

# Application of NMR Metabolomics to Search for Human Disease Biomarkers

Teklab Gebregiorgis and Robert Powers\*

Department of Chemistry, University of Nebraska-Lincoln, Lincoln, NE 68588-0304, USA

**Abstract:** Since antiquity, humans have used body fluids like saliva, urine and sweat for the diagnosis of diseases. The amount, color and smell of body fluids are still used in many traditional medical practices to evaluate an illness and make a diagnosis. The development and application of analytical methods for the detailed analysis of body fluids has led to the discovery of numerous disease biomarkers. Recently, mass spectrometry (MS), nuclear magnetic resonance spectroscopy (NMR), and multivariate statistical techniques have been incorporated into a multidisciplinary approach to profile changes in small molecules associated with the onset and progression of human diseases. The goal of these efforts is to identify metabolites that are uniquely correlated with a specific human disease in order to accurately diagnose and treat the malady. In this review we will discuss recent developments in sample preparation, experimental techniques, the identification and quantification of metabolites, and the chemometric tools used to search for biomarkers of human diseases using NMR.

**Keywords:** Biomarkers, metabolomics, multivariate statistics, NMR.

## INTRODUCTION

Anthropological and folk medical practices have revealed that humans have used body fluids for the diagnosis of disease since antiquity [1]. Hippocrates's theory of disease postulates an imbalance between the four humors of the body: phlegm, blood, black bile and yellow bile [2]. Thus, early medical treatments and diagnosis were based on attempts to understand these humors. Before the 18<sup>th</sup> century, chemistry was not involved in supporting a physician's decision or providing a rationalization in a disease diagnosis. Instead, medics of the era used their senses to analyze urine and other biofluids [3]. Examining body fluids by color, taste, amount, and smell were accepted practices for diagnosing diseases and treating patients in folk, ancient and medieval medicine [1, 4]. But in the 16<sup>th</sup> and 17<sup>th</sup> century, there were reported successes with precipitating proteins from urine using vinegar and heat treatments. Similarly, in the late 18<sup>th</sup> century, the chemical analysis of urine was able to disentangle the mystery between the sweetness of urine and a diabetic patient [5]. By the 19<sup>th</sup> century, chemistry was a vital element in both understanding human physiology and diagnosing diseases [6]. The concept of searching for chemicals to understand human diseases has been enhanced by technology advancements for the analysis of hundreds of metabolites from small quantities of body fluids [7].

Metabolites are end products of gene expression, and are a direct result of enzymatic and protein activity. Thus, metabolites are more proximal to a phenotype or disease than either genetic or proteomics information [8, 9]. Genetics provides a fingerprint of hereditary information, where many diseases are associated with genetic defects. But not all human diseases are a result of an inherited genetic disorder

or mutations at the gene level [10]. Importantly, the presence of a genetic mutation does not necessitate the development of the associated disease or predict all possible occurrences of the disease. For example, only five percent of breast cancer patients carry the BRCA mutation, where carrying a harmful *BRCA1* or *BRCA2* mutation results in a five-fold increase in the likelihood of developing breast cancer [11]. Other genetic modifiers and environmental factors also contribute to the progression of the disease [12]. Clearly, the disconnect between carrying a harmful BRCA mutation and definitively developing breast cancer significantly complicates decisions regarding preventative treatment [13, 14]. Essentially, the presence of a genetic mutation does not necessarily lead to a change in the biological activity of the gene product. As an alternative and complementary approach, proteomics studies the functional and structural elements of gene products, mainly the expression of proteins and enzymes [15, 16]. The up- or down-regulation of proteins has been correlated with the development or progression of a disease [17]. Similarly, posttranslational modifications of proteins have also been shown to be associated with different diseases [18-20]. Unlike genomics, only a small-fraction of the proteome is observed. But similar to genomics, a modulation in a protein's expression or modification does not necessarily correlate with a perturbation in its biological activity. Furthermore, it is challenging to identify the specific protein(s) that are responsible for the disease state. It is difficult to distinguish between biologically relevant proteins and proteins indirectly affected through a complex biological network. For example, hundreds of proteins have been identified in proteomic studies of pancreatic cell lines [21-25]. Clearly, all those proteins are not relevant to cancer. Also, there are serious concerns with mistaken protein identification and inconsistencies between proteomics studies [26, 27].

Metabolomics is the measurement and analysis of metabolites such as amino acids, carbohydrates and lipids, from biofluids, plants and cellular extracts [28, 29]. The

\*Address correspondence to this author at the Department of Chemistry, University of Nebraska-Lincoln, 722 Hamilton Hall, Lincoln, NE 68588-0304, USA; Tel: (402) 472-3039; Fax: (402) 472-9402; E-mail: rpowers3@unl.edu

underlying premise for the field of metabolomics was first described by Pauling in his 1971 publication using gas chromatography to analyze urine and breath metabolites [30]. Metabolic samples have been obtained from a diversity of organisms under various environmental conditions that include drug treatments and diseases. Correspondingly, studying metabolic profiles of living cells has wide applications in many scientific disciplines and systems biology [31-34]. Metabolomics has been used to understand the pathology of disease [35], to diagnose disease [36], to monitor diet [37], and to investigate host vector relationship [38]. The unique value of metabolomics to drug discovery and disease diagnoses is based on the fact that changes in the metabolome are a direct outcome from perturbations in cellular activity.

Mass spectrometry (MS) [39] and nuclear magnetic resonance spectroscopy (NMR) [28] are the primary analytical techniques used in metabolomics. The two techniques have inherently distinct capabilities and limitations, making them ideally complementary [40, 41]. MS is significantly more sensitive than NMR and covers a wider diversity of the metabolome. But, because of the limited molecular-weight dispersion of metabolites, MS requires chromatographic separations that may bias any analysis. Also, there is a significant uncertainty in a specific metabolite's ability to generate a detectable molecular ion peak by MS. This also makes quantitation by MS challenging. Conversely, NMR requires minimal sample handling, allows for easy quantitative analysis of metabolite concentrations, and provides redundant means of metabolite identification. Most metabolites have multiple characteristic NMR resonances and coupling patterns, where peak intensity is directly proportional to concentration. Fundamentally, MS and NMR provide a distinct spectral analysis of the same sample. The complementary nature of MS and NMR has been demonstrated by a number of metabolomic studies using both techniques [42-46].

The growing interest in NMR based metabolomics was initiated by Nicolson's extensive drug activity and toxicity research using NMR analysis of body fluids [47, 48]. NMR metabolomics is currently being used to search for disease biomarkers for infectious diseases like tuberculosis [49],

malaria [50], and pneumonia [51]; and for cancer [52], Parkinson disease [53], neurological disorders [54]; and numerous other human diseases. NMR metabolomics is being used to understand the underlying causes of these diseases, and to identify chemical markers to quickly and readily diagnose the disease. This review will discuss recent developments in sample preparation, experimental techniques, the identification and quantification of metabolites, and the chemometric tools used to search for biomarkers of human disease using NMR.

## NMR METABOLOMICS SAMPLE PREPARATION

NMR metabolomics has been used to study a variety of different types of biofluids that include plasma [55], serum [56], cerebrospinal fluid [57], pus [58], saliva [59], feces [60], cervicovaginal secretions [61], and urine [62]. NMR has also been used to analyze intact tissue samples [63-66]. One of the major advantages of NMR as a tool for metabolomics is its simplicity in sample preparation. Typically, a minimum of 0.1 to 0.5 mL of the biofluid is required to prepare an NMR sample. The most common protocol for preparing an NMR sample from biofluids is to simply add an aliquot of a deuterated buffer to adjust the sample to a common pH value (Table 1) [67]. NMR chemical shifts are sensitive to both pH and temperature changes, which would result in a systematic bias in the data analysis if different samples had different pH values. The deuterated buffer also provides a necessary lock signal.

Storage and handling of biofluid samples affect metabolite stability. Correspondingly, storing urine samples at -80°C has been shown to maximize metabolite stability [68, 69]. A diurnal variation has been observed for both urine and plasma metabolites [70-72]. Preferably, urine samples are collected in the morning (8-hour samples) [73]. The urine is typically more concentrated and contains more metabolites due to the length of time in the bladder. Urine samples are also collected midstream to avoid cellular and microbe contaminants. Preservatives such as NaN<sub>3</sub> and NaF are commonly added to urine samples to prevent bacterial growth [74-76]. The filtration or centrifugation of urine samples is also recommended to prevent bacterial growth

**Table 1. Biofluid Sample Preparation<sup>a</sup>**

Biofluid	Required Sample Handling
<i>Urine</i>	Add deuterated phosphate buffer to 0.2-0.4 mL urine
<i>Blood/plasma/serum</i>	Add deuterium oxide (to lock) Add acetonitrile (for protein precipitation) Add methanol/chloroform extraction (for lipid extraction)
<i>Cerebrospinal fluid</i>	Add deuterium oxide to 0.5 mL of CSF
<i>Expressed prostatic secretions</i>	Add deuterium oxide to 0.03 – 0.10 mL of EPS
<i>Bile</i>	Add deuterium methanol to 0.5 mL of bile
<i>Bronchoalveolar lavage fluid</i>	Add deuterium oxide to 0.5 mL of BALF
<i>Tissue</i>	Add 0.01 mL of deuterium oxide to 3-10 g of tissue in MAS rotor Add perchloric acid extraction on 20-200 g frozen tissue Add methanol/chloroform extraction to 20-200 g frozen tissue

<sup>a</sup>Adapted from Spratlin *et al.* (2009).

[68]. Similarly, plasma samples are preserved by the addition of EDTA or heparin as anti-coagulants [77]. The addition of preservatives can also be applied to metabolomic samples collected from infected tissues. The stability of the biofluid sample and, correspondingly, the quality and value of the NMR spectrum would be detrimentally affected by bacterial growth, especially during long data acquisition times. The bacteria would generate extracellular secretions and utilize available metabolites, changing the state of the sample as a function of time. Blood, serum, and plasma samples contain proteins, in addition to the small molecular-weight metabolites. The proteins need to be removed prior to analysis, which is typically done by the addition of a precipitant or an extractant, such as methanol or acetonitrile, followed by exchange back into an aqueous buffer. Additionally, chemical reactions such as redox reactions and enzymatic degradations are source of metabolite instability in biological samples. Also, some metabolites are simply not stable [78, 79]. Short sample preparation times and long term storage at  $-80^{\circ}\text{C}$  are recommended to maximize the stability of samples for metabolomics study [80]. Another concern is the external influence on the metabolome due to differences in diet. Correspondingly, diet-standardization, fasting or time of sample collection may minimize variability between subjects due to diet differences [81, 82]. Internal NMR standards, such as  $11.1\ \mu\text{M}$  of 3-(trimethylsilyl)propionic acid-2,2,3,3-d<sub>4</sub> (TMSP-d<sub>4</sub>), are routinely added to metabolomic samples to provide for both a chemical shift and concentration reference [83]. The TMSP methyl NMR resonance is referenced to 0.00 ppm and its relative peak integration is set to 9-times the TMSP concentration, since the NMR peak corresponds to 3 methyl groups or a total of 9 hydrogen nuclei.

The goal of applying NMR metabolomics to the analysis of biofluids is to identify potential biomarkers – metabolites correlated with a specific disease. Importantly, a reliable comparative analysis between biofluid samples requires consistency in sample preparation. The observed differences should be biologically relevant as opposed to an artifact or bias from handling the samples. In the case of biofluids, a significant challenge is maintaining a consistency in the source of the biological samples. For patient and animal studies, it is critical to have a similar diet, to be exposed to a common environment, to maintain similar physical activities, and to select participants with comparable medical histories. Numerous factors, such as age, sex, ethnicity, preexisting health conditions, and lifestyle may perturb the metabolome and complicate the identification of biomarkers. For example, a biomarker observed to be correlated with a neurological disease may result from an associated inflammatory response [84].

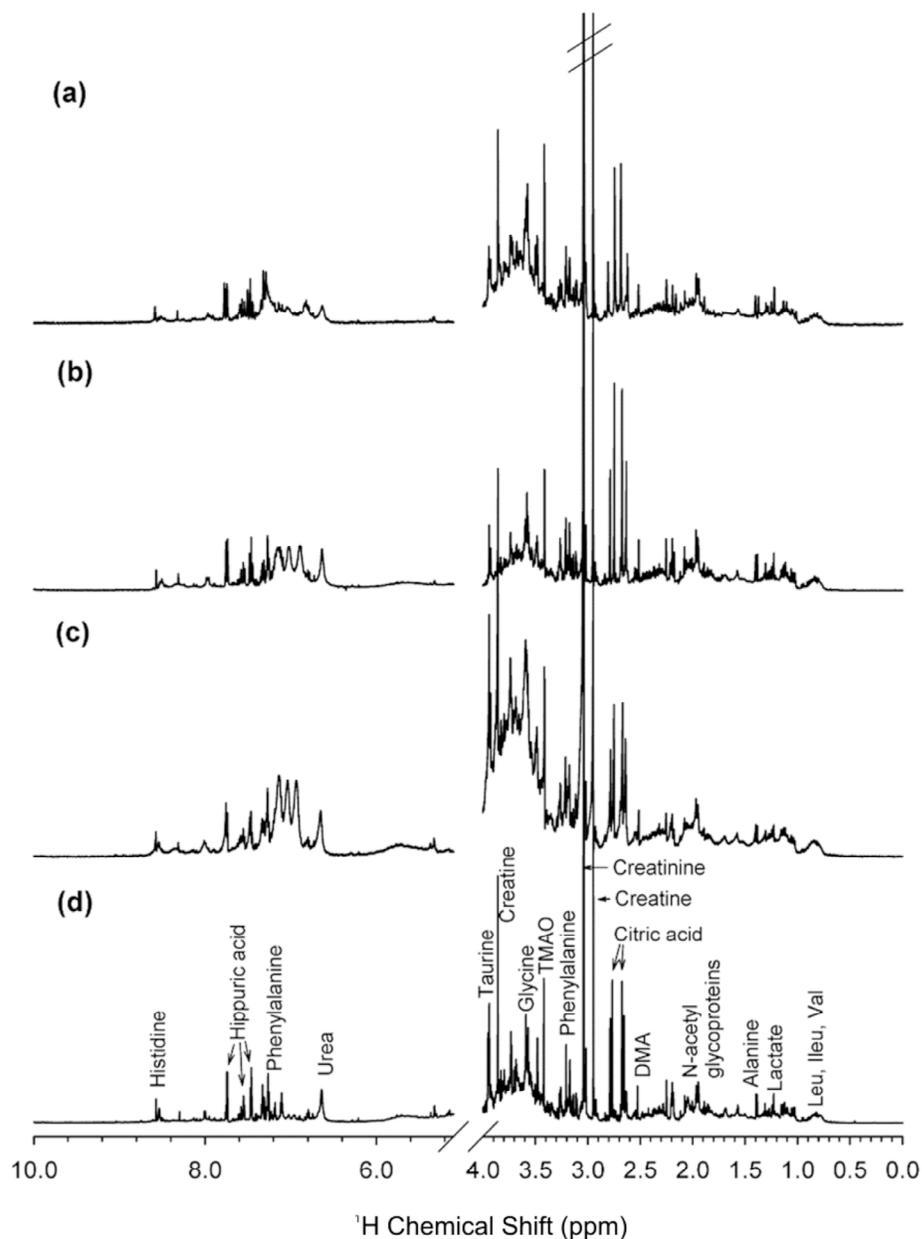
## NMR METABOLOMICS EXPERIMENTS

*One dimensional  $^1\text{H}$  NMR methods.* One-dimensional (1D)  $^1\text{H}$  NMR experiments are routinely used for metabolite biomarker research (Fig. 1) [85]. These experiments are generally fast, robust, easy to implement, and ideally suited for high-throughput screening [86]. The information obtained from 1D  $^1\text{H}$  NMR experiments is used for metabolite identification and for the evaluation of global alterations in metabolites between different groups or classes

[87, 88]. 1D  $^1\text{H}$  NMR experiments provide a global snapshot of the state of the metabolome, but only the most abundant metabolites are observed ( $> 1$  to  $10\ \mu\text{M}$ ). Also, metabolite identification is challenging because of the low chemical shift dispersion and high peak overlap. Biofluids are complex heterogeneous mixtures that contain hundreds of metabolites resulting in an NMR spectrum that is very crowded and suffers from extensive peak overlap, making it difficult to differentiate between multiple peaks. But importantly, the NMR spectrum is also used for quantitative analysis of metabolites by comparing the integral of metabolite peaks with the integral from the internal standard (TMSP) [89].

The Madison Metabolomics Consortium Database [90], the BioMagResBank [91], and the Human Metabolome Database (HMDB) [92] are routinely used to assign metabolites to NMR spectra. The databases contain experimental NMR spectra for hundreds of known metabolites and provide simple user interfaces to match experimental chemical shifts from biofluids data against the spectral database [93]. Typically,  $^1\text{H}$  and  $^{13}\text{C}$  chemical shift tolerances of 0.05 ppm and 0.50 ppm, respectively, are used to identify a match between the experimental and database chemical shifts. Simply, a list of NMR chemical shifts is uploaded to HMDB (<http://www.hmdb.ca/>), which provides a list of potential metabolites with the number of matching NMR resonances. Generally, each metabolite chemical structure contains multiple hydrogen atoms resulting in multiple NMR resonances. Ideally, each NMR resonance for a metabolite should be present in the experimental spectrum. In practice, only a subset of the NMR resonances are observed because of severe peak overlap and low resolution. Thus, the confidence level in a correct metabolite assignment is dependent on the number of matching NMR resonances.

The resolution and sensitivity of the 1D  $^1\text{H}$  NMR spectrum improves as a function of magnetic field strength. Thus, the quality of NMR metabolomics experiments has benefitted from the availability of 800 to 950 MHz NMR spectrometers (Fig. 1) [94]. In addition to the use of high-field NMR instruments, the application of spectral simplification techniques has resulted in improved NMR spectra with better peak separation [95]. Metabolites have relatively long  $T_2$  relaxation times and correspondingly narrow line-widths. Conversely, proteins and other biomolecules have relatively broad peaks and short  $T_2$ s. This significant difference in relaxation times provides an opportunity to manipulate the NMR spectrum to select for small molecular-weight metabolites against a background of protein NMR signals. NMR pulse sequences have been implemented that filter the spectrum based on  $T_2$  relaxation times [96]. Diaz *et al.* (2011) used NMR to search for metabolic biomarkers from urine and plasma that are associated with prenatal disorders (Fig. 2) [97]. An improved separation in metabolite signals from the protein background was achieved by using a  $T_2$  edited experiment. This is accomplished by simply incorporating a Carr-Purcell or Carr-Purcell-Meiboom-Gill (CPMG) sequence into a standard 1D pulse sequence. During this additional  $[\tau-180^{\circ}-\tau]_n$  cycle, NMR peak intensities are decaying proportional to their  $T_2$  relaxation times. The pulse sequence is simply tuned to the minimal number of cycles (n) required to allow the

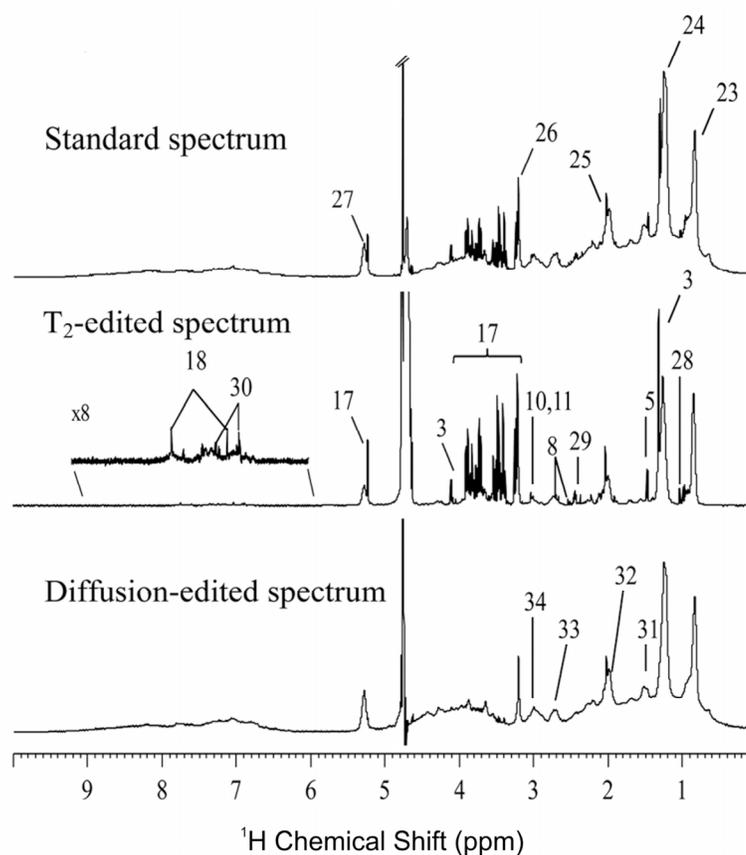


**Fig. (1).**  $^1\text{H}$  NMR spectra of a representative baseline urine sample acquired at (a) 250, (b) 400, (c) 500, and (d) 800 MHz. Key: DMA, dimethylamine, Leu, leucine; Ileu, Isoleucine; Val, valine; TMAO, trimethylamine-*N*-oxide (Reprinted with permission from reference [94], Copyright 2007 by American Chemical Society).

protein NMR resonances to decay to zero with a minimal impact on the intensity of the metabolite signals. Fig. (2) illustrates the metabolites obscured by the protein background using a standard 1D  $^1\text{H}$  pulse sequence. These metabolites are clearly visible in the  $T_2$  edited NMR spectra. The accompanying diffusion edited spectrum has the reverse effect compared to the  $T_2$  edited spectrum; it filters out molecules with rapid diffusion rates. Correspondingly, the diffusion edited spectra attenuated the NMR resonances from the small molecular weight metabolites and emphasizes the protein background peaks.

*Two dimensional NMR methods.* 1D  $^1\text{H}$  NMR spectra obtained from biofluids are very crowded with extensive spectral overlap. As a result, it is often difficult to

unambiguously assign NMR resonances to a specific metabolite. Instead, it is common to identify multiple metabolites that are consistent with a given NMR peak. Two dimensional (2D) NMR experiments overcome this problem by significantly increasing the resolution and dispersing the peaks into two-dimensions (Fig. 3) [98, 99]. 2D  $^1\text{H}$ - $^1\text{H}$ -TOCSY (TOtal Correlation Spectroscopy) and 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC (Heteronuclear Single-Quantum Correlation) are the two most commonly used 2D NMR methods in metabolomics. The 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC experiment correlates the  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts for each C-H pair in a given structure. Similarly, the 2D  $^1\text{H}$ - $^1\text{H}$ -TOCSY experiment correlates the entire network of J-coupled hydrogen nuclei in a metabolite. For example, the  $\text{C}\alpha\text{H}$   $^1\text{H}$  chemical shift for



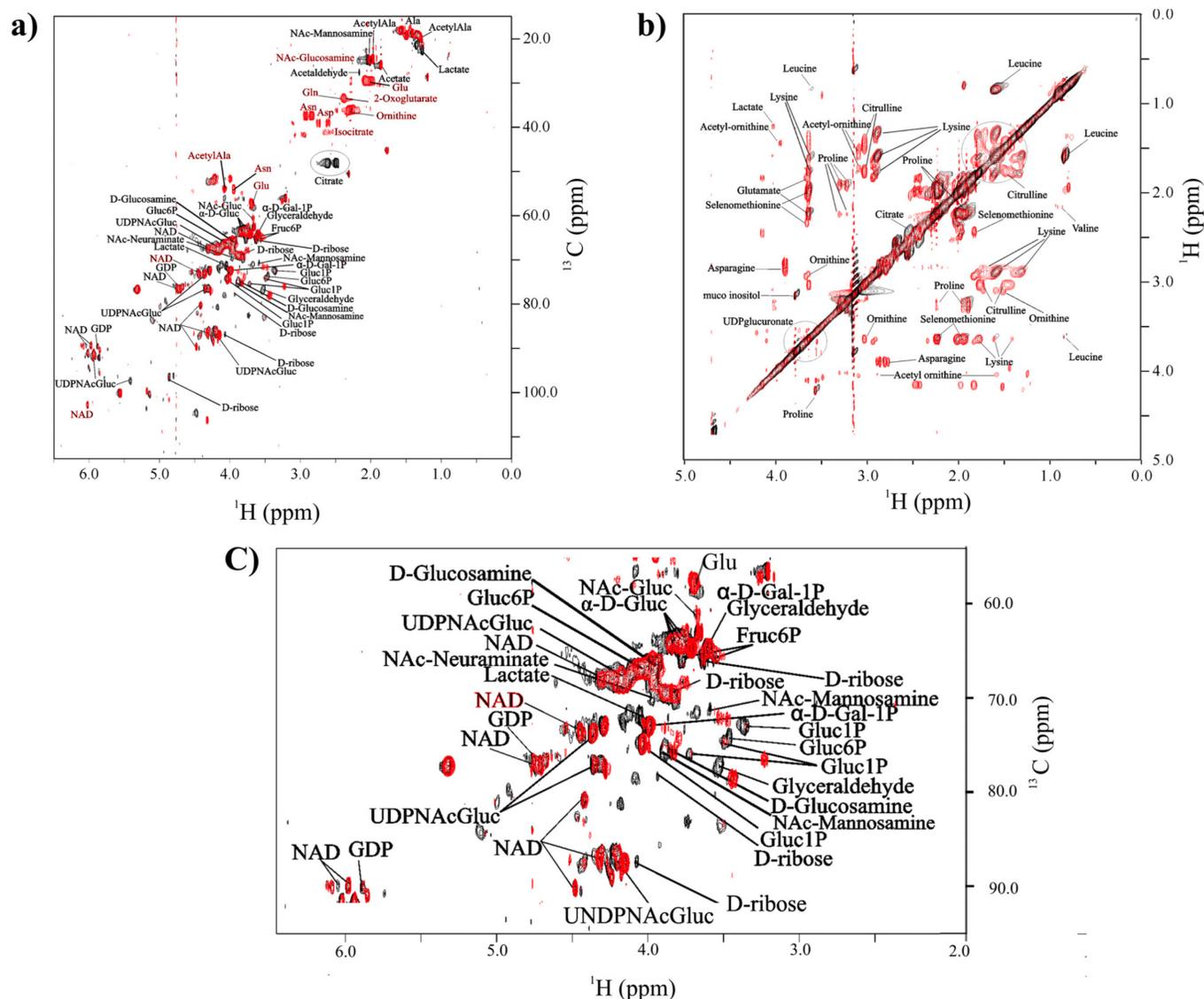
**Fig. (2).**  $^1\text{H}$  NMR spectra (500 MHz) recorded for 2nd trimester urine and plasma of a healthy pregnant woman: blood plasma, standard 1D (top),  $T_2$ -edited (middle) and diffusion-edited (bottom) spectrum. Legend: (1)  $\beta$ -hydroxybutyrate, (2) 3-aminoisobutyrate, (3) lactate, (4) threonine, (5) alanine, (6)  $\gamma$ -aminobutyrate (GABA), (7) succinate, (8) citrate, (9) dimethylamine, (10) creatine, (11) creatinine, (12) trimethylamine N-oxide (TMAO), (13) betaine, (14) glycine, (15) guanidoacetate, (16) trigonelline, (17) glucose, (18) histidine, (19) phenylacetylglutamine, (20) hippurate, (21) formate, (22) N-methyl-nicotinamide, (23) lipid  $\text{CH}_3$ , (24) lipid  $(\text{CH}_2)_n$ , (25) glycoproteins (26) choline, (27) lipid  $\text{CH}=\text{CH}$ , (28) valine, (29) glutamine, (30) tyrosine, (31) lipid  $\text{CH}_2\text{CH}_2\text{CO}$ , (32) lipid  $\text{CH}_2\text{CH}=\text{CH}$ , (33) lipid  $\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}$ , (34) albumin lysyl  $\epsilon\text{CH}_2$ . (Reprinted with permission from reference [97], Copyright 2011 by American Chemical Society).

valine will be correlated with the  $^1\text{H}$  chemical shifts for  $\text{C}\beta\text{H}$ , and the two  $\text{C}\gamma\text{H}_3$  methyls. The dramatic improvement in peak separation along with the direct correlation of pairs of  $^1\text{H}$  or  $^1\text{H}$ - $^{13}\text{C}$  chemical shifts significantly increases the confidence in a correct metabolite assignment. However, 2D NMR experiments require significantly longer acquisition times, upwards of hours per experiment, compared to  $< 10$  minutes per 1D  $^1\text{H}$  NMR experiment [100-102]. Also, the 2D  $^1\text{H}$  -  $^{13}\text{C}$  HSQC spectrum cannot be used for absolute quantitation of metabolite concentrations, but only for relative changes. This limitation arises because peak intensities are not only proportional to concentration, but are also modulated by J-coupling,  $T_1$  and  $T_2$  relaxation, imperfect pulse lengths, and recovery delays. It is impractical to correct for these parameters, since they vary between molecules and also atom type. Recently, a number of methods have been proposed to modify standard 2D HSQC pulse sequences to enable quantitative analysis of metabolite concentrations [103-108]. Hu *et al.* (2011) proposed the use of a time zero 2D  $^1\text{H}$  -  $^{13}\text{C}$  HSQC experiment (Fig. 4) to measure metabolite concentrations [109]. The time zero HSQC spectrum is back extrapolated from a series of HSQC spectra collected with incremental repetition times, the time between the first  $^1\text{H}$  excitation

pulse and the beginning of data acquisition. Essentially, the HSQC spectrum is collected with either one, two or three copies of the standard core HSQC pulse sequence. Peak intensity decreases proportional to the repetition time (Fig. 4b). At time zero, metabolite concentrations are proportional to peak intensities since peak attenuation due to J-coupling, relaxation, etc. are time-dependent and have been effectively removed.

## CHEMOMETRICS FOR BIOMARKER IDENTIFICATION

Multivariate statistical methods are integral to the analysis of NMR metabolomics data [110]. Multivariate statistics provide a clear interpretation of the global alteration in the metabolome resulting from disease, environmental stress, nutrition, or toxins. The analysis is not limited to a few metabolites or specific metabolic pathways, but captures the overall impact on the system from the external factors. A 1D  $^1\text{H}$  NMR spectrum is a complex multivariable data set consisting of upwards of 32K of chemical shifts and intensities. Multivariate statistical techniques reduce the complexity of the NMR data set and present the information in a simple and easily interpreted

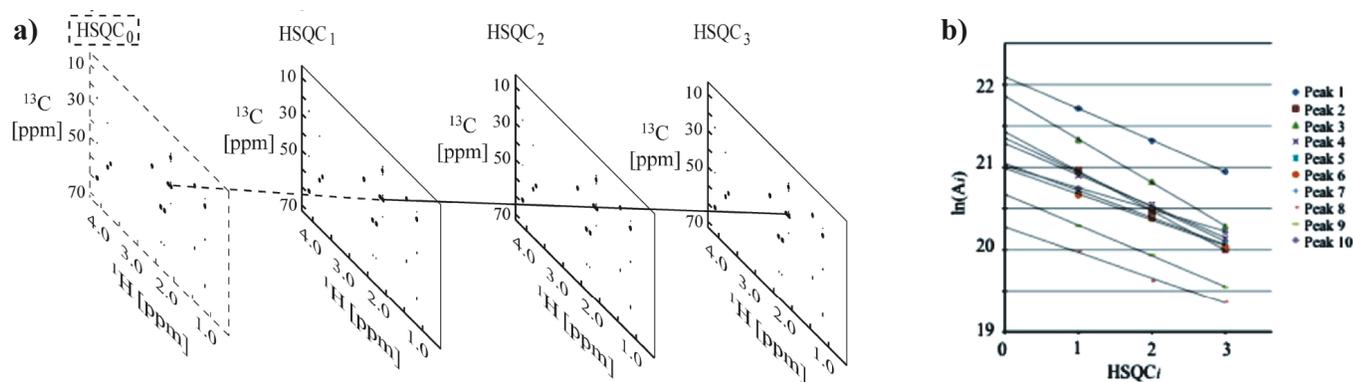


**Fig. (3).** Overlay of (a) 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectra and (b) 2D  $^1\text{H}$ - $^1\text{H}$  TOCSY spectra comparing wild-type *S. epidermidis* strain 1457 (red) and aconitase mutant strain 1457-*acnA::tetM* (black) grown for 6 h in standard tryptic soy broth media augmented with 0.25%  $^{13}\text{C}$ -glucose. NMR resonances corresponding to specific metabolites are labeled, where citrate is circled. (c) Expanded view of the crowded region of the 2D  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectra in (a). (Reprinted with permission from reference [120], Copyright 2011 by American Chemical Society).

format. Typically, the NMR spectrum is reduced to a single data point in a scores plot. Both 1D and 2D NMR spectra have been analyzed using multivariate statistical techniques [111]. Multivariate statistical methods are categorized as either supervised or unsupervised. Supervised methods introduce sample classes, while unsupervised methods are based strictly on inherent variations in the data. Principal component analysis (PCA) is a widely-used unsupervised method for the analysis of NMR metabolomics data [112]. More recently, Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA), a supervised method, has been growing in popularity as an approach to interpret NMR spectra for biomarker identification [113].

Data pre-processing is a vital step in the NMR metabolomics protocol. Pre-processing of the NMR spectrum includes data reduction (binning) [114, 115], scaling [116], and the exclusion of noise [117] and solvent

regions known to bias the analysis. After phase correction to produce pure absorption line shapes and baseline correction of the 1D  $^1\text{H}$  NMR spectrum, the NMR spectrum is divided into uniform bins with a chemical shift range of 0.01 to 0.04 ppm [114] or by using intelligent bucketing (ACD Labs, Toronto, Canada) that uses variable bin sizes to avoid the splitting of peaks between bins [115]. The numerical value within each bin is the integral of the corresponding spectral region. NMR spectra are binned in order to minimize effects from minor and random differences in chemical shifts, phasing, peak shape, baseline, etc. that are inevitable when dealing with replicate biological samples. Similarly, there is a significant and inherent variability in the quality and signal-to-noise of NMR spectra obtained from a collection of biological samples, such as biofluids. It is simply impossible and unreasonable to expect uniformity in biofluid samples obtained from multiple patients, animals or tissues. As a consequence, the NMR spectra needs to be normalized to



**Fig. (4).** (a) Shown are examples of HSQC1, HSQC2, and HSQC3 spectra following Fourier transformation to yield frequency domain spectra. Peak intensities in the virtual HSQC0 spectrum (bordered by the dashed line) are derived from linear  $\ln$  extrapolation of the peak intensities of the corresponding peaks in the HSQC1, HSQC2, and HSQC3 spectra. (b) Extrapolation of the 500 MHz 2D HSQC $_i$  ( $i = 1, 2, 3$ ) peak intensities from integrated peak volumes ( $A_i$ ) to yield  $A_0$  values. (Reprinted with permission from reference [109], Copyright 2011 by American Chemical Society).

eliminate or minimize variability in signal-to-noise due to overall volume or concentration differences between each sample.

Triba *et al.* (2010) used the total sum of the bins for each spectrum as a scaling factor [118]. Simply, the value of each individual bin was divided by the sum of all the bins. This takes into account that variations in the spectral intensity due to different number of cells per sample. Pareto and autoscaling methods are alternative approaches to normalizing NMR spectra to variations in signal intensity [119]. For autoscaling, the NMR bins are mean centered and normalized by the standard deviation of the bins (eqn. 1)

$$Z = \frac{X_i - \bar{X}}{\sigma} \quad (1)$$

where  $\bar{X}$  is the mean of the NMR integrals,  $\sigma$  is the standard deviation of the NMR integrals, and  $X_i$  is the integral associated with bin  $i$ . In Pareto scaling, each bin is still mean centered, but normalization is accomplished through the square-root of the standard deviation.

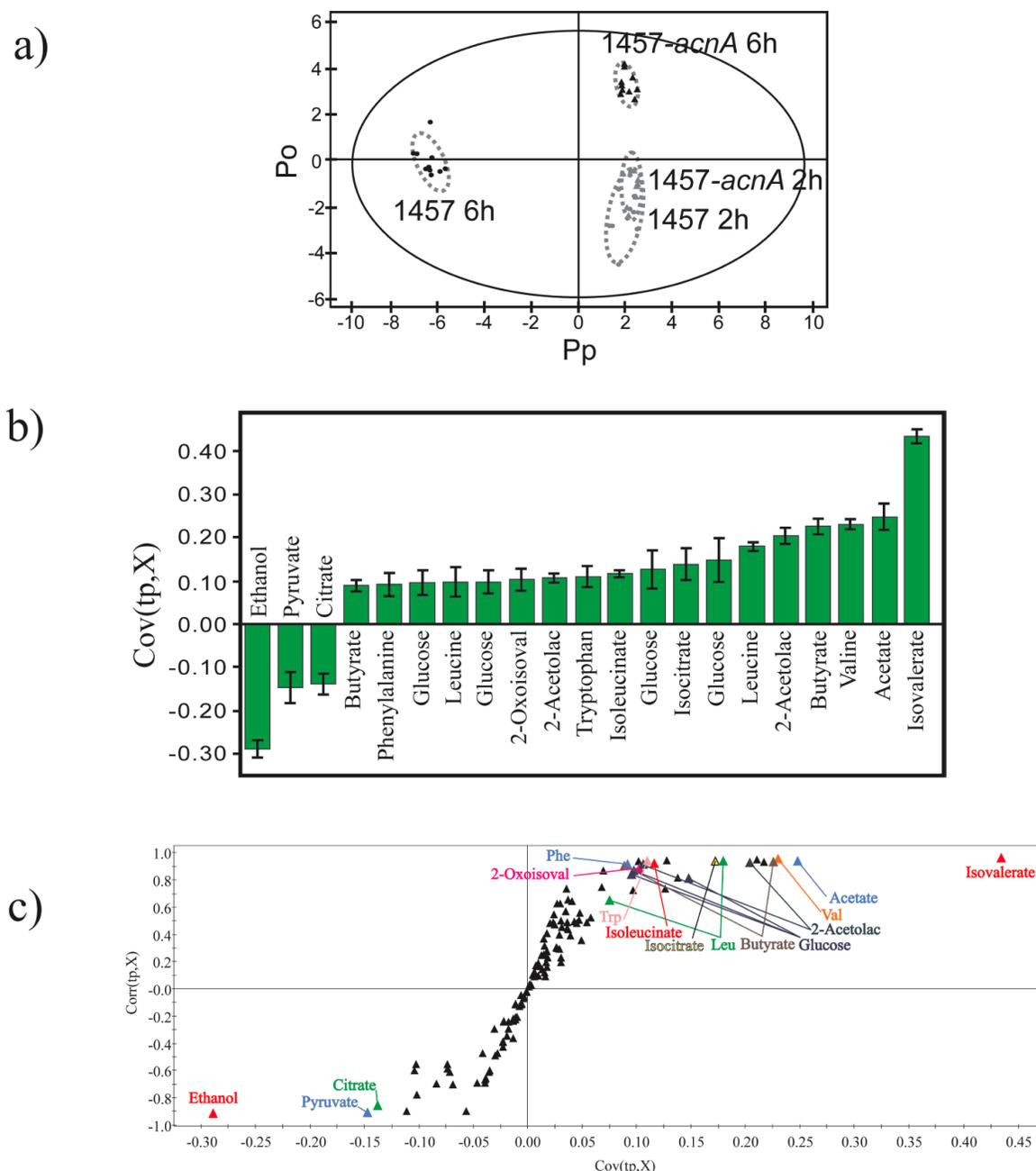
$$Z = \frac{X_i - \bar{X}}{\sqrt{\sigma}} \quad (2)$$

It is important to note, that each approach to data pre-treatment can significantly impact the analysis by emphasizing different aspects of the NMR metabolomics data.

After pre-treatment, the table of NMR integrals (bins) is then subjected to multivariate statistical analysis. PCA is the most common multivariate statistical approach currently applied to NMR metabolomics data. PCA is an unbiased approach and, correspondingly, the information obtained from PCA is a direct result of any correlated structure to the data. PCA projects the multivariable NMR data into simple visual format. The table of NMR integrals is transformed into scores and loadings plots [112]. The PCA scores plot summarizes global similarities and differences between the NMR spectra, where each NMR spectrum is reduced to a single point. The coordinate axis in a PCA scores plot corresponds to the principal components (PC1, PC2, etc.),

which represents the best-fit of each NMR spectrum to a series of orthogonal vectors ( $\bar{P}_1, \bar{P}_2, \text{etc.}$ ). The first vector corresponds to the largest variation in the NMR data set, where each successive vector is in the direction of the next largest variation in the data set. Thus, the PCA scores plot captures the major differences between the NMR spectra resulting from changes in the metabolome. Correspondingly, NMR spectra will cluster in the PCA scores plot based on their relative differences or similarities, which is based on metabolite compositions and concentrations. The PCA loadings plot determines the relative contribution of each NMR bin (chemical shift, metabolite) to the principal components. In essence, the PCA loadings plot identifies the metabolites that primarily contribute to the class separation in the PCA scores plot (Fig. 5b).

The identification of metabolite biomarkers is dependent on determining the latent variables responsible for class separation (e.g., healthy vs disease). PCA identifies the largest variations in the NMR data, but the latent variables (fundamental relationship) responsible for the class separation many not be in the direction of the largest variation [113]. Instead, OPLS-DA is preferred for identifying metabolite biomarkers (Fig. 5a) [120]. OPLS-DA is a regression model that reflects the correlation between multivariate data and dependent variables with class information. In OPLS-DA, a single component is used as a predictor for the class, where the other components are variations orthogonal to the predictive component [121]. In other words, the predictive component describes the between class separation and the orthogonal components describe the within class separation. OPLS-DA emphasizes between class separations and reduces within class separation. As a result, an OPLS-DA scores plot will have tighter clusters with a larger separation relative to PCA. PCA data can be simply changed to OPLS-DA by including a value that discriminates between the classes as a Y direction vector [122]. Typically, a two-class system is used where one data set is designated the control ( $Y = 0$ ) and the remaining data sets are designated as treated ( $Y = 1$ ). For PCA and OPLS-DA, the quality of the model is assessed by the measure of goodness of fit ( $R^2$ ) and quality assessment score ( $Q^2$ ) [123]. A good model has  $R^2$  values  $\geq 0.5$  (range of 0 to 1) and is



**Fig. (5).** (a) 2D OPLS-DA scores plot comparing 2 h growth of wild-type *S. epidermidis* 1457 (●), 2 h growth of aconitase mutant strain 1457-*acnA::tetM* (▲), 6 h growth of wild-type *S. epidermidis* 1457 (●), and 6 h growth of aconitase mutant strain 1457-*acnA::tetM* (▲). The ellipses correspond to the 95% confidence limits from a normal distribution for each cluster. For the OPLS-DA scores plot, the 6 h growth of wild-type *S. epidermidis* 1457 (●) was designated the control class and the remainder of the cells were designated as treated. The OPLS-DA used 1 predictive component and 3 orthogonal components to yield a  $R^2X$  of 0.788,  $R^2Y$  of 0.992 and  $Q^2$  of 0.992. (b) OPLS-DA S-plot comparing the mutant strain 1457-*acnA::tetM* grown for 2 hours and 6 hours. The two conditions are shown to be separated along the PC2 axis in (a). The metabolites identified are associated with variations in the utilization of glucose for cell growth. (c) OPLS-DA loading plot comparing the mutant strain 1457-*acnA::tetM* grown for 2 hours and 6 hours. Negative values indicate a decrease in peak intensity when comparing the wild type to the mutant, while positive values indicate an increase in peak intensity. (Reprinted with permission from reference [120], Copyright 2011 by American Chemical Society).

conceptually similar to simple linear regressions. An ideal value for  $Q^2$  is one, where a typical value for a biological model is  $\geq 0.4$ . OPLS-DA is a supervised method and the model needs to be validated since over-fitting the data is a common concern. The leave-one-out technique is commonly used to validate OPLS-DA models, where a sub-set of the NMR spectra are left out to calculate a model that is then

used to predict the left out data [124]. The predicted data is compared to the original data, where  $Q^2$  determines the consistency between the two data sets and the reliability of the model.

Similar to a loadings plot, an S-plot can also be generated from the OPLS-DA model (Fig. 5c). The S-plot identifies the

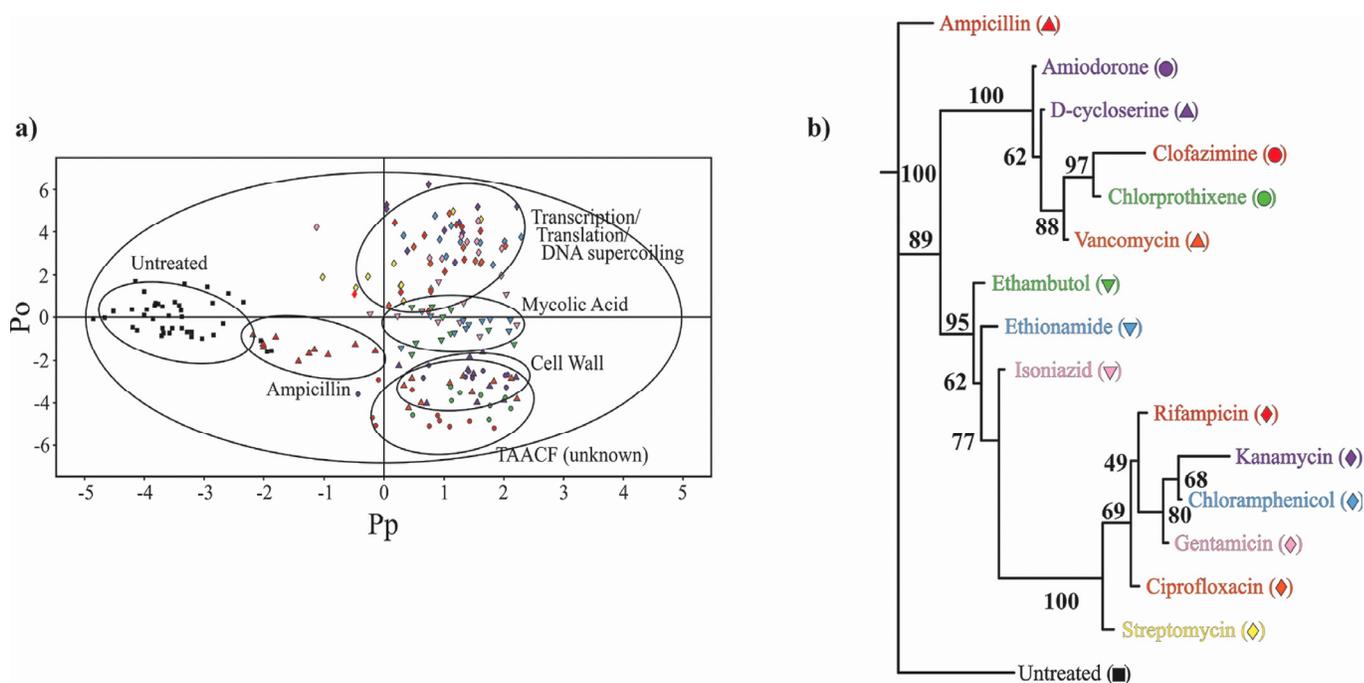
NMR bins (chemical shifts, metabolites) that are correlated ( $X \geq 0.10$  and  $Y \geq 0.8$ ) or anti-correlated ( $X \leq -0.10$  and  $Y \leq -0.80$ ) with the class separation in the OPLS-DA scores plot (Fig. 5a). The S-plot X and Y coordinates represents the correlation and reliability, respectively, of the NMR bins with the OPLS-DA model. NMR bins with the highest correlation and reliability fall in both extremes of the S-plot. These metabolites correspond to potential biomarkers.

Interpretation of PCA and OPLS-DA score plots are generally based on a visual inspection of the relative separation of class clusters. Assigning a statistical significance to this clustering pattern is fundamental to inferring an accurate biological relevance. Werth *et al.* (2010) introduced a new method to analyze PCA and OPLS-DA scores plots based on metabolic tree diagrams with associated bootstrap [125] numbers to quantify the statistical significance of the observed class clustering [126]. The technique is particularly valuable for the accurate analysis of complex datasets that contain multiple classes, where it may be very difficult to see distinct clustering patterns. Simply, the coordinates for the center of each cluster are used to measure a Euclidean distance between each pair of clusters to generate a standard distance matrix. The distance matrix is then used to create a tree diagram using a phylogenetic program such as PHYLIP (<http://www.phylip.com>) [127]. The cluster centers are re-determined by randomly leaving

out class members, resulting in a new distance matrix and tree diagram. The process is typically repeated 100 times, where a consensus tree is determined from the 100 individual trees. The bootstrap value is simply the number of times a node in the consensus tree was observed in the set of 100 tree diagrams, where bootstrap numbers less than 50 indicate a statistically insignificant node. Additionally,  $T_2$  hoteling is used to identify the 95% confidence interval for each cluster, which adds an additional quantitative approach to distinguish between class clusters. An OPLS-DA scores plot shown in Fig. (6) contains ellipses that represent the 95% confidence interval that helps identify the major drug classes [128, 129]. The associated metabolomic tree diagram provides a detailed analysis of the antibiotic activity of fifteen drugs against TB. Specifically, the OPLS-DA scores plot and tree diagram indicate the drugs cluster based on their *in vivo* mechanism of action [129].

## RECENT APPLICATIONS OF NMR FOR BIOMARKER IDENTIFICATION

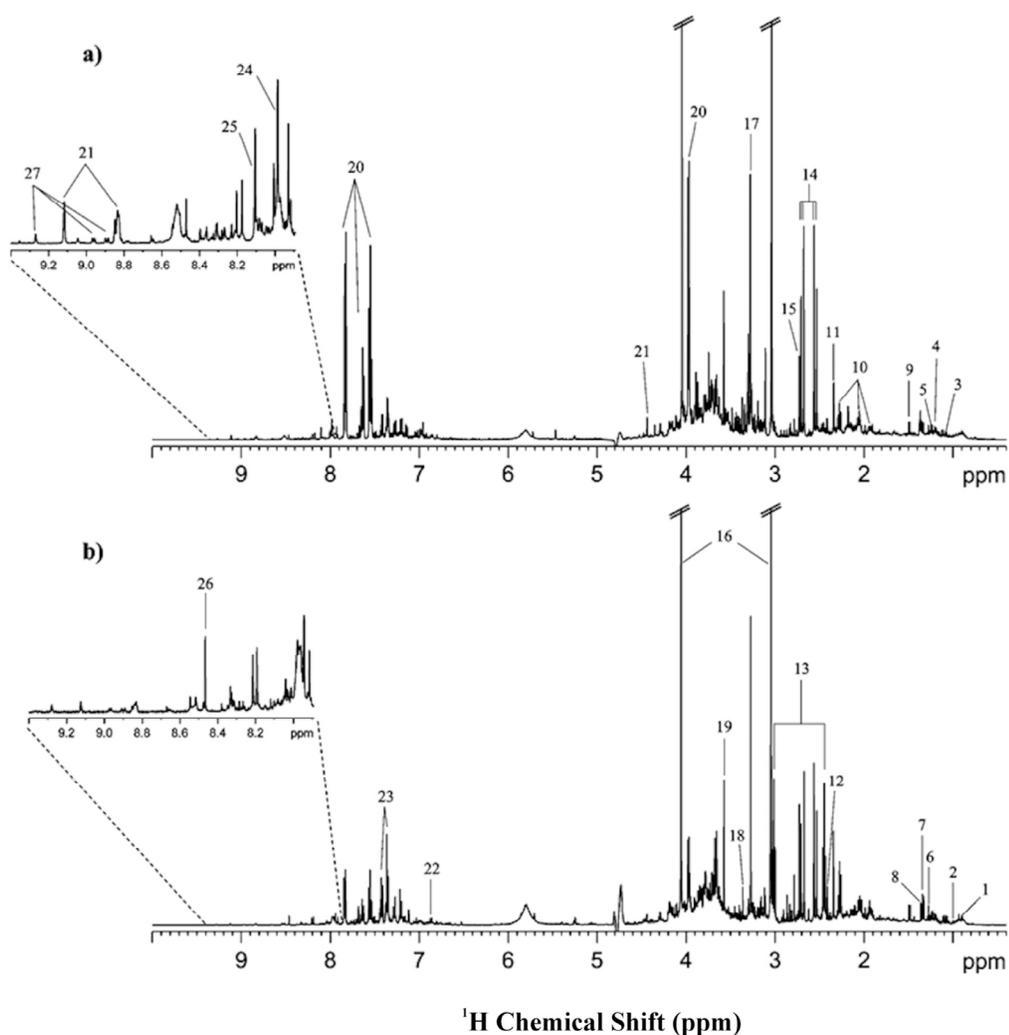
Lung cancer has a low 5-year survival rate of only 15% in the US and 10% in Europe. Early diagnosis is a key factor to increase patient survival, but, unfortunately, current diagnostic methods are inadequate. Carrola *et al.* (2010) utilized NMR metabolomics to analyze urine samples collected from lung cancer patients and healthy individuals.



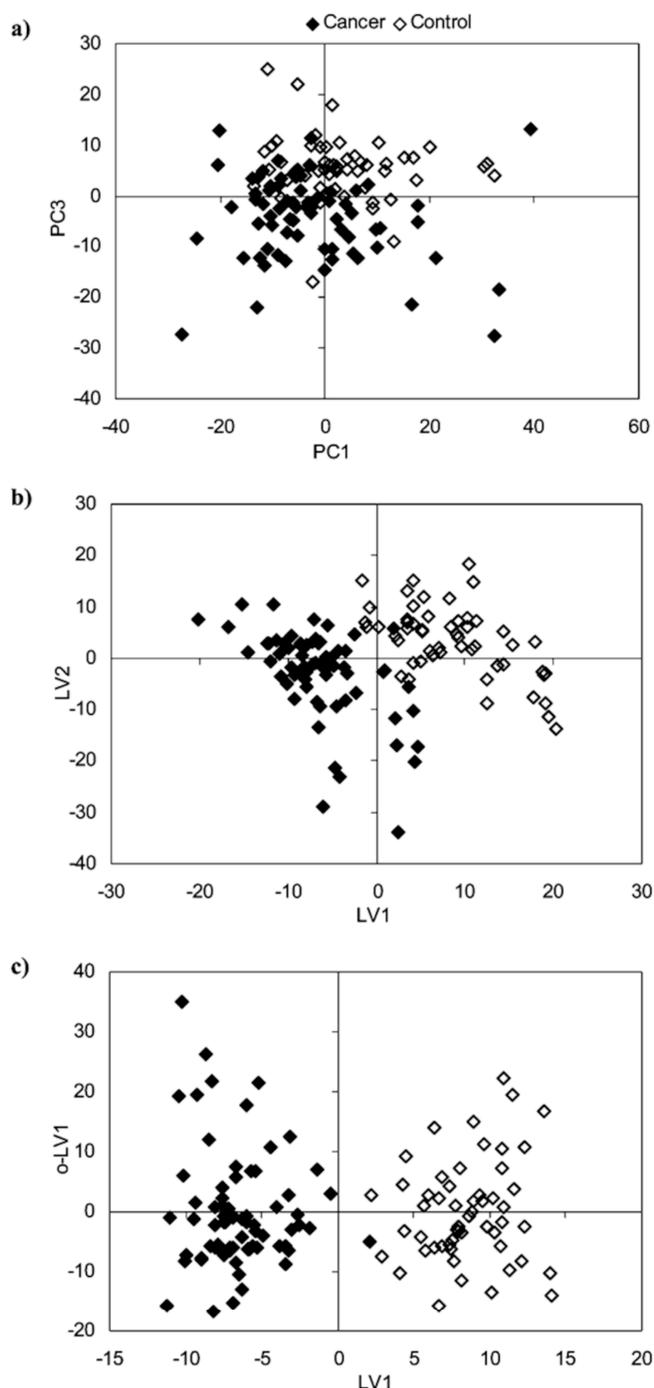
**Fig. (6).** (a) 2D OPLS-DA scores plot demonstrating the clustering pattern for 12 antibiotics with known biological targets and three compounds of unknown *in vivo* activity: untreated *M. smegmatis* cells (black square), chloramphenicol (blue diamond), ciprofloxacin (orange diamond), gentamicin (pink diamond), kanamycin (lavender diamond), rifampicin (red diamond), streptomycin (yellow diamond), ethambutol (green down triangle), ethionamide (blue down triangle), isoniazid (pink down triangle), ampicillin (red up triangle), D-cycloserine (purple up triangle), vancomycin (orange up triangle), amiodorone (purple circle), chlorprothixene (green circle), and clofazimine (red circle) treated *M. smegmatis* cells. The ellipses correspond to the 95% confidence limits from a normal distribution for each cluster. The untreated *M. smegmatis* cells (black square) was designated the control class, and the remainder of the cells were designated as treated. The OPLS-DA used one predictive component and six orthogonal components to yield a  $R^2X$  of 0.715,  $R^2Y$  of 0.803, and  $Q^2$  of 0.671. (b) Metabolomics tree diagram determined from the OPLS-DA scores plot. The coloring scheme for each compound in the tree diagram correlates with the data point colors in the OPLS-DA scores plot. The bootstrap numbers for each node are indicated on the tree diagram. (Reprinted with permission from reference [129], Copyright 2012 by American Chemical Society).

A 600  $\mu\text{L}$  NMR sample was prepared by adding 60  $\mu\text{L}$  of 1.5 M phosphate buffer ( $\text{KH}_2\text{PO}_4$ ) that contains 0.1%  $\text{TMSP-d}_4$  to a 540  $\mu\text{L}$  urine sample to obtain a uniform pH of  $7.00 \pm 0.02$ . The resulting 1D  $^1\text{H}$  NMR spectra indicated a distinct metabolic difference between lung cancer patients and healthy individuals (Fig. 7). The application of PCA, PLS, and OPLS-DA multivariate statistical analysis resulted in an increasing separation between the two classes (Fig. 8). A clear separation was only obtained using OPLS-DA. The impact of age, gender and smoking on the 2D scores clustering pattern was also examined. Interestingly, none of these factors were shown to have a significant impact on the analysis. The corresponding OPLS-DA loading plot was used to identify metabolites responsible for class separation. As a result, hippurate, trigonelline,  $\beta$ -hydroxyisovalerate,  $\alpha$ -hydroxyisobutyrate, *N*-acetylglutamine, and creatinine were shown to have a statistically significant (*P*-value < 0.01) difference between healthy individuals and lung cancer patients [130]. NMR metabolomics has also been

extensively used to study the pathology of cancer cell lines to investigate system-wide process such as transformation, progression, proliferation and metastasis [131]. This effort may lead to the identification of new biomarkers and therapies. For example, MacKinnon *et al.* (2012) describes the metabolomic analysis of an androgen-dependent prostate cancer cell line (LnCAP) using NMR and mass spectrometry [132]. Upon treating LnCAP cells with methyltrienolone (an androgen receptor agonist), a metabolic signature characteristic of aggressive prostate cancer was observed. Specifically, a decrease in myo-inositol, altered glutathione levels, perturbation in amino-acid levels, a decreased level of methionine, a high level of phosphocholine, and an increase in the phosphocholine/glycerophosphocholine ratio were all observed. These potential biomarkers could be used to guide clinical treatments and avoid premature termination of androgen ablation therapy that may lead to the unfortunate induction of an aggressive and incurable form of prostate cancer.



**Fig. (7).** The 500 MHz  $^1\text{H}$  NMR spectra of urine from (a) a healthy (control) subject, and (b) a lung cancer patient. Signal assignment: 1,  $\alpha$ -hydroxybutyrate; 2, valine; 3, isobutyrate; 4,  $\beta$ -aminoisobutyrate; 5, methyl- $\beta$ -hydroxybutyrate; 6,  $\beta$ -hydroxyisovalerate; 7, lactic acid and threonine; 8,  $\alpha$ -hydroxyisobutyrate; 9, alanine; 10, *N*-acetylglutamine; 11, pyruvate; 12, succinate; 13,  $\alpha$ -ketoglutarate; 14, citrate; 15, dimethylamine; 16, creatinine; 17, trimethylamine-*N*-oxide and betaine; 18, scyllo-inositol; 19, glycine; 20, hippurate; 21, trigonelline; 22, *p*-hydroxyphenylacetate; 23, phenylacetyl glycine; 24, histidine; 25, 3-methylhistidine; 26, formate; 27, trigonellinamide. Reprinted with permission from reference [130], Copyright 2011 by American Chemical Society.

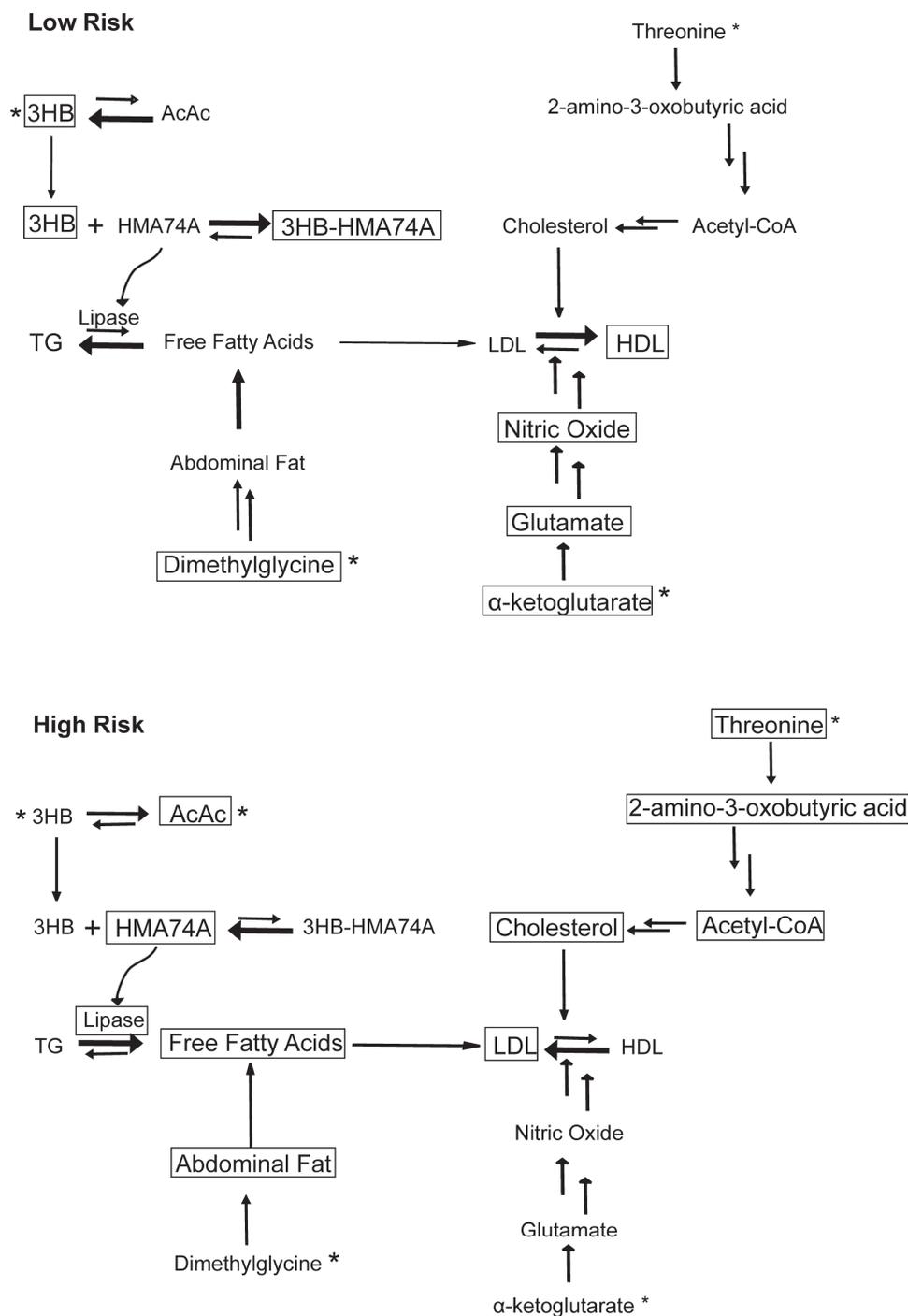


**Fig. (8).** Scores scatter plots (a) PC1 vs PC3, (b) LV1 vs LV2, (c) LV1 vs o-LV1 resulting from applying, respectively, PCA, PLS-DA, and OPLS-DA to the  $^1\text{H}$  NMR spectra of urine ( $\delta$  0.40 to 10.0, excluding 4.55–6.05) from healthy controls (open symbols) and lung cancer patients (full symbols). Reprinted with permission from reference [130], Copyright 2011 by American Chemical Society.

Cardio vascular diseases (CVD) are one of the leading causes of death in the US and the rest of the developed world. Predicting the risk of cardio vascular diseases is an important element of a preventive medical strategy. Bernini *et al.* (2011) conducted an NMR based metabolomics study to identify biomarkers associated with CVD risk in healthy individuals. After a rigorous analysis of 864 plasma samples,

a similar CVD risk assessment was obtained relative to traditional clinical methods. In effect, changes in the metabolome, as determined by NMR, correlated with standard CVD risk factors such as high cholesterol, triglycerides, LDL and HDL. In fact, new metabolite markers, such as 3-hydroxybutyrate,  $\alpha$ -ketoglutarate, threonine, and dimethylglycine, were identified in addition to these commonly known risk markers for CVD. The NMR samples were prepared by simply mixing 300  $\mu\text{L}$  of sodium phosphate buffer with 300  $\mu\text{L}$  of a plasma sample. Standard 1D  $^1\text{H}$  NMR spectra, CPMG diffusion edited spectra and 1D-NOESY spectra were collected for each plasma sample. The primary goal of this diverse set of NMR experiments was to obtain an accurate analysis of known CVD risk factor metabolites to establish a reliable correlation between standard clinical analysis and the NMR results. Furthermore, the statistical analysis was focused on identifying a small set of metabolites that are highly reliable ( $P$ -value  $\leq 0.0001$ ) predictors of CVD risk. This extensive statistical approach enabled the authors to present a CVD risk model based on differently activated metabolic pathways (Fig. 9) [133]. The high risk and low risk pathways are interconnected, where the metabolome of a low risk individual is shifted toward HDL,  $\alpha$ -ketoglutarate, dimethylglycine, and 3-hydroxybutyrate. Conversely, the metabolic pathways for the high-risk individual are shifted toward LDL, threonine and acetoacetate.

Another unique application of NMR metabolomics is demonstrated by Heinzmann *et al.* (2010) [134]. Urine samples collected from eight individuals were analyzed using 1D  $^1\text{H}$  NMR to identify metabolites associated with nutrition. Analysis of the resulting NMR spectra identified proline betaine as a urine biomarker for citrus fruit consumption (Fig. 10). Numerous fruits and fruit juices were similarly analyzed by NMR to quantify the amount of proline betaine in order to establish citrus juice as the potential source of proline betaine in the urine samples. Only citrus juices were shown to contain a significant quantity of proline betaine, with orange juice showing the highest concentration of the metabolite. To further validate proline betaine as a biomarker of citrus fruit consumption, 1D  $^1\text{H}$  NMR was used to study the kinetics of the urinary excretion of proline betaine after consuming orange juice (Fig. 10). Urine was collected from six individuals before (-26 to 0 hrs) and after (+2 to +24 hrs) drinking 250 mL of orange juice. The isolated proline betaine methylene singlet at  $\delta$  3.11 was used to monitor the metabolite's urine concentration as a function of time. The NMR spectrum clearly shows the presence of the metabolite in urine 2 hrs after consuming orange juice. Additionally, integration of the  $\delta$  3.106 – 3.116 spectral region revealed that proline betaine remains in the urine more than 24 hrs after consuming orange juice. After validating the utility of using NMR to monitor proline betaine as a biomarker for citrus fruit consumption, 1D  $^1\text{H}$  NMR spectra were collected from the INTERMAP UK urine collection ( $n > 1000$ ) to classify participants as citrus or non-citrus consumers. INTERMAP is a large-scale epidemiologic study to determine dietary factors associated with high blood pressure. Citric consumers were shown to have healthier diets, and correspondingly to be in better health (lower BMI and blood pressure), and to also be better educated.

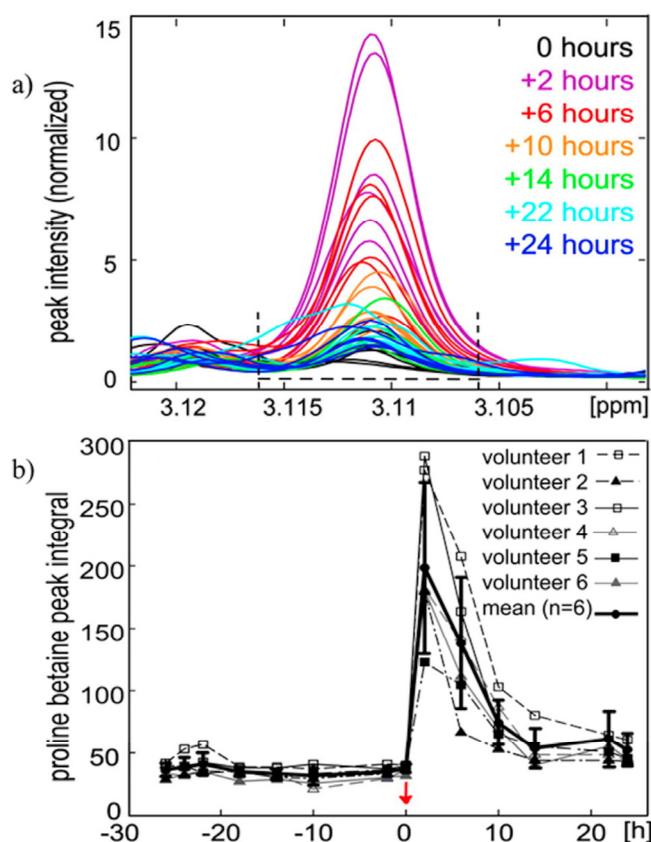


**Fig. (9).** Metabolic fingerprint in low and high risk subjects (metabolites whose amount differs significantly between the low- and high-risk subjects are marked with an asterisk). Reprinted with permission from reference [133], Copyright 2011 by American Chemical Society.

## CONCLUSION

NMR metabolomics continues to make significant contributions to the identification of biomarkers to aid in the diagnosis and treatment of various human diseases. NMR has assisted in identifying biomarkers for various types of cancers, neurological diseases, genetic disorders, and infectious diseases, among others. The appeal of NMR metabolomics is the simplicity in sample preparation and its robust, versatile, and easy application. Global metabolomic

changes can be quickly obtained with relatively routine one-dimensional  $^1\text{H}$  NMR experiments. Specific metabolites correlated with a disease and potential biomarkers can be identified by two dimensional NMR experiments in combination with chemometrics techniques that include S-plots and loading plots. Furthermore, including statistical analysis, such as tree diagrams, bootstrap numbers and  $T_2$  hoteling provides an important level of confidence to the biological interpretation of NMR metabolomics data.



**Fig. (10).** Urinary excretion kinetics of proline betaine after orange juice consumption ( $n = 6$ ). (a) Proline betaine singlet at  $\delta$  3.11 was integrated over the spectral region  $\delta$  3.106–3.116 as shown, where the peak overlap is minimal. (b) Mean and SD proline betaine integral (solid bold line) and the proline betaine integral for each of the 6 volunteers plotted over time. The red arrow indicates the time of orange juice consumption. ppm, parts per million. Reprinted with permission from reference [134], Copyright 2010 by American Society for Nutrition.

## DEDICATION

The authors of this manuscript would like to take this opportunity to acknowledge the remarkable advancements and breakthroughs made by Dr. Atta-ur-Rahman in the field of Chemistry. It would be a great pleasure to dedicate our work to these accomplishments and to celebrate Dr. Atta-ur-Rahman's outstanding contributions to science.

## ACKNOWLEDGEMENT

This manuscript was supported in part by the NIH National Center for Research Resources (P20 RR-17675).

## CONFLICT OF INTEREST

Declared none.

## REFERENCES

[1] Freeman, B. C. *Third-World Folk Beliefs and Practices: Haitian Medical Anthropology*. La Presse Evangélique, Port-au-Prince, 2007.  
 [2] Falagas, M. E. Science in Greece: from the age of Hippocrates to the age of the genome. *FASEB J.*, 2006, 20 (12), 1946-1950.

[3] White, W. I. A new look at the role of urinalysis in the history of diagnostic medicine. *Clin. Chem.*, 1991, 37 (1), 119-125.  
 [4] Nicholson, J.K.; Lindon, J. C. Metabonomics. *Nature*, 2008, 455 (23), 1045-1056.  
 [5] Eknayan, G.; Nagy, J. A history of diabetes mellitus or how a disease of the kidneys evolved into a kidney disease. *Adv. Chronic Kidney D.*, 2005, 12, (2), 223-229.  
 [6] Durner, J. Clinical chemistry: Challenges for analytical chemistry and the nanosciences from medicine. *Angew. Chem., Int. Ed.*, 2009, 49 (6), 1026-1051.  
 [7] Robinson, B.; Pauling, L. Techniques of orthomolecular diagnosis. *Clin. Chem.*, 1974, 20 (8), 961-965.  
 [8] Messlerli, G.; Partovi Nia, V.; Trevisan, M.; Kolbe, A.; Schauer, N.; Geigenberger, P.; Chen, J.; Davison, A. C.; Fernie, A. R.; Zeeman, S. C. Rapid classification of phenotypic mutants of arabidopsis via metabolite fingerprinting. *Plant Physiol.*, 2007, 143 (4), 1484-1492.  
 [9] Fiehn, O. Metabolomics – the link between genotypes and phenotypes. *Plant Mol. Biol.* 2002, 48 (1-2), 155-171.  
 [10] Badano, J. L.; Katsanis, N. Beyond Mendel: an evolving view of human genetic disease transmission. *Nat. Rev. Genet.*, 2002, 3 (10), 779-789.  
 [11] Rebbeck, T. R.; Domchek, S. M. Variation in breast cancer risk in BRCA1 and BRCA2 mutation carriers. *Breast Cancer Res.*, 2008, 10 (108).  
 [12] Mavaddat, N.; Antoniou, A. C.; Easton, D. F.; Garcia-Closas, M. Genetic susceptibility to breast cancer. *Mol. Oncol.*, 2010, 4 (3), 174-191.  
 [13] Nathanson, K. L.; Domchek, S. M. Therapeutic approaches for women predisposed to breast cancer. *Annu. Rev. Med.*, 2011, 62, 295-306.  
 [14] Howard, A. F.; Balneaves, L. G.; Bottorff, J. L.; Rodney, P. Preserving the self: the process of decision making about hereditary breast cancer and ovarian cancer risk reduction. *Qual Health Res.*, 2011, 21 (4), 502-519.  
 [15] Alberio, T.; Fasano, M. Proteomics in Parkinson's disease: An unbiased approach towards peripheral biomarkers and new therapies. *J. Biotechnol.*, 2011, 156 (4), 325-337.  
 [16] Denman, B.; Goodman, S. R. Emerging and neglected tropical diseases: translational application of proteomics. *Exp. Biol. Med.*, 2011, 236 (8), 972-976.  
 [17] Hanash, S. Progress in mining the human proteome for disease applications. *OMICS*, 2011, 15 (3), 133-139.  
 [18] Wang, J.-Z.; Grundke-Iqbal, I.; Iqbal, K. Glycosylation of microtubule-associated protein tau: An abnormal posttranslational modification in Alzheimer's disease. *Nature*, 1996, 2 (8), 871-875.  
 [19] Vucic, D.; Dixit, V. M.; Wertz, I. E. Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death. *Nat. Rev. Mol. Cell Biol.*, 2011, 12 (7), 439-452.  
 [20] Ehrnhoefer, D. E.; Sutton, L.; Hayden, M. R. Small changes, Big impact: posttranslational modifications and function of huntingtin in huntington disease. *The Neuroscientist*, 2011, 17 (5), 475-492.  
 [21] Yamada, M.; Fujii, K.; Koyama, K.; Hirohashi, S.; Kondo, T. The proteomic profile of pancreatic cancer cell lines corresponding to carcinogenesis and metastasis. *J. Proteomics Bioinf.*, 2009, 2 (1), 001-018.  
 [22] Shen, J.; Person, M. D.; Zhu, J.; Abbruzzese, J. L.; Li, D. Protein expression profiles in pancreatic adenocarcinoma compared with normal pancreatic tissue and tissue affected by pancreatitis as detected by two-dimensional gel electrophoresis and mass spectrometry. *Cancer Res.*, 2004, 64 (24), 9018-9026.  
 [23] Chen, R.; Yi, E. C.; Donohoe, S.; Pan, S.; Eng, J.; Cooke, K.; Crispin, D. A.; Lane, Z.; Goodlett, D. R.; Bronner, M. P.; Aebersold, R.; Brentnall, T. A. Pancreatic cancer proteome: The proteins that underlie invasion, metastasis, and immunologic escape. *Gastroenterology*, 2005, 129 (4), 1187-1197.  
 [24] Crnogorac-Jurcevic, T.; Gangeswaran, R.; Bhakta, V.; Capurso, G.; Lattimore, S.; Akada, M.; Sunamura, M.; Prime, W.; Campbell, F.; Brentnall, T. A.; Costello, E.; Neoptolemos, J.; Lemoine, N. R. Proteomic analysis of chronic pancreatitis and pancreatic adenocarcinoma. *Gastroenterology*, 2005, 129 (5), 1454-1463.  
 [25] Grutzmann, R.; Boriss, H.; Ammerpohl, O.; Luttgies, J.; Kalthoff, H.; Schackert, H. K.; Kloppel, G.; Saeger, H. D.; Pilarsky, C. Meta-analysis of microarray data on pancreatic cancer defines a set of commonly dysregulated genes. *Oncogene*, 2005, 24 (32), 5079-5088.

- [26] Biron, D. G.; Brun, C.; Lefevre, T.; Lebarbenchon, C.; Loxdale, H. D.; Chevenet, F.; Brizard, J.-P.; Thomas, F. The pitfalls of proteomics experiments without the correct use of bioinformatics tools. *Proteomics*, **2006**, *6*, (20), 5577-5596.
- [27] Reinders, J.; Lewandrowski, U.; Moebius, J.; Wagner, Y.; Sickmann, A. Challenges in mass spectrometry-based proteomics. *Proteomics*, **2004**, *4*, (12), 3686-3703.
- [28] Powers, R. NMR metabolomics and drug discovery. *Magn. Reson. Chem.*, **2009**, *47*, (S1), S2-S11.
- [29] Zhang, G.-F.; Sadhukhan, S.; Tochtrop, G. P.; Brunengraber, H. Metabolomics, pathway regulation, and pathway discovery. *J. Biol. Chem.*, **2011**, *286*, (27), 23631-23635.
- [30] Pauling, L.; Robinson, A. B.; Teranishit, R.; Cary, P. Quantitative analysis of urine vapor and breath by gas-liquid partition chromatography. *Proc. Natl. Acad. Sci. U S A*, **1971**, *68* (10), 2374-2376.
- [31] Dixon, R. A.; Gang, D. R.; Charlton, A. J.; Fiehn, O.; Kuiper, H. A.; Reynolds, T. L.; Tjeerdema, R. S.; Jeffery, E. H.; German, J. B.; Ridley, W. P.; Seiber, J. N. Applications of metabolomics in agriculture. *J. Agric. Food Chem.*, **2006**, *54* (24), 8984-8994.
- [32] Wang, T. J.; Larson, M. G.; Vasani, R. S.; Cheng, S.; Rhee, E. P.; McCabe, E.; Lewis, G. D.; Fox, C. S.; Jacques, P. F.; Fernandez, C.; O'Donnell, C. J.; Carr, S. A.; Mootha, V. K.; Florez, J. C.; Souza, A.; Melander, O.; Clish, C. B.; Gerszten, R. E. Metabolite profiles and the risk of developing diabetes. *Nat. Med.*, **2011**, *17* (4), 448-453.
- [33] Viant, M. R. Metabolomics of aquatic organisms: the new 'omics' on the block. *Mar. Ecol. Prog. Ser.*, **2007**, *332*, 301-306.
- [34] Bundy, J. G.; Davey, M. P.; Viant, M. R. Environmental metabolomics: a critical review and future perspectives. *Metabolomics*, **2008**, *5* (1), 3-21.
- [35] Vinayavekkin, N.; Homan, E. A.; Saghatelian, A. Exploring disease through metabolomics. *ACS Chem. Biol.*, **2010**, *5* (1), 91-103.
- [36] Gowda, G. A.; Zhang, S.; Gu, H.; Asiago, V.; Shanaiah, N.; Raftery, D. Metabolomics-based methods for early disease diagnostics. *Expert Rev. Mol. Diagn.*, **2008**, *8* (5), 617-633.
- [37] Gu, H.; Chen, H.; Pan, Z.; Jackson, A. U.; Talaty, N.; Xi, B.; Kissinger, C.; Duda, C.; Mann, D.; Raftery, D.; Cooks, R. G. Monitoring diet effects via biofluids and their implications for metabolomics studies. *Anal. Chem.*, **2007**, *79*, 89-97.
- [38] Olszewski, K. L.; Morrisey, J. M.; Wilinski, D.; Burns, J. M.; Vaidya, A. B.; Rabinowitz, J. D.; Llinas, M. Host-parasite interactions revealed by *Plasmodium falciparum* metabolomics. *Cell Host Microbe*, **2009**, *5*, 191-199.
- [39] Drexler, D. M.; Reilly, M. D.; Shipkova, P. A. Advances in mass spectrometry applied to pharmaceutical metabolomics. *Anal. Bioanal. Chem.*, **2011**, *399*, 2645-2653.
- [40] Birungi, G.; Meijie Chen, S.; Pheng Loy, B.; Lee Ng, M.; Fong Yau Li, S. Metabolomics approach for investigation of effects of dengue virus infection using the EA.hy926 cell line. *J. Proteome Res.*, **2010**, *9*, 6523-6534.
- [41] Krishnan, P. Metabolite fingerprinting and profiling in plants using NMR. *J. Exp. Bot.*, **2004**, *56* (410), 255-265.
- [42] Atherton, H. J.; Bailey, N. J.; Zhang, W.; Taylor, J.; Major, H.; Shockcor, J.; Clarke, K.; Griffin, J. L. A combined 1H-NMR spectroscopy- and mass spectrometry-based metabolomic study of the PPAR- null mutant mouse defines profound systemic changes in metabolism linked to the metabolic syndrome. *Physiol. Genomics*, **2006**, *27* (2), 178-186.
- [43] Pan, Z.; Raftery, D. Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. *Anal. Bioanal. Chem.*, **2007**, *387* (2), 525-527.
- [44] Zheng, S.; Zhang, S.; Yu, M.; Tang, J.; Lu, X.; Wang, F.; Yang, J.; Li, F. An 1H NMR and UPLC-MS-based plasma metabolomic study to investigate the biochemical changes in chronic unpredictable mild stress model of depression. *Metabolomics*, **2011**, *7* (3), 413-423.
- [45] Lanza, I. R.; Zhang, S.; Ward, L. E.; Karakelides, H.; Raftery, D.; Nair, K. S. Quantitative metabolomics by 1H-NMR and LC-MS/MS confirms altered metabolic pathways in diabetes. *PLoS One*, **2010**, *5* (5), e10538.
- [46] Gu, H.; Pan, Z.; Xi, B.; Asiago, V.; Musselman, B.; Raftery, D. Principal component directed partial least squares analysis for combining nuclear magnetic resonance and mass spectrometry data in metabolomics: Application to the detection of breast cancer. *Anal. Chim. Acta*, **2011**, *686* (1-2), 57-63.
- [47] Nicholson, J. K.; Buckingham, M. J.; Saldler, P. J. High resolution 1H n.m.r. studies of vertebrate blood and plasma. *Biochem. J.*, **1983**, *211*, (3), 605-615.
- [48] Lindon, J. C.; Holmes, E.; Nicholson, J. K. Metabonomics in pharmaceutical R & D. *FEBS J.*, **2007**, *274* (5), 1140-1151.
- [49] Shin, J.-H.; Yang, J.-Y.; Jeon, B.-Y.; Yoon, Y. J.; Cho, S.-N.; Kang, Y.-H.; Ryu, D. H.; Hwang, G.-S. 1H NMR-based metabolomic profiling in mice infected with mycobacterium tuberculosis. *J. Proteome Res.*, **2011**, *10*, (5), 2238-2247.
- [50] Teng, R.; Junankar, P. R.; Bubb, W. A.; Rae, C.; Mercier, P.; Kirk, K. Metabolite profiling of the intraerythrocytic malaria parasite *Plasmodium falciparum* by 1H NMR spectroscopy. *NMR Biomed.*, **2009**, *22* (3), 292-302.
- [51] Slupsky, C. M. NMR-based analysis of metabolites in urine provides rapid diagnosis and etiology of pneumonia. *Biomarkers Med.*, **2010**, *4* (2), 195-197.
- [52] DeFeo, E. M.; Wu, C.-L.; McDougal, W. S.; Cheng, L. L. A decade in prostate cancer: from NMR to metabolomics. *Nat. Rev. Urol.*, **2011**, *8* (6), 301-311.
- [53] Ahmed, S. S. J.; Santosh, W.; Kumar, S.; Christlet, H. Metabolic profiling of Parkinson's disease: evidence of biomarker from gene expression analysis and rapid neural network detection. *J. Biomed. Sci.*, **2009**, *16*, 63.
- [54] Sinclair, A. J.; Viant, M. R.; Ball, A. K.; Burdon, M. A.; Walker, E. A.; Stewart, P. M.; Rauz, S.; Young, S. P. NMR-based metabolomic analysis of cerebrospinal fluid and serum in neurological diseases - a diagnostic tool? *NMR Biomed.*, **2009**, *23* (2), 123-132.
- [55] Beger, R. D.; Schnackenberg, L. K.; Holland, R. D.; Li, D.; Dragan, Y. Metabonomic models of human pancreatic cancer using 1D proton NMR spectra of lipids in plasma. *Metabolomics*, **2006**, *2* (3), 125-134.
- [56] Bathe, O. F.; Shaykhtudinov, R.; Kopciuk, K.; Weljie, A. M.; McKay, A.; Sutherland, F. R.; Dixon, E.; Dunse, N.; Sotiropoulos, D.; Vogel, H. J. Feasibility of identifying pancreatic cancer based on serum metabolomics. *Cancer Epidem. Biomar.*, **2010**, *20* (1), 140-147.
- [57] Uversky, V. N.; Blasco, H.; Corcia, P.; Moreau, C.; Veau, S.; Fournier, C.; Vourc'h, P.; Emond, P.; Gordon, P.; Pradat, P.-F.; Praline, J.; Devos, D.; Nadal-Desbarats, L.; Andres, C. R. 1H-NMR-based metabolomic profiling of CSF in early amyotrophic lateral sclerosis. *PLoS One*, **2010**, *5* (10), e13223.
- [58] Bharti, S. K.; Jaiswal, V.; Ghoshal, U.; Ghoshal, U. C.; Baijal, S. S.; Roy, R.; Khetrapal, C. L. Metabolomic profiling of amoebic and pyogenic liver abscesses: an *in vitro* NMR study. *Metabolomics*, **2011**, Published online.
- [59] Ramadan, Z.; Jacobs, D.; Grigorov, M.; Kochhar, S. Metabolic profiling using principal component analysis, discriminant partial least squares, and genetic algorithms. *Talanta*, **2006**, *68* (5), 1683-1691.
- [60] Monleón, D.; Morales, J. M.; Barrasa, A.; López, J. A.; Vázquez, C.; Celda, B. Metabolite profiling of fecal water extracts from human colorectal cancer. *NMR Biomed.*, **2009**, *22* (3), 342-348.
- [61] Auray-Blais, C.; Raiche, E.; Gagnon, R.; Berthiaume, M.; Pasquier, J.-C. Metabolomics and preterm birth: What biomarkers in cervicovaginal secretions are predictive of high-risk pregnant women? *International J. Mass Spectrom.*, **2011**, *307* (1-3), 33-38.
- [62] Beckonert, O.; Keun, H. C.; Ebbels, T. M. D.; Bundy, J.; Holmes, E.; Lindon, J. C.; Nicholson, J. K. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. *Nat. Protoc.*, **2007**, *2* (11), 2692-2703.
- [63] Beckonert, O.; Coen, M.; Keun, H. C.; Wang, Y.; Ebbels, T. M. D.; Holmes, E.; Lindon, J. C.; Nicholson, J. K. High-resolution magic-angle-spinning NMR spectroscopy for metabolic profiling of intact tissues. *Nat. Protoc.*, **2010**, *5* (6), 1019-1032.
- [64] Somashekar, B. S.; Kamarajan, P.; Danciu, T.; Kapila, Y. L.; Chinnaiyan, A. M.; Rajendiran, T. M.; Ramamoorthy, A. Magic angle spinning NMR-based metabolomic profiling of head and neck squamous cell carcinoma tissues. *J. Proteome Res.*, **2011**, *10* (11), 5232-5241.
- [65] Chan, E. C. Y.; Koh, P. K.; Mal, M.; Cheah, P. Y.; Eu, K. W.; Backshall, A.; Cavill, R.; Nicholson, J. K.; Keun, H. C. Metabolic profiling of human colorectal cancer using high-resolution magic

- angle spinning nuclear magnetic resonance (HR-MAS NMR spectroscopy and gas chromatography mass spectrometry (GC/MS). *J. Proteome Res.*, **2009**, *8* (1), 352-361.
- [66] Wilson, M.; Davies, N. P.; Brundler, M.-A.; McConville, C.; Grundy, R. G.; Peet, A. C. High resolution magic angle spinning 1H NMR of childhood brain and nervous system tumours. *Mol. Cancer*, **2009**, *8*, 6.
- [67] Spratlin, J. L.; Serkova, N. J.; Eckhardt, S. G. Clinical applications of metabolomics in oncology: A Review. *Clin. Cancer Res.*, **2009**, *15*, 431-440.
- [68] Saude, E. J.; Sykes, B. D. Urine stability for metabolomic studies: effects of preparation and storage. *Metabolomics*, **2007**, *3* (1), 19-27.
- [69] Anthony D. Maher, A.D.; Zirah, S.F.M.; Holmes, E.; Nicholson J.K. Experimental and analytical variation in human urine in 1H NMR spectroscopy-based metabolic phenotyping studies. *Anal. Chem.*, **2007**, *79* (14), 5204-5211.
- [70] Slupsky, C. M.; Rankin, K. N.; Wagner, J.; Fu, H.; Chang, D.; Weljie, A. M.; Saude, E. J.; Lix, B.; Adamko, D. J.; Shah, S.; Greiner, R.; Sykes, B. D.; Marrie, T. J. Investigations of the effects of gender, diurnal variation, and age in human urinary metabolomic profiles. *Anal. Chem.*, **2007**, *79* (18), 6995-7004.
- [71] Minami, Y.; Kasukawa, T.; Kakazu, Y.; Iigo, M.; Sugimoto, M.; Ikeda, S.; Yasui, A.; van, d. H. G. T. J.; Soga, T.; Ueda, H. R. Measurement of internal body time by blood metabolomics. *Proc. Natl. Acad. Sci. USA*, **2009**, *106* (24), 9890-9895.
- [72] Saude, E. J.; Adamko, D.; Rowe, B. H.; Marrie, T.; Sykes, B. D. Variation of metabolites in normal human urine. *Metabolomics*, **2007**, *3* (4), 439-451.
- [73] NCCLS, Urinalysis and Collection, Transportation, and Preservation of Urine Specimens; Approved Guideline-Second Edition. *NCCLS document GP16-A2 [ISBN 1-56238-448-1]*, NCCLS, 940 West Valley Rd, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, **2001**.
- [74] Lauridsen, M.; Hansen, S. H.; Jroszewski, J. W.; Cornett, C. Human urine as test material in 1H NMR-based metabolomics: Recommendations for sample preparation and storage. *Anal. Chem.*, **2007**, *79* (3), 1181-1186.
- [75] Wining, H.; Roldán-Marín, E.; Dragsted, L. O.; Viereck, N.; Poulsen, M.; Sánchez-Moreno, C.; Cano, M. P.; Engelsen, S. B. An exploratory NMR nutri-metabolomic investigation reveals dimethyl sulfone as a dietary biomarker for onion intake. *The Analyst*, **2009**, *134* (11), 2344-2351.
- [76] Winnike, J. H.; Busby, M. G.; Watkins, P. B.; O'Connell, T. M. Effects of a prolonged standardized diet on normalizing the human metabolome. *Am. J. Clin. Nutr.*, **2009**, *90* (6), 1496-1501.
- [77] Bando, K.; Kawahara, R.; Kunimatsu, T.; Sakai, J.; Kimura, J.; Funabashi, H.; Seki, T.; Bamba, T.; Fukusaki, E. Influences of biofluid sample collection and handling procedures on GC-MS based metabolomic studies. *J. Biosci. Bioeng.*, **2010**, *110* (4), 491-499.
- [78] Briscoe, C. J.; Hage, D. S. Factors affecting the stability of drugs and drug metabolites in biological matrices. *Bioanalysis*, **2009**, *1* (1), 205-220.
- [79] Canelas, A. B.; Ras, C.; ten Pierick, A.; van Dam, J. C.; Heijnen, J. J.; van Gulik, W. M. Leakage-free rapid quenching technique for yeast metabolomics. *Metabolomics*, **2008**, *4* (3), 226-239.
- [80] Bernini, P.; Bertini, I.; Luchinat, C.; Nincheri, P.; Staderini, S.; Turano, P. Standard operating procedures for pre-analytical handling of blood and urine for metabolomic studies and biobanks. *J. Biomol. NMR*, **2011**, *49* (3-4), 231-243.
- [81] Rasmussen, L. G.; Savorani, F.; Larsen, T. M.; Dragsted, L. O.; Astrup, A.; Engelsen, S. B. Standardization of factors that influence human urine metabolomics. *Metabolomics*, **2011**, *7* (1), 71-83.
- [82] Van, C. E.; Shapiro, E. T.; Tillil, H.; Polonsky, K. S. Circadian modulation of glucose and insulin responses to meals: relationship to cortisol rhythm. *Am. J. Physiol.*, **1992**, *262* (4, Pt. 1), E467-E475.
- [83] Alum, M. F.; Shaw, P. A.; Sweatman, B. C.; Ubhi, B. K.; Haselden, J. N.; Connor, S. C. 4,4-Dimethyl-4-silapentane-1-ammonium trifluoroacetate (DSA), a promising universal internal standard for NMR-based metabolic profiling studies of biofluids, including blood plasma and serum. *Metabolomics*, **2008**, *4*, (2), 122-127.
- [84] Bone, I. The increasing importance of inflammation in neurological disease. *Curr Opin Neurol*, **2007**, *20*, (3), 331-333.
- [85] Bales, J. R.; P. Higham, D.; Howe, I.; Nicholson, J. K.; Sadler, P. J. Use of high-resolution proton nuclear magnetic resonance spectroscopy for rapid multi-component analysis of urine. *Clin. Chem.*, **1984**, *30* (3), 426-432.
- [86] Jukarainen, N. M.; Korhonen, S.-P.; Laakso, M. P.; Korolainen, M. A.; Niemitz, M.; Soininen, P. P.; Tuppurainen, K.; Vepsäläinen, J.; Pirttilä, T.; Laatikainen, R. Quantification of 1H NMR spectra of human cerebrospinal fluid: a protocol based on constrained total-line-shape analysis. *Metabolomics*, **2008**, *4* (2), 150-160.
- [87] Amathieu, R.; Nahon, P.; Triba, M.; Bouchemal, N.; Trinchet, J.-C.; Beaugrand, M.; Dhonneur, G.; Le Moyec, L. Metabolomic approach by 1H NMR spectroscopy of serum for the assessment of chronic liver failure in patients with cirrhosis. *J. Proteome Res.*, **2011**, *10* (7), 3239-3245.
- [88] Wang, J.; Zhang, S.; Li, Z.; Yang, J.; Huang, C.; Liang, R.; Liu, Z.; Zhou, R. 1H-NMR-based metabolomics of tumor tissue for the metabolic characterization of rat hepatocellular carcinoma formation and metastasis. *Tumor Biol.*, **2010**, *32* (1), 223-231.
- [89] Kang, S.-M.; Park, J.-C.; Shin, M.-J.; Lee, H.; Oh, J.; Ryu, D. H.; Hwang, G.-S.; Chung, J. H. 1H nuclear magnetic resonance based metabolic urinary profiling of patients with ischemic heart failure. *Clin. Biochem.*, **2011**, *44* (4), 293-299.
- [90] Cui, Q.; Lewis, I. A.; Hegeman, A. D.; Anderson, M. E.; Li, J.; Schulte, C. F.; Westler, W. M.; Eghbalian, H. R.; Sussman, M. R.; Markley, J. L. Metabolite identification via the madison metabolomics consortium database. *Nat. Biotechnol.*, **2008**, *26*, (2), 162-164.
- [91] Ulrich, E. L.; Akutsu, H.; Doreleijers, J. F.; Harano, Y.; Ioannidis, Y. E.; Lin, J.; Livny, M.; Mading, S.; Maziuk, D.; Miller, Z.; Nakatani, E.; Schulte, C. F.; Tolmie, D. E.; Kent Wenger, R.; Yao, H.; Markley, J. L. BioMagResBank. *Nucleic Acids Res.*, **2008**, *36*, (Database issue), D402-408.
- [92] Wishart, D. S.; Tzur, D.; Knox, C.; Eisner, R.; Guo, A. C.; Young, N.; Cheng, D.; Jewell, K.; Arndt, D.; Sawhney, S.; Fung, C.; Nikolai, L.; Lewis, M.; Coutouly, M. A.; Forsythe, I.; Tang, P.; Shrivastava, S.; Jeroncic, K.; Stothard, P.; Amegbey, G.; Block, D.; Hau, D. D.; Wagner, J.; Miniaci, J.; Clements, M.; Gebremedhin, M.; Guo, N.; Zhang, Y.; Duggan, G. E.; Macinnis, G. D.; Weljie, A. M.; Dowlatabadi, R.; Bamforth, F.; Clive, D.; Greiner, R.; Li, L.; Marrie, T.; Sykes, B. D.; Vogel, H. J.; Querengesser, L. HMDB: the human metabolome database. *Nucleic Acids Res.*, **2007**, *35*, (Database issue), D521-526.
- [93] Forsythe, I. J.; Wishart, D. S. Exploring human metabolites using the human metabolome database. *Curr Protoc Bioinformatics*, **2009**, Chapter 14, Unit 14.8.
- [94] Bertram, H. C.; Malmendal, A.; Petersen, B. O.; Madsen, J. C.; Pedersen, H.; Nielsen, N. C.; Hoppe, C.; Molgaard, C.; Michaelsen, K. F.; Duus, J. O. Effect of magnetic field strength on NMR-based metabolomic human urine data. comparative study of 250, 400, 500, and 800 MHz. *Anal. Chem.*, **2007**, *79* (18), 7110-7115.
- [95] He, Q.; Ren, P.; Kong, X.; Wu, Y.; Wu, G.; Li, P.; Hao, F.; Tang, H.; Blachier, F.; Yin, Y. Comparison of serum metabolite compositions between obese and lean growing pigs using an NMR-based metabolomic approach. *J. Nutr. Biochemistry*, **2011**, *23* (2), 133-139.
- [96] Rastrelli, F.; Jha, S.; Mancin, F. Seeing through macromolecules: T2-filtered NMR for the purity assay of functionalized nanosystems and the screening of biofluids. *J. Am. Chem. Soc.*, **2009**, *131*, 14222-14224.
- [97] Diaz, S. I. O.; Pinto, J.; Graça, G. a.; Duarte, I. F.; Barros, A. n. S.; Galhano, E. I.; Pita, C.; Almeida, M. D. C. U.; Goodfellow, B. J.; Carreira, I. M.; Gil, A. M. Metabolic biomarkers of prenatal disorders: An exploratory NMR metabolomics study of second trimester maternal urine and blood plasma. *J. Proteome Res.*, **2011**, *10* (8), 3732-3742.
- [98] Lewis, I. A.; Schommer, S. C.; Hodis, B.; Robb, K. A.; Tonelli, M.; Westler, W. M.; Sussman, M. R.; Markley, J. L. Method for determining molar concentrations of metabolites in complex solutions from two-dimensional 1H-13C NMR spectra. *Anal. Chem.*, **2007**, *79*, 9385-9390.
- [99] Jeannerat, D.; Furrer, J. NMR experiments for the analysis of mixtures: Beyond 1D 1H spectra. *Comb. Chem. High Throughput Screen.*, **2012**, *15* (1), 15-35.
- [100] Zhang, F.; Bruschiweiler-Li, L.; Robinette, S. L. Self-consistent metabolic mixture analysis by heteronuclear NMR. application to a human cancer cell line. *Anal. Chem.*, **2008**, *80* (19), 7549-7553.

- [101] Fan, T. W. M.; Lane, A. N.; Higashi, R. M.; Farag, M. A.; Gao, H.; Bousamra, M.; Miller, D. M. Altered regulation of metabolic pathways in human lung cancer discerned by  $^{13}\text{C}$  stable isotope-resolved metabolomics (SIRM). *Mol. Cancer*, **2009**, *8*, 41.
- [102] Moseley, H. N. B.; Lane, A. N.; Belshoff, A. C.; Higashi, R. M.; Fan, T. W. M. A novel deconvolution method for modeling UDP-N-acetyl-D-glucosamine biosynthetic pathways based on  $^{13}\text{C}$  mass isotopologue profiles under non-steady-state conditions. *BMC Biology*, **2011**, *9* (1), 37.
- [103] Heikkinen, S.; Toikka, M. M.; Karhunen, P. T.; Kilpeläinen, I. A. Quantitative 2D HSQC (Q-HSQC) via suppression of J-dependence of polarization transfer in NMR spectroscopy: Application to wood lignin. *J. Am. Chem. Soc.*, **2003**, *125* (14), 4362-4367.
- [104] Henderson, T. J. Sensitivity-enhanced quantitative  $^{13}\text{C}$  NMR spectroscopy via cancellation of  $1\text{JCH}$  dependence in DEPT polarization transfers. *J. Am. Chem. Soc.*, **2004**, *126*, 3682-3683.
- [105] Koskela, H.; Kilpeläinen, I.; Heikkinen, S. Some aspects of quantitative 2D NMR. *J. Magn. Reson.*, **2005**, *174* (2), 237-244.
- [106] Gronwald, W.; Klein, M. S.; Kaspar, H.; Fagerer, S. R.; Nürnberger, N.; Dettmer, K.; Bertsch, T.; Oefner, P. J. Urinary metabolite quantification employing 2D NMR spectroscopy. *Anal. Chem.*, **2007**, *80*, 9288-9297.
- [107] Hu, F.; Furihata, K.; Kato, Y.; Tanokura, M. Nondestructive quantification of organic compounds in whole milk without pretreatment by two-dimensional NMR spectroscopy. *J. Agric. Food Chem.*, **2007**, *55*, 4307-4311.
- [108] Koskela, H.; Heikkilä, O.; Kilpeläinen, I.; Heikkinen, S. Quantitative two-dimensional HSQC experiment for high magnetic field NMR spectrometers. *J. Magn. Reson.*, **2010**, *202* (1), 24-33.
- [109] Hu, K.; Westler, W. M.; Markley, J. L. Simultaneous quantification and identification of individual chemicals in metabolite mixtures by two-dimensional extrapolated time-zero  $^1\text{H}$ - $^{13}\text{C}$  HSQC (HSQC0). *J. Amer. Chem. Soc.*, **2011**, *133* (6), 1662-1665.
- [110] Lindon, J. C.; Nicholson, J. K. Analytical technologies for metabolomics and metabolomics, and multi-omic information recovery. *TrAC Trends in Anal. Chem.*, **2008**, *27* (3), 194-204.
- [111] Parsons, H. M.; Ludwig, C.; Günther, U. L.; Viant, M. R. Improved classification accuracy in 1- and 2-dimensional NMR metabolomics data using the variance stabilising generalised logarithm transformation. *BMC Bioinformatics*, **2007**, *8*, 234.
- [112] Lindon, J. C.; Holmes, E.; Nicholson, J. K. Pattern recognition methods and applications in biomedical magnetic resonance. *Prog. Nucl. Magn. Reson. Spectrosc.*, **2001**, *39* (1), 1-40.
- [113] Bylesjoe, M.; Rantalainen, M.; Cloarec, O.; Nicholson, J. K.; Holmes, E.; Trygg, J. OPLS discriminant analysis: combining the strengths of PLS-DA and SIMCA classification. *J. Chemom.*, **2007**, *20*, 341-351.
- [114] Holmes, E.; Nicholson, J. K.; Nicholls, A. W.; Lindon, J. C.; Connor, S. C.; Polley, S.; Connelly, J. The identification of novel biomarkers of renal toxicity using automatic data reduction techniques and PCA of proton NMR spectra of urine. *Chemom. Intell. Lab. Syst.*, **1998**, *44*, 245-255.
- [115] De Meyer, T.; Sinnaeve, D.; Van Gasse, B.; Tsiporkova, E.; Rietzschel, E. R.; De Buyzere, M. L.; Gillebert, T. C.; Bekaert, S.; Martins, J. C.; Crieckinge, W. V. NMR-based characterization of metabolic alterations in hypertension using an adaptive, intelligent binning algorithm. *Anal. Chem.*, **2008**, *80*, 3783-3790.
- [116] Craig, A.; Cloarec, O.; Holmes, E.; Nicholson, J. K.; Lindon, J. C. Scaling and normalization effects in NMR spectroscopic metabolomic data sets. *Anal. Chem.*, **2006**, *78*, 2262-2267.
- [117] Halouska, S.; Powers, R. Negative impact of noise on the principal component analysis of NMR data. *J. Magn. Reson.*, **2006**, *178* (1), 88-95.
- [118] Triba, M. N.; Starzec, A.; Bouchemal, N.; Guenin, E.; Perret, G. Y.; Le Moyec, L. Metabolomic profiling with NMR discriminates between biphosphonate and doxorubicin effects on B16 melanoma cells. *NMR Biomed.*, **2010**, *23* (9), 1009-1016.
- [119] van den Berg, R. A.; Hoefsloot, H. C. J.; Westerhuis, J. A.; Smilde, A. K.; van der Werf, M. J. Centering, scaling, and transformations: improving the biological information content of metabolomics data. *BMC Genomics*, **2006**, *7*, 142.
- [120] Zhang, B.; Halouska, S.; Schiaffo, C. E.; Sadykov, M. R.; Somerville, G. A.; Powers, R. NMR analysis of a stress response metabolic signaling network. *J. Proteome Res.*, **2011**, *10*, 3743-3754.
- [121] Westerhuis, J. A.; Velzen, E. J. J.; Hoefsloot, H. C. J.; Smilde, A. K. Multivariate paired data analysis: multilevel PLS-DA versus OPLS-DA. *Metabolomics*, **2009**, *6* (1), 119-128.
- [122] Molteni, C. G.; Cazzaniga, G.; Condorelli, D. F.; Fortuna, C. G.; Biondi, A.; Musumarra, G. Successful application of OPLS-DA for the discrimination of wild-type and mutated cells in acute lymphoblastic leukemia. *QSAR Comb. Sci.*, **2009**, *28* (8), 822-828.
- [123] Basant, A.; Rege, M.; Sharma, S.; Sonawat, H. M. Alterations in urine, serum and brain metabolomic profiles exhibit sexual dimorphism during malaria disease progression. *Malaria J.*, **2010**, *9*, 110.
- [124] Westerhuis, J. A.; Hoefsloot, H. C. J.; Smit, S.; Vis, D. J.; Smilde, A. K.; van Velzen, E. J. J.; van Duijnhoven, J. P. M.; van Dorsten, F. A. Assessment of PLS-DA cross validation. *Metabolomics*, **2008**, *4*, 81-89.
- [125] Efron, B.; Halloran, E.; Holmes, S. Bootstrap confidence levels for phylogenetic trees. *Proc. Natl. Acad. Sci. U S A*, **1996**, *93* (23), 13429-13434.
- [126] Werth, M. T.; Halouska, S.; Shortridge, M. D.; Zhang, B.; Powers, R. Analysis of metabolomic PCA data using tree diagrams. *Anal. Biochem.*, **2010**, *399* (1), 58-63.
- [127] Retief, J. D. Phylogenetic analysis using PHYLIP. *Methods Mol. Biol.*, **2000**, *132*, 243-258.
- [128] Zhang, B.; Halouska, S.; Schiaffo, C. E.; Sadykov, M. R.; Somerville, G. A.; Powers, R. NMR analysis of a stress response metabolic signaling network. *J. Proteome Res.*, **2011**, *10* (8), 3743-3754.
- [129] Halouska, S.; Fenton, R. J.; Barletta, R. I. G.; Powers, R. Predicting the *in vivo* mechanism of action for drug leads using NMR metabolomics. *ACS Chem. Biol.*, **2012**, *7* (1), 166-171.
- [130] Carrola, J.; Rocha, C. M.; Barros, A. S.; Gil, A. M.; Goodfellow, B. J.; Carreira, I. M.; Bernardo, J.; Gomes, A.; Sousa, V.; Carvalho, L.; Duarte, I. F. Metabolic signatures of lung cancer in biofluids: NMR-based metabolomics of urine. *J. Proteome Res.*, **2011**, *10* (1), 221-230.
- [131] Chiaradonna, F.; Moresco, R. M.; Airoidi, C.; Gaglio, D.; Palorini, R.; Nicotra, F.; Messa, C.; Alberghina, L. From cancer metabolism to new biomarkers and drug targets. *Biotechnol. Adv.*, **2012**, *30* (1), 30-51.
- [132] MacKinnon, N.; Khan, A. P.; Chinnaiyan, A. M.; Rajendiran, T. M.; Ramamoorthy, A. Androgen receptor activation results in metabolite signatures of an aggressive prostate cancer phenotype: an NMR-based metabolomics study. *Metabolomics*, **2012**, published online.
- [133] Bernini, P.; Bertini, I.; Luchinat, C.; Tenori, L.; Tognaccini, A. The cardiovascular risk of healthy individuals studied by NMR metabolomics of plasma samples. *J. Proteome Res.*, **2011**, *10* (11), 4983-4992.
- [134] Heinzmann, S. S.; Brown, I. J.; Chan, Q.; Bictash, M.; Dumas, M. E.; Kochhar, S.; Stamler, J.; Holmes, E.; Elliott, P.; Nicholson, J. K. Metabolic profiling strategy for discovery of nutritional biomarkers: proline betaine as a marker of citrus consumption. *Am. J. Clin. Nutr.*, **2010**, *92* (2), 436-443.