

## The Theory of HPLC

### Introduction



Wherever you see this symbol, it is important to access the on-line course as there is interactive material that cannot be fully shown in this reference manual.

## Aims and Objectives

### Aims

- To give a brief History of Liquid Chromatography
- To compare and contrast High Performance Liquid Chromatography (HPLC) with Gas Chromatography (GC)
- To introduce Liquid Chromatography (LC) and explain the function of each component of the HPLC system
- To introduce the Chromatogram and explain the information it gives

### Objectives

*At the end of this Section you should be able to:*

- Outline the main separation mechanisms used in HPLC
- Explain the fundamental principles of separation
- Highlight the different 'modes' of chromatography and explain their uses and applications

**Content**

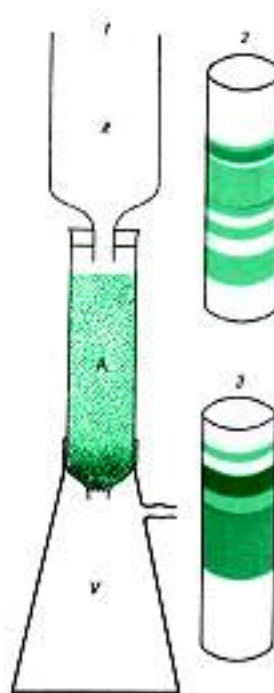
<b>Origins of Liquid Chromatography</b>	<b>3</b>
<b>Why Choose Liquid Chromatography</b>	<b>4</b>
<b>Suitable Samples for HPLC</b>	<b>5</b>
<b>Comparison with Gas Chromatography</b>	<b>6</b>
<b>Typical HPLC Data</b>	<b>7</b>
<b>Chromatographic Separation Mechanisms</b>	<b>8</b>
<b>The Liquid Chromatograph</b>	<b>9-10</b>
<b>The Liquid Chromatographic Process</b>	<b>11</b>
<b>The Chromatogram</b>	<b>12</b>
<b>Modes of Chromatography</b>	<b>13</b>
<b>Glossary</b>	<b>14-16</b>

## Origins of Liquid Chromatography

The Russian botanist Mikhail Tswett first used the term 'Chromatography' (Latin for 'colored drawing') in 1906, to describe the separation that occurred when solutions of plant pigments were passed through columns of calcium carbonate or alumina, using petroleum ether.



Russian Botanist  
Mikhail Tswett (1872-1919)



From Tswett's notebook (1910) on early  
chromatographic experiments

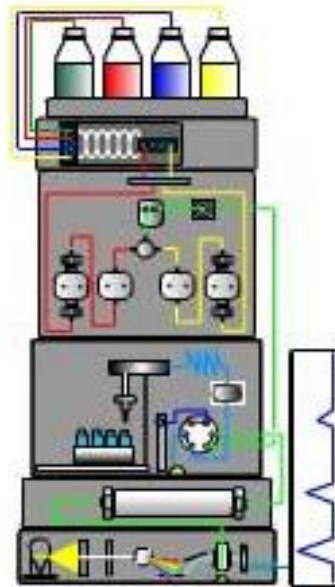
As the mixture seeped downwards, each pigment showed a different degree of readiness to attach itself to the adsorbent, and in this way the pigments became separated as different colored layers in the tube. Tswett called the new technique "chromatography" because the result of the analysis was literally 'written in color' along the length of the adsorbent column. Eventually he found six different pigments.

Just as Tswett had discovered, modern Liquid Chromatography (LC), uses a liquid mobile phase to transport the sample components through a column packed with a solid material; the stationary phase.

The two main chromatographic techniques used in modern analytical chemistry are Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC).



Gas Chromatograph



High Performance  
Liquid Chromatograph

HPLC uses a liquid mobile phase to transport the analytes (sample) through the column, which is packed with a stationary phase material.

In contrast, Gas Chromatography uses a gaseous mobile phase to transport sample components through either packed columns or hollow capillary columns. In most cases, GC columns have smaller internal diameter and are longer than HPLC columns.



Typical HPLC Column  
15 cm x 4.6 mm x 5  $\mu$ m



Typical GC Column  
30 m x 0.25 mm i.d.

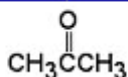
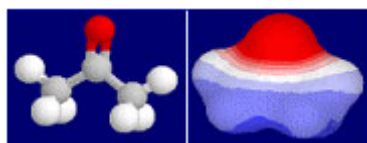
GC has developed into a sophisticated technique since the pioneering work of Martin and James in 1952, and is capable of separating some very complex mixtures. However, due to limitations of volatility and thermal stability, it is only capable of separating around 23% of known substances.

Therefore, under what circumstances would we choose HPLC to separate our sample components?

The following information gives an indication of the type of sample (**analyte**) commonly analyzed by both HPLC and GC, and the relative strengths and limitations of each technique.

### GC

- Samples analyzed by GC must be volatile (have a significant **vapor pressure** below 250 °C)
- **Derivatization** to increase analyte volatility is possible but cumbersome and introduces possible **quantitative** errors
- Most GC analytes are under 500 Da Molecular Weight for volatility purposes



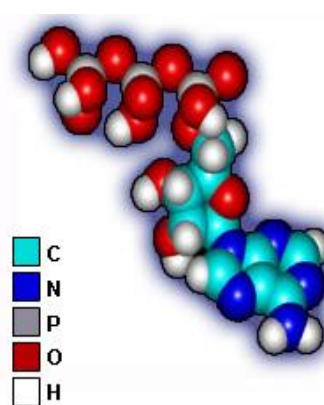
Propanone (acetone) (58 Da)

GC is amenable to smaller more volatile analytes such as Propanone (Acetone) (58 Da).

Regions in red on the ball and stick representation show negative polar functional groups. The topographical representation shows red as negative charge and blue as positive charge.

### HPLC

- HPLC analysis has no volatility issues, however the analyte must be soluble in the mobile phase
- HPLC can analyse samples over a wide polarity range and is able to analyse ionic samples. Mobile phase components are selected to ensure sample solubility
- HPLC has no real upper molecular weight limit and large proteins of many thousands of Daltons may be analysed. Solubility in the mobile phase may preclude the analysis of very large molecules



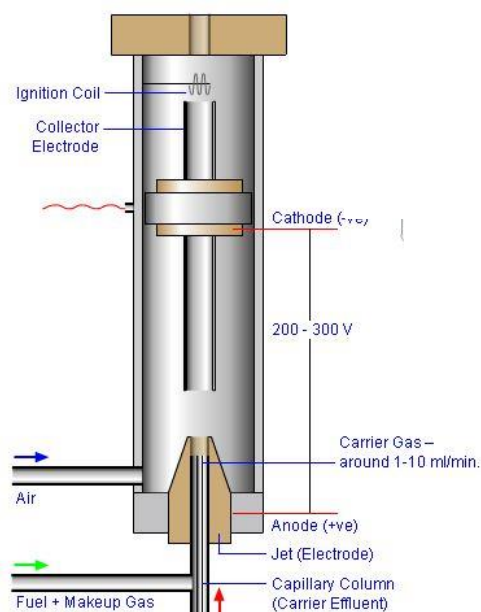
Adenosine Triphosphate (507 Da)

#### Important:

Large proteins and peptides can be analysed by HPLC!!!

### GC

- Temperatures in GC can exceed 350 °C and samples that are thermally unstable (labile) may decompose
- Many GC detectors such as the Flame Ionization Detector (FID) are destructive and the analyte does not survive analysis intact, and therefore, cannot be recovered
- GC samples are prepared in organic solvents and extraction of analytes from aqueous samples will be necessary
- Sample size is usually between 1 and 5 µL with typical detector sensitivity between **nanograms** (ng) and **picograms** (pg) on column

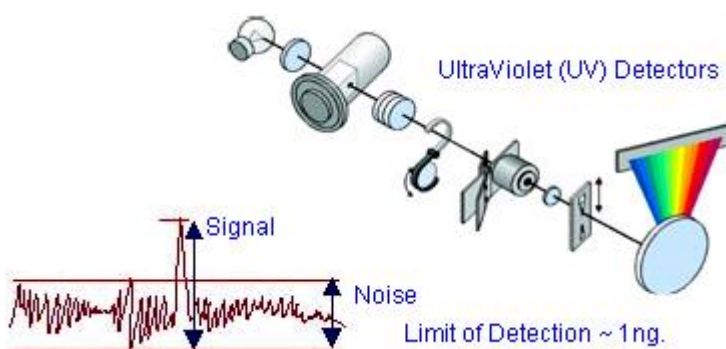


Operates at high temperatures and is destructive toward the analyte.

The Flame Ionization Detector

### HPLC

- HPLC is usually carried out at (or around) room temperature and most HPLC detectors apart from the Mass Spectrometer are non-destructive
- HPLC samples are prepared in a solvent system that has the same or less **organic** solvent than the mobile phase and volumes of 1 to 50 µL are common (1-10 µg of analyte per 1 g of packing material)

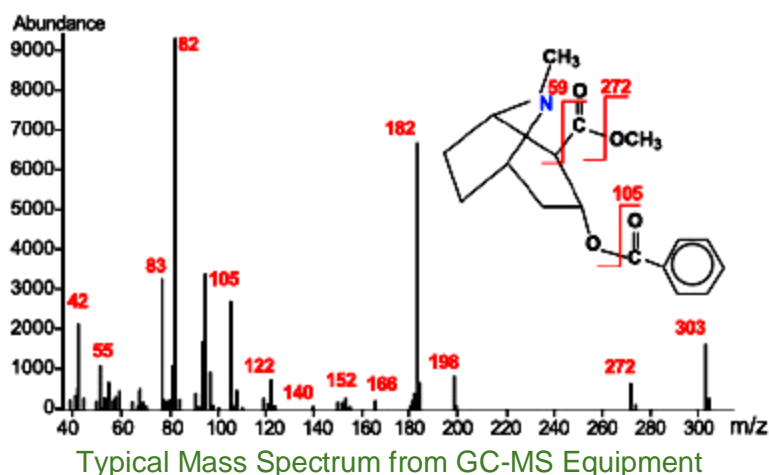


HPLC-Ultra Violet (UV) Detectors operate at room temperature, are non-destructive (the analyte may be recovered) and typical sensitivity is in the order of nanograms.



## Typical HPLC Data

Even though the Flame Ionization Detector (FID) may be more universal and sensitive, the UV detector is non-destructive, relatively sensitive (nanograms of analyte on column), and also has the capability of producing spectra associated with sample components. This can aid **qualitative** analysis and assist with identification of sample components.



HPLC and GC can both use **Mass Spectrometers** (MS) as detection systems to assist with analyte identification – although MS detectors are destructive. HPLC-MS is a less mature technique and there are no spectral libraries available for compound identification as there are with GC instruments. However, HPLC-MS (LC-MS) is a burgeoning technique that can assist in the characterization of sample components in a wide variety of application types.

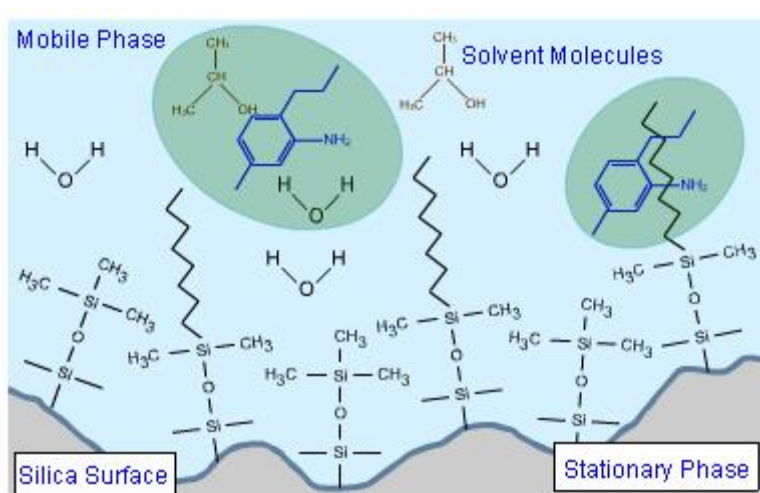
**Summary** - If the sample cannot be analyzed by Gas Chromatography without lengthy sample preparation (indicating issues with volatility), then HPLC should be the technique of choice. HPLC is the best choice for higher molecular weight analytes and analytes which may potentially degrade when heated.

**Mass Spectrometers:** produce spectra that can assist with the identification (qualitative analysis) of sample components for both GC and LC



HPLC separations involve both the **mobile phase** (a liquid) and the **stationary phase** (usually materials of varying hydrophobicity chemically bonded to a solid support). In contrast, GC uses the mobile phase only to carry the analyte through the column.

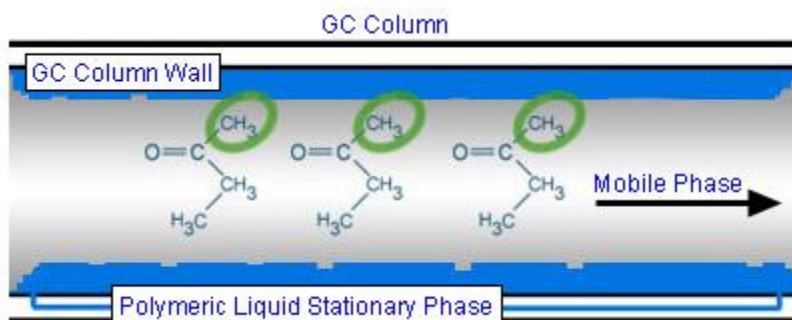
As an illustration, the amount of water in an HPLC mobile phase will determine how strongly a **hydrophobic** analyte is repelled into the stationary phase, and how well it is retained. The chemical nature of the stationary phase will also govern how strongly the analyte is retained. For this reason, HPLC is a technique that is driven by the '**selectivity**' achieved using two interacting phases.



Typical Liquid Chromatographic Separation Mechanism

In HPLC the analyte is able to interact with the mobile and stationary phases; bringing a high degree of **SELECTIVITY**.

In contrast, analytes in a capillary GC column will only be retained due to their interaction with the stationary phase (usually an immobilized liquid of varying hydrophobicity) coated onto the inner walls of the GC column. There are less options for improving selectivity in GC, however, as it is very highly **efficient**; this is often enough to achieve the desired separation.



Typical Gas Chromatographic Separation Mechanism

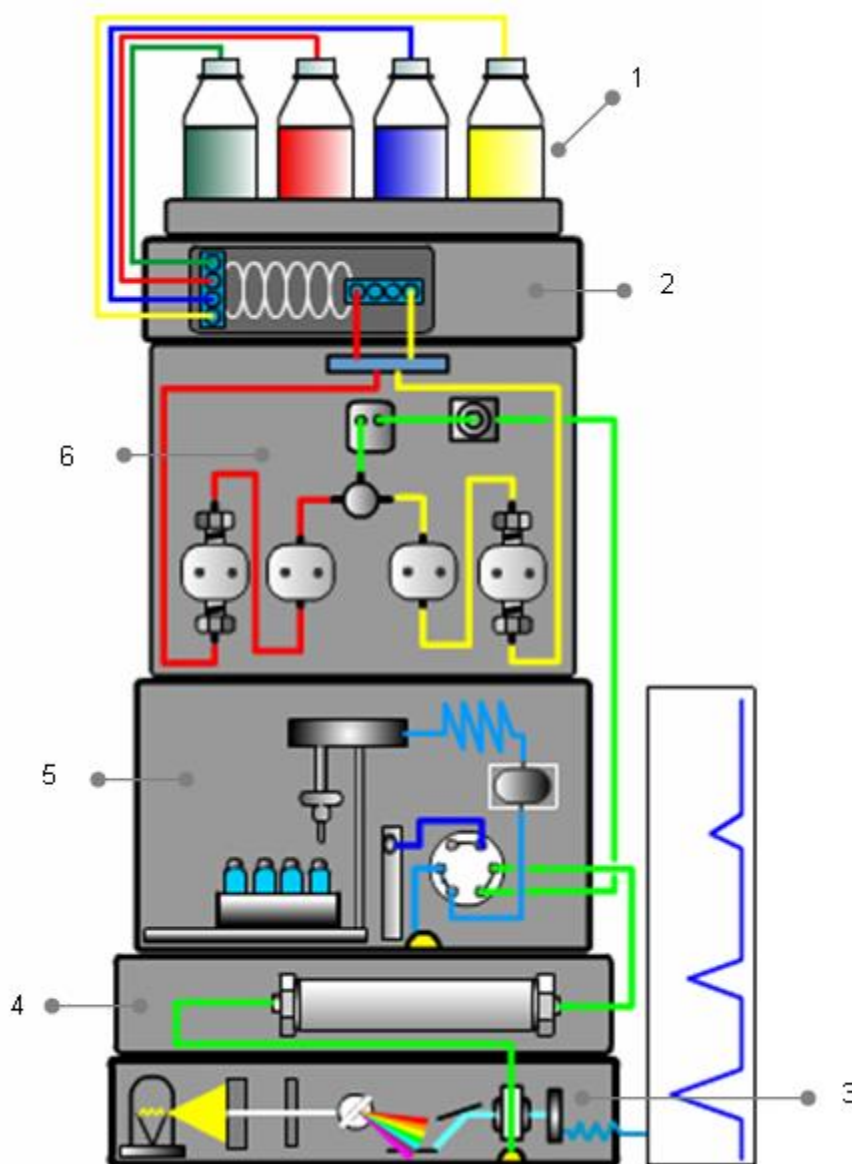
In GC the analyte interacts with the stationary phase only. The technique shows less selectivity but is highly efficient.

## The Liquid Chromatograph

In HPLC, several instrument and column chemistry parameters need to be optimized in order to generate a satisfactory separation.

Each of the following parameters need to be optimized in order to generate a chromatogram that is suitable for qualitative or quantitative purposes.

- Mobile phase composition
- Bonded phase chemistry
- Column and packing dimensions
- Injection volume
- Sample pre-treatment and concentration
- Mobile phase flow rate
- Column temperature
- Detector parameters



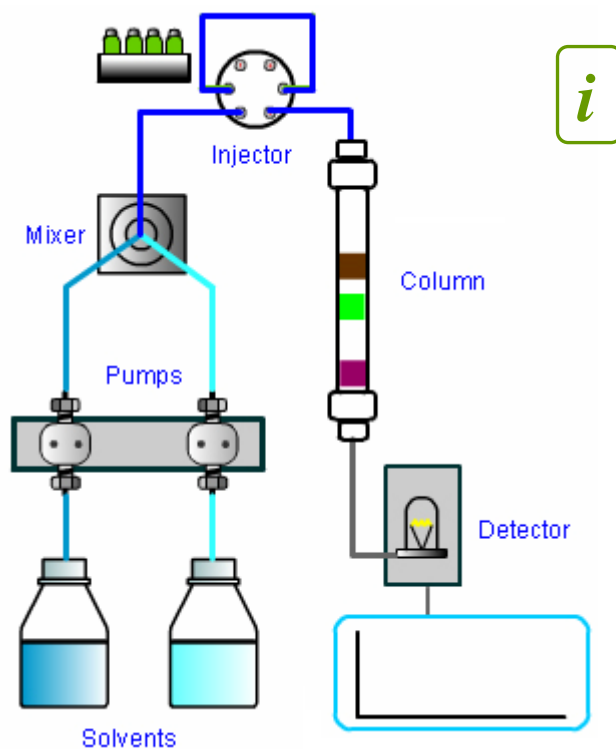
A typical Liquid Chromatograph

**Where:**

- 1:** The mobile phase composition (usually water and an organic solvent plus other additives) needs to be optimized in order to gain good separation.
- 2:** Degassers are often used to remove air from the mobile phase, leading to better chromatographic baselines.
- 3:** The detector conditions are chosen to give the best response to the analytes of interest and to achieve good sensitivity.
- 4:** The column dimensions and stationary phase chemistry are chosen and optimized to give separations of the quality required.
- 5:** The autosampler introduces a plug of sample into the mobile phase flow which is then swept onto the column.
- 6:** Dual reciprocating pumps are used to deliver the mobile phase at back pressures of up to 400 bar. A steady stream of liquid delivered at a constant flow rate is important.

The mobile phase is continuously pumped at a fixed flow rate through the system and mixed (if required) by the pump. The injector is used to introduce a plug of sample into the mobile phase without having to stop the mobile phase flow, and without introducing air into the system.

The mixture of components is carried in a narrow band to the top of the column. Some compounds in the sample mixture will have a greater preference for the stationary phase than the mobile phase and will be retained in the column longer. Those components that are not retained as strongly are carried by the mobile phase down the column. The longer the column, the more opportunities for interaction with the stationary phase, and the greater the separation.

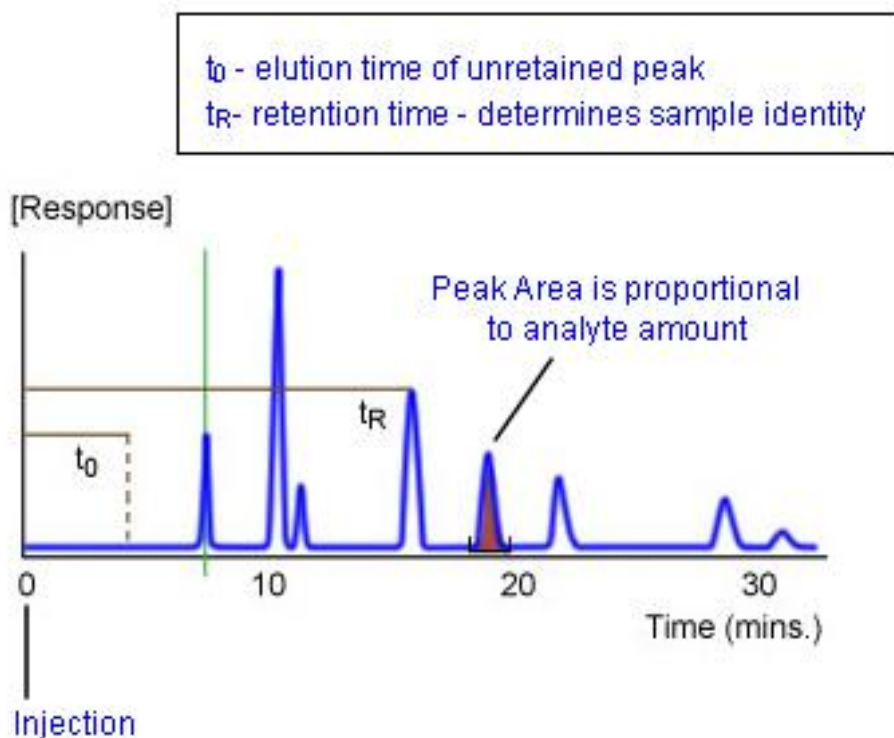


Liquid Chromatographic Process

The detector is then used to respond to a physicochemical property of the analyte (perhaps a UV chromophore consisting of a set of conjugated double bonds or an aromatic group). This response is digitally amplified and sent to a data system where it is recorded as the 'chromatogram'.

## The Chromatogram

As the components elute from the column they pass into a detector – where some physicochemical property of the analyte produces a response from the detector. This response is amplified and plotted against time – giving rise to a ‘chromatogram’.



### The High Performance Liquid Chromatogram

Components (such as the injection solvent) that are not retained within the column **elute** at the ‘dead time’ or ‘hold up time’  $t_0$ . There are various ways of measuring this parameter using un-retained compounds such as **Uracil**. Some workers use the first baseline disturbance caused by the passage of the sample solvent to estimate the value of  $t_0$ .

Those compounds (analytes and sample components) that are retained elute as approximately ‘**Gaussian**’ shaped peaks later in the chromatogram. Retention times provide the qualitative aspect of the chromatogram and the retention time of a compound should be the same under identical chromatographic conditions. The chromatographic peak height or peak area is related to the quantity of analyte. For determination of the actual amount of the compound, the area or height is compared against standards of known concentration.



The 'mode' of chromatography is usually defined by a combination of a certain stationary phase type with a certain mobile phase type.

For example, 'Normal Phase' chromatography has a non-polar mobile phase with a more polar stationary phase. Reversed phase HPLC (as the name implies) uses a system in which the mobile phase is more polar than the stationary phase.

The main modes of chromatography and their uses are described in Table 1.

**Table 1.** The Main Modes of Liquid Chromatography

Types of compounds	Mode	Stationary phase	Mobile phase
Neutrals Weak acids Weak bases	Reversed phase	C18, C8, C4, cyano, amino	Water/Organic Modifiers
Ionics, Bases, Acids	Ion Pair	C18, C8	Water/Organic Ion-Pair reagent
Compounds not soluble in water	Normal Phase	Silica, Amino, Cyano, Diol	Organics
Ionics/Inorganic ions	Ion Exchange	Anion or Cation Exchange Resin	Aqueous/Buffer Counter Ion
High Molecular Weight Compounds, Polymers	Size Exclusion	Polystyrene Silica	Gel Filtration – Aqueous Gel Permeation – Organic

The ability of HPLC to separate such a wide diversity of species leads to its popularity in a wide range of industries. It is important to note that HPLC is capable of separating analytes in the following categories:

- High molecular weight (>2000)
- Low molecular weight (<2000)
- Polar
- Non-polar
- Ionizable
- Cationic
- Anionic



Application Areas of HPLC

## Glossary

**Analyte** - the compound of interest which is analyzed by injection into, and elution from, an HPLC or GC column.

**Vapor pressure** - the absolute pressure at which the vapor contained in a substance is at equilibrium with its liquid or solid phase.

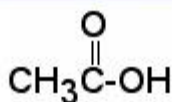
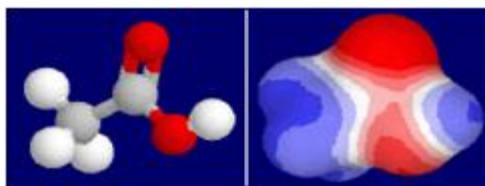
**Derivatization** - to chemically alter an analyte to impart favorable chromatographic behavior (e.g. increase volatility).

**Quantitative** - chemical analysis undertaken to determine (measure) the quantity or amount of each analyte within a mixture.

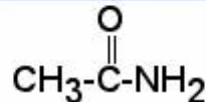
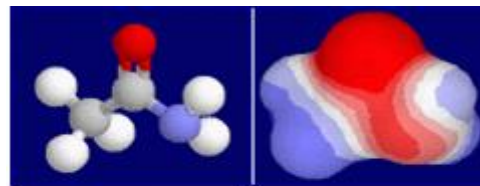
**Polarity** - the greater the difference in electron affinity (electronegativity) between atoms in a covalent bond, the more polar the bond. Partial negative charges are found on the most electronegative atoms, the others are partially positive. The molecular electrostatic potential is the potential energy of a hydrogen ion at a particular location near a molecule.

Negative electrostatic potential corresponds to partial negative charges.

Positive electrostatic potential corresponds to partial positive charges.



Ethanoic (acetic) acid



Ethanamide

**Nanogram** –  $1 \times 10^{-9}$  g

**Picogram** –  $1 \times 10^{-12}$  g

**Organic Solvent** - A solvent is a substance that dissolves another substance or substances to form a solution (a homogeneous mixture). Liquid solutions that do not have water as a solvent are called non-aqueous solutions. A broad and common class of non-aqueous solvents are called organic solvents. In the context of chemistry and materials, 'organic' refers to materials based on carbon (an element abbreviated as C). Additional elements that are commonly found in organic materials are hydrogen (H), nitrogen (N), oxygen (O), phosphorus (P) and sulfur (S).

**Qualitative** – involving distinctions based on qualities; "qualitative analysis attempts to determine the chemical identity of constituents of a substance or mixture".

**Mass Spectrometer** – a mass spectrometer is an instrument that measures the masses of individual molecules that have been converted into ions, i.e. molecules that have been electrically charged.



**Mobile Phase** – A fluid that percolates through or along the stationary bed, in a definite direction. It may be a liquid (Liquid Chromatography) or a gas (Gas Chromatography). In gas chromatography the expression 'Carrier Gas' may be used for the mobile phase. In elution (liquid) chromatography the expression 'Eluent' is also used for the mobile phase.

**Stationary Phase** – The stationary phase is one of the two phases forming a chromatographic system. It may be a solid, a gel, or a liquid. If it is a liquid, it may be distributed on a solid. This solid may or may not contribute to the separation process. The liquid may also be chemically bonded to the solid (Bonded Phase). The expression Chromatographic Bed or Sorbent may be used as a general term to denote any of the different forms in which the stationary phase is used. A stationary phase that is covalently bonded to the support particles or to the inside wall of the column tubing is known as a 'Bonded Phase'.

**Hydrophobic** – Meaning "water fearing". Hydrophobic compounds do not dissolve easily in water, and are usually non-polar. Oils and other long hydrocarbons are hydrophobic

**Selectivity** – also called the Separation Factor ( $\alpha$ ), described the separating 'power' of a chromatographic system (by definition, the value of the separation factor is always greater than one).

$$\alpha = \frac{k_2}{k_1} = \frac{t_{R2} - t_0}{t_{R1} - t_0}$$

Where:

$k$  = retention factor of a selected peak

$t_R$  = retention time of a selected peak

$t_0$  = retention time of an un-retained compound

**Efficiency** – also called Plate Number ( $N$ ), describes the broadening of the chromatographic band by using the chromatographic peak width. Plate number is indicative of column performance and can be calculated from the following equation:

$$N = 16 \left( \frac{t_R}{w_b} \right)^2 = 5.54 \left( \frac{t_R}{w_{1/2}} \right)^2$$

Where:

$t_R$  = retention time of a selected peak

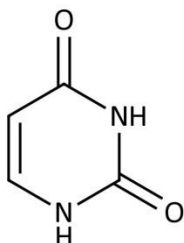
$w_b$  = peak width at the base of the peak

$w_{1/2}$  = peak width at half the peak height

**Elute** – to chromatograph by elution chromatography. The process of elution may be stopped while all the sample components are still on the chromatographic bed or continued until the components have left the chromatographic bed.

**Hold-up time ( $t_0$ ,  $t_M$ )** – The time required for an un-retained compound to be eluted, or the time required for one column volume ( $V_M$ ) of mobile phase to pass through the column. In reversed phase HPLC uracil is often used to measure the hold-up time and hold-up volume.

**Uracil** – a compound often used to determine the hold-up time ( $t_0$ ) of an analytical column (system). Whilst Uracil was not retained on traditional HPLC phases, some modern phases are capable of retaining the compound. Care should therefore be taken when using Uracil to ensure that the  $t_0$  value is correct.



**Gaussian** – following the shape of a ‘normal’ distribution as described by ‘Gauss’ – used to describe a ‘symmetrical’ bell shaped peak.

