

An Overview of Liquid Chromatography-Mass Spectroscopy Instrumentation

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ABSTRACT

Chromatography is a separation technique used to separate the individual compound from a mixture using a stationary and mobile phase. Discovery of chromatography is a milestone event in biomedical research. Chromatographic separation is based on the principles of adsorption, partition, ion exchange, molecular exclusion, affinity and Chirality. There are many types of chromatography available for quantitative and qualitative analysis of pharmaceutical agents, which includes Liquid Chromatography-Mass Spectrometry (LC-MS). Combination of chromatography with spectrometry is first reported in 1967 and first LC-MS system was introduced in 1980s. LC-MS is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography with the mass analysis and mass spectrometry. Mainly the LC-MS contains liquid chromatography assembly, ion generation unit/ ionization source, mass analyzer and mass spectrometric data acquisition. LC-MS is most commonly used in biomedical sciences for pharmacokinetic analysis, genetic analysis, structural elucidation, etc. The main objective of this review is to overview the principle, instrumentation and application of LC-MS.

Key words: Analysis, Chromatography, HPLC-MS, LC-MS.

Chromatography

Chromatography is a separation technique to separate the individual compound from a mixture using a stationary and mobile phase. The chromatography word obtained from Greek, 'chroma' means colour and 'graphein' mean writing, hence the word chromatography means 'colour writing'.^{1,2} Chromatography was first developed by the Russian botanist 'Mikhail Tswett' in 1903; he separated coloured plant pigments through calcium carbonate column.^{3,4}

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DOI: 10.5530/phm.2014.2.2

Types of chromatography

1. Based upon the nature of stationary and mobile phase⁵
 - Gas-solid chromatography
 - Gas-liquid chromatography
 - Solid-liquid chromatography (column chromatography, Thin Layer Chromatography [TLC], High-Performance Liquid Chromatography [HPLC], Liquid Chromatography-Mass Spectrometry [LC-MS])
 - Liquid-liquid chromatography (paper partition chromatography, column chromatography)
2. Based on the principles of separation and type of chromatographic method
 - Adsorption chromatography: The mobile (liquid

or gaseous) phase is adsorbed into the surface of a stationary solid phase. The separation of the compound is based on affinity towards stationary phase. The compounds which have more affinity with stationary phase will be eluted slowly and compounds with less affinity with stationary phase will be eluted fast. Eg. Column chromatography, TLC, HPLC and LC-MS.⁶

- Partition chromatography: Separation of compounds is based on partition of a solute between two solvents.⁷ In this form of chromatography a liquid stationary phase, which is immiscible with the mobile phase, is adsorbed to the surface of the solid adsorbent. Chromatography is carried out as described for adsorption column chromatography. Partition of component of sample between the mobile phase (sample) and stationary phase (liquid on solid support) retards the elution of some components of sample which gives the basis for separation.
- Ion exchange chromatography: The principle involved for this method is reversible exchange of ions, between the ions present in the solution (mobile phase) and ion exchange resin (stationary solid phase). This method can be further classified as cationic exchange chromatography and anionic exchange chromatography.⁸
- Molecular exclusion chromatography: It's otherwise called as gel permeation or gel filtration. This method is used to separate the proteins, peptides and oligonucleotides on the basis of size. The column is packed with inert porous spheres (column media). When a mixture of different sized molecules passed through the column, the smaller molecules will enter the pores of the spheres (column media) and will take more time for elution. On the other hand, the larger molecules could not enter the pores of the spheres and will be eluted faster.⁹

- Affinity chromatography: This method is most selective and used to separate the antibodies, proteins and enzymes from the biological matrix. It is based on biological interactions between two molecules, like enzyme and substrate, receptor and ligand, or antibody and antigen. When a mobile phase containing mixture of proteins/antibodies/enzymes are passed through the stationary phase, only the specific protein binds to its respective ligand in the stationary phase. This protein later can be extracted by changing the ionic strength or pH.¹⁰

- Chiral chromatography: This kind of chromatography used to separate optical isomers (levo and dextro forms) of the molecules.¹¹

3. Based on different type of analytical method

- Capillary electrophoresis
- Chromatography with conventional detectors
- Gas chromatography (GC)
- Liquid chromatography
- Super critical fluid chromatography
- Hyphenated techniques (Mass-spectrometry)
- GC-MS and GC-MS/MS
- LC-MS and LC-MS/MS
- Supercritical Fluid Chromatography-MS (SFC-MS), Capillary Zone Electrophoresis-MS (CZE-MS)

Liquid Chromatography-Mass Spectrometry

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 includes High

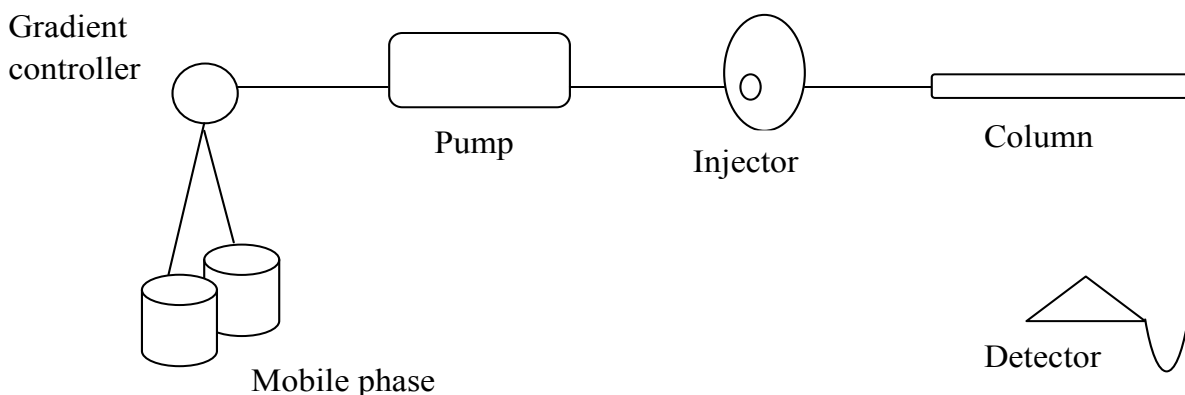


Figure 1: Instrumentation of HPLC

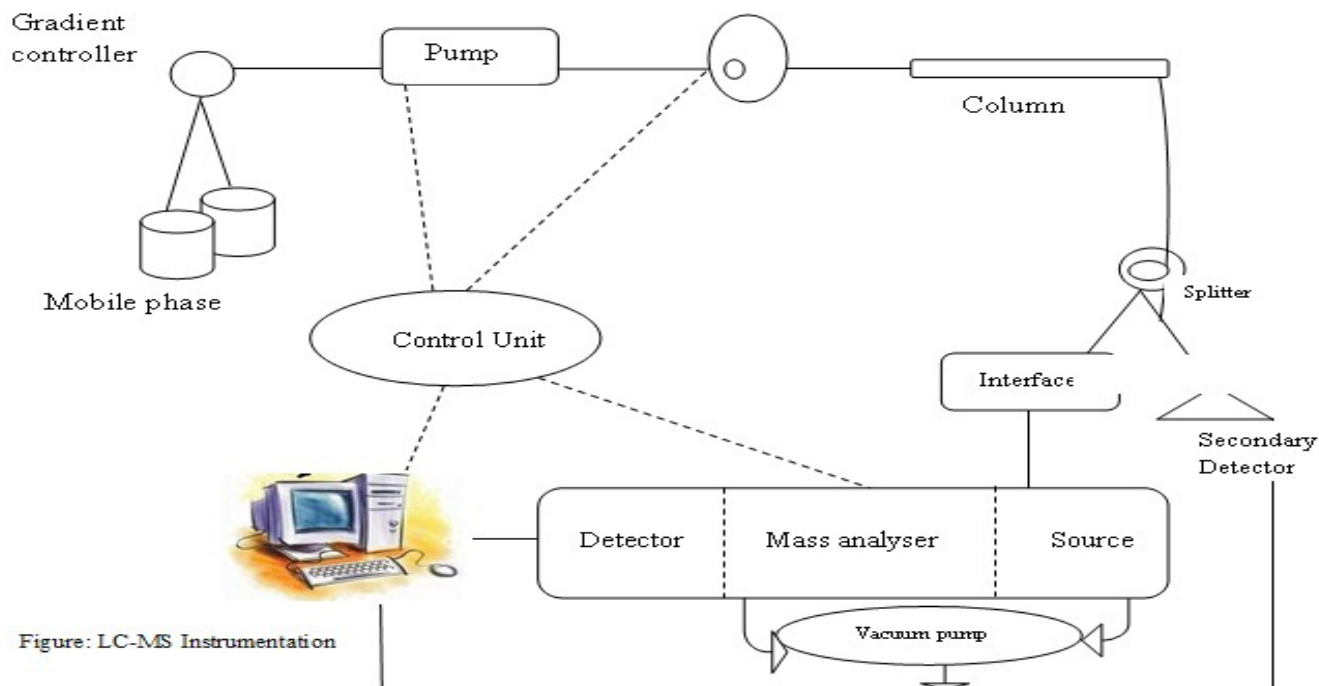


Figure: LC-MS Instrumentation

Figure 2: Instrumentation of LC-MS

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 1967.¹² 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.¹³

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 or isotopic nature of a sample, the masses of particles and of molecules, and to elucidate the chemical structures of molecules.^{14,15}

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 phase columns 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 and integrators (Figure 1).

Detector used in HPLC

1. UV detector
 - Single wavelength (filter)
 - Variable wavelength (monochromator)
 - Multiple wavelengths (Photodiode array detector [PDA])
2. Florescence

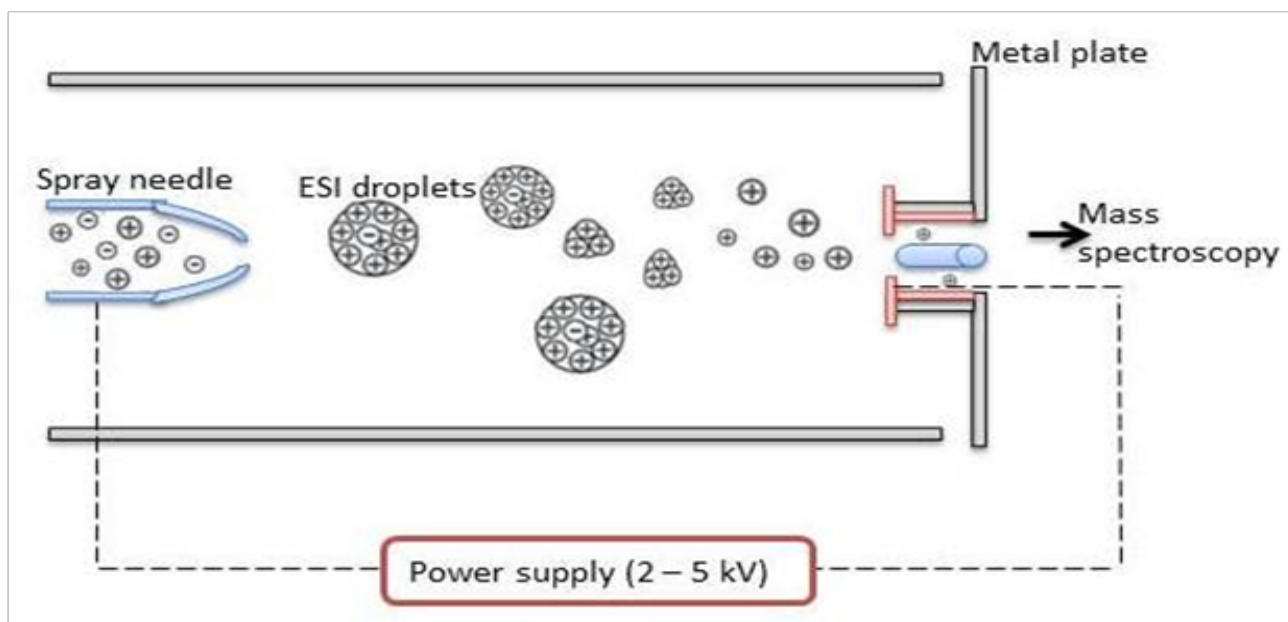


Figure 3: Electrospray ionization

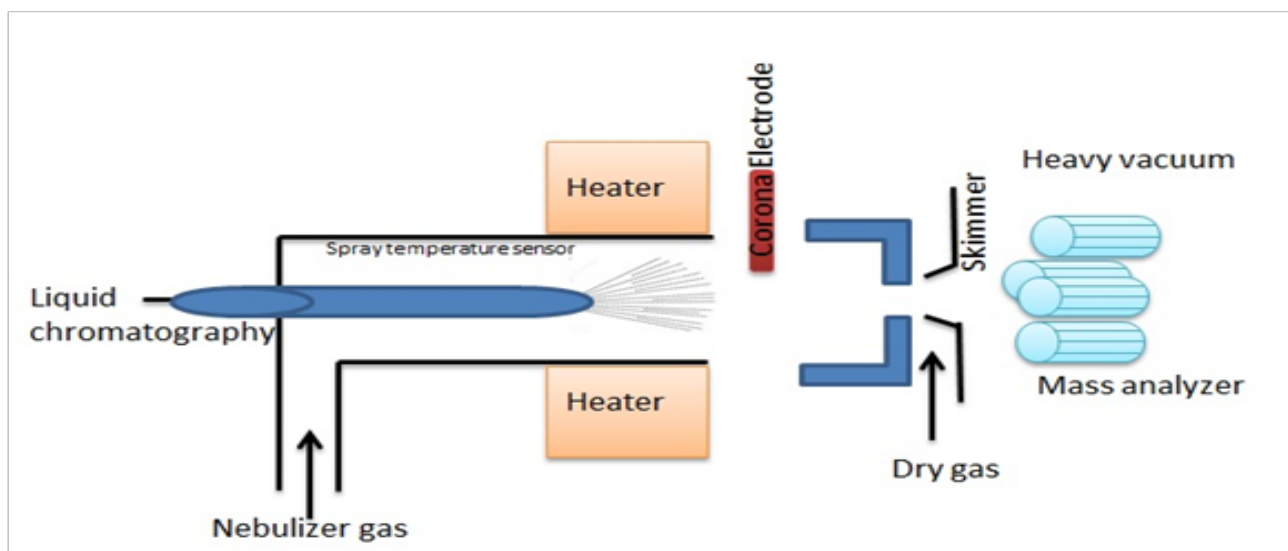


Figure 4: Atmospheric pressure chemical ionization

3. Electrochemical detector

4. Mass spectrometric

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 source

Most common ionization sources are Electrospray ionization (ESI), Atmospheric pressure chemical ionization (APCI) and Matrix-assisted laser desorption/ionization (MALDI). A part from this Electron impact (EI) and Chemical ionization (CI) or negative chemical ionization are also used as ionization source in MS.¹⁸

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

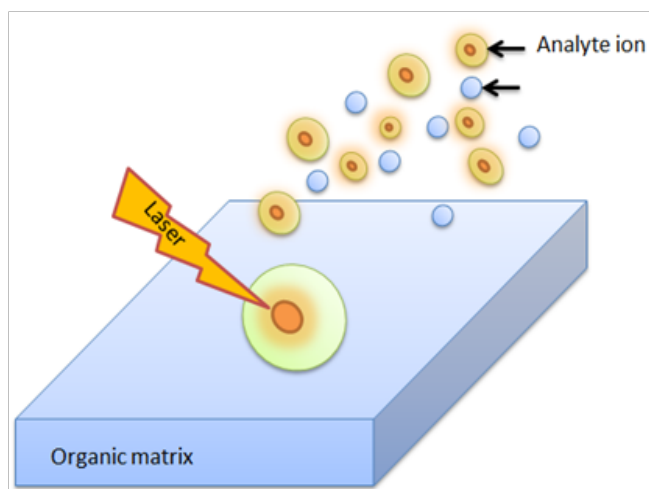


Figure 5: Matrix-assisted laser desorption/ionization (MALDI) evaporation. Increase in charge density resulting from solvent evaporation causes coulombic repulsion to overcome the liquid surface tension, resulting in release of ions from droplets. This is the principle of ion formation using this technique.

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 fragmentation and source is instable (Figure 3).^{19,20}

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.^{21,22}

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 matrix in vacuum to 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).

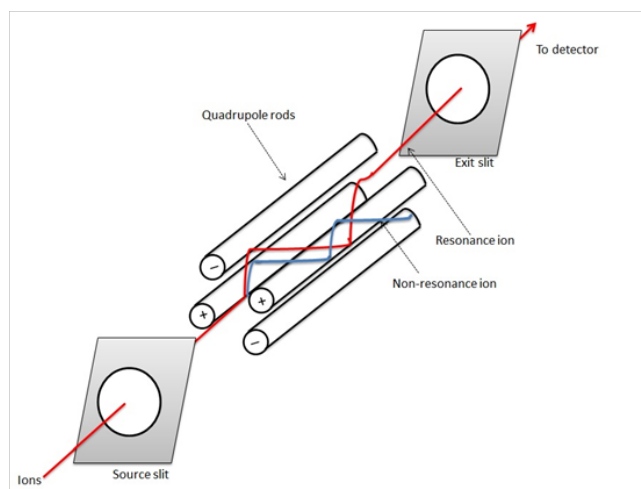


Figure 6: Quadrupole

- Upon impact of laser, Quasithermal evaporation occurs which results from increased molecular motion.
- This causes expulsion of upper lattice layers of matrix (desorption)
- The matrix is then thought to transfer protons to the sample 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 aqueous TFA (solvent/ trifluoroacetic acid), sample and matrix (Figure 5).²³⁻²⁵

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 of the 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.

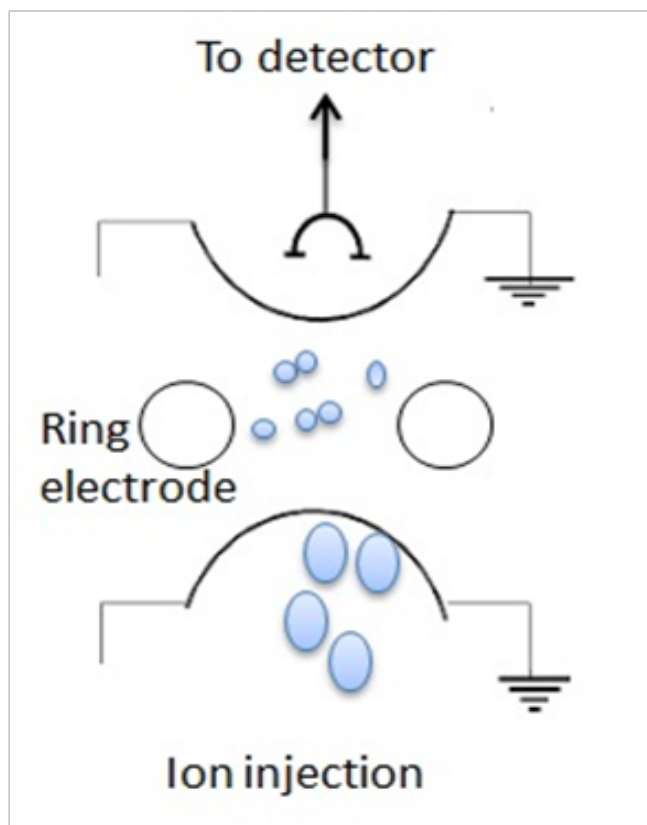


Figure 7: Ion trap analyzer

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 of m/z -values by continuously varying the applied voltage.^{26,27}

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. Non selected 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.^{28,29}

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).³⁰

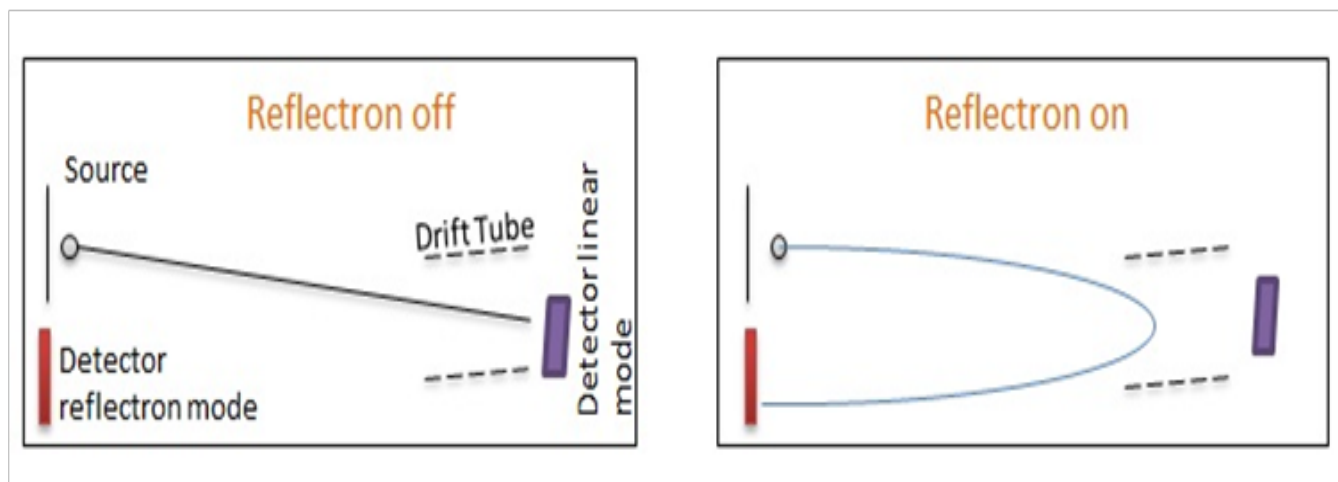


Figure 8: Time of Flight (TOF) analyzer

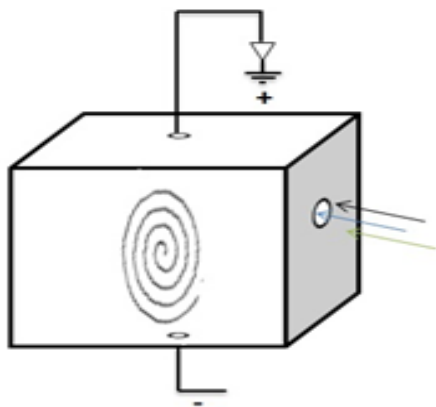


Figure 9: Fourier transform-ion cyclotron resonance (FT-ICR) Principle of the time of flight analyzer is formation of ions from ion source and it will be accelerated to high velocity by an electric field present in the ‘drift tube’ of the instrument. The accelerated ions will be detected by detector by linear mode 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): Ions entering 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-charge ratios. 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).^{31,32}

Detectors

Three different kinds of detectors are used in Mass Spectrometry, i.e. Electron multipliers (Figure 10a), Dynolyte photomultiplier (Figure 10b) and Micro channel

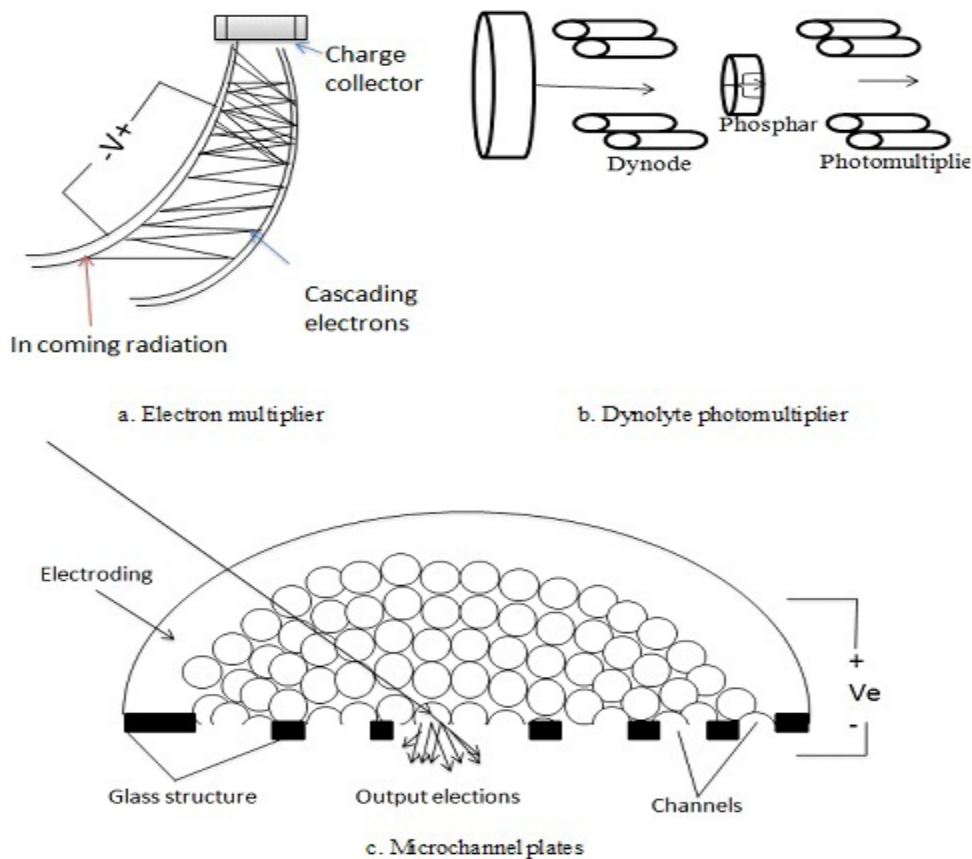


Figure 10: Detectors used in LC-MS

plates (Figure 10 c).³³ 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).

Mass spectrometric data acquisition system

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

- Full mass spectra (library to solve the analytical problem)
- Raw data of a small range (determine of isotope pattern/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.

Applications of LC-MS

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

and qualitative analysis.³⁴⁻³⁶ Applications of LS-MSMS are as follows.

- *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.

CONCLUSION

The advancement of hyphenated techniques, high resolution mass analyzers as well as high throughput separation approaches, quantitative and quantitative analysis of pharmaceutical drugs and metabolites can be achieved with good sensitivity.

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