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Hyphenated Techniques- A Comprehensive Review

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ABSTRACT

The hyphenated technique is the combination or the coupling of the different analytical techniques. Mainly chromatographic techniques are combined with spectroscopic techniques. Then the separated components of the mixture from chromatographic technique will enter into the spectroscopic technique through an interphase. In GC-MS the separated components from gas chromatography enter to MS which is followed by ionization, mass analysis, and detection of mass-to-charge ratios of ions generated from each analyse by the mass spectrometer. Jet/orifice separator, effusion separator, and membrane separator can be used to connect GC with MS. In LC-NMR coupling the analytical flow cell was initially constructed for continuous-flow to NMR. However, the need for full structural assessment of novel natural products has led to the application in the stopped-flow mode in LC-MS. Use of LC-MS-MS is increasing speedily day by day. Hyphenated techniques such as HPLC coupled to UV and mass spectrometry (LC-UV-MS) have been 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 term hyphenated techniques refer to separation, identification, and the hyphenated techniques show better analysis of the samples are components specificity, accuracy, precision.

Keywords: Liquid Chromatography, Gas Chromatography, Infrared Spectroscopy, Mass Spectroscopy, NMR, Capillary Zone Electrophoresis.

INTRODUCTION

A hyphenated technique is combination (or) coupling of two different analytical techniques with the help of proper interface. Mainly chromatographic techniques are combined with spectroscopic techniques (1).In the chromatography, the pure or nearly pure fractions of chemical components in a mixture was separated and spectroscopy produces selective information for identification using standards or library spectra. **"The coupling of the separation technique and an on-line spectroscopic detection technology will lead to a hyphenated technique**. (2)A Hyphenated technique is combination (or) coupling of two different analytical techniques with the help of proper interface **(3).** The term hyphenated techniques range from the combination of separation - separation, separation-identification& identification- identification techniques **(2)**.

The term "hyphenation" was first adapted by **Hirsch Feld** in 1980 to describe a possible combination of two or more instrumental analytical methods in a single run (Hirschfeld, 1980). The aim of the coupling is to obtain an information-rich detection for both identification and quantification compared to that with a single analytical technique **[2].**

Advantages

1. For fast and accurate analysis
2. A Higher degree of automation.
3. Higher sample throughput.

4. Better reproducibility.
5. Reduction 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

- LC-MS
- LC-NMR
- LC-IR
- CE-MS
- GC-IR
- GC-MS
- HPLC-DAD
- GC-FTIR

2. Triple hyphenated techniques

- LC-API-MS
- APCI-MS-MS
- ESI-MS-MS
- LVI-GC-MS
- LC-ESI-MS
- LC-UV-NMR-MS-ESI
- LC-MS-TSPLC-UV-NMR-MS
- LC-NMR-MS
- LC-DAD-API-MS
- LC-PDA-MS
- LC-PDA-NMR-MS
- SPE-LC-MS(2)

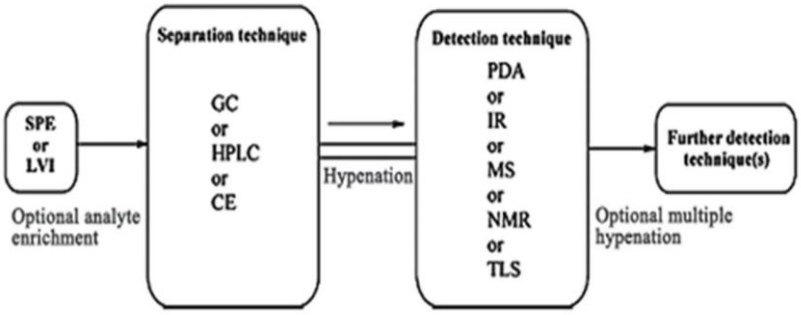

Fig:-1 Schematic presentation of Hyphenation of chromatographic and spectrometric techniques (3)

DOUBLE HYPHENATED TECHNIQUES

1. GC-MS

Gas chromatography

It is a separation technique applicable to compounds which can be volatilised in a gas stream. This is typically done by injection of 0.1-1µL of sample into an injection port heated to 250ºC (4) These two techniques highly compatible with each other, the sample is in the vapour phase in both the techniques. (11)But the incompatibility between two techniques is GC operates at high pressure (760 torrs) and in this the carrier gas is present, whereas in the case of mass spectroscopy it operates at a vaccum10–6 to l0–5torr **(4)**.

Instrumentation and Working

Vaporized analyse when carried through the GC column with the help of heated carrier gas the separation occurs in the column, only the carrier can also be called as the mobile phase(helium). The interactions between analyte, mobile phase, and stationary phase lead to separation of the compounds. The separation of the analyte also depend on the column's dimensions (length, diameter, and film thickness), type of carrier gas, column temperature

(gradient) and the properties of the stationary phase (11). The sample travel through the column the difference in the boiling point and other chemical properties lead to separation of the components of the mixture. The components have differences in elution time and retention time due to their different adsorption or difference in the partition between the mobile phase and the stationary phase resp.(12) 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 analyse 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 ionisation not only ionise the molecule but also break the molecule into the positive or negative modes **(2)**.

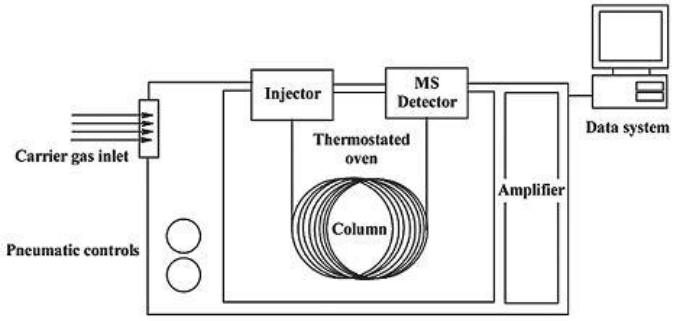

Fig 2. Schematic presentation of GC-MS.

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 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 gasses sample, proper attention is required.(10).
-

2. LC-MS

Instrumentation and working

LC-MS is a chemistry technique that combines the physical separation of liquid chromatography (or HPLC) with the mass spectroscopy. A typical automated LC-MS system (Figure 3) consists of double three-way diverter in-line with an autosampler, LC system, the Mass spectrometer. The diverter generally operates as an automatic switching valve to divert undesired portions of eluting from the LC system to waste before the sample enters the MS.(11) The ionization techniques used in LC-MS are generally soft techniques that mainly display the molecular ion species with only a few fragment ions. The information obtained from a single LC-MS run is not sufficient for confirmation of the identity of the compound. Nevertheless, the problem has now been solved by the introduction of tandem mass spectrometry (MS-MS), which provides fragments through collision-induced dissociation of the molecular ions produced. Use of LC-MS-MS is increasing speedily day by day. Hyphenated techniques such as HPLC coupled to UV and mass spectrometry (LC-UV-MS) have been 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. With these interfaces, various types of analysers, e.g., quadruple, ion trap, or TOF, can be used. Each of these analyser's however, offers a varying degree of mass accuracy and resolution. In the LC-UV-MS mode, thermospray (LC-TSP-MS) and continuous-flow FAB (LC-CF-FAB) interfaces can also be applied. For phytochemical analysis, the TSP has been found to be the most suitable interface as it allows introduction of aqueous phase into MS system at a flow rate (1-2 ml/min) compatible with that usually used in phytochemical analysis.(5)

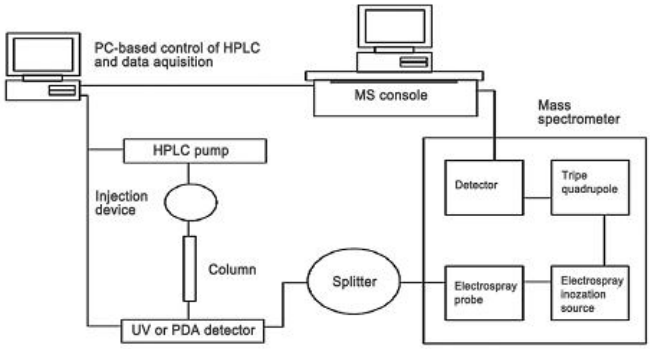

Figure 3: Liquid chromatography- Mass spectroscopy. (2)

3. LC-NMR

Instrumentation and working

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. (12)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 biofluids. (6)

LC-NMR experiments can be performed in both continuous-flow and stop-flow modes. A wide range of bioanalytical 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. 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., the 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. (11)

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. A typical experimental arrangement of LC-NMR is shown in Figure 4. Generally, in the LC-NMR system, the LC unit comprises autosampler, 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 analysed 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: pre-saturation, soft-pulse multiple irradiations or water suppression enhancement through T1 effects (WET) pre-saturation employing a z-gradient. 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 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.(6)

To date, three main types of data acquisition modes have been introduced: continuous-flow acquisition, stopped-flow acquisition, and time-sliced acquisition. Whatever may be the acquisition mode, an optimized HPLC separation is crucial to any LC-NMR analysis. As the sensitivity of LC-NMR is much less than other hyphenated techniques, e.g., LC-MS, or LC-PDA, it is imperative to develop a suitable LC separation where the quantity of the available separated compound is concentrated in the smallest available elution volume. LC-NMR represents a potentially interesting complementary technique to LC-UV-MS for detailed on-line structural analysis. Indeed, recent progress in NMR technology has given a new impulse to LC-NMR, which is now emerging as a powerful analytical tool. The development of efficient solvent suppression techniques enables the measurement of high-quality LC-1H-NMR spectra, both on-flow and stop-flow, with reversed-phase HPLC conditions. Nondeuterated solvents such as Me OH or MeCN can be used, while water is replaced by D2O.

Recent advances in both hardware and software for the direct coupling of LC and NMR have given a new life to this hyphenated technique. These developments include a new coil and flow cell design for high sensitivity, new RF system for multiple solvent suppression and improved dynamic range gradient elution capability, and automatic peak-picking/storing capabilities. As a result, this method is a powerful tool used in many areas such as natural products, organic molecules, biomolecules, drug impurities, by-products, reaction mixtures, and drug degradation products. The potential of HPLC-NMR for the investigation and structural elucidation of novel natural products has been enormously extended by the advent of powerful solvent suppression schemes, and their combination with a series of homo- and heteronuclear 2D NMR experiments such as 2D total correlation spectroscopy (TOCSY) or 2D nuclear Over Hauser enhancement spectroscopy (NOESY). LC-NMR, despite being known for about last two decades, has not quite become a widely accepted technique, mainly because of its lower level of sensitivity and higher cost compared to other available hyphenated techniques. However, the recent advances in technology, especially in relation to the developments in pulse field gradients and solvent suppressions methods, the improvement in probe technology, and the introduction of high-field magnets (800–900 MHz) have offered new impetus to this technique.(6)

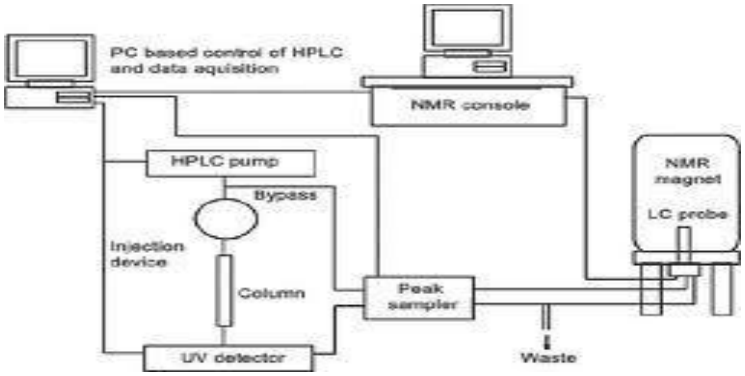

Figure 4: Liquid chromatography- Nuclear Magnetic Resonance (5)

4. LC-IR

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 (Figure 5). 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, a 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. [15] Because FT-IR is an absorbance process, the geometry of the sample during the measurement process matters. For a fixed mass or volume of the analyte, reducing the diameter by a factor of two creates a deposit with four times the thickness and four times the optical density. Because the IR detector is total light limited, this deposit diameter reduction of two improves the signal-to-noise ratio by four times. Therefore, to achieve a useful instrument that produces full mid-infrared spectra, the LC–IR hyphenation process must.

1. Remove the solvent without thermally damaging the analyses or overloading the vacuum system with diluent gas.
2. Have efficient transmission of analyses to the spectrometer.
3. Present analyses to the FT-IR in a thick deposit.
4. Preserve the chromatographic resolution (7)

5. GC-IR

Instrumentation and working

To know the identity of each component in a mixture it is necessary for many analytical scale separations, not only simply measuring retention data for this purpose which is often too ambiguous for the identification of molecule eluting from a capillary gas chromatography(GC) column, which has the capability of resolving several hundred components. Prior knowledge about the chemical structure of the components and spiking of the mixture with one or more reference standards may aid the identification process; however, a less ambiguous identification can be accomplished by interfacing the chromatograph to a sensitive, rapid scanning spectrometer to obtain unique signatures of each component. This instrument should allow each component to be detected in real time without any loss in chromatographic resolution. Mass spectrometry(MS) is the most commonly applied technique for this purpose, but it has certain limitations, in particular for distinguishing between structural isomers, such as *ortho*-, *meta*- and *para*-xylene, whose electron-impact and chemical-ionization mass spectra are identical. For such molecules, a technique complementary to MS is desired. Fourier transform infrared (FT-IR) spectrometry, which yields unique spectra for most structural isomers, has frequently been used as an alternative technique for this purpose.

1. Light-Pipe-Based GC-IR Instruments

Measurement of the Spectrum

The coupling of gas chromatographs and FT-IR spectrometers (GC-IR) has been accomplished by three approaches. In the Rest, and by far the simplest, the GC column is connected directly to a heated So through the cell. For capillary GC, this cell is usually fabricated from a 10-cm length of heated glass tubing with an internal diameter of 1 mm. The inside bore of this tube is coated with a thick enough rim of gold to be highly respective to infrared (IR) radiation. IR transparent windows (for example made of potassium bromide) are attached to both ends of the tube. IR radiation entering one window is multiple resected down the gold-coated interior bore before emerging from the other window, giving rise to the name *light pipe* for this device. The effluent from the GC-column is passed into one end of the tube and out of the other via heated fused silica transfer lines. The entire unit is held at a temperature between 250 and 300C to preclude the condensation of semi-volatile materials. Infrared radiation from an incandescent source, such as a Sic Global, is collimated and passed through a rapid-scanning interferometer so that each wavelength in the spectrum is modulated at a different frequency. The beam of radiation is then focused to the rest window of the light-pipe and the infrared beam emerging from the second window is refocused onto a sensitive detector (typically a liquid nitrogen cooled mercury cadmium telluride (MCT photoconductive detector). A typical system is illustrated schematically in **Figure 5** the signal measured in this way is known as an *interfere-program* and the Fourier transform of the interfere organ yields a single beam spectrum. By calculating the ratio of a single beam spectrum measured when a component is present in the light-pipe to one measured when only the helium carrier gas is present, the transmittance spectrum, T (v), of the component is obtained. (8)The transmittance spectrum is usually immediately converted to an absorbance spectrum, A (v), by the standard Beer's law operation, A (v)"! log10 T(v), as the relative intensities of bands in absorbance spectra, are independent of the concentration of the analyse ,thereby allowing spectral library searching to be performed. For light-pipe-based GC-IR systems, it is rarely necessary to measure spectra at high resolution, as the spectral bands are quite broad. Since most bands in the spectra of molecules in the vapour phase have a width of at least 10-cm_1, the typical resolution at which GC-IR spectra are measured is 8 cm_1.(8)

4. Matrix-Isolation GC-IR

In the Rrst approach, argon is mixed with the helium mobile phase, either as a minor (1%) component in the carrier gas or by addition at the end of the GC column. The column effluent is then sprayed from a heated fused silica transfer line onto a rotating gold-plated disk that is maintained at a temperature of less than 15 K. Helium does not condense at this temperature but argon does. By locating the end of the transfer line an appropriate distance from the cooled disk, argon is deposited as a track approximately 300m in width. Any component emerging from the transfer line at the same time is trapped in the argon matrix. After the separation has been completed, the disk is rotated to a position where the focused beam from an FT-IR spectrometer is transmitted through the track of argon, respects from the gold-coated disk, passes again through the argon and then is collected and focused onto an MCT detector, as shown in **Figure 6**. In principle, if the concentration of any analyte in the argon matrix is low enough, each analyte in the argon matrix is low enough, each analyte molecule will be isolated from similar molecules by the argon matrix. Despite the fact that the concentration is usually a little too high for true matrix isolation to be achieved in GC-IR measurements, this technique none the less is known as *matrix-isolation GC-IR*. By rotating the disk slowly, a series of spectra can be measured that is analogous to the series of spectra that is measured in real-time during a light-pipe-based GC-IR run and either GS or FG chromatograms can be constructed from these data. Each component may be indentured by spectral library searching, but a special library of spectra of Matrix-isolated standards is required. (8)

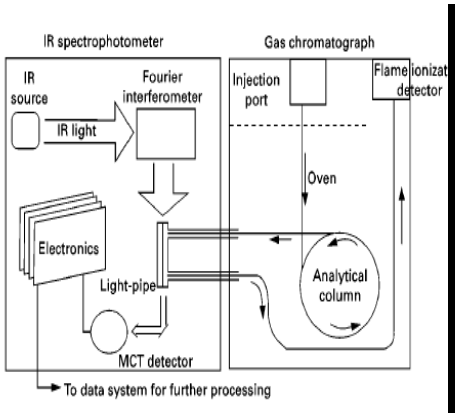

Figure 5

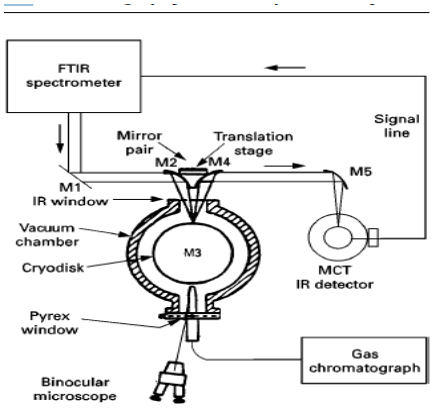

Figure 6

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 widely recognized as a powerful analytical technique in its own right, known for its high separation efficiency, short analysis times and low-volume sample requirements. These characteristics made CZE a popular method for the analysis of peptide mixtures, protein digests, drug substances and biotechnological products. The coupling of CZE with electrospray ionization mass spectrometry (ESI-MS), Rest reported by Olivares *et al*. In 1887, has added further capabilities, in particular for obtaining molecular mass information and structural details when tandem mass spectrometry (MS-MS) is used. However, it can be said that the major advantage of such coupling is that the migration time is not the only parameter used for identifying the eluted components. These times are subjected to variations between runs, yet such variations become irrelevant when, in the same run, highly diagnostic mass spectra are obtained. Depending on the ionization method, CZE can be coupled to a mass spectrometer either directly (online) or indirectly (of Sine). In the latter mode of operation, 252Cf plasma desorption and matrix-assisted laser desorption can be used. The online coupling of CZE is more common and usually performed by electrospray ionization (ESI) or fast atom (ion) bombardment (FAB).Although online CZE/MS is the more common form of **figure 7**application, of Sine analysis, has the advantage of allowing separation in non-volatile buffers, which are highly undesired in ESI. It goes without saying that every analytical technique has its limitations and CZE/MS is no exception. One of the main limitations of this experimental arrangement is its relatively poor sample concentration/ion sensitivity. Approaches to reduce such limitations included online preconcentration, sample stacking, and the increasing use of time-of-flight (TOF) analysers which use ESI and TOF analysers with and without a quadruple in between. The innovative feature of this class of instruments is their fast scanning, which allows the acquisition of a number of full spectra per second. Additionally, as all ions in each spectrum are sampled at the same moment in time, spectra are free of mass discrimination or peak skew typical of slow scanning systems that must scan over a narrow chromatographic/electro phonetic peaks. Capillary electrochromatography (CEC) is another technique which is currently undergoing a rapid phase of advancement and development. This technique was revived by Jorgenson and Lukas in 1981; these authors used 0.005 mol L1 phosphate buffer170 m packed column and 30 kV separation voltage to separate 9-methylanthracene and polyline. This technique has recently become more diffuse because of a number of advances in both CE instruments and detection techniques including electrospray Mass spectrometry. However, on-column UV detection and in-column laser-induced Fluorescence detection remain the most commonly used methods. Despite its high sensitivity, the latter method is subjected to interferences by buffer Fluorescence. (18) In MS detection, the column is commonly packed right up to the point where the sample is injected into the mass spectrometer. The combination of CEC with mass spectrometry provides reliable molecular weights and in many cases structural information, which makes it highly attractive for a wide range of applications. For more details on this topic, the reader is referred to recent extensive reviews, covering the methodology of CEC and its coupling to MS, by Colon's et al. (1997) and Rentel et al. (1999). Interestingly, packed-CEC offers the possibility of higher sample capacity and the utilization of simpler mobile phases, which are more compatible with MS. (9)

MS.

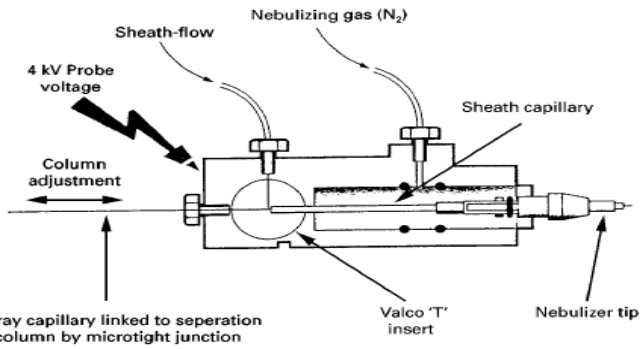

Figure7: The main components of a sheath} flow probe which can be used to couple CZE to a Q-TOF or to single/triple quadruple instruments. (Courtesy of Micro mass, Manchester, UK.).

CONCLUSION

Thus a conclusion can be drawn that hyphenated techniques are far better and useful than normal single techniques. Hyphenation includes both separation and identification which makes the analysis of samples easy. Nowadays the hyphenated techniques are more used than normal spectroscopic or chromatographic techniques.

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