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Research Article

Rapid LC-MRM-MS assay for simultaneous quantification of choline, betaine, trimethylamine, trimethylamine N-oxide, and creatinine in human plasma and urine

There is a growing interest in analyzing choline, betaine, and their gut microbial metabolites including trimethylamine (TMA) and trimethylamine N-oxide (TMAO) in body fluids due to the high relevance of these compounds for human health and diseases. A stable isotope dilution (SID)-LC-MRM-MS assay was developed for the simultaneous determination of choline, betaine, TMA, TMAO, and creatinine in human plasma and urine. The assay was validated using quality control (QC) plasma samples, spiked at low, medium, and high levels. Freeze-thaw stability was also evaluated. The utility of this assay for urine was demonstrated using a nutritional clinical study on the effect of various egg doses on TMAO production in humans. This assay has a wide dynamic range ($R^2 > 0.994$) for all the analytes (choline: 0.122–250 μM; betaine: 0.488–1000 μM; TMA: 0.244–250 μM; TMAO: 0.061–62.5 μM; and creatinine: 0.977–2000 μM). High intra- and inter-day precision (CV < 6%) and high accuracy (< 15% error) were observed from the QC plasma samples. The assay is reliable for samples undergoing multiple freeze-thaw cycles (tested up to eight cycles). The assay also works for urine samples as demonstrated by a clinical study in which we observed a significant, positive linear response to various egg doses for urinary concentrations of all the analytes except creatinine. A rapid SID-LC-MRM-MS assay for simultaneous quantification of choline, betaine, TMA, TMAO, and creatinine has been developed and validated, and is expected to find wide application in nutrition and cardiovascular studies as well as diagnosis and management of trimethylaminuria.

Keywords:
Choline and metabolites / LC/MS/MS / Quantification
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1 Introduction

Choline is a natural quaternary amine and an essential nutrient found in a variety of foods such as meat and egg yolks [1,2]. As a major component of cell membranes and an important methyl donor, choline is vital for brain function [3], liver health [4, 5], reproduction [6], and fetal and infant development [6]. In the body, choline is oxidized to betaine, also known as trimethylglycine, which is another important human nutrient that can be obtained from a variety of foods. Betaine acts as a methyl donor in the liver and as an important osmolyte to protect the cells of medulla in the kidney [7]. In the gut, dietary choline as well as betaine can be metabolized to trimethylamine (TMA) by microbes. TMA is a volatile organic compound, if not sufficiently metabolized, can be released in abnormal amounts in the form of sweat, urine, and breath, causing a strong body odor, a rare metabolic disease known as fish odor syndrome [8]. When TMA is absorbed

Abbreviations: FMO, flavin-containing monoxygenase; HILIC, hydrophilic interaction chromatography; LLOQ, lower LOQ; QC, quality control; SID-LC-MRM-MS, stable isotope dilution coupled to LC-multiple reaction monitoring-MS; TBA, t-butyl bromoacetate; TMA, trimethylamine; TMAO, trimethylamine N-oxide; SD, standard deviation

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by the liver, it can be rapidly oxidized to odorless trimethylamine N-oxide (TMAO) by at least one member of the hepatic flavin-containing monooxygenase (FMO) family of enzymes, FMO3 [9]. TMAO was originally considered nontoxic, but it recently has been associated with increased risk for cardiovascular disease [10]. These results have motivated a number of basic and clinical studies on choline, betaine, TMA, TMAO, and their associations with nutritional status, gut microbiota, and risks in important diseases [1, 9, 11–14]. A rapid, high throughput and cost-effective method for analysis of these molecules would, therefore, facilitate these applications.

Various methods have been established for the measurement of choline and its closely related metabolites in biological samples.Traditionally, choline and betaine were measured either separately or simultaneously by radioenzymatic assays [15, 16], LC with an electrochemical detection [17–19], and GC-MS [20, 21]. Previous methods for TMA and TMAO analysis include GC with a flame ionization detector [22], GC with a nitrogen-phosphorus detector [23], GC-MS [24–27], fast atom bombardment MS [28], and flow injection ESI MS [29]. The above methods have inherent disadvantages including low specificity and tedious sample preparation. In recent years, these metabolites have been frequently detected by metabolomic profiling [10, 30–38], a nontargeted technique being increasingly used to discover novel biomarkers for diseases [39, 40] and drug toxicity [41] by analyzing all small molecules in biological samples. While nontargeted metabolomics is useful to assess the variations of these metabolites with diseases or dietary intervention, it requires the accurate identification of these metabolites and does not offer enough precision and accuracy for their absolute quantity. Targeted metabolomics, using stable isotope dilution coupled to LC-multiple reaction monitoring-MS (SID-LC-MRM-MS), can be optimized to achieve maximum sensitivity and minimum time of absolute quantification of specific molecules and it is very useful to verify candidate biomarkers in a large number of samples after nontargeted metabolomic discoveries. The SID-LC-MRM-MS approach has been used to quantify choline and betaine in various biological samples [42,43], TMA in culture media [44] and microbial samples [45] and TMAO in blood and urine [45,46]. However, there is a critical need to develop assays that permit simultaneous quantification of choline and its microbial metabolites from blood and urine, given that there is a growing interest in determining the relevance of all these metabolites to diseases [1, 8, 10]. While choline, betaine, and TMAO can be quantified after a simple solvent extraction, the quantification of TMA typically requires sample derivatization or preconcentration due to its low MS sensitivity and low blood and urine levels.

In the present work, we report a rapid, targeted metabolomics assay for the simultaneous determination of choline, betaine, TMA, TMAO, and creatinine in human plasma and urine using SID-LC-MRM-MS. To our knowledge, no such multiplexed assays have been reported. This assay makes use of an efficient procedure that integrates solvent extraction, derivatization, hydrophilic interaction chromatography (HILIC), an ultra-performance LC, and triple quadrupole system. In particular, this assay includes creatinine because of two considerations: (i) creatinine is a well-established biological reference used to normalize results when analyzing urine samples (ii) blood creatinine is an important indicator of renal function. The assay was not only fast and efficient, but also robust and reliable. We demonstrated its applicability in a clinical study that aimed to investigate the dose effect of dietary choline (in the form of eggs) on the production of TMAO in humans.

2 Materials and methods

2.1 Standards, reagents, and solvents

Creatinine, TMAO, choline, betaine, and betaine-d9 were purchased from Sigma-Aldrich (St Louis, MO, USA). TMA was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Creatinine-d3 was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). TMAO-d9, TMA-d9, and choline-d9 were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). The derivatization reagent tert-butyl bromacetate was obtained from Sigma-Aldrich Aldrich (St Louis, MO, USA). Acetonitrile (HPLC grade), formic acid (Optima LC-MS grade), ammonium hydroxide, and ammonium formate were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Water used in this method was obtained from a Milli-Q ultrapure water purification system (Millipore, Bedford, MA, USA).

2.2 Plasma and urine collection

Pooled human plasma was purchased from Innovative Research (Novi, MI, USA), which was collected from single donors of age between 18 and 65. The plasma was acidified by adding 1% formic acid v/v. The acidified plasma was stored at –80°C until analysis. The 24h urine samples were collected in a clean container and acidified by adding 1% formic acid v/v. The acidified urine was stored at –80°C until analysis.

2.3 Preparation of standards, internal standards, and quality control samples

The stock solutions of unlabeled and labeled standards were prepared in ultrapure water individually for each compound with a final concentration of 100 mM and then stored at –80°C. To find the linear range, LOD and lower LOQ (LLOQ), standards of 12 different concentrations were made ranging over three orders of magnitude. The calibration standard at the highest concentrations was first prepared in acidified water with 1% formic acid (at a final concentration of 250 μM for choline, 1000 μM for betaine, 250 μM for TMA, 250 μM for TMAO, and 2000 μM for creatinine). The remaining calibration standards were then made by twofold
serial dilutions of the highest concentration standard. The internal standard working solution was prepared by mixing the stock solutions of the labeled standards into acidified water to final concentrations of 20, 20, 20, 20, and 100 μM for choline-d₉, betaine-d₉, TMA-d₉, TMAO-d₉, and creatinine-d₃, respectively, and it was stored at –80°C in small aliquots.

Quality control (QC) samples at three concentration levels were prepared by spiking pooled human plasma samples with stock solutions of choline, betaine, TMA, TMAO, and creatinine to low (LQC; the pooled plasma spiked with 10 μM of choline, 50 μM of betaine, 0.5 μM of TMA, 50 μM of TMAO, and 50 μM of creatinine), medium (MQC; the pooled plasma spiked with 50 μM of choline, 200 μM of betaine, 50 μM of TMA, 50 μM of TMAO, and 200 μM of creatinine), and high (HQC; the pooled plasma spiked with 200 μM of choline, 800 μM of betaine, 200 μM of TMA, 200 μM of TMAO, and 800 μM of creatinine). The QC samples were then aliquoted into polypropylene tubes and stored in the freezer at –80°C. These quality control samples were used for method validation.

2.4 Sample extraction and derivatization

Each 50 μL of acidified plasma/urine or calibration standards was mixed with 10 μL of internal standard working solution, and then extracted and derivatized with 75 μL of 50 mM tert-butyl bromoacetate in acetonitrile and 10 μL of 70% ammonium hydroxide in water. After incubation at room temperature for 30 min, each sample was added with 50 μL of 1% formic acid spiked-acetonitrile. The samples were subjected to centrifugation at 15 000 × g for 5 min at room temperature, and the supernatants were transferred into HPLC vials for analysis.

2.5 LC and MS

Quantification of choline, betaine, TMA, TMAO, and creatinine was performed using an ACQUITY UPLC coupled with TQ Detector mass spectrometry (UPLC-MS/MS) (Waters, Milford, MA, USA). Chromatographic separations were performed on an Atlantis Silica HILIC 3 μm 4.6 × 50 mm column from Waters (Milford, MA, USA). The column was heated to 40°C, and the flow rate was maintained at 1 mL/min. The gradient was 5% A for 0.05 min, to 15% A in 0.35 min, to 20% A in 0.6 min, to 30% A in 1 min, to 45% A in 0.55 min, to 55% A in 0.05 min, at 55% A for 0.9 min, to 5% A in 0.05 min, at 5% A for 1.45 min, where A was 10% acetonitrile/90% water with 10 mM ammonium formate and 0.125% formic acid and B was 90% acetonitrile/10% water with 10 mM ammonium formate and 0.125% formic acid. The analytes and their corresponding isotopes were monitored using characteristic precursor-product ion transitions: 104 > 45 for choline, 113 > 45 for choline-d₉, 118 > 59 for betaine, 127 > 68 for betaine-d₉, 174 > 59 for TMA, 183 > 68 for TMAO-d₉, 76 > 58 (TMAO), 114 > 86 (Creatinine), 117 > 89 (Creatinine-d₃).
Figure 2. Evaluation of the freeze-thaw effect using QC plasma samples (LQC, MQC, and HQC). Top panel: Normalized the average peak area of five analytes to one freeze-thaw cycle; bottom panel: coefficient of variation ($n = 3$).

68 for TMA-d9, 76 > 58 for TMAO, 85 > 66 for TMAO-d9, 114 > 86 for creatinine, and 117 > 89 for creatinine-d3. Concentrations of each analyte in samples were determined from calibration curves using peak area ratio of the analyte to its isotope.

2.6 Method validation

Four analytical runs on separate days (day 1, day 2, day 5, and day 15) were used to evaluate accuracy and precision. Calibration standards were run in duplicate at the beginning and end of each batch. The calibration curve for each analyte was derived using $1/x$ weighted linear regression of the response (the area of the unlabeled standard / the area of the labeled internal standard * the concentration of the labeled internal standard) versus the concentration of the corresponding standard. LOD of each analyte was defined as the concentration at which the signal is three times the standard deviation (SD) of blank samples. LLOQ was defined as the concentration at which the signal is five times the SD of the blank sample. In each analytical run, LQC, MQC, and HQC were analyzed in six replicates.

To evaluate freeze-thaw stability, aliquots of LQC, MQC, and HQC were subjected to one freeze-thaw cycle (used as control), four freeze-thaw cycles and eight freeze-thaw cycles including thawing on ice and freezing at –20°C freezer for at least 12 h. The freeze-thaw aliquots were then analyzed using freshly made calibration standards.

2.7 Clinical application

To demonstrate the utility of this assay, we analyzed the urine samples collected in our recently published clinical study that has demonstrated that the consumption of eggs resulted in
increased plasma and urine TMAO [1]. This study involved six healthy volunteers recruited from a protocol approved by the Institutional Review Board at the University of North Carolina at Chapel Hill (UNC). Details of subject recruitment, subject information, and sample collection were described previously [1]. Briefly, this was a longitudinal, double-blind, randomized dietary intervention study in which six volunteers were fed breakfast doses of zero, one, two, four or six egg yolks. A 24-h urine was collected, acidified, and stored at –80°C freezer.

2.8 Statistical analysis

Pearson’s correlation analysis was performed to evaluate the quantitative relationships between egg dose levels and urinary metabolite concentrations using the R software package version 3.0.2. The statistical significance of the correlation coefficients was tested using a t-test by giving a null hypothesis (H0) of r = 0, where r represents the correlation coefficient. If H0 holds, t = \frac{r \sqrt{n-2}}{\sqrt{1-r^2}} approximately follows the t distribution with degrees of freedom equal to n – 2, where n represents the sample size. A low p value (<0.05) for this test means that there is evidence to reject the null hypothesis in favor of the alternative hypothesis or that there is a statistically significant relationship between the two variables.

3 Results and discussion

This study has focused on developing a single, rapid, and cost-effective assay for choline, betaine, TMA, TMAO, and creatinine in plasma and urine to facilitate basic and clinical studies on nutritional assessment, gut microbiota, and important diseases.

3.1 Method development

There are two potential analytical choices for rapid and simultaneous measurement of choline, betaine, TMA, TMAO, and creatinine. High resolution NMR (500 MHz or higher) is intuitively the first method of choice because it is a highly quantitative, reproducible, and cost-effective analytical approach for metabolite analysis and a single 1H NMR experiment is, in theory, able to simultaneously detect all five given compounds in blood and urine. Because the same nuclei detected in an NMR experiment have the same sensitivity, independent of the properties of metabolite molecules, absolute quantities of different metabolites can be measured using a single proton-containing internal standard rather than using naturally low-abundant stable isotopes. However, serious impediments to the use of NMR for simultaneous quantification of these compounds are insufficient sensitivity, spectral overlap, and complexity. Signals of the metabolites, when present at low concentrations are often buried under nearby strong signals due to insufficient resolution. In addition, TMA and TMAO each has only one single characteristic 1H NMR peak that can shift under various pH and ionic strength, which makes their identification and quantification from biological metrics difficult. The second method of choice is LC-MRM-MS, which is preferred because of the following reasons: (1) MS is a far more sensitive analytical instrument than NMR; (2) the coupling of chromatographic separation and MS/MS ensures its high selectivity; (3) MS allows highly selective and simultaneous detection of multiple target analytes using MRM; (4) MS also permits the use of stable isotopes that can be introduced prior to metabolite extraction and serve to improve analytical accuracy by minimizing preanalytical and analytical variations.

We therefore sought to use the LC-MRM-MS approach for quantification of the five metabolites of interest. As our goal was to develop a rapid and cost-effective assay, we first focused on establishing a rapid LC method that allowed the optimal retention and separation of all these five analytes. We adopted the HILIC separation because the five analytes are all very polar organic compounds and currently there are several choices of commercially available HILIC columns compatible with our UPLC system. A short LC method was established using the Atlantis® Silica HILIC 3 μm 4.6 × 50 mm column and ammonium formate modified mobile phases. This LC method was found very robust because it can generate highly reproducible retention times and peak shapes for all analytes and a HILIC column can be used for at least one thousand injections (samples, blanks, and calibration standards) without peak shifting, distortion, and carry over. Our main effort was to optimize the sample preparation procedure in order that we can accurately and rapidly measure the concentrations of all the five analytes in both plasma and urine. We found that simply extracting plasma/urine with organic solvents such as acetonitrile allowed us to measure the concentrations of choline, betaine, TMAO, and creatinine. However, the procedure was not suitable for TMA because the compound was instrumentally less sensitive and its endogenous concentrations are typically lower than other...
compounds. We then considered using derivatization reagents such as ethyl bromoacetate and 1-butyl bromoacetate (TBA) because these reagents are able to enhance instrumental detection of TMA [29,44]. In most metabolite assays, extraction and derivatization are two separate steps. However, our focus was to develop a simple procedure that allows sample extraction and sample derivatization to be performed at the same time. After a systematic evaluation on derivatization reagents, reaction conditions, and extraction conditions, we finally established a highly efficient and reliable sample preparation procedure that successfully integrated extraction and derivatization into one step (see Materials and methods). This was achieved from the use of TBA because (1) it can significantly enhance the detection of TMA while it does not interfere with the detection of the other four analytes; (2) its reaction with TMA can take place in the mixture of water (from plasma and urine) and acetonitrile (the extraction solvent), therefore permitting a one-step sample extraction and derivatization; and (3) the reaction is fast and reproducible (Supporting Information Fig. 1).

Figure 1 shows the extracted ion chromatograms of choline, betaine, TMA, TMAO, creatinine, and its corresponding internal standards in LQC plasma. Each run took only 5 min and all analytes elute between 1.4 and 2.7 min. Each analyte has a narrow LC peak width (measured as full width at half maximum) smaller than 10 s. The use of isotopically labeled internal standard was to correct the variations from sample preparation and instrumental detection and thus ensure the precision, accuracy, and specificity in measuring these analytes.

### 3.2 Method validation

This assay has a wide linearity of all the analytes in the working concentration ranges with $R^2 > 0.994$. Table 1 summarizes calibration curves, linear range, LOD, and LLOQ. This assay demonstrated a high precision with less than 6% CV for all the analytes (Table 2). In LQC, the intraday and interday CV for all analytes ranged from 0.6 to 5.3% and from 1.4 to 5.8%, respectively. In MQC, the intraday and interday CV for all analytes ranged from 0.7 to 5.9% and from 1.6 to 4.1%, respectively. In HQC, the intraday and interday CV for all analytes ranged from 0.4 to 5.2% and from 1.4 to 5.1%, respectively. Because of the endogenous presence of these analytes in the pooled plasma samples, the accuracy for the measurement of each analyte was determined by comparing the known spiked concentration for each QC sample with its measured spiked concentration, calculated from the subtraction of the measured total concentration in the spiked QC sample to the endogenous concentration measured in the unspiked sample (17.1 ± 0.2 μM for choline, 48.9 ± 0.8 μM for betaine, below LOD for TMA, 4.1 ± 0.2 μM for TMAO, 76.6 ± 1.3 μM for creatinine). This assay also demonstrated a high accuracy with less than 15% error for all the analytes (Table 2). In LQC, the intraday and interday accuracy for all analytes ranged from 90.1 to 113.3% and from 94.1 to 107.3%, respectively. In MQC, the intraday and interday accuracy for all analytes ranged from 92.6 to 102.4% and from 95.6 to 101.1%, respectively. In HQC, the intraday and interday accuracy for all analytes ranged from 94.2 to 104.1% and from 97.2 to 100.2%.

We also evaluated the effects of freeze-thaw cycles on the signal intensity and results are summarized in Fig. 2, showing the averaged peak area (the top panel) and CV (the bottom panel) of individual analytes under three freeze-thaw conditions. We found that the samples were very stable after four freeze-thaw cycles because the variation in peak area was within 5% compared with those of one single cycle and CV values ($n = 3$) were lower than 10%. For all QC samples undergoing eight-thaw cycles, peak area for most analytes were smaller than that of the one-cycle and four-cycle samples; however, the reduction was not significant (less than 12% as compared with one cycle) and the CV values of the five analytes’ peak area ($n = 3$) were also lower than 10%. These data suggest that this assay is suitable for samples undergoing multiple freeze-thaw cycles although the samples undergoing minimal freeze-thaw cycles are preferred.

### 3.3 Clinical application

To demonstrate the utility of this assay, we analyzed the urine samples collected in a longitudinal, double-blind, randomized dietary intervention study in which six volunteers were fed breakfast doses of zero, one, two, four or six egg yolks [1]. The urinary concentrations of choline, betaine, TMA, TMAO, and creatinine were measured. Due to the differences in

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**Table 1. Results of method validation: regression, LOD, LLOQ, and linear range determined from standards of 12 different concentrations, and slope, intercept, and $R^2$ presented as mean ± SD (CV%)**

<table>
<thead>
<tr>
<th></th>
<th>Choline</th>
<th>Betaine</th>
<th>TMA</th>
<th>TMAO</th>
<th>Creatinine</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD (μM)</td>
<td>0.008</td>
<td>0.122</td>
<td>0.122</td>
<td>0.031</td>
<td>0.06</td>
</tr>
<tr>
<td>LLOQ (μM)</td>
<td>0.122</td>
<td>0.488</td>
<td>0.244</td>
<td>0.061</td>
<td>0.977</td>
</tr>
<tr>
<td>Linear range (μM)</td>
<td>0.122–250</td>
<td>0.488–1000</td>
<td>0.244–250</td>
<td>0.061–62.5</td>
<td>0.977–2000</td>
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<tr>
<td>Slope ($n = 8$)</td>
<td>0.809 ± 0.008 (1.0%)</td>
<td>0.599 ± 0.009 (1.5%)</td>
<td>0.961 ± 0.025 (2.6%)</td>
<td>0.824 ± 0.025 (3.1%)</td>
<td>1.116 ± 0.018 (1.6%)</td>
</tr>
<tr>
<td>Intercept ($n = 8$)</td>
<td>0.010 ± 0.011 (116.2%)</td>
<td>0.004 ± 0.009 (241.6%)</td>
<td>0.059 ± 0.033 (61.6%)</td>
<td>0.009 ± 0.004 (42.8%)</td>
<td>0.019 ± 0.022 (118.2%)</td>
</tr>
<tr>
<td>Correlation coefficient ($R^2$, $n = 8$)</td>
<td>0.9996 ± 0.0002 (0.02%)</td>
<td>0.9998 ± 0.0003 (0.03%)</td>
<td>0.9989 ± 0.0019 (0.19%)</td>
<td>0.9942 ± 0.0025 (0.25%)</td>
<td>0.9998 ± 0.0002 (0.02%)</td>
</tr>
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Table 2. Results of method validation: intraday and interday precision and accuracy

<table>
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<tr>
<th></th>
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<th>Betaine</th>
<th>TMA</th>
<th>TMAO</th>
<th>Creatinine</th>
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<td></td>
<td>CV</td>
<td>Accuracy</td>
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<td>Plasma spiked I (LQC)</td>
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<tr>
<td>Intraday (n = 6)</td>
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</tr>
<tr>
<td>Day 1</td>
<td>0.8%</td>
<td>93.0%</td>
<td>0.6%</td>
<td>94.1%</td>
<td>5.3%</td>
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<tr>
<td>Day 2</td>
<td>0.8%</td>
<td>95.2%</td>
<td>1.1%</td>
<td>99.0%</td>
<td>4.4%</td>
</tr>
<tr>
<td>Day 5</td>
<td>1.6%</td>
<td>97.9%</td>
<td>1.8%</td>
<td>100.9%</td>
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<tr>
<td>Day 15</td>
<td>1.2%</td>
<td>97.5%</td>
<td>1.0%</td>
<td>97.3%</td>
<td>4.7%</td>
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<tr>
<td>Interday (n = 24)</td>
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<td></td>
<td>1.4%</td>
<td>95.9%</td>
<td>1.8%</td>
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<td>5.8%</td>
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<tr>
<td>Day 1</td>
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<td>101.4%</td>
<td>0.9%</td>
<td>97.1%</td>
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<tr>
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<td>102.4%</td>
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<td>99.0%</td>
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<td>1.4%</td>
<td>100.9%</td>
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<tr>
<td>Interday (n = 24)</td>
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<td>101.1%</td>
<td>1.8%</td>
<td>97.9%</td>
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<td>Plasma spiked III (HQC)</td>
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<tr>
<td>Day 1</td>
<td>1.3%</td>
<td>98.6%</td>
<td>0.5%</td>
<td>99.2%</td>
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<td>1.1%</td>
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<td>0.4%</td>
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<td>Day 5</td>
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<td>102.0%</td>
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<td>99.6%</td>
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<td>98.2%</td>
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<tr>
<td>Interday (n = 24)</td>
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<td>2.2%</td>
<td>100.2%</td>
<td>1.4%</td>
<td>99.2%</td>
<td>3.1%</td>
</tr>
</tbody>
</table>

Intraday precision and accuracy were measured on four different days (n = 6/each day; 24 determinations total for each QC plasma samples).

Individual baselines, the measured concentrations were normalized to individual baseline levels (with zero dose of eggs) and summarized in Fig. 3. Average baseline concentrations of choline, betaine, TMA, and TMAO of the study subjects were 2.3 ± 0.4, 7.2 ± 2.7, 0.1 ± 0.1, and 21.4 ± 5.1 μmol/mmol creatinine, respectively. The measured concentrations are consistent with the normal urine concentration ranges reported in the Human Metabolome Database [47]. Negligible changes of the urinary creatinine concentrations (Fig. 3) as well as plasma creatinine concentrations (data not shown) [1] were found after all doses of eggs, indicating that the over-consumption of dietary choline, equivalent to approximately 1000 mg/day (for the meal with six eggs), did not perturb kidney function. Urinary excretion of choline and its metabolites increased proportionately after each level of egg intake (choline, betaine, TMA, and TMAO concentrations increased to an average of 118, 114, 119, and 134%, respectively, after the six egg dose). A significant, positive linear response to dose was observed with R² values of 0.99 (p value = 0.00054), 0.95 (p value = 0.0055), 0.97 (p value = 0.0026), and 0.97 (p value = 0.0017) for choline, betaine, TMA, and TMAO, respectively, while there was no significant linear correlation between dose of egg and urinary creatinine concentration (p value = 0.14) (Supporting Information Fig. 2). The significant increase of TMA and TMAO with egg doses clearly suggested that their hepatic FMO3 oxidative capacity was not affected by the increase of eggs. In donor 3, the TMA/TMAO ratio was increased significantly with the egg doses (r = 0.98, p value < 0.0001) and the positive response suggested that the donor had a reduced FMO3 oxidative capacity when increasing the choline load.

4 Concluding remarks

We have described a validated, rapid SID-LC-MRM-MS assay for simultaneous quantification of choline, betaine, trimethylamine, trimethylamine N-oxide, and creatinine. This assay is capable of measuring these five analytes over the concentration range of three orders of magnitude and suitable for both plasma and urine samples. This assay is also cost effective because of its multiplexing five analytes into one single analysis as well as its efficiency in both sample preparation and instrumental analysis. This assay is expected to find wide application in nutrition and cardiovascular studies as well as diagnosis and management of trimethylaminuria.

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5 References


