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Hyphenated Techniques: A Review

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ABSTRACT

The hyphenated techniques are developed from the coupling of a separation technique and an on-line spectroscopic detection technology. The hyphenation of these techniques leads to better analysis of the components. Hyphenated techniques show specificity and sensitivity. The term hyphenated techniques ranges from the combination of separation-separation, separation-identification and identification- identification techniques. The hyphenation of these techniques leads to better analysis of the components. 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-GC, LC-FTIR, LC-NMR, CE-MS, etc have been highlighted.

INTRODUCTION

Hyphenated techniques combine chromatographic and spectral methods to exploit the advantages of both. Chromatography produces pure or nearly pure fractions of chemical components in a mixture. Spectroscopy produces selective information for identification using standards or library spectra.

A couple of decades ago, Hirschfeld introduced the term "hyphenation" in 1980 to refer to the on-line combination of a separation technique and one or more spectroscopic detection techniques[(1).] The hyphenated technique is combination or coupling of two different analytical techniques with the help of proper interface [(2).]

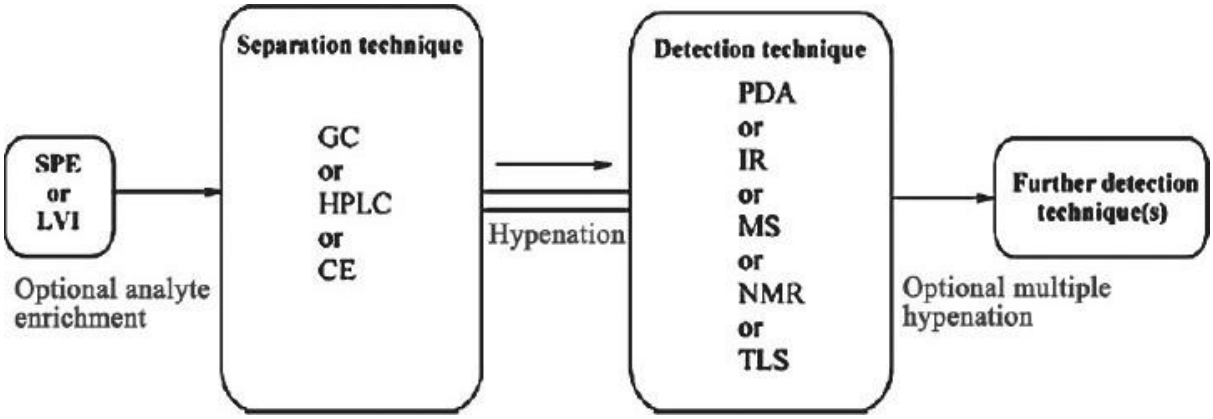

Figure 1: Hyphenated Technique

In recent years, hyphenated techniques have received ever-increasing attention as the principal means to solve complex analytical problems. 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 techniques, e.g., Fourier-transform infrared (FTIR), photodiode array (PDA), UV-vis absorbance or fluorescence emission, mass spectroscopy (MS), and nuclear magnetic resonance spectroscopy (NMR), resulting in the introduction of various modern hyphenated techniques, e.g., CE-MS, GC-MS, LC-MS, and LC-NMR.

Liquid chromatography- nuclear magnetic resonance (lc-nmr)

Principal:-

That combines high-performance liquid chromatography (HPLC) and nuclear magnetic resonance spectrometers (NMR), they have been applied widely in the analysis of complex mixtures that contain unknown components, such as impurities and metabolites in pharmaceuticals, natural products and synthetic polymers[3,4], ever since they were first reported in 1978[5].

History of LC-NMR:-

The first on-line LC–NMR experiments were performed in the late 1970s by Watanabe and Niki who demonstrated stopped-flow measurements of a mixture of known compounds.

The conventional NMR probe was transformed to a flow-through probe by the introduction of a thin-walled Teflon capillary within a standard NMR tube and spectra were recorded with sample rotation.

In theory, the physical coupling of LC with NMR could save a lot of time and be already proposed over 20 years ago. However, a successful and practical LC–NMR coupling has been achieved only in the last decade [6].

Instrumentation and Working:-

The common online LC-NMR system consists out of a standard LC-device connected to a NMR-detection device where a flow probe is inserted. The LC device consists out of a pump system that pushes liquid solvent through the system, a column where the separation takes place and a detector with flow cell that utilizes light to measure the components as they elute. This light-detector can either be an Ultraviolet-/Visible light (UV/VIS)-detector, a refractive index (RI)-detector or Infrared light (IR) detector. Most common nowadays is the use of Diode Array Detector (DAD), a type of UV/VIS-detector that can measure multiple light wavelengths at once.

Basically, any type of detector can be used, as long as it does not alter or destroy the sample. The NMR system consists out of a huge radiofrequency (RF) - magnet where a non-rotating flow cell has been put oriented vertically. This orientation allows for laminar flow and gets rid of bubbles in the mobile phase easily. The RF coil is wrapped around the cell so that a

good filling factor is obtained and the difference in detection volume and coil volume is only the glass that comprises the flow cell[8].

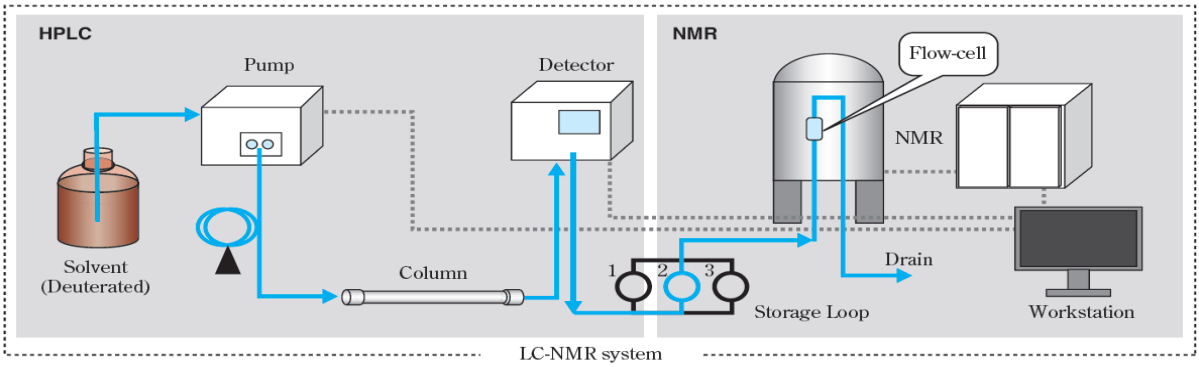

Figure 2: The instrumental setup of a simple LC-NMR system[7].

Liquid chromatography-mass spectroscopy (lc-ms):-

Principal:[9-11]

Liquid chromatography-mass spectrometry is the technique which performs separation by liquid chromatography and mass analysis with the help of the mass spectrometry. With the help of HPLC impurities and degradation products can be separated and Mass Spectrometry allows us to obtain the molecular weight and identification of the same. LC-MS is highly selective and sensitive technique. LC-MS leads to detection and identification of chemicals in presence of other chemicals there for it is called as specific. The flow rate of HPLC is around 1ml/min which is difficult to accommodate in mass spectrometry vacuum system also the diluent which is used has to be vaporized which leads to damage of the thermally labile compounds by excessive heating by hyphenation of these two techniques capabilities of both the techniques were improved.

There are various methods used in Quantitative Analysis which may be broadly classified as

- Chemical/classical Method (Titrimetric, Volumetric and Gravimetric method)
- Instrumental Method (Spectrophotometry, Polarography, HPLC, GC)

LC-MS also plays a role in pharmacognosy especially in the field of molecular pharmacognosy when it comes to the ingredients difference in the aspects of phenotypic cloning. The most important factor that has to be considered is how to make the biggest difference of active ingredients in plant cells between the test group of plants and controlled ones.

Instrumentation and Working :(13-15)

The LC-MS instrument can be interfaced by electrospray, particle beam, Thermospray. Electrospray is most widely used interface. The spray needle is used as bridge to connect the liquid chromatography with that of the mass. But the separate emitter is flexible as well as convenient. LC-MS is mainly separated into the three parts chromatography, interface and spectrometry. In liquid chromatography separation is performed which is detected with the help of Photodiode array, Ultraviolet, fluorescent, etc., detectors. These separated components then transferred to the interface. In interface, the liquid is volatilized and transferred to the MS. With the help of various ionization techniques the compound is ionized and then it is analyzed by mass analyzer. Various mass analyzers are used *viz.* Quadrupoles,

Quadrupole ion traps, time-to-flight (TOF), time-to-flight reflection (TOFR), and ion cyclotron resonance (ICR) mass analyzers.in this technique LC separate due to which clear mass spectra is obtained by suppression of the mutual signal.

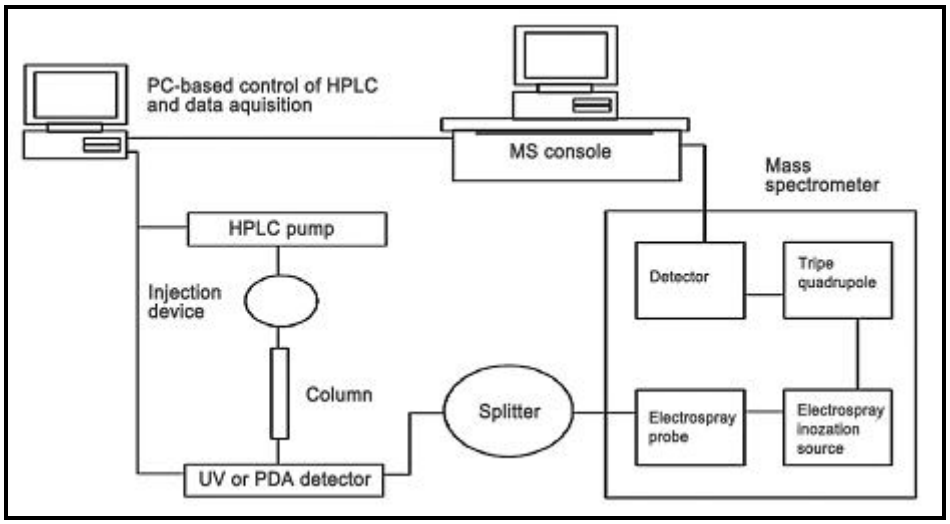

Figure 3: The instrumental setup LC-MS system.

Liquid chromatography-gas chromatography (lc-gc):-

Principal:-(16)

All forms of chromatography involve a ***stationary phase*** and a ***mobile phase***. In all the other forms of chromatography, you will meet at this level, the mobile phase is a liquid. In gas-liquid chromatography, the mobile phase is a gas such as helium and the stationary phase is a high boiling point liquid adsorbed onto a solid.

How fast a particular compound travels through the machine will depend on how much of its time is spent moving with the gas as opposed to being attached to the liquid in some way.

History of LC-GC:-

Chromatography dates to 1903 in the work of the Russian scientist, Mikhail Semenovich Tsw ett. German graduate studentFritz prior developed solid state gas chromatography in 1947. Ar cher John Porter Martin, who was awarded the Nobel Prize for his work in developing liquid (1941) and paper (1944) chromatography, laid the foundation for the development of gas chromatography and he later produced liquid gas chromatography (1950). Erika Cremer laid the groundwork, and oversaw much of Prior's work.

Instrumentation & Working: [16]

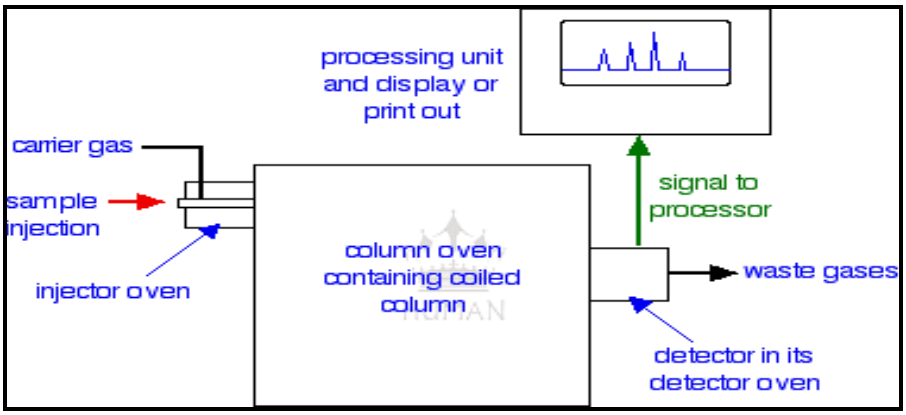

Figure 4: The instrumental setup LC-GC system.

➢ **Injection of the sample: -** Very small quantities of the sample that are to be analyzed are injected into the machine using a small syringe. The syringe needle passes through a thick rubber disc (known as a septum) which reseals itself again when the syringe is pulled out.

The injector is contained in an oven whose temperature can be controlled. It is hot enough so that the entire sample boils and is carried into the column as a gas by the helium (or other carrier gas).

➢ **Working of column:** There are two main types of column in gas-liquid chromatography. One of these is a long thin tube packed with the stationary phase; the other is even thinner and has the stationary phase bonded to its inner surface.

The column is typically made of stainless steel and is between 1 and 4 meters long with an internal diameter of up to 4 mm. It is coiled up so that it will fit into a thermostatically controlled oven.

➢ **Packing Material: -** The column is packed with finely ground ***diatomaceous earth***, which is a very porous rock. This is coated with a high boiling liquid - typically a waxy polymer.

➢ **The column temperature: -** The temperature of the column can be varied from about 50°C to 250°C. It is cooler than the injector oven, so that some components of the mixture may condense at the beginning of the column.

In some cases, as it can be seen below, the column starts off at a low temperature and then is made steadily hotter under computer control as the analysis proceeds.

➢ **Retention time: -** The time taken for a particular compound to travel through the column to the detector is known as its ***retention time***. This time is measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound.

Different compounds have different retention times. For a particular compound, the retention time will vary depending on:

- The boiling point of the compound: A compound which boils at a temperature higher than the column temperature is going to spend nearly all of its time condensed as a liquid at the beginning of the column. So high boiling point means a long retention time.

- The solubility in the liquid phase: The more soluble a compound is in the liquid phase, the less time it will spend being carried along by the gas. High solubility in the liquid phase means a high retention time.

- The temperature of the column: A higher temperature will tend to excite molecules into the gas phase - either because they evaporate more readily, or because they are so energetic that the attractions of the liquid no longer hold them. A high column temperature shortens retention times for everything in the column.

For a given sample and column, there isn't much you can do about the boiling points of the compounds or their solubility in the liquid phase - but you do have control over the temperature.

The lower the temperature of the column, the better the separation you will get - but it could take a very long time to get the compounds through which are condensing at the beginning of the column.

On the other hand, using a high temperature, everything will pass through the column much more quickly - but less well separated out. If everything passed through in a very short time, there isn't going to be much space between their peaks on the chromatogram.

The answer is to start with the column relatively cool, and then gradually and very regularly increase the temperature.

At the beginning, compounds which spend most of their time in the gas phase will pass quickly through the column and be detected. Increasing the temperature a bit will encourage the slightly "stickier" compounds through. Increasing the temperature still more will force the very "sticky" molecules off the stationary phase and through the column.

➢ **Detector: -** There are several different types of detector in use. The flame ionization detector described below is commonly used and is easier to describe and explain than the alternatives.

- **A flame ionization detector:**

In terms of reaction mechanisms, the burning of an organic compound is very complicated. During the process, small amounts of ions and electrons are produced in the flame. The presence of these can be detected.

The whole detector is enclosed in its own oven which is hotter than the column temperature. That stops anything condensing in the detector

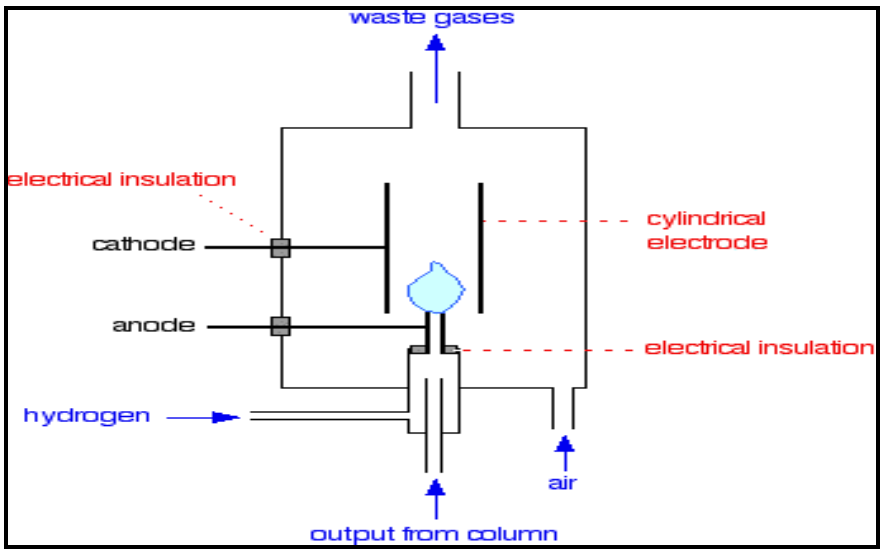

Figure 5: The Flame Ionisation detector.

If there is nothing organic coming through from the column, you just have a flame of hydrogen burning in air. Now suppose that one of the compounds in the mixture you are analyzing starts to come through.

As it burns, it will produce small amounts of ions and electrons in the flame. The positive ions will be attracted to the cylindrical cathode. Negative ions and electrons will be attracted towards the jet itself which is the anode. This is much the same as what happens during normal electrolysis.

At the cathode, the positive ions will pick up electrons from the cathode and be neutralized. At the anode, any electrons in the flame will transfer to the positive electrode; and negative ions will give their electrons to the electrode and be neutralized. This loss of electrons from one electrode and gain at the other will result in a flow of electrons in the external circuit from the anode to the cathode. In other words, you get an electric current.

The current won't be very big, but it can be amplified. The more of the organic compound there is in the flame, the more ions will be produced, and so the higher the current will be. As a reasonable approximation, especially if you are talking about similar compounds, the current you measure is proportional to the amount of compound in the flame.

Disadvantages of the flame ionization detector

The main disadvantage is that it destroys everything coming out of the column as it detects it. If you wanted to send the product to a mass spectrometer, for example, for further analysis, you couldn't use a flame ionization detector.

Applications of hyphenated technique:-

1. Identification of drug degradation products.
2. Low-level impurities can be isolated and identified.
3. This technique is used for tracking pesticides, herbicides and organic pollutant for environmental monitoring.
4. Separation and characterization of peptide libraries
5. Combinatorial chemistry, Photochemical analysis, Drug discovery
6. Identification of drug impurities
7. Characterization of isomers of acid glucuronides and vitamin A derivatives
8. Characterization of endogenous and xenobiotics metabolites directly from biological fluid
9. Combination of LC-NMR and LC-MS
10. Polymer analysis
11. LC-NMR allowed the differentiation of isomers and identification without reference compounds
12. Drug metabolism (to analyze biofluids [i.e., urine or plasma]);

a) 19F (a selective tracer; minimal background); 19F observe of 19F-containing drugs is very selective and clean by NMR

b) We were able to identify 2-hydroxyibuprofen, carboxy ibuprofen, and unmetabolized ibuprofen molecules from a small urine sample after a therapeutic dose of ibuprofen. (Used a micro-coil NMR probe, with an active volume of 3 microliters.)

13. Identification of nine closely eluting and isomeric aporphine alkaloids in the Taiwanese plant *Litsea* genus using 50 times less material compared with conventional NMR experiments using 5 mm tubes.
14. LC–NMR provides rapid multiparametric information on microbial biotransformations as illustrated by the identification of novel warfarin metabolites from *Streptomyces rimosus* and the identification of the antibiotic aristeromycin from broth supernatants of *Streptomyces citricolour*.

15. Identification and separation of chiral compound: photoisomer of azadirachtin extracted from seed of neem tree which is powerful insect antifeedant is separated and characterized by taking $CH_3CN:D_2O$ ratio of 7:13.

16. LC-NMR-MS have identified analogues of vitamin E of palm oil extract

17. Analysis of crude extracts of natural products and plant-derived compounds - the technique is optimized for rapid identification of potential drug candidates in plant products.

18. Detection of bulk drug impurities during drug stability tests - the data from LC-NMR/MS allows full characterization of all impurities present in the drug.

19. Analysis of unstable compounds or compounds formed in situ that cannot be detected or isolated using other techniques.

20. Studying drug metabolism - analysis of biofluids such as plasma or urine. Tracers such as 19F are linked to drugs and they can be tracked clearly by NMR.

21. Analysis of ring-fused and heterocyclic compounds that have a small number of protons - the LC-2D-NMR technique provides carbon shift and bonding information for these compounds, which is very useful for analysis of their structure.

22. Composition profiling to analyze the content and structure of components in a chemical mixture, thus providing valuable insights for the development of a manufacturing process in the chemical industry.

23. Studying polymers – the high resolution of LC-NMR is ideal for analysis of the microstructures in synthetic polymers and biopolymers including proteins.

24. Studying uncharacterized, complex, non-living natural organic matter (NOM) present in the atmosphere, oceans, soil and sediments. LC-NMR and LC-SPE-NMR have been used to study dissolved NOM from freshwater and alkaline soil extracts for the separation and characterization of components in the complex mixture.

25. Metabolomics to determine disease states using body fluids analysis.

26. LC-MS used to detect compounds from polyaromatic (non-polar) to peptide and proteins.

27. LC-MS used for compounds identification and purity.

28. Used for determination of pesticides, herbicides & organic pollutant for environmental monitoring.

29. Proteome analysis is done by this technique

30. Identification and quantification of compounds in environmental samples (eg pesticides, oils and organic pollutants) and medical applications (eg drugs and bioindicators).

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