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NUCLEAR MAGNETIC RESONANCE AND HYPHENATIONS – A REVIEW

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ABSTRACT: Nuclear magnetic resonance spectroscopy is an absorption spectroscopic technique that helps in structural elucidation and has a wide range of applications in various fields like drug discovery and development, metabolomics, combinatorial chemistry, and food sciences. This paper gives information about nuclear magnetic resonance spectroscopy, and clear description of LC-NMR-MS, and a brief description of other hyphenations with NMR like HPLC-NMR, GC-NMR, GPC-NMR, SEC-NMR, SFC-NMR, SFE-NMR, CE-NMR, SPE-NMR, CEC-NMR, and their applications.

INTRODUCTION:

Nuclear Magnetic Resonance Spectroscopy: Nuclear Magnetic Resonance (NMR) spectroscopy is an absorption spectroscopic technique where the energy from an external source is absorbed and brings about resonance to an excited or higher energy state (in which nuclei in a strong constant magnetic field are perturbed by an oscillating magnetic field). The resonance occurs when the oscillation frequency matches the intrinsic frequency of the nuclei. In NMR, the energy required lies in the low energy or long-wavelength radio-frequency end of the electromagnetic spectrum. The emerging radiofrequency gets absorbed in a magnetic field consequent to the magnetic properties of nuclei arising from the axial spin.

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An NMR absorption spectrum comprises of one to several groups of absorption lines in the radio-frequency portion of the electromagnetic spectrum. The locations of the peaks indicate the chemical nature of the nucleus, whereas the multiplet gives information regarding the spatial positions of the neighboring nuclei. Therefore, NMR is also known as Nuclear Spin Resonance (NSR) spectroscopy [1].

History of NMR: The steady progress of NMR spectroscopy can clearly be seen in the list of Nobel Prize winners [2]. Nuclear magnetic resonance was first described and measured in molecular beams by Isidor Rabi in 1938 [3], by extending the Stern-Gerlach experiment in which beams of atoms were separated in an inhomogeneous magnetic field according to the orientation of electron magnetic moment, and in 1944, Rabi was awarded the Nobel Prize in Physics for this work [4]. In 1946, Bloch, Hansen, and Packard tried the experiment with a sample of water, and it worked, and the NMR was born [5]. Purcell, Torrey, and Pound had been able to directly measure the small absorption of radiofrequency energy by the proton

magnetic moments in a block of paraffin [6] for which Bloch and Purcell shared the Nobel Prize in Physics in 1952 [7]. The radar was developed by Purcell during World War II at the Massachusetts Institute of Technology's Radiation Laboratory. By then, the NMR spectroscopy started to become more than a physical experiment. By the discovery of the ‘‘chemical shift’’, the method has become a tool for chemists in structure elucidation [2]. Yevgeny Zavoisky observed the NMR in 1941, before Felix Bloch and Edward Mills Purcell, but dismissed the results as not reproducible.

Russell H. Varian filed the "Method and means for correlating nuclear properties of atoms and magnetic fields", U.S. Patent 2,561,490 on July 24, 1951. The first NMR unit, called NMR HR-30, was developed by Varian associates in 1952. The first useful NMR spectrometers were continuous wave (CW) instruments using permanent or electromagnets. Their utility came to an end with the upcoming superconductor magnets in the 1970s. However, only since Ernst developed the basics of the Fourier transformation (FT) method, the foundation of the modern NMR spectroscopy methods was laid. Since NMR spectroscopy was by then a domain of physicians, Ernst was the first chemist in the list of Nobel Prize winners in 1991. A decade later, Wu¨thrich was the second honoured chemist. He received the Prize in 2002 for the elucidation of three-dimensional structures of macromolecules. The NMR technique has become an important tool in other scientific fields, especially in medicine. It is not surprising that only one year later, the NMR technique was honoured again, and the Nobel Prizes were awarded to Lauterbach and Mansfield for their research in magnetic resonance imaging. Rightly, the NMR community expects further Prizes in one of the widespread application areas of NMR spectroscopy in the future [2].

Principle of NMR: The basic principle in NMR spectroscopy is the characteristic absorption of energy by certain spinning nuclei in a strong magnetic field when irradiated by a second and weaker field perpendicular to it permits the identification of atomic configuration in molecules [8]. The principle of nuclear magnetic resonance is based on the spins of atomic nuclei, which are under the influence of the external magnetic field. A charged spinning nucleus can be regarded as a magnet **Fig. 1**. Any motion of a charged particle has an associated magnetic field, which means a magnetic dipole is created in a magnetic field that corresponds to a magnetic moment ‘μ’. The magnetic moment of a nucleus is connected with intrinsic angular momenta or spin angular momentum. In a simple manner, the magnetic moment ‘μ’ is directly proportional to ‘I’, which is the angular momentum quantum number called the nuclear spin, with a proportionality constant ‘γ’ known as gyromagnetic ratio [9]. The nucleus having a certain magnetic moment when placed in the magnetic field, the phenomenon of space quantization is observed, and for each allowed direction, there will be a slightly different energy level. In order to excite the atoms (usually protons or C-13 atoms), the radiation in the radiofrequency region is used so that their spins are switched from being aligned with to being aligned against an applied magnetic field. The range of frequencies and the complex splitting patterns produced are the characteristic of the chemical structure of the molecule. The frequency at which absorption occurs can be used for qualitative analysis [10].

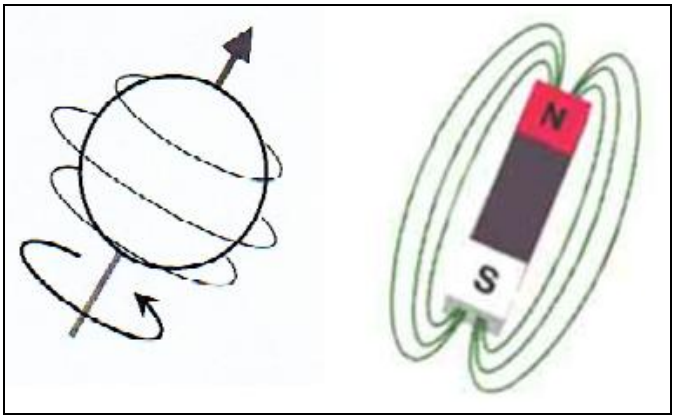


FIG. 1: A SPINNING NUCLEUS CAN BE REGARDED AS A MAGNET

Continuous Wave – Nuclear Magnetic Resonance (CW-NMR) Spectroscopy: Spectrometers used a technique called continuous-wave spectroscopy during the first few decades of nuclear magnetic resonance [11]. In the case of continuous wave-nuclear magnetic resonance spectroscopy, the sample is sweeped through the Larmor condition with a time-varying magnetic field or frequency of irradiation while monitoring the amount of energy that it absorbs. Consider a classical motion of the magnetic moment μ in a

uniform externally applied strong magnetic field B_0 under the condition of constant total energy. The movement of μ traces out a cone about B_0, which is analogous to the motion of a gyroscope running in friction-free bearings under the influence of the earth's field, and such motion is referred to as Larmor Precession [9]. The precession frequency ν_0 is given by

$$\nu_0 = \gamma B_0/2\Pi$$

Investigating the absorption of the RF energy by the ensemble of magnetic moments provides information about their quantity and environment [12]. In simpler, a weak oscillating magnetic field generates a transverse spin magnetization, which is recorded as a function of oscillation frequency or static field strength (B_0). When the oscillation frequency matches the nuclear resonance frequency, the transverse magnetization is maximized, and a peak is observed in spectrum [11].

The most basic CW-NMR experiment was reported in 1949 by Rollins [13] in which RF is supplied by a signal generator through a resistor to a tuned circuit resulting in a constant signal, and the magnetic field in which sample is placed is varied using sweep coils, and the amplitude of RF is monitored during the process. More energy is absorbed once the sample reaches the resonance, and hence the amplitude of the signal becomes lower until the Larmor condition is met. The signal increase back to equilibrium as the field continues to change [12]. The main advantage of continuous wave- nuclear magnetic resonance spectroscopy is that it is cheaper to maintain and operate and can be operated at 60 MHz with correspondingly weaker electromagnets cooled with water rather than liquid helium. There are two coils – One radio coil operated continuously, sweeping through a range of frequencies, while another orthogonal coil receives no radiation from the transmitter but receives signal from nuclei that reoriented in solution. But the spectrum suffers from poor signal-to-noise ratio since the NMR signal is intrinsically weak [14].

Fourier-transform Nuclear Magnetic Resonance (FT-NMR) spectroscopy: In order to promote NMR as a practical tool for molecular structure determination, there should be optimization of the signal-to-noise ratio. The major improvement in the signal-to-noise ratio of NMR spectra was achieved in 1964 by the concept of Fourier transform Spectroscopy [15]. A revolution in NMR occurred when short radio-frequency pulses began to be used, with a frequency centered at the middle of the NMR spectrum. It means a short pulse of a given "carrier" frequency "contains" a range of frequencies centered about the carrier frequency, with the range of excitation (bandwidth) being inversely proportional to the pulse duration, *i.e.*, the Fourier transform of a short pulse contains contributions from all the frequencies in the neighborhood of the principal frequency [16]. The pulsed Fourier transform NMR experiment includes two major steps: (a) the collection of data by a pulse/data acquisition/delay sequence repeated enough times to yield an FID signal possessing the desired S/N ratio. (b) Integration of the FID data and extraction of the frequency/intensity information [17]. The restricted range of the NMR frequencies made it relatively easy to use short (1 - 100 microseconds) radio frequency pulses to excite the entire NMR spectrum. When such type of pulse is applied to a set of nuclear spins it excites all the single-quantum NMR transitions. It corresponds to the tilting of the magnetization vector away from its equilibrium position (aligned along the external magnetic field) in the net magnetization vector. The out-of-equilibrium magnetization vector then precesses about the external magnetic field vector at the NMR frequency of the spins. This oscillating magnetization vector induces a voltage in a nearby pickup coil, creating an electrical signal oscillating at the NMR frequency. This signal is known as the free induction decay (FID), and it contains the sum of the NMR responses from all the excited spins. The time-domain signal (intensity vs. time) must be fourier transformed to obtain the frequency-domain NMR spectrum (NMR absorption intensity vs. NMR frequency). Richard R. Ernst was one of the discoverers of pulsed NMR and won a Nobel Prize in chemistry in 1991 for his work on Fourier Transform NMR and his development of multi-dimensional NMR spectroscopy.

Multi-Dimensional NMR Spectroscopy: In order to obtain different types of information about the molecules in the sample, the pulses of different durations and frequencies should be used. In a multi-dimensional nuclear magnetic resonance spectroscopy, there are at least two pulses, and the

pulse timings are systematically varied, and the oscillations of the spin system are probed point by point in the time domain as the experiment is repeated. Multidimensional Fourier transformation of the multidimensional time signal yields the multidimensional spectrum. There will be one systematically varied time period in the sequence of pulses in two-dimensional nuclear magnetic resonance, which will modulate the intensity or phase of the detected signals whereas in 3D NMR, two time periods will be varied independently, and in 4D NMR, three will be varied [15]. Multi-dimensional NMR spectroscopy helps in determination of three-dimensional structure of biomolecules (proteins, DNA, RNA) by providing the resolution necessary for analyzing the complex spectra [15, 18].

The spectra in one-dimensional NMR results from a Fourier transform (FT) of a directly detected time-domain signal, the free induction decay (FID) is recorded at the end of pulse sequence during the acquisition time [19]. The schematic representation of the one-dimension experiment is shown in **Fig. 2a**.

FIG. 2(A): SCHEMATIC REPRESENTATION OF ONE-DIMENSION EXPERIMENT

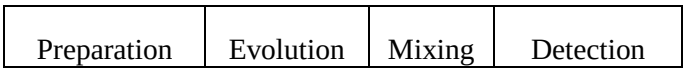
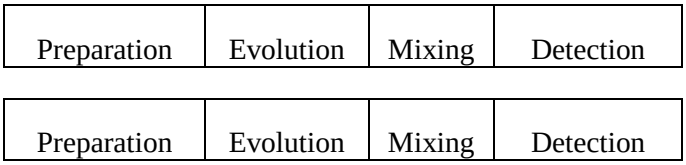


FIG. 2(B): SCHEMATIC REPRESENTATION OF TWO-DIMENSIONAL EXPERIMENT



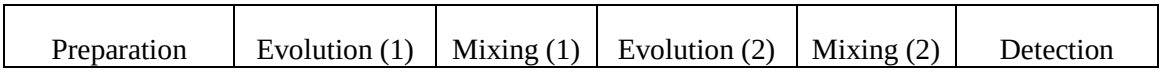


FIG. 2(C): SCHEMATIC REPRESENTATION OF THREE-DIMENSIONAL EXPERIMENT

Two-Dimensional NMR: In two-dimensional NMR it consists of two additional sections, namely- Evolution time and Mixing time apart from preparation and detection. Schematic representation of the two-dimensional experiment is shown in **Fig. 2b**. The indirect detection of an additional time-domain takes place during the evolution time. After completion of the acquisition of the Fourier induction decay (FID), including the repetitions necessary to perform the phase cycle of the experiment and to achieve sufficient signal-to-noise, the experiment is repeated numerous times. A delay in the pulse sequence is incremented systematically from one experiment to the next, and the intensities detected in the FID during the acquisition time are thus modulated according to the length of this delay. The result is a two-dimensional time-domain data set, one dimension from direct detection, one dimension from systematic incrementation of a delay. It is processed using two subsequent Fourier transformations along with both time domains. First the directly detected FIDs are converted into a series of one-dimensional spectra, the inter-ferogram. Then the interferogram is converted into a two-dimensional spectrum that exhibits two independent frequency axes. Both axes will usually contain the chemical shift of nuclei in the sample, the type of chemical shift as well as the appearance of the two-dimensional spectrum results from the design of the mixing period. During the mixing time the magnetization that has been created during the preparation period and has been modulated by a frequency during the evolution time is converted into another type of magnetization that is subsequently detected during the acquisition. This is accomplished using some type of interaction (scalar or J-coupling, dipolar coupling) between one or several spins in sample [19].

The concept of 2D-FT NMR was proposed by Jean jeener, but this idea was largely developed by Richard Ernst who won the 1991 Nobel Prize in Chemistry for his work in FT NMR, including multi-dimensional FT NMR, and especially 2D-FT NMR of small molecules [15]. This 2D- FT NMR

is used for the determination of the structure of biopolymers such as proteins or even small nucleic acids [20]. There is increased resolution in two dimensional spectra when compared with one dimensional spectra. The major disadvantage in NMR spectroscopy is overlapping in the analysis of NMR spectra of biomolecules, so this obstacle can be overcome by the use of two-dimensional techniques.

Three-Dimensional NMR: In order to increase the resolution, three dimensional NMR was introduced. It contains two evolution and two mixing periods, which is shown in **Fig. 2c**. The evolution periods are created by the variation of two separate delays in the pulse sequence. First, a delay is systematically incremented, and the acquisition of the FID is repeated for every increment to create the first indirectly detected dimension. Once the desired number of incrementations has been performed for this delay, it is set back to its original value. The other delay that is used to create the second indirectly detected dimension is now incremented once, and the incrementation of the first delay is repeated, again with the acquisition of FID for every increment. This is done until the variation of the second delay has been completed as well. The resulting three-dimensional data set is processed using a sequence of three Fourier transformations resulting in a three-dimensional spectrum with three independent chemical shift axes. Three-dimensional spectra are classified as as- homonuclear spectra and heteronuclear spectra. A Homonuclear spectrum has the chemical shift of the same nucleus on all three axes, whereas, in heteronuclear spectra, the spectra exhibit two or three different nuclei on the three axes. The disadvantage of homonuclear spectra- two transfer steps involved increases the number of possible correlations in the three-dimensional space. Hetero nuclear-edited NOESY is the combination of two-dimensional heteronuclear correlation and two-dimensional NOESY. In this type of spectra's there is increased resolution [19].

Four-Dimensional NMR: In this four-dimensional NMR there is another delay in pulse sequence, which is incremented independently of the other two delays. It consists of three evolution times and three mixing times. The planes in four-dimensional spectra are planes that correspond to two-dimensional spectra and are extracted by keeping the chemical shift in one or two dimensions constant.

They can be analyzed as two-dimensional spectra, but since the information in the spectrum is spread out over a large number of planes, computer assistance for the analysis of the spectra becomes mandatory. This includes a database to contain all the information about chemical shifts and structurally relevant parameters [19].

Solid-State NMR Spectroscopy: This is a special type of spectroscopy in NMR, which is characterized by the presence of anisotropic (directionally dependent) interactions. Solid-state NMR usually requires additional hardware for high-power radio-frequency irradiation and magic-angle spinning compared to the more common solution NMR spectroscopy. In the case of solid-state NMR, the resonance frequency depends on the strength of the magnetic field at the nucleus, which can be modified by the electron cloud or the proximity of another spin. In media where there is no mobility or little mobility, anisotropic interactions influence the behavior of nuclear spins, whereas in liquid-state NMR, Brownian movement averages anisotropic interactions to zero, so they are not reflected in the NMR spectrum [21]. Due to broadening by chemical shift anisotropy (CSA) and dipolar couplings to other nuclear spins, without special techniques such as magic angle spinning (MAS) or dipolar decoupling by radiofrequency pulses, the observed spectrum is often only a broad Gaussian band for non-quadrupolar spins in a solid [20].

Developments in Solid-State NMR: The progress in solid-state NMR includes developments in NMR methodology such as pulse sequence design or novel correlation techniques, but also advances in sample preparation and hardware instrumentation that allow an unprecedented new array of biophysical problems to be addressed [22]. The use of magic angle spinning (MAS) recorded multi quantum spectra in order to investigate the domain sizes and geometries in disordered biopolymers such as amyloidogenic peptides were proposed by Antzutkin and Tycko. In this case, multi-quantum coherence can be created only between dipolar-coupled nuclei in close spatial proximity, which is

also termed as 'spin counting'. Therefore, the local arrangement of peptides in which isotope labels have been introduced at various carbonyl positions can be investigated. The multiple quantum signals (MQ) involving at least ten quanta of spin angular momentum are observed in nuclear magnetic resonance. With the use of a time-reversible multiple pulse excitation sequence modified specifically for experiments on systems with weak homonuclear dipole-dipole couplings and strong inhomogeneous interactions such as anisotropic chemical shifts [23].

Experiments were also carried out on antibody-bound peptide by Tycko where two-dimensional (2D) magic angle spinning (MAS) exchange experiments and double-quantum filtering experiments were carried out on doubly carbonyl-labeled variants of a 24 residue peptide bound to the Fab fragment of an anti-gp120 antibody. The cross peak intensities of the 2D MAS exchange spectrum depend on the relative orientation of the two labeled carbonyl chemical shift anisotropy (CSA) tensors and can, in conjunction with double quantum filtered recoupling experiments, be used to refine the backbone conformation (*i.e.*, the peptide backbone angles, phi, and psi) of the central Gly–Pro–Gly–Arg (GPGR) motif in the antibody-bound state. The results indicate that the GPGR motif adopts an antibody-dependent conformation in the bound state and a heterogeneous conformation in unbound, full-length gp120 [24].

To determine the molecular orientation in the light-activated chromophore of bacteriorhodopsin, selective labeling has been employed by Watts and co-workers [25]. The first solid-state structure of a uniformly ^{15}N, ^{13}C-labelled peptide has been determined by Opella *et al.*, who worked on the 25-residue functional peptide corresponding to the predicted channel-lining M2 segment of the nicotinic ligand-gated acetylcholine receptor (AchR). The resonance information of fully ^{15}N-labeled M2 peptides in oriented phospholipid bilayers was obtained mostly through comparisons with spectra from selectively ^{15}N-labeled recombinant peptides and specifically labeled synthetic peptides [26]. It has been demonstrated that high-resolution magic angle spinning (MAS) correlation spectra of solid-phase polypeptides with more than 60 residues can be obtained that rival those obtained by solution NMR of large macromolecular systems during the past year [22].

Nuclei Related to NMR Spectroscopy: There is a limitation on the isotopes of various chemical elements that respond to NMR analysis, and some of the nuclei and their relevant applications in NMR Spectroscopy are as follows:

Isotopes/ nuclei	Application
^{1}H	Clinical magnetic resonance imaging
^{2}H	Determining the behaviour of lipids in lipid membranes and other solids or liquid crystals
^{3}He	Studies of endohedral fullerenes
^{13}C	Frequently used for labeling of compounds in synthetic and metabolic studies.
^{14}N	Useful in case of smaller molecules and functional groups with a high degree of symmetry such as the head groups of lipids.
^{15}N	Can be used for labeling compounds
^{17}O	Used in metabolic and biochemical studies in studies of chemical equilibria.
^{31}P	Used in biochemical studies and in coordination chemistry where phosphorus-containing ligands are involved.
^{35}Cl	Use is limited to inorganic and ionic chlorides and very small organic molecules.
^{43}Ca	Used in biochemistry to study calcium binding to DNA, proteins
^{195}Pt	Used in studies of catalysts and complexes.

Note:

a. ^{1}H is the most commonly used spin-1/2 nucleus in NMR investigation, has been studied using many forms of NMR. Hydrogen is highly abundant, especially in biological systems.

b. Deuterium is used in high-resolution NMR spectroscopy in order to monitor the drifts in the magnetic field strength and to improve the homogeneity of the external magnetic field.

c. ^{11}B is more sensitive when compared to ^{10}B as it yields sharper signals.

d. ^{35}Cl is more sensitive and preferred over ^{37}Cl despite its slightly broader signal. Organic chlorides yield very broad signals [20].

Applications of NMR Spectroscopy: There are numerous examples in the literature for nuclear magnetic resonance spectroscopy in various fields. Gang Wu described the latest advancements in ^{17}O NMR spectroscopy of organic and biological molecules in aqueous solution as well as in the solid state [27]. Guzeler *et al*., described about applications of NMR in foods [28]. Ward *et al*., described the applications of NMR spectroscopy in plant metabolomics [29]. NMR also has a wide range of applications in drug discovery and development [30-33]. In the field of food sciences, Almeida *et al.,* determined the composition of beer by ^{1}H NMR Spectroscopy [34,] and various applications are present in literature [35-37]. NMR is also used in quantitative analysis of complex nature samples [38] in the field of biotechnology. Wei Jiang *et al.,* described the application of NMR metabolomics to search for human disease biomarkers in blood [39].

Hyphenation: Hyphenated techniques include a combination of both chromatographic and a spectral method to exploit the advantages of both [40]. Chromatography produces or separates a pure or nearly pure fraction of components from a mixture. Spectroscopy produces relative information or structural information for analytes concerned using standards or library spectra [41]. The online coupling of a separation technique, which may be a LC or GC and one or more spectroscopic detection techniques is referred to as 'hyphenation' [42]. In the past decade, the term 'Hyphenation' was first introduced by 'Tomas Hirschfield' in the year 1980 in order to describe two or more possible combinations of instrumental analytical methods in a single run [43]. The combination or coupling of two different analytical techniques with the help of a proper interface is referred to as 'Hyphenated technique' [44] or when a separation technique is linked to an online spectroscopic detection technology with the help of a proper interface. *e.g.*: GC-MS, LC-MS, LC-NMR, and LC-NMR-MS [45]. The schematic representation of hyphenated techniques is depicted in **Fig. 3**. The major objective of this coupling is to obtain an information-rich detection for both identification and quantification when compared to that with a single analytical technique [46]. The combination of separation technologies with spectroscopic techniques has received increasing attention in recent years and has been demonstrated as a powerful tool for both qualitative and quantitative analysis of compounds in complex natural product extracts or fractions [47].

Advantages of Hyphenation:

- Helps in solving complex analytical problems.
- Time taken for analysis is reduced *i.e;* shorter analysis time.
- Higher degree of automation.
- Higher sample throughput.
- Better reproducibility.
- Reduction of contamination because it is a closed system.
- Enhanced combined selectivity and therefore higher degree of information.
- It provides excellent separation efficiency as well as the acquisition of online complementary spectroscopic data on an LC or GC peak of interest within a complex mixture [47].

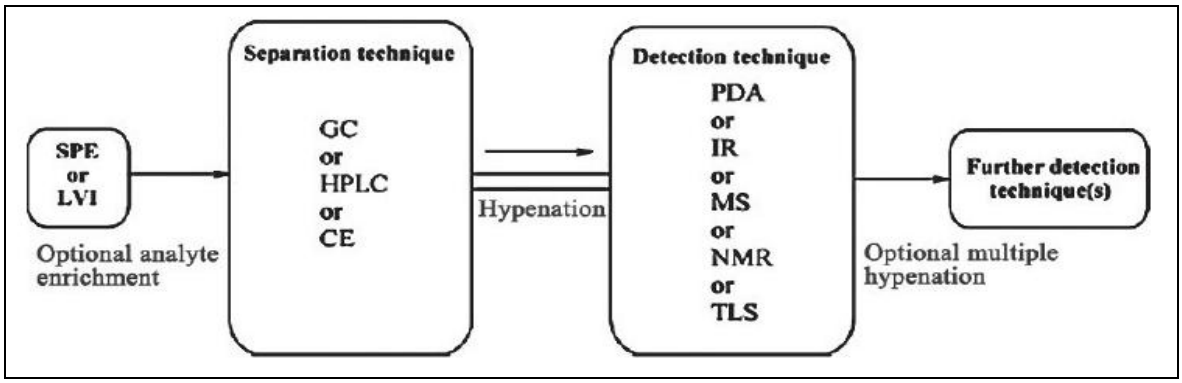


FIG. 3: SCHEMATIC REPRESENTATION OF HYPHENATED TECHNIQUE

LC-NMR-MS:

Introduction: Obtaining NMR and MS data on the same analyte is decisive for structural elucidation. It is not always certain that the NMR and the MS data apply to the same analyte when different isolates such as metabolites are analyzed by NMR and MS. In order to avoid this ambiguity, LC–MS and LC–NMR are combined so that NMR and MS data can be acquired simultaneously from a single chromatographic run [48]. The hyphenation LC-NMR with MS resulted in LC-NMR-MS in the year 1996 [49]. A splitter is incorporated after the HPLC to direct different sample loads to the MS and NMR units, respectively, since MS is more considerably sensitive than NMR [48]. NMR data is alone insufficient for de-novo online or at-line structure determination, and different spectroscopic information has to be collected. LC-NMR-MS systems are the most robust systems, which have evolved into completely automated systems. In case of the LC-NMR-MS system, the MS is used to trigger the trapping of the LC peak of interest for subsequent NMR measurements [50].

MS rapid screening and preliminary structure investigation, followed by supplementary NMR structure determination, has become a typical structure elucidation protocol in clinical, biological and natural product research. Acquisition of MS and NMR data in a single LC run provides inclusive analysis of a complex biological matrix through the real-time comparison and complementation of NMR and MS data.LC-NMR-MS- it has been emerged as a powerful tool for the detection and identification of known compounds and also detection and identification of emerging compounds in complex, clinical, pharmaceutical samples and natural product extracts, due to complementary and comprehensive structural information provided by the two for unambiguous structure elucidation [51, 52]. The different models and manufacturers/suppliers of LC-NMR-MS systems are - Bruker LC-NMR-MS Systems manufactured or supplied by Bruker and Varian LC-NMR-MS systems manufactured by Varian, Inc.

When LC/MS is combined with LC-NMR, it provides both structural and molecular mass information enabling the molecular structure to be determined with greater ease and certainty [53]. The hyphenation of LC with NMR and MS offers the advantage of over simple HPLC-NMR that more structural information is obtained, but this is at the expense of increased system complexity and capital investment. LC-NMR-MS systems are relatively easy to implement, but at most, care should be taken that the eluents used for chromatography should be compatible with both the spectrometers. Generally, LC-NMR-MS is used for the analysis of a mixture of non-steroidal anti-inflammatory drugs, polymer additives, a mixture of pharmaceuticals, ecdysteroid-containing plant extracts using either conventional reverse-phase eluents or superheated D_2O or mobile phase [52-54].

LC-NMR-MS is mainly used in case of expensive samples where the problem cannot be solved in any other ways [55]. This method is helpful in the structural elucidation of the metabolite from biological matrix and requires little sample preparation. Special NMR flow cells/ probes are not required in case of LC-NMR-MS and can be used in determination of compounds in mixtures that may encounter problems during isolation or instability [56]. This LC-NMR-MS gives information regarding structural determination, both fragmentation patterns, with accurate mass instruments, atomic composition data, and shows NMR invisible atoms such as oxygen, halogen, sulphur [57].

LC-NMR-MS is highly adaptable and helps in the identification and determination of active metabolites [49]. For a given LC peak, the MS data and acquired NMR spectra can be directly correlated [50]. But it is very difficult to select a chromatographic solvent system which is compatible with both NMR and mass spectrometers to ensure better separation which is the major disadvantage of LC-NMR-MS. If the buffers used for solvent pH control are non-volatile in nature, the ionization source of MS will get contaminated [57]. Another disadvantage is the high initial cost of capital instruments. There may be issues regarding sensitivity, acquisition time, and solvent compatibility which reflects the suspicions against the effectiveness of coupling all the three instruments together [58]. When MS instrument is physically placed close to the center of the NMR magnet, the main problems arise from sample overloading into the MS systems and due to proton-deuterium

exchange reactions, there is a shift of the molecular ion species.

The problem of sample overloading into the MS systems can be overcome by using an efficient post column splitter and dilution with an appropriate make up flow enables the proton-deuterium back exchange [50].

Instrumentation: The first in-house built consisted of an LC, NMR and a particle beam chemical ionization MS and used for analyzing complex mixture of pharmaceutical interest [58]. The system was developed by certain modifications in LC-NMR system supplied by Bruker instruments. LC columns of 4.6 mm i.d. in order to avoid overloading to the LC system. If columns of smaller i.d. are used there is problem of overloading. These columns supply sufficient material to the NMR spectrometer. In order to meet the requirements of NMR spectrometer without compromising the flow-rates should be in the range of 0.5 to 1.0 mL/min. As electrospray is a concentration-dependent technique, the above flow rates can be used or achieved by modern mass spectrometers. Splitting of the flow to mass spectrometer allowed the mass spectrometer to be operated at optimum sensitivity over a longer period of time. Large volume and high concentration analytes required in order to achieve enough detection sensitivity. The flow should be adjusted in such a way that the chromatographic peak has just passed through mass spectrometer as it fills the NMR spectrometer flow cell. The MS data obtained can be used to direct NMR experiment [59]. The flow rate from the LC needs to split before entering the mass spectrometer as it may affect the mass spectrometer [58]. In order to operate MS detector at optimal conditions without compro-mising the NMR sensitivity is by directing 95% of the LC eluent towards NMR flow cell and the remainder eluent is sent to the mass spectrometer ionization interface due to differences in spectrometer sensitivity. Sometimes the MS is placed after the NMR flow probe by employing in-line configuration which could simplify the plumbing aspects of the system [57-58].

LC-NMR-MS Configuration: Hyphenation of MS and NMR to LC system can either be done in series or in parallel.

A. Series Mode: In series mode, the coupling of NMR and mass spectrometer is arranged by placing the mass spectrometer inlet right after NMR by connecting the inlet tubing of MS to the outlet of NMR flow probe. The sample to be analyzed is first directed towards NMR and then to the mass spectrometer. A flow splitter should be placed in front of the mass spectrometer to control the amount of sample directed towards the MS ionization interface. The flow splitter also helps to release the backpressure generated by interface which may cause NMR probe leakage **Fig. 4**.

Arranging in series mode is simple and robust setup and the peak identification ability of MS is faster [58]. It can be easily disassembled for standalone mass spectrometric studies and free from data synchro-nization problems [60]. Usually, serial configuration only applies to on-flow NMR measurement [54].

Arranging the spectrometers in series and splitting the flow after the NMR spectrometer increases the possibility of peak dispersion and operating the spectrometers in series may cause raise in back pressure in the NMR spectrometer flow cell beyond the operating limits, which will cause considerable down time due to leakage of mobile phase and analyte for NMR spectrometer flow probe [59].

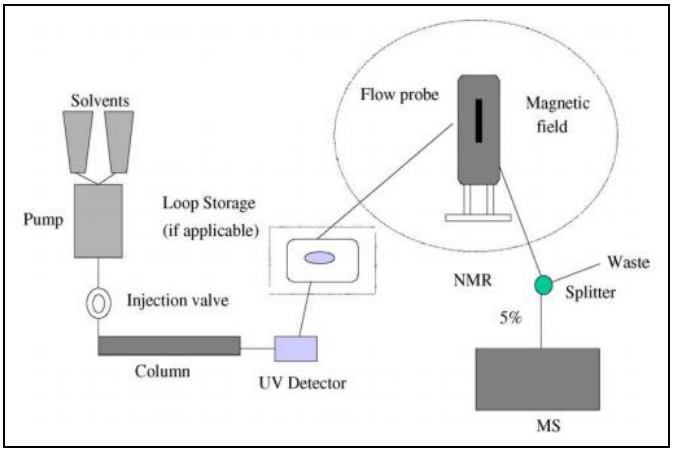


FIG. 4: HYPHENATED LC-NMR-MS SYSTEM IN SERIES MODE

B. Parallel Mode: In case of parallel hyphenated LC-NMR-MS system the LC is interfaced to the two detectors by placing them in parallel and splitting the LC eluent in the ratio of 20:1 or similar in such a way that major portion of the LC eluent flows to the NMR flow probe and minor portion is directed towards the mass spectrometer ionization

interface [58]. The diagram of the hyphenated LC-NMR-MS mode is represented in **Fig. 5**.

The main advantage in this type of configuration is that the MS detector is operated at optimal condition without compromising the NMR sensitivity and also prevents the damage to NMR flow probe by avoiding the concern of backpressure produced by LC-MS interface. Split flows can be adjusted depending on the type of experiment conducted [58]. Adoption of a parallel mode of operation of the hyphenated LC-NMR-MS was much better than operating it in series mode [59].

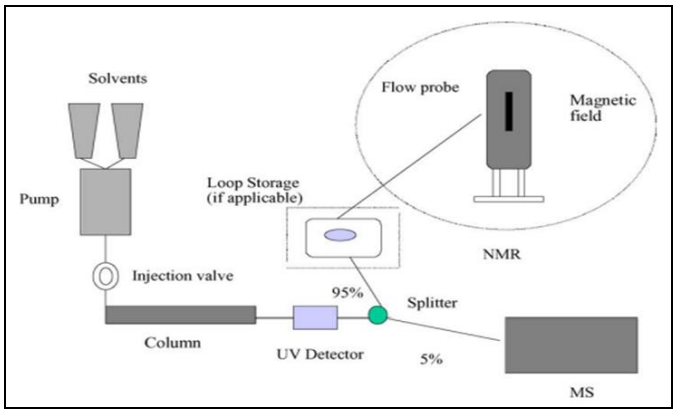


FIG. 5: HYPHENATED LC-NMR-MS SYSTEM IN PARALLEL MODE

Mode of Operation of LC-NMR-MS: The performance of LC-NMR-MS is determined by the performance of LC-NMR as LC-MS is a highly sophisticated technique already. Basically, there are two general working modes of LC-NMR:

a. On-flow (continuous flow)
b. Stop- flow

On-Flow Mode: It is also referred to as continuous flow. In case of on-flow LC-NMR, the LC eluent is directly directed to the NMR flow probe, where the NMR spectra are acquired continuously when the LC eluent is moving through a constant speed [58]. Due to its very short resident time in the NMR flow cell, its approach is limited to 1D NMR spectra acquisition only which can be used to analyse major components of the mixture by ^{1}H and ^{19}F NMR [48, 49]. Instrumentation is simple as no interruption of chromatographic eluent occurs and allows detection of all NMR active compounds under identical conditions. Provides straight forward and real-time NMR and MS comparison of individual compounds.

The major disadvantages of on-flow mode are that it is only limited to acquire high abundance ^{1}H or ^{19}F NMR spectra of major components in a sample due to limited NMR data acquisition time [58], sample amounts in the range of 10-100 µg are commonly needed, which may lead to column overloading and have lower sensitivity and over-lapping of chromatographic peaks [49].

Stop- Flow Mode: In stop-flow mode, it allows NMR spectra of the peak of interest acquired under the static condition in the flow probe, and the peak of interest is delivered online to an NMR flow probe [58]. Stop-flow requires calibration of delay time. Delay time is the time required for the sample to travel from detector to NMR flow cell. Delay time depends on flow rate and also the length of the tubing connecting the HPLC with NMR. NMR data can be obtained for number of chromatographic peaks in series of stops during the chromatographic run without on-column diffusion if the NMR data for each peak can be acquired in short time (30 min. or less if more than 4 peaks have to be analyzed and less than 2 hours for analysis of not more than 3 peaks). In order to improve the sensitivity of stop-flow mode, cryoprobes are used which are available commercially [48]. Stop-flow can be used when there is an insufficient sample for structure determination. The stopped-flow techniques can be used with gradient elution and in this situation, the NMR parameters required to obtain good quality spectra can be optimized on the static peak in the flow cell [61]. Measurement of lower concentration components is possible in case of stop-flow mode [59]. Stop-flow mode allows cautious optimization of acquisition parameters and field homogeneity in order to enhance the exposure time of the peak in the flow cell. This method excludes the dependency of the detector to position the sample in the NMR flow cell, which is the significant role of this approach [49].

The stop-flow splits into two sub-modes based on how the peak is sat in the flow probe.

Direct stop flow (time slicing) provides static sample condition for NMR data acquisition by stopping the flow for a short interval when a peak of interest enters the NMR probe, and the flow resumes after the data acquisition completes and the procedure repeats for the next peak of interest

till the end. It can be used when two analytes have close retention time or when the separation is poor [48]. Time-slicing method is used to obtain spectra from various portions of the peak, useful in estimating peak purity or obtaining spectra from partially resolved ample components [61].

Loop storage (peak picking/ loop collection) is the mode where the LC peaks are stored in a capillary loop and the static NMR measurements begin after the LC separation completes [58]. It acts as interposer between the online and off-line HPLC-NMR [49]. The peaks eluting from the column can be intercepted before they enter into flow probe of NMR spectrometer and stored in capillary loops. The loop storage device actually need not be connected to NMR flow probe during chromatography as there is no need for separation to be performed in same location as NMR spectrometer. It is used when there is more than one chromatographic peak of interest in the same run. The analytes must be stable inside the loops during the extended period of analysis. Capillary tubing should be used to avoid peak broadening and the stored chromatographic peaks can be analysed in different order from the chromatographic run [48].

In order to trigger the temporary LC flow, stop or transfer LC fraction to temporary sample storage unit, an additional device is required which is usually an UV or fluorescence spectrometer in case of LC-NMR. However, it is insufficient for peak selection in case of stop-flow NMR analysis because most of the compounds lacks UV absorbance and fluorescence. MS can be used as a first step for chemical profiling of different clinical and natural product samples, when MS is coupled to an LC-NMR system. The advantage of incorporating mass spectrometer into detector chain is the output obtained can provide a sensitive and selective signal to triggered stop-flow and useful for drug metabolism studies where mass information about the parent compound is known[55]. The chromatographic peaks are trapped online in a 36-loop cassette according to MS detection threshold. The peaks are directed towards the loops and the loop contents are directed towards the NMR flow probe, one-at-a-time for stopped-flow analysis. Loop storage avoids peak diffusion on-column, which reduces NMR sensitivity when several analytes require long NMR acquisition. The MS experiments on loop contents can be obtained when BNMI is integrated while the NMR acquires data. Loop storage is a highly efficient mode of operation [62].

Compounds are identified based on molecular weight and fragmentation information. Radio-activity detectors can be used to trigger the chromatographic peak of interest [48]. Other kinds of in-line detectors used to get more data to trigger the HPLC pump to stop on a peak include UV, fluorescence, radiochemical, refractive index or evaporative light scattering detector [55].

A database helps in the selection of peaks of interest, and upon peak recognition, with a signal from the mass spectrometer manually or automatically to the central control system, the LC pump stops to static NMR analysis. In this case, LC-NMR-MS has to be configured in parallel, and the length of the capillary tubing connecting MS interface needs to be adjusted in such that the LC peak before entering into NMR flow probe enters MS for a certain time. Dedicated software is required to export the obtained mass spectrum of an LC peak and then trigger the pump stop or loop transfer upon being directed to do so [58]. The Bruker NMR- mass spectrometry interface (BNMI) is a computer-controlled splitter which plays an important role in LC-NMR-MS loop storage mode in which a portion of the loop contents, on transfer to the NMR can be stored in a delay loop. Manual, semi-automated, or fully automated operation is possible in both stopped-flow and loop storage modes. It is possible to switch between modes during a run for flexibility [62].

A minimum amount of sample is required in this mode of operation, and it offers unlimited time for static NMR measurement. It gives high-resolution spectra for components that are not concentrated enough for quality on-flow measurements [58]. This method can be used when a detailed structural analysis is desired or analytes of interest are not major components of the sample. Cross-contamination is avoided and longer detection time in case of loop storage [49].

There is a possibility of peak dispersion and carry-over effect in direct stop-flow and also requires an

additional device for peak selection. It is time-consuming [58]. Soluble compounds in capillary loops get degraded on long-term storage [49].

NMR Probe Design: A probe is a sensor placed in the center of the magnet containing the coils that is used to send radiofrequency pulses to the sample and to detect NMR signals returning from the nuclei. A flow probe is the only prerequisite to modify a static NMR to an online LC detector [58]. The first design of NMR probe for LC work was really just a slightly modified tube, as shown in **Fig 6**. The flow from the HPLC passed into the bottom of a NMR tube and back up and out of the tube. Later designs use a capillary around which the coils of the radio frequency generator are tightly wound. There are several types of probe designs now available, but in each case, the eluent from the HPLC passes to a probe that sits within the NMR magnet. The key feature of each design is to wind the coil directly around the cell itself to reduce the gap between the cell and the coil. This maximizes the ratio between the volume of the cell and the volume of the coil, known as the filling factor, and increases the signal to noise ratio [59]. Flow probes usually have active volumes range between 60µL and 240µL using 3-5 mm radiofrequency coils around the detection cell. The introduction of cryoprobes is the most significant advance in NMR probe development [58]. The receiver coils create noise, which is observed in NMR spectra, and in order to increase signal-to-noise(S/N) ratio is to decrease the observed noise. The noise can be reduced by cryogenically cooling the receiver coils and by using pre-amplifiers [63]. In cryogenic probes, the electronic components are cryogenically cooled to -20K whereas the sample remains at ambient temperature. Cryoprobe enables three to four-fold enhancement of the sensitivity in high-resolution NMR [58].

Solvents Used in LC-NMR-MS: The most important consideration for LC-NMR-MS experiments is the mobile phase constituent of LC. The mobile phase or the solvents used in these experiments has to be a compromise between the ideal conditions of the individual instruments[58]. Inorganic buffers such as sodium phosphate can be used for NMR but they are incompatible with electron spray ionization (ESI) which is a principle ionization technique for LC-MS [59]. Non-deuterated formic acid, acetic acid, trifluoroacetic acid are used in LC-NMR-MS applications even though they give some typical NMR signals. Formic acid is best suited for NMR as well as MS.

Trifluoroacetic acid is mostly not recommended for LC-NMR-MS as it was found to suppress the ESI and mask the analyte ions. Mostly the LC separations for LC-NMR-MS are performed on reverse phase columns with binary solvent mixture mobile phase such as acetonitrile/water or methanol/water. LC separation with deuterated solvents was mandatory in early stages of LC-NMR development in order to eliminate the interfering solvent signal. But the disadvantage of deuterated solvents is that they are expensive and may cause problems in MS data interpretation. Solvent interfering problems can be solved by solvent suppression techniques which include pre-saturation, soft-pulse multiple irradiation and WET (water suppression enhanced through T1 effects) pre-saturation employing a z-gradient.

Superheated D_2O is used as the reverse phase LC eluent for most of the LC-MS-NMR experiments and by using this no other buffer systems or organic modifiers are necessary. D_2O is available in high state purity and is cheap when compared to deuterated solvents. NMR signals originating from the principal impurity of D_2O, non-deuterated water (0.1%), can be suppressed using conventional NMR pulse techniques. Though it has not been widely applied in LC–NMR–MS, superheated D_2O provides a promising alternative to other expensive deuterated organic solvents[58].

Applications of LC-NMR-MS: There are various examples in the literature for the application of LC–MS–NMR in various fields. LC-NMR-MS has been extensively applied in the area of natural products, this technique has been applied as a rapid screening method of searching unknown marine natural products in chromatographic fractions[64-65] and for the separation and characterization of natural products from plant origin [66-69]. In the field of drug metabolism, LC–MS–NMR has been extensively applied for the identification of metabolites and in pharmaceutical research [70-73]. LC-NMR-MS is also used extensively in food industries and also helps in different organic contaminants and compounds in nature [74-76]. LC-

MS-NMR has been employed in two major drug discovery and development areas, which include combinatorial chemistry and impurity profiling [77-79].

Other Hyphenations of NMR: There are other separation techniques that have been hyphenated with NMR detection.

1. High-Performance Liquid Chromatography-Nuclear Magnetic Resonance (HPLC-NMR): The coupling of LC effluent to NMR was first reported in 1978. In order to improve the sensitivity and resolution, the modern LC-NMR systems are associated with multiple technological advancements like the use of strong field magnets, microprobes and cryoprobes technology [80].

In HPLC-NMR, an HPLC system is first connected to a UV detector and then to an LC-NMR flow probe inside an NMR magnet through a capillary tubing. The UV detector is used to monitor the eluted components for NMR detection. The outlet of the UV detector is connected to an LC-NMR probe head via a polyethylene ketone (PEEK) capillary. There are flow cells of different sizes are available for LC-NMR probe head where the outer diameters of these flow cells range from 3 mm to 5 mm. Various flow cells of different sizes are available for a typical LC-NMR probe head. The selection of the flow cell size depends on the size of the LC column used, which in turn depends on the sample size to be analyzed. In general, a 400–750 MHz NMR spectrometer is required for an analytical LC-NMR system to obtain decent NMR spectra. It can be operated in three modes: on-flow, stop-flow and loop- collection [81]. LC-NMR is mainly used in the characterization of impurities and degradation products [82-84] and also in the natural product analysis [85].

2. Gas Chromatography-Nuclear Magnetic Resonance (GC-NMR): The first attempt of coupling GC and NMR using specific NMR tubes for direct sample trapping was carried out by Brame in 1965. Another hyphenated GC-NMR instrument was discovered by Buddrus and Hertzog in the year 1981 and they analyzed a mixture of terpenoic compounds with boiling points as high as 200 ºC. In this year, it was demonstrated by converting the NMR tube into a flow cell with both sides opened with the coil around the tube placed inside the magnet of the NMR instrument and one end of the open NMR tube connected to the GC instrument and the other end to waste but the data showed lesser resolution because the NMR tube was not spinning [86]. The hyphenation of GC-NMR has followed a development that is similar to the LC-NMR. The currently used GC-NMR instrument consists of an NMR flow cell that consists of a custom-made solenoildal microcoil around a fused-silica capillary tube inside an NMR magnet that is connected to a GC instrument through capillary tubing. It is not available commercially, but it is in the developmental stage, which is used in industries. There is the potential use of this technique if there is enough demand to solve the structural problems of gases that require NMR structural determination [87].

3. Gel Permeation Chromatography- Nuclear Magnetic Resonance (GPC-NMR): It is the hyphenation of Gel Permeation Chromatography with Nuclear Magnetic Resonance Spectroscopy, which is the determination of molecular weight and structure of polymers in complex mixtures [88]. The polymers analyzed by this method include polystyrene, polybutyl acrylate, and polybutadiene mixtures. GPC-NMR contributes to the molecular dependence of tacticity as it contributes to the understanding of the mechanism of sterol regulation in polymerization. The main advantage of this method is it takes very less time for analysis of sample (*i.e.*; 60 min for 1 mg sample). The major disadvantage of this method is the low sensitivity of NMR when compared to other techniques and the short T_1 for polymers so they need to have sufficient scans in order to acquire a reasonable signal-to-noise ratio.

Hatada *et al*., investigated poly (methyl metha-crylate) (PMMA) and poly (butyl methacrylate) copolymers prepared with t-C4H9MgBr in toluene by on-flow GPC-NMR to determine their molecular weight and polymer size. This is done by calculating the average number of monomers based on the ratio of the areas of the end terminal monomer unit with a signal from the polymer by ^{1}H NMR. Hiller *et al*., developed the GPC-NMR experiments on olefin homopolymers and copolymers as well as polyolefin bends in order to

separate them and to determine the sizes and compositions at 130 ºC [87].

4. Size Exclusion Chromatography- Nuclear Magnetic Resonance (SEC-NMR): The coupling of size exclusion chromatography with nuclear magnetic resonance spectroscopy has been widely used in molecular weight determination and also analysis of polymers in complex mixtures, which helps in understanding the polarization mechanism and facilitates the understanding of the relationship between polymer structure and its material properties [87].

Chloroform-d is used as an eluent for most of the organic polymers and ethanol-d6 in the amount of Ca 0.5% is used as a stabilizer. Non-deuterated solvents containing a small amount of a deuterated solvent for the deuterium lock can also be used as an eluent where the eluent signals can be eliminated by applying WET water suppression enhanced through T1 effects in combination with the LC–NMR probe with PFG coils. The NMR data are collected over the entire chromatographic peak and stored as a consecutive series of n-coadded scans. The post-analysis of SEC-NMR data by the multivariate curve resolution can resolve the individual components when the chromatographic separation of a polymer mixture is insufficient and thus enable one to extract each NMR spectrum from the SEC–NMR data set according to intensity profiles [89]. Online SEC-NMR is an efficient method to determine the tacticity of the molar mass dependence of co-monomer composition in a polymer sample. Ute *et al.*, applied SEC-NMR to determine the molecular weight distribution of poly(methyl methacrylate) (PMMA) quantitatively [87]. Hiller *et al*., described the Online Size Exclusion Chromatography-NMR for the Determination of Molar Mass Distributions of Co-polymers [90].

5. Supercritical Fluid Chromatography- Nuclear Magnetic Resonance (SFC-NMR): The hyphenation of SFC-NMR has an inherent advantage when compared to the LC-NMR analytical technique. In supercritical fluid chromatography, supercritical carbon dioxide is used as a mobile phase. In SFC-NMR, the supercritical carbon dioxide will not give ^{1}H NMR spectra so cleaner spectra are obtained. In the SFC-NMR setup, the SFC was directly coupled to the NMR probe through a flow control system, and a back pressure regulator at 120 bars is used to control the pressure of the SFC system and also the pressure of the tube going to the NMR. Sometimes the system was extended with an in-line concentrator as the concentration of the sample in the plug was too low to detect by NMR spectroscopy within a reasonable amount of time. In our initial SFC-NMR setup, in which SFC was coupled directly to the NMR probe through a flow control system, the pressure of the SFC system and the pressure of the tubing going to the NMR probe were controlled by the same backpressure regulator at 120 bars. The tubing going to the NMR probe is connected to a different manual backpressure regulator with an operating range between 20 and 103 bars, and this backpressure regulator allows us to lower the pressure of a sample fraction, which was selected after SFC. An extra stainless steel tube is added before the NMR probe head, which allows the separation of phases. The sample is injected into a CO_2 flow and loaded onto a column foe separation at high pressure, which is followed by UV detection, after which the sample goes through the backpressure regulator to the waste. The sample, after passing through UV detector valve 1 is switched on where the loop is filled with sample, supercritical carbon dioxide, and a co-solvent. Once the loop is filled, the valve switches back by inserting the sample plug into a flow of incompressible and immiscible medium where this separate water flow line is leading to the NMR probe, which is kept at low pressure for expansion of supercritical carbon dioxide and the sample flow to the stripline of detection. After the NMR detection, the sample will flow to the waste through a back pressure regulator [91]. SFC-NMR has been applied to the separation and structural analysis of monomeric acrylates [92], hydrocarbons [93,] and separation and identification of the cis/trans isomers of vitamin A by on-flow methodology [93, 87]. SFC-NMR has been used in food analysis for the decomposition of lipids targeting triglycerides from 40 commercial margarine products[87].

6. Super Fluid Extraction-Nuclear Magnetic Resonance (SFE-NMR): The hyphenation of superfluid extraction (SFE) with NMR is mainly used for monitoring ^{1}H NMR supercritical fluid extraction processes with CO_2 as the eluent of the

extraction. The operation is similar to that of SFC-NMR, which can be operated in on-flow as well as stop-flow modes. The main advantage of this SFE-NMR is that this method does not require solvent suppression and by using flow cells that can handle the SFE-high pressure system. Analysis of the components of coffee extract and black pepper extract in the on-flow and stop-flow modes with the acquisition of two-dimensional COSY45 and plastifiers from poly (vinyl chloride) (PVC) [87].

7. Capillary Electrophoresis- Nuclear Magnetic Resonance (CE-NMR): The hyphenation of capillary electrophoresis with NMR spectroscopy offers the separation capability of CE, and the superior detection of NMR. The NMR microcoil probe can be coupled to the capillary CE system with no major modification systems to the existing CE instrumentation. The magnetic objects must be kept several meters away from the magnet depending on the magnetic field strength. The non-magnetic plastic buffer vials and Pt electrodes can be positioned within the magnetic bore. The solenoid microcoil was either wrapped around the capillary or the capillary was housed in the saddle coil. In this case, the capillary acts as a sample holder as well as coil forms. Thin-walled capillaries should not be used as it may affect line-shapes as well as S/N ration when the coil comes in closer contact with the sample. The length of NMR active volume depends on the number of turns on the solenoidal RF coil and the diameter of the capillary. The sensitivity at the expense of separation efficiency can be improved by the use of an expanded detection cell. In CE-NMR, the solenoidal coils orthogonal to the static magnetic field (B_0) forms the most mass sensitive RF coils. In this configuration, the electrophoretic current induces a second local magnetic field gradient that perturbs the magnetic field homogeneity. The first hyphenation of CE-NMR reported the separation of arginine, cysteine, and glycine under static conditions with a detection limit of 50 ng. Continuous flow CE-NMR is being used for the separation of a mixture of histidine and lysine in phosphate buffer. It is also used in analyses of major metabolites of paracetamol in human urine[94].

8. Solid Phase Extraction- Nuclear Magnetic Resonance (SPE-NMR): Solid- Phase Extraction has been extensively used for NMR analysis, and it provides two benefits by eliminating the undesirable protonated solvents and additives from extraction or separation processes and concentration of the analytes of interest prior to NMR analysis by NMR or LC-NMR. In this, the analytes of interest are trapped in SPE cartridges which are eluted and sent to the NMR flow cell in deuterated solvent(s), eliminating or minimizing the need for solvent suppression techniques. This method does not require deuterated solvents for chromatographic separation when SPE is used to pre-concentrate the analytes of interest after chromatographic separation. The main advantage of SPE is that deuterated solvents are not needed for chromatographic separation when SPE is used to pre-concentrate the analytes of interest after chromatographic separation. The slowest step in SPE-NMR is the drying process of the samples in the cartridges when an individual analyte is trapped in a cartridge prior to NMR analysis. It takes overnight to dry several cartridges, which is the disadvantage of SPE-NMR. SPE-NMR facilitates the structural elucidation of a small amount of analytes in samples with complex mixtures. SPE-NMR has been used widely as a hyphenated technique in the fields of natural products for the structural analysis of major and minor components present in natural products [95-97], drug development [98], environmental concerns [99,] and drug degradation products [100-101].

9. Capillary Electrochromatography- Nuclear Magnetic Resonance (CEC-NMR): It is a hyphenated technique which combines the separation efficiency of capillary electrophoresis and selectivity of liquid chromatography. In this, there is more sample loading capacity, which increases the sensitivity of NMR. The separation is performed using capillary LC columns with electroosmotic flow instead of pressure flow. It is a promising tool for analyzing complex mixtures. The instrumentation of CEC-NMR is a hybrid between CE-NMR and cLC-NMR. Pressurized CEC-NMR separated and identified a mixture of unsaturated fatty acid esters. Continuous flow CEC-NMR is used as a tool for analyzing drug metabolites in human urine [94].

Multiple Hyphenations of NMR: There are other systems where the NMR is hyphenated with more than one spectroscopic method which is referred to

as multiple hyphenation. Louden *et al.*, reported a HPLC-DAD-MS-NMR system supplemented with an IR spectrometer in which the eluent from HPLC was directed to IR spectrometer via DAD detector from which it enters NMR and MS through a flow splitter [102-103] or the IR Spectrometer was placed alternatively after the splitter online with UV detector followed by NMR spectrometer [104]. There is a hyphenation of LC- SPE-NMR with MS, which is referred to as LC-SPE-MS-NMR (referred to as SPE-MS-NMR) in order to obtain a complete set of data for a comprehensive structural elucidation analysis of the analytes of interest from complex mixtures where the SPE unit is placed before the NMR instrument and after splitting the flow to trap the chromatographic peaks of interest and concentrate them in SPE cartridges after several injections before NMR analysis. As the SPE unit concentrates and dries the chromatographic peaks of interest, the HPLC unit does not need to use deuterated solvents for its solvent system, which eliminates having deuterated molecular ions for the analytes of the mixture in the MS. The main advantage of SPE-MS-NMR is that it minimizes the timing of the traditional method of isolation before NMR and MS analysis and accelerate the structural analysis. Seger *et al*., used SPE-MS-NMR to elucidate the structures of four minor components from the root tuber extract of Harpagophytum procumbens DC daliaceae, a potential phytomedicine plant for the treatment of rheumatism, polyarthritis, and osteoarthritis [105]. Another example is the application of SPE-MS-NMR for the identification of the three drugs-amlodipine (calcium channel blocker agent), indapamine (diuretic), and valsartan (angiotensin II receptor blocker) based Chinese medicine gold nine soft capsules [106].

CONCLUSION: The improvements in NMR hyphenations have made NMR analysis of complex mixtures more easy and rapid without any laborious preparative and purification procedures. The versatility of various hyphenated techniques can be exemplified by the number of applications listed in this paper. There will be an increased number of hyphenated techniques commercially available with the development in technological processes. The development of practically useful and cost-effective hyphenated HPLC-NMR systems requires that problems with NMR detector sensitivity, related to loading capacities in the chromatographic step, are solved. The automated liquid handling devices in the future may shift the routine NMR hyphenation from data acquisition using flow probes to NMR microtubes. However, the ultrafast data acquisition techniques may render on-flow LC-NMR very attractive in some areas. Extended hyphenations involving the specialized detectors are considered an interest of choice. The present hyphenated system involves the use of low-resolution mass spectrometers, and these spectrometers are affordable, so they are included in future routine analysis. The combination of hyphenated NMR with bioactivity detectors, such as flow-through bioreactors with immobilized proteins or automated bioassay systems that employ well plates, is another exciting possibility. NMR sensitivity enhancement through hyperpolarization is one of the rapidly developing areas. There should be the development of NMR special databases and intelligent spectra interpretation systems for structural elucidation or classification to get high-quality 1D and 2D NMR spectra from multiple chromatographic peaks. The hyphenation of gas chromatography with NMR has to be developed as there is the potential use of this technique if there is enough demand to solve structural problems of gases that require NMR structural determination.

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