Accred Qual Assur (2006) 11: 130–137
DOI 10.1007/s00769-005-0006-7

GENERAL PAPER

Analytical Methods Committee

Report by the analytical methods committee: evaluation of analytical instrumentation Part XVI Evaluation of general user NMR spectrometers

Received: 1 November 2004
Accepted: 31 March 2005
Published online: 5 April 2006

Electronic Supplementary Material Supplementary material is available for this article at http://dx.doi.org/10.1007/s00769-005-0006-7

Analytical Methods Committee (□)
The Royal Society of Chemistry,
Burlington House, Piccadilly,
London, W1V 0BN
e-mail: vandenewman@tiscali.co.uk

The Analytical Methods Committee has received and approved the following report from the Instrument Criteria Sub-Committee.

Introduction

This report was compiled by the above Sub-Committee of the AMC which consisted of Professor S Greenfield (Chairman), Dr M Barnard, Dr C Burgess, Professor S J Hill, Dr K E Jarvis, Dr M Sargent and Mr D C M Squirrell with Mr C A Watson as Honorary Secretary. The initial input of the features for consideration and the reasons for their consideration was undertaken by a working party of Dr R Fletton, Dr P Sidebottom and Dr A Kenwright to whom the committee express their thanks.

$I = 1/2$ nuclei include ^{1}H, ^{3}H, ^{13}C, ^{15}N, ^{19}F and ^{31}P while ^{2}H and ^{14}N have $I = 1$. Nuclei with $I = 0$, such as ^{12}C and ^{16}O, have no spin angular momentum and do not give NMR spectra. The great majority of NMR studies relate to spin half nuclei (very largely ^{1}H, ^{13}C, ^{19}F and ^{31}P).

Nuclei with $I / 1/2$ in addition possess a *nuclear quadrupole moment*. In many cases this causes severe line broadening through its effect on relaxation processes (see below) and signals are consequently difficult to observe.

Since they are charged particles, nuclei with a spin angular momentum also possess a

signals into multiplets with structures dependent on the number and spatial disposition of the interacting nuclei. For an unknown molecular structure this effect provides valuable insight into the relative locations of chemical groups which have been identified from their chemical shifts.

Magnetic nuclei also interact directly through space (like two bar magnets) – an effect called *direct* or *dipolar coupling*. These interactions may be large but in solution they are not directly observable in the spectrum since on the NMR timescale they are averaged to zero by molecular motions. The fluctuating magnetic field generated by the modulation of dipolar interactions through molecular motion is however a major contributor to relaxation processes (*dipolar relaxation*).

In the solid state, where molecular motions are much reduced, this averaging does not occur; dipolar interactions are then a major obstacle to the observation of spectra and must be overcome by special techniques.

Spin decoupling

Since ^{13}C is only about 1% abundant ^{13}C NMR signals are usually split only by spin couplings to ^{1}H (or other abundant magnetic nuclei) and are difficult to observe. It is therefore usual to remove this effect by simultaneously exciting proton resonances while observing ^{13}C (*heteronuclear spin decoupling*). This improves detection limits through collapse of the coupled multiplets to singlets but information helpful in the assignment of spectra is thereby lost. Several specialised experiments are available for recovery of this.

As an aid to assignment in the spectra of abundant nuclei *homonuclear spin decoupling* has long been employed. In this one multiplet in a spin coupled spectrum is subjected to specific excitation while rest of the spectrum is recorded in the usual manner. If a signal has a splitting due to spin coupling in common with the irradiated group this will be removed in the decoupled spectrum and the signal multiplicity reduced. This is of value in simplifying complicated spectra and, particularly, in resolving ambiguities as to which signals exhibit a common spin splitting.

In practice information on homonuclear coupling is usually obtained on modern instruments by *COSY* experiments (see below).

Nuclear overhauser effect

Where two protons are in spatial proximity in a molecule the fluctuation in their magnetic dipolar interaction caused by molecular tumbling provides an efficient mechanism for nuclear relaxation (see above). In these circumstances if the signal of one nucleus is irradiated with a low power while the other signal is observed there will be a change in the intensity of the latter. This effect is termed the *Nuclear Overhauser Effect* and it is brought about by a transfer among the energy levels of the population disturbance caused by the irradiation.

For typical organic molecules this effect is positive and the maximum change possible is 50%. The utility of the NOE arises from the fact that its initial rate of build up with irradiation time is strongly dependent on the separation of the nuclei involved. By running a spectrum with irradiation of one signal it is possible to determine which signals arise from close nuclei and get an estimate of their relative separations.

This is of great value in distinguishing between isomers and determining molecular conformations. The actual size of the nuclear Overhauser enhancement is dependent on molecular mobility and declines with increasing molecular weight. Thus for molecules of about 1000 MW NOEs are commonly near zero while at molecular weights of a few thousand NOEs become negative. To get over this difficulty experiments have been devised to measure *rotating frame NOEs* (*ROEs*) which do not suffer from this sign variation with molecular weight. Such experiments are mainly of interest for specialized protein and peptide studies.

Quantitative aspects of NMR

If NMR spectra are recorded under conditions which avoid saturation the intensity of a signal is directly related to the number of nuclei giving rise to it. Thus in the ^{1}H spectrum of ethanol the signals due to CH_3, CH_2 and OH have relative intensities of 3:2:1.

In addition the signal intensity is independent of the molecular environment; thus, for example, a CH_3 group will give a signal of the same intensity whether it is part of an alkane, an ether or an ester.

This quantitative aspect when combined with chemical shifts to identify molecular sub-units and spin coupling to deduce their connectivity gives NMR particular power as a tool for elucidating molecular structures.

In the special case of ^{13}C NMR, which is almost always used for the purpose of structural elucidation, it is usual to shorten experiment times by recording spectra under conditions where signal intensities are not strictly quantitative.

Pulsed fourier transform NMR (PFTNMR)

Scanning a spectrum to observe signals sequentially (*continuous wave spectroscopy*) is an inefficient method for spin 1/2 NMR, where linewidths are usually much smaller than the total spectral width. It is also totally unsuitable for recording spectra of nuclei like ^{13}C which, by reason of its low natural abundance and smaller nuclear magnetic moment, is *ca.* 6000 times less sensitive than ^{1}H.

All modern NMR spectrometers therefore utilise *pulsed excitation*

The response of the sample to the pulse is a *free induction decay (FID)*, a composite signal containing contributions from all the excited resonances and decaying away through relaxation processes. This is sampled and digitised for computer processing.

To convert this *time domain* signal to a conventional (*frequency domain*) spectrum the digitised FID is subjected to *Fourier transformation*, a mathematical process which in effect picks out the individual frequency components from the FID. Commonly the FID may be multiplied by appropriate weighting functions to enhance spectral resolution or signal-to-noise ratio.

In almost all NMR spectroscopy the process of inputting a pulse of radiation and sampling the FID is repeated many times and Fourier transformation is applied to the summed FIDs. In this way the signal-to-noise of the resulting spectrum (as compared with that of the spectrum from a single pulse) is increased as the square root of the number of FIDs acquired.

By this means ^{13}C spectra on 10 mg samples of most organic molecules can be obtained in about 1 h and signals of sensitive nuclei like ^{1}H and ^{19}F observed from microgramme size samples.

Dynamic range and solvent suppression

The detected FID is converted to digital form for computer processing using an A/D converter. For ^{1}H NMR, in particular, this may present a problem of *dynamic range* since both large and small signals may be present in the spectrum and must both be detected. It is therefore important that the A/D converter has sufficient resolution. In practice most modern instruments use 16 bit A/D converters although lower resolution converters may be encountered on old spectrometers.

A problem may still arise, however, where the FID is dominated by a very strong signal. This is most commonly met with in ^{1}H NMR where the solution has a substantial content of H_2O (it is also sometimes necessary to record spectra in a solution which is largely H_2O).

It is not permissible for the FID to overflow the A/D converter since this causes a distortion of all of the resulting spectrum. Modern spectrometers therefore adjust the gain to fit the FID into the digitiser but, in this case, the scaling may be such that background noise is insufficient to trigger the least significant bit of the digitiser. It then becomes impossible to recover a small signal buried in the noise by the accumulation of many FIDs.

To get over this difficulty *solvent (or signal) suppression* techniques are employed - these are often used in 2D as well as 1D spectroscopy (see below).

The simplest and most widely used of these methods *presaturation* can be implemented on all FT spectrometers. In this technique an irradiating field (rather like spin decoupling) is applied at the appropriate frequency for several seconds to saturate the solvent response. The irradiation is then gated off before application of the observing pulse and detection of the FID. Since the solvent response takes several seconds to recover from saturation through relaxation processes its contribution to the FID is much reduced. The sequence is then repeated as many times as necessary.

It should be noted that any signals in the vicinity of the solvent signal will also be lost or attenuated by this method. If, however, a signal in another part of the spectrum is undergoing exchange with the solvent (this is not uncommon with H_2O) it may be possible to saturate this and suppress the solvent response without loss of adjacent signals.

A number of other methods for suppressing or not exciting the solvent have been developed but may require more advanced instrumentation (e.g. selective excitation or pulsed field gradients).

NOE measurements by FT methods

In most cases the measurement of steady state NOEs, where one signal in the spectrum is continuously irradiated, is not very useful for structural purposes since static NOE values cannot be directly related to internuclear distances.

A much more useful experiment (*Truncated Driven NOE*) involves irradiating selected signals for a series of short periods and thus studying NOE buildup rates. Results are usually presented as difference spectra obtained by subtracting a spectrum with irradiation outside the spectral range from the on-resonance spectrum (*NOEDIFF*). Difference spectra ideally show signals only for the nuclei affected by the irradiation. The advantage of this technique is that it can be implemented on all modern NMR instruments.

On instruments with pulsed field gradient and shaped pulse facilities more sophisticated experiments giving higher quality results may be undertaken. An important recent development is the

Two dimensional NMR

Pulsed excitation offers much scope for experimental innovation. Thus it is possible to disturb the nuclear spins before applying the observe pulse and sampling the FID and to use this to study various internuclear interactions within the molecule (*two dimensional* and *multidimensional NMR*).

Multidimensional NMR is used almost exclusively to investigate biopolymers and commonly requires isotopically enriched samples and advanced instrument configurations but two dimensional methods are widely used on spectrometers of the General User type.

The most widely used 2DNMR methods involve detection of homonuclear spin coupling (*Correlation Spectroscopy – COSY)* or heteronuclear spin coupling (*Heteronuclear Correlation Spectroscopy – HETCOR*). Several variants offering advantages over these basic experiments have been developed e.g. *COSY45*, *phase-sensitive COSY*, *double quantum filtered COSY* and *COLOC*, *HMQC* and *HMBC*.

NOESY and *ROESY* are two dimensional methods for detecting, respectively, conventional and rotating frame NOEs. The latter experiment is of value under circumstances where the conventional NOE is near zero (see above).

In these experiments the system of nuclear spins is first disturbed by a sequence of pulses and the development of the disturbance under the influence of spin coupling interactions observed as a function of time; in essence a 2D experiment is a series of 1D acquisitions differing in the delay between the disturbance and the observing pulse used to generate the FID. This delay provides a second time domain which is converted by Fourier transformation to a second frequency axis in the resulting 2D spectrum.

A 2D spectrum is normally represented as a map with the proton chemical shifts along both axes (COSY) or with the proton shifts along one axis and the heteronucleus shifts along the other (HETCOR). The presence of a spin coupling interaction between two signals is indicated by a *cross peak* with the coordinates of the two chemical shifts.

Since 2D NMR involves the acquisition of many FIDs experiments tend to be time consuming; methods have therefore been developed to ease this problem.

In the case of COSY the time needed by an experiment is often determined not by poor signal-to-noise but by the need to sequentially cycle signal phases in the detection system to eliminate unwanted components of the NMR response. The use of *pulsed field gradients* removes these components without the need for phase cycling and allows the length of experiments to be determined only by sensitivity requirements. With typical sample quantities (a few mg.) a several fold reduction in experiment times is commonly achieved. Pulsed field gradients require additional equipment but are widely applicable in advanced NMR experiments.

The basic HETCOR experiment involves the detectiontheich issampl
along6* [ecu2tcehs(83.9(is)T* [ecu2.st)T* [ecuinst TD sssb24

non-destructive: the total sample is recoverable with more or less difficulty depending on the NMR solvent used.

Owing to the smallness of the nuclear magnetic moment NMR is at least an order of magnitude less sensitive than IR spectroscopy and several orders of magnitude less sensitive than uv/visible spectroscopy and mass spectrometry. Sample sizes routinely used are around 1 mg for ^{1}H and around 10 mg for ^{13}CNMR. Smaller samples may be run at the expense of longer acquisition times.

Provided an NMR acquisition is run under suitable conditions signals will be present in the resulting spectrum for all magnetic nuclei of the observed species present in the sample and the intensity of each signal will be directly proportional to the number of nuclei giving rise to it. These features give the technique a unique advantage over other common forms of spectroscopy.

For spin 1/2 nuclei the spectral dispersion is similar that of high resolution mass spectrometry and increases with the strength of the magnetic field. Most NMR spectra therefore provide a high level of accessible information and spectra can commonly be fully interpreted. NMR is the most versatile of the common spectroscopic methods and may offer several approaches for the solution of a problem.

For example it may be possible to record the spectra of several different nucleides on a particular sample and thereby gain complementary information. Where more than one spectrometer is available it may be helpful to record spectra on a higher fieldstrength instrument to increase spectral dispersion.

With the pulsed excitation used on all modern instruments many experimental variants are available providing information such as connectivity in molecules (for structural elucidation), proximity of atoms (for molecular conformation), conformational and chemical exchange, molecular diffusion etc. Much of this work cannot be easily undertaken by other techniques.

In consequence NMR spectroscopy with General User instruments, often but not necessarily in combination with mass spectrometry and IR spectroscopy, has been, for many years, the main method for determining the structures of organic molecules of synthetic or natural origin. If samples are adequately soluble and contain suitable nuclear species the method is equally applicable to covalent inorganic compounds.

In contrast to X-ray diffractometry, the definitive method of structural determination, NMR does not require a pure or crystallisable sample and is thus more widely applicable. Variable temperature NMR is widely employed for the study of processes like tautomerism and conformational exchange.

Through its interpretability and quantitativeness NMR is of particular value in chemical development where the products of a reaction and the major solvents and impurities can often be identified and the relative amounts approximately estimated from inspection of a simple 1D spectrum.

NMR methods have considerable potential for monitoring processes in chemical production but, for reasons of low instrumental sensitivity, expense and the environmental sensitivity of the magnet, have so far been little exploited. With the introduction of actively shielded magnets and the recent large advances in sensitivity achieved with cryogenic probes this situation may change.

In recent years advances in instrumentation have extended the use of NMR well beyond small molecule chemistry. The use of NMR methods to determine the structure of small proteins utilises sophisticated high field instruments but spectrometers of the General User type are often employed in the study of cell suspensions (e.g. for following cell metabolism using labelled substrates) and the investigation of ligand-to-protein binding (by observing changes in the spectrum of the small ligand molecule or in the protein if suitably isotopically labelled).

New methods of sample introduction requiring modifications to conventional probe geometry have been developed for particular purposes e.g. HPLC probes for on-line analysis of chromatographic fractions and flow injection probes for use with a multisampler for mass screening of combinatorial chemistry products. High resolution MAS probes (already mentioned) have been much used in combinatorial chemistry for the on-bead analysis of bound products.Of these three types of probe only the latter is easily accommodated in a General User environment.

NMR spectroscopy, which separates the signals of nuclei according to chemical shift, is to be distiguished from Magnetic Resonance Imaging (MRI), a technique with important medical and non-medical applications. In MRI the combined NMR signal of all nuclei of a particular species (in medical imaging normally ^{1}H) is detected from a restricted volume in the intact sample, which is selected by applying an appropriately shaped external magnetic field gradient. An image of the total sample is built up from the signals of different volumes selected sequentiechniq selected m

(2) effectiveness of solvent suppression in recording ^{1}HNMR spectra of aqueous solutions
(3) effectiveness of autosampler - test by running a number of samples in a variety of solvents and look for any mechanical problems, the ease with which field/frequency lock is established, the time required to adjust resolution and the quality of the resulting spectrum in terms of signal-to-noise, lineshape and phase correction.
(4) if ^{19}FNMR spectra are likely to be required only occasionally this facility can often be provided by retuning the coil of a ^{1}H probe. If this feature is likely to be of interest check for the quality of the results obtained,