

FIELDS OF APPLICATION AND SAFE MANAGEMENT OF NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY IN BIOMEDICINE FOR NON-MEDICAL PURPOSES

Iacono Maria¹, Trinchese Ciro², Colombo Giovanni³, Iacono Chiara⁴, Ruggiero Roberto⁵

¹*Dott.ssa in Biologia*

²*Ingegnere Meccanico - Energetico*

³*TSRM presso AO dei Colli – Ospedale Monaldi – UOC Radiodiagnostica - Napoli*

⁴*TSRM presso Ospedale Pediatrico Giovanni XXIII di Bari*

⁵*TSRM presso ASL Bari*

KEYWORDS: NMR, safety, magnetic fields, biology, spectrometer

ABSTRACT

Nuclear magnetic resonance (NMR) spectroscopy has assumed a leading position among the instrumental methods used in the analysis and characterization of organic and non-organic molecules, in addition to the application of ¹³C and ¹H high-resolution Fourier transform techniques for structural analysis of lipid components, peptides and proteins and carbohydrates. Thanks to the increasingly higher magnetic fields of the spectrometers, there has been a significant increase in resolution which has allowed us to move on to larger molecules and increasingly sophisticated techniques. Today multidimensional spectra such as the correlation spectra between ¹³C, ¹H and ¹⁵N are routine analysis whose most common acronyms are FOCSSY, NOESY and TROSY. Most of these experiments are based on the NOE (Nuclear Overhauser Effect) phenomenon which provides information on the dipolar interaction between magnetic nuclei. We will also deal with the various types of installations in relation to the safety standards that these equipment require for correct use.

INTRODUCTION

Since its discovery in the 1940s, nuclear magnetic resonance (NMR) spectroscopy has become a powerful and interdisciplinary method for investigating matter. Since then, NMR has been used in various ways in physics, chemistry and biology. For the study of biological systems it is convenient to distinguish between three types of applications:

- study the structure and function of macromolecules;
- study the metabolism;
- Obtain in vivo images of the anatomical structure and functional (physiological) states.

NMR, before finding wide applications in medicine thanks to its great sensitivity, was widely used in the field of scientific research, in particular that aimed at the analysis of chemical compounds and organic molecules, whose complex structure is revealed mainly through the interpretation of spectra. These results are mainly obtainable through direct investigations, which do not require pre-treatments, and which have the undoubted advantage of being non-destructive for the analyzed sample and are repeatable over time.

In fact, there are many applications in pharmaceutical chemistry, for example the study of the relationship between the molecular structure and their biological activity of a given compound, in particular in drugs capable of interacting with human biomolecular receptors. NMR allows to investigate the real capacity of action of the molecule studied, allowing to give greater impact to the study of new molecular struc-

tures to be used for pharmacological use.

The first applications of NMR were related only to the hydrogen nucleus, but subsequently moved on to the analysis of a larger number of magnetically active nuclei, thus evolving towards increasingly sophisticated and refined methods, capable of carrying out increasingly complex and meticulous, obtaining information that, correlated to each other, allow to give structural indications in several dimensions, even in relation to very complex molecular structures.

MATERIALS AND METHODS

Today we can find mainly two types of NMR spectrometers: the more obsolete but still in operation, and the decidedly more recent ones. The oldest installations in Italy are mostly 200 - 300 MHz spectrometers (associated with 4.7 and 7 tesla static magnetic fields), with liquid helium contents of several tens of liters.

The new generation equipment, on the other hand, ranges on average from 400 to 600 MHz, with some that can even reach up to 800 MHz (about 19 tesla). Most of the studies on metabolite profiles reported in the literature are performed with high field intensity spectrometers (≥ 600 MHz) to obtain a good spectral dispersion. The helium contents of these devices range on average from 35 liters, for the smallest spectrometers, up to over 1300 liters for the largest, of course the presence of large quantities of helium is justified in relation to the performance of these spectrometers. A 1200 MHz (28 Tesla) spectrometer, containing several hundred liters of helium, is installed at the Cerm (Magnetic Resonance Center) in Florence.



Fig. 1 - Plug valve which, upon reaching a maximum pressure value, and in case of overheating of the magnet, opens, releasing helium into the environment.

Similarly for the equipment used in medical imaging, dedicated pipes are provided for the possible evacuation of cryogenic gases, oxygen sensors for detecting their possible leaks into the environment, as well as a dedicated air exchange system in normal and emergency, able to evacuate the air dispersion of cryogenes to the outside, which occurs both physiologically (process called boil-off) and in cases of operating anomalies (in particular for helium the phenomenon is called quench).

In case there is an abnormal and massive release of these cryogenes in the room, these massive quantities of liquid helium present various dangerous conditions if not carefully monitored:

- frost damage: splashes on the skin cause damage similar to burns, the eyes are particularly vulnerable;
- suffocation: an O₂ concentration <17-18% is not sufficient for human respiration, at room temperature (20 °C) 1 liter of liquid helium produces approximately 750 liters of gaseous helium;
- oxygen condensation: The low temperature of the liquid cryogen container can cause the con-

densation of oxygen or oxygen-enriched air, with additional risk of fire even if helium itself is not combustible.

Small and medium-sized NMR equipment, especially of older construction, are generally not equipped with the safety systems typical of superconducting magnets used for medical imaging. Often there is no quench piping to convey the helium outside the room, the accessory safety devices (oxygen sensor) or the ventilation system for expelling the air containing the gaseous helium that may have escaped inside the laboratory.

More recent spectrometers have at least two or more turrets on the top: one intended for nitrogen refilling, and another for helium refilling operations, with at least one plug valve associated with the quench (see Figure 1). Generally, the smaller turrets are nitrogen and the larger ones are helium.

As prescribed in the most recent regulations, especially in the most modern and powerful NMR installations, the identification and confinement of the risk area associated with the magnetic field up to a value of 0.5 millitesla (5 Gauss) is envisaged: in fact ≤0.5 millitesla there cannot be fixed workstations, laboratory activities, or in any case activities that involve the permanence of authorized personnel beyond the time necessary to carry out the operations necessary for the operation and set-up of the equipment, such as for example: sample introduction, tuning, cryogen refilling operations, etc.

DISCUSSION

Nuclear magnetic resonance (NMR) spectroscopy is an instrumental analytical technique that allows to obtain detailed information on the structure of molecules by observing the behavior of atomic nuclei in a magnetic field. After immersing the molecule under examination in a strong magnetic field, the absorption of a radio frequency radiation (from 100 to 1000 MHz) is measured, which causes nuclear spin transitions in particular atoms such as ¹H or ¹³C.

An NMR spectrometer consists of a superconducting

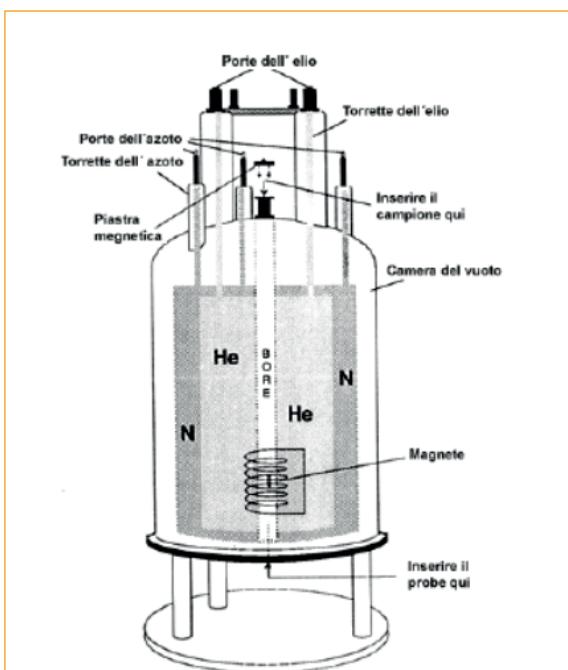


Fig. 2 - simplified cross-section in coronal view of a spectrometer

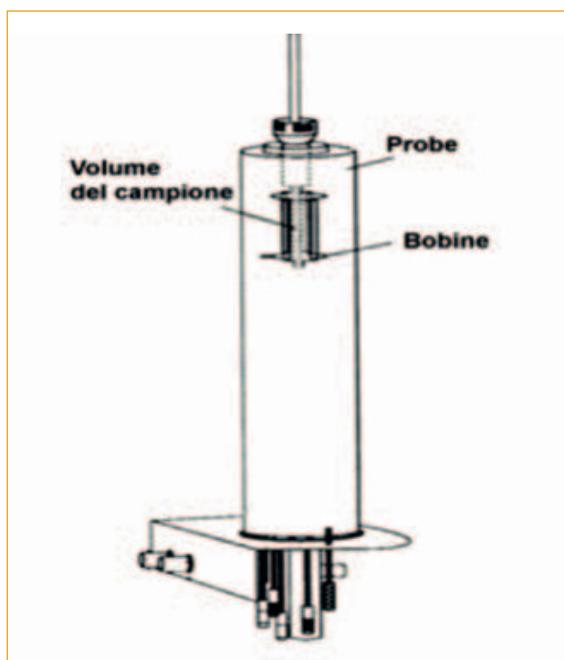


Fig. 3 - simplified cross-section in coronal view of a probe

Isotope	Frequency values in MHz for		% Natural Abundance	Relative sensitivity	Spin value
	14,082 kG	23,487 kG			
¹ H	60,000	100,000	99,985	1	1/2
² H	9,210	15,351	0,015	9,65 x 10 ⁻³	1
¹¹ B	19,250	32,084	80,420	0,165	3/2
¹³ C	15,087	25,144	1,108	1,59 x 10 ⁻²	1/2
¹⁵ N	4,334	7,224	0,367	1,01 x 10 ⁻³	1
¹⁷ O	8,134	13,560	0,037	2,91 x 10 ⁻²	5/2
¹⁹ F	56,446	94,077	100,000	0,833	1/2
³¹ P	24,288	40,481	100,000	0,066	1/2

Tab. 1 - ¹H and ¹³C spectra of a virgin olive oil

winding immersed in a liquid helium jacket which is itself surrounded by a liquid nitrogen jacket (see Figure 2).

The use of liquid nitrogen is necessary in order to lower the thermal gradient of the jacket of the dewar containing the liquid helium and therefore to decrease its evaporation; obviously the consumption of nitrogen is greater than that of helium by about 10 times. At the center of the spectrometer you can find the empty cylinder, called bore, where the static field is present, inside the bore you can slide the probe (see Figure 3), that is a sort of cylindrical container where it is housed inside the sample to be analyzed in a specific test tube.

There are various types of probes that can be used, especially in relation to the type and quantity of sample to be analyzed, and they also differ according to the number and type of internal coil supplied, which identifies their specificity.

The sensitivity of the NMR spectrometer depends not only on the strength of the magnet applied to the field, but mainly on the intrinsic sensitivity of the probe. Several probes are available to detect different cores with optimal signal-to-noise results.

For metabolite profiling studies where the endogenous molecules in the biofluids are mostly quite diluted, tubes that directly contain the sample are used which are inserted into the probe, the NMR observation volume of the tube must be completely filled with the sample to allow maximum sensitivity. The volume of the sample that resides in the active sensing region of the coil is called the observation volume. It is always smaller than the total volume of the sample needed to fill the tube, so that the sample extends evenly over both ends of the coil. There are various tubes available with different coil sizes, ranging from 10mm to 1mm coil diameter and with corresponding sample volumes from a few ml up to 2 µl. The probe head is usually equipped with a 5 mm coil and requires 550–600 µl sample volume.

■ APPLICATIONS OF NMR

This methodology has established itself in recent years as the most powerful and fastest technique for the identification of reaction products or unknown or-

ganic compounds. Although the costs are much higher than other analytical techniques, spectrometry has spread not only in university laboratories, but also in private ones and in industry, as the ability to examine and couple the results with other analytical techniques (also considering its character of non-destructive technique), are often decisive for examining very complex substances, especially of an organic-biological nature. In this case, it allows the study, in addition to the chemical composition and sequence order of the different functional groups, of the three-dimensional structure of the investigated macromolecule (as in the case of proteins).

Another important use is that linked to the research of food sophistication, therefore to the need to carry out the analysis of compounds such as wine, oil and many other natural products, whose real quality, possible sweetening and / or state of conservation. The technique has the advantage, as for applications in imaging, of being “non-destructive”, of requiring very small quantities of sample and, in most cases, of not requiring separations and / or preliminary treatments. The sample is examined without altering its characteristics; the only limit is that it has dimensions compatible with the device probe.

¹³C spectroscopy is widely applied to the study of fatty acids and triglycerides. It is also used in the analysis of the quality of olive oils and is being tested in the analysis of other dietary fats such as butter and lard. Table 1 shows the ¹H and ¹³C spectra of a virgin olive oil with the most significant resonance assignments.

Thanks to the ¹³C spectrum, obtained on the deuterated chloroform sample, we can obtain various information on the nature of the oil. In particular, from the measurement of the intensity of the resonances it is possible to obtain the ratio between saturated, mono-unsaturated and polyunsaturated aliphatic chains, the ratio between cis and trans fatty acids, the quantity (expressed in molar fractions) of triglycerides, monoglycerides and diglycerides, the ratio 1.2 / 1.3 diglycerides and the ratio between free and esterified fatty acids. The ratio between mono and polyunsaturated fatty acids (oleic / linoleic) allows to discriminate olive oils from seed oils with a high content of

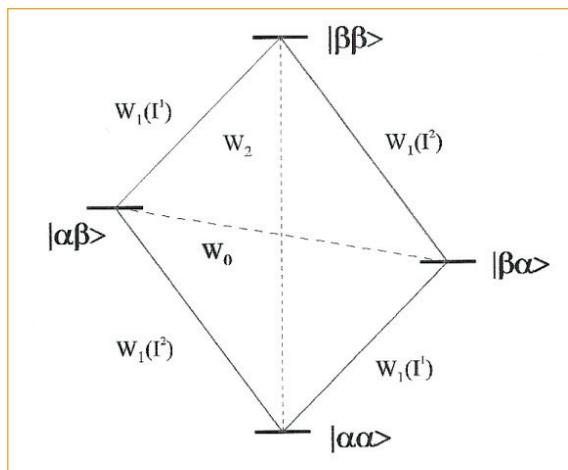


Fig. 4 - A dipolarly coupled two-spin scheme (I^1 and I^2) showing the relaxation processes of the NOE phenomenon. W_i is the relaxation rate that connects the different energy levels involving transitions to 0, 1 or 2 quanta of energy.

polyunsaturated fats. Even the presence of high temperature rectified or neutralized fats can be identified by detecting the resonances of trans fatty acids and a high proportion of partial glycerides compared to the acidity of the fat.

NMR spectroscopy continues to play a very important role in the study of biopolymers and macromolecules of biological interest, in fact it is essential for the analysis of the structure of molecules and macromolecules in physiological solution, as well as for the study of the dynamic processes that regulate for example the activity of enzymes and the transport of nutrients across cell membranes. Together with other techniques, such as X-ray diffractometry with synchrotron light, neutron diffractometry and electron microscopy, NMR has made it possible to answer many questions concerning the link between molecular structure and biological function.

The first studies in this field were based on the analysis of only one-dimensional spectra of ^{13}C and ^1H NMR of oligopeptides in aqueous solution using spectrometers of just 100 MHz. The increase in the resolution of the spectra, due both to the use of increasingly higher magnetic fields and to the better computerized analysis of the spectra, has made it possible to analyze larger molecules and with increasingly sophisticated techniques.

Today, the analysis of multidimensional spectra such as those of correlation between ^{13}C , ^1H and ^{15}N whose most common acronyms are FOCSSY, NOESY and TROSY, are routine. Most of these experiments are based on the NOE (Nuclear Overhauser Effect) phenomenon which provides information on the dipolar interaction between magnetic nuclei. If two nuclei are relatively close in space, the dipole-dipole interaction, analogous to that of the classical description, can be exploited as a relaxation mechanism, favoring the suppression of some signals in the spectrum or simply to derive from the decrease or increase in the intensity of the signals the actual distance between two nuclei of the same molecule. It is in this way, by comparing the data obtained with the NMR with those of the simulations, that we can trace the structure of the macromolecules in question.

Figure 4 shows the scheme of energy and population exchanges for a system of two spins (I^1 and I^2) where

the transfer or relaxation speeds between one level and another are indicated with the terms W_i , with i equal to the type of transition (0 = zero quanta, 1 = one quantum, 2 = two energy quanta). Altering the population of one of the four levels induces a variation in the ratio between the W_i speeds, giving rise to the NOE phenomenon which is realized precisely in the variation of the intensity of the NMR signals.

Analysis of solid and / or semi-solid substances

NMR is a technique that, in addition to analyzing molecules in solution, can also be used for the study of solid and semisolid samples. A solid state sample, in the presence of a magnetic field, is characterized by very large signals, due to the presence of dipolar interactions; this type of interactions are mediated by the mobility of the molecules in the case of samples in solution. To study solid samples it is therefore necessary to eliminate these interactions with the MAS technique, i.e. the fast rotation of the sample which is placed at an angle of about 54° from the main magnetic field.

Generally for solid samples it is necessary to increase the sensitivity and reduce the times with the Cross Polarization technique. In this case, the technique is called CP-MAS and requires high power amplifiers (up to 1kWatt).

In the solid state, the nuclei of ^{13}C , ^2H , ^{31}P , ^{29}Si are mainly studied, as well as quadrupolar nuclei such as ^{27}Al , ^{23}Na . Solid state applications range from organic chemistry to inorganic chemistry, from physics to biophysics and biochemistry, from hydrocarbons to soil chemistry. Using the MAS technique, semisolid samples are also analyzed (such as food, biological tissues, cells, resins, gels, etc.). In this case the technique is referred to as high resolution MAS or HR-MAS.

Safety

Especially in recent years, the purely medical use of magnetic resonance imaging has been specifically regulated with the obligation to adhere to safety standards, regulated by art. 2 of the d.p.r. 542/1994, or the provisions of the Ministerial Decree 10/08/2018. But nothing specific exists for non-medical applications. This lack has now become a stable gap in daily practice, also because the current technologies offered by the market make available equipment characterized by very high levels of static magnetic field. Safe management cannot be determined by a system of standards that is not adequate for the performance of this equipment.

All this takes on even more critical value, when one reflects on the fact that NMR spectrometers are more widespread for research purposes and the users are postgraduates, fellows and university contractors. These users cannot be configured as employees but rather as scientific users, sometimes even occasional, often very competent in the scientific application of the technique, but generally not very sensitive to the safety aspects, because they are unaware, or poorly trained and sensitized about the risks associated with the use of high static magnetic fields. They might mistakenly think that the use of NMR spectrometers today involves less exposure for operators, since the introduction of samples, once the probe is installed,

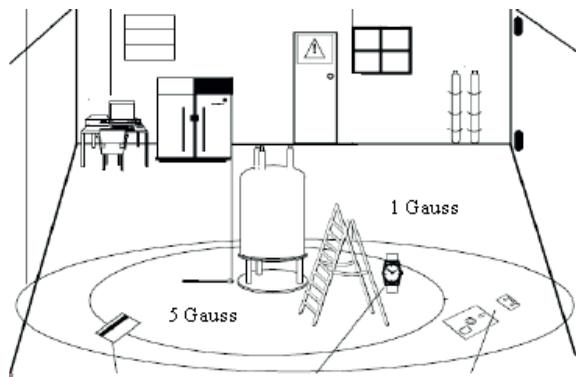


Fig. 5 - diagram of the risk areas with mapping of the intensity of the magnetic field in an ideal installation that respects the safety conditions.

generally takes place for much shorter times than in other applications and even in systems dedicated to serial analysis, this also occurs through the use of automated sample insertion systems.

However, for NMR investigations, an additional level of exposure is added that cannot be traced in other cases and related to the tuning adjustment process which, in most cases, must necessarily be carried out by placing under the equipment. Having to adjust the tuning for each type of sample introduced (except for repetition of analyzes on the same types of samples), the residence time at high levels of static magnetic field represents a decidedly significant and very important factor in terms of protection.

The final result is therefore a scarce sensitivity towards safety, supported by the lack of a specific reference regulatory framework: this makes it necessary to try to codify these additional scenarios of application use of magnetic resonance and, therefore, to propose operational indications that may be useful for the assessment and minimization of risk, having as a single regulatory reference that of Legislative Decree 81/2008 and subsequent amendments, as amended and supplemented by Legislative Decree 159/2016 as regards the exposure of workers to the fields electro-

magnetic. In most cases, the workstation of the operators, the control area of the equipment and the chemical laboratory used to prepare the samples, are located in the same single room where the NMR spectrometer is installed or in the immediate vicinity.

This is a condition that certainly deserves attention and, unfortunately, testifies how the technological and scientific evolution of NMR applications in recent years has not gone hand in hand with the guarantee of safety. This consideration in itself can be easily confirmed by observing how most of the spectrometers installed in Italy, even if of the latest generation, often respond to the same management standards that were applied to installations thirty-five years ago, which presented considerably overall levels of risk, lower than the current ones.

It is important to visually and practically trace the risk areas around the spectrometer (see Figure 5), in order to correctly manage an emergency situation. It would be ideal if a free walkway were left around the installation of the equipment: this means that the use of ladders or similar devices to carry out the operations envisaged on the magnet head must include a procedure for their removal at the end of each operation, leaving the space around the magnet is always free.

CONCLUSIONS

NMR spectrometry certainly represents a valid ally in the study of organic and non-organic matter. It allows to perform non-destructive analyzes on samples to understand which basic elements they are composed of and in what percentage.

On the other hand, the potential risks that these equipment entail if they are not managed correctly cannot be overlooked. It is therefore important to raise awareness in today's context of those who use them and to intervene so that there are regulatory changes that make the installations of these equipment concretely safe and regulated, without leaving the management of safety to the common sense of installers and end users.

REFERENCES

1. D.G. Gadian, (1995) NMR and its application to living systems Oxford University Press Oxford.
2. Campanella F, Mattozzi M, Di Luigi M, et al. (2020) Utilizzo in sicurezza della spettroscopia NMR. INAIL
3. <https://www.unifi.it/art-4736-ricerca-operativa-al-cerm.html> Visitato l'ultima volta il 04/09/2021
4. "NMR in the post-genomic era: from molecular structure to biological function", (2000) report per la Training and Mobility of Researchers (TMR) "NMR in Concert".
5. Decreto Legislativo 81/2008 e s.m.i - Titolo VIII Capo IV Protezione dei lavoratori dai rischi di esposizione a campi elettromagnetici.
6. Decreto Legislativo d.lgs. 159/2016 e s.m.i