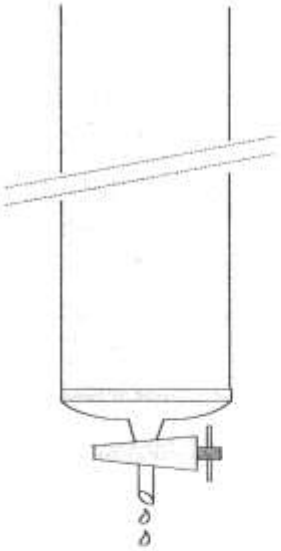
HPLC (Developed in the late-1960 s)



Liquid Chromatography

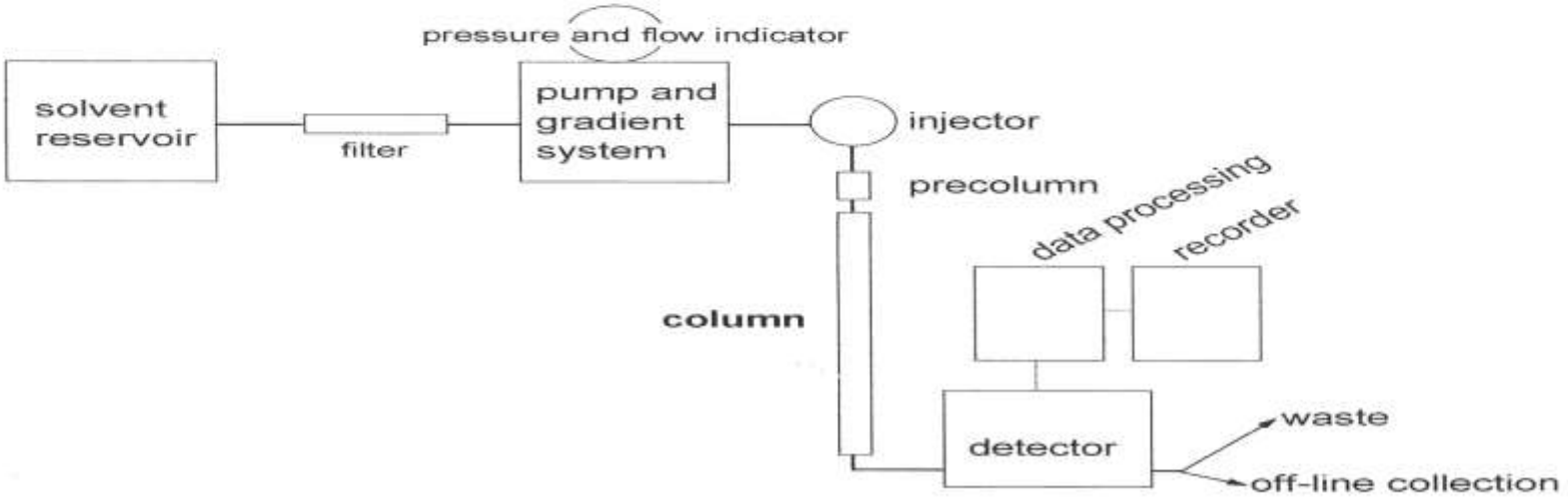
- long columns (> 1m)
- low flow rates (mL/hr)
- packing particles with large diameters (150 μm)
- low efficiency separations

Gas Chromatography

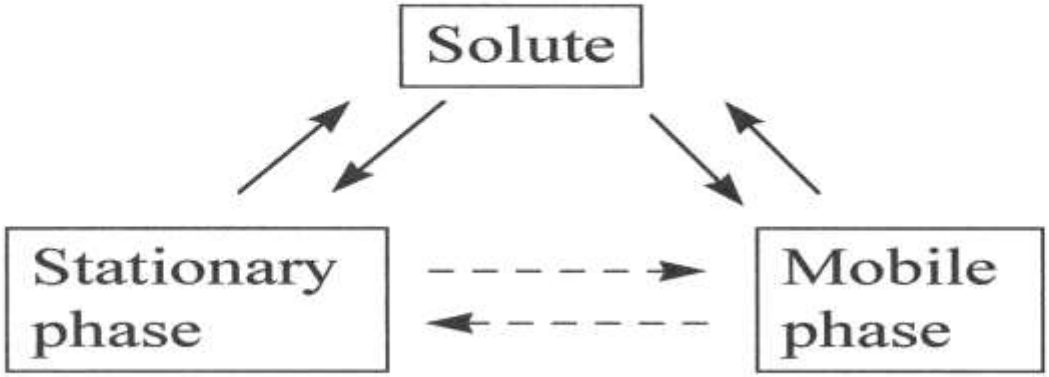
- only 20% compounds are suitable for direct GC (not good for thermally labile and polar compounds)
- gas chromatographic theory (efficiency can be improved by reducing particle size)

Main advancements in HPLC over the last 30 years:

- development of small diameter particles (10, 5, 3, 1 μm)
- improvement in packing narrow columns (4.6-0.32mm i.d.)
- bonded stationary phases
- delivery of stable mobile phase flows



Solute retention in LC depends on interactions between:



Interionic and Intermolecular Forces:

	Type of interaction		Dependence of force on distance
Ions and Ions	Ion – ion		$1/r^2$
Ions and polar molecules	Ion – dipole		$1/r^3$
Ions and nonpolar molecules	Ion – induced dipole		$1/r^5$
Polar molecules and polar molecules	Dipole – dipole	Intermolecular forces (van der Waals forces)	$1/r^7$
Polar molecules and Nonpolar molecules	Dipole – induced dipole		
Nonpolar molecules and Nonpolar molecules	Induced dipole – induced dipole (London forces)		

Normal-phase HPLC

(type of adsorption chromatography)

The stationary phase is more polar than the mobile phase

Sationary phase (adsorbent): mostly inorganic polymers

hydrated silica-oxygen polymer (silica or silica gel)

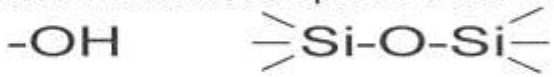
hydrated aluminium-oxygen polymer (alumina)

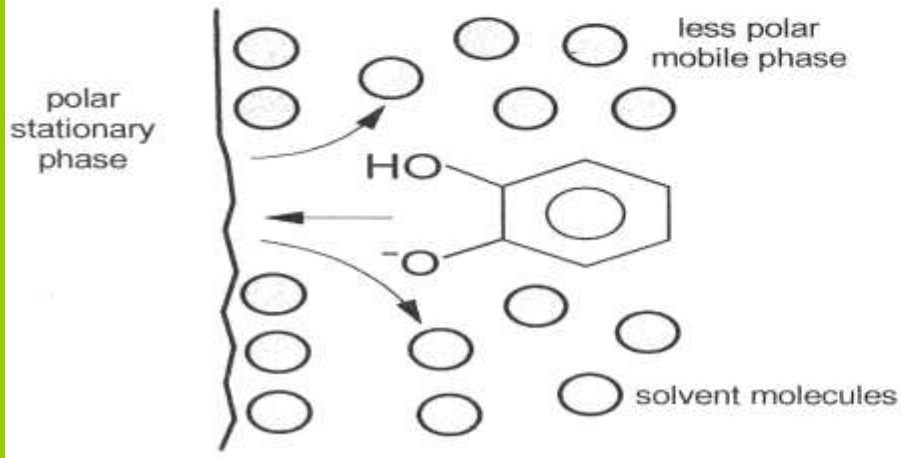
Bonded phases: silica particles with R groups bonded to the surface; R= $-CH_3$ (C-1), $-(CH_2)_3CN$

(advantages include: less tailing, rapid changes to mobile phase composition, generally better reproducibility)

Mobile phase: less polar than the stationary phase

Mechanism of retention: solute and solvent molecules compete for "sites" on the stationary phase; to be adsorbed, the solute molecules must first displace a solvent molecule. Silica has discrete adsorption sites:





Molecules with polar functional groups or capable of H-bonding or polarisable molecules have a strong affinity for the adsorbent surface and will be strongly retained.

Silica gel
(hydrated silicon – oxygen polymer)

Preferred for a number of practical reasons:

- Allows for higher sample loadings
- Less likely to catalyse the decomposition of any sample component

Factors that affect sample retention:

1. Surface consists of discrete adsorption sites (-OH)
   -Si-OH grouping is known as a silanol group

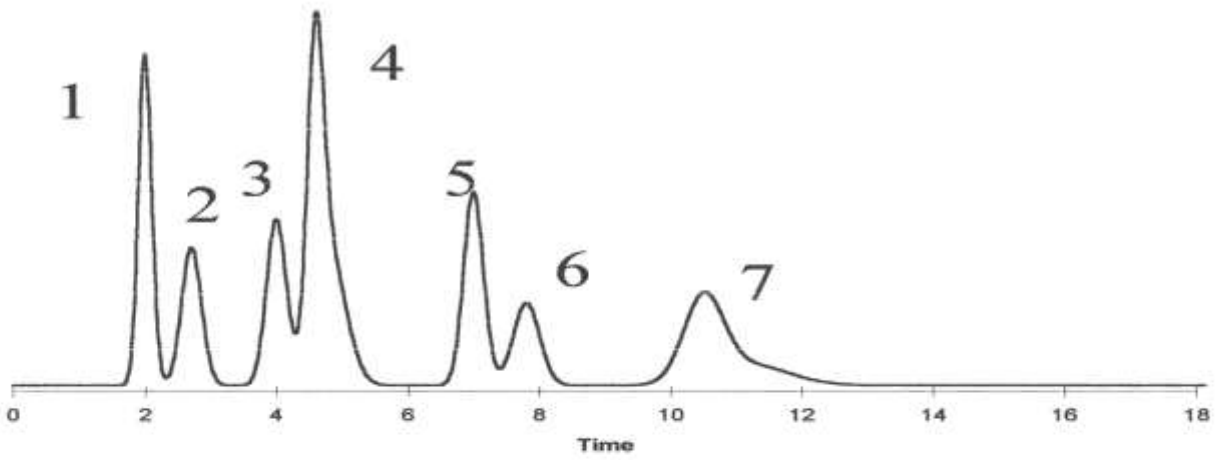
2. Surface area and average pore diameter
   Porous silica: 100-400 m^2/g for spherical particles (3-10 μm)
   Mean pore diameter 5 – 400 nm
   Surface area within pores >>>> external surface area

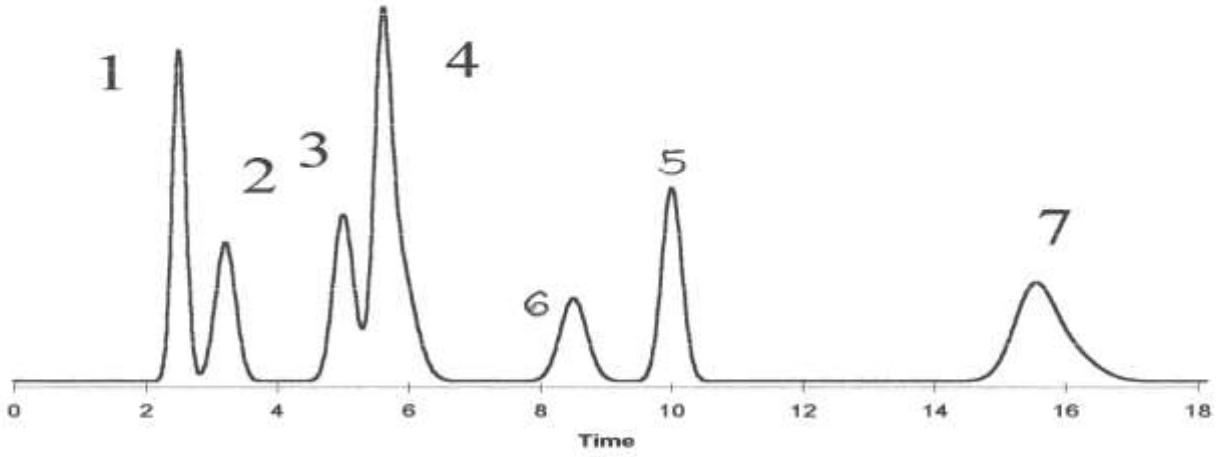
Limitations:

1. Limited pH range (typically used between pH 2-7)

2. Retains basic compounds very strongly (i.e. amines)

Normal phase separation of phthalates





10 micron silica, 30 cm x 4 mm
2 ml/min; UV detection 254 nm
A: ethyl ethanoate/iso octane 5:95
B: butyl ethanoate/iso octane 5:95

Reversed-phase HPLC

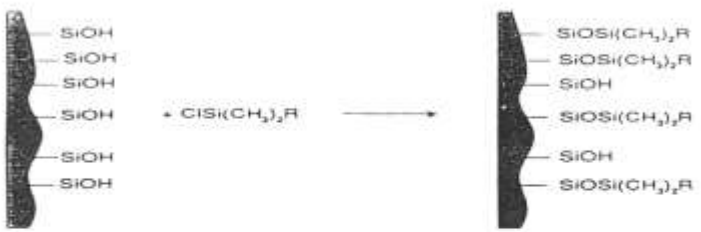
(partition/adsorption chromatography)

The stationary phase is less polar than the mobile phase

Sationary phases:

1. silica particles with hydrocarbon groups (R) bonded to the surface; R= $-CH_3$ (C-1), $-C_8H_{17}$ (C-8), $-C_{18}H_{37}$ (ODS or C-18), $-C_6H_5$

Monomeric Synthesis

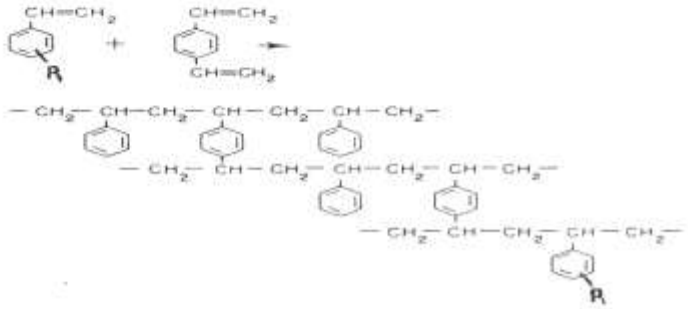


Polymeric Synthesis

end capping residual silanol groups

2. Styrene-divinylbenzene resin (copolymerizing styrene and divinylbenzene) containing suitable hydrocarbon functional groups: C-1 or C-18

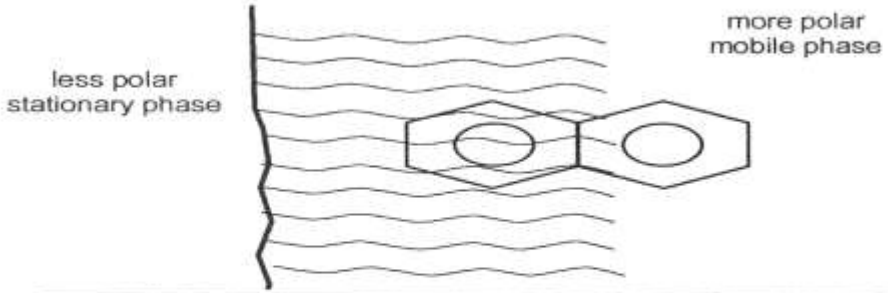
stability over a wide pH range



3. Hypercarb (a porous graphitic carbon, introduced by Shandon in 1988); stable throughout the whole pH range.

Mobile phase: more polar than the stationary phase, e.g. CH_3OH/H_20.

Mechanism of retention: Solutes are generally eluted in order of polarity, the most polar first. We can think of a reversed phase separation as being a partitioning process.



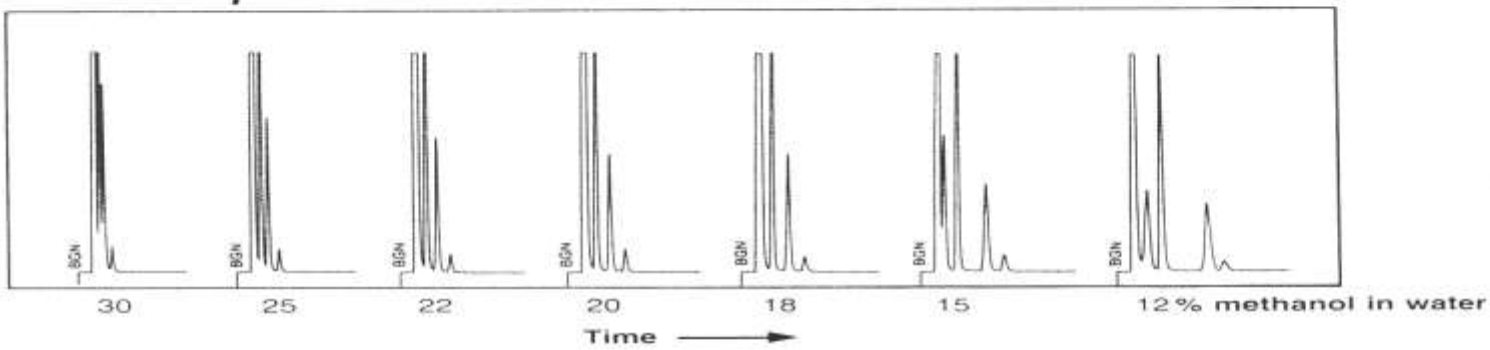
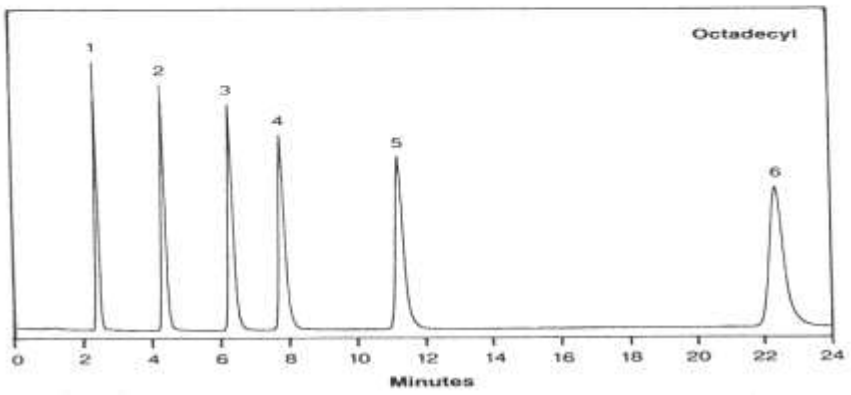
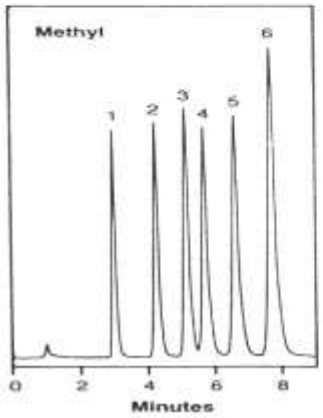


Illustration of the effects of changing solvent conditions on a reversed-phase column. The sample is an analgesic preparation. Each run is isocratic, and the eluents range from high (30%) methanol to low (12%) methanol : water mixtures. The methanol is a better solvent for these materials than is the water. Conditions: ODS column 0.26 × 25 cm; eluent methanol (% shown)/water + 0.5% H_3PO_4; flow rate 1.0 mL/min; detection ultraviolet light absorption at 250 nm. [Data courtesy The Perkin–Elmer Corporation.]





1. uracil
2. phenol
3. acetophenone
4. nitrobenzene
5. methyl benzoate
6. toluene

50/50 methanol/water
1 ml/min

Reversed phase operation with bonded phases has achieved wide popularity because it has the following advantages:

1. The method has a very broad scope that allows samples with wide ranges of polarity to be separated. There is the possibility of using many different bonded phases, producing a very flexible separating system.
2. The method uses relatively inexpensive mobile phases, and equilibration of the mobile phase with the column is rapid.
3. The mode is generally experimentally easier, faster and more reproducible than other HPLC modes.
4. It can be applied for the separation of ionic or ionizable compounds by the use of ion pairing or ion suppression techniques

Ion Suppression HPLC

Ion suppression is used for the separation of weak acids or bases on reversed phase columns.

Principle: suppress the ionization of an acid or the protonation of a base by adjusting the pH, then chromatograph the sample on a reversed phase column (C-18) using methanol or acetonitrile plus an aqueous buffer solution as the mobile phase.

Ion Exchange HPLC

The surface contains ionizable sites: sulphonic acid or quaternary ammonium

$$R^+Y^- + X^- \rightleftharpoons R^+X^- + Y^-$$

(sationary phase) (solution) (stat.phase) (solution)

$$R^-Y^+ + X^+ \rightleftharpoons R^-X^+ + Y^+$$

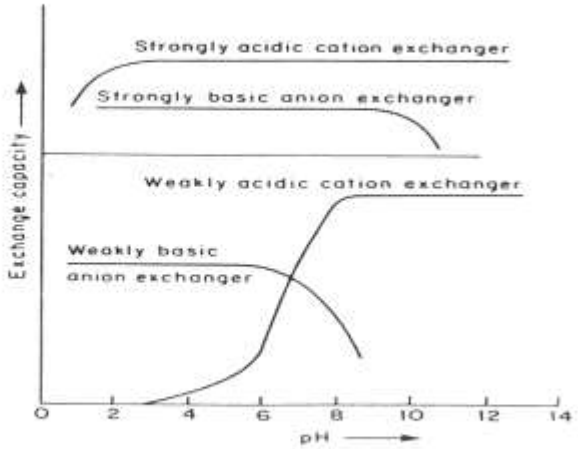
(sationary phase) (solution) (stat.phase) (solution)

Sationary phase:

	Styrene–DVB	Porous layer beads	Bonded silica
Particle size, μm	5–20	30–50	5–10
Capacity	high	low	high
Sample loading	large	small	moderate
pH range	2–14	2–9	2–8
Packing method	slurry	dry	slurry
Efficiency	low →		high

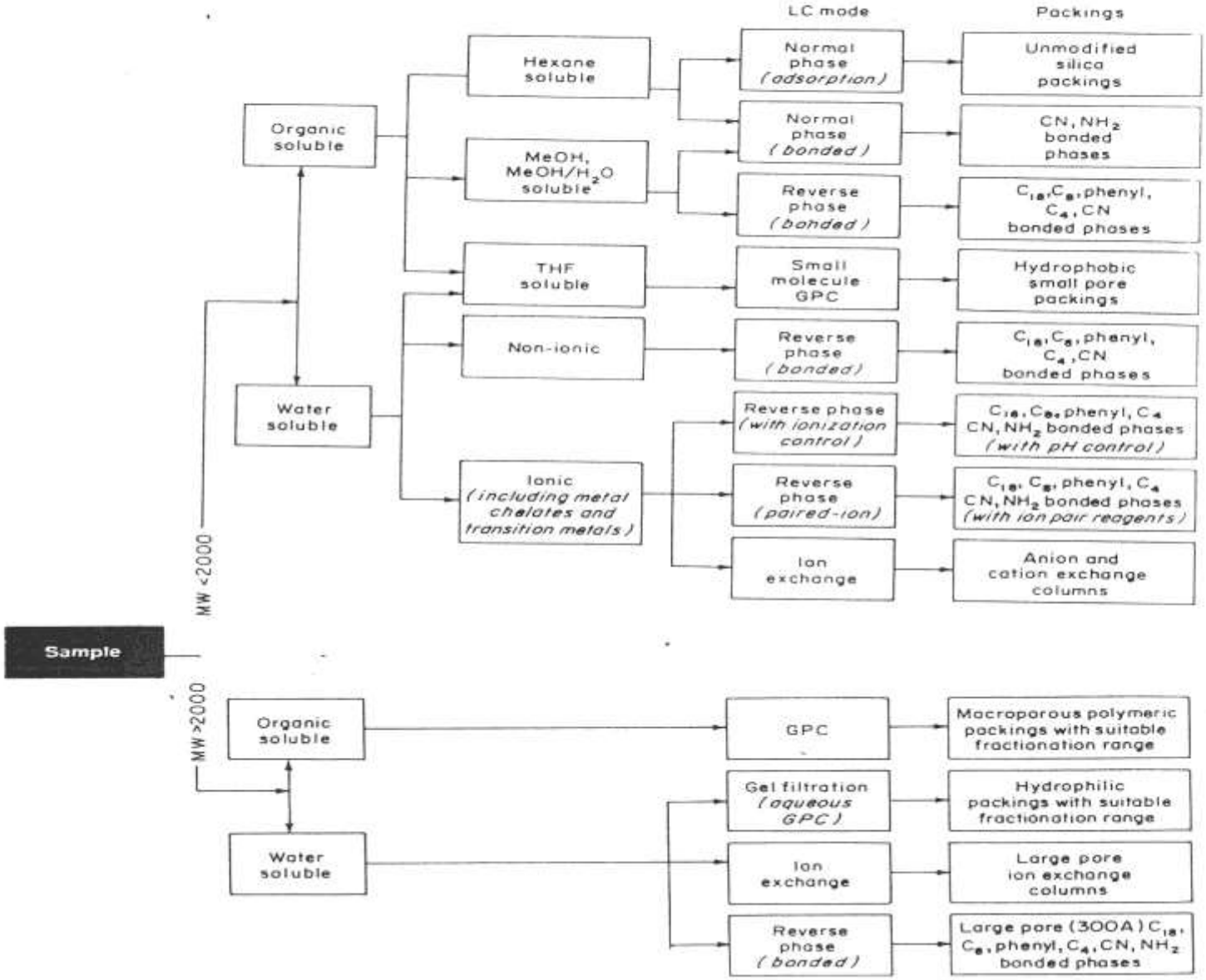
Ion Exchange Groups

Type	Active Group	pH Range of Operation	Application Example
Strongly acidic cation exchanger	$—SO_3^-$	1–14	Amino acids, inorganic separations
Weakly acidic cation exchanger	$—COO^-$	5–14	Transition elements, organic bases
Strongly basic anion exchanger	For example, $—N(CH_3)_3^+$	1–12	Alkaloids, fatty acids
Weakly basic anion exchanger	For example, DEAE, $—C_2H_4N(C_2H_5)_2$	1–9	Organic acids, amino acids



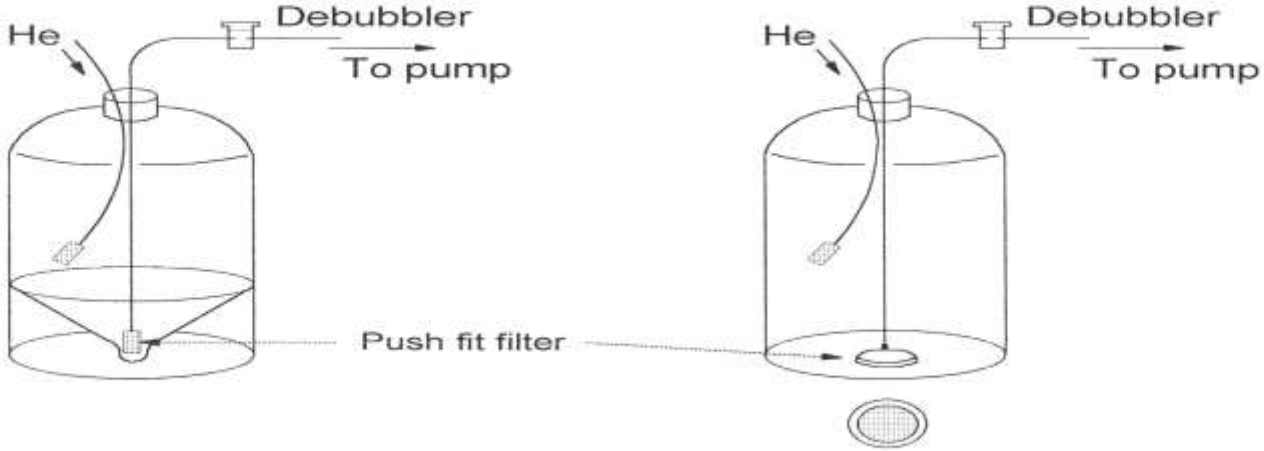
Mobile phase: pH buffer or pH buffer + organic modifier

Selection of HPLC method



Solvent Delivery and Sample Injection

Solvent reservoir



Pressure, Flow and Temperature

HPLC: 25-100 bar; pressure depends on column length, partcile size, viscosity and flow rate of mobile phase.

Pressures in HPLC do not present a hazard (precautions should be taken when packing columns).

Table 1: Pressure units. The official SI unit is the pascal.
1 pascal (Pa) = 1 newton (N) / m^2
1 bar = 10^6 dyn/cm^2 = 10^5 Pa
1 millibar (mbar)= 10^{-3} bar = 10^2 Pa
1 microbar (μbar) = 10^{-6} bar = 10^{-1} Pa
1 nanobar (nbar) = 10^{-9} bar = 10^{-4} Pa
1 atmosphere (atm) = 1.013 bar = 101 308 Pa
1 Torr = 1 mmHg = 1.333 mbar = 133.3 Pa
1 psi = 1 pound/square inch = 0.07 atm

1 Kg/cm² = 0.981 bar = 14.2 psi

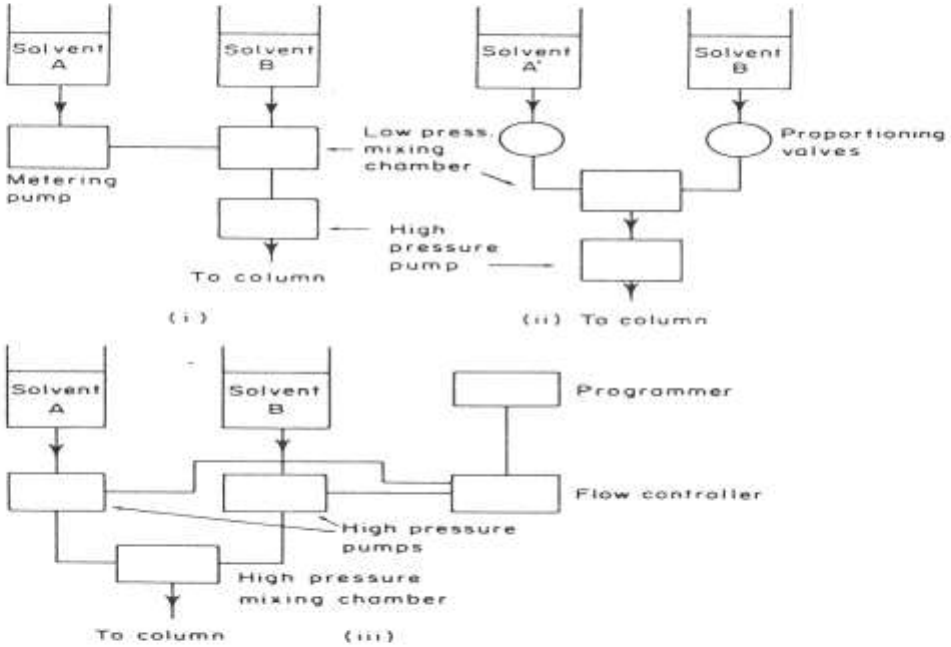
Temperature control is important for reproducible retention times.

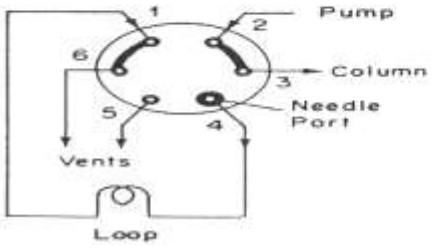
Forced air oven, 0.1 °C stability from ambient to 100 °C

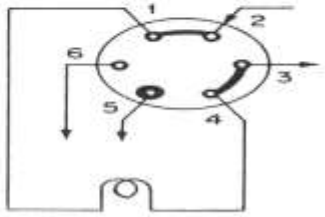
Safety considerations

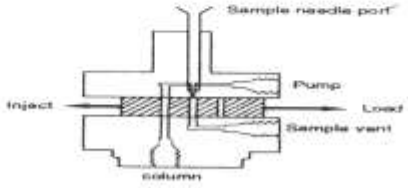
Controlling mobile phase and sample temperature is also important

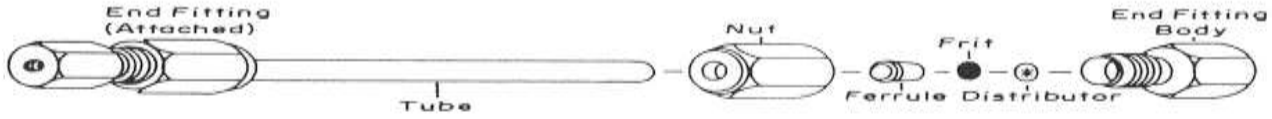
Model	Shimadzu LC-9A	ISCO LC 500	Stanstead A9512 LC
Type	constant flow twin reciprocating	constant flow syringe type	constant pressure pneumatic amplifier
Maximum output pressure, bar	392	250	500
Flow rate range, $cm^3\ min^{-1}$	0.001–5	1.3×10^{-4}–3.34	up to 200
Capacity, cm^3	continuous pumping	375	continuous pumping

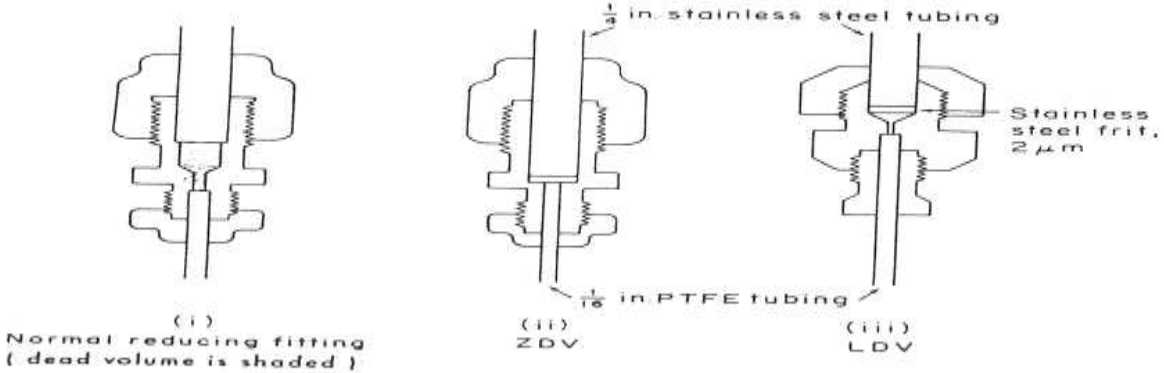


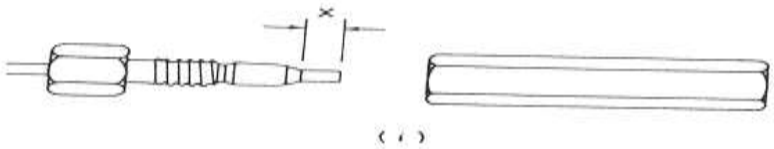


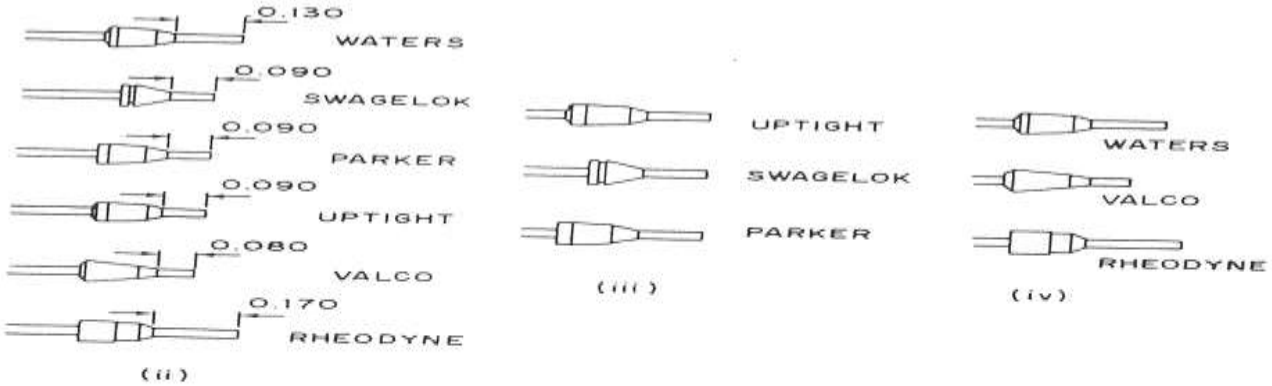





End Fitting
(Attached)
Nut
Frit
End Fitting
Body
Tube
Ferrule
Distributor


1/4 in stainless steel tubing
Stainless
steel frit,
2 μm
1/16 in PTFE tubing
(i)
Normal reducing fitting
(dead volume is shaded)
(ii)
ZDV
(iii)
LDV


x
(i)


0.130
WATERS
0.090
SWAGELOK
0.090
PARKER
0.090
UPTIGHT
0.080
VALCO
0.170
RHEODYNE
(ii)
UPTIGHT
SWAGELOK
PARKER
(iii)
WATERS
VALCO
RHEODYNE
(iv)


(v)
(vi)

Detectors for HPLC

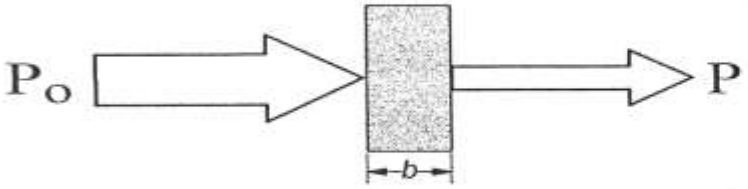
Important characteristics (listed in random order):

a. High sensitivity
b. Low limit of detection (negligible baseline noise)
c. Large linear dynamic range
d. Universal or selective responce
e. Low dead volume
f. Non-destructive of the sample
g. Inexpensive to purchase and operate, reliable and easy to operate
h. Capable of providing information on the identity of the solute
i. Response independent of mobile phase composition

Detector	Response	C_N g cm^{-3}	Linear range	Flow cell volume, μl
UV-vis absorption	S	10^{-8}	10^4-10^5	1-8
Fluorescence	S	10^{-12}	10^3-10^4	8-25
Conductivity	S	10^{-7}	10^3-10^4	1-5
Amperometric	S	10^{-10}	10^4-10^5	0.5-5
Mass spectrometry	S	10^{-10}		---
Refractive Index	G	10^{-6}	10^3-10^4	5-15

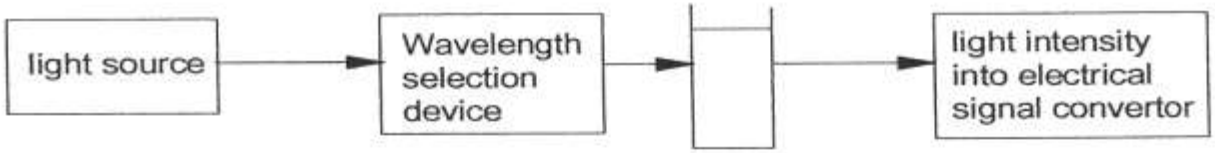
UV/Vis light absorbance detectors

Beer-Lambert Law: $A = \varepsilon bc = \log \frac{P_o}{P}$ ⊛



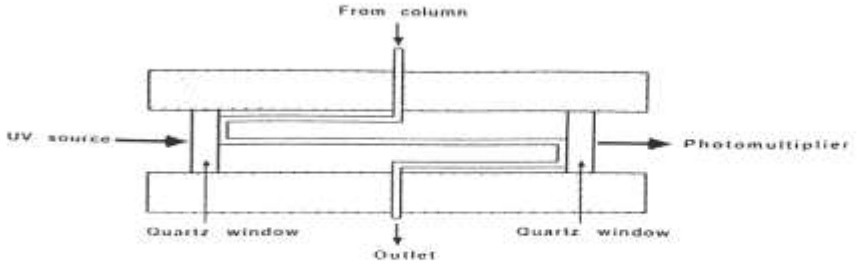
A: absorbance of solution in cell
ε: molar absorptivity [L cm^{-1} mol^{-1}]
b: optical path-length through the cell [cm]
c: molar concentration of the solute
P_o: light intensity (or power) focused onto the cell
P: light intensity transmitted

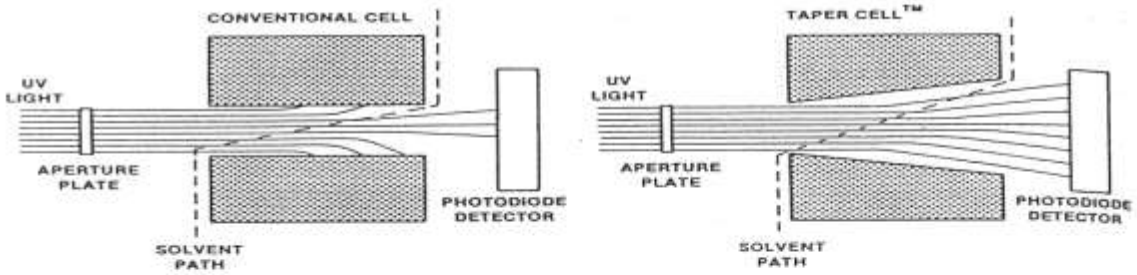
⊛ : applies only to monochromatic radiation



Radiation Source	Wavelength (nm) of emmision lines or emission range
Mercury	**254**, 313, 365, 405, 436, 546, 578
Cadmium	229, 326
Zinc	214, 308
Magnesium	206
Deuterium	190-350 (continuum)
Tungsten	190-700 (continuum)

Cell design for UV/vis light detectors





Elimination of the liquid lens effects with a tapered flow-cell

Operating characteristics of UV/vis light detectors

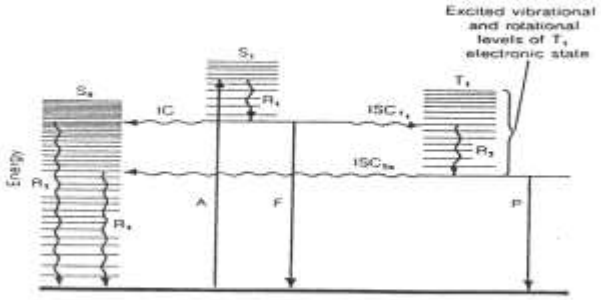
- imperative that the background absorbance of the mobile phase be kept as low as possible if direct detection is to be used (requires knowledge of the UV cut-off wavelengths)

Table 5.3 u.v. Cut-offs for some common solvents and buffers.

Solvent	u.v. Cut-off (nm)	Buffer	u.v. Cut-off (nm)
n-Pentane	190	Acetic acid, 1%	230
Carbon tetrachloride	265	Triethylamine, 1%	235
Methanol	205	Sodium citrate, 10 mM	225
Tetrahydrofuran	230	Sodium acetate, 10 mM	205
Chloroform	245	Tris HCl, 20 mM	204
Acetonitrile	190	Potassium phosphate, 10 mM	190
Dioxane	215	Ammonium bicarbonate, 10 mM	190
Ethanol	210	Sodium chloride, 1 M	208
Ethyl acetate	256	EDTA, disodium, 1 mM	190
Petroleum ether	210	Sodium dodecyl sulfate, 0.1%	190

- absorption spectrum of the solute depends, to some extent, on the composition of the mobile phase
- derivatisation reactions for producing coloured products

Fluorescence detectors



$$F = f(\theta)\, g(\lambda)\, I_o\, \theta_f\, \varepsilon\, l c$$

$f(\theta)$: geometry factor related to the positioning of the detector

$g(\lambda)$: wavelength response characteristics of the detector

I_o: intensity of the incident radiation

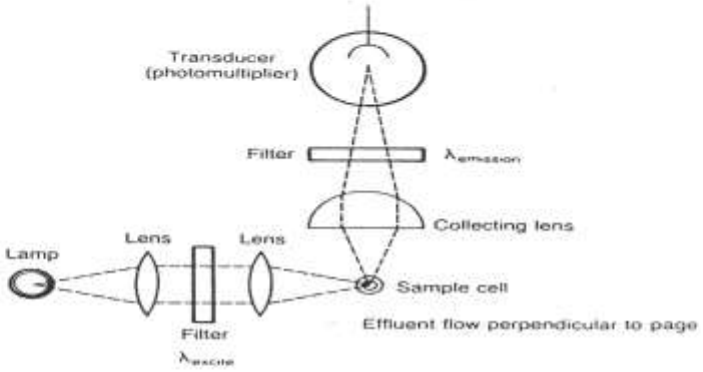
θ_f: quantum yield of the analyte molecule

ε: molar absorptivity of the analyte

l: optical path length

c: molar concentration of the analyte

Physical processes which can follow the absorption of a photon by a molecule. S denotes a singlet state and T denotes a triplet state. Solid arrows represent processes involving photons, while wavy arrows denote radiationless transitions. A, absorption; F, fluorescence; P, phosphorescence; IC, internal conversion; ISC, intersystem crossing; R, vibrational relaxation.



Disadvantages:

Dependence of fluorescence signal on a range of experimental parameters:

- mobile phase pH,
- nature of components of the mobile phase,
- temperature,
- concentration of analyte,
- and quenching effects.

Advantage:

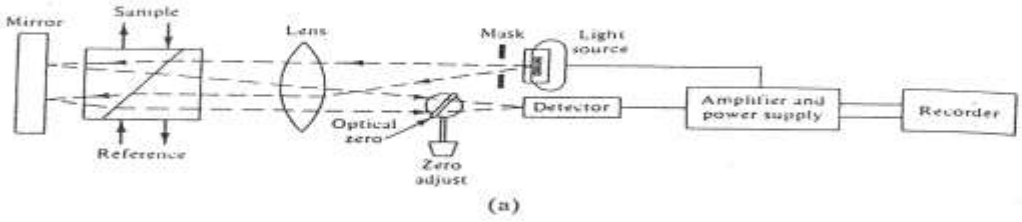
- Affords detection limits which are often 100 times lower than those achieved with UV/vis detection.

Refractive Index detectors

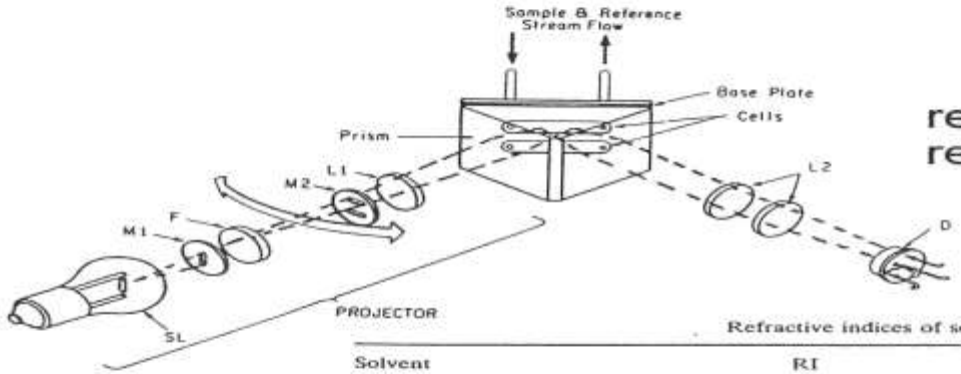
(closest to the ideal universal detector)

Principle: comparison of the RI of the pure mobile phase with the column effluent will indicate the presence of an eluted solute.

-differential refractometers



deflection type refractometer



reflection (Fresnel) type refractometer

Refractive indices of some common solvents.

Solvent	RI	Solvent	RI
Methanol	1.329	Nitromethane	1.394
Water	1.330	Cyclopentane	1.406
Acetonitrile	1.344	Tetrahydrofuran	1.408
n-Pentane	1.358	*n*-Decane	1.412
Ethanol	1.361	Dioxane	1.422
Acetic acid	1.372	Methylene chloride	1.424
Isopropanol	1.380	Ethylene glycol	1.427
n-Propanol	1.380	Chloroform	1.443
Methylethylketone	1.381	Carbon tetrachloride	1.466
Methylisobutylketone	1.394	Toluene	1.496

Characteristics:

moderate sensitivity

temperature sensitive (0.001 oC will give a change of 10-6 RI units)

not sensitive to pressure changes

generally unsuitable for gradient elution chromatography

Applications:

carbohydrates, alcohols, polymers