A Multiplexed Targeted Assay for High-Throughput Quantitative Analysis of Serum Methylamines by Ultra Performance Liquid Chromatography Coupled to High Resolution Mass Spectrometry

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Short title: Multiplexed Analysis of Methylamines by UPLC-MS

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Abstract

Methylamines are biologically-active metabolites present in serum and urine samples, which play complex roles in metabolic diseases. Methylamines can be detected by proton nuclear magnetic resonance (NMR), but specific methods remain to be developed for their routine assay in human serum in clinical settings. Here we developed and validated a novel reliable "methylamine panel" method for simultaneous quantitative analysis of trimethylamine (TMA), its major detoxification metabolite trimethylamine-N-oxide (TMAO), and precursors choline, betaine and L-carnitine in human serum using Ultra Performance Liquid Chromatography (UPLC) coupled to High Resolution Mass Spectrometry (HRMS). Metabolite separation was carried out on a HILIC stationary phase. For all metabolites, the assay was linear in the range of 0.25 to 12.5µmol/L and enabled to reach limit of detection of about 0.10µmol/L. Relative standard deviations were below 16% for the three levels of concentrations. We demonstrated the strong reliability and robustness of the method, which was applied to serum samples from healthy individuals to establish the range of concentrations of the metabolites and their correlation relationships and detect gender differences. Our data provide original information for implementing in a clinical environment a MS-based diagnostic method with potential for targeted metabolic screening of patients at risk of cardiometabolic diseases.

Keywords: Trimethylamine, Trimethylamine-N-oxide, choline, betaine, L-carnitine, Cardiometabolic diseases

1. Introduction

Metabolic profiling technologies enable detection and quantification of low molecular weight compounds in biological samples to enhance our understanding of gene function, disease mechanisms and drug treatments [1]. They represent powerful high-throughput and high-density molecular phenotyping tools to uncover diagnostic and prognostic metabolic biomarkers [2]. Technological and methodological advances in the field provide opportunities in clinical settings to profile patient metabolism [3] and in genetic research to identify metabolites associated with complex diseases [4, 5].

There is increasing interest in methylamines in clinical and fundamental research. Variations in trimethylamine (TMA), the product of microbial metabolism of choline, and its detoxification metabolite, trimethylamine-*N*-oxide (TMAO), have been associated with nephrotoxin treatments [6], microbial colonization of germ-free rats [7], insulin resistance in mice [8] and cardiovascular disease in humans [9-11]. Butyrobetaine, another metabolite in this pathway derived from dietary L-carnitine, has also been proposed as a marker for atherosclerosis [12]. These findings suggest that the search for disease-associated metabolite biomarkers and gut microbial-mammalian co-metabolites should be extended beyond TMAO to other metabolites in the methylamine pathway.

Untargeted high field proton nuclear magnetic resonance (NMR) spectroscopy is widely used for TMAO and choline analysis [13, 14] but detection limits (around 0.5×10^{-5}M) make TMA quantification difficult in blood compared to mass spectrometry (MS). LC-MS/MS methods have been developed to individually assay TMAO [15], choline and betaine [16] and L-carnitine and acylcarnitines compounds [17]. In addition, methods for quantitative analysis of TMA/TMAO and L-carnitine (Fast Atom Bombardment mass spectrometry (FAB-MS) [18, 19], Metastable Atom Bombardment (MAB-MS) [20, 21], Matrix-Assisted Laser Desorption-Time-of-Flight (MALDI-TOF) [22]) use analytical instruments uncommon in clinical

laboratories. GC-MS has also been used for indirect quantification of TMAO [23-25], but it requires TMA reduction and derivatization and is therefore time-consuming. Finally, flow injection electrospray ionization-mass spectrometry (FIA-MS) [26, 27] prevents addition of other compounds in the method without prior chromatographic technique.

Here we developed a multiplexed MS-based method, which can be applied in both research and clinical settings for simultaneous quantitative analysis of TMA and four quaternary amine compounds (betaine, choline, TMAO and L-carnitine). It is based on HILIC ultra performance liquid chromatography coupled with high-resolution mass spectrometry (UPLC-HRMS). We assessed the sensitivity and reliability of the method, and tested its applicability in a group of healthy individuals.

2. Material and Methods

2.1.Chemicals and reagents

Certified pure trimethylamine hydrochloride (TMA), trimethylamine-*N*-oxide dihydrate (TMAO), betaine hydrochloride, L-carnitine hydrochloride, and choline chloride were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). The corresponding stable isotopes used as internal standards of trimethylamine-^{13}C, ^{15}N, choline-d_4, betaine-d_3 and L-carnitine-methyl-d_3 were also obtained from Sigma-Aldrich. d_3 labelled L-carnitine was purchased from Cluzeau Info Labo (Sainte Foy La Grande, France) and d_9 labelled TMAO from Euriso-tope (Saint-Aubin, France). Formic acid and ammonium formate were LC-MS Chromasolv® Fluka, HPLC quality and purchased from Sigma-Aldrich. Ultra pure water (resistivity: 18 mΩ) was obtained with a Milli-Q Integral purification system (Millipore, Molsheim, France) with a 0.22 µm filter. Human and bovine sera were obtained from Life technologies (Saint-Aubin, France).

2.2. Preparation of standard and calibration solutions

Stock standard solutions were prepared in acetonitrile (CH_3CN) for TMA, TMAO, choline and betaine at a concentration of 20mmoL/L. L-carnitine was dissolved in a mixture of acetonitrile/water (95:5) at a concentration of 20 mmol/L. Working solutions of the reference compounds mixture were prepared at concentrations of 100, 50 and 25µmol/L. Individual solutions for each stable isotope, associated to each native compound were prepared in the same conditions. A working solution mixture of stable isotope standards was set at a concentration of 100µmol/L. All standard solutions were stored at -20°C.

2.3.*Serum sample collection and preparation*

Human serum samples were prepared from blood of 67 healthy individuals (39 males and 28 females aged 24-59 years) who were recruited from Boston area clinics and community health care centers. This study was approved by the institutional review board of Massachusetts

General Hospital and all individuals involved provided informed consent to participate. Work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Samples were stored at -80°C until analysis. Sample preparation was based on protein precipitation and liquid-liquid extraction with acetonitrile (1:9, *v:v*). Proteins were eliminated by centrifugation and the supernatant was injected for analysis. Experiments were carried out with 50µL serum aliquots which were spiked with 100µL of a mixture of internal standards before completing to 500µL with acetonitrile. Samples were vortexed at 2500 rpm during 3 minutes with an automatic shaker (Heidolph©, VWR, Fontenay-sous-bois, France) and centrifuged at 4100g. Sample extracts were then transferred into vials for injection on the analytical system.

2.4.Liquid chromatography

Liquid chromatography was carried out on a Waters Acquity UPLC® (Waters Corp, Saint-Quentin en Yvelines, France) equipped with a sample manager, a binary solvent delivery system and a PDA detector. The flow rate was 750µL/min and the injection volume was 5µL. The autosampler vial tray and the column temperatures were set at 5°C and 50°C, respectively. The needle was washed with a mixture of acetonitrile, isopropanol and water (1:2:1 *v:v:v*). The system was fitted with an Acquity BEH HILIC column (2.1×150 mm, 1.7µm) and a corresponding guard column (ACQUITY BEH HILIC 1.7µm) purchased from Waters®. The mobile phase consisted of 10mM ammonium formate and 0.6% of formic acid (v/v) in water (A) or in 95:5 (v/v) CH_3CN:Water (B). Mobile phase for HILIC chromatography was prepared by dissolving the appropriate amount of ammonium formate in water before mixing with acetonitrile. The HILIC gradient started at an initial composition of 100% solvent A for 2 mins, followed by a 4 mins linear gradient up to 30% of solvent A, which was held for 1 min before returning to initial conditions in 1 min. The column was

thoroughly conditioned during 6 mins until the next injection, for a total chromatographic run time of 14 mins.

2.5.Mass spectrometry

The chromatographic system was coupled with a Q-Exactive™ hydrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Illkirch, France). Instrument calibration was performed by infusing a calibration mixture (caffeine, MRFA and Ultramark® 1621). A heated-electrospray HESI-II interface was used with the following parameters : S-Lens 80 V, Sheath gas: 50, Auxiliary gas: 20 arbitrary units, capillary voltage 3.5kV, capillary temperature 255°C and vaporization temperature 320°C. The maximum target capacity of the C-trap (AGC) target was defined as 3e6 and the maximum injection time was set to 200ms. Full scan was acquired in positive ion mode with a resolution of 70 000 FWHM, in the scan range of *m/z* 50-400. The Xcalibur Quanbrowser software (Thermo Fisher Scientific, Illkirch, France) was used for quantification. Targeted analyte signals were extracted with a mass window accuracy < 0.5ppm.

2.6.Method validation and matrix effect assessment

The optimized method was validated by assessing the following parameters: linearity, limit of detection and quantification, precision, recoveries, accuracy and stability. Linearity was assessed with concentrations ranging from 0.25 to 12.5µmol/L in triplicate analysis. Instrumental limits of quantification (ILOQ) were determined by analyzing solutions from serial dilution of standards in ACN and were expressed as the lowest concentration yielding the signal-to-noise ratio of 10. Instrument limits of detection (ILOD) were defined by the lowest concentration detected in serial diluted solvent standards at a signal-to-noise ratio of 3. Method limits of quantification (MLOQ) and method limits of detection (MLOD) were evaluated by the lowest concentration, which could be detected in samples submitted to the entire analytical process with the same criteria as ILODs. To estimate precision, serum

143 samples spiked at 3 concentrations (1, 5 and 10µmol/L) were injected 10 times to evaluate
144 repeatability. As no certified reference material exists for our specific method, accuracy was
145 assessed by comparing spiked concentrations to effectively measured concentrations.
146 Absolute recoveries were evaluated by triplicate analysis of serum spiked at a concentration
147 of 5µmol/L. Solvent and serum calibration curves were compared. The matrix effect was
148 characterized by either enhancement or suppression of an ion. Slopes of the calibrations
149 curves were then compared to determine matrix effect. For stability studies, four serum
150 sample aliquots kept frozen at -80°C were treated either immediately upon thawing or
151 following one, two or three thaw-freeze cycles. Two further series of aliquots were stored at
152 room temperature or refrigerated for 7 days. The latter was frozen at -80°C, thawed and
153 extracted. All aliquots were then extracted and analysed.

154 *2.7.Statistical analysis*

155 Statistical analyses were performed using the Statistica® software (version 8.0) and R
156 programming language. Descriptive statistics were performed for data treatment. Shapiro-
157 Wilk test of normality of the data was used before implementing the t-student test to evaluate
158 precision.

159 ## 3. Results

160 ### *3.1.Method development for HILIC-HRMS*

161 To optimize simultaneous assay of the selected metabolites by liquid chromatography prior to
162 detection by MS, we considered primarily their physico-chemical properties and
163 chromatographic separation and retention features. Betaine, choline, L-carnitine, TMA and
164 TMAO have similar chemical structures containing a common trimethylamine group.
165 Trimethylamine and the quaternary ammonium compounds studied here are polar compounds
166 with a partition coefficient (log P) ranging from -4.52 to 0.06 (Table 1). For this reason, TMA
167 and TMAO are poorly retained on the general stationary phase like C18, CN and phenyl
168 columns and derivatisation is either necessary when using RP-HPLC [28], or not required
169 when using polar stationary phases [15, 29]. Therefore Hydrophilic Interaction Liquid
170 Chromatography (HILIC) appeared to be the most suitable column for their separation. HILIC
171 stationary phase has been used for individual separation of TMAO, TMA and other nitrogen
172 compounds [29]. The main advantages of HILIC in our study for simultaneous analysis of
173 five low molecular weight compounds characterized by similar behavior in liquid
174 chromatography, are its ability to separate polar compounds without derivatisation and to be
175 coupled with mass spectrometry, thus allowing high sensitivity.

176 Some source fragmentations could occur on all the compounds during ionization processes
177 leading to the loss of the trimethylamine residue. Therefore chromatographic separation is
178 required in order to avoid any contribution of any fragment signal to the trimethylamine
179 residue initially present in the extract. To optimize the composition of the gradient, retention
180 behavior of individual analytes was determined in isocratic mode by varying the amount of
181 acetonitrile. In these conditions, TMA, TMAO, choline and L-carnitine started to be
182 separated around 90%, but a better separation was achieved at 95% with a highest dispersion
183 of retention factors k (Fig. 1). Thus, experiments were carried out with 95% of acetonitrile as

184 an initial gradient composition. Liquid chromatography separation was further optimized by
185 testing various mobile phases adjusted at 3 different pH. A compromise between sensitivity,
186 peak shape and retention time was found for the 5 compounds at a pH adjusted to
187 approximately 2. We determined that optimal conditions of flow rate (0.75mL/min) and
188 column temperature (50°C) allowed separation of the analytes in less than 5 min (Fig. 2). To
189 avoid retention time shift, a minimum of 5 min reconditioning was recommended by the
190 supplier, which considerably extended the final run time whilst ensuring reliable
191 reproducibility.

192 Following chromatographic separation, compounds were analysed by mass spectrometry
193 using a Q-Exactive instrument (quadrupole coupled with an orbitrap system), which allows
194 the application of various acquisition modes. In our experiments, acquisition was performed
195 in full scan mode and mass spectrometry diagnostic signal was extracted with a mass
196 accuracy < 0.5ppm. To optimize mass spectrometry detection parameters, such as S-lens, gas
197 pressure and temperature, each standard compound prepared in ACN/H_2O (50:50, v/v) at a
198 concentration of 1 µg/mL, was directly infused in a mobile phase flow using a Tee system
199 while varying parameters until highest sensitivity was obtained. A compromise was chosen to
200 ensure sufficient sensitivity for each metabolite (parameters given in Experimental).

201 *3.2.Method validation*

202 To characterize performances of the method, we evaluated the following criteria: linearity,
203 limits of detection (LOQ), precision, accuracy and recovery (see Experimental). Experiments
204 were carried out with spiked human serum, which is a matrix containing naturally various
205 amounts of the analytes. Therefore initial concentrations of the analytes in the non-spiked
206 matrix were always subtracted from the calculated concentration of the spiked samples. All
207 validation parameters are summarized in table 2.

208 The linearity was determined using linear regression model. For each compound the ratio
209 analyte/internal standard was plotted against the spiked concentrations. The slope, intercept
210 and determination coefficient were measured. The calibration curves showed excellent linear
211 response with a mean coefficient of determination higher than 0.99 for all compounds tested
212 (Table 2). The relative standard deviations calculated on the slopes for the triplicate analysis
213 were <2% for TMAO, choline, L-carnitine and betaine and were therefore outstanding. Even
214 though relative standard deviation for TMA linearity was higher (15%) it was considered as
215 acceptable. ILOD ranged from 2nmol/L for choline to 10nmol/L for TMA, L-carnitine and
216 betaine. Values of LODs were in good agreement with those found by other research groups
217 on comparable instruments [16, 17, 30, 33].

218 The precision of the method was determined by comparing measures of spiked human serum
219 at 3 concentrations obtained by two different operators. Repeatability was assessed by
220 determining the relative standard deviations (RSD), which were all below 12%, with the
221 exception of those of choline compounds which nevertheless remained <16%. This
222 difference on repeatability is due to the high endogenous concentration already present in the
223 human serum sample used. RSD slightly reduced with increasing spiked concentrations, with
224 a drop from 16% to 3% at 10µmol spiked concentration. Fetal bovine serum was also tested
225 as blank matrix, but initial concentration of the 5 targeted analytes was considered too high.
226 Accuracy of the method was assessed by comparing spiked concentrations to real
227 concentrations determined after analysis, for 3 different levels of concentration.

228 For all samples that underwent thaw and freeze cycles, RSD of compound signals varied
229 between 1.5 and 5.1% and were not significantly altered by the number of thaw and freeze
230 cycles. All analytes were stable when serum was kept refrigerated for 7 days. All analytes but
231 choline were stable in serum left at room temperature.

232 *3.3.Matrix effect*

To test our method for matrix effects, we carried out a post-extraction addition approach to compare retention times of native compounds and deuterium labeled internal standards. Since matrix effect can occur at different levels of concentrations, we compared both concentrations measured at each level of the calibration range with or without the matrix and correlation coefficients of the calibration curves. We showed that for each of the five metabolites tested, the slopes obtained were identical for native metabolites and deuterium labeled internal standards, thus demonstrating that the correction of matrix effects was achieved (Fig. 3A-E). Analysis of regression models showed a significant association of intensity ratio with concentrations in solvent and serum for all compounds (Fig. 3F).

To assess similarity between the regression lines for each compound, we applied two different linear models (Table 3). In model 1, the ratio intensity is modeled as the dependent variable with matrix-type variable as the factor and concentration as the covariate. We did not find evidence of significant interaction between the two variables, suggesting that the slope of the regression between "concentration" and "intensity ratio" is similar for solvent and serum. Model 2 is more parsimonious and does not account for interaction. Results obtained with Model 2 show that the matrix type has a significant effect on TMAO (3.0×10^{-7}), choline (1.1×10^{-16}), L-carnitine (2.6×10^{-15}) and betaine (5.0×10^{-15}) (Table 3) which can be interpreted as significant differences in intercepts between the regression lines of solvent and serum as illustrated in Figure 3. Comparisons of the two models using ANOVA show that withdrawal of the interaction does not significantly affect the fit of the model (Table 3). These data show that Model 2 is the most parsimonious and thereby regression lines are parallel (similar slope) for solvent and serum for all cases. Therefore the isotopic dilution coupled to UPLC-HRMS method enables to overcome the matrix effects.

3.4.Application of targeted quantification of methylamines in human serum samples

To validate our analytical method by demonstrating its application in a clinical context, we profiled serum samples from 67 healthy subjects according to the analytical process we developed. There were no significant differences in age in males (44.2 ± 1.2 years) and females (47.2 ± 1.1 years). No significant correlations between metabolite concentrations and individuals' age were found. Serum levels of L-carnitine, choline and TMAO were similar in males and females, whereas significant gender differences were observed for TMA and betaine. Serum concentrations of these metabolites were significantly more elevated in males than in females for betaine (6.97 ± 0.39 μmol/L and 5.84 ± 0.33 μmol/L; p=0.03) and TMA (0.73 ± 0.09 μmol/L and 0.34 ± 0.01 μmol/L; p<0.001). The range of serum metabolite levels is illustrated with box and whisker plots distribution of betaine, L-carnitine, choline, TMA and TMAO (Fig. 4). Even though serum levels were similar to those reported in the literature [16, 33], we were able to demonstrate the broad ranges of concentrations of betaine (3.01-15.84 μmol/L), L-carnitine (2.75-9.96 μmol/L), choline (0.91-2.98 μmol/L), TMAO (0.16-17.52 μmol/L) and TMA (0.29-1.66 μmol/L) in healthy individuals.

Serum concentrations of the 5 metabolites measured in 67 subjects were further investigated by principal component analysis (PCA). The biplot for principal components 1 (PC1) and 2 summarized 52.4% of the variation and was primarily influenced by betaine, L-carnitine and TMA (Fig. 5A). This indicates close correlation between serum concentrations for betaine and L-carnitine, which was assessed by regression analysis (p=0.0009) (Supplementary Fig. 1). In contrast, variations in L-carnitine and betaine were almost orthogonal to concentrations of choline, TMA and TMAO, which is consistent with the fact that L-carnitine and betaine are minor substrates compared to choline for gut bacterial synthesis of TMA[41]. Choline and TMAO were modestly correlated whereas serum TMA and TMAO concentrations were clearly anti-correlated (Supplementary Fig. 1). The PC3/PC4 biplot summarized 36% of variance and was mainly influenced by choline and TMAO (Fig. 5B).

4. Discussion

We have developed and validated a single method for MS-based simultaneous quantitative analysis of five methylamines involved in a metabolic pathway underlying functional cross-talk between gut microbiome and host genome and involved in various metabolic disease processes. Unexpected anticorrelation between concentrations of TMAO, associated with cardiovascular risk in several studies, and TMA, which is a product of gut microbial metabolism, provides a biological rationale for broadening quantitative methylamine profiling at pathway level in order to gain more detailed biological information on these important compounds.

Components of the methylamine metabolic pathway illustrate the importance of functional symbiotic relationships between gut microbes and the host and their contribution to mammalian metabolic regulations and genome expression in health and disease. TMA can be generated by intestinal microbial transformation of dietary constituents, including L-carnitine, choline and betaine. TMA is then absorbed by the host and metabolized into TMAO in the liver by a flavin monooxygenase (FMO3) [42]. A targeted analytical strategy designed to monitor in clinical environment coordinated changes in serum concentration of these compounds simultaneously has therefore great potential to investigate global regulations of the relevant pathway in patients.

We have demonstrated the specificity, sensitivity and robustness of our MS method coupled with HILIC chromatography. HILIC is a variant of normal phase liquid chromatography (NP-LC) for analytes eluted near the void on reverse phase (RP-LC). In contrast to RP-LC, compounds are eluted by increasing the percentage of polar mobile phase in HILIC columns. Among all the advantages of using HILIC over conventional RP-LC and NP-LC, the use of expensive ion pair reagents in mobile phases is not required and it can be conveniently coupled with mass spectrometry. For these reasons HILIC has become increasingly popular

because it has progressively been found useful for the analysis of polar drugs, metabolites and biologically important compounds in complex matrices [43] including quaternary ammonium compounds [29, 33].

Metabolite compounds can be detected in MS [29] or MS/MS [16, 30] experiments with single or triple quadrupole instruments and recent breakthrough in LC-MS/MS has promoted the use of this type of instrument in clinical laboratories [31]. When compounds do not fragment specifically or not at all, LC-MS/MS can be limited when compared to high-resolution mass spectrometry (HRMS). Many studies have compared analytical performances of HRMS and tandem mass spectrometry, finding similar advantages in terms of linearity, limits of detection and precision for both systems, but higher specificity for low molecular compounds for HRMS [32]. Despite good performances of LC-MS/MS for routine diagnostic, LC-HRMS offers higher mass resolution than quadrupole systems, avoiding false negative. Moreover, it offers the opportunity to screen targeted analytes as well as non-a-priori selected substances with high-selectivity.

We have also carefully evaluated matrix effect, which is frequently described in MS-based analyses [34], even though the exact underlying mechanisms remain unknown. Reduced analyte response with increasing compound concentration was the first evidence of this well recognized phenomenon [35]. One of the hypotheses to explain matrix effect is that it is due to competition between the targeted compound and co-eluting endogen components. The main techniques to assess matrix effect are post-column infusion and post-extraction addition [36, 37]. The latter, which we chose to apply, is based on analyte response comparison of spiked serum samples processed according to the sample preparation protocol and standard solutions prepared in the mobile phase.

Matrix effects can be minimized by improving sample cleanup procedures or by altering chromatographic conditions to separate analytes of interest from matrix interferences [38].

However, the most efficient way to overcome ionization effects associated with electrospray mass spectrometry experiment is through the utilization of stable isotopically labeled internal standard analogues with identical chemical and physical properties to the native analyte [39]. Since internal standards follow all steps of the sample preparation process, it helps correct for variations in sample preparation and compensates for variability during ionization. However, in some cases, a deuterium labeled internal standard has demonstrated differing ionization potential compared to the analyte, due to the slight shift in retention time between analyte and internal standard, and the retention relationship with the co-eluting endogenous material [40]. Optimized chromatographic gradients are therefore required to overcome this issue, as described by Zhang and Wujci [39]. In our study, the chromatographic gradient ensured identical retention times between analyte and its deuterium internal standard.

In conclusion, we propose a UPLC-HRMS method for methylamine assay in biological samples, which shows performance in good agreement with observed concentrations in human serum and was successfully applied to metabolite quantification in clinical samples to establish the range of serum concentrations in control individuals. The high-throughput nature of this method should facilitate clinical applications of biomarker quantitative analyses in various analytical matrices and human disease contexts.

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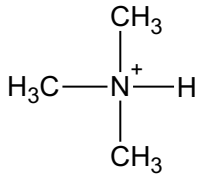
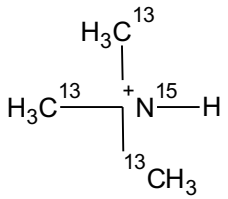
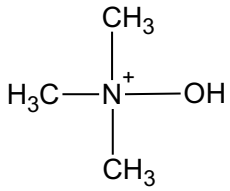
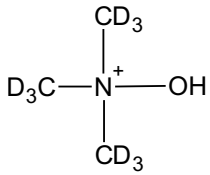
423

424 **Table 1**: Chemical structures and properties of target analytes, and their corresponding isotope labeled standards.

425

Compound name and abbreviation	Standard type	Molecular formula	Chemical structure	log P
Trimethylamine (TMA)	Target analyte	C_3H_9N		0.06
Trimethylamine-$^{13}C_3$,^{15}N (TMA-$^{13}C_3$,^{15}N)	Isotope-labeled internal standard	$^{13}C_3H_9{}^{15}N$		-
Trimethyamine N-Oxide (TMAO)	Target analyte	$C_3H_{10}NO$		-2.57
Trimethylamine-oxide D_9 (TMAO-D_9)	Isotope-labeled internal standard	$C_3H_1D_9NO$		-

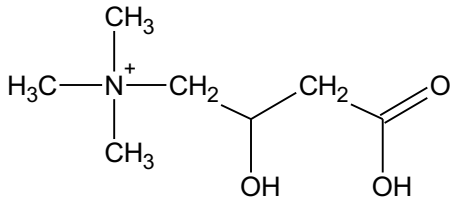
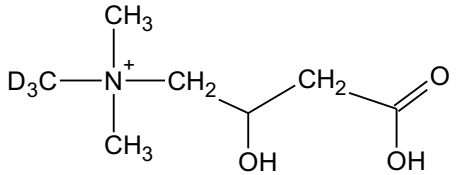
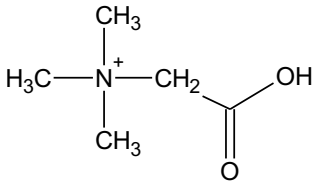
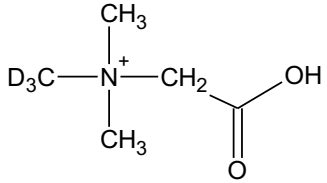
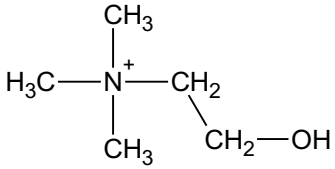
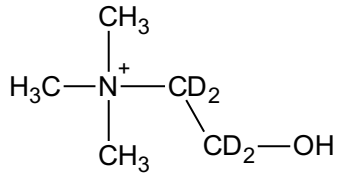
L-Carnitine	Target analyte	$C_7H_{16}NO_3$		-4.52
L-Carnitine-D_3	Isotope-labeled internal standard	$C_6H_{13}CD_3NO_3$		-
Betaine	Target analyte	$C_5H_{12}NO_2$		-3.25
Betaine-D_3	Isotope-labeled internal standard	$C_4H_9CD_3NO_2$		-
Choline	Target analyte	$C_4H_{12}NO_2$		-3.22
Choline-1,1,2,2-D_4	Isotope-labeled internal standard	$C_4H_{12}D_4NO_2$		-

Table 2: **Analysis of spiked human serum demonstrates the performance of the methylamine method**. Linearity, limit of detection and quantification, recovery, precision (repeatability and reproducibility), accuracy were assessed. ILOD, instrument limits of detection; MLOD, method limits of detection; RSD, Relative standard deviation.

Compound		TMA	TMAO	Choline	L-Carnitine	Betaïne
Linearity	Mean intercept	0.016	0.001	0.003	-0.001	0.008
	Mean slope	0.025	0.020	0.032	0.039	0.036
	RSD (%)	14.324	1.507	1.397	1.513	1.530
	Mean R^2	0.996	1.000	1.000	0.999	1.000
Limit of detection	ILOD (µmol/L)	0.010	0.005	0.002	0.010	0.010
	MLOD (µmol/L)	0.050	0.025	0.010	0.050	0.050
Mean recovery (RSD %)	5 µmol/L	93 (2)	111 (3)	87 (10)	97 (4)	98 (7)
Repetability (RSD %)	Concentration spiked	RSD (%)				
	1 µmol/L	6	5	16	8	12
	5 µmol/L	2	3	10	4	7
	10 µmol/L	3	3	3	3	4
Accuracy	Endogenous (µmol/L)	0.66	1.00	17.61	5.29	6.22
	Concentration spiked	Concentration measured (RSD%)				
	1 µmol/L	0.80	0.99	0.80	1.10	1.01
	5 µmol/L	4.65	5.56	4.36	4.86	4.91
	10 µmol/L	10.79	11.95	9.60	10.06	10.00
Stability (freeze-thaw cycles)	RSD (%)	1.5	3.6	4.6	4.8	5.1

Table 3: Demonstration of statistically significant matrix effects in quantitative analysis of all five metabolites tested in the mass spectrometry assay. Data from serum samples from 67 control individuals were used.

	TMAO	TMA	Choline	L.carnitine	Betaine
Model 1	0.092	0.576	0.155	0.583	0.799
Model 2	2.97e-07	0.887	1.085e-16	2.60e-15	4.95e-15
comparison	0.092	0.576	0.155	0.583	0.799

Legends to Figures

Figure 1: Outlined representation of the methylamine pathway and Vant'hoff plot showing separation of TMA, TMAO, choline and L-carnitine from 90% acetonitrile (ACN). k retention factors are shown for L-carnitine, betaine, choline, TMA and TMAO as a function of the ACN percentage in the mobile phase.

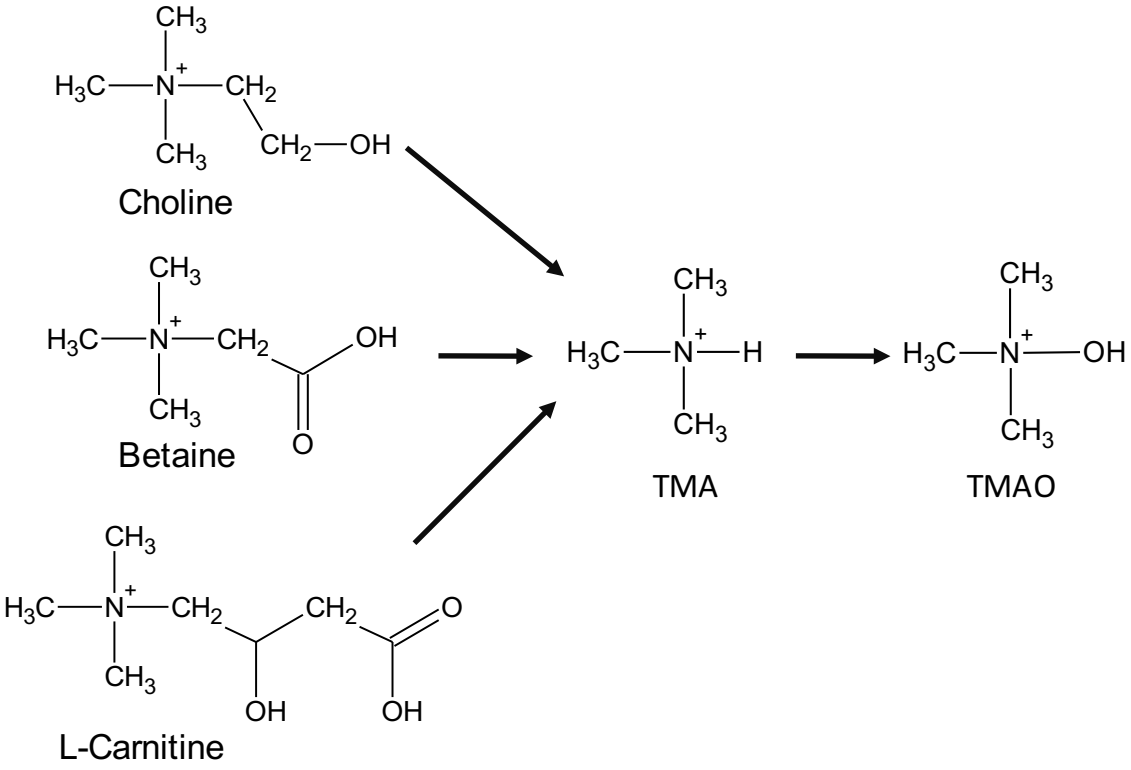
Figure 2: Illustration of chromatographic separation of serum L-carnitine, betaine, choline, TMA and TMAO. Mass spectrometry and chromatographic features are shown.

Figure 3: Regression models following isotopic dilution for the five compounds of interest (A: TMAO, B: TMA, C: L-Carnitine, D: Betaine and E: Choline) demonstrate matrix effects. The area ratios of each analyte to the internal standard are shown on the y-axes and concentrations are on the x-axes. Data are shown for serum (●) and solvent (○). Results from analyses of regression models based on intensity ratio and metabolite concentration in solvent and serum are shown (E).

Figure 4: Physiological variability of the metabolites in human serum. Ranges of serum concentration of betaine, L-carnitine, choline, TMA and TMAO were determined in human serum from 67 control subjects.

Figure 5: TMA and TMAO concentrations are anti-correlated in human serum from control individuals. Correlation comparisons are shown for concentrations of betaine, L-carnitine, choline, TMAO and TMA in human serum. Data were obtained from serum samples from 67 control individuals. Biplots following principal component analysis are shown for PC1 vs. PC2 (A) and for PC3 vs. PC4 (B).

Figure 1


Choline
Betaine
TMA
TMAO
L-Carnitine

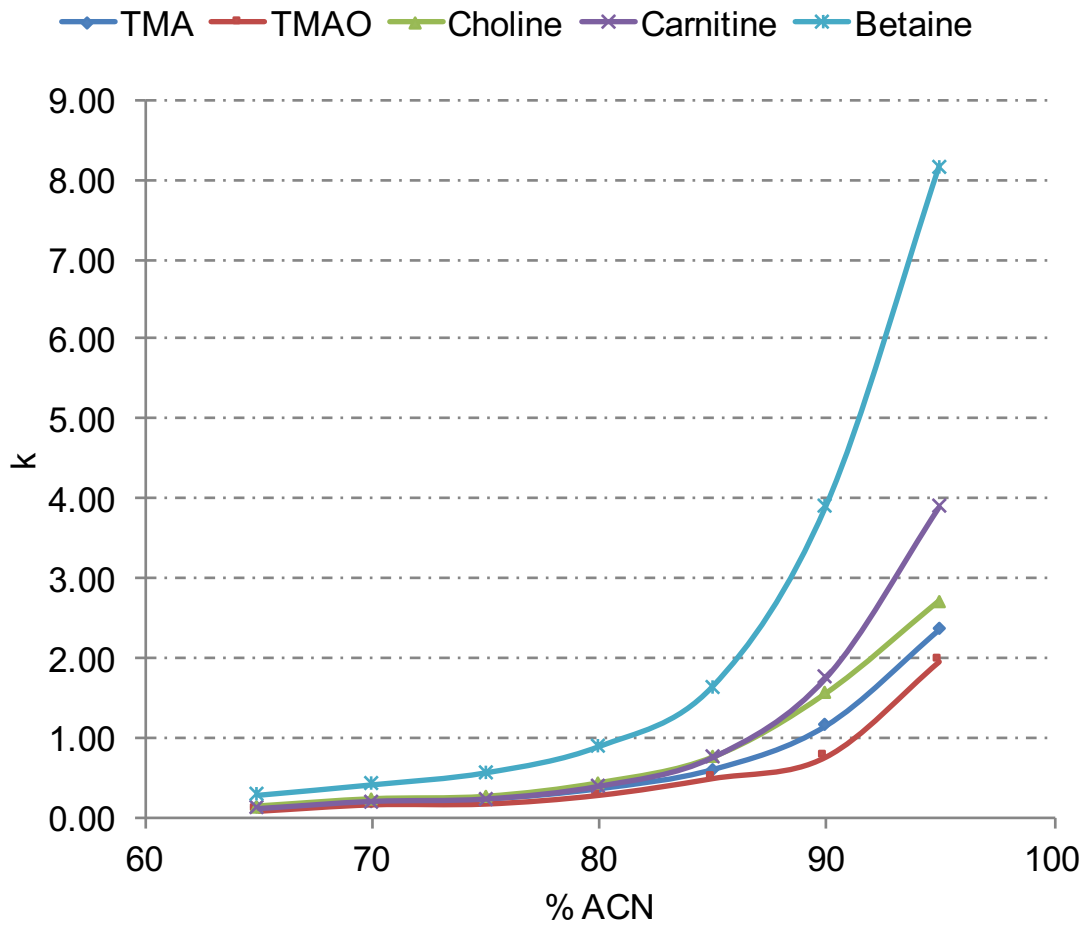

TMA
TMAO
Choline
Carnitine
Betaine
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
k
60
70
80
90
100
% ACN

Figure 2

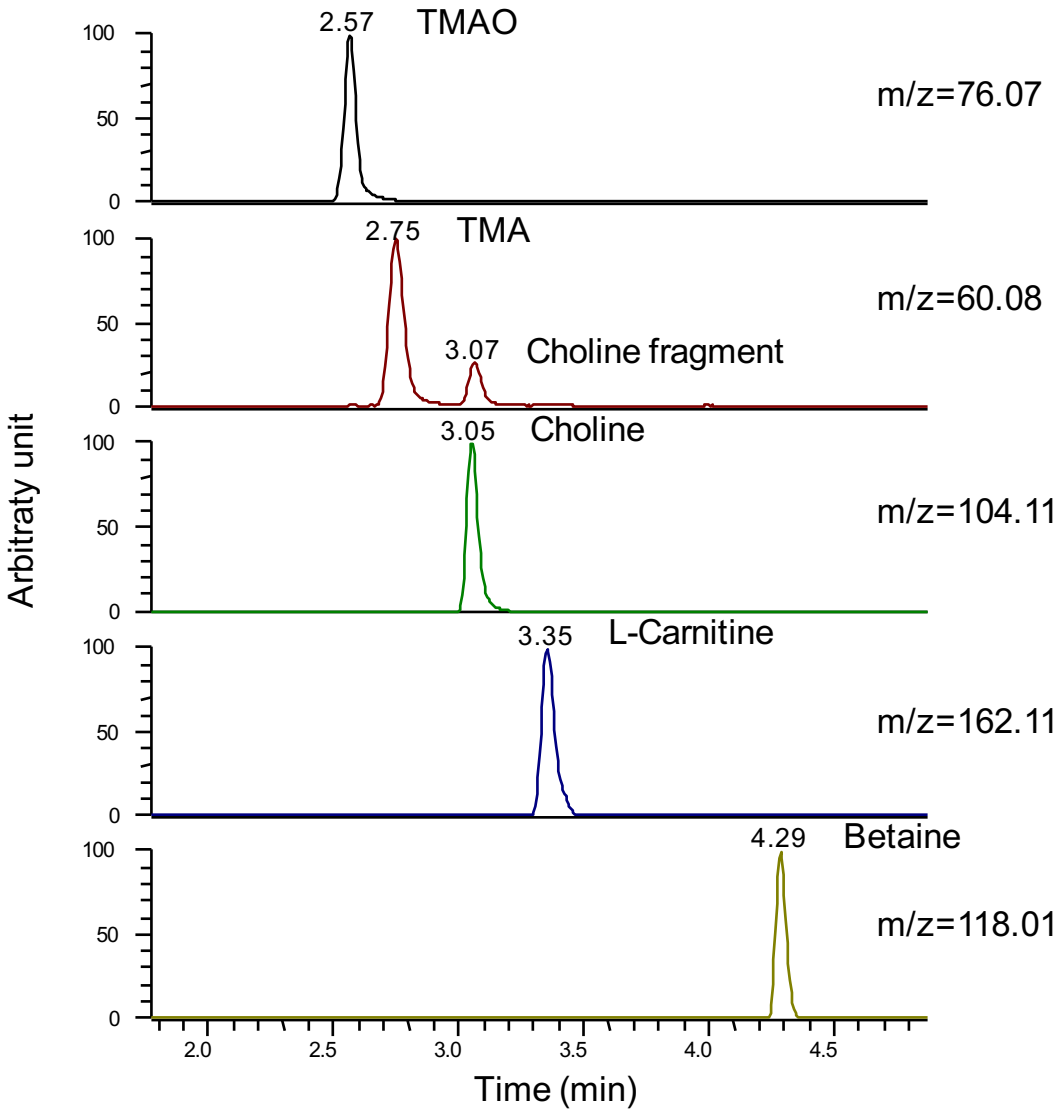
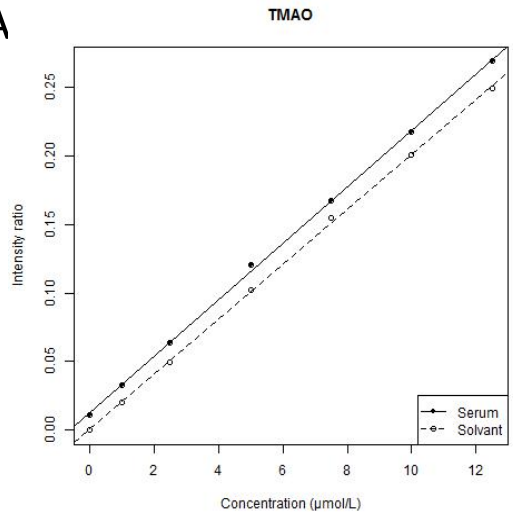
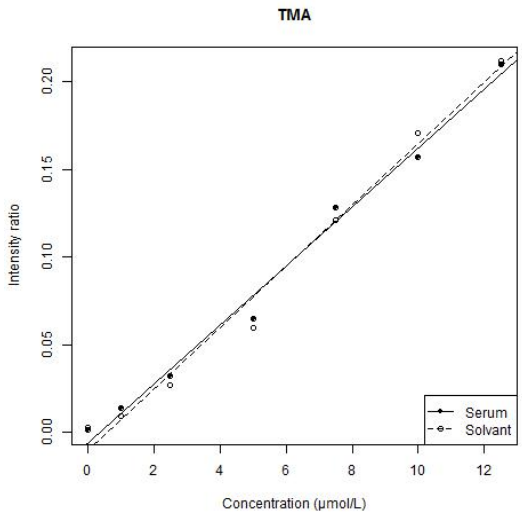

2.57
TMAO
m/z=76.07
2.75
TMA
m/z=60.08
3.07
Choline fragment
3.05
Choline
m/z=104.11
3.35
L-Carnitine
m/z=162.11
4.29
Betaine
m/z=118.01
Arbitraty unit
Time (min)
100
50
0
2.0
2.5
3.0
3.5
4.0
4.5

Figure 3

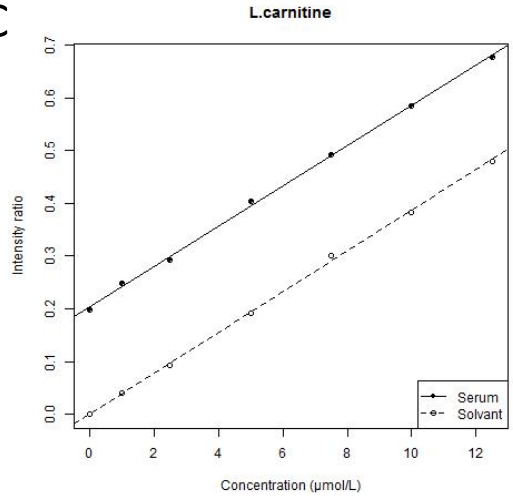
A



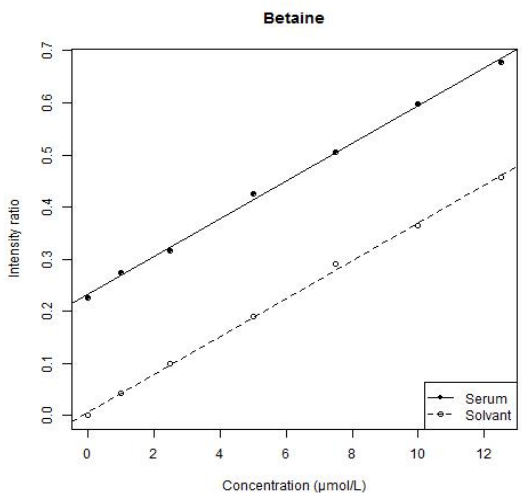
B



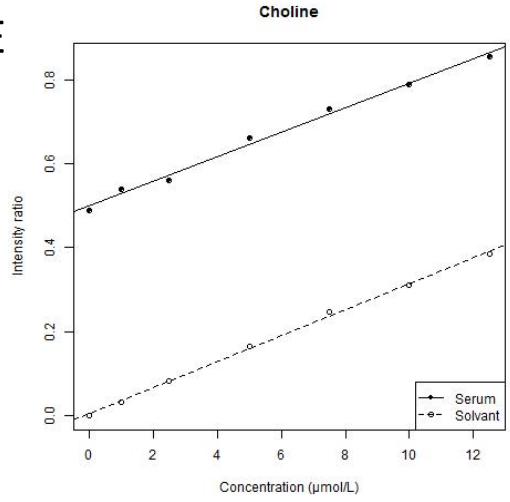
C



D



E



F

	coefficient	intercept	P-value
serum_TMAO	0.0205	0.013	1.548e-09
solvant_TMAO	0.02	0.0009	2.142e-09
serum_TMA	0.0168	-0.0056	3.481e-06
solvant_TMA	0.0175	-0.0101	7.679e-06
serum_choline	0.029	0.5006	1.922e-06
solvant_choline	0.031	0.0046	2.864e-08
serum_L.carnitine	0.0381	0.204	1.743e-08
solvant_L.carnitine	0.0385	0.0007	6.859e-09
serum_Betaine	0.0361	0.232	5.967e-08
solvant_Betaine	0.0364	0.00576	2.109e-08

Figure 4

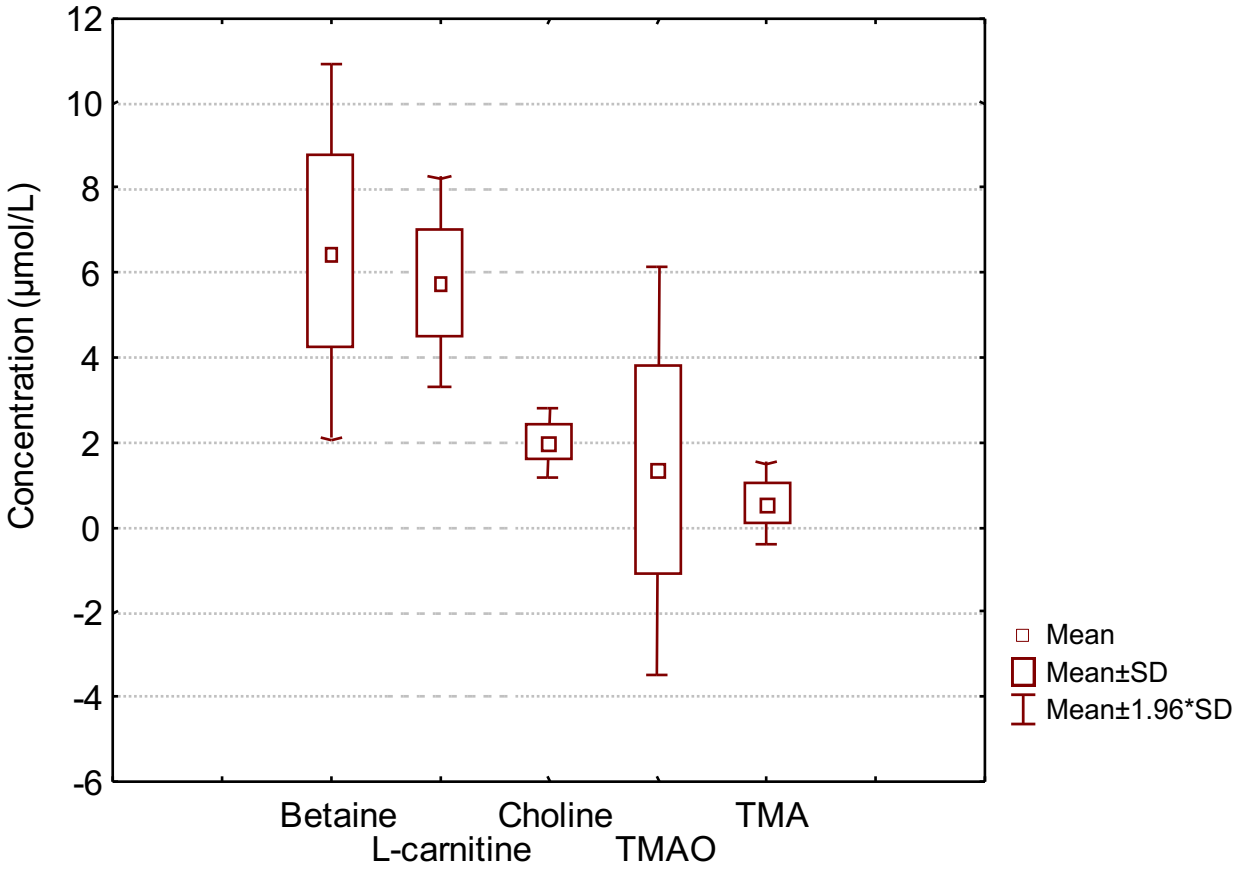
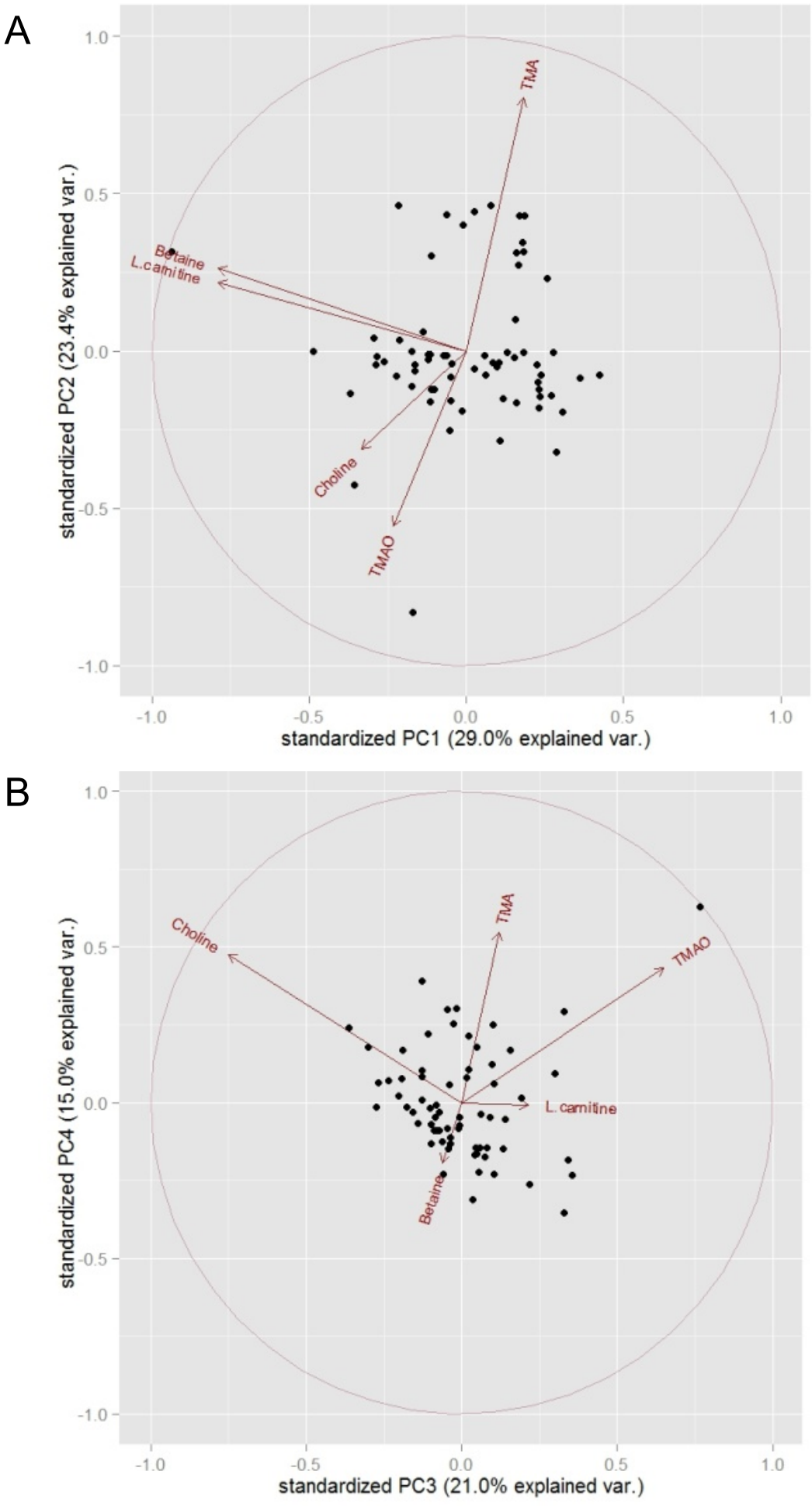
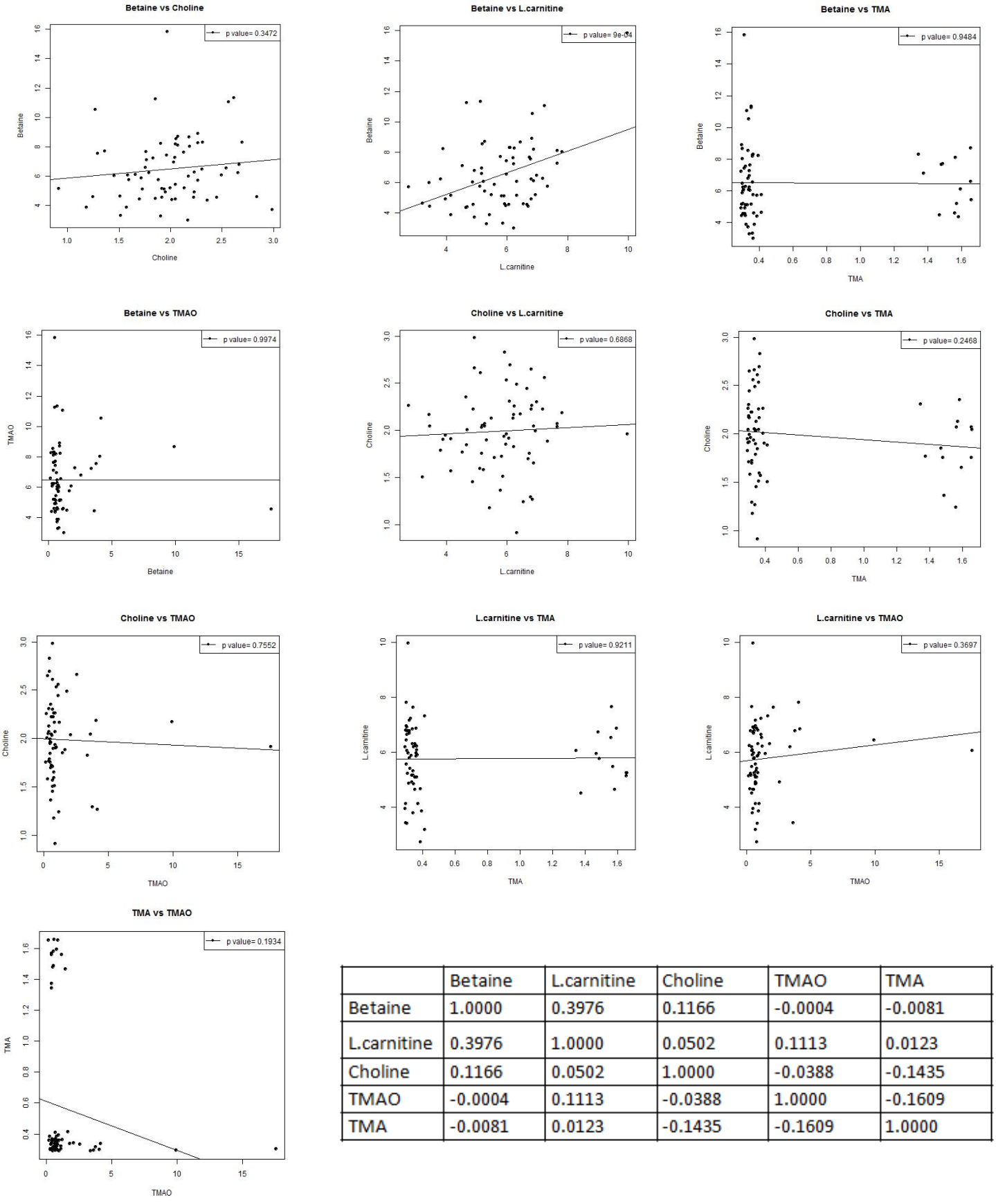

12
10
8
6
4
2
0
-2
-4
-6
Concentration (µmol/L)
Betaine
L-carnitine
Choline
TMAO
TMA
Mean
Mean±SD
Mean±1.96*SD

Figure 5


A
TMA
Betaine
L.carnitine
Choline
TMAO
standardized PC2 (23.4% explained var.)
standardized PC1 (29.0% explained var.)
1.0
0.5
0.0
-0.5
-1.0
B
Choline
TMA
TMAO
L.carnitine
Betaine
standardized PC4 (15.0% explained var.)
standardized PC3 (21.0% explained var.)

Supplementary Figure 1. Regression analysis of the concentration of betaine, L-carnitine, choline, TMAO and TMA in human serum. Data were obtained from serum samples from 67 control individuals. Correlation matrix (Pearson) was calculated.



	Betaine	L.carnitine	Choline	TMAO	TMA
Betaine	1.0000	0.3976	0.1166	-0.0004	-0.0081
L.carnitine	0.3976	1.0000	0.0502	0.1113	0.0123
Choline	0.1166	0.0502	1.0000	-0.0388	-0.1435
TMAO	-0.0004	0.1113	-0.0388	1.0000	-0.1609
TMA	-0.0081	0.0123	-0.1435	-0.1609	1.0000