Review
doi.org/10.1002/ansa.202100021
Chemistry Europe
European Chemical Societies Publishing

Received: 29 March 2021
Revised: 9 July 2021
Accepted: 7 September 2021

A conversation between hyphenated spectroscopic techniques and phytometabolites from medicinal plants

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Funding information
CSIR, Grant/Award Number: MLP0159

Abstract

Medicinal plant metabolomics has emerged as a goldmine for the natural product chemists. It provides a pool of bioactive phytoconstituents leading to accelerated novel discoveries and the elucidation of a variety of biosynthetic pathways. Further, it also acts as an innovative tool for herbal medicine's scientific validation and quality assurance. This review highlights different strategies and analytical techniques employed in the practice of metabolomics. Further, it also discusses several other applications and advantages of metabolomics in the area of natural product chemistry. Additional examples of integrating metabolomics with multivariate data analysis techniques for some Indian medicinal plants are also reviewed. Recent technical advances in mass spectrometry-based hyphenated techniques, nuclear magnetic resonance-based techniques, and comprehensive hyphenated technologies for phytometabolite profiling studies have also been reviewed. Mass Spectral Imaging (MSI) has been presented as a highly promising method for high precision in situ spatiotemporal monitoring of phytometabolites. We conclude by introducing GNPS (Global Natural Products Social Molecular Networking) as an emerging platform to make social networks of related molecules, to explore data and to annotate more metabolites, and expand the networks to novel "predictive" metabolites that can be validated.

KEYWORDS
herbal medicine, hyphenated techniques, multivariate data analysis, phytometabolite profiling, plant metabolomics

1 | INTRODUCTION

The last few decades have witnessed a continuous demand and growth in the technologies required to profile medicinal plants and biological systems. Metabolomics has emerged as a science, representing analytical technology's potential in symbiosis with a biological system. The utilization of fully developed analytical techniques in association with biological systems has emerged as goldmines for the natural product chemist as it identifies a pool of bioactive phytoconstituents. This latest advancement has brought a revolution in natural product chemistry as it requires minimum time and labor compared to the tedium of conventional isolation methods.

Within the main discipline of omics science that includes genomics, transcriptomics, and proteomics, metabolomics deals with the simultaneous characterization and quantification of the biological metabolites originating from microbes, plants, humans, and animal sources,

Abbreviations: CE-MS, capillary electrophoresis – mass spectrometry; GC-MS, gas chromatography- mass spectrometry; HPLC-MS, high performance liquid chromatography-mass spectrometry; LC-MS, liquid chromatography–mass spectrometry; MS, mass spectrometry; NMR, nuclear magnetic resonance; UPLC-Tandem MS, ultra performance liquid chromatography-tandem mass spectrometry

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and that further helps to uncover the aspects related to their structural and functional knowledge.[1] Indeed, the linkages between the macromolecular Omics components viz. genomics, transcriptomics, and proteomics, and the small molecular weight metabolomics are intimate and vital for the living state. This intimacy is evident from the fact that while macromolecular proteins in the form of enzymes catalyze the synthesis of small molecular weight metabolites, it is the latter that regulate gene expression as well as activities of different enzymes. Although the concept of metabolomics originated during the 1960s and 1970s, it has grabbed greater attention only recently. The evolution of chromatographic and spectrometric techniques in the late 1960s became the catalyst for the initiation of metabolite fingerprinting.[2] In 1971, Horning and Horning successfully applied the GC-MS technique to investigate metabolites in urine samples.[3,4] Pauling and Robinson had also developed the quantitative method for metabolite investigation of biofluids using combined gas chromatographic and mass spectrometric techniques.[5,6] By the end of the 20th century, the development in analytical techniques, viz. NMR, MS, and multivariate statistical analysis helped to establish metabolomics as a universal technique for the study of natural products. Although metabolomics is a boon for systems biology research, it has also played a crucial role in various disciplines such as drug discovery and development, food science, toxicology, molecular and cell biology, and agriculture and environmental science.[5,7] The utilization of omics sciences in phytomedicine research has helped in the development of evidence-supported phytotherapeutics along with greater quality-based acceptance due to proper toxicological, pharmacological, and chemical standardization.[8] This review presents the latest advancements in the analytical platforms and experimental methodologies related to phytometabolites profiling of medicinal plants.

Since ancient times, medicinal plants have been the fundamental components of almost all systems of medicine (Ayurveda, Siddha, Unani, Traditional African and Chinese Medicine, etc.) prevalent worldwide. By virtue of harboring a diverse class of specialized secondary metabolites,[9] medicinal plants have been the primary sources of raw material for developing novel drugs and therapeutics. Globally, most of the prescribed therapeutics agents, such as anticancer, antidiabetic, anti-rheumatism, and so on, contain nature or nature-derived active moieties.[10] However, due to lack of scientific information and validation guidelines related to the phytochemical composition, traditional medicines, despite their countless ethnopharmacological[11] uses, have always been considered as somewhat dubious alternative medicine systems. Unlike the high degree of standardization in the pills of modern medicine, the major limitation in medicinal plants research and development is the polypharmacies that all plants are on the one hand and the lack of advanced experimental techniques for isolation, identification, and characterization of novel phytometabolites on the other. The conventional approach for phytometabolite isolation deals with fractionation or partitioning of plant extracts using chromatographic separations, which is often time, - laborintensive and low yielding. Moreover, the conventional approach does not involve dereplication of natural products where rapid identification of previously known metabolites present in a mixture is done based on comparison of chromatographic and spectroscopic data of compounds with molecular characteristics of standard compound libraries to prevent re-isolation of known natural compounds.[12]

Addressing the lacunae of the traditional approach, metabolomics has recently emerged as the omics technology for the comprehensive and quantitative estimation of metabolites either for a targeted group of compounds or global analysis, for the wide range of applications such as the development of phytomedicines, disease diagnosis, development of disease models, drug discovery and development, biomarker discovery, and study of human cancer.[8,12–17] It deals with the investigation of molecules using high throughput technologies that enable the comprehensive investigation of metabolite cellular state considering the ambient environment and crucial factors such as gene regulation and expression, dynamic kinetic activity, alteration in metabolic reactions, enzyme regulation, and expression.[18] Metabolomics, originated only after the three main omics components viz. genomics, transcriptomics, and proteomics, had established itself as the key component of the systems biology approach.[19] Further, it has also been accepted as a critical platform for the metabolite profiling of medicinal plants, and drug and biomarker discovery and development. Over time, phytometabolite profiling studies are being considered as the most authentic approach mainly due to reproducibility as well as scientific validation of the metabolomics results.[20,21]

Plants synthesize multiple structurally diverse classes of specialized biochemical compounds such as alkaloids, flavonoids, peptides, polyphenols, terpenes, glycosides, coumarins, chalcones, tannins, and so on, all of which possess great ethnopharmacological uses. There are several success stories of drug discovery and development from medicinal plants such as paclitaxel (Taxol) from *Taxus brevifolia*, aspirin from *Salix* species (Willow tree), podophylotoxin (teniposides, etoposides) from *Podophyllum hexandrum*, atropine from *Atropa belladonna*, camptothecins (topotecan, irinotecan) from *Camptotheca acuminate*, quinine from *Cinchona officinalis*, and artemisinin from *Artemisia annua* that provide consistent motivation for the isolation of new drug-like molecules from them.[22–24] Traditionally, most of the research in natural product chemistry is focused on the principle of bioactivity-guided fractionation of plant extracts, which has successfully provided numerous active molecules. However, deficits in the exploration of maximum information in terms of minor secondary metabolites and high rediscovery rate make this approach ineffective.[25]

The application of metabolomics to natural product chemistry has revolutionized the entire field. In coupling with the latest chemometric tools,[7] metabolomics has helped to identify all targeted and untargeted phytometabolites using modern analytical platforms. In summary, a metabolomics-based approach for phytochemical investigation is of paramount usage since it helps the researcher to achieve a bird's eye view of the entire set of metabolites present in a particular sample. Advances in metabolomics data acquisition and data handling computational tools have also made it a significant breakthrough technique in the phytochemical fingerprinting and profiling studies for botanical's quality assurance. Nowadays, the researcher's focus is shifting from a targeted approach to the untargeted collective pool of molecules with increasing acceptance of handling big data scientifically

doi.org/10.1002/ansa.202100021

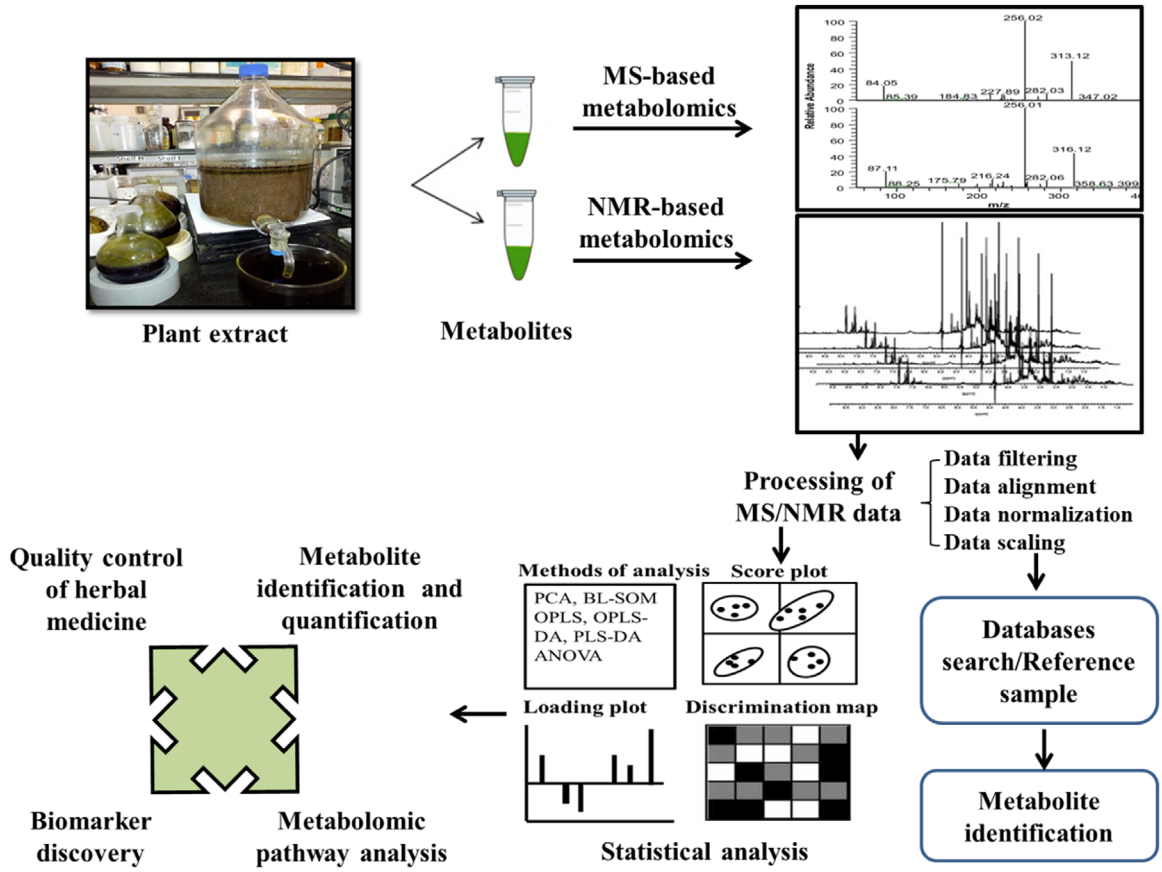

FIGURE 1 Schematic workflow and potential applications of metabolomics

to attain maximum output. Metabolomics can help in the prediction of plant response toward different physiological factors even before any visible phenotypic change in the plant[26,27] In addition to prediction of plant response, metabolomics has also been explored in the development of personalized drug treatment[28] because it can also predict the individual response or interindividual variations to a drug treatment based on the patient's genetically encoded metabolic profile.

Rapidly developing technologies with regard to metabolomics have completely altered the perspective toward medicinal plants and the traditional system of medicine prevalent worldwide as they have helped to either scientifically validate or to quickly identify potent constituents present in them. Although metabolomics has achieved exponential growth in the past few decades, it also poses a great challenge of handling a large and diverse phytochemical space. In addition to the biochemical complexity, there also exists great diversity in the architecture of metabolites, concentration, stability, and polarity that makes the identification of metabolites extremely difficult through a single analysis. Therefore, sometimes the term metabolomics is used interchangeably with metabolic profiling (which is focused on a specific group of metabolites), as practically only a fraction of metabolite analysis is achieved compared to the whole metabolome.[29] It is also said that the comprehensive analysis of all metabolites is not possible yet in a real sense as identification of the unknown metabolites continues to be difficult and remains a major bottleneck in the metabolomic workflow.

As shown in the schematic diagram of metabolomics workflow (Figure 1) metabolomics employs two analytical processes, viz. analytical chemistry and computational work for the comprehensive analysis of a sample of interest. Analytical chemistry focuses on complete, high-throughput, and precise metabolite investigation of plant extract fraction(s) and accurate detection using different hyphenated analytical platforms such as LC-MS, UPLC-Tandem MS, CE-MS for nonvolatile components, and GC–MS for volatile metabolites for simultaneous separation and detection/identification of metabolites. The 1D/2D solution state and solid-state NMR spectroscopy are also some of the most common techniques of which LC-NMR is widely used to detect, identify, and quantitate metabolites. Computational work deals with processing, analyzing, and reducing huge analytical data by employing statistical tools such as multivariate statistical analysis techniques. It is a crucial step as it helps to assign identity or chemical structures to compounds of interest. Reduction of data is done by employing different components of multivariate analysis such as principal component analysis (PCA), discrimination map analysis, batch learning–self organizing map (BL-SOM), and discriminant analysis (DA) (includes partial least square [PLS-DA] and orthogonal projections to latent structure [OPLS – DA]). The multivariate analysis helps in generating discrimination maps and loading or score plots, which ultimately produce signals corresponding to the chromatographic and spectroscopic analysis in the form of separation data, chemical shift, or *m/z* values for NMR

and mass analysis, respectively.[30] These metabolomic tools can help to screen/identify both the major and the minor bioactive fractions by simultaneous bioactivity screening. This can be achieved using chemometrics strategies by identifying signals in the metabolomic dataset (chromatographic and NMR/MS plots) and developing a correlation with the bioactivity.

Hence, metabolomics is a multidisciplinary technology that helps to gather meaningful information from a set of metabolites pool to the complete metabolite profile of a biological system by hyphenating different techniques including chromatography, spectroscopy, and statistical analysis in the backdrop of diverse biological screens.

2 | DIFFERENT STRATEGIES IN METABOLOMICS

Metabolomic studies can be performed by following both targeted and untargeted strategies for the analysis of phytometabolites.

2.1 | Targeted metabolomics

In this approach, a particular set of metabolites is simultaneously analyzed for both qualitative and quantitative parameters. This approach mainly works on the predeveloped hypothesis based on prior knowledge of compounds of interest and their biological responses. The prime focus of targeted analysis is the absolute quantification of compounds of interest.[31] Qualitative and quantitative estimations of targeted specialized phytometabolites from Indian medicinal plants such as *Trillium govanianum*,[32] *Cissampelos pareira*,[33] *Zanthoxylum* species,[34] *Rumex* species,[35] *Narcissus tazetta*,[36] and so on have been successfully accomplished using hyphenated chromatographic and spectroscopic techniques. Although several analytical techniques are used for the characterization of selective metabolites, targeted MS has emerged as the most frequently used analytical platform. In targeted MS, multiple reaction monitoring (MRM, also called selective reaction monitoring) is mostly preferred using a tandem mass spectrometer such as triple quadrupole mass spectrometer (TQMS). MRM is a targeted MS approach for the simultaneous quantification of numerous small molecular weight metabolites in a complex mixture. MRM is usually performed using a triple quadrupole tandem mass spectrometer (MS/MS or MS^n). Both precursor ions and characteristics product ions are monitored in every sample. MRM-MS has come up as a great tool particularly in the field of proteomics and in biomarker discovery for the quantification of less abundant molecules in a sample.[37,38] It is a highly sensitive and specific spectroscopic technique as it can be utilized to quantify specific metabolites in a pool of different compounds leading to the analysis of hundreds of metabolites simultaneously due to the excellent efficiency of the TQMS system.[39,40] Although this quantitative analytical approach has several advantages but it has generally been used to quantify only pre-known metabolites or targeted class of compounds. However, most recently, it is heartening to see that MRM is being used for the quantitative profiling of untargeted low abundance compounds in biological studies.[41]

It must be remembered that quantitative analysis of specialized metabolites via targeted metabolomics requires reference standards of the metabolite of interest.[30] However, the success rate of quantitation of identifiable metabolites in targeted metabolomics is less due to the limited availability of reference standards. By way of examples, a targeted metabolomics approach was employed for identifying the anti-hepatocarcinogenic compounds using UHPLC–HRMS method from ethnopharmacologically relevant Indian medicinal plants viz. *Andrographis paniculata*, *Oroxylum indicum*, *Orthosiphon aristatus*, and *Willughbeia edulis*.[42] This approach revealed the chemical entities responsible for anti-proliferative activity using the dereplication approach in combination with a bibliographic score system, to generate a direct correlation between ethnopharmacological use and its molecular basis. Similarly, antioxidant or cardioprotective potential and metabolomics profiling have been done for different medicinal plants using LC-MS analysis.[43] Targeted metabolomics offers the additional advantage of rapid and accurate analysis due to the availability of initial information and relevant data related to medicinal plants. This approach has helped to explore ways of quickly identifying active plant metabolites, potential biomarkers, and elucidation of their biosynthetic metabolic pathways. In recent years, natural product researchers have initiated the widely targeted metabolomics approach by which nearly a thousand phytometabolites can be quantified based on MRM methods using quadrupole-trap mass spectrometry. This technique has been successfully applied in the identification of the nutritional composition of black sesame[44] and also in the broad identification and quantification of metabolites present in rice.[45]

2.2 | Untargeted metabolomics

The untargeted metabolomics approach mainly focuses on a comprehensive analysis of a species' complete metabolome, which includes both known and unknown metabolites having varying physical and chemical properties.[46] In contrast to targeted metabolomics, there is no prior knowledge of metabolites; hence, untargeted metabolomics is categorized as unbiased and comprehensive metabolite profiling. These studies are always the first choice and remain crucial in the initial research phase, where information about different types of metabolites is indispensable for further research. Although there are many techniques available for untargeted metabolomics, the most frequently used technique is high-resolution MS (HRMS). The identification of small molecules is done by matching the MS/MS fragment spectra with reference spectra available at different sources across the web. It provides complete information of molecular mass for the complete set of metabolites present in the sample of interest. Metabolite profiling in the case of unbiased/global metabolite detection is better elucidated with mass spectral data. Simultaneously, it generates very complex data sets with restricted repeatability and limited linear ranges; therefore, data handling in an untargeted approach is a major challenge.[31,47] The global metabolite analysis complexity is minimized by employing chemometric tools, viz. multivariate data analysis via supervised or unsupervised techniques.[48,49] Hierarchical clustering

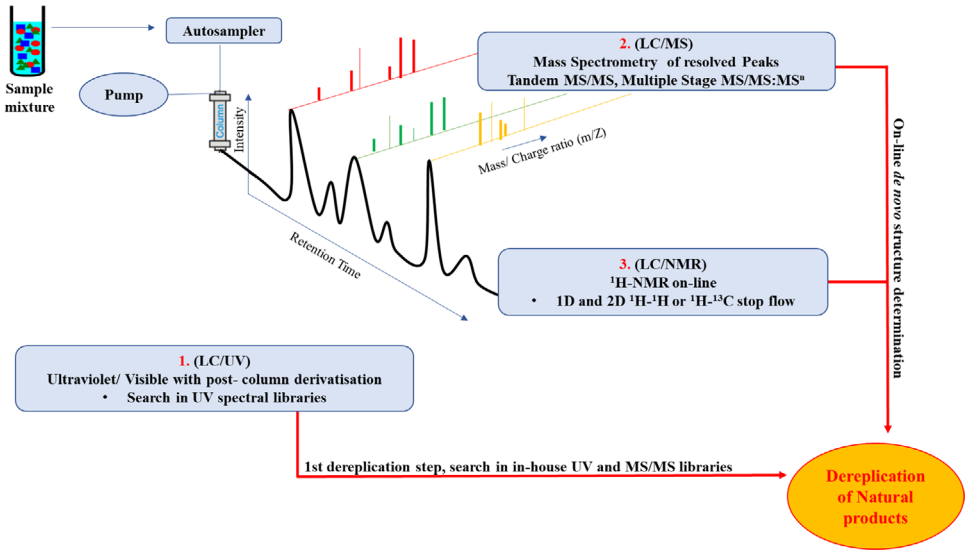

FIGURE 2 Schematic representation of outcomes obtained from chromatography – hyphenated techniques. 1. Acquisition of online LC/UV data and simultaneous screening in the in-house UV and MS/MS libraries. 2. Acquisition of online LC-mass spectrometric data of the resolved peaks and simultaneous structure determination of the compound. 3. Acquisition of online LC-NMR data and simultaneous structure determination of the compound. Taken from *Ibekwe, Nneka N et al*[61]

analysis, self-organizing maps, and PCA are unsupervised techniques that work mainly by the principle of pattern recognition or similarity analysis, whereas supervised methods include partial least square, which deals with the identification of features leading to variation.[50,51]

Recently, UPLC-HR-ESI-MS/MS untargeted metabolomics strategy was applied to assess antioxidant and anti-aging properties of five different plant species viz. *Camellia sinensis*, *Lavandula officinalis*, *Rosmarinus officinalis*, *Matricaria camomilla*, and *Pelargonium graveolens*.[52] Metabolomic analysis provides secondary metabolites quantification data that further helps to select potent plant species in terms of quantity and economy of the metabolite of interest. Using UPLC-MS/MS method, a greater accumulation of paclitaxel in *T. yunnanensis* was reported by a comparative metabolomics study employing integrated targeted and untargeted approaches for two endangered species of Himalayan *Taxus* viz. *T. yunnanensis* and *T. fauna*.[53] Untargeted metabolomics studies also aid in the identification of variation in metabolite profile within a plant species due to different geographical origins, environmental factors, genetic modification, and altitude-related factors. For example, a comprehensive metabolite discrimination study of *Catharanthus roseus* leaves infected with phytoplasma has been done using 1D and 2D NMR spectroscopy together with PCA.[54] The effect of genetic modifications on the metabolite profile of *Arabidopsis thaliana* using techniques such as LC/DAD, LC-MS, and NMR, followed by PCA has also been reported.[55]

3 | ANALYTICAL TECHNIQUES EMPLOYED IN METABOLOMICS

Unlike proteomics or genomics, metabolomics is usually performed with a combination of analytical techniques. A metabolomics analysis includes four main steps: sample collection, preparation, analysis, and data handling. It is important to take meticulous care in performing sample preparation steps, *i.e.*, sample collection, preservation, and preparation, as they all play a vital role in a metabolomics analysis.[56,57] The most popular analytical platforms for metabolomics are NMR and MS that are used in hyphenation with chromatographic techniques for the simultaneous separation and analysis of analytes of interest. Liquid phase (HPLC and UPLC) and gas phase (for volatile metabolites) chromatographies are the most commonly used techniques for separating metabolites. The hyphenated techniques mentioned above are extensively used in phytometabolite metabolomics with the aim to prevent re-isolation of the compounds based on the principle of dereplication of natural products. Several natural product databases such as AntiBase,[58] Dictionary of Natural Products,[59] and MarinLit,[60] are available to assist dereplication studies. A schematic representation of outcomes obtained from LC-hyphenated systems for rapid dereplication of phytometabolites from plant extracts is shown below (Figure 2). Different types of hyphenated techniques employed for the analysis are discussed in detail in the subsequent sections.

3.1 | MS-based hyphenated techniques: GC-MS, LC-MS, CE-MS

MS is a universal, most sensitive, and most commonly used analytical technique in the metabolomics of medicinal plants. MS-based hyphenated techniques are indispensable in the field of analytical chemistry for routine high-throughput analysis of phytometabolites. In hyphenated system, the MS detector is usually coupled with a chromatographic instrument. Thus chromatographic separation techniques such as liquid chromatography (LC), gas chromatography (GC), and

capillary electrophoresis (CE) are coupled with an MS instrument for metabolite analysis.[62] The selected method's efficiency depends on two parameters *viz*. resolution of the separation technique and sensitivity of the detection instrument. Such hyphenated techniques enable complete chromatographic isolation and relative or absolute quantification of the molecules of interest, which further helps in attaining the sample analyte structural information. These hyphenated platforms can be utilized for both untargeted and targeted metabolomics.[63] LC-MS is the most powerful analytical technique and has supersensitivity to the analyte as its limit of detection (LOD) is upto 0.5 nmoles.[64] It involves chromatographic separation across a broad range of polarities to enable simultaneous separation of maximum possible compounds along with high sensitivity MS detection. Chromatographic separation is also instrumental in providing enhanced sensitivity by virtue of reduction or elimination of ion competition which is known to decrease sensitivity. Practically, estimation of the diverse range of metabolites is impossible in a single run[65]; thus, both normal phase and reverse phase separations with polarity gradient separation are used to resolve the complete range of metabolites in a sample. Different compounds have dissimilar tendencies to get ionized in positive *vs* negative modes; hence both positive and negative electrospray ionization (ESI) modes are mandatory for comprehensive detection. Furthermore, some metabolites such as fatty acids and triglycerides, possess higher ionization potential, which can be detected using atmospheric pressure chemical ionization (APCI) techniques.[66] So to achieve comprehensive LC-MS metabolomics profiling of any targeted sample based on all these combinations, one must do chromatographic and detection mode scouting to optimize the method for each sample.[67]

GC-MS is also a prevalent technique in plant metabolomics. In fact, the GC-MS technique came in metabolomics science prior to the LC-MS technique and is a potent tool for estimating volatile compounds. There are different standard spectral libraries such as Wiley, Fiehn metabolomics library, and NIST, which are available as aids to the metabolite identification process by GC-MS analysis. High-resolution instrumentation, for example, GC-TOF-MS, is now gaining more popularity in phytometabolites research due to its greater identification efficiency and accuracy in the analysis.[68] Apart from plant metabolomics, GC-MS has a wide range of applications in the analysis of oils and fossil fuels, biomedical and microbial research, and in doping and illegal substance control.[67,69–72] This analytical platform offers several advantages such as high resolution, great sensitivity, reproducibility, robustness, excellent fragmentation, and low cost compared to other available analytical platforms. The major challenge in GC-MS analysis is the requirement of derivatization in the case of non-volatile analytes during the sample preparation step. It is this step that introduces chances of errors and lower reproducibility or erroneous quantification due to incomplete derivatization of the sample.[73] However, the issue can be addressed by data rectifying strategies and by using derivatized standard metabolites.

CE-MS is also an important technique mainly used to analyze polar or charged compounds as they are separated based on charge to mass ratio. In contrast to other analytical techniques, CE offers various advantages such as rapid isolation and identification, minimum sample pre-processing, minimum organic solvent utilization, and low cost due to the use of inexpensive silica capillaries in place of expensive GC/LC columns. Both targeted and untargeted metabolite profiling of samples from bacterial, plant, biomedical and clinical research[74–77] can be performed using this analytical technique. Even though CE-MS has not been widely used as an analytical technique, it is considered an important alternative platform for metabolite profiling. However, CE-MS suffers from the significant disadvantage of lower reproducibility with regard to peak area, retention time, and system stability profile due to alteration in the capillary walls affecting molecular sorption phenomenon.[67] As remedial measures, certain reports have suggested use of coated capillaries to overcome this system's drawbacks. Thus polybrene-dextran sulfate coated capillaries have been reported to give better performance for the CE-MS based metabolic profiling of human urine.[78]

Imaging MS (IMS) is a fascinating recent advancement in the MS technique. IMS has its role in the spatiotemporal metabolomics that deals with visualization of spatial location, temporal distribution, and production of phytometabolites in intact tissues. The immense connotations of spatiotemporal data make IMS particularly fascinating for developmental biological studies where understanding complexities of biology can be fathomed only in the context of dynamic qualitative and quantitative changes in time and space. IMS, which includes desorption ESI-MS (DESI-MS), matrix-assisted laser desorption ionization–MS (MALDI-MS), and secondary ion MS (SIMS) molecular imaging techniques, is a technique that complements all the pre-established omics fields.[79] This technique allows the mapping of metabolites in 2D or 3D space by merging analytical chemistry information with molecular imaging data. The technique offers an additional advantage of minimal sample preparation protocols for spatial analysis of several phytometabolites in a single sample.[80] The special feature of IMS is the fact that liquid extraction procedures are avoided to keep spatial information intact.[81] Analysis of specialized secondary metabolites accumulated in specialized plants cells such as trichomes (Sunflower)[82] or laticifers has been done using this technology. The spatiotemporal localization of alkaloids present in the nightshades has also been studied using IMS.[83] IMS enables examination of molecular characteristics and metabolic status of the plant tissue in a single analysis such as accumulation of defense compounds in tissues, flower pigmentation pattern,[84] UV fluorescence of specialized metabolites, and so on.[81] In IMS, the ionization technique works by ionizing and then desorbing the sample from its surface. The mass spectral image of the sample is visualized at the ionization spot. IMS is operated in full scan mode where a lot of images are generated representing the spatial distribution of different compounds in a scan. As reported by Sugahara *et al* the pigmentation of flowers[84] can be studied by using IMS together with chromatographic techniques. IMS has achieved great success in the past decade and continuous advancement in imaging techniques can act as a potential platform for a better understanding of diverse biological phenomena.

doi.org/10.1002/ansa.202100021

3.2 | NMR-based techniques: 1D NMR, 2D NMR, and solid-state NMR spectroscopy

As a principal analytical technique for the high throughput metabolomics study of natural products, NMR-based metabolite profiling is also a very prevalent technique in the study of plant metabolomics.[85] NMR-based profiling outcomes are robust, which is the major advantage of this technique in comparison to MS-based technique. NMR-based techniques, e.g., 1D, 2D, and solid-state NMR, possess multidisciplinary applications in the field of science.[86] As stated earlier, the huge number of phytometabolites and their vast complexity make the identification process extremely difficult in a single step. However, NMR as a technique exhibits excellent potential to identify and detect a diverse class of phytometabolites such as terpenoids, glycosides, flavonoids, saponins, alkaloids, and so on, even when present as a mixture. This technique provides a great advantage of signifying the relative molar concentrations of metabolites as being directly proportional to the signal intensities obviating the need for calibration curve generation for each metabolite. More specifically, NMR spectroscopy together with a standard moles–NMR peak intensity graph depicts the actual molar concentrations of metabolites in plant extracts due to the intrinsic quantitative nature of NMR signals.[1,85] Indeed NMR spectroscopy is the only well-established analytical platform for the structural elucidation of synthetic or natural product compounds. Metabolite profiling of *Strychnos nux-vomica* and its two other species has been reported by using high-resolution proton NMR spectroscopy and multivariate statistical analysis approach.[87] Though NMR is a quantitative analytical platform, however as stated earlier, standards are required to annotate already known compounds on the basis of the pattern recognition method.

Nonetheless, there are certain limitations while using the NMR in metabolomics studies related to lower sensitivity (LOD = 5 μmoles) and overlapping of different signals. A much higher amount of sample is required for NMR detection than in the case with other analytical techniques, for example, MS. Overlapping signals create problems in peak identification and integration, but these can be resolved using 2D-NMR spectroscopy.[85]

Starting in the early 2000s, proton-NMR spectroscopy has been widely applied to the metabolomics study of biological samples.[88] This method is non-invasive, non-destructive, and highly reproducible.[89] In contrast to GC-MS that is most suited for identifying only volatile nonpolar compounds, it goes to the credit of NMR that can detect all kinds of compounds.[86] As shown in Figure 3, the issue of overlapping signals and reduced sensitivity can be well addressed using 2D-NMR spectroscopy. This method also enables the determination of spin multiplicity, peak intensity, and coupling constants.[81] These 2D techniques help identify compounds that remain undetected using the 1D-NMR technique and help in complete structure elucidation by confirming peak allocation using spin connectivity properties.[90] Most frequently employed 2D-NMR spectroscopic techniques with wide applications in metabolomics are homonuclear correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), heteronuclear multiple quantum correlation (HMQC), nuclear overhauser effect spectroscopy

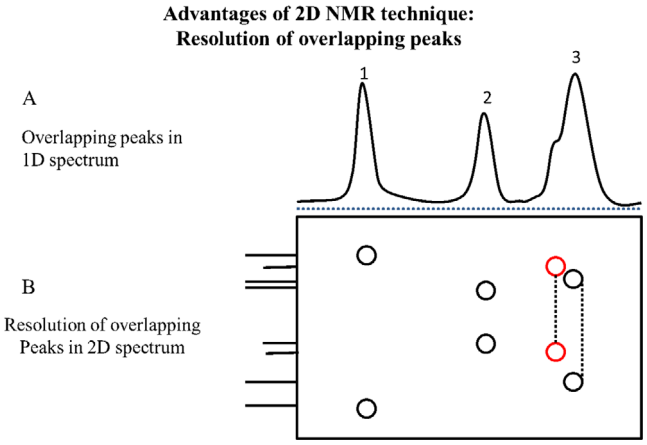

FIGURE 3 Resolution of overlapping signals using 2D NMR spectroscopy. Signals on the two axes represent 1D spectra with ^{13}C peaks on the vertical axis and ^{1}H peaks on the horizontal axis. This figure represents the utility of 2D NMR for resolution of overlapping peaks at ^{1}H NMR peak no 3. The black and red circles shown in 2D NMR spectrum represent the heteronuclear correlations between ^{1}H-^{13}C of the respective peaks. Herein, peak 3 is showing two distinct heteronuclear correlations (depicted as Red and Black circles) which suggests the presence of two distinct protons

(NOESY), double quantum filtered shift – correlated spectroscopy (DQF-COSY), heteronuclear multiple bond coherence (HMBC), total correlation spectroscopy (TOCSY), statistical TOCSY (STOCSY), and 2D-J-resolved NMR spectroscopy. There are further subtypes of these listed methods which are used for further exploration of metabolomics data and for comprehensive analysis of metabolome.[86] Different 2D-NMR techniques applicable in metabolomics analysis are summarized in Table 1.[89] The spectral databases/libraries available for aiding metabolite annotation using NMR spectral information include Small Molecule Accurate Recognition Technology (SMART 2.0) and Chemical Shift Multiplet Database (CSMDB).[91]

Solid-state NMR spectroscopy has been used to analyze solid powders, tissue samples, or samples with solubility issues in general NMR solvents. This is a nondestructive technique where there is minimum or no sample preparation required. In the case of biological samples, it provides the additional advantage of using the same sample for biological estimations, following spectroscopic analysis.[89] Although solid-state NMR spectroscopy has multiple advantages, it has not yet been much explored for metabolomic studies. Comprehensive metabolite profiling of four different cultivars of *Solanum tuberosum* potato has been reported using MS and solid-state ^{13}C-NMR spectroscopy (Figure 4).[92] Native periderms of the four potato cultivars were compared using PCA and hierarchical cluster analysis. Cultivar discrimination analysis was also performed to distinctly characterize them on the basis of composition of marker metabolite using OPLS-DA. Polar and nonpolar compounds were characterized using MS whereas solid polymeric residues were characterized by solid-state ^{13}NMR spectroscopy.

NMR-based metabolomics studies have a wide range of applications in diverse fields. For instance, it has been used for the identification and characterization of several Indian medicinal plants including

TABLE 1 2D-NMR techniques to facilitate metabolomics studies

Serial Number	Experiment and its subtypes	Working procedure	Application
1.	COSY (Homonuclear correlation spectroscopy) DQF-COSY (Double Quantum Filtered Shift-Correlated Spectroscopy) COCONOSY (NOE & shift-correlated spectroscopy)	Identifies nuclear spins that are coupled to each other due to scalar couplings	The pattern of three methylene protons in GABA.
2.	TOCSY (Total Correlation Spectroscopy)	Homonuclear, shift-correlated 2D NMR experiment similar to COSY in which all the correlation between a spin and all other spins from the same spin system are observed.	Correlations of the amide proton in case of lysine
3.	STOCSY (Statistical TOCSY) SHY-Statistical Hetero Spectroscopy	Generates a pseudo-2D spectrum, which shows the relationships between the intensities of different peaks	For the multi-spectroscopic data analysis of large number of samples
4.	HSQC (Heteronuclear Single Quantum Coherence) gHSQC, QOCCAHSQC (Offset-compensated, CPMG-adjusted HSQC), Q-HSQC, QQ-HSQC (shorter total acquisition time), Q-CAHSQC HILIC, HSQC0 (time-zero 2D spectrum).	Uses coherence transfer between nuclei, usually between ($^{1}H–^{13}C$) noise artifacts present are also present.	Confirmation of amino-acids such as proline, isoleucine, phenylalanine leucine
5.	HMQC (Heteronuclear Multiple-Quantum Correlation)	Homonuclear proton coupling resulting in broad peaks with poor resolution	Assigning peaks of 1- and 3-methylhistidine
6.	NOESY (Nuclear Overhauser Effect Spectroscopy) EXSY (for studying Configuration or chemical change)	Gives correlation between coupled nuclei that are in near vicinity in space (4-5 A) but are not directly connected by bonds	For confirmation of tertiary butyl groups in 12,14-ditbutylbenzo[g]chrysene.
8.	HMBC (Heteronuclear Multiple Bond Coherence)	Uses coherence transfer between nuclei ($^{1}H–^{13}C$) that are not directly connected but are 2,3,4 bond away.	Long-range correlations of ^{1}H-^{15}N for cyclosporin A
9.	INADEQUATE (Incredible Natural Abundance Double Quantum Transfer Experiment)	Homonuclear [^{13}C-^{13}C] shift correlated spectra	Powerful experiment useful for highly substituted compounds having low proton density.
10.	2D JRES NMR (Two-dimensional J-resolved NMR spectroscopy)	Measures isotopic patterns of compounds labeled with ^{13}C carbon Suitable for reproducible isotopic profiling	Isotopomers of Alanine

Ephedra,[93] Ginseng,[94] *Hypericum perforatum*,[95] *Cannabis sativa*,[96] and *Strychnos* species[87] using a high-throughput metabolomics approach. This technique is also used for the quality assessment and quality control of medicinal plants, which is also a vital parameter to achieve uniform and consistent pharmacological effects. Presently, quality assessment of medicinal plants is limited only to one active pharmaceutical ingredient or a few main active components of a medicinal plant preparation. Thus, as studied for *Senecio*, *Brassica rapa*, and *Arabidopsis thaliana*, NMR-based metabolomics is a great and innovative tool for a comprehensive analysis of medicinal plant preparations. NMR techniques are also helpful in studying different plant-related metabolic, physiological, or environment-induced alterations such as the effect of plant infection or herbivory on metabolite profile, plant's response to internal or external stress, and also the effect of preservation on plant metabolism.[97–102] Table 2 shows analytical and statistical techniques used in metabolomic studies of several Indian medicinal plants.

3.3 | Comprehensive hyphenated technologies: 2D-GC×GC-MS, LC-MS-NMR

Hyphenation of major chromatographic analytical techniques such as liquid or gas chromatography with NMR, MS, and other advanced detection techniques has greatly enabled metabolomics studies in both biomedical and pharmaceutical research. Separation-detection hyphenation has the great advantages of enhanced sensitivity with economy of time and labor. The major hyphenated techniques used in metabolomics research are 2D-GCxGC-FID, 2D-GCxGC-MS,

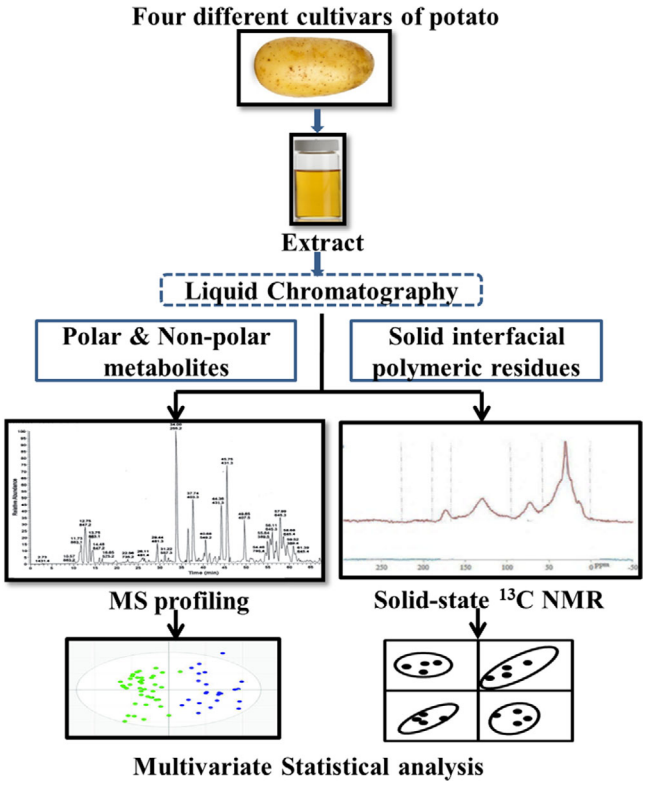

FIGURE 4 Metabolomic profiling of *Solanum tuberosum* using MS and Solid-state NMR. Taken from Huang Wenlin et al.[92]

LC-NMR, and LC-MS-NMR.[116] Of these the triply hyphenated LC-MS-NMR enables separation as well as simultaneous detection of each metabolite using both MS and NMR spectroscopic techniques. These integrated analytical techniques allow real-time analysis of the metabolome. Figure 5 is a pictorial representation of an integrated quadruply hyphenated UPLC-DAD-MS-NMR system. The utilization of superfast NMR instrumentation enables rapid data acquisition, which as stated earlier, reduces the time of analysis and enhances the efficiency of the system.[117] 2D-GC×GC-TOF-MS is also an excellent approach for the analysis of a pool of metabolites. It offers several advantages like enhanced separation, increased sensitivity, amplified accuracy, and precision in results.[116] LC-SPE-NMR is a hyphenated technology where solid phase extraction (SPE) is employed as an interface between separation and analysis.[118] It works by selective adsorption of the compound of interest on a solid adsorbent followed by online subjecting of the eluted sample to NMR system.

4 | APPLICATIONS AND ADVANTAGES OF MEDICINAL PLANTS METABOLOMICS

Metabolomics offers multidisciplinary applications, especially in phytometabolite research. It helps to provide a comprehensive analysis of metabolites that generates complete data of the metabolite profile of a sample. There are numerous applications of metabolomics of which some are discussed below:

- The USP of metabolomics is that it enables deciphering the whole phytometabolome together with characterization and structure information of each metabolite sourced from medicinal plants.[87] Complete metabolome profile in turn helps in revealing the main bioactive constituents of a plant and also the possible mechanism of biological actions of the plant extract. There are a number of herbal preparations or plant extracts whose phytometabolite profiles, physiological and toxicological effects are still unknown due to the lack of full molecular exploration of most medicinal plants. Hence, evaluation of metabolome is of utmost importance to guarantee the safety and pharmacological efficacy of herbal products.
- It has excellent applications in drug discovery and development, starting with the identification of a lead compound to the post-marketing surveillance of newly approved drugs. Globally speaking, the rate of the number of newly approved drugs is low due to huge drug discovery and development costs.[120,121] The pharmaceutical R&D sector is now facing a big challenge of coming up with some new chemical entities for rapidly emerging diseases of diverse kinds including pathogen caused, genetic, life-style related, or of idiopathic nature. Moreover, the number of potential lead drug candidates that reach phase-I clinical trial is one of 30, and only one of six molecules reaches the consumer.[122] This high rate of attrition is because a molecule must be endowed with many qualities such as high selectivity, good pharmacokinetics, high potency, and affordability before it can qualify as a drug. Hence the cost of failure in the drug discovery process is huge. However, failures can be minimized to a certain extent by optimizing the approaches adopted on the path to drug discovery. Metabolomics is a powerful technique that aids in the identification of lead compounds and their corresponding targets, helps in the assessment of safety, associated risks, and efficacy, illustrates the mechanism of drug action, reveals ADME parameters, and many others.[123]
- Metabolomics by virtue of its ability for the quantitative and structural identity of every metabolite plays a significant role in biomarker discovery. In identifying every metabolite, Metabolomics enables the identification of the biological character of each metabolite including toxicological biomarkers present in natural products. In the biomedical field, both early diagnostic tests and disease studies rely on the evaluation of each molecule as an indicator of the disease condition. Greater than 80% of diagnostic tests measure small molecules levels as biomarkers in the biological system.[124] Similarly, metabolomics can be used to assess the efficacy of drugs in individual patients allowing the benefits of personalized medicine in place of one regimen for all.
- Metabolomics studies can be used to study physiological, environmental, and chemical changes (such as the effect of stress, age, plant infection, climate, and herbivory) on medicinal plants. Genotype and phenotype alterations occurring at a cellular level are also well revealed by plant metabolomics. Plant breeds and growth conditions can be optimized using metabolomics for optimum therapeutic effects of medicinal plants.[97] Metabolite profiling of genetically modified plants, and the distinction of wild or transgenic plant

TABLE 2 Summary of metabolomic studies of Indian medicinal plants using computational and hyphenated techniques

Serial Number	Indian Medicinal plant	Purpose	Analytical technique	Statistical analysis	Reference
1.	*Centella asiatica*	Anti-diabetic activity	NMR	PCA*	103
2.	*Ephedra pachyclada* *E. gerardiana* *E. americana*	Metabolite profiling	UPLC-Q-TOF-MS	PCA BL-SOM*	104
3.	*Salix sp.*	Studied interaction of herbal medicines with human intestinal bacteria	UHPLC-HRMS		105
4.	*Artemisia annua* *A. afra*	Quality control	NMR	PCA	106
5.	*Andrographis paniculata*	Anti-hepatocarcinogenicity	UHPLC-HRMS	PCA, OPLS* regression analysis	42
6.	*Oroxylum indicum*	anti-hepatocarcinogenicity	UHPLC-HRMS	PCA, OPLS regression analysis	42
7.	*Orthosiphon aristatus*	Anti-hepatocarcinogenicity	UHPLC-HRMS	PCA, OPLS regression analysis	42
8.	*Willughbeia edulis*	Anti-hepatocarcinogenicity	UHPLC-HRMS	PCA, OPLS regression analysis	42
9.	*Rauvolfia serpentina*	Cardioprotective activity	LC-MS	Two way- ANOVA*	43
10.	*Terminalia arjuna*	Cardioprotective activity	LC-MS	Two way- ANOVA	43
11.	*Elettaria cardamomum*	Cardioprotective activity	LC-MS	Two way- ANOVA	43
12.	*Piper nigrum*	Cardioprotective activity	LC-MS	Two way- ANOVA	43
13.	*Strychnos nux-vomica*	Metabolite profiling	^{1}H NMR	PCA	87
14	*Withania somnifera*	Comprehensive metabolite profiling	LC-MS, GC-MS, HPTLC, and NMR	PCA	107–109
15.	*Aegle marmelos*	Metabolite profiling, anti-oxidant, anti-inflammatory activity	HPLC, GC-MS, NMR	One-way ANOVA	110
16.	*Moringa oleifera*	Hypoglycemic activity	HPLC, GC-MS	Two-way ANOVA, PCA	111
17.	*Cuminum cyminum*	Analyses of physio-biochemical composition and metabolite profiling	LC-TOF-MS	ANOVA, PCA and Heat map analysis	112
18.	*Cannabis sativa C. indica*	Chemotaxonomic mapping of cannabis varieties	GC-FID	PCA OPLS-DA*	113
19.	*Terminalia catappa (fruit peels)*	Metabolite profiling and antioxidant activity	GC-QTOF-MS LC-QTOF-MS	Standard deviation, Linear regression analysis	114
20.	*Swertia chirayita S. mussotii*	Metabolite discrimination analysis	Quantitative ^{1}HNMR	PLS-DA*, PCA	115

*PCA, Principal Component Analysis; BL-SOM, Batch learning–Self Organizing Map; OPLS, Orthogonal Projections to Latent Structure; ANOVA, analysis of variance; OPLS-DA, Orthogonal Projections to Latent Structure-Discriminant Analysis; PLS-DA, Partial Least Squares-Discriminant Analysis.

species have also been performed using metabolomics by applying chromatography-NMR hyphenated techniques.[125–129]

- As stated earlier, the latest advancements and increasing access to metabolomics technology have offered the development of precision medicine as a new application of metabolomics. There are reports that suggest the use of metabolomics in analyzing individualized patient bio-metabolites profile or individualized (personalized) metabolic phenotyping. Precision medicine aims to provide customized and personalized medication by analyzing the patient's omics profile.[130–132]
- Metabolomics has been considered a very powerful analytical technique for standardization, quality control, and evaluation of plant extracts and herbal formulations including Ayurvedic preparations. For instance, the quality control of herbal preparations containing *Artemisia annua* or *Artemisia afra* has been performed using NMR in combination with PCA.[106] Quality control, standardization, and scientific validation of medicinal preparations are of utmost importance to fulfil market demand criteria. These standardization studies help in the validation of ethnopharmacological use, and rationalizes the usage of an herbal product for medicinal use. Now regulatory bodies across the world have made it mandatory to provide scientific validation regarding every aspect of a formulation. Hence, the latest analytical platforms can be used to make products having maximum purity, potency, and safety.[20]

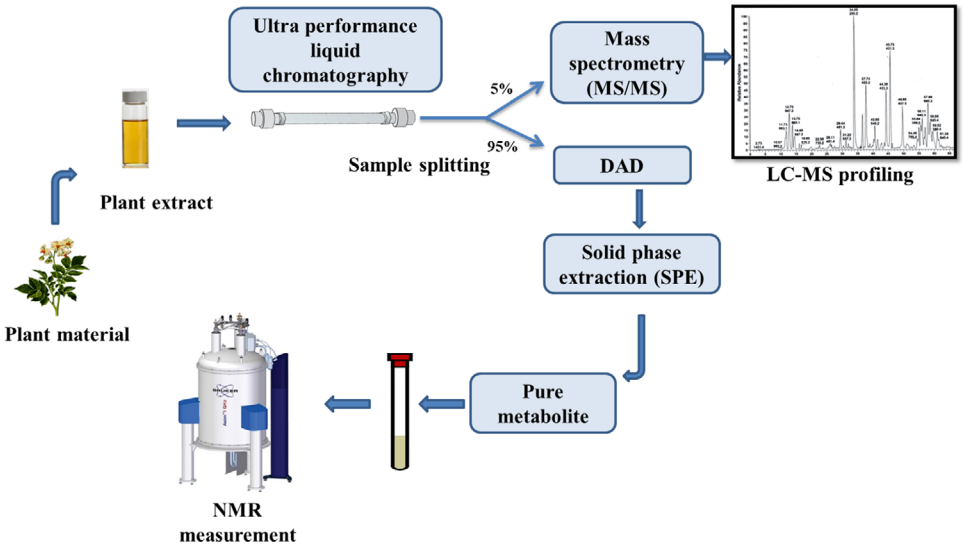

FIGURE 5 Schematic representation of an integrated UPLC-DAD-MS-NMR system. Taken from Gathungu Rose M. et al[119]

In addition to this, metabolomics has many applications in the field of biology, such as in drug metabolism studies, safety and toxicity studies, clinical trial and post-approval surveillance studies, and so on. Hence, metabolomics is a versatile and potential analytical tool in natural product chemistry and needs continual advancement in technologies for better exploration of untapped flora.

5 | CURRENT CHALLENGES AND FUTURE PERSPECTIVES IN METABOLOMICS

Being a relatively new entrant to the other established omics disciplines, metabolomics is facing initial challenges of proving its potential, especially for herbal drugs. Diversity rich phytochemistry and limited understanding of metabolic pathways is the main challenge in plant metabolomics. In terms of chemical space, phytometabolites exhibit enormous diversity. For instance, it has been reported that a single part of the plant such as tobacco leaves can contain about 3000 different metabolites.[133] It is this diversity that makes data analysis more challenging in metabolomics studies.[134] Another major challenge is the limited availability of standard metabolites for quantitative studies. Hence, at present, it is not feasible to screen a complete set of metabolites in a single scan. However, complete metabolome analysis is expected to become feasible in the coming times with continuous advances in data analysis and interpretation technologies. Molecular networking has emerged as the latest approach in the field of metabolomics and drug discovery. Although untargeted MS is one of the key strategies in the metabolomic study, annotation of chemical entities remains a challenging task.[135] Molecular networking is a computational visualization strategy for the untargeted mass spectrometery analysis. It provides the mapping of chemical relationships between molecular ions detected in MS experiments by organizing MS fragmentation data in the form of relational spectral networks.[136,137] In essence, molecular networking reveals the degree of spectral similarity in all MS/MS spectra given in a data pool. Global Natural Products Social Molecular Networking (GNPS) is a data-sharing web-based molecular networking tool that enables users to take advantage of this strategy to unravel motif relationships of a new molecule with molecules in the database.[138] With knowledge sharing at its core, GNPS is a data-directed approach where tandem MS data is stored and analyzed. Thus, the GNPS platform allows public sharing of the raw MS/MS data, processing, and annotation of entered data. It provides a collective information processing from reference spectra and experimental inputs present in the spectral library. GNPS enables the analysis and comparison of raw data with publicly accessible data. Both data deposition and its retrieval can be done via Mass spectrometric Interactive Virtual Environment (MassIVE) data repository. GNPS provides an additional advantage of online dereplication studies[139–141] and crowd sourced tandem MS curation. There are several GNPS spectral libraries such as NIH natural product library, GNPS Sigma's Mass Spectrometry Metabolite Library (MSMLS), Massbank Spectral Library, EMBL Metabolomics Core Facility (EMBL MCF), NIST, and ReSpect, some of which are available online.[142] MetaboLights[143] and metabolomic workbench[144] are the repositories of the metabolomic data. In a nutshell, GNPS provides a global platform for researchers working in different laboratories to get connected via data sharing to assist or shorten natural products annotation procedures. It fulfils the need for global collaboration for collective sharing of data towards reusable knowledge.

Furthermore, it is highly desirable for these hyphenated technologies, computational tools, and molecular networking approaches to become common bench side research platforms to thoroughly understand the deeper insights of fundamental processes of medicinal plants and their chemistry. Lastly, there should be interactions among the various research groups working with these hyphenated techniques to facilitate learning from each other and honing techniques to comprehend the messages inherent in the structure of every phytometabolite.

ACKNOWLEDGEMENT

The authors are thankful to the Director, CSIR-IHBT, for continuous inspiration. The work described in this manuscript is financially supported by CSIR (MLP0159). Ms. Shivani thanks CSIR for GPAT-JRF research fellowship. CSIR-IHBT communication no. for this manuscript is 4833.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Puri S, Sahal D, Sharma U. A conversation between hyphenated spectroscopic techniques and phytometabolites from medicinal plants. *Anal Sci Adv*. 2021;2:579–593. https://doi.org/10.1002/ansa.202100021