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# The ABRF Metabolomics Research Group 2013 Study: Investigation of Spiked Compound Differences in a Human Plasma Matrix

*Amrita K. Cheema,[1,2] John M. Asara,[4] Yiwen Wang,[3] Thomas A. Neubert,[5] Vladimir Tolstikov,[6] and Chris W. Turck[7]*

*[1]Department of Oncology and [2]Department of Biochemistry, Molecular and Cellular Biology, and [3]Department of Biostatistics, Bioinformatics and Biomathematics, Georgetown University Medical Center, Washington, DC, USA; [4]Division of Signal Transduction, Beth Israel Deaconess Medical Center, and Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA; [5]Department of Biochemistry and Molecular Pharmacology, New York University School of Medicine, New York, New York, USA; [6]Berg, New York, New York, USA; and [7]Max Planck Institute of Psychiatry, Munich, Germany*

Metabolomics is an emerging field that involves qualitative and quantitative measurements of small molecule metabolites in a biological system. These measurements can be useful for developing biomarkers for diagnosis, prognosis, or predicting response to therapy. Currently, a wide variety of metabolomics approaches, including nontargeted and targeted profiling, are used across laboratories on a routine basis. A diverse set of analytical platforms, such as NMR, gas chromatography-mass spectrometry, Orbitrap mass spectrometry, and time-of-flight-mass spectrometry, which use various chromatographic and ionization techniques, are used for resolution, detection, identification, and quantitation of metabolites from various biological matrices. However, few attempts have been made to standardize experimental methodologies or comparative analyses across different laboratories. The Metabolomics Research Group of the Association of Biomolecular Resource Facilities organized a "round-robin" experiment type of interlaboratory study, wherein human plasma samples were spiked with different amounts of metabolite standards in 2 groups of biologic samples (A and B). The goal was a study that resembles a typical metabolomics analysis. Here, we report our efforts and discuss challenges that create bottlenecks for the field. Finally, we discuss benchmarks that could be used by laboratories to compare their methodologies.

**Key Words:** targeted metabolomics, untargeted metabolomics, mass spectrometry, NMR

## METABOLOMICS: OPPORTUNITIES AND CHALLENGES

Metabolomics involves the comprehensive detection and identification of endogenous metabolites representing the "metabolome." Small molecule metabolites are substrates or products in metabolic processes. Metabolomics provides a systems biology snapshot of the net expression of metabolites from several known pathways and is hence, a powerful tool for biomedical research. Moreover, as minor perturbations can lead to major metabolic changes, the metabolome is an attractive target for analysis. The interrogation of the relative abundance of these metabolites not only provides a snapshot of the metabolic phenotype but also offers insights into pathway perturbations in health and disease.[1–4] However, procedural, technical, and instrumental limitations have been major barriers for widespread use of this technology for basic, clinical, and translational research.

Recent developments in ultra-performance liquid chromatography (UPLC) coupled with highly sensitive time-of-flight (TOF) and Orbitrap mass spectrometry (MS) have given investigators improved chromatographic resolution and sensitivity for detection of metabolites in a biologic sample.[5–10] Identification of small molecule metabolites to delineate new molecular mechanisms has been enhanced with the application of multivariate data analysis methods for chemometric data.[7–12] Metabolomics has been used for a variety of studies, such as development of injury and disease biomarkers,[13–17] functional genomics, biomarker discovery, and integrative systems biology.[18–20] Metabolomics technology is a central component of the personalized medicine paradigm that intends to understand the rewiring of molecular networks in health and disease.[21, 22] A standardized metabolomics design would consider the use of an optimal number of replicates, quality control samples, batch randomization, and reference standards, which in turn, would permit

Address correspondence and reprint requests to: Amrita K. Cheema, Depts. of Oncology and Biochemistry and Molecular and Cellular Biology, GC2, Pre-Clinical Science Bldg., Georgetown University Medical Center, 3900 Reservoir Rd., N.W., Washington, DC 20057, USA (E-mail: akc27@georgetown.edu; Phone: 202-687-2756; Fax: 202-687-8860).
doi: 10.7171/jbt.15-2603-001

reuse of the data to interpret, confirm, or validate research findings.[23–25] Although sample collection and handling are critical for maintaining molecular integrity, there is a paucity of specific protocols that will help minimize the analytical error for comprehensive or targeted metabolomic profiling.[26, 27] Metabolomics research that uses bio-banked samples is challenging as a result of pre- and postanalytical variables that affect the quality of resultant data. Because metabolomics is still an emerging field, many parameters, such as sample stability, matrix effects, ion interference, and reproducibility of sample processing, need optimization. The tremendous chemical diversity of metabolites from different biologic matrices makes broad-range metabolite extraction a challenging process.[28, 29] Furthermore, in-depth metabolomic analysis requires optimization of chromatographic separation and MS parameters.[30, 31] Finally, the front-end improvements, in addition to innovations in data pre- and postprocessing methods, are critical for improving difference detection among the sample groups.[32, 33] Metabolomics research poses several challenges for ensuring data quality, metabolome coverage, and the reuse of the rich information generated for hypothesis testing or verification. This requires interlaboratory replicative studies, availability of public data, protocols and methodologies that would provide a framework for qualification, and applications of systems biology outcomes.[34]

## THE ABRF MRG 2013 STUDY: LESSONS LEARNED

The Metabolomics Research Group (MRG) wished to conduct an interlaboratory study to assess the ability of metabolomics laboratories to conduct successful untargeted and targeted metabolomics analyses. The overall MRG 2013 study design goal was to replicate an initial quantitative metabolomics discovery experiment that either a core facility or a research laboratory would perform. A typical metabolomics study involves 2 or more study groups with multiple samples/group ($n > 1$) to provide statistical power to the measured outcomes. As metabolomics data sets tend to have highly variable backgrounds, the number of biological replicates is critical for reliable and reproducible biomarker discovery and validation efforts.

An American Society for Mass Spectrometry survey done in 2010 revealed that blood was the most commonly analyzed tissue or biofluid used in metabolomics studies (67% of the respondents had used serum or plasma, followed by 49% urine). Therefore, we chose National Institute of Standards and Technology (NIST) plasma, which has been thoroughly evaluated for the concentrations of 60 metabolites by use of selected reaction monitoring.[35] As our goal was to recapitulate a biologic study, the sample set was made up of 2 groups, A and B, containing plasma spiked with 17 compounds at different concentration levels (**Table 1**). Furthermore, for each

**TABLE 1**

*Schema for metabolite spiking in plasma*

| Compound name | MF | MW | Target conc A (μM) | Target conc B (μM) | Unspiked concentration (μM) | Ratio A/B |
|---|---|---|---|---|---|---|
| Sarcosine | $C_3H_7NO_2$ | 89.10 | 10 | 20 | Probably negligible | 0.5 |
| Betaine | $C_5H_{11}NO_2$ | 117.15 | 50 | 100 | 33–88 | [0.62,0.73] |
| Taurine | $C_2H_7NO_3S$ | 125.15 | 50 | 100 | 55–162 | [0.68,0.81] |
| Nicotinic acid (niacin) | $C_6H_5NO_2$ | 123.11 | 50 | 100 | 49–53 | [0.66,0.67] |
| Creatine | $C_4H_9N_3O_2$ | 131.14 | 50 | 100 | 30–55 | [0.62,0.68] |
| Suberic acid | $C_8H_{14}O_4$ | 174.20 | 5 | 10 | 3.6 | 0.63 |
| Quinolinic acid | $C_7H_5NO_4$ | 167.12 | 3 | 6 | 0.47 | 0.54 |
| Acetaminophen | $C_8H_9NO_2$ | 151.06 | 5 | 20 | Dose dependent | 0.25 |
| Acetylcarnitine | $C_9H_{17}NO_4$ | 203.12 | 16 | 8 | 6 | 1.57 |
| Caffeine | $C_8H_{10}N_4O_2$ | 194.08 | 8.50 | 48.50 | Dose dependent 2–10 mg/l | 0.18 |
| Creatinine | $C_4H_7N_3O$ | 113.06 | 69.98 | 9.98 | 70 | 1.75 |
| DL-Indole-3-lactic acid | $C_{11}H_{11}NO_3$ | 205.07 | 4.2 | 1.2 | 2.8 | 1.75 |
| L-Arginine | $C_6H_{14}N_4O_2$ | 174.11 | 3.7 | 48.7 | 80 | 0.65 |
| L-Isoleucine | $C_6H_{13}NO_2$ | 131.09 | 54.5 | 4.5 | 60–80 | [1.59,1.78] |
| Xanthosine | $C_{10}H_{12}N_4O_6$ | 284.08 | 7.00 | 2 | 5 | 1.71 |
| Urea | $CH_4N_2O$ | 60.06 | 4000 | 8000 | ND | 0.5 |
| Indoxyl sulfate | $C_8H_7NO_4S$ | 213.01 | 2 | 18 | ND | 0.11 |

MRG 2013 study sample spiked-in compounds and concentrations (conc). The bracketed values denote the expected ratios for all 17 metabolites. For endogenous metabolites with a concentration range in plasma, the values reflect low and high ends of the expected concentration ratio. MF, molecular formula.

group, 3 subgroups (A1, A2, and A3 and B1, B2, and B3) were created by spiking compounds with a 10% variation in concentration, such that the replicates would be representative of biologic variability (**Fig. 1*A***). The expected ratios for the spiked metabolites are illustrated in Fig. 1*B*. The spiked plasma samples were measured in aliquots (100 μl) and lyophilized to enable shipping at room temperature. Lyophilization did not interfere significantly with sample analysis or perturb input compound concentrations if the samples were frozen at −80°C after lyophilization until reconstituted (**Fig. 2**). Four criteria were used for compound selection: 1) most of the spiked-in compounds should be endogenous with known concentrations in NIST plasma; 2) compounds should be selected, such that they are well distributed in terms of ability to be analyzed by a particular technique; for example, some compounds should be detectable by electrospray ionization $(ESI)^+$, whereas others with $ESI^-$, electron ionization (EI), or atmospheric pressure chemical ionization; 3) compounds should be selected with a range of difficulty regardless of the technique used; and finally, 4) high-purity compounds must be chosen. These criteria were expected to keep the analysis open with respect to the analytical platform used while allowing the capture of a reasonable cross-section of metabolites. Furthermore, we anticipated that a combination of platforms would yield broader and deeper metabolome coverage. Each participating laboratory received 100 μl lyophilized aliquots of 6 plasma samples spiked with different concentrations of 17 compounds (Table 1). The end users were provided with an instruction sheet for reconstitution and data reporting format (Supplemental Data). The choice of the analytical platform was left to the end user's discretion. The participants were asked to report back metabolites that were deemed to be significantly different between the 2 biological groups with the help of analytical platforms and bioinformatics analyses that are routinely used in their laboratories. The participants were given the option to carry out the analyses blinded (nontargeted metabolomics) or with the knowledge of the spiked-in compounds (targeted metabolomics). An international group of participants expressed interest in receiving and analyzing samples. A total of 14 participants returned data, with some by use of multiple analytical platforms, for a total of 25 analyses (see Supplemental Table 1).

Overall, liquid chromatography (LC)-MS was found to be the most commonly used platform to analyze study samples (**Fig. 3**), which is consistent with metabolomics studies reported in PubMed. With respect to the LC-MS platforms, the metabolite detection accuracy was found to be dependent on the protocol used for sample processing, as well as the analytical conditions (column chemistry, mobile phase, etc.). Furthermore, the quantification trends were quite consistent across laboratories that use LC-MS platforms (**Fig. 4**). For example, the quantitative data for taurine, suberic acid, caffeine, and creatinine were most consistent across laboratories and analytical platforms (**Table 2**). It is noteworthy that nearly all of the

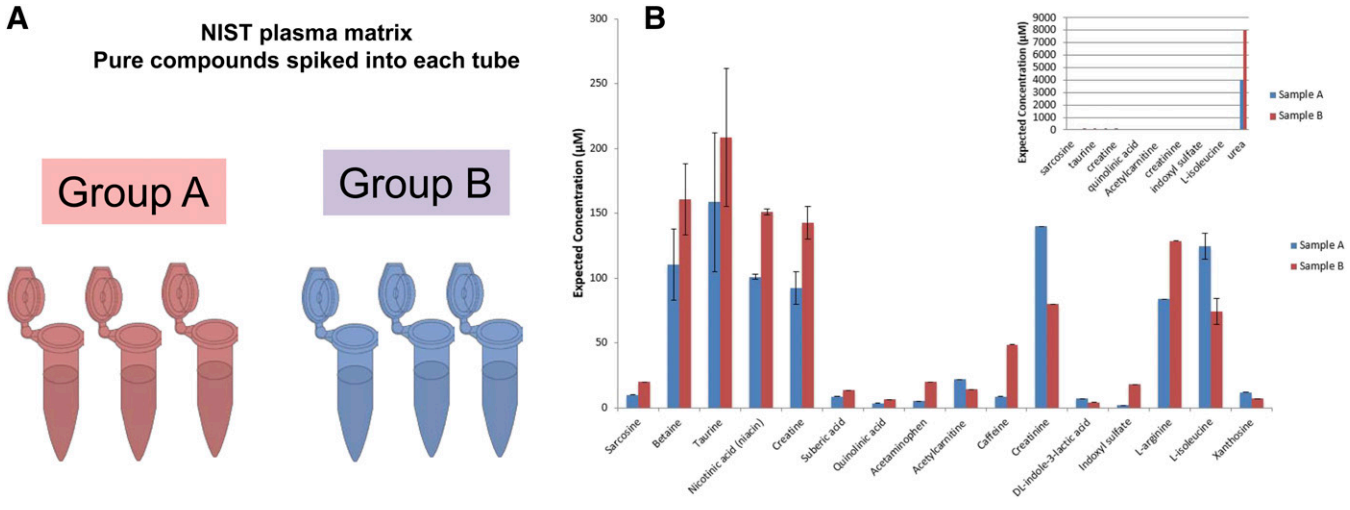


**FIGURE 1**

MRG 2013 study design. *A*) The sample was made up of 2 groups, A and B, containing NIST plasma spiked with 17 compounds at different levels (Table 1). For each group, 3 subgroups (A1, A2, and A3 and B1, B2, and B3) were created by spiking compounds at concentrations that would be representative of biologic variability. *B*) Spiking scheme for the 17 spiked metabolites.

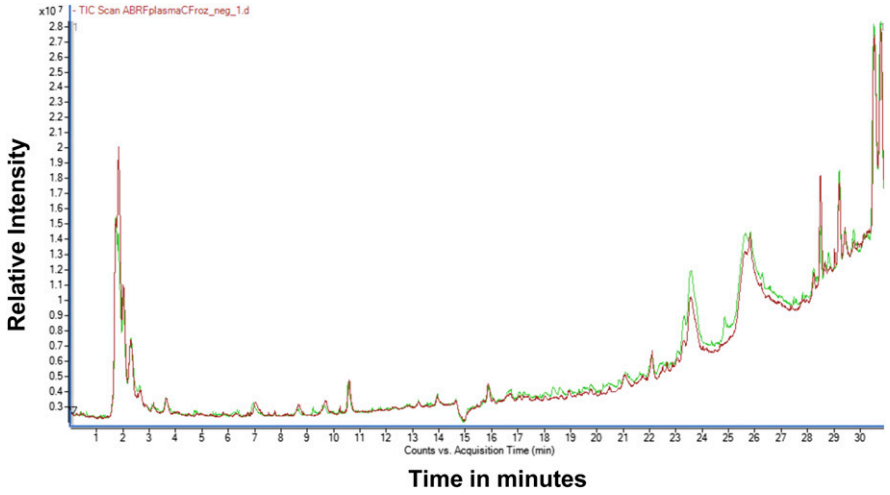


**FIGURE 2**

Total ion chromatograms of frozen (green) and lyophilized (red) MRG 2013 study samples. Plasma samples were processed, frozen or lyophilized, reconstituted in MS-compatible buffer, resolved by use of LC, and analyzed on an electrospray-TOF mass spectrometer. The total ion currents (TICs) were overlaid to examine visual differences in chromatographic elution patterns and peak intensities.

participants determined the same quantitation trends (greater A or B sample amounts), sometimes as predicted by reported spiked amounts (Table 1) but more often in the opposite direction (**Fig. 5**). This result suggests the possibility of an error at some stage in sample preparation for the study, such as reversing the A and B amounts for some compounds. However, such an error would not affect the overall goals of the study, which was to determine which compounds (of the spiked compounds) differed between samples and then to determine the identity of those compounds. Quantification of metabolites with high endogenous plasma concentrations, such as L-arginine, L-isoleucine, and betaine, turned out to be the most challenging. Although urea and indoxyl sulfate were spiked at relatively high concentrations (Table 1 and Fig. 5), they were not detected by any of the participants or MRG members. In the initial test-runs by the MRG members, we found that samples resolved on a C18 reverse-phase (RP) column with a water:acetonitrile (ACN) gradient were able to detect all metabolites reported, whereas the hydrophilic interaction LC amide column was more sensitive for detection of polar metabolites, although the metabolome coverage was inferior to the C18 RP column. The identification and detection of all spiked metabolites were challenging for most participants. As shown in Supplemental Table 1, participants who used only 1 analytical platform have fewer identifications compared with those who used >1 platform, thus providing broader metabolome coverage. In addition, sample processing, gradient conditions, and column chemistry are likely to affect the resolution and signal of metabolites that were detected (Fig. 3). In addition, room-temperature shipping of lyophilized samples (done to keep the cost of the study low) could lead to metabolite degradation as a result of atmospheric oxidation or high temperature, thus leading to some of the observed inconsistencies in the quantitation of metabolites. Our data show that the use of 2 platforms provides complementary information that helped increase metabolome coverage. We also found that the quantitation of spiked metabolites with high endogenous levels was not as accurate compared with those that had lower endogenous levels. Metabolite identification remains an important bottleneck in the field. Finally, given the current constraints of data-processing workflow for untargeted metabolomics data, most of the participants found it difficult to identify many of the spiked metabolites. However, when the identity was made available, the detection and quantitation accuracy increased, demonstrating a practical challenge for use of this high-throughput technology.

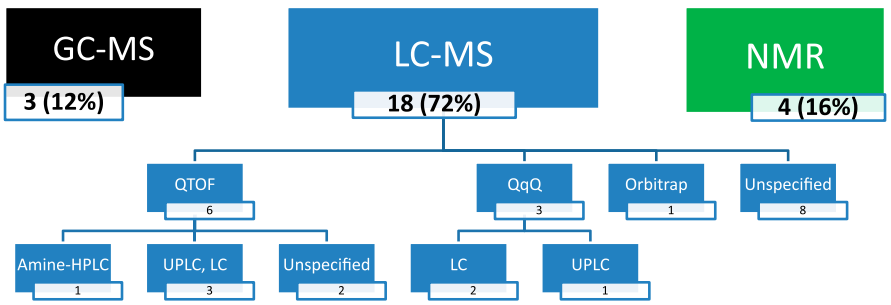


**FIGURE 3**

Analytical platforms used by the participants. Fourteen laboratories that returned data performed a total of 25 analyses on a variety of platforms. QTOF, quadrupole TOF; QqQ, triple quadrupole.

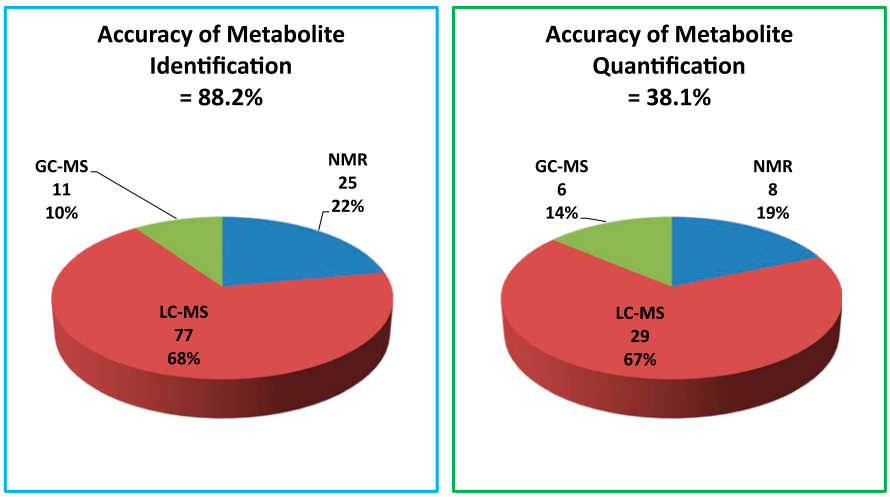


**FIGURE 4**

Accuracy of metabolite detection and quantification. The participant data were analyzed for accurate metabolite identification and expected ratios between the 2 groups, per the spiking design. Participants who used LC-MS showed higher correlation with the expected results, partially as it was the most commonly used analytical platform.

## SUMMARY AND CONCLUSIONS

Despite extensive technological advancements, several challenges still remain, restricting routine use of metabolomics technology in laboratories. Untargeted analysis, which requires accurate detection and relative quantitation of many unknown analytes, followed by the identification of those that differ significantly between study groups, is especially difficult. Some questions to consider include the following: what is the optimal way of sample collection, handling, and storage for long-term bio-banking? How do preanalytical variables affect data quality? What factors should be considered for designing a metabolomic experiment? How can chromatographic and mass spectrometric parameters be improved for reproducible metabolome coverage? How is metabolite recovery impacted by matrix effects, and how can this be addressed? Finally, what is the minimum information associated with a metabolomics experiment that needs to be reported for standardized publishing? Controlled metabolomics experiments are difficult to report in a manner that ensures their cross-platform/laboratory reproducibility. We believe that consolidation of validated metabolomics methodologies

**TABLE 2**

*MRG 2013 member group results*

| Lab ID | Member 1 | Member 2 | Member 3 | | |
|---|---|---|---|---|---|
| Platform used | QTOF | TQ | QTOF1 (5600 Triple TOF) | QTOF2 (Synapt G2-S) | Orbitrap |
| Platform type | Untargeted | Targeted | Untargeted | Untargeted | Untargeted |
| Sample name | Fold change (A/B) | Fold change (A/B) | Fold change (A/B) | Fold change (A/B) | Fold change (A/B) |
| Sarcosine | 1.08 | 0.97 | 1.38 | 1.40 | 1.00 |
| Betaine | 3.53 | 0.81 | 2.92 | 1.84 | 0.59 |
| Taurine | 0.84 | 0.28 | | 0.35 | 3.99 |
| Nicotinate | 5.11 | 0.28 | 5.52 | 9.38 | 4.08 |
| Creatine | 0.79 | 0.50 | 1.54 | 2.07 | 0.87 |
| Suberic acid | 0.17 | 1.19 | | 0.19 | 0.53 |
| Quinolinic acid | 0.37 | 0.90 | | | 0.38 |
| Acetominophen | 8.78 | 8.06 | 8.68 | 8.09 | |
| Acetylcarnitine DL | 0.72 | 0.43 | 0.62 | 0.48 | |
| Caffeine | 0.78 | 0.15 | 0.29 | 0.20 | 1.69 |
| Creatinine | 1.61 | 1.78 | 1.55 | 1.65 | 0.90 |
| DL-Indole-3-lactic acid | 0.42 | 0.51 | | 0.20 | 1.12 |
| Arginine | 0.17 | 2.10 | 1.71 | 1.99 | 1.26 |
| Leucine-isoleucine | 0.86 | 0.59 | 0.75 | 0.49 | 0.47 |
| Xanthosine | 0.16 | 0.60 | | 0.12 | 0.61 |

TQ, triple quadrupole

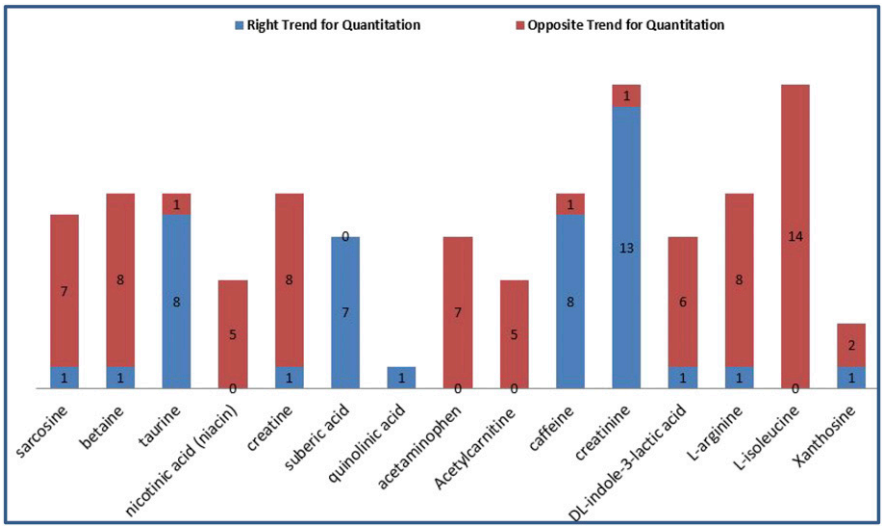


**FIGURE 5**

Detection rates and trends for the spiked metabolites. The figure illustrates the observed ratios (A/B) of spiked compounds correlated with the expected ratios, after adjusting for endogenous concentration of each metabolite in plasma.

and benchmarking standards of use and reporting will augment routine and widespread use of this powerful and cost-effective technology.

## ACKNOWLEDGMENTS

The authors thank former members of the MRG, Pavel Aronov, Vladimir Shulaev, and William Wikoff, for their participation in planning the MRG 2013 study. The authors acknowledge the Proteomics and Metabolomics shared resource at the Georgetown University Medical Center, supported in part by the U.S. National Institutes of Health National Cancer Institute Grant P30-CA051008.

## REFERENCES

1. Blow N. Metabolomics: biochemistry's new look. *Nature* 2008; 455:697–700.
2. Nicholson JK, Lindon JC. Systems biology: metabonomics. *Nature* 2008;455:1054–1056.
3. Serkova NJ, Glunde K. Metabolomics of cancer. *Methods Mol Biol* 2009;520:273–295.
4. Johnson CH, Gonzalez FJ. Challenges and opportunities of metabolomics. *J Cell Physiol* 2012;227:2975–2981.
5. Plumb R, Castro-Perez J, Granger J, Beattie I, Joncour K, Wright A. Ultra-performance liquid chromatography coupled to quadrupole-orthogonal time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* 2004;18:2331–2337.
6. Wang C, Kong H, Guan Y, Yang J, Gu J, Yang S, Xu G. Plasma phospholipid metabolic profiling and biomarkers of type 2 diabetes mellitus based on high-performance liquid chromatography/electrospray mass spectrometry and multivariate statistical analysis. *Anal Chem* 2005;77:4108–4116.
7. Chen C, Meng L, Ma X, Krausz KW, Pommier Y, Idle JR, Gonzalez FJ. Urinary metabolite profiling reveals CYP1A2-mediated metabolism of NSC686288 (aminoflavone). *J Pharmacol Exp Ther* 2006;318:1330–1342.
8. Giri S, Idle JR, Chen C, Zabriskie TM, Krausz KW, Gonzalez FJ. A metabolomic approach to the metabolism of the areca nut alkaloids arecoline and arecaidine in the mouse. *Chem Res Toxicol* 2006;19:818–827.
9. Giri S, Krausz KW, Idle JR, Gonzalez FJ. The metabolomics of (+/−)-arecoline 1-oxide in the mouse and its formation by human flavin-containing monooxygenases. *Biochem Pharmacol* 2007;73:561–573.
10. Ma X, Idle JR, Krausz KW, Tan DX, Ceraulo L, Gonzalez FJ. Urinary metabolites and antioxidant products of exogenous melatonin in the mouse. *J Pineal Res* 2006;40:343–349.
11. Weckwerth W, Morgenthal K. Metabolomics: from pattern recognition to biological interpretation. *Drug Discov Today* 2005;10:1551–1558.
12. Major HJ, Williams R, Wilson AJ, Wilson ID. A metabonomic analysis of plasma from Zucker rat strains using gas chromatography/mass spectrometry and pattern recognition. *Rapid Commun Mass Spectrom* 2006;20:3295–3302.
13. Sreekumar A, Poisson LM, Rajendiran TM, Khan AP, Cao Q, Yu J, Laxman B, Mehra R, Lonigro RJ, Li Y, Nyati MK, Ahsan A, Kalyana-Sundaram S, Han B, Cao X, Byun J, Omenn GS, Ghosh D, Pennathur S, Alexander DC, Berger A, Shuster JR, Wei JT, Varambally S, Beecher C, Chinnaiyan AM. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. *Nature* 2009;457:910–914.
14. Patterson AD, Li H, Eichler GS, Krausz KW, Weinstein JN, Fornace AJ Jr, Gonzalez FJ, Idle JR. UPLC-ESI-TOFMS-based metabolomics and gene expression dynamics inspector self-organizing metabolomic maps as tools for understanding the cellular response to ionizing radiation. *Anal Chem* 2008;80: 665–674.
15. Tyburski JB, Patterson AD, Krausz KW, Slavík J, Fornace AJ Jr, Gonzalez FJ, Idle JR. Radiation metabolomics. 1. Identification of minimally invasive urine biomarkers for gamma-radiation exposure in mice. *Radiat Res* 2008;170:1–14.
16. Patti GJ, Yanes O, Shriver LP, et al. Metabolomics implicates altered sphingolipids in chronic pain of neuropathic origin. *Nat Chem Biol* 2012;8:232–234.
17. Fiehn O, Garvey WT, Newman JW, Lok KH, Hoppel CL, Adams SH. Plasma metabolomic profiles reflective of glucose homeostasis in non-diabetic and type 2 diabetic obese African-American women. *PLoS ONE* 2010;5:e15234.
18. Goodacre R, Vaidyanathan S, Dunn WB, Harrigan GG, Kell DB. Metabolomics by numbers: acquiring and understanding global metabolite data. *Trends Biotechnol* 2004;22:245–252.
19. Coy SL, Cheema AK, Tyburski JB, Laiakis EC, Collins SP, Fornace A Jr. Radiation metabolomics and its potential in biodosimetry. *Int J Radiat Biol* 2011;87:802–823.
20. Zhou D, Jiang X, Ding W, et al. 2014. *Impact of bariatric surgery on ghrelin and obestatin levels in obesity or type 2 diabetes mellitus rat model*. J Diabetes Res p. 569435.

21. Tian Q, Price ND, Hood L. Systems cancer medicine: towards realization of predictive, preventive, personalized and participatory (P4) medicine. *J Intern Med* 2012;271:111–121.
22. Auffray C, Charron D, Hood L. Predictive, preventive, personalized and participatory medicine: back to the future. *Genome Med* 2010;2:57–59.
23. Sansone SA, Fan T, Goodacre R, Griffin JL, Hardy NW, Kaddurah-Daouk R, Kristal BS, Lindon J, Mendes P, Morrison N, Nikolau B, Robertson D, Sumner LW, Taylor C, van der Werf M, van Ommen B, Fiehn O; MSI Board Members. The metabolomics standards initiative. *Nat Biotechnol* 2007;25: 846–848.
24. Fiehn O, Kristal B, van Ommen B, Sumner LW, Sansone SA, Taylor C, Hardy N, Kaddurah-Daouk R. Establishing reporting standards for metabolomic and metabonomic studies a call for participation. *OMICS* 2006;10:158–163.
25. Castle AL, Fiehn O, Kaddurah-Daouk R, Lindon JC. Metabolomics Standards Workshop and the development of international standards for reporting metabolomics experimental results. *Brief Bioinform* 2006;7:159–165.
26. Zivkovic AM, Wiest MM, Nguyen UT, Davis R, Watkins SM, German JB. Effects of sample handling and storage on quantitative lipid analysis in human serum. *Metabolomics* 2009;5: 507–516.
27. Johnson CH, Gonzalez FJ. Challenges and opportunities of metabolomics. *J Cell Physiol* 2012;227:2975–2981.
28. Taymaz-Nikerel H, de Mey M, Ras C, ten Pierick A, Seifar RM, van Dam JC, Heijnen JJ, van Gulik WM. Development and application of a differential method for reliable metabolome analysis in Escherichia coli. *Anal Biochem* 2009; 386:9–19.
29. Sheikh KD, Khanna S, Byers SW, Fornace A Jr, Cheema AK. Small molecule metabolite extraction strategy for improving LC/MS detection of cancer cell metabolome. *J Biomol Tech* 2011;22:1–4.
30. Patti GJ. Separation strategies for untargeted metabolomics. *J Sep Sci* 2011;34:3460–3469.
31. Yanes O, Tautenhahn R, Patti GJ, Siuzdak G. Expanding coverage of the metabolome for global metabolite profiling. *Anal Chem* 2011;83:2152–2161.
32. Bueschl C, Kluger B, Berthiller F, et al. MetExtract: a new software tool for the automated comprehensive extraction of metabolite-derived LC/MS signals in metabolomics research. *Bioinformatics* 2012;28:736–738.
33. Blekherman G, Laubenbacher R, Cortes DF, et al. Bioinformatics tools for cancer metabolomics. *Metabolomics* 2011;7: 329–343.
34. Ioannidis JP, Khoury MJ. Improving validation practices in "omics" research. *Science* 2011;334:1230–1232.
35. Phinney KW, Ballihaut G, Bedner M, Benford BS, Camara JE, Christopher SJ, Davis WC, Dodder NG, Eppe G, Lang BE, Long SE, Lowenthal MS, McGaw EA, Murphy KE, Nelson BC, Prendergast JL, Reiner JL, Rimmer CA, Sander LC, Schantz MM, Sharpless KE, Sniegoski LT, Tai SS, Thomas JB, Vetter TW, Welch MJ, Wise SA, Wood LJ, Guthrie WF, Hagwood CR, Leigh SD, Yen JH, Zhang NF, Chaudhary-Webb M, Chen H, Fazili Z, LaVoie DJ, McCoy LF, Momin SS, Paladugula N, Pendergrast EC, Pfeiffer CM, Powers CD, Rabinowitz D, Rybak ME, Schleicher RL, Toombs BM, Xu M, Zhang M, Castle AL. Development of a standard reference material for metabolomics research. *Anal Chem* 2013;85: 11732–11738.