Paper 9: TECHNIQUES USED IN MOLECULAR BIOPHYSICS-I

Module -27: Instrumentation in Mass Spectrometer

Ionization methods, mass separation using quadruples, detectors and integrators in mass spectroscopy

Dr.T.Velpandian,
Professor,
High Precision Bio-Analytical Laboratory,
Ocualr Pharmacology and Pharmacy Division,
Dr.RPCentre, AIIMS, New Delhi

Electron

"At first there were very few who believed in the existence of these bodies smaller than atoms. I was even told long afterwards by a distinguished physicist who had been present at my [1897] lecture at the Royal institution that he thought I had been pulling their legs"
(J J Thmpson)

1. Introduction

Mass Spectrometry is a powerful technique for identifying unknowns, studying molecular structure, and probing the fundamental principles of chemistry. Mass spectrometry is essentially a technique for "weighing" molecules. Mass spectrometry is based upon the motion of a charged particle, called an ion, in an electric or magnetic field. The mass to charge ratio (m/z) of the ion affects this motion. Since the charge of an electron is known, the mass to charge ratio represents a measurement of an ion's mass. Typical mass spectrometry research focuses on the formation of gas phase ions, the chemistry of ions, and applications of mass spectrometry.

2. Objective

This chapter explains you further detail about sample introduction methods, ionization methods, mass separation methods, detectors and integrators in mass spectroscopy.

Sample source

In the previous generation gas chromatography coupled mass spectrometers; the molecule was introduced in the gas phase which was easy to undergo electron impact ionization in the electron gun. Whereas the tremendous developments in liquid chromatography techniques like HPLC showed the versatile, safe, easy utility of diverse compounds in a single run. Therefore, analysts had a problem of coupling HPLC's mobile phase where the ions in liquid state to be introduced into the quadrupole analyzers of the LC-MS/MS. Thus sample introduction was an early challenge in mass spectrometry during the transformation. Therefore, first the sample is introduced at a normal atmospheric pressure (760 torr), ionized and the ionized species are introduced while preserving the vacuum at ~10-6 torr. It is either achieved by probes like Electron Spray Ionization (ESI) or Atmospheric Pressure Chemical Ionisation (APCI) or Atmospheric Pressure Photo-Ionization (APPI). Apart from this, the direct sample modes like MALDI are seldom used for ionization with the help of LASER. Whereas in the instruments like GS-MS electron impact ionization, fast atomic bombardment ionization techniques are used. In the soft ionization process like ESI, the molecule subjected to get charged in a suitable atmosphere (with the pH of the mobile phase along with the applied electric potential) without making any structural deformity. Whereas in the electron impact ionization process the molecule is fragmented into ionic form before gets subjected for the analysis using mass analyzer. Therefore, soft ionization process gained much of importance for quantification as well as structural elucidation process of proteins and other molecules.

Extensive information regarding liquid chromatography techniques is beyond the scope of this chapter as it has been dealt separately. Liquid chromatography inlets are used to

introduce thermally labile compounds not easily separated by gas chromatography. These inlets have undergone considerable development and are now fairly used in routine. Because these inlets are used for temperature sensitive compounds, the sample is ionized directly from the condensed phase. This section focuses on ionization techniques, quadrupole separation, concept of MRM and detector used in triple quadrupole tandem mass spectrometers.

Ionization Source:

A variety of ionization techniques are used for mass spectrometry. Most ionization techniques excite the neutral analyte molecule which then ejects an electron to form a radical cation (M). Other ionization techniques involve ion molecule reactions that produce adduct +1 ions $(M+H^+)^+$. The most important considerations are the physical state of the analyte and the +1 ionization energy. Electron ionization and chemical ionization are only suitable for gas phase ionization. Fast atom bombardment, secondary ion mass spectrometry, electrospray, and matrix assisted laser desorption are used to ionize condensed phase samples. Ionization source is required to vaporize the molecule into gaseous state and charging it by removing or giving the charge to the molecule. There are different type of ionization sources are available which help in this process. Following are the different type of ionizing probe. Some ionization techniques are very soft and only produce molecular ions, other techniques are very energetic and cause ions to undergo extensive fragmentation. Although this fragmentation complicates the mass spectrum, it provides structural information for the identification of unknown compounds.

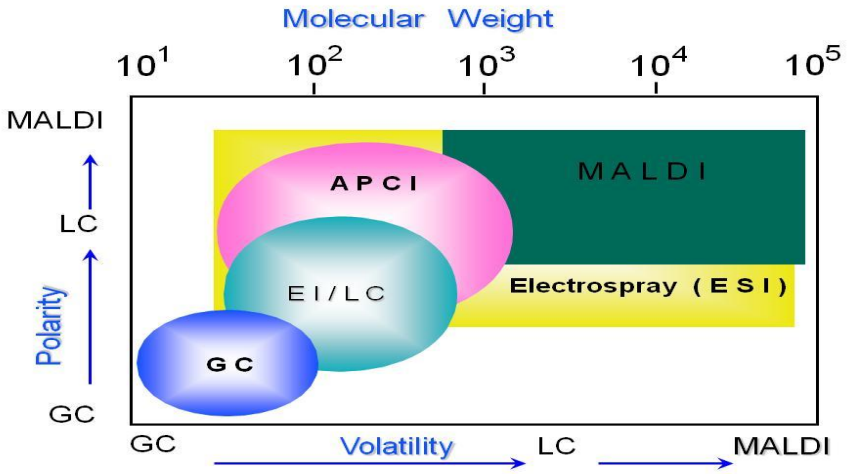
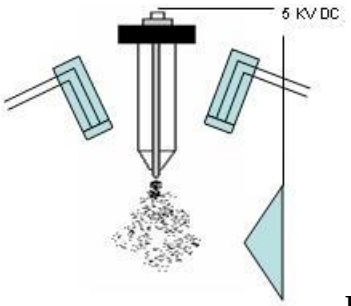


Fig.1 The above diagram shows the choice of ionization probes according to the polarity and molecular weight of compounds.

1) Electro Spray Ionisation (ESI)

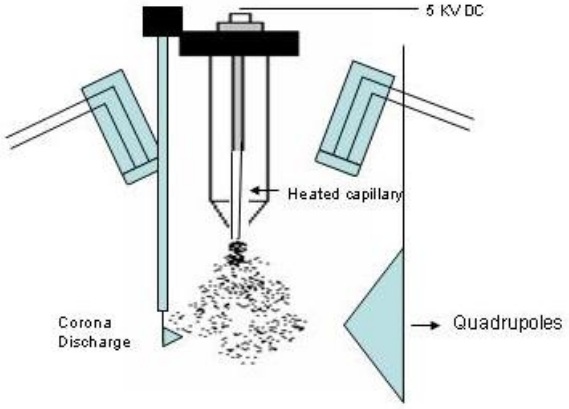


ESI Probe (Fig.2)

In ESI probe to enhance ionization process often mild acid conditions are used with the help of weak acids like formic, acetic acids etc. ESI probe ionization can be operated at positive and negative polarities to add a proton (adding a hydrogen) or removal of the proton (removal of the hydrogen) respectively. With the pressure of solvents from HPLC as well as source gas pressure the particles produced in a normal ESI probe would be having approximately 1µm in diameter where as in nanospray techniques, the diameter may be reduced further to 100-200nm.

Under a high electrical field, conducting fluid is getting automized each droplet leaves the needle with an electrical gradient in the shape of "Taylor Cone". The tip of the Taylor cone protrudes as a filament until the liquid reaches the Rayleigh limit where the surface tension and electrostatic repulsion are equal and the highly charged droplets are attracted to the entrance of the mass spectrometer due to the high opposite voltage at the mass analyzer's entrance. As the droplet moves towards the analyzers, the coulombic repulsion on the surface exceeds the surface tension; the droplet explodes into smaller droplets ultimately releasing ions (Fig. 2). Typically a suitable temperature gas flow in the probe (based on the type of solvent mixture used) enhances the evaporation of solvent droplets containing the analyte of interest to increase charge overcrowding. This evaporation causes shrinkage of the droplet size leading to coulombic explosion where the molecule of interest exists in ionized form and attracted towards the mass analyzer. This method is also used for ionizing proteins, peptides and thermo-labile compounds without thermal degradation as the evaporating solvent decreases the temperature reaching around the molecule. However, the process of gas as well as temperature parameters needs to be optimized for each method. This process is suitable for molecules having higher polarity. This method is also highly useful in understanding the exact molecular weight of peptides due to their nature of having multiply charged.

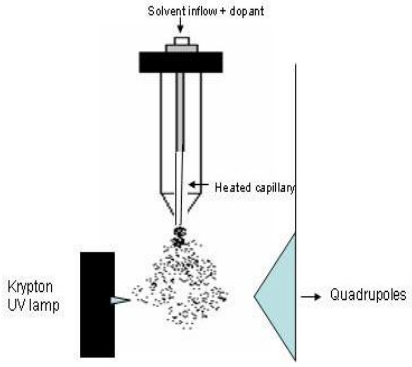
2) Atmospheric Pressure Chemical Ionization (APCI)



APCI Probe (Fig.3)

The sprayer probe consists of 100 µm (0.004") ID stainless steel tubing surrounded by a flow of nebulizer gas (Fig.3). The liquid sample flow is pumped through the sprayer where it is nebulized into a quartz tube surrounded by a heater. The inner wall of the quartz tube is maintained at a temperature of about 100–250 °C. When the liquid sample is pumped into the quartz tube, the sample and solvent are vaporized and when the heated vapor reaches down a corona discharge needle pointing towards the gas flow causes proton transfer. Since the solvent ions are present at atmospheric pressure conditions, chemical ionization of analyte molecules is very efficient; at atmospheric pressure analyte molecules collide with the reagent ions frequently. Proton transfer (for protonation MH^+ reactions) occurs in the positive mode, and either electron transfer or proton loss, ($[M-H]^-$) in the negative mode. The moderating influence of the solvent clusters on the reagent ions, and of the high gas pressure, reduces fragmentation during ionization and results in primarily intact molecular ions. This method is used for compounds which are moderately polar having ionization problem in ESI mode.

3) **Atmospheric Pressure Photo Ionization (APPI)**



APPI Probe (Fig. 4)

This method uses the similar set up of APPI where the corona needle is replaced Krypton ultraviolet (UV) lamp. The high energy UV radiation (a typical krypton light source emits at 10.0 eV and 10.6 eV) causes the DuPont to exit and transfer the charge to the adjacent

analyte (Fig.4). APPI is much more sensitive than ESI or APCI and has been shown to have higher signal-to-noise ratios because of lower background ionization.

5) Matrix Assisted Laser Desorption/Ionization

Matrix Assisted Laser Desorption/Ionization (MALDI) is used to analyze extremely large molecules. This technique directly ionizes and vaporizes the analyte from the condensed phase. MALDI is often used for the analysis of synthetic and natural polymers, proteins, and peptides. Analysis of compounds with molecular weights up to 200,000 dalton is possible and this high mass limit is continually increasing. In MALDI, both desorption and ionization are induced by a single laser pulse. The sample is prepared by mixing the analyte and a matrix compound (cinnamic acid derivatives) chosen to absorb the laser in ultraviolet wavelength. This is placed on a probe tip and dried. A vacuum lock is used to insert the probe into the source region of the mass spectrometer. A laser beam is then focused on this dried mixture and the energy from a laser pulse is absorbed by the matrix. This energy ejects analyte ions from the surface so that a mass spectrum is acquired for each laser pulse. However, this ionization probe is only in MALDI TOF instruments and doest have provision to handle liquid analytes.

Curtain gas, curtain plate and suction assembly for sample introduction

The curtain gas is pure nitrogen which is allowed to pass through the curtain plate. While the ions enter into the mass analyzer, the curtain gas flows against the entry of ionized species. This process reduces the inlet of uncharged solvent molecules to reduce the background noise in the mass spectrum. As in most of the applications HPLC solvents are pumped at the rate varying form 0.4-1ml/minute this process is essential to avoid solvent induced background noise. The curtain plate having a central hole is directed to the ion guide where the ions are accelerated and focused towards the Q1 mass analyzer quadrupole.

In the ionizing probe, bottom of the probe is usually connected with a vacuum pump to remove the vapors of the mobile phase immediately to maintain the ionization head at normal atmospheric pressure. It is usually achieved by using vacuum devices which are working with Bernale's principle where a compressed air is allowed to create vacuum.
Separation based on m/z (Quadrupole-1)

Mass Analyzers:

Analyzers are typically described as either continuous or pulsed. Continuous analyzers include quadrupole filters and magnetic sectors. These analyzers are similar to a filter or monochromator used for optical spectroscopy. They transmit a single selected m/z to the detector and the mass spectrum is obtained by scanning the analyzer so that different mass to charge ratio ions are detected. While a certain m/z is selected, any ions at other m/z ratios are lost, reducing the S/N for continuous analyzers. Single Ion Monitoring (SIM) enhances the S/N by setting the mass spectrometer at the m/z for an ion of interest. Since the instrument is not scanned the S/N improves, but any information about other ions is lost. Pulsed mass analyzers are the other major class of mass analyzer.

1) Quadruple:
The attachment of more than one mass analysers in series is called tandem mass spectrometry. The quadrupole mass spectrometer is the most common mass analyzer. Its

compact size, fast scan rate, high transmission efficiency, and modest vacuum requirements * are ideal for small inexpensive instruments. Most quadrupole instruments are limited to unit m/z resolution and have a mass range of m/z 1000. Many bench top instruments have a mass range of m/z 500 but research instruments are available with mass range up to m/z 4000. Typically, in LC-MS/MS one mass analyzer first separates a particular molecular weight molecule in the vacuum using its mass to charge ratio. This is typically by applying radio frequency and high power direct current applied alternatively between two pairs of quadrupoles. The charged molecule generated by the ionization technique is brought into the quadrupoles where the separation is typically based on the type of its resonance between radio frequency and high volt DC current and given momentum in circular path makes the charged molecular guided towards the second quadrupole.

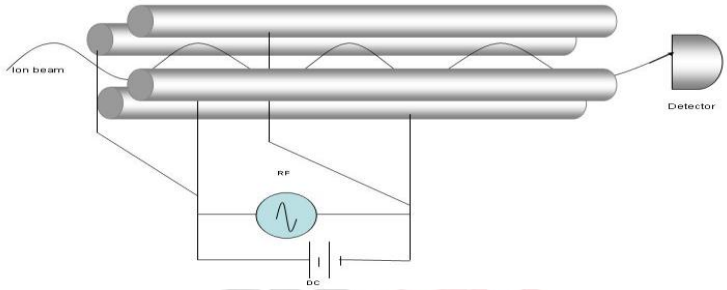


Fig. 5

Tandem mass spectroscopy

Tandem mass spectrometry enables a variety of experimental sequences. Many commercial mass spectrometers are designed to expedite the execution of such routine sequences as single reaction monitoring (SRM), multiple reaction monitoring (MRM), and precursor ion scan. In SRM, the first analyzer allows only a single mass through and the second analyzer monitors for a single user defined fragment ion. MRM allows for multiple user defined fragment ions. SRM and MRM are most often used with scanning instruments where the second mass analysis event is duty cycle limited. These experiments are used to increase specificity of detection of known molecules, notably in pharmacokinetic studies. Precursor ion scan refers to monitoring for a specific loss from the precursor ion. The first and second mass analyzers scan across the spectrum as partitioned by a user defined *m/z* value. This experiment is used to detect specific motifs within unknown molecules.

Let us see the sequence of events in the typical tandem mass spectroscopy

Separation of compounds (Quadrupole 1)

This quadrupole is set for a desired value of mass (which has been set according to the reported literature or decided as a result of mass experiments in tuning mode). Therefore, this quadrupole is set to transmit only the molecular ions having desired molecular weight towards the quadrupole-2 which otherwise called as Collision cell.

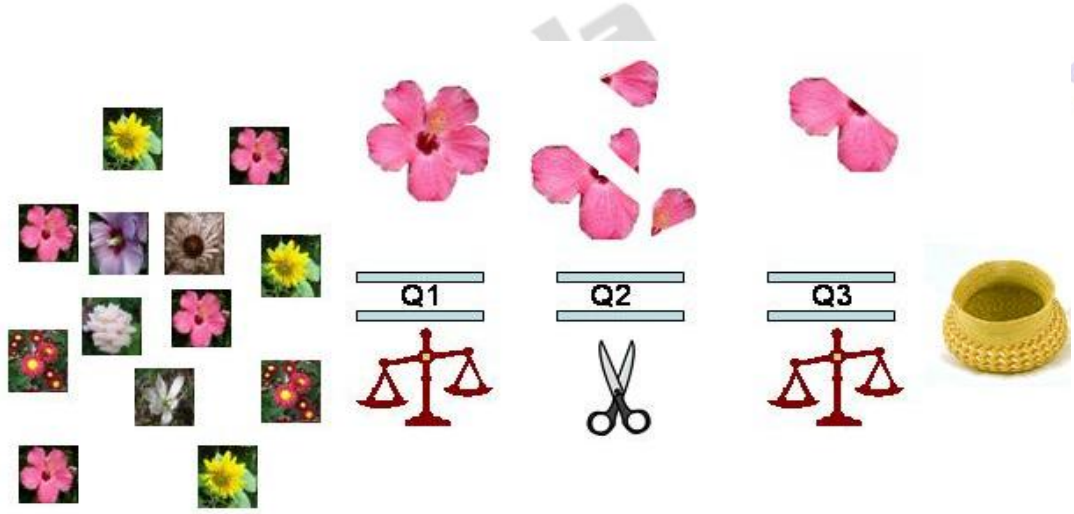
Fragmentation using neutral gas molecules (Quadrupole-2)

The second quadrupole collects the molecule and makes it to collide with neutral gas molecular like argon or nitrogen where the chemically weaker bonds like C→ N or C→O bonds are broken and give fragmented ions called daughter ions. This process is called CID

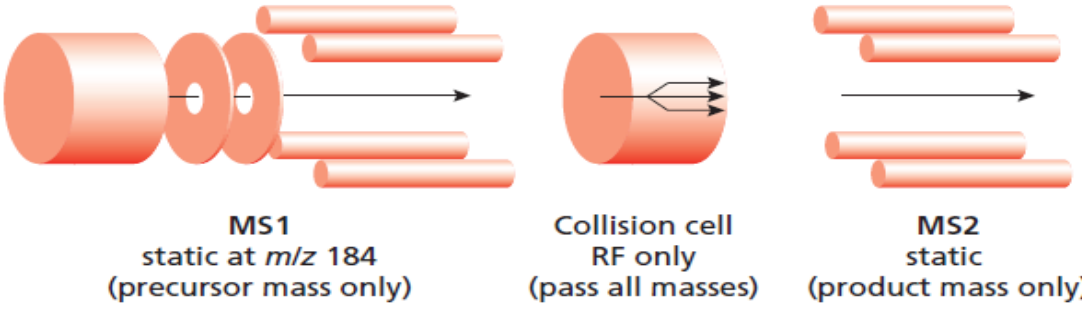
(Collision –Induced-Dissociation). This process is enabled by using collision potentials set in the rods along with RF and DC voltages for a preset set time usually in nanoseconds. CID is popularly used in most of the modern commercial mass spectrometers. However, there are many other methods available for fragmenting the molecules viz. electron capture dissociation (ECD), electron transfer dissociation (ETD), infrared multi photon dissociation (IRMPD) and blackbody infrared radioactive dissociation (BIRD). An important application using tandem mass spectrometry is in protein or molecular identification.

How does this process work?

In this bunch of flowers you wish to select a particular flower. However, in its weight there could be many therefore fragmentation helps to double confirm the particular species only. The simple (artistic view of the process) and real working process are depicted in the Figures



An artist's view of the principle involved in Tandem mass spectroscopy **(Fig. 6)**



The realistic view of the working principle in Tandem mass spectroscopy **(Fig. 7)**

Separation of the fragmented ion (Quadrupole-3)

The third quadrupole separates a particular broken piece of fragmented ion to guide towards detector having opposite charge.

In some of the mass spectrometers specially designed Trap functions are used to do fragmentation state-by-stage for structure elucidation and to study metabolism of xenobiotics.

2) Time-of-Flight type separators

The time-of-flight mass analyzer separates ions in time as they travel down a flight tube. This is a very simple mass spectrometer that uses fixed voltages and does not require a magnetic field. The greatest drawback is that TOF instruments have poor mass resolution, usually less than 500. These instruments have high transmission efficiency; no upper m/z limit, very low detection limits, and fast scan rates. For some applications these advantages outweigh the low resolution.

Detectors used in mass spectrometers

Various detectors are used for capturing the ionized species in the mass spectrometer. Phosphor coupled Photomultiplier, Electron multiplier, multichannel plate detectors. Solid state detectors utilize dynodes to convert into ion signal secondary to the amplification. Many detectors uses an additional ion acceleration step which is called postaccelation prior to detection. This step increases ion velocity and improves sensitivity.

1) Electron Multiplier:

Electron multipliers provide signal detection through ion conversion and subsequent amplification. When an ion impacts on the conversion dynode, it converts the ions to secondary particles, such as electron and secondary particles are accelerated into an electron multiplier, on which DC voltage is applied. The conversion dynode can be configured to work in any polarity. The active lifetime of an electron multiplier is a function of surface deactivation-ion, such as caused by the absorption of water or contaminants to the multiplier surface, and can range from 1-2 years. Electron multiplier is the commonly used detectors in quadruple mass analyzers and quadruples ion traps.

2) Photomultipliers:

The conversion dynode of a photomultiplier detector generates electrons that impinge on a phosphor, which subsequently generates photons that are detected and amplified by a photomultiplier. Photomultiplier has advantage over electron multiplier as their shelf life is around 10 years. Photomultiplier is encased in a glass and hence not susceptible from damage through water molecules or contaminants. One disadvantage of photomultiplier is, that it is very sensitive to light background and thus mass spectrometer has these detectors should be kept away from the ambient light.

3) Micro channel Plate detectors:

Micro channel Plate detector (MCP) is one type of the solid state array detectors commonly used in mass spectrometry. Micro channel plates are flat waferlike detectors of leaded glass consisting of an array of 10^4-10^7 electron multipliers in parallel, each channel of which is 10-100 µm in diameter. Micro channel plate detectors are particularly useful in time-in-flight mass spectrometry, as they are flat and minimize the time spread and enhance subsequent mass resolution. In addition, they have reasonable gain and has fast response rate (100-psec time resolution). The major limitation of this detector is that it requires recovery time to recharge.

Other modified types of quadrupole and separation facilities

Quadrupole Ion Trap

The Quadrupole ion storage trap mass spectrometer is a recently developed mass analyzer with some special capabilities. Several commercial instruments are available and this analyzer is becoming more popular. These are very sensitive, relatively inexpensive, and scan fast enough for GC/MS experiments. The sensitivity of the results from trapping and then analyzing all the ions produced in the source. Since all the ions are detected, the S/N is high.

Ion Cyclotron Resonance

The Ion Cyclotron Resonance (ICR) mass spectrometer uses a superconducting magnet to trap ions in a small sample cell. This type of mass analyzer has extremely high mass resolution and is also useful for tandem mass spectrometry experiments. These instruments are very expensive and are typically used for specialized research applications. The ICR traps ions in a magnetic field that causes ions travel in a circular path. This is similar to the path of an ion in a magnetic sector, but the ions are not traveling as fast and the magnetic field is stronger. As a result the ions are contained in the small volume of the trap.

Vacuum System

All mass spectrometers operate at very low pressure (high vacuum). This reduces the chance of ions colliding with other molecules in the mass analyzer. Any collision can cause the ions to react, neutralize, scatter, or fragment. All these processes will interfere with the mass spectrum. To minimize collisions, experiments are conducted under high vacuum conditions, typically 10 to 10 Pa (10 to 10 torr) depending upon the geometry of the instrument. This -2 -5 -4 -7 high vacuum requires two pumping stages. The first stage is a mechanical pump that provides rough vacuum down to 0.1 Pa (10 torr). The second stage uses diffusion pumps or -3 turbo molecular pumps to provide high vacuum.

Data Collection system and Instrument control

Mass spectrometers heavily rely on integrated computer control system for instrument control, data collection and data processing. Early mass detectors used to have manual operating system and the analog data collection system, beside it used to have oscilloscope or chart recorder for the acquisition of the data. Software technology has revolutionized the mass detector operation and data acquisition.

Specialized software

Most system utilizes the personal computers running instrument control software locally, and thus software often contains the specialized applications and post processing capacities. This type of application has expanded the mass spectrometry horizon for variety of usage and ease of operation.

Accessories for Mass Spectrometry

For the proper functioning of the mass spectrometer requires accessories such as source for compressed air to produce ultrapure nitrogen and hydrocarbon free air. It also required dehumidifier, sample infusion assembly or chromatography assemblies.