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A Review on Hyphenated Separation Techniques Used in Pharmaceutical Analysis

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Abstract: *Hyphenated techniques combine chromatographic and spectral methods to exploit the advantage of both. Chromatography produces pure or nearly pure fractions of chemical components in a mixture.The development of the pharmaceuticals brought a revolution in human health. Pharmaceuticals would serve their intent only if they are free from impurities and are administered in an appropriate amount. To make drugs serve their purpose various chemical and instrumental methods were developed at regular intervals which are involved in the estimation of drugs.The review of hyphenated technique includes various techniques which are used nowadays for analysis. Chromatographic techniques GC,LCetc., are used for separation and spectroscopic techniques such as NMR,MS,IR used for identification purpose. Pharmaceuticals may develop impurities at various stages of their development, transportation and storage which makes the pharmaceutical risky to be administered thus they must be detected and quantitated. For thisanalytical instrumentation and methods play an important role. This review highlights the role of the analytical instrumentation and the analytical methods in assessing the quality of the drugs. The review highlights a variety of hyphenated analytical techniques applied in the analysis of pharmaceuticals.*

I. Introduction

In the past ten years, demands on analytical support for drug discovery have intensified. As a result, new technology is continually evolving to meet these challenges. In addition, the use of more established methodologies is being enhanced by incremental improvements in technology and protocol. Hyphenation (combination) of analytical techniques (1,2) is onesuch approach adopted by modern pharmaceuticalanalysts in meeting the needs of today'sindustry.

The new dimension in the area of hyphenated techniques that offers somevery significant benefits in pharmaceutical analysis is that of multi-dimensionalchromatography. Various set-ups involving coupling GC, HPLC and CE systemstogether in different configurations have been studied for analysing manydifferent sample types.(3) Examples include Size exclusion chromatography coupled with RP-HPLC, CE andGC coupled with LC. Since, RP-HPLC and CE techniques are capable ofhigh resolution separation with orthogonal separation mechanisms, combiningboth techniques in a two-dimensional mode can produce very high peakcapacities and extremely high resolving power, particularly useful for complexmixtures.(4)

This hyphenated technique not only provides appropriate sensitivity but also unique capabilities for identification and confirmation of the species of interest.

Summary of hyphenated separation techniques used in pharmaceutical analysis

Separation technique	Hyphenated mode
Liquid chromatography	Liquid chromatography-Mass spectrometry (LC/MS)
	Liquid chromatography-Fourier-transform infrared spectrometry (LC-FTIR)
	Liquid chromatography-Nuclear magnetic resonance spectroscopy (LC/NMR)
	Liquid chromatography-Inductively coupled plasma mass spectrometry (LC-ICPMS)
Gas chromatography	Gas chromatography-Mass spectrometry (GC/MS)
	Gas chromatography-Fourier-transform infrared (GC-FTIR)
	Gas chromatography-FTIR-MS (GC-FTIR-MS)
	Gas chromatography-Inductively coupled plasma mass spectrometry (GC-ICPMS)
Capillary electrophoresis	Capillary electrophoresis-Mass spectrometry (CE/MS)
	Capillary electrophoresis-Nuclear magnetic resonance spectrometry (CE/NMR)
	Capillary electrophoresis-Surface enhanced Raman spectrometry (CE-SERS)
Thin layer chromatography (TLC)	Thin layer chromatography-Mass spectrometry (TLC/MS)
	Thin layer chromatography-Surface enhanced Raman spectrometry (TLC-SERS)
Supercritical fluid chromatography/ extraction (SFC/SFE)	Supercritical fluid extraction-Capillary gas chromatography mass spectrometry (SFE-CGC-MS)
	Supercritical fluid-Fourier transform infrared (SFC-FTIR)

In Pharmaceutical analysis mass spectrometric detection is always preceded by some kind of separation that enables the qualitative and quantitative analysis of the different species by separating them from each other and also from matrix interferences. This kind of techniques are called coupled or hyphenated analytical techniques (5). They consist of two main parts: a separation technique (GC, HPLC or electrophoresis) and a detector (UV, AAS, ICP-MS or ESI-MS) that are connected by an interface. In selenium speciation studies, the most frequently applied coupled analytical techniques are HPLC-ICP-MS, HPLC-ESI-Q-TOF-MS and HPLC-Orbitrap-MS (6).

Application

- Rapid identification and characterization of known and new natural products directly from plant and marine sources without the necessity of isolation and purification
- Isolation and analysis of natural products
- Chemical fingerprinting and quality control of herbal medicine
- Application of coupled analytical techniques became exceptionally popular due to their unmatched advantages. For instance, the separation of target compounds from matrix interferences can remarkably improve the signal-to-noise ratio and therefore enables lower detection limits.
- In this way, absolute detection limits as low as sub-picogram level in GC and femtogram level in LC separations can be achieved (7). In addition to improved quantification, the information about the retention time of target compounds allows their qualitative analysis.
- The coupling of elemental mass spectrometry to HPLC was less complicated than the development of HPLC coupled molecular mass spectrometric techniques. Soon after the development of ICP-MS (8),
- The HPLC-ICP-MS coupling was also introduced by Dean and coworkers(9). It was possible because the flow rate of HPLC separations was compatible with ICP nebulizers that made the direct introduction of the HPLC eluent feasible.

II. Advantages of Hyphenated Technique

The biggest advantage of hyphenated speciation techniques is the ability to detect species other than the pre-conceived compounds. This has been found to be especially true in the analysis of drinking and wastewater, drug discovery, biochemistry and biotechnology, where focus on research is maximum the world over.

Hyphenated techniques offer

- Shorter analysis time
- Higher degree of automation
- Higher sample throughput
- Better reproducibility
- Reduction of contamination because it is a closed system
- Enhanced combined selectivity and therefore higher degree of information

III. Review of Hyphenated Techniques

1. LC/MS:

The coupling of HPLC separation to mass spectrometry for the analysis of non-volatile compounds remained to be unresolved for decades due to technical issues. Developing an adequate interface for the efficient ionization of non-volatile and thermally instable compounds and overcoming the problems of solvent and flow rate incompatibility of HPLC and MS turned out to be quite a challenge (10).

Liquid Chromatography/Mass Spectrometry (LC/MS) is fast becoming the preferredtool of liquid chromatographers. It is a powerful analytical technique that combinesthe resolving power of liquid chromatography with the detection specificity of massspectrometry. Liquid chromatography (LC) separates the sample components andthen introduces them to the mass spectrometer (MS). The MS creates and detectscharged ions. The LC/MS data may be used to provide information about the molecularweight, structure, identity and quantity of specific sample components.

LC-MS or HPLC-MS refers to the coupling of an LC with a mass spectrometer (MS).The separated sample emerging from the column can be identified on the basis of its mass spectral data. A switching valve can help make a working combination of the two techniques. A typical automated LC-MS system consists of double three-way diverter in-line with an auto-sampler, an LC system, and the mass spectrometer. The diverter generally operates as an automatic switching valve to divert undesired portions of the elute from the LC system to waste before the sample enters the MS.

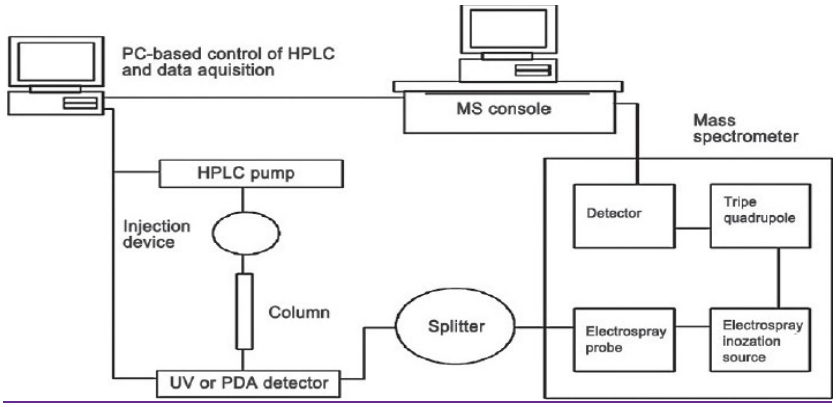

Fig-1: Schematic diagram of an LC-MS (electrospray ionization interface) system

This qualitative analysis makes it possible to reconstruct an unknown compound from MS data. The ionization techniques used in LC-MS are generally soft ionization techniques that mainly display the molecular ion species with only a few fragment ions. Hence, the information obtained from a single LC-MS run, on the structure of the compound, is rather poor. However, this problem has now been tackled by the introduction of tandem mass spectrometry (MS-MS), which provides fragments through collision-induced dissociation of the molecular ions produced (11). The use of LC-MS-MS is increasing rapidly. Hyphenated techniques such as HPLC coupled to UV and mass spectrometry (LC-UV-MS) have proved to be extremely useful in combination with biological screening for a rapid survey of natural products.

Nowadays, various types of LC-MS systems incorporating different types of interfaces are available commercially. The interfaces are designed in such a way that they offer adequate nebulization and vaporization of the liquid, ionization of the sample, removal of the excess solvent vapour, and extraction of the ions into the mass analyser. The two most widely used interfaces, especially in relation to natural product analysis, are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). The latter is considered as "the chromatographer's LC-MS interface" because of its high solvent flow rate capability, sensitivity, response linearity, and fields of applicability. (12)

2. GC-MS:

With MS as the preferred detection method, and single- and triplequadrupole, ion trap and time-of-flight (TOF) mass spectrometers as the instruments most frequently used, both LC-MS and GC-MS are the most popular hyphenated techniques in use today. (13) GC-MS, which is a hyphenated technique developed from the coupling of GC and MS, was the first of its kind to become useful for research and development purposes. Mass spectra obtained by this hyphenated technique offer more structural information based on the interpretation of fragmentations. The fragment ions with different relative abundances can be compared with library spectra. Compounds that are adequately volatile, small, and stable in high temperature in GC conditions can be easily analyzed by GC-MS. Sometimes, polar compounds, especially those with a number of hydroxyl groups, need to be derivatized for GC-MS analysis. The most common derivatization technique is the conversion of the analyte to its trimethylsilyl derivative. In GC-MS, a sample is injected into the injection port of GC device, vaporized, separated in the GC column, analyzed by MS detector, and recorded. The time elapsed between injection and elution is called "retention time" (R_t). The equipment used for GC-MS generally consists of an injection port at one end of a metal column (often packed with a sand-like material to promote maximum separation) and a detector (MS) at the other end of the column.

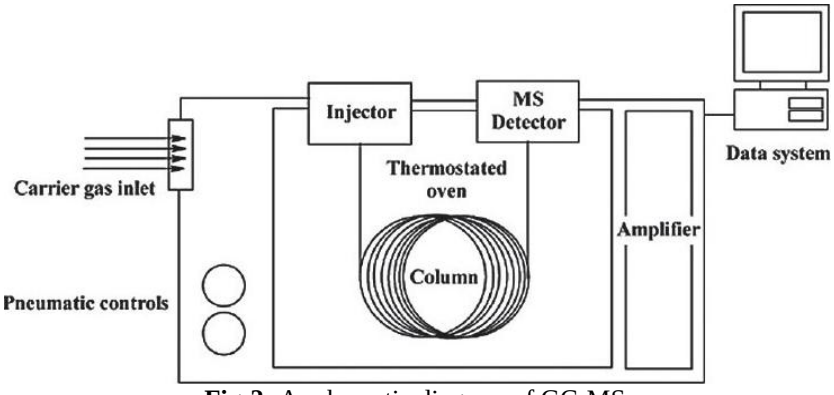

Fig-2: A schematic diagram of GC-MS

A carrier gas (argon, helium, nitrogen, hydrogen, to name a few) propels the sample down the column. The GC separates the components of a mixture in time and the MS detector provides information that aids in the structural identification of each component.

The GC-MS columns can be of two types: capillary columns, macrobore columns and packed columns. The following points need to be considered carefully regarding the GC-MS interface.

1. The interface transports efficiently the effluent from the GC to MS.
2. The analyte must not condense in the interface.
3. The analyte must not decompose before entering the MS ion source.
4. The gas load entering the ion source must be within the pumping capacity of the MS.

The most extensively used interfaces for a GC-MS are electron impact ionization (EI) and chemical ionization (CI) modes. However, in modern GC–MS systems, various other types can be used that allow identification of molecular ion. For example, an orthogonal TOF mass spectrometry coupled with GC is used for confirmation of purity and identity of the components by measuring exact mass and calculating elemental composition. Nowadays, a GC-MS is integrated with various on-line MS databases for several reference compounds with search capabilities that could be useful for spectra match for the identification of separated components.

3. LC-FTIR:

The hyphenated technique developed from the coupling of an LC and the detection method infrared spectrometry (IR) or FTIR is known as LC-IR or HPLC-IR. While HPLC is one of the most powerful separation techniques available today, the IR or FTIR is a useful spectroscopic technique for the identification of organic compounds, because in the mid-IR region the structures of organic compounds have many absorption bands that are characteristic of particular functionalities, e.g., –OH, –COOH, and so on. However, combination of HPLC and IR is difficult and the progress in this hyphenated technique is extremely slow because the hyphenated technique's 237 absorption bands of the mobile phase solvent are so huge in the mid-IR region that they often obscure the small signal generated by the sample components.

In addition, as a detection technique, IR is much less sensitive compared to various other detection techniques, e.g., UV and MS. The recent developments in HPLC-IR technology have incorporated two basic approaches based on interfaces applied in HPLC-IR or HPLC-FTIR. One is a flow-cell approach and the other is a solvent-elimination approach. The approach used with the flow cell in LC-IR is similar to that used in UV–Vis and other typical HPLC detectors. In this case, absorption of the mobile phase induces the interference of the detection of sample component absorption bands, but some transparent region of the mid-IR range produces detection possibility. For example, if one uses a mobile phase of a deuterated solvent such as heavy water or deuterated methanol, IR can monitor many organic compounds that have C–H structures in the molecules. The solvent-elimination approach is the preferred option in most of the LC-IR operations. After the mobile phase solvent is eliminated, IR detection is carried out in some medium that has a transparency for IR light.

Generally, KBr or KCl salts are used for the collection of sample components in the eluent, and heating up the medium before IR detection eliminates the volatile mobile phase solvents. There are two types of interfaces for the solvent-elimination approach: diffuse-reflectance infrared Fourier transform (DRIFT) approach and buffer-memory technique. (14,15) A unified interface for GC, HPLC, and SFC hyphenation to FTIR applying IR microscopic technique is also available today.(16)

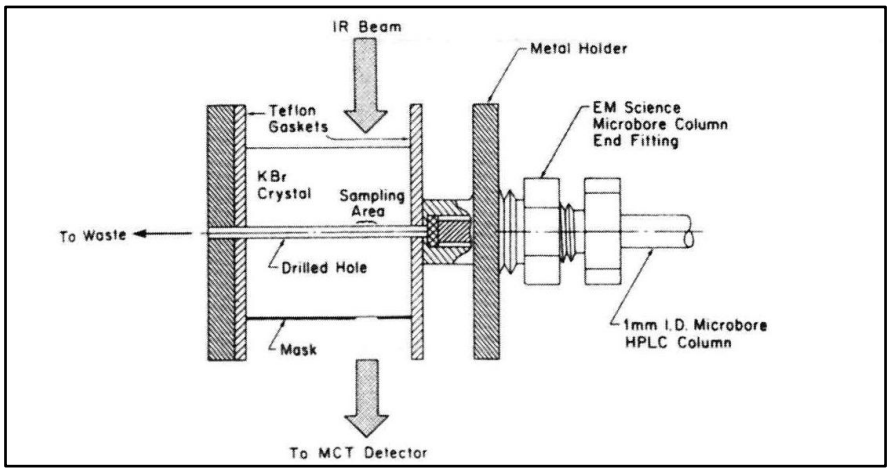

Fig-3: A schematic diagram of LC-FTIR

4. GC-FTIR:

Infrared spectroscopy is considered a confirmation method for theidentification of organic compounds due to the uniqueness of infraredspectra for very similar organic molecules. Gas chromatography coupledwith transform Fourier infrared spectrometry is capable of obtainingInfrared spectra from the peaks as they elute from the capillary columnsthus combining the separation power of gas chromatography with theidentification power of infrared spectrometry.

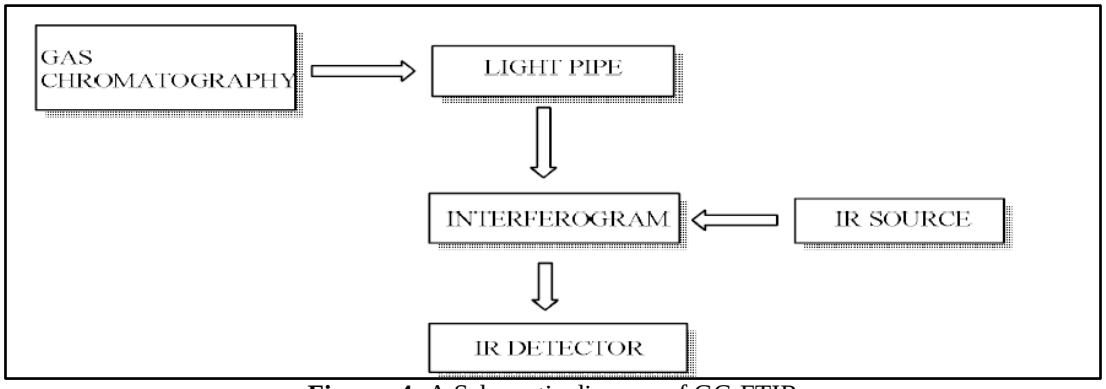

Figure- 4: A Schematic diagram of GC-FTIR

5. LC-NMR:

Among the spectroscopic techniques available to date, NMR is probably the least sensitive, and yet it provides the most useful 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. 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. 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.

LC-NMR experiments can be performed in both continuous-flow and stop-flow modes. A wide range of bio analytical problems can be addressed using 500, 600, and 800 MHz systems with 1H, 13C, 2H, 19F, and 31P probes. The main prerequisites for on-line LC-NMR, in addition to the NMR and HPLC instrumentation, are the continuous-flow probe and a valve installed before the probe for recording either continuous-flow or stopped-flow NMR spectra. (17). A UV–Vis detector is also used as a primary detector for LC operation. Magnetic field strengths higher than 9.4 T are recommended, i.e., 1H resonance frequency of 400 MHz for a standard HPLC-NMR coupling. The analytical flow cell was initially constructed for continuous-flow NMR acquisition. However, the need for full structural assignment of unknown compounds, especially novel natural products, has led to the application in the stopped-flow mode.

In fact, the benefits of the closed-loop separation–identification circuit, together with the prospect of using all presently available 2D and 3D NMR techniques in a fully automated way, have prompted the development of stopped-flow modes, e.g., time-slice mode.

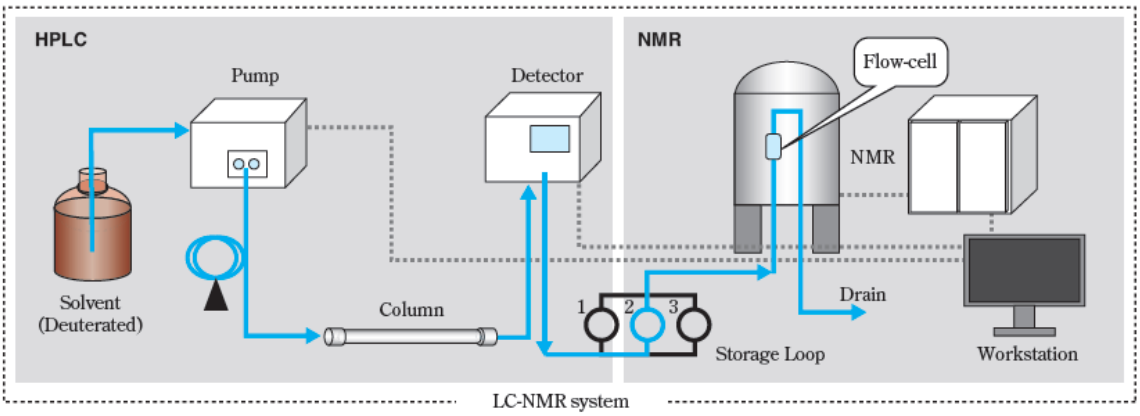

Fig -5:A Schematic diagram of LC-NMR system

Generally, in LC-NMR system, the LC unit comprises auto-sampler, LC pump, column, and a non-NMR detector (e.g., UV, DAD, EC, refractive index, or radioactivity). From this detector, the flow is guided into the LC-NMR interface, which can be equipped with additional loops for the intermediate storage of selected LC peaks. The flow from the LC-NMR interface is then guided either to the flow-cell NMR probe-head or to the waste receptacle. Following passage through the probe-head, the flow is routed to a fraction collector for recovery and further investigation of the various fractions analyzed by NMR. An MS can also be attached to the system via a splitter at the output of the LC-NMR interface.

In most of the LC-NMR operations, reversed-phase columns are used, employing a binary or tertiary solvent mixture with isocratic or gradient elution. The protons of the solvents of the mobile phase cause severe problems for obtaining an adequate NMR spectrum. The receiver of the NMR spectrometer is not quite able to handle the intense solvent signals and the weak substance signals at the same time. To overcome this problem, solvent signal suppression can be achieved by one of the three major methods: presaturation, soft-pulse multiple irradiation or water suppression enhancement through T1 effects (WET) presaturation employing a z-gradient. (17) This problem can also be minimized by considering the following guidelines:

1. Using eluents that have as few 1H NMR resonances as possible, e.g., H_2O, ACN, or MeOH.
2. Using at least one deuterated solvent, e.g., D2O (approx $290/L), ACN-d3 (approx $1600/L), or MeOD (approx $3000/L).
3. Using buffers that have as few 1H NMR resonances as possible, e.g., TFA or ammonium acetate.
4. Using ionpair reagents that have as few 1H NMR resonances as possible, e.g., ionpairs with *t*-butyl groups create an additional resonance.

6. CE-MS:

CE is an automated separation technique introduced in the early 1990s. CE analysis is driven by an electric field, performed in narrow tubes, and can result in the rapid separation of many hundreds of different compounds. The versatility and the many ways that CE can be used mean that almost all molecules can be separated using this powerful method. It separates species by applying voltage across buffer-filled capillaries, and is generally used for separating ions that move at different speeds when voltage is applied, depending on their size and charge. The solutes are seen as peaks as they pass through the detector and the area of each peak is proportional to their concentration, which allows quantitative determinations. Analysis includes purity determination, assays, and trace level determinations. When an MS detector is linked to a CE system for acquiring on-line MS data of the separated compound, the resulting combination is termed as CE-MS.

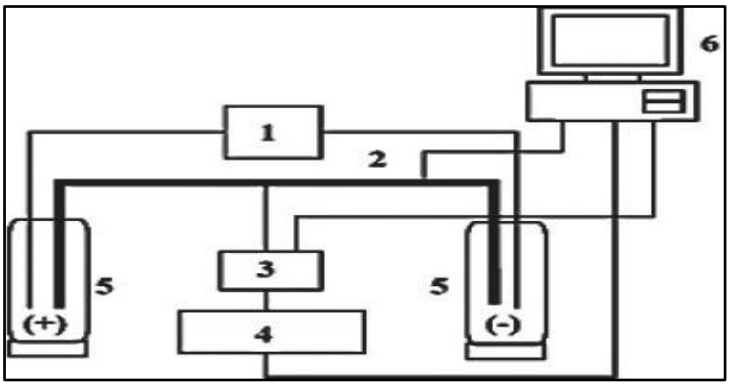

Fig-6:A Schematic diagram of CE-MS system.

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

Separation is achieved through channels etched on the surface of the capillary (connected to an external high-voltage power supply) that delivers sample to ESIMS. This technique runs in full automation and offers high degree of sensitivity and selectivity. A new type of interface, known as coaxial sheath liquid CE-MS interface, has been developed recently, which allows the use of both LC-MS and CE-MS alternatively on the same mass spectrometer.(18) The necessary sheath liquid is delivered by a pump that floats on the ion sprayer of the MS, avoiding any current flow toward ground. LC-MS and CE-MS modes can be switched within minutes. To obtain a stable ion spray and to avoid electrical problems, the CE power supply is used to produce the potential for the CE separation and the ESI sprayer tip simultaneously. ESIMS detection technique is generally used in most of the CE-MS systems because ESI is considered to be one of the most powerful on-line tools for the analysis of biomolecules, including natural products, providing both the molecular weight and structural characterization of analyte.(19) The optimization of the interfacing of CE with MS can be a real challenge because of the low flow rates (10–100 mL/min) required in CE, which is achieved by a make-up liquid.

7. GC with ICPMS Detection:

Many element species of interest in environmental specification analysis are non-volatile and cannot be converted into such by means of derivatization. They include virtually all the coordination complexes of trace metals but also many truly organometallic compounds.

The choice of the hyphenated setup depends primarily on the research objective. The separation component of the coupled system becomes a particular concern when a high degree of species specifically required. It may even be necessary to combine two or more separation techniques in series to assure that a unique species arrive at a certain time at the detector. (20-22)

In contrast to speciation analysis of the anthropogenic pollutants realised mostly by GC-ICP MS for which analytical standards are available. The majority of species of interest in biological environmental trace analysis have not yet been isolated sufficiently pure to be used as retention time standards.

8. LC with ICP MS Detection:

The principal HPLC mechanisms used in environmental speciation analysis include size exclusion, ion-exchange and reversed phase chromatography. Capillary electrophoresis is less mature but offers exciting possibilities for speciation analysis owing to the high separation efficiency, the nanolitre sample requirement and absence of packing susceptible to interact with metals and to affect the Complexation equilibria(23-24).

9. GC–FourierTransform IR–MS:

This brief review is intended to summarize, recent applications of GC– FT-IR–MS analysis. The main requirement for mention here is that both IR and MS spectra were collected from each component separated by GC, thus excluding papers where a separation stage does not precede spectral collection. Although it was required that both IR and MS spectra be obtained, it was not necessary that they be obtained simultaneously,i.e. we have included consideration of applications where separate GC–MS analysis and GC–FT-IR analyses were conducted. Because we recently reviewed this area (25), overlap is intentionally kept to a minimum. Although a great deal of work involving GC–MS and GC–FT-IR individually been reported since the last review, research involving joint use of both methods of detection is more limited.

10. CE-NMR :

The low-mass sample requirements with high separation efficiencies and fast separations are keys to the success of CE. Combined CE–NMR can offer the separation capability of CE and the superior detection of NMR, although the smaller volumes and shorter residence time of analytes makes NMR detection problematic in CE.

Analyte peaks in CE typically contain low nanoliter volumes. High resolution CE electro-pherograms and NMR spectra can be obtained using nano-litre volumes; however the obtainable NMR sensitivity often precludes the use of small NMR active volumes. CE–NMR experiments histidine in phosphate buffer. In this report a have been successfully performed with microcoils detection limit of 336ng (2.3nmol) has been having observe volumes as small as 5nl. A high filling factor is one of the advantages of using microcoils directly wrapped on CE capillary. How-ever, this excludes the ability to change the observe volume.

These problems have been solved with sleeve probe technology in which the solenoidalcoilis wrapped around a polyimide sleeve which canhouse different sizes of CE capillaries. Highresolutionspectra with linewidths of 1–2 Hz can beobtained with this new coil fabrication approach.With a saddle coil NMR probe as the online NMRdetector, CE–NMR data can be recorded in acontinuous mode.

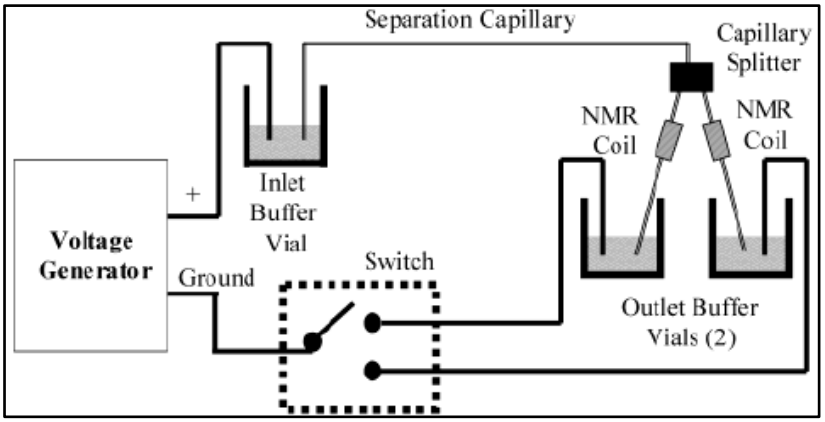

Fig-7: A Schematic diagram of CE-NMR system.

11. TLC-MS:

TLC to be combined with mass spectrometry (MS)--one of the most efficient analytical tools for structural elucidation. So far, many different TLC-MS techniques have been reported in the literature; some are commercially available. According to differences in their operational processes, the existing TLC-MS systems can be classified into two categories:

(i) Indirect mass spectrometric analyses, performed by scraping, extracting, purifying, and concentrating the analyte from the TLC plate and then directing it into the mass spectrometer's ion source for further analysis;

(ii) Direct mass spectrometric analyses, where the analyte on the TLC plate is characterized directly through mass spectrometry without the need for scraping, extraction, or concentration processes. Conventionally, direct TLC-MS analysis is performed under vacuum, but the development of ambient mass spectrometry has allowed analytes on TLC plates to be characterized under atmospheric pressure.

Thus, TLC-MS techniques can also be classified into two other categories according to the working environment of the ion source: vacuum-based TLC-MS or ambient TLC-MS. This review article describes the state of the art of TLC-MS techniques used for indirect and direct characterization of analytes on the surfaces of TLC plates.(27)

12. SFC-FTIR:

Super critical fluid chromatography coupled to Fourier transform infrared spectroscopy (SFC-FTIR) combines high resolution chromatographic separation of thermally labile, high molecular weight materials with online finger printing of eluted components by infra-red spectroscopy. With supercritical xenon as the mobile phase, the system becomes a powerful and cost effective analytical tool with high sensitivity.

The most successful approach to coupling SFC and FTIR has been solvent elimination. The eluent is deposited on a cooled IR transmitting window and subsequently analysed spectorscopically, using an Infrared microscope.

The technique has the vantage of allowing multiple scans of small samples with a corresponding increase in sensitivity. (28)

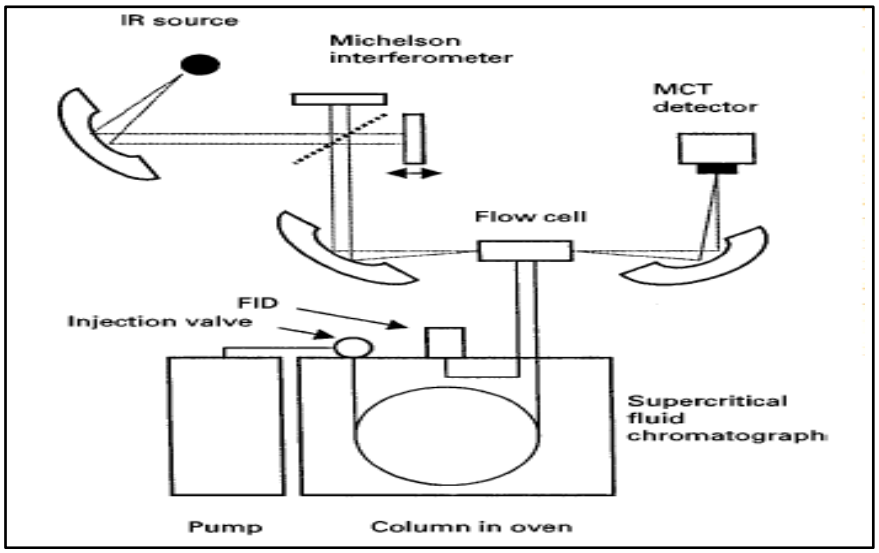

Fig-8: A Schematic diagram of SFC-FTIR system.

IV. Conclusion

The technique developed from the coupling of a separation technique and an on-line spectroscopic detection technology is known as hyphenated technique. The remarkable improvements in hyphenated analytical methods over the last two decades have significantly broadened their applications in the analysis of biomaterials, especially natural products. In this article, recent advances in the applications of various hyphenated techniques, e.g., GC-MS, LC-MS, LC-FTIR, LC-NMR, CE-MS, etc. in the context of pre-isolation analyses of crude extracts or fraction from various natural sources, isolation and on-line detection of natural products, chemotaxonomic studies, chemical fingerprinting, quality control of herbal products, dereplication of natural products, and metabolomic studies are discussed with appropriate examples. Particular emphasis is given on the hyphenated techniques that involve LC as the separation tool.

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