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A REVIEW ON BIOANALYTICAL TECHNIQUES

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ABSTRACT

Quantitative determination of active pharmaceutical ingredient and its degradative products in biological fluids is a challenging task for an analyst due to difficulties and uniqueness of the sample. A systematic study of pharmaceutical products on human subjects whether patients or non-patient volunteers in order to discover or verify the clinical, pharmacological (including pharmacodynamics/ pharmacokinetics), adverse effects, with the object of determining their safety or efficacy and correcting plasma level with therapeutic action.

Keywords: Bioanalytical method, Recent Analytical Techniques, LC-MS/MS.

INTRODUCTION

Mass spectrometers also generate three-dimensional data. In addition to signal strength, they generate mass spectral data that can provide valuable information about the molecular weight, structure, identity, quantity, and purity of a sample. Mass spectral data add specificity that increases confidence in the results of both qualitative and quantitative analysis.

Some mass spectrometers have the ability to perform multiple steps of mass spectrometry on a single sample. They can generate a mass spectrum, select a specific ion from that spectrum, fragment the ion, and generate another mass spectrum; repeating the entire cycle many times. Such mass spectrometers can literally deconstruct a complex molecule piece by piece until its structure is determined [1].

For most compounds, a mass spectrometer is more sensitive and far more specific than all other LC detectors. It can analyze compounds that lack a suitable chromophore. It can also identify components in unresolved chromatographic peaks, reducing the need for perfect chromatography.

Mass spectral data complements data from other LC detectors. While two compounds may have similar UV spectra or similar mass spectra, it is uncommon for them to have both. The two orthogonal sets of data can be used to confidently identify, confirm, and quantify compounds.

Liquid chromatography-mass spectrometry (LC-MS, or alternatively HPLC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used for many applications which has very high sensitivity and selectivity. Generally its application is oriented towards the specific detection and potential identification of chemicals in the presence of other chemicals (in a complex mixture) [2-6].

Scale

A major difference between traditional HPLC and the chromatography used in LC-MS is that in the latter case the scale is usually much smaller, both with respect to the internal diameter of the column and even more so with respect to flow rate since it scales as the square of the diameter. For a long time, 1 mm columns were typical for LC-MS work (as opposed to 4.6 mm for HPLC). More recently 300 µm and even 75 µm capillary columns have become more prevalent. At the low end of these column diameters the flow rates approach 100 nl/min and are generally used with nanospray sources [7].

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Flow splitting

When standard bore (4.6 mm) columns are used the flow is often split ~10:1. This can be beneficial by allowing the use of other techniques in tandem such as MS and UV. However splitting the flow to UV will decrease the sensitivity of spectrophotometric detectors. The mass spectrometry on the other hand will give improved sensitivity at flow rates of 200 µl/min or less.

The Basic Liquid Chromatograph

The basic liquid chromatograph consists of six basic units. The mobile phase supply system, the pump and programmer, the sample valve, the column, the detector and finally a means of presenting and processing the results. A block diagram of the basic liquid chromatograph is shown in figure 1.

NEED OF LC SYSTEM INTERFACING WITH MASS SPECTROMETER [8-10]

The separation is done prior to mass analysis because the mass spectrometer is incapable of directly determining every analyte in all type of sample. The liquid chromatography can be regarded as apart of preparative procedure required for sample cleanup which improves linearity, accuracy and better sensitivity.

Mass spectrometer provides greater selectivity and sensitivity for chromatographic level because endogenous matrix can coelute with analyte yet not interferes as long as these component posses precursor masses.

The main purpose of interface is to evaporate the mobile phase and transfer the analyte from the higher pressure atom at which chromatographic separation is achieved to the lower pressure required for the mass analysis. LCMS is highly effective interface for coupling liquid chromatograph to the mass spectrometer. Mass spectrometer used as a detector system while liquid chromatogragh taken as separation system [11].

INSTRUMENTATION

SAMPLE INLET SYSTEM

The sample introduced as neutral species through a controlled vaccum peak followed by ionization in vaccum chamber.

Create the ion at atmospheric pressure and then introduce the ion into mass spectrometer through a controlled vaccum leak with aid of electrostatic this process is called as API provides best way when a dynamic coupling of liquid chromatogragh done.

Mass spectrometers work by ionizing molecules and then sorting and identifying the ions according to their mass-to-charge (*m/z*) ratios. Two key components in this process are the ion source, which generates the ions, and the mass analyzer, which sorts the ions. Several different types of ion sources are commonly used for LC/MS. Each is suitable for different classes of compounds. Several different types of mass analyzers are also used. Each has advantages and disadvantages depending on the type of information needed.

Ion Sources

Much of the advancement in LC/MS over the last ten years has been in the development of ion sources and techniques that ionize the analyte molecules and separate the resulting ions from the mobile phase. Earlier LC/MS systems used interfaces that either did not separate the mobile phase molecules from the analyte molecules (direct liquid inlet, thermospray) or did so before ionization (particle beam). The analyte molecules were then ionized in the mass spectrometer under vacuum, often by traditional electron ionization. These approaches were successful only for a very limited number of compounds [18].

The introduction of atmospheric pressure ionization (API) techniques greatly expanded the number of compounds that can be successfully analyzed by LC/MS. In atmospheric pressure ionization, the analyte molecules are ionized first, at atmospheric pressure. The analyte ions are then mechanically and electrostatically separated from neutral molecules. Common atmospheric pressure ionization techniques are:

- Electrospray ionization (ESI)
- Atmospheric pressure chemical ionization (APCI)
- Atmospheric pressure photoionization (APPI)

ELECTRO SPRAY IONISATION

Electrospray relies in part on chemistry to generate analyte ions in solution before the analyte reaches the mass spectrometer. The LC eluent is sprayed (nebulized) into a chamber at atmospheric pressure in the presence of a strong electrostatic field and heated gas.

The electrostatic field causes further dissociation of the analyte molecules. The heated drying gas causes the solvent in the droplets to evaporate. As the droplets shrink, the charge concentration in the droplets increases. Eventually, the repulsive force between ions with like charges exceeds the cohesive forces and ions are ejected (desorbed) into the gas phase. These ions are attracted to and pass through a capillary sampling orifice into the mass analyzer. Some gas-phase reactions, mostly proton transfer and charge exchange, can also occur between the time ions are ejected from the droplets and the time they reach the mass analyzer.

Electrospray is especially useful for analyzing large biomolecules such as proteins, peptides, and oligonucleotides, but can also analyze smaller molecules like benzodiazepines and sulfated conjugates [5].

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Large molecules often acquire more than one charge and to this multiple charging, electrospray can be used to analyze molecules as large as 150,000 u even though the mass range (or more accurately mass-to-charge range) for a typical LC/MS instruments is around 3000 *m/z*. For example: 100,000 u / 10 z = 1,000 *m/z* When a large molecule acquires many charges, a mathematical process called deconvolution is often used to determine the actual molecular weight of the analyte. Dielectric capillary entrance Nebulizer gas Solvent spray Ions heated nitrogen drying gas.

ATOMIC PRESSURE CHEMICAL IONISATION

In APCI, the LC eluent is sprayed through a heated (typically 250°C – 400°C) vaporizer at atmospheric pressure. The heat vaporizes the liquid. The resulting gas-phase solvent molecules are ionized by electrons discharged from a corona needle. The solvent ions then transfer charge to the analyte molecules through chemical reactions (chemical ionization).

The analyte ions pass through a capillary sampling orifice into the mass analyzer. APCI is applicable to a wide range of polar and nonpolar molecules. It rarely results in multiple charging so it is typically used for molecules less than 1,500 u. Due to this and because it involves high temperatures, APCI is less well-suited than electrospray for analysis of large biomolecules that may be thermally unstable. APCI is used with normal-phase chromatography more often than electrospray is because the analytes are usually non polar.

ATOMIC PRESSURE PHOTO IONISATION

Atmospheric pressure photoionization (APPI) for LC/MS is a relatively new technique. As in APCI, a vaporizer converts the LC eluent to the gas phase. A discharge lamp generates photons in a narrow range of ionization energies. The range of energies is carefully chosen to ionize as many analyte molecules as possible while minimizing the ionization of solvent molecules. The resulting ions pass through a capillary sampling orifice into the mass analyzer.

APPI is applicable to many of the same compounds that are typically analyzed by APCI. It shows particular promise in two applications, highly nonpolar compounds and low flow rates (<100 µl/min), where APCI sensitivity is sometimes reduced.

In all cases, the nature of the analyte and the separation conditions has a strong influence on which ionization technique: electrospray, APCI, or APPI, will generate the best results. The most effective technique is not always easy to predict.

Mass Analyzers

Although in theory any type of mass analyzer could be used for LC/MS, four types:

- Quadrupole
- Time-of-flight
- Ion trap

Quadrupole mass analyzer

The quadrupole mass analyzer is one type of mass analyzer used in mass spectrometry. As the name implies, it consists of 4 circular rods, set highly parallel to each other.In a quadrupole mass spectrometer (acronym QMS) the quadrupole is the component of the instrument responsible for filtering sample ions, based on their mass-to-charge ratio (m/z). Ions are separated in a quadrupole based on the stability of their trajectories in the oscillating electric Principle of operation

The quadrupole consists of four parallel metal rod pair rods. Each opposing 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 or allows the operator to scan for a range of m/z-values by continuously varying the applied voltage.

Ideally the rods are hyperbolic. Circular rods with a specific ratio of rod diameter-to-spacing provide an easier-to-manufacture adequate approximation to hyperbolas. Small variations in the ratio have large effects on resolution and peak shape. Different manufacturers choose slightly different ratios to fine-tune operating characteristics in context of anticipated application requirements. In recent decades some manufacturers have produced quadrupole mass spectrometers with true hyperbolic rods.
Fields that are applied to the rods [12].

Applications

These mass spectrometers excel at applications where particular ions of interest are being studied because they can stay tuned on a single ion for extended periods of time. One place where this is useful is in liquid chromatography-mass spectrometry or gas chromatography-mass spectrometry where they serve as exceptionally high specificity detectors. Quadrupole instruments are often reasonably priced and make good multi-purpose instruments.

Triple quadrupoles

A linear series of three quadrupoles can be used; known as a triple quadrupole mass spectrometer. The first (Q_1) and third (Q_3) quadrupoles act as mass filters, and the

middle (q_2) quadrupole is employed as a collision cell. This collision cell is an RF only quadrupole (non-mass filtering) using Ar, He or N gas (~10^{-3} Torr, ~30 eV) to induce collision induced dissociation of selected parent ion(s) from Q_1. Subsequent fragments are passed through to Q_3 where they may be filtered or scanned fully.

This process allows for the study of fragments (daughter ions) which are crucial in structural elucidation. For example, the Q_1 may be set to "filter" for a drug ion of a known mass, which is fragmented in q_2. The third quadrupole (Q_3) can then be set to scan the entire *m/z* range, giving information on the sizes of the fragments made. Thus, the structure of the original ion can be deduced.

The arrangement of three quadrupoles was first developed by Jim Morrison of LaTrobe University, Australia for the purpose of studying the photodissociation of gas-phase ions.The first triple-quadrupole mass spectrometer was developed at Michigan State University by Dr. Christie Enke and graduate student Richard Yost in the late 1970's.

Advantages

- Inexpensive
- Easily Interfaced to Many Ionization Methods

Disadvantages

- Low Resolution (<4000)
- Low Accuracy (>100ppm)
- MS/MS requires multiple analyzers
- Low Mass Range (<4000)
- Slow Scanning

Quadrupole ion trap

A **quadrupole ion trap** exists in both linear and 3D (**Paul Trap, QIT**) varieties and refers to an ion trap that uses constant DC and radio frequency (RF) oscillating AC electric fields to trap ions. It is a component of a mass spectrometer that would use such a trap to analyze ions. The invention of the 3D quadrupole ion trap itself is attributed to Wolfgang Paul who shared the Nobel Prize in Physics in 1989 for this work.

Theory

The 3D trap itself generally consists of two hyperbolic metal electrodes with their foci facing each other and a hyperbolic ring electrode halfway between the other two electrodes. The ions are trapped in the space between these three electrodes by AC (oscillating, non-static) and DC (non-oscillating, static) electric fields. The AC radio frequency voltage oscillates between the two hyperbolic metal end cap electrodes if ion excitation is desired; the driving AC voltage is applied to the ring electrode. The ions are first pulled up and down axially while being pushed in radially. The ions are then pulled out radially and pushed in axially (from the top and bottom). In this way the ions move in a complex motion that generally involves the cloud of ions being long and narrow and then short and wide, back and forth, oscillating between the two states. Since the mid-1980s most 3D traps (Paul traps) have used ~1 mtorr of helium. The use of damping gas and the mass-selective instability mode developed by Stafford et al. led to the first commercial 3D ion traps [16].

Scheme of a Quadrupole ion trap of classical setup with a particle of positive charge (dark red), surrounded by a cloud of similarly charged particles (light red). The electric field *E* (blue) is generated by a quadrupole of endcaps (a, positive) and a ring electrode (b). Picture 1 and 2 show two states during an AC cycle.

The quadrupole ion trap has two configurations: the three dimensional form described above and the linear form made of 4 parallel electrodes. A simplified rectilinear configuration has also been used.The advantage of the linear design is in its simplicity, but this leaves a particular constraint on its modeling. To understand how this originates, it is helpful to visualize the linear form. The Paul trap is designed to create a saddle-shaped field to trap a charged ion, but with a quadrupole, this saddle-shaped electric field cannot be rotated about an ion in the centre. It can only 'flap' the field up and down. For this reason, the motions of a single ion in the trap are described by the Mathieu Equations. These equations can only be solved numerically or equivalently by computer simulations.

The intuitive explanation and lowest order approximation is the same as strong focusing in accelerator physics. Since the field affects the acceleration, the position lags behind (to lowest order by half a period). So the particles are at defocused positions when the field is focusing and vice versa. Being farther from center, they experience a stronger field when the field is focusing than when it is defocusing.

Linear ion trap

The linear ion trap uses a set of quadrupole rods to confine ions radially and a static electrical potential on end electrodes to confine the ions axially. The linear form of the trap can be used as a selective mass filter, or as an actual trap by creating a potential well for the ions along the axis of the electrodes.Advantages of the linear trap design are increased ion storage capacity, faster scan times, and simplicity of construction (although quadrupole rod alignment is critical, adding a quality control constraint to their production. This constraint is additionally present in the machining requirements of the 3D trap).

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Cylindrical ion trap

Cylindrical ion traps have a cylindrical rather than a hyperbolic ring electrode.This configuration has been used in miniature arrays of traps.

Advantages

- Inexpensive
- Easily Interfaced to Many Ionization Methods
- MS/MS in one analyzer

Disadvantages

- Low Resolution (<4000)
- Low Accuracy (>100ppm)
- Space Charging Causes Mass Shifts
- Low Mass Range (<4000)
- Slow Scanning

Time-of-flight

Time-of-flight is a method in which ions are accelerated by an electric field of known strength.This acceleration results in an ion having the same kinetic energy as any other ion that has the same charge. The velocity of the ion depends on the mass-to-charge ratio. The time that it subsequently takes for the particle to reach a detector at a known distance is measured. This time will depend on the mass-to-charge ratio of the particle (heavier particles reach lower speeds). From this time and the known experimental parameters one can find the mass-to-charge ratio of the ion [13-15].

Theory

The potential energy of a charged particle in an electric field is related to the charge of the particle and to the strength of the electric field:

$$E_p = qU \quad [1]$$

where E_p is potential energy, q is the charge of the particle, and U is the electric potential difference (also known as voltage).

When the charged particle is accelerated into time-of-flight tube by the voltage U, its potential energy is converted to kinetic energy. The kinetic energy of any mass is:

$$E_k = \frac{1}{2}mv^2 \quad [2]$$

In effect, the potential energy is converted to kinetic energy, meaning that equations [1] and [2] are equal

$$E_p = E_k \quad [3]$$

$$qU = \frac{1}{2}mv^2 \quad [4]$$

The velocity of the charged particle after acceleration will not change since it moves in a field-free time-of-flight tube. The velocity of the particle can be determined in a time-of-flight tube since the length of the path (d) of the flight of the ion is known and the time of the flight of the ion (t) can be measured using a transient digitizer or time to digital converter.

Thus,

$$v = \frac{d}{t} \quad [5]$$

and we substitute the value of v in Eqn [5] into Eqn [4].

$$qU = \frac{1}{2}m\left(\frac{d}{t}\right)^2 \quad [6]$$

Rearranging Eqn [6] so that the flight time is expressed by everything else:

$$t^2 = \frac{d^2}{2U}\frac{m}{q} \quad [7]$$

Taking the square root of the time

$$t = \frac{d}{\sqrt{2U}}\sqrt{\frac{m}{q}} \quad [8]$$

These factors for the time of flight have been grouped purposely. $\frac{d}{\sqrt{2U}}$ contains constants that in principle do not change when a set of ions are analyzed in a single pulse of acceleration. Eqn 8 can thus be given as:

$$t = k\sqrt{\frac{m}{q}} \quad [9]$$

where k is a proportionality constant representing factors related to the instrument settings and characteristics.

Eqn [9] reveals more clearly that the time of flight of the ion varies with the square root of its mass-to-charge ratio (m/q).

Consider a real world example of a MALDI time-of-flight mass spectrometer instrument which is used to produce a mass spectrum of the tryptic peptides of a protein. Suppose the mass of one tryptic peptide is 1000 daltons (Da). The kind of ionization of peptides` produced by MALDI is typically +1 ions, so q = e in both cases. Suppose the instrument is set to accelerate the ions in a U = 15,000 volts (15 kilovolt, or 15 kV) potential. And suppose the length of the flight tube is 1.5 meters (typical). All the factors necessary to calculate the time of flight of the ions are now known for Eqn [8], which is evaluated first of the ion of mass 1000 Da:

$$t = \frac{1.5\ \text{m}}{\sqrt{2(15000\ \text{V})}}\sqrt{\frac{(1000\,Da)(1.672621\times10^{-27}\,\text{kg Da}^{-1})}{+1.602\times10^{-19}\,\text{C}}}$$

[10]

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Note that the mass had to be converted from daltons (Da) to kilograms (kg) to make it possible to evaluate the equation in the proper units. The final value should be in seconds:

$$t = 2.792 \times 10^{-5}\text{s}$$

which is about 28 microseconds. If there were a singly charged tryptic peptide ion with 4000 Da mass, and it is four times larger than the 1000 Da mass, it would take twice the time, or about 56 microseconds to traverse the flight tube, since time is proportional to the square root of the mass-to-charge ratio.

Advantages:
- Extremely High Mass Range (>1 MDa)
- Fast Scanning

Disadvantages:
- Low Resolution (4000)
- Low Accuracy (>200ppm)
- MS/MS not possible

Detectors [16-18]

A large number of LC detectors have been developed over the past thirty years based on a variety of different sensing principles. However, only about twelve of them can be used effectively for LC analyses and, of those twelve, only four are in common use. The four dominant detectors used in LC analysis are the *UV detector* (fixed and variable wavelength) the electrical conductivity detector, the fluorescence detector and the refractive index detector. These detectors are employed in over 95% of all LC analytical applications. These four detectors will be described and for those readers requiring more information on detectors are referred to Liquid Chromatography Detectors. The subject of detector specifications will not be discussed here but will also be dealt with in detail there. Detector sensitivities and detector linearity will, however, be given for each of the four detectors.

The UV Detector

The UV detector is by far the most popular and useful LC detector that is available to the analyst at this time. This is particularly true if multi-wavelength technology is included in this class of detectors. Although the UV detector has some definite limitations (particularly for the detection of non polar solutes that do not possess a UV chromaphores) it has the best combination of sensitivity, linearity, versatility and reliability of all the LC detectors so far developed [15].

Most compounds adsorb UV light in the range of 200-350Å including all substances having one or more double bonds (electrons) and all substances that have unshared (non bonded) electrons; *e.g.* all olefins, all aromatics and all substances containing >CO, >CS, -N=O and $-\mathrm{N}\equiv\mathrm{N}-$ groups. The relationship between the intensity of UV light transmitted through the detector cell and solute concentration is given by "Beers' Law,

$$I_T = I_o e^{-kLc}$$

where, (I_o) is the intensity of the light entering the cell,
(I_T) is the intensity of the transmitted light,
(L) is the path length of the cell,
(c) is the concentration of the solute,
(k) is the molar extinction coefficient of the solute for the specific wavelength of the UV light.

The Electrical Conductivity Detectors

The electrical conductivity detector can only detect those substances that ionize and consequently, are frequently used in the analysis of inorganic acids, bases and salts. It has also found particular use in the detection of organic acids and bases that are frequently required in environmental studies and in biotechnology applications. The sensor is the simplest of all the detectors consisting of only two electrodes situated in a suitable flow cell.

An example of an electrical conductivity sensing cell is shown in figure 8. It consists of two electrodes situated in a suitable flow cell as depicted in the upper diagram. The electrodes are arranged to constitute one arm of a Wheatstone Bridge. When ions enter the detector cell, the electrical resistance changes and the out of balance signal is fed to a suitable amplifier.

The output from the amplifier is either digitized, and the binary number sent to a computer for storage, or the output is passed directly to a potentiometric recorder. The detector actually measures the electrical resistance between the electrodes which by suitable non-linear amplification, can be made to provide an output that is linearly related to solute concentration.

It is essential that an AC voltage is used across the electrodes to measure the cell impedance to avoid electrode polarization. The frequency of the AC potential across the electrodes is usually around 10 kHz.

Fluorescence Detector

The fluorescence detector is one of the most sensitive LC detectors and for this reason is often used for trace analysis. Unfortunately, although the detector is very sensitive, its response is only linear over a relatively limited concentration range. In fact, the response of the detector can only be assumed to be linear over a concentration range of two orders of magnitude.

Unfortunately, the majority of substances do not naturally fluoresce which is a serious disadvantage to this type of detector. It follows, that in many instances fluorescent derivatives must be synthesized to render the substances of interest detectable. There are a number of regents that have been developed specifically for this purpose but derivatizing procedures will be discussed in detail in a later Book. A diagram of the Fluorescence Detector is shown in figure 9.

The Refractive Index Detector

The refractive index detector is one of the least sensitive LC detectors. It is very sensitive to changes in ambient temperature, pressure changes, flow-rate changes and cannot be used for gradient elution.

Despite these many disadvantages, this detector is extremely useful for detecting those compounds that are nonionic, do not adsorb in the UV, and do not fluoresce. There are many optical systems used in refractive index detectors (9) but one of the most common is the differential refractive index detector shown diagramatically in figure 10.

MODES OF LCMS MONITERING

Typically the mass spectrometer is set to scan a specific man range. This can be wide as in the full scan analysis or can be very narrow as in ion monitoring. A single mass can take anywhere from10ms to 5ms depending upon the type of scan. Many scans acquired during an LCMS analysis.

LCMS and MS data is represented by adding up ion current has an intensity point against time. The resulting plot is a plot that looks very much like an HPLC UV trace.

The most common modes acquiring lcms data are

-Selected Ion Monitoring [SIM]

-Selected Reaction Monitoring [SRM] or Multiple Reaction Monitoring [MRM].

SELECTED ION MONITERING

In this mass spectro meter is set to scan over a very small mass range typically one mass unit. The SIM plot is a plot of the ion current resulting from this very small mass range.Only com pounds with the selected mass are detected and plotted the resulted plots look very different.

The reason is that the peaks seen in the SIM plot may be very minor component in the TLC plot above.The SIM plot is a more specific plot than the full scan TLC plot.

Many compounds have the same mass and in electrospray where there are multiply charged species is even higher for a compounds having the same m/z value.

The SIM is more sensitive than full scan because the mass spectrometer can elute for a longer time over a smaller mass range.

SELECTED REACTION MONITORING

Selected reaction monitoring is the method used by the majority of scientist performing mass spectrometric quantitation. SRM delivers a unique fragment ion that can be monitored and quantified in the midest of the very complicated matrix. SRM plots a very simple usually containing only a single peak.

SRM accomplished by specifying the parent mass of the compound for ms/ms fragmentation and then specifically monitoring for a single fragment ion.

APPLICATIONS

LC/MS is suitable for many applications, from pharmaceutical development to environmental analysis. Its ability to detect a wide range of compounds with great sensitivity and specificity has made it popular in a variety of fields.

Molecular Weight Determination

One fundamental application of LC/MS is the determination of molecular weights. This information is key to determining identity.

Examples:

- Differentiation of similar octapeptides
- Determining the molecular weight of green fluorescent protein

Structural Determination

Another fundamental application of LC/MS is the determination of information about molecular structure. This can be in addition to molecular weight information or instead of molecular weight information if the identity of the analyte is already known.

Examples:

- Structural determination of ginsenosides using MSn analysis

Pharmaceutical Applications

Rapid chromatography of benzodiazepines

The information available in a mass spectrum allows some compounds to be separated even though they are chromatographically unresolved. In this example, a series of benzodiazepines was analyzed using both UV and MS detectors. The UV trace could not be used for quantitation, but the extracted ion chromatograms from the MS could be used. The mass spectral information provides additional confirmation of identity. Chlorine has a characteristic pattern because of the relative abundance of the two most abundant isotopes [19].

Identification of bile acid metabolites

The MSn capabilities of the ion trap mass spectrometer make it a powerful tool for the structural analysis of complex mixtures. Intelligent, data-dependent

acquisition techniques can increase ion trap effectiveness and productivity. They permit the identification of minor metabolites at very low abundances from a single analysis. One application is the identification of metabolic products of drug candidates.

Biochemical Applications

Rapid protein identification using capillary LC/MS/MS and database searching

Traditional methods of protein identification generally require the isolation of individual proteins by two-dimensional gel electrophoresis. The combination of capillary LC/MS/MS with intelligent, data-dependent acquisition and probability-based database searching makes it possible to rapidly identify as many as 100 proteins in a single analysis.

Clinical Applications

High-sensitivity detection of trimipramine and thioridazine

For most compounds, MS is more sensitive than other LC detectors. Trimipramine is a tricyclic antidepressant with sedative properties. Thioridazine is a tranquilizer. These compounds in a urine extract at a level that could not be detected by UV. To get the maximum sensitivity from a single-quadrupole mass spectrometer, the analysis was done by selected ion monitoring.

Food Applications

Identification of aflatoxins in food

Aflatoxins are toxic metabolites produced in foods by certain fungi. The total ion chromatogram from a mixture of four aflatoxins. Even though they are structurally very similar, each aflatoxin can be uniquely identified by its mass spectrum.

Determination of vitamin D3 in poultry feed supplements using MS3

Vitamin D is an essential constituent in human and animal nutrition. Livestock diets deficient in vitamin D can cause growth abnormalities. Traditional GC/MS analysis methods for vitamin D3 in feed extracts require extensive and time-consuming sample preparation and derivatization prior to analysis. Atmospheric pressure chemical ionization with ion trap detection provides a sensitive analytical method without the need for extensive sample preparation and derivatization.

Environmental Applications

Detection of phenylurea herbicides

Many of the phenylurea herbicides are very similar and difficult to distinguish with a UV detector. Monuron and diuron have one benzene ring and differ by single chlorine. Chloroxuron has two chlorines and a second benzene ring attached to the first by oxygen.

The UV-Vis spectra are similar for diuron and monuron, but different for chloroxuron. When analyzed using electrospray ionization on an LC/MS system, each compound has a uniquely identifiable mass spectrum.

Detection of low levels of carbaryl in food

Pesticides in foods and beverages can be a significant route to human exposure. Analysis of the carbamate pesticide carbaryl in extracts of whole food by ion trap LC/MS/MS proved more specific than previous analyses by HPLC fluorescence and single-quadrupole mass spectrometry. The protonated carbaryl molecule (*m/z* 202) was detected in full scan mode using positive ion electrospray. A product ion at *m/z* 145 generated by collisioninduced dissociation provided confirmation of carbaryl and was used for subsequent quantitative analysis.

CE/MS Applications

Analysis of peptides using CE/MS/MS

Capillary electrophoresis (CE) is a powerful complement to liquid chromatography. Different selectivity and higher chromatographic resolution are its biggest advantages when analyzing clean samples such as synthetic peptides.

Figure 1. Basic chromatograph

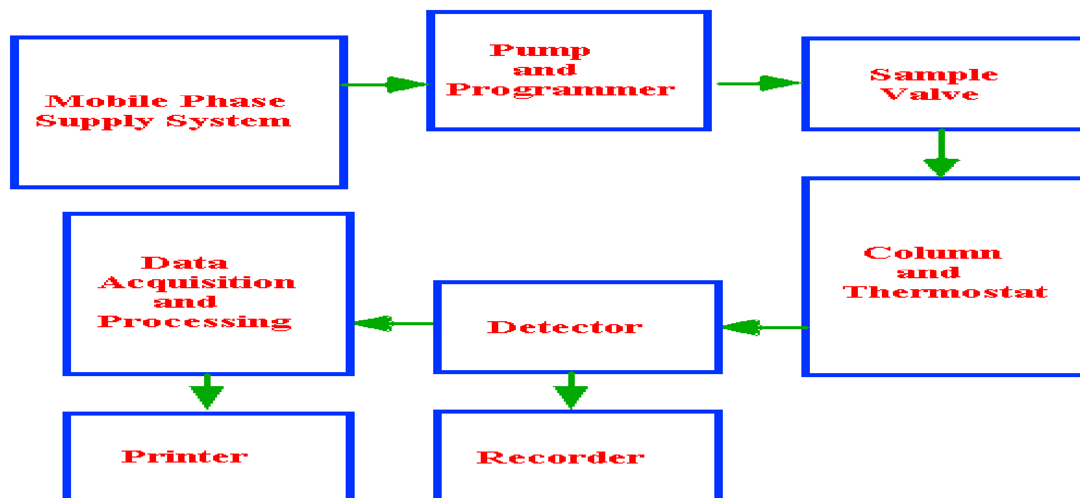

Figure 2. Electrospray Ionization

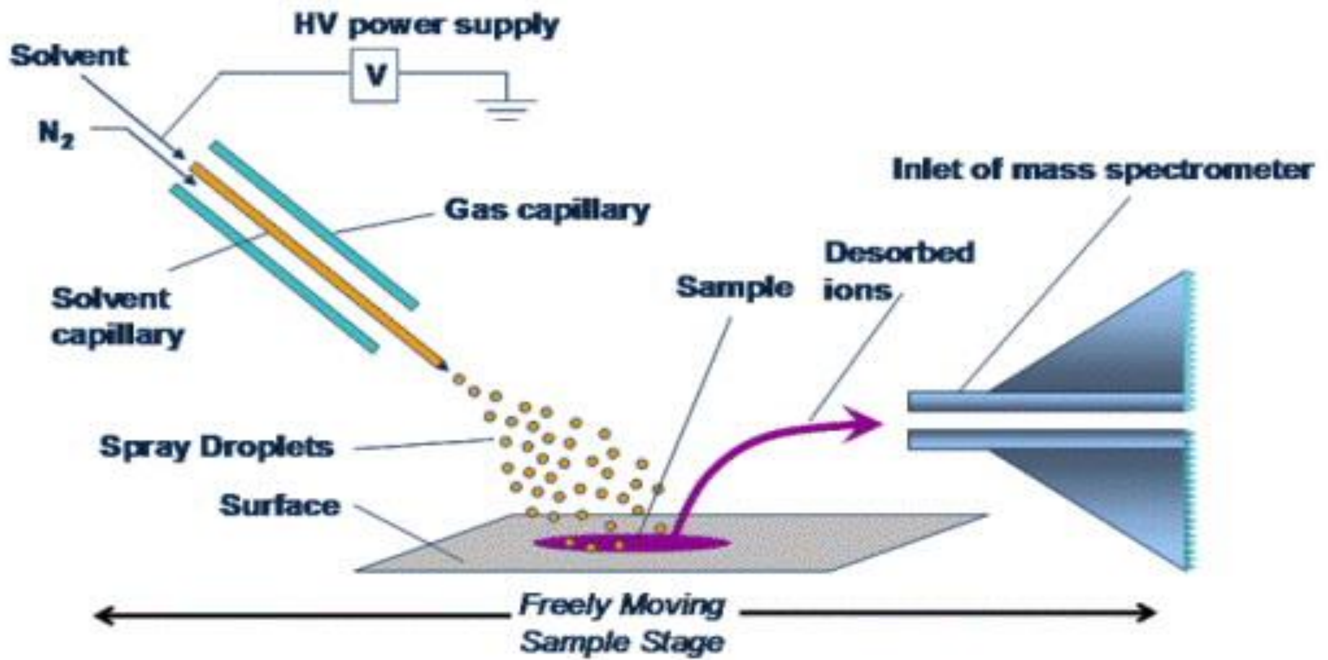

Figure 3. Atmospheric Pressure Chemical Ionization

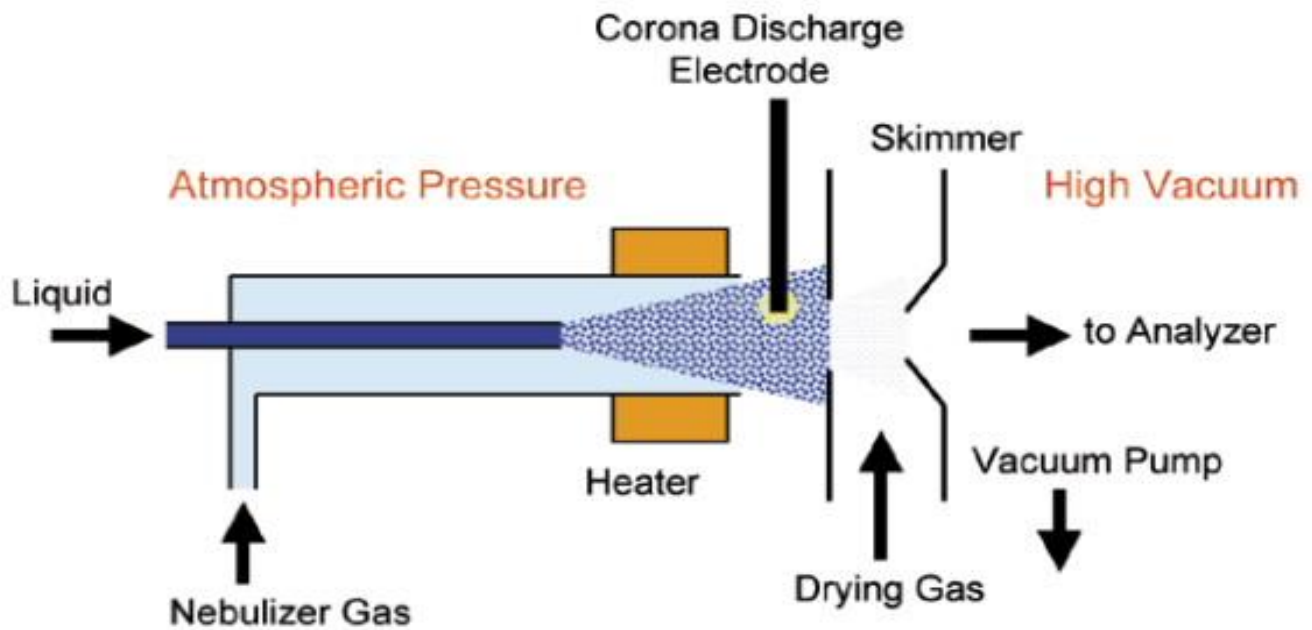

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Figure 4. Atmospheric pressure photoionization

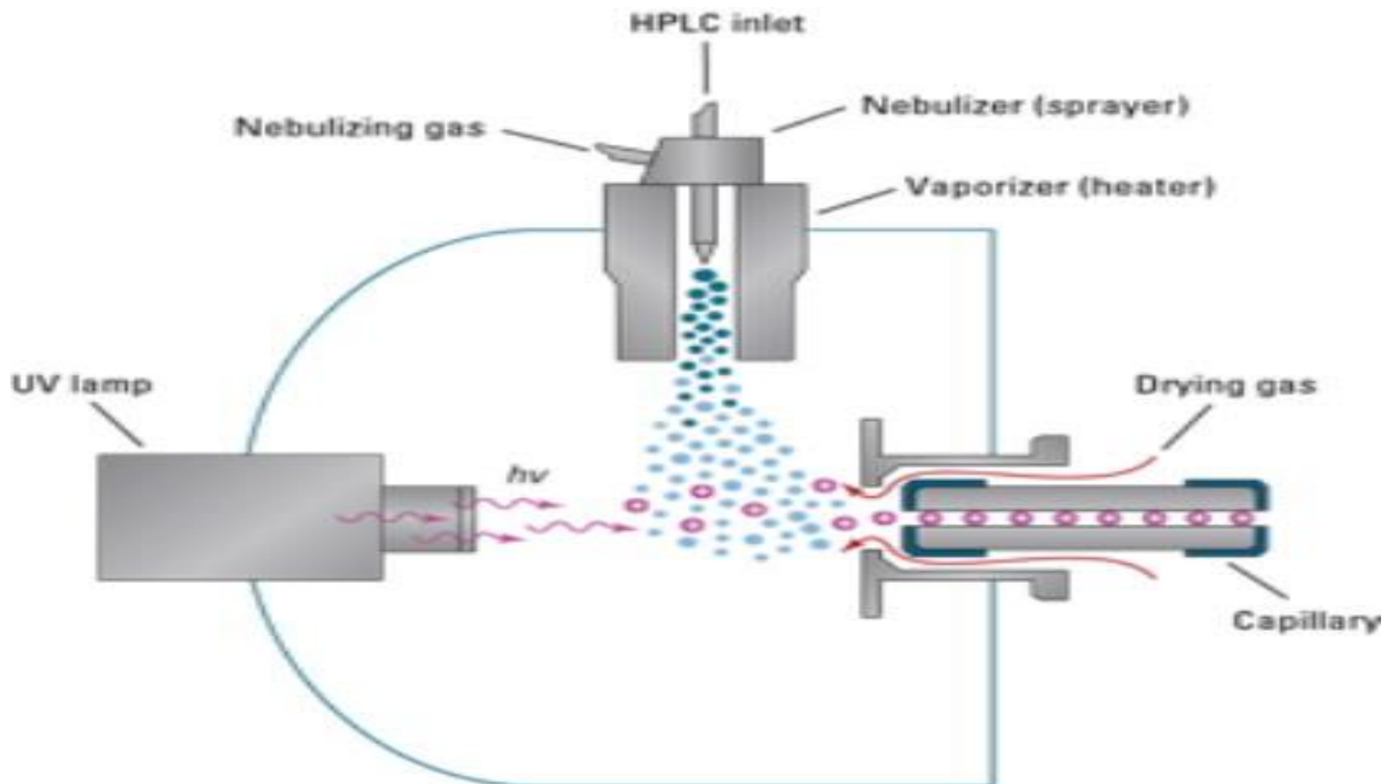

Figure 5. Quadrupole mass analyzer

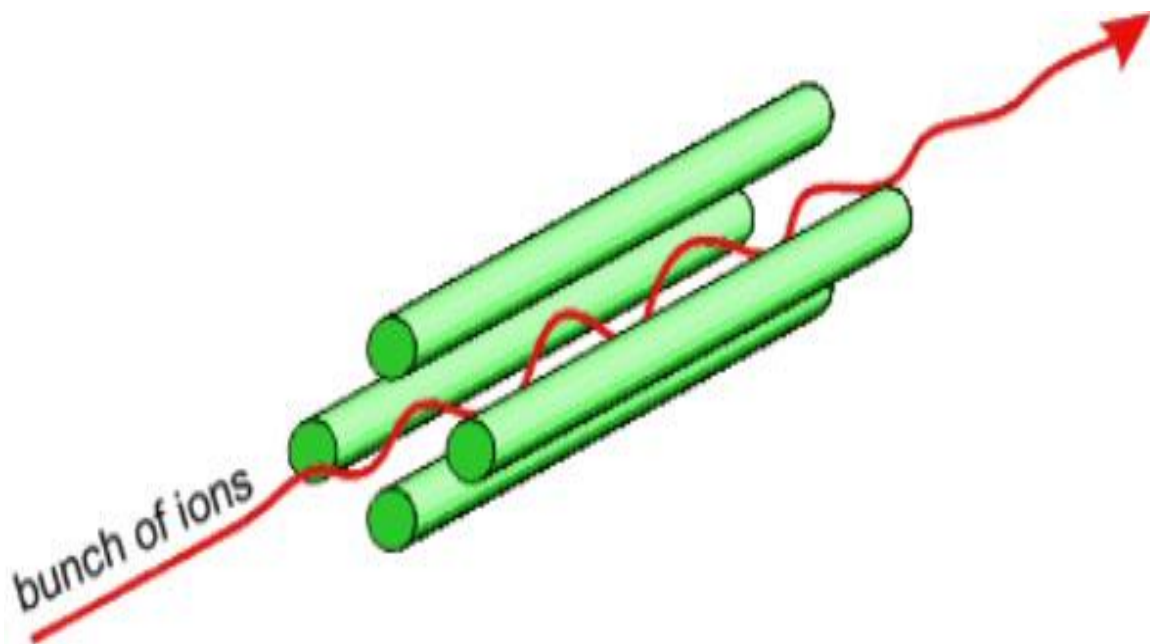

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Figure 6. Apparatus for Separating Charged Particles of Different Specific Charges

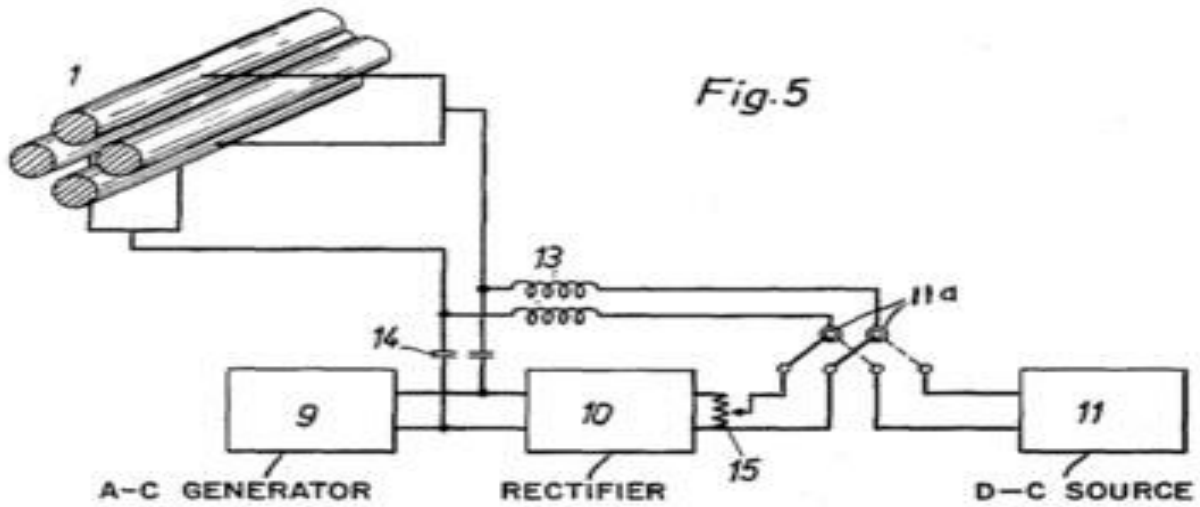


Figure 7. Quadrapole ion trap

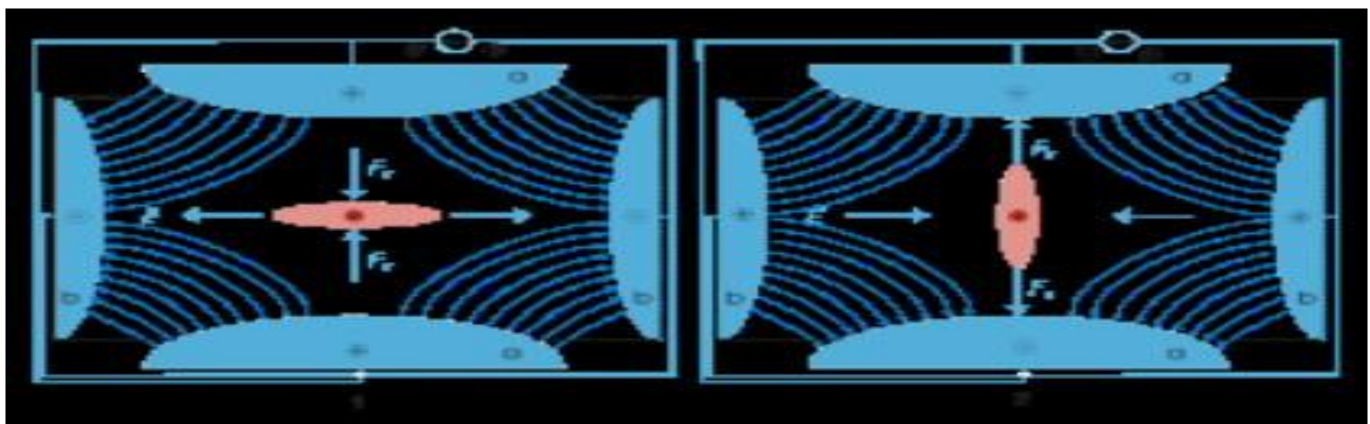


Figure 8. Electrical Conductivity detector

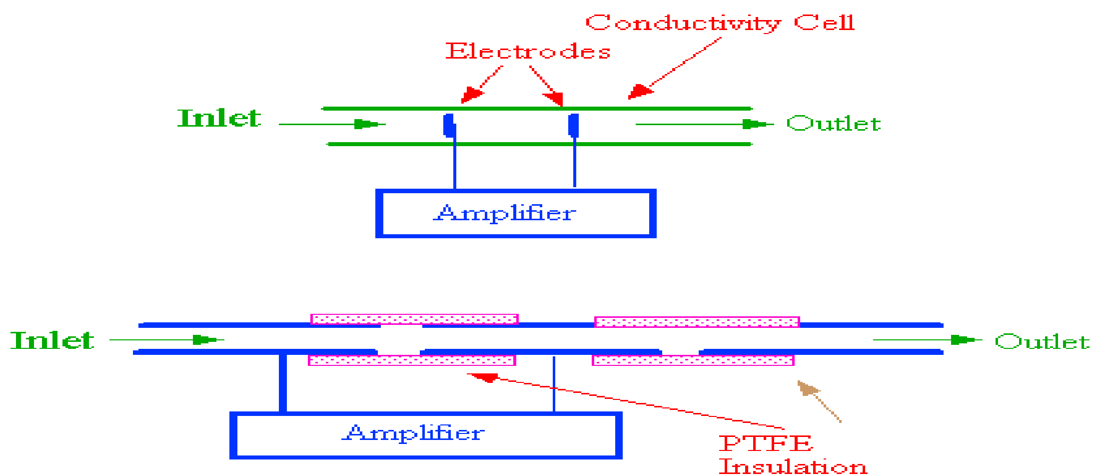


Figure 9. Fluorescence Detector

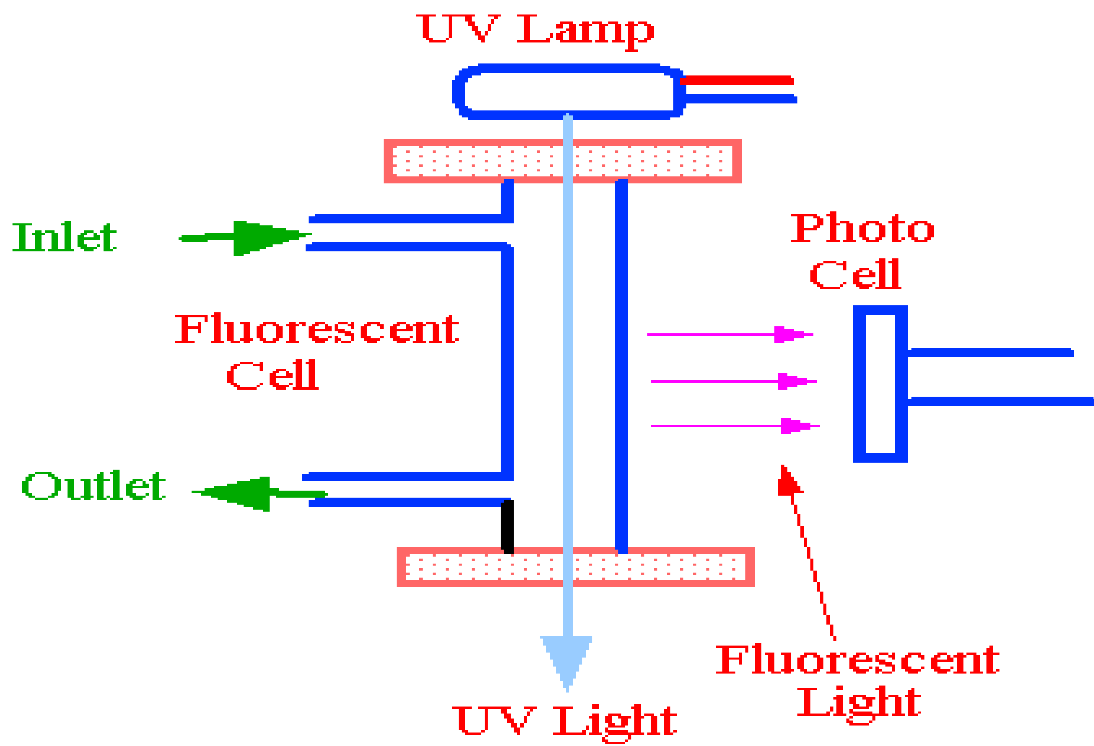


Figure 10. Refractive index Detector

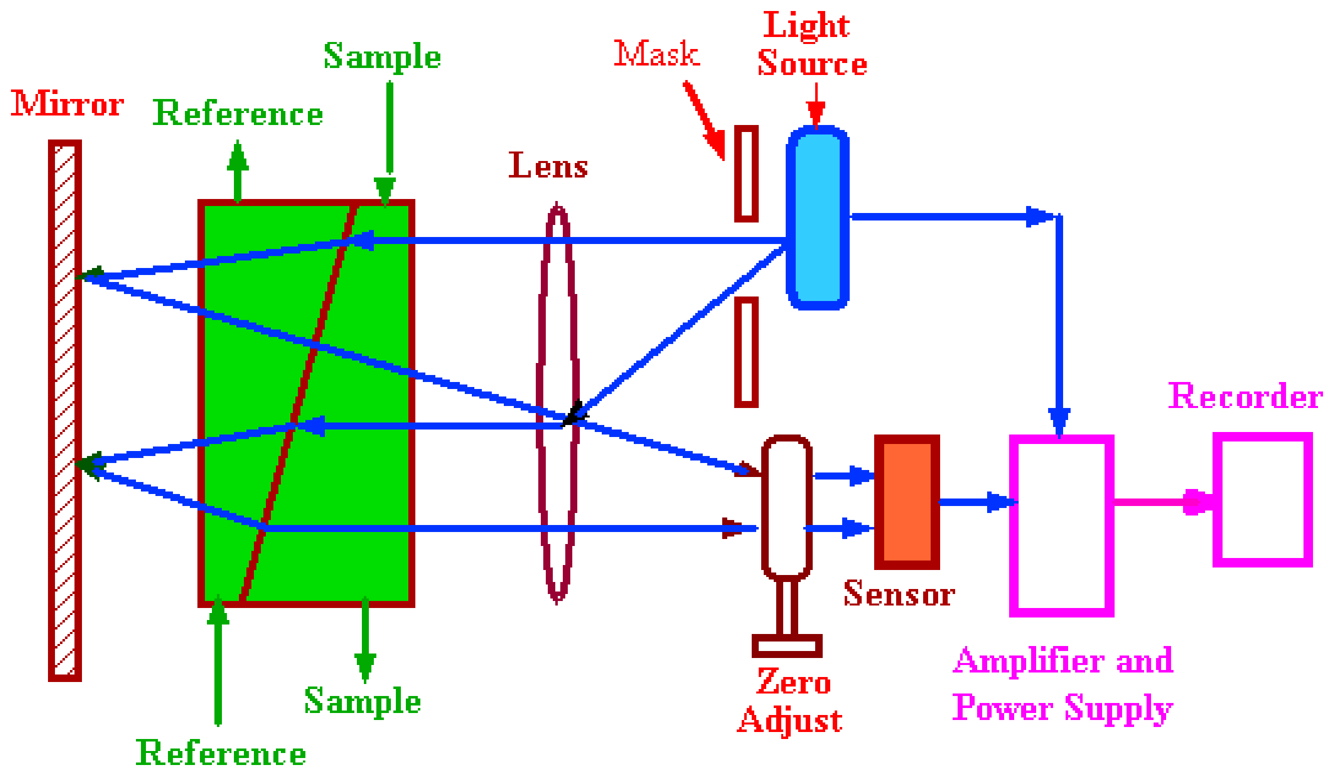
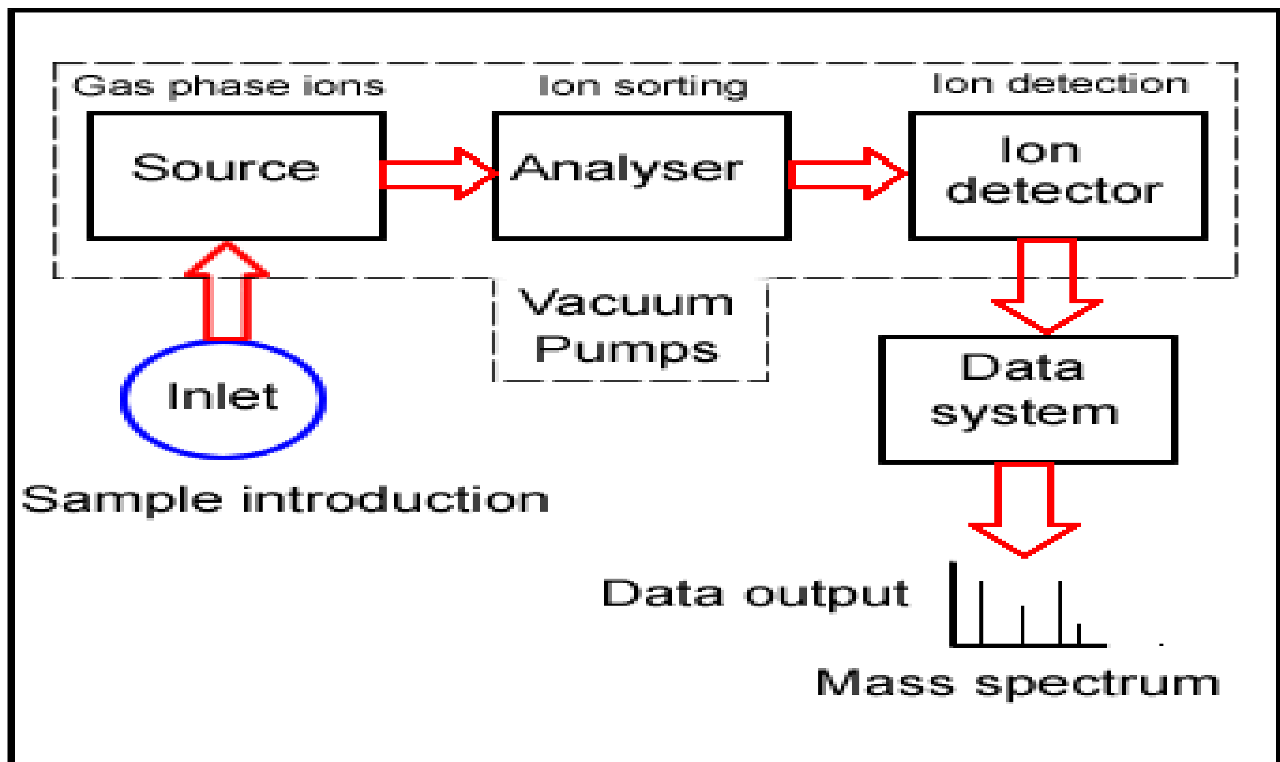


Figure 11. LC-MS/MS Equipment

Figure 12. Block Diagram of LC-MS/MS



CONCLUSION

LC-MS/MS is most commonly used for proteomic analysis of complex samples.LC-MS/MS is used to determine accurate concentrations of drug and metabolites in the biological matrix. The major challenges in LC-MS/MS are establishment of sample preparation technique and P^H of the mobile phase may harm the system because impurity of sample and P^H degrade the columns.

LC-MS/MS is frequently used in the drug development at many different stages including peptide mapping, glycoprotein mapping, bio affinity screening, metabolic stability screening, impurity identification, degradant identification, quantitative bio analysis and quality control.

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