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Tailored sensitivity reduction improves pattern recognition and information recovery with a higher tolerance to varied sample concentration for targeted urinary metabolomics

Zhixiang Yan[a,b], Ru Yan[a,b,*]

[a] State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Taipa, Macao, China
[b] UM Zhuhai Research Institute, No.1 Software Road, Zhuhai Hi-tech Zone, Guangdong, China

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ABSTRACT

Variation in total metabolite concentration among different samples has been a major challenge for urinary metabolomics. Here we investigated the potential of tailored sensitivity reduction of high abundance metabolites for improved targeted urinary metabolomics. Two levels of sensitivity reduction of the 21 predominant urinary metabolites were assessed by employing less sensitive transition or collision energy with level 1 (reduced 1) and 2 (reduced 2) exhibiting 30–90% and 2–20% of the optimal sensitivity, respectively. Five postacquisition normalization methods were compared including no normalization, probabilistic quotient normalization, and normalization to sample median, creatinine intensity, and total intensity. Normalization to total intensity with reduced 2 gave the best pattern recognition and information recovery with a higher tolerance to varied sample concentration. Pareto scaling could improve the performance of tailored sensitivity reduction (reduced 2) for targeted urinary metabolomics while data transformation and autoscaling were susceptible to varied sample concentration. Using controlled spike-in experiments, we demonstrated that tailored sensitivity reduction revealed more differentially expressed markers with higher accuracy than did the conventional optimal sensitivity. This was particularly true when the differences between the sample groups are small. This work also served as an introductory guideline for handling targeted metabolomics data using the open-source software MetaboAnalyst.

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1. Introduction

Variation in total metabolite concentration among different samples has been a major barrier towards reliable and accurate relative quantification of the metabolome changes in liquid chromatography mass spectrometry (LC–MS)-based metabolomics. This problem is especially acute for urinary metabolomics since urine volume and solute concentrations vary greatly depending on a variety of factors, such as water consumption, physiological conditions, dietary and behavioral habits, and environmental factors [1–3]. Up to 15-fold changes in urine volume are commonly observed [4] and this may introduce unwanted variation in sample concentration and lead to erroneous conclusions unless a proper normalization method is applied.

There are continuing efforts to develop novel strategies to improve urinary metabolomics data analysis, primarily through postacquisition [5–8] and preacquisition methods [9–11]. For postacquisition approach, probabilistic quotient normalization [5] and normalization to constant sum [6] have been proposed to reduce sample variance for NMR-based urinary metabolomics. For LC–MS-based metabolomics, normalization to creatinine content or total intensity is the predominant normalization method by most non-experts in the field of statistics, mainly due to its ease and availability in most of the vendor-specific metabolomics software packages such as MarkerLynx (Waters) and MarkerView (AB SCIEX). Normalization to total intensity of ions that are common to all samples (MSTUS) has also been applied to avoid xenobiotics and artifacts caused bias [7].

Recently, median fold change normalization combined with log transformation has been demonstrated to reduce the high variability of high-abundance metabolites [8]. However, missing data derived from peak picking or from unstable detection of trace metabolites [12,13] are prohibitive for log transformation and there has been no consensus on how to handle the missing values in metabolomics [13]. Log transformation may also inflate the

* Corresponding author at: State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Taipa, Macao, China.
E-mail address: ruyan@umac.mo (R. Yan).

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standard deviations of low-abundance metabolites due to their large relative standard deviation [14].

Preacquisition methods take more initiative by differential dilution before analysis based on different corrective factors such as MSTUS [9], creatinine level [10], and specific gravity [11]. However, these methods require prior analysis of urine samples to measure the corresponding corrective factors, which may introduce additional errors and is impractical for large-scale sample analysis. Furthermore, these methods bring all urine samples to the lowest urinary density measured. For instance, a 56-fold (7-fold normalized followed by 8-fold un-normalized) dilution has been reported for certain samples in specific gravity based normalization [11]. Paradoxically, such a high dilution level would actually lose much information about low-abundance metabolites although the dilution initially aims at improved information recovery. There are many more low abundance metabolites than the high abundance metabolites in human urine [15] and these border-line metabolites are detectable only in the most concentrated samples [16].

Another factor that reduces the performance of metabolomics data analysis is the large differences in metabolite concentrations within each sample. Current postacquisition and preacquisition methods could not fundamentally solve this problem. From a biological point of view, metabolites present in high concentrations are not necessarily more important than those present at low concentrations. However, high abundance molecules account for the majority of the total ion intensities and the corresponding normalization results, consequently reducing the chemometric sensitivity required for monitoring trace metabolites. In this regard, tailored sensitivity reduction of abundant metabolites is conceptually attractive to achieve intensity uniformity across the entire metabolome. This strategy, however, could not be readily performed in untargeted metabolomics which blindly record all detectable ions in a full scan mode. In contrast, it can be easily and flexibly implemented in multiple reaction monitoring (MRM) based targeted metabolomics. MRM is the superior approach for monitoring trace but critical metabolites. Recently MRM is undergoing a renaissance within the metabolomics community [17–22] for its higher sensitivity and repeatability, and wider linear dynamic range than untargeted metabolomics [22,23].

In untargeted metabolomics, the MS parameter optimization for each metabolite is impossible, which is the unique feature of MRM-based targeted metabolomics. Although MRM has been widely employed to monitor trace metabolites in metabolomics, it has been seldom used to reduce the signal intensities of predominant metabolites. In a recent targeted metabolomics study of human urine, samples were 5-fold diluted and the highest intensity MS/MS ion peak of each precursor ion was optimized for MRM quantification [22]. However, predominant metabolites do not require specific optimization for ultrahigh sensitivity. Rather, they should be monitored at reduced sensitivity to avoid detector saturation and to make their intensities more comparable for low abundance metabolites to facilitate chemometric analysis. Although sensitivity reduction has been employed to increase the linear dynamic range for MRM quantitative bioanalysis [24,25], the implementation of this strategy has not been evaluated in metabolomics. In the present study, we demonstrate the advantage of tailored sensitivity reduction for MRM-based targeted urinary metabolomics.

2. Materials and methods

2.1. Reagents and materials

Acetonitrile and formic acid were from Merck (Darmstadt, Germany). Standards of 5-hydroxy-indole-3-acetic acid (5-HIAA), β-alanine, γ-aminobutyric acid, choline, dopamine, guanine, hippuric acid, indole-3-acetic acid (IAA), L-arginine, L-alanine, L-carnitine, L-histidine, L-leucine, L-isoleucine, L-methionine, L-phenylalanine, L-threonine, L-tryptophan, L-tyrosine, L-serine, spermidine, thiamine, urea, and xanthine were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other phytochemicals for the spiking experiment were obtained from Shanghai Forever Biotech Co., Ltd., (Shanghai, China).

2.2. Sample preparation

Random urine samples of 31 healthy individuals collected at any time of day without individual point were provided by Kiang Wu hospital (Macau, China) according to a protocol approved by the Medical Department, Kiang Wu Hospital and the Internal Ethical Committee of the Institute of Chinese Medical Sciences, University of Macau, respectively. Aliquots were stored at −80 °C immediately after collection. An equal amount of random urine samples of 6 healthy volunteers (age range 25–29 years; 3 females and 3 males) in our laboratory was mixed as quality control (QC) samples. The QC samples and all individual samples were processed in the same way. Briefly, 100 μL of urine were mixed with 100 μL of water, vortexed and centrifuged at 14,000 *g* for 10 min (4 °C). The supernatant (2-fold diluted) was further added with different volumes of water to obtain 4–40-fold diluted samples and filtered through a 0.22 μm membrane before LC–MS analysis.

2.3. Untargeted metabolite characterization using Q-TOF

The 4-fold diluted QC urine sample was first analyzed by a SYNAPT G2-Si HDMS Q-TOF mass spectrometer (Waters Corporation, Milford, MA) to acquire accurate *m*/*z* value, retention time, and MS/MS for untargeted metabolite characterization. Chromatographic separation was achieved using an ACQUITY UPLC system (Waters Corporation, Milford, MA). The column was a ZORBAX SB-C18 (100 mm × 2.1 mm, 1.8 μm; Agilent, Palo Alto, CA, USA). The flow rate was 200 μL min^{-1}, the column temperature 40 °C, and the injection volume 5 μL. Mobile phase A was 100% H_2O and mobile phase B was 100% ACN, both containing 0.1% formic acid. The following elution gradient was used: 0–4 min, 95% A; 8 min, 50% A; 16 min, 0% A; 21 min, 0% A; 23 min, 95% A; 30 min, 95% A. Mass spectrometric detection was carried out in positive ion mode with the following parameters: capillary voltage of 3000 V, cone voltage of 35 V, desolvation temperature of 350 °C, source temperature of 100 °C, desolvation gas flow of 600 L/h. The scan range of MS and MS/MS was *m*/*z* 50–800. Improved data-dependent acquisition for MS/MS was achieved using staggered gas-phase fractionation (sGPF) [26]. Precursor ions with a good chromatographic peak shape and corresponding MS/MS spectra were selected for targeted metabolomics. Adducts, isotopes, and in-source CID fragments were excluded to minimize data redundancy. Metabolites were characterized by comparing the MS/MS with standards, MS/MS databases (MassBank, Metlin, and HMDB) and literatures.

2.4. Targeted metabolomics using QTRAP

Targeted metabolomics were carried out using an Agilent 1200 RRLC system coupled with a 4000 QTRAP® system in MRM mode. The LC conditions were the same as those used in LC-Q-TOF based untargeted metabolite profiling. Given that the retention time on the ACQUITY UPLC system is not exactly identical to that on the 1200 RRLC system, we transformed the major 7 production ions (if available) in Q-TOF MS/MS spectra to multiple MRM transitions to localize metabolites in the LC-QTRAP system. The obtained retention time were then used for scheduled MRM (sMRM) to increase the dwell time of each transition and increase sensitivity. The width of each retention window was 140 s and the scan

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