Liquid Chromatography

HPLC

HPLC: High Performance Liquid Chromatography

- The mechanisms of partitioning and adsorption primarily exploited employed (LC)
- HPLC (*high pressure utilized*)
- Analytes – high molecular mass, low volatility

Normal phase chromatography - NP

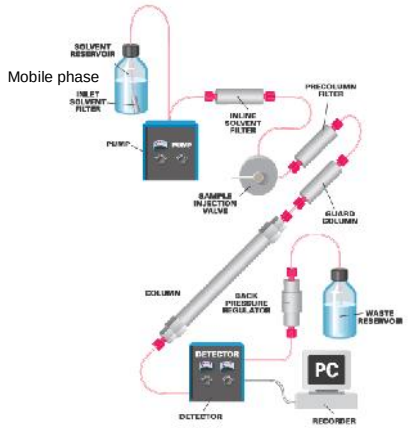
Reversed phase chromatography - RP

Normal phase chromatography:

More polar stationary phase and a less polar mobile phase. Bonded-phase silica are used to separate weakly polar compounds

Reversed phase chromatography:

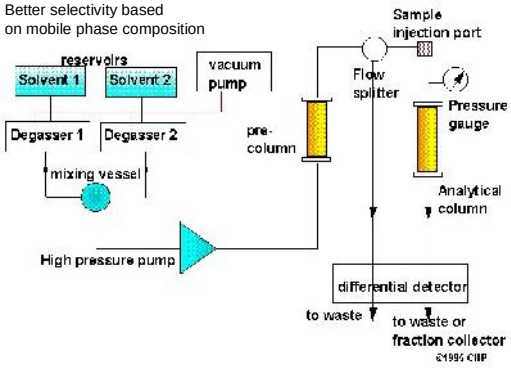
Non-polar stationary phase and a polar mobile phase



http://www.upchurch.com/TechInfo/hplcIntro.asp#

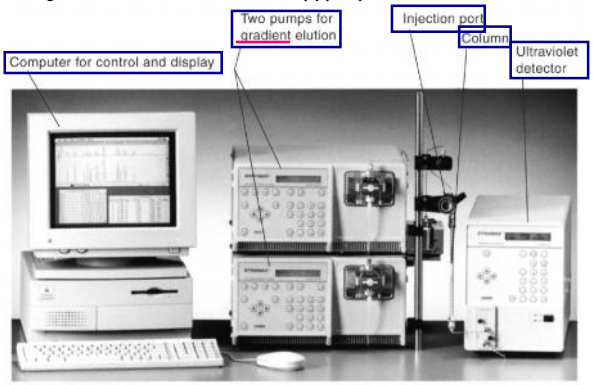
Instrument Components

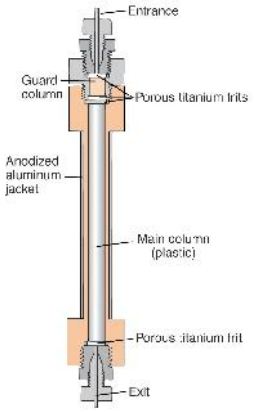
Better selectivity based on mobile phase composition



http://www.chemistry.adelaide.edu.au/external/soc-rel/content/hplc.htm

Configurations with more than 2 solvent(s) pumps available



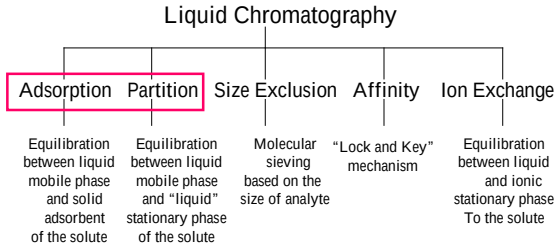


HPLC uses packed columns predominantly
d_p = 1-5µm small particles
L = 5 – 30 cm short
i. d. = ~0.5 cm

a Pressure: 50-350 bar (UHPLC >400bar)
Higher temperatures (~10ºC) lowers viscosity, lowers pressure used, lowers retention times, may increase stationary phase bleeding, needs preheating of mobile phase.

$$\Delta P = f\frac{u\eta L}{\pi r^2 d_p^2}$$

The classification of chromatographic modes according to the retention mechanism...



- Capacity Factor

$$k_R^{'} = \frac{t_R - t_M}{t_M} = \frac{v_R - v_M}{v_M}$$

- Selectivity (Relative retention)

$$\sim = \frac{t_2 - t_M}{t_1 - t_M} = \frac{v_2 - v_M}{v_1 - v_M} = \frac{k_2^{'}}{k_1^{'}}$$

- Efficiency $N = \frac{L}{H}$

- Resolution $R = \frac{\sqrt{N}}{4}\frac{\sim - 1}{\sim}\frac{k^{'}}{1+k^{'}}$

Van Deemter Equation: (Packed Column)

$$H_{column} = A + Bu^{-1} + Cu$$

$$H_{col} = \left({}^{a}d_p\right) + \frac{\left(2\gamma D_M\right)}{u} + \left(\frac{qk'}{(1+k')^2}\frac{d_f^2}{D_S} + \frac{k'^2 d_p^2}{96 D_M (1+k')^2}\right)u$$

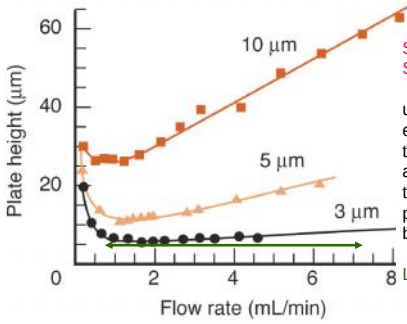
$$H_{col} = {}^{a}d_p + \frac{2\gamma D_M}{u} + \frac{qk'}{(1+k')^2}\frac{d_f^2}{D_S}u + \frac{k'^2 d_p^2}{96 D_M (1+k')^2}u$$

Diffusion coefficient, D_m, is low in LC, equilibration slow; m.p. being a liquid use of capillary columns is not possible (pressure considerations). Large s.p. surface preferred, coated on inert particles, both d_f, d_p non-zero.

Small inert particles, d_p (increases the s.p. surface and minimize multiple paths and hence minimize term A) requires very high pressures to move the m.p. through the column.

Lower d_f is preferred; faster equilibration.

$$H_{col} = \left({}^{a}d_p\right) + \frac{\left(2\gamma D_M\right)}{u} + \left(\frac{qk'}{(1+k')^2}\frac{d_f^2}{D_S} + \frac{k'^2 d_p^2}{96 D_M (1+k')^2}\right)u$$



Small d_p, low A
Small d_p d_f; low C-slope.

uniform flow (A) and fast equilibrations because of the smaller distances analyte molecules would travel due to better packing of smaller, bonded s.p, particles (C).

Larger optimum flow rate.

However these systems require a **high pressure** pump to push the mobile phase through the column.

$$\Delta P = f\,\frac{u\eta L}{\pi r^2 d_p^2}$$

f depends on particle shape and packing

Table 25-1 Performance as a function of particle diameter

Particle size d_p (μm)	Retention time (min)	Plate number (*N*)	Required pressure (bar)
5.0	30	25 000	19
3.0	18	42 000	87
1.5	9	83 000	700
1.0	6	125 000	2 300

NOTE: Theoretical performance of 33-μm-diameter × 25-cm-long capillary for minimum plate height for solute with capacity factor k' = 2 and diffusion coefficient = 6.7 × 10^{-10} m^2/s in water-acetonitrile eluent.

SOURCE: J. E. MacNair, K. D. Patel, and J. W. Jorgenson, "Ultrahigh-Pressure Reversed-Phase Capillary Liquid Chromatography with 1.0-μm Particles," Anal. Chem. 1999, 71, 700.

Table 25-1
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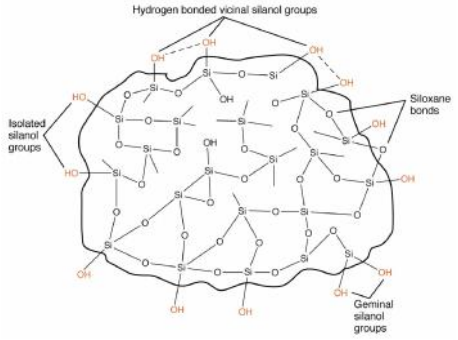
Stationary Phase

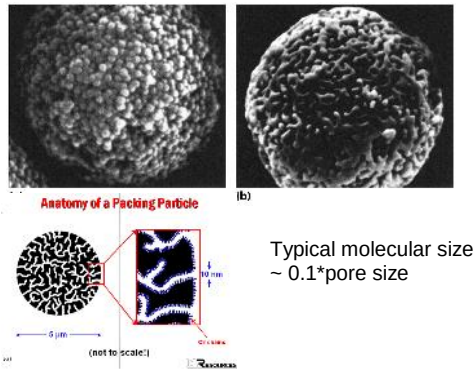
Column support: small, pure, spherical, micro-porous particles of silica or polymer particles.

Often these particles are ***coated*** with s.p. or the s.p is *chemically* ***bonded*** to the surface. Stationary phase coatings on silica particles are not very durable.

Sometimes silica particles are used (as is) for LC (adsorption mechanism, and **normal mode**).

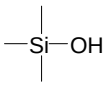
Highly polar





Typical molecular size ~ 0.1*pore size

Stationary phase **coated or bonded** onto the μm size silica/polymer particles (base 'inert' particles).



Adsorption chromatography, NP

pH 2-3, Si-OH
pH>3 de-protonation may occur
Si-O^-, resulting in tailing of positively charged analyte species
pH > 8, silica dissolves
use polymeric supports

pH 2-8 optimum range (silica)

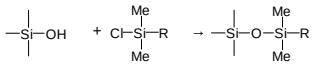
Partition chromatography on bonded phases:

Chemically bonding the hydroxy groups of silica particles would generate ***a rugged*** s.p. and most importantly the ***thinnest possible*** (monolayer) coating, tiny d_f.

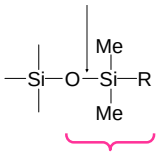
Bonded carbon chains forms the primary s.p. material $C_4 \rightarrow C_{18}$ (RP). Derivatization of the terminal carbons allows us to 'tailor' s.p.'s with different polarities.

Such derivatized s.p. are used in the reverse phase mode, generally.

Bonded phases



Hydrolyses at pH <2

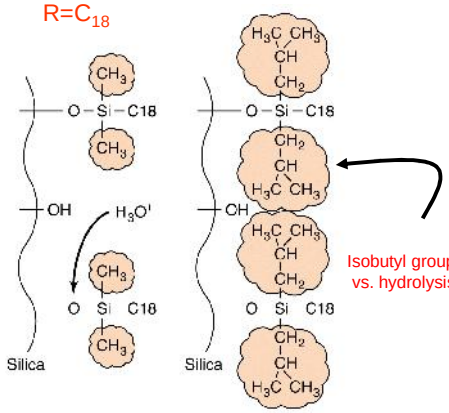


s.p.
Monolayer!

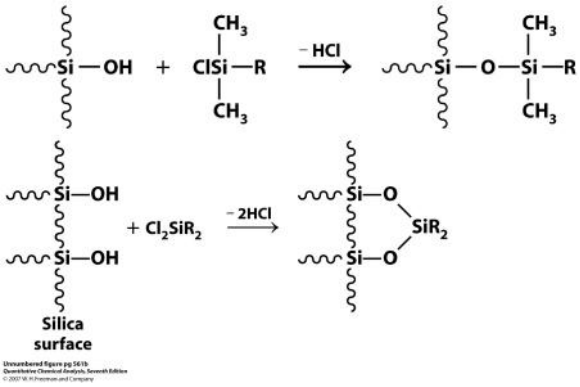
R = long C chain with/without functional groups.

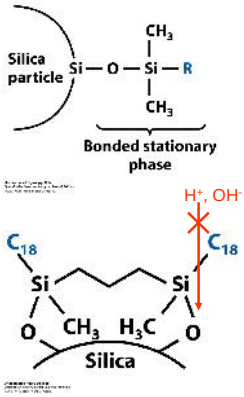
Partition chromatography, R=n-C18 RP

R=C_{18}



Isobutyl groups better protects vs. hydrolysis;





Well protected against hydrolysis

Some common R groups

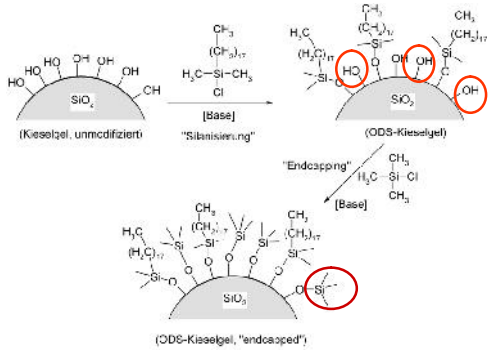
$(CH_2)_{17}CH_3$	octadecyl	nonpolar RP
$(CH_2)_7CH_3$	octyl	
$(CH_2)_3C_6H_5$	phenyl	
$(CH_2)_3NH_2$	amino	polar NP
$(CH_2)_3CN$	cyano	
$(CH_2)_2OCH_2CH(OH)CH_2OH$	diol	

End-capping

A procedure following the primary bonding process of the stationary phase onto the base 'inert' packing. This secondary bonding step to covers unreacted silanols on the silica surfaces.

This process typically helps increase a column's overall CARBON LOAD.

The replacement of accessible silanol groups in a bonded stationary phase by $Si(CH_3)_3$ groups prevents the any tailing associated with a polar analyte peaks, increases the durability of the column even with an alkaline mobile phases.



http://commons.wikimedia.org/wiki/File:Silica_gel_endcapping.png

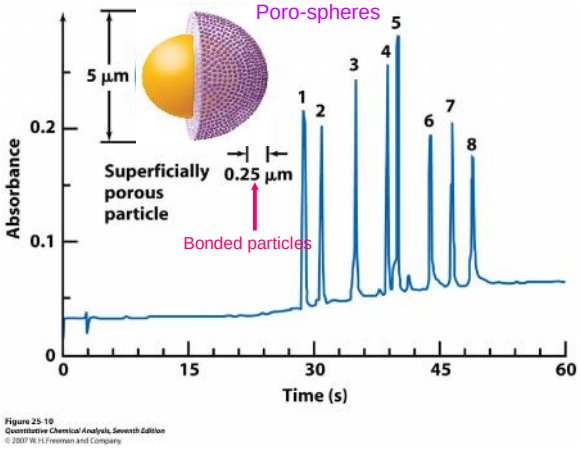


Figure 25-10
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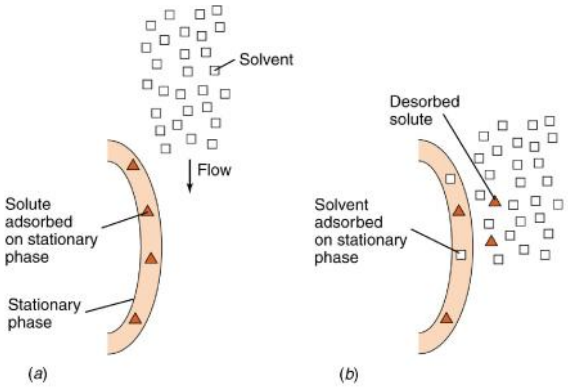
Mobile Phase:

Unlike in GC, the m.p. in LC influences heavily on the separation process by 'actively participating' in the elution of analytes from the s.p.

Both s.p. and m.p. must be considered in determining the separation process.

Modes of LC; normal phase - NP
(s.p. > m.p. polarity)
reversed phase - RP
(s.p. < m.p. polarity)

Adsorption Chromatography



Isocratic Elution: use of a constant-composition mobile phase in liquid chromatography during the entire run.

The mode is "isocratic" if only a single composition solvent mixture or pure solvent is used!

Various solvents can be mixed to give mixture with a suitable polarity for the experiment, which depend on the ratio of the solvents combined to form the solvent mixture.

Eluent Strength of solvent (m.p.)

The ability to draw analytes to the mobile phase (solvent) – depends on the m.p. polarity, pH etc.

Eluotropic series - a *relative* ranking of HPLC solvents (m.p.) ranging from non-polar to very polar. *Property defined based on silica s.p..*

Polarity effects are due, in part, to dielectric constant, dipole moment and hydrophobic / hydrophillic properties of the mobile phase.

Adsorption LC / **Normal phase LC**

Table 25-2 Eluotropic series and ultraviolet cutoff wavelengths of solvents for adsorption chromatography on silica

Solvent	Eluent strength (ϵ°)	Ultraviolet cutoff (nm)
Pentane	0.00	190
Hexane	0.01	195
Heptane	0.01	200
Trichlorotrifluoroethane	0.02	231
Toluene	0.22	284
Chloroform	0.26	245
Dichloromethane	0.30	233
Diethyl ether	0.43	215
Ethyl acetate	0.48	256
Methyl *t*-butyl ether	0.48	210
Dioxane	0.51	215
Acetonitrile	0.52	190
Acetone	0.53	330
Tetrahydrofuran	0.53	212
2-Propanol	0.60	205
Methanol	0.70	205

The ultraviolet cutoff for water is 190 nm.

SOURCES: L. R. Snyder, in *High-Performance Liquid Chromatography* (C. Horváth, ed.), Vol. 3 (New York: Academic Press, 1983); *Burdick & Jackson Solvent Guide*, 3rd ed. (Muskegon, MI: Burdick & Jackson Laboratories, 1990).

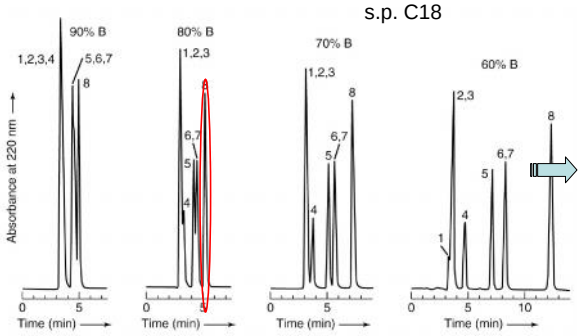
Stronger eluents minimizes (blocks) the interaction of solutes with the stationary phase.

In RP chromatography the strength of the eluting power of the solvent is in reverse order; less polar solvent elute is the stronger solvent.

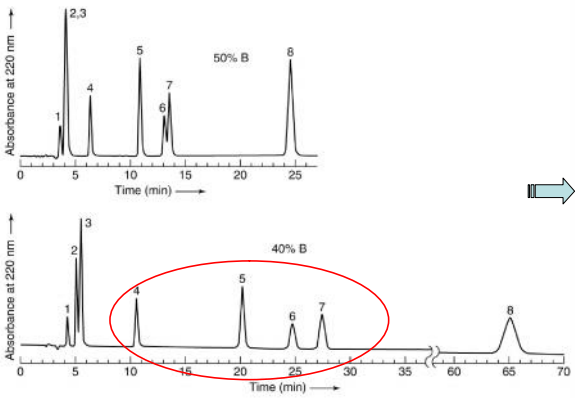
Gradient Elution: mode where the mobile phase composition is altered over the duration of the chromatographic separation. Also known as solvent programming.

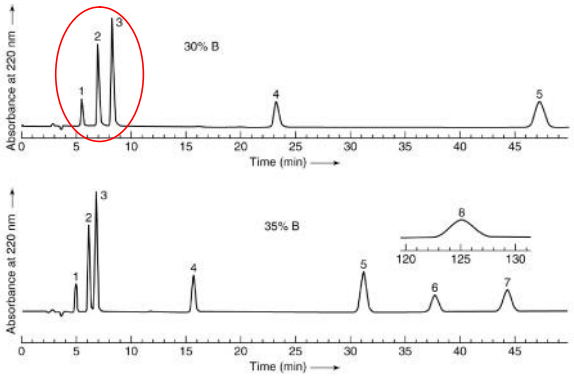
Gradients can be continuous or stepwise. Binary, ternary, and quaternary solvent gradients have been used routinely in HPLC.

Eg: Aromatic analytes (8) s.p. C18

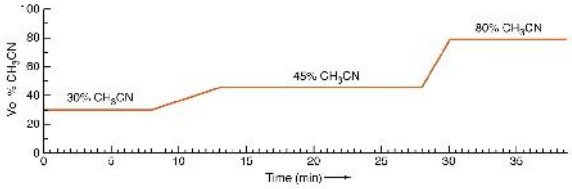


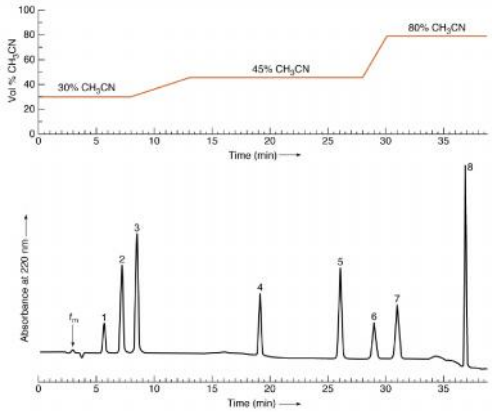
Solvent: A+B A = aqueous buffer; B = acetonitrile





Based on the observations of the above isocratic runs, a gradient program for decreasing separation time by changing mobile phase composition over time during the chromatographic separation.





Read material under the subheadings
'Solvents' and 'Maintaining Symmetric Band shape'
Harris 8th Ed

Temperature Stability and HPLC

Retention (k' ↔ K) is temperature-dependent. Temperature variations makes retention times and peak heights unreliable /variable.

Stability of analytes - especially biological compounds such as enzymes or proteins, may not be stable at room temperature or higher.

Temperature programming is an available option (max T = ~60°C), but stabilization of temperature is critical. Diffusion coefficients of analytes in s.p. and m.p. would determine the quality of the separation of analytes and the time of separation, however.

Requirements: High temperature and Temperature Programming in HPLC

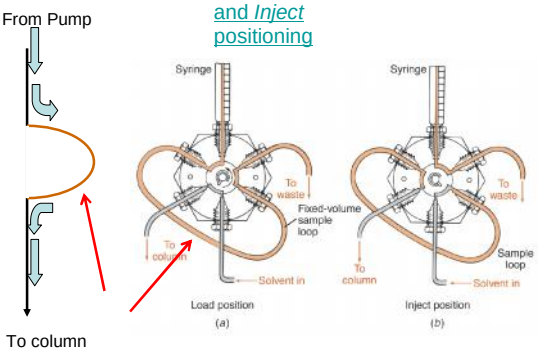
Successful implementation of HPLC at elevated temperatures depends on;

- Preheating the mobile phase to avoid band broadening related to thermal mismatch across the column
- Ability to efficiently heat both the exterior space around the column and the fluid entering the column to allow rapid temperature programming.
- Columns must be stable at elevated temperatures.

Injection Valves

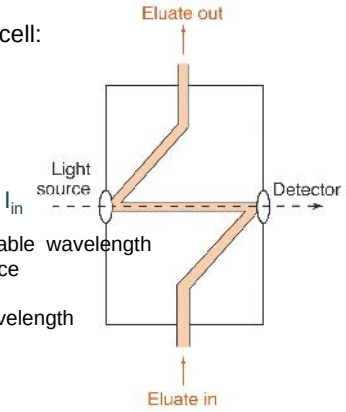
Load and *Inject* positioning

From Pump



To column

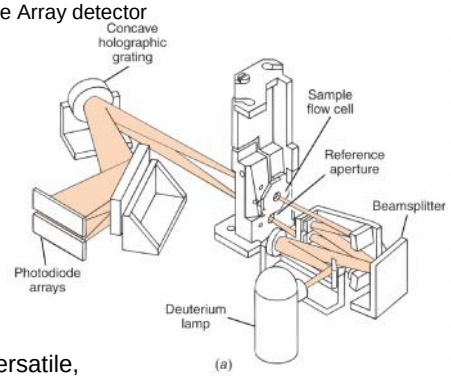
Flow cell:



Fixed/variable wavelength Light source

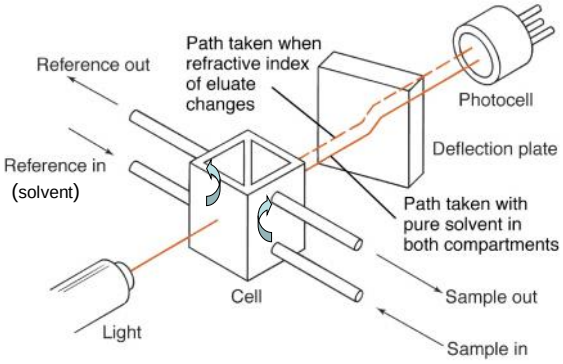
Single wavelength

Photodiode Array detector

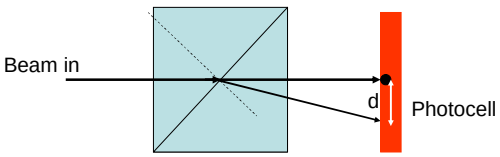


More versatile,
full spectrum allows identification of analyte.

Refractive index detector: isocratic mode only



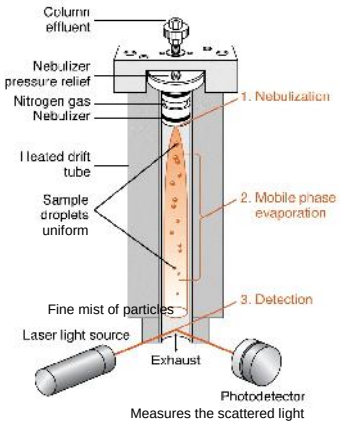
solvent – low μ



solution; *analyte in solvent* – high μ

$d \leftrightarrow (\mu_{solution} - \mu_{solvent}) \leftrightarrow C_{analyte}$

Evaporative light scattering detector:

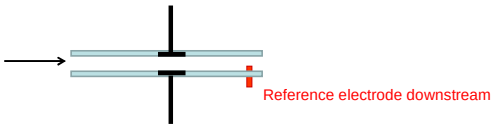


Response, R ↔ mass,m
nonlinear curves
volatile buffers/solvents only
no solvent peak
Compatible with gradient elution

Log R = a + b log m

Electrochemical detectors

The mobile phase is flowing past the electrodes, the solvent will be continuously replaced as the peak passes through the detector.



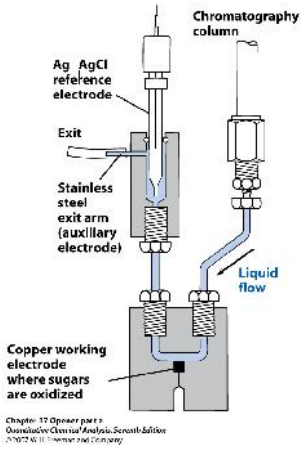
The electrochemical detector responds to substances that are either oxidizable or reducible. The detector normally has three electrodes, the working electrode (oxidation/reduction occur), the auxiliary electrode and the reference electrode). The electrical output results from an electron flow caused by the chemical reaction that takes place at the surface of the electrodes.

There are two modes of operation in electrochemical detection; coulometric detection and amperometric detection.

Coulometric detection: If the reaction at the electrode surface exhausts all the reactant and the current becomes zero, the total charge that passes will be proportional to the mass of solute detected.

Amperometric detection: While there is solute present between the electrodes, a current will be maintained (albeit varying in magnitude).

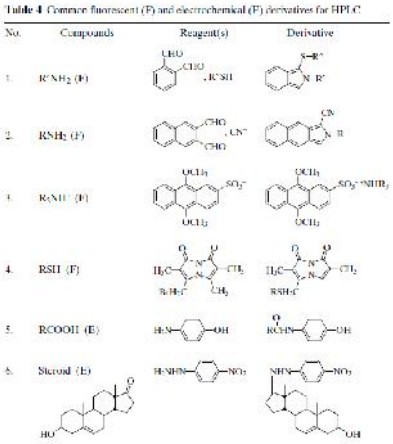
Details later.



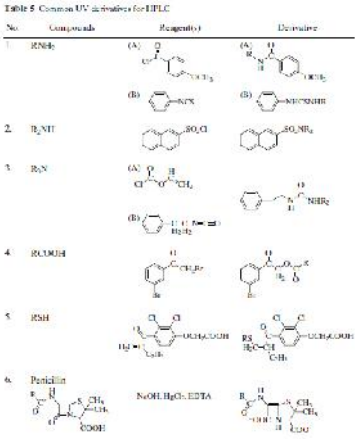
Derivatizing of samples has become vitally important in making the analytes conducive for detection.

Derivatization of reactive analytes for HPLC shows following advantages:

increase analyte stability
higher selectivity and sensitivity
changed polarity/functional group that leading to better chromatographic separation and/or detection.

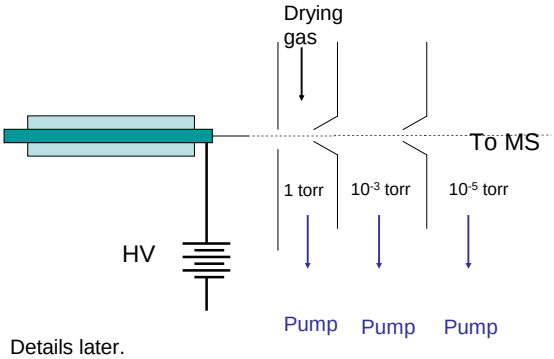


No.	Compounds	Reagent(s)	Derivative
1.	$RCNH_2$ (F)		
2.	RNH_2 (F)		
3.	R_2NH (F)		
4.	RSH (F)		
5.	RCOOH (E)		
6.	Steroid (E)		

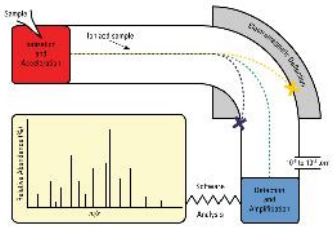


No.	Compounds	Reagent(s)	Derivative
1.	RNH_2		
2.	R_2NH		
3.	R_3N		
4.	RCOOH		
5.	RSH		
6.	Penicillin	NaOH, $HgCl_2$, EDTA	

MS Detection



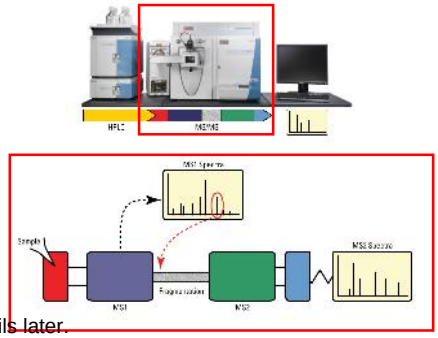
Details later.



Details later.

http://www.piercenet.com/browse.cfm?fldID=33C6C4ED-4B0D-49FA-ABD2-23BCB0FADEC0#chromatography

MS/MS Detection



Details later.

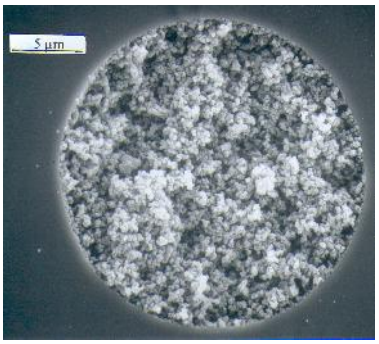
http://www.piercenet.com/browse.cfm?fldID=33C6C4ED-4B0D-49FA-ABD2-23BCB0FADEC0#chromatography

Monolithic Columns:

Monolithic columns consist of a ***single***, rigid or semi-rigid, porous ***rod***. Monolithic columns approach fast analysis by bypassing the limitations imposed by pressure by using *through-pores*, which ***allow higher flow rates*** than particulate columns at reasonable column back-pressures.

Two types of monolithic columns have been developed for chromatography: *organic polymers* based on polymethacrylates, polystyrenes or polyacrylamides, and *inorganic polymers* based on silicates.

SEM Micrographs of a Monolithic Stationary Phase



http://www.northeastern.edu/barnett2/research/group_pages/kargerpages/monolithic.html

Monolithic Stationary Phases are made by polymerizing and/or precipitating the stationary phase within the capillary tube.

It is a continuous interconnected skeleton with large through-pores. The resulting flow channels have both a higher permeability and a lower impedance to bulk flow (i.e. lower back pressure), than the 'stacked' spheres of particulate materials.

This physical structure ***reduces*** the Eddy-diffusion path. The integrity of the structure enhances the mechanical strength.