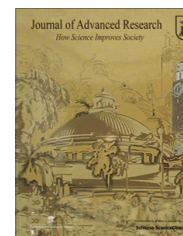




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REVIEW

Two dimensional NMR spectroscopic approaches for exploring plant metabolome: A review



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ABSTRACT

Today, most investigations of the plant metabolome tend to be based on either nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS), with or without hyphenation with chromatography. Although less sensitive than MS, NMR provides a powerful complementary technique for the identification and quantification of metabolites in plant extracts. NMR spectroscopy, well appreciated by phytochemists as a particularly information-rich method, showed recent paradigm shift for the improving of metabolome(s) structural and functional characterization and for advancing the understanding of many biological processes. Furthermore, two dimensional NMR (2D NMR) experiments and the use of chemometric data analysis of NMR spectra have proven highly effective at identifying novel and known metabolites that correlate with changes in genotype or phenotype. In this review, we provide an overview of the development of NMR in the field of metabolomics with special focus on 2D NMR spectroscopic techniques and their applications in phytomedicines quality control analysis and drug discovery from natural sources, raising more attention at its potential to reduce the gap between the pace of natural products research and modern drug discovery demand.

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Introduction

NMR and plant secondary metabolites

The comparison of metabolite composition of biological systems (known as metabolomics) is now a mature field that has been increasingly applied to investigate a range of problems in plant and crop science [1,2]. A wide variety of analytical techniques have been employed in metabolomics, and each has its own advantages and drawbacks. The analytical techniques used to collect metabolomic data can be, broadly, split into two categories—those which separate the components of the crude solvent extracts prior to detection and those which directly analyze crude, unfractionated mixtures (detection is usually made by mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR)). Direct analysis by NMR is ideally suited to high-throughput metabolomics applications and has the advantage of detecting a wide range of metabolites in an inherently quantitative and unbiased manner. Compared to MS, NMR spectroscopy has a larger dynamic range for detection and is less biased since results of MS-based analyses greatly depend on choice of ionization conditions and the specific instrumentation used [3]. Albeit, NMR is less sensitive than other spectroscopic methods and can suffer from problems with signal overlap. The use of multidimensional NMR spectra can help in that regard by overcoming many of the problems encountered with one dimensional NMR and providing more detailed structural information [4].

One additional strength of NMR lies in its utility for the identification of unknown or unexpected compounds in a complex mixture. In initial plant metabolomics experiments, NMR use was mostly focused on the metabolic profiling of mixtures, and not yet being accepted as an appropriate tool for the definitive identification of novel or unexpected metabolites in a mixture. Only recently and driven in part by increases in NMR spectrometers sensitivity, extensive 2D NMR experiments and advances in data processing, NMR spectroscopic methods have begun to play a larger role in the identification of previously unknown small-molecules in complex mixtures. Such application is of great value in situations where some compounds are inaccessible, for example compounds that are prone to chemical decomposition and thus cannot be isolated [5]. Furthermore, it has become

apparent that NMR spectroscopy-based metabolome analyses can be highly effective in identifying novel and known metabolites that correlate with changes in genotype or phenotype [6]. The present review provides the first overview on the advances made in the field of developing 2D NMR technologies to meet with applications in the field of plant metabolomics.

NMR spectroscopy: a historical perspective

Since its development in the middle of the past century, NMR has been an indispensable tool in the discovery of natural products largely replacing all traditional chemical degradation methods that were used for structural elucidation. Compared to other spectroscopic tools, NMR offers detailed structure information that can be surpassed only by X-ray crystallography while NMR remains much less demanding in terms of purity and sample preparation [7]. As a result, NMR spectrometers, despite their relative high cost, have become a core part in research laboratories and one of the main tools in natural product discovery. This wide spread use of NMR has led to fast improvement of both NMR hardware as well as supporting software. Advances in NMR spectroscopy have been remarkably accelerated during the past few decades driven, at least in part, by the demand to use NMR in the analysis of mixtures especially with the establishment of metabolomics as a new scientific discipline with myriad useful applications in both human and plant biology [7–10].

Since NMR spectroscopy measures the properties of nuclei and not molecules, response to NMR is uniform across all chemical classes and under certain experimental conditions, NMR enables absolute quantitation of metabolites through the integration of their corresponding ^1H NMR signals [11,12]. This method remains as the only acceptable approach to determine the concentration of plant chemical constituents in a crude extract without the need to use reference standard for each single constituent [13,14]. In addition to its value as a tool for metabolites quantification, NMR is a nondestructive technique from which the sample can be completely recovered for further analysis. The nondestructive nature of NMR as well as the minimal samples preparation for NMR acquisition poses this technique as being less prone to artifacts than other techniques commonly used in metabolomics. Moreover, with the introduction of autosamplers, ^1H NMR can be used as a high throughput technique since the acquisition time per sample is very short [15]. Nevertheless, the relative low sensitivity of NMR and the complexity of its generated spectra remain as the two main deterrents for wider application of NMR-based metabolomics.

Recent developments in NMR

The first NMR spectrometers were equipped with either electromagnets or permanent magnets and operated at a resonance frequency not higher than 60 MHz for proton [8]. Since then, sensitivity and resolution of NMR spectrometers have been greatly improved by the use of superconducting magnets that can operate at field resonance of up to 1 GHz [7,16]. Another major improvement in NMR

sensitivity was the reduction of thermal noise through the use of cryogenically cooled probes that operate at very low temperatures achieving 3.5 folds enhancement in sensitivity [7,17]. Another approach to increase NMR sensitivity is the miniaturizing of sample probe head, so that it is now possible to analyze samples in 10 μ l solution using the microvolume probe compared to 600 μ l that are typically required to analyze samples using the traditional 5 mm probes [18,19]. Lately, analysis of intact tissues became possible through the implementation of high resolution solid state magic angle spinning HR-MAS NMR that has been successively used in analysis of food samples [20–22].

Apart from the most commonly used one dimensional NMR experiments (1D NMR), multiple 2D NMR experiments are available and are routinely used in the structure elucidation of natural products including ^1H – ^1H homotropic experiments measuring either scalar or spatial dipolar coupling between similar nuclei ^1H – ^1H or ^{13}C – ^{13}C as well as heteronuclear experiments measuring scalar coupling between dissimilar nuclei such as ^1H – ^{13}C or ^1H – ^{15}N and many more that are actively being added to a growing list of NMR experiments [23–25]. Generally, since ^1H – ^1H homonuclear experiments measure couplings between nuclei of high natural abundance, these experiments are more sensitive and require shorter acquisition times compared to other experiments that measure the resonance of nuclei of low natural abundance (1% in case of ^{13}C isotope). In fact, homonuclear C–C experiments require exceptionally long acquisition times and thus are not used in metabolomics studies. Recently, it has become possible to acquire 2D ^1H – ^{13}C NMR spectra in fraction of a second by using a single scan 2D NMR technique developed by Frdyman et al. also known as Ultrafast NMR [26,27]. Such development led to the successful incorporation of NMR spectroscopy in new fields such as real time monitoring of protein folding and chemical reactions [28,29] and proved to be especially useful in coupling NMR with HPLC as will be discussed [30].

Application of ^1H NMR in plant metabolomics

The use of NMR spectroscopy in both human and plant metabolomics was simultaneously launched in 1991 and even before the term metabolomics was coined [31,32]. Pattern recognition methods were used to detect metabolites in human urine and cerebrospinal fluid around the same time when Schripsema and Verpoorte reported using ^1H NMR for investigating the effect of variable experimental conditions on the metabolites produced in different plant cell cultures [31–34]. Nevertheless, it was not until the next decade when the use of NMR in plant metabolomics was adopted by many research groups in several applications of plant science including monitoring growth stage of plant, measuring stress response of plant to different stimuli, chemotaxonomic classification, determination of geographical origin of plant sample, establishing substantial equivalence of genetically modified plants and more recently the quality control of nutraceuticals [35–38].

As previously mentioned, the recent developments in NMR have significantly improved its sensitivity to the extent that ^1H NMR spectrum of less than 1 μ g of a small molecule

can be derived in a reasonable experimental time [39]. Still considered of much lower sensitivity than mass spectrometry (MS), NMR has the great advantage of being a universal detector that can identify all molecules with the same efficiency [40]. Also, when many structural isomers are being analyzed in a single extract, NMR plays an indispensable role in the discriminations of isomer type especially when reference standard materials are not available. Generally, about 30–150 metabolites can be simultaneously identified in the ^1H NMR spectrum of a given plant extract. The chemical shift and the integration values of the peaks observed in that spectrum are used to create a multivariate data set that can be subsequently analyzed using suitable multivariate data analyses such as hierarchical cluster analysis (HCA), orthogonal projections to latent structures (O-PLS) or principal component analysis (PCA). These chemometric methods perform the function of grouping most similar samples and providing some level of segregation between the least similar ones [13,41,42]. Since most signals in the ^1H NMR are related to primary metabolites, ^1H NMR is most useful when primary metabolites are targeted such as in the case of food analysis where NMR is rapidly replacing LC/MS as the technique of choice. Moreover, identification of NMR peaks of most primary metabolites can be easily achieved through several online databases that will be discussed later. Such databases, although incomprehensive for plant metabolites, are useful in the assignment of primary metabolites as they all allow search queries using both proton and carbon chemical shifts [43,44].

Application of multidimensional NMR in plant metabolomics

In most cases, proton NMR spectra of crude plant extracts are crowded with overlapping peaks making accurate peaks assignment difficult and in most cases unattainable. Two main strategies are employed to untangle NMR overlapping signals. The first strategy aims at simplifying the ^1H NMR spectrum through creating a projection of ^1H broadband decoupled spectrum like in J resolved experiment (JRES NMR) [45] or through the selective suppression of signals from certain compounds using relaxation or diffusion filters as in the case of diffusion ordered-NMR spectroscopy (DOSY) [46]. While both JRES and DOSY are two dimensional techniques, the most useful aspects in both experiments are the ability to extract simplified proton spectrum with less peak overlapping rather than collecting the information displayed in the second dimensions such as coupling constant and translational diffusion coefficient, respectively.

The second strategy to handle ^1H NMR peaks overlapping is to spread the crowded peaks in a second dimension using the different 2D NMR experiments especially ^1H – ^1H homonuclear experiments which have relative short acquisition time, however, some peaks overlapping might still exist as the second dimension spans only a region of 10 ppm. On the other hand, ^1H – ^{13}C heteronuclear NMR experiments have much longer acquisition time but allow resolving the overlapping signals in a second dimension that spans a region of more than 200 ppm units which could successfully eliminate peaks overlap [47]. As heteronuclear NMR became more popular in NMR metabolomics, there is growing interest in the application of 2D NMR for the characterization

Table 1 Two-dimensional NMR applications for analysis of plant extracts.

Purpose of the study	Technique(s)	Metabolites reported	Ref.
Classification of <i>Ilex</i> species	¹ H, JRES NMR	Caffeoyl esters	[48]
Classification of grapes cultivars	¹ H, JRES NMR	Phenolic acids	[51]
Classification of licorice species	¹ H, 2D ROESY	Triterpenoid saponins	[66]
Classification of hops cultivars	2D HMBC	Bitter acids	[82]
Classification of <i>Hypericum</i> species	¹ H, HSQC, HMBC	Phloroglucinol derivatives	[68]
Classification of <i>Salix</i> species in relation to activity assay	LC-SPE-NMR DPPH	Salicin, phenolic compounds	[102]
Classification of <i>Verbascum</i> species	¹ H, JRES NMR	Iridoid glycosides	[52]
Effect of jasmonates treatment on <i>B. rapa</i>	¹ H, JRES NMR	Phenyl propanoids	[49]
Effect of jasmonates treatment on <i>B. rapa</i>	¹ H, HSQC, HMBC	Phenyl propanoids	[67]
Effect of pesticides on lettuce	HR-MAS	Primary metabolites	[20]
Effect of agronomical practice on olive	HR-MAS	Fatty acids, phenolic compounds	[21]
Geographical discrimination of garlic	HR-MAS	Amino acids, organosulfur compounds	[84]
Geographical discrimination of pistachio	¹ H, 2D TOCSY	Amino acids, organic acids	[64]
Different extraction methods for cannabis	¹ H, 1D DOSY	Cannabinoids	[55]
Screening for new metabolites in <i>T. cylindrosporum</i> culture	¹ H, 2D COSY	Indole alkaloid	[60]
Monitoring fruit ripening in tomato	HR-MAS	Primary metabolites amino acids, fatty acids	[85]
Quality control of apple and grape juice	¹ H, 2D DOSY	Sugars, amino acids, phenolic acids	[57]
Quality control of ginseng commercial preparations	¹ H, JRES NMR	Phenolic compounds, amino acids	[50]
Quality control of <i>Ginkgo biloba</i> commercial preparations	LC-PDA-MS-SPE-NMR	Flavonoids, terpene trilactone	[100]
Quality control of herbal preparations for erectile dysfunction	3D COSY-DOSY	Different adulterants (amino acids, synthetic compounds)	[58]

of unknown metabolites in crude extracts without any chromatographic step or extensive fractionation. Details on the value of these techniques and their application in plant metabolomics along with a brief discussion on the use of computational approaches for NMR based compound identification from mixtures principally are the focus of the next sections (see Table 1). Provided below is a series of two-dimensional NMR experiments of common use in plant metabolomic projects with its advantages and current limitations.

2D J-Resolved NMR

To date, JRES NMR remains the most popular among 2D experiments to be used for peak assignment due to its simplicity and short acquisition time. In 2D JRES NMR experiment, the proton spectrum is presented in one dimension and the coupling constant (J value) of each signal is represented in a second dimension [45]. Through JRES experiment, a simplified projection of the proton spectrum in which all multiplet peaks appear as singlet can be extracted resulting in great reduction in the complexity of the spectrum and solving most of the peak overlapping problems. Based on the information displayed in 2D JRES NMR, connection between neighboring protons can be established. In addition, information about the J value of each signal can be used to distinguish between some isomers such as α and β anomers of sugars and glycosides or *cis* and *trans* isomers of olefinic compounds.

In the field of plant metabolomics, the use of JRES NMR was adopted by many researchers including its application to differentiate between caffeoyl esters in 11 *Ilex* species as described by Kim et al. [48]. In another study, metabolic response of *Brassica rapa* leaves following treatment with jasmonates was studied by Liang et al. who reported the

utility of 2D JRES NMR in the differentiation between *cis* and *trans* sinapyl acid esters as well as between α and β glycosides as well [49]. 2D JRES NMR was also used to aid in the classification of different commercial preparations of ginseng and to help in the assignment of different phenolic compounds found in different Greek grape varieties [50,51]. Despite the utility of JRES NMR in simplifying heavily congested ¹H NMR spectra, structural information provided by JRES spectra is rather quite limited. Moreover, values of coupling constants obtained from the spectrum cannot be used to search for metabolites in any of the available metabolomic databases. Hence, the effective use of JRES spectra in metabolite assignments requires prior knowledge of the chemical composition of the studied extract and preferably reference spectra of the main chemical constituents, a strategy that was successfully adopted in the study of *Verbascum* species where comparison with spectra of authentic standards of some primary metabolites such as sucrose, fructose, α and β glucose in addition to four iridoid glycosides: aucubin, ajugol, harpagide and harpagoside were used to detect and quantify these metabolites in different *Verbascum* species crude extracts using JRES NMR [52]. A complete review on JRES NMR including recommendation for the best acquisition parameters is available elsewhere [45].

Application of 2D and 3D DOSY

Diffusion ordered spectroscopy or DOSY depends on the difference in the transitional diffusion coefficient between molecules of different molecular sizes [53]. For any plant extract, constituents of different molecular weights ranging from high molecular weight polymers to simple sugars or amino acids coexist in different concentrations. DOSY experiments seem to be exceptionally suited for analysis of plant

extract as the proton spectrum of each compound (or actually compounds of the same molecular weight) can be recorded separately achieving what can be described as “NMR chromatography” [46]. Diffusion experiments can be performed in one dimension as in 1D DOSY or more commonly in two dimensions as in 2D DOSY. In 1D DOSY, two or more ^1H NMR experiments are acquired each targeting a set of compounds of specific diffusion coefficient (certain molecular size, *i.e.*, mono-, oligo- and polysaccharides). This strategy is often utilized in the metabolomics study of biological fluids such as human plasma to separate high molecular weight proteins from organic metabolites [54]. To a less extent, such approach was applied in plant metabolomics including examining extraction type effect on cannabis resin chemical composition [55]. Authors did not only compare between different types of water extracts and tinctures but they provided semi quantitation of the amount of Δ^9 -tetrahydrocannabinol (Δ^9 -THC) and Δ^9 -THC acid based on the diffusion edited spectra [55]. An interesting application of 1D DOSY is its use for the suppression of NMR signals from low molecular weight solvents such as water or ethanol without affecting other NMR signals that belong to the phytochemical constituents in plant extract in the same ppm range [56]. This simple concept was used for the direct NMR analysis of commercial herbal tinctures of *Echinacea purpurea*, *Hypericum perforatum*, *Ginkgo biloba* and *Valeriana officinalis* [56].

In two dimensional diffusion experiment (2D DOSY), the proton spectrum is plotted in one dimension and the diffusion coefficient related to each NMR signal is displayed on the second dimension. In theory, constituents of different molecular weights should be separated each by their diffusion coefficient, but in reality full separation of all plant extract chemical constituents is quite difficult to be achieved. Instead, it is always possible to obtain a good degree of separation between compounds that differ substantially in their molecular weights. Despite the growing number of reports that used 2D DOSY experiments in the fields of polymer chemistry, inorganic chemistry and human metabolomics, this experiment has very limited applications in the field of plant metabolomics. Several reports have been published as preliminary studies to show the utility of 2D DOSY in the assignment of NMR signals observed in certain fruit juices, wine and beer. Gil et al. reported that 2D DOSY can aid in the assignment of the anomeric protons of mono-, di- or oligosaccharides in apple and grape juices [57]. With the use of 2D DOSY experiments, 14 and 11 metabolites could be identified and completely assigned in apple and grape juice, respectively including sugars, phenolic acids and amino acids.

One of the major problems with 2D DOSY experiment is that the diffusion coefficient for an overlapped signal is displayed as the average between the value of the two coefficients associated with the two different metabolites that participate to the intensity of this signal which add ambiguity to the acquired data [46]. Thus, new pulse sequences for DOSY were developed to overcome the problem of peaks overlap by spreading the proton signals in a second proton dimension as in 3D COSY-DOSY and 3D TOCSY-DOSY, or spreading the overlapping proton peaks in a second carbon dimension as in 3D DOSY-HMQC. These previous experiments have been introduced only few years ago, so their suitability for plant metabolomics studies is not clear

yet. Balaysac et al. reported using 3D DOSY-COSY in a “half day experiment” to study the composition of 17 herbal supplements used for erectile dysfunction and collected from different countries. They used 3D DOSY along with LC/MS to identify several synthetic phosphodiesterase inhibitors in addition to some amino acids, vitamins and sugars in these herbal preparations [58]. Wider use of these experiments cannot be foreseen without significant improvement in NMR instrumentation that allows for acquisition in a much shorter time.

Homonuclear ^1H - ^1H NMR

2D ^1H - ^1H correlation spectroscopy (COSY) is one of the experiments often performed to assign ^1H NMR peaks to their corresponding metabolites when signal overlapping in the ^1H NMR is severe. The main advantage of COSY experiment is its relatively short acquisition time since it records only coupling between proton nuclei with inherited high natural abundance. In fact, with the great improvement in the NMR sensitivity and miniaturization of sample volumes, high quality COSY spectra can be acquired for 10 μg of sample [59]. In an interesting application, Schroeder et al. reported the use of double quantum filtered COSY (DQF-COSY) to screen for new metabolites produced by the fungus *Tolyposcladium cylindrosporium* when it was grown in different growth media [60]. The acquired COSY spectra of all different cultures in addition to the control media were overlaid and subsequent differential analysis of these spectra was performed which identified few cross-peaks that were discriminative for certain growth conditions. Careful analysis of these cross-peaks followed by more extensive NMR studies suggested the presence of two novel indole alkaloids in the unfractionated extract [60]. The structure of the two alkaloids was then confirmed through their successful isolation from the growth media in which their specific COSY cross-peaks were most notable. The authors acknowledged that 2D COSY was well suited for the purpose of screening for new metabolites but it may not be suitable for other metabolic studies where full assignment of signals or metabolites quantification is required.

Another powerful ^1H - ^1H correlation spectroscopy is the Total Correlation Spectroscopy or TOCSY. In this type of experiment, all protons participating in the same spin system are detected and displayed either in 1D fashion or most commonly in the form of 2D TOCSY. Generally, 2D TOCSY is used in the structural elucidation of carbohydrates and peptides since all protons belonging to the same sugar residue or to a single amino acid will appear correlated. Accordingly, utility of 2D TOCSY has been demonstrated in the virtual separation of 5 inositol derivatives in a mixture using a 2 h experiment as reported by Johnson et al. [61]. It should be noted that, unlike DOSY, correlations observed in TOCSY spectra indicate a single spin system not a single molecule, so that a specific molecule may be expressed by one or more spin systems and the connections of these systems together cannot be achieved through a TOCSY experiment. For such reason, TOCSY application in plant metabolomics is quite limited. As an example of the application of TOCSY includes the work of Consonni et al. applying selective 1D TOCSY to prove that certain NMR peaks observed in aged samples of

balsamic vinegar belong to the same spin system and hence to a single molecule [62]. However, authors did not follow up with more NMR studies and this compound was not fully identified. 2D TOCSY experiments were also used to determine that 5-hydroxy furfural can be used as a quality marker for instant coffee through a metabolomic study involving 98 samples of instant coffee [63]. In a recent report, extensive analysis of TOCSY spectra was used to provide complete assignments for ^1H NMR signals of amino acids, sugars and nucleotides found in pistachio seeds. These metabolites were further quantified in the different pistachio samples using the integration values of their corresponding ^1H NMR peaks. Statistical analysis of the data generated was then used to classify pistachio samples based on their geographical origin [64].

Other than NMR experiments designed to detect scalar coupling between protons, some 2D experiments can selectively identify protons in close proximity through recording their through-space dipolar coupling. These experiments are based on the phenomenon known as nuclear Overhauser effect or NOE and they include 1D NOE, 2D NOESY and 2D ROESY. Using 2D NOESY to detect the spatial arrangement of amino acids protons in a certain protein is a common practice by structural biologists to determine the 3D structure of small to medium size proteins. In phytochemistry, ROESY and NOESY experiments are used to determine the stereochemistry of compounds with one or more chiral centers and are especially useful in the structural determination of polycyclic compounds such as steroids, triterpenes and saponins [65]. In cases where a herbal extract enriched in triterpenes or saponins is under metabolomics investigation, 2D ROESY or NOESY ought to be considered to assign the stereochemistry of the individual metabolites. For example, 2D ROESY was used to determine the isomer-type of the major aglycon in the saponin rich extract of licorice root (glycyrrhizin). Analysis of 4 different *Glycyrrhiza* species extracts showed that the 18β form of glycyrrhizin is the only naturally occurring form and that the presence of the 18α -glycyrrhizin suggests possible degradation or partial chemical decomposition of the extract [66].

Heteronuclear 2D NMR

The use of 2D ^1H - ^{13}C heteronuclear NMR in metabolomics may be necessary to identify metabolites present in a herbal extract especially when the ^1H NMR is heavily congested. Using 2D correlation spectroscopy such as Heteronuclear Single Quantum Coherence HSQC and Heteronuclear Multiple Bond Correlations HMBC, full structural information about all major metabolites becomes available and so structural isomers could be differentiated. In fact, when both HMBC and HSQC are used for the assignment of NMR signals to the corresponding metabolites, identification of new chemical entities becomes more possible provided that these metabolites are present in a concentration within the detection limits of the NMR spectrometer. For example, a novel phenyl propanoid, 5-hydroxyferuloyl malate, was identified in the crude extract of methyl jasmonate treated *B. rapa* leaves [67]. In a similar fashion, a metabolomics investigation of several *Hypericum* species led to the identification of a novel phloroglucinol derivative designated as hyperpoly-

phyllirin in the flowers of *H. polyphyllum* [68]. Other reports of new natural products identified in complex mixtures without prior fractionation using 2D NMR include the identification of several sulfated nucleosides in the venom of certain spiders [69] and a novel monoterpene (Parectadial) from the walking stick insect *Parectatosoma mocquerysi* [70]. The continuous implementation of 2D heteronuclear NMR for the analysis of plant crude extracts will result in a gradual shift in the common routine adopted for the discovery of novel natural products to replace the classic method of fractionation and chromatographic isolation or at least assist in speeding up the process. 2D NMR spectral analysis of crude extract can also be employed as an efficient tool for de-replication in natural products drug discovery that should save time and effort spent in re-isolation of compounds that have already been identified [71].

2D NMR for metabolites quantification

For the past two decades, ^1H NMR proved to be an excellent tool for absolute quantification of compounds in a mixture due to the direct correlation between the molar concentration of any compound and the area under the curve (integration) of each of its corresponding signals, as long as all of the compounds are soluble in the NMR solvent and sufficiently stable under the conditions of analysis. However, in the case of 2D spectra, this direct linear correlation no longer exists as other factors such as "resonance specific signal attenuation" contribute to the intensity of the cross-peaks and their corresponding integration volumes. Multiple approaches have been suggested by different research groups to overcome these factors and enable extraction of the quantitative NMR data from 2D spectra [72–75]. Lewis et al. described a short HSQC experiment (12 min) that can be used to determine the molar concentration of a certain metabolite in a crude extract based on its calibration curve acquired under the same experimental conditions [76]. Using a different approach, Hu et al. eliminated the need to use calibration curves for each metabolite to be quantified by acquiring a series of HSQC spectra at different repetition time and extrapolating the data to construct a time zero spectrum in which signal intensity is directly proportional to metabolite concentration [77]. Giraudeau and coworkers have further investigated the possibilities and limitations of quantitative homo and heteronuclear 2D NMR in the fast and ultrafast modes [73,75,78,79]. New phase modulated pulse sequence has also been proposed such as Q-OSCA-HSQC to enable the use of ^1H - ^{13}C HSQC spectra for the quantitative determination of metabolites in plant extracts [80]. Nevertheless, it has yet to be seen if any of these proposed methods will withstand repetitive application by researchers and can be widely implemented.

To date, few investigators explored the possibility of creating multivariate data sets based on 2D NMR experiments. Lolli et al. were the first to explore the possibility of using the integration volumes of 2D ^1H - ^{13}C HMBC cross-peaks to create a multivariate data set. For using 2D HMBC spectra for the quality control of honey, they have manually integrated a subset of HMBC cross-peaks and used the integration volumes obtained to create a multivariate data set that was statistically analyzed using principal component

analysis PCA to segregate honey samples based on their botanical origin [81]. More recently, Farag et al. described a more comprehensive analysis of 2D HMBC spectra using an R-script that divides the 2D spectrum into thousands of squares of fixed size to generate what is called “a pixel-map” [82]. The integration volume associated with each square is automatically calculated to create a large data set that was subsequently analyzed using PCA to classify different hop cultivars [82]. In both of the previously mentioned examples, the authors acknowledged the limitation of 2D spectra for quantitative analysis and that the integration data were rather used for comparative analysis and not for absolute metabolites quantification. It is feasible, with the growing trend to transform the traditional ^1H - ^{13}C experiments to generate quantifiable spectra, that 2D NMR is supposed to be employed more frequently in plant metabolomics not only for the purpose of identifying metabolites but rather as the main metabolomic platform used when ^1H NMR fails to provide unambiguous interpretation of data.

High resolution magic angle spinning HR-MAS

The ultimate goals of a metabolomic study were to (1) reflect the real repertoire of metabolites within an extract, cell, tissue or living system, (2) eliminate sample preparation steps to reduce artifacts, (3) exclude human errors, (4) enhance reproducibility and increase the possibility of high throughput analysis. The development of high resolution magic angle spinning HR-MAS can achieve the most direct analysis of a plant material as no sample manipulation is required but rather a single step of mixing the sample to be analyzed with minimal volume of solvent. Acquisition of ^1H NMR spectrum by HR-MAS requires only a high field NMR spectrometer with a special HR-MAS probe head that allows the sample to be spun with a high speed (more than 2 KHz) at the magic angle 54.7° [83]. Also, special pulse sequence such as Carr, Purcell, Meiboom and Gil pulse sequence known as CPMG has to be used to reduce band broadening.

The technique is still undervalued by researchers in the field but has started to receive considerable attention for different applications in food chemistry. It was successfully applied for the traceability of Italian garlic collected from different regions. In this report, HR-MAS followed by partial least squares projections to latent structures discrimination analysis (PLS-DA) led to the development of a statistical model that was successfully used to determine samples geographical origin [84]. In a similar perspective, HR-MAS followed by PCA analysis was used to discriminate among olive varieties that were grown either as conventional or organic crop [21]. Similarly, Pereira et al. described significant changes in the metabolome of lettuce leaves due to treatment with mancozeb pesticide including alteration in the concentration of Krebs' cycle intermediates and phospholipids [20]. In the aforementioned examples, proton HR-MAS spectra were used to identify different classes of metabolites including amino acids, organic acids, fatty acids, organo-sulfur compounds, carbohydrates and phospholipids. Given its successful application in plant and food chemistry [20,21,85], HR-MAS is expected to be soon in the quality control of herbal medicines where the

direct analysis of powdered plant material can be envisioned with no need to use extraction procedures that can limit results reproducibility.

Computational tools for analysis of 2D NMR spectra

In order to achieve the maximum utility from the plethora of information generated by 2D NMR experiments, it is necessary to develop the computational tools that can facilitate data interpretation and extraction of structural information from such experiments. Computational tools should be of utmost value in two main domains: (1) creation of 2D maps that are amenable to chemometric analysis, and (2) deconvolution of the NMR spectra to aid in peak assignments and metabolites identification. In the first area, OPLS analysis of data generated by different types of 2D NMR was proposed by Hedenström et al. who demonstrated the utility of such approach in OPLS analysis of ^1H - ^{13}C HSQC spectra acquired for different plant pectins [86]. Recently, a different approach of reducing the 2D NMR spectra to pixel maps was used to generate data sets that can be subjected to chemometric analysis as previously mentioned [77].

The more challenging task, however, remains in the development of efficient computational tools that can be used in 2D NMR spectrum deconvolution. DemixC is a computational platform that was developed for deconvoluting ^1H - ^1H TOCSY spectra into several ^1H NMR spectra representing different spin systems that can be identified in the mixture [87]. The first applications of DemixC were shown in a relatively simple mixtures of 4–7 components [88,89], thus its utility in plant metabolomics studies has yet to be thoroughly examined. Recently in an ambitious study, DemixC platform was further used in the deconvolution of ^1H - ^{13}C HSQC-TOCSY spectra acquired for human prostate cancer cell line extracts [88,90]. By using available metabolomics databases, glutathione, glycerol and some amino acids were identified as the major metabolites in the spectra which were acquired using cryogenic 5 mm probe in 70 h experiment time [88]. An alternative deconvolution platform was proposed by Nicholson and colleagues given the term statistical correlation spectroscopy or STOCSY [91,92] in which signals belonging to one molecule appear correlated regardless of their spatial proximity or contribution in different spin systems. This approach makes use of the quantitative response of NMR across all signals, meaning that signals which do belong to the same molecule would increase or decrease in intensity by the same value when compared among different samples. To date, all applications of STOCSY are limited to human metabolomics studies and none has been examined for plant or food type metabolomic projects. By applying the same concept, 2D NMR spectra can be deconvoluted provided that they have been acquired by using a pulse sequence as well as parameters that assure the quantitative nature of the acquired spectra. Two platforms for such strategies have been proposed by Chylla et al. and Chikayama et al. [93,94].

In certain applications where comparative metabolomics are to be performed, *i.e.*, functional genomics where the metabolic product of a certain gene can be discovered by comparing NMR spectra of the wild type and deletion mutant, simple differential analysis (DANS) can be easily performed. This

approach uses a simple algorithm that overlay different 1D or 2D NMR spectra to highlight peaks that are discriminatory among this set of spectra. DANS has been successfully applied in screening for new secondary metabolites from fungus culture and was also used to elucidate the polyene antibiotic “bacillaene” structure from *Bacillus subtilis* [6]. DANS analysis of 2D spectra has a great potential as a metabolomics tool that can be directed for natural products drug discovery or for depicting bioactive natural products biosynthetic pathways *in planta*. Its simplicity and applicability to all types of 2D experiments promises for more future applications especially in the field of natural product discovery [95].

Hyphenated NMR techniques

As mentioned before, the major problems in NMR based metabolomics are its inability to detect low abundant metabolites and the complexity of the spectra produced due to peak overlapping hindering peak assignment. One strategy to aid in peak assignments and subsequent metabolite identification is to combine the benefits of NMR as an excellent structural determination tool with the superior separation features of high pressure liquid chromatography (HPLC). The idea of NMR coupled to LC started in the 1980s for the purpose of de-replication and chemical screening for novel natural products [96,97]. However, the early applications of LC-NMR, either in the continuous flow or stopped flow mode, revealed many problems associated with the technique such as its low sensitivity, inefficient solvent suppression and long experimental time. Later development in LC-NMR to overcome these problems included the introduction of LC-NMR-MS which allows the simultaneous identification of each eluted compounds via both mass spectrometry and NMR spectroscopy [98]. A significant reduction in the acquisition time of LC-NMR has been achieved after the introduction of “Ultrafast NMR” which allows acquisition of 2D NMR spectra of the eluted compounds in seconds thus achieving “real time separation” of the components of mixture [30]. Another important development in hyphenated NMR techniques is the introduction of solid phase extraction (SPE) at the interface between LC and NMR in the system known as LC-SPE-NMR [97]. The online coupling of SPE to the LC instrument prior to NMR acquisition allows for capturing eluted component on a solid adsorbent. After evaporation of the LC mobile phase, the adsorbed component can be then eluted in a minimal volume of deuterated solvents hence eliminating the need for solvent suppression and enhancing NMR sensitivity. A greater increase in NMR sensitivity to the nanogram range level was achieved through the use of capillary liquid chromatography coupled to NMR with miniaturized probe head in the technique known as capLC-NMR [15,99].

Hyphenated NMR techniques have been integrated as a versatile platform that can be extended to include multiple hyphenated approaches that became useful in many applications. HPLC-PDA-MS-SPE-NMR was used for the assessment of 16 *G. biloba* preparations [100]. Following the initial investigation of the ginkgo preparations by ^1H NMR, the multihyphenated approach was used for the identification and unequivocal assignment of 8 flavonol glycosides. More recently, Zhang et al. described the coupling of

HPLC-PDA-SPE-NMR to online high resolution radical scavenging assay such as DPPH or ABTS assay allowing for the simultaneous characterization of both the chemical and pharmacological fingerprint of a given plant extract [101]. Alternatively, HPLC-PDA-SPE-tube transfer NMR (HPLC-PDA-SPE-*tt*NMR) was used with offline high resolution ABTS assay for the targeted quantitative analysis and metabolomic classification of six *Salix* species [102]. In that report by Agnolet et al. were able to identify a total of 16 metabolites using the HPLC-PDA-SPE-*tt*NMR system including salicin, cinnamic acid esters and benzoic acid derivatives. The advantage of this technique over the totally integrated online system developed by Zhang et al. is that it does not require dedicated instrument that may be available in few research centers while providing the same data acquired through the former approach. An alternative new strategy for the fast structural elucidation of metabolites in small volume plant extracts involves automated MS-guided LC-MS-SPE-NMR in which NMR spectra of plant metabolites, automatically trapped and purified from LC-MS traces, were successfully obtained, leading to the structural elucidation of the metabolites. The MS-based trapping enabled a direct link between the mass signals and NMR peaks derived from the selected LC-MS peaks [103], thereby decreasing the time needed for elucidation of the metabolite structures.

NMR databases

Despite the fact that NMR application in plant and human metabolomics has started in the same year, plant metabolomics studies are still lagging behind if compared with NMR-based analytical studies of the human metabolome. In fact, most of the new developments in NMR were first applied in human metabolomics and some of these new advances have yet to be introduced for plant metabolomics, mostly attributed to the greater structural diversity displayed in plant extracts. One important aspect that keeps plant metabolomics lagging is the lack of comprehensive NMR spectroscopy database dedicated to plant metabolites. After the completion of the human genome project, researchers in human metabolomics were fast to adapt an approach similar to that used for the human genome projects. Many data depositories were initiated for human metabolites including the Human Metabolome DataBase which was developed as part of the human metabolome project launched in 2005 [104]. This database is dedicated to human metabolites and in its latest version, HMDB contained more than 41,000 entries for human metabolites that can be searched by either metabolite name, proton and carbon chemical shifts and also using x, y co-ordinates of ^1H - ^1H and ^1H - ^{13}C spectra [105]. Other metabolomics databases include Madison Metabolomics Consortium Database (MMCD) that contain more than 20,000 entries [106], the RIKEN platform for both MS and NMR metabolomics (PRIME) [107] and the Swedish NMR metabolome database at Linkoping (MDL) [108]. The presence of such comprehensive and easily searchable databases has encouraged researchers in human metabolomics to make the most benefit from data generated by using NMR. Consequently, novel experiments, algorithms and data servers were developed to aid in the identification of metabolites detected in biological fluids. COLMAR web server developed in 2008 was designed to search chemical shift query against different

databases [70]. The availability of such databases can be the first step toward automatic assignment of NMR spectra. Of the few attempts targeting automatic assignment of ^1H NMR spectra of metabolites include a web server application, called MetaboHunter [109], which implements three efficient methods to search for metabolites in manually curated data from two reference libraries HMDB and MMCD, such software has yet to be developed for metabolite assignments in 2D NMR spectra.

In the meantime, interpretation of NMR data generated from plant extracts requires tedious work and prior knowledge of the possible chemical composition of the plant extract under investigation. Assignment of ^1H NMR peaks, as discussed above, can hardly be achieved through database search since most NMR databases serve for mostly synthetic compounds such as the freely available SDBS database of AIST of Japan, spectral similarity search webpage that allows only ^{13}C -search query and NMR shift Database (NMR shift DB). Using software for NMR prediction is not always very accurate, thus assignment of ^1H NMR peaks typically requires thorough examination of multiple 2D NMR spectra acquired for the plant extract under investigation and if possible reference standards. It was only recently, when a new NMR spectroscopy database for plant metabolites was introduced under the name MetIDB which encompass spectral data of plant phenolic compounds. More than 5500 plant metabolites are deposited in the MetIDB with more than 21,000 spectra that were acquired in different solvents to account for variation in the chemical shift values with the change of the NMR solvent [110]. Similar databases should be added in the near future to meet the demands needed for NMR based approaches in plant metabolomics studies.

Concluding remarks

NMR spectroscopy is indeed a powerful analytical tool that has not been fully utilized or even tailored by researchers in the field of plant metabolomics. Despite the availability of many pulse sequences and different experimental approaches, only few experiments are being routinely used probably due to the lack of expertise to run the relatively more sophisticated NMR experiments. Most recent advances in plant metabolomics have been developed by researcher in the field of human metabolomics. Techniques that can achieve “in tube separation” like different types of DOSY and relaxation edited experiments have been utilized to study different biological fluids such as plasma, cerebrospinal fluid, amniotic fluid and bile [111–113]. However, only few applications have been reported for the study of plant extracts despite the fact that plant polysaccharides are good candidates for these types of experiments given the high medicinal value of these polymers. Perhaps, the ongoing development of simple new pulse sequences that depend on the use of relaxation or diffusion filters such as TOSY and TOSY shall encourage plant science researchers to incorporate these experiments in their NMR-metabolomics protocols [46].

Another undervalued NMR approach is the HR-MAS technique which has been almost ignored for its use in the quality control of herbal medicines. In HR-MAS both low and high molecular substances, water and fat soluble molecules can be simultaneously detected. The minimal sample

manipulation required prior to NMR acquisition, allows for the detection of highly unstable metabolites that can be degraded or chemically modified by the process of extraction or even due to direct light exposure. The recent increasing reports of HR-MAS use in food chemistry may be the beginning of a new trend to be used for other natural herbal drugs.

Problems with the low sensitivity of NMR compared to other spectroscopic techniques have been widely acknowledged and for that reason, most of the advances made in NMR spectroscopy during the past few decades were focused on increasing NMR sensitivity. However, a more serious challenge still exists which is the lack of a comprehensive NMR database that could aid in the identification of plant metabolites in crude extracts. To accomplish such a goal, a collective effort from many research groups around the world should be orchestrated to produce a freely available database that can be accessed by all scientists working in the field. Mihaleva et al. have initiated the effort to develop a comprehensive NMR metabolomics database by introducing their own MetIDB database for flavonoids. Similar efforts by other groups are certainly still needed with different teams focusing on one class of plant metabolites *i.e.*, terpenes, alkaloids etc. in order to compile the most accurate and useful data resource.

The second significant challenge for NMR based metabolomics is the lack of a suitable computing tool that can aid in the complex NMR spectral deconvolution. Several attempts were made toward developing algorithms that can help perform spectral deconvolution but none have yet proved to be successful enough to be adapted by researchers in the field. More tools will be developed especially as 2D NMR spectra of mixtures are now acquired alongside ^1H NMR and the information provided by 2D NMR can be of great aid in the deconvolution process. It cannot be envisioned, however, how such computing tools could be useful without the availability of comprehensive NMR spectral databases. It remains to be seen if such development will be the breakthrough needed for the wider application of NMR-based metabolomics. In general and for current authors opinion, the most future challenges for NMR metabolomics lies in the developments in 2D NMR spectroscopy technology that provide improvement in signal detection and quantification and also the facility to use shared databases.

Conflict of interest

All authors declare no conflict of interests.

Compliance with Ethics Requirements

Authors declare that this study does not include work on patients or animals and does not need the approval by the appropriate Ethics Committee or IRB.

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