The future of NMR-based metabolomics

John L Markley[1], Rafael Brüschweiler[2,3], Arthur S Edison[4],
Hamid R Eghbalnia[1], Robert Powers[5], Daniel Raftery[6] and
David S Wishart[7,8]

CrossMark

The two leading analytical approaches to metabolomics are
mass spectrometry (MS) and nuclear magnetic resonance
(NMR) spectroscopy. Although currently overshadowed by MS
in terms of numbers of compounds resolved, NMR
spectroscopy offers advantages both on its own and coupled
with MS. NMR data are highly reproducible and quantitative
over a wide dynamic range and are unmatched for determining
structures of unknowns. NMR is adept at tracing metabolic
pathways and fluxes using isotope labels. Moreover, NMR is
non-destructive and can be utilized *in vivo*. NMR results have a
proven track record of translating *in vitro* findings to *in vivo*
clinical applications.

Addresses
[1] Biochemistry Department, University of Wisconsin-Madison,
433 Babcock Drive, Madison, WI 53706, USA
[2] Department of Chemistry & Biochemistry, The Ohio State University,
151 W. Woodruff Ave., Columbus, OH 43210, USA
[3] Department of Biological Chemistry & Pharmacology, The Ohio State
University, 151 W. Woodruff Ave., Columbus, OH 43210, USA
[4] Department of Genetics and Biochemistry, Institute of Bioinformatics
and Complex Carbohydrate Center, University of Georgia,
315 Riverbend Rd, Athens, GA 30602, USA
[5] Department of Chemistry, University of Nebraska-Lincoln,
722 Hamilton Hall, Lincoln, NE 68588, USA
[6] Department of Anesthesiology & Pain Medicine, 850 Republican St,
University of Washington, Seattle, WA 98109, USA
[7] Department of Biological Sciences, University of Alberta, Edmonton,
AB, Canada T6G 2E8
[8] Department of Computing Science, University of Alberta, Edmonton,
AB, Canada T6G 2E8

Corresponding author: Markley, John L (jmarkley@wisc.edu)

Introduction

The metabolic state of an organism depends on its
genome, transcriptome, proteome, epigenome, micro-
biome, and exposome (environment). Thus, metabolo-
mics, the study of small molecules (<1500 Da) in living
systems, provides information with a high potential for
accurately describing the physiological state of an organ-
ism. The two most successful approaches to determining
the metabolic state of an organism have been mass
spectrometry (MS) and nuclear magnetic resonance
(NMR) spectroscopy. Several years ago, the number of
publications utilizing the two approaches were compara-
ble; more recently, however, MS-based metabolomics has
clearly overtaken NMR-based metabolomics. This state
of affairs prompted the organization of a workshop to
review the current state of NMR-based metabolomics, to
assess its strengths and weaknesses, and to envision its
future potential. As reported here, this workshop ('NMR-
Based Metabolomics,' held in the Discovery Building,
Morgridge Institute for Research, Madison, Wisconsin,
USA, on June 10, 2016) highlighted a number of benefits
of NMR-based metabolomics that appear to be currently
underappreciated. MS and NMR offer different
strengths, which can be used synergistically. The work-
shop stressed the need for more extensive small molecule
databases and improved standards at each step of a
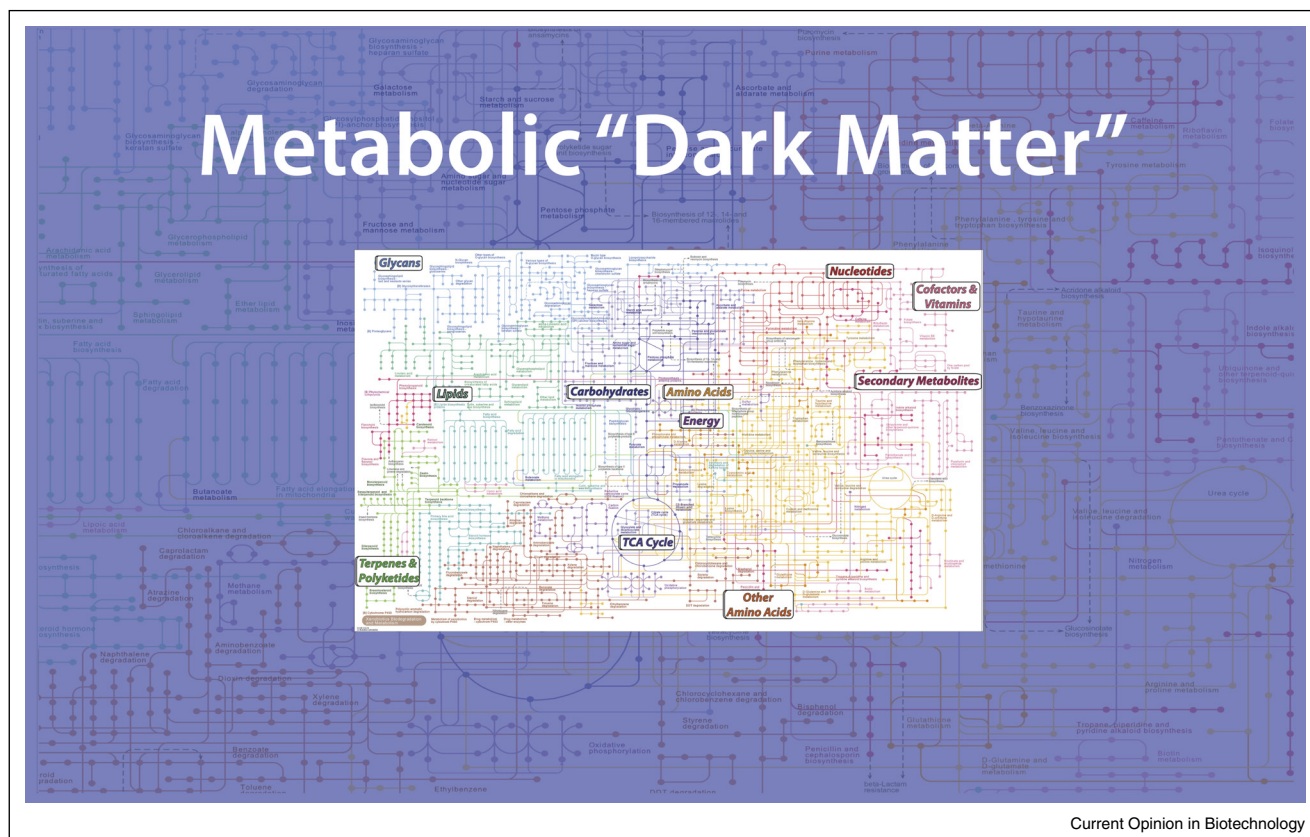metabolomics study.

The metabolome

The two major fields of chemical research on biological
small molecules, metabolomics and natural product dis-
covery, have the similar goals of identifying and charac-
terizing small molecules, either in their isolated active
state (natural product chemistry) or as mixtures (meta-
bolomics) [1]. The number of small molecules of impor-
tance to humans is far greater than those currently
represented on metabolic charts, with the excess consti-
tuting current 'metabolic dark matter' (Figure 1). The
swapping of metabolites between pathways in humans
and those of organisms in the human microbiome
increases the network of relevant reactions by a stagger-
ing amount. The HMDB [2] lists 42 000 metabolites and
the number of lipid variants is on the order of 100 000;
thus, a lower limit of expected endogenous and exoge-
nous human metabolites is around 150 000, but the actual
number of metabolites could be much higher. Of this vast
number of metabolites, only 1500 may be identified from
global profiling, 200–500 from targeted profiling, and far
fewer are routinely subjected to quantitative analysis.

NMR and its advantages

Despite its lower sensitivity, NMR spectroscopy offers
many unparalleled advantages over MS [3••,4••]. NMR

Figure 1

Current Opinion in Biotechnology

Schematic representation of metabolic (blue shaded area).

offers a window into observing and rigorously quantifying all of the more abundant compounds present in biological fluids, cell extracts, and tissues without the need for elaborate sample preparation or fractionation. NMR offers advantages for compounds that are difficult to ionize or require derivatization for MS. NMR allows the identification of compounds with identical masses, including those with different isotopomer distributions. NMR is the mainstay for determining structures of unknown compounds. Through the use of stable isotope labels, NMR can be used to elucidate the dynamics and mechanisms of metabolite transformations and to explore the compartmentalization of metabolic pathways. NMR has advantages in drug screening [5]. Finally, site-specific NMR imaging and spectroscopy offer approaches for metabolic studies in living organisms.

Strategies for the identification of metabolites in complex mixtures from NMR data have been reviewed recently [6••]. The most important nuclei in biomolecular NMR studies are ^{1}H (proton), ^{13}C, ^{15}N, and ^{31}P. Of these, ^{1}H is the most sensitive followed by ^{31}P; both are present at near 100% natural abundance. ^{31}P NMR is useful for studies of cellular energy states *in vivo* and *ex vivo*, but a

limitation is that the ^{31}P signals from most phosphorylated compounds overlap. One-dimensional (1D) ^{1}H NMR is the most widely used NMR approach in metabolomics. Signals are either binned and then analyzed or fitted to patterns of signals corresponding to the metabolites expected to be present in the mixture. The latter approach can be problematic in that many ^{1}H signals overlap in ways that offer alternative fitting solutions, a problem that can be overcome by standardizing the analysis in terms of biofluid, solution conditions, data collection protocol, and by employing probabilistic fitting (Bayesil) [7••]. ^{13}C NMR signals cover a 200 ppm range compared with 10 ppm for ^{1}H and as a consequence are better resolved; however, the low sensitivity of ^{13}C (less by a factor of 8 or more) is compounded by its low natural abundance (1.1%). Two dimensional (2D) NMR methods offer improved approaches for unambiguous identification of metabolites in mixtures. These 2D methods include ^{1}H-^{1}H COSY (correlated spectroscopy), ^{1}H–^{1}H TOCSY (total correlation spectroscopy), and ^{1}H–^{13}C HSQC (heteronuclear single-quantum correlation). A widely used software package (rNMR) matches regions of interest in spectra of standards to those in experimental mixtures for compound identifications [8]. Software is

available for automating metabolite identification from combined TOCSY and HSQC data [9$^{\bullet\bullet}$,10$^{\bullet}$]. By setting tolerances for the matching of ^1H and ^{13}C signals, one can maximize compound identification while minimizing false positives [11]. This approach has been extended to a calculated confidence level for compound identifications from NMR data [12]. Another approach for connecting signals from individual compounds in mixtures is based on searching for statistical correlations among the intensities of NMR signals from various samples [13]. Nuclei present at low natural abundance ^2H (deuteron), ^{13}C, and ^{15}N serve as ideal metabolic tracers [14$^{\bullet\bullet}$].

Need for standards in NMR metabolomics

Standard NMR spectra and associated information on small biological molecules are available from freely-accessible databases, including HMDB [2], BMRB [15], TOCCATA [9], and COLMAR [10], but they still cover only a fraction of relevant compounds. A repository has been established for results of metabolomics studies from the NIH Common Fund Centers [16$^{\bullet}$]. The Coordination of Standards in Metabolomics (COSMOS) Initiative is developing a robust data infrastructure and new data exchange standards (http://nmrml.org) for metabolomics data and metadata to support workflows metabolomics applications [17$^{\bullet}$]. One of the COSMOS projects is a website (http://metabolomexchange.org) that federates data available from the leading metabolomics data repositories. Best practices and standards have been published for metabolic phenotyping of biological fluids [18$^{\bullet\bullet}$,19]. An open-source platform for complete NMR metabolomics data handling (MVAPACK) has been developed as a step toward establishing best practices for the analysis of metabolic fingerprinting data [20$^{\bullet}$].

Sample preparation

Certain biofluids, for example, cerebrospinal fluid, require little or no preparation for NMR. Others, such as plasma contain proteins and lipids that interfere with NMR spectral quality. Treatment with methanol at solvent-to-serum ratio of 2:1 (v/v) has been shown to remove lipoproteins and minimize the loss of metabolites [21] enabling the detection of about 67 different compounds [22$^{\bullet\bullet}$]. Another promising protocol utilizes the removal of protein by added silica nanoparticles [23$^{\bullet\bullet}$].

Tagging

An approach for compounds with overlapping ^1H signals or present at lower concentration is to tag them with an NMR-active label. Nitrogen-15 with attached hydrogen is an attractive tag because 2D ^1H–^{15}N signals can be acquired at high sensitivity without interference from signals from unlabeled compounds owing to the low natural abundance of ^{15}N [24,25$^{\bullet}$]. Such tags also provide a permanent positive charge for MS analysis.

Combining NMR and MS

As reviewed recently [26$^{\bullet\bullet}$], advances in NMR-based and MS-based metabolomics, including the combination of the two approaches, promise to greatly improve the identification and quantitation of compounds in mixtures. One example is the simultaneous analysis of DI-ESI-MS and 1D ^1H NMR spectral data to yield accurate mass measurements and class separation scores [27]. Other approaches filter data from one approach against the other to increase the number of compounds confidently identified [28$^{\bullet}$,29$^{\bullet}$]. Another method identifies compounds by exploiting the principle that abundance/intensity ratios are relatively constant for the same metabolite in different samples [30]. Combined NMR and MS has advantages for isotope tracing experiments and metabolic flux analysis. MS generally quantifies isotopic labeling distributions but even with MS/MS often does not give the specific labeling position, which is available from NMR.

Quantification

If 1D ^1H NMR peaks from a compound are well resolved with acceptable signal-to-noise, their intensities correlate linearly with its relative concentration. To determine absolute concentrations, one adds a standard of known concentration. The cross peak intensities of the same 2D ^1H–^{13}C HSQC spectrum, however, do not correlate linearly with concentration. One can collect spectra of mixtures with known concentrations bracketing those of the unknowns and use these to determine factors that translate peak intensity to concentration [31]. Peak intensities in 2D ^1H–^{13}C HSQC spectra can also be converted to concentration from the slopes generated by spectra utilizing different replicates of the pulse sequence module and the linear extrapolation back to zero time of the peak intensities following the delays from one and two modules yields the 'HSQC$_0$ spectrum' whose peak intensities are proportional to concentration [32]. Spectral overlaps can be accounted for by methods such as FMLR (fast maximum-likelihood reconstruction) [33]. A new experiment (^1H–^{13}C QUIPU HSQC) aims to quantify in one map a complex mixture composed of low concentrated metabolites [34]. Another approach, one that requires full ^{13}C labeling, achieves quantification through the collection of ^{13}C–^{13}C CT-TOCSY spectra and the application of analytical approximations based on the known carbon-backbone topologies [35].

Applications of metabolomics

Applications of metabolomics include disease diagnosis, monitoring the effects of medical interventions including drugs, detection of adulteration of food, and analysis of biochemical pathways and their perturbations resulting from mutations, aging, diet, exercise, or life style. A recent study showed how *ex vivo* 1D ^1H NMR spectroscopy can be used for the simultaneous identification and quantification of co-enzymes that report on cellular function [36$^{\bullet\bullet}$]. Another study used this approach to investigate

alterations in the energy/redox-metabolome in dopaminergic cells exposed to environmental/mitochondrial toxins [37]. Studies of the metabolomics of model organisms are both timely and important for understanding of their different biology [38•]. Protocols have been described for studies of the metabolomics of bacteria [39] and plants [40]. Metabolomics, along with activity-guided fractionation followed by structural analysis, constitutes a powerful approach for identifying biologically active compounds for studies in chemical ecology [41•]. Metabolomics is used regularly in drug discovery programs to uncover the efficacy, specificity, or toxicity of lead compounds [42]. Metabolomics can provide information on the *in vivo* mechanism of action and to eliminate compounds likely to cause problems with side effects [43•]. Recent studies have utilized metabolomics to search for biomarkers for colon cancer [44] and multiple sclerosis [45].

Future technology

All technologies that increase NMR sensitivity are of extreme importance as are improvements in sample preparation [46••]. Approaches to high sensitivity include NMR spectrometers with ultra-high-field magnets operating at ^1H resonance frequencies of 1.2 GHz or higher. The first such systems are scheduled for delivery in 2017. Small high-temperature superconducting coils can maximize the signal per sample mass: a ^{13}C-optimized 1.5-mm high temperature superconducting NMR probe has enabled novel ^{13}C NMR studies of natural products [47], and this has been followed up with a ^1H–^{13}C dual-optimized NMR probe based on double-tuned high temperature superconducting resonators [48•]. These probes take advantage of the excellent peak dispersion of ^{13}C spectra [49], which can be augmented by further 2D ^{13}C–^{13}C experiments, such as INADE-QUATE [50].

Hyperpolarization offers an approach for enhanced sensitivity with even higher potential. The underlying physics utilizes the magnetic moment of the unpaired electron, which is roughly 2800 times that of ^{13}C and 6900 times that of ^{15}N, to polarize nuclear spins. First demonstrated by Golman and coworkers [51], studies utilizing hyperpolarized ^{13}C to increase sensitivity are becoming routine. An exciting advance is the discovery [52••] of an efficient and inexpensive method for hyperpolarizing ^{15}N spins at room temperature. Enhancements are on the order of >10 000 enabling the detection of NMR signals for over an hour [52••]. It may become possible to use this approach to tag a range of compounds for metabolomics studies *in vivo*.

Conclusions

The workshop demonstrated that NMR-based metabolomics promises to continue to play an important role in the studies of complex mixtures of small biological molecules, their metabolic networks, and their interactions with biomacromolecules. Although the development of new and better methods continues to be an integral part, the field needs to focus on developing standardized, enlarged, and integrated databases of NMR data on small molecules as well as archives representing the NMR metabolic fingerprints of standard biological fluids and tissue extracts from humans and model organisms. Standardization of best practices for sample preparation, data collection and analysis should enhance the reproducibility of results within the metabolomics community, while at the same time avoiding the risk of adhering to methods and protocols that are suboptimal in a field that is very much in flux. In order to overcome skepticism in omics [53•], it will be advisable for metabolomics to build the ideas of reproducibility and data sharing into every tool and database. Future work is expected to build upon core strengths of NMR spectroscopy, which includes its versatility and specificity in the form of 1D and higher dimensional spectra, its reproducibility, its quantitative ability, its capability for following chemical reactions and flux, its ability to identify compounds and deduce structures of unknowns, and its growing potential for collecting metabolomics data *in vivo*.

Acknowledgments

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Robinette SL, Brüschweiler R, Schroeder FC, Edison AS: **NMR in metabolomics and natural products research: two sides of the same coin**. *Acc Chem Res* 2012, **45**:288-297.

2. Wishart DS, Knox C, Guo AC, Eisner R, Young N, Gautam B, Hau DD, Psychogios N, Dong E, Bouatra S *et al.*: **HMDB: a knowledgebase for the human metabolome**. *Nucleic Acids Res* 2009, **37**:D603-D610.

3. Fan TW, Lane AN: **Applications of NMR spectroscopy to**
•• **systems biochemistry**. *Prog Nucl Magn Reson Spectrosc* 2016, **92-93**:18-53.
Comprehensive review of the role of NMR in metabolic research.

4. Nagana Gowda GA, Raftery D: **Can NMR solve some significant**
•• **challenges in metabolomics?** *J Magn Reson* 2015, **260**:144-160.
The authors present their perspectives on emerging trends in NMR-based metabolomics and NMR's continuing role in the field with an emphasis on recent and ongoing research from their laboratory.

5. Tiziani S, Kang Y, Choi JS, Roberts W, Paternostro G: **Metabolomic high-content nuclear magnetic resonance-based drug screening of a kinase inhibitor library**. *Nat Commun* 2011, **2**:545.

6.   Bingol K, Bruschweiler-Li L, Li D, Zhang B, Xie M, Brüschweiler R:
••   **Emerging new strategies for successful metabolite identification in metabolomics**. *Bioanalysis* 2016, **8**:557-573.
Review of recent strategies for the identification of metabolites in complex biological mixtures.

7.   Ravanbakhsh S, Liu P, Bjorndahl TC, Mandal R, Grant JR,
••   Wilson M, Eisner R, Sinelnikov I, Hu X, Luchinat C *et al.*: **Accurate, fully-automated NMR spectral profiling for metabolomics**. *PLoS One* 2015, **10**:e0124219.
The authors present a publicly-accessible system, BAYESIL, for the automated generation of the most probable metabolic profile from 1H NMR spectra of serum or cerebrospinal fluid. BAYESIL provides quantitative NMR spectral profiling with an accuracy meeting or exceeding the performance of trained experts.

8.   Lewis IA, Schommer SC, Markley JL: **rNMR: open source software for identifying and quantifying metabolites in NMR spectra**. *Magn Reson Chem* 2009, **47**:s123-s126.

9.   Bingol K, Bruschweiler-Li L, Li DW, Brüschweiler R: **Customized**
••   **metabolomics database for the analysis of NMR ^{1}H-^{1}H TOCSY and ^{13}C-^{1}H HSQC-TOCSY spectra of complex mixtures**. *Anal Chem* 2014, **86**:5494-5501.
The authors introduce the TOCCATA database, which contains complete ^{1}H and ^{13}C chemical shift information on individual spin systems and isomeric states of common metabolites. The database supports identifications of compounds present in complex metabolic mixtures at ^{13}C natural abundance from 2D ^{1}H TOCSY and 2D ^{13}C-^{1}H HSQC-TOCSY spectra.

10. Bingol K, Li DW, Bruschweiler-Li L, Cabrera OA, Megraw T,
•   Zhang F, Brüschweiler R: **Unified and isomer-specific NMR metabolomics database for the accurate analysis of ^{13}C-^{1}H HSQC spectra**. *ACS Chem Biol* 2015, **10**:452-459.
The Complex Mixture Analysis by NMR (COLMAR) ^{13}C-^{1}H HSQC database, which treats slowly exchanging isomers a metabolite as separate species, permits improved detection of compounds present at low concentration. The software is accessible from an interactive web interface.

11. Cui Q, Lewis IA, Hegeman AD, Anderson ME, Li J, Schulte CF, Westler WM, Eghbalnia HR, Sussman MR, Markley JL: **Metabolite identification via the Madison Metabolomics Consortium Database**. *Nat Biotechnol* 2008, **26**:162-164.

12. Everett JR: **A new paradigm for known metabolite identification in metabonomics/metabolomics: metabolite identification efficiency**. *Comput Struct Biotechnol J* 2015, **13**:131-144.

13. Wei S, Zhang J, Liu L, Ye T, Gowda GA, Tayyari F, Raftery D: **Ratio analysis nuclear magnetic resonance spectroscopy for selective metabolite identification in complex samples**. *Anal Chem* 2011, **83**:7616-7623.

14. Fan TW, Lane AN, Higashi RM: **Stable isotope resolved**
••   **metabolomics studies tissue slices**. *Bio Protoc* 2016:6.
The authors present an elegant study of the effect of tumor microenvironment on metabolic flux.

15. Ulrich EL, Akutsu H, Doreleijers JF, Harano Y, Ioannidis YE, Lin J, Livny M, Mading S, Maziuk D, Miller Z *et al.*: **Bio Mag Res Bank**. *Nucleic Acids Res* 2008, **36**:D402-D408.

16. Sud M, Fahy E, Cotter D, Azam K, Vadivelu I, Burant C, Edison A,
•   Fiehn O, Higashi R, Nair KS *et al.*: **Metabolomics Workbench: an international repository for metabolomics data and metadata, metabolite standards, protocols, tutorials and training, and analysis tools**. *Nucleic Acids Res* 2016, **44**:D463-D470.
The Metabolomics Workbench, part of the data coordinating effort of the National Institute of Health (NIH) Common Fund's Metabolomics Program and available at www.metabolomicsworkbench.org, is a public repository for metabolomics metadata and experimental data spanning various species and experimental platforms, metabolite standards, metabolite structures, protocols, tutorials, and training material and other educational resources. The Metabolomics Workbench provides data from the Common Fund's Metabolomics Resource Cores, metabolite standards, and analysis tools to the wider metabolomics community and seeks data depositions from metabolomics researchers across the world.

17. Salek RM, Neumann S, Schober D, Hummel J, Billiau K, Kopka J,
•   Correa E, Reijmers T, Rosato A, Tenori L *et al.*: **COordination of Standards in MetabOlomicS (COSMOS): facilitating integrated metabolomics data access**. *Metabolomics* 2015, **11**:1587-1597.

The authors announce an effort sponsored by the Framework Programme 7 EU Initiative 'coordination of standards in metabolomics' (COSMOS) to develop a robust data infrastructure and exchange standards for metabolomics data and metadata.

18. Emwas AH, Roy R, McKay RT, Ryan D, Brennan L, Tenori L,
••   Luchinat C, Gao X, Zeri AC, Gowda GA *et al.*: **Recommendations and standardization of biomarker quantification using NMR-based metabolomics with particular focus on urinary analysis**. *J Proteome Res* 2016, **15**:360-373.
The authors review published studies on NMR-based urine metabolic profiling with the aim of identifying key variables that may affect the results of metabolomics studies. From this survey, they identify a number of issues that require either standardization or careful accounting in experimental design and provide recommendations for urine collection, sample preparation and data acquisition.

19. Dona AC, Jimenez B, Schafer H, Humpfer E, Spraul M, Lewis MR, Pearce JT, Holmes E, Lindon JC, Nicholson JK: **Precision high-throughput proton NMR spectroscopy of human urine, serum, and plasma for large-scale metabolic phenotyping**. *Anal Chem* 2014, **86**:9887-9894.

20. Worley B, Powers R: **MVAPACK: a complete data handling**
•   **package for NMR metabolomics**. *ACS Chem Biol* 2014, **9**:1138-1144.
The authors report a newly developed open-source platform (MVAPACK) for complete NMR metabolomics data handling and describe its application to metabolic fingerprinting.

21. Nagana Gowda GA, Gowda YN, Raftery D: **Massive glutamine cyclization to pyroglutamic acid in human serum discovered using NMR spectroscopy**. *Anal Chem* 2015, **87**:3800-3805.

22. Nagana Gowda GA, Gowda YN, Raftery D: **Expanding the limits**
••   **of human blood metabolite quantitation using NMR spectroscopy**. *Anal Chem* 2015, **87**:706-715.
Through investigations of pooled human serum, the authors developed a protocol for removing protein while minimizing the removal of metabolites, which enables the routine identification of 67 blood metabolites including amino acids, organic acids, carbohydrates, and heterocyclic compounds,. The proposed protocol, which utilizes 2:1 (v/v) methanol:-serum to precipitate protein, was found to be far superior to the previously established acetonitrile treatment in retaining metabolites.

23. Zhang B, Xie M, Bruschweiler-Li L, Bruschweiler R: **Nanoparticle-**
••   **assisted removal of protein in human serum for metabolomics studies**. *Anal Chem* 2016, **88**:1003-1007.
The authors demonstrate how serum protein can be efficiently removed at physiological pH through attractive interactions with silica nanoparticles. The authors conclude that the combination of nanoparticle treatment and ultrafiltration has a minimal effect on the metabolite content and permits the collection of clean homo-nuclear and heteronuclear NMR spectra of the serum metabolome.

24. Tayyari F, Gowda GA, Gu H, Raftery D: **^{15}N-cholamine — a smart isotope tag for combining NMR- and MS-based metabolite profiling**. *Anal Chem* 2013, **85**:8715-8721.

25. Lane AN, Arumugam S, Lorkiewicz PK, Higashi RM, Laulhe S,
•   Nantz MH, Moseley HN, Fan TW: **Chemoselective detection and discrimination of carbonyl-containing compounds in metabolite mixtures by ^{1}H-detected ^{15}N nuclear magnetic resonance**. *Magn Reson Chem* 2015, **53**:337-343.
The authors describe a carbonyl-selective aminooxy probe incorporating ^{15}N in the aminooxy functional group that specifically reacts with free keto and aldehyde functions, but not carboxylates. This probe enables the exclusive detection by ^{15}N-edited NMR of metabolites that contain a free carbonyl function with differential identification of aminooxy adducts of ketones and aldehydes by their very different chemical shifts. By utilizing 2-bond or 3-bond ^{15}N-^{1}H couplings more than 30 carbonyl-containing compounds could be detected by the ^{15}N-edited NMR analysis. As the aminooxy probe contains a permanently charged quaternary ammonium group, the adducts are also optimized for detection by mass spectrometry.

26. Bingol K, Brüschweiler R: **Two elephants in the room: new**
••   **hybrid nuclear magnetic resonance and mass spectrometry approaches for metabolomics**. *Curr Opin Clin Nutr Metab Care* 2015, **18**:471-477.
The authors review approaches for combining metabolomics data from mass spectrometry with those from NMR spectroscopy.

27. Marshall DD, Lei S, Worley B, Huang Y, Garcia-Garcia A, Franco R, Dodds ED, Powers R: **Combining DI-ESI-MS and NMR datasets for metabolic profiling**. *Metabolomics* 2015, **11**:391-402.

28. Bingol K, Brüschweiler R: **NMR/MS translator for the enhanced**
• **simultaneous analysis of metabolomics mixtures by NMR spectroscopy and mass spectrometry: application to human urine**. *J Proteome Res* 2015, **14**:2642-2648.
The authors present an approach for increasing the number of metabolites detected in urine by combining information from NMR and MS data.

29. Bingol K, Bruschweiler-Li L, Yu C, Somogyi A, Zhang F,
• Brüschweiler R: **Metabolomics beyond spectroscopic databases: a combined MS/NMR strategy for the rapid identification of new metabolites in complex mixtures**. *Anal Chem* 2015, **87**:3864-3870.
In the SUMMIT MS/NMR approach, the chemical formulas of the mixture components are first identified from accurate masses by MS and then all feasible structures (structural manifold) consistent with these chemical formulas are generated. Next, NMR spectra of each member of the structural manifold are predicted and compared with the experimental NMR spectra in order to identify the molecular structures that match the information obtained from both the MS and NMR techniques.

30. Gu H, Gowda GA, Neto FC, Opp MR, Raftery D: **RAMSY: ratio analysis of mass spectrometry to improve compound identification**. *Anal Chem* 2013, **85**:10771-10779.

31. Lewis IA, Schommer SC, Hodis B, Robb KA, Tonelli M, Westler WM, Sussman MR, Markley JL: **Method for determining molar concentrations of metabolites in complex solutions from two-dimensional ^1H–^{13}C NMR spectra**. *Anal Chem* 2007, **79**:9385-9390.

32. Hu K, Ellinger JJ, Chylla RA, Markley JL: **Measurement of absolute concentrations of individual compounds in metabolite mixtures by gradient-selective time-zero ^1H–^{13}C HSQC with two concentration references and fast maximum likelihood reconstruction analysis**. *Anal Chem* 2011, **83**:9352-9360.

33. Chylla RA, Hu K, Ellinger JJ, Markley JL: **Deconvolution of two-dimensional NMR spectra by fast maximum likelihood reconstruction: application to quantitative metabolomics**. *Anal Chem* 2011, **83**:4871-4880.

34. Mauve C, Khlifi S, Gilard F, Mouille G, Farjon J: **Sensitive, highly resolved, and quantitative (1)H-(13)C NMR data in one go for tracking metabolites in vegetal extracts**. *Chem Commun (Camb)* 2016, **52**:6142-6145.

35. Bingol K, Zhang F, Bruschweiler-Li L, Brüschweiler R: **Quantitative analysis of metabolic mixtures by two-dimensional ^{13}C constant-time TOCSY NMR spectroscopy**. *Anal Chem* 2013, **85**:6414-6420.

36. Nagana Gowda GA, Abell L, Lee CF, Tian R, Raftery D:
•• **Simultaneous analysis of major coenzymes of cellular redox reactions and energy using *ex vivo* ^1H NMR spectroscopy**. *Anal Chem* 2016, **88**:4817-4824.
The authors show how ^1H NMR can be used to simultaneously deterimine levels of key coenzymes: oxidized and reduced forms of nicotinamide adenine dinucleotide (NAD$^+$ and NADH), oxidized and reduced forms of nicotinamide adenine dinucleotide phosphate (NADP$^+$ and NADPH), and adenosine triphosphate (ATP) and its precursors, adenosine diphosphate (ADP) and adenosine monophosphate (AMP).

37. Lei S, Zavala-Flores L, Garcia-Garcia A, Nandakumar R, Huang Y, Madayiputhiya N, Stanton RC, Dodds ED, Powers R, Franco R: **Alterations in energy/redox metabolism induced by mitochondrial and environmental toxins: a specific role for glucose-6-phosphate-dehydrogenase and the pentose phosphate pathway in paraquat toxicity**. *ACS Chem Biol* 2014, **9**:2032-2048.

38. Edison AS, Hall RD, Junot C, Karp PD, Kurland IJ, Mistrik R,
• Reed LK, Saito K, Salek RM, Steinbeck C *et al.*: **The time is right to focus on model organism metabolomes**. *Metabolites* 2016:6.
The authors propose a grand challenge for metabolomics studies of model organisms representing microbes, animals and plants in which all metabolites are identified and mapped onto metabolic pathways and quantitative metabolic models are developed. These should enable the analysis of metabolic pathways within the context of evolutionary metabolomics (phylometabolomics).

39. Halouska S, Zhang B, Gaupp R, Lei S, Snell E, Fenton RJ, Barletta RG, Somerville GA, Powers R: **Revisiting protocols for the NMR analysis of bacterial metabolomes**. *J Integr OMICS* 2013, **3**:120-137.

40. Sumner LW, Lei Z, Nikolau BJ, Saito K: **Modern plant metabolomics: advanced natural product gene discoveries, improved technologies, and future prospects**. *Nat Prod Rep* 2015, **32**:212-229.

41. Edison AS, Clendinen CS, Ajredini R, Beecher C, Ponce FV,
• Stupp GS: **Metabolomics and natural-products strategies to study chemical ecology in nematodes**. *Integr Comp Biol* 2015, **55**:478-485.
As illustrated with results from studies of *Caenorhabditis elegans* and related free-living nematodes, this review describes the two complementary approaches used to identify biologically active compounds for studies in chemical ecology: activity-guided fractionation and metabolomics. A new liquid chromatography–mass spectrometry-based method called isotopic ratio outlier analysis is described that can be used as an adjunct to NMR spectroscopy.

42. Forgue P, Halouska S, Werth M, Xu K, Harris S, Powers R: **NMR metabolic profiling of *Aspergillus nidulans* to monitor drug and protein activity**. *J Proteome Res* 2006, **5**:1916-1923.

43. Powers R: **The current state of drug discovery and a potential**
• **role for NMR metabolomics**. *J Med Chem* 2014, **57**:5860-5870.
The authors propose ways in which the inclusion of metabolomics as a routine component of the drug discovery process may improve its efficiency and effectiveness. Metabolomics can be used to eliminate compounds with potential efficacy and side effect problems while prioritizing well-behaved leads with druglike characteristics.

44. Chen C, Deng L, Wei S, Nagana Gowda GA, Gu H, Chiorean EG, Abu Zaid M, Harrison ML, Pekny JF, Loehrer PJ *et al.*: **Exploring metabolic profile differences between colorectal polyp patients and controls using seemingly unrelated regression**. *J Proteome Res* 2015, **14**:2492-2499.

45. Gebregiworgis T, Nielsen HH, Massilamany C, Gangaplara A, Reddy J, Illes Z, Powers R: **A urinary metabolic signature for multiple sclerosis and neuromyelitis optica**. *J Proteome Res* 2016, **15**:659-666.

46. Ardenkjaer-Larsen JH, Boebinger GS, Comment A, Duckett S,
•• Edison AS, Engelke F, Griesinger C, Griffin RG, Hilty C, Maeda H *et al.*: **Facing and overcoming sensitivity challenges in biomolecular NMR spectroscopy**. *Angew Chem Int Ed Engl* 2015, **54**:9162-9185.
The authors review strategies for overcoming the inherent low sensitivity of biomolecular NMR spectroscopy.

47. Ramaswamy V, Hooker JW, Withers RS, Nast RE, Brey WW, Edison AS: **Development of a ^{13}C-optimized 1.5-mm high temperature superconducting NMR probe**. *J Magn Reson* 2013, **235**:58-65.

48. Ramaswamy V, Hooker JW, Withers RS, Nast RE, Edison AS,
• Brey WW: **Development of a ^1H–^{13}C dual-optimized NMR probe based on double-tuned high temperature superconducting resonators**. *Ieee Transact Appl Superconduct* 2016:26.
The authors report a novel NMR probe design simultaneously optimized for ^1H and ^{13}C detection. The probe employs novel double-tuned HTS resonators designed for optimal magnetic field homogeneity and minimal electric field that generate strong, uniform, and mutually orthogonal magnetic fields at the ^1H and ^{13}C NMR frequencies.

49. Clendinen CS, Lee-McMullen B, Williams CM, Stupp GS, Vandenborne K, Hahn DA, Walter GA, Edison AS: **^{13}C NMR metabolomics: applications at natural abundance**. *Anal Chem* 2014, **86**:9242-9250.

50. Clendinen CS, Pasquel C, Ajredini R, Edison AS: **^{13}C NMR metabolomics: inadequate network analysis**. *Anal Chem* 2015, **87**:5698-5706.

51. Ardenkjaer-Larsen JH, Fridlund B, Gram A, Hansson G, Hansson L, Lerche MH, Servin R, Thaning M, Golman K: **Increase in signal-to-noise ratio of >10,000 times in liquid-state NMR**. *Proc Natl Acad Sci U S A* 2003, **100**:10158-10163.

52. Theis T, Ortiz GX Jr, Logan AW, Claytor KE, Feng Y, Huhn WP,
•• Blum V, Malcolmson SJ, Chekmenev EY, Wang Q *et al.*: **Direct and cost-efficient hyperpolarization of long-lived nuclear spin states on universal $^{15}N_2$-diazirine molecular tags**. *Sci Adv* 2016, **2**:e1501438.
The authors demonstrate how a recently developed method (SABRE-SHEATH) can be used to directly hyperpolarize $^{15}N_2$ magnetization and $^{15}N_2$ singlet spin order, with signal decay time constants of 5.8 and

23 minutes, respectively. The approach generates >10 000-fold enhancements with detectable nuclear MR signals that last for over an hour from molecular tags that can be incorporated into a wide range of biomolecules.

53. Ioannidis JP, Greenland S, Hlatky MA, Khoury MJ, Macleod MR,
•   Moher D, Schulz KF, Tibshirani R: **Increasing value and reducing waste in research design, conduct, and analysis**. *Lancet* 2014, **383**:166-175.
Potential solutions are proposed for the problems of irreproducible scientific studies including improvements in protocols and documentation, consideration of evidence from studies in progress, standardization of research efforts, and scientific workforce development.