Research Article

# A Technique Expanding the Limits of Structure Elucidation: LC-NMR

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**ABSTRACT**

In general, LC-NMR is an efficient analytical technique for the identification of components in pharmaceutical mixtures. To increase its efficiency it can be further combined with MS. There are various NMR probes that can be used for increasing the efficiency of LC-NMR. This review explains the balancing of both LC and NMR for attaining the sensitivity and accuracy of both the technique. There is some practical experiment explanation for the better understanding. The two online detectors that are complementary to each other and equally provides the structural identification of known expected components as well as unknown substances. The use of LC-NMR techniques is lowered due to its major drawback of lower sensitivity. However new upcoming challenges in the future can be solved by using this technique, due to the development of the new cryogenic LC-NMR probes which coupled with the recent interface enhancement and higher magnetic field strengths.

**Keywords:** LC-NMR, LC-NMR Coupling, NMR probes.

## INTRODUCTION

### LC-NMR techniques

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 detection.[1]

This techniques 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 manufacturers.[2-6]

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 leads 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 lies in two areas. The first is the suppression of signals from the commonly employed solvents used in solvents, whereas the second is gaining the suitable sensitivity and spectral resolution.[7]

### 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.[8]

The first real sample to be analyzed by LC-NMR technique was a military jet fuel using the normal phase columns and deuterated chloroform and Freon.[9]

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.[8]

Recently, there are various LC-NMR system available, and data acquisition can be accommodated with the help of different modes depending on the status of the sample during investigation. Therefore, objective of this article is to offer summary of LC-NMR and give information about, different mode of analysis of LC-NMR instrumentation, as well as their applications.

**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 deuterated solvent. This sample solution is introduced in a cylindrical sample tube and placed in a conventional NMR probe within the NMR magnet.[10] 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 factor 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.

**On-flow LC-NMR (continuous flow)**

In this mode, similar to UV and MS detectors in a 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 $C_{18}$ column with $CH_3CN:D_2O$ (methyl cyanide and deuterated 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 $R_f$ 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 ID 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 $R_f$ 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-NMR[9]**

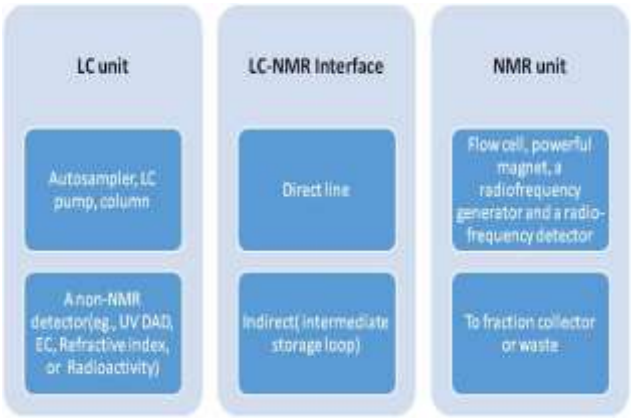


**Figure 1:** Instrumentation of LC-NMR

**Direct coupling:** It include 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 NMR-NMR 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 Probes[11]**

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 uses 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 allows 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 depend 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 reduces 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 µg 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 are approximately 100 ng for 60-240 µl flow probe cell.

For highly concentrated analytes in 1.5 µl 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.

$^{13}C$ sensitivity 246:1, line shape 0.7/2.3, resolution 0.05 Hz.

***Experimental Arrangement of LC-NMR Coupling[12]***

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 cyromagnets 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 cyromagnets 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.

Figure 4 shows an experimental arrangement of LC±NMR coupling which is presently employed in many analytical laboratories. 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 × 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 ($i_d$, 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.

**Practical Considerations, Solvent Peak Suppression Techniques, Gradient Elution and Purity of LC Solvents**

In real-life application of LC±NMR, three major types of data procurement have been established, namely continuous-flow procurement, stopped-flow procurement, and time-sliced procurement with the help

of storage loops. For all of these acquisition techniques the major prerequisite is an optimized LC separation. As sensitivity is still the fundamental point of this coupling technique it is extremely important to improve a chromatographic separation in which the amount of the obtainable separated compound is concentrated in the smallest available eluted volume. This need necessitates the development of stationary phases which exhibit optimum separation characteristics, together with the capability to tolerate column overloading. The newly developed $C_{30}$ phases are representatives of these types of columns, which is evidenced by the separation of tocopherol isomers. Figure 3(a) and 3(b) clearly shows that it is possible to overload a $C_{30}$ column by a factor of 200 without losing any chromatographic resolution. The large amount of LC separations is performed with reversed-phase columns employing two or three solvent mixtures with isocratic or gradient elution method. The protons of the solvents of the mobile phase can lead to the problem in NMR determination of compound. The detector of the NMR instrument (either a 12-bit or a 16-bit analog ± digital converter (ADC)) is not able to detect both the intense signals of the solvents and the weak signals of the sample substance simultaneously.

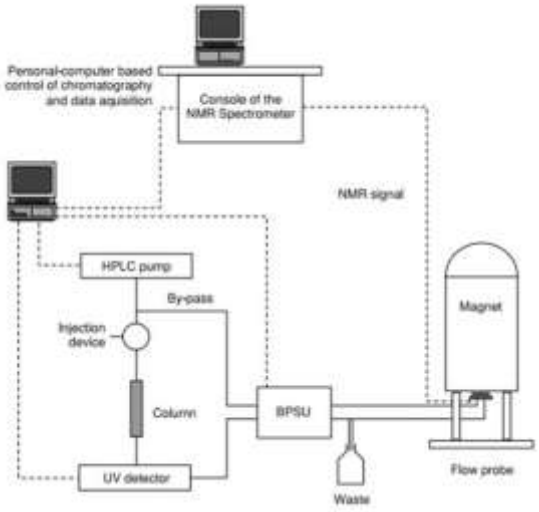


**Figure 2:** Schematic Diagram Of The Experimental Set-Up Used For Lc-Nmr Coupling: Bspu Bruker Peak Sampler Unit, (-) Capillary Junctions, (-----) Electronic Junctions.

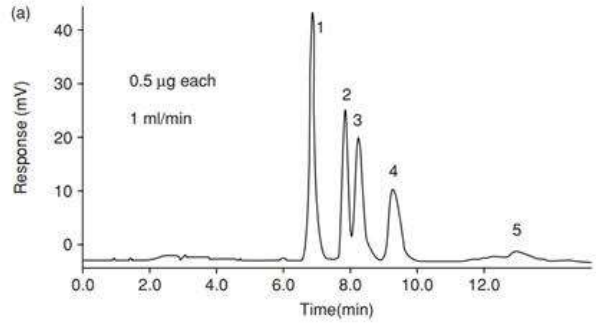


**Figure 3(A):** UV Chromatogram of the Separation of Tocopherol Isomers by Analytical Separation

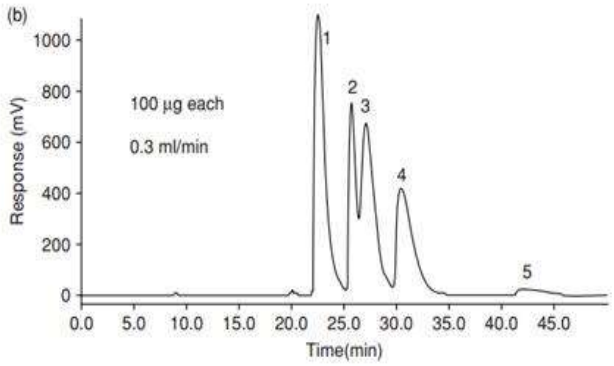


**Figure 3(B):** UV Chromatogram of the separation of Tocopherol Isomers with A 200 Fold Amount of Sample.

Figure 4 shows (a) the free induction decay (FID) and (b) the transformed spectrum of a 0.01 % sample of butyl benzyl phthalate in acetonitrile (ACN):$D_2O$ (80/20). The FID is dominated by the methyl group signal from the acetonitrile. As we wish to get an undistorted spectrum, a small receiver gain has to be chosen, to get small signal-to-noise (S/N) value for the sample signals.

Conventional $^1H$ NMR spectrum of a 0.01 % sample of butyl benzyl phthalate in acetonitrile (ACN):$D_2O$ (80/20) is given below:

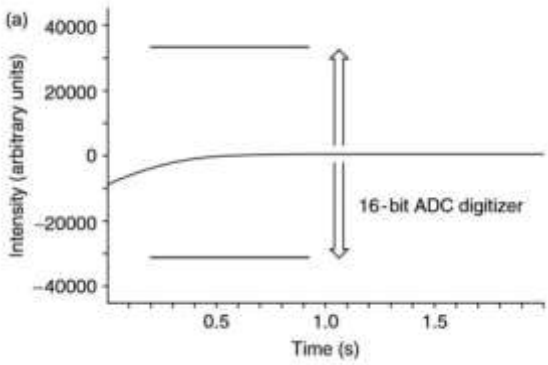


**Figure 4(A):** Free Induction Decay

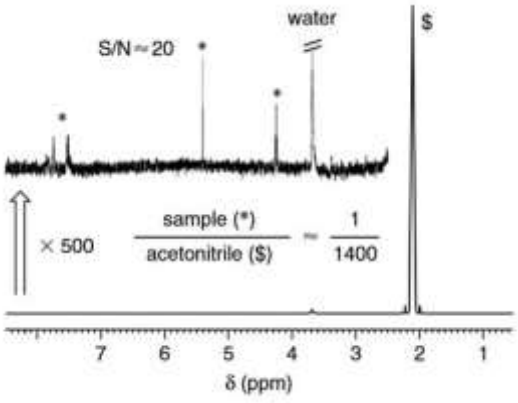


**Figure 4(B):** Transformed Spectrum

An increase in receiver gain does not lead to the desired result. Figure 5(a) and 5(b) shows the effect of overloading the receiver with solvent signals. By the `clipping' of the FID, the transformed spectrum is

distorted and thus useless for interpretation and the sensitivity of detection is severely decreased. Therefore to avoid this problem, the signal intensity of the solvent signals has to be reduced.

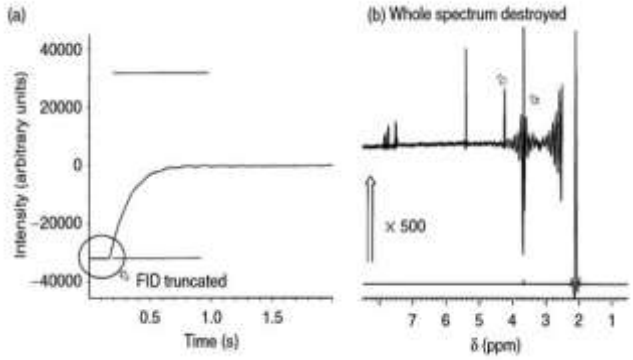


**Figure 5:** Increase In Receiver Gain Without Solvent Signal Separation: (A) Free Induction Decay (B) Resulting NMR Spectrum

Now the receiver gain can be increased and adjusted to the small FID without any problems. Figure 6(a) and 6(b) shows the FID and the resulting $^{1}$H NMR spectrum of the same sample after reducing the solvent signal intensity. There maining signals of the suppressed methyl group resonance of acetonitrile can be seen at 2.1 ppm. The signal of butyl benzylphthalate shows a much higher S/N of 320:1. The 16 fold enhancement of the signal-to-noise value corresponds to a saving factor of 256.

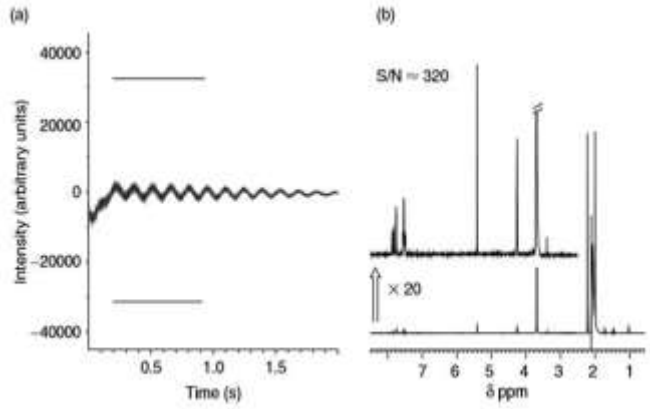


**Figure 6:** Optimized Receiver Gain with Solvent Signal Separation: (A) Free Induction Decay (B) Resulting NMR Spectrum

**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 techniques[9]:

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

**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.

**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.

**WET pre-saturation**

The WET sequence (Water Suppression Enhanced) uses four solvent selective pulses of variable lengths. Each selective $R_f$ pulse is followed by a dephasing field gradient pulse. By varying the tip angle of the selective $R_f$ 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 $^{13}$C decoupling to remove the $^{13}$C 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 50-100 milliseconds. So, it is used for on flow method. This method is used for on flow mode.

**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 method.[9]

**Disadvantages of LC-NMR**

- This technique is involving high costs.
- Capital also include high equipment costs,
- Longer time required for experimental works.
- It also include the use of deuterated solvents (partial use).
- Skilled professionals required therefore operator training requirements.
- Difficulty in solvent selection.[9]

**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 betacyanin 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 corticotropin-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 mesotrione: 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]
- Solvolysis of 14,17-etheno-bridged 16 a-nitroestratrienyl acetateand lactam formation pathways studied by LC–NMR and LC–MS. Structures of minor products.

## 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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