Simultaneous quantification of trimethylamine oxide and its precursors from gut microbial metabolic pathway

Using the QTRAP 4500MD system

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Trimethylamine N-oxide (TMAO), a gut microbiota-derived metabolite, is found in higher concentrations in plasma after ingestion of L-carnitine and phosphatidylcholine. Ingestion of choline, L-carnitine, betaine and foods such as red meat cause significant increase in trimethyl amine (TMA) due to the enzymatic activity of gut microbiota. TMA is absorbed into the bloodstream and metabolized by flavin-containing mono oxygenase (FMO) enzymes to trimethylamine oxide (TMAO) in the liver. TMAO has been implicated in the pathology of multiple diseases, including heart failure.[1]

Therefore, establishing a rapid and accurate quantitative method for routine monitoring of metabolites involved in TMAO production is needed to support gut microbiome research and screening of hundreds samples from a large research cohorts.

Here, an LC-MS/MS method for the simultaneous quantification of acetyl-carnitine, carnitine, choline, TMAO and betaine in plasma has been developed. All target compounds were extracted using a simple sample extraction procedure then diluted before analysis using LC-MS/MS.[2]

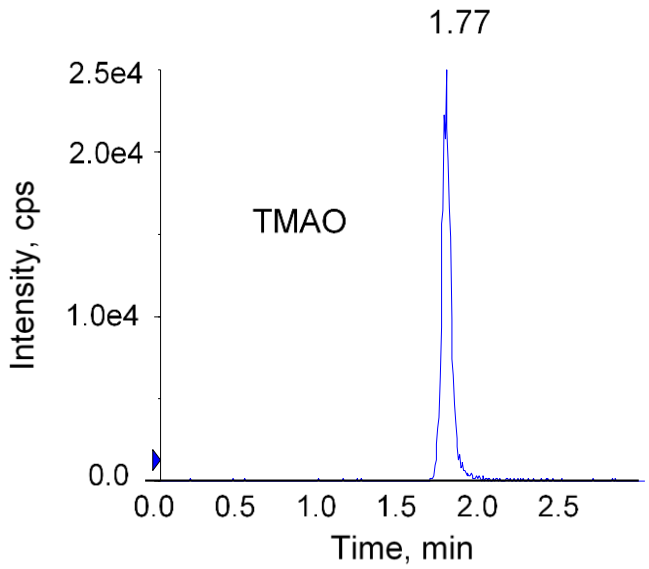

Figure 1. Fast chromatography. A chromatographic peak of trimethylamine N-oxide Q1/Q3 76.1/58.1 using a rapid 5.5 min method for quantification.

Key features of QTRAP 4500MD system for gut microbiome studies

- Robust LC-MS/MS system for routine quantification studies with the Turbo V ion source and Curtain Gas interface
- Simplified plasma sample preparation, using a one-step extraction and dilution
- Fast, reversed-phase chromatography provides good separation of the target analytes
- High accuracy and precision values for all analytes monitored, meeting bioanalytical requirements.

Methods

Sample preparation: A 20 µL plasma sample was extracted with 100 µL of acetonitrile and further spiked with 100 µL of a 5 ng/mL deuterated internal standard mixture. The sample mixture was centrifuged at 14000 rpm for 5 min, then 5 µL of the supernatant was injected on the column for analysis. The intra-day and inter-day variability over 5 days of the assay was evaluated.

Chromatography: A Jasper HPLC system (SCIEX) with a Phenomenex Kinetex C18, 2.6 µm, 2.1x100 mm column (00D-4462-AN) was used for sample separation using gradient conditions (Table 1) and a flow rate of 0.6 mL/min. A 5 µL injection volume was used and the column temperature was maintained at 40°C throughout the analysis. The total run time was 5.5 min.

Mass spectrometry: A QTRAP 4500MD system with a Turbo V ion source was used in positive ionization mode. All source and compound parameters were optimized and are outlined in the SCIEX How method.[3] The MS method included optimized MRM transitions for multiple analytes.

Data processing: The method development and data acquisition was carried out using Analyst MD software. Data processing was performed using MultiQuant MD software.

Table 1. Fast gradient conditions.

Time	Mobile phase A	Mobile phase B
0	*10*	*90*
3.5	*50*	*50*
3.6	*10*	*90*
5.5	*10*	*90*

Mobile phase A – water with 0.1% formic acid in 10 mM ammonium formate.
Mobile phase B - 90% acetonitrile with 0.1% formic acid in 10 mM ammonium formate

Rapid analysis of TMAO and related species

First, phosphate buffered saline (PBS) was used to dilute the standards to make a standard calibration curve and obtain a first rough assessment of sensitivity. The concentration range evaluated and the linearity of the calibration curves is outlined in Table 2 along with the observed LLOQ concentrations for TMAO, betaine, L-carnitine, acetyl-carnitine, and choline. All analytes showed good gaussian peak shape (Figure 2).

Triplicate injections of TMAO, betaine, L-carnitine, acetyl-carnitine and choline were each analyzed at different concentrations and across 5 days, as outlined in Table 3. The intra-day reproducibility was analyzed across triplicate injections from a single day. The accuracy for each metabolite ranged from 89.87% to 112.03% and the %RSD ranged from 1.81% to

Table 2. Concentration curves. Compounds were diluted with phosphate buffered saline (PBS) to create standard concentration (ng/mL) curves for each analyte. Good linearity was achieved for each of the standards. Observed lower limits of quantification in buffer are highlighted in bold, using standard bioanalytical requirements of <20% CV and ±20% accuracy.

Series	Acetyl-carnitine	Carnitine	Betaine	Choline	TMAO
STD1	***0.03***	***0.05***	***0.05***	***0.05***	***0.13***
STD2	*0.05*	*0.1*	*0.1*	*0.1*	*0.25*
STD3	*0.1*	*0.2*	*0.2*	*0.2*	*0.5*
STD4	*0.25*	*0.5*	*0.5*	*0.5*	*1.25*
STD5	*1.25*	*2.5*	*2.5*	*2.5*	*6.25*
STD6	*2.5*	*5*	*5*	*5*	*12.5*
STD7	*5*	*10*	*10*	*10*	*25*
STD8	*10*	*20*	*20*	*20*	*20*
Linear equation	*Y=1.54803x-0.009*	*Y=1.43472x+0.04162*	*Y=0.63080X+0.00622*	*Y=0.93725X-0.00116*	*Y=9.55724x-0.54357*
Correlation coefficient	*0.9999*	*0.99118*	*0.99713*	*0.99987*	*0.99792*

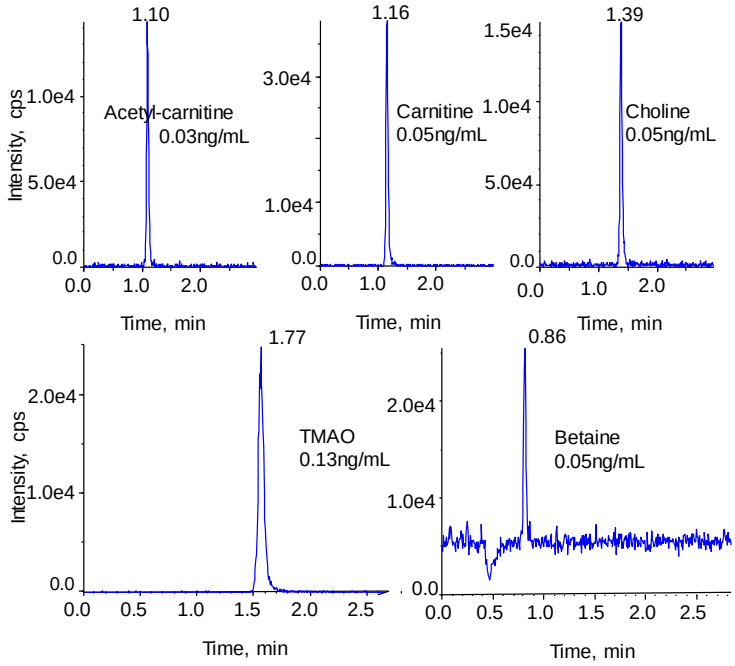

Figure 2. Lower limits of quantification (LLOQ) for acetyl-carnitine, L-carnitine, choline, TMAO and betaine. Good peak shape and signal/noise were observed at the LLOQ for each analyte in PBS matrix.

8.87%. The inter-day reproducibility was measured by comparing results from triplicate injections across days. The inter-day accuracy ranged from 92.93% to 107.15% and the %RSD ranged from 1.77% to 8.01%. The LLOQs observed for each analyte are marked in bold in Table 2. Standard bioanalytical requirements were followed.

Next, this rapid LC-MS method was tested in plasma. A simple extraction protocol was used, which provided good detection and good signal/noise of TMAO and its precursors analytes in plasma (Figure 3).

Table 3. Quantification results. Inter-day and intra-day results for the analysis of acetyl-carnitine, carnitine, choline, TMAO and betaine in PBS. Precision and accuracy results for a few of the low concentrations points are shown.

Compound Name	Theoretical concentration	Inter-day			Intra-day		
	ng/mL	Mean (ng/mL)	RSD%	Accuracy%	Mean (ng/mL)	RSD%	Accuracy%
Acetyl-carnitine	*0.08*	*0.08*	*4.88%*	*95.97%*	*0.078*	*4.29*	*97.32*
	0.15	*0.15*	*6.44%*	*96.96%*	*0.141*	*4.91*	*94.33*
	7.5	*7.36*	*7.01%*	*98.14%*	*6.74*	*3.16*	*89.87*
Carnitine	*0.15*	*0.16*	*8.01%*	*103.33%*	*0.143*	*7.89*	*95.57*
	0.3	*0.31*	*6.78%*	*103.22%*	*0.291*	*8.28*	*97.03*
	15	*13.94*	*1.77%*	*92.93%*	*13.971*	*1.97*	*93.14*
Choline	*0.15*	*0.16*	*4.53%*	*107.15%*	*0.166*	*3.45*	*110.55*
	0.3	*0.32*	*5.07%*	*105.63%*	*0.336*	*2.04*	*112.03*
	15	*14.91*	*2.88%*	*99.40%*	*14.509*	*2.3*	*96.73*
TMAO	*0.38*	*0.38*	*5.18%*	*100.35%*	*0.402*	*2.93*	*105.9*
	0.75	*0.71*	*3.53%*	*94.71%*	*0.716*	*3.75*	*95.52*
	37.5	*37.06*	*6.91%*	*98.83%*	*35.023*	*1.81*	*93.4*
Betaine	*0.15*	*0.15*	*7.89%*	*101.26%*	*0.15*	*8.87*	*100.04*
	0.3	*0.32*	*5.45%*	*106.87%*	*0.327*	*2.27*	*108.99*
	15	*14.14*	*3.95%*	*94.26%*	*13.788*	*4.11*	*91.92*

Conclusions

Here, a method for the rapid quantification of TMAO, betaine, L-carnitine, acetyl-carnitine and choline in plasma has been developed on the QTRAP 4500MD system. This method has the advantages of high specificity, linearity and high accuracy. As shown, this method is suitable to support rapid monitoring of TMAO metabolites:

- Rapid 5.5-minute run time provided good peak separation for detection in a shorter run time than several recently published methods for faster screening.[4]
- Consistent quantification results were obtained for all standards tested, with correlation coefficients ranging from 0.991 to 0.999
- Inter- and intra-day reproducibility and accuracy meet standard bioanalytical requirements of %CV less than ≤ 15% in for all analytes analyzed in plasma
- Method was evaluated in plasma matrix using a simple extraction protocol and good signal/noise was observed.

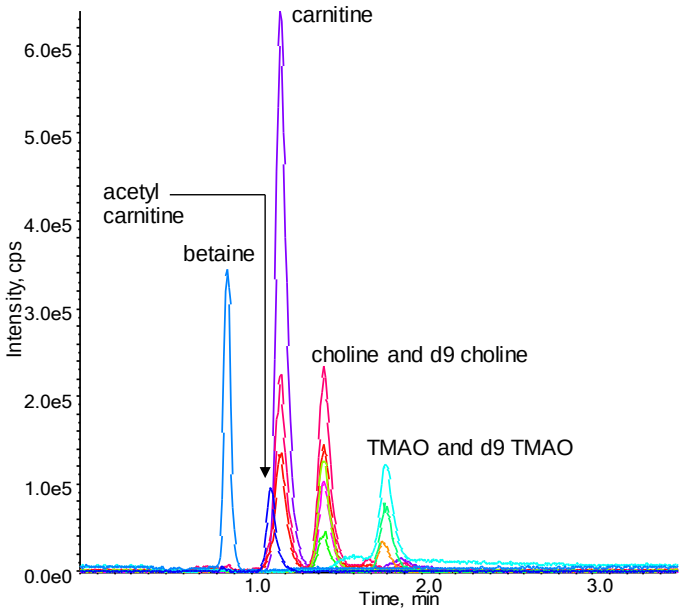

Figure 3. Detection in plasma. The extracted ion chromatograms (XIC) show the separation of TMAO and its precursors in 3.5 min run time from extracted from a plasma sample.

References

1. The gut, the heart, and TMAO. Cleveland clinic website.
2. Velasquez MT, et al. (2016) Trimethylamine N-oxide: The good, the bad and the unknown. *Toxins* **8**, 326.
3. SCIEX How link
4. *Katrina et al.,* (2021) A simplified LC-MS/MS method for the quantification of the cardiovascular disease biomarker trimethylamine-N-oxide and its precursors. *J. Pharma. Anal.* **11,** 523-528.

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