

A Review: Liquid Chromatography-Nuclear Magnetic Resonance Spectroscopy (LC-NMR) and Its Applications

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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. 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.

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

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I. INTRODUCTION

Whenever the chromatographic techniques and the spectrometric methods are combined online, they are termed as the hyphenated techniques. The review that is going to be discussed i.e. LC-NMR, is a hyphenated technique which is the combination of the high performance liquid chromatography (LC) and nuclear magnetic resonance (NMR) spectrometers.

One of its type i.e. Capillary LC-NMR also significantly lowers the detection limits to nanogram ranges with the integration of the capillary LC with NMR detection¹.

This technique has been widely applied in the analysis of complex mixtures that contains number of unknown components such as various metabolites and impurities in the pharmaceuticals, natural as well as synthetic polymers. On other hand it is also used in the research and development at manufacturers²⁻⁶.

Perhaps, even more useful than MS is NMR, which is more arguably the most powerful tool for structure elucidation. It is therefore the reason for upcoming of the hyphenation of LC and NMR, despite the slow development of the technique. The basic requirement of the LC-NMR is the LC system together with an accessible NMR.

One of the key limitations that restricted the development of the LC-NMR technique, was the insight to analyze samples by the NMR the sample must be rotated so as to remove magnetic field in homogeneities. However with the magnetic field produced by the modern instruments employing the cyromagnets, field homogeneity is high and as a consequence the samples need not be rotated. In fact rotations of the sample lead to distortions in the 2D NMR. The first reported online coupling of LC and NMR utilizes the U-shaped tube as a sample holder located in NMR probe body. This early design demonstrated the excellent NMR resolution which can be obtained without rotation.

In comparison with the coupling of LC to MS, the physical unification of LC to NMR is straightforward. But the limitations of the techniques lie in two areas. The first is the suppression of signals from the commonly employed solvents used in solvents, where as the second is gaining the suitable sensitivity and spectral resolution⁷.

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 rotation⁸.

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 Freon⁹.

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 into⁸.

II. PRINCIPLES

2.1 Principle of HPLC:

HPLC is mainly based on the mechanism of either adsorption or partition. Other than these, HPLC is also based on some other types of technique chosen for the process.

2.1.1 Adsorption:

Adsorption is the adhesion of atoms, ions or molecules from a gas, liquid or dissolved solid to a surface (adsorbent). In this, separation depends on the relative affinities of the solute molecules towards the stationary phase (solid). The solute molecules having greater affinity towards the stationary phase take longer time to get eluted from the column while those having less affinity elute faster.

2.1.2 Partition:

Partition is a process where the solute molecules are partitioned between two solvents. In this, both stationary phase and mobile phase are in liquid state. The stationary phase is immobilized by the substance in column or by the paper (Whatmann filter paper).

The other types of techniques are: size-exclusion/gel permeation, affinity chromatography, ion-pair chromatography, chiral phase chromatography.

2.2 Principle Of-Nuclear Magnetic Resonance Spectrometry:

The principle behind NMR is that many nuclei have spin and all nuclei are electrically charged. If an external magnetic field is applied, an energy transfer is possible between the base energy to a higher energy. 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.

3. 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 versus 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.

On-flow LC-NMR (continuous flow)

In this mode, similar to UV and MS detectors in chromatographic systems here the NMR spectrometer is used. Since the sample is measured without the stoppage of the flow, it is called on-flow LC-NMR. The results obtained are typically displayed as a two-dimensional (2D) time-frequency plot consisting of a set of frequency domain versus retention time. The optimum flow rate for continuous-flow NMR is usually decided as a compromise between the flow rate required for the best chromatographic resolution and the best NMR sensitivity. The time of measurement for each analyte is limited due to the short residence time within the RF coil at the normal flow rates used and this results in a poor S/N ratio for the NMR spectra obtain. To increase the

retention time we need to reduce the flow rate, in this two cases arises. Firstly, in reduction of the flow rate by a factor of 3 to 10, increases the residence time and hence the time of measurement as well as S/N ratio of individual components. Secondly, diffusion at slow flow rates can reduce the efficiency of the chromatographic separation of the individual eluting peaks from the LC column or the NMR flow-cell. Therefore, the flow rates should be optimum balancing both resolutions as well as the S/N ratio of the components.

The limit of detection provided is not less than 10 μg (compounds with molecular weight 300 to 500) and the technique is limited to the quick 1D experiments. Nevertheless, it is still used as a method of getting the preliminary overview of major constituents in the samples. They are widely used for the studies of natural products. The majority of studies carried out indicated the use of gradient based reversed phased C18 column with $\text{CH}_3\text{CN}:\text{D}_2\text{O}$ (methyl cyanide and duteriated water) as mobile phase.

LC-NMR under static condition

To carry out static or non-flowing conditions in NMR measurements, there are two methods available:

- When the sample to be analyzed reaches the flow cell volume within the Rf coil, then valve can be used to stop the elution, this technique mode is called the stopped flow mode.
- Sample storage loops can be used to store the individual fraction of the analyte that are obtained from the chromatographic separation.

In both the cases the analyte can be determined using the more time demanding 1D and 2D experiments.

To perform the stopped flow mode experiments we need to determine the delay time i.e. time required for transport of analyte from the detector of the LC system to the specified position in the flow cell corresponding to the Rf coil. This delay time depends on the flow rate for chromatographic separation and diameter and length of the tube that connect the LC system detector with the NMR flow cell. Once the delay time is calibrated, the software can be set to work automatically i.e. stop the chromatographic run elution for the delay time after the passage of analyte through the LC detector. Once the NMR data is acquired, the chromatographic run is restarted and same procedure is repeated for the next analyte to be determined. A large number of the chromatographic peaks can be studied with this sequence of stopped flow data acquisition mode. But, these frequent stops may disturb the quality of separation and highly concentrated compounds may lead to the spoilage of the NMR detection cell (memory effects). Therefore, this stopped flow mode is preferable for the analysis of mixture of relatively small number of chromatographically resolvable components. So, for the determination of natural products, the combination of the on-flow mode and stopped-flow mode has been extensively used.⁸

Instrumentation of LC-NMR⁹

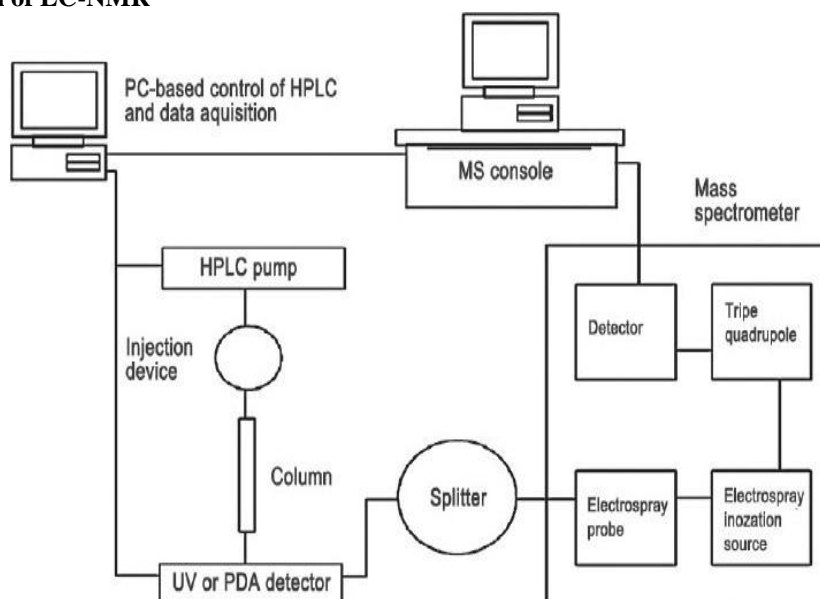


Figure 1: Instrumentation of LC-NMR

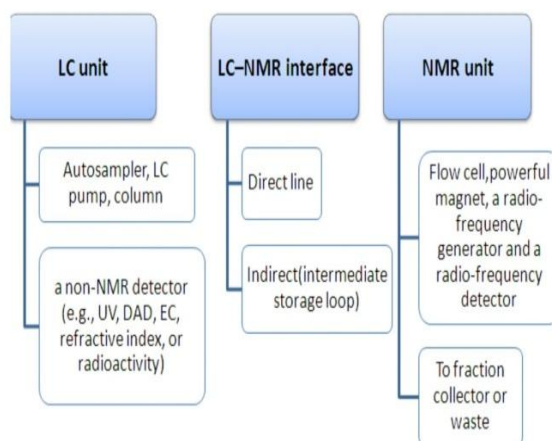


Figure 2: Interfaces of LC-NMR

Direct coupling:

It includes direct flow of LC effluent into NMR flow cell and continuously recording of spectra. Following can be used for this type of NMR:

- Post column splitter
- Valve switching interface i.e. BNMI (Bruker NMRNMR spectrometry interface)

Indirect coupling:

- Intermediate storage loop which transfer outlet of LC to NMR flow cell at a fixed specified time interval.
- SPE unit.

NMR Probes¹¹

The probe is the part of an NMR spectrometer that does most of its work, in terms of exciting the nuclear spins, and the sample is inserted inside the probe to perform the NMR experiments. These probes fundamentally consist of the radiofrequency coils which are tuned at the definite nuclei in a particular magnetic field. The probe also have be provided with some necessary hardware parts to control the sample temperature (with combined with an external temperature controller).

The probes are constructed with two coils, one closer to sample is termed inner coil and one far from coil is termed outer coil. This arrangement allows the probe to reply to multiple frequencies, and also to allow the excitation/irradiation of multiple nuclei. The nuclei that use the inner coil are detected with higher sensitivity.

The probes can be designed to accommodate various sizes of NMR tubes. In general, large volume tubes are best suited in case where the sample is solubility limited or concentration limited. Larger volume allows more sample to be contained in the coil. Smaller volume tubes allow the concentration of the sample to be increased when solubility is not the limiting factor. Small volume probes (i.e. 3 mm, nano or capillary coil) give the maximum sensitivity when very minor amount of highly soluble material are under study.

Modern NMR probes also contain an actively-shielded pulsed field gradient (PEG) coil, which allow the application of field-gradient pluses.

Probes of solid state NMR also contain the hardware which are necessary to spin the sample solution rapidly, at a precise angle to the magnetic field.

The probes that are used in the conventionally used Helmholtz (saddle) type radiofrequency coils in LC-NMR are matching with the dimensions of the analytical LC. Within the last 20 years the experimental setups have changed with gradual improvement from on-flow experiments to the stopped flow experiments and loop storage devices for obtaining peaks.

During the past few years the use of on-flow experiments have been vanished from the literature as this experiments showed insensitivity and referencing problems whenever the solvent conditions are changing i.e. the when the gradient solvents are used. In the gradient solvents the stopped flow and the loop storage experiments are widely used in the natural product chemistry and pharmaceutical chemistry.

The LC-NMR system is comprised of an NMR spectrometer console, a superconducting magnet, a workstation, and a flow probe, all under the operation of specialized LC-NMR software. The LC is comprised of an LC pump, LC column(s), variable-wavelength UV detector and/or photo diode array detector, and LC workstation that may be set up for stopped-flow or continuous-flow operation. Timing for movement of a peak

between the different positions in the hyphenated system must be carefully calibrated. The time required for a peak to reach the NMR probe or a designated collection unit depends upon the void volume between the LC unit and the collection units or probe flow cell.

This will depend upon flow rate. In order to allow selection of desired peaks, the separation is monitored by the LC detector, usually the UV detector, which displays the chromatogram of separation. The chromatography software allows certain positions in the chromatogram to manually or automatically select for further measurement. The NMR probe or the storage compartment is located downstream of the UV detector and is estimated to be reached about 10 to 40 sec after the first peak appears in the LC detector. The software calculates the appropriate delays to capture the peak as its desired position, the needed actions for storage or measurement are initiated. Software is commercially available from the instrument vendors that allows automated or interactive selection of peaks from the chromatogram and automatic calculation of the time delays.

Design of the on-flow probes

The design of the LC-NMR probes has always resulted from a compromise between the needs of the chromatography and NMR. The volume of the flow cell has indeed to be as small as possible for optimum chromatography and as high as possible for NMR detection. In order to significantly reduce the volume and maintain a good sensitivity, the Rf coil of the NMR probe has been directly fixed onto the NMR flow cell wall. Thus, in contrary to the conventional NMR measurements, it is impossible to spin the sample in LC-NMR. Spinning was needed to improve the RF field homogeneity in the sample but this seems not to be a problem because the homogeneity is good in the small cell volumes of the flow-probes, notably because modern computer methods for optimizing the field homogeneity reduce the requirement for spinning. Thus, LC-NMR cell consist of a non-rotating glass tube surrounded by the Rf coil and connected at both ends with LC tubing.

Sensitivity of LC-NMR

On flow mode

Sensitivity and resolution are limited by the short residence time of analyte at the flow rate of 0.5-1.5 ml/min and typically greater 10 μ g per analyte are needed for quality results at the 1H observation frequency of 500MHz.

Stopped flow mode/loop storage mode

The limit of detection at the 1H observation frequency of 600 MHz for analyte is approximately 100 ng for 60-240 μ l flow probe cell.

For highly concentrated analytes in 1.5 μ l NMR active flow probe volume, detection limit are in 5 ng range.

Accuracy limit

1H sensitivity in 382:1, line shape 2.2/4.4, resolution 0.22 Hz. 13C sensitivity 246:1, line shape 0.7/2.3, resolution 0.05 Hz.

Experimental Arrangement of LC-NMR Coupling¹²

The main pre-requisites for online LC-NMR, also involves the continuous-flow probes and a valve that is to be installed that too before registration of the either the stopped flow probes or the continuous flow probes along with basic NMR and LC instrumentation parts.

Due to the current development of cryomagnet technology, no bench top like cryomagnets will be available with a magnetic field strength between 9.4 and 14T in the next few years.

The position of the LC instrument is dependent upon the magnitude of the stray magnetic field of the used cryomagnet.

Thus, the conventionally installed LC instrument is located at the distance of 1 to 2 m from the cryomagnet, whereas with new available shielded cryomagnets the LC instruments can be directly attached to the cryomagnet.

The analytical NMR flow-cell was originally developed for continuous-flow NMR procurement, but the need to determine complete structural assessment of unknown compound led to the development and usage of the stopped-flow mode. Here, the supports of the closed loop separation-identification path, together with the possibilities to use all types of presently available 2D as well as 3D NMR techniques in a entirely automated way, has convinced a lot of application chemists.

In many laboratories, unshielded magnets are used at the instant, and thus the LC instrument, comprising of an injection device, LC pumps composed of a gradient unit, an LC column (4.6 \times 250 mm) and UV detector, is situated at a distance of 1.5 m from the cryomagnet. The outlet of the UV detector is either attached via a stainless steel capillary (id, 0.25 mm) to a valve or to a peak sampling unit which are attached to

the continuous-flow probe in the cryomagnet. With either the valve or the peak sampling unit being under software control, stopped-flow NMR acquisition of all peaks of an LC separation can be accomplished due to the triggering of the UV signal. For a proper timing and recording of chromatographic peaks, the transfer time of peak passage between the UV detector and the NMR flow cell has to be judiciously recorded.

This experimental design has the advantage that it can be easily accomplished. The operation mode of the NMR instrument from routine NMR data acquisition to the LC±NMR mode can be easily improved by removing the routine probe from the room-temperature bore of the cryomagnet and inserting the continuous-flow probe by setting two screws. The magnetic field homogeneity of the continuous-flow probe can be freely altered by using standard reference files.

The transfer volume of the capillaries between the LC instrument and the NMR probe is about 150ml. For minimum peak dispersion, the insertion of the LC column into the probe body of the continuous-flow probe would be required. This experimental arrangement was proposed by Wilkins and co-authors. As the use of shielded cryomagnets is increased, the distance between the LC and NMR instruments will be reduced, thus rendering the need for inserting the LC column within the probe body unnecessary.

Solvent Signal Suppression

Solvent signal suppression is very important in order to attain a reduction of the NMR signal entering the detector for analyzing small analyte signals. In the case of a free induction decay much larger signals from the mobile phase are present.

Solvent signal suppression is efficiently performed by using three techniques⁹:

- a) Pre-saturation (NOESY pre-saturation)
- b) Soft-pulse multiple irradiation
- c) WET pre-saturation employing a z-gradient

a) Pre-saturation

The principle of pre-saturation relies on the phenomenon of that nucleus which is unable to relax, because their population in the ground state and the excited state is the same. These do not contribute to the free induction decay and after pulse irradiation. Prior to data acquisition, a highly selective low power pulse irradiates the desired solvent signals for 0.5 to 2.0 s, thus leading to the saturation of the solvent signal frequency. During this data acquisition, no irradiation should occur. NOSEY-type of an effective pulse sequence of pre-saturation.

b) Soft pulse multiple irradiation

Here, saturation is performed with the use of shaped pulses, which have a broader excitation profile. This method is therefore a better method to be used for the suppression of the multiplets of the solvents. Its advantage is that it is easy to apply and easy to implement with most of the NMR experiments, and multiple pre-saturation is possible, and that it is very effective. The disadvantage is that transfer of saturation can occur (in aqueous solution) to slowly exchanging protons that would be detectable without saturation. Another drawback is that spins with resonance close to solvent frequency will also be saturated and 2D cross peaks will be present.

C) WET pre-saturation

The WET sequence (Water Suppression Enhanced) uses four solvent selective pulses of variable lengths. Each selective Rf pulse is followed by a dephasing field gradient pulse. By varying the tip angle of the selective Rf pulse, the WET sequence can be optimized. This approach provides a fast and highly efficient saturation of multiple solvent frequencies. It can be combined with ¹³C decoupling to remove the ¹³C satellites of the solvents.

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 attainment to cancel common signals, such as solvent and solvent impurities. This technique is based on a principle of dual beam background subtraction, where the reference signal and sample signal are collected simultaneously and subtracted from each other automatically. No software manipulation, pulse sequence modification, or any change in spectrometer is required. Therefore the technique does not lengthen the pulse sequence but it reduces experimental time. It takes 50100 milliseconds. So, it is used for on flow method. This method is used for on flow mode.

Other possible steps for solvent suppression

1. Using eluents that have as few ¹H NMR resonances as possible, e.g: H₂O, ACN, or MeOH.
2. Using at least one deuterated solvent, e.g., D₂O, ACN-d₃, or MeOD.
3. Using buffers that have as few ¹H NMR resonances as possible, e.g., TFA or ammonium acetate.

- Using ion pair reagents that have as few ¹H NMR resonances as possible, e.g., ionpairs with t-butyl groups create an additional resonance.

Advantages of LC-NMR:

- The information that is provided by this techniques are orthogonal to each other which means they works very differently without interfering with other techniques i.e. LC methods is helping in separation of the complex mixtures whereas NMR helps in determination of the structure (through different experiments).
- The NMR can determine whether the peak is for pure compound or impure compound.
- NMR data can be taken without complete separation of mixture.
- It is non-destructive technique.
- Sample can be stored for analysis by another method⁹.

Disadvantages of LC-NMR :

- This technique is involving high costs.
- Capital also includes high equipment costs.
- Longer time required for experimental works. It also include the use of duteriated solvents (partial use).
- Skilled professionals required therefore operator training requirements.
- Difficulty in solvent selection⁹.

Applications :

- Use of LC-MS/TOF, LC-MSⁿ, NMR and LC-NMR in characterization of stress degradation products: Application to cilazapril.¹³
- The use of LC/MS, GC/MS, and LC/NMR hyphenated techniques to identify a drug degradation product in pharmaceutical development.¹⁴
- The application of LC-NMR and LC-MS for the separation and rapid structure elucidation of an unknown impurity in 5-aminosalicylic acid.¹⁵
- 4-Hydroxyphenylacetic acid derivatives of inositol from dandelion (*Taraxacumofficinale*) root characterized using LC-SPE-NMR and LC-MS techniques.¹⁶
- Structural investigations on beta cyanin pigments by LC NMR and 2D NMR spectroscopy.¹⁷
- Structural elucidation of in vivo metabolites of isobavachalcone in rat by LC-ESI-MSⁿ and LC-NMR.¹⁸
- 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.¹⁹
- Application of directly coupled LC-NMR-MS to the structural elucidation of metabolites of the HIV-1 reverse-transcriptase inhibitor BW935U83.²⁰
- Application of evolving factor analysis to on-flow LC- NMR data using spectral windows.²¹
- Application of LC-NMR for the study of the volatile metabolite of MK-0869, a substance P receptor antagonist.²²
- Application of LC- MS and LC- NMR Techniques for Secondary MetaboliteIdentification.²³
- Application of LC-NMR and HR-NMR to the characterization of biphenyl impurities in the synthetic route development for vestipitant, a novel NK1 antagonist.²⁴
- Application of LC-NMR and LC-MS to the identification of degradation products of a protease inhibitor in dosage formulations.²⁵
- Biodegradation pathway of mesotriprone: Complementarities of NMR, LC-NMR and LC-MS for qualitative and quantitative metabolic profiling.²⁶
- Characterization of triacetone triperoxide (TATP) conformers using LC-NMR.²⁷
- Detection of methyl quinoline transformation products in microcosm experiments and in tar oil contaminated ground water using LC-NMR.²⁷
- LC-NMR identification of a novel taurine-related metabolite observed in ¹H NMR-based metabonomics of genetically hypertensive rats.²⁸
- Solvolyis of 14, 17-etheno-bridged 16 anitroestratrienyl acetate and lactam formation pathways studied by LC-NMR and LC-MS. Structures of minor products.²⁹
- Separation and characterization of peptide libraries.
- Combinatorial chemistry, photochemical analysis, drug discovery.
- Identification of drug impurities.
- Characterization of isomers of acid glucuronides and vitamin A derivatives.
- Characterization of endogeneous and xenobiotics metabolites directly from biological fluid.

23. LC-NMR allowed the differentiation of isomers and identification without reference compound .
24. 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.
25. 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*.
26. 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 CH₃CN:D₂O ratio of 7:13 .
27. LC-NMR MS have identified analogues of vitamin E of palm oil extract.

III. CONCLUSION

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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