Name of Unit:	Hyphenated Techniques
Course/Subject Name:	Advanced Instrumentation Techniques
Course/Subject Code:	BP811ET
Class: B. Pharm. Semester	VIII
Faculty:	Ms. Manpreet Kaur
Email id:	manipreet663@gmail.com
Mobile No.	9592378971

Learning Outcome of Module-V

LO	Learning Outcome (LO)	Course Outcome Code
LO1	To understand the basic concept of Hyphenated Techniques	BP811.1, BP811.6
LO2	To know about the components of LC - MS / MS instrument	BP811.1, BP811.6
LO3	To know about the components of GC-MS/MS instrument	BP811.1, BP811.6
LO4	To know about the components of HPTLC-MS instrument	BP811.1, BP811.6

TABLE OF CONTENT

Liquid Chromatography-Mass Spectrometry (LC-MS)

Liquid Chromatography-Mass Spectrometry (LC-MS) or High Pressure Liquid Chromatography-Mass Spectrometry (HPLC-MS) is an analytical technique that coupled high resolution chromatographic separation with sensitive and specific mass spectrum detection. This

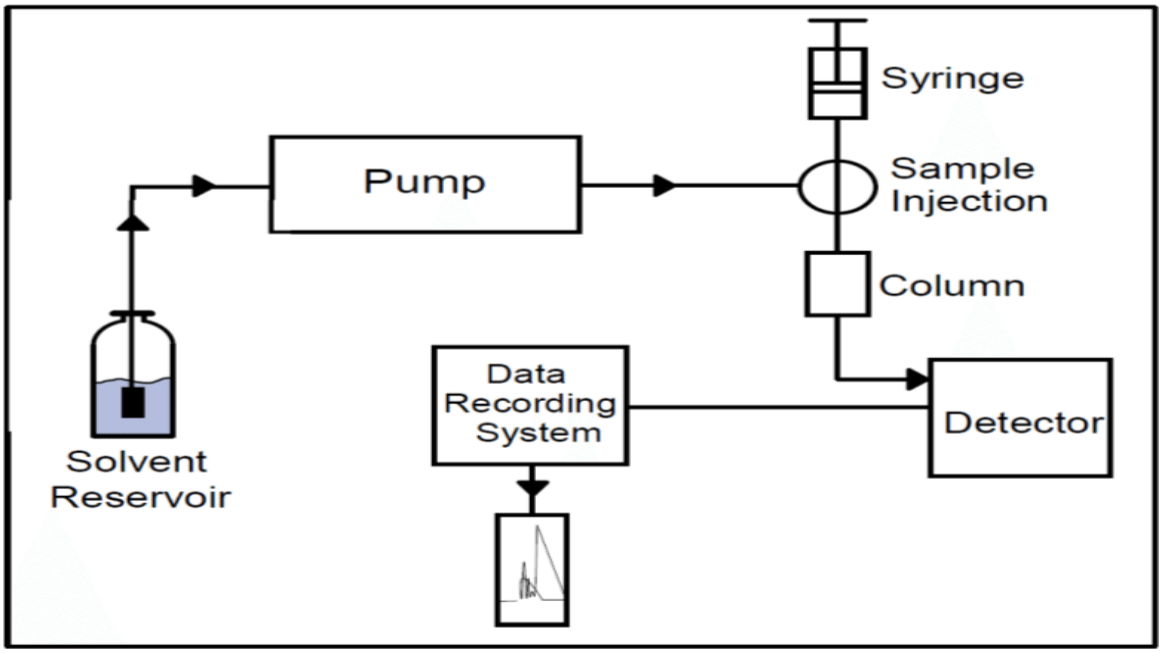

Figure 1: Instrumentation of HPLC

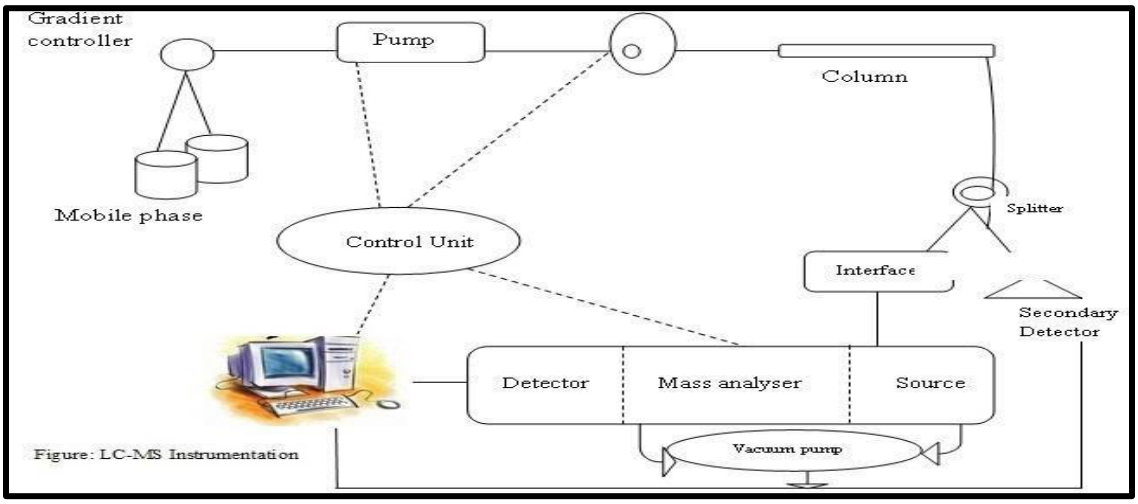

Figure 2: Instrumentation of LC-MS

includes High Performance Liquid Chromatography (HPLC)-MS, Capillary Electrophoresis (CE)-MS and Capillary Electrochromatography (CEC)-MS. The combination of Gas Chromatography and Mass Spectrometry (MS) was first reported in 1958 and made available commercially in 1967. Combination of LC with MS is an important development in the history of chromatography (1980s). Mass spectrometry in LC-MS helps to determine the elemental composition and structural elucidation of a sample.

Principle of LC-MS

Typical LC-MS system is combination of HPLC with MS using interface (ionization source) (Figure 1). The sample is separated by LC, and the separated sample species are sprayed into atmospheric pressure ion source, where they are converted into ions in the gas phase. The mass analyzer is then used to sort ions according to their mass to charge ratio and detector counts the ions emerging from the mass analyzer and may also amplify the signal generated from each ion. As a result, mass spectrum (a plot of the ion signal as a function of the mass-to-charge ratio) is created, which is used to determine the elemental orisotopic nature of a sample, the masses of particles and of molecules, and toelucidate the chemical structures of molecules.

Requirement of LC

Usually LC used in LC-MS is HPLC. The principle of separation in HPLC, is normal phase mode or reverse phase mode of adsorption. Normal phase constricts with polar stationary phase with non-polar solvent/mobile phase and reverse phase constricts with non-polar stationary phase with polar solvent/mobile phase. Normal phase mode not widely used in biomedical research and not advisable for pharmaceutical applications since most of the drug are polar in nature and takes longer time to be elute and detected. Reverse phase mode have wide range of pharmaceutical application. Examples for reverse phasecolumns are octadecylsilane (ODS)/ C18,C8,C4, etc.

HPLC Instrumentation

Mainly HPLC instrument contains pump, mixing unit (solvent degassing system), injector (manual/auto), guard column, analytical columns, detectors, recorder andintegrators (Figure 1).

Detector used in HPLC

1. **UV detector**

- Single wavelength (filter)

- Variable wavelength (monochromator)
- Multiple wavelengths (Photodiode array detector[PDA])

2. Florescence

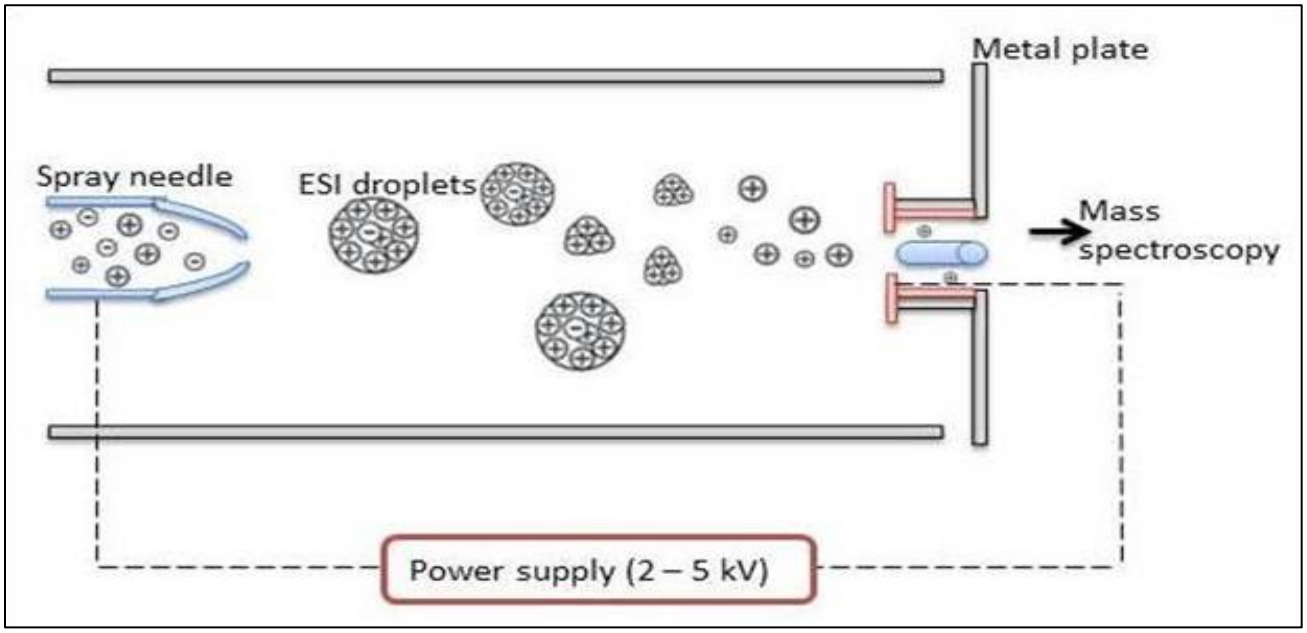

Figure 3: Electrospray ionization

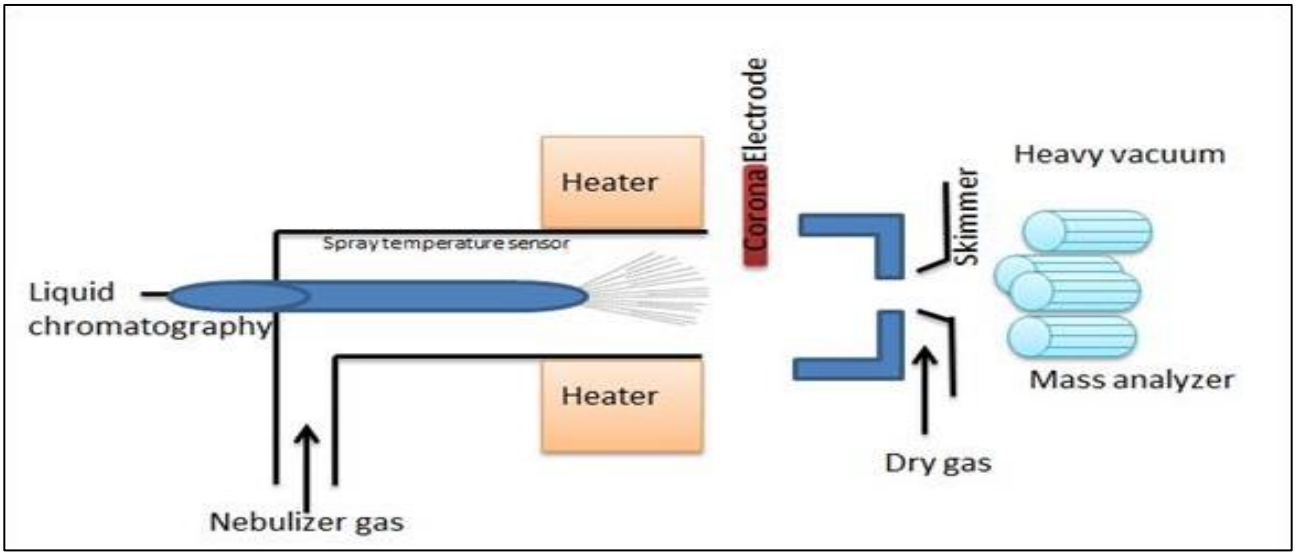

Figure 4: Atmosheric pressure chemical ionization Ionization source

3. Electrochemical detector

4. Mass spectrometric

Most common ionization sources are Electrosprayionization (ESI), Atmospheric pressure chemical

Requirements of LC-MS instrumentation

Mainly the LC-MS contains liquid chromatography assembly, ion generation unit/ ionization source, mass analyzer and mass spectrometric data acquisition (Figure 2). The effluent mobile phase with separated compound from the liquid chromatography is interfaced with the ionization source of the Mass spectrometer. ionization (APCI) and Matrix-assisted laser desorption/ ionization (MALDI). A part from this Electron impact (EI) and Chemical ionization (CI) or negative chemicalionization are also used as ionization source in MS.

Electrospray ionization (ESI): Electrospray is produced by applying a strong electric field to a liquid passing through a capillary tube with a weak flux. This produces charged large droplets which is then subjected to solvent

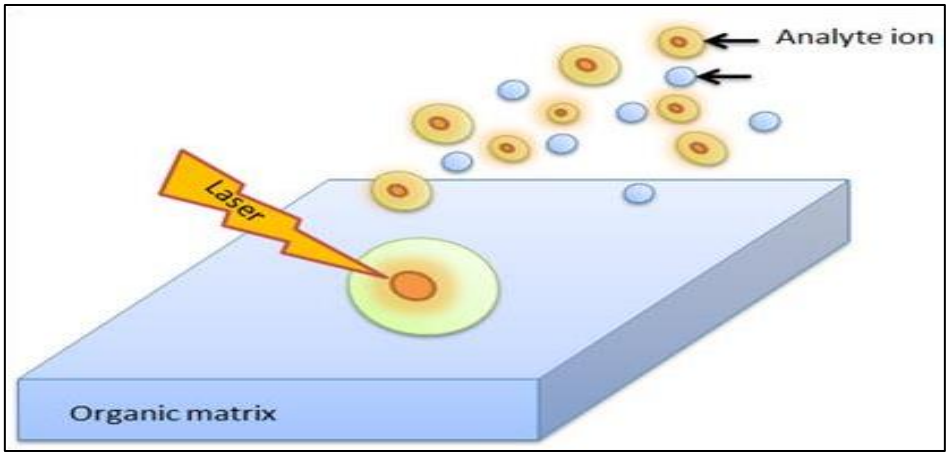

Figure 5: Matrix-assisted laser desorption/ionization (MALDI)

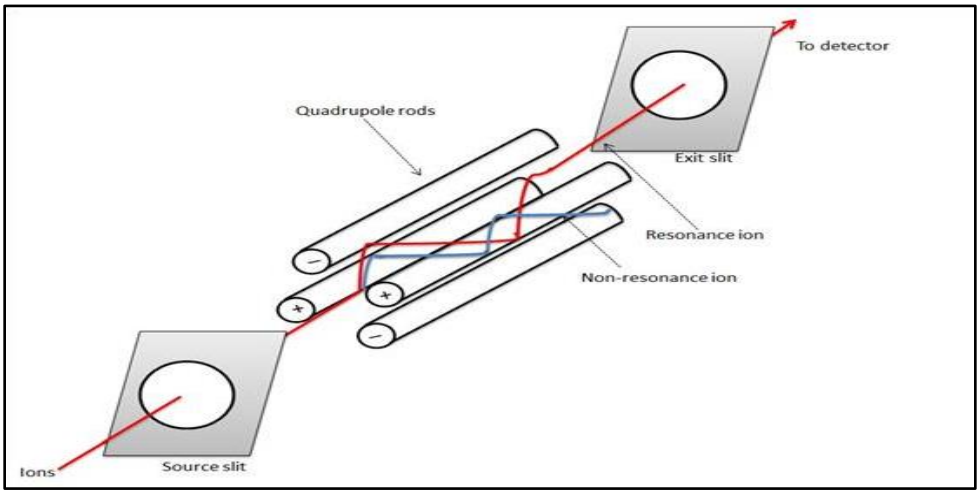

Figure 6: Quadrupole

evaporation. Increase in charge density resulting from solvent evaporation causes coulombic repulsion to overcome the liquid surface tension, resulting in release of ions from droplets. This is the principle of ion formation using this technique. Detection sensitivity in this technique is limited to 10-8 µl, and needs large volume to increase the sensitivity of the detection. By using ESI high mass sample, non-volatile molecules, liquids can be ionized and disadvantage of this source of ionization is poor sensitivity, low fragmentationand source is instable (Figure 3).

Atmospheric pressure chemical ionization (APCI): Principle of this technique involves nebulization of the mobile phase with nitrogen gas and vaporization by heating it to relatively high temperature (above 400°C). The resulting vapor is then subjected to a corona discharge electrode to create ions.

APCI (Figure 4) is most commonly used ionization source used in LC-MS. APCI are used for analysis of pharmaceutical, environmental, toxicological, clinical and chemical industrial/ laboratory samples.

Matrix-assisted laser desorption/ionization (MALDI): MALDI is an ionization technique for large and/or labile molecules such as peptides, proteins, polymers, dendrimers, and fullerenes. This technique involves embedding analytes in a matrix which absorbs energy at the wavelength of the laser. The nitrogen ultraviolet (UV) lasers (337 nm) is applied over the matrixin vacuumto generate ions of analyte. The mechanism for ionization is not clear, but three different models are postulated to explain desorption of the matrix-sample materials from the crystal surface (Matrix will be crystalline in nature under vacuum).

- Upon impact of laser, Quasithermal evaporation occurswhich results fromincreased molecular motion.
- This causes expulsion of upper lattice layers of matrix(desorption)
- The matrix is then thought to transfer protons to thesample molecules, thus charging the analyte.

Preparation of matrix/sample perpetration for MALDI mass spectra: MALDI mass spectra samples are usually prepared by dried-droplet method, thin layer method and sandwich method. In dried-droplet method matrix-to-sample ratio is of about 5000:1 and an aliquot (0.5–2.0 µL) of this mixture is then applied to the sample target where it is allowed to dry. In thin layer method the sample will be applied in the matrix and it will be absorbed by the matrix. This method yields good sensitivity, resolving power and mass accuracy. In thin layer method Nitrocellulose (NC) is

used as a matrix. In sandwich method, thin layer matrix crystals is prepared followed by subsequent addition of droplets of aqouse TFA (solvent/ trifluroacetic acid), sample and matrix (Figure 5).

The matrix performs two important functions as follows,

- It absorbs photon energy from the laser beam and transfer into excitation energy of the solid system
- It serves as a solvent for analyte, so that the intermolecular force are reduced and aggregation ofthe analyte molecules will be minimum.

The mass spectrometer analyzer

The analyzer is component of the mass spectrometer that takes ionized molecules and separates them based on charge to mass ratios and outputs them to the detector where they are detected and later converted to a digital output.

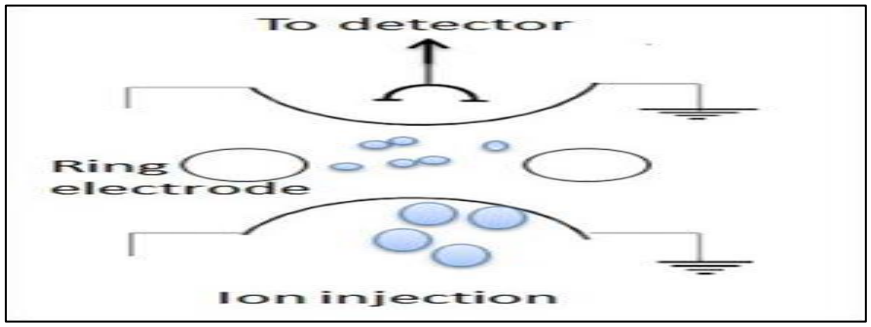

Figure 7: Ion trap analyzer

Quadrupole Mass analyzer: Quadrupole (Figure 6) and triple quadrupole are the most widely used analyzer because it is easy to operate and it will cover wide mass range (10 to 4000 A.M.U./atomic mass unit). Quadrupole gives good linearity for quantitative work and good resolution (up to 4000), quality of mass spectra, scanning speed (5000 A.M.U per second) and mass accuracy (0.1 to 0.2 A.M.U.).

The working principle involves usage of electric fields to separate ions based on its mass to charge ratio when they are passed along the central axis of four parallel equidistant poles. Ions are separated in a quadrupole based on the stability of their trajectories in the oscillating electric fields that are applied to the rods.

Quadrupole is composed of two pairs of metallic rods. Each opposing rod pair is connected together electrically, and a Radio Frequency (RF) voltage is applied between one pair of rods and the other. A direct current voltage is then superimposed on the RF voltage. Ions travel down the

quadrupole between the rods. Only ions of a certain mass- to-charge ratio (m/z) will reach the detector for a given ratio of voltages. Other ions have unstable trajectories and will collide with the rods. This permits selection of an ion with a particular m/z and allows the operator to scan for a range ofm/z-values by continuously varying the applied voltage.

Ion trap analyzer: This analyser is also known as the quadrupole ion trap analyser (QIT) (Figure 7). Mostly it will be used on GC/MS rather than LC/MS. The principle of the trap is to store the ions in a device (ion trap) consisting of a ring electrode and two end cap electrodes. These ions are manipulated by using applied DC and RF fields. The amplitude of the applied voltages enables the analyser to trap ions of specified mass to charge ratio within the analyzing device. Nonselected ions are given a trajectory by the electrostatic field that causes them to exit the trap. By filling the trap with inert gas fragmentation of selected ions is possible. This is useful when structural information is required.

Time of Flight (TOF) analyzer: This analyzer is commonly called the TOF (Figure 8) and this is used in single MS systems. In MS/MS configuration, the TOF is associated to a quadrupole (QTof), or to another TOF (TOF-TOF) or to an Ion Trap (QIT/TOF).

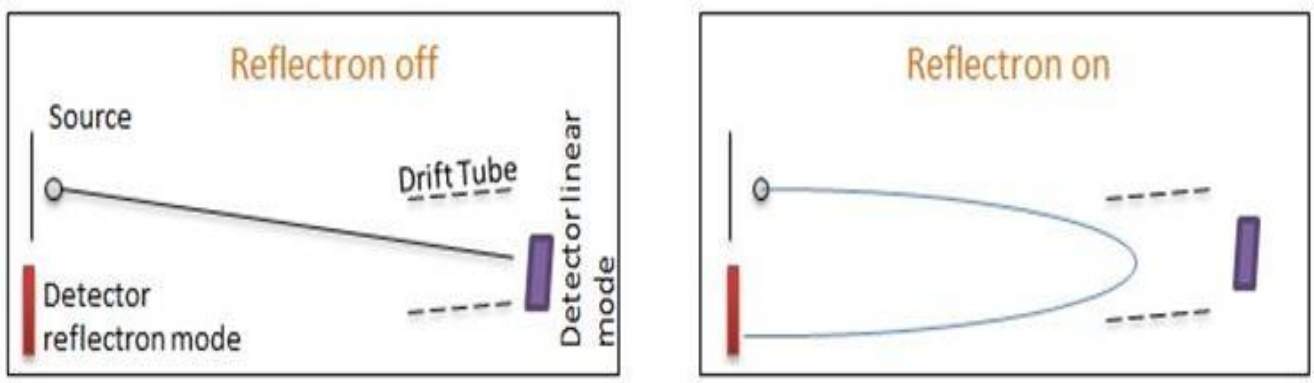

Figure 8: Time of Flight (TOF) analyzer

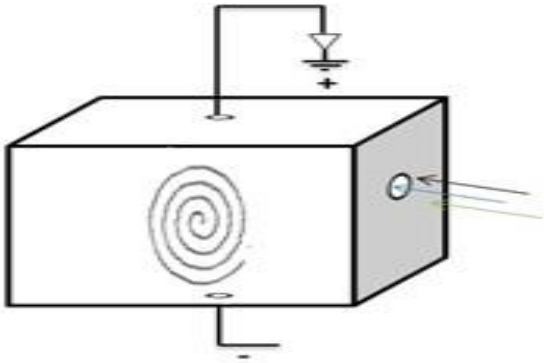

Figure 9: Fourier transform-ion cyclotron resonance (FT-ICR)

Principle of the time of flight analyzer is formation of ions from ion source and it will be accelerated to high velocity by an electric field present in the „drift tube" of the instrument. The accelerated ions will be detected by detector by linearmode or reflection mode.

Magnetic Sector Mass Analyser: In Magnetic sector analyzers, ions are accelerated through a flight tube by magnetic field, where the ions are separated by charge to mass ratios. As moving charges enter a magnetic field, the charge is deflected to a circular motion of a unique radius in a direction perpendicular to the applied magnetic field. Ions in the magnetic field experience two equal forces; force due to the magnetic field and centripetal force. When similar ions pass through the magnetic field, they all will be deflected to the same degree and will all follow the same trajectory path. Other will collide with either side of the flight tube wall or will not pass through the slit to the detector.

Fourier transform-ioncyclotron resonance (FT-ICR): Ionsentering a chamber are trapped in circular orbits by powerful electrical and magnetic fields. When excited by a Radio-frequency (RF) electrical field, the ions generate a time dependent current. This current is converted by Fourier transform into orbital frequencies of the ions which corresponds to their mass-to-chargeratios. FT-ICR mass analyzers can perform multiple stages of mass spectrometry without additional mass analyzers. They also have a wide mass range and excellent mass resolution (Figure 9).

Detectors

Three deferent kinds of detectors are used in Mass Spectrometry, i.e. Electron multipliers (Figure 10a), Dynolyte photomultiplier (Figure 10b) and Micro channel plates (Figure 10c). Electron multipliers dynode is used to convert either –ve, +ve ions into electrons, that will be amplified and detected. This will be widely used in quadrupole and ion trap instruments.

The dynode of Dynolyte photomultipliers converts the charged ions into electrons. These electrons sticks to a phosphor and emits photons, and that photons are made to strike the photomultiplier to achieve multiplied signals for recording.

Microchannel Plate (MCP) are commonly employed in ToF spectrometers. This will have very low time response and high degree of sensitivity (<1 ns and single ion signal >50 mV respectively).

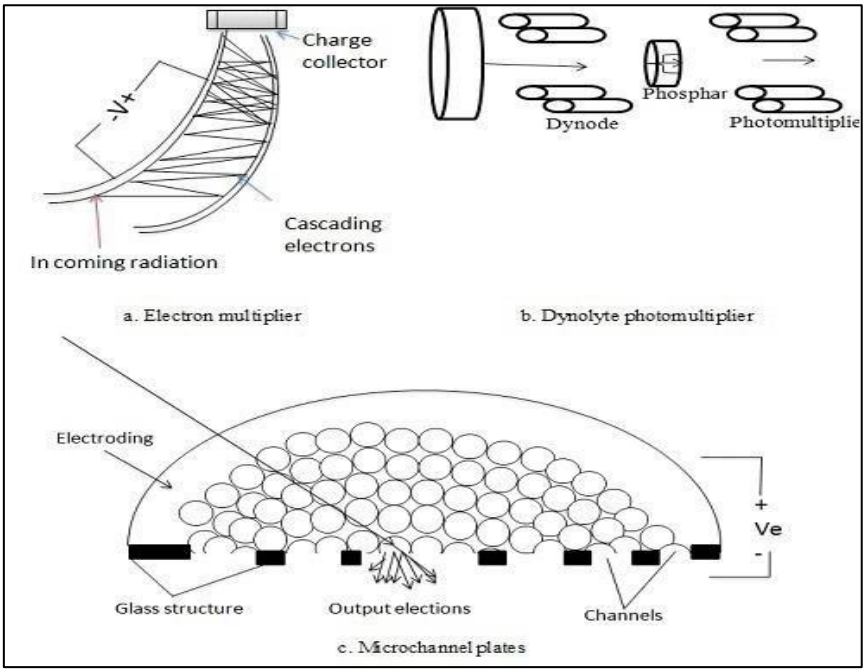

Figure 10: Detectors used in LC-MS

Mass spectrometric data acquisition system

The data acquisition system is designed to digitalize electrical signals from the detector and transfer them tothe data system in a compatible format.

- Full mass spectra (library to solve the analyticalproblem)
- Raw data of a small range (determine of isotopepattern/molecular weight)
- Mass chromatograms of selected ions (quantification)
- MS/MS experiments with Collisionally Activated Decompositions (CAD), such as daughter ion, parent ion and neutral loss scans structural information.

LC-MS/LC-MSMS are most widely used in food industries, pharmaceutical and chemical industries for quantitative and qualitative analysis.

Applications of LS-MS/MS

- Molecular weight determination: Able to determine the molecule weight of chemical substance, pharmaceutical substances, proteins, etc.
- Structural determination/elucidation: Tandem mass spectrometry used to determine structural information using mass spectral fragmentations.
- Pharmaceutical applications: It‟s used to determine the pharmacokinetic profile of the pharmaceuticals like drug, drug metabolites/degradation product, impurities and chiral

impurities. The separation and detection of chiral impurities in pharmaceuticals are of great importance because the D-isomer of a drug can have different pharmacological, metabolic and toxicological activity from the L-isomer.

- Clinical and biochemical applications: MALDI-TOF MS is used in SNP genotyping, quantification of DNA, gene expression analysis, DNA and RNA sequencing.
- Food and Environmental applications: use to identify aflatoxins (toxic metabolic product in certain fungi), determine the vitamin D3 in poultry fed supplements, etc.
- Capillary electrophoresis/MS applications: Used for analysis of peptides.

Gas Chromatography-Mass Spectrometry (GC-MS)

Gas chromatography-mass spectrometry (GC-MS) is a **hyphenated** technique consisting of two analytical procedures coupled in series that is a Gas Chromatography (GC) for separation of volatile compounds and Mass Spectrometry (MS) for identification and quantification of molecular weight of each separated component. The GC helps in separation of multiple components mixture so that they can reach the mass spectrometry one at a time. Thus, the molecular mass and other fragments mass values describe only for a single component at a time. The GC-MS instrument has two independent techniques as follows:

Gas Chromatography (GC)

The GC is based on chromatographic method used in the separation of volatile organic and/or inorganic mixtures. It is broadly classified into two types depending upon mobile phase (MP) and stationary phase (SP) used. When MP is a gas and SP is a liquid called Gas-Liquid Chromatography (GLC) while when SP is a solid then called Gas- Solid Chromatography (GSC). The GC uses generally a high-resolution fused silica capillary column coated with SP as solid or nonvolatile liquid, housed in a temperature- controlled oven. Temperature of the oven is controlled to optimize the vaporization of mixture without decomposition which led to separation. When a sample solution is injected into the injection port where it get vaporized immediately because of the high temperature (upto ~300 C) under low pressure (~1-7-10 torr). Then, the sample (mixture) is transported through the column by continuous addition of MP. This process is called *elution*. As the sample travel through the column, the components physically interact with the SP as well as MP material of varying degrees depending on mixture components affinity. As a result, different compounds will travel with different speeds through the capillary column and will exit from the column after a distinct **retention time**. When the components present in mobile phase travels through the column and when reached to the detector, a signal is produced depending on the concentration and sensitivity of a particular component. A plot of signal vs. time generates a series of peaks in a chromatogram depending upon the number of components present in the sample. For example, Fig.1 shows that the mixture has five different components in it.

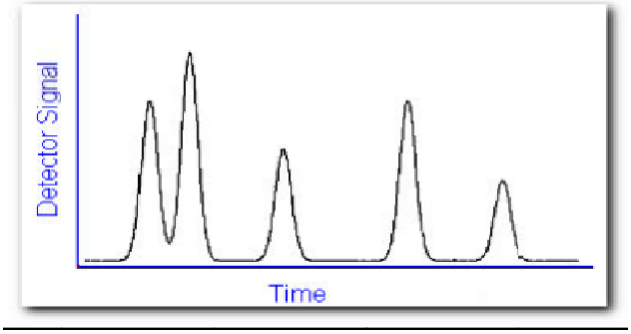

Fig. 1. GC-chromatogram

Thus, a GC chromatogram provides information like sample composition, retention time of the component and quantity from the heights of the peaks or the area under the peaks. In principle, the components would exit the column one after another depending upon retention time (shorter the retention time faster will be elution, i.e. less interaction with SP). However, sometime due to less interaction with MP (gas phase) and/or high interaction with SP results in peaks overlapping or peak broadening.

Instrumentation:

There are five major parts as follow:

- Injector : Samples are vaporized
- Carrier gas: Nitrogen (N_2), Helium (He) or Hydrogen (H_2)
- Oven: Isothermal or gradient temperature
- Columns: Capillary Column and Packed column
- Detectors: Different detectors are used depending upon selectivity and/or sensitivity of detection for specific compound. For example,

Flame-ionization detector (FID): FID is extremely sensitive with a large dynamic range. It has only disadvantage to destroy the sample.

Electron-capture detector (ECD): ECD is sensitive like FID, but has a limited dynamic range. Mostly used in the analysis of electronegative atoms containing organic molecules such as halogens, phosphorous and nitro groups.

Flame-photometric detector (FPD): The determination of sulfur or phosphorus containing compounds

Theory: The distribution of components between MP and SP is a dynamic equilibrium process which can be explained in terms of Gaussian probability distribution method. For example, analyte A is in equilibrium between the two phases:

$A_{mobile} \rightleftharpoons A_{stationary}$

The equilibrium constant (K) is termed as *partition coefficient* or *distribution constant* is defined as the degree of the solute molar concentration (C_M) distributes themselves between the mobile and stationary phases.

$K = [C_M] / [C_M]$

S M

Where, $[C_M]$ is molar concentration of the analyte in the SP and $[C_M]$ is molar S M

Concentration in the MP. If K is constant over a wide range of analyte concentrations, then $[C_M]$ is directly proportional to $[C_M]$ and chromatographic peak is symmetrical, S M Gaussian distribution and retention time are independent of the amount of analyteinjected

Retention Times of an analyte is defined as the time it takes after sample injection for the analyte to reach the detector and elute. The time for non-interacted species with SP to reach the detector is defined as the dead time (t_D), if taken in volume called dead volume (V_D). Thus.the rate of migration of a non-interacted species is the same as the rate of motion of the mobile phase expressed as,

Rate $= L / t$ .....(i)
M D

Where, L is the column length and t is the retention time of the mobile phase/

D non-interacted species. Similarly, the linear rate of a solute molecule is calculated by dividing the columnlength by the retention time t , R

Rate S $= L / t$ ..... (ii)
R

The linear rate of a solute molecule can be expressed as a function of the rate of migration of the unretained species,

Rate $_S$ = Rate x S (t).....(iii)

M M

Where, S (t) is the fraction of time the solute spends in the mobile phase.

M

Generally, we need to know about movements of solutes through a gas chromatographic column. The efficiency of the column itself is described by the **height equivalent to theoretical plates (HETP)**,

HETP = L / N.....(iv)

Where, N is the **number of theoretical plates**. Obtaining the number of theoretical plates requires some manipulation of the chromatogram,

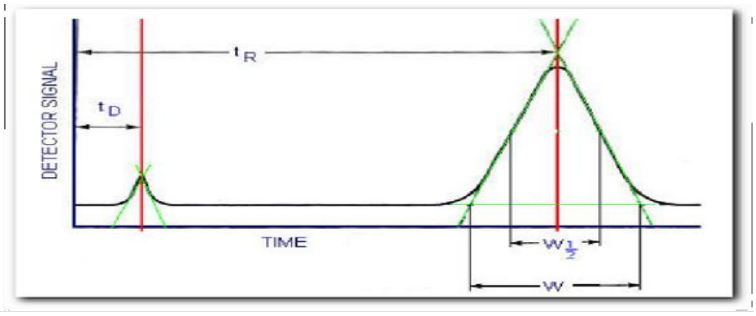

Fig. 2. GC-chromatogram

Determining the number of theoretical plates: From Fig. 2, the number of theoretical plates can be calculated,

$N = 16 (t R^2 / W) = 5.55(t R/ W) ½ ^2$ (v)

Where, W is the peak width measured in the same units as t and W width measured at half the peak height.

The **resolution** of a column is defined as its ability to separate a mixture of compounds. For example, compounds Ψ and Θ were separated by the GC column as shown in Fig. 3.

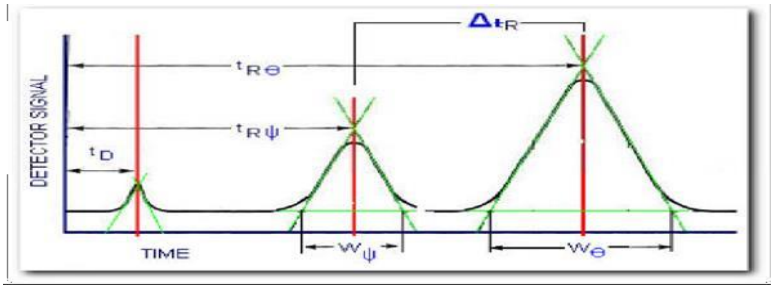

Fig. 3. GC-chromatogram

Determining the column resolution: After some mathematical manipulation, the required parameters to compute the column resolution.

The chromatogram generally plotted in detector signal vs. time, gave Gaussian- shaped (or bell

shaped) peaks. In reality, non-Gaussian peak shapes will often occur. The front side of a peak might be drawn out while the tail on the right is steep called **fronting**, might be due to large amount of a sample is introduced into the column or wrong combination of packing material. The right side of the peak is drawn out while the tail on the left is steep called **tailing** and usually occurs when the solute has a concentration dependent distribution coefficient. Fronting and tailing will result in less accurate quantitative analysis and peaks broadening.

Equipment calibration: The GC-MS instrument needed to be tuned before each analysis. The calibration consists of injecting a known volume of a standard and measuring the time between injection and elution. The retention time of a compound remained the same for a given set of variables (temperature, flow rate and column length). If the quantitative analysis is required, it might need to compare peak heights or areas of the peak with that of one of the standards. The most ancient method for quantitative analysis is the **internal standard calibration method** (ISTD) and involves the preparation of a series of standards that approximate the composition of unknown. In this procedure, a measured quantity of an internal standard is introduced into each standard and sample. Chromatograms for the standards are generated and peak areas are plotted as a function of analyte amount or concentration. Such plot should yield a straight line passing through the origin and gives qualitative and quantitative analysis of different components present in the mixture (Fig. 4).

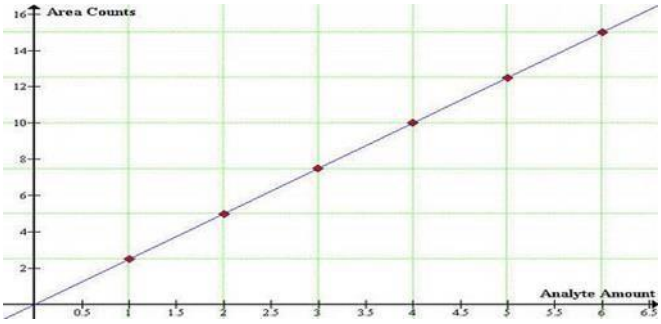

***Fig. 4. An ISTD calibration curve (2.5 area counts = 1.0 units of compound amount) Mass* Spectrometry (MS)**

In GC-MS technique, GC produces „pure" fractions after separating a mixture. Followed by each component sends to the mass spectrometry where they undergo ionization and fragmentation in ion source region, mass separation in ion analyzer and

detection processes, respectively. For example, a mixture of three components got separated in GC followed by MS gave three mass spectra for each component (Fig. 5). GC and MS used together

to give better understanding of substance in identification and composition independently.

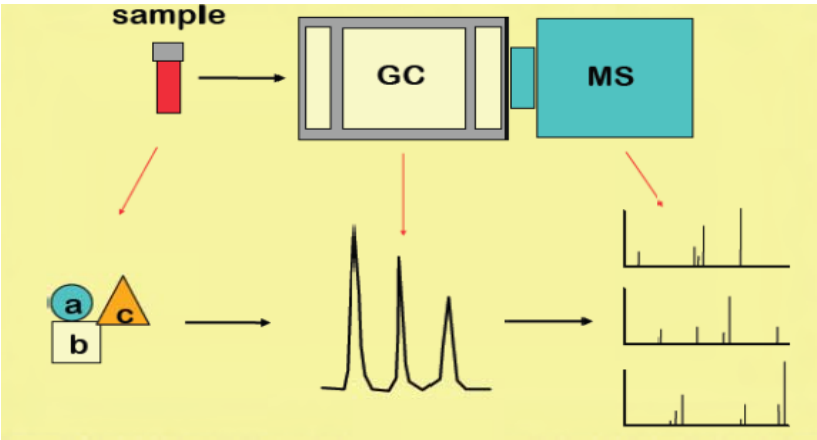

Fig. 5. GC and MS chromatograms

Applications

Water monitoring and analysis

GC-MS is highly sensitive analytical tool which can separate and quantify organic compounds at ppb level. Therefore, this can be used in water quality monitoring and assessment for the total organic carbons (TOCs) that include pesticides, endocrine disruptors, chlorofluoro-compounds, protein degradation and radioactive products for drinking water. Very low levels of total organic carbon are essential with a typical resistivity of 18.2 MΩ. cm, a very low TOC value of less than 2 ppb and bacteria levels below 0.1 CFU/ml – is highly recommended for ultra pure water which can be achieved with GC-MS analysis.

Environmental monitoring and clean-up

GC-MS is used as the tool of choice for tracking organic pollutants in the environment. There are some compounds for which GC-MS is not sufficiently sensitive, including certain pesticides and herbicides, but for most organic analysis of environmental samples, including many major classes of pesticides, it is very sensitive and effective.

Criminal forensics

GC-MS can analyze the particles from a human body in order to help link a criminal to a crime. The analysis of fire debris using GC-MS is well established. There is even an established American Society for Testing Materials (ASTM) standard for fire debris analysis. GCMS/MS is especially

useful here as samples often contain very complex matrices and results, used in court, need to be highly accurate.

Law enforcement

GC-MS is used for detection of illegal narcotics and supplant drug-sniffing dogs. It is also commonly used in forensic toxicology to find drugs and/or poisons in biological specimens of suspects, victims, or the deceased.

Sports anti-doping analysis

GC-MS is the main tool used in sports anti-doping laboratories to test athletes' urine samples for prohibited performance-enhancing drugs, for example anabolic steroids.

Food, beverage and perfume analysis

Foods and beverages contain numerous aromatic compounds, GC-MS is extensively used for the analysis of these compounds. It is also used to detect and measure contaminants from spoilage or adulteration which may be harmful for human,for example pesticides.

Astrochemistry

Several GC-MS have left earth. Two were brought to Mars by the Viking program. Venera 11 and 12 and Pioneer Venus analyzed the atmosphere of Venus with GC-MS. The Huygens probe of the Cassini-Huygens mission landed one GC-MS on Saturn's largest moon, Titan. The material in the comet 67P/Churyumov-Gerasimenko will be analyzed by the Rosetta mission with a chiral GC-MS in 2014.

Medicine

Dozens of congenital metabolic diseases also known as “In born” errors of metabolism are now detectable by gas chromatography–mass spectrometry. GC-MS can determine compounds in urine even in minor concentration. It is now possible to test a newborn for over 100 genetic metabolic disorders by a urine test at birth based on GC- MS and compared with healthy condition.

In combination with isotopic labeling of metabolic compounds, the GC-MS is used for determining metabolic activity. Most applications are based on the use of ^{13}C as the labeling and the measurement of ^{13}C-^{12}C ratios with an **isotope ratio mass spectrometer** (**IRMS**); an MS with a detector designed to measure a few select ions and return values as ratios.

High Performance Thin Layer Chromatography Mass Spectrometery (HPTLC-MS)

Introduction

Thin layer chromatography is the basic planar chromatography method is used for the separate the volatile and non-volatile substances. High performance thin layer chromatographic method was developed for estimation of crude drug, synthetic, and semi synthetic drug. Chromatographic technique is used to separate volatile as well as non-volatile compound (mixtures). This chromatography performed on the sheet of aluminum foil, glass or plastic which is coated with a thin layer of adsorbent material usually silica gel, cellulose and aluminum oxide. Adsorbent layer is called as a stationary phase (S.P.). Applied sample on the plate by injection, a solvent or solvent mixture (mobile phase) is drawn up the plate via capillary action. Because different compounds having different separation time. Also requirement of different mobile phase for different mixture or drug. Different rates of separation are archived by TLC technique.

Chromatographic technique can be used to monitor reaction, identify compounds present in a given mixture and determine the purity of a compound. Specific examples of these applications include analyzing crude drug, detection of impurities in compound, pesticides or insecticides from food, radiochemical purity from radiopharmaceuticals, medicinal constituents in the plants (herbal) and water, detection of the dye composition from fibers in forensics.

Separation of compounds depends on the competition of the compound (solute), mobile phase and stationary phase. silica gel is used in normal phase chromatography as a stationary phase it is polar in nature. Two or more than two elute present in the mixture which different polarity. Those more polar elute last and less polar elute first. The more polar compound has a stronger interaction with the silica and is therefore more capable to disappear the mobile phase from the binding places.

Less polar solvent or mobile phase gives higher R_f value. If the mobile phase is changed to a more polar solvent or mixture of solvents, it is more capable of disappearing solutes from the silica binding places and all compounds on the TLC plate will move higher up the plate and R_f value not more than one. It is commonly said that "strong" solvents (eluents) push the analyzed compounds up the plate, while "weak" eluents only move them.

Basic principle of HPTLC

Similar to other chromatographic methods, thin layer chromatography is also based on the principle of separation.

i. The separation depends on the relative affinity of compounds towards stationary and the mobile phase.

ii. The compounds under the influence of the mobile phase (driven by capillary action) travel over the surface of the stationary phase. During this movement, the compounds with higher affinity to stationary phase travel slowly while the others travel faster. Thus, separation of components in the mixture is achieved.

iii. Once separation occurs, the individual components are visualized as spots at a respective level of travel on the plate. Their nature or character are identified by means of suitable detection techniques.

Table 1: Trouble Shooting In HPTLC

Sr. No.	Cause	Remedy
1.	Poor band quality (Linomat applicator) Gas flow not optimal. Wrong distance between needle tip/ TLC layer Damaged or clogged needle.	a) Check gas pressure and adjust to 2-3 bars. b) Check distance and adjust to 1 mm. c) Remove syringe and fill it with solvent. Force out by hand.
2.	Bad reproducibility	Limit switch for sample dosage syringe misadjusted.
3.	Poor accuracy (linomat applicator). a) Damaged spray head due to wrong sample dosage syringe type. b) Leaky syringe because of destroyed glass barrel.	a) Replace the spray head. b) It happen because of high pressure, replace the sample dosage syringe.
4.	Poor band quality a) Check gas supply for spraying. b) Check nozzle for clogs. c) Check distance capillary/nozzle and	a) Check the regulating gas flow. b) Check cleaning the spray nozzle. c) Check adjusting the capillary

	capillary/object. d) Application position is overloaded with sample.	d) Dissolve sample in more suitable solvent.
5.	Leakage a) If air bubbles are formed, when the syringe piston has a leak. b) Another reason for leaks could be caused by the O-ring gasket in the connection between syringe and capillary.	Check and replacing the o-ring (gasket) as well as syringe piston.

Interfaces

High performance thin layer chromatography (Camag, Muttanz, Switzerland) consisted of an auto sample applicator Linomat V connected to a nitrogen cylinder, TLC scanner attached to a PC running win-CATS software (version 1.4.4), TLC Visualizer, and Camag twin-trough chambers were used in analysis. HPTLC-MS detailed examination was carried out by TLC-MS interface using acetonitrile as eluting agent at a flow rate of 1 ml/min. It remove/take out circular zones in the form of bands from the developed HPTLC plate. The eluted material was transferred automatically to single-quadrupole mass spectrometer, and mass spectra was recorded. Surveys have shown that not all samples may be processed by HPTLC-MS or HPTLC-DAD or HPTLC-MALDI or low delectability of the compounds or impurities in the UV range, a heavy matrix load or a deficiency of MS compatible solvents. On the other hand HPTLC is another very fast and suitable method to separate samples. In the past unknown substances were scraped off from the TLC/HPTLC plate, eluted into a tube with solvent and transferred into the MS. Now a very suitable and universal TLC-MS Interface is available which can semi-automatically extract zones of interest and direct them online into any brand of HPLC-MS system. The interface is fast and simply connected (by two fittings) to any LC-coupled mass spectrometer without adjustments or mass spectrometer modifications. Questioned materials are directly extracted from a TLC/HPTLC plate and sensitive mass spectrometric signals are obtained within a minute per substance zone. The interface extracts the complete material zone with its depth profile and thus allows detections

comparable to HPLC down to the pg/zone range. The interface has been demonstrate to be one of the most reliable and versatile interfaces for TLC/HPTLC-MS coupling.

Principle of HPTLC-MS

The versatile instrument is used to isolate unknown compounds from a HPTLC/TLC plate and transfer them into a mass spectrometer for identification or structure elucidation. TLC/MS Interface can be bring together to any brand of LC-coupled mass spectrometer. Pluga nd play installation by two HPLC fittings at a given HPLC-MS system Semi-automatic instrument involving automatic piston movement for pressure seal the HPTLC/TLC zone on both glass plates and aluminum foils take out directly from the plate using a suitable solvent delivered by the HPLC pump Online transfer into the mass spectrometer. Automatic cleaning of the piston between the extractions.

Key features

- Rapid and contamination-free elution of selected zones.
- Online transfer into the mass spectrometer.
- Compatible with all conventional HPLC-MS systems.
- Identification of unknown substances at a limit of detection as known from HPLC-MS.
- Low solvent consumption

Instrumentation of HPTLC-MS

Materials:

Plates or aluminum foils up to 20 x 20 cm can be positioned precisely and analyzed zone by zone.

Automation:

Semi-automatic instrument including automatic piston movement, automatic cleaning of the piston, manual positioning and switching.

Weight:around 11 kg

Size:around 23 x 50 x 25 cm with optional size

large table is 40 x 50 x 25 cm (w x d x h)

Requirements: 5 bar compressed air or N2, HPLC pump or HPLC-MS system.

Structure identification and elucidation

Following proper isolation using proper chromatographic technique, the next step is the characterization and elucidation of the unknown isolated bioactive materials. Typically, hyphenation is selected to provide the most relevant information as mandatory. In this case spectra can be recorded directly from zones of interest on an HPTLC plate through the flexible TLC-MS interface. Nuclear Magnetic Resonance (NMR) has been connected with several bio autography. Even on analytical range, it has been applied for structure confirmation. Diffuse Reflectance Infrared Fourier Transform (DRIFT) spectroscopy is also a well-known method for characterization. It has discovered some application in natural product research and has been attached with chromatography. Surface Enhanced Raman Spectroscopy (SERS) has also been connected with chromatography and could find some applications in natural product research. UV/VIS/FLD spectrometry connected with chromatography is also a useful method in bioactive materials identification. It is usually coupled with planar chromatography from which images can be grabs directly on the plate. Most of these experiments can be calculated directly from analytical plates. Important samples are introduced directed to mass spectrometer (MS) or high-resolution mass spectrometer (HRMS) for structure confirmation.

Applications

1. HPTLC-MS method can be used for the routine analysis of pharmaceutical product/formulation.
2. It play important role in the study of the drug metabolism, discovery of new drug candidate and the analysis, identification and characterization of impurities and degraded products in drug substances.
3. HPTLC-MS have proved to be an extremely sensitive and specific technique for the analysis of pharmaceutical products

IMPORTANT QUESTIONS

Long answer type Questions (10 Marks)

1. Write in detail working of LC-MS
2. Explain working of HPTLC-MS
3. Write in detail working of GC-MS
4. Explain instrumentation of GC-MS

Short answer type questions (5Marks)

1. Draw a well labeled diagram of GC-MS
2. Explain in detail the interfaces used in HPLC-MS
3. Give advantages of Hyphenated techniques.
4. Draw a well labeled diagram of LC-MS
5. Explain in detail the interfaces used in HPTLC-MS

Very Short answer type questions (2 Marks)

1. What is meant by hyphenated techniques?
2. Enlist interfaces used in GC-MS
3. Enlist the interfaces used in LC-MS
4. Enlist the interphases used in HPTLC-MS
5. Types of analyzers used in GC-MS
6. Principle of HPLC-MS.