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| Abstract | Mass spectrometry (MS) and nuclear magnetic resonance (NMR) have evolved as the most common techniques in metabolomics studies, and each brings its own advantages and limitations. Unlike MS spectrometry, NMR spectroscopy is quantitative and does not require extra steps for sample preparation, such as separation or derivatization. Although the sensitivity of NMR spectroscopy has increased enormously and improvements continue to emerge steadily, this remains a weak point for NMR compared with MS. MS-based metabolomics provides an excellent approach that can offer a combined sensitivity and selectivity platform for metabolomics research. Moreover, different MS approaches such as different ionization techniques and mass analyzer technology can be used in order to increase the number of metabolites that can be detected. In this chapter, the advantages, limitations, strengths, and weaknesses of NMR and MS as tools applicable to metabolomics research are highlighted. |
| Keywords (separated by " - ") | NMR - MS - LC-MS - GC-MS - Metabolomics - Spectroscopy - Metabonomics |

# Chapter 13

## The Strengths and Weaknesses of NMR Spectroscopy and Mass Spectrometry with Particular Focus on Metabolomics Research

[AU1] Abdul-Hamid M. Emwas


### Abstract

Mass spectrometry (MS) and nuclear magnetic resonance (NMR) have evolved as the most common techniques in metabolomics studies, and each brings its own advantages and limitations. Unlike MS spectrometry, NMR spectroscopy is quantitative and does not require extra steps for sample preparation, such as separation or derivatization. Although the sensitivity of NMR spectroscopy has increased enormously and improvements continue to emerge steadily, this remains a weak point for NMR compared with MS. MS-based metabolomics provides an excellent approach that can offer a combined sensitivity and selectivity platform for metabolomics research. Moreover, different MS approaches such as different ionization techniques and mass analyzer technology can be used in order to increase the number of metabolites that can be detected. In this chapter, the advantages, limitations, strengths, and weaknesses of NMR and MS as tools applicable to metabolomics research are highlighted.


**Key words** NMR, MS, LC-MS, GC-MS, Metabolomics, Spectroscopy, Metabonomics



## 1 Introduction

[AU2]

Metabolomics is a technology-driven approach whereby recent developments in analytical tools, software, and statistical data analysis push the field forwards. Various analytical techniques have been used, but nuclear magnetic resonance (NMR) and mass spectrometry (MS) are the most common analytical tools in metabolomics research [1–14]. The high reproducibility of NMR-based techniques and the high sensitivity and selectivity of MS-based techniques mean that these tools are superior over other analytical techniques. Figure 1 shows the increase in the number of both NMR-based and MS-based metabolomics publications during the past 12 years.

Metabolomics analyses can be separated into the categories of targeted or untargeted analysis. Untargeted analysis focuses on the metabolic profiling of the total complement of metabolites

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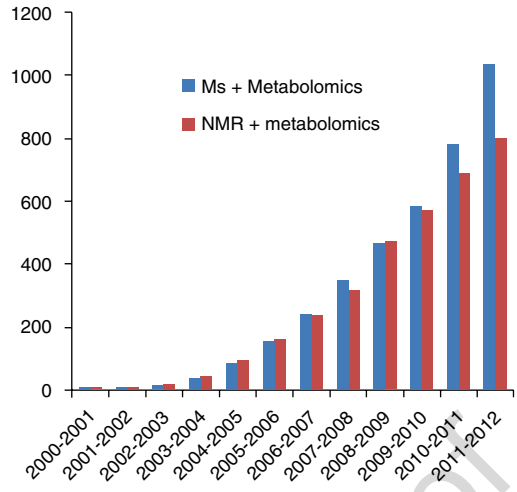


**Fig. 1** Number of NMR metabolomics and MS metabolomics publications; literature review was conducted using Web of Knowledge (http://apps.webofknowledge.com) with the keywords (**a**) (metabolomics and NMR) and (**b**) (metabolomics and MS)

("fingerprint") in a sample. NMR is commonly used in metabolomics fingerprinting studies. The targeted metabolomics approach focuses on the quantification and identification of selected metabolites, such as those involved in a particular metabolic pathway or others as the direct product of drug administering or of food intake. In targeted analysis, the metabolites under investigation are usually known, and the preparation of samples can be adjusted to reduce the effects of interference from associated metabolites. An MS-based metabolomics approach is usually the optimum method for targeted analysis.

Continuous development of MS methodology and machinery presents a highly specific analytical tool that can provide chemical information such as accurate mass, isotope distribution patterns for the determination of elemental formulae, structural elucidation though the characteristics of parent and fragment ions, identification of chemicals using spectral matching to authentic compound data, and comparative concentration levels of different chemicals in mixed samples. In fact, MS-based metabolomics techniques provide an exceptional combination of sensitivity and selectivity offering a powerful platform for a wide range of metabolomics research. Compared with NMR spectroscopy, MS is superior in allowing analysis of secondary metabolites where the detection level is of picomole to femtomole [15, 16]. Moreover, the different MS technologies provide an array of operational principles that can be applied, such as different ionization techniques, so increasing the number of metabolites that can potentially be detected.

The high reproducibility along with the nondestructive and noninvasive characteristics of NMR spectroscopy represents significant

advantages for employing NMR in metabolomics research. Further, NMR can be employed for in vivo studies, referred to as magnetic resonance spectroscopy (MRS); any in vitro metabolite pathway investigated by NMR spectroscopy can be pursued by in vivo studies using MRS. An NMR-based metabolomics approach involving isotopically labeled nuclei such as $^{13}C$ and $^{15}N$ can be used to obtain useful information about the balance of metabolites in a biological system and to monitor the flow of compounds through metabolic pathways. The high number of metabolites that can be detected simultaneously in a short time period of only a few minutes represents further advantage of using NMR spectroscopy in metabolomics research. For example, a single proton NMR spectrum can quantify around 100 metabolites in a sample of human urine, this providing a comprehensive picture of human metabolic status at a given point in time [17]. Moreover, high-resolution magic-angle spinning (HRMAS) NMR spectroscopy can be used in the study of intact tissue samples whereby metabolites present in a tissue can be detected without the need for pre-preparation steps such as extraction [18–24].

Finally, it is crucial to remember that there is no single analytical platform that can perform a complete quantification and identification of all molecules within a sample. Table 1 demonstrates the advantages and the limitations of NMR spectroscopy compared to MS spectrometry. Thus, employing different techniques such as different ionization methods coupled with liquid chromatography–mass spectrometry (LC-MS) and gas chromatography–mass spectrometry (GC-MS) in addition to one- and two-dimensional NMR experiments is necessary to maximize the identification of different metabolites within a complex sample. For example, Wishart et al. employed different analytical platforms such as NMR, GC-MS, direct flow injection mass spectrometry (DFI/LC-MS/MS), inductively coupled plasma mass spectrometry (ICP-MS), and high-performance liquid chromatography (HPLC) to identify the human urine metabolome. The results show that a total of 209 different metabolites can be identified by NMR, in addition to 179 by GC-MS, 127 by DFI/LC-MS/MS, 40 by ICP-MS, and 10 by HPLC [25].

In this chapter, the advantages, limitations, strengths, and weaknesses of NMR and MS as tools applicable to metabolomics research are highlighted.

## 2 Materials

Compared with MS, NMR spectroscopy requires highly skilled and trained manpower operators, and it is more expensive to purchase and maintain; it also demands a large space for the instrumentation (Fig. 2). Consequently, MS instruments are much more commonly found in clinical centers and hospitals compared with NMR spectrometers.

**Table 1**
**The advantages and limitations of NMR spectroscopy and MS spectrometry as an analytical tool for metabolomics research adapted from ref. [14]** [AU3]

| | NMR | Mass spectrometry |
|---|---|---|
| Sensitivity | Low but can be improved with higher field strength, cryo- and microprobes, and dynamic nuclear polarization | High and detection limit reach nanomolar |
| Selectivity | Even though few selective experiments are available such as selective TOCSY, it is in general used for nonselective analysis | Can be used for both selective and nonselective (targeted and nontargeted) analyses |
| Sample measurement | All metabolites that have NMR concentration level can be detected in one measurement | Usually need different chromatography techniques for different classes of metabolites |
| Sample recovery | Nondestructive; sample can be recovered and stored for a long time; several analyses can be carried out on the same sample | Destructive technique but need a small amount of sample |
| Reproducibility | Very high | Moderate |
| Sample preparation | Need minimal sample preparation | More demanding; needs different columns and optimization of ionization conditions |
| Tissue samples | Yes, using HRMAS NMR tissue samples analyzed directly | No, requires tissue extraction |
| Number of detectable metabolites in urine sample | 40–200 depending on spectral resolution | Could be more than 500 using different MS techniques |
| Target analysis | Not relevant for targeted analysis | Superior for targeted analysis |
| In vivo studies | Yes—widely used for $^{1}H$ magnetic resonance spectroscopy (and to a lesser degree $^{31}P$ and $^{13}C$ | No—although suggestion that desorption electrospray ionization (DESI) may be a useful way to sample tissues minimally invasively during surgery |

### 2.1 Samples

Different biological samples are commonly used in metabolomics analysis including:

- Bio-fluids.
- Cell extracts.
- Bacterial extracts.
- Animal/human intact tissues.
- Plant extracts.

**Fig. 2** Typical NMR laboratory (KAUST NMR laboratory) where sufficient, separated space is required for location of NMR spectrometer (*top*); GC-MS where the GC-MS is usually small enough to be allocated on lab bench top (*bottom*)

[AU4]

### 2.2 Chemicals

1. The chemicals most commonly used in metabolomics analyses include HPLC grade solvents such as methanol, ethanol, chloroform, acetonitrile, and isopropanol.
2. Phosphate buffer is usually required for NMR analysis.
3. Solvents usually contain hydrogen atoms and the solvent $^{1}H$ NMR peak would overwhelm the solute $^{1}H$ peaks. Consequently, deuterated solvents should be used for NMR

measurements; those most frequently used in metabolomics studies are $D_2O$ or (90:10 $H_2O$: $D_2O$) for polar metabolites and $CDCl_3$ for lipid samples. In the case of strong solvent residual peaks such as (90:10 $H_2O$: $D_2O$), the "solvent suppression" experimental technique would be necessary to suppress the residual solvent peak.

4. References for NMR spectra: 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) or its sodium salt and 3-trimethylsilylpropionic acid (TSP) are normally used for samples dissolve in $D_2O$ or 90:10 $H_2O$: $D_2O$, and tetramethylsilane (TMS) is the most commonly used in organic solvents such as chloroform.

### 2.3 NMR Equipment

1. NMR magnets: Even though an ultrahigh magnetic field NMR spectrometer, such as one set at 950 MHz, will offer better resolution and higher sensitivity, such equipment is prohibitively expensive and the maintenance is similarly costly. Consequently, most NMR-based metabolomics studies have been conducted using 600 and 500 MHz NMR spectrometers that are commonly available in research institutes and offer a good compromise between resolution, sensitivity, and cost.
2. NMR probes: Different NMR probes can be used for particular applications and for the detection of different nuclei. Examples of commercial NMR probes that have commonly been used in metabolomics research include:
   (a) Double Resonance Broad Band Probes (BBO and BBOF) often called Broad Band Inverse Probes. These are widely used in experiments such as heteronuclear 2D experiments involving single-quantum coherence (HSQC), heteronuclear multiple-quantum correlation (HMQC), and heteronuclear multiple bond correlation (HMBC).
   (b) Triple Resonance Broad Band Probe (TBI).
   TBI can be used for all BBO applications, the extra channel bringing the advantage of being able to study another X-nucleus, so providing the capability for study of organometallic compounds and metalloproteins using $^{1}H$, $^{13}C$, and another metal-nucleus.
   (c) Double Resonance Broadband Probe (BBI). Tunable to a wide range of frequencies; used for detection of multinuclear experiments optimized for $^{1}H$ applications.
   (d) Triple Resonance Broad Band Probe (TBO). TBO probe has features similar to those of BBI probe but with one extra channel.
   (e) Triple Resonance Probe (TXI). Provides the opportunity to pulse for up to three or four nuclei in one experiment; used extensively for NMR structural determination of biological macromolecules.

(f) Triple Resonance CryoProbe (TCI) (used mainly for protein applications but can also be used for metabolomics). The Probe is composed of three completely independent channels (in addition to a lock channel) that can be used for simultaneous decoupling on multiple nuclei, such as $^{15}N$ and $^{13}C$. It is a powerful probe for multidimensional experiments, whereby pulsing up to four nuclei in one experiment is possible. Consequently, it is used mainly for NMR structural determination of biological macromolecules such as proteins.

(g) MicroProbes (1 mm TXI, 1.7 mm TXI)—small volume probes (useful for samples of a small size).

### 2.4 MS Equipment

1. MS components: the MS instrument consists of three major components.
   (a) *Ion source*: ionization part (produces gaseous ions).
   (b) *Analyzer*: main function is to separate ions into distinctive mass components on the basis of mass-to-charge ratio ($m/z$).
   (c) *Detector system*: detects ions based on their $m/z$ and records relative abundance of each resolved ion.

   The MS instrument also has other important components including a system to insert samples into the ion source and a computer to capture and analyze the data, as well as compare the spectra to reference MS database libraries. There are several ionization methods including EI (electron impact), CI (chemical ionization), MALDI (matrix-assisted laser desorption ionization), ESI (electrospray ionization), FAB (fast-atom bombardment), SIMS (resonance ionization), PD (plasma-desorption ionization), LIMS (laser ionization), and RIMS (resonance ionization). Application of each of these methods brings its own advantages and limitations. Sample analysis could be carried out using different types of analyzer such as time of flight (TOF), quadrupole, ion trap, magnetic sector, and Fourier transform mass spectrometry. It is beyond the scope of this chapter to give a detailed description of the application, advantages, and disadvantages of each method of ionization or mass analysis. Examples of LC-MS and GC-MS equipments that are available in our analytical core lab are presented in the following sections.

2. LC-MS equipment.
   (a) TSQ Vantage, triple quadruple MS.
   (b) Loading pump, transcend system.
   (c) Valve interface model.
   (d) Accela PDA detector.
   (e) CTC autosampler equipped with injector valve.

3. GC-MS equipment.
   (a) 7890 GC.
   (b) 7683 Autosampler.
   (c) 5975 C quadrupole MSD.
   (d) Trace GC ultra.
   (e) Triplus autosampler.
   (f) TSQ Quantum, triple quadrupole MS.
   (g) Microtof II MS.
   (h) GC Capillary Column: DB-5MS.

## 3 Methods

### 3.1 NMR Spectroscopy

NMR spectroscopy is a powerful analytical tool that has been used mainly in chemistry for the identification and quantification of the chemical composition of a given sample. The applications of NMR spectroscopy are not limited to liquid samples but can also be used on solid [26–31], gas phase [32–36], and tissue samples [28, 29, 37–49]. Moreover, as well as its main applications in molecular identification and structural elucidation, NMR can also be used to study the physical and chemical properties of molecules, such as electron density and molecular dynamics [50–57]. Consequently, NMR has become the main tool for structural biology studies as it enables researchers to study molecular structures as well as molecular dynamics under biological conditions. Moreover, NMR spectroscopy has been employed in a wide range of research areas including structural biology, organic chemistry, inorganic chemistry, biochemistry, physics, biology, polymers, and drug discovery [9, 41, 58–75]. NMR spectroscopy has been proposed as one of the most relevant methods in metabolomics applications, for example, as powerful diagnostic method for a wide range of human diseases [11, 14, 76–87]. Low sensitivity is the inherent disadvantage and the foremost challenge for the application of NMR in biomedical research. Continuous developments in the relevant machinery such as a higher magnetic field strength [88], cryogenically cooled probes [89], and microprobes [90] have significantly enhanced the sensitivity of NMR. The dynamic nuclear polarization (DNP) approach is one of the most efficient developments [91] that has been used successfully to enhance NMR sensitivity in imaging and spectroscopy [92–95].

#### 3.1.1 High-Resolution Magic-Angle Spinning (HRMAS) NMR Spectroscopy

The applications of NMR spectroscopy are not limited to liquid and solid samples, but extend to intact tissue samples with use of high-resolution magic-angle spinning (HRMAS) NMR spectroscopy. By spinning samples at an angle of 54.74°—the "magic

angle"—to the magnetic field and at high speed, spectra can be obtained with a resolution comparable to that of solution-state NMR spectra. Using this method, the chemical composition of tissue samples can be detected spontaneously without the need for pre-preparation steps such as extraction [18, 96]. In an NMR-based metabolomics approach, this technique is helpful in offering a correlation between the metabolic profiling of bio-fluids and the histology of specific tissues. Consequently, HRMAS NMR spectroscopy has been used to study the metabolomic balance of small intact tissue samples [97] including brain [98], kidney [99], liver [100], and testicular tissues [101]. HRMAS has recently been used in meningioma biopsies as a potential diagnostic tool for the differentiation of typical meningiomas and benign tissues [102]. Recently, Ying-Lan Zhao and coworkers have employed HRMAS spectroscopy in conjunction with principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal projection to latent structure with discriminant analysis (OPLS-DA) to investigate the metabolic profile of human rectal cancer tissue collected from 127 patients and compare these with 47 samples collected from healthy control subjects [103]. The results revealed a clear separation between the samples from patients and those from the healthy control subjects. Several distinguishing metabolites were identified and correlated to different stages of rectal cancer tissues, so demonstrating the possibility of using metabolite biomarkers to follow the progression of rectal cancer. Moreover, a total of 38 differential metabolites were successfully identified, and 16 of them were found to be closely correlated with a particular stage of rectal cancer. The results demonstrate that, compared with healthy control samples, the concentration levels of several metabolites including lactate, threonine, acetate, glutathione, uracil, succinate, serine, formate, lysine, and tyrosine are found to be elevated in cancer tissue samples from patients, whereas the levels of other metabolites such as turrine, creatine, betaine, myo-inositol, phosphocreatine, and dimethylglycine are found to decrease [103].

[AU5]

### 3.1.2 One-Dimensional (1D) NMR Spectroscopy

Although NMR spectroscopy has been used in numerous multidimensional experiments with different detectable nuclei, one-dimensional (1D) proton ($^1$H) NMR remains the most usable and useful technique, especially for metabolomics studies. However, due to the narrow range of chemical shift (10 ppm), $^1$H NMR spectra from overlapped signals usually persist, and this leads to uncertainty in the spectral assignments. Figure 3 shows the proton NMR spectrum of a sample composed of two simple molecules, *n*-propanol and *n*-butanol. As can be seen from the figure, the signals for the methyl groups for both *n*-propanol and *n*-butanol are observed at 0.92 ppm as an overlapped signal and cannot be

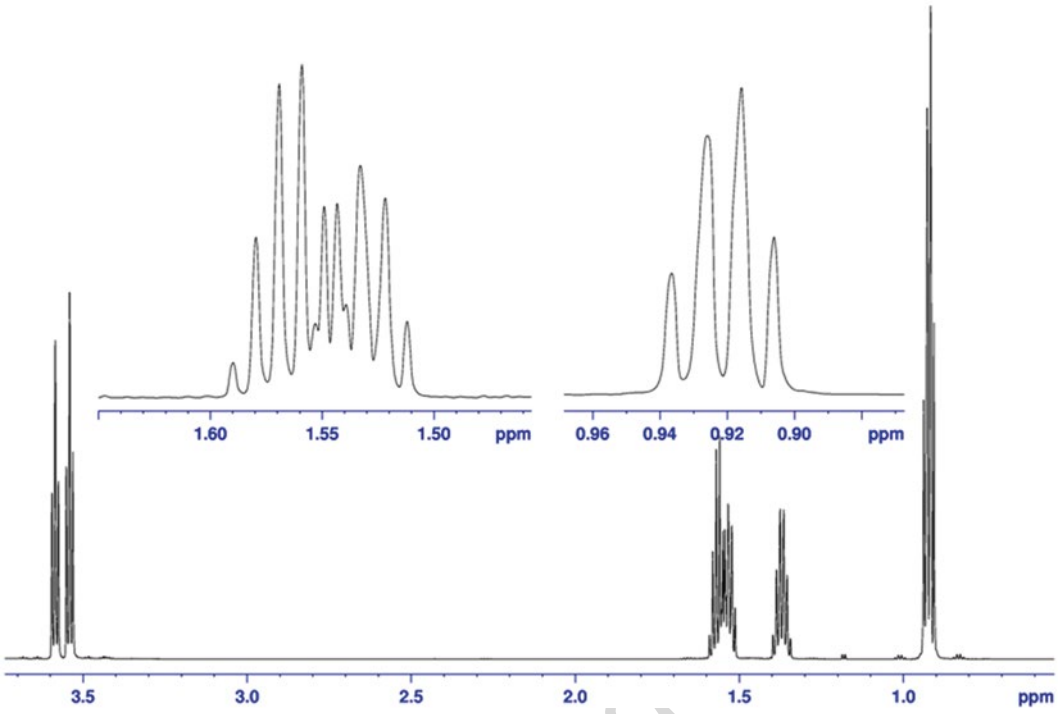


**Fig. 3** 700 MHz proton NMR spectrum of a sample composed of two simple molecules, *n*-propanol and *n-butanol*, dissolved in $CDCl_3$ recorded at 298 K



resolved by simple 1D NMR spectroscopy. Moreover, overlapping signals were also obtained at 1.55 ppm. These overlapping signals are related to the -$CH_2$-groups from the two molecules, but it is not possible to ascribe a particular signal to a particular molecule based on the simple 1D proton NMR spectrum. Other nuclei such as carbon and nitrogen have a wider range of NMR chemical shift but bring other limitations. For example, one-dimensional phosphorous ($^{31}$P) NMR spectroscopy has a few advantages such as the 100 % natural abundance of the $^{31}$P nuclei, a wide chemical shift range, and high sensitivity. Thus, $^{31}$P NMR spectroscopy is commonly used to study phospholipids and metabolites involved in energy metabolism [104, 105]. However, the fact that most metabolites do not contain phosphorus represents the main limitation of $^{31}$P NMR spectroscopy. Generally, the spectral width of one-dimensional carbon ($^{13}$C) NMR spectra is more than 200 ppm leading to wider spectral dispersion. $^{13}$C NMR spectroscopy is particularly informative in molecular identification and for structural elucidation. However, the low natural abundance of the $^{13}$C nuclei (1.1 %) as well as a low sensitivity has hindered the use of this isotope in NMR-based metabolomics applications. Different NMR approaches have been developed to enhance the $^{13}$C NMR signals. For example, Distortionless Enhancement by Polarization Transfer

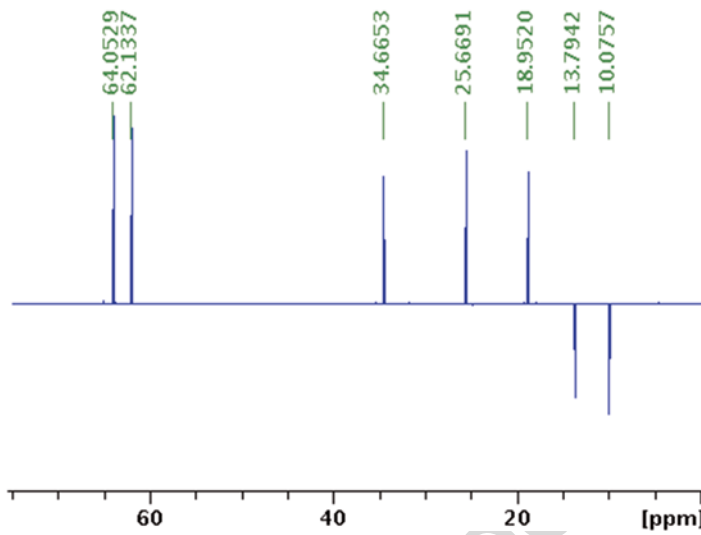




**Fig. 4** DEPT-135 $^{13}C$ NMR spectrum of a mixed sample composed of *n*-propanol and *n*-butanol in $CDCL_3$ at 298 K. The $CH_3$ signals in the opposite direction of the $CH_2$ signals providing a powerful approach to resolve the $CH_3$ signals from $CH_2$ ones are illustrated



(DEPT) is a powerful means of increasing the sensitivity of NMR spectra whereby the $^{13}C$ signal intensity can be enhanced by a factor of four. DEPT NMR experiments are also useful for distinguishing between $CH_2$ and (CH, $CH_3$), as the $^{13}C$ NMR spectrum of DEPT-135, for instance, yields $CH_2$ peaks with a negative intensity and CH and $CH_3$ peaks with a positive intensity (Fig. 4).

$^{15}N$ NMR spectroscopy is very useful in structural biology including the study of proteins, RNA, and DNA structure and dynamics and also investigation of protein–metal coordination, protein–protein, and protein–ligand interaction [106–112]. However, due mainly to the low natural abundance of $^{15}N$ at only 0.37 % and a low sensitivity, this approach is expected to be less useful in metabolomics studies.

### 3.1.3 Two-Dimensional (2D) NMR Spectroscopy

Two-dimensional (2D) NMR spectroscopy can be used to overcome the problem of overlapping resonances in proton 1D NMR spectra, leading to the detection and assignment of a greater number of metabolites than is possible with the 1D method. 2D NMR spectroscopy is based on through space spin coupling or through bond coupling, the former being used mainly for structural elucidation and the latter for molecular identification. Through bond correlation, NMR spectroscopy divides into two main categories: homonuclear, being mostly ($^1H$–$^1H$) such as correlation spectroscopy (COSY) [113] and total correlation spectroscopy (TOCSY) [114], and heteronuclear, such as ($^1H$–$^{13}C$). Both the homonuclear

and heteronuclear categories have been used in NMR-based metabolomics studies for signal isolation and to support the assignment of metabolites [115–119]. Other 2D methods, such as two-dimensional J-resolved NMR spectroscopy (J-Res) [120], and diffusion-ordered spectroscopy (DOSY) [121] have been used in NMR-based metabolomics studies. A combination of COSY and DOSY was employed to study metabolic changes in dystrophic heart tissue for samples collected from a mouse model of muscular dystrophy [122]. Heteronuclear 2D experiments involving single-quantum coherence (HSQC), heteronuclear multiple-quantum correlation (HMQC), and heteronuclear multiple bond correlation (HMBC) have a high degree of resolution in the second dimension mostly using ($^{13}C$) and have been employed for metabolite discrimination and identification [123].

Although 2D NMR experiments improve the dispersing of the NMR signals, increased acquisition time, data size, and complexity in data analysis limit frequent use of this approach. However, the continuous development of NMR machinery and new faster NMR method of signal acquisition and data processing are leading to the increased use of 2D techniques in metabolomics studies [113, 124–126].

#### 3.1.4 Correlation Spectroscopy (COSY)

COSY was the first technique of 2D homonuclear correlation spectroscopy and has been used over many years for molecular identification and for structural elucidation [127–130]. COSY has been employed in metabolomics research as it benefits from a relatively short experimental time with the possibility of running a 2D spectrum in only a few minutes and providing far more information than is gained from 1D NMR spectra. The simplest COSY pulse sequence consists of a single 90° RF pulse followed by evolution time (t1) and then a second 90° pulse followed by a measurement period (t2). The COSY spectrum comprises a homonuclear, mostly ($^{1}H$–$^{1}H$), correlation spectrum in which the cross peaks in the 2D spectrum indicate through bond couplings between pairs of nuclei. The cross peaks represent through bond magnetization transfer between two nuclei. This provides a powerful tool for the identification of peaks that belong to the same molecule in samples composed of many molecules, as would be the case for metabolites in biological samples. As through bond correlation occurs only within the same molecule, COSY NMR spectroscopy has been used in a wide range of NMR-based metabolomics applications [131–135]. Figure 5 shows the 2D COSY NMR spectrum from which the correlation between coupled protons can be used to assign an NMR signal and to identify the corresponding molecule. However, for multiple overlapped signals, 2D COSY is not powerful enough to allow assignment of the individual signals. Other 2D NMR experiments, such as total correlation spectroscopy (TOCSY), can be used to assist with signal assignment.

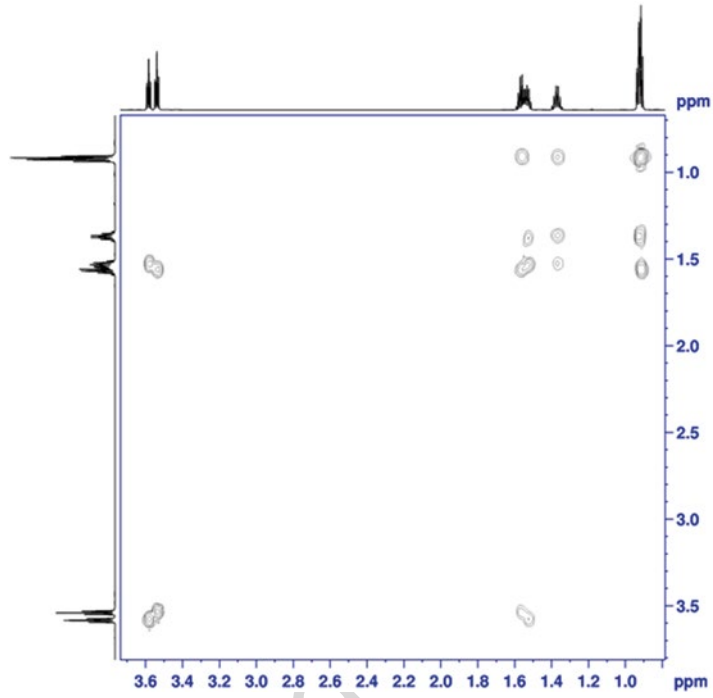


**Fig. 5** 700 MHz 2D COSY NMR spectrum of *n*-butanol and *n*-propanol in $CDCl_3$



Total Correlation Spectroscopy (TOCSY)

*TOCSY or HOHAHA* (Homonuclear Hartmann Hahn) is a similar approach to COSY, whereby the chemical shift of a given nucleus such as H is correlated with the chemical shift of other Hs of the same compound which are within the spin system (unbroken chain of couplings) of the atom. Similar to COSY, whereby the correlation between pairs of atoms (protons) in nearby carbon atoms that are connected by scalar coupling would be observed, the TOCSY spectrum shows the cross peaks not only for protons which are directly coupled but also for protons which are connected by a chain of couplings. For example, if proton A is coupled with proton B and proton B coupled with proton C, the COSY spectrum would express only the coupling A with B, whereas the TOCSY spectrum would display the coupling of A with both B and C. Figure 6 shows the stack plot of TOCSY (blue) and the COSY spectrum (red): more blue peaks can be observed, these representing every proton signal coupled with proton signals related to the same molecule (*n*-propanol and *n*-butanol). This shows that TOCSY spectrum can be used for resolving overlapped peaks that belong to different molecules. For instance, Fig. 7 shows an extended region of Fig. 6. Clearly, propanol peaks can be resolved from butanol peaks by simply detecting the signals that share four cross-correlation peaks (butanol, green arrow) compared with signals

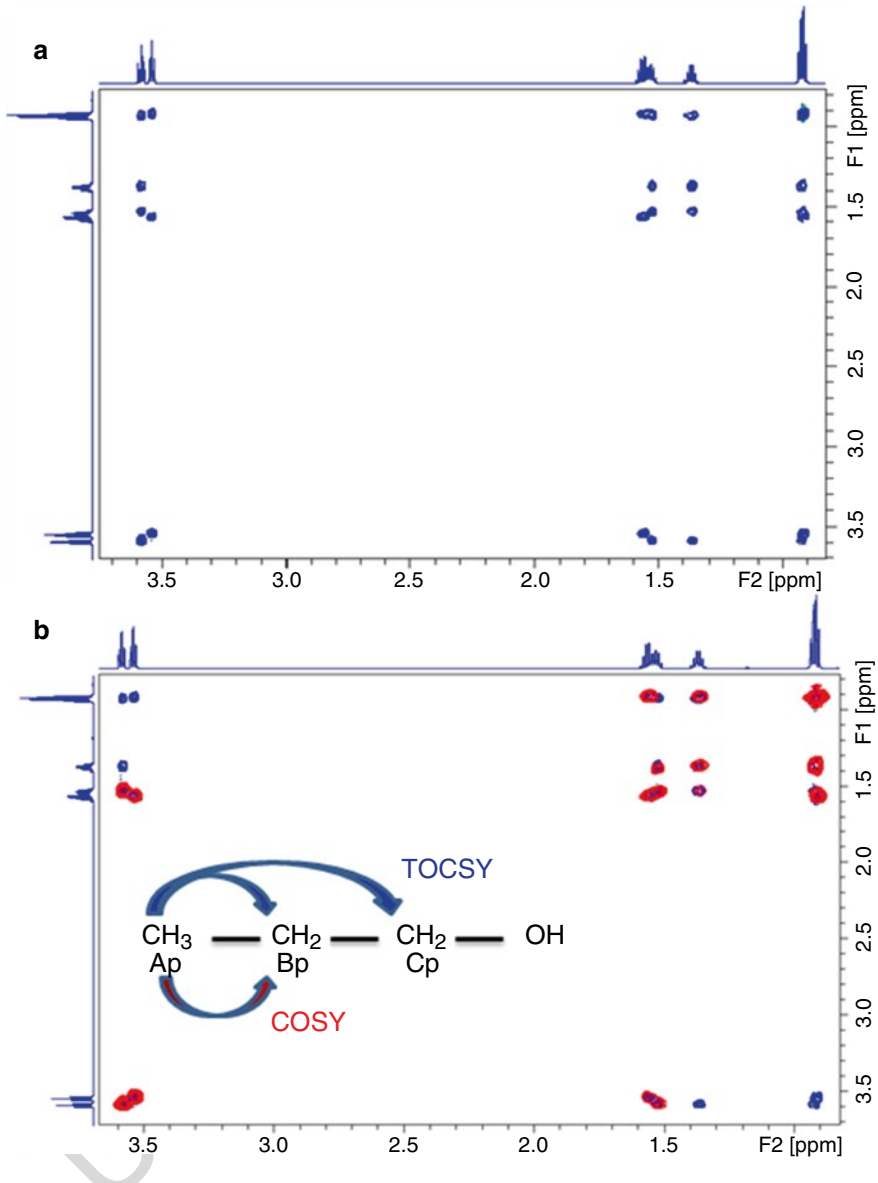


**Fig. 6** (**a**) TOCSY NMR spectrum and (**b**) stack plot of TOCSY (*blue*) and COSY spectrum (*red*) for a mixed sample of *n*-propanol and *n*-butanol in $CDCL_3$. A greater number of *blue* peaks (TOCSY) can be observed than COSY peaks (*red*). As anticipated, the COSY spectrum shows only the coupling between Ap and Bp, while the TOCSY spectrum displays the coupling of Ap with both Bp and Cp

that share only three cross peaks (propanol, black arrow). Assignment of peaks is accomplished by drawing a projection from the cross peak to 1D spectrum in the plot axis (Fig. 8). The arrow projection that connects the green arrow (butanol) with the signal at 3.584 ppm confirms that this peak refers to butanol, not propanol, and the second projection that correlates the signal at 1.564 ppm with the black arrow confirms that this peak corresponds to the

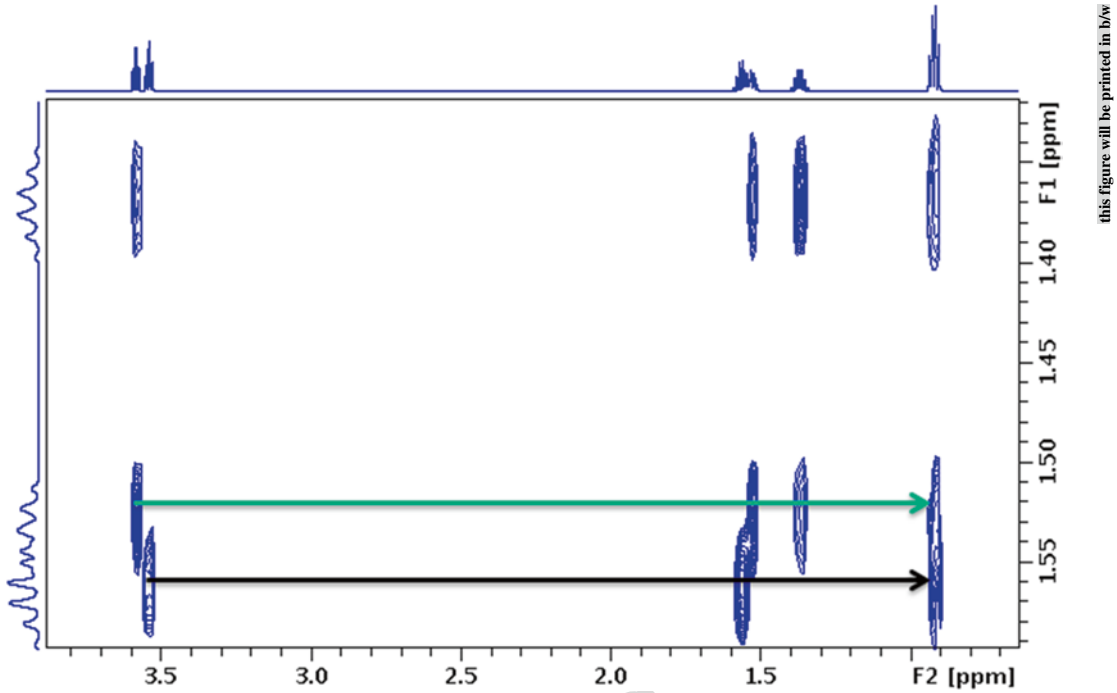




**Fig. 7** Extended region of Fig. 6a



propanol molecule (Fig. 7). Confirmation of one peak as propanol and another peak as butanol would be sufficient to complete the assignment of all signals for both molecules using the TOCSY spectrum (Fig. 8). The assignment of $^{1}H$ NMR signals can be used in combination with other 2D NMR techniques, such as heteronuclear correlation spectroscopy. This will assist in the assignment of other nuclei signals such as those of the carbon NMR spectrum.

### *3.1.5 Heteronuclear Single-Quantum Correlation Spectroscopy (HSQC)*

Bond correlation can also be used for correlation between two different types of nuclei (commonly $^{1}H$ with $^{13}C$ or $^{15}N$), which are separated by one bond. For example, the $^{1}H$–$^{13}C$ HSQC spectrum coordinates the chemical shift of protons and the corresponding bonded carbon, whereby only one cross peak will be obtained per pair of coupled atoms. Thus, HSQC offers a particularly informative approach for the assignment of signals, especially for the assignment of overlapping proton signals. Figure 9 shows the $^{1}H$–$^{13}C$ HSQC of a mixture of *n*-butanol and *n*-propanol spectra in $CDCL_3$. The figure demonstrates the efficacy of HSQC in resolving overlapped proton signals. For example, the extended region (A) resolved the overlapped proton signal at 0.91 ppm. Moreover, HSQC spectra can be used to assign both proton and carbon NMR spectra.

HSQC is also a useful technique to reduce the experimental time for nuclei with low sensitivity and low natural abundances,

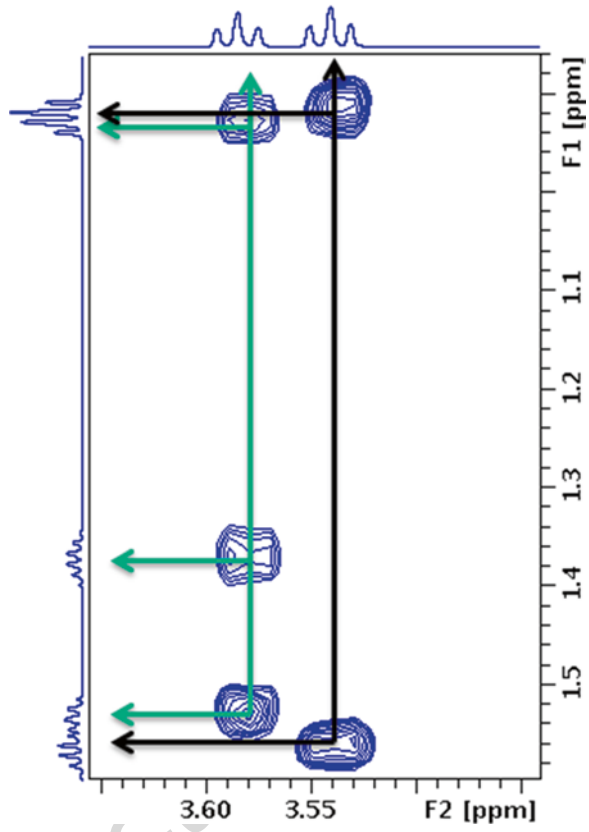


**Fig. 8** Projection of the arrow that connects the *n*-butanol (*green*) and *n*-propanol (*black*) TOCSY cross peak with the corresponding 1D proton NMR spectrum

such as $^{13}C$ and $^{15}N$, whereas the magnetization from the more sensitive nucleus I (usually $^{1}H$) transferred to the less sensitive nucleus, such as $^{13}C$ and $^{15}N$. $^{1}H$–$^{15}N$ HSQC spectroscopy is one of the most important and common experimental techniques in the assignment of protein signals, as the assignment of NMR signals is the prerequisite step for the study of protein structure and dynamics [136–139]. Two-dimensional multiple-quantum correlation spectroscopy (HMQC) is a 2D heteronuclear correlation NMR approach similar to HSQC spectroscopy and provides identical information with a similar spectrum but uses different methods. Both HSQC and HMQC have been used in NMR-based metabolomics research, with HSQC being considered superior for larger molecules such as proteins [117, 133, 140].

*HMBC* (Heteronuclear Multiple Bond Correlation) is a 2D heteronuclear correlation technique that correlates the chemical shift of two different types of nuclei (i.e., $^{13}C$ and $^{1}H$) that are separated from each other by two or more chemical bonds. The chemical shift of one nucleus, such as $^{1}H$, is usually detected in the directly measured dimension (F2), and the chemical shift of the

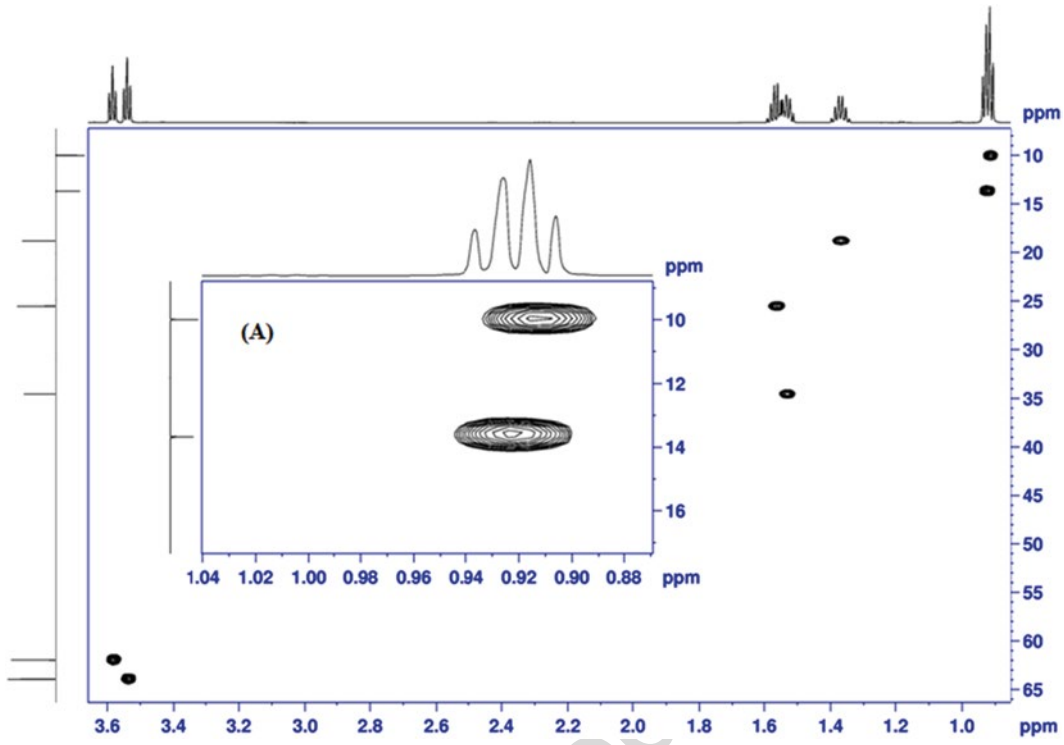


**Fig. 9** $^{1}H–^{13}C$ HSQC spectrum of a mixture of *n*-butanol and *n*-propanol dissolved in $CDCl_3$ recorded using 700 MHz Bruker Avance (III) NMR spectrometer. The extended region (A) of the methyl group signals at 0.92 ppm indicates that this signal was resolved into two $^{13}C$ signals in the second dimension, one at 10 and the other at 13.85 ppm

other nucleus, such as $^{13}C$ (X-nucleus or heteronucleus), is recorded in the indirect dimension, as shown in Fig. 10. In this approach, a low-pass filtration is used to eliminate the single bond correlation known as "single-quantum coherence" corresponding with single bond interactions. For example, ($^{1}H–^{13}C$) HMBC eliminates the single C–H bond correlation while correlating the chemical shift of H with C when separated by two or three bonds and, in some cases, with even more distant ones. Thus, ($^{1}H–^{13}C$) HMBC is usually used for the assignment of signals of quaternary and carbonyl carbons. The combination of HMBC with HSQC or HMQC provides a powerful approach for assignment of signals. Figure 11 shows both HMQC (red) and HMBC (blue), the seven red cross peaks being associated with the seven carbons in both *n*-propanol and *n*-butanol and the sixteen blue cross peaks being associated with the long bond correlation interactions. This figure demonstrates the power of combining and integrating the information from 2D NMR experiments for spectral assignment. The HMQC spectrum can be used to distinguish an overlapped signal from a separated one. For example, the proton multiplet signals observed around 0.92 ppm coupled with the two carbon peaks at 10.07 and

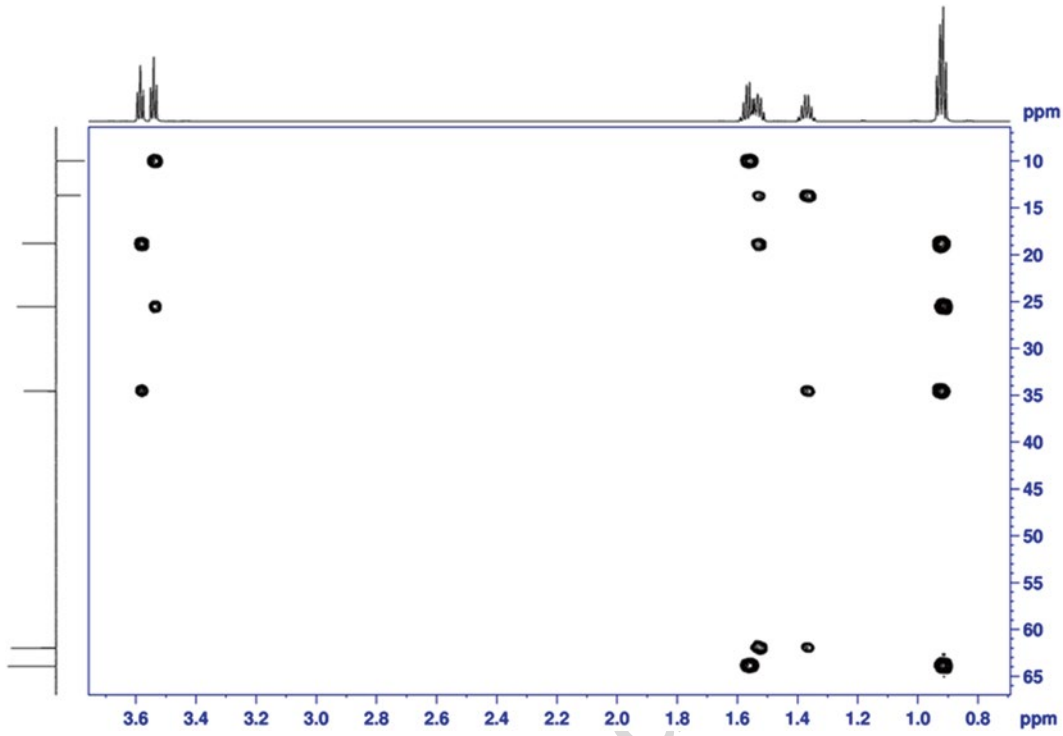


**Fig. 10** $^{13}C$-$^{1}H$ HSQC HMBC spectrum of a mixed sample composed of *n*-propanol and *n*-butanol in $CDCl_3$. The long correlation between the 1D proton spectrum (*top* projection) and the $^{13}C$ DEPT-135 spectrum (*see* Fig. 4, *left* projection) is illustrated



13.79 ppm confirm that the proton NMR signal is indeed two overlapping ones. Similarly, the proton multiplet around 1.55 ppm is coupled with two carbon signals, while each one of the remaining proton signals at 1.37, 3.54, and 3.58 ppm is coupled with only one carbon signal. Moreover, HMBC and HSQC spectra can be used to separate and assign *n*-butanol peaks from *n*-propanol ones. For instance, the carbon HSQC cross peak (red) connecting the carbon signal at 25.67 ppm with proton resonance at 1.564 is aligned with two HMBC cross peaks (black arrows), so assigning the three peaks to the *n*-propanol molecule while the HSQC (red) aligned with three more blue HMBC cross peaks assigns these peaks to the *n*-butanol molecule (green arrows; *see* Fig. 12). Figure 13 demonstrates example models of chemical bond connections of *n*-butanol that can be studied by using 2D experiments for both homonuclear correlation and heteronuclear correlation. Details of the way in which these 2D experiments can be used to assign the proton and carbon NMR spectra of *n*-propanol and *n*-butanol are presented and the complete proton and carbon assignments provided in Table 2.

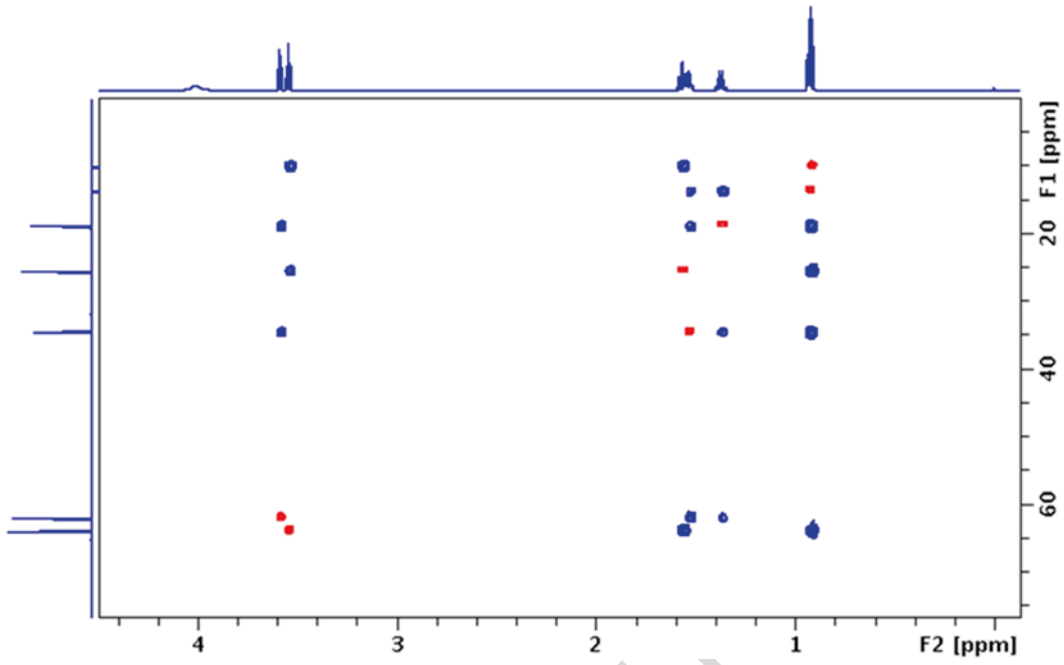


**Fig. 11** Stack plot of $^{13}C$-$^{1}H$ HSQC spectrum (*red*) and HMBC spectrum (*blue*) of a mixed sample of *n*-propanol and *n*-butanol in $CDCl_3$

### *3.2 Mass Spectrometry (MS)*

Mass spectrometry (MS) is a powerful technique used mainly for the identification of unknown compounds and for the quantification of known molecules within a sample [71, 141–146]. As for NMR [28, 57, 147–152] and X-rays [153–160], it can also be used for structural elucidation and for study of the chemical properties of materials under investigation [161–166]. Due to its high sensitivity and selectivity, MS provides an important analytical platform for profiling metabolites in mixed samples, such as biological samples. Moreover, MS can detect ions that do not contain protons or carbon, such as metal ions. However, no MS method is perfect for the detection of all classes of metabolites, and so more than one method must be employed for comprehensive metabolic profiling. Figure 14 shows the main components of the MS instrument with the different sources of ionization and types of mass analyzer that can be used for the detection of different classes of molecule. The advantages of using GC-MS, for example, include high separation efficiency and reproducible retention times that may be exchanged between different laboratories for data comparison using the retention index concept with retention time as a marker [167]. However, the inherent limitation of GC-MS is the fact that it detects only volatile compounds or compounds that can be derivatized to become volatile. Furthermore, MS cannot detect all metabolites, as some metabolites do not ionize with certain

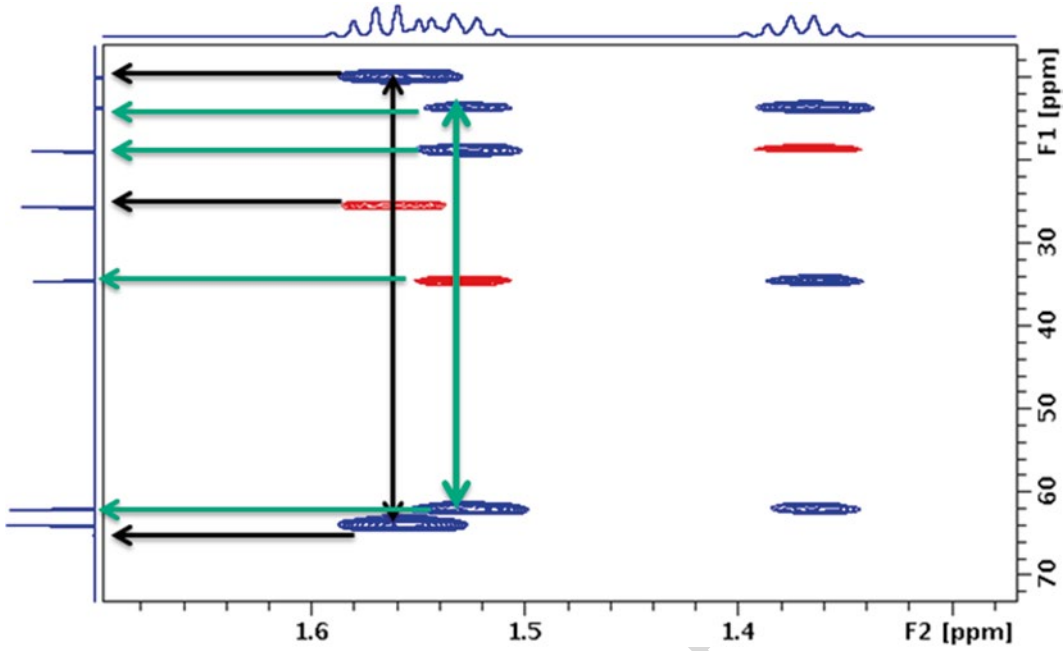


**Fig. 12** Extended region of Fig. 11

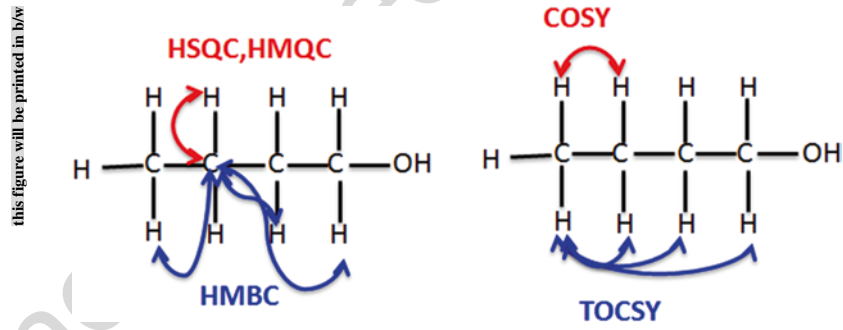


**Fig. 13** The bond connection of *n*-butanol that can be studied by using different 2D NMR experiments for both a homonuclear correlation and a heteronuclear correlation

ionization methods. The dynamic range of the MS detector is still only three to four orders of magnitude, whereas the range of metabolite concentration is usually much larger, and no detector exists that can detect all metabolites. A general challenge in the metabolic proofing of biological samples is the fact that many metabolites have not yet been fully identified. For example, among the 869 different metabolites that have been detected in tomato, 494 are not found in the common metabolite databases [168].

The number and class of metabolites that can be detected by mass spectrometry depend on the choice of ionization mode. No single ionization method can cover all metabolite classes, such as

t2.1 **Table 2**

t2.2 **Complete assignment of $^{1}H$ and $^{13}C$ NMR peaks for both *n*-propanol and *n*-butanol**

t2.3 **using data from a number of 1D and 2D NMR experiments**

| t2.4 | | Chemical shift of $^{1}H$ NMR signal ppm | Chemical shift of $^{13}C$ NMR signals ppm |
|---|---|---|---|
| t2.5 | $A_p$ | 0.916 | 10.01 |
| t2.6 | $A_b$ | 0.926 | 13.79 |
| t2.7 | $B_b$ | 1.370 | 18.95 |
| t2.8 | $C_b$ | 1.533 | 34.56 |
| t2.9 | $B_p$ | 1.563 | 25.67 |
| t2.10 | $C_p$ | 3.539 | 64.05 |
| t2.11 | $D_b$ | 3.584 | 62.13 |

t2.12 $CH_3–CH_2–CH_2–OH$ (*n*-propanol) $A_p$ $B_p$ $C_p$

$CH_3 - CH_2 - CH_2 - OH$ (n-propanol)

t2.13 $CH_3–CH_2–CH_2–CH_2–OH$ (*n*-butanol) $A_b$ $B_b$ $C_b$ $D_b$

$CH_3 - CH_2 - CH_2 - CH_2 - OH$ (n-butanol)

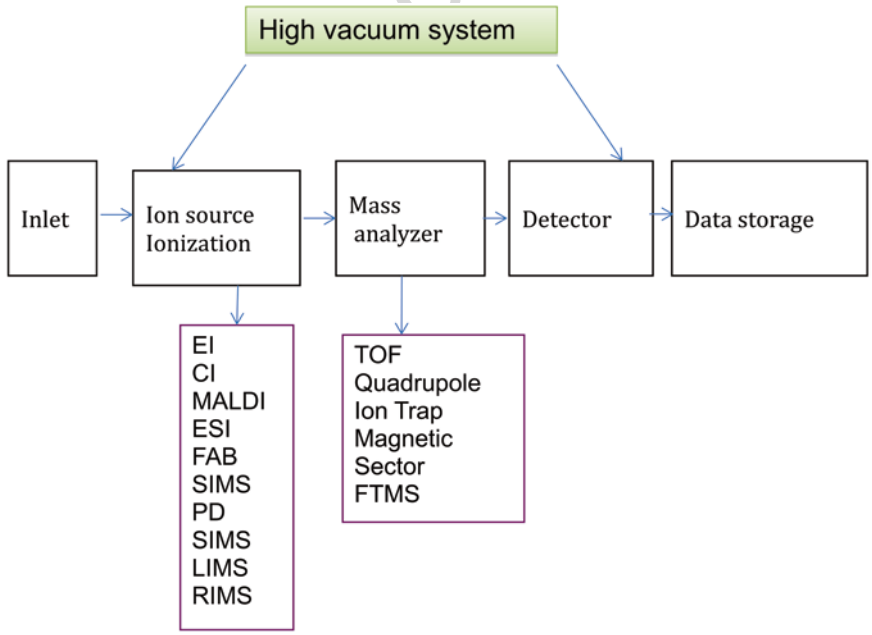


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**Fig. 14** Schematic plot of MS components including EI (electron impact), CI (chemical ionization), MALDI (matrix-assisted laser desorption ionization), ESI (electrospray ionization), FAB (fast-atom bombardment), SIMS (resonance ionization), PD (plasma-desorption ionization), LIMS (laser ionization), and RIMS (resonance ionization)

polar, nonpolar neutral, and ionic. Consequently, different ionization methods should be used independently to maximize the number of metabolites detected. For example, in LC-MS analysis, electrospray ionization (ESI) in positive mode is the most common mode that can effectively ionize a wide range of medium-sized polar molecules, whereas the negative ionization mode is more powerful for certain metabolite classes, such as carbohydrates and organic acids. For example, it was reported that use of both atmospheric-pressure chemical ionization (APCI) and ESI increased the coverage of the erythrocyte metabolome by 34 %. It has been reported that by using a set of different complementary methods of GC-MS and LC-MS up to 100–500 metabolites can be detected in a targeted analytical approach for blood samples, and about 600–1,000 can be detected in a fingerprinting mode [169–171]. It is important to note that the strategy for metabolite identification in LC-MS is different from that in GC-MS, in which usually only the molecular ion is detected and additional MS/MS experiments are required to gain information about the identity and structure of the metabolites.

#### *3.2.1 Liquid Chromatography–Mass Spectrometry (LC-MS)*

LC-MS comprises two powerful analytical tools, high-performance liquid chromatography (HPLC, known as high-pressure liquid chromatography) and mass spectrometry. When combined, LC-MS represents a very powerful analytical tool for the separation, identification, and quantification of molecules in a mixed sample. The HPLC technique separates molecules first based on different physical and chemical properties such as molecular size, charge, polarity, and affinity toward other molecules. As for other chromatography techniques, HPLC consists of a stationary phase and a mobile phase. The stationary phase involves the use of materials such as silica gel that slow down the movement of molecules to varying extents according to molecular size, so allowing separation of molecules based on size differences. The mobile phase comprises the solution containing the sample mixture, and this travels through the stationary phase (chromatography column) where separation of molecules occurs. Column chromatography can be used to purify individual chemical compounds from mixtures. Different samples require different columns, proteins, and peptide samples, for example, requiring different columns from those needed for samples of small molecules typical of metabolomics studies. Once the analytes are separated, they pass through the mass spectrometer analyzer where they are detected based on the mass-to-charge ratio, and the intensity of each resultant line corresponds to relative concentration of each molecule.

On the basis of its ability to separate and detect a wide range of molecules, LC-MS is probably the most widely used mass spectrometry technology, especially in the biosciences. LC-MS is a very adaptable

tool for carrying out the majority of metabolite profiling studies, allowing both quantitative and structural information to be obtained with high level of sensitivity. Different separation methods can be used to separate different classes of metabolite. For example, the reversed-phase (RP) gradient chromatography method has been the most commonly used separation method in LC-MS studies for global metabolite profiling [145, 172]. However, this is not the most appropriate method for polar and/or ionic species, which includes many important metabolites (organic acids or amino acids). These metabolites represent highly significant components in biochemical pathways, and their evaluation may be important in detecting critical metabolic states, such as inborn errors of metabolism and metabolic syndrome. Hydrophilic interaction chromatography (HILIC) is an alternative method that can be used to ionize polar metabolites, so increasing the breadth of metabolites detected [173]. In order to maximize the coverage of metabolites being profiled, the sample can be analyzed twice, either using RP and HILIC separately or using a column-switching approach of two-dimensional analysis in an "orthogonal" combination of HILIC and RP-LC [174–177]. Although the combined use of RP and HILIC is the preferable ionization method for many metabolites, this approach does not cover the whole range of metabolite polarities for biological samples such as urine [145]. Consequently, other ionization methods such as positive and negative electrospray ionization (ESI) modes and atmospheric-pressure chemical ionization (APCI) are recommended in order to maximize the breadth of detection of different metabolites in a biological sample [178]. Considering all these possibilities, analyses in eight different modes (eight separate runs) are required for comprehensive profiling of metabolites. These combinations widen the applications of LC-MS in metabolomics, and in fact, both targeted and nontargeted metabolomics analyses have increasingly been conducted using different methods of LC-MS [172, 179–188].

### 3.2.2 Gas Chromatography–Mass Spectrometry GC-MS

GC-MS is a novel tool for the analysis of volatile molecules, with a high-resolution and reproducible chromatographic separations due to the modern capillary GC, and these features render it well suited for the analysis of complex metabolic mixtures. As for LC-MS, GC-MS consists of two powerful analytical methods, gas chromatography and mass spectrometry. Together, these methods provide one of the most powerful methods of separation that can be used to provide qualitative and quantitative information about volatile compounds. The sample first goes through the gas chromatography unit where high-resolution separation of volatile organic compounds in a mixture is accomplished in the gas phase. The GC unit is composed mainly from columns, basically a tube which generally varies in length from less than 2 m up to 60 m or more, with a diameter ranging from 10 to 30 cm.

Different kinds of GC columns exist, such as packed and capillary tubes, which are designed to separate different kinds of samples. Packed tubes may be of stainless steel, glass, or fused silica and are usually formed as coils in order that they fit into an oven for high-temperature experiments, at around 250 °C. An inert gas, such as helium, is blown through the column; as the sample is inserted into the column, it becomes vaporized and the volatile molecules are pushed though the column by the helium. At the beginning, all the molecules move together but certain of them move slower than others, based on molecular weight and size. Smaller molecules travel faster than larger molecules, and as they progress through the column, the molecules continue to separate from each other and eventually emerge from the column as different components, so providing an effective approach to separation.

As the molecules exit the GC column, they are introduced into the MS unit where they are ionized using an ionization method such as an electron beam. The ions formed from a specific molecule will depend on the nature of that molecule, and both ionized molecules and ion fragments of the molecule are useable for distinguishing and identifying the components of a mixture at the molecular level based on the mass-to-charge ratio. Moreover, qualitative information about the components of a mixture can be obtained by measuring the absolute intensity of the peaks, where the highest peak is taken to represent 100 % abundance and used as reference for other peaks. Thus, GC-MS is the preferred analytical tool for the analysis of volatile metabolites and has been employed in different areas of metabolomics research including plant metabolomics and screening for inborn errors of metabolism [189–192]. In addition to well-established databases such as the Fiehn Metabolomics library, GC-MS provides good reproducibility and a highly reproducible fragmentation so offering a potent tool for the identification of metabolites. Other advantages include high sensitivity and resolution, low cost, and ease of use of instruments. The main limitation of GC-MS analysis is that it is limited to small volatile molecules, which means that this approach is of only limited application in global metabolic profiling studies. Moreover, the preparation of biological samples, such as bio-fluids, may be time-consuming and repetitive, this potentially leading to experimental error. Other problems such as product formation and degradation could occur during the ionization process. Moreover, during the derivatization reaction, nonvolatile metabolites could be converted into different forms of derivatives, leading to production of fragments, so that different forms of the same parent metabolite exist together. While analyzing real samples such as human urine which has a high variability in terms of metabolite content, derivatization may occur at different rates of conversion depending on the different properties of metabolites, so potentially

affecting reproducibility and overall results dramatically [193]. To overcome problems such as inaccurate quantification, a standard compound may be used for both derivatized standard compounds and for data correction processes, such as normalization.

To generate reproducible mass spectra and highly transferable EI-MS spectral libraries, use of standardized MS electron ionization energy of 70 eV is recommended to allow identification of compounds through mass spectral library matching, such as NIST and FiehnLib [194, 195].

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