

Pressure
or

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

What is HPLC?

HPLC is a chromatographic technique used to separate components of mixture for the purpose to identify, quantify or purify the individual components of the mixture. This is widely used in field of biochemistry and analytical chemistry.

Principle of HPLC

"In column chromatography the mobile phase moves slowly through the stationary phase under the influence of force of gravity. This requires much time for the separation of the components. In HPLC the mobile liquid phase is pumped through the column packed with the adsorbent, therefore the separation takes place rapidly. The pressure mechanical pump ensures the rapid solvent flow. The flow rate of the solvent affects the resolution of the sample components. As each component passes through the column, the detector notes its elution

Types of HPLC

There are following variants of HPLC, depending upon the phase system (stationary) in the process :

- | | Phase | HPLC |
|-------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| 1. Normal | This method separates analytes on the basis of polarity. NP-HPLC uses polar stationary phase and non-polar mobile phase. Therefore, the stationary phase is usually silica and typical mobile phases are hexane, methylene chloride, chloroform, diethyl ether, and mixtures of these. Polar samples are thus retained on the polar surface of the column packing longer than less polar materials. | HPLC |
| 2. Reverse | The stationary phase is non-polar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. It works on the principle of hydrophobic interactions hence the more non-polar the material is, the longer it will be retained. | HPLC |
| 3. Size-exclusion | The column is filled with material having precisely controlled pore sizes, and the particles are separated according to their molecular size. Larger molecules are rapidly | HPLC |

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washed through the column; smaller molecules penetrate inside the porous of the packing particles and elute later.

4. Ion-Exchange

HPLC

The stationary phase has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time.

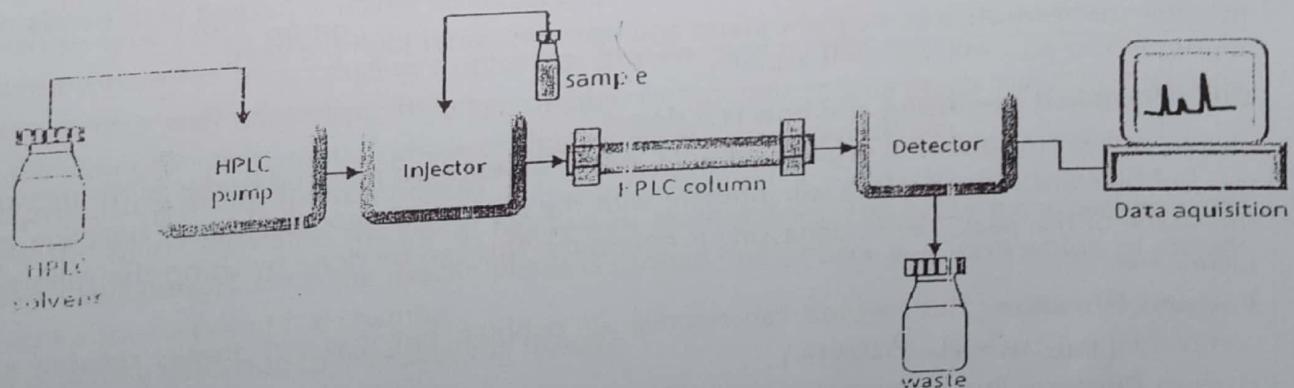
basis of
(on composition
of solvent)

Based upon elution technique HPLC can be classified into following types:

1. Isocratic HPLC: In this technique, the same mobile phase combination is used throughout the process of separation. The polarity or elution strength is maintained throughout the process.

Better Resolution ✓
2. Gradient HPLC: In this technique, mobile phase combination of lower polarity or elution strength is used followed by gradually increasing the polarity or elution strength.
polarity (change)
• Resolution (good)
• Separation (J)

INSTRUMENTATION OF HPLC



As shown in the schematic diagram in Figure above, HPLC instrumentation includes a pump, injector, column, detector and integrator or acquisition and display system. The heart of the system is the column where separation occurs.

Working of Instrumentation

- The operation of HPLC involves the column equilibration with the solvent (mobile phase).
- This requires the solvent to flow through the column for a few minutes.
- At the same time, the detector is turned on and a base line is established on the recorder.
- Then the sample dissolved in least possible polar solvent is injected by a microlitre syringe. The solvent is allowed to flow.
- Elution of each component of the sample appeared as a peak on the recorder.
- A computing integrator can also be connected to the detector to print the composition of the sample mixture

Now we shall discuss about the components of instrumentation in detail;

1. Solvent Reservoir: Mobile phase contents are contained in a glass reservoir. The mobile phase, or solvent, in HPLC is usually a mixture of polar and non-polar liquid components whose respective concentrations are varied depending on the composition of the sample.

What is Mixing unit in HPLC?

Mixing unit is used to mix the solvents in different proportions and pass through the column. There are two types of mixing units. They are low pressure mixing chamber which uses helium for degassing solvents. High pressure mixing chamber does not require helium for degassing solvents. Mixing of solvents is done either with a static mixer which is packed with beads or a dynamic mixer which uses magnetic stirrer and operates under high pressure.

What is solvent Degassing and how is it done?

Several gases are soluble in organic solvents. When solvents are pumped under high pressure, gas bubbles are formed which will interfere with the separation process, steady baseline and the shape of the peak. Hence degassing is necessary. This can be done by using the following techniques:

Vacuum Filtration: This method can remove air bubbles, but it is not always reliable and complete. (Puts through Pressure)

Helium Purging: By passing helium through the solvent. This is very effective but expensive method.

Ultrasonification: By using ultrasonicator, this converts ultra high frequency to mechanical vibrations. This causes the removal of air bubbles.

2. Pumps in HPLC :

- HPLC pumps form the basic part of **HPLC instrumentation**.
- Unlike other chromatography techniques, HPLC needs to generate pressure by pumps.
- Without a proper pump, HPLC analysis is impossible and the instrument is not operable.
- As the name, HPLC i.e. high-performance liquid chromatography indicates, one needs to generate pressure.
- HPLC pumps generate pressure on the solvent so as to pass through the dense column.
- Since the column particles are very small, the surface area is high and even separation is efficient.
- But the pressure required for the mobile phase to flow is very high. So pumps able to generate such high pressure are needed in HPLC.
- In general, the operable pressures in HPLC are 500 to 5000psi.
- In UPLC, i.e. Ultra pressure liquid chromatography the pressure might go up to 10,000psi or even more. So the HPLC pumps must be tough to generate such high pressures.
- As a general rule, HPLC pumps used should have following properties:
 - a) It should generate sufficient pressure through the column.
 - b) It should be suitable to the wide range of solvents used.
 - c) It should create uniform pressure without fluctuations.
 - d) It must deliver constant flow rate i.e. the volume of solvent pumped per minute.
 - e) It should be easy to use and operable for long duration.
 - f) It must be preferably inexpensive.

There are three types of pumps used in HPLC

- Screw-driven syringe pump
- Reciprocating pumps and
- Pneumatic pumps

Syringe type HPLC pump:

This pump works by producing a pulse-less delivery. And by this, the flow rate of the mobile phase is very well controlled. They are suitable for isocratic mobile phase HPLC run. Isocratic means using the same mobile phase or single solvent throughout the run.

Reciprocating pump (piston pump)

As the name indicates, the pump has two or more pistons. This is the most common and widely used pumps. Here the mobile phase is pushed due to back and forth action of pistons present about a cylindrical chamber.

It is widely used due to the following reasons.

- Good pressure generation.
- Constant flow rate.
- Useful for both gradient and isocratic runs.
- Space occupancy is low.

The **pneumatic pump** is another simplest pumps. As the name indicates (pneumatic) there is the use of gas to pressurize the mobile phase present in solvent container. They have some advantages like low-cost or inexpensive to purchase, pulse-free and also simple.

- * But are not widely used due to disadvantages like low-pressure generation, low capacity to dispel the mobile phase and also the pumping rate varies with the viscosity of the mobile phase used.
- * Reciprocating pumps are used in most of the instruments. They are used as a single pump or dual or multiple pumps to meet the needs of the analysis like isocratic or gradient elution etc. They are present in most advanced instruments so they are controlled by software. The pressure, rate of flow of mobile by the pump are regulated by software making it hassle-free.
- * Even though the pumps generate sufficient pressure, due to air bubbles the pressure might drop and fluctuate. So the HPLC mobile phase should be free from gas bubbles.

3. **Sample Injector:** The injector can be a single injection or an automated injection system. An injector for an HPLC system should provide injection of the liquid sample within the range of 0.1-100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).

4. **Columns in HPLC:** Columns are usually made of polished stainless steel, are between 50 and 300 mm long and have an internal diameter of between 2 and 5 mm. They are commonly filled with a stationary phase with a particle size of 3–10 μm . Columns with internal diameters of less than 2 mm are often referred to as microbore columns. Ideally the temperature of the mobile phase and the column should be kept constant during an analysis.

Normally, columns are filled with silica gel because its particle shape, surface properties, and pore structure help to get a good separation. Silica is inert to most compounds and has a high surface activity which can be modified easily with water and other agents. Silica can be used to separate a wide variety of chemical compounds, and its chromatographic behavior is generally predictable and reproducible.

The different types of columns used in HPLC are as follows:

➤ **Normal phase columns**

In this column type, the retention is governed by the interaction of the polar parts of the stationary phase and solute. For retention to occur in normal phase, the packing must be more polar than the mobile phase with respect to the sample.

➤ **Reverse phase Columns**

In this column the packing material is relatively non-polar and the solvent is polar with respect to the sample. Retention is the result of the interaction of the non-polar components of the solutes and the non-polar stationary phase. Typical stationary phases are non-polar

hydrocarbons, waxy liquids, or bonded hydrocarbons (such as C₁₈, C₈, etc.) and the solvents are polar aqueous-organic mixtures such as methanol-water or acetonitrile-water.

➤ *Size exclusion Columns*

In size exclusion the HPLC column is consisted of substances which have controlled pore sizes and is able to be filtered in an ordinarily phase according to its molecular size. Small molecules penetrate into the pores within the packing while larger molecules only partially penetrate the pores. The large molecules elute before the smaller molecules.

➤ *ion exchange Columns*

In this column type the sample components are separated based upon attractive ionic forces between molecules carrying charged groups of opposite charge to those charges on the stationary phase. Separations are made between a polar mobile liquid, usually water containing salts or small amounts of alcohols, and a stationary phase containing either acidic or basic fixed sites.

5. Detectors in HPLC

The detector senses the presence of the individual components as they leave (elute) the column. The detector output after amplification is traced on a recorder. The duration of the intervals is usually a single second or even less than that. Hence the detector is considered to be the brain of the instrument. The detector converts a change in effluent into an electric signal that is recorded by data system.

Ideal properties of a detector:

The detectors used in both GC and HPLC should have following ideal properties

- High sensitivity
- Good stability and reproducibility.
- A linear response to solutes.
- Negligible base line noise.
- Should be inexpensive.
- Capable of providing information on the identity of solute.
- A temperature range from room temperature to at least 400°C.
- A short response time independent of flow rate.
- High reliability and ease of operation.
- The detector should be non-destructive.
- Response independent of mobile phase composition.

The function of the detector used in HPLC is to monitor the mobile phase it emerges from column..

The detectors used in HPLC are of majorly two types:

(a) Selective detectors (solute property):

- ~ Absorbance detectors
- ~ Fluorescence detectors
- ~ Electrochemical detectors
- ~ Mass spectrometric detectors

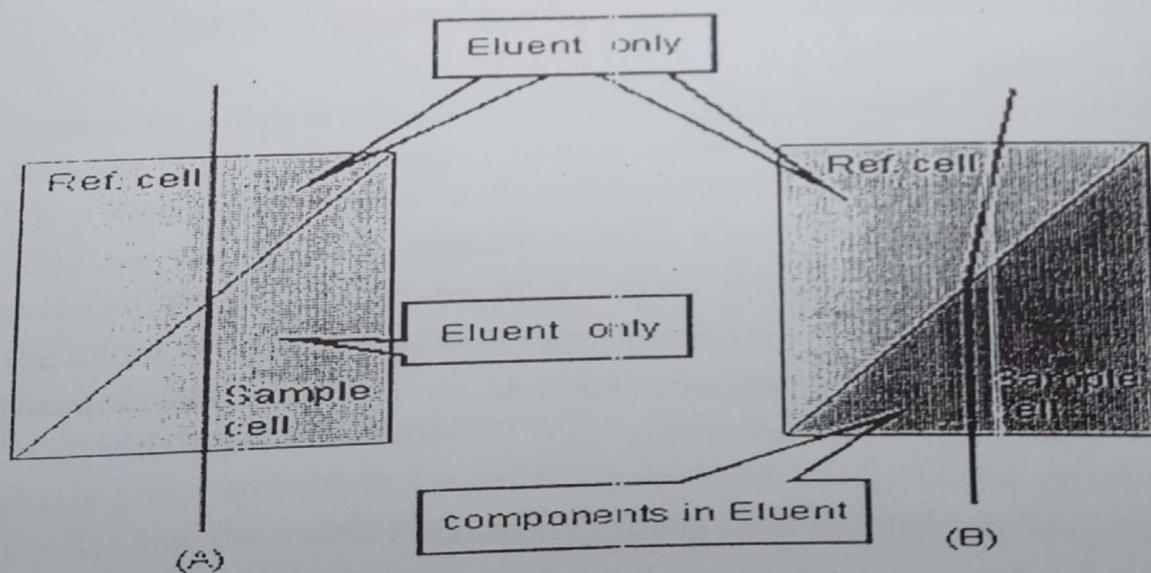
(b) Universal detectors(bulk property):

- ~ Refractive index detectors
- ~ Evaporative light scattering detectors

There are details about some of the important detectors used in HPLC

> **Refractive-index detectors:**

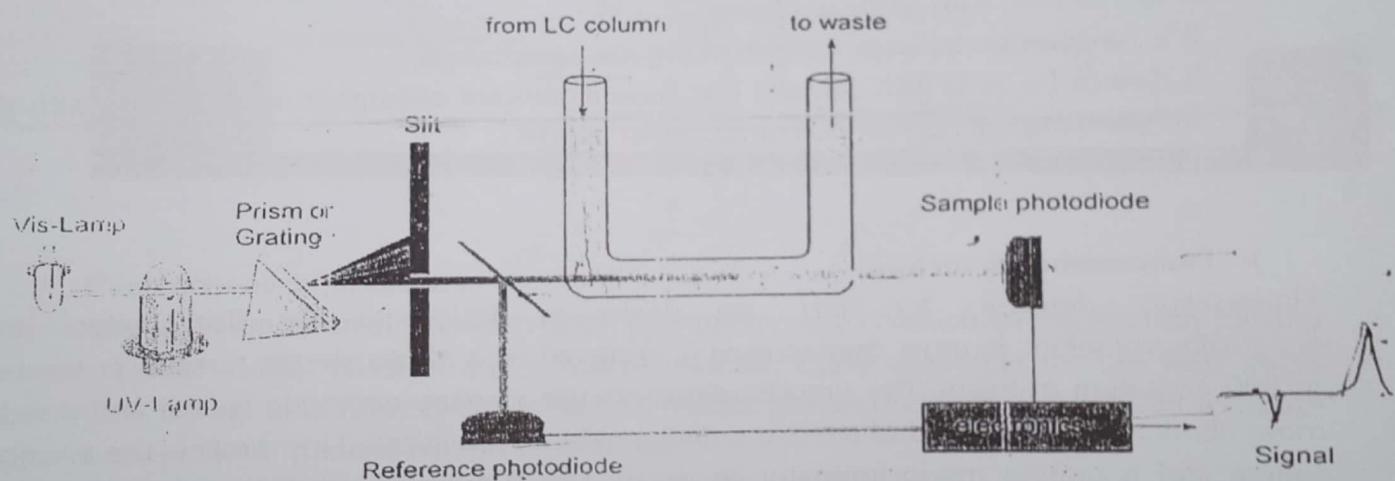
- ~ The ability of a compound or a solvent to deflect light provides a way to detect it.
- ~ RI is a measure of molecule's ability to deflect light in a flowing mobile phase in a flow cell relative to a static mobile phase contained in a reference cell.
- ~ The amount of deflection is proportional to concentration.
- ~ The RI detector is considered as a universal detector but it is not very sensitive.
- ~ To achieve high sensitivity, in practice solvents are selected that have a very high or very low refraction index.
- ~ The detection limit is in the range of 10^{-5} to 10^{-8} g/ml.
- ~ This type of detector cannot be used effectively with gradient elution due to a change in the baseline nor when the solvent has an index of refraction close to that of solute.
- ~ This detector is very much sensitive to temperature changes.



➤ UV Absorption Detector

UV and visible absorption detectors are used to detect compounds that contain double bonds that absorb at appropriate wavelengths. Typically, a "monochromatic" band of wavelengths is passed through a cell, and the fraction of light transmitted (T) is measured and converted to an absorption ($A = -\log T$). Variable wavelength UV absorption detectors can scan to take spectra, which provide additional information about components. Diode array detectors measure absorption at all wavelengths in parallel and can provide continuous spectra of the eluting components. UV detection is limited to absorbing samples and to methods whose eluents do not absorb themselves.

Many analytes absorb UV-light, typically in the 200-300 nm range. The UV detector is therefore popular since it can detect compounds that contain conjugated or isolated double bonds, i.e., compounds with free or conjugated electron pairs. Some 'UV'-detectors offer both UV and visible light sources. The principle is the same regardless of the wavelength of light used.



A light beam with a known wavelength (λ) and intensity (I) is transmitted through a sample cell. The intensity of the transmitted light (I_s) is compared to the intensity transmitted by an empty reference cell (I_r).

As long as the two intensities are the same, no signal is generated. If an absorbing solute is present in the sample cell, the reference intensity and the sample intensity are no longer equal and a signal is generated. The ratio of the measured intensities I_s/I_r is called transmittance (T). The logarithm of $1/T$ is called the absorbance, ($A = -\log T$), and is linearly proportional to the concentration of the solute in the eluent.

According to Beer's Law, the absorbance (A) depends on the concentration of the compound (c), the pathlength of the cell (b), and on the molar extinction coefficient (ϵ) of the analyte: $A = \epsilon bc$ (Beer's law)

Obviously, the wavelength of the light and path length of the cell must be kept constant during the measurement in order to produce meaningful data.

Most compounds show the best absorbance in the UV-region (200 - 300 nm). When choosing the eluent, it is important to make sure that the eluent itself does not absorb light at the selected detection wavelength. In other words we must make sure that absorption is caused mainly or exclusively by sample components. Otherwise the detected absorbance signal caused by the analyte will become too low, resulting in a lower sensitivity.

- The ultraviolet detector has much better sensitivity, about 0.01 ppm.
- It is not temperature sensitive.
- It is relatively inexpensive.
- It can be used with gradient elution.
- It is sensitive to the large number of organic compounds.
- It cannot be used with solvents that have significant absorption in the UV or with the sample components that do not absorb in the UV.

➤ Fluorescence detectors

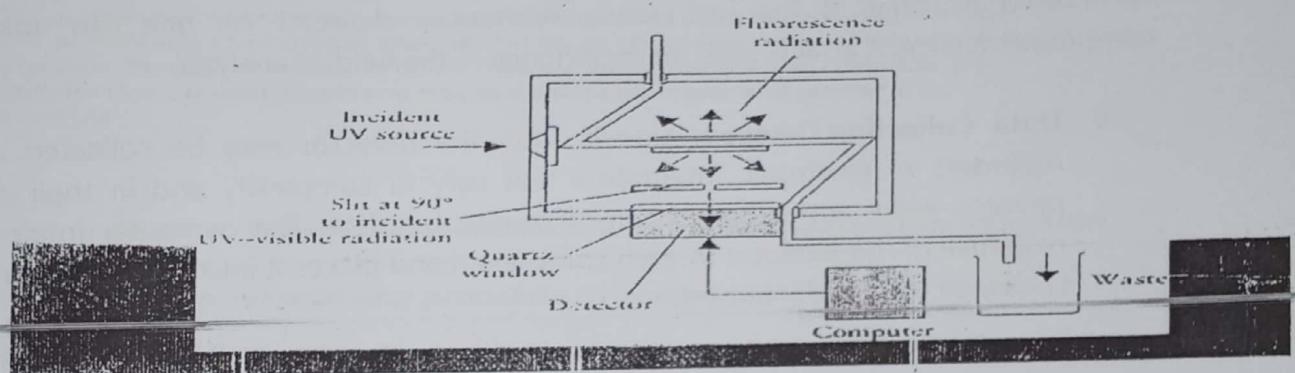
Fluorescence detectors for HPLC are similar in design to the fluorometers and spectrofluorometers. In most, fluorescence is observed by a photo electric transducer located at 90° excitation of beam. The simplest detectors use mercury excitation source and one or more filters to isolate a band of emitted radiation. More sophisticated instruments use a xenon source and a grating monochromator to isolate the fluorescence radiation. Laser-induced fluorescence is also used because of its sensitivity and selectivity. An inherent advantage of these detectors is their high sensitivity, hence used in LC for separation and determination of components of samples that fluoresce.

Fluorescent compounds can be obtained by treating with reagents that form fluorescent derivatives. For example, dansylchloride forms fluorescent compounds with primary amines, secondary amines, amino acids and phenols, hence widely used for detection of amino acids in protein hydrolyzates.

Fluorescent detector

HPLC Specific Property Detectors

Fluorescence Detector



> Electrochemical detectors

This is based on amperometry, voltammetry, coulometry, and conductometry. Electron transfer processes offer highly selective and sensitive method. Easily adaptable for use with micro columns As background noise is dependent on mobile phase conditions, it is difficult to utilize these detectors with gradient elution separations.

> Evaporative Light Scattering Detector:

ELSD can outperform traditional detectors when analyzing non-chromophoric samples by HPLC. Traditional HPLC detectors such as UV and RI have limited capabilities. UV and RI are not compatible with a wide range of solvents .RI detection is not gradient compatible. Different analytes produce different UV responses depending on their extinction co-efficient. ELSDs can detect anything that is less volatile than the mobile phase. ELSD is universal and compatible with a wide range of solvents

Three steps are involved in detection:-

- Nebulization
- Mobile Phase Evaporation
- Detection

Nebulization: Column effluent passes through nebulizer needle. It mixes with the nitrogen gas. Dispersion of droplets is formed.

Mobile phase evaporation: The above formed droplets are passed through a heating zone. Mobile phase evaporates from the particles.

Detection: Sample particles pass through an optical cell. Sample particles interrupt laser beam and scattered light. Photodiode detects the scattered light. ELSD is an effective replacement or a perfect complement to existing LC detectors.

Detector is the key element that is present in any device that is used for the identification and estimation of any compound. It detects in a faster rate i.e. within seconds hence it is considered as brain of the instrument. Without a detector no one can analyze the compound. Hence, it attains such an importance in the field of analysis.

6. Data Collection Devices: Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret.

HPLC DERIVATIZATION

DERIVATIZATION

- Derivatization is a technique used in chemistry which transforms a chemical compound into a product (the reaction's derivative) of similar chemical structure called a derivative.
- Generally, a specific functional group of the compound participates in the derivatization reaction and transforms it to a derivative of deviating reactivity, solubility, boiling point, melting point, aggregate state, or chemical composition.
- Resulting new chemical properties can be used for quantification or separation of the compound

RATIONALE BEHIND DERIVATIZATION

□ In liquid chromatography, fluorescent derivatives can be prepared to render the substances specifically detectable at high sensitivity.

□ To prepare fluorescent derivatives of phenols, and primary and secondary amines, dansyl chloride (5-dimethyl aminonaphthalene-1-sulphonyl chloride) is strongly recommended.

□ To render involatile substances volatile for GC analysis, organic acids can be esterified using boron trifluoride as a catalyst or directly with diazomethane.

□ The polarity of a solute needs to be drastically reduced to improve its chromatographic behavior and reduce tailing. Polarity reduction can often be achieved for amino, hydroxyl and thiol groups by acylation.

□ In LC analyses, UV-chromophores and fluorophores are often introduced into sample molecules to increase their sensitivity to UV absorption and fluorescence detection. Benzoyl chloride, m-toluol chloride and p-nitrobenzoyl chloride are reagents that can add a benzene ring to a solute molecule and render it UV absorbing.

SEVERAL CHARACTERISTICS ARE DESIRABLE FOR A DERIVATIZATION REACTION

□ The reaction is reliable and proceeds to completion.

□ Less unreacted starting material will simplify analysis.

□ Also, this allows a small amount of analyte to be used.

□ The reaction is general, allowing a wide range of substrates,

□ yet specific to a single functional group,

□ reducing complicating interference.

□ The products are relatively stable, and form no degradation

□ products within a reasonable period, facilitating analysis.

PRE-COLUMN OFF-LINE DERIVATIZATION

When there is a mixture of many compounds they may interfere in the separation and resolution in such case we can derivatize the compound of interest to change its properties so that it can be separated.

Merits :

- (a) Requires no modification to the instrument i.e., a plus point when compared to the post-column methods
- (b) Imposes fewer limitations with regard to reaction-time and conditions.

Demerits :

- (a) Formation of a stable and well-defined product is an absolute necessity.
- (b) Presence of excess reagent or by products may invariably interfere with separation.
- (c) Very often derivatization may altogether change the chromatographic properties of the sample which facilitated separation.

Nineteen amino acids in a standard mixture were separated using a GROM-SIL OPA-1 column after precolumn derivatization with OPA / mercaptopropionic acid and detected by fluorescence spectroscopy at 330 and 450nm.

Nineteen amino acids in a standard mixture were separated using a GROM-SIL OPA-1 column (150 x 4mm, Part No. GSOP10308S1504) after precolumn derivatization with OPA/mercaptopropionic acid by gradient elution with

25mM sodium phosphate, pH 7.2 and THF 995/5

25mM sodium phosphate, pH 7.2, methanol and acetonitrile 50/35/15

Time - 41 minutes

Detection - Fluorescence spectroscopy at 330 and 450nm.

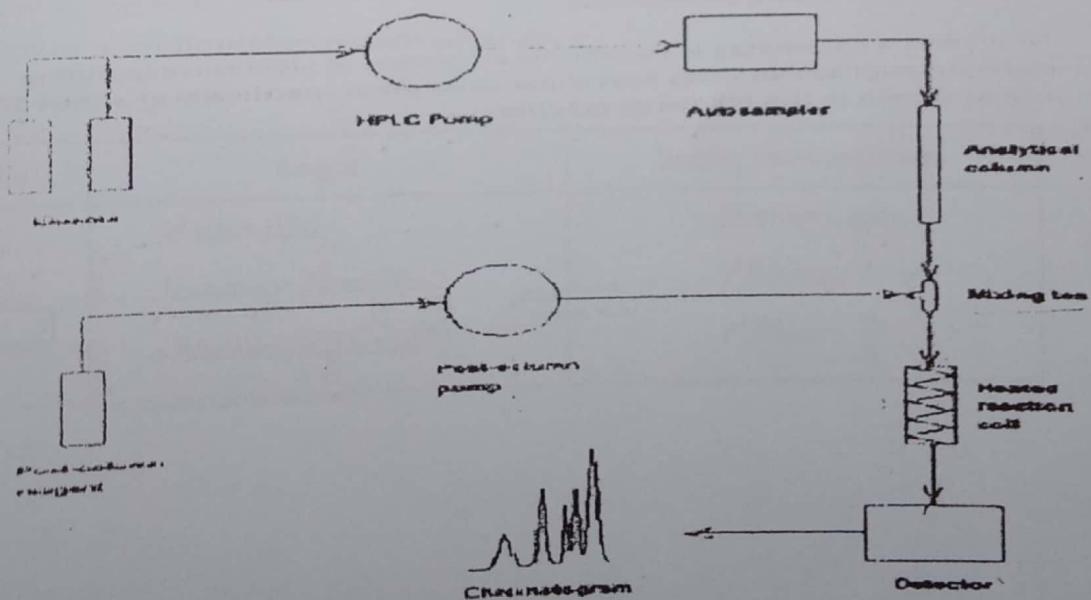
The amino acids separated were aspartic acid, glutamic acid, asparagine, serine, glutamine, glycine, threonine, histidine, citrulline, 3-methylhistidine, alanine, taurine, arginine, alpha-aminobutyric acid, tyrosine, valine, methionine, nor-valine and tryptophan.

POST-COLUMN ON-LINE DERIVATIZATION

The following experimental parameters should be maintained

- (a) Derivatization performed in a special-reactor strategically positioned between the column and the detector.
- (b) Reaction must be completed rapidly at moderate temperatures.
- (c) Derivatization reaction need not even go to completion provided it can be made reproducible.
- (d) No detector-response should exist due to any excess reagent present.
- (e) Reaction must be carried out in a medium other than the mobile-phase.

Merit : The main merit of post-column-on-line derivatization is that ideally the separation & detection processes can be optimized individually



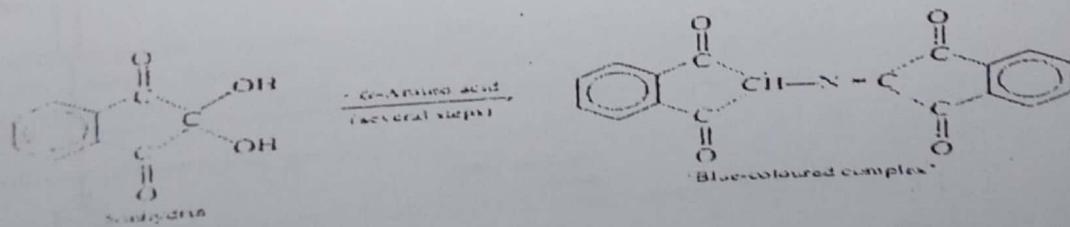
IMPORTANT CONSIDERATIONS IN POST COLUMN DERIVATIZATION

- Reactants and conditions must be chosen so that conversion to the desired product(s) takes place rapidly (usually < 1 minute) and reproducibly.
- PCD system must ensure good mixing
- If the conversion is not sufficiently rapid, it may be necessary raise the temperature, incorporate a catalyst or in some other way accelerate the rate of derivatization.
- Completeness of reaction is not always a required result.
- In many qualitative applications it may be sufficient that each analyte derivative be formed in abundance great enough to generate a detectable signal.

REAGENTS FOR DERIVATIZATION

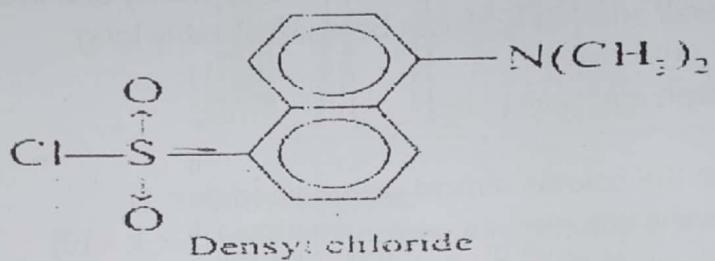
Derivatization for UV Detectors:

Ninhydrin a chromatag is commonly employed to yield corresponding derivatives of amino acids that show absorption specifically at about 570 nm as shown in the following reaction :



Derivatization for Fluorescence Detectors

Dansyl Chloride (a fluorotag) is invariably used to obtain fluorescent derivatives of proteins, amines and phenolic compounds, the excitation and emission wavelengths being 335 to 365 nm and 520 nm respectively.



Reagents for fluorescent derivatization

S. No.	Reagent	Reacting Functional Groups
1	Ce ⁴⁺ -salts + H ₂ SO ₄	Dicarboxylic acids
2	Fluorescamine [Fluram ^(R)]	1 ^o -Amines
3	o-Phthalaldehyde [Fluoropac ^(R)]	1 ^o -Amines

GRADIENT ELUTION & RELATED PROCEDURES

Gradient method is recommended for samples that cannot be easily separated by isocratic methods because of their wide k range. In gradient elution the eluent strength is increased during the separation by changing the composition of the mobile phase. As a result, the

analysis time is reduced and the quality of the separation is improved as well as the detection limit. Binary linear gradients are the most common and the easiest to handle. Although gradient elution is a more complex technique than isocratic elution, a good understanding is not difficult to attain.

Complex samples containing many compounds with widely differing polarities will give complex chromatograms, where retentions will be widely and irregularly spaced. With isocratic methods, the overall analysis time can be unacceptably long.

In terms of retention and selectivity:

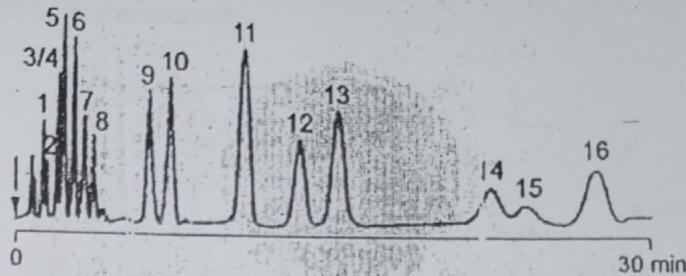
1. Some leave the column almost un-retained ($k < 1$)
2. some leave the column in a reasonable time ($1 < k < 10$)
3. some are strongly retained ($k >> 10$)
4. some are not completely separated from each other (inadequate selectivity or resolution)
5. The nearly un-retained components can merge together due to their short retention or they can simply stay hidden under the solvent peak. Determining the retention time is a problem and determining peak area is even more difficult. Strongly retained components show large peak broadening which smears peaks out and reduces peak height. If broadening is particularly bad, the peak height can decrease to the point that the detector will not detect these components and one could wrongly conclude that such components are absent from the sample.

In the event of widely varying component affinities for the stationary phase, we may need to change the composition of the eluent over the course of the analysis. This technique is called liquid-gradient elution and has the same effect as temperature-gradient elution in gas chromatography. Gradient methods can considerably improve the quality of the separation and the analysis time.

An example of the need for gradient elution: The analysis of PAH's

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Column : Chromspher PAH, 100 x 3.0 mm (L x ID)
Mobile Phase : Acetonitrile/water 70/30
Flow rate : 1.0 ml/min
Detector : UV 254
Concentration : 0.01 - 0.1 mg/ml



Peak Identification:

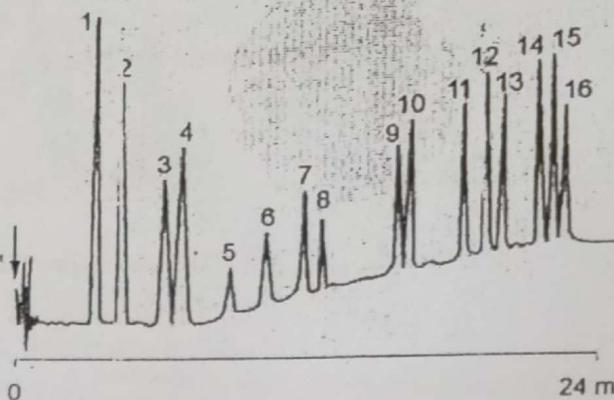
- | | |
|-------------------|----------------------------|
| 1. naphthalene | 9. benzo(a)anthracene |
| 2. acenaphthylene | 10. chrycene |
| 3. acenaphthene | 11. benzo(b)fluoranthene |
| 4. fluorene | 12. benzo(k)fluoranthene |
| 5. phenanthrene | 13. benzo(a)pyrene |
| 6. anthracene | 14. dibenz(a,h)anthracene |
| 7. fluoranthene | 15. benzo(g,h,i)perylene |
| 8. pyrene | 16. indeno(1,2,3,cd)pyrene |

PAH analysis by isocratic elution

Isocratic conditions are clearly unsuitable for the analysis of the sixteen PAHs listed as water priority pollutants: the figure above this text shows a poor resolution for the less retained solutes and small peak heights for the highly retained ones. In contrast, these problems disappear when using a gradient elution and furthermore, the analysis time is significantly decreased.

Column : Chromspher PAH, 100 x 3.0 mm (L x ID)
 Mobile Phase : Acetonitrile/water 0-5 min: 50/50
 5-20min: 100/0
 20-30min: 100/0

Flow rate : 1.0 mL/min
 Detector : UV 254
 Concentration : 0.01 - 0.1 mg/mL



PAH analysis by gradient elution

CHROMATO

Optimization of Column Performance

Introduction

Optimisation of chromatographic separations is achieved by varying the experimental conditions of the run until the components of the mixture are separated cleanly in a reasonable amount of time. There are two aspects to achieving good separation:

- The components in the mixture need to migrate or travel down the column at sufficiently different rates.
- The peaks for the components need to be relatively sharp and uniform (as components migrate, they tend to broaden or spread out such that they can overlap each other, thereby compromising detection/accuracy).

Obviously just altering conditions with no knowledge of the underlying theory of chromatographic separations would be very time consuming, wasteful and unlikely to lead to good separations.

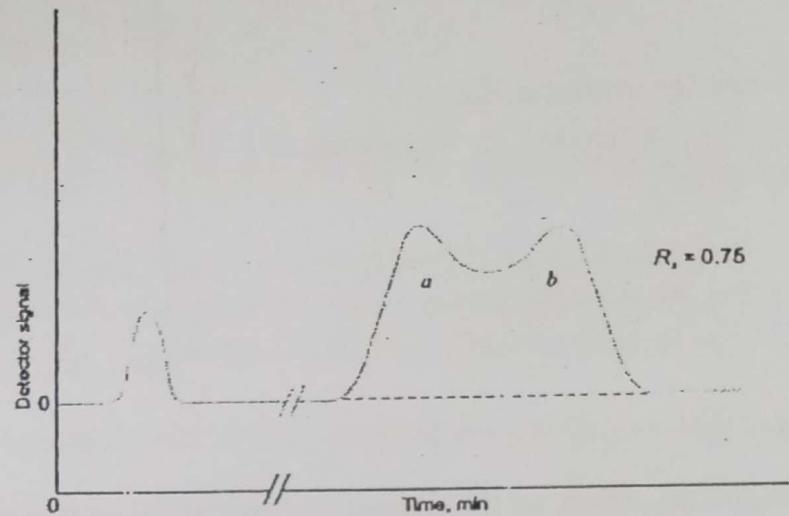
Optimisation has two aims:

- Reduction of zone broadening (the component moves through the column as a

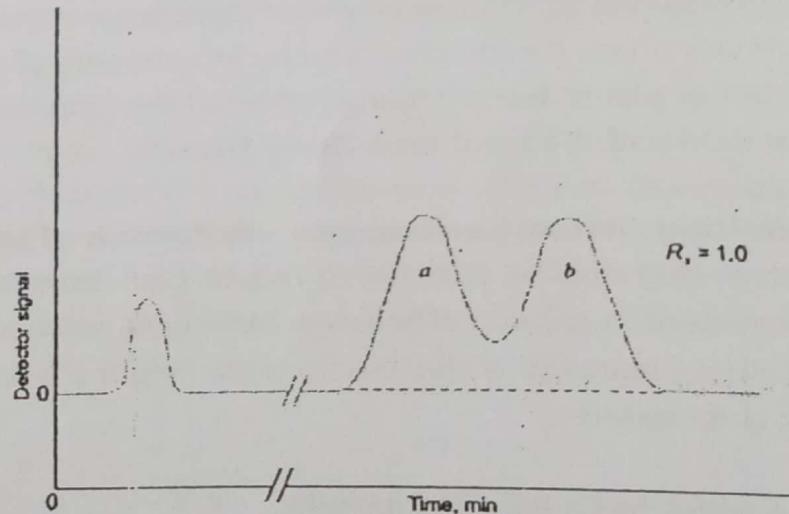
zone which is detected by the detector and translated into a peak).

- Altering the migration rates of the components.

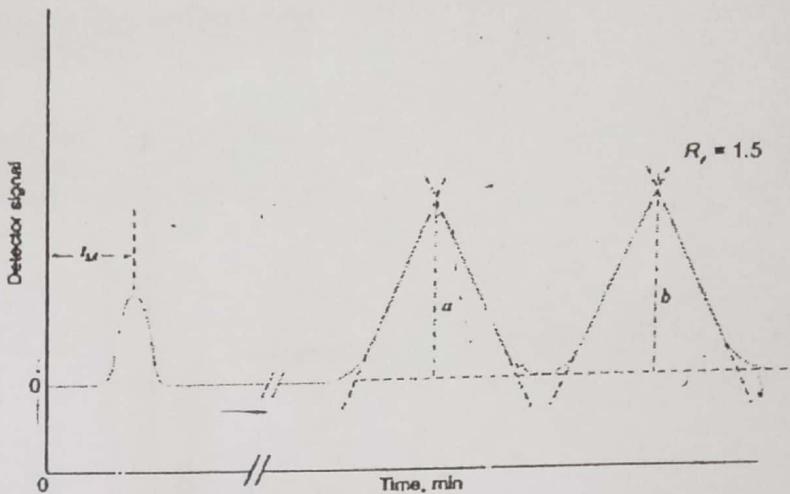
A poor separation of components A and B.



An improvement in resolution leads to a partial separation of the peaks.



A further improvement in resolution has led to separation of the peaks.



Resolution refers to the extent of separation of the peaks of interest or a chromatogram.

Column resolution

Resolution R_s is a quantitative measure of the ability of the column under specific operating conditions to separate (or resolve) two analyte peaks. The resolution for a given stationary phase can be improved by lengthening of the column thus increasing the number of plates, but a negative effect of this approach is the increased time required for separation and the band broadening that will occur.

Optimising column performance - Reduction of peak broadening

Variables that may be adjusted to reduce peak broadening are those that lead to an improvement in column **efficiency**. Making adjustments to increase the number of theoretical plates (*N*) or decreasing plate height (*H*) will therefore reduce the tendency of peaks to broaden.

The variables to be adjusted include:

- linear velocity of the mobile phase (too low and too high reduces efficiency)
- diameter of packing particle (smaller is generally better)
- thickness of the liquid coating of the stationary phase (HPLC)
- column temperature (GC)
- diameter of the column (narrower is better).

Optimising column performance – Altering migration rates

Migration rates are altered with the aim of ensuring that the components of interest all

elute from the column within a reasonable time and are sufficiently separated from each other. The extent of separation in this regard indicates how effective the chromatographic column is.

The variables to be adjusted that influence migration rates include:

- column temperature (GC)
- mobile phase composition (HPLC) eg 70% methanol/30% water → 50%/50%, gradient elution
- chemical composition of the stationary phase – use a different type of column
- special chemical effects – incorporating a species in the stationary phase that interacts with one or more of the sample components to enhance resolution.

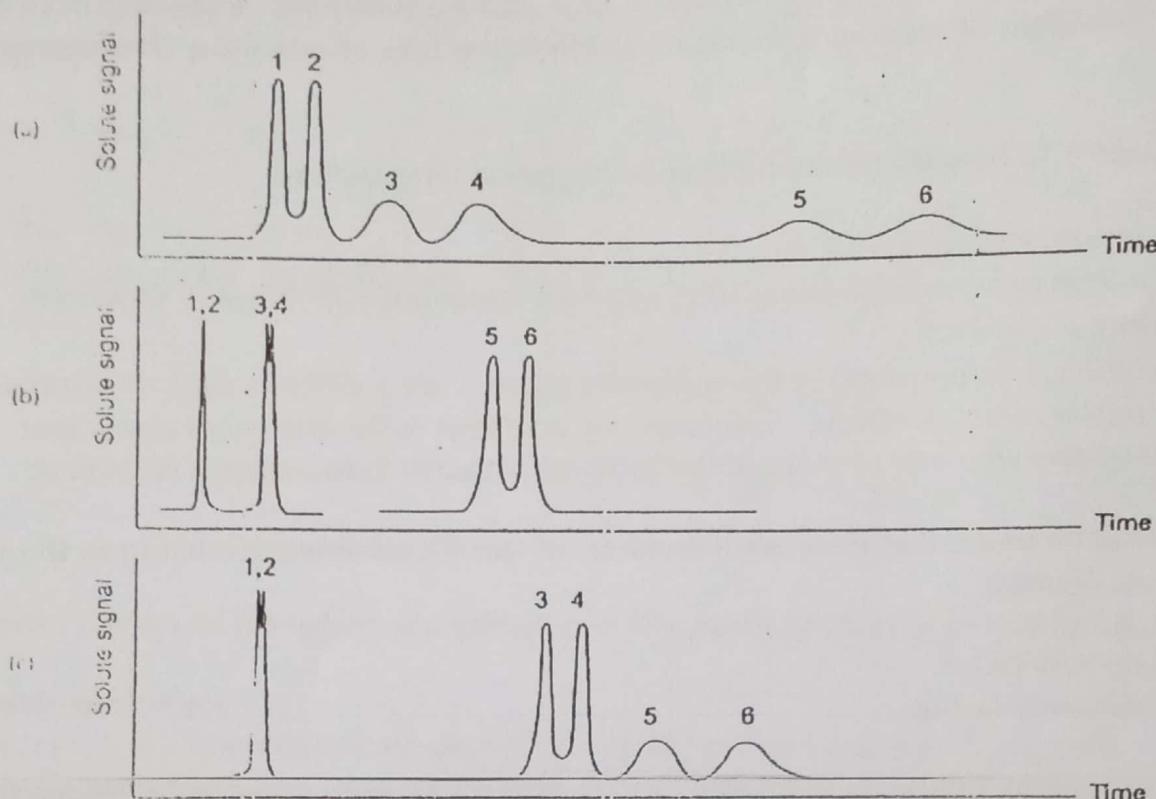
You should be aware that there are a numbers of specific parameters relating to the study of migration rates:

- Capacity factor
- Selectivity factor.

If you are working on the development or optimisation of chromatographic techniques you may need to find out about these terms.

The general elution problem

This is best illustrated in the following diagram of a six-component mixture - three pairs of closely migrating peaks.



Look at the three panels (a), (b) and (c), which represent separations under three different conditions. Note that in each panel only two of the peaks are well resolved with minimal broadening. The other two pairs are either not resolved (overlap) or are broad and diffuse.

This is a common problem in chromatography and may be overcome by the following two approaches:

- Set up the run so that only the peaks of interest are well resolved and not worry about the others. For example, if peaks 3 and 4 are those of interest then set up the run with the same conditions as panel (c).
- Change conditions during the separation to achieve the required resolution of all 6 peaks. That is, use a gradient or stepwise elution. For HPLC, change the composition of the mobile phase and for GC use temperature increases (temperature programming) to induce the appropriate changes. These will be discussed in the HPLC and GC sections later.

Interfacing HPLC with Mass Spectrometry

- High performance liquid chromatography mass spectrometry is an extremely versatile instrumental technique
- As the name suggest the instrumentation comprises a high performance liquid chromatography (HPLC) attached, via a suitable interface, to a mass spectrometer (MS).
- The primary advantage HPLC/MS has over GC/MS is that it is capable of analyzing a much wider range of components. Compounds that are thermally labile, exhibit high polarity or have a high molecular mass may all be analyzed using HPLC/MS, even proteins may be routinely analyzed.
- Solutions derived from samples of interest are injected onto an HPLC column that comprises a narrow stainless steel tube (usually 150 mm length and 2 mm internal diameter, or smaller) packed with fine, chemically modified silica particles. Compounds are separated on the basis of their relative interaction with the chemical coating of these particles (stationary phase) and the solvent eluting through the column (mobile phase).
- Components eluting from the chromatographic column are then introduced to the mass spectrometer via a specialized interface. The two most common interfaces used for HPLC/MS are the electrospray ionization and the atmospheric pressure chemical ionization interfaces.

(a) Electrospray ionization

- In electrospray ionization the analyte is introduced to the source at flow rates typically of the order of $1\mu\text{l min}^{-1}$.
- The analyte solution flow passes through the electrospray needle that has a high potential difference (with respect to the counter electrode) applied to it (typically in the range from 2.5 to 4 kV).
- This forces the spraying of charged droplets from the needle with a surface charge of the same polarity to the charge on the needle.
- The droplets are repelled from the needle towards the source sampling cone on the counter electrode (shown in blue).
- As the droplets traverse the space between the needle tip and the cone, solvent evaporation occurs. This is circled on the Fig.1 and enlarged upon in Fig.2. As the solvent evaporation occurs, the droplet shrinks until it reaches the point that the surface tension can no longer sustain the charge (the Rayleigh limit) at which point

a "Coulombic explosion" occurs and the droplet is ripped apart. This produces smaller droplets that can repeat the process as well as naked charged analyte molecules.

- These charged analyte molecules (they are not strictly ions) can be singly or multiply charged. This is a very soft method of ionization as very little residual energy is retained by the analyte upon ionization.
- It is the generation of multiply charged molecules that enables high molecular weight components such as proteins to be analyzed since the mass range of the mass spectrometer is greatly increased since it actually measures the *mass to charge ratio*.
- The major disadvantage of the technique is that very little (usually no) fragmentation is produced although this may be overcome through the use of tandem mass spectrometric techniques such as MS/MS or MSⁿ.

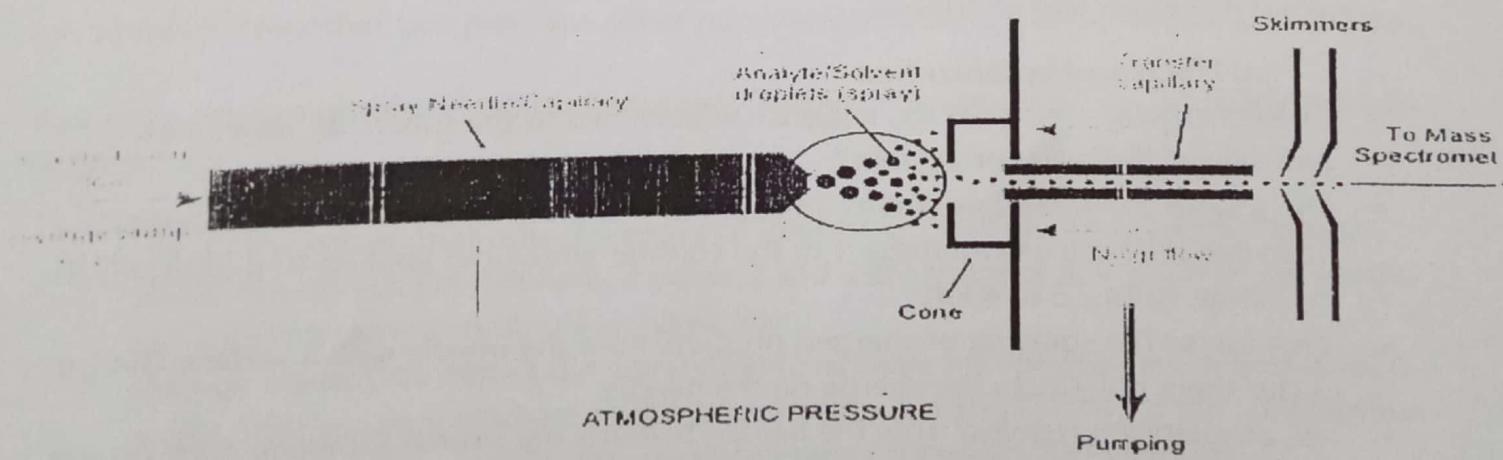


Figure A schematic of an ESI interface

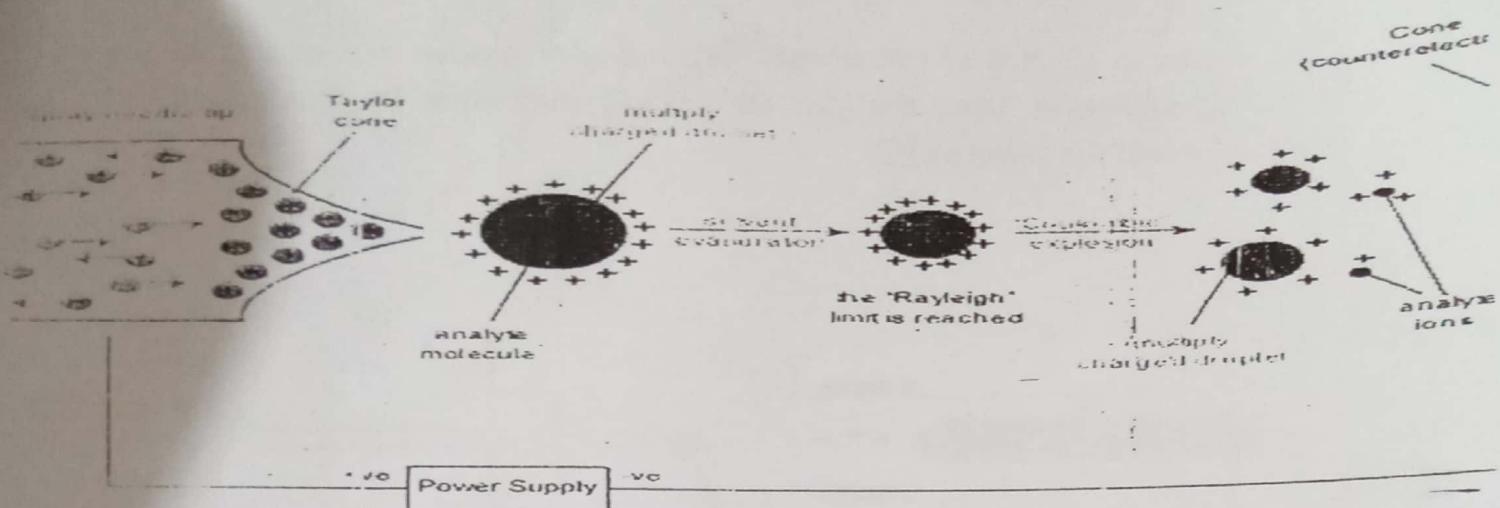


Figure 2 A schematic of the mechanism of ion formation

(b) Atmospheric pressure chemical ionization

- Atmospheric pressure chemical ionization (APCI) is an analogous ionization method to chemical ionization (CI).
- The significant difference is that APCI occurs at atmospheric pressure and has its primary applications in the areas of ionization of low mass compounds (APCI is not suitable for the analysis of thermally labile compounds).
- The general source set-up (see Fig. 3) shares a strong resemblance to ESI. Where APCI differs to ESI, is in the way ionization occurs. In ESI, ionization is brought about through the potential difference between the spray needle and the cone along with rapid but gentle desolvation.
- In APCI, the analyte solution is introduced into a pneumatic nebulizer and desolvated in a heated quartz tube before interacting with the corona discharge creating ions.
- The corona discharge replaces the electron filament in CI - the atmospheric pressure would quickly "burn out" any filaments - and produces primary N_2^+ and N_4^+ by electron ionization.
- These primary ions collide with the vaporized solvent molecules to form secondary reactant gas ions - e.g. H_3O^+ and $(\text{H}_2\text{O})_n^{\text{H}+}$ (see Fig. 4). These reactant gas ions then undergo repeated collisions with the analyte resulting in the formation of analyte ions.
- The high frequency of collisions results in a high ionization efficiency.

- This results in spectra of predominantly molecular species and adduct ions with very little fragmentation. Once the ions are formed, they enter the pumping and focusing stage in much the same as ESI.

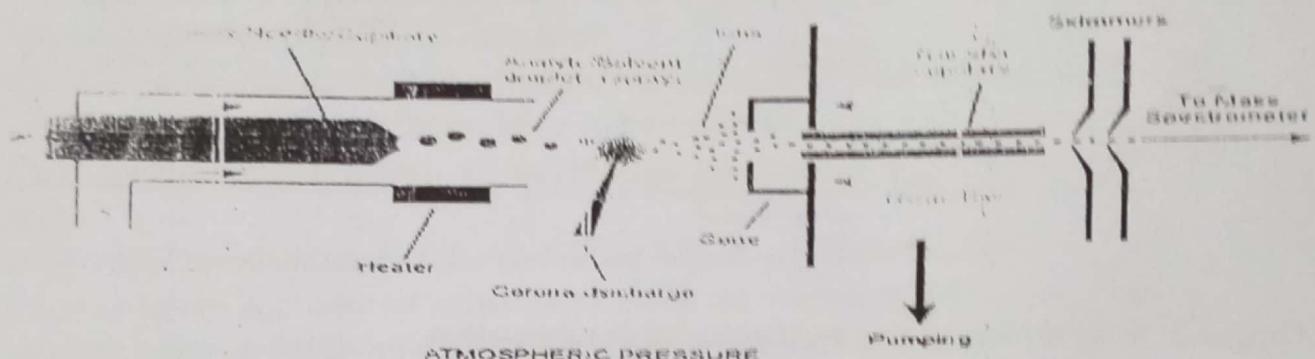


Figure 3 A schematic of the components of an APCI source

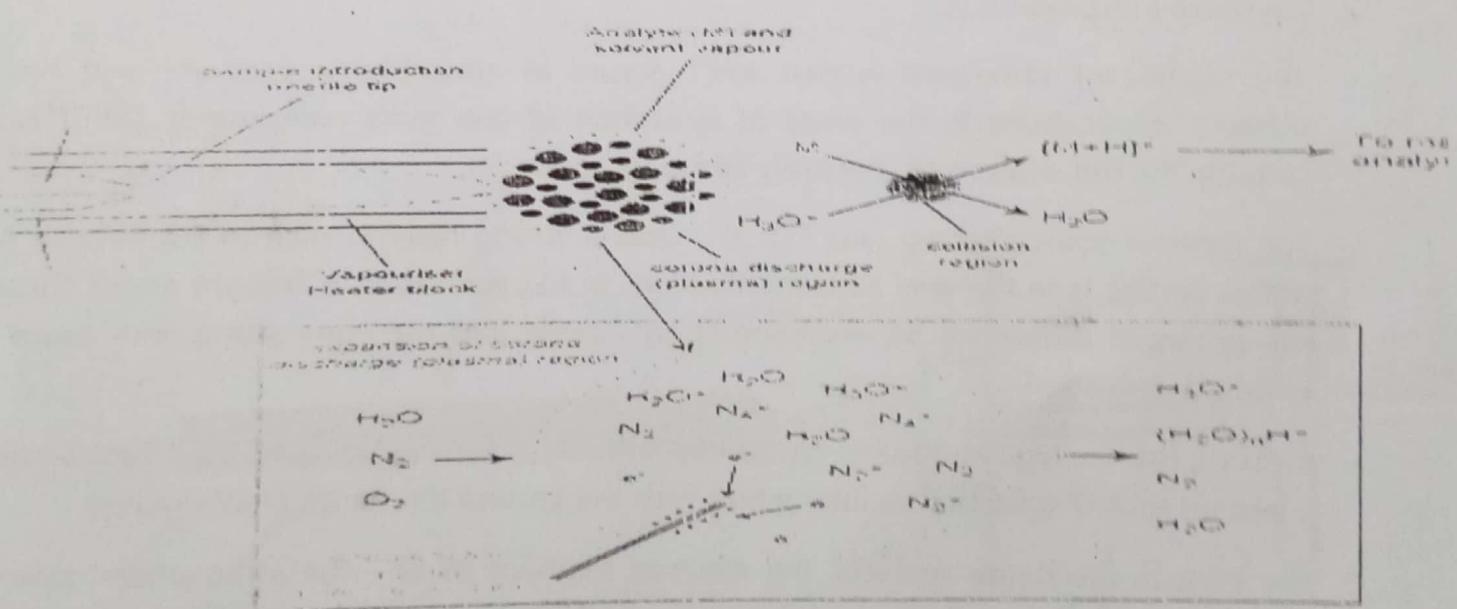


Figure 4 A more detailed view of the mechanism of APCI