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NMR Based Metabolomics

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Abstract

Nuclear Magnetic Resonance (NMR) spectroscopy is a major analytical method used in the growing field of metabolomics. NMR is relatively less sensitive relative to mass spectrometry, the other major analytical platform in the metabolomics field. However, numerous characteristics of NMR including its high reproducibility and quantitative abilities, its non-selective and non-invasive nature, and the ability to identify unknown metabolites in complex mixtures and trace the downstream products of isotope labeled substrates *ex vivo, in vivo or in vitro* offer numerous benefits to the metabolomics field. The analysis of highly complex biological mixtures has benefitted from advances in both NMR data acquisition and analysis methods. Although metabolomics applications span a wide range of disciplines, a majority have focused on understanding, preventing, diagnosing, and managing human diseases. This chapter describes NMR based methods relevant to the rapidly expanding metabolomics field.

Keywords

Nuclear Magnetic Resonance (NMR); Metabolomics; 1D NMR; 2D NMR; quantitation; isotope tagging; fast NMR methods; data analysis; statistical analysis

2.0 Metabolomics

The field of metabolomics involves the quantitative and simultaneous analysis of large numbers of metabolites in biological systems. Metabolites provide information on action, inaction or over action of the upstream molecular species such as genes, transcripts and proteins, in health and diseases. Analysis of complex metabolite data in combination with univariate and multivariate statistical methods, as well as mapping of altered pathways enables understanding of biological phenotypes, deciphering mechanisms, and identifying biomarkers or drug targets for a variety of conditions (Lindon and Nicholson 2014; Rhee and Gerszten 2012; Griffin et al. 2011; Nagana Gowda and Raftery 2013; Dang et al. 2014). Applications of metabolomics span a wide range of disciplines including health and various diseases, pharmacology, drug development, toxicology, environment, plants, and food and

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nutrition. However, a majority of the studies are focused on improving the mechanistic understanding, along with prevention, early diagnosis, and management of human health and diseases.

3.0 Analytical Methods

Analytical chemical methods are a major component of metabolomics research. Numerous types of analytical techniques have been used; however, nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are the two most commonly employed methods in the metabolomics field. MS is a highly sensitive method and it enables the analysis of several hundreds to thousands of metabolites from a single measurement and on a routine basis. In MS analysis, often, metabolites are subjected to chromatographic separation using liquid chromatography, gas chromatography or electrophoresis prior to detection. A variety of MS methods are often used for analysis of different classes of metabolites from the same samples to achieve a wider coverage of the metabolome. NMR spectroscopy, on the other hand, is often used without combining with any sample preprocessing or separation techniques and provides data complementary to MS. Peaks in the NMR spectra can be reliably assigned to specific metabolites and peak intensities are directly proportional to the number of contributing nuclei. Thus, NMR provides a wealth of information on both the identity and quantity of many metabolites in parallel.

4.0 Characteristics of NMR Spectroscopy

NMR spectroscopy exhibits numerous unique and favorable characteristics that are beneficial to the field of metabolomics (Nagana Gowda and Raftery 2014a, 2015, 2017a). Some of these important characteristics include:

1. NMR is highly reproducible and quantitative.
2. A single internal reference is sufficient for absolute quantitation of all metabolites in the spectrum.
3. NMR enables establishment of the identity for unknown metabolites, which is important considering that advances in analytical technologies have enabled the detection of an increasing number of signals in complex biological mixtures and many of them are unknown.
4. NMR enables the analysis of intact bio-fluids and tissue with no need for sample separation or preparation, which is important considering that sample preparation and separation processes contribute significantly to the analytical variability.
5. NMR is non-destructive, which means the sample remains intact after the analysis and can be used for reanalysis using NMR at a later time or other methods such as MS.
6. NMR enables tracing of metabolic pathways and measuring metabolic fluxes utilizing a variety of stable isotope labeled precursors.
7. NMR has the ability to detect metabolites through one or more types of atomic nuclei such as ^{1}H, ^{13}C, ^{31}P or ^{15}N.

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8. NMR does not involve harsh sample treatment prior to or during the analysis, which is important for analysis of metabolites such as glutamine and coenzymes that are fragile or sensitive to ionization voltage as used in the MS analysis (Trammell and Brennera 2013; Kumar et al. 2013, Purwaha et al. 2014; Nagana Gowda et al. 2015, 2016).

5.0 Workflow for NMR Based Metabolomics

Figure 1 shows a schematic diagram indicating a general workflow involved in NMR based metabolomics. Briefly, biological samples such as blood plasma or serum, urine, and tissue from humans, animal models or cells are used for metabolomics studies. Important steps involved are detection of metabolite signals, metabolite identification using a combination of 1D and 2D NMR methods, database searching and spiking with authentic compounds, and finally quantifying the identified metabolites using a single internal standard. Metabolite concentrations are then used for distinguishing diseases from controls; this is done generally based on univariate or multivariate statistical analysis, developing and validating classification models, and testing the sensitivity and specificity of the models based on the area under the receiver operating characteristic (ROC) curve. Additionally, metabolite concentrations are used for identifying altered metabolic pathways, which help provide a mechanistic understanding of cellular function including information on drug targets for therapeutic development, and translational opportunities for preventing or curing diseases.

6.0 Biological Samples

NMR based metabolomics studies use a wide variety of biological specimens. The most widely used biological specimen for investigation of virtually all human diseases is blood. The clinical relevance of blood arises from its close association with essentially every living cell in the human body combined with its relatively easy access for routine investigations. Generally, blood samples from overnight fasted subjects are preferred to avoid confounding effects from the diet. Conventional human blood metabolomics studies employing blood serum or plasma provide a wealth of metabolic information on health and diseases (Beckonert et al. 2007; Psychogios et al. 2011; Nagana Gowda et al. 2015). Blood serum and plasma metabolomics, however, lacks the ability to measure and evaluate important metabolites such as redox and energy coenzymes, as well as antioxidants, which are generally present in high concentrations in red blood cells. An important alternative to serum/plasma metabolomics is a whole blood metabolomics approach using NMR (Nagana Gowda and Raftery 2017b). Whole blood metabolomics enables access to a wider and complementary pool of metabolites and also avoids the potential confounding effects of hemolysis often encountered in serum/plasma based metabolomics.

Other biological specimens used in NMR-based metabolomics include urine (Emwas et al. 2015, 2016), saliva (Aimetti et al. 2012), cerebrospinal fluid (Wishart et al. 2008), gut aspirate (Bala et al. 2006), bile (Nagana Gowda 2011), amniotic fluid (Graca et al. 2008), synovial fluid (Lacitignola et al 2008), exhaled breath condensate (Bertini et al. 2014), intact tissue (Dietz et al. 2017), and tissue extracts (Kumar et al. 2014). Specimens from animal

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models, cell lines, yeast (Airoldi et al. 2015), bacteria (Lussu et al. 2017), tumor cells (Lane et al. 2017), and tumor spheroids have also been analyzed by NMR (Kalfe et al. 2015).

Urine is the most widely used biological specimen, apart from blood. Interest in using urine for NMR based metabolomics stems from the fact that it is a rich source of disease biomarkers and it can be obtained non-invasively. In addition, unlike blood, urine has a relatively low concentration of proteins and large number of low molecular weight compounds (metabolites); hence metabolomics studies of urine are relatively simple in terms of both sample preparation and NMR analysis.

Metabolic profiling of intact tissue has gained increased interest for investigations of human diseases. A major advantage of using tissue is that disease biomarkers are considered to be highly concentrated in tissue due to their close association with the pathological source, such as tumors. Importantly, biomarkers identified using tissue can be translated to biomarkers detection in the relatively easily accessible biofluids such as blood and urine. Technological advancements in NMR have reduced the amount of tissue needed to as little as a few nanoliters, which is beneficial for analysis of mass limited samples (Wong et al. 2012).

7.0 Sample Processing

One of the advantages of NMR is its ability to analyse intact samples with no need for sample processing. Specimens such as serum/plasma and urine have thus been widely used for the analysis without processing.

7.1 Blood Serum/Plasma

A major challenge for metabolite profiling of intact serum/plasma is the interference from a massive amount of serum/plasma proteins (6-8 g/dL). The unwanted macromolecular signals from proteins are typically suppressed using NMR experiments that use a T_2 (transverse relaxation) filter, such as the CPMG sequence (Beckonert et al. 2007); metabolites generally exhibit longer T_2 relaxation times compared to macromolecules and hence they are selectively retained by the CPMG sequence. Intact blood serum and plasma metabolomics has thus exploited the CPMG based NMR experiment for many years. While the analysis of intact serum/plasma is attractive, numerous limitations as shown below make this approach less suitable for metabolomics studies:

1. The number of metabolites detected using intact serum/plasma is restricted to about 30 or less, which is far fewer compared to the actual number of blood metabolites present in the sample (Psychogios et al. 2011).
2. Concentrations of many metabolites detected in intact serum or plasma are grossly underestimated due to the attenuation caused by metabolite binding to serum/plasma proteins (Figure 2(a)) (Nicholson and Gartland 1989, Chatham and Forder, 1999; Bell et al. 1988, Nagana Gowda and Raftery 2014b).
3. Residual macromolecules signals cause distorted spectral baseline in the NMR spectra, which deleteriously affect metabolite quantitation.

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4. Massive amounts of serum/plasma proteins cause reduced T_2 relaxation times for metabolite signals, which results in broader NMR peaks and affects spectral resolution.

5. The exchange between protein-bound and free metabolites results in significantly broadened NMR peaks, which adds to the line broadening and affects quantitative accuracy.

An alternative approach to overcome challenges arising from the interference of proteins involves physically removing serum/plasma proteins. Numerous methods have been explored to achieve the protein removal, which include using ultra-filtration, solid phase extraction, or protein precipitation using an organic solvent such as methanol, acetonitrile, acetone, perchloric acid or trichloroacetic acid (Wevers et al 1994; Daykin et al. 2002; Tiziani et al. 2008; Fan 2012). Such protein removal approaches enable significant improvements in the number of metabolites identified in blood. For example, based on the analysis of ultra-filtered serum, as a part of the investigation of the human serum metabolome, 49 metabolites could be analysed (Psychogios et al. 2011). In another study, based on ultra-filtered human plasma from NIST SRM (National Institute of Standards and Technology Standard Reference Material), 39 metabolites were identified (Simón-Manso et al. 2013).

Realizing the need to process serum/plasma before the analysis, additional efforts were focused on the development of a method for both optimal recovery of metabolites as well as expanding the number of quantifiable metabolites. A detailed quantitative assessment of the performance of protein precipitation methods and the ultrafiltration approach was made based on a comprehensive analysis using various NMR techniques. Both the protein precipitated and ultrafiltered serum detected metabolites with comparable reproducibility. However, nearly half of the metabolites in ultrafiltered serum exhibited lower concentrations ranging from nearly 10–75% (Nagana Gowda and Raftery 2014b) (Figure 2). Further experiments indicated that protein precipitation using methanol offers a more optimal approach for NMR-based metabolomics of blood serum/plasma. In addition, comparison of the serum NMR spectra obtained after protein precipitation using methanol and acetonitrile revealed a surprisingly poor performance for protein precipitation using acetonitrile (Nagana Gowda et al. 2015). Nearly one-third of the detected metabolites were attenuated up to nearly 70% compared to methanol precipitation at the same solvent to serum ratio of 2:1 (v/v). A further attenuation of nearly two-third of the metabolites (by up to 65%) was observed upon increasing acetonitrile to serum ratio to 4:1 (v/v). Therefore, serum or plasma precipitation using methanol to serum/plasma ratio of 2:1 (v/v) is recommended for NMR based metabolomics studies.

7.2 Urine

For urine analysis, no sample preprocessing is required due to the absence of macromolecules and hence, intact urine samples are generally used. It is of significance that the pH of normal human urine varies widely, from approximately 5 to 8 (Hernandez et al. 2001; Rylander et al. 2006; Welch et al. 2008). Many peaks in the urine NMR spectra are sensitive to the pH variation, and thus sample to sample variation in pH is therefore a

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major challenge in the analysis of urine. Therefore, urine samples are generally mixed with a phosphate buffer solution in D_2O typically in a 1:1 (v/v) ratio (at pH = 7.4). A detailed procedure for urine analysis by NMR, is provided in a comprehensive article published previously (Beckonert et al. 2007).

7.3 Cells and Tissue

Metabolites in biological specimens such as cells and tissue can be analyzed using two different methods. In one method, intact cells or tissue can be analyzed with no need for sample processing. Although this method is attractive, it has two major issues: (1) poor spectral resolution due to magnetic susceptibility inhomogeneity across the sample, as well as the undesired intra and intermolecular interactions; and (2) altered metabolite profiles due enzyme activity. To alleviate the first issue, sample tubes containing the biological specimens are spun at the magic angle (54.7° with respect to the magnetic field) during NMR data acquisition. To alleviate the second issue, the sample is generally maintained at low temperature (4° C) during the analysis. A different approach for analysis of cells and tissues is to extract the metabolites before analysis. This approach alleviates major challenges associated with the analysis of intact samples. Typically, the cells or tissue samples are homogenized in a cold water and methanol solvent mixture to extract aqueous metabolites. Addition of chloroform to the water-methanol mixture enables extraction of both aqueous and lipid metabolites, in a single step. This three solvent mixture forms two phases; aqueous metabolites dissolve in the top phase, which contains water and methanol, whereas lipid metabolites are dissolved in the bottom phase, which contains methanol and chloroform. The two phases are separated and solvents removed by drying. The dried residue containing aqueous metabolites is then dissolved in D_2O, whereas the residue containing lipids is dissolved in a mixture of deuterated chloroform ($CDCl_3$), deuterated methanol (CD_3OD) and water (D_2O) typically in 16:7:1 (v/v/v) ratio (which does not phase separate) for analysis using NMR.

8.0 NMR Experiments

Many NMR active nuclei such as ^{1}H, ^{13}C, ^{31}P and ^{15}N can be used to analyze metabolites in biological mixtures (Nagana Gowda and Raftery 2017a, Shanaiah et al. 2007; Ye et al. 2009; Tayyari et al 2013; Desliva et al 2009). However, ^{1}H NMR is most widely used because (1) ^{1}H is present virtually in all the metabolites and (2) it has a higher NMR sensitivity relative to other nuclei.

8.1 1D NMR Methods

One-dimensional (1D) NMR experiments are the most widely used in the metabolomics field, owing to the ease of use and high-throughput. The 1D NOESY (nuclear Overhauser enhancement spectroscopy) and CPMG (Carr-Purcell-Meiboom-Gill) are the most popular NMR experiments and are complementary in nature. 1D NOESY is used for samples that provide narrow line shapes such as urine, cells and tissue extracts, due to their low macromolecular content. The CPMG experiment, on the other hand, is useful for samples such as blood serum/plasma, which contain macromolecules such as proteins and lipids.

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Signals from the macromolecules are suppressed selectively by this experiment as these signals are not of interest for metabolomics studies.

8.2 2D NMR Methods

Two dimensional (2D) NMR experiments are increasingly used in metabolomics. Two major areas of 2D NMR metabolomics applications are unknown identification and improved metabolite quantitation. Unknown metabolite identification is a major issue in the metabolomics field and NMR represents the gold standard method. 2D NMR experiments are particularly well suited for the identification of unknown compounds. In addition, due to the fact that 2D NMR experiments significantly improve the spectral resolution and alleviate the peak overlap problem for complex biological samples, 2D NMR offers improved accuracy for metabolite quantitation. Statistically relevant changes in low abundant metabolites can be better characterized using 2D NMR compared to 1D NMR (Van et al. 2008). The most commonly used 2D NMR experiments involving only ^{1}H nuclei are correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY). 2D *J*-resolved spectroscopy is another type of 2D experiment used in metabolomics; it provides no additional peaks compared to 1D NMR, unlike COSY and TOCSY, but it greatly simplifies the NMR spectrum. Important 2D experiments that involve heteronuclei such as ^{13}C or ^{15}N are heteronuclear single quantum coherence spectroscopy (HSQC), heteronuclear multiple quantum correlation spectroscopy (HMQC) and heteronuclear multiple bond correction spectroscopy (HMBC). A challenge for heteronuclear 2D experiments is the low natural abundance of the ^{13}C and ^{15}N; while the 2D experiments involving the natural abundance ^{13}C require significantly increased data acquisition time, those involving the natural abundance ^{15}N are currently largely impractical to use in the metabolomics field owing to its extremely lower sensitivity. Nevertheless, isotope labeled experiments (described below) do provide opportunities to measure ^{15}N containing compounds. When compared to 1D NMR, 2D NMR experiments generally involve longer data acquisition times, larger data size and less convenience for data analysis.

8.3 NMR Techniques for Analysis of Mass Limited Samples

Use of micro-coil probes offer additional sensitivity for NMR detection and are particularly useful for mass limited samples (Lacey et al. 1999; Ravi et al. 2010, Grimes and O'Connell 2011; Bird et al. 2012). Various analysis methods using mirco-coil NMR include online detection of eluted fractions from the liquid chromatography (LC), LC followed by online pre-concentration and micro-coil NMR detection, and LC followed by offline detection (Cloarec et al. 2007; Djukovic et al. 2006; 2008). Commercially available micro-coil probes integrated with automation enable high throughout analysis and are well suited for large cohorts of small volume samples. Recently, as an important alternative to conventional 5 mm and 3 mm NMR probes, a commercially available 1.7 mm micro-coil probe is gaining interest for metabolomics applications. Cryoprobes offer further enhancement to the sensitivity by a factor of 3–4 compared to room temperature probes. A combination of cryoprobe and micro-coil technologies offers an order of magntitude reduction in the data acqusion time. It is important, however, to remember that sample preparation for micro-coil NMR experiments can be challenging as sample pre-concentration can result in the loss of linear response among the metabolites due to their varied solubilities (Grimes and O'Connell

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2011). Thus, while the use of micro-coil NMR offers significant enhancement in sensitivity, care should be exercised while concentrating samples, online or offline, for enhancing the sensitivity.

8.4 Fast Data Acquisition Methods

A number of approaches have been used to speed the acquisiton of NMR data. Important developments in fast acqusition methods include non-linear sampling and forward maximum entropy resconstruction, which offers significant reduction in data acquisition times (Hyberts et al. 2007, 2012). Using this approach for 2D HSQC experiments, a reduction in acquisition time of an order of magnitude was achieved (Hyberts et al. 2007). Separately, nonlinear sampling and forward maximum entropy reconstruction was applied to 2D HSQC experiments in combination with J-compensation to achieve more than a 20-fold reduction in data acquisition time [Rai and Sinha 2012]. Another approach that speeds up the data acqusition is the SOFAST (band-selective optimized flip angle short transient) technique, in which 2D data are acquired in a few seconds; fast acqusition in SOFAST is achieved through the enhancement of the steady state magnetization by combining an accelerated T_1 relaxation time and optimized flip angle (Pervushin et al. 2002; Ernst et al. 1987). SOFAST HMQC (heteronuclear multiple quantum correlation) with its capability to acquire data within 15 s enables real time metabolism studies in live cells (Motta et al. 2010). Somewhat recently, the SOFAST HMQC was combined with nonlinear sampling to acquire serum and urine spectra at natural ^{13}C abudance with seven fold reduced time compared to the conventional hetronuclear 2D experiment (Ghosh et al. 2017). Covariance NMR spectroscopy is another fast acquisition approach, which provides high resolution 2D NMR spectra with minimal data points in the indirect dimension (Bruschweiler and Zhang 2004). An altogether different class of NMR experiments that speeds up the data acqusition is the so-called utrafast NMR techniques. Here, the 2D NMR spectrum is acquired in a single scan with a sub-second data acquisition based on the application of field gradients that divide the sample into different segments; the NMR signals from these segments are then acquired in parallel using magnetic resonance imaging type acquisition. Applications of this approach to areas including metabolomics have been demonsrated (Giraudeau and Frydman 2014).

8.5 Ultra Sensitive NMR Methods

Hyperpolarization methods such as optical pumping of ^{3}He or ^{129}Xe, parahydrogen induced polarization (PHIP), and dynamic nuclear polarization (DNP) are shown to boost NMR sensitivity by several orders of magnitude. Of these, PHIP and DNP have been shown to be promising for metabolomics applications. Parahydrogen produced by PHIP can be transferred to other spins by chemical synthesis using an unsaturated compound or by transfer of magnetization to metabolites via a catalyst (Adams et al. 2009; Reile et al. 2016). PASADENA (parahydrogen and synthesis allow dramatically enhanced nuclear alignment) is a commonly used PHIP method (Bhattacharya et al. 2007, Chekmenev et al. 2009; Shchepin et al. 2012). DNP is an especially promsing signal enhancement method for metabolomisc applications because, unlike PHIP, DNP enables hyperpolarization of a very wide range of substrates. DNP uses paramagnetic centers to transfer polarization from electron spins to nuclear spins of substrates (Frydman and Blazina 2007; Mishkovsky and Frydman 2008; Saunders et al. 2008). The dissolution DNP approach starts with nuclear

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spin polarization in the solid state at low temperature, after which the sample is liquified, transported and then injected into a high resolution NMR spectrometer for detection. This approach promises new avenues for real time metabolism studies (Ardenkjaer-Larsen et al. 2003, Ardenkjaer-Larsen 2016). Some drawbacks of DNP are the long hyperpolarization preparation time, the need for an expensive polarizer, and short relaxation times of a number of biologically interesting substrates (which limits signal intensities for metabolic tracer studies). Progress is being made on multi-sample polarization approaches that promise high-throuput studies using dissolution DNP (Ardenkjaer-Larsen 2016)

9.0 Isotope Labeling Methods

NMR methods involving isotope incorporation *in vivo* or *ex vivo* offer unique opportunities to the metabolomics field. These methods offer a combination of selectivity, sensitivity and resolution, and alleviate major challenges in NMR experiments involving low natural abundant nuclei. Numerous isotope labeling studies using nuclei such as ^{13}C, ^{15}N, ^{2}H and/or ^{31}P have so far been reported.

9.1 Isotope Labeling in Flux Measurements

Isotope labeling *in vivo* enables the tracing of metabolic pathways and measurement of fluxes through specific pathways. Using this approach, the same metabolite that appears and flows through multiple pathways can be identified with a particular flux or pathway, unlike the traditional metabolic profiling appraoch that measures the overall metabolite intensitiy and lacks such an ability. As an illustration, lactate can be formed by the catabolism of glucose or through pathways unconnected to glycolysis. Pyruvate arising from glycolysis can be distinguished from that arising from several other pathways by treating cells with ^{13}C labeled glucose, for example, and measuring the ^{13}C labeled pyruvate. Numerous pathways including glycolysis, glutaminolysis and TCA cycle can thus be investigated using NMR and isotope labeled substrates such as ^{13}C-glucose and $^{13}C/^{15}N$-glutamine (Wise et al. 2008; Lloyd et al. 2004; Lane and Fan 2007). Understanding the alterations of these pathways under different conditions and diseases is important owing to the fact that catabolism of glucose and glutamine is critical for the viability and growth of mammalian cells (Coles and Johnstone 1962; Eagle 1955). Cancer cells have been shown to depend on high rates of glucose and/or glutamine uptake and metabolism to maintain their viability (DeBerardinis et al. 2007; Wise et al. 2008). While, *in vivo* isotope labeled studies using cells enable understanding of metabolic pathways under controlled conditions, the use of animal models or humans can translate the findings from cell studies to the pathogenesis in the relevant organs (Fan et al. 2009; 2011; Locasale et al. 2011; Lane et al 2011).

9.2 Isotope Labeling in Plants/Organisms

Generally, the high level of biological complexity continually demand new approaches for unraveling such complexity. NMR methods combined with isotope labeling *in vivo* in plants and organisms such as bacteria and yeast offer significant enhancement to spectral resolution and the detection sensitivity (Zhang et al. 2012; Chikayama et al. 2008; Bingol et al. 2013; Bingol et al. 2012). In particular, *in vivo* labeling enables a systematic analysis of a large number of metabolites (including novel metabolites) using conventional high-resolution 2D

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NMR experiments such as HSQC. In addition, owing to the uniform labeling of metabolites using nuclei such as ^{13}C, the approach also enables characterization of metabolites based on homonuclear 2D ^{13}C NMR experiments, whereas it is generally impractical to perform such experiments under natural ^{13}C abundance. Carbon-bond topology networks obtainable from the homonuclear 2D ^{13}C experiments provide additional avenues for unknown metabolite identification (Chikayama et al. 2008; Bingol et al. 2012).

9.3 *Ex vivo* Isotope Labeling

Isotope labeling *ex vivo* selectively targets different classes of metabolites based on the specific functional group (Shanaiah et al. 2007; Desilva et al 2009; Ye et al. 2009). Derivatization of metabolites using substrates containing isotope labeled nuclei, such as ^{13}C, ^{15}N and ^{31}P offers benefits in terms of both sensitivity and resolution, owing to the high isotopic abundance and wide chemical shift dispersion of tagged heteronuclei (Figure 3). 2D NMR experiments involving heteronuclei generally provide a single peak for each tagged metabolite, devoid of multiplicity, which further adds to the sensitivity and resolution. Numerous substrates for isotope tagging have been used to date focusing on metabolite classes such as amines, carboxylic acids and hydroxyls (Shanaiah et al. 2007; Desilva et al 2009; Ye et al. 2009). The “smart isotope tag” ^{15}N-cholamine targets carboxylic acids containing metabolites and can detect the same metabolites using both NMR and MS methods (Tayyari et al. 2013). This is because, the smart isotope tag possesses an NMR sensitive isotope (^{15}N) that offers good chemical shift dispersion, and a permanent positive charge that improves MS sensitivity. Use of this smart isotope tag approach enables direct comparison of NMR and MS data for the same samples and hence allows exploitation of the combined strengths of the two analytical platforms.

10.0 Data Analysis

Analysis of complex NMR data in metabolomics is made using one of the two major approaches: One is a global chemometric analysis and the other is quantitative analysis, referred to as quantitative metabolomics (Djukovic et al. 2013).

10.1 Chemometric Analysis

Chemometric analysis is a traditional method in untargeted or global metabolomics, in which metabolites are not identified initially. Instead, the complex data are directly used for statistical analysis. Prior to the analysis, the data are subjected to preprocessing such as baseline correction, peak alignment and solvent peak removal. Finally, the data are subjected to multivariate analysis. A challenge to the chemometric approach, however, is that often sample classes are differentiated based on minor spectral features, which needs to be addressed by appropriate data scaling or filtering. In addition, imperfect peak alignments and spectral baselines pose significant challenges to the analysis. Peak misalignment is particularly pronounced for biological samples such urine, for which peak positions are sensitive to sample conditions such as pH, ionic strength, temperature and concentration of macromolecules (Lauridsen et al. 2007; Asiago et al 2008).

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Multivariate statistical approaches are broadly classified into two categories: (1) unsupervised analysis and (2) supervised analysis. In unsupervised analysis, the sample class identity is not known, whereas in supervised methods the sample class information (example, disease or control) is provided as an input prior to the analysis. Detailed descriptions of multivariate statistical analyses are widely available (Johnson and Wichern 2007; Zhou et al. 2001; Brereton, 2010; Krzanowksi, 2000).

10.1.1 Unsupervised Analysis—Unsupervised analysis is often used in the exploratory research for hypothesis generation. Several methods including principal component analysis (PCA), hierarchical cluster analysis (HCA), k-nearest neighbor (KNN), and factor analysis are used (Brereton 2003). However, PCA is the most widely used among these in the metabolomics field (Lindon et al. 2001). Using PCA, it is relatively straightforward to detect potential outliers and clusters in the whole sample set. It transforms metabolites data into a set of ranked principal components (PCs). The variance in PCs can then be visualized through the 'scores' plot and specific variables that cause such variance are visualized through the "loadings" plot. The variables' identities may not be known and hence further analysis can be required to establish their identities (Gu et al. 2007). HCA, another unsupervised method, is most useful for comparing a small number of variables. It defines natural clusters based on the distances between pairs of samples or variables within the data set. The smallest distances between samples imply that this subset of samples share similar metabolite levels and signify that the samples exhibit similar physiological properties or disease states.

10.1.2 Supervised Analysis—Supervised statistical analysis methods are used for developing predictive models; they take into account the sample class (for example, disease or control), which are used as dependent variables, as well as the metabolites used as independent variables. Partial least squares discriminant analysis (PLS-DA) (Barker and Rayens 2003), often combined with orthogonal signal correction (Beckwith-Hall et al. 2002), is by far the most popular method used in metabolomics. This is in part because PLS-DA can handle well the inherent correlation among metabolite variables. Other methods, such as logistic regression, soft independent modeling of class analogies (SIMCA), random forests, and neural networks are also used as supervised methods in metabolomics. Somewhat similar to PCA, in PLS-DA, each orthogonal axis is referred to as a latent variable (LV) and the LVs contain the combinations of weights of each metabolite variable. Based on the LVs, putative biomarker variables can be identified. Supervised methods need extensive validation using a "set-aside" part of the same data set or, ideally, a separate data set to test the predictive model. The model is evaluated, typically, by single or multiple cross validation steps, to test the robustness of putative variables (biomarker candidates) (Johnson, and Wichern 1999). The successful use of additional sets of samples, preferably from independent sources, which are sufficiently large to yield statistically significant results is a current challenge in metabolomics.

10.2 Quantitative Analysis

Quantitative analysis, on the other hand, involves metabolite identification and quantitation, which may then be followed by multivariate statistical analysis. Pathway analyses is also

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made based on the obtained distinguishing metabolites. Quantitative analysis is generally a targeted method wherein the metabolites are first identified based on the literature or databases of standard compounds. The identified metabolite peaks are then quantified using internal or external reference compounds. Such quantitative data become the input variables for multivariate statistical analysis. A major benefit of the quantitative analysis approach compared to global chemometric analysis is that it can reduce potential errors arising from factors such as baseline distortions, strong solvent signals and peak misalignments. Hence the quantitative metabolomics approach promises numerous benefits including reliable insights into the mechanistic understanding of diseases.

Recent advances have expanded the pool of metabolites quantifiable by NMR in various biological specimens including blood serum/plasma, whole blood, tissue and cells, and thus offer new avenues in the quantitative metabolomics field.

10.2.1 Quantitative Analysis of Metabolites in Serum, Plasma and Whole Blood—Metabolite profiling of human blood serum/plasma is of major interest for the investigations of virtually all human diseases. Despite this importance, for many years metabolomics analysis of blood was largely restricted to serum/plasma. A significant challenge for widespread quantitative metabolomics of blood by NMR was limited number of metabolites that could be identified and quantified. Limited resolution and sensitivity combined with the challenges associated with unknown metabolite identification have long restricted both the number and the quantitative accuracy of blood metabolites. The origin for such a limitation was due to the practice of performing blood serum/plasma analysis in their intact form, which invariably met with interference from a vast amount of serum/plasma proteins. In contrast, removal of the proteins, physically, prior to the analysis improved resolution and sensitivity, dramatically (Wevers et al 1994; Daykin et al. 2002; Tiziani et al. 2008; Fan 2012) and enabled significant improvement to quantitation in terms of the number of metabolites. Subsequent developments, have enabled optimized protein removal methods (Nagana Gowda and Raftery 2014b) (Figure 2). Use of this optimized method has resulted in the identification of the vast majority of peaks in the NMR spectrum and identification and quantitation of nearly 70 blood serum/plasma metabolites from a single 1D NMR experiment (Nagana Gowda et al. 2015). Characteristic peaks for the identified metabolites were annotated in the NMR spectrum to enable their identification and quantitation, routinely, even for beginners to the metabolomics field (Figure 4). The ability to analyze such a vast pool of metabolites by NMR, quantitatively, promises significant advances in the quantitative metabolomics area. Further, and more recently, quantitative analysis of blood serum/plasma by NMR was extended to whole blood (Nagana Gowda and Raftery 2017b). Whole blood analysis now enables analysis of many major coenzymes and antioxidants, as described in the following section, in addition to the other metabolites, and has extended the total number of metabolites quantified in blood to nearly 80.

10.2.2 Quantitative Analysis of Major Coenzymes/Antioxidants in Blood, Tissue and Cells—Recently, NMR analysis has been extended to the measurement of a series of coenzymes, including coenzyme A, acetyl coenzyme A, coenzymes of cellular redox reactions and cellular energy, as well as antioxidants in blood, tissue and cells

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on one step (Nagana Gowda et al. 2016; Nagana Gowda and Raftery 2017b; Nagana Gowda 2018; Nagana Gowda et al. 2019). These species include: the major cellular redox coenzymes NAD^+ (oxidized nicotinamide adenine dinucleotide), NADH (reduced nicotinamide adenine dinucleotide), $NADP^+$ (oxidized nicotinamide adenine dinucleotide phosphate) and NADPH (reduced nicotinamide adenine dinucleotide phosphate); major energy coenzymes ATP (adenosine triphosphate), ADP (adenosine diphosphate) and AMP (adenosine monophosphate); and antioxidants GSSG (oxidized glutathione) and GSH (reduced glutathione). Increased interest to develop methods to analyze the coenzymes/antioxidants in one step stems from the fact that they are fundamental to the function of all living cells and hence are extremely relevant to mechanistic studies in health and virtually all human diseases. Analysis of these coenzymes in one step using the highly sensitive method of mass spectrometry is challenging owing to factors such as ion suppression, unit mass difference between many coenzymes, and in source fragmentation (Nagana Gowda et al. 2016). Another major challenge unconnected to the analytical platform is the extremely unstable nature of the coenzymes; many coenzymes, depending on the sample harvesting and extraction procedure used, evade detection altogether or their levels attenuated significantly. Recent methodological developments in both sample harvesting, processing and NMR analysis have alleviated the major challenges and enabled their analysis in one step (Nagana Gowda et al. 2016; Nagana Gowda and Raftery 2017b; Nagana Gowda et al. 2019) (Figure 5). For blood, the coenzymes and antioxidants were detected only in whole blood and not in serum or plasma as shown in Figure 5 indicating that they are endogenous to the blood cells. Nearly half of the blood volume is cells and more than 99% of these are red blood cells (RBCs) and hence the measured coenzymes in whole blood represent their levels in RBCs. The newly reported method offers numerous opportunities in the metabolomics field. An additional advantage of measuring the coenzymes/antioxidants by NMR is that the method also provides quantitative data for a large pool of other metabolites with little additional effort.

11.0 Summary

In summary, due to its unique capabilities, NMR spectroscopy plays a key role in the growing metabolomics field, despite its lower sensitivity and resolution compared to the other widely used analytical platform, mass spectrometry. NMR based metabolomics offers opportunities to understand systems biology, discover biomarkers and potential therapy targets, and translate laboratory findings to clinical applications. Numerous efforts focused on alleviating the sensitivity and resolution bottlenecks in NMR have enabled identification and quantitation of an expanded pool of metabolites and led the developments that now promise monitoring of metabolism in real time. NMR based metabolomics approaches, however, are not devoid of limitations. Owing to the increasingly realized complexity of biological mixtures, reliable detection, unknown identification and quantitation continue to pose major challenges. However, continued, multifaceted efforts to boost sensitivity, resolution and the speed of data acquisition, and to improve quantitative accuracy promise to alleviate the current challenges. With constant advances in the field, NMR-based metabolomics is anticipated to continue greatly impacting the understanding of systems

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biology and to help make progress in the treatment and management of a range of human diseases.

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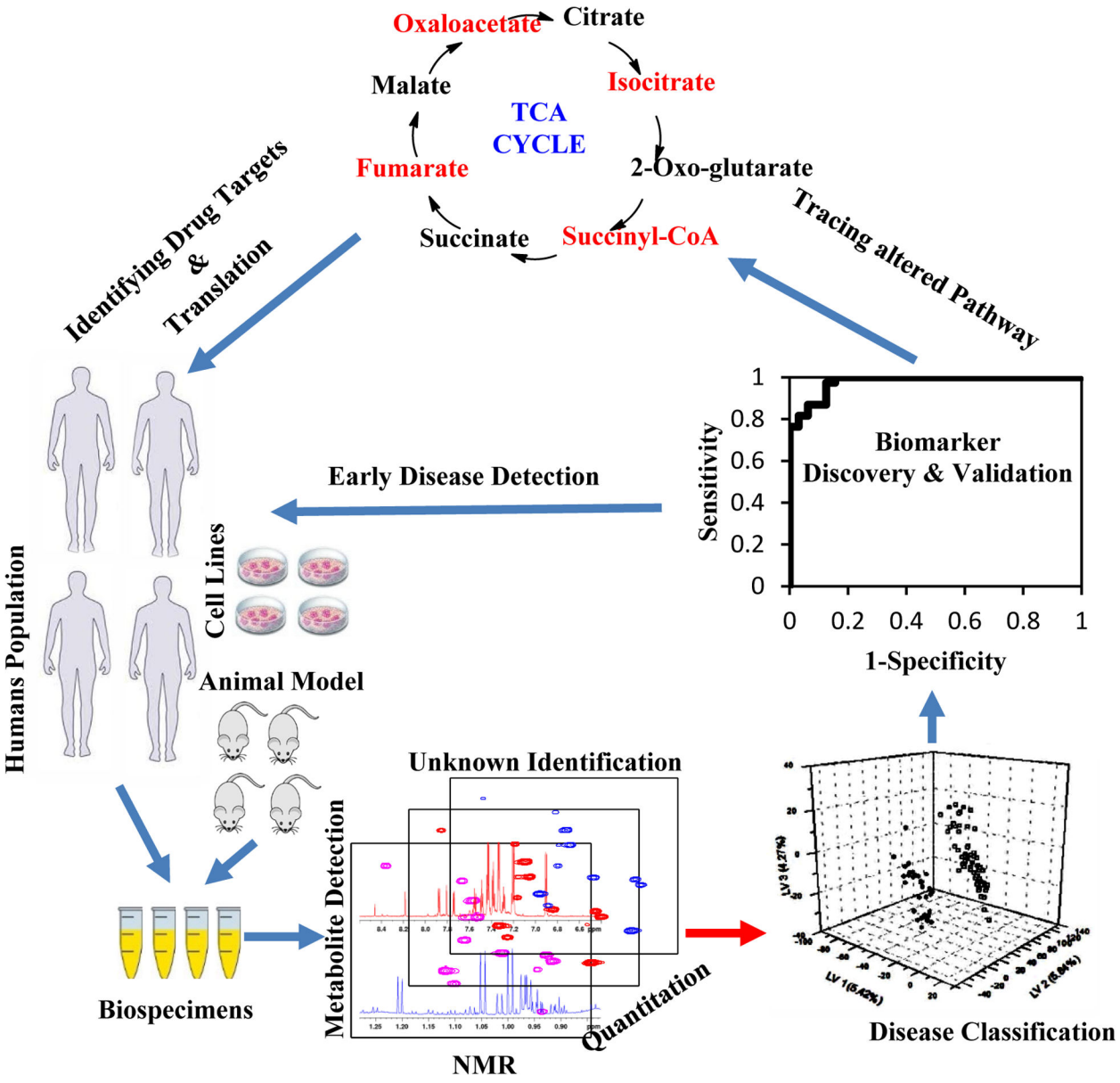

Figure 1.
Schematic diagram describing the workflow of NMR based metabolomics

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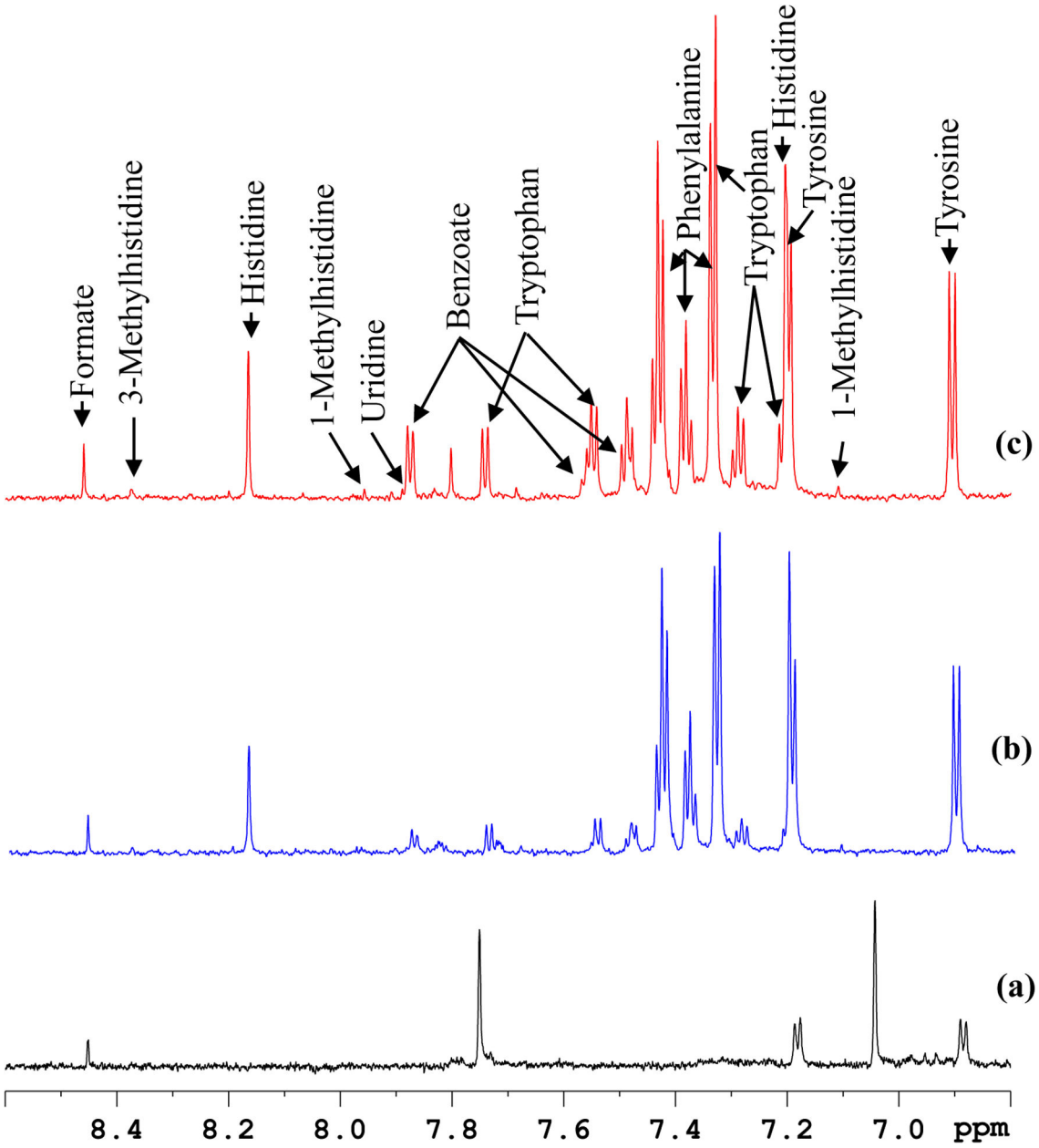

Figure 2:

Comparison of a portion of ^{1}H NMR spectra of the same pooled human serum sample obtained by suppressing protein signals by **(a)** T_2 filtering using the CPMG pulse sequence; **(b)** ultrafiltration using a 3 kDa molecular weight cut-off filter; and **(c)** protein precipitation using methanol (1:2 v/v). In **(a)** most of the metabolite signals are missing or significantly attenuated, while in **(b)** many metabolites including tryptophan, benzoate and formate are significantly attenuated when compared to **(c)** (Reproduced with permission from Nagana Gowda et al. 2014b).

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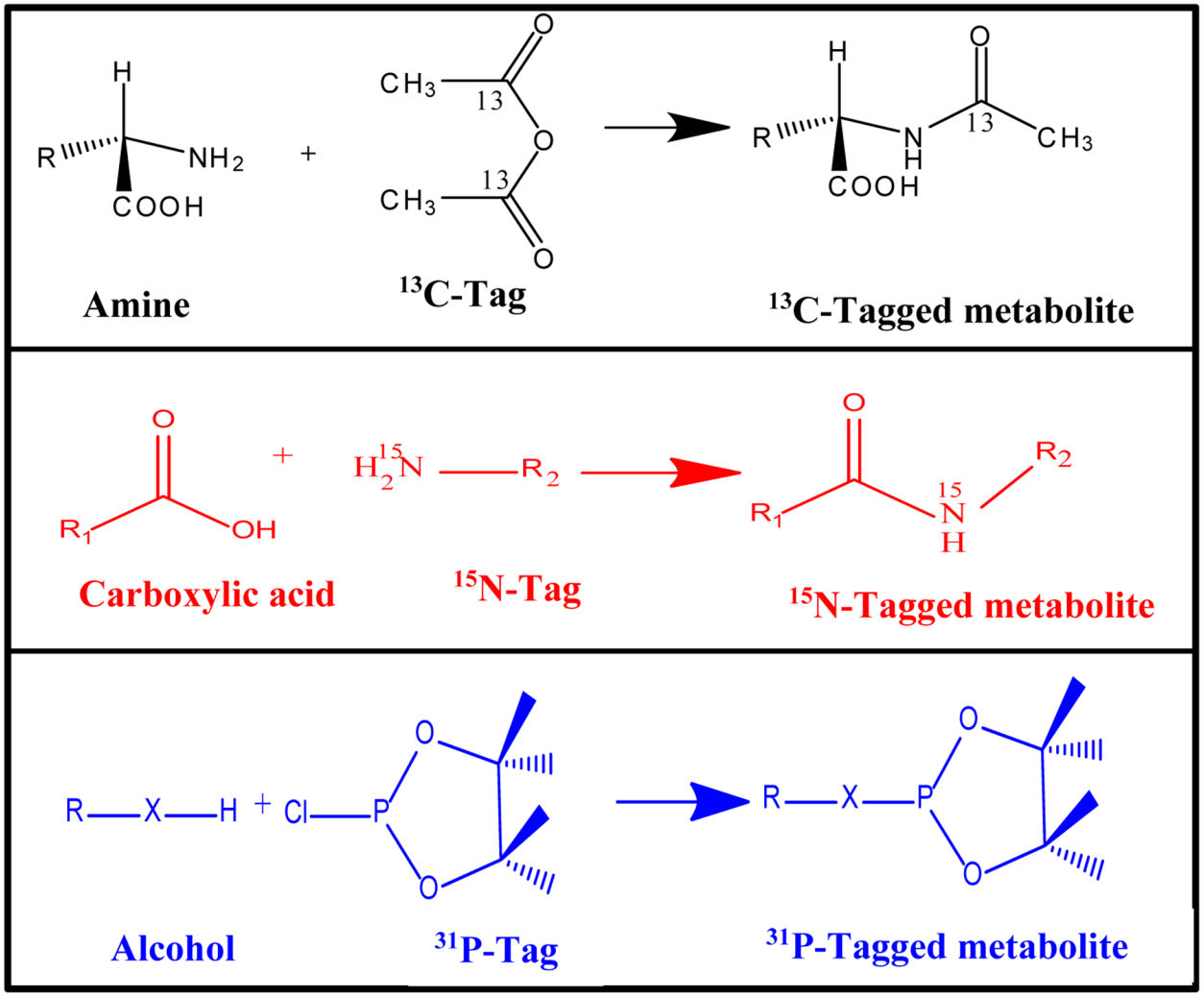

Fig. 3:

Reaction schemes for chemical derivatization using ^{13}C, ^{15}N or ^{31}P tags to target amine, carboxylic acid or hydroxyl group containing metabolites in complex biological mixtures that enable detection of metabolites by NMR with enhanced resolution and sensitivity (Reproduced with permission from Nagana Gowda and Raftery 2015).

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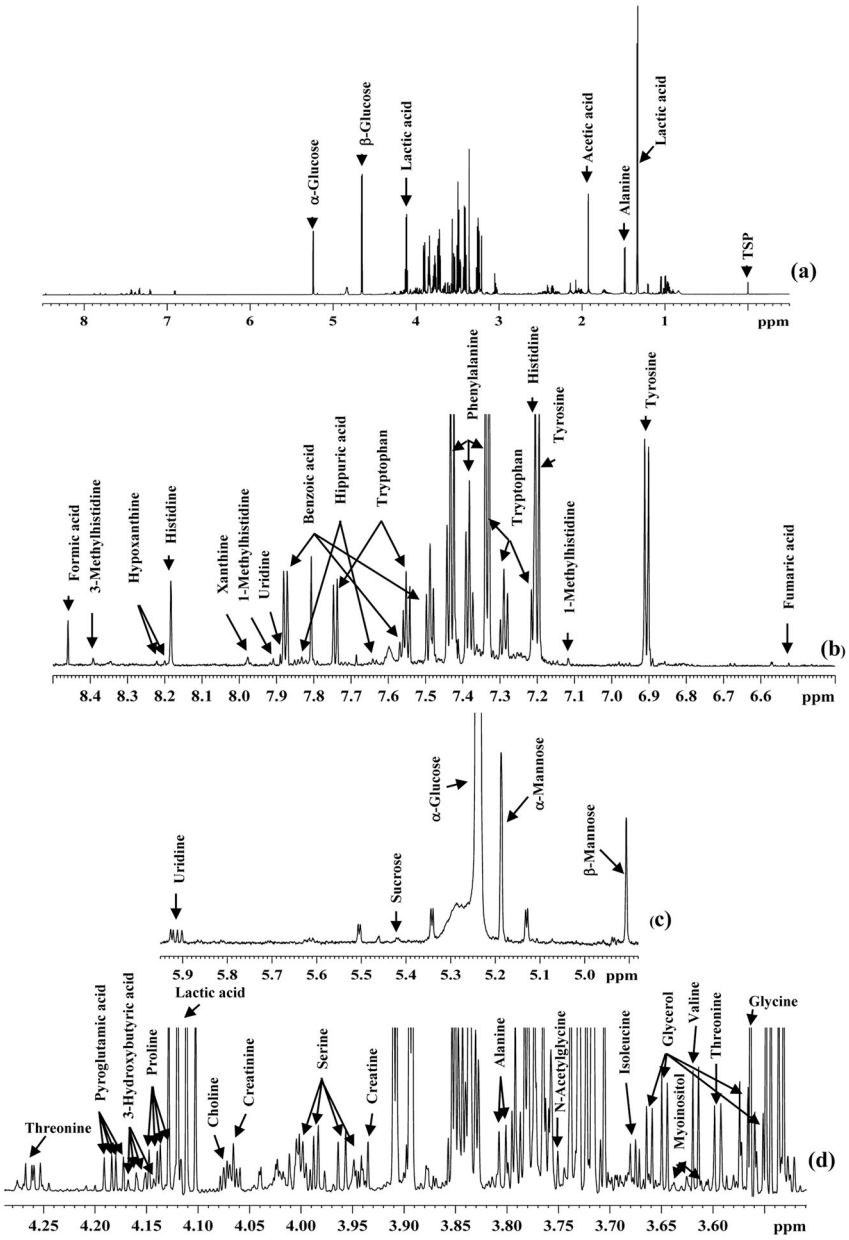

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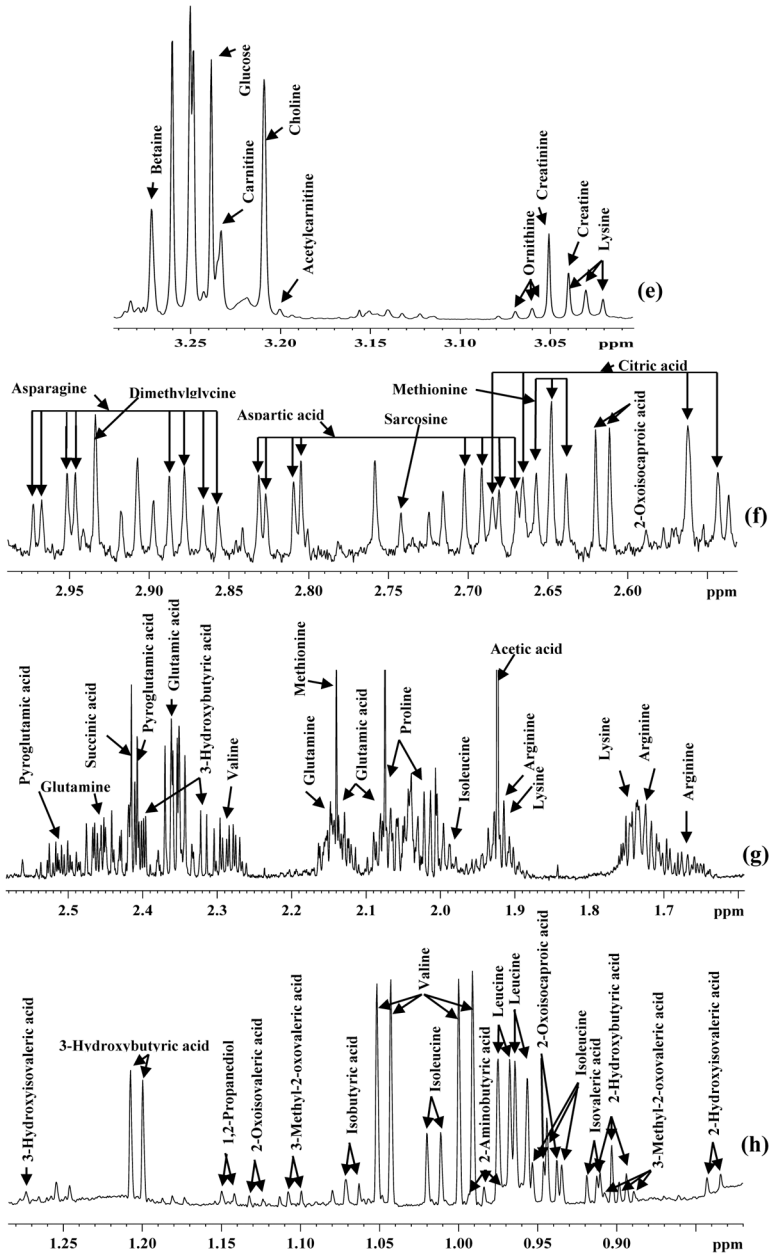

Figure 4.

(a) A typical 800 MHz 1D CPMG ^{1}H NMR spectrum of a human serum obtained after protein precipitation using methanol with expanded regions (b–h) and annotations for all identified metabolites (modified from Nagana Gowda et al. 2015)

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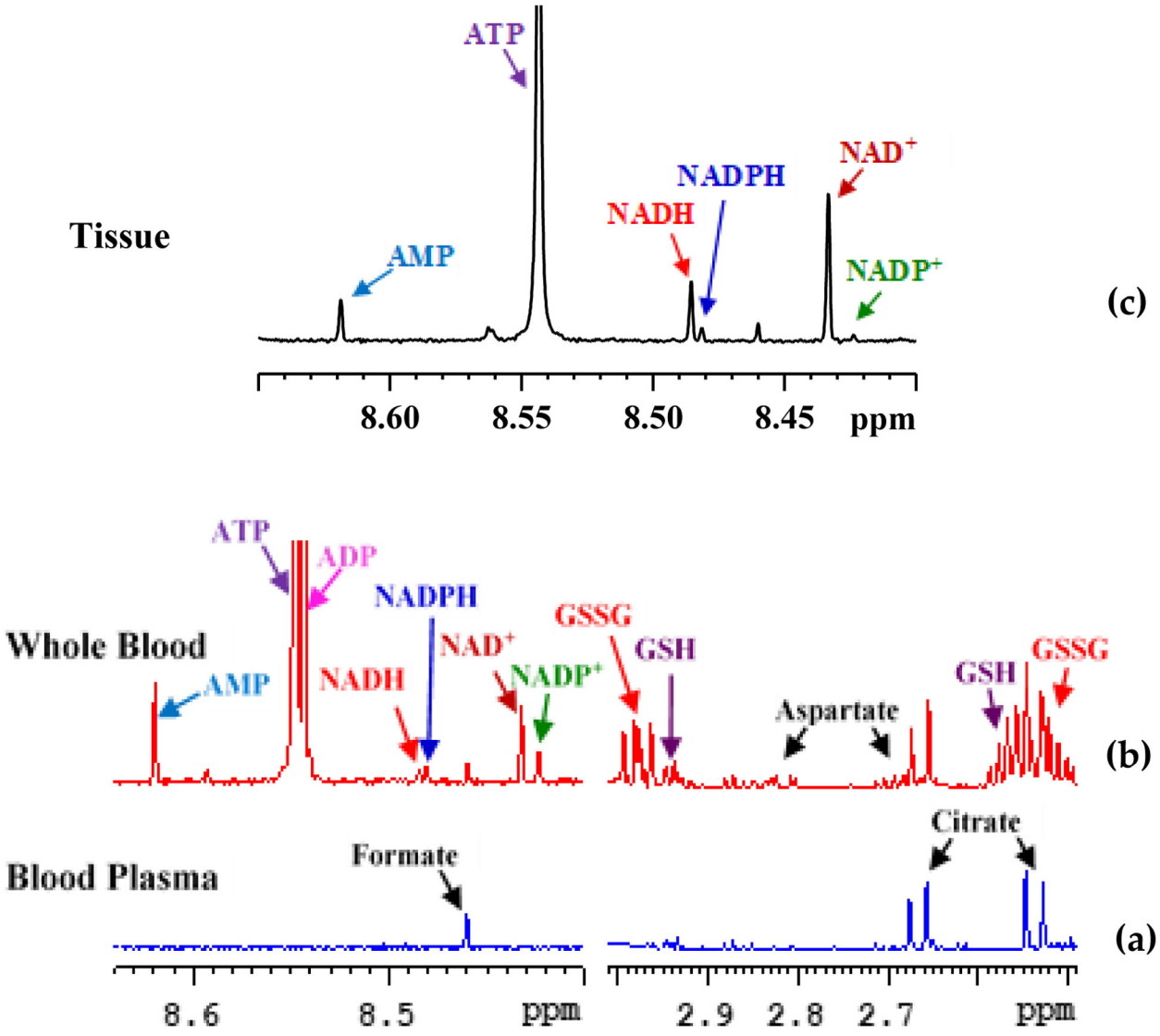

Figure 5.

Portions of typical 800 MHz ^{1}H NMR spectra of extracts of **(a)** blood plasma, **(b)** whole blood and **(c)** mouse heart tissue. Identification of redox and energy coenzymes and antioxidant in the extract of whole blood/tissue is indicated. NAD^{+}: nicotinamide adenine dinucleotide, oxidized; NADH: nicotinamide adenine dinucleotide, $NADP^{+}$: nicotinamide adenine dinucleotide phosphate, oxidized; NADPH: nicotinamide adenine dinucleotide phosphate, oxidized; ATP: adenosine triphosphate; ADP: adenosine diphosphate; AMP: adenosine monophosphate; GSH: glutathione, reduced; and GSSG: glutathione, oxidized. Note: none of these compounds were detected in blood plasma.