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Development and Application of a UPLC–MRM–MS Method for Quantifying Trimethylamine, Trimethylamine-N-Oxide, and Related Metabolites in Individuals with and Without Metabolic Syndrome

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Abstract: Trimethylamine-N-oxide (TMAO) is associated with various chronic diseases. TMAO is a downstream oxidative metabolite of trimethylamine (TMA) that is generated by the gut microbiota from dietary choline, carnitine, and betaine. Current analytical methods predominantly target TMAO only, due to the challenge of efficiently extracting and quantifying TMA. The present study demonstrates a simple and rapid UPLC–MRM–MS method for concurrent quantification of TMAO, TMA, and related precursors (choline, betaine, and various carnitines) following a methanol extraction from plasma and derivatization using iodoacetonitrile (IACN). Pure methanol resulted in a higher extractability of TMA (up to two-fold) compared to both pure acetonitrile and various methanol/acetonitrile mixtures. The quantification method showed high linearity within the tested range of 0.0625–100 µmol/L (determination coefficient > 0.999) and an intra- (n = 3) and inter-day (n = 9) precision of 2–8% along with an average recovery of above 94% for all metabolites (TMAO, TMA, choline, betaine, L-carnitine, acetyl-L-carnitine, and propionyl-L-carnitine). The method's applicability was confirmed through a comparison of TMAO and its precursor concentrations in plasma samples of overnight-fasted subjects with (n = 12) and without (n = 21) metabolic syndrome.

Keywords: methylamines; TMAO; TMA; plasma samples; extraction; UPLC–MRM–MS

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1. Introduction

TMAO has been recognized as a significant risk factor that is associated with increased adverse outcomes of several chronic diseases, such as cardiovascular disease, colorectal cancer, diabetes, and chronic kidney disease [1–4]. TMAO is a small metabolite that can be directly obtained from one's diet, e.g., seafood [5], or derived from the oxidation of TMA by hepatic flavin monooxygenases into TMAO [6]. TMA is produced by the gut microbial enzymes from nutrients in animal-based foods, e.g., carnitine(s) and choline [7], through two primary pathways (choline and betaine pathways). The key enzymes involved are choline TMA-lyase (CutC/D) and carnitine-oxygenase (CntA/B) [8,9]. Betaine can also be converted to TMA by an enzyme complex (YeaW/X) [8,9]. Thus, the quantification of TMA levels can provide insight into the composition and the activity of the gut microbiota, as well as the activity of flavin monooxygenases. Deficiency of the latter leads to trimethylaminuria (fish odor syndrome) [10]. Establishing a rapid and accurate quantitative method for the

quantification of TMAO and its related metabolites is needed for research advancement and clinical diagnosis.

Liquid chromatography, coupled with mass spectrometry, is the most common method for analyzing TMAO, TMA, and related metabolites [11–16]. Unlike TMAO and the other metabolites, which can be measured directly using MS/MS, TMA typically requires derivatization [12,14,15,17,18] due to its low mass (59 Da), which results in poor fragmentation using UPLC–MRM–MS [18]. Additionally, ionization of TMA, without fragmentation, produces a weak signal of protonated ions at m/z = 60 [18]. Consequently, this additional derivatization step limited the development of a UPLC–MRM–MS method for TMA quantification, highlighting a gap in TMA-specific methodological optimization. This has led to TMAO becoming the primary focus of several UPLC–MRM–MS methods developed to study the role of TMAO in health and disease. To address this, this study focused on systematically optimizing the extraction procedure and chromatographic conditions to develop ultra-performance liquid chromatography–multiple reaction monitoring–tandem mass spectrometry (UPLC–MRM–MS) for the analysis of TMA, TMAO, and related metabolites in plasma.

The method was internally validated and applied on a subsample of plasma samples from overnight fasted subjects (both with and without metabolic syndrome) to determine and compare TMAO, TMA, and related metabolites between the two groups. Metabolic syndrome is a cluster of conditions—including obesity, high blood pressure, dyslipidemia, and insulin resistance—that occur together, thus increasing the risk of cardiovascular disease and type 2 diabetes [19]. TMAO has been implicated in cardiovascular diseases and metabolic disorders, rendering it a relevant biomarker [6].

2. Materials and Methods

2.1. Reagents and Solutions

TMAO, TMA, betaine, choline, L-carnitine, propionyl-L-carnitine, acetyl-L-carnitine, and L-carnitine chloride-d9 (Table 1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other deuterated compounds (TMA-d9, TMAO-d9, betaine-d11, L-carnitine-d3, acetyl-L-carnitine-d3) (Table 1) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). All the other chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals in this study were of LCMS grade, except iodoacetonitrile (IACN), which was of p.a. grade. Water (MQ) was purified using a Milli-Q Water Purification System (Merck, Darmstadt, Germany).

Table 1. Mass transition collision energy of analyzed metabolites.

Metabolite	Precursor Ion	Quantifier Ion/ Qualifier Ion	Collision Energy (eV)
Trimethylamine-acetonitrile	99.01	58.1 59.1	29 17
Trimethylamine-N-oxide	76.01	58.1 59.1	21 13
Choline	104.01	60.1 45.1	17 25
Betaine	118.01	58.2 59.2	33 21
Acetyl-L-carnitine	204.01	85.1 29.2	21 50

Table 1. *Cont.*

Metabolite	Precursor Ion	Quantifier Ion/ Qualifier Ion	Collision Energy (eV)
L-Carnitine	162.01	43.2 60.2	33 17
Propionyl-L-carnitine *	218.2	85.1 29.2	21 49
Internal standards (ISs)			
Trimethylamine-acetonitrile-d9	108.01	66.2 68.2	33 17
Trimethylamine-N-oxide-d9	85.01	66.2 68.1	25 13
Choline-d9	113.01	69.2 45.2	21 25
Betaine-d11	129.01	66.2 68.2	37 21
Acetyl-L-carnitine-d3	204.01	85.1 29.3	21 50
L-Carnitine-d3	165.01	43.2 103.1	41 17

* D_3-L-carnitine was used as an IS for the quantification of propionyl-L-carnitine. Dwell time was 50 milliseconds (ms), cell accelerator voltage 4 V, and fragmentor voltage 166 V.

Individual stock solutions of TMAO, TMA, betaine, choline, L-carnitine, propionyl-L-carnitine, and acetyl-L-carnitine were prepared in MQ water at a concentration of 10 mmol/L and stored at −30 °C. Aqueous calibration solutions were prepared using serial dilution to contain 0.0625, 0.125, 0.25, 0.5, 1, 2, 5, 10, 20, 50, and 100 µmol/L of the individual compounds and stored at −30 °C. Individual stock solutions of internal standards (ISs) were prepared in MQ water at a concentration of 10 mmol/L and stored at −30 °C. A mixture of IS (10 µmol/L of each compound) was prepared in water and stored at −30 °C.

2.2. Optimization of Chromatographic Conditions

A standard solution containing a mix of external standards (50 µmol/L) and ISs (10 µmol/L) was used to optimize the UPLC method and the ion source and to establish mass transition settings. All analysis was carried out using a UPLC 1260 II Infinity system (Agilent Technologies, Santa Clara, CA, USA) coupled with a triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) that was equipped with an AJS-ES source (Agilent Jet Stream ionization source).

Methylamines were separated on a neutral UPLC–HILIC column (ACE 1.7 HILIC-N, 75 × 2.1 mm) (Chromtech ACE, Scantec Nordic, Aberdeen, Scotland), along with a guard column(ACE 1.7 HILIC-N, 3.0 × 2.1 mm)(Chromtech ACE, Scantec Nordic, Aberdeen, Scotland). Isocratic elution was performed using a mobile phase consisting of 70% ammonium formate (10 mmol/L) in MQ water (solvent A) and 30% acetonitrile (ACN) (solvent B) as described earlier [14]. The flow rate was set at 0.2 mL/min and the injection volume was 1 µL. The total run time was 6 min, and the first 0.5 min was diverted to waste. The column was thermostatically controlled at 25 °C, while the autosampler temperature was kept at 5 °C. The injector was washed using 20% methanol in water.

Optimization of ion source parameters was carried out using Source Optimizer Software (version B.08.00, Mass Hunter workstation, Agilent Technologies, Santa Clara, CA, USA). A wide range of the following parameters was optimized: capillary voltage (0–2000 V, step size 500), nozzle voltage (0–2000 V, step size 500), drying gas temperature (100–280 °C, step size 30), drying gas flow (10–20 L/min, step size 1), sheath gas temperature (225–400 °C, step size 25), sheath gas flow (5–12 L/min, step size 1), nebulizer (15–60 psi, step size 5), high-pressure flow (70–210 V, step size 20), and low pressure flow (40–160 V, step size 20). The optimized parameters were applied during the analysis of plasma samples and were set as follows: drying gas temperature 160 °C, drying gas flow 11.0 L/min, nebulizer pressure 35 psi, sheath gas temperature 400 °C, sheath gas flow 12 L/min, capillary voltage +2000 V (positive ionization), nozzle voltage 0 V, iFunnel high pressure 110 V, and low pressure 60 V (positive mode).

AJS-ES was used and operated in Multiple Reaction Monitoring (MRM) mode. Mass transitions were also optimized using method optimizer software (version 10.1, Mass Hunter workstation, Agilent Technologies, Santa Clara, CA, USA). Four product ions were acquired, and a wide range (5–50 eV) of collision energy was tested (Table 1). The most intense transition was used for the quantification (quantifier transition) (Table 1), and the second was used for confirmation (qualifier transition).

2.3. Optimization of Extraction Conditions and Derivatization of TMA

Optimization of extraction conditions was carried out using a non-fasted human plasma from a single donor stored in aliquots of 250–500 µL at −80 °C. Before the analysis of TMAO and its related metabolites, the plasma was thawed at room temperature. Various extraction solvent mixtures of MeOH/ACN (100:0, 90:10, 50:50, 10:90, 0:100) were evaluated using a fixed volume (25 µL) of the plasma sample. Two different solvent-to-sample ratios were examined (100:25 and 200:25), and a fixed amount of IS (10 µL, containing 10 µmol/L of each deuterated compound) was added. Different extraction times (5, 10, and 20 min) were also checked.

The optimized extraction conditions were applied to all plasma samples. In brief, 25 µL of plasma samples were transferred to a 1.5 mL Eppendorf tube and mixed with 100 µL of pure methanol, and 10 µL of an IS (containing 10 µmol/L of each deuterated compound) was added. To generate the calibration curve, 25 µL of each external standard level was transferred to a 1.5 mL Eppendorf tube and mixed with 100 µL of pure methanol and 10 µL of the IS. Both plasma samples and standards were then vortexed, shaken for 10 min at room temperature, and centrifuged (13,000× *g*, 8 min). After extraction, samples were derivatized as previously described [14,20] with minor modifications. Briefly, 100 µL of the supernatant was then transferred to a 1.5-mL microcentrifuge tube, and 890 µL of ACN, 5 µL of concentrated NH_4OH, and 5 µL of IACN were added. Samples were vortexed and shaken (10 min at room temperature). The reaction was stopped by adding 2 µL formic acid. Samples were centrifuged (13,000× *g*, 5 min). The supernatant was transferred into HPLC vials.

2.4. Quality Control of Analytical Method

To control for within- and between-day variations, plasma samples were analyzed (four analysis occasions, duplicate samples). Recovery was carried out by spiking the plasma samples with TMA, TMAO, betaine, choline, L-carnitine, acetyl-L-carnitine, and propionyl-L-carnitine each at the levels of 5, 10, 20, 50, and 100 µmol/L (5 levels as duplicate samples, n = 10). The linearity of the added standards was investigated using linear regression. The limit of detection (LOD) and limit of quantification (LOQ) were estimated from calibration curve data using LOD = (3.3 × SD)/b; LOQ = (10 × SD)/b,

where SD is the residual standard deviation of the linear regression and b is the slope of the regression line.

2.5. Method Application on Clinical Plasma Samples

To demonstrate the utility of the method, plasma samples from the 33 subjects with (n = 12) and without (n = 21) metabolic syndrome, aged 57 ± 6.7, after overnight fasting (≈12 h), were analyzed to determine and compare TMAO, TMA, and related metabolites in both groups. The analyzed plasma samples represent a subsample of plasma samples from subjects participating in an intervention trial to determine the effects of animal food ingestion (meat and eggs) on postprandial plasma concentrations of methylamines. Blood samples were collected from January to May 2020 and from December 2022 to May 2023 (the interruption was due to the COVID-19 pandemic). Blood samples (3 mL) were withdrawn using an intravenous catheter into EDTA K2 vacutainers, which were centrifuged (2000× g, 10 min) for plasma separation. Subsamples from the collected plasma (250 µL) were stored at −80 °C until analysis. The human trial was approved by the Swedish Ethical Review Authority (Dnr: 2019-04354).

2.6. Calculations and Statistics

The coefficient of variation (CV) for between-day variation was calculated for clinical samples (n = 8, four analysis occasions as duplicate samples). The recovery (R) was calculated according to the following Equation (1):

$$R\ (\%) = \left(\frac{\left(C_{found} - C_{sample}\right)}{C_{added}} \right) \times 100 \quad (1)$$

where C_{found} is the measured concentration in the spiked sample, C_{sample} is the measured concentration in the sample before spiking, and C_{added} is the added concentration.

The linearity of each calibration curve was determined using regression analyses. Extraction efficacy of different solvents was compared using a one-way analysis of variance (ANOVA). Data of methylamines in clinical plasma samples were expressed as mean ± standard deviation (SD). A t-test was performed at a 0.05 level of significance to examine the difference in methylamine concentrations between subjects with and without metabolic syndrome. Statistical analyses were carried out using GraphPad Prism (Prism 10, GraphPad, La Jolla, CA, USA) at a significance level of $p < 0.05$.

3. Results

3.1. Extraction and Derivatization

Pure methanol or methanol/ACN (9:1) demonstrated similar higher efficacy in extracting TMA, both yielding up to 2-fold higher, compared to pure ACN and other various mixtures of methanol/ACN (Figure 1). The extractability of the other metabolites did not differ significantly when using methanol, ACN, or various mixtures of methanol/ACN (Figure 1). A volume of 100 µL of methanol was found to be adequate for the extraction of 25 µL plasma, as no significant difference was observed when compared to using 200 µL of solvent. IACN proved to be a suitable derivatizing reagent in the presence of 5 µL NH_4OH, and a duration of 10 min was sufficient for complete derivatization of TMA. Formic acid is necessary to terminate the reaction and to stabilize the derivatized TMA.

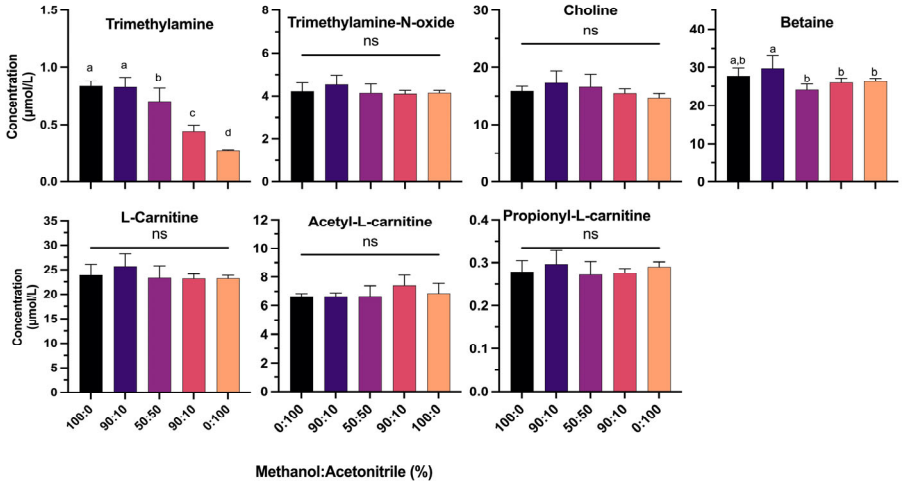


Figure 1. The effect of different methanol/acetonitrile mixtures (100:0, 90:10, 50:50, 10:90, 0:100) as extraction solvents on the extractability of TMAO and related metabolites (µmol/L) from plasma (n = 5 replicates). Different letters a–d indicate significant differences, ns, not significant.

3.2. Optimized Chromatographic Conditions

Isocratic elution with a mobile phase consisting of 70% mobile phase A (10 mmol/L ammonium formate in MQ water) and 30% mobile phase B (pure ACN) showed a satisfying resolution of TMAO, TMA, choline, betaine, and propionyl-, acetyl-, and L-carnitine using a UPLC–HILIC-N (neutral) column (Figure 2) at 0.2 mL/min flow rate.

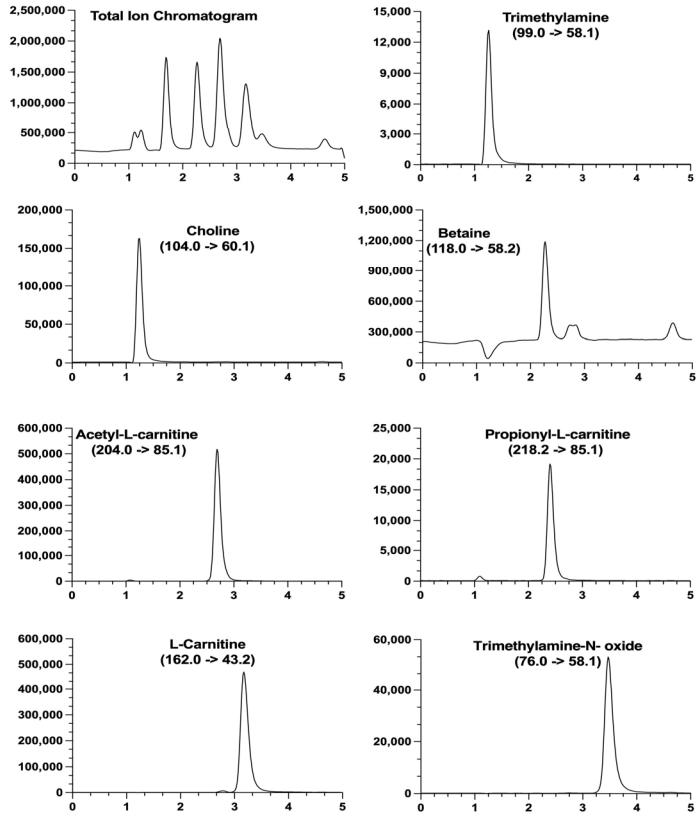


Figure 2. Typical total ion chromatogram and MRM spectrum (positive ionization mode) of methylamines in a plasma sample. The concentrations (µmol/L) were as follows: 0.5 for trimethylamine, 2.1 for trimethylamine-N-oxide, 9.7 for choline, 46.7 for betaine, 4.6 for acetyl-L-carnitine, 0.4 for propionyl-L-carnitine, and 37.4 for L-carnitine.

Mass transitions (Table 1) and ion source parameters were optimized using two product ions as quantifiers (the most abundant ion) and qualifiers (the second most abundant) for confirmation purposes. The optimized conditions resulted in a total run time of 6 min, with good sensitivity (Table 2) and reproducibility. The calibration curves of all metabolites were linear up to the tested level of 100 µmol/L ($R^2 > 0.9991$) (Table 2). The LOQs ranged from 0.06 µmol/L for L-carnitine to 0.24 µmol/L for propionyl-L-carnitine, which proved suitable for clinical samples. The average recovery of individual metabolites after standard addition (duplicate samples at each addition level, five levels, $n = 10$) ranged from 94% for choline to 114% for TMA (Table 3) and resulted in coefficients of determination R^2 above 0.9953 (Figure 3). The between-day variation (CV%, $n = 8$, duplicate samples at four consecutive days) for all metabolites in plasma samples was below 10%.

Table 2. Method quality control parameters of aqueous calibration curve, limit of detection, and limit of quantification ($n = 3$, three different occasions).

Metabolites	Calibration			Limits (µmol/L)	
	Slope	Intercept	R^2	LOD	LOQ
TMA	0.0591	−0.018	0.9996	0.06 ± 0.01	0.18 ± 0.02
TMAO	0.0855	0.0003	0.9997	0.03 ± 0.02	0.09 ± 0.08
Choline	0.0575	−0.0095	0.9999	0.03 ± 0.00	0.09 ± 0.01
Betaine	0.0769	−0.0192	0.9998	0.05 ± 0.03	0.1 ± 0.06
Acetyl-L-carnitine	0.0630	−0.0372	0.9991	0.08 ± 0.01	0.26 ± 0.02
L-carnitine	0.0604	0.0011	0.9999	0.02 ± 0.01	0.06 ± 0.02
Propionyl-L-carnitine	0.2505	−0.0969	0.9996	0.08 ± 0.03	0.24 ± 0.01

TMA: trimethylamine, TMAO: trimethylamine-N-oxide, R^2: coefficient of determination; LOD = limit of detection, LOQ = limit of quantification. LOD and LOQ were based on the residual standard deviation (R), and the slope (b) of the regression line using the following equation: LOD = (3.3 × SD)/b; LOQ = (10 × SD)/b.

Table 3. Concentrations of methylamines (µmol/L) in spiked and non-spiked plasma samples ($n = 2$).

Level of Addition (µmol/L)	Average Concentration (µmol/L)						Average Recovery (% ± SD)
		0	5	10	20	50	100	
TMA	C_{found}	0.4	6.4	11.7	19.5	64.1	113.0	
	Recovery (%)		118	113	95	128	112	114 ± 1
TMAO	C_{found}	1.9	7.3	11.9	18.9	52.9	103.6	
	Recovery (%)		105	99	85	101	101	95 ± 5
Choline	C_{found}	10.6	15.3	19.7	29.7	58.2	104.6	
	Recovery (%)		93	91	96	96	94	94 ± 2
Betaine	C_{found}	41.9	46.4	51.1	61.2	93.5	132.3	
	Recovery (%)		104	99	100	105	91	98 ± 5
Acetyl-L-carnitine	C_{found}	4.8	9.8	14.9	21.3	63.4	113.2	
	Recovery (%)		101	100	82	117	108	102 ± 3
L-carnitine	C_{found}	30.6	35.8	40.9	51.3	82.9	128.5	
	Recovery (%)		104	103	104	105	98	103 ± 3
Propionyl-L-carnitine	C_{found}	0.3	5.3	9.8	19.3	51.2	103	
	Recovery (%)		98.9	95.4	95.2	102	103	98 ± 8

Recovery trials were carried out using a non-fasted human plasma from a single donor stored in aliquots of 250–500 µL at −80 °C, with duplicate samples ($n = 2$) at each addition level (five levels). Variation between measured concentrations in duplicates was £ 10%. Recovery was calculated according to Equation (1).

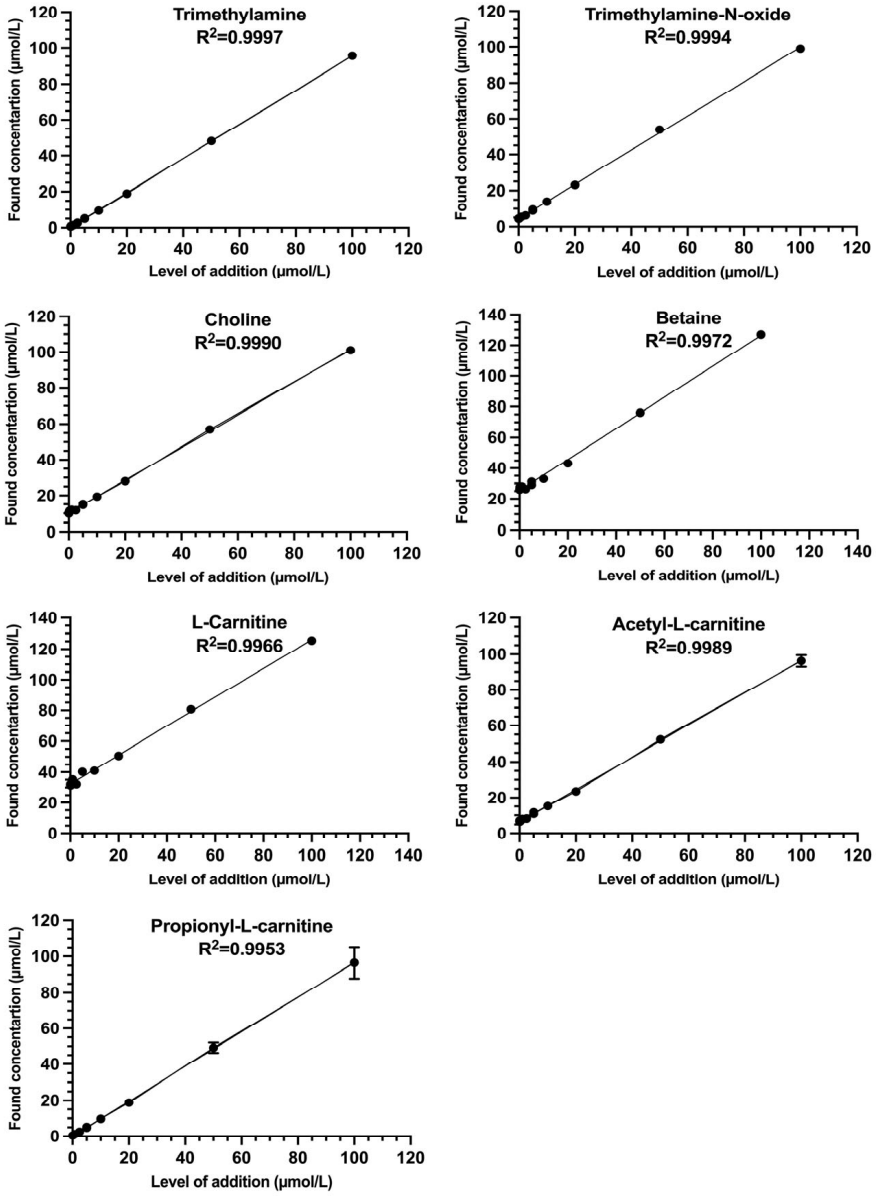


Figure 3. Coefficients of determination of added trimethylamine (TMA), trimethylamine-N-oxide (TMAO), choline, betaine, L-carnitine, acetyl-L-carnitine, and propionyl-L-carnitine to a plasma sample (n = 3). The intercept of linearity lines is not at zero due to baseline concentrations of methylamines in the unspiked sample (TMA 0.4, TMAO 1.9, choline 10.6, betaine 41.9, acetyl-L-carnitine 4.8, L-carnitine 30.6, and propionyl-L-carnitine 0.3 µmol/L).

3.3. Method Application

The method was successfully applied to determine methylamines in fasted plasma samples of subjects with (n = 12) and without (n = 21) metabolic syndrome. In all plasma samples, the metabolites trimethylamine N-oxide, trimethylamine, choline, betaine, and acetyl-, propionyl-, and L-carnitine were simultaneously quantified. TMA concentrations were the same in both groups; however, TMAO, propionyl-L-carnitine, and L-carnitine concentrations were higher in individuals with metabolic syndrome. Other metabolites did not differ between both groups (Table 4).

Table 4. Concentrations of methylamines (µmol/L) in plasma of 21 healthy subjects, collected on two occasions (n = 42), and 12 subjects with metabolic syndrome, collected on two occasions (n = 24).

Variable	Healthy (n = 21)	Metabolic Syndrome (n = 12)	Unpaired t-Test
Trimethylamine	1.6 ± 0.3	1.6 ± 0.3	ns
Trimethylamine N-oxide *	4.6 ± 6.3	9.5 ± 14.4	0.03
Choline	9.1 ± 1.9	9.4 ± 2.1	ns
Betaine	29.1 ± 6.5	30.2 ± 8.8	ns
Acetyl-L-carnitine	6.6 ± 2.2	6.8 ± 1.9	ns
L-Carnitine	30.4 ± 5.7	36.0 ± 6.7	0.0005
Propionyl-L-carnitine	0.27 ± 0.1	0.33 ± 0.1	0.02

ANOVA repeated measurement (mixed model) was used to analyze the individual variation across the two time-points and between the two groups. As no significant differences were observed within the groups over time, time was not considered in the model. Instead, an unpaired t-test was used to compare the groups. All variables were normally distributed except for TMAO; therefore, the data were log transferred. * TMAO values presented in the table show the untransformed and not the log-transformed data. ns, not significant.

4. Discussion

4.1. Analytical Findings

The method was partially validated in alignment with ICH guidelines [21], following approaches established in our previous work and other published studies [11–14,18]. This study emphasizes TMA extraction and detection, as the current literature mainly targeted its oxidized metabolite, TMAO (a hepatic flavin monooxygenase-derived metabolite), highlighting a gap in TMA-specific methodological optimization. Quantifying TMA, however, offers unique insights into gut microbial activity and host metabolic function and might enable the diagnosis of trimethylaminuria and therapeutic strategies to disrupt the TMA-TMAO axis. Challenges in TMA quantification are mainly due to difficulties in detection and here we demonstrate that the selection of extraction solvent significantly impacts TMA extractability. The low molecular weight of TMA led to poor fragmentation, with fragments appearing in a mass region filled with the interferences, which resulted in a noisy baseline and low sensitivity. We also observed that analysis of TMA without fragmentation (CE = 0) produced a weak signal at m/z = 60 ($C_3H_9N^+$ H); similar observations were reported by others [18]. Additionally, ACN can form adducts with ammonium ions producing a signal at m/z = 59, which may interfere with the TMA detection [20]. ACN can also be hydrolyzed to produce acetamide (mass 59.1) [20], which contributed to the high background signal observed when monitoring the molecular ion expected for TMA at m/z = 60.1. Therefore, derivatizing TMA is necessary to achieve reliable and accurate results. In this study, TMA was derivatized using IACN, which proved to be a suitable and fast reaction, as previously described [14]. However, we observed that the addition of formic acid was crucial to terminate the derivatization reaction, thus ensuring the samples' stability in the autosampler. The absence of formic acid resulted in the formation of a peak that shares the same retention time as derivatized TMA but possesses different qualifier ratios. Previously, we showed that the alkaline conditions employed during the derivatization maintained TMAO in an uncharged state, preventing any cross-reactions with TMAO or other methylamines [14].

Moreover, ensuring a high extractability of TMA was a challenging aspect of this study, as the sample extraction method affects the metabolite profile and data quality [22]. Indeed, during this study, we found that the use of the previously chosen extraction medium methanol/ACN (10:90) [14] resulted in lower TMA extractability. In contrast, using pure methanol significantly improved the extractability (up to two-fold increase) of TMA (Figure 1) and the reproducibility. This finding aligns with previous studies [22,23] and indicates a lower protein interference, a more comprehensive metabolite profile, and greater reproducibility when methanol was used as the extraction solvent compared to

ACN. Another contributing factor could be the higher polarity of methanol, a protic solvent that can form hydrogen bonds by donating protons (H+), which facilitates the dissolution and extractability of highly polar compounds (e.g., TMA). In contrast, ACN, an aprotic solvent, lacks the ability to form hydrogen bonds, relying mainly on dipole–dipole interactions, which may be less effective for extracting highly polar compounds compared to the hydrogen bonding mechanism facilitated by methanol.

The limit of quantification for all methylamines in the present study (ranging from 0.06 µmol/L for L-carnitine to 0.24 µmol/L for propionyl-L-carnitine) is comparable to those obtained by others using UPLC–MRM–MS [12,15]. This allows an accurate quantification of physiological concentrations of TMA, which exists in very low concentrations in plasma, as observed in this study. The quantification using MRM is highly selective and shows less interference from co-eluting compounds, which eliminates the need for complete chromatographic separation, whereas the use of a UPLC–HILIC column results in nearly complete separation of all metabolites in a short run time of 6 min. The use of labeled ISs ensured the precision of the quantification by correcting for variation from sample preparation, as well as instrumental detection [24].

The optimized extraction procedure (pure methanol, 10 min), derivatization (IACN, 10 min), and chromatographic conditions resulted in significant improvements in the quantification, particularly of TMA.

4.2. Method Application

In line with previous studies [25,26], we observed higher TMAO and L-carnitine concentrations in subjects with metabolic syndrome. Previous studies have also found a positive correlation between plasma TMAO and BMI in adults [4] and children [27]. Andraos et al. [28] reported a positive correlation between TMAO precursors and metabolic syndrome in children and adults, but notably not with TMAO itself. The increased levels of L-carnitine and particularly TMAO in subjects with metabolic syndrome were assumed to reflect underlying metabolic disturbances and an altered gut microbiota profile [29]. Further, the high L-carnitine concentrations observed in individuals with metabolic syndrome are well in line with data reported in other studies [26,28]. L-carnitine plays an important role in fatty acid metabolism by facilitating the transport of long-chain fatty acids into the mitochondria for β-oxidation; hence, L-carnitine supplement has been used in weight treatment [30].

There is also research to suggest that a diet high in L-carnitine is linked to increased body fat and negative health effects [26], which may, however, not be observed for L-carnitine supplements [26]. The relationship between the origin of dietary L-carnitine (from food versus supplement), plasma levels, and the condition of metabolic syndrome warrants further investigation.

To summarize, an MRM triple quadrupole mass spectrometry (UPLC–MRM–MS) method suitable for quantifying TMA, TMAO, betaine, choline, and acetyl-, propionyl- and L-carnitine was developed. The method involves a robust and fast extraction and derivatization step and was successfully applied to quantify TMAO and related methylamines in the plasma of both healthy subjects and subjects with metabolic syndrome.

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Abbreviations

The following abbreviations are used in this manuscript:

ACN	Acetonitrile
IACN	Iodoacetonitrile
IS	Internal Standards
LOD	Limit of Detection
LOQ	Limit of Quantification
R	Recovery
R^2	Coefficient of determination
SD	Standard Deviation
TMA	Trimethylamine
TMAO	Trimethylamine-N-oxide
UPLC–MRM–MS	Ultra Performance Liquid Chromatography–Multiple Reaction Monitoring–Mass Spectrometry

References

1. Wang, Z.; Klipfell, E.; Bennett, B.J.; Koeth, R.; Levison, B.S.; DuGar, B.; Feldstein, A.E.; Britt, E.B.; Fu, X.; Chung, Y.-M.; et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature* **2011**, *472*, 57–63. [CrossRef] [PubMed]
2. Qi, J.; You, T.; Li, J.; Pan, T.; Xiang, L.; Han, Y.; Zhu, L. Circulating trimethylamine N-oxide and the risk of cardiovascular diseases: A systematic review and meta-analysis of 11 prospective cohort studies. *J. Cell. Mol. Med.* **2018**, *22*, 185–194. [CrossRef] [PubMed]
3. Heianza, Y.; Ma, W.; Manson, J.E.; Rexrode, K.M.; Qi, L. Gut Microbiota Metabolites and Risk of Major Adverse Cardiovascular Disease Events and Death: A Systematic Review and Meta-Analysis of Prospective Studies. *J. Am. Heart Assoc.* **2017**, *6*, e004947. [CrossRef] [PubMed]
4. Barrea, L.; Annunziata, G.; Muscogiuri, G.; Di Somma, C.; Laudisio, D.; Maisto, M.; De Alteriis, G.; Tenore, G.C.; Colao, A.; Savastano, S. Trimethylamine-N-oxide (TMAO) as Novel Potential Biomarker of Early Predictors of Metabolic Syndrome. *Nutrients* **2018**, *10*, 1971. [CrossRef]
5. Wang, Z.; Tang, W.H.W.; O'Connell, T.; Garcia, E.; Jeyarajah, E.J.; Li, X.S.; Jia, X.; Weeks, T.L.; Hazen, S.L. Circulating trimethylamine N-oxide levels following fish or seafood consumption. *Eur. J. Nutr.* **2022**, *61*, 2357–2364. [CrossRef]
6. Janeiro, M.H.; Ramírez, M.J.; Milagro, F.I.; Martínez, J.A.; Solas, M. Implication of Trimethylamine N-Oxide (TMAO) in Disease: Potential Biomarker or New Therapeutic Target. *Nutrients* **2018**, *10*, 1398. [CrossRef]
7. Evans, M.; Dai, L.; Avesani, C.M.; Kublickiene, K.; Stenvinkel, P. The dietary source of trimethylamine N-oxide and clinical outcomes: An unexpected liaison. *Clin. Kidney J.* **2023**, *16*, 1804–1812. [CrossRef]
8. Craciun, S.; Balskus, E.P. Microbial conversion of choline to trimethylamine requires a glycyl radical enzyme. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 21307–21312. [CrossRef]

9. Koeth, R.A.; Levison, B.S.; Culley, M.K.; Buffa, J.A.; Wang, Z.; Gregory, J.C.; Org, E.; Wu, Y.; Li, L.; Smith, J.D.; et al. γ-Butyrobetaine Is a Proatherogenic Intermediate in Gut Microbial Metabolism of L-Carnitine to TMAO. *Cell Metab.* **2014**, *20*, 799–812. [CrossRef]
10. Messenger, J.; Clark, S.; Massick, S.; Bechtel, M. A review of trimethylaminuria: (fish odor syndrome). *J. Clin. Aesthet. Dermatol.* **2013**, *6*, 45–48.
11. Yu, W.B.; Xu, C.C.; Li, G.M.; Hong, W.P.; Zhou, Z.Y.; Xiao, C.X.; Zhao, Y.Q.; Cai, Y.F.; Huang, M.; Jin, J. Simultaneous determination of trimethylamine N-oxide, choline, betaine by UPLC-MS/MS in human plasma: An application in acute stroke patients. *J. Pharm. Biomed. Anal.* **2018**, *152*, 179–187. [CrossRef] [PubMed]
12. Zhao, X.Q.; Zeisel, S.H.; Zhang, S.C. Rapid LC-MRM-MS assay for simultaneous quantification of choline, betaine, trimethylamine, trimethylamine N-oxide, and creatinine in human plasma and urine. *Electrophoresis* **2015**, *36*, 2207–2214. [CrossRef] [PubMed]
13. Wang, Z.N.; Levison, B.S.; Hazen, J.E.; Donahue, L.; Li, X.M.; Hazen, S.L. Measurement of trimethylamine-N-oxide by stable isotope dilution liquid chromatography tandem mass spectrometry. *Anal. Biochem.* **2014**, *455*, 35–40. [CrossRef]
14. Hefni, M.E.; Bergstrom, M.; Lennqvist, T.; Fagerstrom, C.; Witthoft, C.M. Simultaneous quantification of trimethylamine N-oxide, trimethylamine, choline, betaine, creatinine, and propionyl-, acetyl-, and L-carnitine in clinical and food samples using HILIC-LC-MS. *Anal. Bioanal. Chem.* **2021**, *413*, 5349–5360. [CrossRef] [PubMed]
15. Xu, C.; Zhang, M.; Zhang, S.; Wang, P.; Lai, C.; Meng, D.; Chen, Z.; Yi, X.; Gao, X. Simultaneous determination of choline, L-carnitine, betaine, trimethylamine, trimethylamine N-oxide, and creatinine in plasma, liver, and feces of hyperlipidemic rats by UHPLC-MS/MS. *J. Chromatogr. B* **2024**, *1243*, 124210. [CrossRef] [PubMed]
16. Korytowska-Przybylska, N.; Michorowska, S.; Wyczałkowska-Tomasik, A.; Pączek, L.; Giebułtowicz, J. Development of a novel method for the simultaneous detection of trimethylamine N-oxide and creatinine in the saliva of patients with chronic kidney disease—Its utility in saliva as an alternative to blood. *J. Pharm. Biomed. Anal.* **2023**, *234*, 115519. [CrossRef]
17. Cho, C.E.; Taesuwan, S.; Malysheva, O.V.; Bender, E.; Tulchinsky, N.F.; Yan, J.; Sutter, J.L.; Caudill, M.A. Trimethylamine-N-oxide (TMAO) response to animal source foods varies among healthy young men and is influenced by their gut microbiota composition: A randomized controlled trial. *Mol. Nutr. Food Res.* **2017**, *61*, 1600324. [CrossRef]
18. Valdivia-Garcia, M.A.; Bi, Y.; Abaakil, K.; V Li, J. Derivatization to reduce background interferences for simultaneous quantitation of trimethylamine (TMA) and trimethylamine-N-oxide (TMAO) using liquid chromatography with tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2025**, *252*, 116480. [CrossRef]
19. Rochlani, Y.; Pothineni, N.V.; Kovelamudi, S.; Mehta, J.L. Metabolic syndrome: Pathophysiology, management, and modulation by natural compounds. *Ther. Adv. Cardiovasc. Dis.* **2017**, *11*, 215–225. [CrossRef]
20. McEntyre, C.J. Liquid Chromatography—Mass Spectrometry Methods for Investigating Osmolytes and Related One-Carbon Metabolites in Health and Disease. Ph.D. Thesis, University of Canterbury, Christchurch, New Zealand, 2016.
21. European Medicines Agency. *ICH Guideline M10 on Bioanalytical Method Validation and Study Sample Analysis*; European Medicines Agency: Amsterdam, The Netherlands, 2022.
22. Yang, Y.; Cruickshank, C.; Armstrong, M.; Mahaffey, S.; Reisdorph, R.; Reisdorph, N. New sample preparation approach for mass spectrometry-based profiling of plasma results in improved coverage of metabolome. *J. Chromatogr. A* **2013**, *1300*, 217–226. [CrossRef]
23. Lepoittevin, M.; Blancart-Remaury, Q.; Kerforne, T.; Pellerin, L.; Hauet, T.; Thuillier, R. Comparison between 5 extractions methods in either plasma or serum to determine the optimal extraction and matrix combination for human metabolomics. *Cell. Mol. Biol. Lett.* **2023**, *28*, 43. [CrossRef]
24. Wu, J.; Wiegand, R.; LoRusso, P.; Li, J. A stable isotope-labeled internal standard is essential for correcting for the interindividual variability in the recovery of lapatinib from cancer patient plasma in quantitative LC–MS/MS analysis. *J. Chromatogr. B* **2013**, *941*, 100–108. [CrossRef]
25. León-Mimila, P.; Villamil-Ramírez, H.; Li, X.S.; Shih, D.M.; Hui, S.T.; Ocampo-Medina, E.; López-Contreras, B.; Morán-Ramos, S.; Olivares-Arevalo, M.; Grandini-Rosales, P.; et al. Trimethylamine N-oxide levels are associated with NASH in obese subjects with type 2 diabetes. *Diabetes Metab.* **2021**, *47*, 101183. [CrossRef]
26. Gao, X.; Tian, Y.; Randell, E.; Zhou, H.; Sun, G. Unfavorable Associations Between Serum Trimethylamine N-Oxide and L-Carnitine Levels With Components of Metabolic Syndrome in the Newfoundland Population. *Front. Endocrinol.* **2019**, *10*, 168. [CrossRef]
27. Mihuta, M.S.; Paul, C.; Borlea, A.; Roi, C.M.; Pescari, D.; Velea-Barta, O.-A.; Mozos, I.; Stoian, D. Connections between serum Trimethylamine N-Oxide (TMAO), a gut-derived metabolite, and vascular biomarkers evaluating arterial stiffness and subclinical atherosclerosis in children with obesity. *Front. Endocrinol.* **2023**, *14*, 1253584. [CrossRef]
28. Andraos, S.; Jones, B.; Lange, K.; Clifford, S.A.; Thorstensen, E.B.; Kerr, J.A.; Wake, M.; Saffery, R.; Burgner, D.P.; O'Sullivan, J.M. Trimethylamine N-oxide (TMAO) is not Associated with Cardiometabolic Phenotypes and Inflammatory Markers in Children and Adults. *Curr. Dev. Nutr.* **2021**, *5*, nzaa179. [CrossRef]

29. Liu, Y.; Dai, M. Trimethylamine N-Oxide Generated by the Gut Microbiota Is Associated with Vascular Inflammation: New Insights into Atherosclerosis. *Mediat. Inflamm.* **2020**, *2020*, 4634172. [CrossRef]
30. Talenezhad, N.; Mohammadi, M.; Ramezani-Jolfaie, N.; Mozaffari-Khosravi, H.; Salehi-Abargouei, A. Effects of L-carnitine supplementation on weight loss and body composition: A systematic review and meta-analysis of 37 randomized controlled clinical trials with dose-response analysis. *Clin. Nutr. ESPEN* **2020**, *37*, 9–23. [CrossRef]

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