

Review Article

Hyphenated Techniques of Drug AnalysisMd. Rageeb Md. Us man^{1*}, Swap nil R. Badgular¹, Tanvir Y. Shaikh²¹Department of Pharmacognosy, Smt. Sharadchandrika Suresh Patil College of Pharmacy Chopda, Maharashtra, India²Department of Pharmaceutics, Smt. Sharadchandrika Suresh Patil College of Pharmacy Chopda, Maharashtra, India***Corresponding author**

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Abstract: A Hyphenated technique is combination or coupling of two different analytical techniques. Which is used as a separation techniques and an online spectroscopic detection technology? . The remarkable improvements in hyphenated analytical methods over the last two decades have significantly broadened their applications in the analysis of biomaterials, natural products, elemental species, explosives, trace elements, etc. and show specificity and sensitivity. in this article recent advance application about hyphenated techniques and information about analytical techniques, GC-MS, CE-MS, LC-MS, LC-FTIR, LC-NMR, etc. the different areas like forensic science, environment, biotechnology, geography, pharmaceutical etc. are discussed with appropriate examples for various purpose.

Keywords: Hyphenated technique, GC-MS, LC-MS, LC-FTIR, LC-NMR, natural products, separation technique, Chromatographic Techniques, Spectroscopic Techniques.

INTRODUCTION

A couple of decades ago, Hirschfield introduced the term "hyphenation" to refer to the on-line combination of a separation technique and one or more spectroscopic detection techniques. This technique, developed from a marriage of a separation technique and a spectroscopic detection technique, is nowadays known as hyphenated technique [1]. Hyphenated separation techniques refer to a combination of two or more techniques to separate chemicals from solutions and detect them. Most often the other technique is some form of chromatography. Hyphenated techniques are widely used in chemistry and biochemistry. A slash is sometimes used instead of hyphen, especially if the name of one of the methods contains a hyphen itself. Combinations of the above techniques produce "hybrid" or "hyphenated" techniques. Several examples are in popular use today and new hybrid techniques are under development. For example, gas chromatography-mass spectrometry, LC-MS, GC-IR, LC-NMR, LC-IR, CE-MS, ICP-MS, and so on. Hyphenated techniques combine chromatographic and spectral methods to exploit the advantages of both. [2-5] Chromatography produces pure or nearly pure fractions of chemical components in a mixture. Spectroscopy produces selective information for identification using standards or library spectra. [6] (Figure 1).

ANALYTICAL TECHNIQUES {HYPHENATED TECHNIQUES

The coupling of a separation technique and an on-line separation technique leads to the development of hyphenated technique. A hyphenated technique in analytical chemistry is 'the marriage of two separate analytical techniques via appropriate interfaces, usually with backup of a computer tying everything together'. "Hyphenation" term was first coined by Hirschfield although the idea itself began with coupling of GC & MS in the early 1970's. In recent years, hyphenated techniques have received ever-increasing attention as the principal means to solve complex analytical problems. The power of combining separation technologies with spectroscopic techniques has been demonstrated over the years for both quantitative and qualitative analysis of unknown compounds in complex natural product extracts or fractions. To obtain structural information leading to the identification of the compounds present in a crude sample, liquid chromatography (LC), usually a high-performance liquid chromatography (HPLC), gas chromatography (GC), or capillary electrophoresis (CE) is linked to spectroscopic detection. Fourier-transform infrared (FTIR), photodiode array (PDA) UV-vis absorbance or fluorescence emission, mass spectroscopy (MS), and nuclear magnetic resonance spectroscopy (NMR), are sum of the modern hyphenated techniques. A variety of hyphenated techniques such as LC-MS, GC-MS, LC-NMR, ICP-MS, and CE-MS have been applied in the

analysis of pharmaceuticals. The determination of drugs in biological materials is an important step in drug

discovery and drug developments [7, 8] (Figure 2).

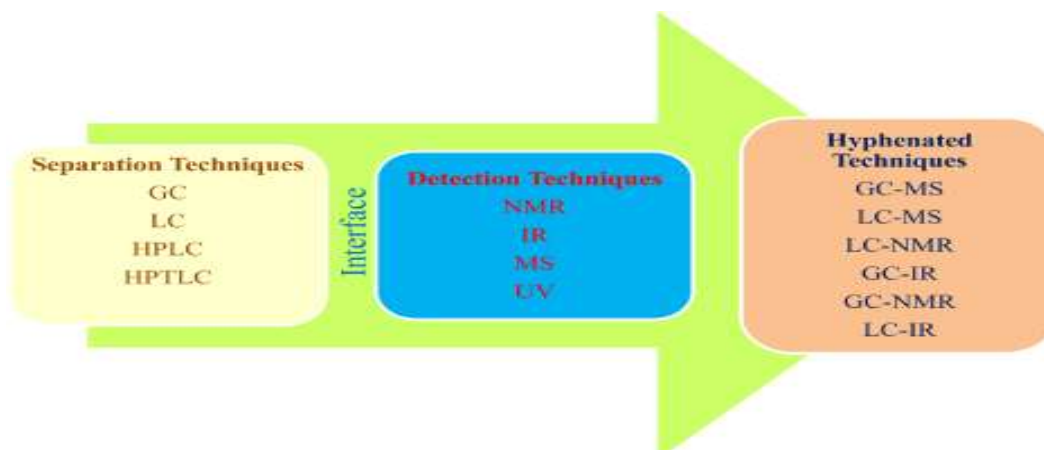


Fig.No.1: Hyphenated techniques for Separation and Detection techniques

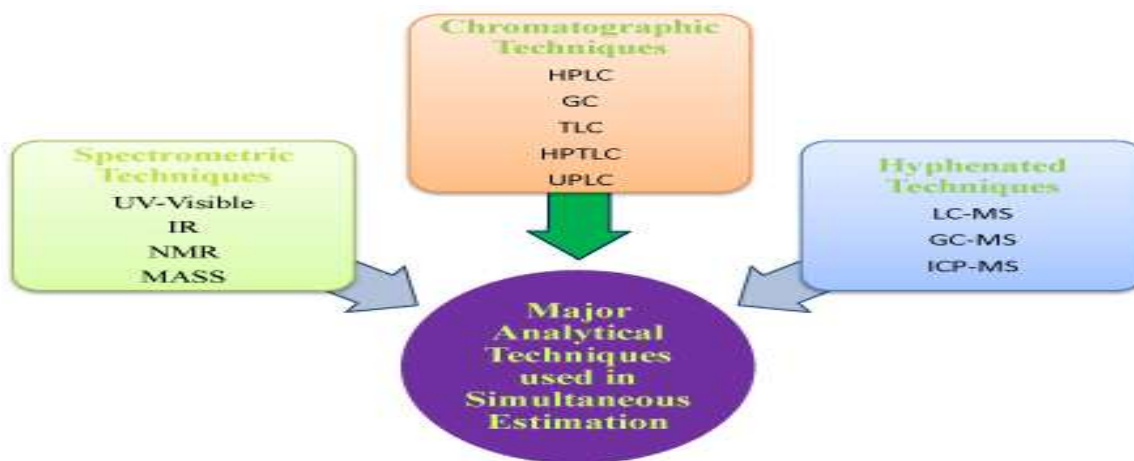


Fig.No.2: Hyphenated techniques for Spectrometric and Chromatographic techniques

METHODS

GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

In 1957, Holmes and Morrell (Holmes & Morrell, 1957) demonstrated the first coupling of gas chromatography with mass spectrometry shortly after the development of gas-liquid chromatography and organic mass spectrometry. Years later, improved GC-MS instruments were commercialized with the development of computer-controlled quadrupole mass spectrometer for fast acquisition to accommodate the separation in gas chromatograph. Since then, its applications in various areas of sciences have made it a routine method of choice for (bio) organic analysis. As its name suggested, a GC-MS instrument is composed of at least the following two major building blocks: a gas chromatograph and a mass spectrometer. GC-MS separates chemical mixtures into individual components (using a gas chromatograph) and identifies / quantifies the components at a molecular level (using a MS detector). It is one of the most accurate and efficient tools for analyzing volatile organic samples. [1] Ionization techniques and interfaces

The carrier gas that comes out of a GC column is primarily a pressurized gas with a flow about mL/min for capillary columns and up to 150 mL/min for packed columns. In contrast, ionization, ion transmission, separation and detection in mass spectrometer are all carried out under high vacuum system at approximately 10⁻⁴ Pa (10⁻⁶ Torr). For this reason, sufficient pumping power is always required at the interface region of a GC-MS in order to provide a compatible condition for the coupling [3]. When the analytes travel through the length of the column, pass through the transfer line and enter the mass spectrometer they can be ionized by several methods. Fragmentation can occur alongside ionization too. After ion separation by mass analyzer, [11] they will be detected, usually by an electron multiplier diode, which essentially turns the ionized analytes/fragments into an electrical signal...[9] The two well-accepted standard types of the ionization techniques in GC-MS are the prevalent electron impact ionization (EI) and the alternative chemical ionization (CI) in either positive or negative modes.[10]

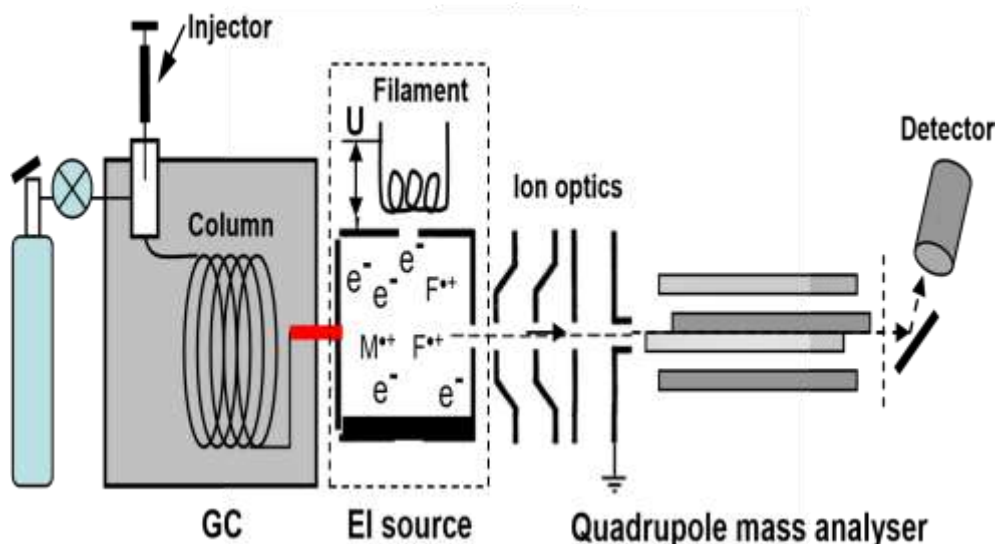


Fig. No.3: Schematic drawing of a GC-MS with EI ionization and quadrupole mass analyser

ELECTRON IMPACT (EI) IONIZATION:

The EI source is an approximately one cubic centimeter device, which is located in the ion source housing as shown in Figure 1. The ion source is open to allow for the maximum conductance of gas from the ion source into the source housing and then into the high vacuum pumping system. The EI source is fitted with a pair of permanent magnets that cause the electron beam to move in a three-dimensional helical path, which increases the probability of the interaction between an electron and an analyte molecule. Even under this condition, only about 0.01~0.001% of the analyte molecules are actually ionized (Watson, 1997). During the EI ionization, the vaporized molecules enter into the MS ion source where they are bombarded with free electrons emitted from a heated filament (such as rhenium). The kinetically activated electrons (70-eV) collide with the molecules, causing the molecule to be ionized and fragmented in a characteristic and reproducible way. [2]

Chemical ionization (CI):

CI is a less energetic process than EI. In the latter case, the ionizing electrons accelerated to have some kinetic energy collide directly with gas-phase analyte molecules to result in ionization accompanied by simultaneous fragmentation. Molecular weight, molecular ion, exact mass and isotope distribution For a singly charged analyte, the mass-to-charge ratio of its molecular ion indicates the molecular weight (a.m.u.) of the analyte. The nominal mass of an ion is calculated using the integer mass by ignoring the mass defect of the most abundant isotope of each element. This is equivalent as summing the numbers of protons and neutrons in all constituent atoms Some molecular ions can be obtained using EI ionization. If not, the use of soft-ionization techniques (CI, FI) would help to facilitate identification of the molecular ion. Accurate masses can be determined using high resolution instruments such as magnet/sectors, reflector TOFs

Fourier-Transform ion cyclotron resonance (FT-ICR) and Orbitrap mass spectrometers

Typical mass analyzers and ms detectors in gc-ms

MS instruments with different mass analyzers, e.g. magnet/electric sectors, quadruples (linear), ion traps (Paul traps & quadruple linear ion trap), FT-ICR and time-of-flight (TOF) mass analyzers have all been implemented for the coupling of GC and MS. Since its conception, linear quadruples have been dominating the GC-MS applications

Quadrupole mass analyzer:

A single-stage linear quadrupole mass analyzer can be considered as a mass filter and it consists of four hyperbolic metal rods placed parallel in a radial array Ion trap (it) mass analyzer:

An ion trap mass spectrometer uses a combination of electric or magnetic fields to capture and store ions in a vacuum chamber (Figure 3)

Time-of-flight (tof) mass analyzer:

Time-of-flight mass spectrometry (TOF-MS) is based on a simple mass separation principle in which the m/z of an ion is determined by a measurement of its flight time over a known distance (Cotter, 1994; Stephens, 1946). Pulsed ions are initially accelerated by means of a constant homogeneous electrostatic field of known strength to have the same kinetic energy (given they have the same charge). Therefore, the square of the velocity of an ion is reversely proportional to its m/z, $E_k = (1/2) mv^2$ and the time of arrival t at a detector directly indicates its mass

$$t = d \times (m/q)^{1/2} \times (2U)^{-1/2} \dots \dots \dots$$

where, d is the length of the flight tube, m is the mass of the ion, q is the charge, U is the electric potential difference used for the acceleration.[1]

INSTRUMENTATION AND WORKING

Vaporized analyte when carried through the GC column with the help of heated carrier gas the separation occurs in column only. Carrier can also be called as the mobile phase e.g. helium. Distinguishable interactions of analyte between mobile phase and stationary phase lead to separation of the compounds. The separation of the analyte is also depend on the column's dimensions (length, diameter film thickness), type of carrier gas, column temperature (gradient) and the properties of the stationary phase. The sample travel through the length of column the difference in the boiling point and other chemical properties lead to separation of the components of the mixture. The components will be having differences in elution time and retention time due to their different adsorption or difference in the partition between mobile phase and the stationary phase resp. Then the separated components of the mixture will enter into the MS through an interphase. This is followed by ionization, mass analysis and detection of mass-to charge ratios of ions generated

from each analyte by the mass spectrometer. An interface like effusion separator, jet/orifice separator & membrane separator can be used to connect GC with MS. The process of ionization not only ionize the molecule but also break the molecule into the fragments and detect these fragments with the help of electron impact ionization and chemical ionization The molecular ion of analyte form a finger print spectrum which is different from other analytes. GC-MS is important tool in analytical chemistry because these techniques accurately separate, identify and provide information about structure and composition from very less sample. The advantage of this technique is sometimes two different analyte will have same mass spectrum but the retention time of both the analytes is different so such type of analytes can be separated or analyses with the help of GC-MS. Two widely used Ionization techniques in GCMS are the electron impact ionization (EI) and the alternative chemical ionization (CI) in either positive or negative modes. [1]

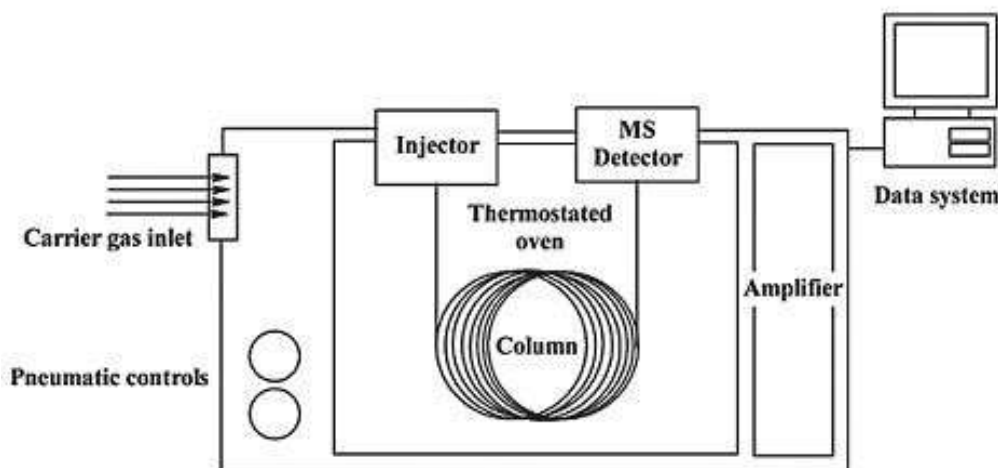


Fig. No. 4: GC-MS

APPLICATION

Thermally labile compounds by excessive heating

1. Quantization of pollutants in drinking and. By hyphenation of these two techniques waste water using official U.S. capabilities of both the techniques were Environmental Protection Agency (EPA) improved methods.
2. Quantization of drug in metabolites and urine is done for the pharmacological and The LC-MS instrument can be interfaced by forensic use electro spray, particle beam, Thermo spray.
3. Identification of unknown organic Electro spray is most widely used interface. The compounds in hazardous waste dumps spray needle is used as bridge to connect the and reaction products by synthetic organic liquid chromatography with that of the mass. But chemistry. the separate emitter is flexible as well as

Used for drug analysis, pesticide convenient [1]

LC-MS

Liquid chromatography-mass spectrometry is the. Which is detected with the help of Photo diode technique which performs separation by liquid array, Ultraviolet, fluorescent etc. detectors. These chromatography and mass analysis with the help separated components then transferred to the of the mass spectrometry. With the help of HPLC interface. In interface the liquid is volatilized and impurities and degradation products can be transferred to the MS. With the help of various separated and Mass Spectrometry allows us to ionization techniques the compound is ionized obtain the molecular weight and identification of and then it is analyzed by mass analyzer. Various the same. LC-MS is highly selective and sensitive mass analyzers are used *viz.* Quadruples, technique. LC-MS leads to detection and quadruple ion traps, time-to-flight (TOF), time identification of chemicals in presence of other to-flight reflection (TOFR), and ion cyclotron chemicals there for it is called as specific. The

resonance (ICR) mass analyzers. In this technique flow rate of HPLC is around 1ml/min which is LC separate due to which clear difficult to accommodate in mass spectrometry vacuum system also the diluents which is used has to be vaporized which leads to damage of the mass spectra is obtain by suppression of the mutual signal.(Figure 5)

LC-MS used to detect compounds from polyaromatic (non-polar) to peptide and proteins. LC-MS used for compounds identification and purity. Used for determination of pesticides, herbicides & organic pollutant for environmental monitoring .

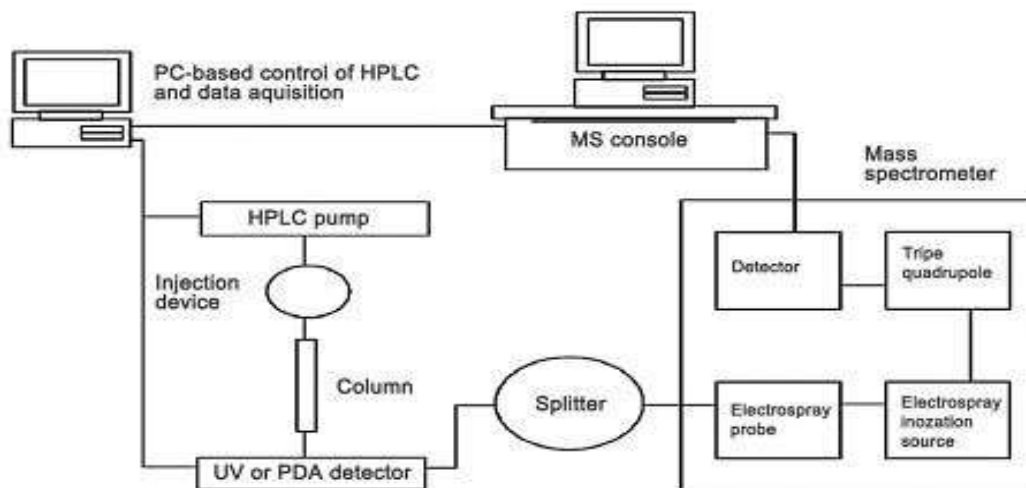


Fig. No. 5: LC-MS

LC-NMR

It is the hyphenated technique in which HPLC is combined with the NMR. This technique is widely used for the analysis of complex mixtures which contain unknown impurities, natural products and synthetic polymers. In LC-NMR LC does the separation and NMR does the identification of the separated components. [12, 13] Among the spectroscopic techniques available to date, NMR is probably the least sensitive, and yet it provides the most useful structural 240 Sarker and Nahar information toward the structure Elucidation of natural products. Technological developments have allowed the direct parallel coupling

of HPLC systems to NMR, giving rise to the new practical technique HPLC-NMR or LC-NMR, which has been widely known for more than last 15 years. [14] The first on-line HPLC-NMR experiment using superconducting magnets was reported in the early 1980s. However, the use of this hyphenated technique in the analytical laboratories started in the latter part of the 1990s only.(Figure 6)

LC-NMR promises to be of great value in the analysis of complex mixtures of all types, particularly the analysis of natural products and drug-related metabolites in bio fluids [15-17].

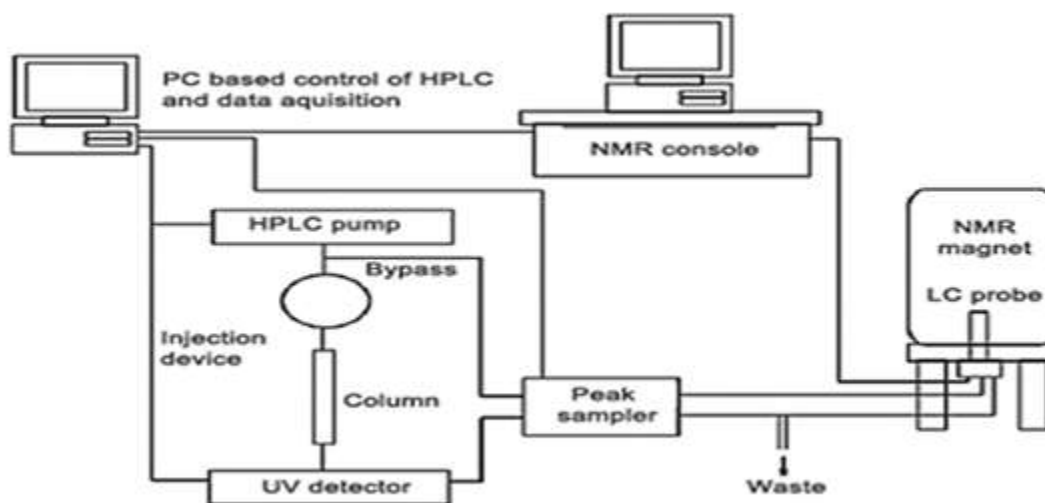


Fig. No. 6: LC-NMR

INSTRUMENTATION AND WORKING

LC-NMR is the sensitive method because the sensitivity of the NMR can be increased by use of highly magnetic field magnets and highly sensitive probes, and maturation of peripheral technologies, such as solvent elimination technology and automatic measurement software suitable for multicomponent analysis. Stronger the external magnetic field is, the higher the sensitivity is the improvement in sensitivity has made for a large reduction in measurement time. The compounds showing complex spectra can also be easily analyzed with the help of increase in magnetic field which will improve the signal resolution.

APPLICATION

1. Identification of drug degradation products.
2. Low level impurities can be isolated and identified.
3. This technique is used for tracking pesticides, herbicides & organic pollutant for environmental monitoring.
4. Differences in electrophoretic motilities and structural information. Capillary electrophoresis (CE) is used for the separation of the components and mass will identify the components separated by CE. This technique is speedy efficient and low solvent and sample is required for the analysis. The CE is connected to the MS with the help of the long capillaries which will increase the analysis time also there is lack of suitable volatile buffer which has to be compatible with MS.[1]

EC-MS

EC-MS is the combination of electrochemistry (EC) mass spectrometry (MS). In this technique by EC one can do the electrochemical oxidation which will directly be introduced into the ESI interface for monitoring short lived intermediated with the help of MS. [2]

APPLICATION

EC/MS provides an easy and rapid means to predict possible oxidation products of xenobiotics in the liver.

More recent applications include the bio affinity screening of electro generated oxidation products, and attempts to predict the allergenic potential of xenobiotics by investigating the peptide and protein adduct formation of their electro-generated products. [1]

CE-MS

CE-MS is online separation technique in which molecules are distinguished according to their differences in electrophoresis motilities and structural information [22]. Capillary electrophoresis (CE) is used for the separation of the components and mass will identify the components separated by CE. This technique is speedy efficient and low solvent and sample is required for the analysis. The CE is connected to the MS with the help of the long capillaries which will increase the analysis time also there is lack of suitable volatile buffer which has to be compatible with MS.[2](Figure 7)

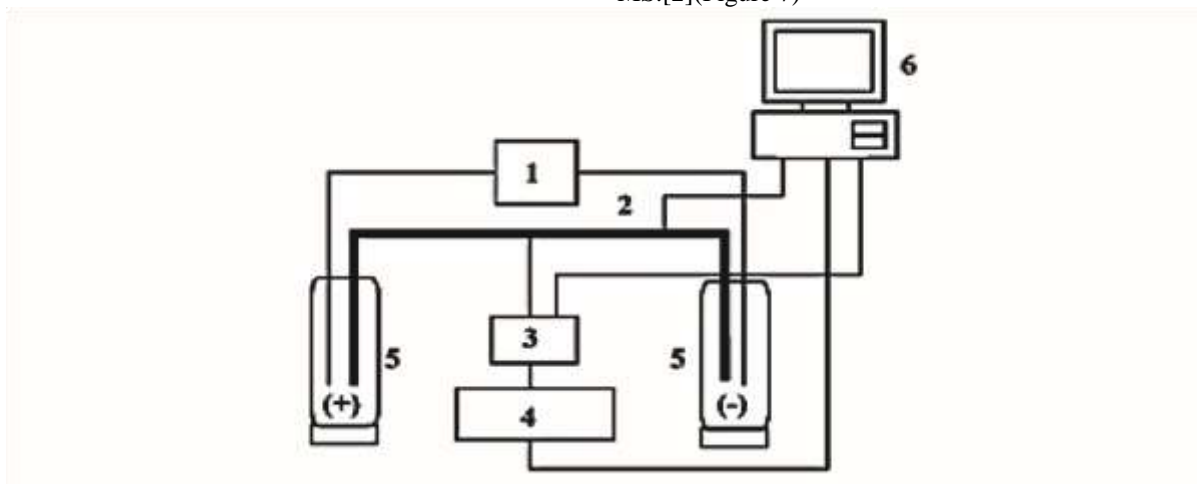


Fig. No. 7: Atypical CE-MS system.

1= High-Voltage Supply; 2= Capillary; 3= UV-vis or PDA detector; 4 = MS detector; 5= Buffer solution; 6= PC control

INSTRUMENTATION & WORKING

The detectors used for the CE analysis are UV and DAD and the electrolytes used in this technique are inorganic and non-volatile. This technique has some drawbacks which include lack of reproducibility, repeatability, selectivity. These problems can be counteracted by applying the buffer system which will coat the inner wall of the fused silica capillaries with double layer. Very small volumetric flow is passed

through the interfaces into the MS. Ionization interfaces used are electro spray; fast atom bombardment interface and ion spray ionizer. Detectors used in mass spectroscopy are TOF, ion trap and Quadruple. Main advantage of quadruple detector is sensitivity.

APPLICATION

Using non-aqueous CE and non-aqueous CE-MS basic and acidic compounds can be identified.

Complex arabino oligosaccharides are analyzed. CE-MS is a tool for drug bio analysis& biomarker

discovery Summary of on-line information obtained from hyphenated techniques [1].

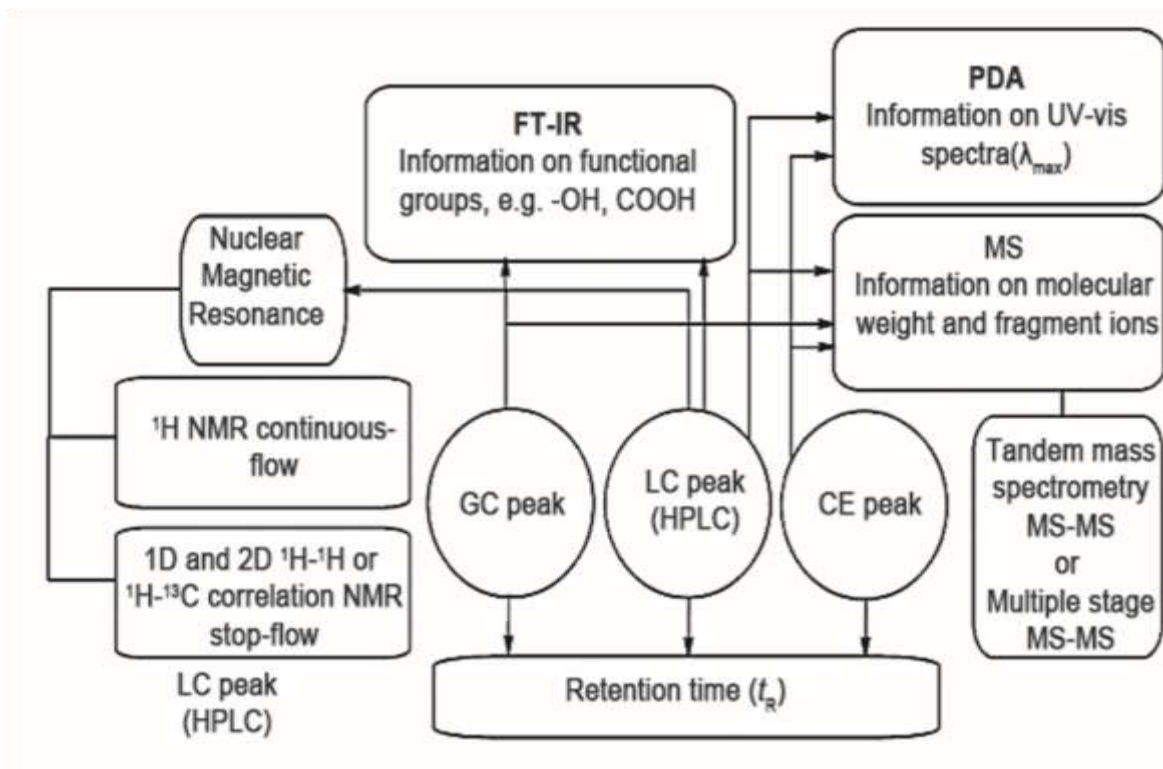


Fig.No.8: Summary of on-line information obtained from hyphenated techniques

GC-IR: Technique is hyphenation of gas chromatography and Infrared spectroscopy. This technique is very sensitive, very expensive, sample recovery is also possible because IR is non-destructive

technique in this technique the GC does the separation part where as IR perform the function of identification.[18-19] (figure 9)

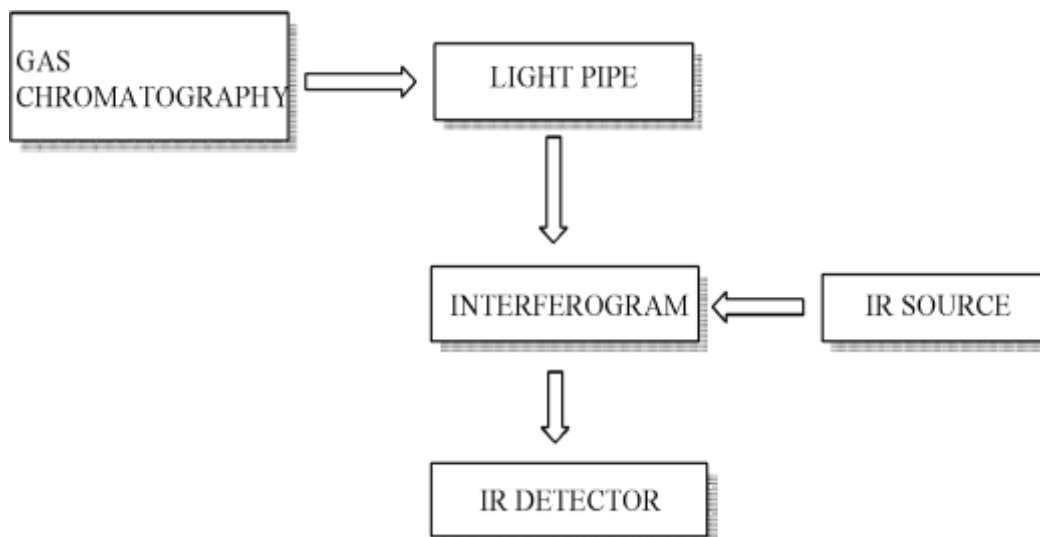


Fig. No. 9: Schematic presentatin of GC-IR

INSTUMENTATION & WORKING

Gas chromatography separate's components of the analyte. These components will travel through the column. These two techniques are linked through glass column or vacuum tubes.

Interface used in this technique is internally gold coated small glass pipe connected to column by narrow tubing. Light pipe is heated in order to rid

condensation and maximize path length for enhanced sensitivity

Application

- 1 Pharmaceutical, Industrial
- 2 DNA Analysis of blood samples, other fluids [1]

LC-MS/ (MS)

LC-MS/ (MS) can detect over 300 compounds of different class by low injection volume. Minimal sample pre-treatment is required and it also reduce the time of analysis. LC-MS is the first step of LC-MS/MS. This technique is more sensitive and specific than that of the LC-MS. it around 20-100 times sensitive than LC-MS. More specific because in this technique second filtering process is also involved the time of analysis. LC-MS is the first step of LC-MS/MS. This technique is more sensitive and specific than that of the LC-MS. it around 20-100 times sensitive than LC-MS. More specific because in this technique second filtering process is also involved [20]

GC-MS/MS

In this technique the gas chromatography is coupled with tandem mass spectrometry. This technique is sensitive as well as specific and can be used for ultra-trace analysis. For qualitative identification with MS/MS, product ion scan, precursor ion scan and neutral loss with a triple quadruple or product scan with an ion trap can be used. In recent years the sensitivity of the quadruples is increased instead of loss also the scanning speed is also high.

Application

- Identification of trace unknown impurities.

- Used for determination of contaminations in environment and foods such pesticides and PCBs in foods and biological samples [21]

GC×GC-MS

In this technique two dimensional GC is coupled with mass spectroscopy. This coupling will lead to the better resolution of the peaks in GC. Sometimes in one dimensional GC the analyte is unable distributed along the whole retention time. The two dimensional GC lead to the better resolution. with the help of two dimensional GC components of the analytes are properly separated due to which in the MS even trace amount components will be identified.

Application

- Large number of samples can be analyzed at the same time.
- Analysis of petroleum, PCBs, complex extracts and food samples is performed by this technique. [22]

GAS CHROMATOGRAPHY-FOURIER TRANSFORM INFRARED SPECTROSCOPY (GC-FTIR)

For novel structures or new chemical entities, it is possible that no matched reference spectra can be found in MS databases. Manual interpretation of mass spectra requires sound knowledge on organic mass spectrometry and dedicated experience and often not possible to suggest any candidate structures. With the help of the molecular spectroscopy FT-IR, information on functional groups or structure moieties with specific infrared absorptions is complementary to MS and can be very valuable for structure elucidation, as already being used as stand-alone.

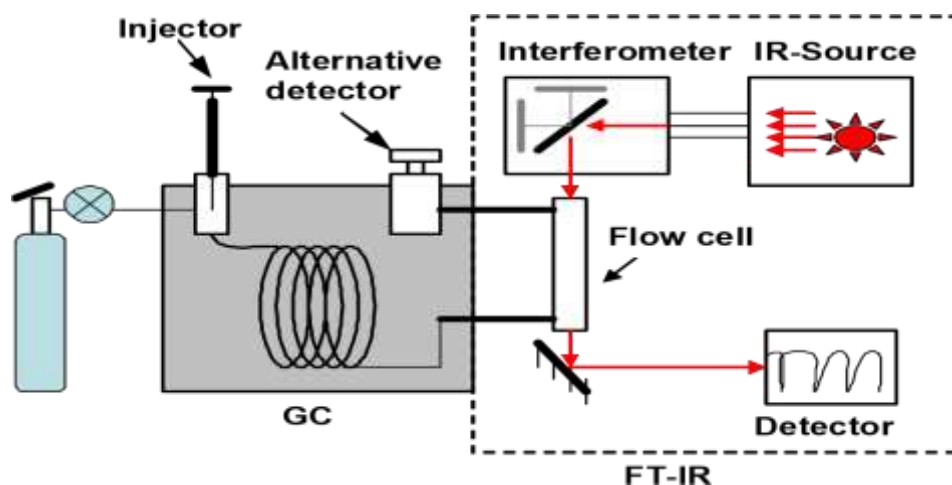


Fig.No.10: Schematic drawing of a typical GC-FTIR

GC-AES

This technique is combination of gas chromatography with atomic emission spectroscopy. Atomic emission spectroscopy is one of the elemental analysis techniques. GC performs the separation of the components and with the help of AES the elemental identification of the components is performed.

Elemental composition of every peak separated by GC is determined. [23]

INSTRUMENTATION

In this process, analytes are first atomized using either ICP or microwave irradiation (high temperatures), where the atoms are transferred to

electronically excited state. Then these electrons are return to the lower energy levels at that time photons are emitted at certain wavelengths that are characteristic of the particular element. In both the techniques sample is in gas phase so the techniques are complementary to each other. The GC effluent is directly introduced into the Quartz atomization furnace via heated nickel transfer line. The interface is simple but in practice the conditions has to be optimised. Quartz furnace, heating with flame or a thermostat, or using the graphite furnace as the atomization device for obtaining good sensitivity and selectivity. [24]

GAS CHROMATOGRAPHY-ATOMIC ABSORPTION SPECTROSCOPY (GC-AAS) AND -ATOMIC EMISSION SPECTROSCOPY (GC-AES)

Atomic absorption spectrometry (AAS) is a well-established detection technique in elemental analysis. In AAS, when the UV light with the right wavelength irradiates on a ground-state free atom, the atom may absorb the light to enter an excited state in a process known as atomic absorption. The wavelength of the light is somehow specific for an element. By measuring the amount of light absorbed, a quantitative determination of the amount of analyte element present can be made. A careful selection of wavelengths allows the specific quantitative determination of individual elements in the presence of others. By supplying enough thermal energy (such as in a flame), analyte compounds dissociate into free atoms. The ease and speed, at which precise and accurate determinations can be achieved, have made AAS one of the most popular methods for the analysis of metals (25-28).

CONCLUSION

The aim of this project is to supply information taken from sources believed to be valid and reliable. This is not an attempt to render any type of professional advice, or analysis, nor is it to be treated as such. While much care has been taken to ensure veracity and currency of the information presented within, neither the publisher, nor its authors bear any responsibility for any damage arising from inadvertent omissions, negligence or inaccuracies (typographical or factual) that may have found their way into this project.

RESULT AND DISCUSSION

A Hyphenated technique is combination or coupling of two different analytical techniques with the help of proper interface. The term hyphenated techniques ranges from the combination of separation-separation, separation-identification & identification-identification techniques. The hyphenation of these techniques leads to better analysis of the components. With the help of this technique we can analyse the drug in short period of time.

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