WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 9, Issue 12, 647-662. Review Article ISSN 2277– 7105

A REVIEW ON THE LIQUID CHROMATOGRAPHY- NUCLEAR MAGNETIC RESONANCE (LC-NMR) AND IT'S APPLICATION IN PHARMACY

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Article Received on
11 August 2020,

Revised on 01 Sept. 2020,
Accepted on 22 Sept. 2020,

DOI: 10.20959/wjpr202012-18839

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ABSTRACT

The hyphenated technique is developed from the coupling of a separation technique an on-line spectroscopic detection technology. 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. 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. LC-NMR is the hyphenated technique in which HPLC is combined with the NMR. There are various NMR probes that can be used for increasing the efficiency of LC-NMR. This technique is widely used for the analysis of complex mixtures which contain unknown impurities, natural products and synthetic polymers. In LC-NMR LC does the separation and NMR does the identification of separated components. Further hyphenated technique such as LC-NMR/MS are also described.

KEYWORDS: Hyphenated technique, LC-NMR, LC-NMR Coupling, Separation technique.

INTRODUCTION

Whenever analytical method connects chromatographic techniques and spectrometric method called as a hyphenated technique.[1] Nuclear magnetic resonance (NMR) spectroscopy is a technique for the structure elucidation of large and small compounds. It provides detailed information about the structure, reaction state, and the chemical environment of the molecule. Demand of NMR spectroscopy increased due to multiple application in different fields. The

technique has been completely applied in different area such as structure elucidation unknown compound and confirmation of known compound; characterization of impurities in pharmaceutical substance and degradation products in pharmaceutical products. It also has been applied in study of natural products, metabolism research, study of bio-molecule (like carbohydrates, nucleic acid, proteins. bio-medical sciences; crystallography, etc. On other hand it is also used in the research and development at manufacturers.[2-6]

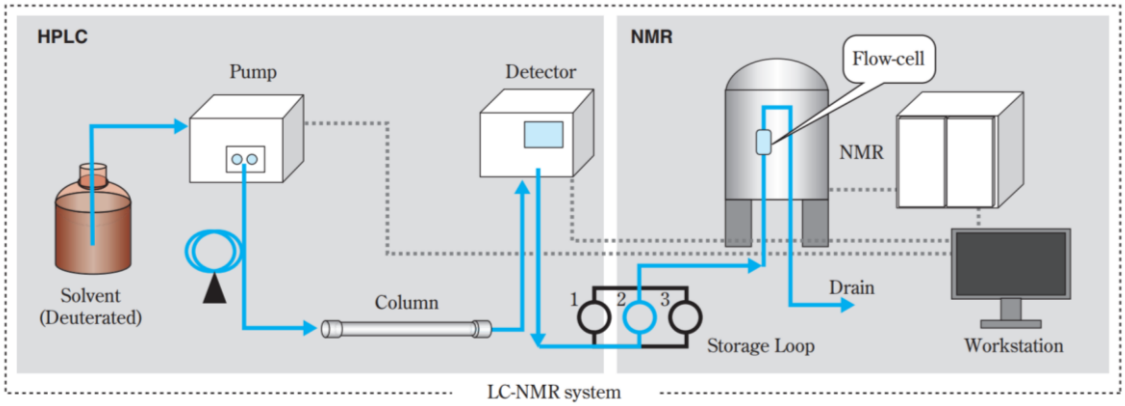

Figure 1: Schematic view of conventional LC-NMR system.

High-performance liquid chromatography (HPLC) is the most commonly used analytical separation technique for the qualitative and quantitative determination of the compound. Mass Spectrometry (MS) and Nuclear magnetic resonance (NMR) are the analytical techniques that provide the structural information of the molecule. The connection of HPLC (LC) and MS (LC-MS) or NMR (LC-NMR) increase the ability of solving the structural problems of mixture of unknown compounds. LC-MS has been widely applied hyphenated technique because MS has higher sensitivity as compare to NMR.[7-9] Recent advances in NMR, LC-NMR and even LC-MS-NMR these techniques become a powerful tool in routine analysis in many laboratories. The article provides an overview of LC-NMR and LC-MS-NMR techniques and the example of these techniques.

History of LC-NMR

In spite of the fact known that this approach is time consuming and technically demanding, both the LC and NMR have been and are still routinely used in the mixture analysis. In theory, the physical coupling of LC and NMR could save a lot of time and was proposed over 30 years ago. Even then the successful and applicable coupling of LC-NMR was achieved in past three decade.

The first on-line LC-NMR experiments were performed in late 1970s by Watanabe and Niki who demonstrated stopped-flow measurements of mixture of known compounds. The conventionally used NMR probes was converted to the flow-through probe by the use of the thin-walled Teflon capillary within a standard NMR tube and spectra were recorded with sample rotation.[10]

The first real sample to be analyzed by LC-NMR technique was a military jet fuel using the normal phase columns and duteriated chloroform and Freon.[11]

After the advances made the combination of LC-NMR was made. LC-NMR and LC-MS are considered to be the most valuable techniques for the structure elucidation of the unknown compound in wide field of application. This technique is essential for analysis of products obtained from natural sources because; various closely related substances are present in their extracts, which are difficult to separate. It is important to note that substances derived from plant origin are almost containing 40 % of newly registered compound present in the drug discovery program. Thus, there is the need for development of new innovative technique that can describe the profile of each and every component of complex mixture and that to in a very simple way as well as fast procedure, this has become a challenge and this is to be looked forward into.[10]

Basic Principals of LC-NMR

High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography which is also known as High Pressure Liquid Chromatography. It is a popular analytical technique used for the separation, identification and quantification of each constituent of mixture. HPLC is an advanced technique of column liquid chromatography. The solvent usually flows through column with the help of gravity but in HPLC technique the solvent will be forced under high pressures up to 400 atmospheres so that sample can be separated into different constituents with the help of difference in relative affinities.[11-18]

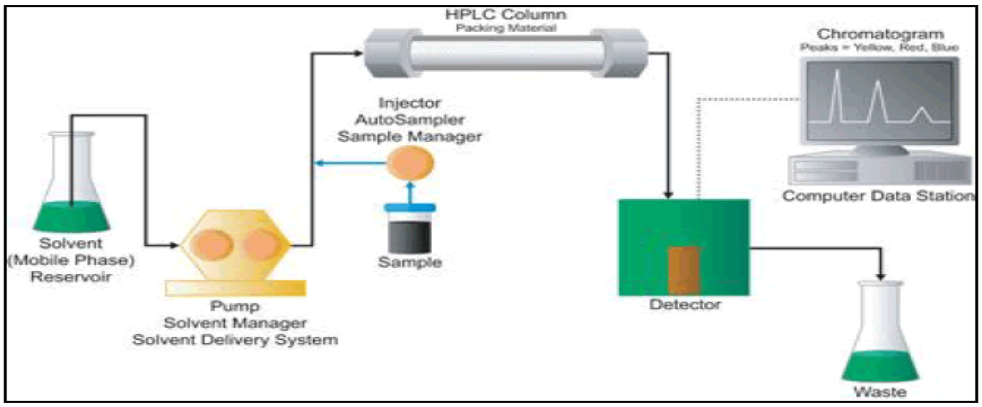

Figure 2: Block Diagram of HPLC.

Nuclear magnetic resonance (NMR)

The principle behind NMR is that many nuclei have spin and all nuclei are electrically charged.[19] If an external magnetic field is applied, an energy transfer is possible between the base energy to a higher energy.[19] The energy transfer takes place at a wavelength that corresponds to radio frequencies and when the spin returns to its base level, energy is emitted at the same frequency. The signal that matches this transfer is measured in many ways and processed in order to yield an NMR spectrum for the nucleus concerned.[20]

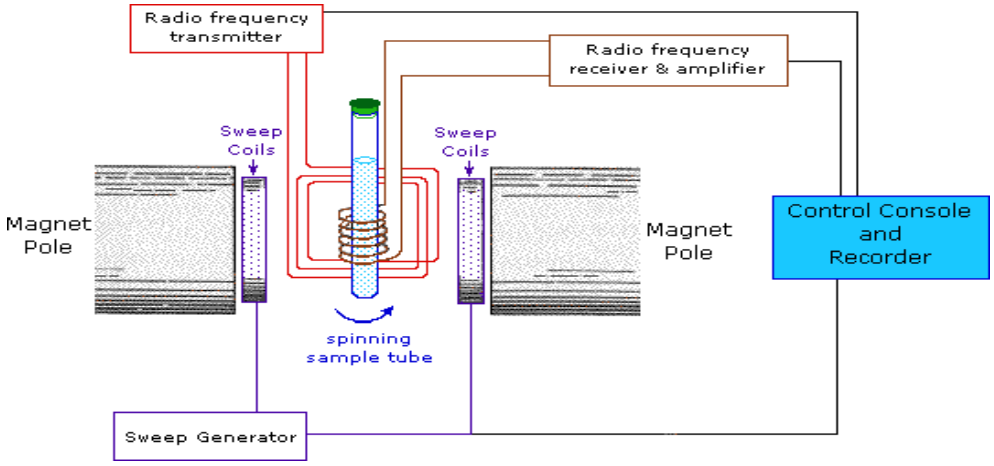

Figure 3: block Diagram of NMR.

Coupling of the LC and NMR

The proper learning and developing skills of a conventional NMR spectrum necessitates the dissolving of the sample to be tested in the duteriated solvent. This sample solution is introduced in a cylindrical sample tube and placed in a conventional NMR probe within the NMR magnet. As already described, that it requires a probe that must be modified to allow the continuous flow of the solution that is under study. The LC-NMR coupling technique

should involve the appropriate interface of LC and NMR, flow through sampling probe design and many other factors such as solvent suppression, NMR sensitivity, LC and NMR compatible solvents and volume of chromatographic peak verses the volume of the NMR flow cell. Different modes of operations for LC-NMR are used which can be distinguished based on the status of the samples during measurement. For example, the sample under observation is flowing continuously through the NMR flow cell during acquisition than mode of operation is the on-flow mode.[20]

Instrumentation of LC-NMR

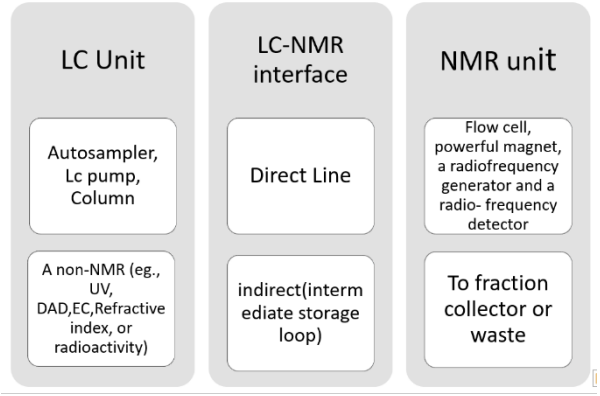

Figure: 4 Interfaces of LC-NMR.

Direct coupling: It include direct flow of LC effluent in to NMR flow cell and continuous recording of spectra

- post-column splitter
- valve-switching interface i.e BNMI (Bruker NMR-Mass Spectrometry Interface)

Indirect coupling: Intermediate storage loop which transfer outlet of lc to NMR flow cell at specified time interval. This can be done by solide phase extraction.

Modes of LC-NMR

- **Continuous Flow (on flow):** Eluent sampled in "real time" as flowing through NMR Detection Coil
- **Stopped Flow:** Pump is stopped at desired location and data acquired
- **Time Slices:** Regions, or "time-slices" of interest are analyzed
- **Peak Parking:** Peaks of interest are "parked" in off-line sample loops
- **Peak Trapping:** Solid Phase Extraction cartridges are used to "re-concentrate" samples.

Continuous flow[21]

The outlet of the LC-detector is connected directly to the NMR probe. While the peaks are eluting, NMR spectra are continuously acquired.

The chromatographic system is used to move the samples/peaks through the NMR cell

Equipment: Any HPLC system, which delivers a stable pulse free flow. LC-NMR Probe, LCNMR interface not required.

Stopped flow

The outlet of the LC-detector is connected directly to the NMR probe. A LC-detector (normally UV) is used to detect peaks eluting from the column When a peak is detected, the flow continues until the peak arrives in the NMR cell. At this time, the chromatography (pump, data acquisition, gradient) stops and the NMR experiments are performed Once the NMR experiments are completed, the chromatography resumes until the next peak is found. This process can be repeated several times within one chromatogram.

Equipment: HPLC system, LC-NMR Probe, Controlling station.

Time slice method

It include to stop the flow at short interval over the chromatography peak to time slice different part of chromatography run .It is useful if there is poor chromatography separation or if compound under study have poor or no UV chromophore or if the exact chromatography retention time is unknown. The data from such a time slice experiment referred as a total NMR chromatogram(tNMRc).

Peak parking method

The outlet of the LC-detector is connected to the sample loops of the BPSU-36 or BPSU-12. A LC-detector (normally UV) is used to detect peaks eluting from the column.

A detected peak is moved into one of the sample loops without interrupting the chromatography. When the chromatography is completed, the HPLC pump is used to transfer the peaks from the loops into the NMR probe.

Equipment: Any HPLC system, Pump under control for transfer, LC-NMR Probe, Controlling station.

Peak Trapping method

The outlet of the LC-detector is connected to the SPE unit. A LC-detector (normally UV) is used to detect peaks eluting from the column.

A detected peak is moved trapped on a SPE cartridge without interrupting the chromatography. When the chromatography is completed, the chromatography solvents are removed and the peak is transfer with fully deuterated solvents into the NMR probe.

Equipment: Any HPLC system - pump under control for transfer, LC-NMR Probe, SPE system, Controlling station.

Technology to improve sensitivity of LC-NMR method

1) LC method

a) On line SPE method

b) On line column trapping method

c) Use of semi micro column

2) NMR method

a) high strength magnetic field

b) high sensitivity probe

3) Solvent supression method

a) presaturation

b) soft pulse multiple irradiation

c) WET method

LC method

- **On line SPE method**

It is important to eliminate unnecessary fractions by efficient pre-treatment, introducing only the targeted component to the column and controlling overloading. The SPE cartridge absorbs the desired peak After the sample is dried with N_2 gas and the contents are finally eluted from the cartridge into the NMR flow probe.

- **On line column trapping**

In this method, after separation using a conventional column, concentration is first done in a trap column, and the sample is separated again using a semi-micro column then introduced to NMR. Concentration by this technique is highly effective. Once sufficient sample has been collected on the trap, the flow reversed and the solute is transported to the NMR for further analysis.

- **Use of semi micro column**

The highest sensitivity is provided when all of the components separated by HPLC are introduced to the flow-cell of NMR.

However, the peak volume separated by HPLC is greater than the flow-cell capacity (normally about 30 μL to 120 μL) therefore, only part of the component is actually the target of measurements.

The method of using columns with an internal diameter of around 2 mm, known as semi-micro columns, is a peak concentration method suited to LC-NMR.

The volume of a semi-micro column is around 1/5 of a conventional column, and since the required amount of solvent is reduced in proportion to the elution, highly concentrated sample solutions can be introduced to LC-NMR.

NMR methods

- **High strength magnetic field**

NMR detection sensitivity is proportional to the magnetic field strength to the 3/2 power,and the stronger the external magnetic field is, the higher the sensitivity.

Currently, the magnetic field strength has reached 1000 MHz. Magnetic fields being generated by modern instruments employing cryomagnets, field homogeneity is high and as a consequence the sample need not be rotated.

- **High-sensitivity probe**

It is also known as a cryogenic probe that reduces the heat noise arising during NMR signal detection by cooling the coil using superconductor materials. This will eliminates the thermal electronic noise associated with the initial stages of signal detection and increases the coil quality factor. This leads to an improvement in the S/Nratio by a factor of 3-4.

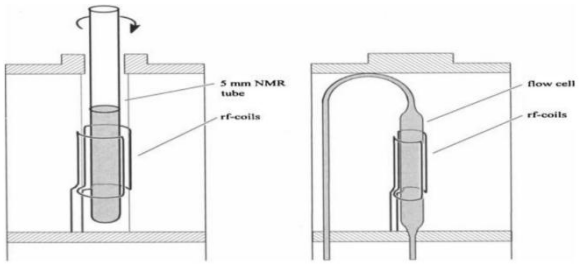

Figure: 5 a) Conventional NMR probe. b) Continuous flow NMR probe.

Solvent supression methods

- **Presaturation**

It depend on the phenomenon that nuclei which are unable to relax because their population in ground state and exited state is same, do not contribute to free induction decay after pulse irradiation Before the data acquisition, a highly selective low power pulse irradiates the desired solvent signal for 0.5 to 2 s. This leading to saturation of solvent signal frequency. During data acquisition, no irradiation should occur. This method is used for stopped flow mode.

- **Soft pulse multiple irradiation**

Here, presaturation is performed with the use of shaped pulse which has a broader excitation profile. This method is better suitable for supression of multiplets.

- **WET method**[22]

This technique contains NMR difference probe. This difference probe consists of a dual coil probe that contains two sample coils in a resonant circuit that switches between parallel excitation and serial acquisition to cancel common signals, such as solvent and solvent impurities.

Essentially, this technique is based on a dual beam background subtraction, where the reference signal and sample signal that are collected simultaneously are subtracted from each other automatically. No software manipulation, pulse sequence modification, or spectrometer alteration is necessary. Hence the technique does not lengthen the pulse sequence but it reduces experimental time. It takes 50-100 ms. So it is used for on flow method. This method is used for on flow mode.

New Technology Combining LC-NMR with Other Detectors

In addition to high-performance LC-NMR, integral structural analysis techniques that combine LC-NMR with other detectors have been practical. These methods give information that cannot be obtained by LCNMR alone from different detectors, and as a result multifaceted structural analysis become possible. Here, we will give an introduction focusing on LC-NMR/MS which is a combination with MS, which is particularly useful in the analysis of fine chemicals.

LC-NMR/MS

If NMR and MS measurements can be made at the same time, it is possible to unambiguously determine the structures of most organic compounds. Therefore, the advent of LC-NMR/MS was comparatively early, and a research group at Pfizer reported on it in the latter half of the 1990s.[23] However, the first LC-NMR/MS was a system where a splitter was installed downstream of the LC, and the NMR spectrum and mass spectrum were obtained at the same time. Since deuterated solvents are present as the mobile phase, molecular ions where the hydrogen atoms of compounds having exchangeable protons were replaced by deuterium atoms were observed, and the interpretation of the obtained mass spectra was difficult.

Subsequently, this problem was solved by the development by Exarchou et al of LC-SPE-NMR/MS (Figure.6) that incorporated SPE.[24] If this method is used, the deuterated solvent is only used in the elution from SPE to NMR, and a normal mass spectrum with no deuterium substitution can be obtained at the same time as the NMR spectrum.

There are not a few cases where structural information that could not be obtained by LC-NMR alone can be obtained by using this system. A fine example is the obtaining of useful information about compounds having NMR-silent halogen atoms from the mass spectrum. The species and number of the halogen atoms contained in the halogen compounds can be identified from the characteristic isotope patterns.[25] In addition, it is difficult to distinguish among compounds having repeated structures with NMR, but it is comparatively easy to make estimates from molecular weight information. Therefore, it is expected that the combination of NMR and MS will become the main current in hyphenated techniques.

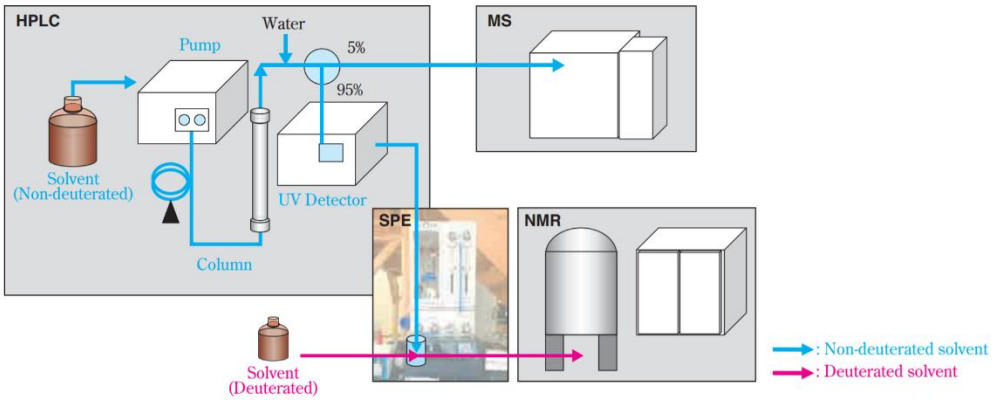

Figure 6: Schematic diagram of the LC-UV-SPE-NMR/MS.

Applications

1. Use of LC–MS/TOF, LC–MSn, NMR and LC–NMR in characterization of stress degradation products: Application to cilazapril.[26]
2. The use of LC/MS, GC/MS, and LC/NMR hyphenated techniques to identify a drug degradation product in pharmaceutical development.[27]
3. The application of LC–NMR and LC–MS for the separation and rapid structure elucidation of an unknown impurity in 5-aminosalicylic acid.[28]
4. 4-Hydroxyphenylacetic acid derivatives of inositol from dandelion (Taraxacumofficinale) root characterized using LC–SPE–NMR and LC–MS techniques.[29]
5. Structural investigations on beta cyanin pigments by LC NMR and 2D NMR spectroscopy.[30]
6. Structural elucidation of in vivo metabolites of isobavachalcone in rat by LC–ESI-MSn and LC–NMR.[31]
7. A multi-technique approach using LC–NMR, LC–MS, semi-preparative HPLC, HR–NMR and HR–MS for the isolation and characterization of low-level unknown impurities in GW876008, a novel corticotrophin release factor 1 antagonist.[32]
8. Application of directly coupled LC–NMR–MS to the structural elucidation of metabolites of the HIV-1 reverse-transcriptase inhibitor BW935U83.[33]
9. Application of evolving factor analysis to on-flow LC– NMR data using spectral windows.[34]
10. Application of LC-NMR for the study of the volatile metabolite of MK-0869, a substance P receptor antagonist.[35]
11. Application of LC– MS and LC– NMR Techniques for Secondary MetaboliteI dentification.[36]
12. Application of LC-NMR and HR-NMR to the characterization of biphenyl impurities in the synthetic route development for vestipitant, a novel NK1 antagonist.[37]
13. Application of LC-NMR and LC-MS to the identification of degradation products of a protease inhibitor in dosage formulations.[38]
14. Biodegradation pathway of mesotrione: Complementarities of NMR, LC–NMR and LC–MS for qualitative and quantitative metabolic profiling.[39]
15. Characterization of triacetone triperoxide (TATP) conformers using LC-NMR.[40]
16. Detection of methyl quinoline transformation products in microcosm experiments and in tar oil contaminated ground water using LC-NMR.[40]

17. LC-NMR identification of a novel taurine-related metabolite observed in 1H NMR-based metabolomics of genetically hypertensive rats.[41]
18. Solvolysis of 14, 17-etheno-bridged 16 anitroestratrienyl acetate and lactam formation pathways studied by LC–NMR and LC–MS. Structures of minor products.[42]
19. Separation and characterization of peptide libraries.
20. Combinatorial chemistry, photochemical analysis, drug discovery
21. Identification of drug impurities.
22. Characterization of isomers of acid glucoronides and vitamin A derivatives.
23. Characterization of endogeneous and xenobiotics metabolites directly from biological fluid.
24. LC-NMR MS have identified analogues of vitamin E of palm oil extract
25. 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.
26. LC-NMR allowed the differentiation of isomers and identification without reference compound.

CONCLUSION

Hyphenated technique like LC-NMR is seen as a highly optimized powerful tool for studying and determine various unknown compounds in drug development and plant metabolism. Use of hyphenated techniques is by far the most powerful strategy that an analyst can use to study complex mixtures of products. LC–NMR coupling is well established with a series of available options for solving a variety of analytical problems. The different possible modes of operation available to ensure that the restrictions on the applicability of LC–NMR can be reduced to a minimum. LC-NMR-MS is used to determine vary complex compound and also optimize choice of instrumentation. After looking to the history, development and application of the LC-NMR that took place in past years, we can conclude that these techniques can be used for the characterizations of many new upcoming molecules, detection of the impurities, determination of the unknown compounds from unknown sources, degradation products, etc.

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