**Applications of Hyphenated Techniques in Pharmaceutical Sciences**

Lata Potey,\*1 Ashwini Armarkar,1 Shilpa Chapekar,1 Yogini Shete,1Pooja Birade,1Suchita Waghmare2

1Shree Sainath College of Pharmacy, Dawalameti, Nagpur, Maharashtra, India-440023

2Department of Pharmaceutical Sciences, Nagpur, Maharashtra, India-440033

e-mail: latapotey@rediffmail.com

**ABSTRACT**

The hyphenation term was introduced by Thomas Hirschfield in 1980. Hyphenated techniques are exponentially to deal with various complicated challenges faced by analyst in analytical chemistry. It is the fusion of chromatographic methods and spectrometric or spectroscopic methods. Chromatographic techniques are methods of component separation in complicated mixtures like Liquid Chromatography, Capillary Electrophoresis, High-Performance Liquid Chromatography, and Gas Chromatography. Spectroscopy use to elucidate the structure, molecular mass of compounds like UV, IR, Mass, Raman, Fluroescence spectroscopy. Over the past two decades, hyphenated analytical methods have seen amazing advancements that have greatly expanded their applications in the study of biomaterials, particularly natural goods. Today, a variety of hyphenated methodologies including multiple interface types are commercially accessible to demonstrate a better analysis of the samples' specificity, accuracy, and precision. In this chapter, we have emphasized on most common types of double and triple hyphenated techniques like LC-FTIR, GC-MS, LC-NMR, LC-NMR-MS, etc. and their applications in the field of pharmaceutical sciences.

**Keywords-**Hyphenated Technique; GC-MS; LC-MS; LC-FTIR; LC-NMR.

1. **INTRODUCTION**

A hyphenated approach is the integration of two different analytical techniques (or their coupling) using an appropriate interface. Spectroscopic techniques are typically paired with chromatographic procedures. [1] Chemical components in a mixture were separated into their pure or nearly pure fractions using chromatography, and spectroscopy produces selective information for identification using standards or library spectra. “A hyphenated technique will result from the combination of the separation approach and an online spectroscopic detection technology. [2]A hyphenated approach is the combining of two distinct analytical methods with the use of an appropriate interface. [3]The term "hyphenated techniques" refers to a variety of techniques that combine separation-separation, separation-identification, and identification-identification. [4]Hirsch Feld coined the term "hyphenation" in 1980 to refer to the potential fusion of two or more instrumental analysis procedures in a single run (Hirschfeld, 1980). When compared to a single analytical approach, the coupling's goal is to obtain an information-rich detection for both identification and quantification. [5] See table 1 for the classified hyphenated techniques.

**Table 1: Various types of Hyphenated techniques**

|  |  |  |
| --- | --- | --- |
| **Sr.no** | **Separation Technique** | **Hyphenated Mode** |
|  1. | Liquid Chromatography | 1. Liquid Chromatography—Fourier Transform Infrared Spectrometry (LC-FTIR)
 |
| 1. Liquid Chromatography-Mass Spectrometry (LC/MS)
 |
| 1. Liquid Chromatography-Nuclear Magnetic Resonance Spectroscopy (LC/NMR)
 |
|  2.  | Thin layer Chromatography | 1. Thin layer Chromatography-Mass spectrometry (TLC/MS)
 |
| 1. Thin layer Chromatography-Surface enhanced Raman Spectroscopy (TLC-SERS)
 |
|  3. | Gas Chromatography | 1. Gas Chromatography- Mass Spectrometry (GC/MS)
 |
| 1. Gas Chromatography- FTIR-MS (GC-FTIR-MS)
 |
| 1. Gas Chromatography- Fourier Transform Infrared Spectrometry (GC-FTIR)
 |
| 1. Gas Chromatography-Inductively coupled Plasma Mass Spectrometry (GC-ICPMS)
 |
| 4. | Capillary Electrophoresis | 1. Capillary Electrophoresis-Nuclear Magnetic Resonance Spectrometry (CE/NMR)
 |
| 1. Capillary Electrophoresis- Surface enhanced Raman Spectrometry (CE-SERS)
 |
|  c) Capillary Electrophoresis- Mass Spectrometry (CE/MS) |
| 5. | Supercritical Fluid Chromatography Extraction (SFC/SFE) | 1. Supercritical Fluid Chromatography Extraction -Fourier Transform Infrared (SFC-FTIR)
 |
| 1. Supercritical fluid chromatography extraction – Capillary Gas Chromatography Mass Spectrometry (SFE-CGC-MS)
 |

**TYPES OF HYPHENATED TECHNIQUES**

**Double hyphenated techniques**

The hyphenated techniques with the combination of two analytical techniques at a particular interface are called as double hyphenated techniques.

1. **GC-MS**

This method is introduced by the combination of gas chromatography and mass spectrometry. Most popular method applied for the research and development purpose in the field of analysis. [6] It is used in the detection of drug sample, fire examination, explosive exploration, environmental research, food examination, flavour analysis, unknown sample testing, additionally it can be used in trace element detection, it allows analysis and detection even of minute quantity of a substance. [7, 8,] This technique is used to analyze volatile, small compounds which can be stable at high temperature, compound with polar substituents like OH group want to be easily derivatized for analysis by this technique. [9] The most common method of derivatization is to convert analyte with polar group to their trimethylsilyl derivatives. The actual process involves injecting the sample into the GC device's injection port, vaporizing it, separating it in a GC column, analyzing it using a mass spectrometry detector, and recording the results on a recorder.[10] On the basis of the interpretation of fragmentations, the spectra produced by this hyphenated approach provide accurate structural elucidation. The library spectra of the fragment ions with various relative abundances can be compared [11]

 The most common type of detector used in this GCMS technique is the quadrupole mass spectrometer, also called as ‘Mass Selective Detector’ referred by the Hewlett-Packard. Ion trap mass spectrometers are another frequently used detector in MS. A magnetic sector mass spectrometer may also be included, although these tools are costly and huge, and therefore are not frequently encountered in high-throughput service laboratories. Other detectors could includes tandem quadrupoles (MS-MS), time of flight (TOF), or ion trap MSn, where n denotes the number of mass spectrometry stages.[12] (The flow diagram of GCMS has been shown in figure 1)



**Figure 1: Schematic diagram of GC-MS.**

1. **LC-MS**

 This hyphenated technique is the fusion of liquid chromatography (LC) with mass spectrometry (MS) used to examine inorganic, organic, biochemical and natural compounds. [13] In addition to pharmaceutical field, it is found useful in food processing, agriculture, cosmetic, and biotechnology. [14] It begins to be used in clinical applications also. This system is consists of an interface which can move the compound separated from LC column into MS ion source. [15]The LC-MS interface is a mechanically simple component which helps in preserving the chemical identity of the product separated by LC by removing maximum solvent used in chromatography while transferring analyte. For practical need, the interface should not interfere with the ionizing efficiency and vacuum conditions of the MS system. [16]

 The most frequently utilized atmospheric pressure ionization (API)-based LC-MS interfaces, including atmospheric pressure chemical ionization (APCI), electron ionization (ESI), and atmospheric pressure photoionization (APPI), have been discovered. [17]These more recent MS ion sources ease the transition from high pressure (HPLC) to high vacuum (required at the MS analyzer) conditions. Despite being individually detailed, these interfaces can also be purchased commercially as dual ESI/APCI, ESI/APPI, or APCI/APPI ion sources. While there have been other deposition and drying methods employed in the past (such as moving belts), off-line MALDI deposition has been the most popular. A nano HPLC system and a mass spectrometer with electron ionization capabilities are combined in a novel technique still in development called direct-EI LC-MS interface.[18](The flowchart of LCMS has been shown in figure 2)



**Figure 2:Schematic diagram of LC-MS.**

1. **LC-IR**

It is an analytical technique which comprises of liquid chromatography (LR) and infrared spectroscopy (IR) which permit easy in sample detection and identification. [19] LC is a separation method and IR spectroscopy is helpful to characterize the compound by the presence of functional group in the structure, also detect the structural isomers. This technique (LC-MS) was developed on the basis of two approaches. The first approach is a very simple that consists of flow cell through which effluents are passes from LC column to IR detector to give the IR spectra.[20] There are so many advantages of this approach like simple operation, low maintenance, real time detection. Eliminating the LC solvent prior to IR detection is another strategy. In this method, the eluent is evaporated and the separated compounds are deposited onto a substrate appropriate for infrared detection at an interface. [21] The ability to capture the analytes' complete spectra and the significantly higher sensitivity compared to flow-cell detection are the main benefits of solvent-elimination LC/IR. The evaporation interfaces are frequently somewhat complex because typical LC solvents, especially aqueous eluent, are difficult to remove. Literature have shown that many analyst only prefer to use electrospray interface for this coupling technique which is flexible interface used for the analysis of both micro molecules and macromolecules, cationic and anionics and for the thermally labile compounds. There are other interfaces also like and atmospheric pressure photoionization (APPI) and atmospheric pressure chemical ionization (APCI). [22] LC/IR is more and more being recognized as a feasible and rewarding technique.LC-IR offers a variety of possible uses because it can distinguish between various compounds and separate complicated mixtures. One such application is in environmental assessments, where it is necessary to find and examine trace levels of toxins.

. LC-IR has been used to identify trace amounts of herbicides in water samples, with solvent elimination applied to enhance sensitivity. [23] Characterizing bacteria is another use, which has military, medical, food, agricultural, and other uses. Researchers were able to get clear spectra of cellular components by combining the LC's separation capability with the traditional FT-IR technique. That enables a better knowledge of the chemical makeup of various bacteria. [24]

1. **LC-NMR**

This hyphenated technique is a combination of liquid chromatography (LC) and nuclear magnetic resonance spectroscopy (NMR) which involve separation followed by sample detection by NMR spectroscopy. LC-NMR is an influential analytical technique helpful to resolve complex mixture. [25] It has wide range of applications. As a microanalytical technique, HPLC-NMR enables the detection of a variety of natural compound groups and other biomolecules at nanogram or even picogram scales, and can thus aid in the resolution of biochemical, physiological, and chemo ecology research issues.[26]It is a useful instrument for analyzing natural products. The online method is typically used to give a brief summary of the key elements present in plants and other sources of natural products. The more sensitive stopped-flow approach permits the utilization of various homo- and heteronuclear correlation NMR investigations as well as the identification and structural assignment of even small components.[27]However, information from other analytical methods, particularly MS, is needed for the unambiguous structure assignment of new compounds with unexpected structural types. It is feasible to fully elucidate a structure along with stereochemical information using numerous online combinations, including NMR, is possible but currently is rather the exception.[28] Rapid development in [analytical chemistry](https://www.sciencedirect.com/topics/chemistry/phase-composition) is expected to overcome present limitations of HPLC-NMR.LC-NMR is also fused with other analytical methods such as solid phase extraction or SPE (LC-NMR-SPE), Mass spectroscopy or MS (LC-MS-NMR), two-dimensional NMR measurements (LC-2D NMR), shows efficient separation and identification with more sensitivity. [29](The flow diagram of LC-MS has been shown in figure 3)



**Figure 3: Schematic diagram of LC-NMR**

**Triple hyphenated techniques**

The hyphenated techniques with a combination of three different analytical techniques at particular interfaces are called as triple hyphenated techniques.

1. **LC-NMR-MS**

This technique combines three analytical techniques to give hybrid platforms combining LC with NMR and MS allows a rapid and more accurate characterization of unknown compounds in complex clinical and pharmaceutical samples, and even [natural product](https://www.sciencedirect.com/topics/chemistry/occurrence-in-nature) extracts. [30] Therefore, the complementary information produced by the two detectors can be used to clearly reveal the structure of predicted analytes without the need for prior component isolation. The identification and characterization of degradation products, contaminants, metabolites, natural compounds, etc. are hence applications of LC-NMR-MS. LC-NMR-MS systems have been used increasingly frequently to examine the pure components from the difficult to enrich and produce synthetically mixture of natural product extracts and in vivo matrices including metabolites.[31] LC-NMR-MS is highly helpful in the elucidation of metabolites having confusing structures, for example, when the position of [hydroxylation](https://www.sciencedirect.com/topics/chemistry/hydroxylation) could not be fixed because of availability of several sites. This fusion provides mass and NMR information in a single run without any isolation and enrichment. Various literatures have reported the application of LC-NMR-MS for the identification of metabolites in biological fluids like Urine.[32]

1. **LC-API-MS**

This technique has been produced by the combination of high performance liquid chromatography (HPLC), atmospheric pressure ionization (API), and Mass Spectrometry. It is developed to identify and characterize individual components in complicated biological mixtures. [33] Direct identification of a numerous proteins can be done by LC-API-MS in the biological assay. It is also used in analysis of protein in urine and blood plasma. Such applications require efforts by analyst while preparation of samples. Common methods for the sample preparation in various biological assays are liquid-liquid extraction (LLE), size exclusion chromatography (SEC), hydrophilic and hydrophobic solid phase extraction (SPE), affinity chromatography (AC), isoelectric focusing chromatography (IFC). [34]

The samples were separated with the help of liquid chromatograph HPLC 1100 micro series (Agilent, Waldbronn, Germany) and analyzed by means of an electrospray-coupled Q-ToF mass spectrometer (Ultima, Waters-Micromass, Manchester, UK). The HPLC system was controlled by ChemStationsoftware (Rev. B01.01, Agilent, Waldbronn, Germany) and the mass spectrometer by MassLynx 4.0 software (Waters-Micromass, Manchester, UK). The data analysis was also performed by the MassLynx 4.0 software. [35]

1. **LC-ESI-MS**

LC-ESI-MS ionization (ESI) interface. Chromatographic separation carried out through a Venusil HILIC column (150 mm × 4.6 mm, 5 µm; Agela, USA) and is a hyphenated mass spectrometry method. This technique consists of the fusion between the elution of High-Performance Liquid Chromatography (HPLC) method with the greater Mass accuracy of the Mass Spectrometer. It is powerful technique used for the analysis of complex oligonucleotides mixture very efficiently and readily. [32]The instrumentation part of LC-ESI-MS/MS system consisted of an Agilent 1200 HPLC System (Santa Clara, CA, USA) and a Thermo Finnigan TSQ triple-quadrupole mass spectrometer (San Jose, CA, USA) equipped with an electrospray isocratic elution with the mobile phase of acetonitrile/water/formic acid (75:25:0.1, v/v/v). [36]

**III.APPLICATIONS OF HYPHENATED TECHNIQUES**

1. **Natural product Analysis**

 Hyphenated method used for the quick structural analysis of natural compounds in crude plant extracts and also used to identify previously isolated substances present in the extract. There are various online identification strategies that can be combined with hyphenated procedures. Online chemical analysis of Erythrinavogelii (Leguminosae) elements and online assessment of their antifungal potential study of unbalanced natural compounds using stop-flow and on-flow techniques. LC-NMR along with being a useful technique for reproducing natural compounds in crude plant extracts, LC-NMR can also be used in conjunction with conventional in-mixture NMR investigations to analyze the structural composition of thermolabile products in simple fractions. Study of the antioxidant compounds from Eriophorumscheuchzeri (Cyperaceae) by a combination of on-flow LC-NMR and online bioassay and complementary at-line CAP-NMR measurements as stated before, on-flow LC-NMR did not always provide sufficient information for the structure determination of de novel natural products and, thus, at-line spectra can be recorded with more sensitive probes such as CAP-LC-NMR. This aspect is illustrated here by the investigation of the antioxidant compounds of the swiss alpine plant Cyperaceae Eriophorumscheuchzeri. [37,38]

1. **Structural Elucidation of Impurities**

Structures based on MS Fragmentation in most cases, pseudomolecular ions are producedusing atmospheric pressure ionization procedures, but only fragments can reveal information about the structure of the underlying substance. As a result, fragments must be formed in a subsequent phase. Depending on the instrument being utilized, this process is referred to as MS/MS or MS. The pseudomolecular ion is chosen and separated In the first step of MS/MS. Injecting the ion into a collision cell with an inert gas at a little higher pressure is one way to give the ion energy. Through several collisions, the ion is energized and fragmented into smaller pieces. These daughter ions are discovered in the final phase. [39]

1. **Chiral amino acid analysis with MS detection**

After the peptide has been hydrolyzed, a chiral amino acid study is often conducted to characterize diastereomeric peptides. The analysis can be carried out by converting the respective amino acid enantiomers into diastereomers that can be differentiated using reversed phase chromatography after conversion of chemical compound into the product of similar structure of the amino acids using Marfey's reagent (Bruckner and Keller-Hoehl, 1990). It would be necessary to have access to reference materials in order to identify the corresponding pairs of diastereomers. Additionally, optimizing the separation may be quite difficult, particularly with a higher number of amino acids. [40]

1. **In food analysis**

The analysis of food samples is done for a variety of reasons. Scientists work in all key areas of the food industry, analyzing food and food components. As a result, this network consists of food producers, suppliers of food ingredients, as well as governmental, academic, and service laboratories. To ensure food safety comes first. For instance, if a food contains harmful substances like pesticides, herbicides, or poisonous metals, harmful bacteria or other microbes like salmonella, or offensive materials like glass, wool, or insects, it may be deemed unhealthy. [41]

Despite playing a significant role in food analysis, Due to the complexity of food matrices, MS cannot be utilized as a stand-alone method. On the other hand, sensitive, exacting, and repeatable methods for applications requiring food analysis are provided by HPLC, GC, SFC, and CE in combination with an MS detector. While GC-MS is particularly suitable for the analysis of volatile organic compounds in food and food products, SFC-MS aids in the research of thermally unstable and nonvolatile food components that are difficult to examine by GC-MS. SFC-MS, on the other hand, makes it more difficult to analyze polar molecules. The use of HPLC-MS ensures various benefits, including extraction processes that are completed more quickly and with less effort. Additionally, the capacity of HPLC-MS can recognize and quantify a wider variety of Food ingredients that are polar, semipolar, and nonpolar with a wide variety of molecular Sizes enable this method to outperform GC-MS or SFC-MS. Furthermore, peptide masses could cross over even when utilizing a high-resolution mass spectrometer when doing proteome analysis on complex dietary samples, which is where LC-MS/MS is most frequently used. For the quick and extremely accurate separation of weakly ionic, strongly polar, and ionic substances, electromigration techniques like CE-MS are particularly well suited. Low cost, quick analysis, and minimal sample and reagent usage are the key benefits. It is environmentally favorable because there is a decrease in the use of organic solvents. The fundamental disadvantage of CEUV detectors is their low sensitivity; however, sensitivity can be increased by CE-MS coupling. Moreover, pre concentration methods and The sensitivity of CEMS can be raised even higher by using high-resolution mass spectrometry. However, LC-MS or GC-MS platforms are more reliable and stable than CE-MS platforms. [42]

1. **Glycoprotein analysis by LC-EIS-MS**

Due to its speed, high sensitivity, accuracy, and suitability for automation, mass spectrometry (MS) has emerged as the method of choice for proteomics and associated study fields including glycemic and glycoproteomics. A broad range of biomolecules can be analyzed using a variety of mass spectrometers and ionization/fragmentation procedures, offering considerable adaptability in a variety of scenarios.[43]

1. **Coupling of Liquid Chromatography and Mass Spectrometry as Interface Technique**

HPLC-MS coupling became a success story with the introduction of the so-called atmospheric pressure ionization (API) interfaces some 10 years ago. The HPLC eluent is evaporated outside the mass spectrometer via API interfaces. At atmospheric pressure, the analyte is also ionized outside the spectrometer, and only the ions formed are delivered into the mass spectrometer, so that no huge gas freight has to be pumped off. As a result, mass spectrometric detectors can now easily be combined with liquid chromatographic separations. Electrospray ionization is the most widely used interface technique (ESI) followed by atmospheric pressure chemical ionization (APCI) [44]

1. **Drug Discovery**

For identification of a drug and determining its structural composition the most specialized analytical techniques Nuclear magnetic resonance (NMR) is used. It is also important in the discovery and development of drugs, quantitative 1H NMR spectroscopy. It discusses the fundamentals of quantitative NMR (qNMR), the physiological and chemical factors that influence qNMR, and the most recent quantification referencing techniques. The precise application of qNMR at several stages of drug discovery and development, such as investigations of natural products, dosage form quantification, drug metabolism, impurity profiling, and solubility evaluations, is described in detail. NMR tests are employed in the drug discovery and development process due to their non-destructive nature, adaptability, robustness, and great intra- and inter-subject variability.

Nuclear magnetic resonance (NMR) is one of the most specialized analytical techniques for this purpose since it allows for the identification of a drug and the structural determination of that molecule, which are the two most vital steps in the process of discovering new drugs. Drug research and development using quantitative 1H NMR spectroscopy. It covers the principles of quantitative NMR (qNMR), the physiochemical factors impacting qNMR, and the most recent quantification referencing approaches. The precise use of qNMR during many stages of drug discovery and development, such as natural product research, drug quantification in dosage forms, drug metabolism investigations, and impurity profiling and solubility assessments, is elaborated. Because NMR tests are non-destructive, adaptable, and robust with high intra- and inter-subject variability, they are used in the drug discovery and development processes used to find new therapeutic drugs, and MR techniques are the key to this process.

NMR-based procedures are simpler and faster as compared to other approach, which typically require real reference standards for measurement. [45]

Several drug formulation and purification methods can make accurate use of quantitative 1H NMR technology. By using 1H NMR techniques, rapid quantitative measurements can be made while developing an API (active pharmaceutical ingredient) for a potential medicine. Recently, NMR has been capable to maintain the clinical validation phase of drug development due to the development of qNMR into the study of the metabolome. [46]

1. **Identification of Functional Group**

Using GC in conjunction with IR and UV detectors, functional groups in pharmaceutical compounds can be detected using a multiple techniques. Functional group identification was carried out utilizing GC-IR prior to the creation of fast FTIR instruments. In this instance, a sample that was injected into the GC after being separated using a column is deposited on a salt window in the IR instrument, causing the functional groups in the sample to absorb IR light. This uses infrared spectroscopy as a separation method, then identification, similar to GC-MS. [47]

1. **In Clinical Toxicology**

Clinical toxicology benefits from the use of GC due to the creation of molecular ions, the availability of a wider variety of substances that may be analyzed, its high sensitivity, and faster analysis. Supersonic GC-MS is frequently used in clinical toxicology. In some circumstances, this is also used to confirm or reject LC-MS analysis findings. This technique is typically used to identify and measure toxins and venoms.[48]

1. **Drugs Abuse Analysis**

GC-MS: Headspace combined is a useful analytical tool for the analysis of illegal drugs. examining the metabolites of amphetamines in urine and determining the amount of nicotine in prescription medicines are examples of this type of analysis. In SIM mode, GC-MS with chemical ionization and traditional headspace will provide greater sensitivity by roughly 20 times. It is simpler to compare discovered chemicals with library data thanks to GC MS's consistent ionization [ 49,50]

1. **In fingerprint analysis**

A physical technique for separating mixtures is chromatography,used for identification of certain chemicals those found in the herbal extract of herbal medicine (HM). For the purpose of analyzing herbal medicine's fingerprints, chromatographic methods such as, gas chromatography (GC), liquid chromatography (LC), capillary electrophoresis (CE), thin layer chromatography (TLC), etc. were applied. The most often used technique is LC hyphenated techniques, which have the traits of large applicability, full automation, great selectivity, sensitivity, and resolution. The biggest benefit of LC is the ability to choose detector arrays based on the physical or chemical characteristics of intended substances. Additionally, to obtain more fingerprint details from a complicated biological material, several studies were performed in that the data fusion of complementary detectors and the multi-wavelength combination of high-performance liquid chromatography-diode array detection (HPLC-DAD) were discovered.[51]

**IV. ADVANTAGES OF HYPHENATED TECHNIQUES**

1. It is use for rapid and accurate analysis
2. Greater automation
3. More samples are processed
4. Better reproducibility
5. Separation and quantification are achieved simultaneously by using a hyphenated approach.
6. Its closed system reduces contamination. [52]

**V. DISADVANTAGES OF HYPHENATED TECHNIQUES**

1. GC-MS not suitable for non-volatile and thermo unstable compound. It required derivatization depending on the type of molecule that are analyzed and derivatization can mask the result
2. Fragmentation pattern in LC-MS are poorly reproducible, therefore the database are problematic, the entire compound in plant extract will ionize under the same condition.
3. Most important disadvantage of LC-NMR is that it is time consuming process. And duteriated solvent which has partial use are used for analysis, there is difficulty in solvent selection
4. The LC-NMR technique involving elevated charges and costly equipment .
5. Skilled professional required therefore there is a requirement of operator training. [53]

**CONCLUSION**

A hyphenated method is a coupling (or) combination of two various analytical techniques with the help of appropriate interface. Hyphenated techniques combine the strength of separation and quantification for more in-depth analysis, in turn solving more complicated problems. A variety of double and triple hyphenated techniques, including LC-MS, LC-IR, LC-NMR, LC-ESI-MS and LC-NMR-MS and, have been utilized because of their several benefits, including increased sample throughput, higher levels of automation and separation reproducibility, quicker analysis, and simultaneous quantification. The significant advancements in hyphenated analytical techniques have greatly expanded their use in the analysis of biomaterials, especially natural products over the last two decades.

**ACKNOWLEDGEMENT**

The authors wish to thank the Principal and Management of Shree Sainath College of Pharmacy ,Nagpur, India for providing opportunity for this book chapter.

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