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A COMPREHENSIVE REVIEW ON HYPHENATED TECHNIQUES

Y. Venkateswara Rao*[1], J.Chanti Babu[1], D.Rupa[1], K.V.S Tarun Kumar[1], P.Srinivasa Babu[2]

[1]Department of Pharmaceutical Analysis, Vignan Pharmacy College, Vadlamudi, Guntur, 522213, Andhra Pradesh, India.

[2]Department of Pharmaceutics, Vignan Pharmacy College, Vadlamudi, Guntur, 522213 Andhra Pradesh, India.

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Abstract

The hyphenated technique refers to the combination (or) fusion of distinct analytical methodologies. Spectroscopic techniques are mostly paired with chromatographic techniques. After that, an interphase will allow the mixture's separated components from the chromatographic approach to enter the spectroscopic technique. In GC-MS The separated components from the gas chromatography process are introduced into the mass spectrometer, where they undergo ionization, mass analysis, and measurement of the mass-to-charge ratios of the ions produced by each analysis. GC and MS can be connected by a membrane separator, jet/orifice separator, or effusion separator. The analytical flow cell in LC-NMR coupling was originally designed for continuous flow to NMR. However, the application in the stopped-flow mode in LC-MS has resulted from the requirement for a thorough structural study of novel natural compounds. The use of LC-MS-MS is growing daily at a rapid rate. When used with biological screening, hyphenated techniques like mass spectrometry (LC-UV-MS) and HPLC coupled to UV have proven to be very helpful for a quick analysis of natural products. Currently, a wide range of LC-MS systems with diverse interface options are offered for sale. Hyphenated procedures are methods of separation and identification that demonstrate improved sample analysis through the use of specificity, accuracy, and precision as constituents.

Keywords: HPLC, LC-MS/MS, NMR, GC-MS, IR, FT-IR.

***Corresponding Author**

Y. Venkateswara Rao

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Introduction

Hyphenated technique is about combining two distinct analytical approaches with the aid of an appropriate interface are known as a hyphenated methodology [1]. Spectroscopic techniques are primarily paired with chromatographic procedures The pure or almost pure portions of the chemical components in a combination were separated and selective information is used for identification using standards or library spectra is produced by spectroscopy. A hyphenated technique will result from the combination of the separation process with an online spectroscopic detection technology [2].

A hyphenated technique fuses the two distinct analytical methods with the aid of an appropriate interface [3].The combination of separation-separation, separation-identification, and identification-identification approaches are all included in the phrase "hyphenated techniques."

Hirsch Feld used the term "hyphenation" in 1980 to refer to the potential fusion of two or more instrumental analytical techniques in a single run (Hirschfeld, 1980). In comparison to using a single analytical method, the coupling aims to provide an information-rich detection for both identification and quantification.

Advantages:

1. It is used for fast and accurate analysis
2. It has a higher degree of automation.
3. Higher sample throughput
4. Better reproducibility.
5. It helps in reducing of contamination due to its closed system.
6. Separation of quantification at the same time [20]

Types of Hyphenated Techniques:

1. Double hyphenated techniques.
2. Triple hyphenated techniques.

1. Double hyphenated techniques

Liquid chromatography-mass spectrometry (LC-MS)

Liquid chromatography – Nuclear magnetic resonance spectroscopy (LC-NMR) Liquid chromatography-Infrared spectroscopy (LC-IR)
Continuous emission-monitoring system (CE-MS)
Gas chromatography-infrared spectroscopy (GC-IR)
Gas chromatography-mass spectrometry (GC-MS)
High performance liquid chromatography-photodiode-array Detection (HPLC-DAD) Gas chromatography- Fourier transform infrared spectroscopy (GC-FTIR)

2. Triple Hyphenated Techniques

Liquid chromatography-Atmospheric pressure ionization mass spectrometry (LC-API- MS)
Atmospheric pressure chemical ionization mass spectroscopy monitoring system (APCI- MS-MS)
Electron spray ionization mass spectroscopy (ESI-MS-MS)
Large volume injection gas chromatography mass spectrometry (LVI-GC-MS)
Liquid chromatography Electron spray ionization mass spectroscopy (LC-ESI-MS)
Liquid chromatography mass spectroscopy electron spray ionization (LC-UV-NMR-MS- ESI)
Liquid chromatography mass spectroscopy nuclear magnetic resonance mass spectroscopy (LC-MS-TSPLC-UV-NMR-MS)
Liquid chromatography nuclear magnetic resonance mass spectroscopy (LC-NMR-MS)
Liquid chromatography photodiode array detection mass spectroscopy (LC-DAD-API-MS)
Liquid chromatography Photodiode array detection mass spectroscopy (LC-PDA-MS)
Liquid chromatography Nuclear magnetic resonance mass spectroscopy (LC-PDA-NMR-MS SPE-LC-MS)

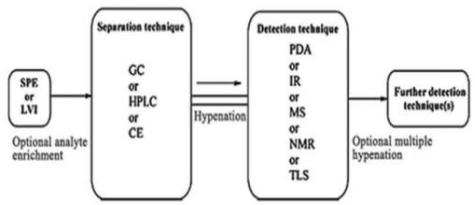

Fig:-1 Schematic presentation of Hyphenation of chromatographic and spectrometric techniques (3)

Double Hyphenated Techniques:

1. GC-MS:

Gas chromatography:

Gas chromatography is mainly used when the substance that is being used has the potential to become volatile in gas stream. Usually, to accomplish this, 0.1–1µL of sample is injected into an injection port that has been heated to 250ºC [4]. The sample is in the vapour phase in both of these methods, which make them quite compatible. However, the inconsistency and the difference between the two approaches is that mass spectroscopy operates at a vacuum between 10^-6 and 10^-5 torr, while gas chromatography runs at a high pressure of 760 torrs, where the gas carrier is present.

Instrumentation and Working:

The separation occurs in the GC column when the vaporized air gas is carried through the heated air gas; this carrier is also referred to as the mobile phase (helium). Due the results of various interaction between the analyte, mobile phase, stationary phase the chemicals will seperate. The characteristics of the stationary phase, the kind of carrier gas, the gradient temperature of the column, and the column's length, diameter, and film thickness all affect the analyte's separation. As the sample moves through the column, the mixture's constituent will separate eventually due to variations in boiling points and other chemical characteristics. As there is difference in absorption or difference in partition coefficient the components will have differences in elution time and retention time [10]. After that, through an interphase which will allow the mixture's separated components to enter the MS. After that follow is the ionization, mass analysis and detection of mass-to-charge ratios of ions generated each analyte by the mass spectrometer. The GS and MS can be connected by forming an interface like effusion separator, jet/orifice separator and membrane separator. This type of ionisation not only breaks the molecule but also divides the molecule into positive and negative modes.

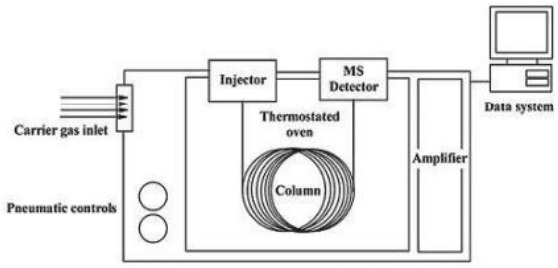

Fig 2. Schematic presentation of GC-MS.

Advantages of GC-MS:

- GC having the high resolution power to compare other methods.
- This method having high sensitivity when used with thermal detectors.
- This technique having separation and analysis of sample very quickly samples with less quality is and also operated. This technique having relatively good accuracy and precision.

Disadvantages:

In GC only volatile samples (or) the sample with can be made volatile and are separated by this method.
During injection of the gas sample, proper attention is required.

2. LC-MS:

Instrumentation and working:

It is a chemical technique that combines physical separation of liquid chromatography of HPLC with mass spectrometer. The components of a standard automated LC-MS system are an autosampler, mass spectrometer, LC system, and double three-way diverter. The diverter typically functions as an automatic switching valve to direct undesirable portions of the eluting to waste before

the sample enters into MS. These ionization techniques are generally soft techniques which mainly display the molecular ions with only a few fragment ions. Confirmation of the compound's identity cannot be achieved with the data obtained from a single LC-MS run. This problem can be simply solved by introducing tandem mass spectrometry (MS-MS) which helps to produce molecular ions which are produced through collision induced dissociation. The use of LC-MS-MS is growing daily at a rapid rate. Hyphenated techniques such as HPLC coupled to UV and mass spectrometry (LC-UV-MS) in combination with biological screening have proven to be incredibly beneficial for a quick survey of natural compounds. These days, a variety of commercially available LC-MS systems with diverse interface types are available. The interfaces are engineered to provide sufficient liquid nebulization and vaporization, ionization of the sample, extraction of the ions into the mass spectrometer, and elimination of the surplus solvent vapour. The two most popular interfaces are atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), particularly in the context of natural product analysis. The latter is referred regarded as "the chromatographer's LC-MS interface" due to its broad applicability, sensitivity, linear response, and high solvent flow rate capabilities. Different types of analysers, such as quadruple, ion trap, or TOF, can be utilized with these interfaces. However, the mass accuracy and resolution provided by each of these analyser's may varies. Thermospray (LC-TSP-MS) and continuous-flow FAB (LC-CF-FAB) interfaces can also be used in the LC-UV-MS mode. The TSP interface has been determined to be the most appropriate for phytochemical analysis because it enables the entrance of an aqueous phase into the MS system at a flow rate (1-2 ml/min) that is consistent with the typical flow rate used in phytochemical analysis [5].

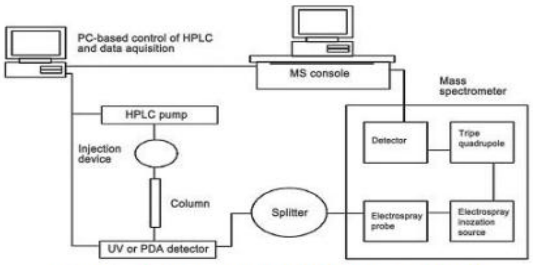

Figure 3: Liquid chromatography- Mass spectroscopy. (2)

3. LC-NMR:

Instrumentation and working:

NMR has the least sensitivity, despite that it helps mainly in understanding the natural product structures (240 Sarker and Nahar)[10]. Due to the technological advancements, HPLC systems and NMR can now be coupled directly in parallel, creating the useful new method known as HPLC-NMR or LC-NMR, which has gained widespread recognition during the past fifteen years. In 1980s, the first HPLC-NMR experiment conducted online with super conducting magnets had a report. In 1990s this hyphenated technique was used in analytical laboratories. LC-NMR played a main role in the investigation of natural products and drug related metabolites in biofluids. LC-NMR was done in stop flow & continuous flow modes. The broad variety of bioanalytical issues was handled by the 1H, 13C, 2H, 19F and 31P probes 500, 800 and 900 MHZ systems. Apart from NMR and HPLC equipment, the basic requirements for online LC-NMR are a valve input in front of the continuous-flow probe that allows it to record stopped-flow or continuous-flow NMR spectra. For LC operation, a UV–visible detector also used as the primary detector. Use of magnetic field strengths more than 9.4 T or 400 MHz for a typical HPLC-NMR coupling is advised, which is the 1H resonance frequency. The main aim of the analytical flow cell's construction was continuous-flow NMR acquisition. Nonetheless, the purpose for complete structural assignment of unidentified compounds—particularly novel natural products has prompted the use of the stopped-flow method. In reality, the development of stopped-flow modes, such as time-slice mode, has been spurred by the advantages of the closed-loop separation–identification circuit as well as the potential for fully automated use of all currently existing 2D and 3D NMR techniques. Figure depicts a typical LC-NMR setup.

The auto sampler, LC pump, column, and a non-NMR detector (such as UV, DAD, EC, refractive index, or radioactivity) often make up the LC unit in an LC-NMR system.LC-NMR has the capacity to accommodate extra loops that why the flow will be sent tom the LC-NMR interface for the analysis of particular peaks. The flow from the LC-NMR interface is then directed either to the NMR probe head of the flow-cell or to the waste receptacle. After that for the recovery and additional study of the different fractions the flow will be directed to the fractional collector and it will be examined by passing through the NMR probe head. An additional splitter is placed to connect the LC-NMR system to the MS.

Reversed-phase columns with binary or tertiary solvent mixtures and isocratic or gradient elution are utilized in the majority of LC-NMR activities. Having protons in the mobile phase will make it hard to get good NMR spectra. The NMR spectrometer's receiver is not nearly capable of handling the simultaneous presence of the weak drug signals and the strong solvent signals. One of the three main approaches pre-saturation, soft-pulse repeated irradiations, or water suppression enhancement through T1 effects (WET) pre-saturation using a z-gradient can be used to tackle this issue and suppress the solvent signal. Additionally, this issue can be reduced by taking into account the following Guidelines.

1. Using eluents that have as few 1H NMR resonances as possible, e.g., H2O, ACN, or Me OH.
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 ion pair reagents that have as few 1H NMR resonances as possible, e.g., ion pairs with t-butyl groups create an additional resonance.

Time-sliced acquisition, stopped-flow acquisition, and continuous-flow acquisition are the three primary forms of data acquisition modalities that have been introduced thus far. For any LC-NMR analysis, an optimal HPLC separation is essential, regardless of the acquisition modality. Since other hyphenated techniques, such as LC-MS or LC-PDA, have significantly greater sensitivity than LC-NMR, it is essential to design an appropriate LC separation where the amount of the smallest possible elution volume contains the concentrated separated chemical that is available. In addition to LC-UV-MS, LC-NMR offers a potentially intriguing method for in-depth online structural investigation. In fact, LC-NMR has received a boost from recent advancements in NMR technology and is currently showing promise as a potent analytical instrument. The development of effective solvent suppression methods makes it possible to measure high-quality LC-1H-NMR spectra under reversed-phase HPLC conditions, both on-flow and stop-flow. Water can be substituted with D2O, and nondeuterated solvents like Me OH or MeCN can be utilized instead of this hybrid method has been revitalized by recent developments in LC and NMR direct connection hardware and software. A novel RF system for multiple solvent suppression and enhanced dynamic range gradient elution capability, a new coil and flow cell design for high sensitivity, and automated capacity for peak-picking and storing. Consequently, this technique is a potent instrument that finds use in numerous domains, including natural products, organic compounds, biomolecules, drug impurities, reaction mixtures, by-products, and drug degradation products. The possibility of using HPLC-NMR to examine and clarify the structure of natural products.

The field of natural products has experienced significant growth because to the development of potent solvent suppression techniques and their application in both homo- and heteronuclear 2D NMR investigations, including 2D nuclear Over Hauser enhancement spectroscopy (NOESY) and 2D total correlation spectroscopy (TOCSY). Despite being around for the previous 20 years, LC-NMR has not yet gained widespread acceptance. This is mostly due to its higher cost and inferior sensitivity when compared to other hyphenated techniques. But in any case, the recent technological breakthroughs, particularly with regard to the creation of solvent suppression techniques and pulse field gradients, the enhancement of probe technology, and the advent of high-field magnets (800–900 MHz) have given this technique fresh life.

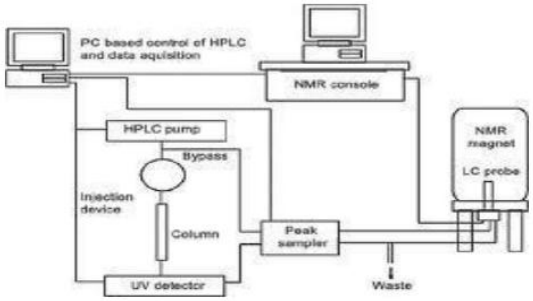

Figure 4: Liquid chromatography- Nuclear Magnetic Resonance (5)

4. LC-IR:

A hybrid technique known as LC-IR or HPLC-IR is a combination of a liquid chromatography (LC) and an infrared spectroscopy (IR) or FTIR (Figure 5) . Even though HPLC is one of the most powerful separation techniques available today, IR and FTIR are useful spectroscopic approaches for identifying organic materials. substances, since the structures of organic compounds have numerous absorption bands in the mid-IR region that are indicative of specific functions, such as -OH, -COOH, and so forth. However, the hyphenated approach's 237 absorption bands of the mobile phase solvent are so large in the mid-IR region that they frequently make combining HPLC and IR challenging, and advancement in this technology is quite slow. that they frequently obfuscate the weak signal that the components of the sample are generating. The form of the sample is significant throughout the measuring process since FT-IR measures absorbance. For a given mass or volume of the analyte, a two-fold reduction in diameter yields a deposit that is four times thicker and four times more optically dense. Since the IR detector is totally light-limited, this two-fold decrease in deposit diameter results in a four-fold increase in signal-to-noise ratio. Consequently, the LC-IR hyphenation method must be improved in order to produce a useful instrument that produces entire mid-infraredspectra.

1. Eliminate the solvent without overflowing the vacuum system with diluent gas or thermally harming the analysis.
2. Ensure that analyses are transmitted to the spectrometer efficiently.
3. In a thick deposit, present analyses to the FT-IR.
4. Maintain the resolution of the chromatography [7].

5. GC-IR:

Instrumentation and working:

Many times, when we're trying to separate stuff in a mixture, it's really important to know what each part is. Just measuring retention data isn't enough. It often gets too complicated to figure out the molecules that come out of a capillary gchromatography (GC) column. This column can actually handle hundreds of different substances. Sometimes, if we already know the chemical structures of the components, it can help. Spiking the mixture with reference standards may also give us some clues. But,

there's an even better way By connecting a chromatograph to a cool, fast-scanning spectrometer.With this setup, we should be able to identify every single component in real-time without messing up the chromatographic resolution. The most common technique used is mass spectrometry (MS). It's great but has some issues, especially when it comes to telling apart structural isomers like ortho-, meta-, and para-xylene.The electron impact and chemical ionization mass spectra for ortho, meta, and para-xylene look the same. A complementary approach to mass spectrometry is needed for these compounds. One alternative method is Fourier transform infrared (FT-IR) spectroscopy. It produces unique spectra for most structural isomers

1. Light-Pipe-Based GC-IR:

Instruments Measurement of the spectrum;

Gas chromatographs and FT-IR spectrometers can be connected using three methods. The GC column is connected directly to the heated SO through the cell in the Rest, which is the most straightforward method. This cell is commonly used for capillary GC using a 10-cm length of heated glass tubing with an internal diameter of one millimeter. Infrared (IR) radiation is very responsive to the inner bore of this tube because it has a layer of gold coating thick enough to make it very responsive. Through heated fused silica lines, the effluent from the GC column enters one of the tube and exits from the opposite end. The entire setup is kept at temperatures between 250 and 300°C to avoid the condensation of semi-volatile substances. A rapid-scanning interferometer is employed to collimate and transmit infrared light from a source such a Sic Global, enabling each wavelength in the spectrum to be modulated at a unique frequency. The resulting radiation beam is directed towards the light-pipe's first window, and the infrared beam that comes out of the second window is then aimed at a sensitive detector, commonly a mercury cadmium telluride (MCT) cooled with liquid nitrogen. A typical system is illustrated schematically in Figure 5. The signal generated through this measurement process is known as an interfere-program, and the Fourier transform of the interfere organ results in a single beam spectrum. This component's transmittance spectrum, T(v), is derived by dividing the single beam spectrum recorded with the component in the light pipe by the single beam spectrum taken when only helium carrier gas is present. The standard operation of Beer's law, A(v), generally converts the transmittance spectrum directly into an absorbance spectrum, A(v). The relative intensities of the bands in absorbance spectra, or log10 T(v), remain independent of the concentration of the analyte, which facilitates spectral library searches. Due to the wide spectral bands in light-pipe-based GC-IR systems, it's seldom necessary to analyze spectra at high resolution. Typically, GC-IR spectra are acquired at a resolution of 8 cm^{-1}, as most bands in the vapor phase molecular spectra have a width of at least 10 cm^{-1}.

4. Matrix-Isolation GC-IR:

In the first approach, argon is introduced to the helium mobile phase either at the end of the gas chromatography (GC) column or as a minor1% component of the carrier gas Subsequently, the heatedluent from the fused silica transfer is sprayed onto a rotating gold-plated maintained at a temperature below 15 K. At this low temperature, argon condenses, whereas helium remains in gas form. The cooled disk serves as the location where the end of the transfer line is positioned to deposit argon in a track approximately 300 meters wide. All components that emerge simultaneously from the transfer line are trapped within the argon matrix. The disk is then rotated to where the focused beam from a Fourier-transform infrared (FT-IR) spectrometer passes through the argon track, reflects off the gold-coated disk, traverses the argon again, and is collected. Once the separation is complete, the collected beam is directed to an MCT detector, as illustrated in Figure 6. The underlying idea is that, ideally, the argon matrix will isolate each analyte molecule from similar molecules, provided the concentration of any analyte is sufficiently low. This technique, referred to as matrix isolation, is employed despite the typical concentration being somewhat too high for true matrix isolation to occur in GC-IR analyses. A series of spectra can be gathered by gradually rotating the disk, resembling the spectra obtained in real-time during a GC-IR run with a light pipe, allowing for the creation of GS or FG chromatograms from the gathered data. Spectral library searches can identify each component; however, a library of matrix-isolated standard spectra is essential.

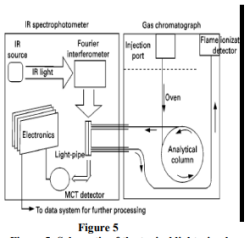

Figure 5

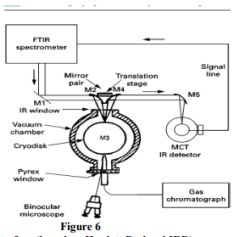

Figure 6

Figure 5: Schematic of the typical light-pipe-based GC-FTIR interface (based on Hewlett Packard IRD).
Figure 6: Schematic diagram of matrix-isolation GC-FTIR interface (Based on Mattson Instruments Collect).

6. CE-MS:

Instrumentation and working:

Capillary zone electrophoresis (CZE) is a very effective analytical method that is well acknowledged for its remarkable separation efficiency, short analysis periods, and low sample volume needs. These characteristics have led to the widespread use of CZE as a technique for the analysis of biotechnology products, protein digests, peptide mixtures, and pharmaceutical substances. As noted by Olivares et al. in 1987, the usefulness of CZE has been further increased by combining it with electrospray ionisation mass spectrometry (ESI-MS). When tandem mass spectrometry (MS-MS) is utilised to gather structural and molecular mass data, this combination is especially helpful. The use of extra parameters to identify eluted components beyond migration time—which varies across

runs—is the main advantage of this connection. When a run of highly diagnostic mass spectra is completed, Depending on the ionisation method used, CZE can be directly (online) or indirectly (offline) coupled to a mass spectrometer. The latter technique enables 252Cf plasma desorption matrix-assisted laser desorption. Online coupling of CZE is increasingly prevalent and is often accomplished by electrospray ionisation (ESI) or rapid atom (ion) bombardment (FAB). The most popular use is online CZE/MS, however it has the benefit of allowing separation in non-volatile buffers, which is less ideal for ESI.Like every analytical technique, CZE/MS has drawbacks, though, most notably low sample concentration and ion sensitivity. Time-of-flight (TOF) monitoring, sample stacking, and online preconcentration are methods to lessen these restrictions. The benefit of using TOF analysers with or without a quadrupole is the ability to get numerous full spectra. This type of instruments is unique in that it can get many complete spectra per second due to its rapid scanning speed. Furthermore, spectra lack the peak skew and mass discrimination common to slow scanning systems that must scan over a small range of chromatographic or electro phonetic peaks since every ion in each spectrum is collected at the same time. Another method that is rapidly developing and advancing at the moment is capillary electrochromatography (CEC). In 1981, Jorgenson and Lukas brought this method back to life. They utilized a 30 kV separation, a 170 m packed column, and 0.005 mol L1 phosphate buffer. voltage to distinguish between polyline and 9-methylanthracene. Due to several developments in CE instruments and detection methods, such as electrospray mass spectrometry, this technology has recently spread more widely. Nonetheless, the most widely utilized techniques are still in-column laser-induced fluorescence detection and on-column UV detection. The latter approach is susceptible to buffer fluorescence interferences despite its high sensitivity. [17] In mass spectrometry (MS) detection, the column is typically packed all the way to the site of sample injection. When mass spectrometry and CEC are combined, reliable molecular weights and frequenter structural information, make it very useful for additional information on this topic, the reader is referred to recent thorough studies that address the methodology of CEC and its coupling to MS by Colon et al. (1997) and Rentel et al. (1999). It's interesting to note that packed-CEC offers the possibility of using bigger sample capacities and simpler mobile phases that are better suited for MS [9].

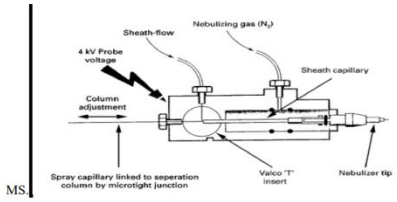

Figure7: The main components of a sheath] flow probe which can be used to couple CZE to a Q-TOF or to single/triple quadruple instruments. (Courtesy of Micro mass, Manchester, UK.).

Conclusion:

Therefore, hyphenated approaches are much more effective and beneficial than single techniques. Hyphenation includes both identifying and separating, which helps analyze samples better. Nowadays, hyphenated methods are used more often than just chromatographic or spectroscopic methods.

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Not Declared.

Conflict of Interest

No Conflict of Interest.

Informed Consent and Ethical Considerations

Not Applicable.

Author Contribution

All authors are contributed equally.

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