision between hplc and uplc

Parameters	HPLC Assay	UPLC Assay
Column	XTerra,C18,50 × 4.6mm	AQUITY UPLC BEH C18,50 ×2.1mm
Particle size	4µm particles	1.7µm particles
Flow rate	3.0 ml per min	0.6 ml per min
Injection volume	20 µl	3 µl partial loop fill or 5 µl full loop fill
Total run time	10 min	1.5 min
Theoretical Plate count	2000	7500

Parameters	HPLC Assay	UPLC Assay
Lower limit of quantization	0.2 µg/ml	0.054µl/ml
Total solvent consumption	Acetonitrile:10.5ml, water:21ml	Acetonitrile:0.53ml, water:0.66ml
Delay volume	720 µl	110 µl
Column temperature	30 °C	65 °C
Maximum back pressure	35-40 Mpa less	103.5 Mpa more
Resolution	Less	High
Method development cost	High	Low

application

- ❖Drug Discovery
- ❖Clinical Analysis
- ❖Proteomics
- ❖Forensic Chemistry
- ❖Drug Metabolism study
- ❖Environmental chemistry
- ❖Diagnostic studies
- ❖Cosmetic analysis
- ❖Determination of Green Florescent Protein
- ❖Structural Determination
- ❖Pharmaceutical Applications
- ❖Identification of Bile Acid Metabolite
- ❖Clinical Applications
- ❖Biochemical Genetics
- ❖qualitative and quantitative analysis
- ❖Therapeutic Drug Monitoring