Pharmaceutical Analysis

(Separation methods)

Chromatography

الأستاذ الدكتور جمعه الزهوري (دكتوراه صيدلة-ألمانيا 1991)

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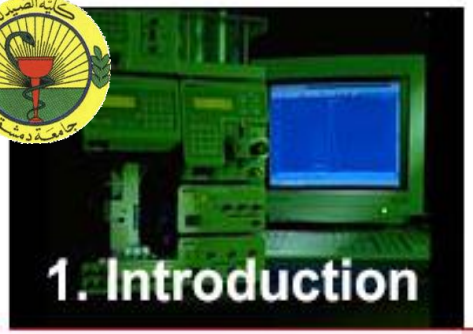
High-Performance Liquid Chromatography

HPLC

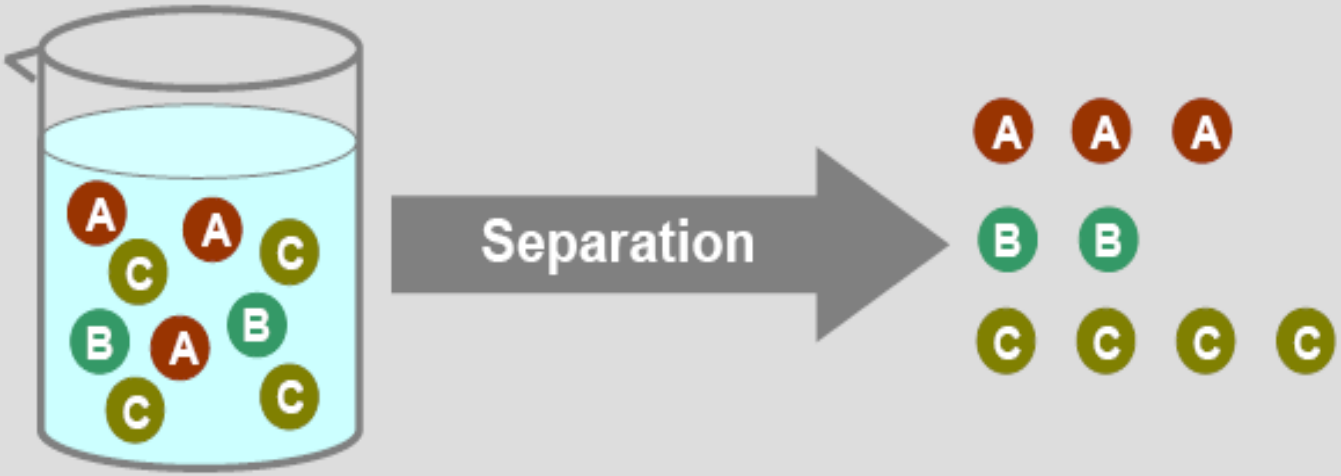


What is HPLC ?

H : High
P : Performance (Pressure)
L : Liquid
C : Chromatography

GC : Gas chromatography
TLC : Thin layer chromatography

Separation and Analysis



Qualitative analysis

What are components A, B and C ?

Quantitative analysis

What is the concentration of components A, B and C ?

High-Performance Liquid Chromatography

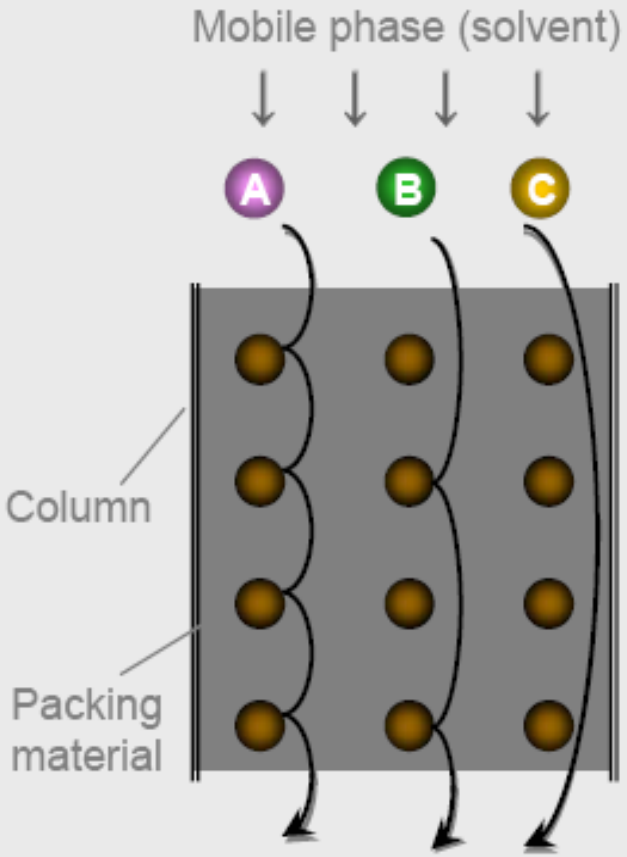
- HPLC is probably the most important analytical technique available to the pharmaceutical product to day.
- A solute is distributed between two phases as a result of the molecular forces
- The stronger the forces between the solute molecules and those of the stationary phase, the greater will be the amount of solute held in that stationary phase under equilibrium conditions.
- Conversely , the stronger the interactions between the solute molecules and those of the mobile phase ,the greater the amount of solute that will be held in the mobile phase.

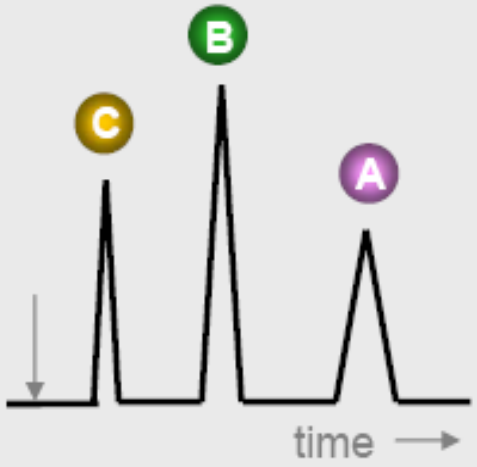
Separation Mechanism

Separation is determined by column (packing material) and mobile phase (solvent).

Mobile phase elutes components.

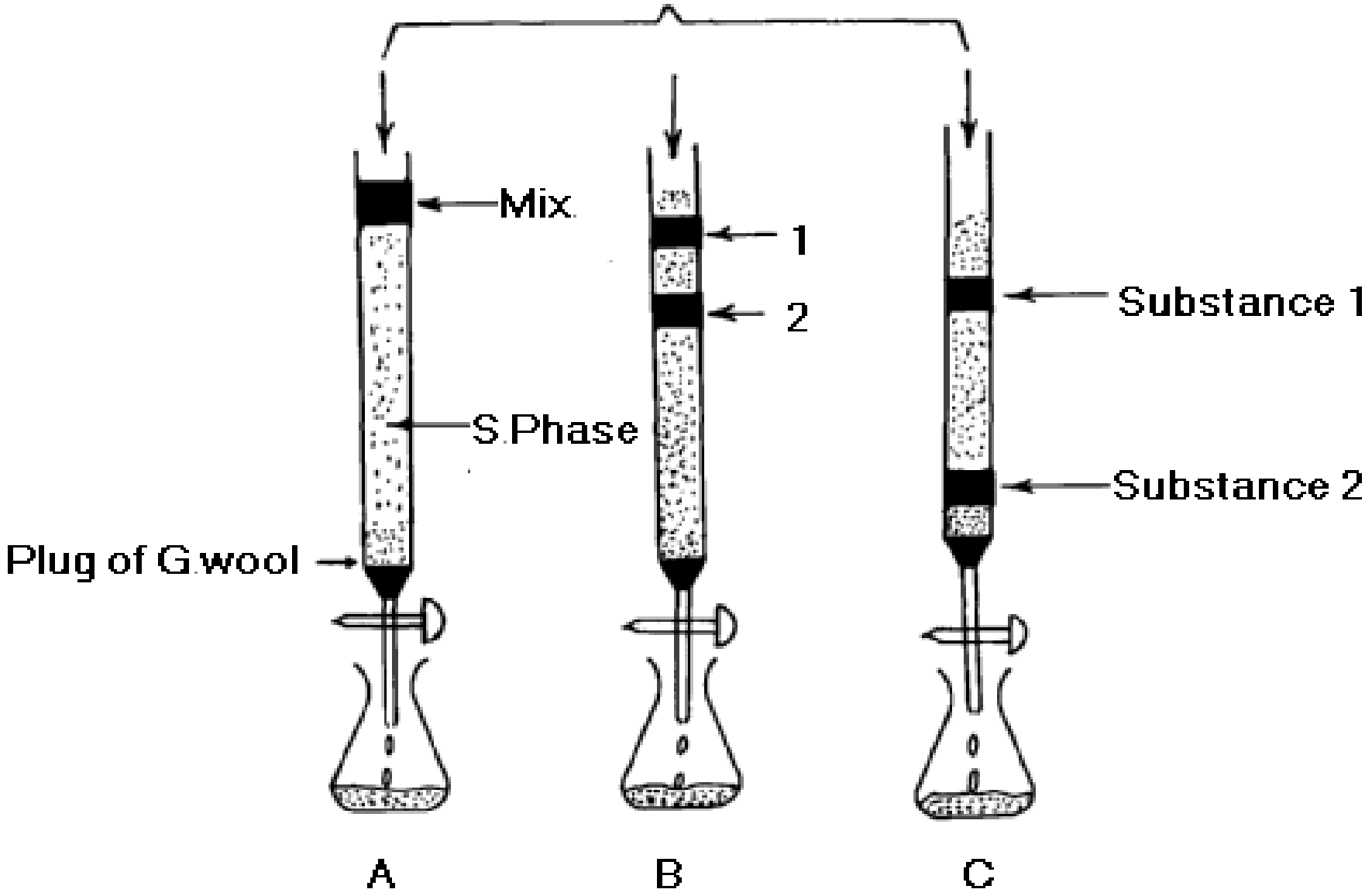
Packing materials retain components in the column.





C > B > A

Tswett 1903



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Elution

A solute partitions between two phases (equilibrium).

Separation is based on relative retention.

Making the column longer will increase the degree of separation.

	LC (Tswett)	HPLC
Columns	Glass d=1-5 cm L=50-500 cm	Stainless steel 2- 5 mm 5- 30 cm
Particles size	150-200 µm	3-10 µm
Flow rate	Too low (a few tenth ml/min	High
Separation time	Too long (several hours)	Short (a few min.)
Pressures	Normal	very high
Detectors	Absence	Present (several types)

High-Performance liquid Chromatography

- Chromatography has been Classically defined as a separation process that is achieved by the distribution of substances between two phases, a stationary phases and a mobile phase.
- The name high-performance liquid chromatography (HPLC) is employed to distinguish these newer procedures from the basic methods, which are still used for preparative purposes.
- HPLC is the liquid chromatography analogue of GC. The secret to its success is small uniform particles to give small eddy diffusion and rapid mass transfer.

High Performance Liquid Chromatography

HPLC is characterized by the use of high pressure to push a *mobile phase* solution through a column of *stationary phase* allowing separation of complex mixtures with high resolution.

High-Performance liquid chromatography

- HPLC is the most widely used of all of the analytical separation techniques.

The reasons for the popularity of the method is :

1- Its sensitivity

2- its ready adaptability to accurate quantitative determination.

3- its suitability for separating nonvolatile species or thermally fragile ones.

Basic HPLC equipment

Unlike GC equipment, many HPLC systems have a modular design - can simply add a new 'box' to change/extent capabilities.

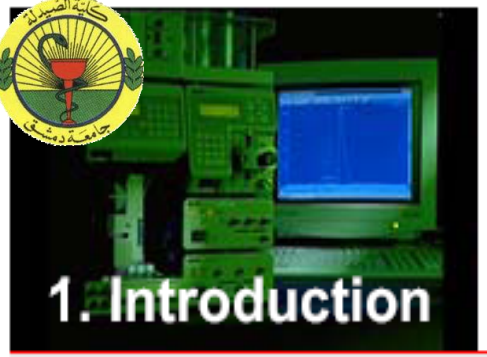
There is also a wider range of how to do things like produce a flow or gradient.

We'll cover some of the basic approaches.

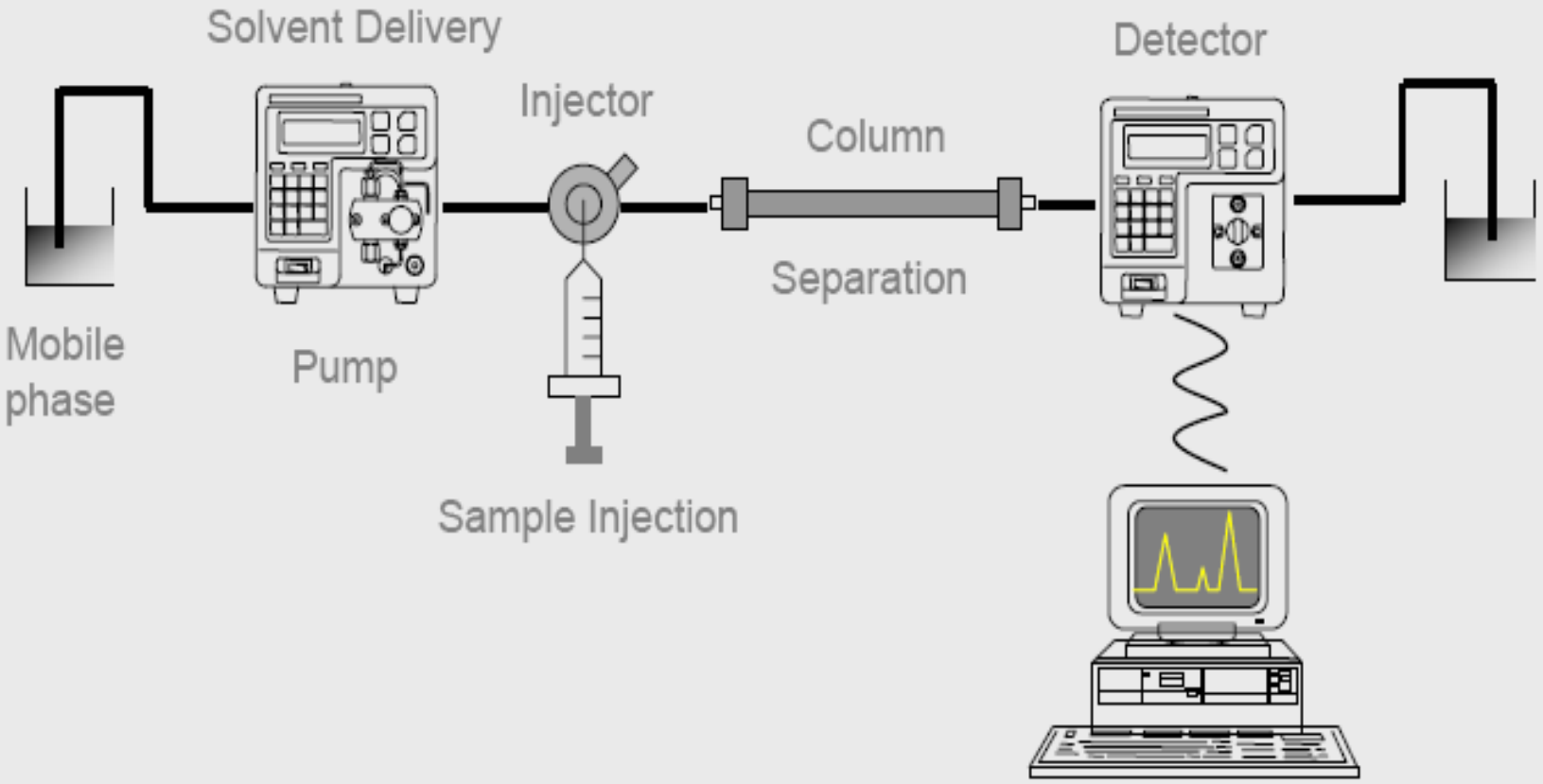
Instruments for liquid Chromatography

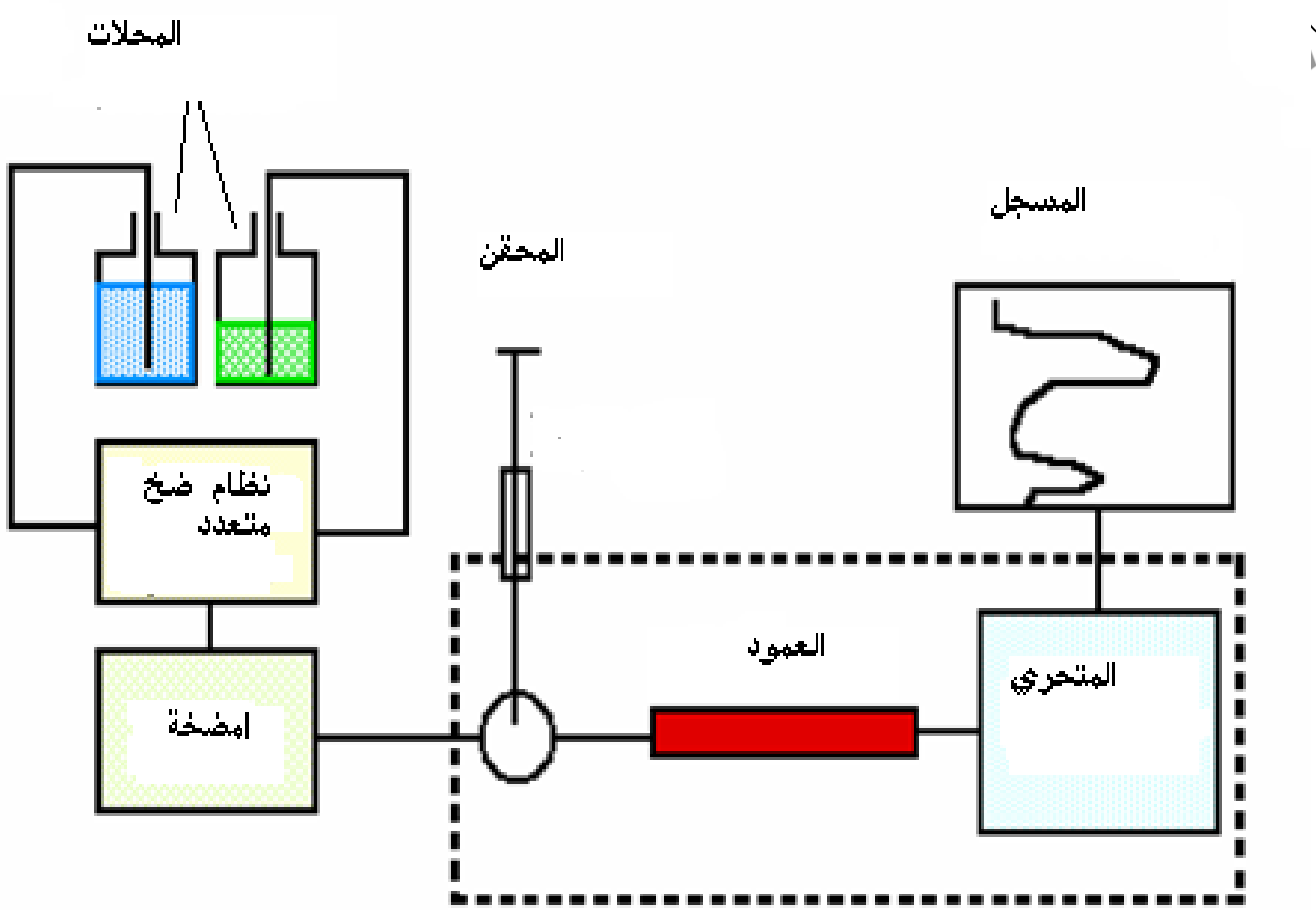
- The basic HPLC consists of seven essential parts :

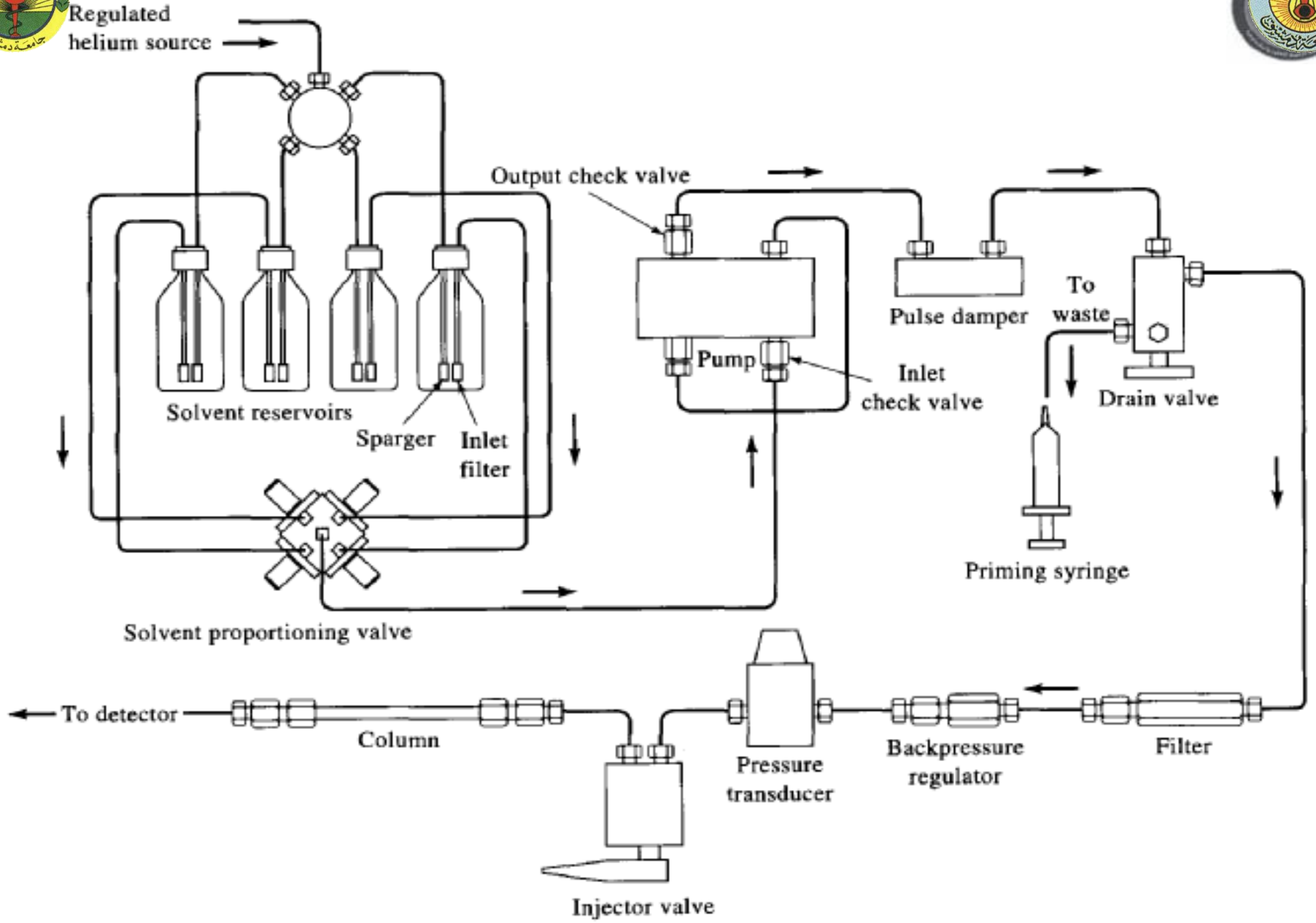
1. Mobile Phase Supply System.
2. Pump and Programmer.
3. Sample Valve.
4. Column and Thermostat.
5. Detector.
6. Data Acquisition and Processing.
7. Chart Recorder or Printer.



HPLC Basic Instrumentation







Schematic of an apparatus for HPLC.

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Parameters used in HPLC

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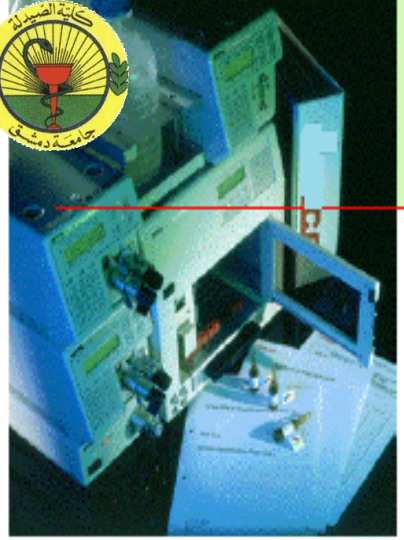
A symmetry Factor (Tailing Factor) :

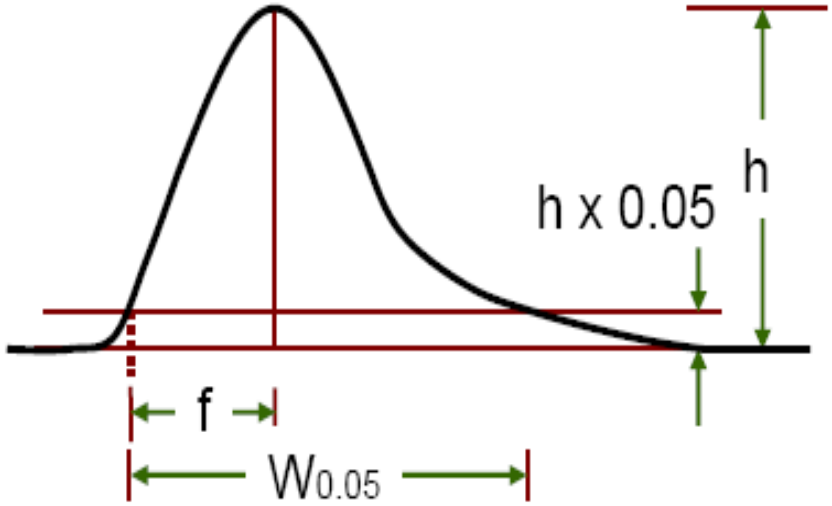
describing the shape of a chromatographic peak. Theory assumes a Gaussian shape and that peaks are symmetrical.

The peak asymmetry factor is the ratio (at 5% of the peak height) of the distance between the peak apex and the back side of the chromatographic curve to the distance between the peak apex and the front side of the chromatographic curve. A value > 1 is a tailing peak, while a value <1 is a fronting peak .(Leading)



Peak symmetry

S : Symmetry factor (T : Tailing factor)



$$S = \frac{W_{0.05}}{2f}$$

S = 1 : The peak is completely symmetric.
S > 1 : Tailing
S < 1 : Leading

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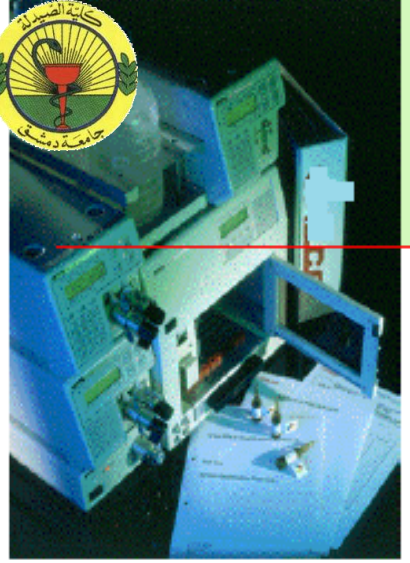
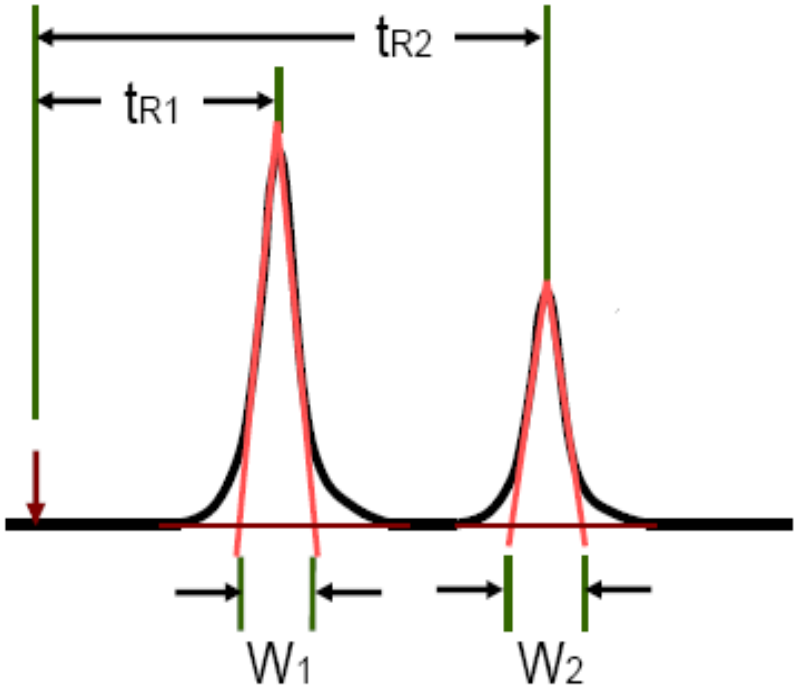
Resolution Rs

A measure of how completely two neighboring peaks are separated from each other. Also it is show the ability of a column to separate chromatographic peaks



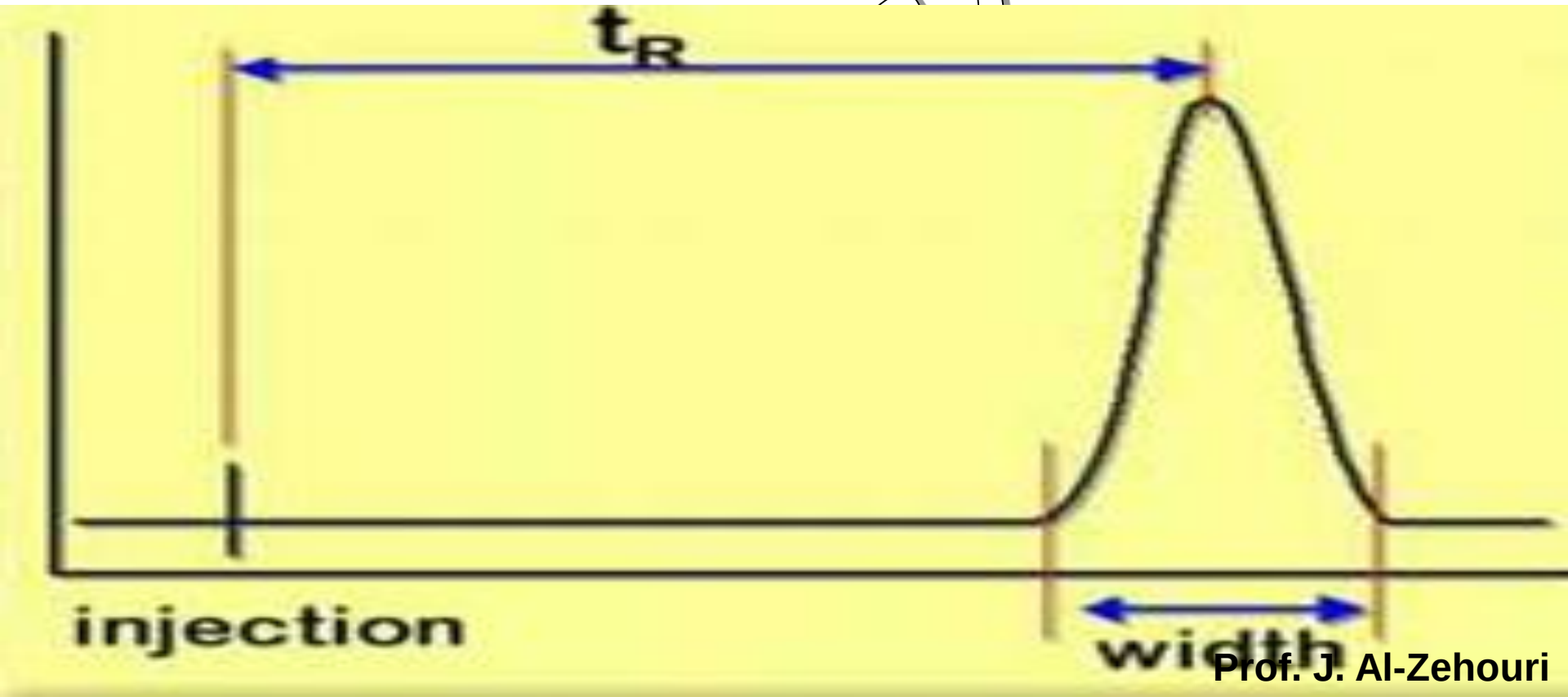
Degree of separation



$$\text{Resolution}: \quad R_s = 2 \times \frac{t_{R2} - t_{R1}}{W_1 + W_2}$$

Retention Time t_R

The time between injection and appearance of the peak maximum



How to calculate Relative Retention Time (RRT)

Divide the retention time of the peak of interest by the retention time of the main peak .

RRT < 1 the peak elute before the main peak.

RRt >1 the peak elute after the main peak

Potassium clavulanate (USP)

Chromatographic system (see Chromatography ‹ 621 ›)— The liquid chromatograph is equipped with a 220-nm detector and a 4-mm × 30-cm column that contains 3- to 10-µm packing L1. The flow rate is about 2 mL per minute. Chromatograph the *Standard preparation,* and record the peak responses as directed for *Procedure:* the column efficiency determined from the analyte peak is not less than 550 theoretical plates; the tailing factor for the analyte peak is not more than 1.5; and the relative standard deviation for replicate injections is not more than 2.0%. Chromatograph the *Resolution solution,* and record the peak responses as directed for *Procedure:* the relative retention times are about 0.5 for clavulanic acid and 1.0 for amoxicillin; and the resolution, *R,* between the amoxicillin and clavulanic acid peaks is not less than 3.5.

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Separation mode

Column and mobile phase solvent

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Chromatography

There are several types of interaction that have been used to separate eluents.

Major Categories

surface adsorption

solvent partitioning

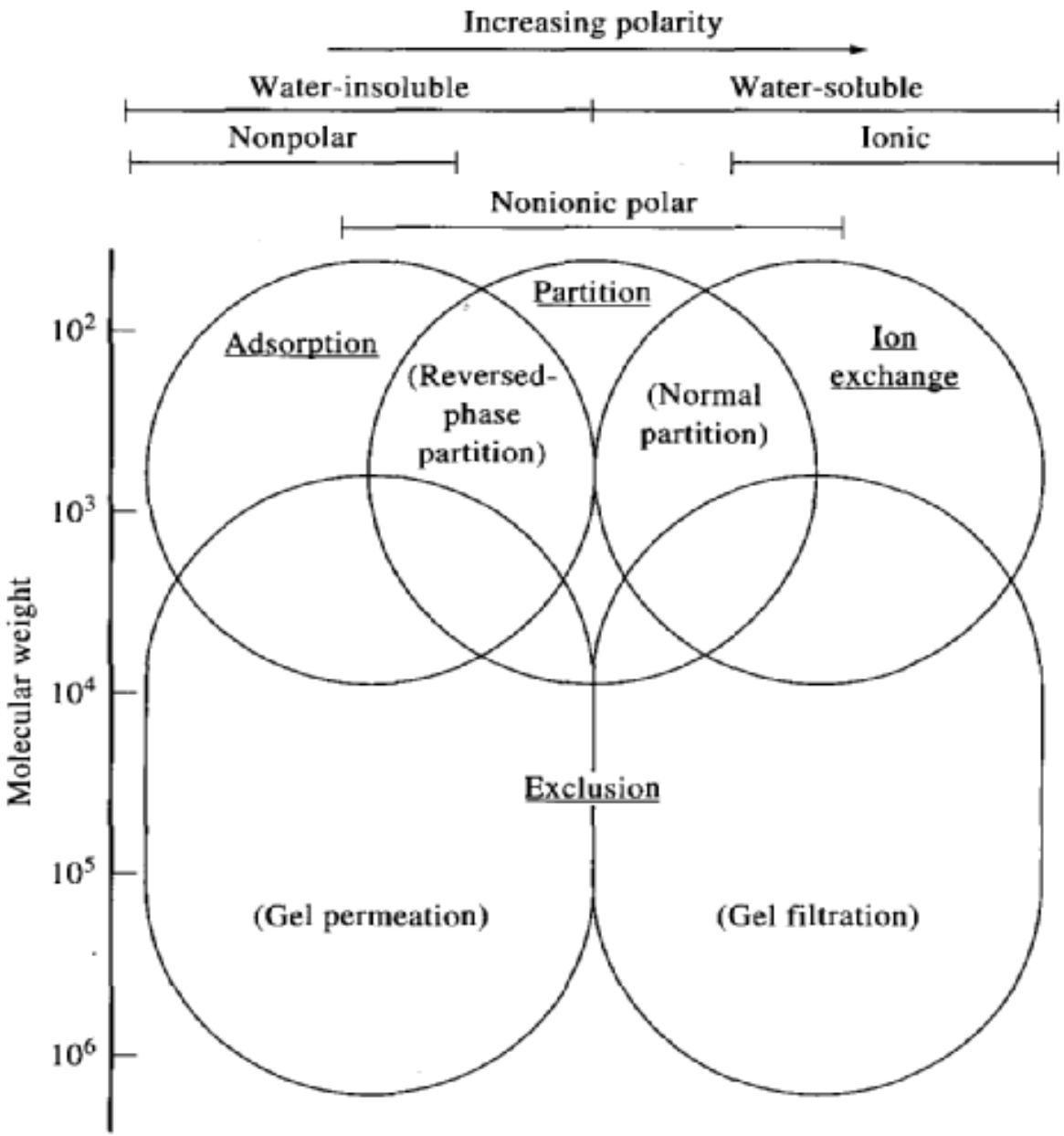
ion exchange

relative solute size (Exclusion)

Affinity

Five modes in HPLC

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



Applications of liquid chromatography.

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- **For solutes having molecular weight greater than 10 ,000, Exclusion chromatography is often used.**

- **For lower – molecular –weight ionic species, ion-exchange chromatography is widely used.**

- **Small polar but nonionic species are best are best handled by partition methods.**

Partition Chromatography

- The concentration of the solute in the mobile phase is inversely proportional to the distribution coefficient of the solute with respect to the stationary phase:

$$K = \frac{X_s}{X_m}$$

K= Distribution Coefficient

X_s= Concentration of the solute in the stationary phase.

X_m= Concentration of the solute in the mobile phase.

Molecular Interactions

- Molecular interactions are the direct effect of intermolecular forces that occur between the solute and solvent molecules.
- There are four basic types of molecular force that can control magnitude of the distribution Coefficient of a solute between two phases. Theses forces are :

1- Chemical Forces

2- Ionic Forces

3- Polar Forces

4- Dispersive Forces

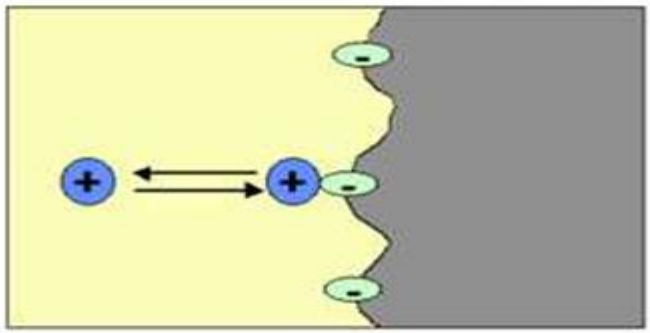
Chemical Forces

- Chemical forces are normally **irreversible** in nature (at least in Chromatography) and thus, The distribution coefficient of the solute with respect to the stationary phase is **infinite** .**Affinity chromatography** is an example of the use of chemical forces in a separation process. The stationary phase is formed in such a manner that it will chemically interact with **one unique solute** present in the sample and thus, **exclusively extract** it form the other materials present. **The technique of affinity chromatography is, therefore ,an extraction process more than a chromatographic separation.**

Ionic Forces

- Ionic forces are electrical in nature and result from the net charge on an atom or molecule caused by ionization.
- Ionic interactions are exploited in ion chromatography
- In the analysis of organic acids ,it is the negatively charged acid anions that are separated.

The stationary phase has an ionically charged surface, opposite that of the eluents.



Polar Force

- Polar forces also arise from electrical charges on the molecule but in this case from permanent or induced dipoles. Example of substances with permanent dipoles are alcohols, esters , aldehyeds and so on.
- In practice, silica gel is a highly polar stationary phase.

Dispersive Forces

- Dispersive forces ,although electric in nature, result from random charge fluctuations rather than permanent electrical charges on the molecule. Example of purely dispersive interactions are the molecular forces that exist between saturated aliphatic hydrocarbon molecules.
- Saturated aliphatic hydrocarbons are not ionic, have no permanent dipoles, and are not polarizable.
- n-heptan is not a gas , but a liquid .This is a result of the collective effect of all the dispersive interactions that hold the molecules together as a liquid.

Dispersive Force

- To retain solutes solely by dispersive interactions , the stationary phase must contain no polar or ionic substances

The Thermodynamic Explanation of Retention

Solute retention can also be explained on a thermodynamic basis by considering the change in free energy that occurs when a solute is moved from the environment of one phase to that of the other. The classic thermodynamic expression for the distribution coefficient K of a solute between two phases is given by

$$RT \ln K = -\Delta G_0$$

where R is the gas constant, T is the absolute temperature, and ΔG_0 is the excess free energy.

Now,

$$\Delta G_0 = \Delta H_0 - T\Delta S_0$$

where ΔH_0 is the excess free enthalpy, and ΔS_0 is the excess free entropy.

Thus,

$$\ln K = -\left(\frac{\Delta H_0}{RT} - \frac{\Delta S_0}{R}\right)$$

or,

$$K = e^{-[(\Delta H_0/RT)-(\Delta S_0/R)]}$$

Effect of Particle Size of Packing

- Result :

  The efficiency of an HPLC column should improve dramatically as the particle size is decreased.

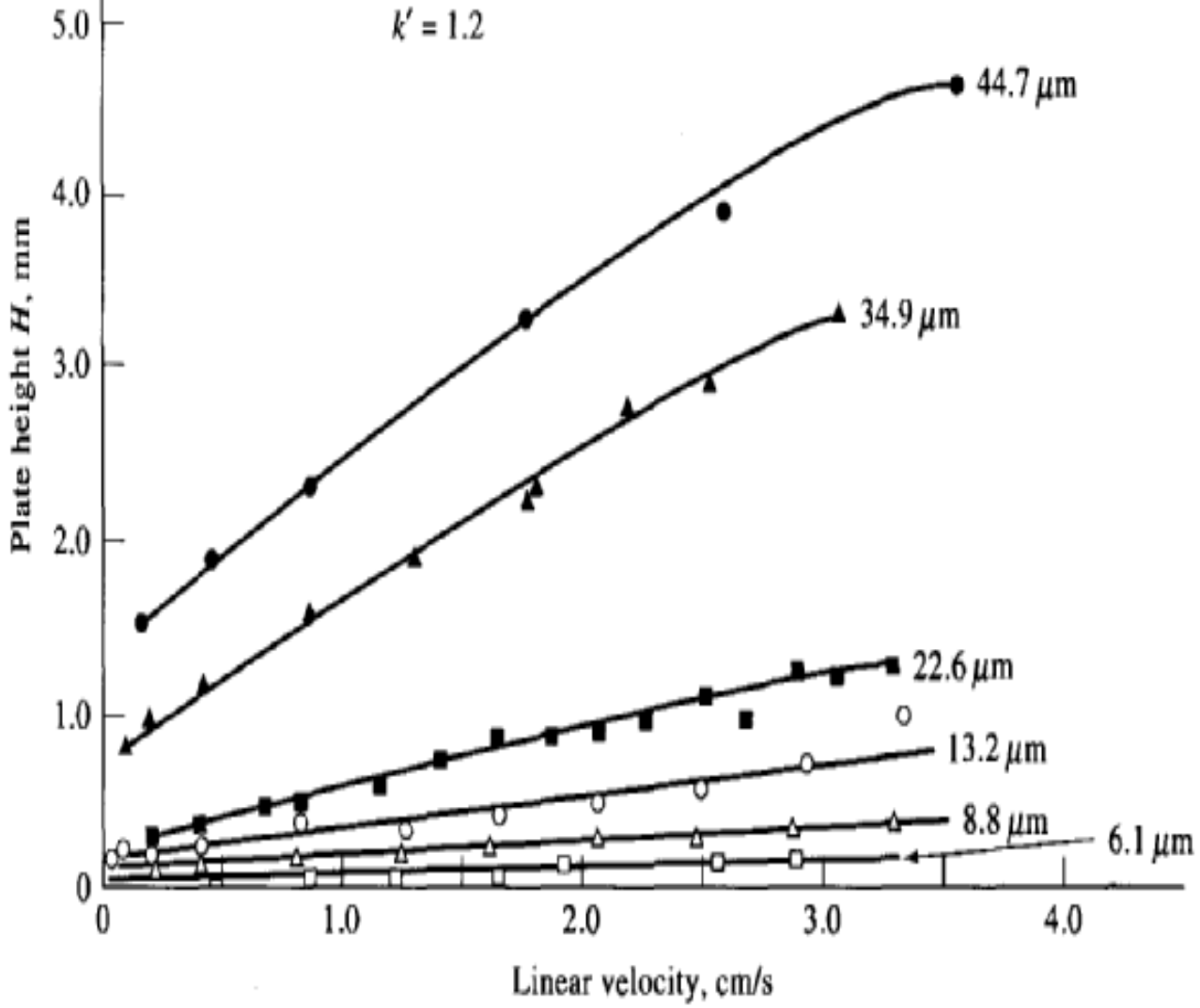
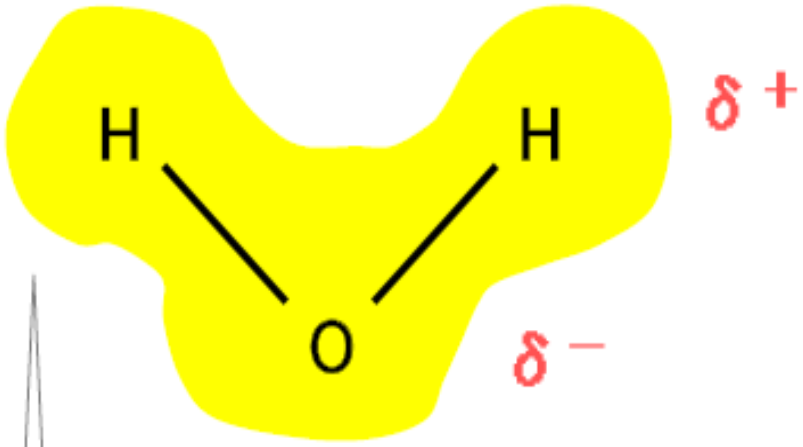


Figure 28-2 Effect of particle size of packing and flow rate upon plate height H in liquid chromatography. Column dimensions: 30 cm × 2.4 mm. Solute: N,N-diethyl-*n*-aminoazobenzene. Mobile phase: mixture of hexane, methylene chloride, isopropyl alcohol. *(From R. E. Majors, J. Chromatogr. Sci., **1973**, 11, 92. With permission.)*

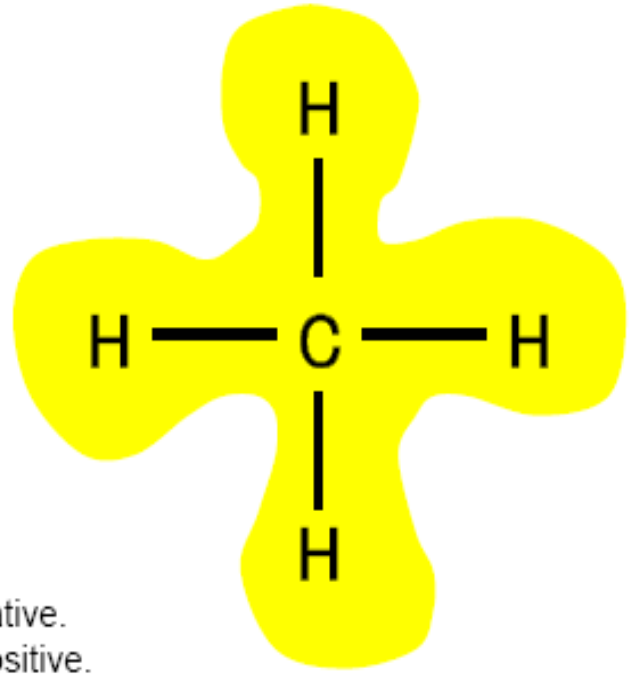
eparation mode

Polar compounds

Polar compound



Non-polar compound



Bonding electrons are not shared evenly.
The end of the bond with electrons becomes partially negative.
The end of the bondwithout electrons becomes partially positive.

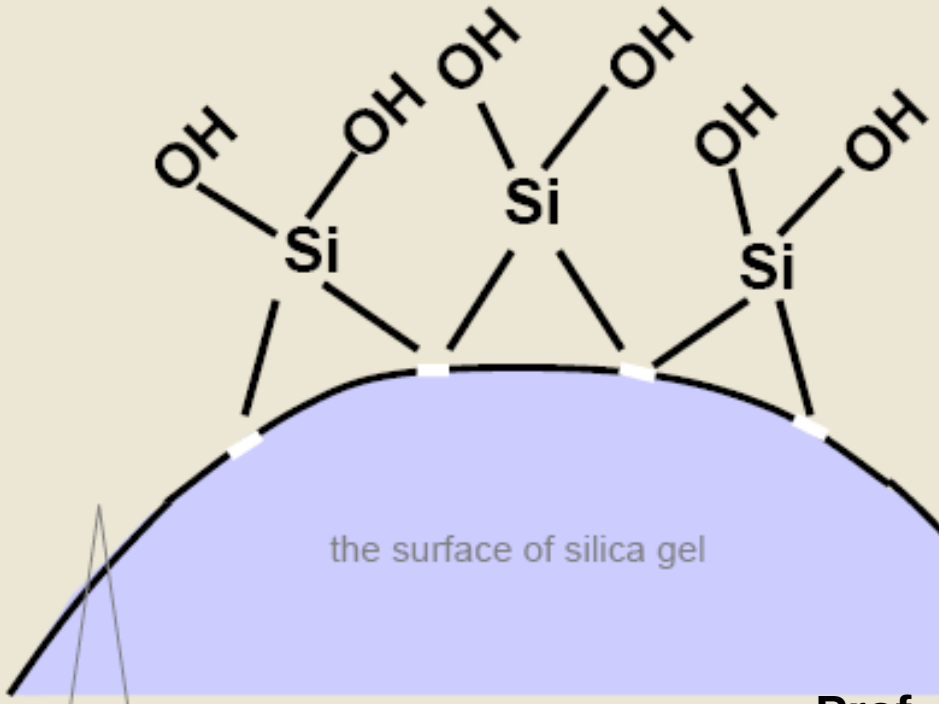
Polar compounds are soluble in polar solvents.
Non-polar compounds are soluble in non-polar solvents.

Separation mode

Normal Phase Chromatography

Packing material

The most popular packing material is silica gel.
It is believed that silanol radicals (-Si-OH) on the surface of silica gel act as the active site and the sample is separated.



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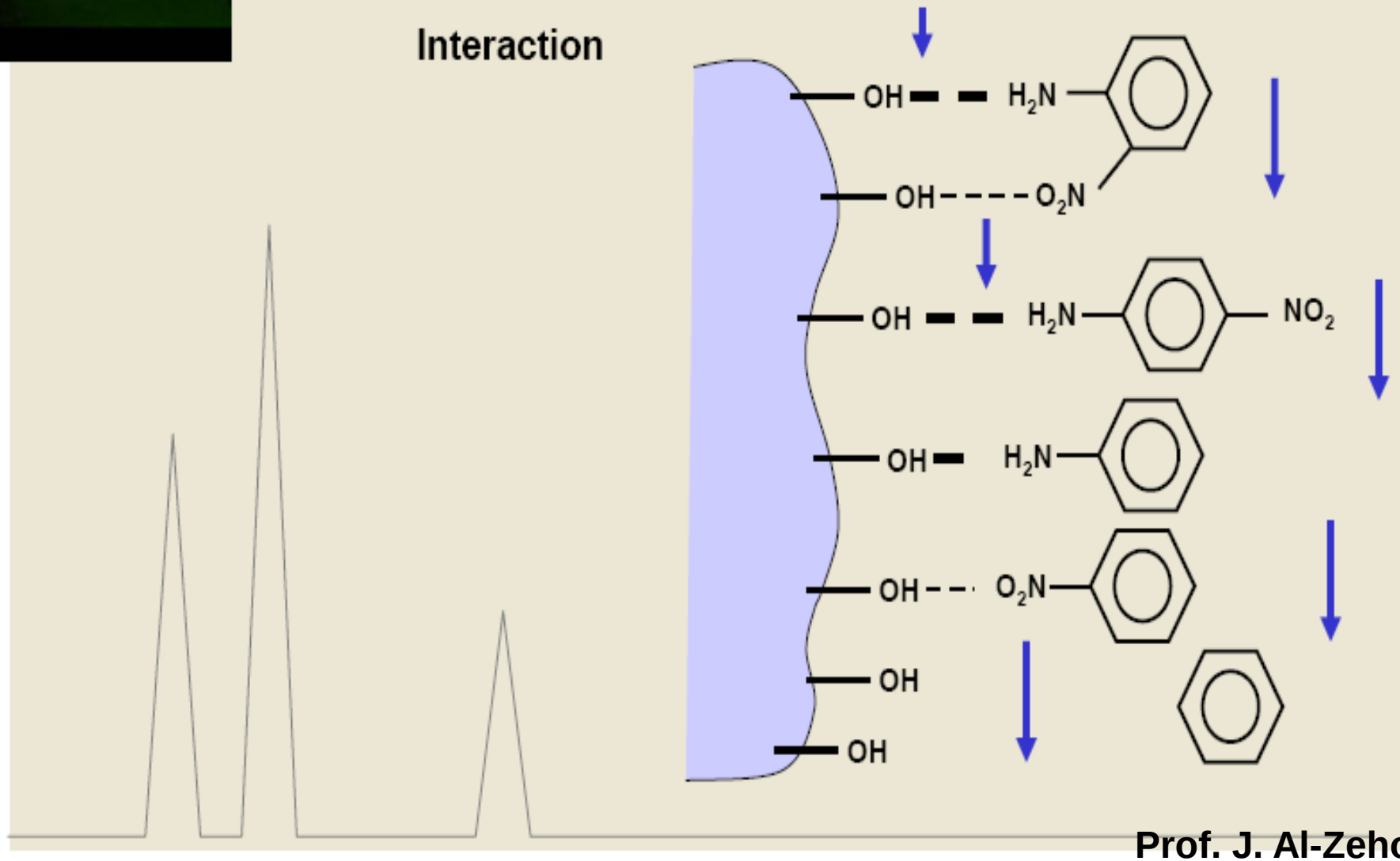
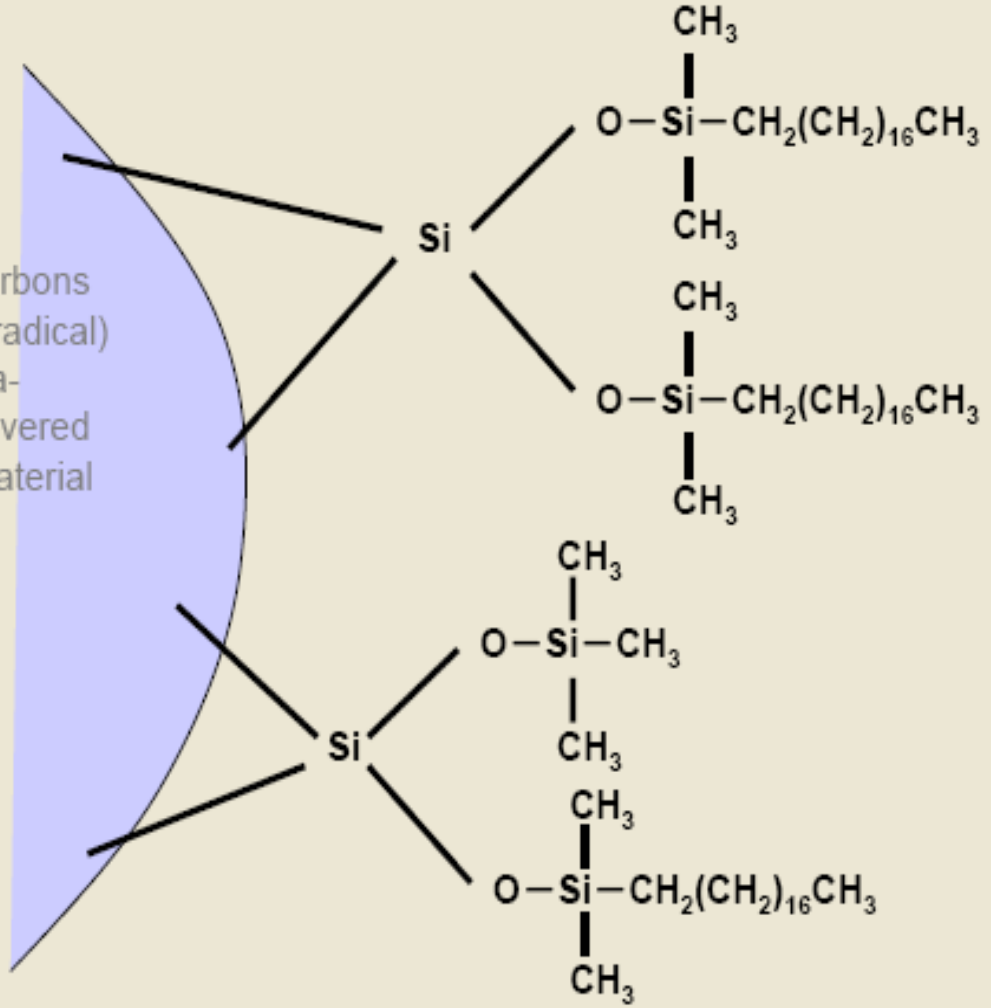
Normal Phase Chromatography



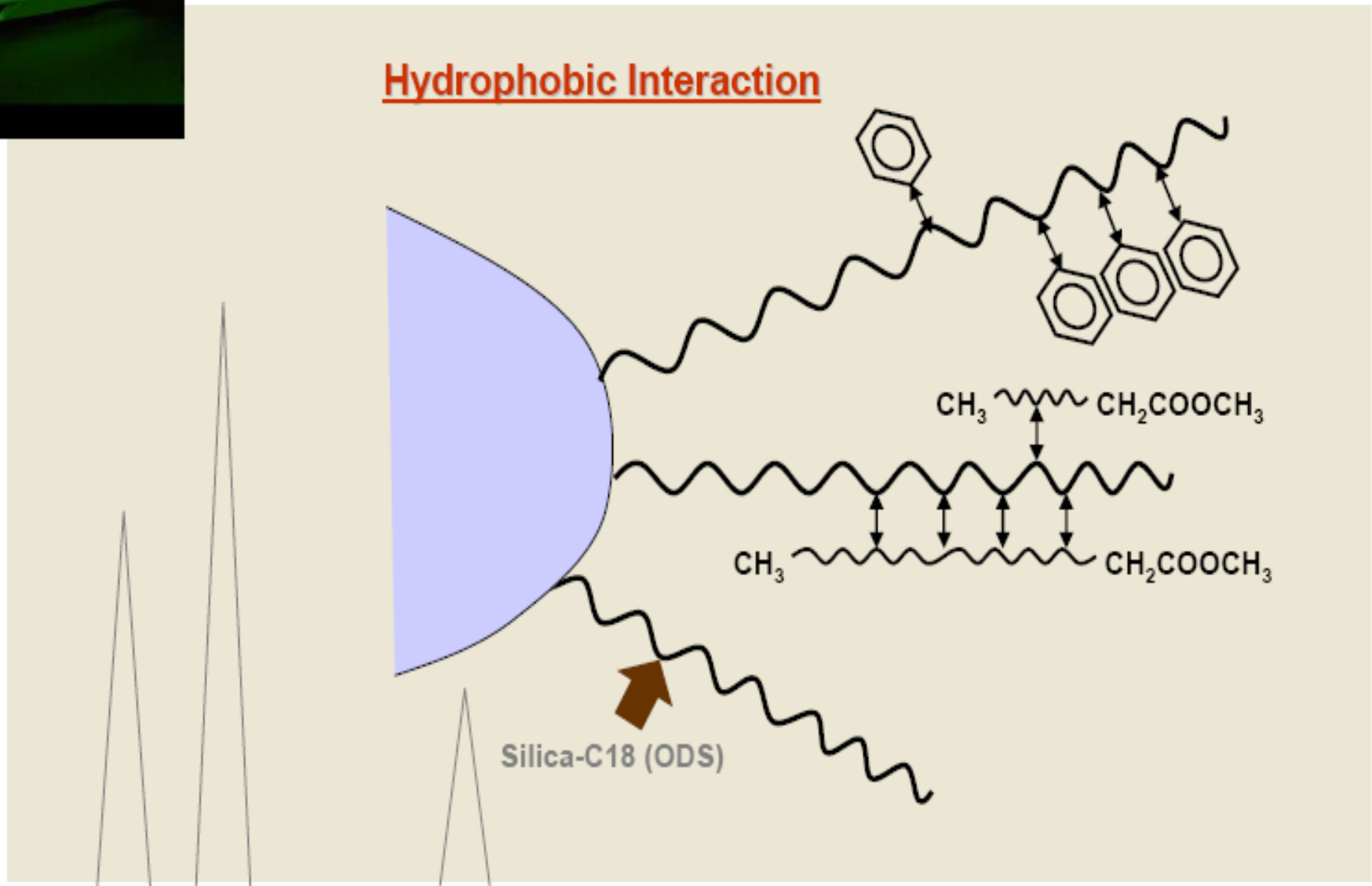
Reversed Phase Chromatography

Silica-C18 Packing materials

Commonly used packing materials are hydrocarbons having 18 carbon atoms (called the Octadecyl radical) which are chemically bonded to silica gel (Silica-ODS).Since the surface of the Silica-ODS is covered with hydrocarbon, the polarity of the packing material itself is very low.



Reversed Phase Chromatography



Normal Phase Chromatography

Mobile phase solvents

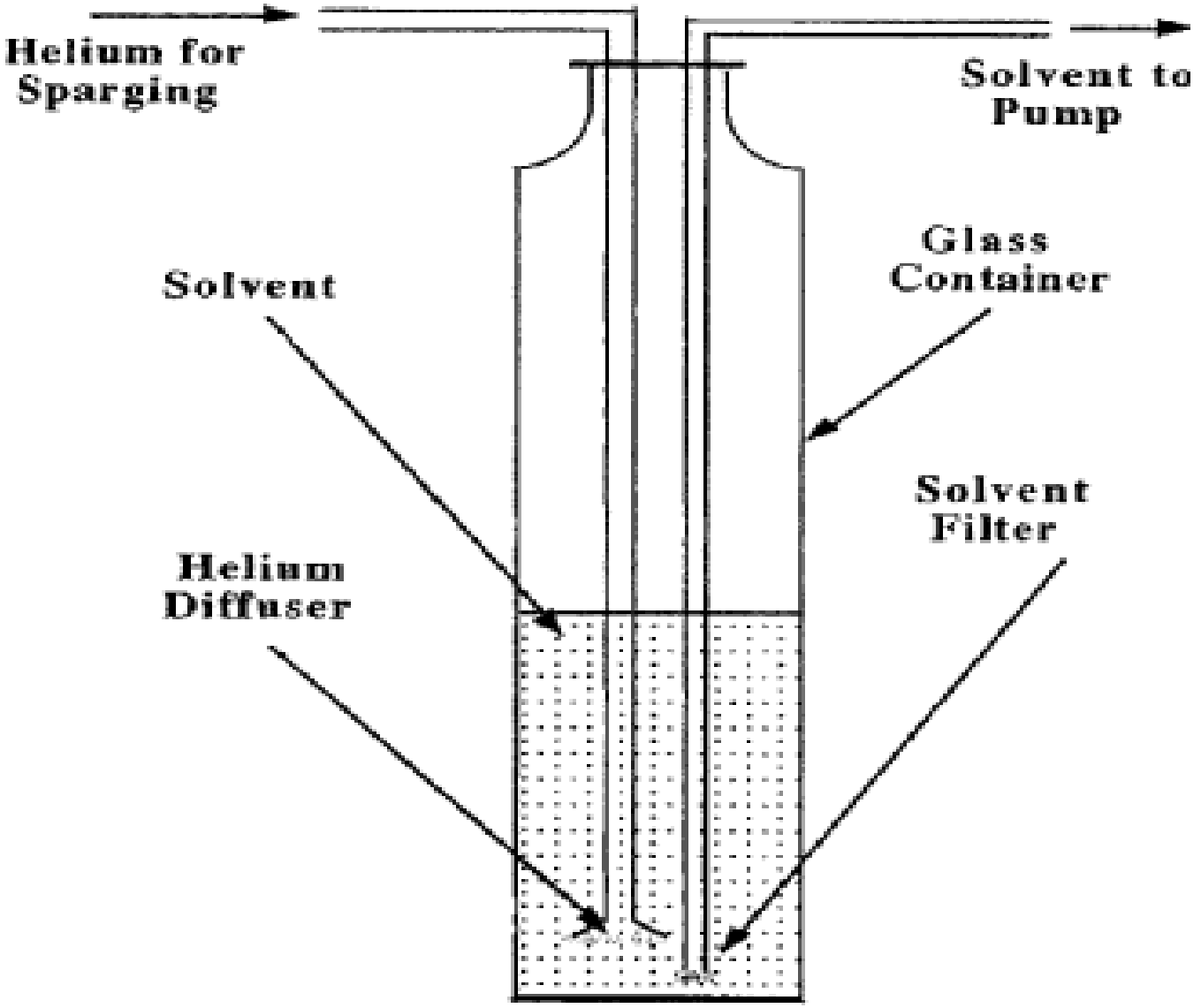
n-Hexane	(n-Hex)
iso-Octane	(iso-Oct)
Chloroform	($CHCl_3$)
Dichloromethane	(CH_2Cl_2)
Ethylacetate	(AcOEt)
Isopropylalchol	(IPA)
Tetrahydrofran	(THF)
Dioxane	
Acetonitrile	(CH_3CN)
Ethanol	(EtOH)
Methanol	(MeOH)
Amines	
Acids	

Mobile-Phase Reservoirs and Solvent Treatment System

- Solvent reservoirs were originally made of either glass or stainless steel ,but glass is now the preferred material as stainless steel tends to corrode in the presence of certain buffers and dilute chloride solutions.
- All solvent mixtures that are to be used as a mobile phase should be filtered before placing in the reservoir but, as an extra precaution, it is wise to have a filter placed at the inlet to the tube that leads to the pump .A 10 -20 µm filter is usually adequate.

- One of the problems met in (HPLC) is caused by air(N_2,O_2) dissolved in the mobile phase which can evolve inside the column forming bubbles .These bubbles produce serious and completely unacceptable noise as they pass through the detector.
- The dissolved air can be removed by bubbling helium through the solvent in the reservoir(sparging) or by vacuum pumping or by a distillation system
- The Procedure called (Degasses).

High-Performance Liquid Chromatography



Solvent reservoir.

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Solubilities of Gases in Water

Gas	Solubility (mL/100 mL of cold water) (15°C)
Helium	0.94
Nitrogen	2.37
Oxygen	4.89

It is seen from the table that Oxygen is the gas with the highest solubility in water but the solubility of helium is much smaller than either that of nitrogen or that of oxygen. The continuous stream of helium leaches out all the dissolved air and the residual dissolved helium appears to cause no problem in the column or detector.

- It should be remembered ,however ,that if the sparging is stopped and the solvent comes again in contact with air, the solvent will rapidly become saturated with oxygen and nitrogen again.
- Other degassing procedure (vacuum or heating) are not recommended.

Solvents

All solvents should be 'HPLC' grade.

This is a type of reagent grade material.

It has been filtered using a 0.2 µm filter.

You can purchase it or produce it yourself.

Filtered solvent helps extend pump life by preventing scoring. It also reduces the chances of a column plugging.

Solvent degassing

All solvents should be degassed prior to use. This reduces the chances of bubbles being formed in the column or detector. Oxygen present at high pressure can also cause a problem.

Methods that can be used

- → Displacement with a less soluble gas
- → Applying a vacuum
- → Heating the solvent.

Gradient elution method

Solvent Programmers

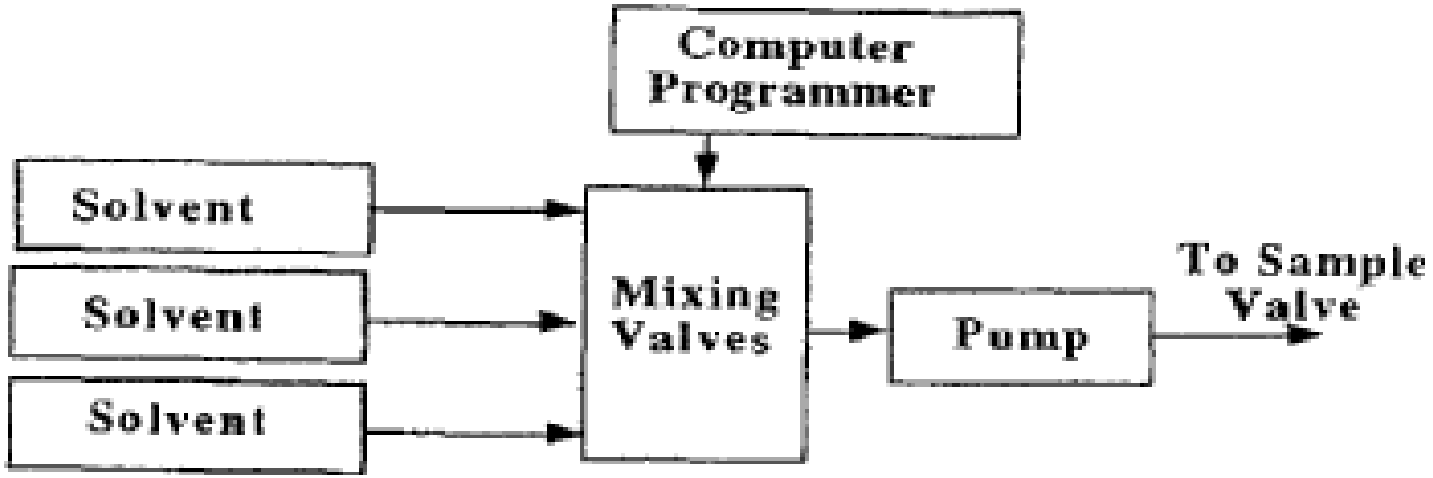
:

A separation that employs a single solvent of constant composition is termed an isocratic elution.

Here two or three solvent systems that differ significantly in polarity are employed which done with low pressure or high pressure solvent program.

1. The Low Pressure Solvent Program :

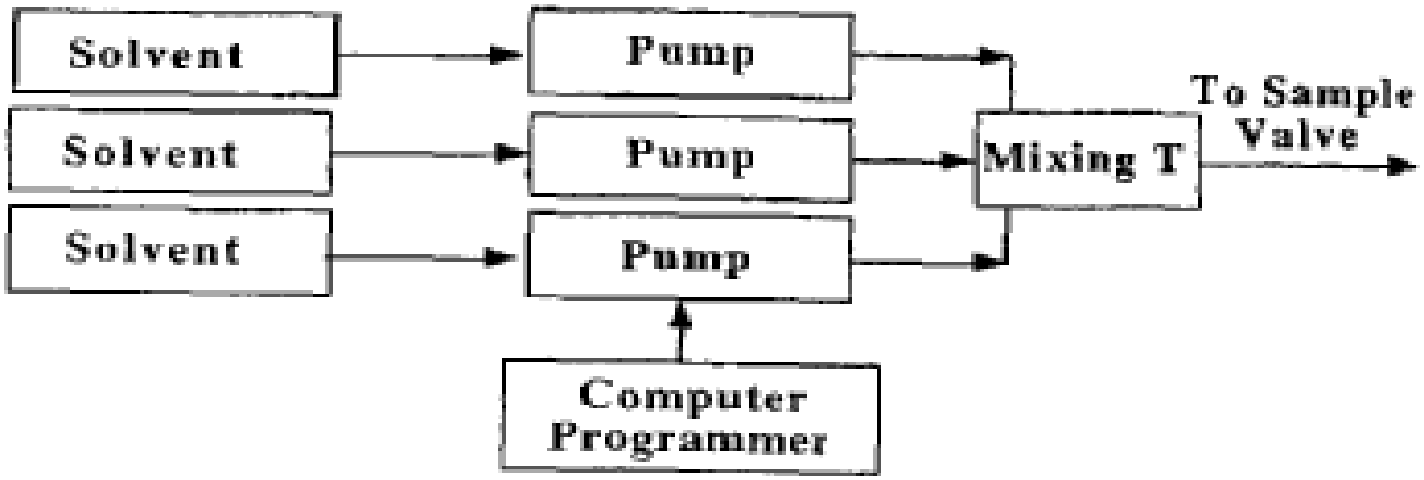
Consists of three solvent reservoirs(or more if required), each connected to a valve which is controlled by a computer.



The low pressure solvent programer.

1. The High Pressure Solvent Programmers

High pressure solvent programmers utilize a pump for each solvent which is independently controlled by the computer.



The high pressure solvent programer.

Pumping Systems

- The requirements for an HPLC pumping system are severe and include:

  1- The generation of pressures .(6000-10000 psi

  2- Pulse –free output

  3- flow rates raining from 0.1 to 10 ml/min.

  4- flow control

  5- corrosion-resistant components

1. Reciprocating Pumps المضخة التبادلية

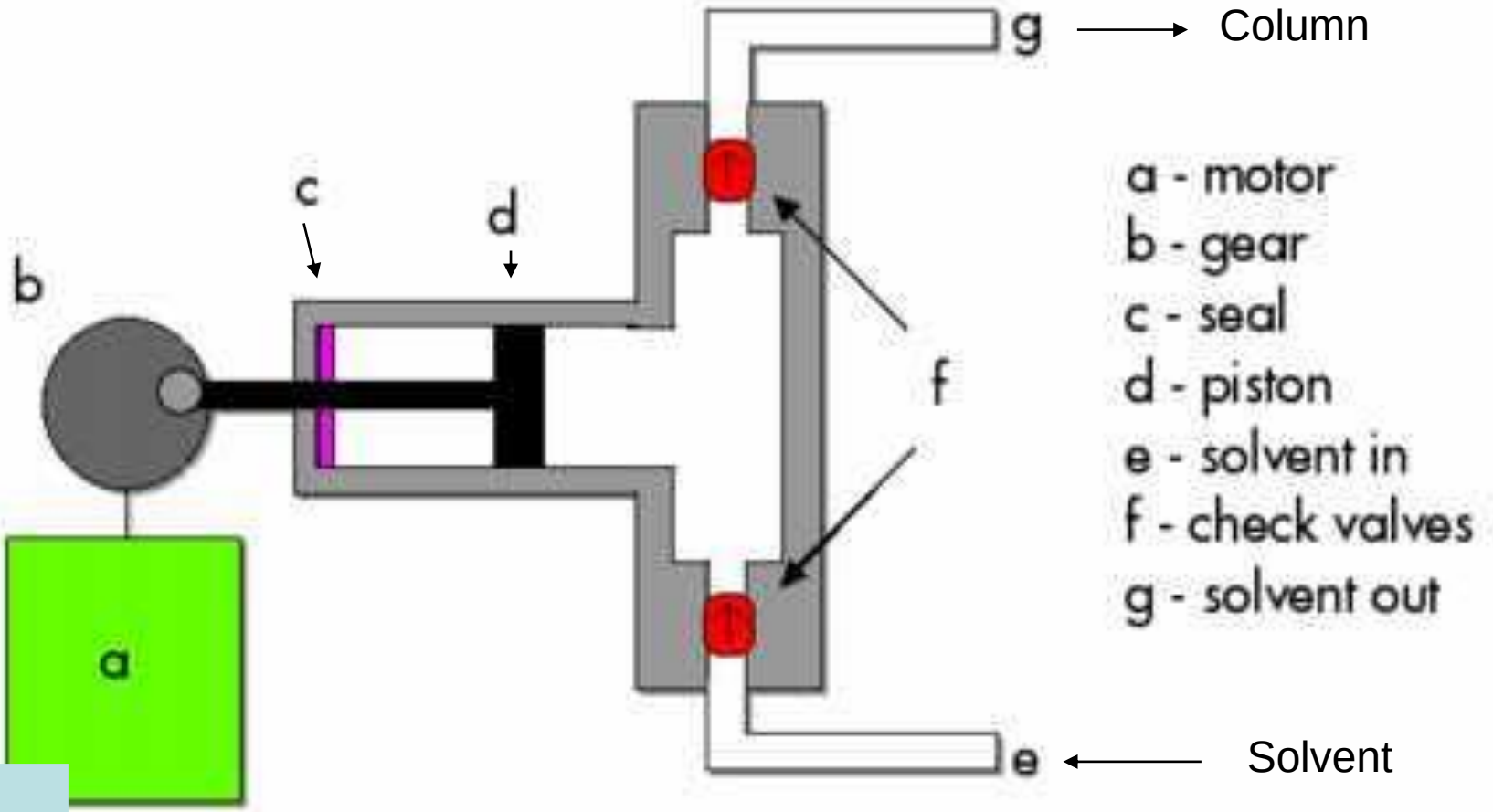
- 90% of the commercially available HPLC used this system.
- Usually consist of a small chamber in which the solvent is pumped by the back and forth motion of a motor-driven piston.
- Two ball check ,which open and close alternately, control the flow of solvent in to and out of a cylinder.
- The solvent is in direct contact with the piston.

Types of pumps

- Reciprocating pumps have the disadvantage of producing a pulsed flow ,which must be damped because its presence is manifested as base line noise on the chromatogram.
- The advantages of reciprocating pumps include their small internal volume (35-400µl) ,their high output pressures (up to 10,000 psi) ,their ready adaptability to gradient elution, and their constant flow rates ,which are largely independent of column backpressure and solvent viscosity.

Reciprocating pump

Single Piston

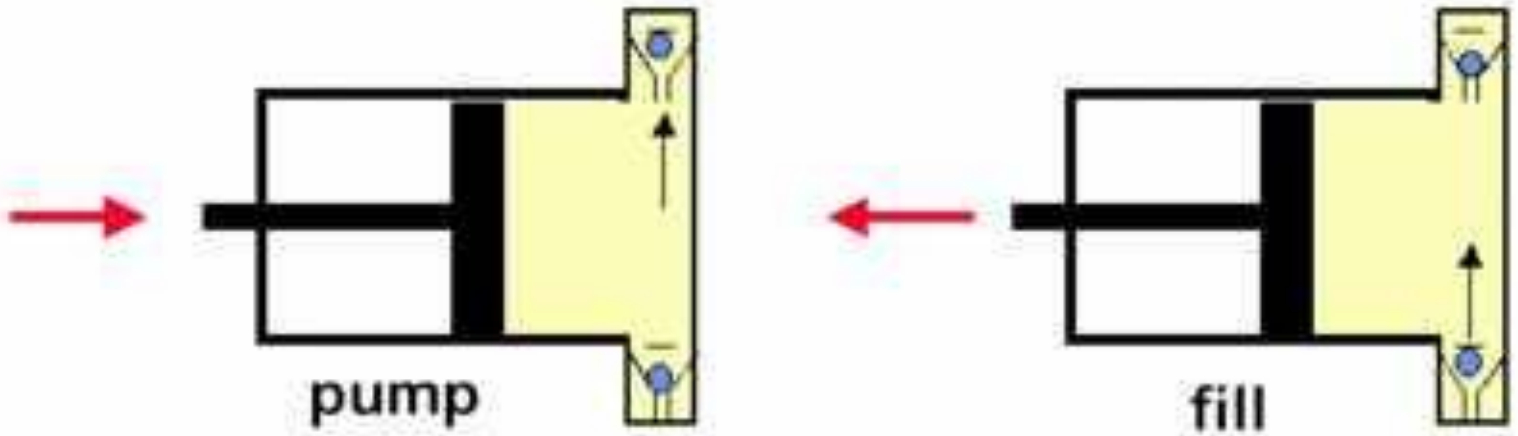


Reciprocating pump

One of the most common type of systems.

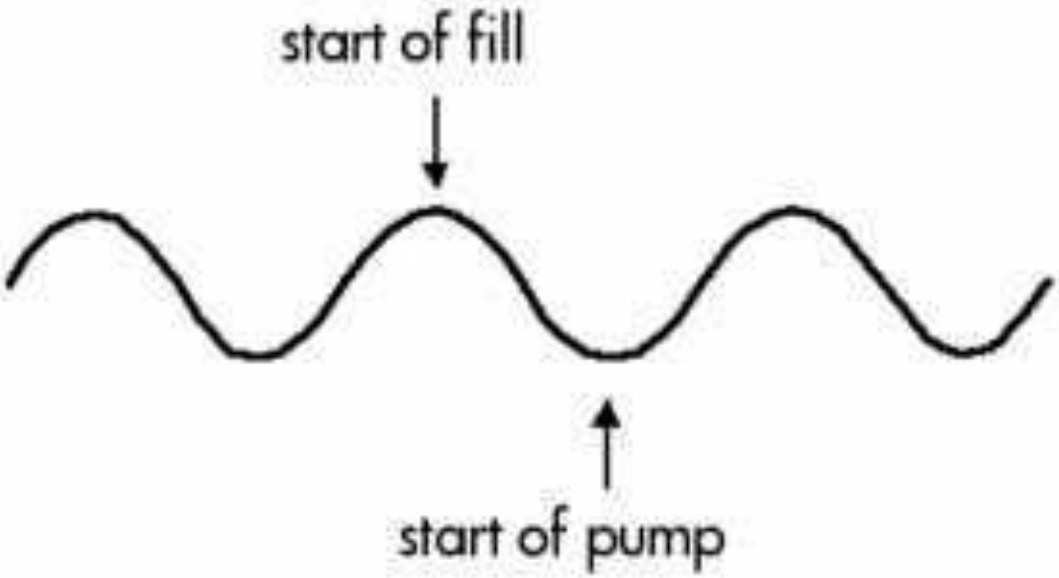
Unlimited reservoir system but expensive.

Another problem is that it produces variable pressure - must reverse stroke to refill.



Reciprocating pump

Since the pump must spend at least a portion of its time filling, the is a pressure drop during this phase.



This effect must be minimized or your peaks will all have pulses in them.

That would greatly affect your sensitivity and detection limit

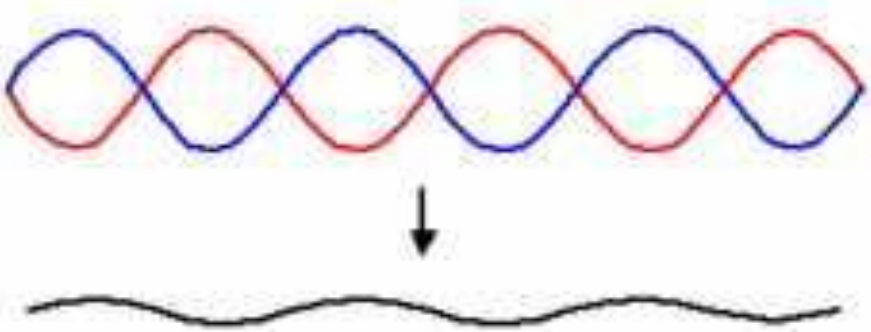
Reciprocating pump

One approach is to have a more rapid fill cycle compared to the pump cycle.

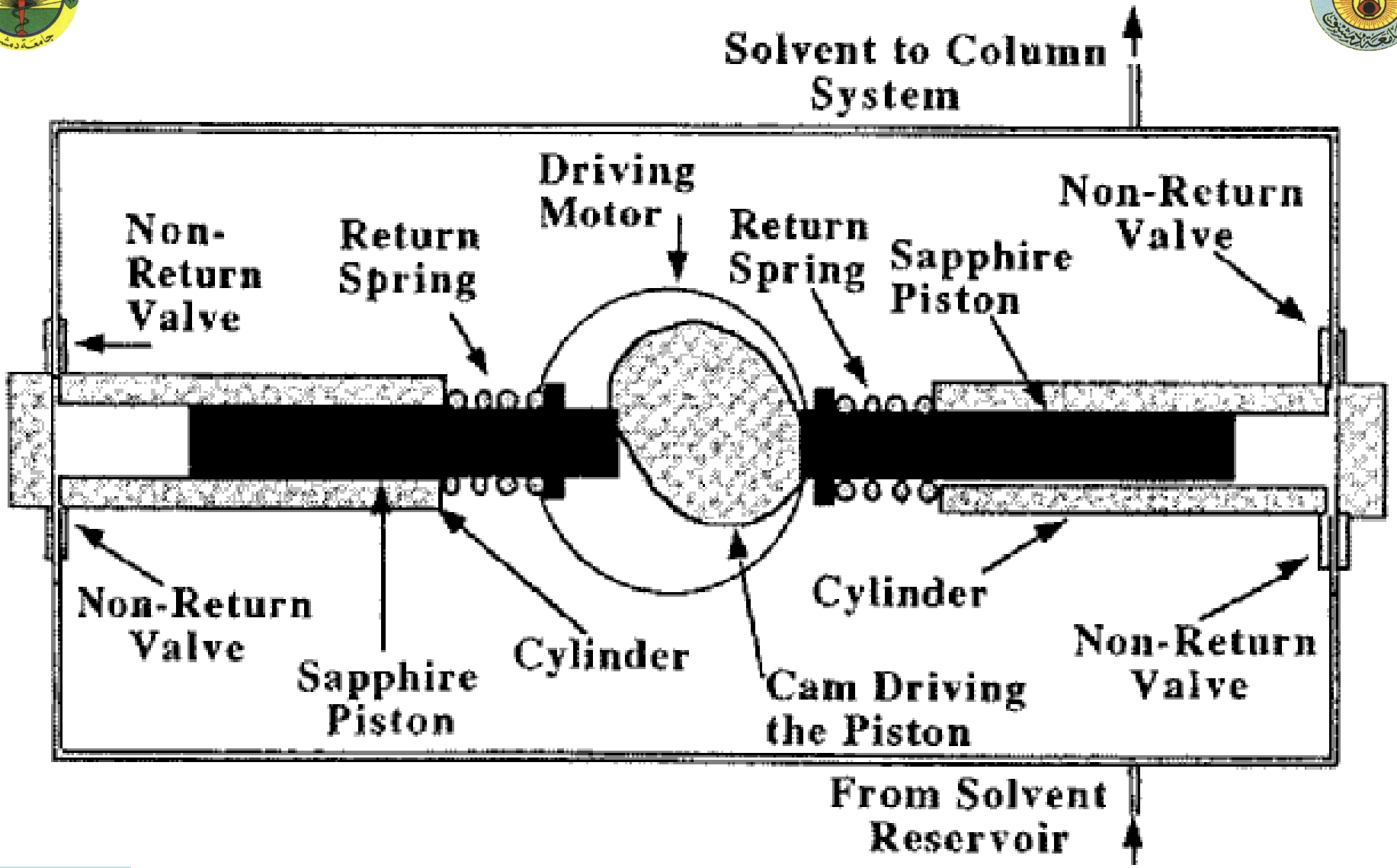
This does not eliminate the problem but does reduce it.

Reciprocating pump

One could also use two or more pumps working in tandem.



This is a more expensive option.

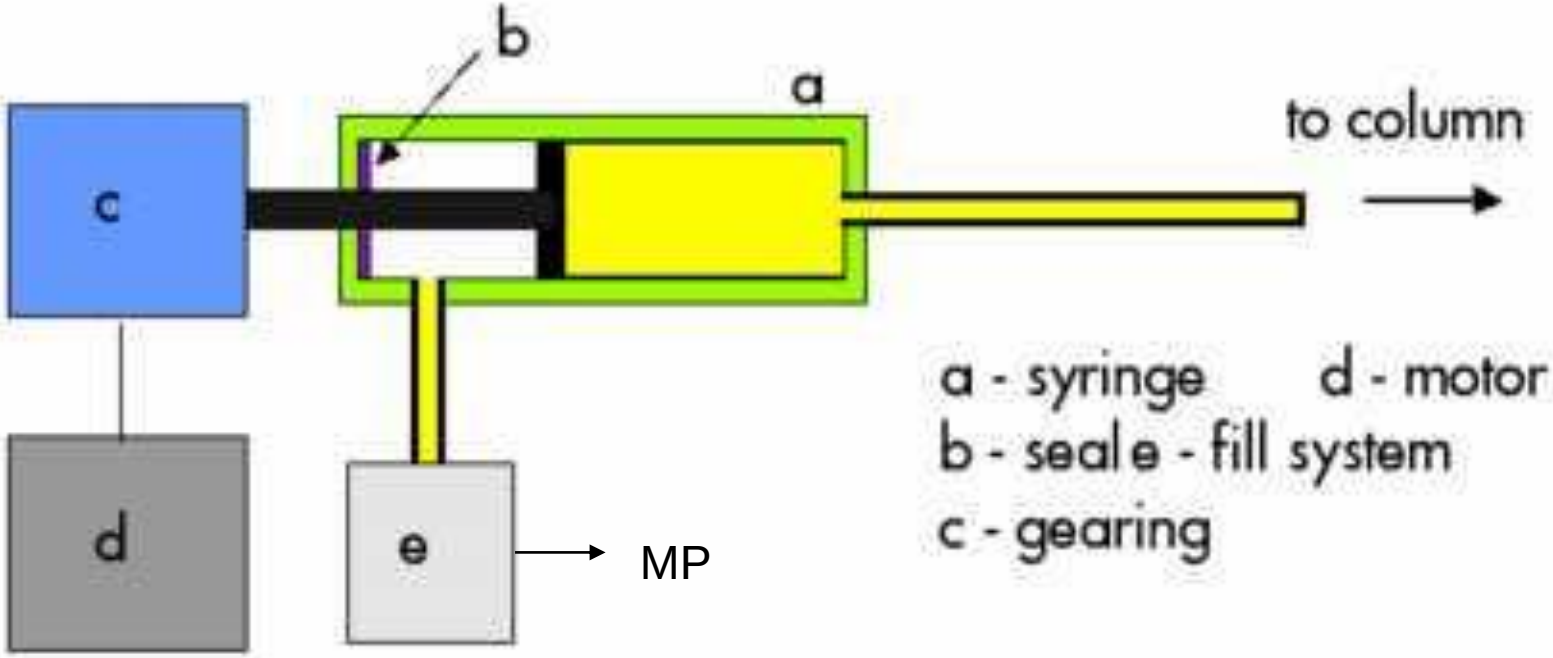


The dual piston reciprocating pump.

2. Displacement Pumps (Syringe pump)

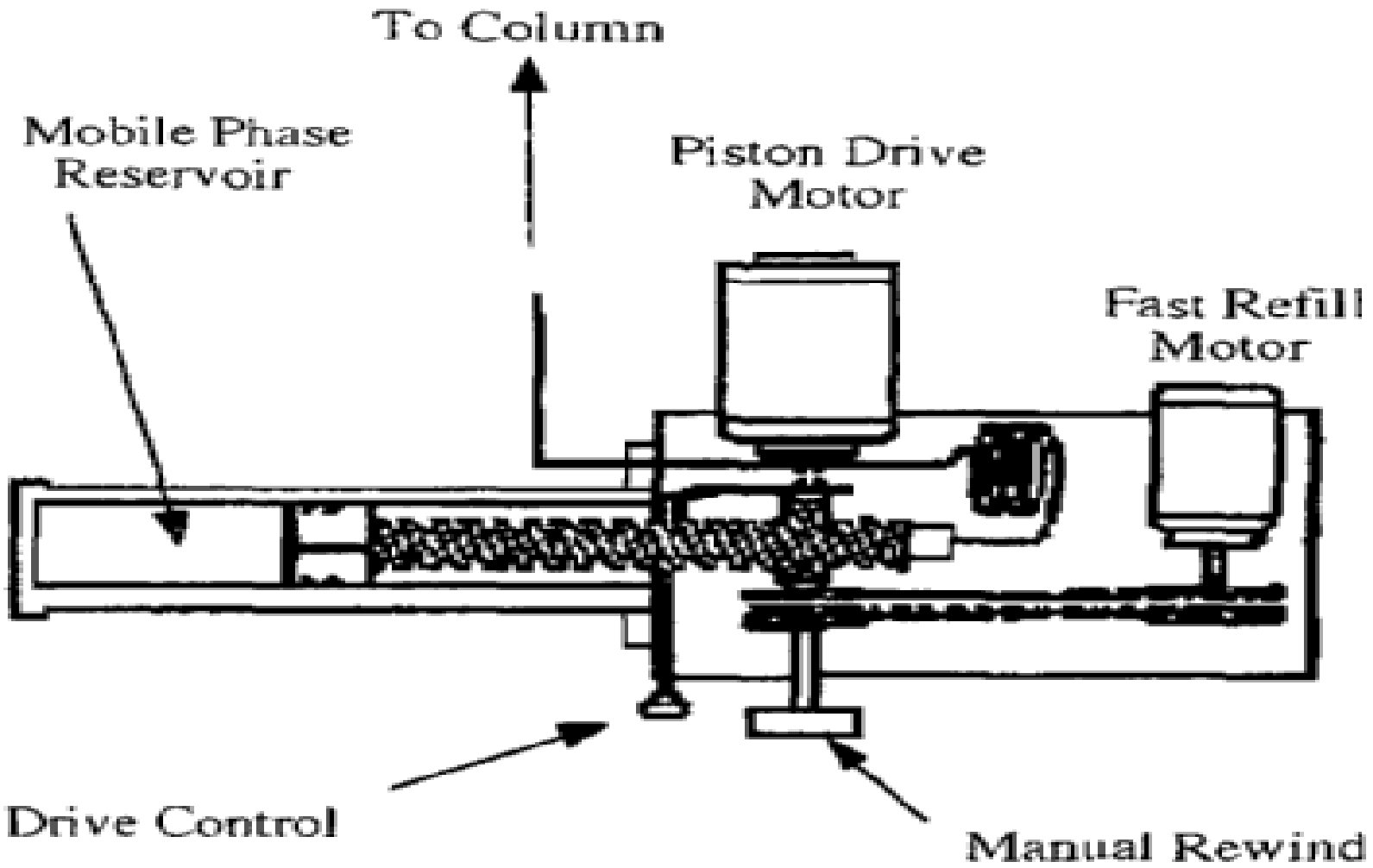
Displacement pumps usually consist of large, syringe like chambers equipped with a plunger that is activated by a screw-driven mechanism powered by a stepping motor. Displacement pumps also produce a flow that tends to be independent of viscosity and back pressure. In addition the output is pulse free. Disadvantages include limited solvent capacity(≈250 ml) and considerable inconvenience when solvent must be changed.

Motor driven syringe



Another non-pulsating system with a limited reservoir. Stepper motor/gear system allows for very fine flow control.

gh-Performance Liquid Chromatography



Courtesy of the Perkin Elmer Corporation

The syringe pump.

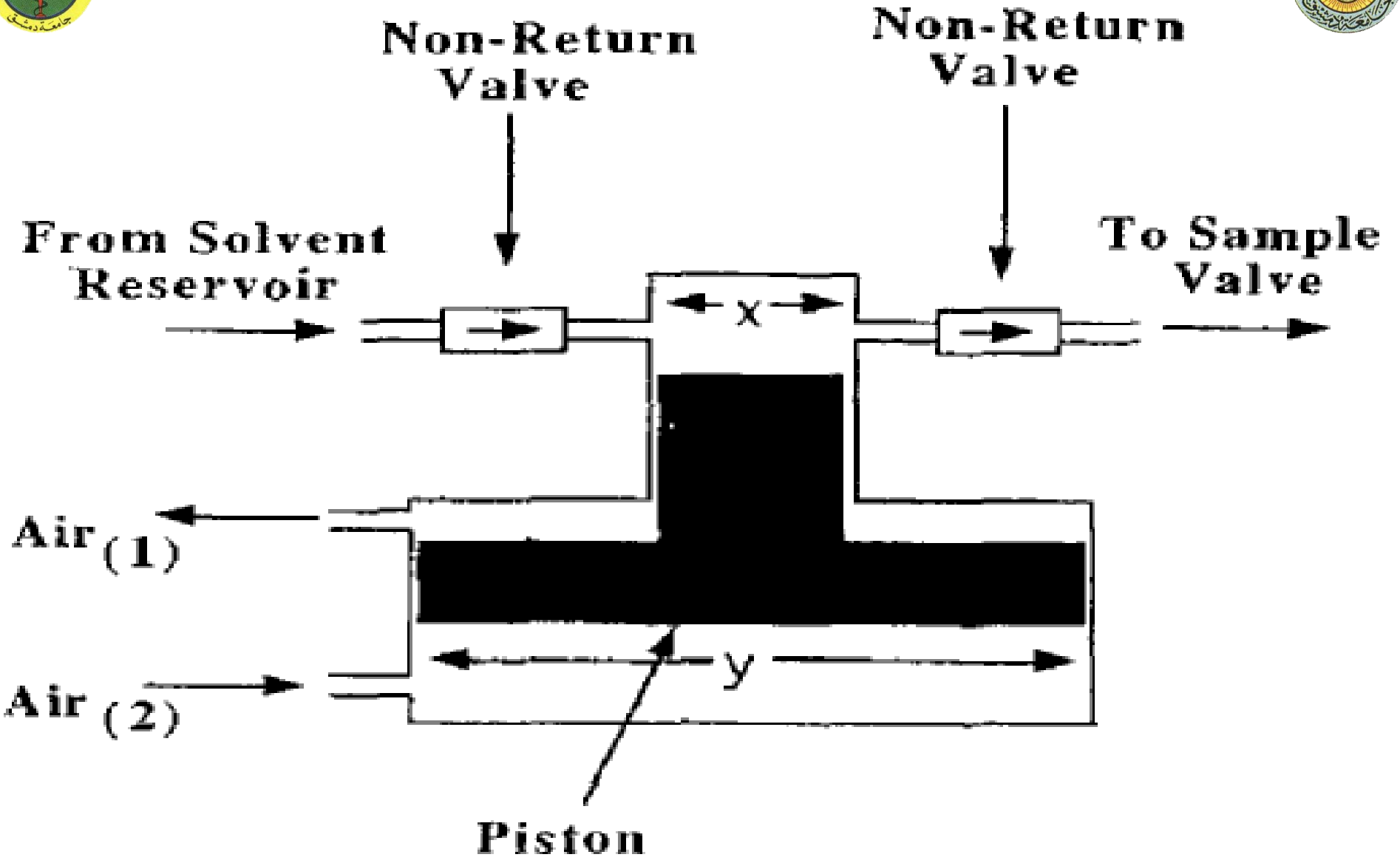
The Syringe Pump

It can provide only a limited pressure and the volume of mobile phase available is restricted to the pump volume.

The pump consist of a large metal syringe, the piston being rappelled by an electric motor and driven by a worm gear.

3- Pneumatic Pumps المضخة الهوائية

In the simplest pneumatic pumps , the mobile phase is contained in a collapsible container housed in a vessel that can be pressurized by a compressed gas. Pumps of this type are inexpensive and pulse free, they suffer from limited capacity and pressure output as well as a dependence of flow rate on solvent viscosity and column back-pressure. In addition, they are not amenable to gradient elution and are limited to pressures less than about 2000 psi.



The pneumatic pump.

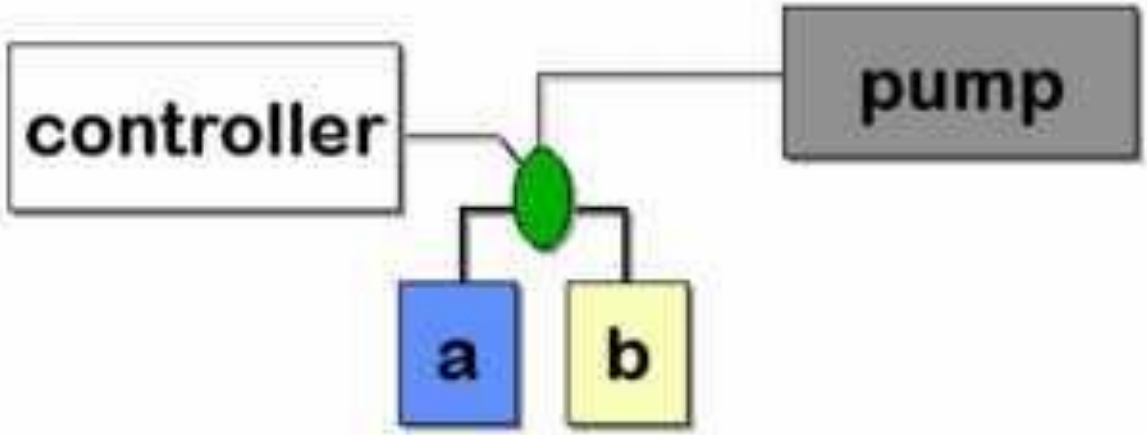
Constant flow

- Motor driven syringes
- Piston
- Reciprocating
- Multie reciprocating

Gradient controller

Single reciprocating pump systems

The gradient is produced by controlling a valve. The valve determines the relative amounts of each solvent pulled into the pump.



Gradient elution

Unlike GC, variations in temperature have minimal effect on an LC separation.

However, variations in solvent polarity can greatly affect retention.

This can be accomplished by altering the solvent mix during an analysis.

Gradient elution

Advantages of gradient elution

- total analysis time is reduced
- overall resolution of a mixture is improved
- better peak shapes are possible
- improved sensitivity

Gradients can result in baseline drift.

Common reverse phase solvents

All are

- low viscosity
- available in high purity
- UV transparent
- miscible in each other

Solvent classes

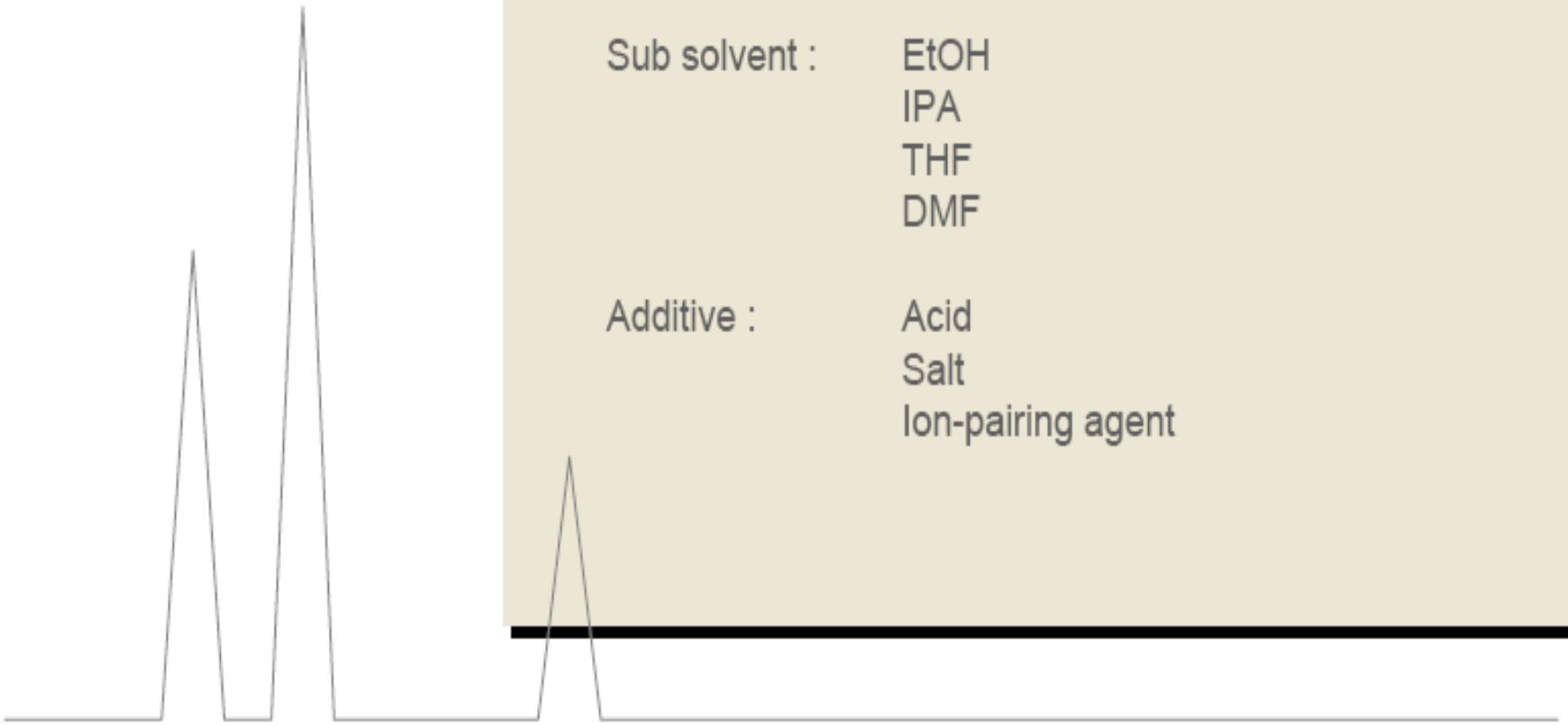
Not all solvents are truly usable.

- Can't be mixed at any proportion
- May interact chemically
- UV absorption or viscosity is too high
- Toxic, too flammable
- High vapor pressure
- Too expensive

Reversed Phase Chromatography

Mobile phase solvents

Main solvent :	MeOH - H2O
	CH3CN - H2O
Sub solvent :	EtOH
	IPA
	THF
	DMF
Additive :	Acid
	Salt
	Ion-pairing agent



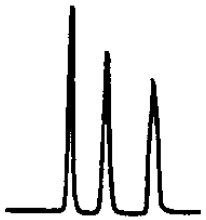
Mode selection

In general

- If sample is water insoluble or non-polar - use normal phase
- If sample is water soluble or not soluble but polar - use reverse phase.
- It's not always this cut and dry but represents a good starting point.

Effect of Solvent

75 % MeOH / 25 % H_2O **FAST**



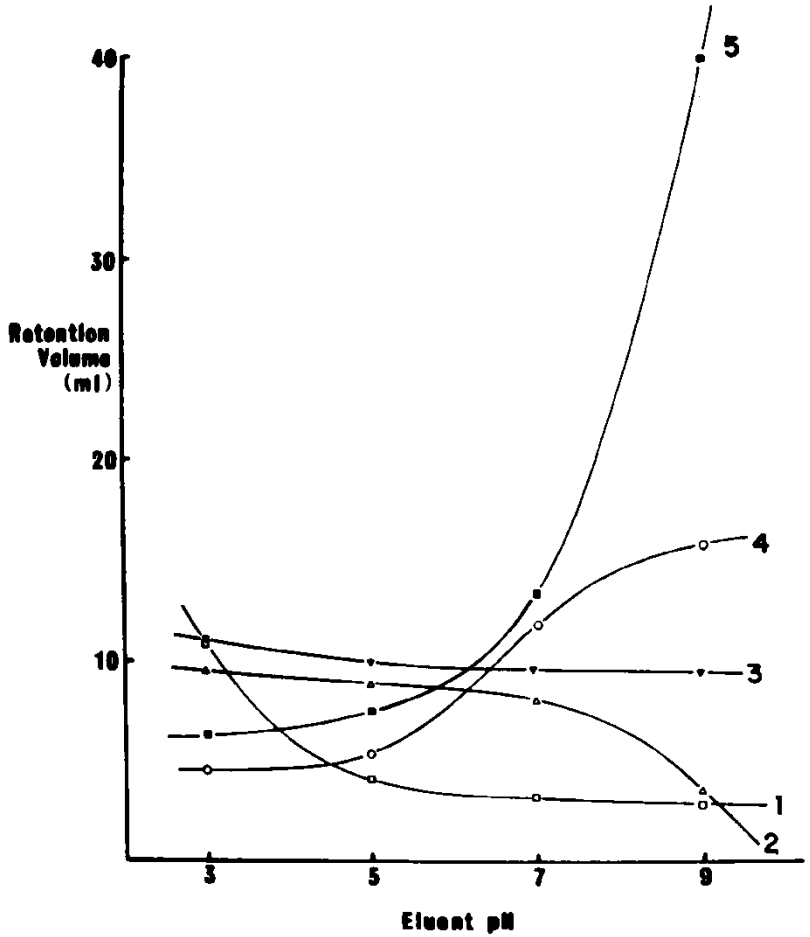
60 % MeOH / 40 % H_2O **SLOW**

50 % MeOH / 50 % H_2O **SLOWEST**

pH

- Affects ionizable compounds
  - organic acids
  - organic bases
- In reversed phase we need to suppress ionization as much as possible
- May need very precise pH control (buffer)

pH Effect on Retention



1. Salicylic acid
2. Phenobarbitone
3. Phenacetin
4. Nicotine
5. Methylampohetamine

30x0.4 cm C-18, 10 μm, 2 mL/min, UV 220 nm

Snyder and Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, 1979, p. 288.

1- Manually injection:

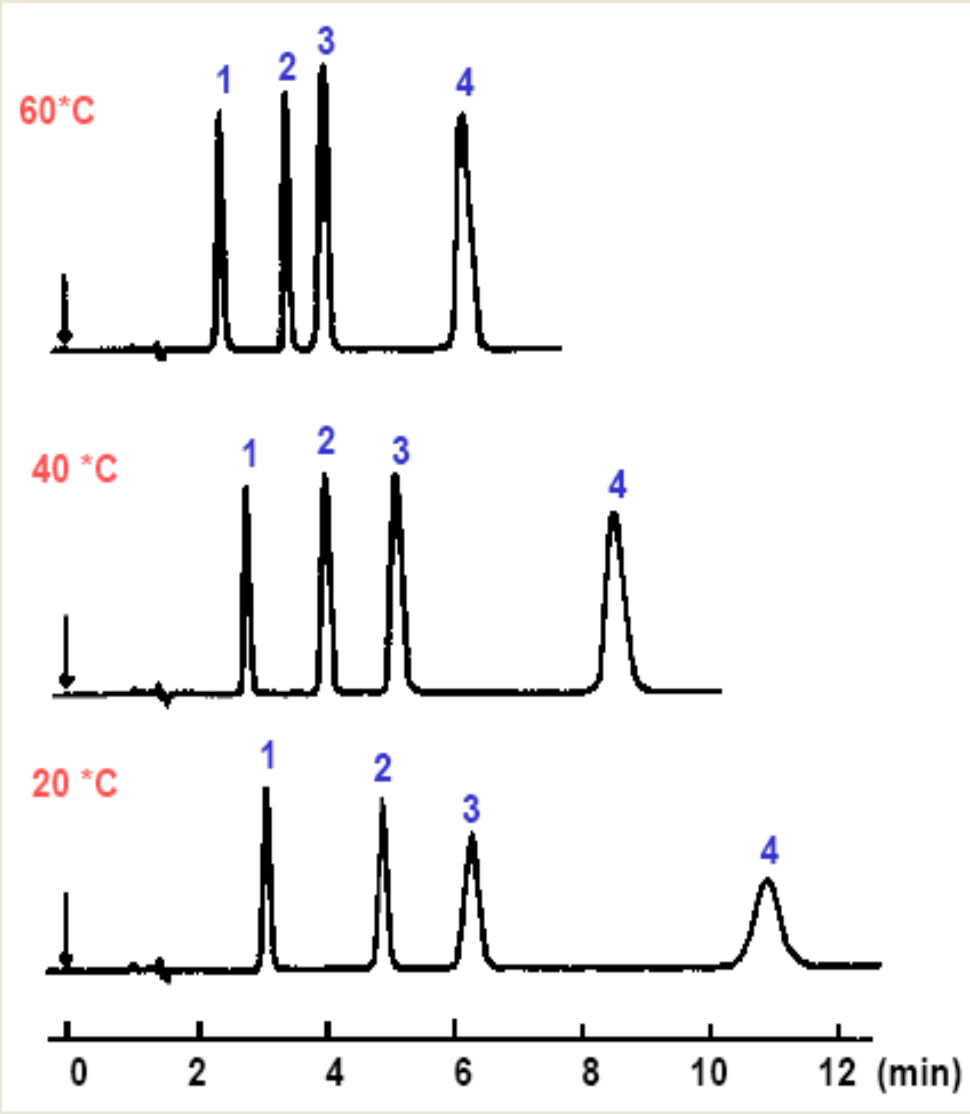
- micro syringes are used
- In stop-flow injection (the flow of solvent is stopped momentarily.
- A fitting at the column head is removed and the sample is injected directly on to the head of the column packing.
- After replacing the filling ,the system is a gain pressurized.
- Advantage is simplicity.
- Disadvantage the reproducibility is worse .

Temperature effect

زيادة درجة الحرارة تنقص من زمن الأحتباس

Gradient elution method

Effect of temperature on retention time



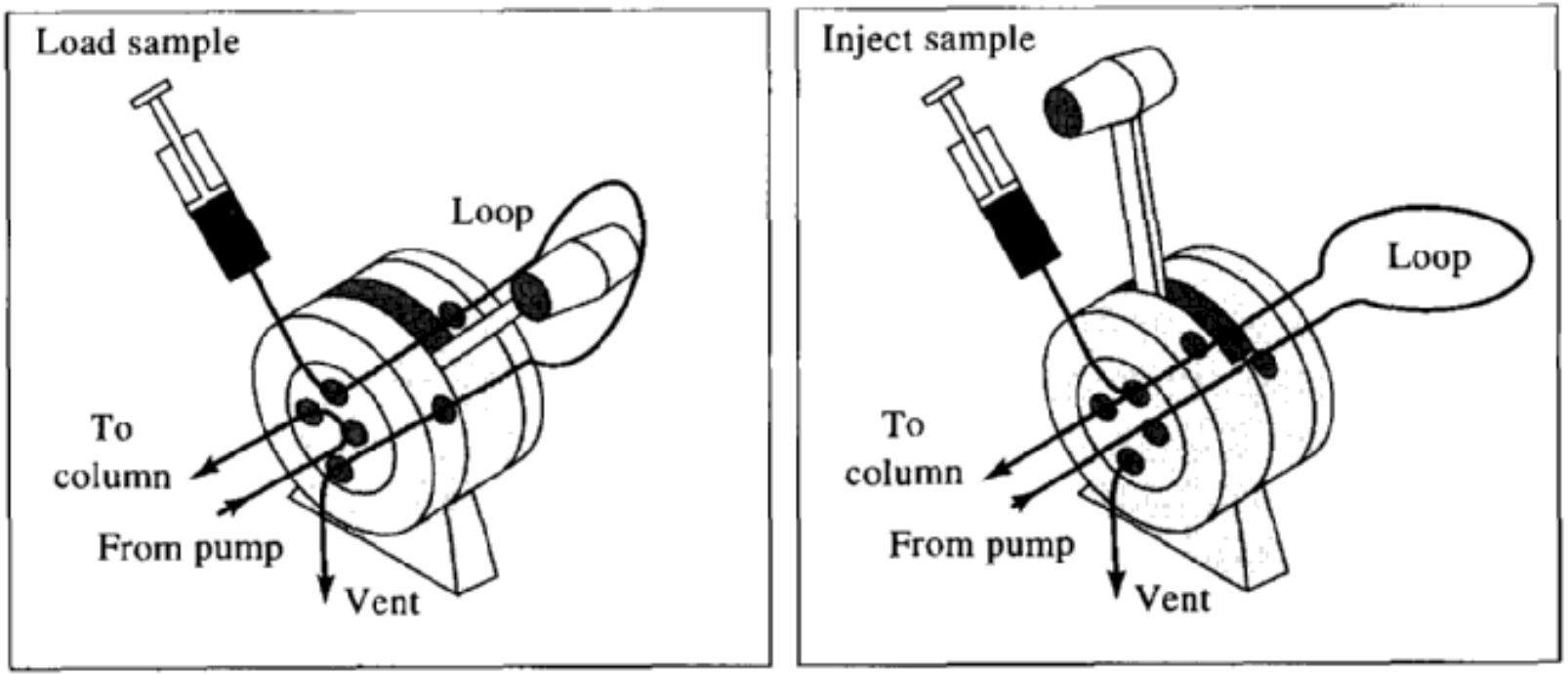
Finepak SIL C18

CH_3CN/H_2O(90/10)

Sample:

1. Benzene
2. Anthracene
3. Pyrene
4. Benz(a)pyrene

2- Sampling loops :

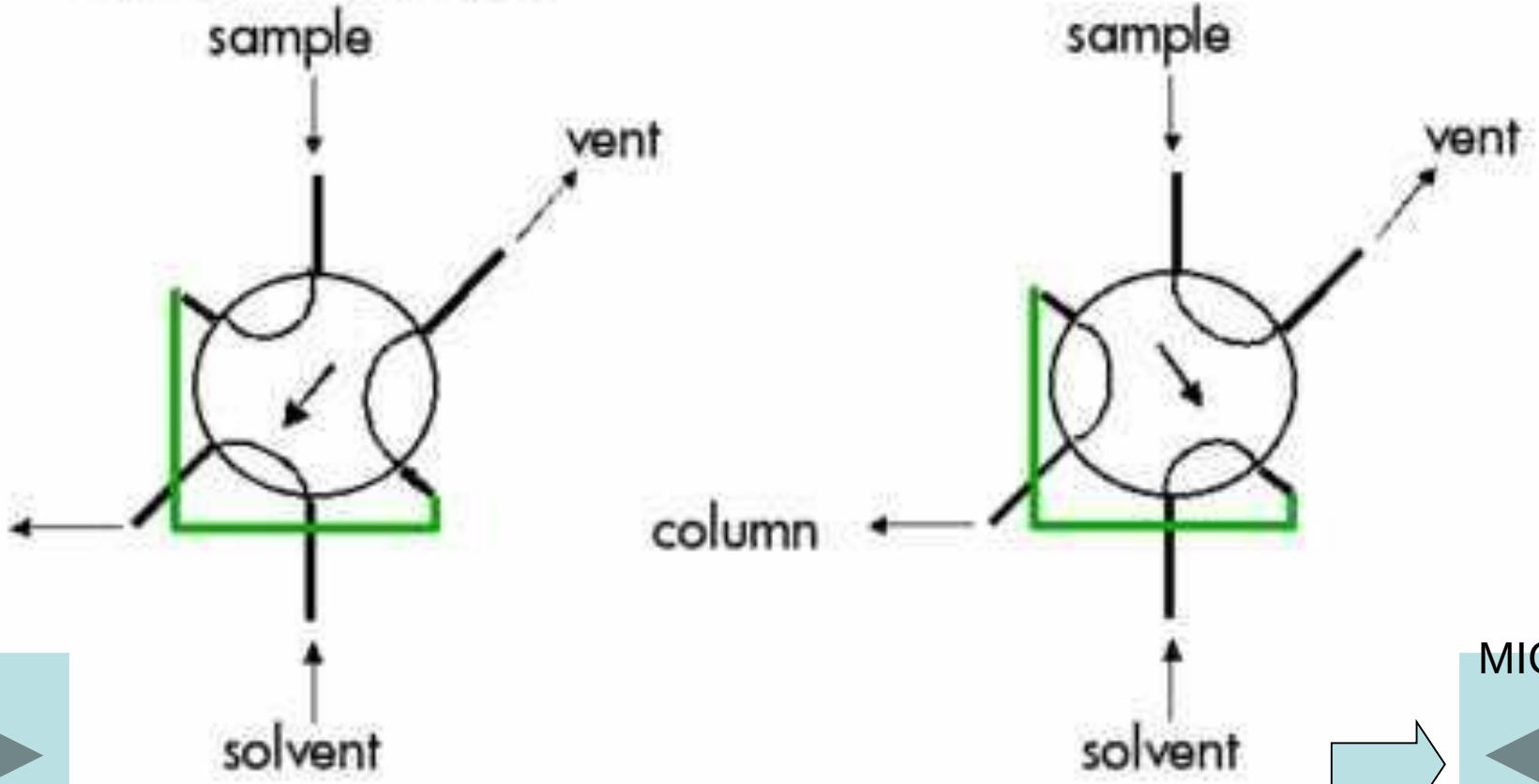


A sampling loop for liquid chromatography.

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.

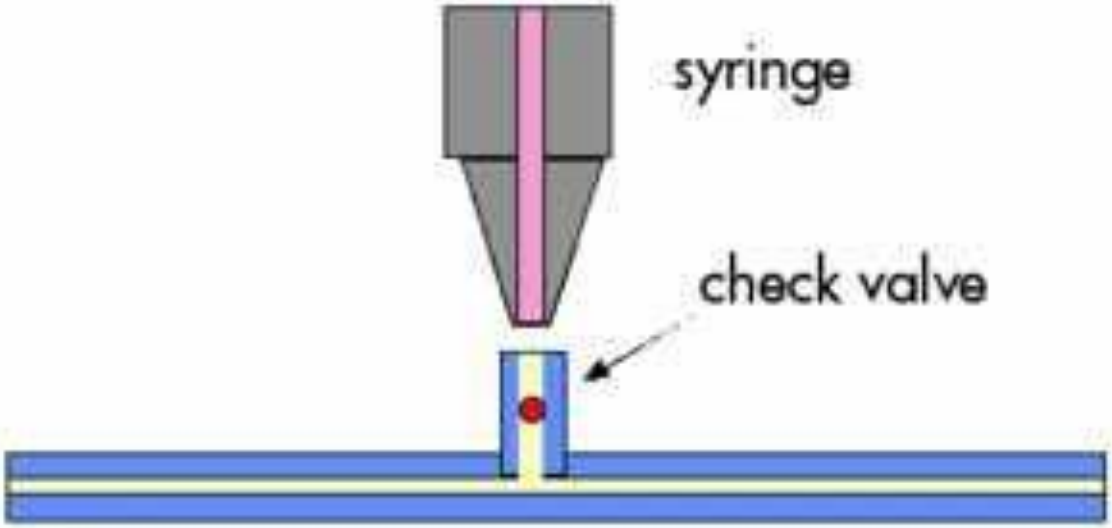
Injection systems

A very common approach is the use of sampling valves and loops.



Injection systems

3- Automated syringes



This method allow for adjustment of sample size. The motor driven syringe can provide sufficient pressure to inject sample past the check valve.

HPLC-Columns

Prof. J. Al-Zehouri

HPLC-Columns

- Ordinarily constructed from smooth-bore stainless steel tubing.
- Length 10 -30 cm .
- Straight.
- The inside diameter 4-10 mm.
- The most common particle size of packings is 5 or 10 µm.
- The most common column currently in use is one that is 25 cm in length and 4.6 mm in inside diameter and packed with 5 µm.
- Columns of this type contain 40000-60000 plate/meter.
- Recently manufactures have been producing high-speed HPLC- column (100000 plat/meter).

The column

HPLC has seen significant improvement over the last 10 years primarily due to improved column technology.

Packings are more uniform and smaller.

Phases are commonly chemically bound to the packing.

Packing methods have improved.

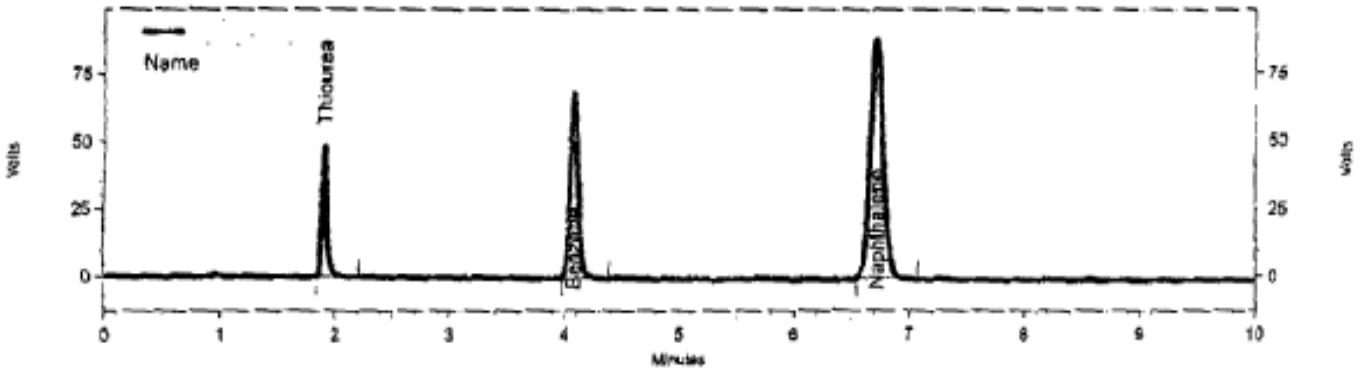
X

HPLC Test Certificate

Column Specifications:		Test Conditions:	
Order No.	25WE181ESJ (I6Y535)	**Mobile Phase**	Methanol/Water 8:2 (v/v)
Batch No.	B195969	**Flow (mL/min)**	1.0
Length x ID	250 x 4 mm with precolumn	**Pressure (MPa)**	15.7
Packing Material	Eurospher 100-5 C18	**Temperature**	ambient
Type of Column	Vertex Column	**Detector**	UV, 254 nm
Serial No.	WB 77	**Injection Volume**	10 µl

Sample Compounds:

1. Thiourea 0,01 mg/mL
2. Benzene 1,0 mg/mL
3. Naphthalene 0,1 mg/mL



No.	Retention Time [min]	Asymmetry	Theoretical plates (DAB)	Capacity	Selectivity
1	1,907	1,45	9282	0,00	0,00
2	4,080	1,11	16958	1,14	0,00
3	6,717	1,03	17600	2,52	2,21

Guard column

A small column added between the injection system and the analytical column.

It helps prevent entry of materials that might want to stay on the column from your sample or solvent.

Used to extend column life

Should be the same packing as the analytical column.

Guard Columns

MIC

MIC X

Packings

Originally, these were irregular silica and alumina. A range of synthetic, regularly shaped packings are now available.

Porous - channels through packing

Superficially porous - rough surface

Smooth - bead like.

Packing size

As packing size is decreased, efficiency and pressure requirements are increased.

Common diameters for analytical work

diameter	plates
10 μm	5000
5 μm	9000
3 μm	15,000

All are for a 15 cm x 4.6 mm id column

Column body

Typically consist of stainless steel with a high precision internal bore.

Some manufacturers offer column inserts

- don't need to repurchase the column fittings.

Others offer columns where the external body can be compressed to improve packing efficiency.

Size exclusion phases

Gels - organic or aqueous based

Controlled-pore - silica or glass

Must be selected based on pressure requirements and size range required for your application.

Column stationary phases

Today, most packing fall into four classes.

Silica or alumina

Bound phases on either alumina or silica.

Gels

Controlled-pore glass or silica

Absorption phases

alumina

common mobile phases

hexane, chloroform, 2-propanol.

example application - amines.

silica

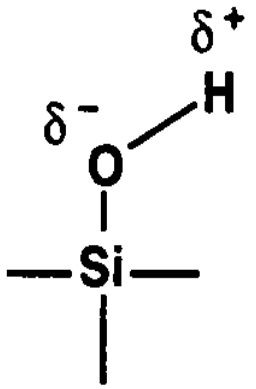
common mobile phases

hexane, chloroform, 2-propanol.

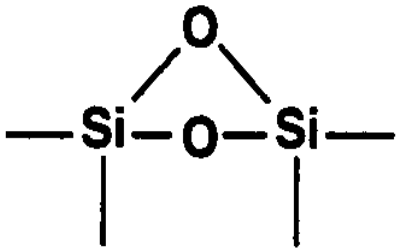
example applications - ethers, esters, porphyrins, fat-soluble vitamins.

Silica Surface

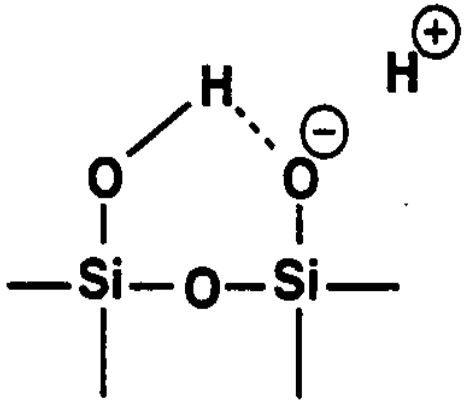
The surface of silica gel is *nonhomogeneous*, being comprised of several different types of functional groups:



Normal Silanol



Inert Siloxane Bridge



Active "hot spot"

Partition phases

Can be broken down into

Normal phase - polar materials bound to the support.

Reverse phase - non-polar materials bound to the support.

Mixed phase - may have some of each.

Partition phases

Normal

Amino ($-NH_2$)

Cyano (-CN)

Diol (glycidoxy-ethylmethoxysilane)

Reverse

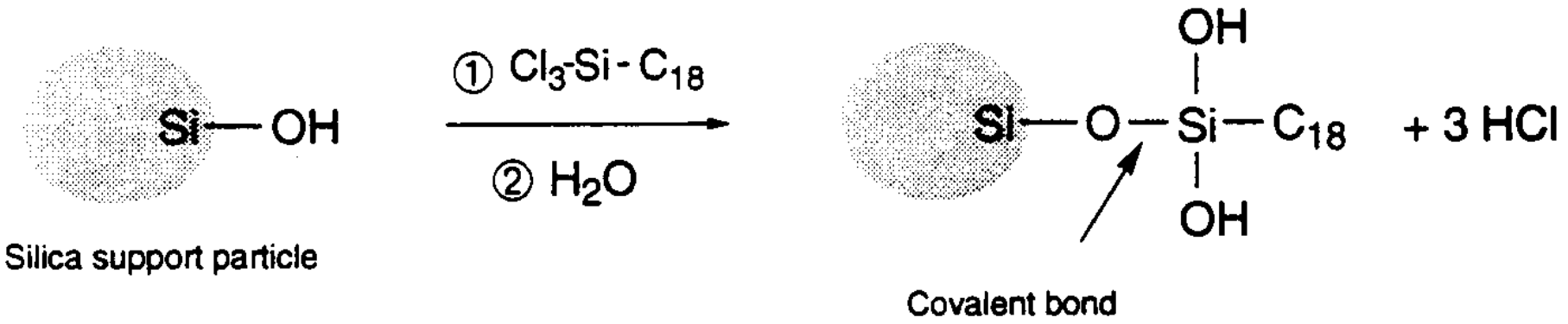
C-2 or RP-2 ($-Si-CH_2CH_3$)

C-8 or RP-8 ($-Si-(CH_2)_7CH_3$)

C-18 or RP-18 ($-Si-(CH_2)_{17}CH_3$)

Increasing the C number results in a thicker, more retentive phase

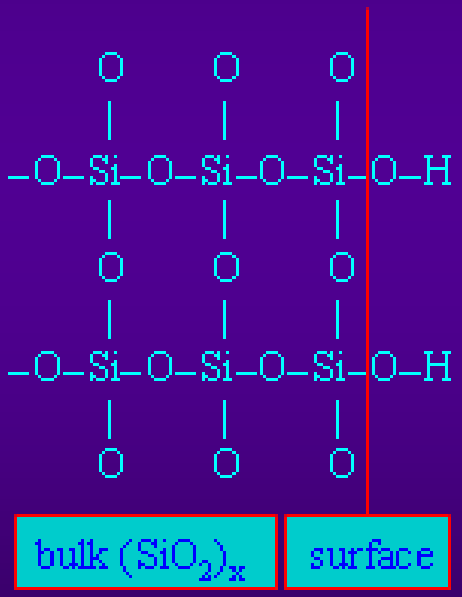
Synthesis of RP Packings



- **Covalent attachment of the stationary phase yields a thermally and hydrolytically stable *bonded phase*.**

Chromatography Stationary Phases

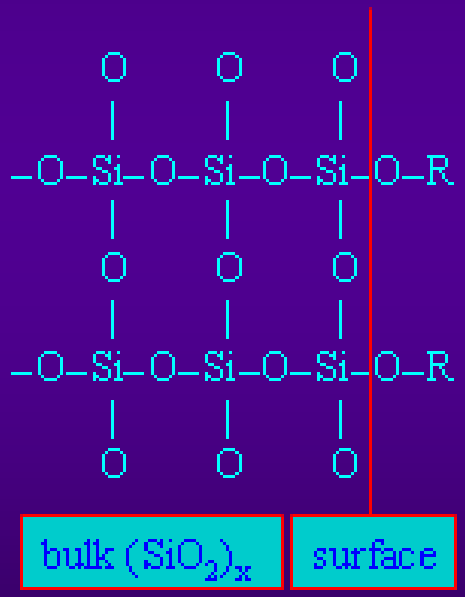
Silica Gel



relatively *polar* surface

"normal phase"

Derivatized Silica Gel

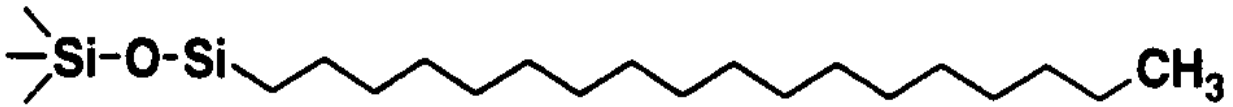


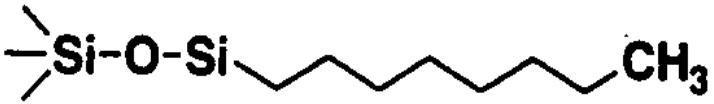
relatively *nonpolar* surface

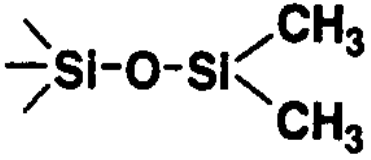
"reversed phase"

Where R = $C_{18}H_{37}$ hydrocarbon chain (octadecylsilyl deriv. silica or "C18")

Common RP Packings

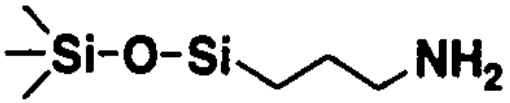
Octadecyl - 

Octyl - 

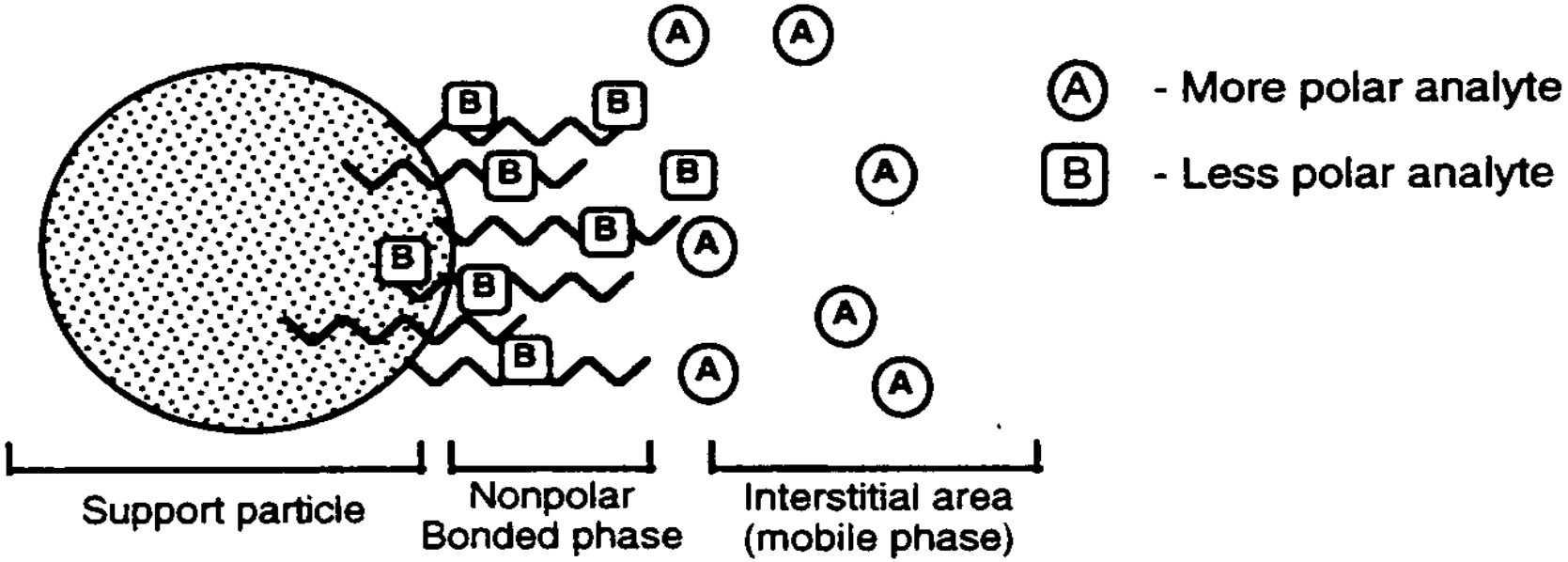
Dimethyl - 

NORMAL PHASE:

Cyanopropyl - 

Aminopropyl - 

RP Mechanism (Simple)



- **Less polar (more hydrophobic) analytes are more attracted to the hydrophobic bonded phase...**
- **...more hydrophobic spends more time associated with the bonded phase...**
- **...and are eluted last. Methanol is active solvent.**

Ion exchange phases

Strong cation	- sulfonic acid group
Strong anionic	- quarternary amine
Weak anion	- primary amine
Weak cation	- COOH

Detector

Detector Systems

Virtually every chemical and physical property that can be measured in solution has been look at.

Detectors fall roughly into two classes

Bulk property - measures an overall change in the mobile phase.

Solute property - measures a solute specific property.

Properties of a good detector

A detector must provide
high sensitivity, low detection limits,
linearity, reproducibility.

This is true for any detector.

Each detector will have specific advantages
and will vary as to peak shape and
spread, noise and flow/temperature
dependence they have.

Detectors

- Unlike gas chromatography ,liquid chromatography has no detectors that are as universally applicable and as reliable as the flame ionization and thermal conductivity detectors.

Performances of LC Detectors

LC Detector	Commercially Available
Absorbance	Yes[c]
Fluorescence	Yes[c]
Electrochemical	Yes[c]
Refractive index	Yes
Conductivity	Yes
Mass spectrometry	Yes[d]
FT–IR	Yes[d]
Light scattering[e]	Yes
Optical activity	No
Element selective	No
Photoionization	No

HPLC detectors

HPLC detectors

UV-VIS(Absorption)

PDA (Absorption)

Differential refractometer(Refractive index)

Fluorometric (Florescence)

Electrochemical (ECD) (Oxidation -reduction)

Conductivity

Mass

Chiral (OR)

Circular dichroism (CD)

HPLC detectors

UV/Vis detector

- Selective detection minimizing effects from other components

- High sensitivity detection at maximum absorption wavelength

X

X

UV/Vis detector

A solute property detector.

Sample must exhibit absorption in UV/Vis range.
Solvent must not absorb significantly at the measured wavelength.

Types Filter photometer - single λ
Variable wavelength
Multiwavelength.

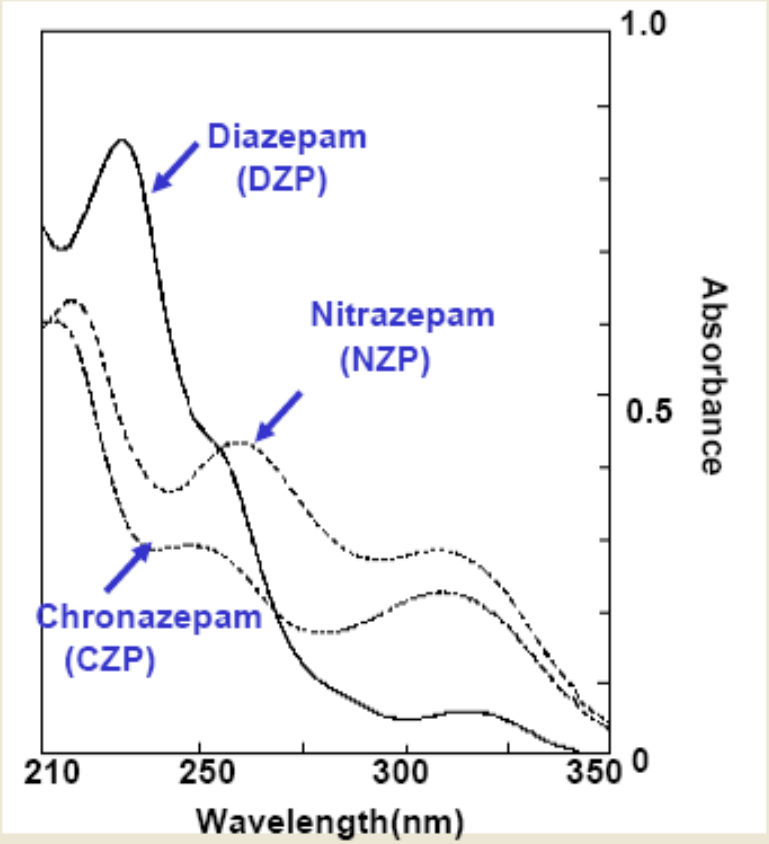
Fixed wavelength

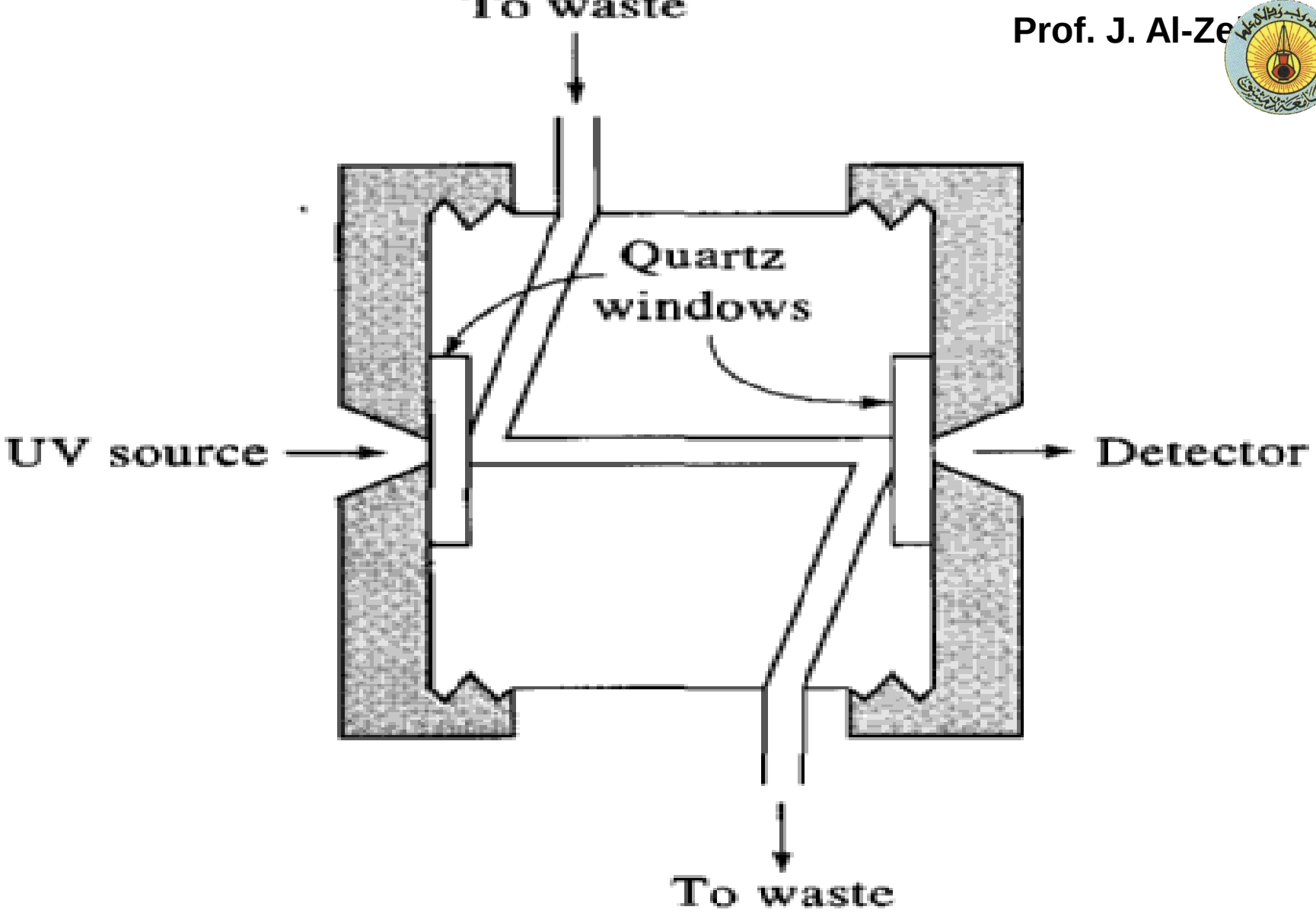
Or diode array

UV detectors are predominantly

HPLC detectors

UV spectrum measurement
to find wavelength effective for wavelength programming



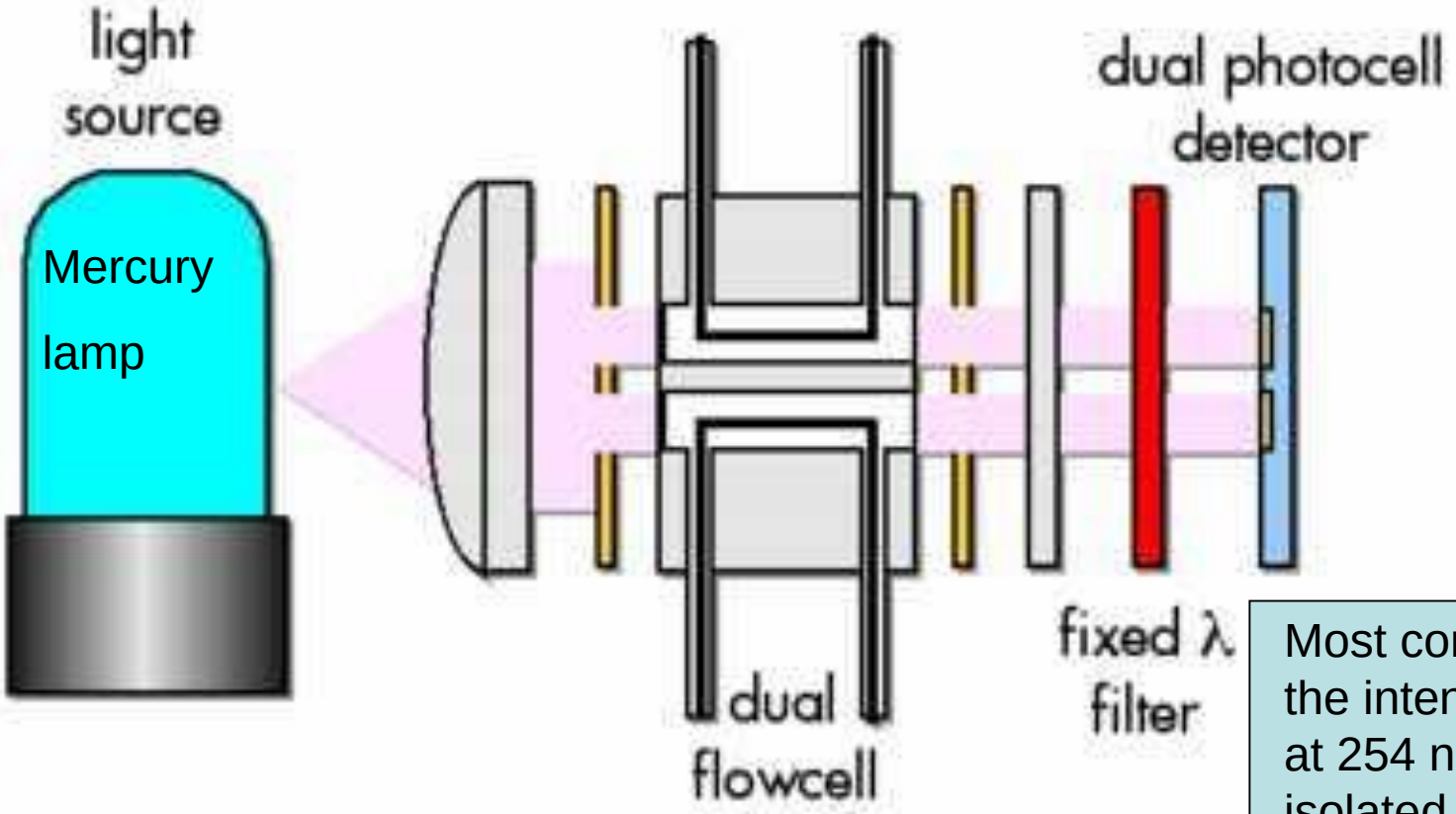


Ultraviolet detector cell for HPLC.

Absorbance Detectors

- Z-shaped, flow through cell for absorbance measurements on eluents from a chromatographic column.
- In order to minimize extra –column band broadening, the volume of such a cell is kept as small as possible. Thus, typically ,volumes are limited to 1 to 10 µl and cell lengths to 2 to 10 mm.
- Most cells of this kind are restricted to pressures no greater than about 600 psi.

UV/Vis detector - filter type



Most commonly the intense line at 254 nm is isolated by filter

If the filter is replaced by a monochrometer, you end up with a variable wavelength UV/Vis system

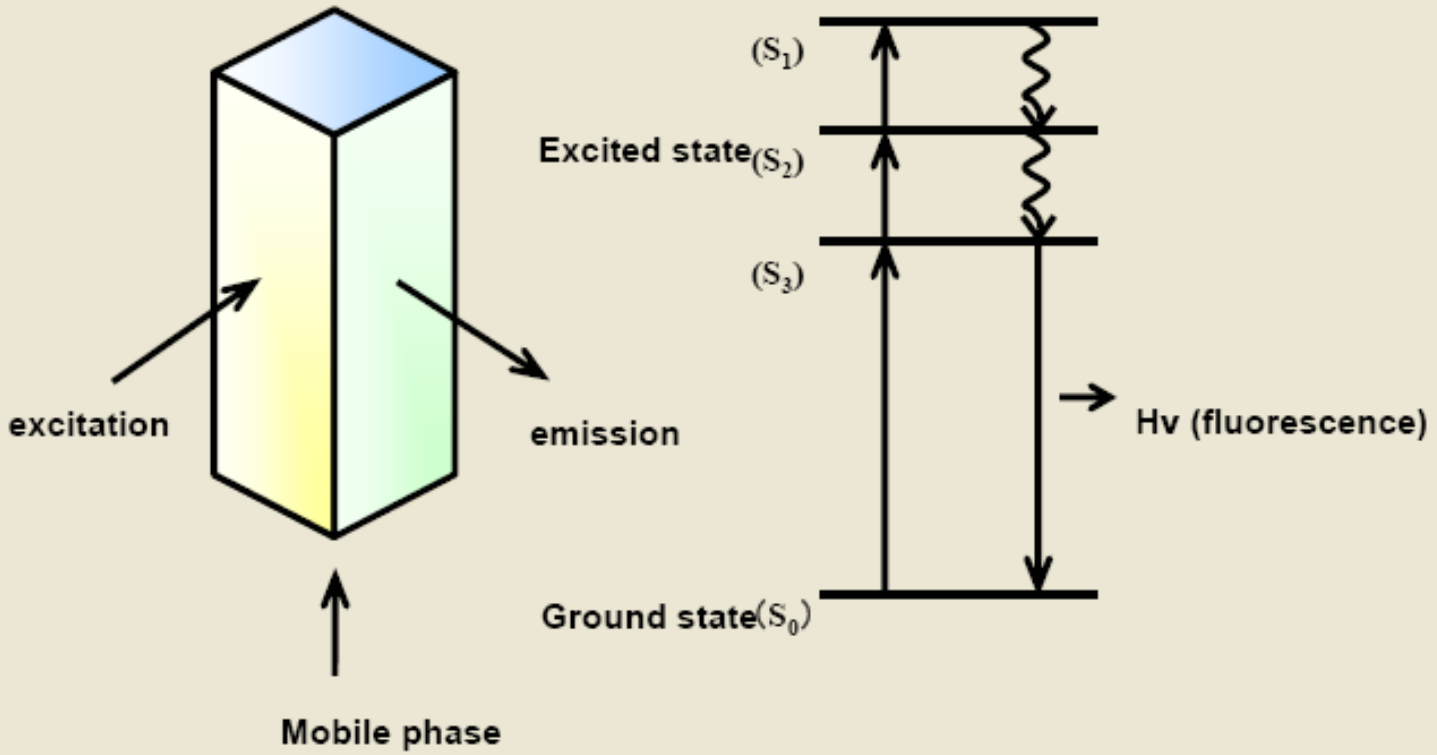
(250,313,334 ,365)

UV/VIS detector with filters

- It is restricted to solutes that absorb at on of these wavelengths.(254 ,250,313,334and 365).
- Some modern instrument s are equipped with filter wheels containing several filters that can rapidly switched to detect various species as they are eluted. (Often the filter changes are computer controlled.

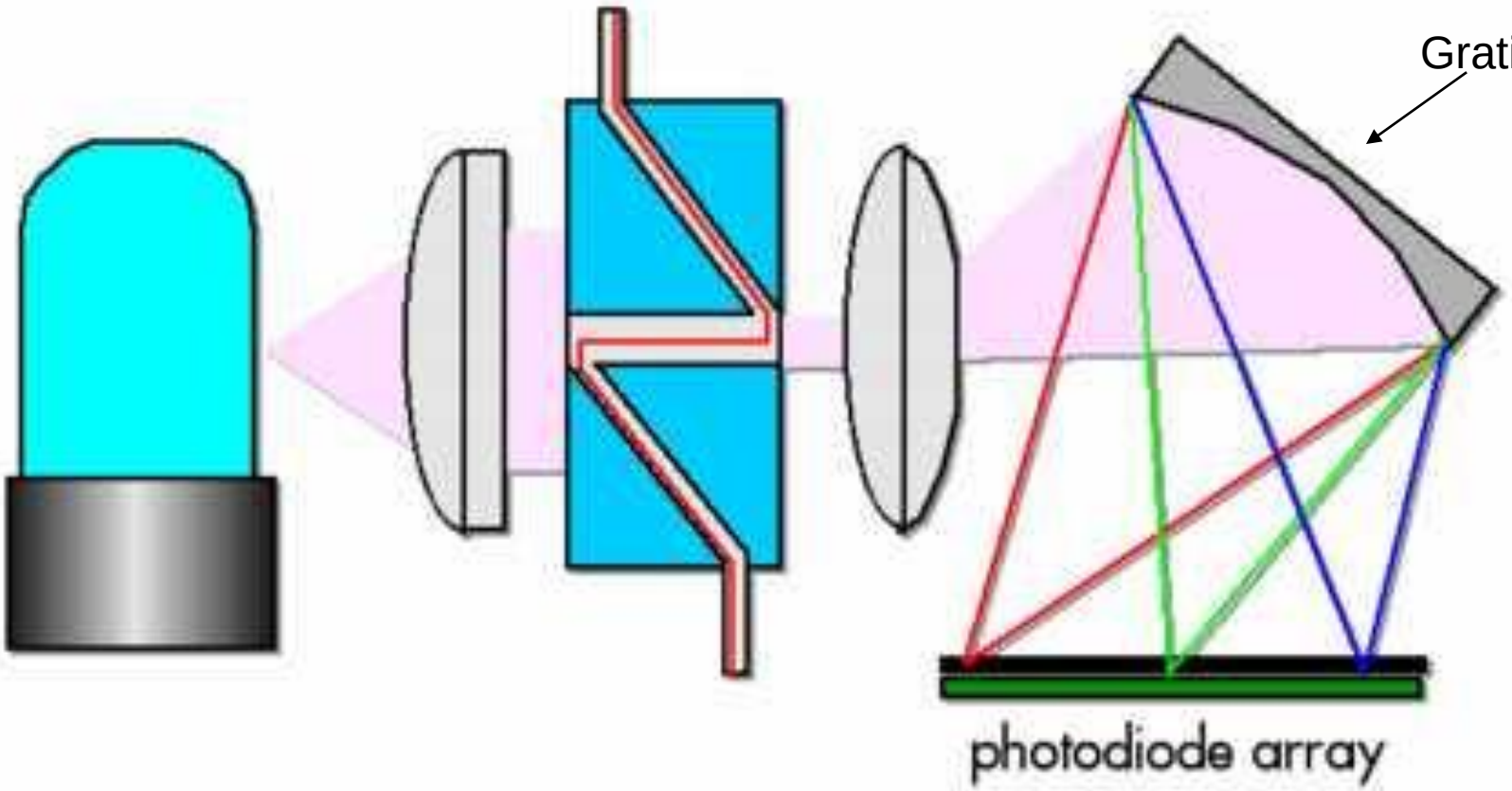
HPLC detectors

Principle of Fluorescence detector



Photodiode array detector

PDA

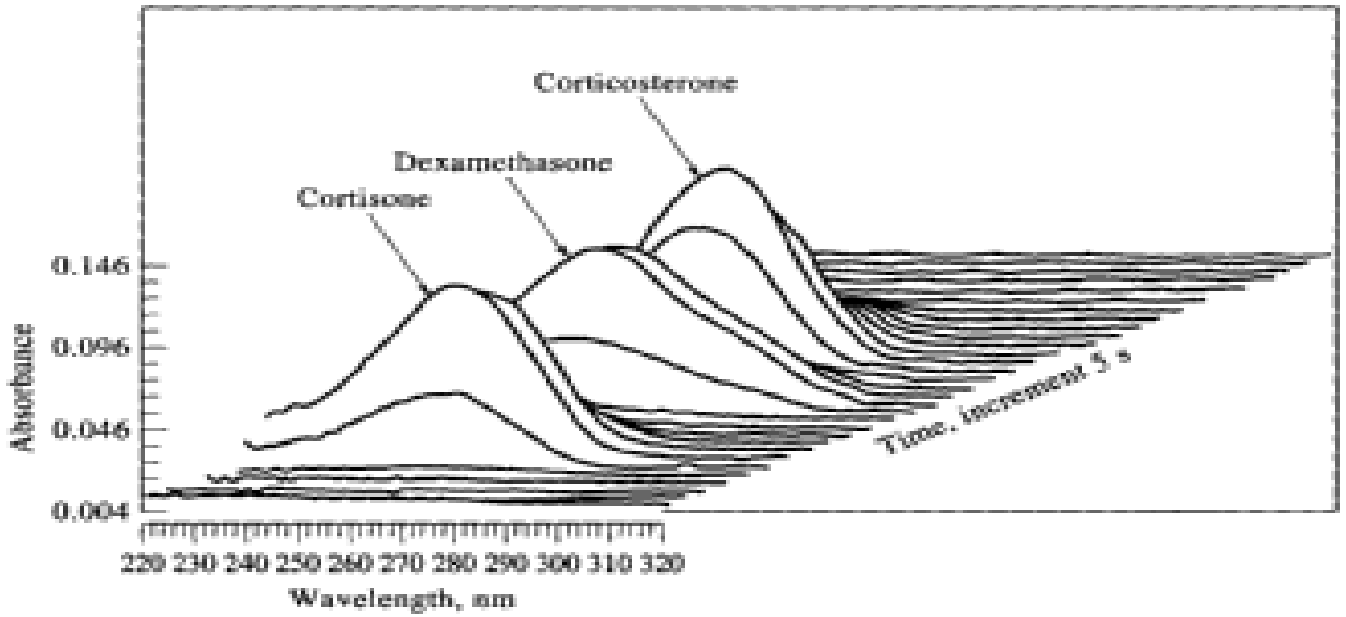


The photodiode array allow you to simultaneously monitor a range of λ or obtain complete spectra.

Photodiode array detector

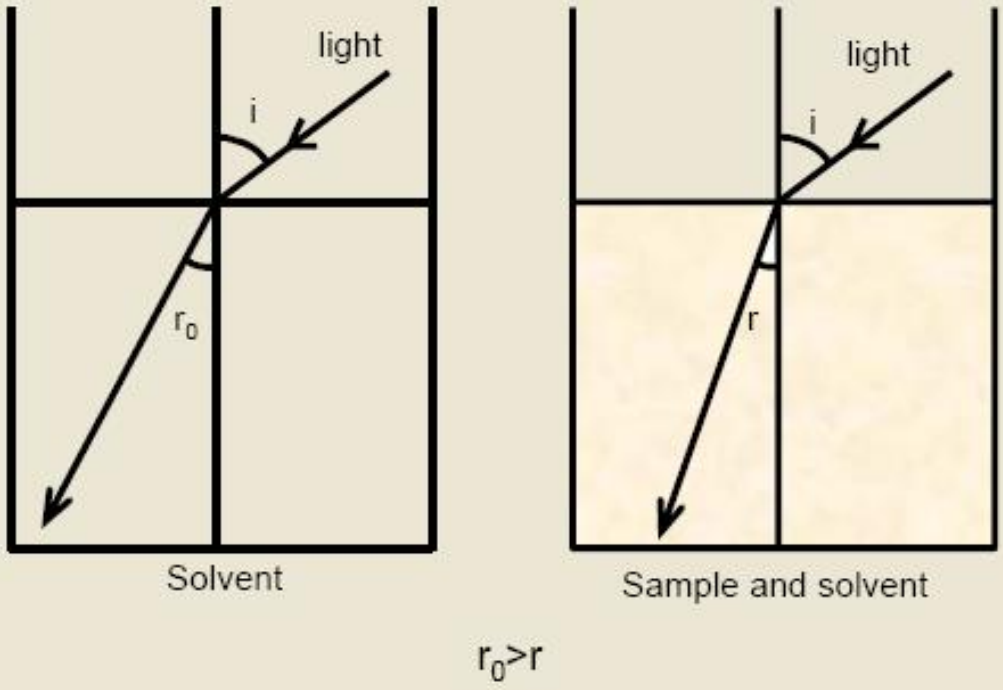
PDA

- It is permit collection of data for an entire spectrum in approximately 1 second.
- Can gives three –dimensional plot.



HPLC detectors

Principle of RI detection



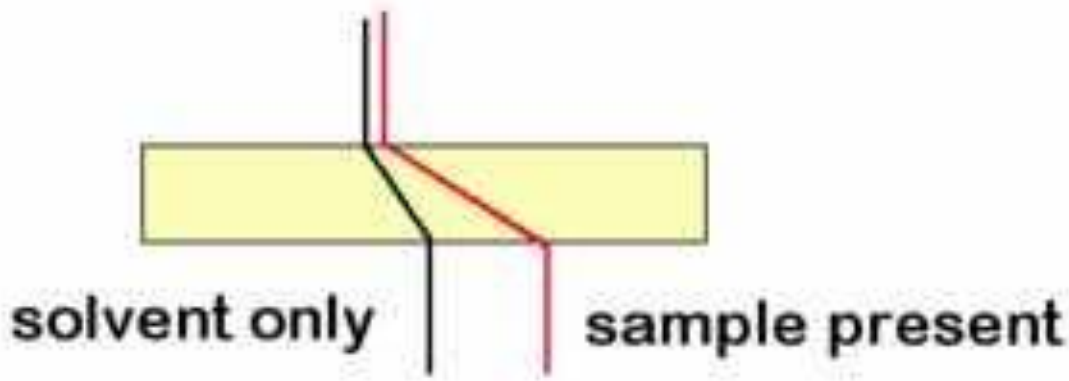
$r_0 > r$

Refractive index detector

Bulk property detector - general purpose.

Based on refraction of light as it passes from one media to another. Presence of a solute changes the refractive index of the solvent.

التغير في قرينة الأنكسار



Refractive index detector

Temperature effect

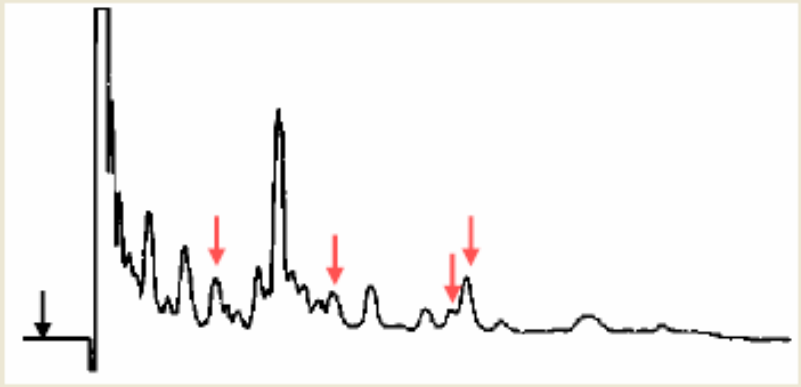
Dependent on magnitude of refractive index and thermal expansion coefficient of solvent.

Temperature must be maintained to +/- 0.0001 °C for optimum performance.

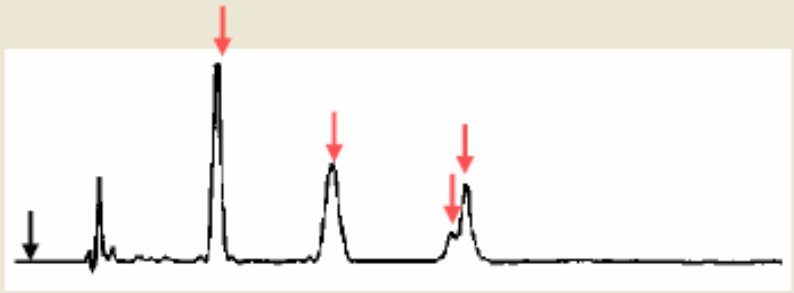
This requirement can be relaxed somewhat if a reference cell is used.

HPLC detectors

UV detector and RI detector



RI detector

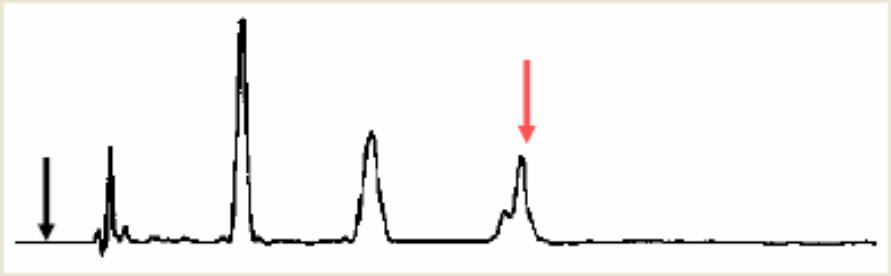


UV detector

HPLC detectors

Selectivity of UV detector and Fluorescence detector

UV detector



جزىء مستقر + أشعة λ1 ⟵ جزىء مثار ⟵ جزىء مستقر + أشعة تألقية λ2

(λ 1 < λ 2)

HPLC detectors

Detectors

	UV	Fluorescence	RI
Sensitivity	ng	pg	μ g
Detection selectivity	selective	highly selective	universal
Temperature Influence	small	small	large
Gradient elution	possible	possible	impossible

Electrochemical detectors

A number of properties have been evaluated

Detector types

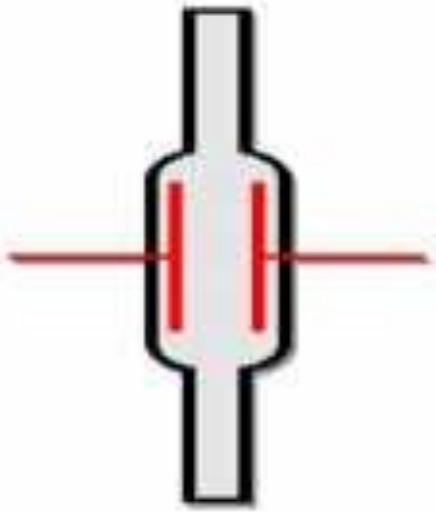
- Dielectic constant
- Amperometric
- Conductometric
- Polarographic
- Potentiometric

We'll only look briefly at the first three.

Dielectric constant detector

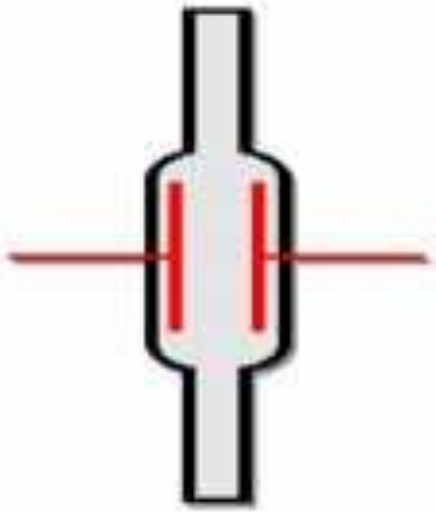
Bulk property detector.

Measures changes in polarity of the liquid phase passing through the cell.



Conductometric detector

Measures conductivity of the solvent. Useful for solutions of ions



Amperometric detectors

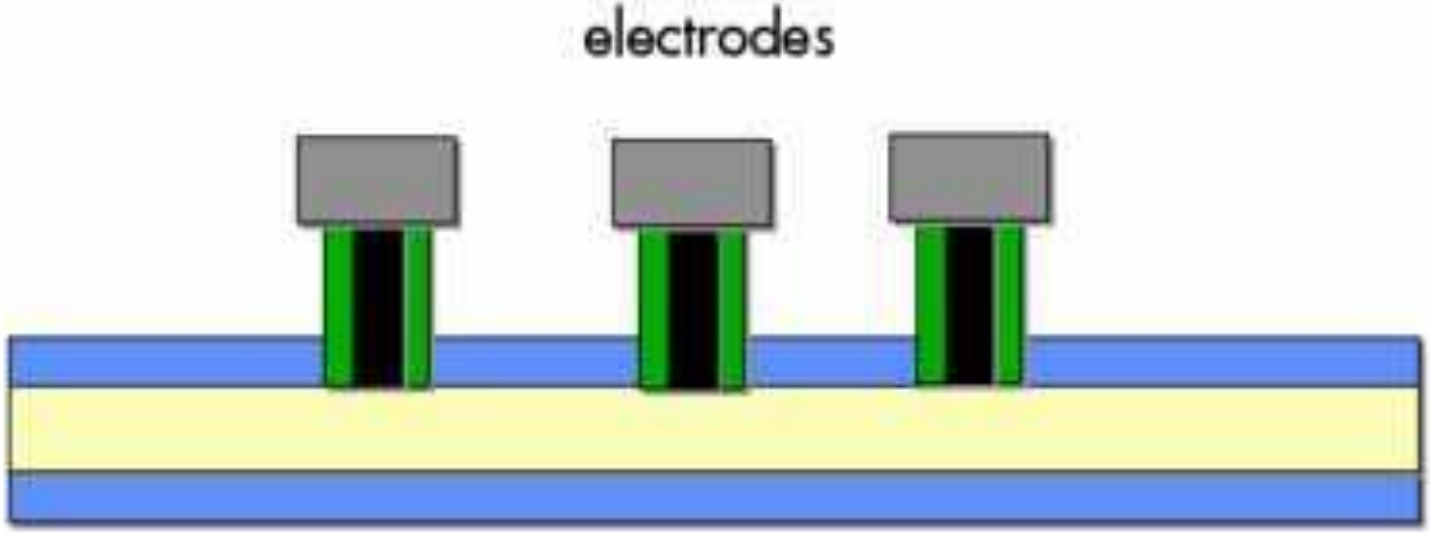
Most frequently applied of electrochemical detectors.

A known potential is applied across a set of electrodes - typically a glassy carbon type.

Ability to oxidize or reduce a species can be measured.

Typically limited to working with a specific class of materials per analysis.

Amperometric detectors



Several electrodes and combinations can be used. Allows for some interesting data.

HPLC detectors

Label method طريقة الاشتقاق

Samples absorb less UV/Vis light .
Samples do not fluoresce.

↓

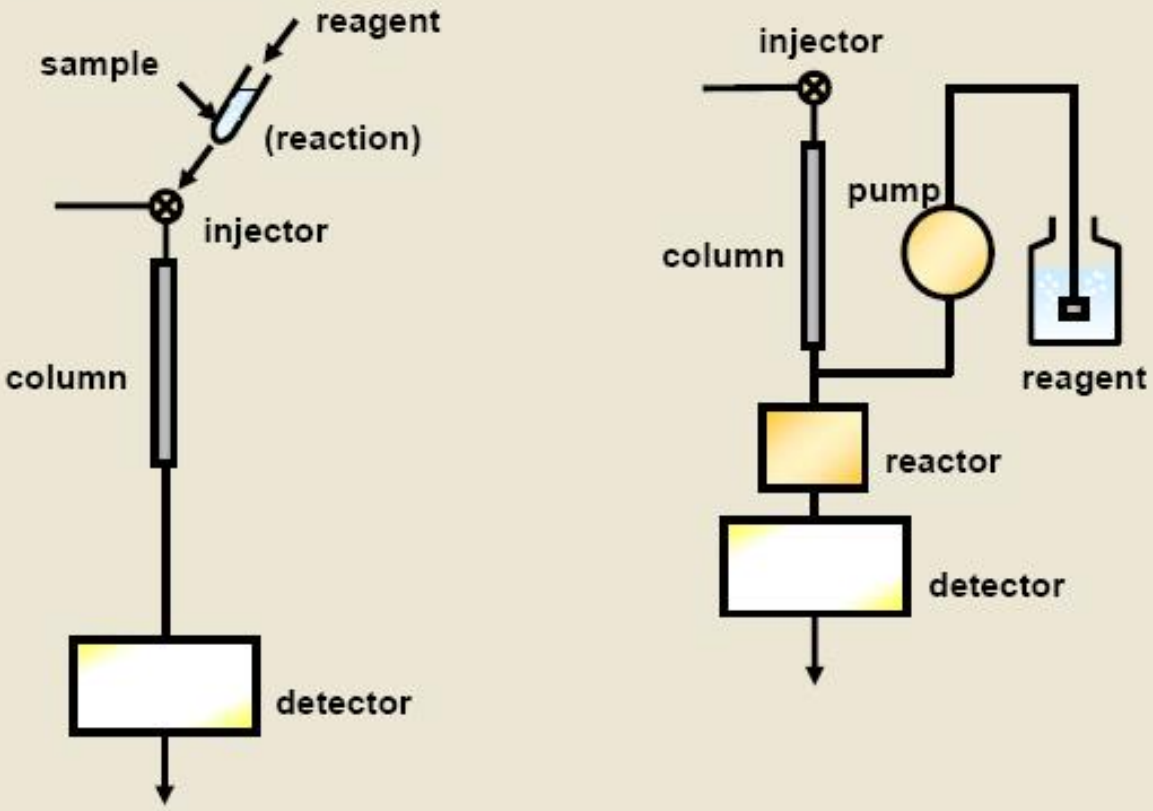
Improved sensitivity and selectivity required

↓

Label method

HPLC detectors

Label method



HPLC detectors

Label method

Ortophtalaldehyde

Post-label method

Aminoacid	0PA ninhydrine	Fluorescence Absorption in Visible range
Sugar	guanidine	Fluorescence
Organic acid	brom thymol blue	Absorption in Visible range
Catecholamine	ethylenediamine THI	Fluorescence Fluorescence
Bile acid	NAD HSD	Fluorescence Fluorescence

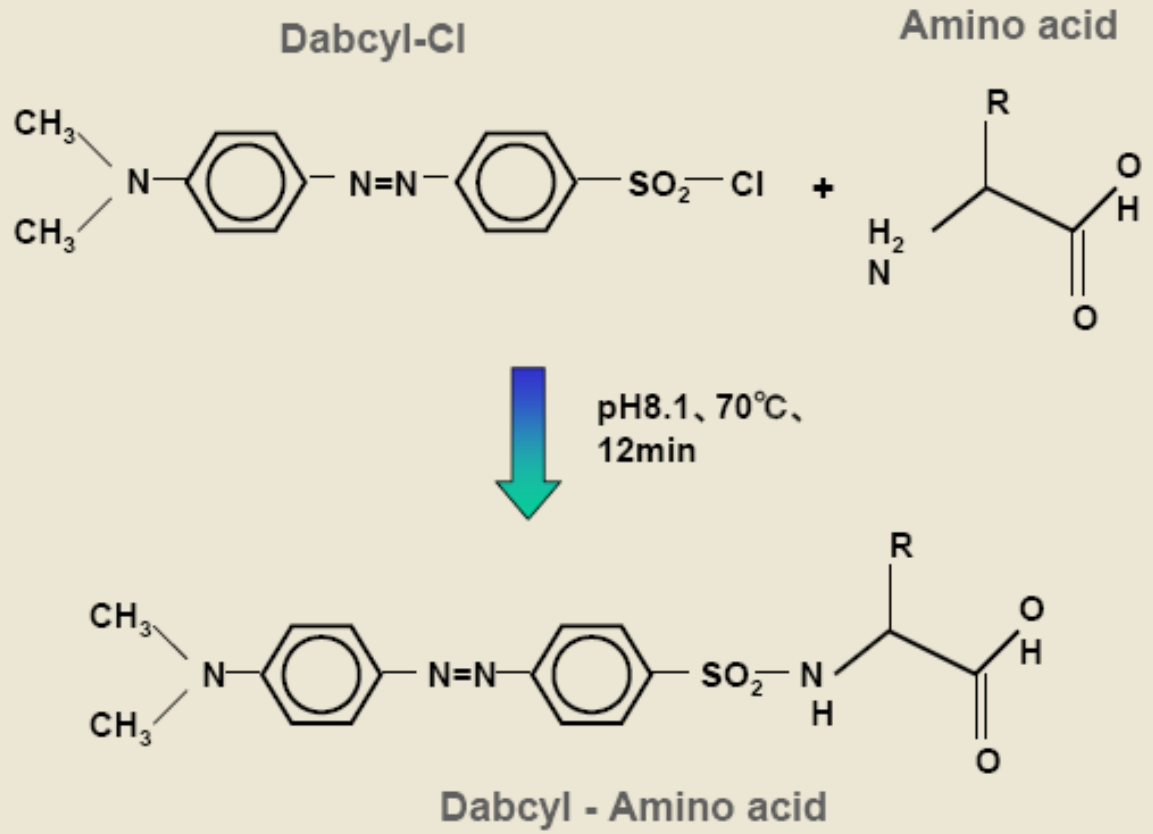
HPLC detectors

Pre and Post column derivatization method

	Pre-column	Post-column
LC system required	Standard system	Reaction system is required
Reproducibility	less than post-column	good
Operation	for all samples	only reagents
Reagents	wide range	limited
Applicability	spot	routine

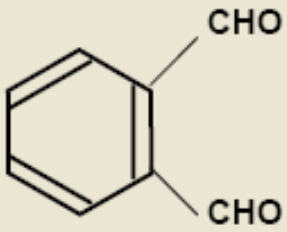
HPLC detectors

Pre-column derivatization method



HPLC detectors

Post-column derivatization method

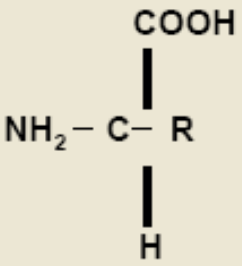


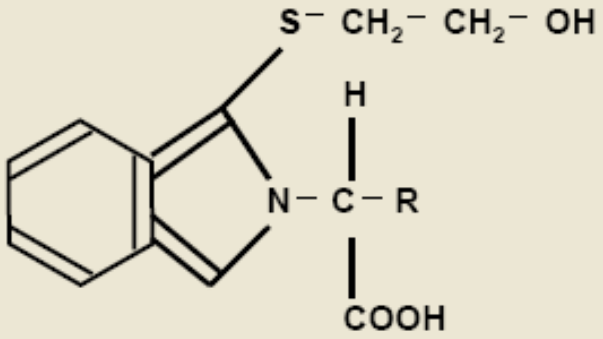
orthophthalaldehyde (OPA)

2-mercapto ethanol

+ HS– CH_2– CH_2 – OH +

Amino acid





Derivative compound

Detectors and peak shapes

Based on type of detector used.

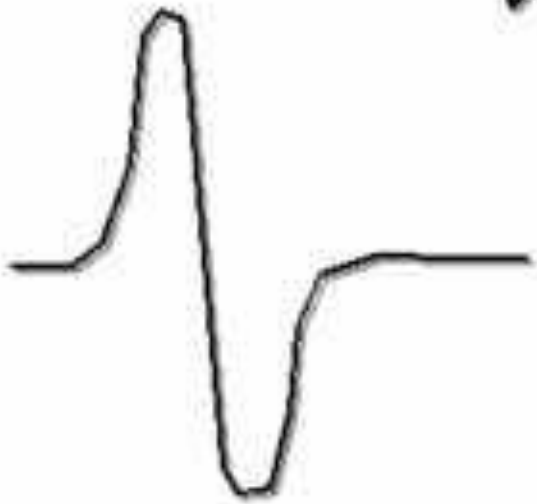
A UV or UV/Vis detector gives typical Gaussian shaped peaks. Absorption is proportional to concentration.

This is not true for all detectors.

Detectors and peak shapes

RI detector

Absorption detector



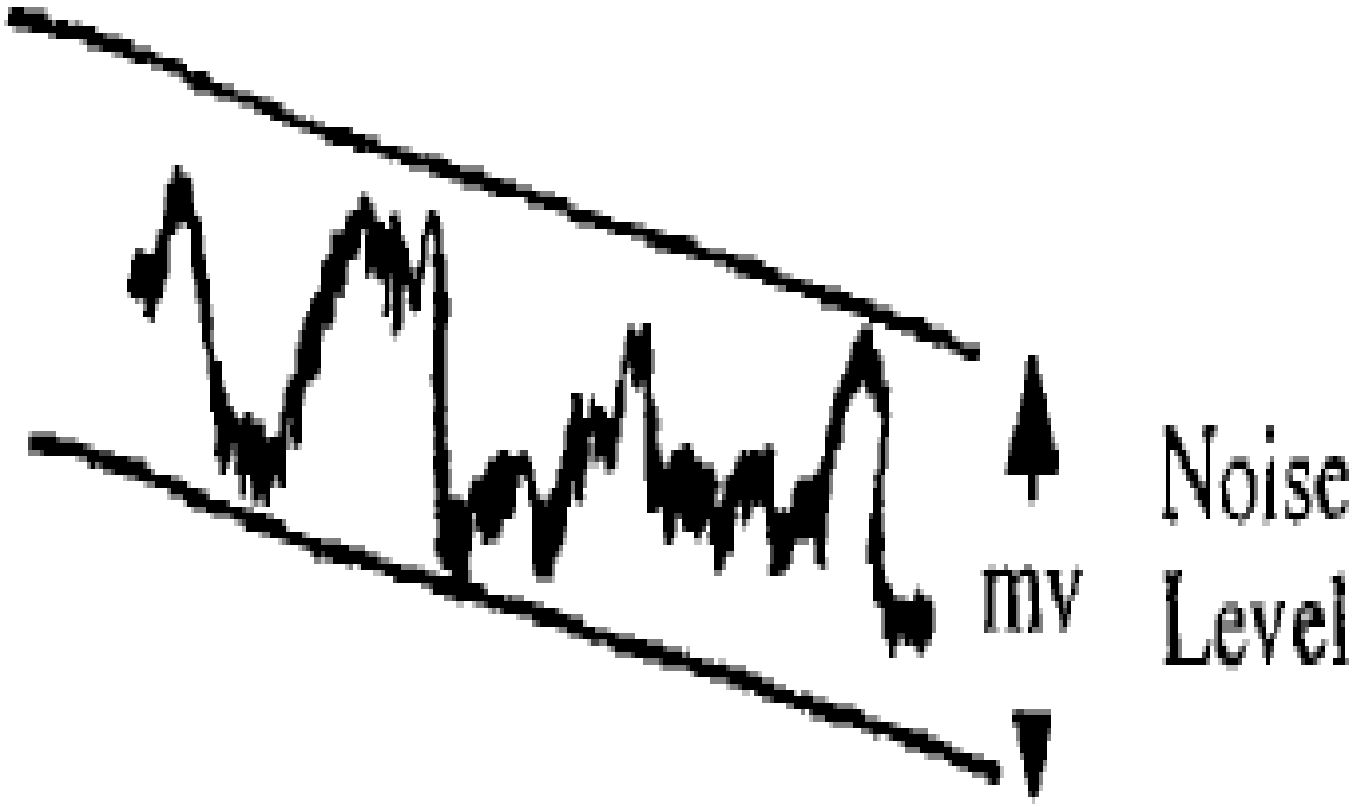
Your data system must be able to correct for this

Short Term Noise

Long Term Noise

Drift

Total Noise



Method for measuring detector noise.

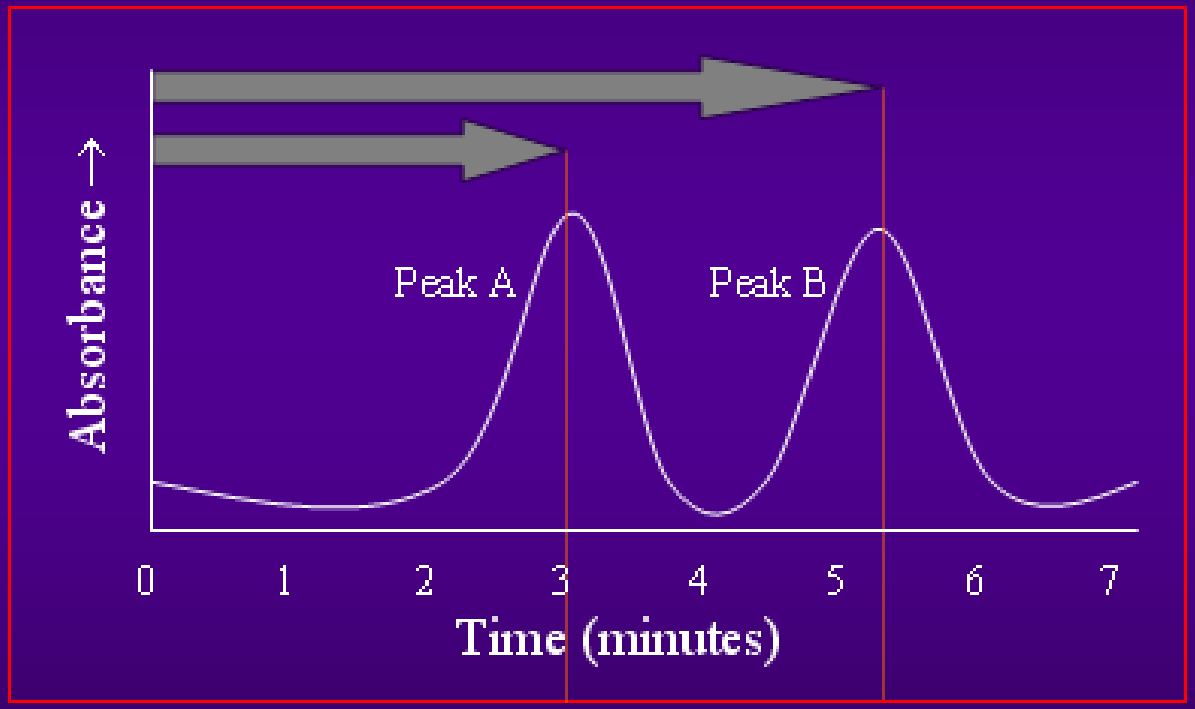
TLC vs. HPLC

Type of Analysis	qualitative only	qualitative & quantitative
Stationary Phase	2-dimensional thin layer plate	3-dimensional column
Instrumentation	minimal!	much! with many adjustable parameters
Sample Application	spotting (capillary)	injection (Rheodyne injector)
Mobile Phase Movement	capillary action (during development)	high pressure (solvent delivery)
Visualization of Results	UV lightbox	"on-line" detection (variable UV/Vis)
Form of Results	spots, R_f's (retention factors)	peaks, R_t's (retention times)

Normal vs. Reversed Phase Chromatography

	Normal Phase	Reversed Phase
Stationary phase	Polar (silica gel)	Non-polar (C18)
Mobile phase	Non-polar (organic solvents)	Polar (aqueous/organic)
Sample movement	Non-polar fastest	Polar fastest
Separation based on	Different polarities (functionality)	Different hydrocarbon content

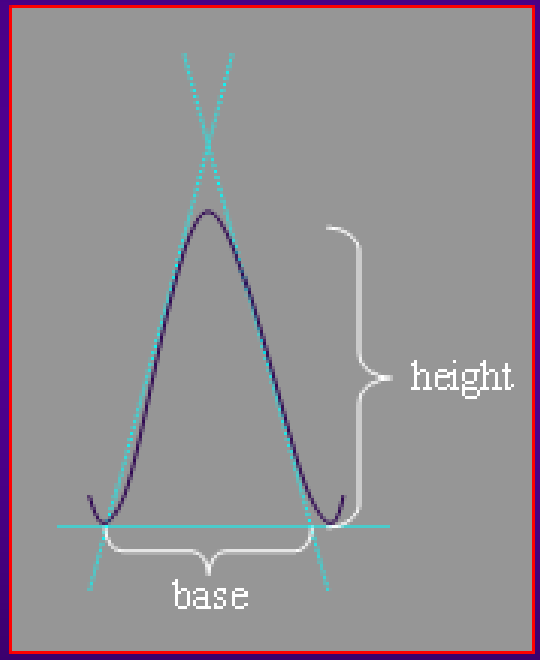
HPLC Chromatograms



R_t = 3.0 min.
faster moving
less retained

R_t = 5.2 min.
slower moving
more retained

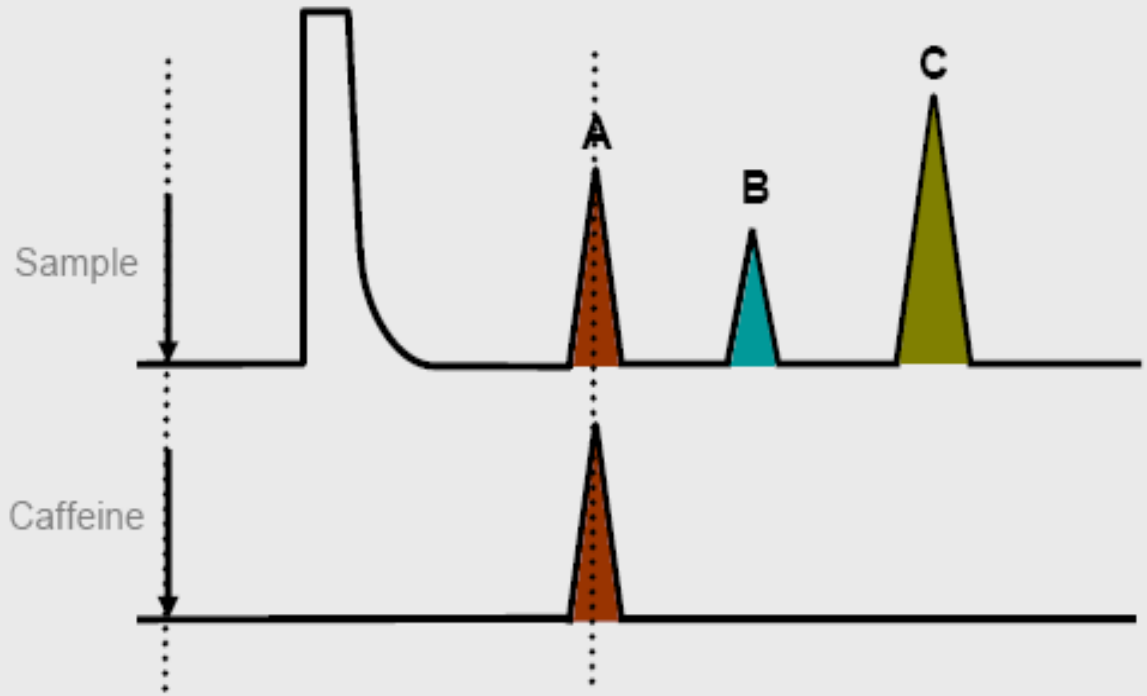
Approximation of peak area by **triangulation**



$$\text{Area} = \frac{\text{base} \times \text{height}}{2}$$

Identification

What is component A?

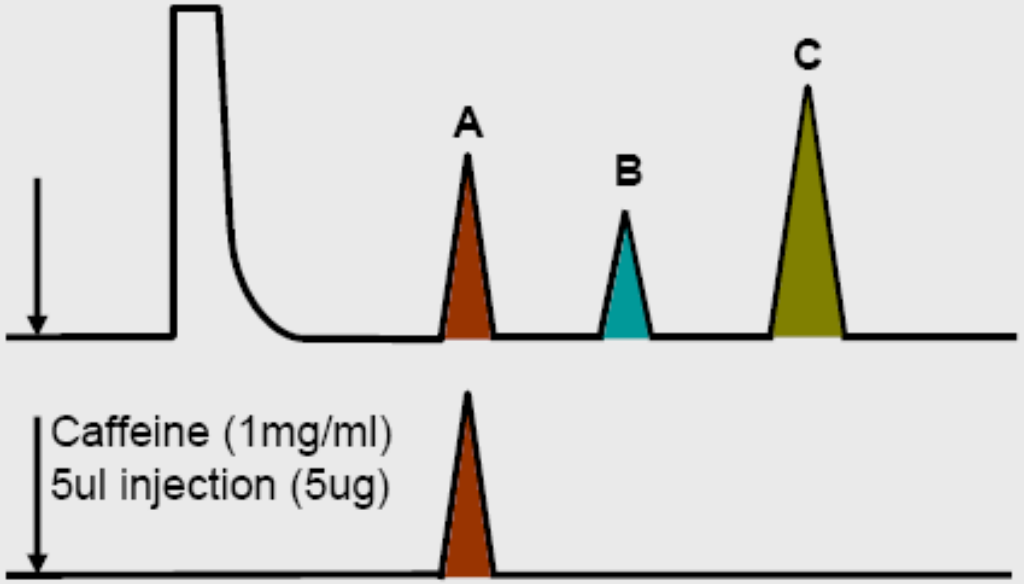


Component A elutes the same time as a caffeine peak.

Component A is identified as caffeine.

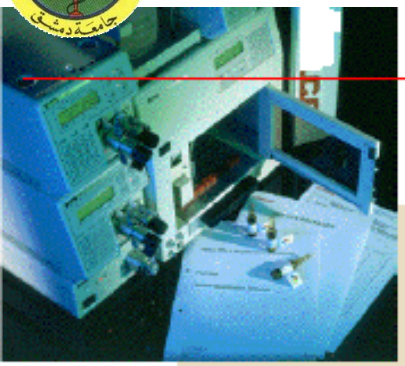
Determination

What is the concentration of component A?

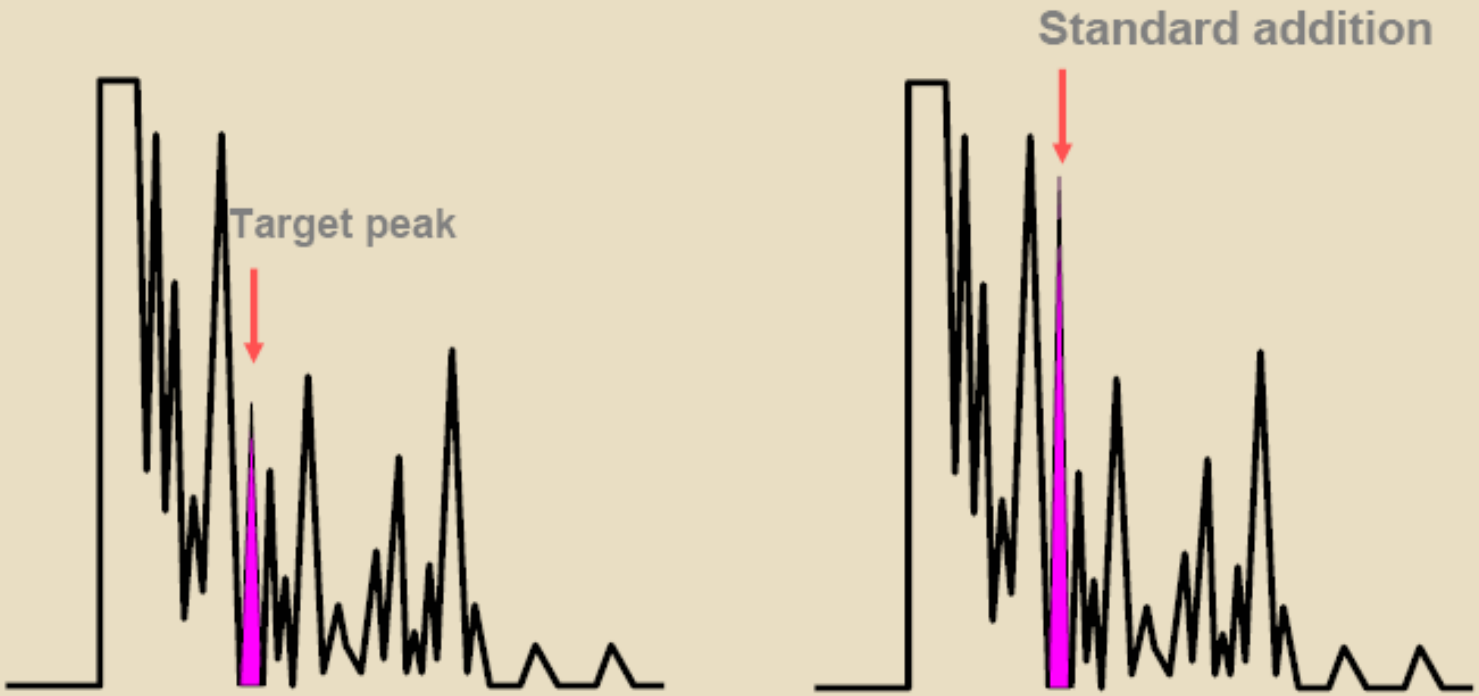


Peak area (or height) is proportional to the concentration (or amount) of the component.

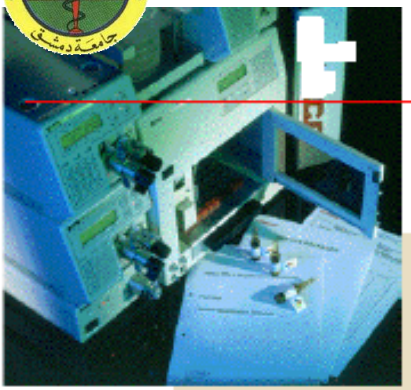
The concentration of component A(caffeine) is determined by comparing the peak area with that of the standard caffeine peak.



Standard addition method

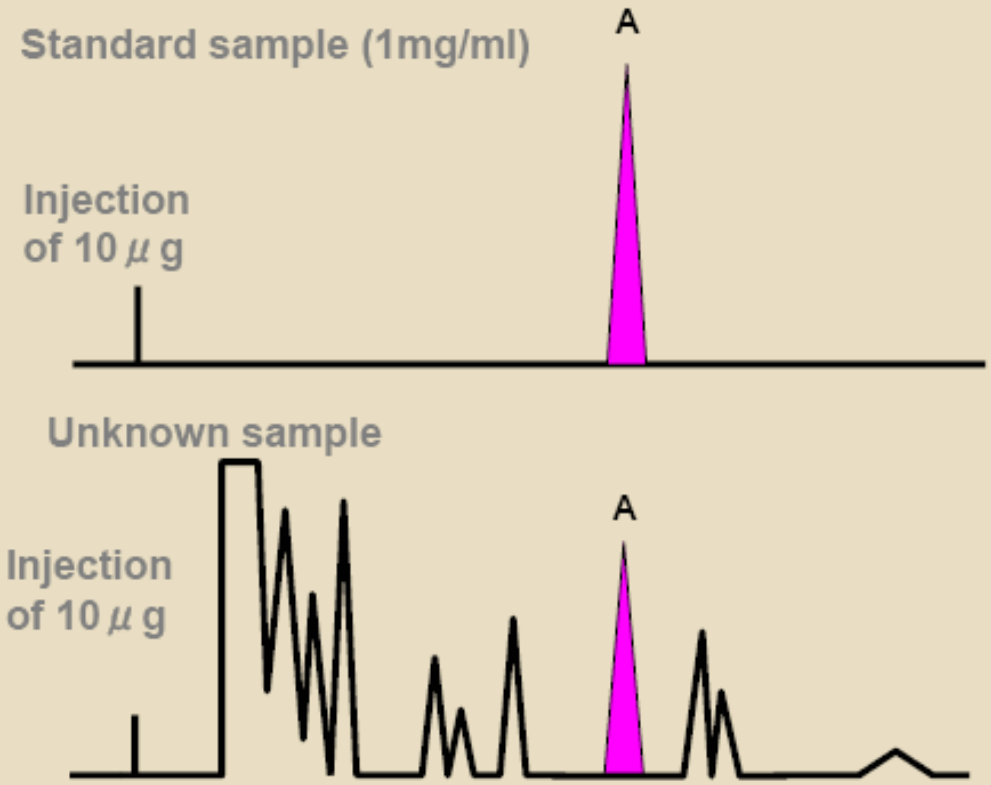


Quantitative analysis



The amount of a component can be calculated from the peak height and peak area of the chromatogram.

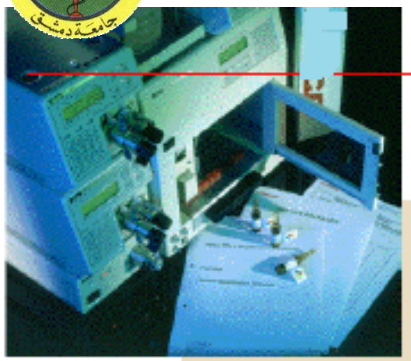
How much component A ?



Calibration method

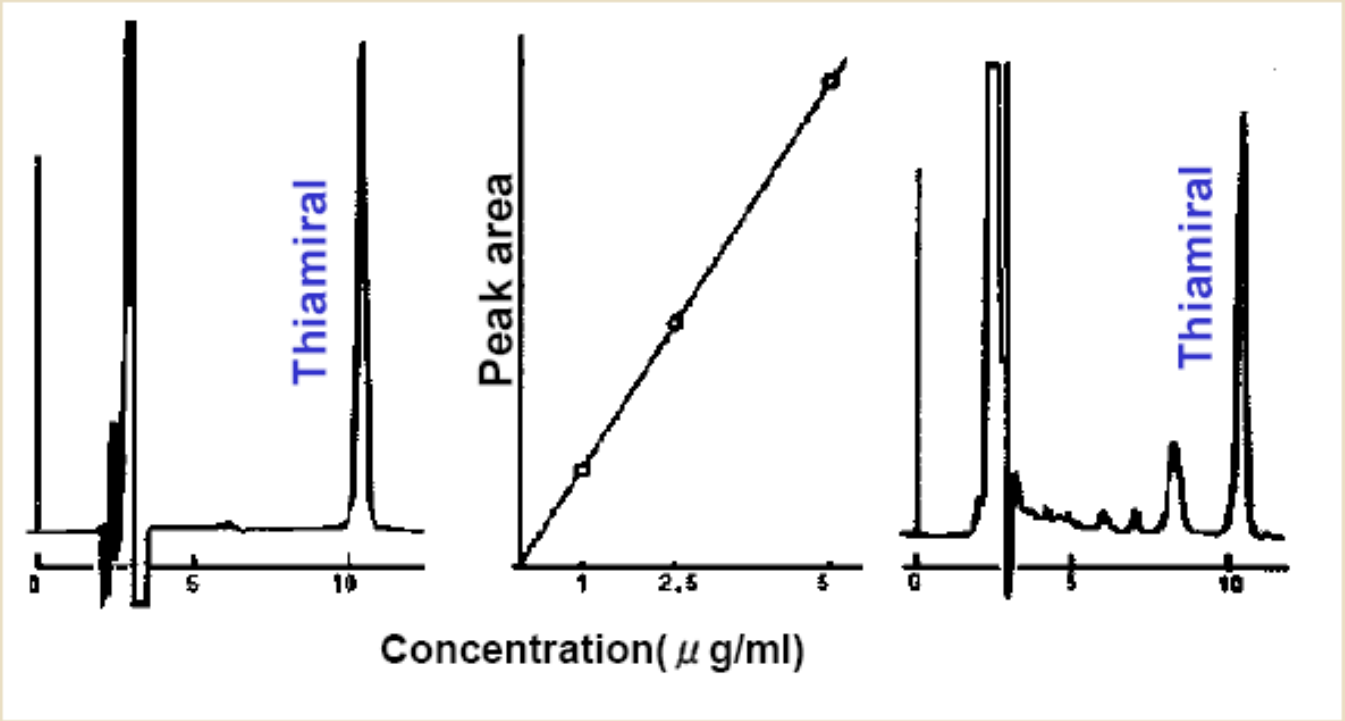
External standard sample

Internal standard sample

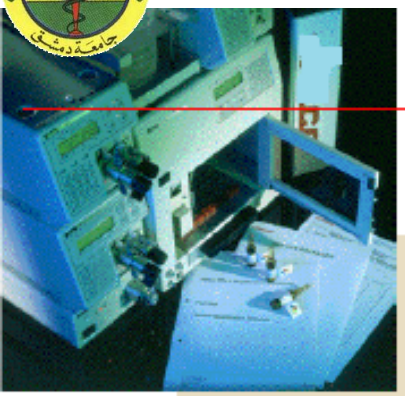


External standard sample

Thiamiral in serum



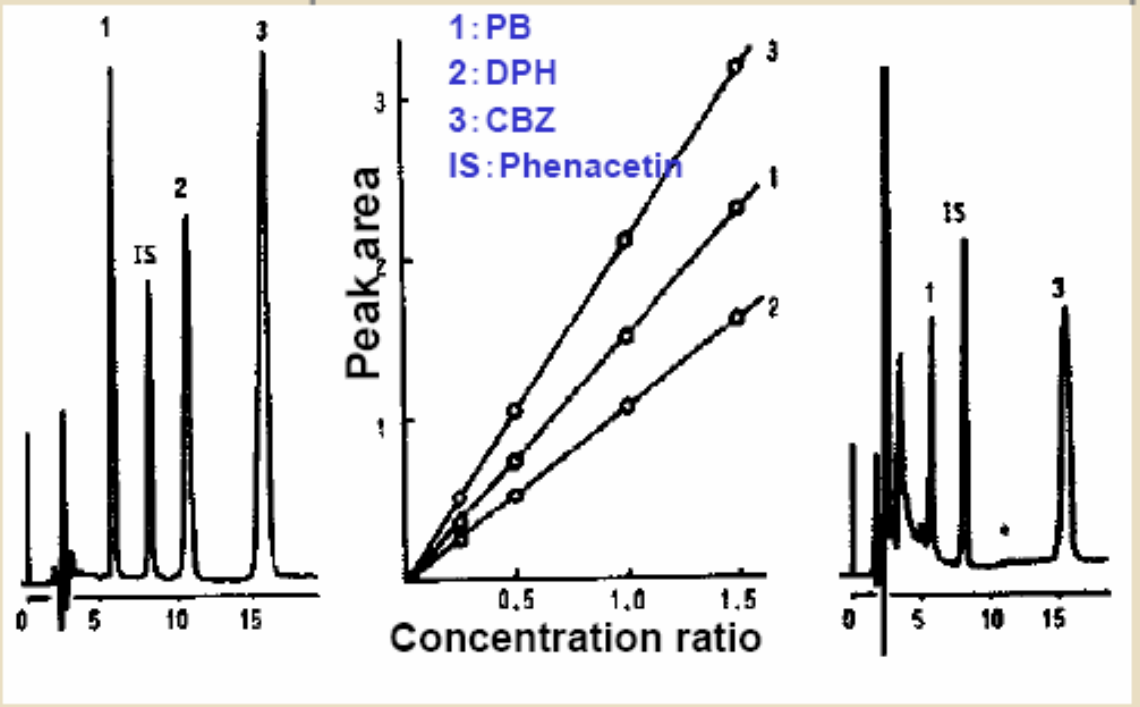
Finepak SIL C18T-5、CH_3CN/10mM KH_2PO_4 aq. (50:50)
UV 288nm



Internal standard sample

Anticonvulsants in serum

Standard sample | Calibration curve | Unknown sample



Finepak SIL C18T、CH_3CN/5mM KH_2PO_4 aq.

HPLC

Pharmaceutical Application

Starting conditions for reversed-phase chromatography

Stationary phase:	C_{18} or C_8 on 5-μm-diameter spherical silica particles. Less acidic Type B silica (Figure 25-7) is preferred. For operation above 50°C, sterically protected silica (Figure 25-8) is preferred.
Column:	0.46 × 15 cm column for 5-μm particles[a] 0.46 × 7.5 cm column for 3.5-μm particles (shorter run, same resolution)
Flow rate:	2.0 mL/min
Mobile phase:	CH_3CN/H_2O for neutral analytes CH_3CN/aqueous buffer[b] for ionic analytes 5 vol % CH_3CN in H_2O to 100% CH_3CN for gradient elution
Temperature:	35°–40°C if temperature control is available
Sample size:	25–50 μL containing ~25–50 μg of each analyte

a. A 0.30 × 15 cm column reduces solvent consumption to $(0.30/0.46)^2 = 43\%$ of the volume required for 0.46-cm diameter, reducing the flow to (0.43)(2.0 mL/min) = 0.86 mL/min.

b. Buffer is 25–50 mM phosphate/pH 2–3 made by treating H_3PO_4 with KOH. K^+ is more soluble than Na^+ in organic solvents and leads to less tailing. Add 0.2 g sodium azide per liter as a preservative if the buffer will not be used quickly.

Application

Pharmaceutical Quantitative application

$$\frac{A_1 \text{ X } C\% \text{ X } W_1}{A_2 \text{ X } W_2}$$

Where: A1= Peak area or peak ht or peak ratio for sample

A_2 = Same as above but for std.

W_1 = Average weight of Dosage form.

W_2 = weight taken

C% = concentration in g% of the stock standard solution.

Phenytoin Oral Suspensions

Assay—

Mobile phase—Prepare a filtered and degassed mixture of water, methanol, acetonitrile, 0.5% triethylamine in water, and 1.74 N acetic acid (191:100:40:1.3:1). Make adjustments if necessary.

Standard preparation—Dissolve an accurately weighed quantity of USP Phenytoin RS in methanol, and dilute quantitatively, and stepwise if necessary, with *Mobile phase* to obtain a solution having a known concentration of about 0.625 mg per mL.

Assay preparation—Transfer an amount of Oral Suspension, equivalent to about 125 mg of phenytoin, into a 200-mL volumetric flask, rinse the pipet with 40 mL of methanol, and add the rinsings to the flask. Add about 50 mL of *Mobile phase*, dilute with methanol to volume, mix, sonicate, and filter.

Chromatographic system (see *Chromatography* ⟨621⟩)—The liquid chromatograph is equipped with a 229-nm detector and a 4.6-mm × 25-cm column that contains packing L1. The flow rate is about 1.5 mL per minute. Chromatograph the *Standard preparation*, and record the peak responses as directed for *Procedure:* the tailing factor is not more than 1.5; and the relative standard deviation for replicate injections is not more than 2.0%.

Procedure—Separately inject equal volumes (about 10 µL) of the *Standard preparation* and the *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. Calculate the quantity, in mg, of phenytoin ($C_{15}H_{12}N_2O_2$) in the portion of Oral Suspension taken by the formula:

$$200C(r_U/r_S),$$

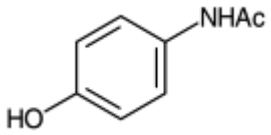
in which C is the concentration, in mg per mL, of USP Phenytoin RS in the *Standard preparation;* and r_U and r_S are the peak responses obtained from the *Assay preparation* and the *Standard preparation*, respectively.

USP-Packings

Symbol	Chemical .co	Particles µm
L1	Octadecyl silane ODS(C18)	3-10
L3	Porous silica non-bonded	5-10
L6	Strong cation-exchange-sulfonated	30-50
L7	Octylsilane (C8)	3-10
L8	Aminopropyl $-(CH_2)_3NH_2$ bonded to silica	10
L11	Phenyl C6H5- bonded to silica	5-10
From L1 to L60		

Paracetamol tablet 250 mg

Action and use Analgesic and antipyretic.



151.2 C8H9NO2

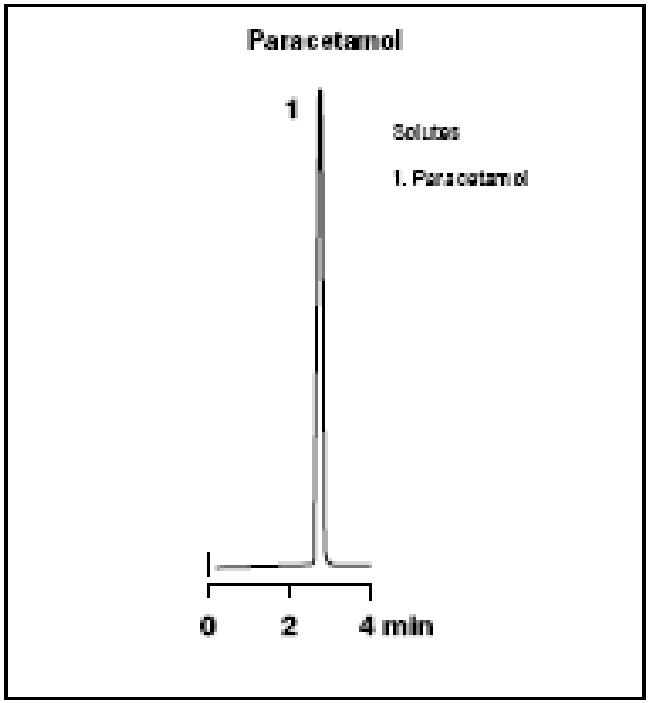
Suppose the average wt. Of tablet 0.31 g is assayed by HPLC and following data are available:

A1(sample) = 0.503

A2(std) = 0.510

C % =0.05

W1 = 0.062



Column	Hypersil BDS C18, 5µ,150x4.6mm
Eluent	Acetonitrile:0.05M Phosphate Buffer, pH4.5 (30:70)
Flow Rate	1.0ml/min
Detection	UV 254nm

$$\frac{A_1 \times C\% \times W_1}{A_2 \times W_2}$$

$$\frac{0.503 \times 0.05 \times 0.310}{0.510 \times 0.062}$$

$$= 0.246$$

$$\frac{0.246}{0.250} \times 100 = 98\ \%$$

HPLC

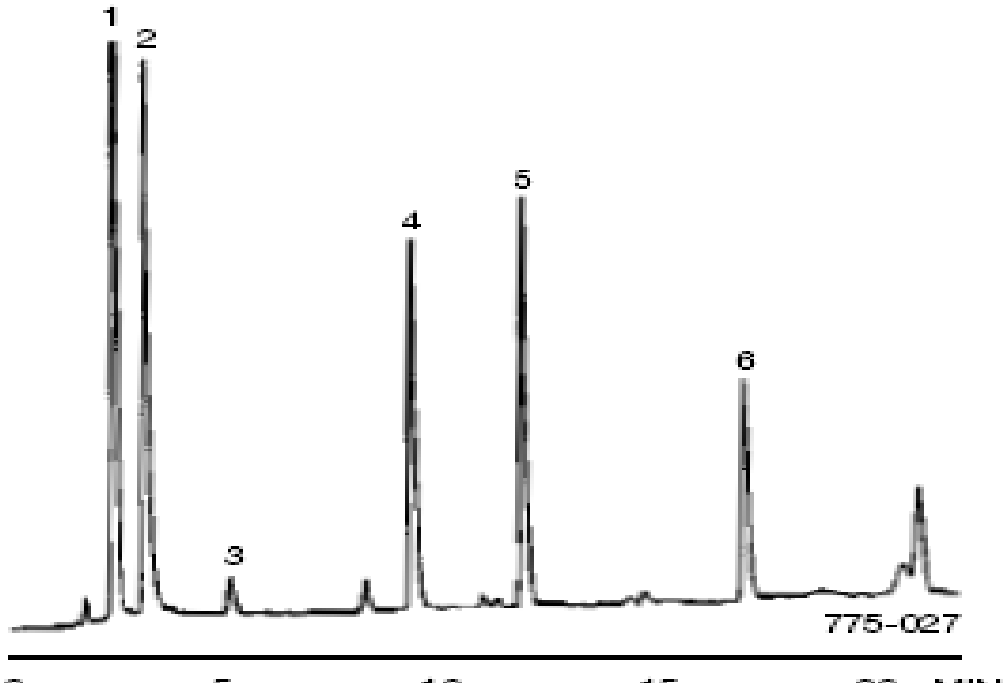
Pharmaceutical

Application

Vitamins

Sample: Solgar Tablet Solution

1. Vitamin C
2. Vitamin B_1
3. Vitamin B_6
4. Vitamin B_3
5. 4-Aminobenzoic Acid (Preservative)
6. Vitamin B_2



AQUASIL C18, 5µm, 150x4.6mm

Gradient: A: 5% MeOH / 95% 0.25M NaH_2PO_4, pH 3.5 (H_3PO_4)
B: 60% MeOH / 40% 0.25M NaH_2PO_4 pH 3.5 (H_3PO_4)

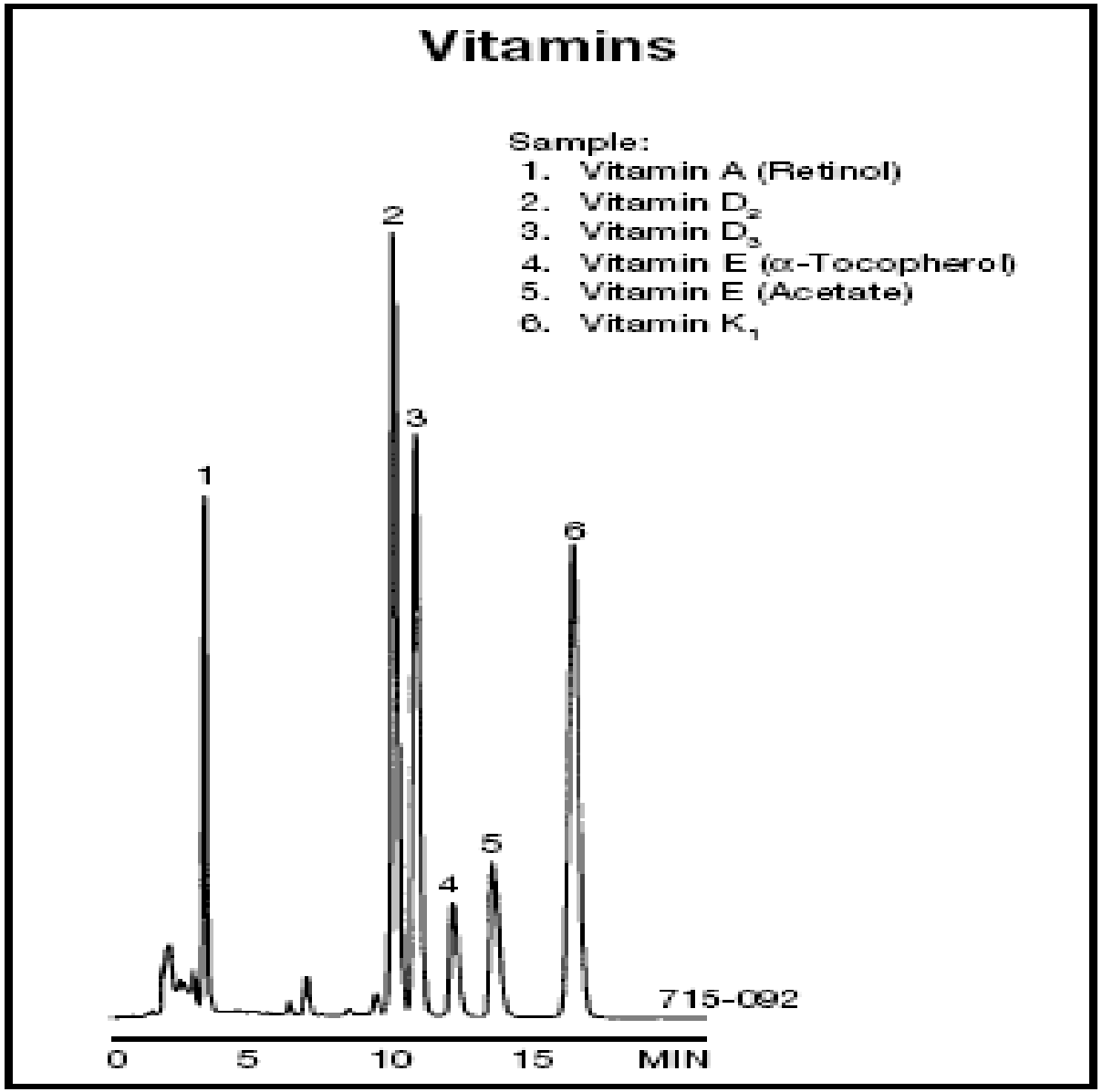
0-100% B in 20 min.

Flow: 1.0 mL/min

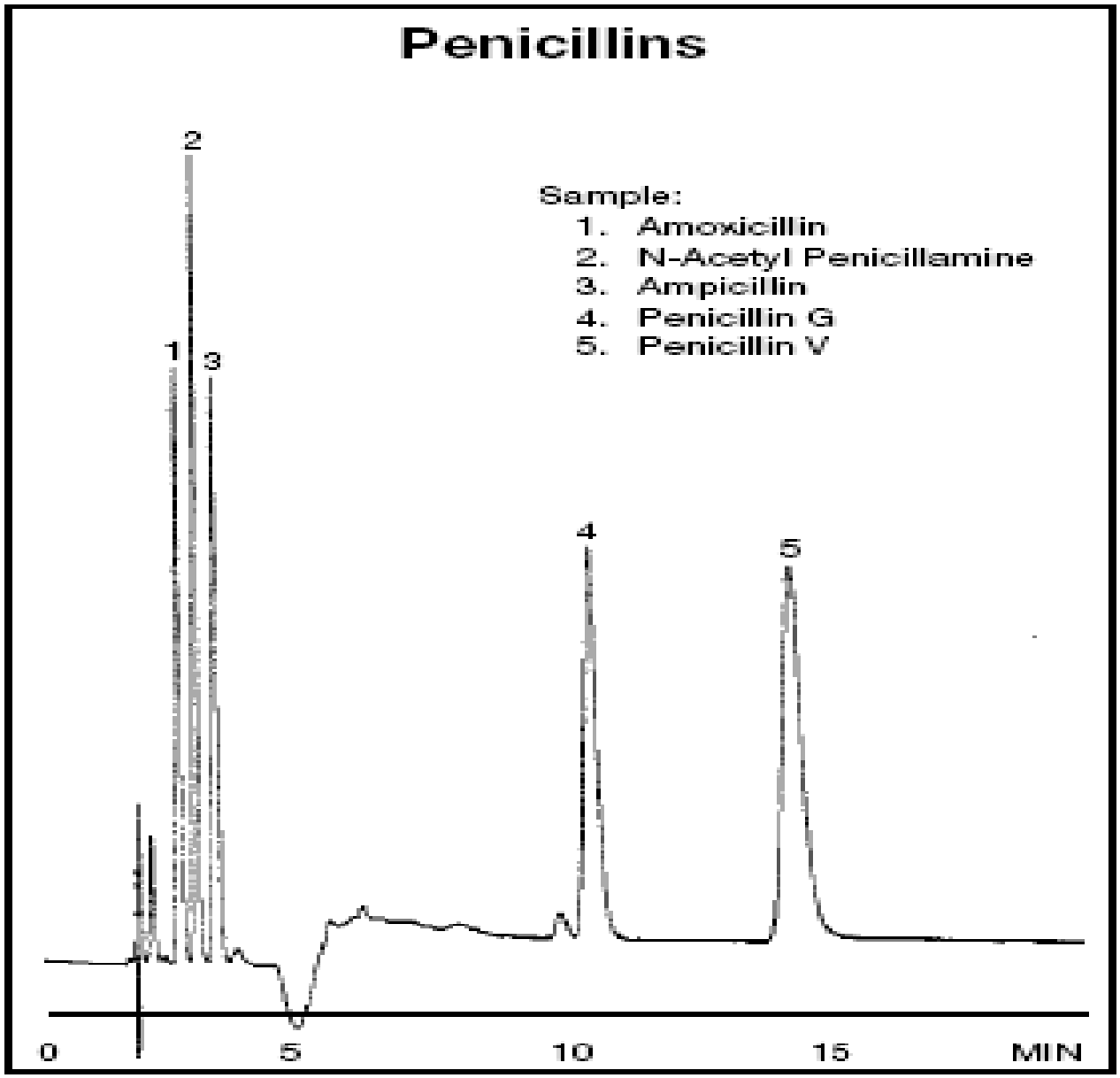
Detector: UV @ 254

Temp: 22°C

Prof. J. Al-Zehouri



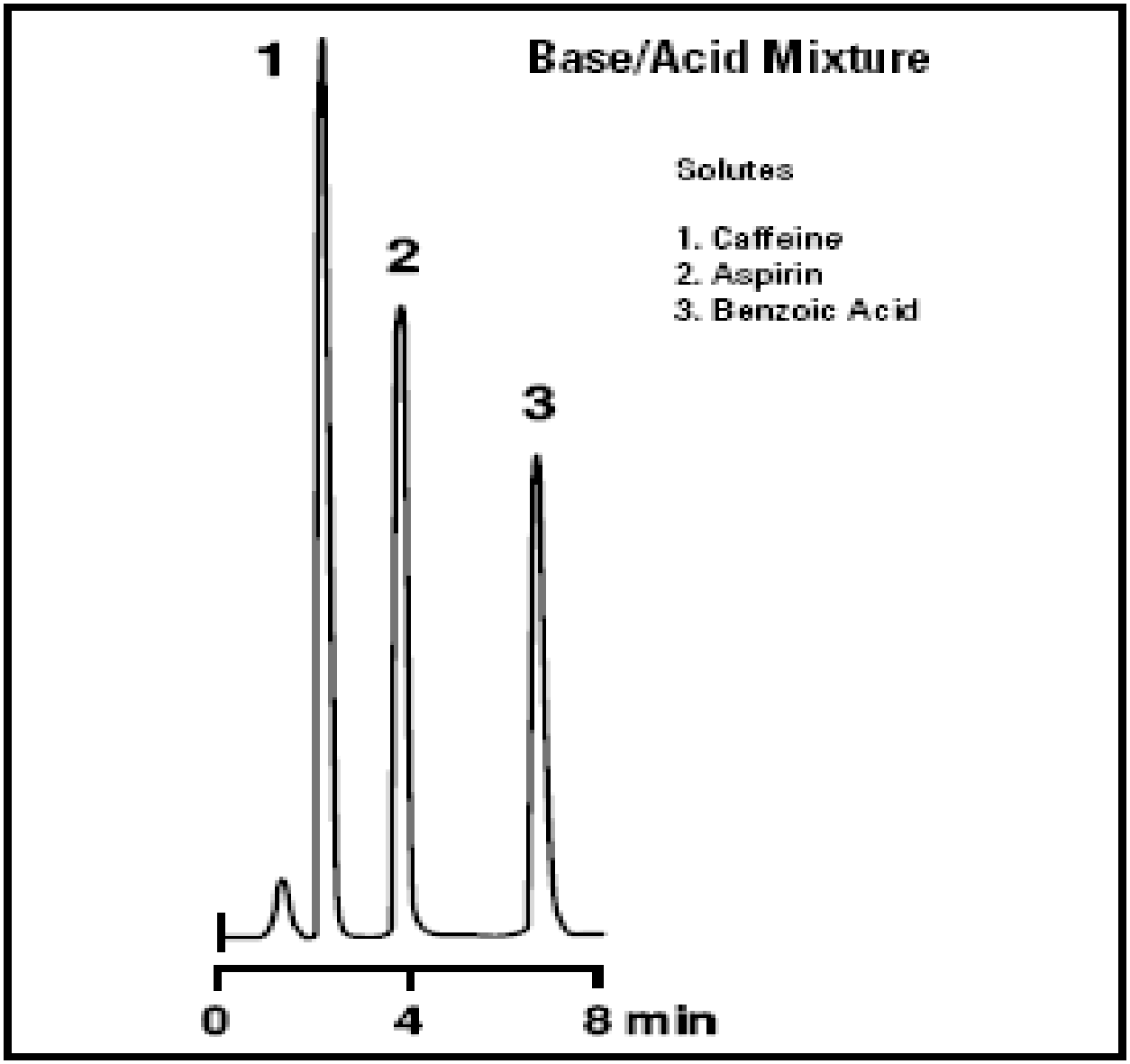
BetaBasic 18, 5µm, 150x4.6mm
Eluent: 75% ACN / 25% MeOH
Flow: 1.0 mL/min
Detector: UV @ 280



BetaBasic CN, 5µm, 150x4.6mm
Gradient: 0.01M KH_2PO_4 / H_3PO_4 Adj pH 3.0
Step at 1.0 min to 90/10 Buffer/ACN
Flow: 1.2 mL/min
Detector: UV @ 220

HPLC Pharmaceutical Application

Prof. J. Al-Zehouri



Column	Hypersil BDS C18, 5µ,150x4.6mm
Eluent	Methanol:0.05M Phosphate Buffer, pH3.5 (40:60)
Flow Rate	1.0 ml/min
Detection	UV 254nm

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Ephedrine Hydrochloride Tablets

Assay Weigh and powder 20 tablets. Carry out the method for *liquid chromatography*, Appendix III D, using the following solutions. For solution (1) shake a quantity of the powdered tablets containing 50 mg of Ephedrine Hydrochloride with 30 ml of *methanol* for 10 minutes, add sufficient *water* to produce 50 ml, filter through glass-fibre paper (Whatman GF/C is suitable) and use the filtrate. Solution (2) contains 0.1% w/v of *ephedrine hydrochloride BPCRS* in *methanol (60%)*.

The chromatographic procedure may be carried out using (a) a stainless steel column (20 cm × 4.6 mm) packed with *stationary phase C* (10 µm) (Nucleosil C18 is suitable), (b) 0.005M *dioctyl sodium sulphosuccinate* in a mixture of 65 volumes of *methanol*, 35 volumes of *water* and 1 volume of *glacial acetic acid* as the mobile phase with a flow rate of 2 ml per minute and (c) a detection wavelength of 263 nm.

Calculate the content of C10H15NO,HCl using the declared content of C10H15NO,HCl in *ephedrine hydrochloride BPCRS*.

Separation of enantiomers by chiral HPLC

فصل المماكبات باستخدام الأستشراب المتخايل

- يتم باستخدام أطوار ثابتة متخايلة Chiral Stationary Phase بحيث يتمكن أحد المتخايلات أن يرتبط معه بثلاث نقط .

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Q&A