HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

PRESENTED BY

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INTRODUCTION

TYPES OF HPLC TECHNIQUES

PRINCIPLE

INSTRUMENTATON

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APPLICATIONS

1. INTRODUCTION:

In 1941 Martin and Synge, described the discovery of liquid-liquid partition chromatography and also laid the foundation of Gas liquid chromatography and High performance liquid chromatography. They also introduced the concept of the Height Equivalent to the Theoretical Plate , which has since been adopted as the measure of Chromatographic efficiency.

In Classical Column Liquid Chromatography, the mobile liquid phase flows slowly through the column by means of gravity. The method is generally characterized by low column efficiencies and long separation times. Since about 1969, there has been a very marked revival of interest in the technique of liquid column chromatography because of the development of HPLC by Kirkland and Huber. They proposed high pressure systems capable of operating at pressures up to 3000psi. In HPLC, small diameter columns(1-3mm) with support particle sizes in the region of 30μm are used and the eluent is pumped through the column at a high flow rate. It has been fond that separation by HPLC may be effected about 100 times faster than by the use of conventional liquid chromatography.

by operating at high pressures these instruments for liquid chromatography over come the effect of higher liquid viscosities relative to gas viscosities and gave analysis times comparable with GLC.

Sample clean up is usually much less of a problem with HPLC than GLC and biological fluids can often be directly onto an HPLC column. Much sample pretreatment can also be avoided because aqueous solvents can be used in HPLC.

because of all these advantages, HPLC has already made a significant impact in pharmaceutical, clinical, forensic and environmental analysis and it is now an ideal complementary technique to GLC.

2. TYPES OF HPLC TECHNIQUES:

A. Based on modes of chromatography

1. Normal phase mode
2. Reverse phase mode

B. Based on principle of separation

1. Adsorption chromatography
2. Ion exchange chromatography
3. Ion pair chromatography
4. Size exclusion(or)Gel permeation chromatography
5. Affinity chromatography
6. Chiral phase chromatography

C. Based on elution technique

1. Isocratic separation
2. Gradient separation

D. Based on the scale of operation

1. Analytical HPLC
2. Preparative HPLC

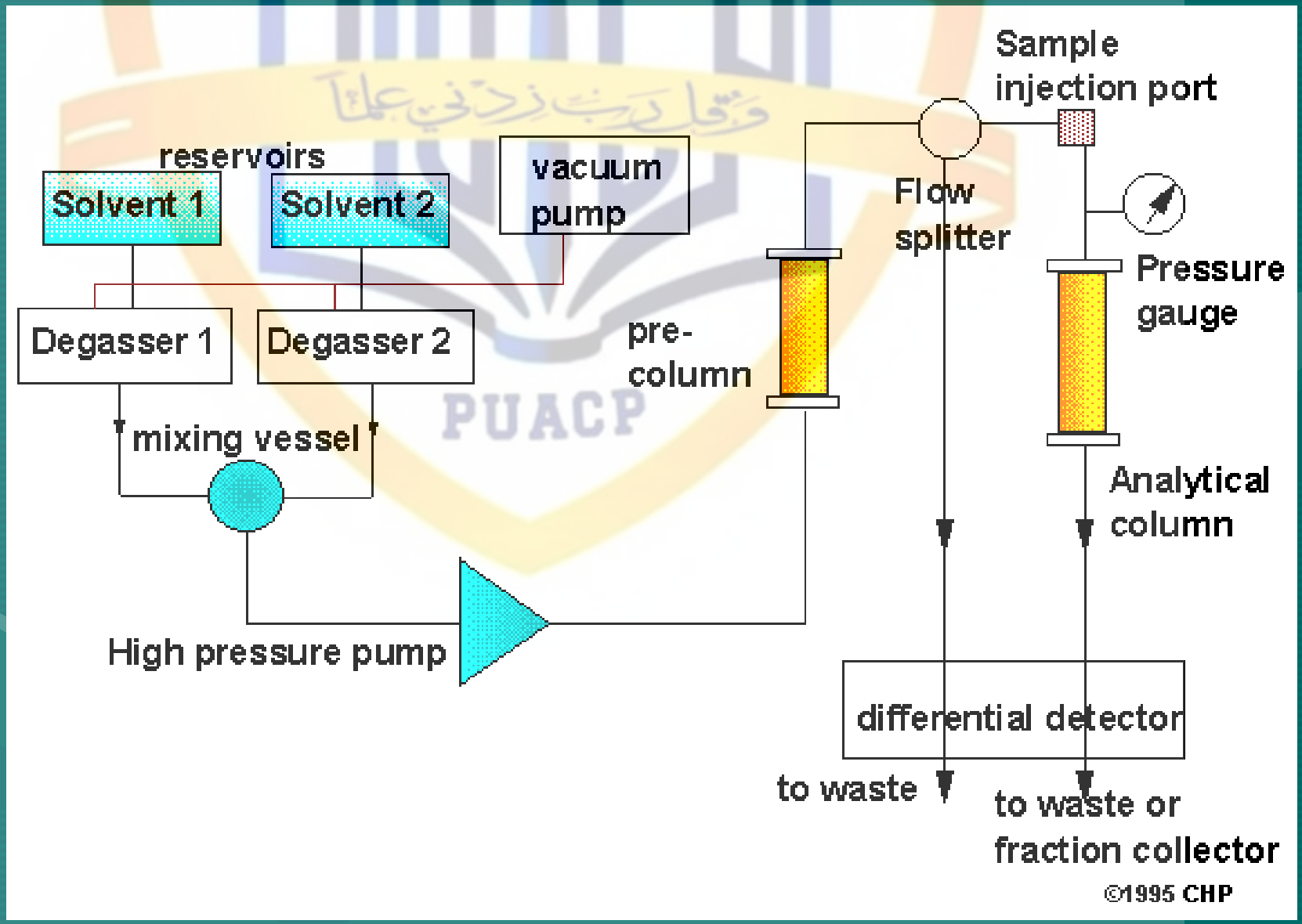
E. Based on the type of analysis

1. Qualitative analysis
2. Quantitative analysis

3. PRINCIPLE:

The principle of separation in normal phase mode and reverse phase mode is adsorption. When a mixture of components are introduced into a HPLC column, they travel according to their relative affinities towards the stationary phase. The component which has more affinity towards the adsorbent, travels slower. The component which has less affinity towards the stationary phase travels faster. Since no 2 components have the same affinity towards the stationary phase, the components are separated.

High-Performance Liquid Chromatography (HPLC)



4. INSTRUMENTATON:

1. Solvent delivery system
2. Pumps
3. Sample injection system
4. Column
5. Detectors
6. Recorders and Integrators

SOLVENT DELIVERY SYSTEM:

The solvents or mobile phases used must be passed through the column at high pressure at about 1000 to 3000 psi. this is because as the particle size of stationary phase is few µ (5-10µ), the resistance to the flow of solvent is high. Hence such high pressure is recommended.

The choice of mobile phase is very important in HPLC and the eluting power of the mobile phase is determined by its overall polarity, the polarity of the stationary phase and the nature of the sample components.

Mixing unit is used to mix the solvents in different proportions and pass through the column. There are 2 types of mixing units.

They are low pressure mixing chamber and high pressure mixing chamber. Mixing of solvents is done either with a static mixer or a dynamic mixer.

In an isocratic separation, mobile phase is prepared by using solvent of same eluting power or polarity. But in gradient elution technique, the polarity of the solvent is gradually increased and hence the solvent composition has to be changed. Hence a gradient controller is used when 2 or more solvent pumps are used for such separations.

Several gases are soluble in organic solvents. When solvents are pumped under high pressure, gas bubbles are formed which will interfere with the separation process, steady base line and the shape of the peak. Hence degassing of solvent is important. This can be done by using Vacuum filtration, Helium purging, Ultrasonication.

In normal phase chromatography hexane, iso octane, di ethyl ether are the mobile phases. In reverse phase chromatography water, methanol, acetonitrile are the mobile phases.

PUMPS:

2 types of pumps are there.

1. Syringe Pumps
2. Reciprocating Pumps

Pumping systems:

– Requirement: high P (6kpsi), pulse-free, constant F(0.1 10mL/min.),reproducibility(0.5%), resistant to corrosion

– **Displacement pump** (Screw-driven syringe pump): pulse free, small capacity (250 mL), no gradient elution. Limited mobile phase capacity.

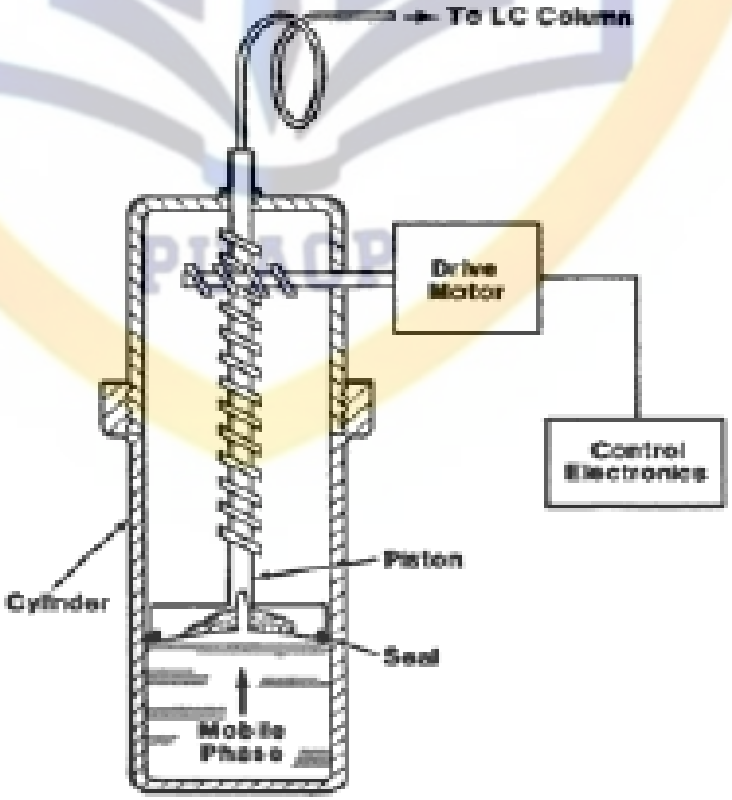
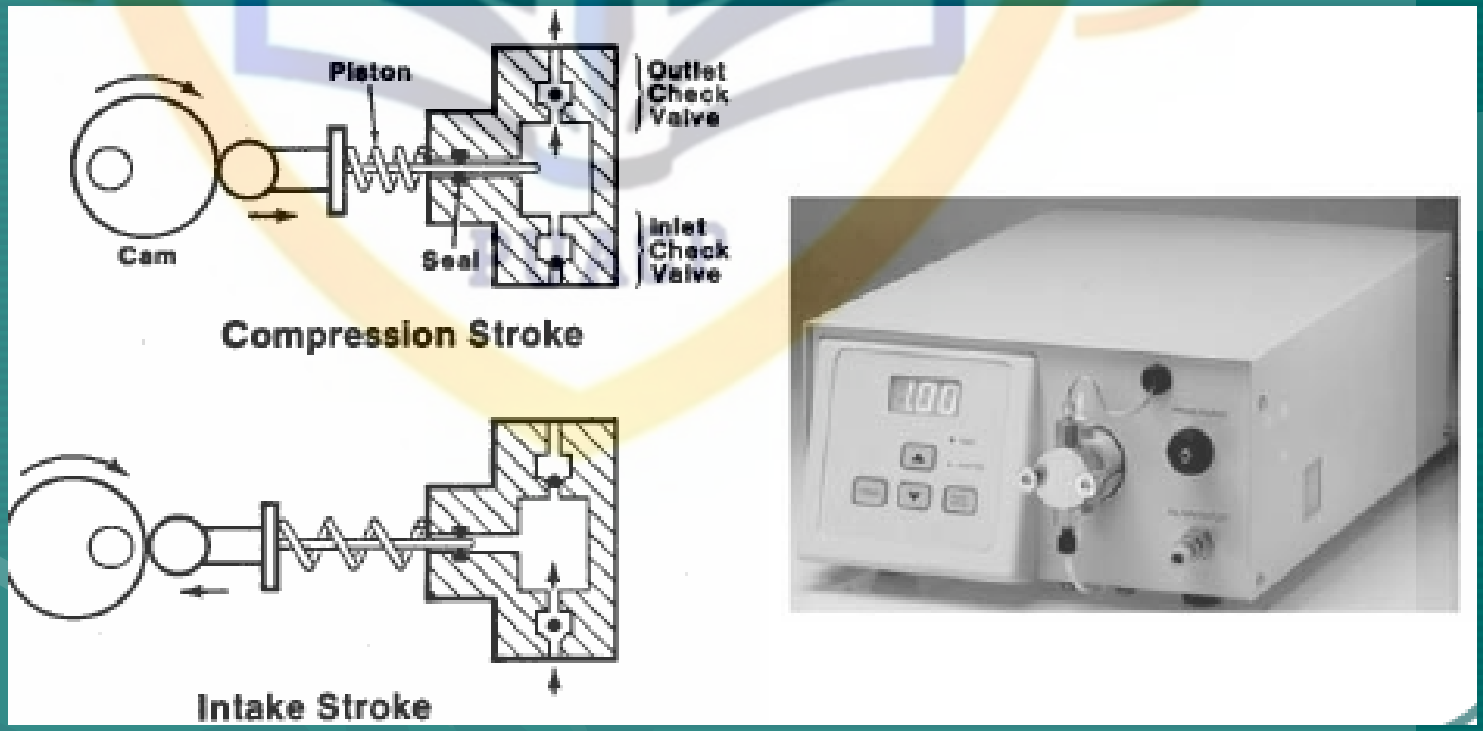


Fig. 26.6 Cross section of a syringe pump. The piston drive motor forces the piston down into the reservoir cylinder by means of a screw drive. The mobile phase is forced out through a channel up the center of the screw gear.

Reciprocating pump: Most widely used. Small internal volume (35 ~ 400 µL), high-pressure (105 psi), gradient elution, constant flow. Need pulse damper.



SAMPLE INJECTION SYSTEM:

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

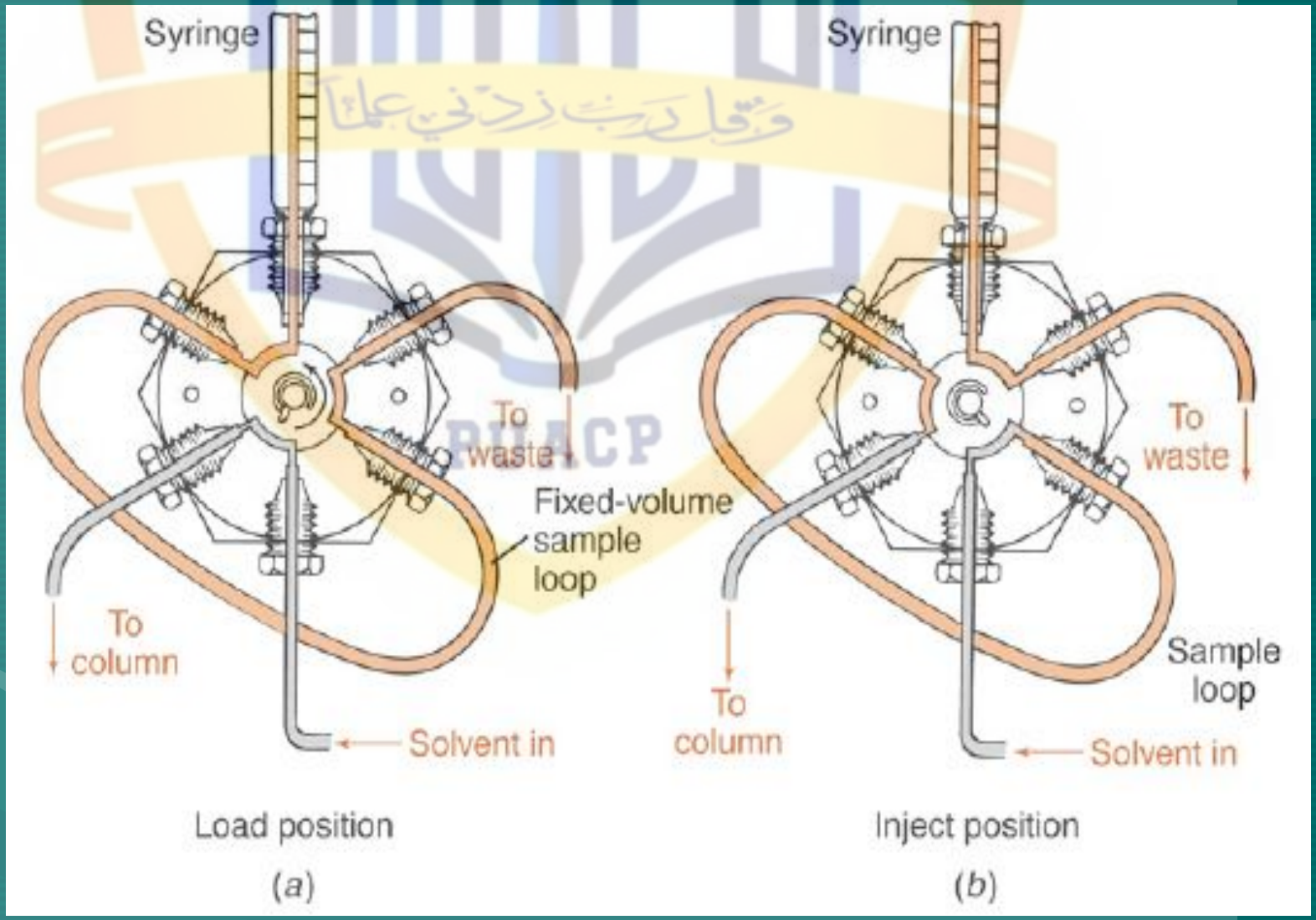
Different devices are: 1.Septum injectors

2. Stop flow injectors

3. Rheodyne injectors (loop valve type)

Rheodyne injector is the most popular injector. This has a fixed volume loop like 20 µl or 50 µl or more. Injector has 2 modes. Load position and Inject mode.

– Limit of precision of HPLC
– Sample size: 0.5 ~ 500 µL
– No interference with the pressure
– Based on a sample loop, 1 ~ 100 µL, Reproducibility: 0.1%, P < 7000 psi
– Auto sampler: inject continuously variable volume 1 µL – 1 mL
Controlled temperature environment for derivatization reaction.


Syringe
To waste
Fixed-volume sample loop
To column
Solvent in
Load position
(a)
Syringe
To waste
Sample loop
To column
Solvent in
Inject position
(b)

COLUMNS:

– Stainless steel tubing for high pressure
– Heavy-wall glass or PEEK tubing for low P (< 600 psi)
– **Analytical column**: straight, L(5 ~ 25 cm), dc(3 ~ 5 mm), dp(35 μm). N (40 k ~ 70 k plates/m)
-**Micro column**: L (3 ~ 7.5 cm), d (1 ~ 5 mm), dp: 3 ~ 5 μm, N: ~100k plates/m, high speed and minimum solvent consumption
– **Guard column**: remove particulate matter and contamination protect analytical column, similar packing
– T control: < 150 °C, 0.1 °C
-**Column packing:** silica, alumina, a polystyrene-di vinyl benzene synthetic or an ion-exchange resin
– Pellicular particle: original, Spherical, nonporous beads, proteins and large biomolecules separation (dp: 5 μm)
– Porous particle: common used, dp: 3 ~ 10 μm. Narrow size distribution, porous micro particle coated with thin organic films

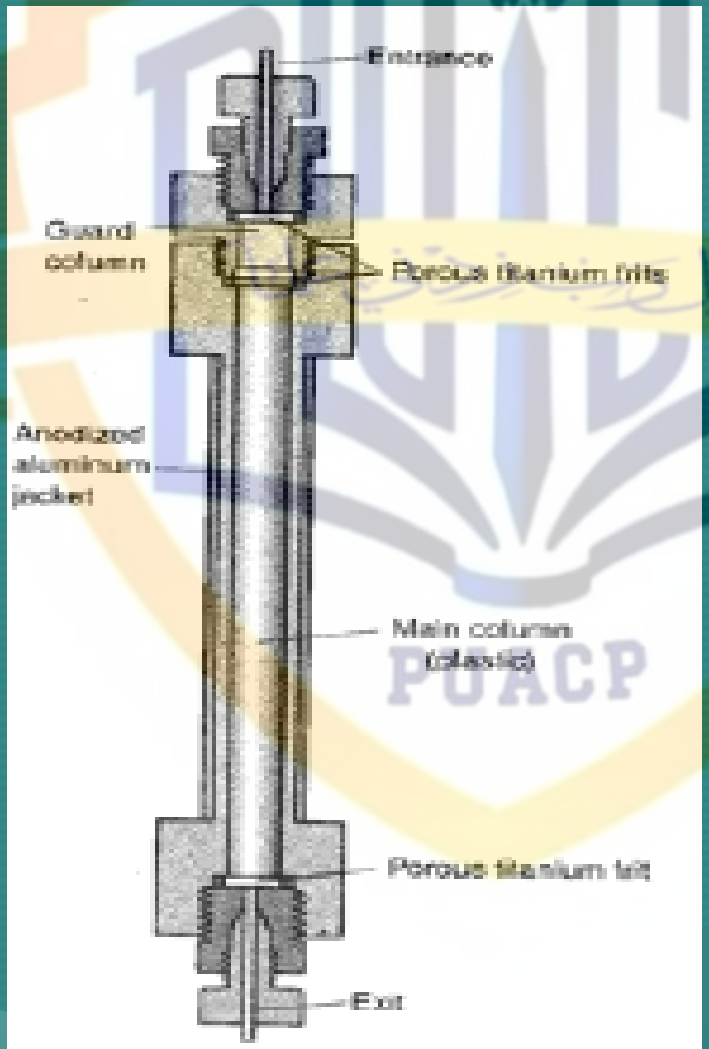
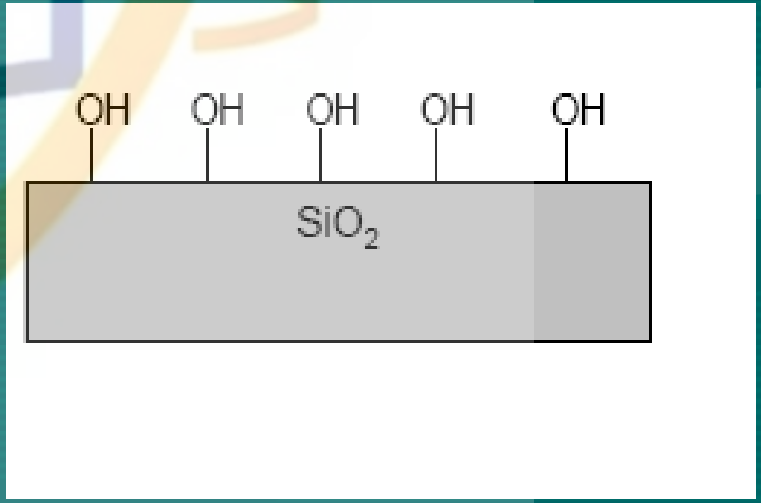


Figure 25-4 HPLC column with replaceable guard column to collect irreversibly adsorbed impurities. Titanium frits distribute the liquid evenly over the diameter of the column. [Courtesy Upchurch Scientific, Oak Harbor, WA.]



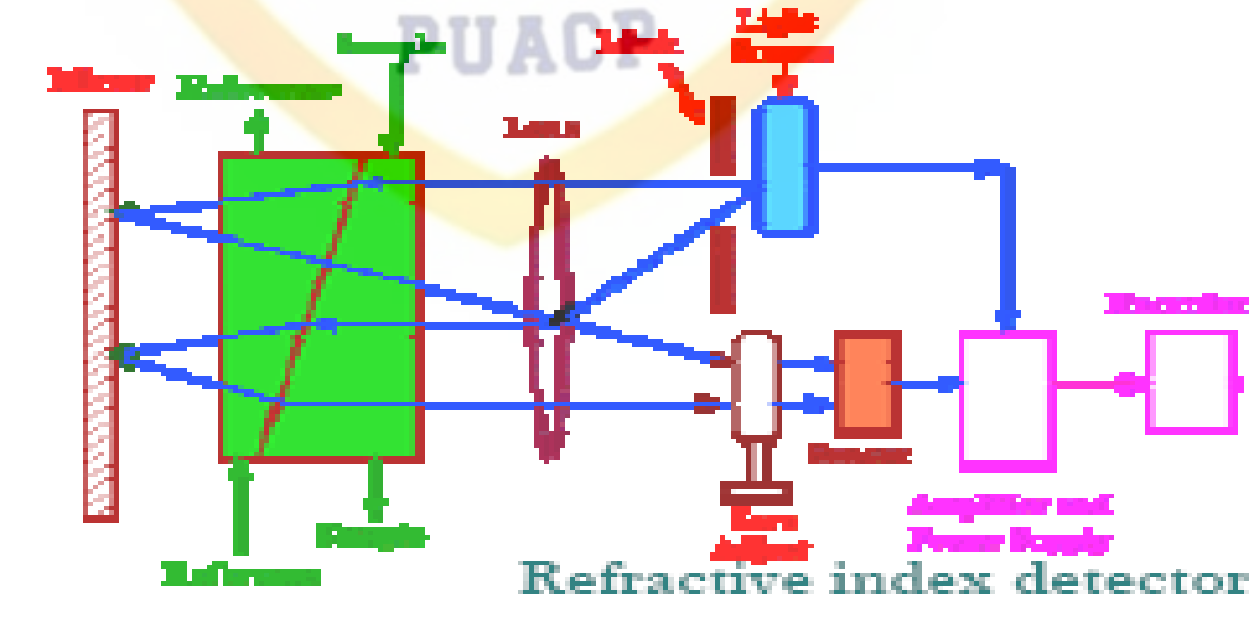
DETECTORS:

Detectors used depends upon the property of the compounds to be separated. Different detectors available are:

1. Refractive index detectors
2. U.V detectors
3. Fluorescence detectors
4. Electro chemical detectors
5. Evaporative light scattering detectors
6. IR detectors
7. Photo diode array detector:

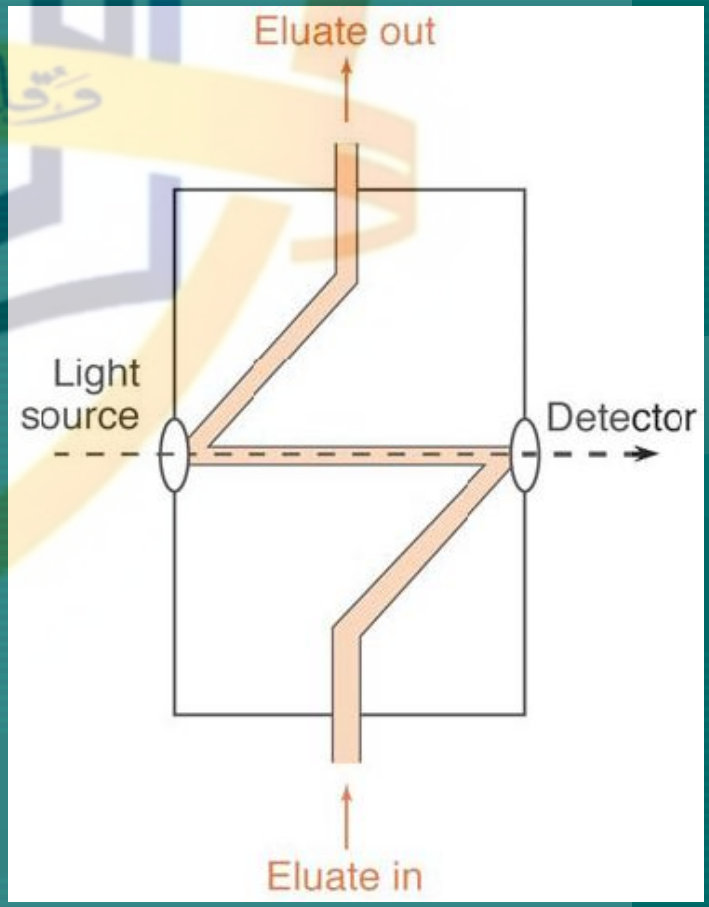
1. Refractive index detectors:

- Nearly universal but poor detection limit
- Passes visible light through 2 compartments, sample & reference.
- When the solvent composition are the same the light passed through the compartments the light beam that passes through is recorded as zero.
- When a solute is in the sample compartment, refractive index changes will shift the light beam from the detector.
- Limit of detection (LOD) 10 ng of solute



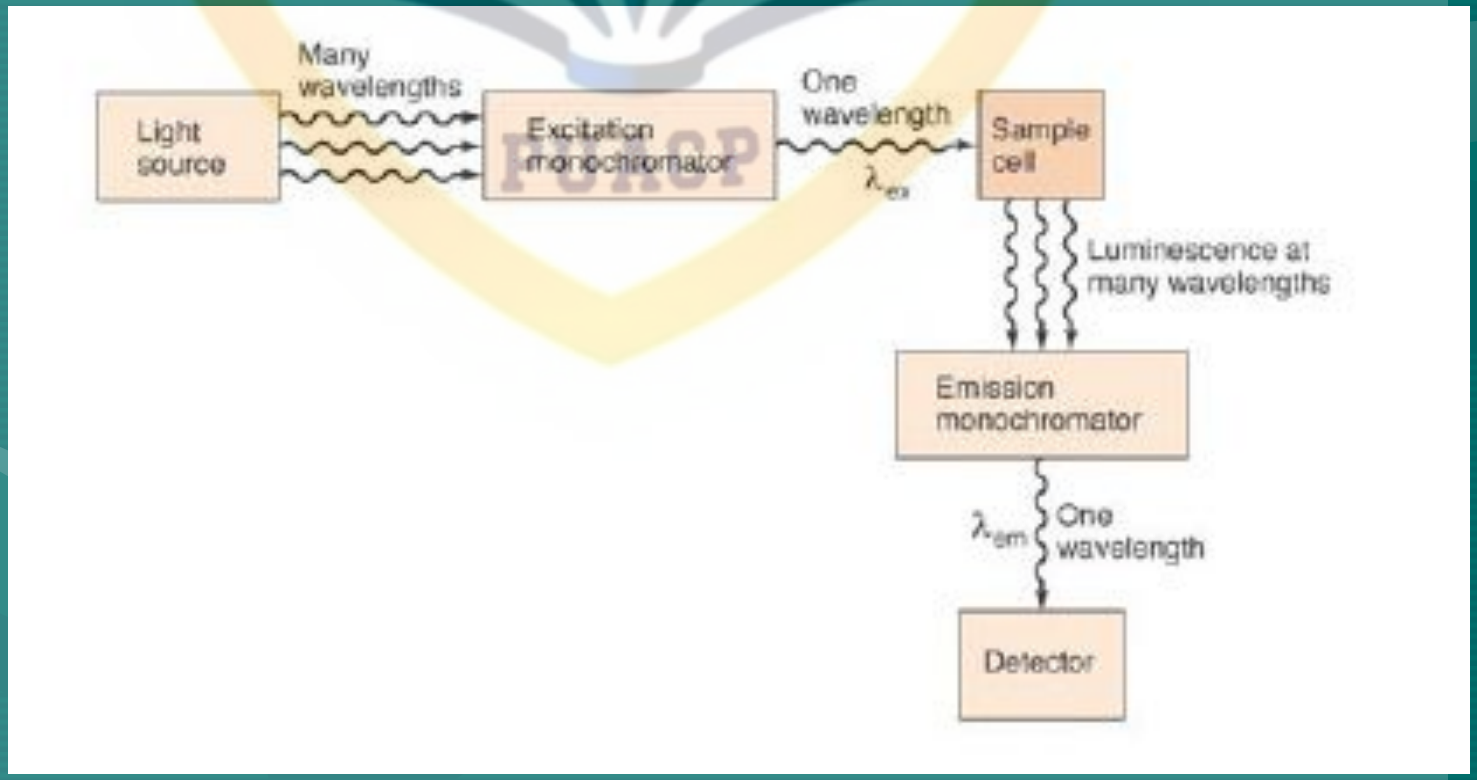
2. U.V detectors:

• Based on electronic transitions within molecules.
• Most common type of detector for LC
• Fixed wavelength, Hg lamp 254 nm ($\pi \Rightarrow \pi^*$)
•Tunable wavelength, selectable for specific wavelengths, monochromators or filters. Still limited to single wavelengths.
• - 1 pg LOD
•Solvent limitations with UV-vis abs. Detectors
•Z-shape, flow-through cell (V, 1 ~ 10 µL and b, 2 ~ 10 mm)
• Spectrophotometer: more versatile



3. Fluorescence detectors:

- Review - based on emission of excited state molecules.
- Detector 900 from excitation axis.
- LOD 10 fg
- Hg or Xe lamp
- Fluorometer and spectrofluorometer
- Fluorescing species or fluorescent derivatives



4.Electro chemical detectors:

•Based on amperometric response of analyte to electrode usually held at constant potential.
•If the analyte is electro active, can be highly sensitive since response is based on a surface phenomenon rather than a solution bulk property (e.g. UV-vis absorbance)
•simplicity, convenience and wide-spreading application
• Thin-layer flow cell of Teflon : 50μm thick, 1 ~ 5 μL volume
• Indictor E: Pt, Au, C
• Multi-electrode: simultaneous detection or sample purity indication.

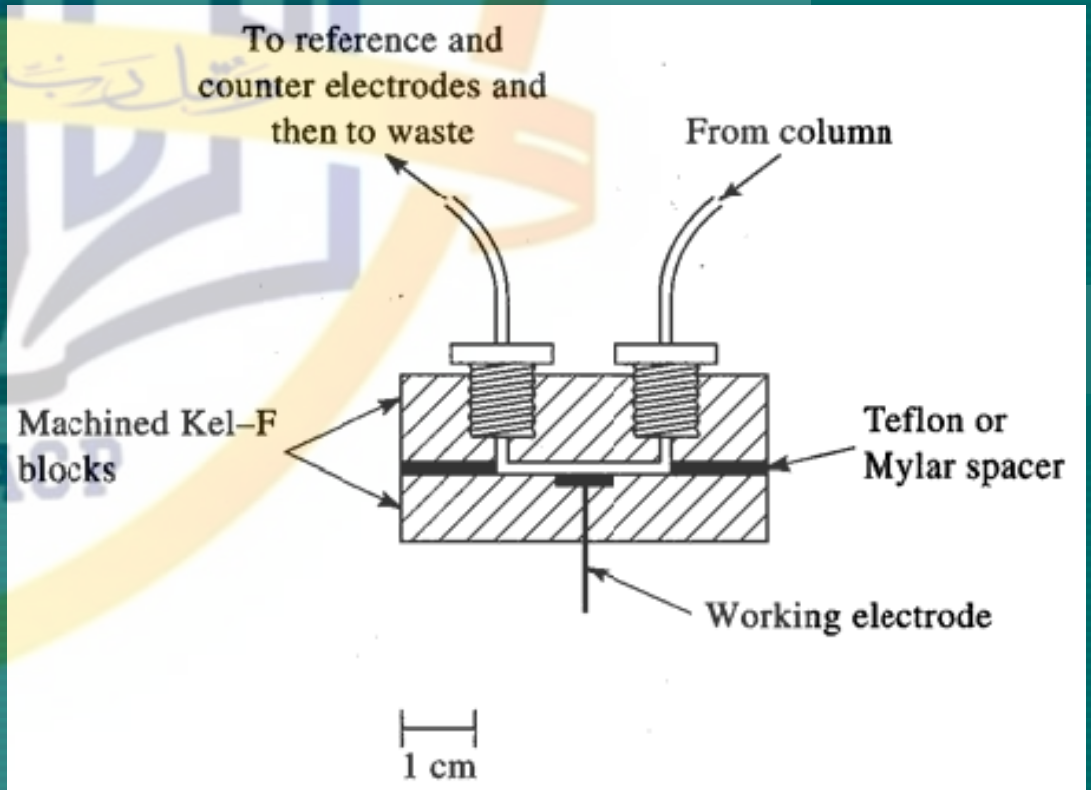
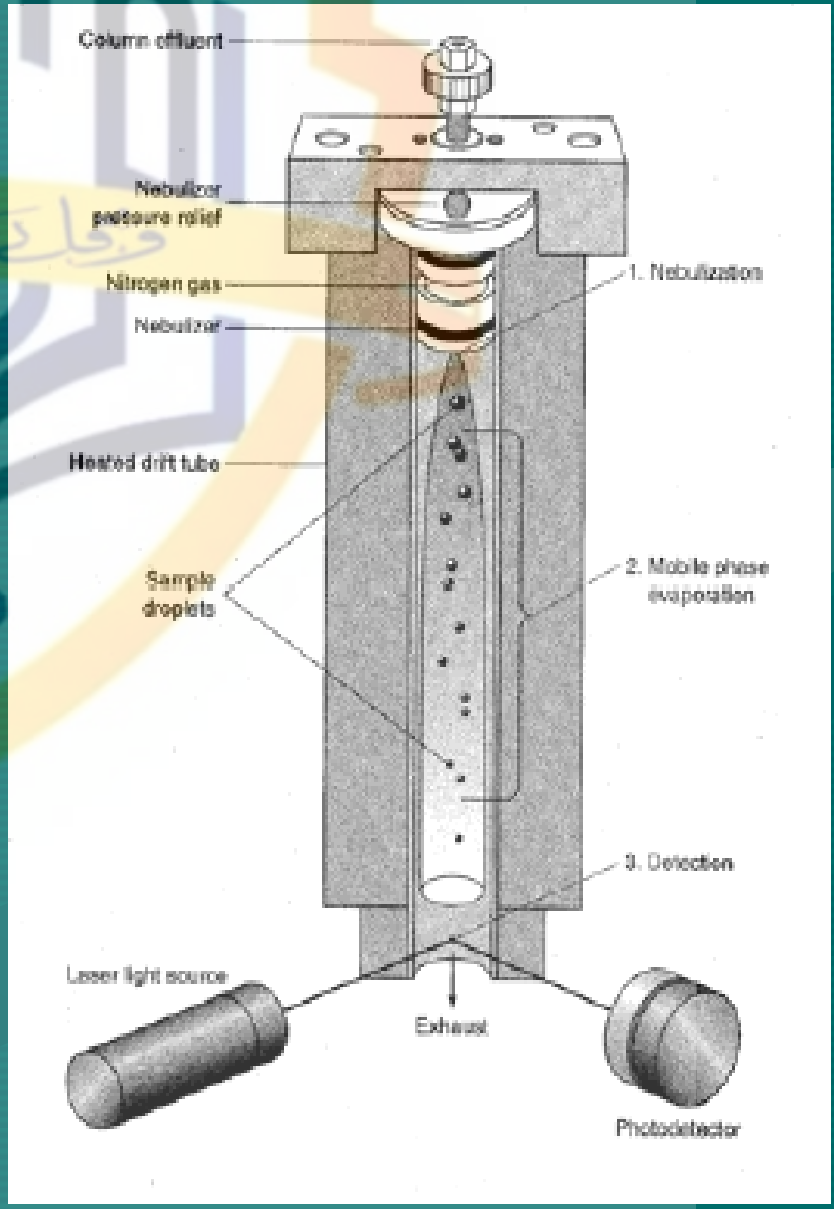


Figure 28-13 Amperometric thin-layer detector cell for HPLC.

5. Evaporative light scattering detectors:

- Responds to any analyte that is significantly less volatile than the mobile phase.
- Eluate is mixed with N2(g) and forms a fine mist.
- Solvent (m.p.) evaporates leaving fine particles of analyte. The particles themselves are detected by light scattering.
- Response is proportional to analyte mass.



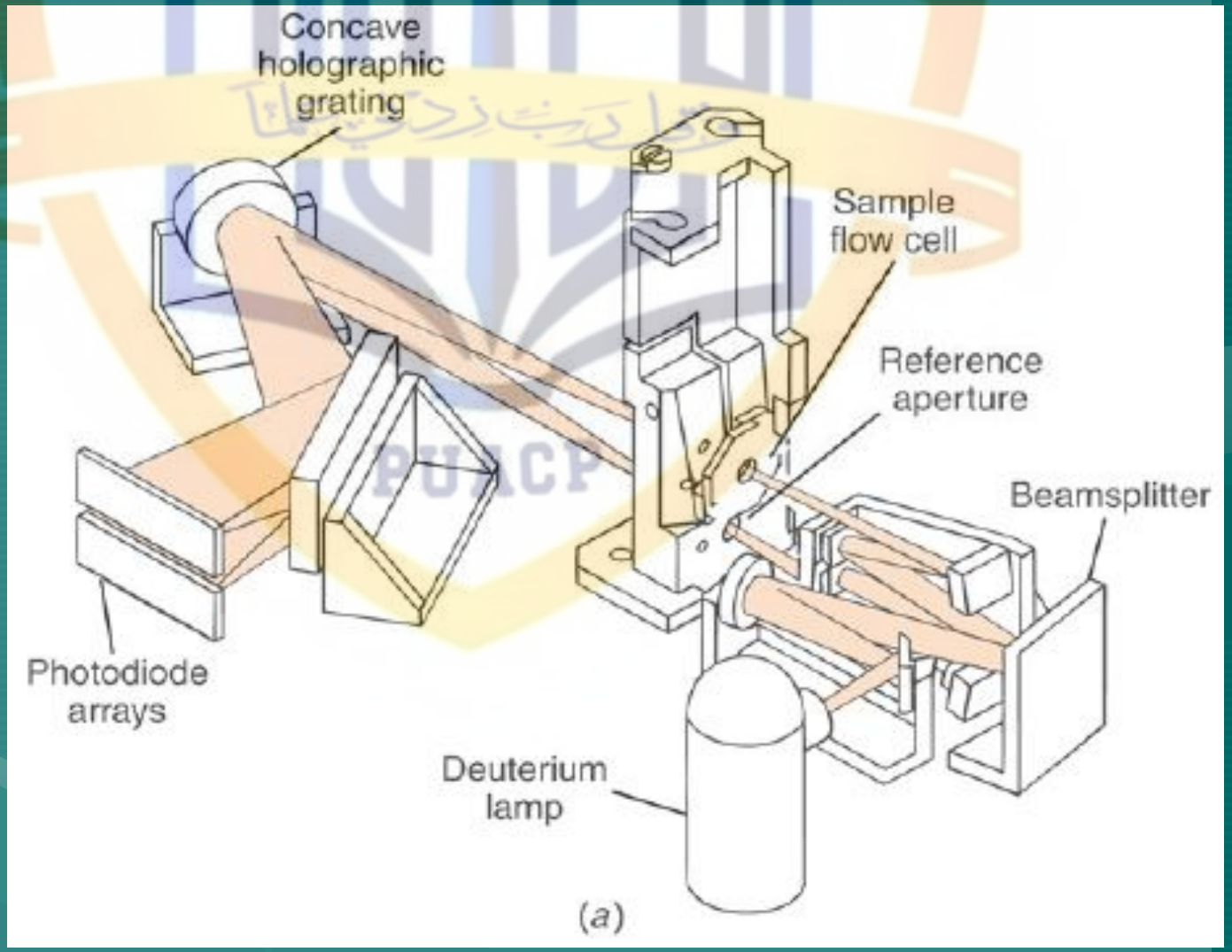
6. IR detectors:

• filter instrument or FTIR
• Similar cell (V, 1.5 ~ 10 µL and b, 0.2 ~ 1.0mm)
• Limit: no suitable solvent, special optics
• FT-IR allows for spectrum records of flowing systems analogous to the diode array system.
• Water/alcohols can be major interferences to solute detection
• LOD 100 ng

7. Photo diode array detector:

This is a recent one which is similar to U.V detector which operates from 190-600 nm. Allows for the recording of the entire spectrum of each solute as it passed through the diode array detector. The resulting spectra is a 3-D or three dimensional plot of Response Vs Time Vs Wave length.

Photo diode array detector



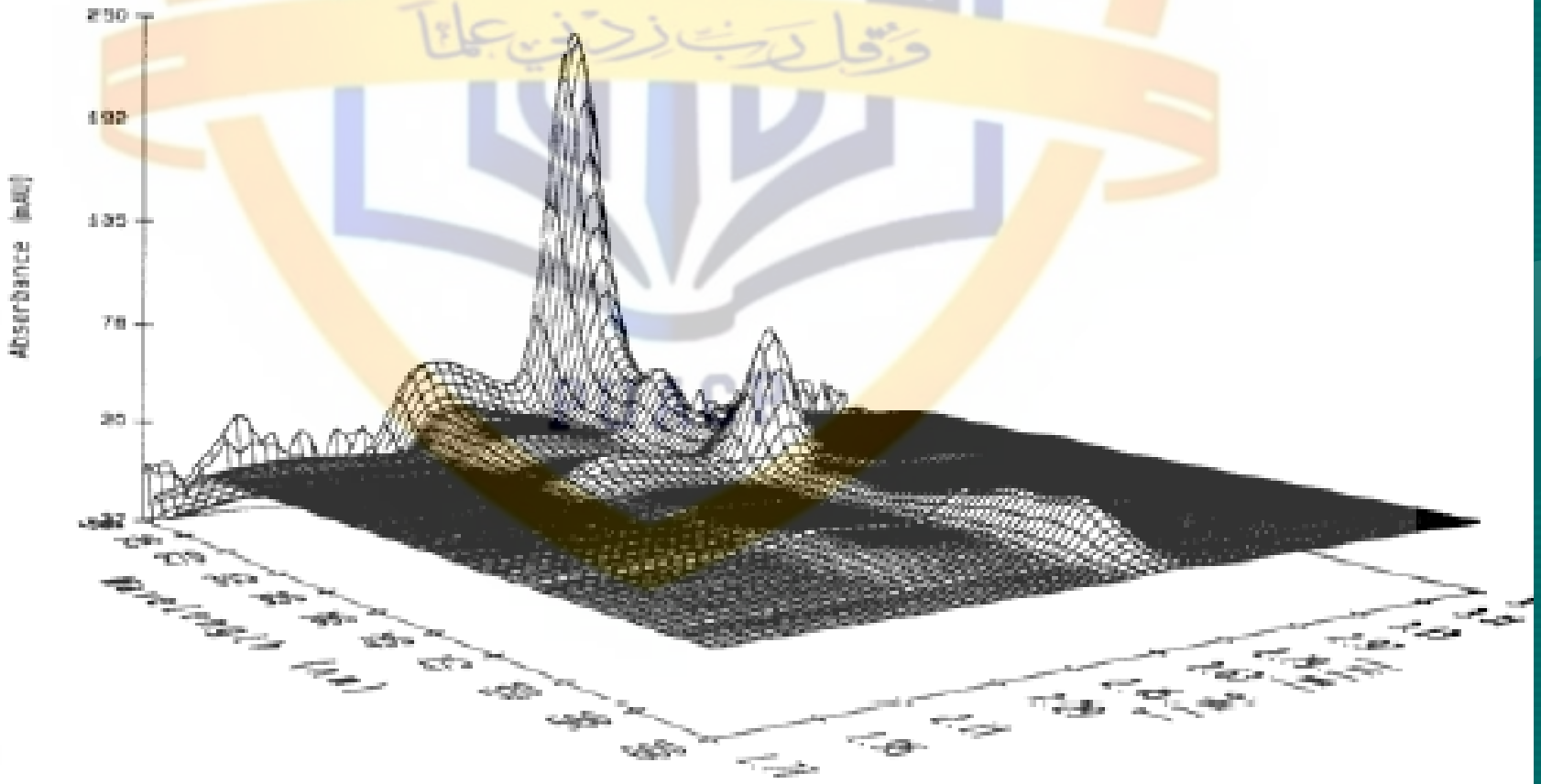


FIGURE 21.21. *Typical three-dimensional plot (absorbance versus wavelength versus time) for a liquid chromatograph. Separation of hydroxycobalamine (peak 1, at about 2.5 min) and cyanocobalamine (peak 2, at about 3.0 min). Courtesy of the Hewlett-Packard Company.*

RECORDERS AND INTEGRATORS:

Recorders are used to record the responses obtained from detectors after amplification. They record the base line and all the peaks obtained, with respect to time. Retention time for all the peaks can be found out from such recordings, but the area of individual peaks cannot be known.

Integrators are improved version of recorders with some data processing capabilities. They can record the individual peaks with retention time, height and width of peaks, peak area, percentage of area, etc. Integrators provide more information on peaks than recorders. Now a days computers and printers are used for recording and processing the obtained data and for controlling several operations.

5. PARAMETERS USED IN HPLC:

1.Retention time

2.Retention volume

3.Seperation factor

4. Resolution

5. Height Equivalent to a Theoretical Plate (HETP)

6. Efficiency

7. Asymmetry factor

1.Retention time:

Retention time is the difference in time between the point of injection and appearance of peak maxima. It is also defined as time required for 50% of a component to be eluted from a column. It is measured in minutes and seconds.

2.Retention volume:

Retention volume is the volume of carrier gas required to elute 50% of the component from the column. It is the product of retention time and flow rate.

$$\text{Retention volume} = \text{Retention time} \times \text{flow rate}$$

3.Seperation factor:

Separation factor is the ratio of partition coefficient of the 2 components to be separated.

$$S=Ka/Kb=(tb-to)/(ta-to)$$

Where to = Retention time of unretained substance

Ka, Kb = Partition coefficients of a, b

ta, tb = Retention time of substance a, b

If there is a more difference in partition coefficient between 2 compounds, the peaks are far apart and the separation factor is more. If the partition coefficient of 2 compounds are similar, then the peaks are closer and the separation factor is less.

4. Resolution:

Resolution is the measure of extent of separation of 2 components and the base line separation achieved.

$$Rs = 2 (Rt_1-Rt_2) / w_1+w_2$$

5. Height Equivalent to a Theoretical Plate (HETP):

A theoretical plate is an imaginary or hypothetical unit of a column where distribution of solute between stationary phase and mobile phase has attained equilibrium. It can also be called as a functional unit of the column.

A theoretical plate can be of any height, which describes the efficiency of separation. If HETP is less, the column is more efficient. If HETP is more, the column is less efficient.

HETP = length of the column/ no. of theoretical plates

HETP is given by Van deemter equation

$$HETP = A+B/u+Cu$$

Where A = Eddy diffusion term or multiple path diffusion which arises due to the packing of the column. This can be minimized by uniformity of packing.

B = Longitudinal diffusion term or molecular diffusion.

C = Effect of mass transfer.

u = flow rate or velocity of the mobile phase.

6. Efficiency:

Efficiency of a column is expressed by the theoretical plates.

$$n = 16\ Rt^2/\ w^2$$

Where n = no of theoretical plates

Rt= retention time

w = peak width at base

Rt and w are measured in common units (cm or mm , min or sec). No of theoretical plates

Is high, the column is said to be highly efficient. For GLC, a value of 600/metre is sufficient. But in HPLC, high values like 40,000 to 70,000/ meter are recommended.

7. Asymmetry factor:

A chromatographic peak should be symmetrical about its centre and said to follow Gaussian distribution. But in practice due to some factors, the peak is not symmetrical and shows tailing or fronting.

Fronting is due to saturation of stationary phase and can be avoided by using less quantity of sample. Tailing is due to more active adsorption sites and can be eliminated by support pretreatment.

Asymmetry factor (0.95 to 1.05) can be calculated by AF = b/a (b, a calculated by 5% or 10% of the peak height).

Broad peaks occur due to the more conc. of sample, large injection volume, column deterioration.

Ghost peaks occur due to the contamination of the column, compound from earlier injections.

Negative peaks occur if mobile phase absorbance is larger than sample absorbance.

Peak doubling occurs due to the co- elution of interfering compound, column over load, channeling in column.

Base line spikes occur due to the air bubbles in the mobile phase and/or detector, column deterioration.

6.ADVANTAGES OF HPLC:

1. Separations fast and efficient (high resolution power)
2. Continuous monitoring of the column effluent
3. It can be applied to the separation and analysis of very complex mixtures
4. Accurate quantitative measurements.
5. Repetitive and reproducible analysis using the same column.
6. Adsorption, partition, ion exchange and exclusion column separations are excellently made.

7. HPLC is more versatile than GLC in some respects, because it has the advantage of not being restricted to volatile and thermally stable solute and the choice of mobile and stationary phases is much wider in HPLC

8. Both aqueous and non aqueous samples can be analyzed with little or no sample pre treatment

9. A variety of solvents and column packings are available, providing a high degree of selectivity for specific analyses.

10. It provides a means for determination of multiple components in a single analysis.

7.DERIVATISATION IN HPLC:

In order to increase the detectability of various classes of compounds (for which sensitive detectors are not available) derivatisation is carried out in HPLC. A good amount of work has been performed on the labelling of compounds with chromophores and flurophores for detection using UV spectrometers and fluorimeters respectively.

There are 2 important types of derivatisation. These are

1. Pre column derivatisation
2. Post column derivatisation

PRE COLUMN DERIVATISATION:

In pre column derivatisation there are no restrictions on the solvents, reagents, or reaction rates chosen and excess of reagents can be removed before the injection. However, artifact formation, if present, can be checked by positive identification of the eluted peaks. For example, in the derivatisation of a triketone with more than one functional group capable of being derivatised there is a possibility of range of derivatives being formed from one solute. It is clearly necessary to check that the derivatisation reactions are quantitative or the sample derivatisations proceed in a manner analogues to the derivatisation of standards.

Examples of pre column derivatisation to form UV chromophores include the treatment of ketosteroids with 2,4, DNP and the benzoylation of hydroxy steroids or the esterification of fatty acids. Similarly, fluorophores have been introduced into amino acids, biogenic amines, and alkaloids by treatment with dansyl chloride.

POST COLUMN DERIVATISATION:

It is carried out on the separated solutes as they emerge from the chromatographic column. In HPLC, this places serious restriction on the derivatisation reactions, because dilution of the eluent peak must be minimized. Consequently, very fast reactions must be used and the reagents and mobile phase must be compatible.

Examples of post column derivatisation reactions for use with UV detectors include:

A. Reaction of amino acids with ninhydrin and fluorescamine.

B. Reaction of fatty acid with ortho nitro phenol.

C. Reaction of ketones with 2, 4, DNP.

D. Thermal or acid treatment of carbohydrates.

An oxidation detector for the fluorimetric analysis of carbohydrates in body fluids using Ce (III) flourescence has also been reported.

APPLICATIONS:

HPLC is one of the most widely applied analytical separation techniques**.**

Pharmaceutical:

Tablet dissolution of pharmaceutical dosages.

Shelf life determinations of pharmaceutical products.

Identification of counterfeit drug products.

Pharmaceutical quality control.

Environmental

Phenols in Drinking Water.
Identification of diphenhydramine in sediment samples.
Biomonitoring of PAH pollution in high-altitude mountain lakes through the analysis of fish bile.
Estrogens in coastal waters - The sewage source.
Toxicity of tetracyclines and tetracycline degradation products to environmentally relevant bacteria.
Assessment of TNT toxicity in sediment.

Forensics

A mobile HPLC apparatus at dance parties - on-site identification and quantification of the drug Ecstasy.
Identification of anabolic steroids in serum, urine, sweat and hair. Forensic analysis of textile dyes.
Determination of cocaine and metabolites in meconium.
Simultaneous quantification of psychotherapeutic drugs in human plasma.

Clinical

Quantification of DEET in Human Urine.
Analysis of antibiotics.
Increased urinary excretion of aquaporin 2 in patients with liver cirrhosis.
Detection of endogenous neuropeptides in brain extracellular fluids.

Food and Flavor

Ensuring soft drink consistency and quality.
Analysis of vicinal diketones in beer.
Sugar analysis in fruit juices.
Polycyclic aromatic hydrocarbons in Brazilian vegetables and fruits.
Trace analysis of military high explosives in agricultural crops.
Stability of aspartame in the presence of glucose and vanillin.

THANKYOU