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Chapter 5

1H and 13C NMR for the Profiling of Natural Product Extracts: Theory and Applications

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Abstract

Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are the principal methods of metabolomics, the branch of '-omics' that deals with small molecules. Although MS is gaining popularity in metabolomics, NMR enjoys a number of key advantages because it is nondestructive, unbiased, quantitative, does not require separation or derivatization, and is amenable to compounds that are difficult to analyze by gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). There are two general approaches to the use of NMR for profiling studies: an untargeted approach, which uses chemometric analysis; and a targeted approach, which aims to quantify known compounds in the extract. These approaches, however, are not mutually exclusive and will likely converge in the future. This paper will describe the basic theoretical principles that should be considered to develop NMR into a standard quantitative method. Although 1H NMR is more sensitive, 13C NMR spectra are simpler with less overlapping signals and are less affected by different magnetic field strengths. Various applications of 1H and 13C NMR for the profiling of natural products are described. The use of two-dimensional 1H NMR has been used to overcome problems of spectral overlap. The standardization of the NMR protocol will make it a more useful tool for the profiling of natural products extracts.

Keywords: nuclear magnetic resonance, 1H NMR, 13C NMR, natural products profiling, metabolomics, chemometrics

1. Introduction

The objective of this paper is to review the applications of 1H and 13C nuclear magnetic resonance (NMR) for the quantitative profiling of plant natural products extracts and the theoretical parameters that should be considered, if it is to become a more useful tool.

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NMR and mass spectrometry (MS) are the principal methods of metabolomics, the branch of '-omics' that deals with small molecules. The Metabolomics Society describes metabolomics as: "the comprehensive characterization of the small molecule metabolites in biological systems" [1]. NMR has a number of characteristics that meet the requirements of metabolomics: it is accurate, quantitative, comprehensive, unbiased, and is able to provide information that can be used to determine molecular structure. The review will discuss these aspects in detail.

1.1. NMR and MS

Although MS techniques, such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS), are most commonly used in metabolomics, NMR still enjoys a number of key advantages. In particular, NMR is nondestructive, unbiased, quantitative, does not require separation or derivatization, and is amenable to compounds that are difficult to analyze by GC-MS and LC-MS. For example, GC-MS often requires derivatization of compounds, such as sugars and amines. LC-MS, on the other hand, generally requires sample preparation, chromatographic separation, specific experimental and ionization conditions, instrumentation and operator skill [2]. These make it difficult to standardize MS analysis. In contrast, NMR does not require elaborate sample preparation and fractionation, is highly reproducible, and is able to provide both qualitative and quantitative information on chemically diverse compounds [3, 4]. The standardization of the NMR protocol will further improve the usefulness of NMR as a tool for the profiling of natural products extracts. Because NMR is able to detect compounds only down to 0.1% level, it is not suitable for the detection of trace components. NMR is less sensitive than MS, which can detect compounds down to parts per million (ppm) levels. Because of the distinct advantages of each method, NMR and MS are considered as complementary techniques.

NMR is a quantitative spectroscopic tool because the intensity of the peaks is directly proportional to the number of nuclei. With improvements in electronics and the use of higher magnetic field strengths, the sensitivity and resolving power of NMR has improved. However, the lack of standardized protocols has limited its quantitative application and many consider NMR mainly as a qualitative method, mainly for chemical structure determination and molecular dynamics [5].

The use of NMR as a quantitative method has been expanding, giving rise to the term "quantitative NMR" (qNMR). The pharmaceutical industry, which has stringent requirements of analysis, has been turning to the use of qNMR in early drug development to address the need for rapid, selective, and accurate analysis without requiring expensive and tedious chromatographic methods. It is also worth noting that qNMR meets the stringent regulatory standards of the pharmaceutical industry, including the International Conference on Harmonization. qNMR has been applied mainly to 1H nuclei although 19F and 31P NMR have also been used where appropriate because of their 100% isotopic abundance [6]. The main advantages of qNMR are its accuracy, reproducibility, and flexibility with respect to the nature of the analyte, the only requirement being the presence of protons and carbon, and its ability to simultaneously quantify multiple analytes, especially when validated using external calibration. Quantitative 1H NMR (qHNMR) has been shown to have an accuracy and precision of ±1%

and an uncertainty of measurement of less than 0.1%. This makes it suitable as a metrological technique for the certification of purity of organic compounds [7].

There are two general approaches to the use of NMR for profiling or metabolomics studies. In the first approach, only the spectral patterns (chemical shifts and intensities) are recorded and are used to compare and group samples. In this approach, compounds are not initially identified. Because statistical tools, such as principal components analysis (PCA) are used, this is sometimes called a chemometric approach. In the second approach, particular compounds which are known to be present in the extract are identified and quantified using a reference spectral library. This approach is referred to as quantitative or targeted metabolomics [8]. These approaches, however, are not mutually exclusive and will likely converge in the future with improved statistical tools and bigger NMR spectral databases.

Because of the large amount of data that are produced, statistical methods, known as chemometrics, are applied to reduce the number of variables. Chemometrics is a family of statistical techniques that are applied to large sets of chemical data, such as NMR chemical shift peaks, with the objective of gaining insights into the characteristics of the samples through the use of graphical representation [9]. Because chemometrics is able to process large amounts of data, it is an ideal tool for NMR which produces a lot of data (chemical shifts). This can be used to find patterns of groupings and correlations among natural product samples which can be used for quality control and standardization [10]. Since chemometrics started to be applied to NMR around the year 2000, progress has been very rapid. Chemometrics has been used to classify whole plant samples based on their NMR profiles according to species, origin, processing treatment, age, and various quality parameters [11].

2. 1H and 13C NMR as profiling methods

In a talk given during the William Draper Harkins Lecture, University of Chicago in 1991, Alexander Pines mentioned that his organic chemistry colleagues at Berkeley consider two vital instruments in a research laboratory: a balance and an NMR spectrometer. His view is not surprising as decades of improvement in both instrumentation and techniques had rendered the NMR spectrometer as a tool of choice in characterizing molecules, from the structures of natural products and synthetic organic compounds to biomolecules and organometallic complexes. NMR spectroscopy takes advantage of the interaction between nuclei that are acting as tiny magnets and an external magnetic field and this provides a powerful means of probing the chemical bonding and environment of the nucleus. These phenomena are key to the applicability of 1H and 13C NMR to natural products.

2.1. 1H NMR spectroscopy

Hydrogen is present in almost every organic molecule, and its major isotope, 1H, has an abundance of 98.985%. The 1H nucleus reports a frequency specific to its immediate vicinity in an NMR spectrum. This frequency is extremely sensitive to the electronic environment thus giving each 1H nucleus in an organic compound a type of identification number, called the

NMR chemical shift. Magnetic nuclei, such as 1H, also interact with each other. In solution or liquid-state NMR spectroscopy, these interactions, called couplings, are observed as "splitting" of lines in an NMR spectrum. The magnitude of these couplings not only depends on the number and type of bonds separating the interacting pair of 1H nuclei but also on the spatial orientation between the nuclei. Both NMR chemical shifts and coupling constants provide immense information regarding structure and environment. Hence, NMR spectroscopy has become a powerful tool for the determination of organic structure.

These NMR interactions (chemical shifts and coupling constants), although very sensitive, are quite weak such that improvements in their detection have been one of the primary goals of developments in NMR instrumentation. Such limitations are no longer severe. The use of pulses and data processing by Fourier transformation, first introduced by Ernst and Anderson [12] and the availability of high-field superconducting magnets have allowed for efficient signal averaging such that nowadays, with an 11 T magnet (500 MHz), a 1H NMR spectrum can be obtained even from very dilute solutions (micromolar concentration).

Pulse Fourier transform NMR spectroscopy, as in other spectroscopic methods, involves transitions between energy levels. However, unlike other spectroscopic methods, the transition probability in an NMR excitation is the same regardless of chemical environment. NMR spectroscopy does not need to consider oscillator strengths or extinction coefficients, which are important for infrared and UV-visible spectroscopy, respectively. The intensity of an NMR signal is determined solely by the excitation pulse, strength of the external magnetic field, and temperature. The magnetic field strength and temperature determine the Boltzmann population difference between the two energy levels while the excitation pulse dictates the extent of the transition. Since only one pulse is often used to excite all of the 1H nuclei in a sample, the extent of transitions is the same for all. Furthermore, the NMR chemical shift, which reflects the differences in resonance frequencies of inequivalent 1H nuclei, is very small: the differences are in parts per million (ppm). Hence, the Boltzmann distribution for the two spin states is essentially the same for every 1H in a molecule. Indeed, as early as 1963, the area under each peak in a 1H NMR spectrum has been shown to correspond proportionally to the number of hydrogen atoms sharing the same environment in a given compound [13]. This quantitative aspect applies not only to pure substances but also to mixtures. In fact, during the same year, a successful quantitative analysis by 1H NMR spectroscopy of a mixture of aspirin, phenacetin, and caffeine was demonstrated [14].

2.2. 13C NMR spectroscopy

13C also has a spin of ½ and is therefore likewise NMR active. However, because the 13C isotope occurs at only 1.108%, it is difficult to observe. (The major carbon isotope, 12C, is not NMR-active.) David Grant and coworkers published a series of papers on 13C NMR spectroscopy that spanned two decades [15, 16]. In the first paper of this series, inherent difficulties in observing 13C NMR spectra were addressed by proton decoupling and sample spinning. Since carbon atoms are frequently attached to hydrogen atoms in organic compounds, 13C-1H coupling is present and leads to splitting of 13C resonances. Proton decoupling removes this interaction, consolidating multiple 13C peaks into a single taller peak.

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Moreover, additional enhancement of 13C signals is observed when the 1H spin populations are perturbed, similar to the effect observed by Overhauser with electron spins [17]. Taking advantage of both the nuclear Overhauser effect (NOE) and the increased signal due to the collapse of multiple peaks, measurement of 13C NMR spectra became routine and easy to interpret. Being in the proximity of more than one pair of electrons, 13C nuclei offer a much wider range of chemical shifts than 1H (200 ppm for 13C versus 10 ppm for 1H). In addition, since the probability that a 13C nucleus is attached to another 13C nucleus is very small (about 0.0001), 13C-13C couplings are usually not observed thereby providing a much simpler 13C NMR spectrum.

Using 13C NMR spectroscopy as a powerful analytical tool can be easily appreciated by considering the three isomers of a simple hydrocarbon C_5H_{12} (see **Figure 1**). n-Pentane ($CH_3CH_2CH_2CH_2CH_3$), produces three peaks with a 1:2:2 intensity ratio, 2-methylbutane ($(CH_3)_2CHCH_2CH_3$) displays four peaks with a 1:1:2:1 intensity ratio, and neopentane ($(CH_3)_4C$) gives two peaks of 4:1 intensity ratio. For the above reasons, a qualitative and quantitative analysis that is nondestructive and requires no separation is possible with 13C NMR spectroscopy [18]. All that one needs is a library of 13C NMR spectra of all possible components, a good spectral prediction software, and an efficient algorithm that can do the search and construct a simulated spectrum that matches the observed spectrum. All of these requirements are already available today. A similar treatment has been shown to be feasible in determining the acyl profile in various vegetable oils [19] and in characterizing the various sesquiterpenes in essential oils from juniper, rosemary, cedarwood, and ginger [20].

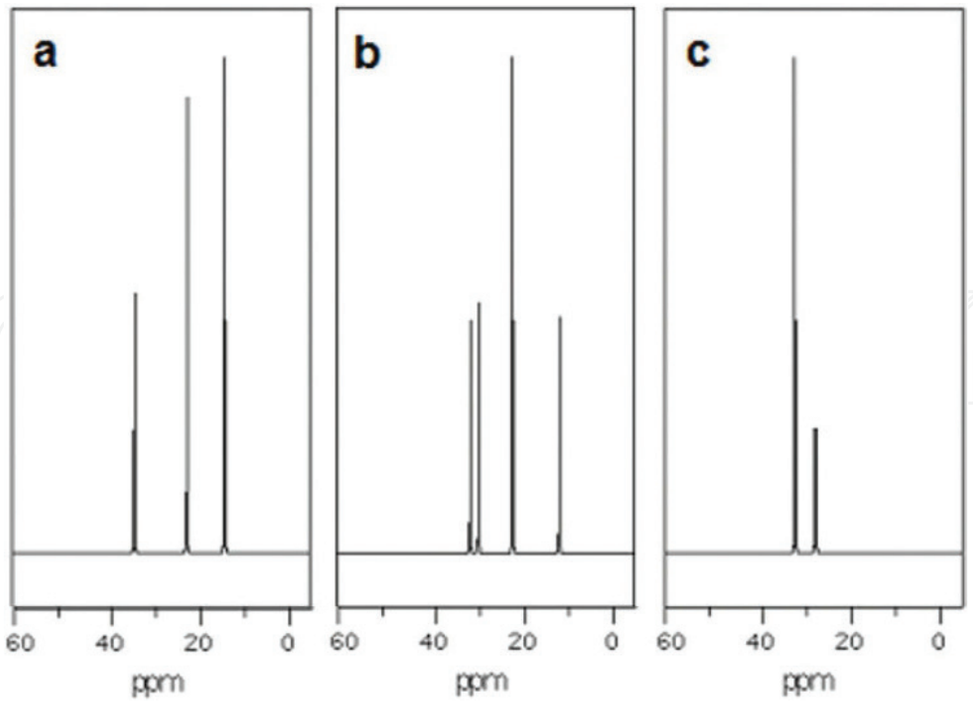

Figure 1. 13C NMR spectra of (a) n-pentane, (b) 2-methylbutane, and (c) neopentane.

The promise of a wealth of information that NMR spectroscopy offers, however, comes also with challenges. Since the frequencies observed depend on the magnetic field strength, the peaks' shapes and widths are sensitive to the homogeneity of the magnetic field throughout the sample. Experimentally, corrections to field homogeneity are done through a process called shimming, which involves adding small magnetic field gradients. Shimming used to be an art and both symmetry and narrowness of an NMR peak depended on the expertise of the NMR operator. Fortunately, with new superconducting magnets and automated shimming, 13C NMR spectra can now be made reproducible and comparable regardless of who operates the spectrometer. However, there are still numerous factors that are independent of the NMR operator which can affect the appearance of an NMR spectrum.

2.3. NMR chemical shifts and coupling constants

Since the frequencies (in hertz) observed for each NMR-active nucleus are dependent on the field strength, chemical shifts are reported in dimensionless units of parts per million (ppm), which then becomes independent of the magnetic field strength. Interactions between nuclei, on the other hand, are independent of field strength, so these are recorded in units of frequency, hertz. Since the ppm equivalent of a hertz is determined by the strength of the magnetic field, splittings will appear narrower in a high-field magnet than in a low-field magnet. When the coupling interactions are of the same magnitude as the chemical shift differences, the coupling pattern is complex [21]. A hypothetical example for 1H NMR is shown in **Figure 2**, where the coupling constant is equal to the chemical shift difference in a spectrometer operating with a 1H frequency of 100 MHz. As the strength of the magnetic field increases, chemical shift differences (in hertz) also increase, which can dramatically change the appearance of the spectrum. In this particular example, the spectrum only begins to appear simpler with a spectrometer operating at 1 GHz, in which the chemical shift difference is now 10 times bigger than the coupling constant; this is called a first-order spectrum. Thus, 1H spectra taken at different magnetic field strengths appear different. On the other hand, 13C spectra appear similar at different magnetic field strengths. This is because 13C-13C coupling is not observed due to low natural abundance, 13C-1H couplings, although present, are always several orders of magnitude lower than the frequency difference between these two nuclei, and proton-decoupled 13C NMR spectra are singlets. Therefore, although 13C presents detection challenges due to its lower frequency and low natural abundance, 13C has the advantage over 1H with regard to simplicity of NMR spectra.

Absolute frequencies for NMR transitions are seldom used since these numbers are dependent on the strength of the external magnetic field. Chemical shift differences are instead reported in ppm, which is the ratio of the absolute frequency with respect to the frequency of a reference compound, such as tetramethylsilane (TMS). Alternatively, the solvent can be utilized as internal reference. Due to the sensitivity of the NMR chemical shift, intermolecular effects are also frequently observed [22]. Since solvents are known to induce shifts, it is important that when comparing different spectra, the same solvent should be used. Since 13C has a much wider chemical shift range, the effect of solvent on chemical shift is smaller for 13C (2/200) than that of 1H (0.7/10). Furthermore, since carbon atoms, unlike hydrogen atoms, reside on the interior of the molecule, 13C is generally shielded from environmental effects, such as intermolecular interactions and

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solvent effects. This is one reason why 13C NMR chemical shifts are nearly exclusively dependent only on its covalent bonding interactions [23]. The greater susceptibility of 1H NMR chemical shifts to solvent effects makes 13C NMR spectroscopy a better alternative in profiling natural products. Solvent effects on both 1H and 13C NMR chemical shifts are expected to be dominated by van der Waals interactions with the solvent. These interactions are largely nonspecific thus an approximation that the solvent simply causes a constant offset on all resonances may be valid. Using an internal reference can therefore easily remove effects of the medium on the observed chemical shifts. Attention, however, is still required for sites that can participate in electrostatic interactions and hydrogen bonding. 13C in carbonyl groups is one example [24].

Temperature can also affect observed chemical shifts through changes in the density of the sample as well as changes in the internal motions of the molecule [25]. For a fair comparison of library and sample spectra, it is important that spectra are taken at the same temperature.

Lastly, a quantitative 13C NMR spectrum requires uniform excitation of all 13C nuclei. The wider chemical shift range and lower frequency for 13C necessitate excitation pulses with much higher power with a pulse that is less than 15 μs long so that the entire chemical shift

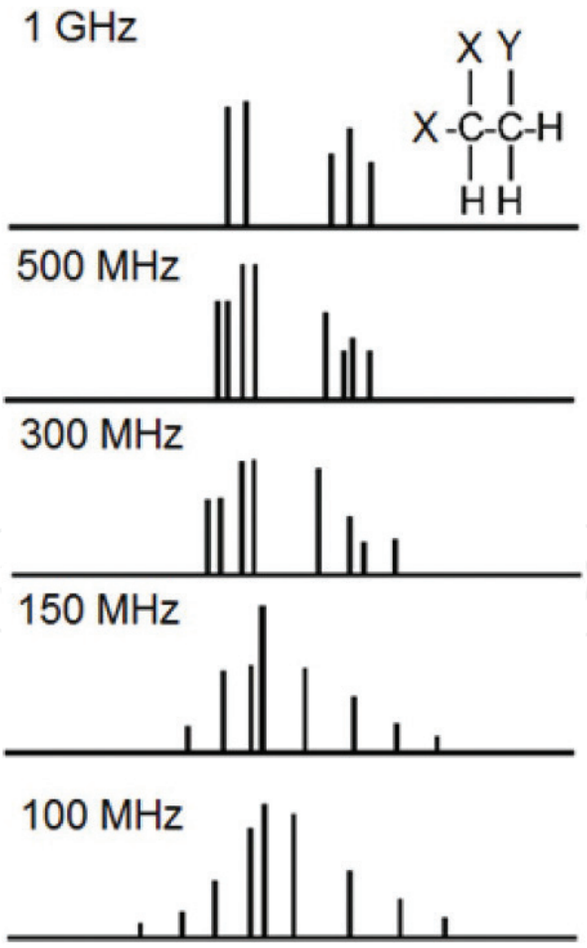

Figure 2. 1H NMR spectra of strongly coupled nuclei at various magnetic field strengths.

range is uniformly irradiated [26]. Furthermore, proton decoupling is also regularly used to collapse multiple 13C peaks, but this can lead to NOEs which enhance 13C nuclei that are directly bound to protons, making 13C NMR no longer uniform for carbon nuclei with different numbers of attached protons. The inverse-gated 13C NMR experiment can be used to overcome these problems. This involves turning the proton decoupler on only during acquisition and providing adequate time for all the 13C nuclei to relax [27]. 13C nuclei are most often relaxed by a nearby 1H nucleus. Thus, the needed relaxation time (equal to $5 \times T_1$) can be quite long for compounds that contain quaternary carbons. These quaternary 13C nuclei may require minutes to relax and this dramatically increases the time required for NMR experiments. Because running such a lengthy 13C NMR experiment is not practical, it is normal practice to run proton-decoupled 13C NMR using standard conditions and to use the resulting spectra for pattern recognition but not for quantitation.

2.4. Reproducibility of NMR spectra

The use of a library of NMR spectra in the analysis of a mixture of natural products requires reproducibility of both chemical shift and peak intensity. Since samples of natural products are often dissolved in either dimethyl sulfoxide or methanol, confining both library and sample data to these two solvents can easily ameliorate the confounding effects of the solvent on the observed chemical shifts. Modern NMR spectrometers are normally equipped with temperature control so the measurements can be made at a given temperature, which also avoids the temperature dependence of NMR chemical shifts, thus eliminating this problem. The reproducibility of peak intensities, however, requires additional considerations.

The robustness of current NMR instrumentation is evident in successful indirect detection methods during which resonances from 1H nuclei bound to 12C are separated from those attached to 13C [28]. Indirect detection is possible only if the scans or transients are highly reproducible such that these can be added to extract the desired resonances and remove completely the unwanted signals. However, this robustness only entails the reproducibility of an NMR experiment from one transient to the next. It does not address the reproducibility of NMR experiments among different laboratories. Thus, there is a need to standardize both NMR acquisition conditions and processing parameters.

The intensity of an NMR peak depends on the duration of the pulse. Equalizing the m = +½ and m = −½ spin populations requires what is called in NMR spectroscopy as a 90° pulse. Peak intensities are at a maximum with this pulse. Since all nuclei in a sample are subject to the same pulse, it is not necessary that a 90° pulse is always employed. For an NMR spectrum to be quantitative, the relative, not the absolute, peak intensities are sufficient. However, the extent of the pulse determines how much time is required for relaxation between transients. For signal averaging to be effective, one still needs to make sure that the spins have reached equilibrium before applying the next pulse so as to avoid saturation, which leads to loss of signal [29]. When a 90° pulse is employed, the time between transients must be at least five times as long as the relaxation time. The time required between pulses can be reduced by using a smaller flip angle. For example, a 30° pulse requires a delay that is three times shorter. This reduces the peak intensity for each scan but reduces the delay time required between scans enabling the acquisition of more scans for the same amount of time. Another

parameter that can affect the appearance of an NMR spectrum is acquisition time, which determines spectral resolution. What is directly acquired from an NMR experiment is a free induction decay (FID), which still needs to be processed to produce the frequency spectrum. During processing, apodization, zero-filling, and baseline and phase corrections are normally applied. All of these can significantly alter the integrated areas under the peaks of an NMR spectrum. Thus, a list of universal parameters for quantitative NMR has been established by national and international round robin tests [30] which includes temperature (300 K), pulse angle (30°), preacquisition delay (5 s), acquisition time (3.4 s), relaxation delay (7/3 of relaxation time), and line broadening (0.3 Hz). For processing, careful manual phase and baseline corrections are recommended since automated features of popular NMR processing software packages are unreliable. This validation has been performed with 1H NMR, but these can be applied to 13C. With 13C, relaxation times are appreciably longer so relaxation agents such as paramagnetic compounds have been used as in the earlier work on petroleum distillates [31].

2.5. Sensitivity and dynamic range

For the unbiased profiling of natural products extracts, one needs to consider the problems of sensitivity and dynamic range. A natural product extract typically contains major and minor components. Oftentimes, in order to detect minor components, it is necessary to employ separation techniques, such as successive fractionation and chromatography which have the effect of increasing sensitivity to minor constituents and improving dynamic range. However, this introduces bias.

Limits of detection and quantification are often given in terms of signal to noise ratios. The International Conference on Harmonization of Technical Requirements recommends a signal to noise ratio of 3 and 10 for the detection limit and quantification limit, respectively (ICH Expert Working Group, 1994). In practice, for error values less than 1%, a signal to noise ratio of 150 is recommended [30]. The signal-to-noise ratio (S/N) in NMR spectroscopy however depends not only on concentration but also on other factors [32]:

$$\frac{S}{N} \approx \frac{N \gamma_n^{\frac{5}{2}} B_0^{\frac{3}{2}} T_2 \sqrt{ns}}{T} \tag{1}$$

In this equation, N is concentration, γ_n is the magnetogyric ratio of the nucleus, B_0 is the strength of the external field, T_2 is the transverse relaxation time, ns is the number of transients, and T is temperature. Considering both magnetogyric ratio and natural abundance, one can therefore estimate that the detection limit for 13C will be orders of magnitude higher than that of 1H. Since the number of transients depends on how much time is available for data acquisition, one can improve S/N by simply taking more scans for 13C measurements. Since nuclei with longer relaxation times give sharper lines, these likewise yield higher S/N, making the detection limit dependent on the size of the molecule and the solvent. The above equation does not include factors dependent on the spectrometer's probe, receiver, and filters. In an analysis of diesel fuel, detection limits of 0.01 and 0.5 mol% are cited for 1H and 13C, respectively [33].

Another consideration is dynamic range. For 1H NMR, signals arising from the solvent, in particular water, can easily use up most of the higher bits in a spectrometer's digitizer thereby

decreasing the precision of signals coming from the natural product constituents. This can be alleviated by suppressing solvent resonances, but this introduces the problem of reproducibility between runs and remains a problem for components which have signals near the solvent.

A quantitative comparison using three magnetic field strengths—300, 400, and 500 MHz—showed that there was no difference in the sensitivity and that the standard protocol could differentiate plant samples which were spiked with 0.2 mg/mL of rutin (MW 610.5; 328 μM). This is due to the mild dependence of S/N on the field strength.

For the application of 1H NMR for pattern recognition, the use of the magnitude spectrum has been suggested [34]. The standard 1H NMR spectrum utilizes the phase-corrected real component of the Fourier transform of the free induction decay (FID), discarding the imaginary component. This yields the absorption spectrum which is useful for normal qualitative analysis due to its good peak resolution. However, this procedure sacrifices reproducibility. The use of the magnitude spectrum, which utilizes the absolute value of both the real and imaginary components of the FID improves the reproducibility of the spectra thereby improving its accuracy for pattern recognition. This method is applicable to one-dimensional 1H NMR.

Peak integrals in an NMR spectrum unfortunately are also sensitive to data processing. Apodization, zero-filling, phase and baseline corrections, and the integration itself can affect the signal-to-noise ratio of an NMR spectrum. Thus, the current limit in the sensitivity of NMR-based metabolomics is not due to magnetic field strength, but is due to the current data processing methodology which uses spectral binning (alternatively called bucketing) and PCA. The usual bin size for 1H NMR is 0.04 ppm. This divides a 10 ppm 1H spectrum into 250 bins, which effectively becomes the resolution of the method. A smaller bin size can be used if the variability in the chemical shift can be minimized. Another problem observed is the effect of different solvent (see below) to move the position of chemical shifts, which will make identification using database comparisons difficult [35].

2.6. Effect of solvent

Because plant samples contain a wide variety of compounds with corresponding differences in polarity, the solvent used for extraction and the NMR analysis is very important. The solvent system must balance the ability to perform a comprehensive extraction with solvent complexity and reproducibility. In particular, multi-component solvent systems are prone to variation, and if there is a wide difference in vapor pressures (boiling points), the solvent composition may change if care is not taken. Acetone and acetonitrile are effective solvents but their use is limited by their low boiling points. The use of methanol-D_4 in combination with deuterated water (1:1) have been reported. By using these deuterated solvents, the extracts can be measured directly after extraction without need for evaporation and reconstitution. However, use of water will introduce a strong water peak in the 1H NMR spectrum that must be irradiated. This becomes a source of variability around the water peak across different operators and instruments. To avoid shifts due to differences of pH in 1H NMR measurements a buffer, such as KH_2PO_4, is used [36].

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3. Recent applications

NMR is capable of providing simultaneous access to both qualitative (chemical structure) and quantitative information. Unfortunately, NMR has been more generally associated with multidimensional qualitative NMR used in structural analysis and qNMR has been living under this shadow. Fan (1996) pointed out that comprehensive metabolite profiling of complex food products can be done using one- and two-dimensional NMR analysis [37]. However, it is in the use of NMR combined with chemometric methods that the extraordinary potential of both the qualitative and quantitative applications have been realized [38].

In view of its ability to be used as an exhaustive molecular fingerprinting technique, 1H NMR has been found to be a suitable method for the identification, quality control, and fraud detection of essential oils, a function normally reserved for GC-MS [39]. NMR fingerprinting involves obtaining 1H or 13C spectra of whole solvent extracts under standardized conditions and ignoring, at least initially, the assignment of peaks. Multivariate statistical methods, such as PCA, are used to compare spectra from the samples to identify clusters so that inferences can be drawn about the classification of individual plant samples. The identities of metabolites responsible for differences between groups can be investigated from loadings plots generated by PCA [40]. The following section will cover applications of 1H NMR in one- and two-dimensions and 13C NMR together with the statistical tools.

3.1. Metabolomic profiling using 1H NMR

One-dimensional 1H NMR (1D HNMR) can be used in the untargeted and targeted mode. The earliest use of 1D HNMR for the profiling of complex extracts had the objective of monitoring the major components of exudates of plants, such as its root system. The relative increase or decrease of primary metabolites, such as lactate, ethanol, and certain amino acids, could be observed [41]. However, its application to natural product compounds is more challenging due to their more complex structures and lower concentrations. Because of its simplicity and speed, 1D HNMR in the untargeted mode can be used by itself or as a first-pass screening to obtain cluster and profile information using HCA and PCA [42]. The majority of HNMR studies combine 1D HNMR for PCA analysis with two-dimensional homonuclear (1H-1H) or heteronuclear (1H-13C) NMR methods for identification of natural product metabolites.

3.1.1. One-dimensional 1H NMR

This section discusses applications that make use of 1D HNMR alone. The number of such studies is limited because of the presence of overlapping signals and the need for high magnetic fields. 1D HNMR at 500 MHz was used to authenticate grapes for wine making by analyzing their skin and pulp at maturity. Spectral data were reduced by binning using 0.04 ppm bin size and normalized to generate 183 variables to describe each spectrum. Chemometric methods, in particular PCA and partial least squares (PLS), enabled the identification of compounds that contributed to differences between berries, due to the sugars (glucose, fructose,

and sucrose), organic acids (tartaric, malic, citric, and succinic acids), and amino acids (proline, arginine, gamma-aminobutyric acid, valine, alanine, leucine, and isoleucine) [43].

A set of green teas selected from a Japanese tea contest were analyzed by 1D HNMR at 750 MHz to classify tea quality with respect to that judged by tea tasters and to propose a quality prediction model. PCA metabolomics profiling revealed a separation between the high- and the low-quality green teas. The taste marker compounds contributing to the discrimination of tea quality were identified from 1D HNMR as caffeine, theanine, epigallocatechin-3-gallate, epigallocatechin, epicatechin-3-gallate, and epicatechin [44].

The use of the magnitude spectrum showed good reproducibility in the analysis of 4 diverse natural product samples (12 tea extracts, 8 liquor samples, 9 hops extracts, and 25 cannabis extracts) using 1D HNMR at 500-MHz and various statistical tools [45].

3.1.2. Two-dimensional 1H NMR

Because of problems of signal overlaps in 1D HNMR spectra, two-dimensional NMR techniques are usually used to overcome these limitations. The 2D methods include 1H *J*-resolved NMR (2D JNMR), 1H-1H correlation spectroscopy (2D COSY) and total correlation spectroscopy (2D TOCSY), 1H-13C heteronuclear single quantum coherence (2D HSQC), and 1H-13C heteronuclear multiple bond coherence (2D HMBC).

1D and 2D NMR at 600 MHz together with chemometric analysis were used to differentiate the origin, purity, and processing methods of chamomile flowers which were obtained from three different countries. The extracts were dissolved in D_2O phosphate buffer adjusted to pH 7.4. 1D NMR data were analyzed by PCA analysis to determine the groupings by pattern recognition and 2D COSY and 2D TOCSY pulse sequences were used to assign the resonances and identify constituents [46].

Several NMR-based metabolomic studies have been done on green tea (*Camellia sinensis*, L.). In one study, 191 green tea samples from different countries were analyzed using 1D HNMR and 2D NMR at 400 MHz to determine origin, quality, effects of climate and season, growth conditions, and even plucking position. The highest quality Chinese tea showed higher levels of theanine, gallic acid, caffeine, epigallocatechin gallate, and epicatechin gallate and lower levels of epigallocatechin when compared with other teas. These new markers were suggested to be useful for the authentication of tea [47]. In another study, the effects of climatic conditions (temperature, sun exposure, and precipitation) and plucking positions on the tea plant were investigated using 1D HNMR profiling combined with multivariate pattern recognition methods. Assignment of NMR signals was done using 2D TOCSY, 2D HMBC, and 2D HSQC. The variations in the composition of specific tea compounds were obtained [48, 49]. The sensitivity of the NMR method at 400 MHz was demonstrated in a study on three different varieties of green tea. 1D HNMR, 2D JNMR, and 2D COSY spectra were run and identification of constituents was done using MestRenova version 11.0.0. The following compounds were identified: theanine, alanine, threonine, succinic acid, aspartic acid, lactic acid, caffeine, and derivatives of epigallocatechin [50].

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The same strategy was used for chemotaxonomic classification of 11 South American *Ilex* species. Data from 1D HNMR at 600 MHz were combined with PCA, partial least square-discriminant analysis (PLS-DA), and hierarchical cluster analysis (HCA) to reveal four distinct groups. 1H signal overlaps were addressed using 2D JNMR and 2D HSQC. The combined use of 1D- and 2D-NMR and chemometric analysis enabled unambiguous chemotaxonomic discrimination of the *Ilex* species and varieties [51].

1D HNMR fingerprinting followed by 2D TOCSY and 2D HSQC methods were used to distinguish four Asian and four Korean ginseng products, as well as their commercial products. In this way, the major metabolites—glutamine, arginine, sucrose, malate, and myo-inositol—were identified as chemical markers for quality assurance [52]. In a study on Indian ginseng, *Withania somnifera* (L.) Dun., 1D HNMR profiling was performed on the leaves, stems, and roots to obtain a profile of this plant. PCA and hierarchical cluster analysis (HCA) were performed to group samples which were collected from six different regions of India. 2D JNMR, 2D COSY, 2D HSQC, and 2D HMBC, were then used to identify specific metabolites. The ratio of two withanolides was found to be a key discriminating feature of *W. somnifera* leaf samples from different regions [53].

This NMR-based metabolomic strategy was applied to analyze seven spices used in traditional Mediterranean cuisine and to detect metabolic changes over different seasons. Both primary and secondary metabolites were identified and quantified. The major secondary metabolites identified were polyphenols, including flavonoids (apigenin, quercetin, and kaempferol derivatives) and phenylpropanoid derivatives (chlorogenic and rosmarinic acid). This study was performed using a 300 MHz NMR instrument [54].

The application of NMR-based metabolomics method in plant breeding has been reported. Using a 500 MHz instrument, the NMR-based metabolomics was applied to the identification of sugar beet (*Beta vulgaris* L.) genotypes which were susceptible to the *Cercospora* leaf diseases of sugar beet plants worldwide. This approach was able to successfully profile foliar metabolites without inoculation tests which would have required a significant amount of time and effort. In this study, field-grown leaves which had different levels of resistance were collected from 12 sugar beet genotypes at 4 growth time points. The aqueous extracts were studied using 1D HNMR, 2D COSY, 2D TOCSY, and 2D HSQC. Thirty metabolites were identified and annotated using the SpinAssign program from the PRIMe web service. PCA of the NMR data revealed clear differences among the growth stages, in terms of the content of sugar, glycine betaine, and choline [55].

3.2. Metabolomic profiling using 13C NMR

Because of its lower sensitivity and longer acquisition time, 13C NMR is used less often than 1H NMR. However, 13C NMR spectra are simpler, have less severe problems with overlapping peaks, are more comparable across different magnetic field strengths, and are less susceptible to solvent effects. In addition, the singlet nature of 13C NMR signals makes it easier to determine the identity of individual compounds in a mixture.

13C NMR methodology was used to study the triacylglycerols of the oil extracted from the seeds of *Moringa oleifera*, Lam. It was able to simultaneously detect specific unsaturated acyl chains according to their positions on the glycerol backbone through carboxylic, olefinic, and methylene carbons [56]. However, at this time, its use was not specifically identified as a profiling method. Later, 13C NMR was applied to the fingerprinting of lipids for the authentication of marine and fish oils. In this work, 13C NMR was combined with chemometrics and database information and compared with relevant authentic samples [57]. 13C NMR in combination with multivariate data analysis have been used in the analysis of lipids from various fishes. In one application, this method was used to discriminate between farmed and wild Atlantic salmon (*Salmo salar*, L.), between samples from different geographical origins [58], and to detect mislabeling and adulteration [59].

13C NMR was used in a dereplication strategy for the identification of natural product compounds directly from plant extracts. The whole extract was first separated into fractions of simpler composition, which were then analyzed by 13C NMR. The 13C spectra of all the fractions were aligned and subjected to pattern recognition by HCA. This yielded correlations among 13C signals within each fraction which were visualized as chemical shift clusters, which were assigned to specific compounds in a 13C database. This strategy was applied to the analysis of 5 g of a bark extract from the African tree *Anogeissus leiocarpus* which resulted in the unambiguous identification of seven major compounds [60].

Chemical profiling and standardization of the methanol extract from the leaves of *Vitex negundo*, L. were carried out using 13C NMR followed by chemometric analysis. Because PCA analysis gave an explained variability of only 41% for PC1 and PC2, an alternative method, called k-means clustering, was employed. This was able to successfully differentiate samples that were deliberately allowed to degrade. The multivariate control chart, which is analogous to the analytical control chart method, classified samples whose quality exceeded the upper control limit (UCL). The plant samples were also analyzed by quantitative thin layer chromatography (qTLC) using agnuside as marker compound. Comparison of the univariate qTLC results with the multivariate control chart showed poor correspondence: some samples that gave high agnuside values exceeded the UCL while others that had low agnuside values were below the UCL. This means that a univariate analysis of a plant sample using a marker compound does not adequately represent the overall plant profile [61].

13C NMR is being used more often for dereplication of natural product extracts without fractionation. This approach is being enhanced by availability of 13C NMR databases and predictive software which list compounds that are most likely to be present in the extract. These results have been found to be comparable to those obtained using LC-MS and GC-MS, which require fractionation and sample preparation [62].

The combined use of high-resolution 1H and 13C NMR analysis has the potential to reveal more details that are not available using only one technique. This combined approach was employed to detect and quantify a wide range of triacylglycerols and their component fatty acids in marine cod liver oil supplements. The combination of 1H and 13C spectra permitted the detailed analysis of components, including sn-1 monoacylglycerols, sn-1,2- and sn-1,3-diacylglycerol adducts, and other minor components, such as trans-fatty acids, free

glycerol and cholesterol, and added vitamins A and E and synthetic compounds, such as ethyl docosahexaenoate or eicosopentaenoate. The identity of each compound was confirmed using 2D COSY [63].

4. Future prospects

The use of 1H and 13C NMR for the profiling of natural products extracts is a rapidly growing branch of metabolomics. It will further accelerate with the increasing use of NMR in quality management, the growth of NMR databases, the development of portable and benchtop NMR instrumentation, and advances in the use of statistical analysis. Despite its considerable potential, the routine application of this method is limited by the lack of expertise to run sophisticated NMR experiments and the lack of computational tools for NMR spectral deconvolution, in particular of 1H spectra [64].

4.1. NMR in quality management

NMR has been used for the monitoring and quality management of foods, beverages, cosmetics, and pharmaceuticals. The same can be done for the profiling of natural products. In order to ensure reproducibility and reliability and to minimize experimental artifacts, the entire process—from sample collection and storage, extraction, NMR measurement and data processing, and statistical analysis—should be optimized and standardized [65, 66]. The NMR solvent is of particular importance because of its influence on the chemical shift positions of protons in phenolic compounds [67] and other solvent effects. This problem is more severe for 1H as compared with 13C NMR.

It has been claimed that periodic calibration can deliver accuracy as high as 99.9% and precision as good as 0.59%, and if calibration is performed with each study, the accuracy and precision can reach 100 and 0.35%, respectively [68]. The various experimental parameters are listed below:

- Sample preparation: homogeneity of sample, extraction solvent, extraction method, and NMR solvent.
- Acquisition parameters: temperature, acquisition time, pulse angle, number of data points, time delay (relaxation time), and electronic amplification.
- NMR data processing: smoothing, phase correction, baseline correction, and signal integration.

4.2. NMR databases in natural products

The usefulness of NMR databases is premised on the reproducibility of the NMR experiment—starting with sample preparation, NMR acquisition, and processing—across different laboratories. It is important to avoid conditions that alter the position of chemical shifts, which will make identification using database comparisons difficult. Open-access and user-contributed 1H and 13C NMR spectral databases have a high potential as a useful tool for natural

products researchers provided that sample preparation, instrumentation, and acquisition parameters are standardized. For sample preparation, only selected NMR solvents should be used. Magnetic field strength is more critical for 1H than 13C NMR. Acquisition and processing parameters should be standardized. As of 2015, 1829 1H NMR and 1383 13C NMR spectra have been available in open-access chemical databases. To further promote participation by researchers, the entire process, from data acquisition, conversion of vendor-specific raw data files, and data deposition have to be simplified and standardized [69].

4.3. Portable and benchtop NMR instrumentation

NMR is usually considered to be an expensive analytical technique which is used for research purposes only. However, for NMR to become more useful for the natural products industry where many of the companies are small to medium in size, more affordable instrumentation is needed. There have been numerous announcements regarding the development of portable and benchtop NMR instruments with full spectrum 1H and 13C NMR capability using microcoils with small portable magnets of up to 2 T (approximately 85 MHz 1H) [70]. Although these are limited in capability and reproducibility compared with a full laboratory NMR instrument, they can be used in the field or production site where cryogenic liquids and stable power are not available. Because there is a demand for such instrumentation for other purposes, such as forensic investigation, detection of explosives, and medical diagnostics, their development is certain to accelerate. This will expand the use of NMR for the profiling of natural products.

4.4. Advances in the use of statistical analysis

Although the use of NMR in the analysis of biological extracts was already being done in the 1980s, it was the application of statistical methods that enabled researchers to make use the large amount of NMR data to find patterns and correlations. The first step usually involves the simplification of large NMR data sets to find relationships, groupings, or dependencies using PCA. Second, the groups can be classified with or without a training set which has known information or characteristics against which other sample sets are compared. Linear discriminant analysis (LDA) and soft independent modeling of class analogy (SIMCA) are used for this purpose. For quantitative analysis of constituents, in particular for strongly overlapping peaks, principal component regression (PCR) or PLS regression can be used [71]. Although these statistical techniques are now commonly used, new ones continue to be developed and reported.

One of the most exciting areas of development is the use of statistical methods to correlate NMR signals with biological activity. Since the NMR signals can be related to specific compounds, this in effect allows one to correlate specific compounds with biological activity. Although it has to be emphasized that correlation is not proof of biological activity, this strategy nevertheless allows one to shortcut the process of discovering bioactive compounds in a complex natural product mixture. This also allows one to detect multiple active compounds.

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5. Conclusions

1H and 13C NMR are rapidly expanding it role from its traditional use mainly as a qualitative spectroscopic technique for the determination of chemical structure to a quantitative tool for the metabolomic study of natural product extracts, whether for quality control of phytomedicine products, analysis of the metabolome for plant profiling, identification of constituents as plant markers, or for plant biotechnology. A major enabler for the use of NMR for metabolomic studies is the application of various statistical techniques which are able to find patterns and correlations in the large NMR data sets. The continued expansion of the use of NMR for the metabolomic profiling of natural product extracts will likely depend on the further development of statistical methods and the availability of NMR databases for both 1H and 13C nuclei. It is likely that more compounds will be identified as techniques are improved.

An NMR spectrum is quantitative. An understanding of the physical principles of NMR provides the theoretical basis for its use as a quantitative tool. NMR spectroscopy does not require a standard for each component since the intensity of each signal is directly proportional to the number of nuclei being observed regardless of environment. NMR spectroscopy also offers detailed information regarding molecular structure. Using NMR spectroscopy as a tool in the profiling of natural product extracts therefore not only provides accurate and precise composition, but also structural evidence for each of the components. Since the NMR signal dependence on various factors is already well known, resonance positions and intensities are highly reproducible. These are important characteristics which give NMR a unique advantage over other analytical methods.

Abbreviations

1D HNMR	One-dimensional 1H NMR
2D COSY	Two-dimensional 1H-1H correlation spectroscopy
2D JNMR	Two-dimensional 1H J-resolved spectroscopy
2D HMBC	Two-dimensional 1H-13C heteronuclear multibond coherence
2D HSQC	Two-dimensional, 1H-13C single quantum coherence
2D TOCSY	Two-dimensional total correlation spectroscopy
GC-MS	Gas chromatography-mass spectrometry
HCA	Hierarchical cluster analysis
LC-MS	Liquid chromatography-mass spectrometry
MS	Mass spectrometry
NMR	Nuclear magnetic resonance

PCA	Principal components analysis
PLS	Partial least squares
PLS-DA	Partial least squares-discriminant analysis
qNMR	Quantitative NMR
qHNMR	Quantitative proton (1H) NMR
qTLC	Quantitative thin layer chromatography

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