High Performance Liquid Chromatography

Introduction:

High Performance Liquid Chromatography (HPLC) is basically a high improved form of column chromatography. Instead of solvent being allowed to drip through a column under gravity, it is forced through under high pressure up to 400 atmospheres. That makes it much faster. In HPLC it is also allows you to use a very much smaller particle size (3-5μm) for column packing. Material which gives a much greater surface area for interactions between the stationary phase And the mobile phase it. This allows the better separation of the components of the complex mixture. This in turn Increase the elution by over 100 folds. The other major improvement over column chromatography concerns the Detection methods which can be used. These methods are highly automated and extremely sensitive.

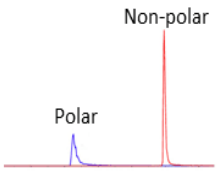
Ultra Performance Liquid Chromatography (UPLC) Further advances in instrumentation and column technology were made to achieve very significant increases in resolution, speed, and sensitivity in liquid chromatography.

Please refer to (GC) notes for principals of Chromatography

HPLC Types:

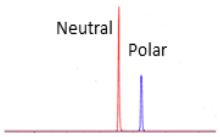
1) **Reversed-Phase Chromatography**
Polar mobile phase
water with water miscible organic modifier

Non-polar stationary phase
C_{18} , C_8 , C_4 , Phenyl



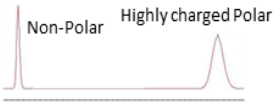
2) **HILIC**
Polar mobile phase
High organic content and water

Polar stationary phase
Amino, Mixed mode, Zwitterionic



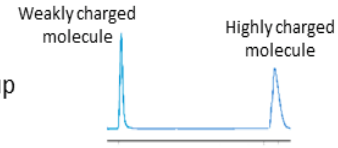
3) **Normal-Phase Chromatography**
Non-polar mobile phase

Polar stationary phase
Silica, Amino, Diol, Cyano



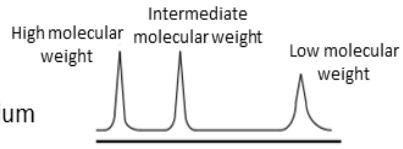
4) **Ion-Exchange Chromatography**
Highly aqueous buffers/salts

Packing material containing ionic group
Sulfonate, Tetraalkylammonium

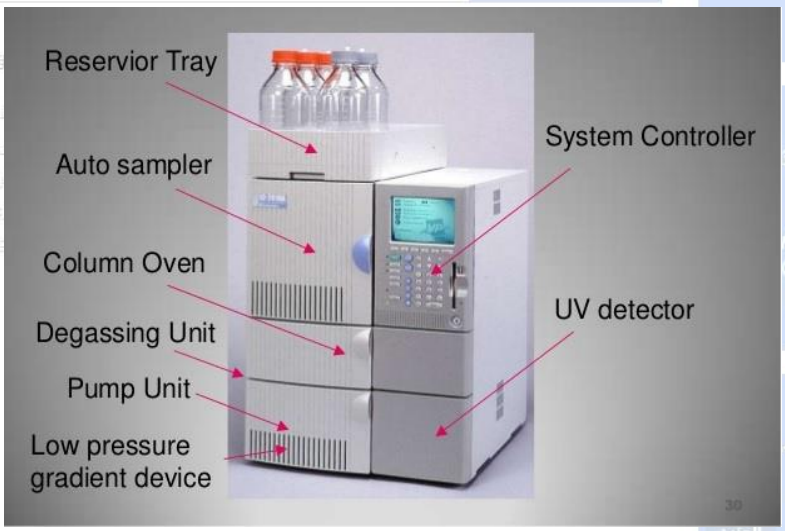


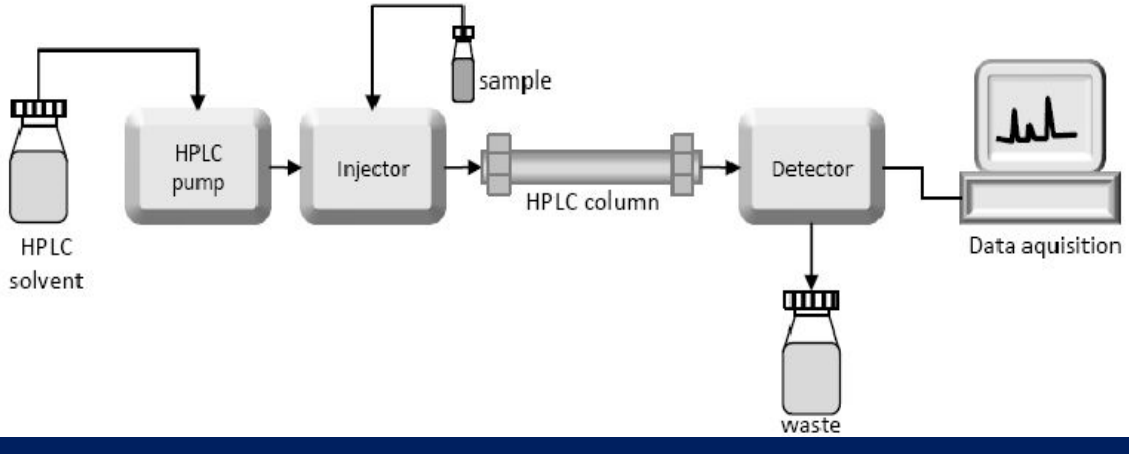
5) **Gel Permeation/ Size Exclusion Chromatography**
Non-aqueous, aqueous

Porous polymeric or silica medium



Instrument & Instrumentation:





Mobile Phase Reservoir:

The solvent or the mobile phase is placed in a glass reservoir. It is usually a blend of polar and non-polar liquids whose concentrations depend on the sample composition.

Pump:

The solvent in the mobile phase is aspirated by a pump from the reservoir and forced through the HPLC column and then the detector.

Injection Port:

The sample is injected into the column by an injector which is capable of handling sample volumes in the range of 0.1 - 100mL under high pressures of up to 4000psi.

Column:

HPLC columns are normally made of stainless steel and are 50-300mm long with an internal diameter of 2 - 5mm. They are filled with the adsorbents (stationary phase) of particle size 3 – 10µm.

- Internal Pressure:

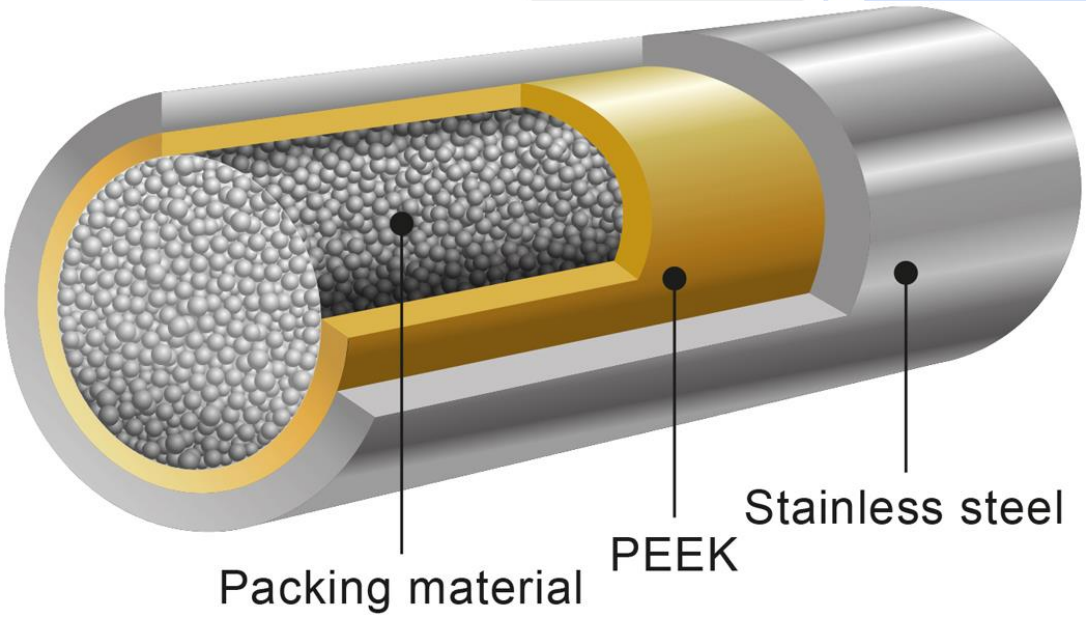
Stationary phases with particle sizes of 5 µm or larger can be used routinely at up to 40 MPa (6,000 psi) without any problem. HPLC Plus phases with particle sizes of 3 µm can be used at up to 60 MPa (8,700 psi). UHPLC columns with particle sizes of 2 µm or smaller and inner column diameter of 2 mm can be used at up to 100 MPa (14,500 psi). Stationary phases based on silica with a larger pore size of > 200 A like Eurosil Bioselect are not as mechanically stable as materials with smaller pore sizes. The pressure limit lies in the range of 200 bar for these materials.

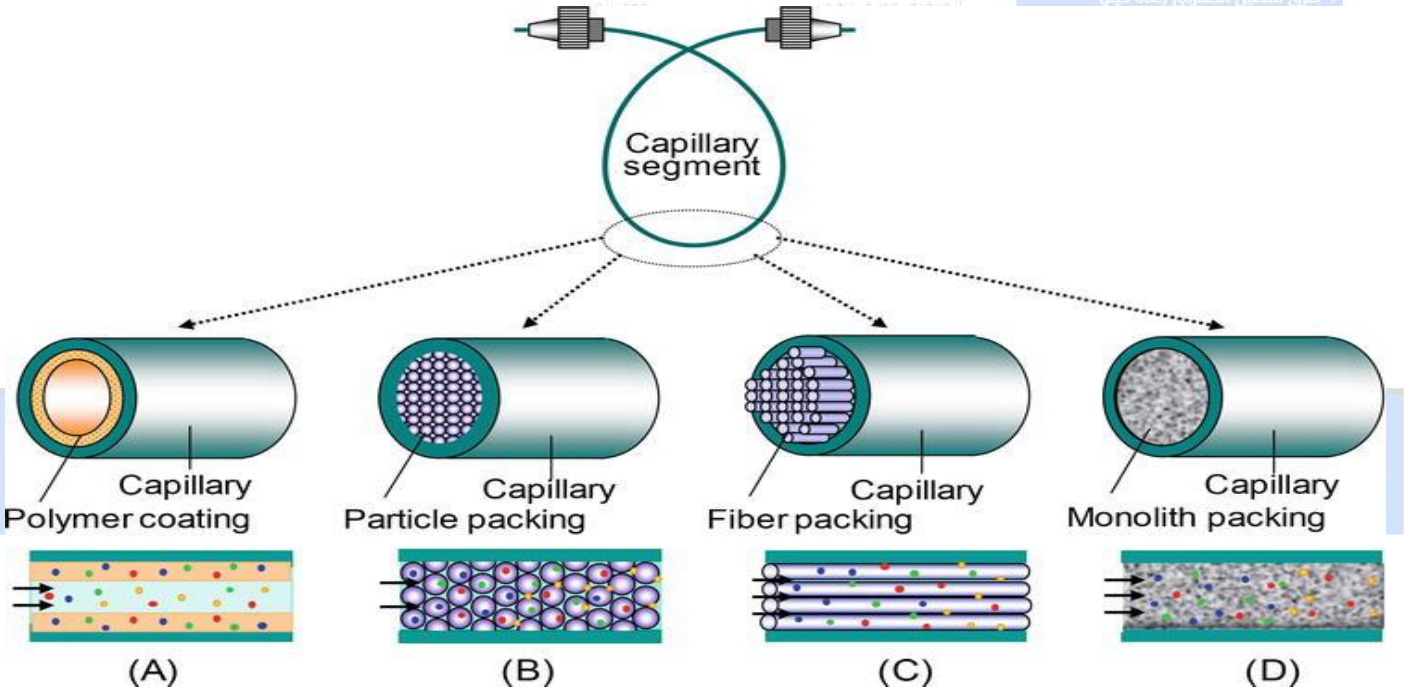
For preparative HPLC columns based on silica gel, the pressure limit highly depends on the column hardware: The maximum pressure is dependent on the diameter of the column (16 and 20 mm ID 400bar, 30 mm ID 300 bar, 50 mm ID 200 bar).

It is always recommended to work below the maximum allowed pressure range to guarantee a longer column lifetime. However, pressure shocks to the column should be avoided. Pressure shocks can lead to channeling in the bed column, which may result in peak splitting in the corresponding chromatogram.

Polymer based Eurokat columns shall not exceed 100 bar as the polymer matrix will collapse at higher backpressures.

- **Structure:**





- Dimensions:

Standard columns for reversed phase, normal phase, and ion exchange chromatography typically range from 3.9 to 4.6 mm internal diameter and 15, 25, and 30 cm in length.

- Temperature:

Temperature control for separations is important for long-term retention reproducibility, one factor of method ruggedness. Controlling temperature at 35 - 40°C is normally sufficient for good method reproducibility and ruggedness. In addition, the use of elevated temperature can have other benefits. First, it reduces the system operating pressure by reducing the viscosity of the mobile phase. Second, it will reduce analysis time, which can substantially increase productivity. Third, temperature may change the selectivity of a separation. Not all compounds have the same response to temperature so the selectivity of a separation can change dramatically when temperature is increased or decreased.

Sample:

Sample matrices can be broadly classified as organic, biological or inorganic, and may be further subdivided into solids, semi-solids (including creams, gels, suspensions, and colloids), liquids, and gases. For nearly every matrix, some form of sample pre-treatment, even if it is just simple dilution, will be required prior to chromatographic analysis. Gaseous samples usually are analyzed by GC, rather than HPLC. Techniques such as canister collection, direct sampling via sample loops, headspace sampling and purge and trap are used to collect and inject gases.

Diameter [mm]	Sample Volume [mL]	Hold Up Volume [µL]	Effective Filter Area [cm^2]
30	1-50	<50	5.1
25	1-30	<30	3.5
13	1-10	<10	0.75
3	<1	<7	0.0

Detectors:

The detector in a HPLC system is located at the end of the column and it detects the components of the sample that elute from the column. Different types of detectors such as fluorescence, mass-spectrometric, UV-spectroscopic, and electrochemical detectors are used.

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

Ultraviolet (UV)

- This type of detector responds to substances that absorb light.
- The UV detector is mainly to separate and identify the principal active components of a mixture.
- UV detectors are the most versatile, having the best sensitivity and linearity.
- UV detectors cannot be used for testing substances that are low in chromophores (colorless or virtually colorless) as they cannot absorb light at low range.
- They are cost-effective and popular and are widely used in industry

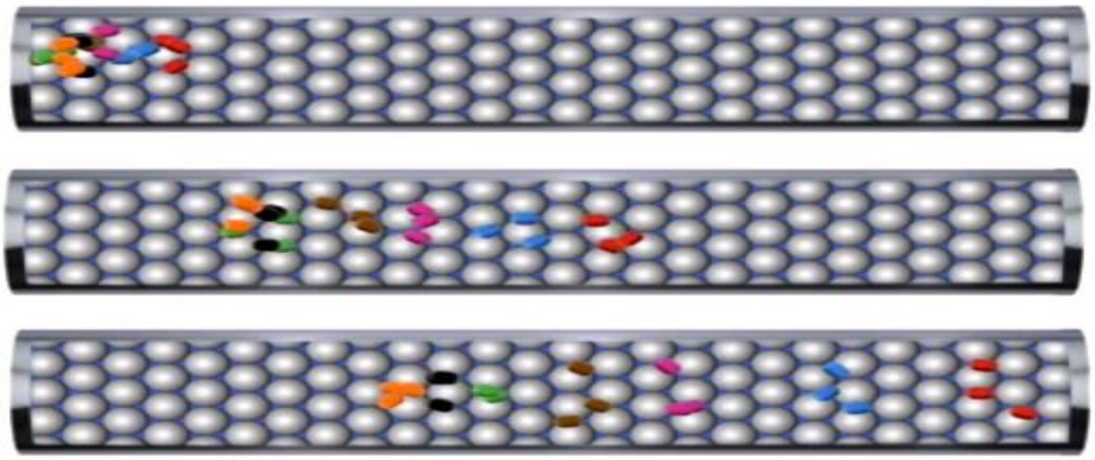
Fluorescence

- This is a specific detector that senses only those substances that emit light. This detector is popular for trace analysis in environmental science
- As it is very sensitive, its response is only linear over a relatively limited concentration range. As there are not many elements that fluoresce , samples must be syntesized to make them detectable.

Mass Spectrometry

- The mass spectrometry detector coupled with HPLC is called HPLC-MS. HPLC-MS is the most powerful detector,widely used in pharmaceutical laboratories and research and development.
- The principal benefit of HPLC-MS is that it is capable of analyzing and providing molecular identity of a wide range of components.

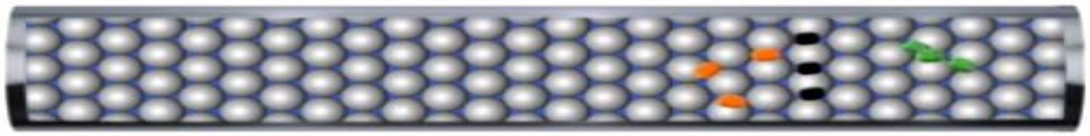
Retention Time:

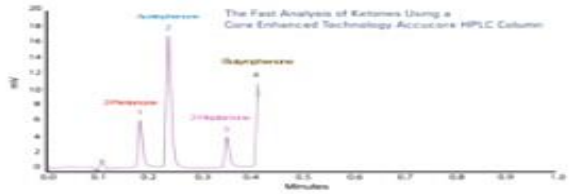


Detection:

- Analytes leave the column and pass into a detector giving a response
- The chromatogram is a plot of detector response vs time

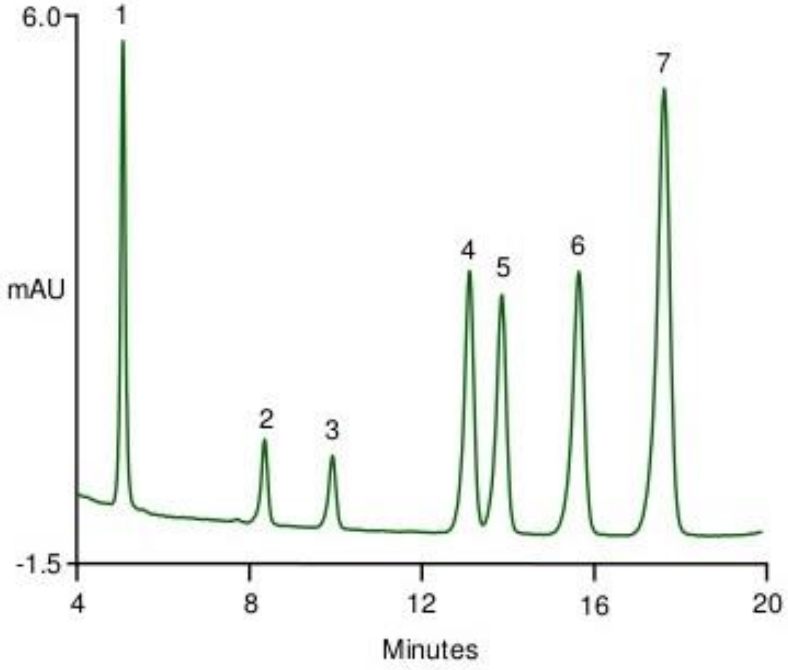
Retention Time	Compound
0.15.10 mins	2-Pentanone
0.15.11 mins	Acetophenone
0.15.11 mins	2-Heptanone
0.15.11 mins	Butyrophenone
0.15.11 mins	Hexanophenone
0.15.11 mins	Octanophenone
0.15.11 mins	Decanophenone





Retention Time	Compound
0.18 mins	2-Pentanone
0.24 mins	Acetophenone
0.35 mins	2-Heptanone
0.42 mins	Butyrophenone
0.43.05 mins	Hexanophenone
0.43.03 mins	Octanophenone
0.43.05 mins	Decanophenone

Response & Chromatogram:



Column:	Dionex Acclaim RSLC PolarAdvantage II column, 2.1 × 150 mm
Inj. Volume:	10 µL
Column Temp.:	25 °C
Detection:	UV 214 nm
Sample:	Standard mix

Peaks:

		R_s (USP)
1. Estriol	1.0 µg/mL	14.2
2. Androstenedione	0.5	5.4
3. Testosterone	0.5	8.8
4. Equilin	1.0	1.8
5. Estrone	1.0	4.0
6. Estradiol	1.0	3.9
7. Ethynylestradiol	1.7	--

Applications:

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- The information that can be obtained by HPLC includes resolution, identification and quantification of a compound. It also aids in chemical separation and purification. The other applications of HPLC include :

- Pharmaceutical Applications

1. To control drug stability.
2. Tablet dissolution study of pharmaceutical dosages form.
3. Pharmaceutical quality control.

- Environmental Applications

1. Detection of phenolic compounds in drinking water.
2. Bio-monitoring of pollutants.

- Applications in Forensics

1. Quantification of drugs in biological samples.
2. Identification of steroids in blood, urine etc.
3. Forensic analysis of textile dyes.
4. Determination of cocaine and other drugs of abuse in blood, urine etc.

Practical:

Determination of Caffeine in Coffee beverage