Full Title: High mass accuracy assay for trimethylamine *N*-oxide using stable-isotope dilution with liquid chromatography coupled to orthogonal acceleration time of flight mass spectrometry with multiple reaction monitoring

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Abstract.

Background: Trimethylamine *N*-oxide (TMAO) has attracted interest as circulating levels have reported prognostic value in patients with cardiovascular conditions, such as heart failure. With continual advances in accurate mass measurements, robust methods that can employ the capabilities of time of flight mass spectrometers would offer additional utility in the analysis of complex clinical samples.

Methods: A Waters Acquity UPLC was coupled to a Waters Synapt G2-S high resolution mass spectrometer. TMAO was measured in plasma by stable-isotope dilution-hydrophilic interaction liquid chromatography-time of flight mass spectrometry with multiple reaction monitoring (LC-ToF-MRM). Two transitions were monitored; *m/z* 76.1 to 58.066/59.073 and *m/z* 85.1 to 66.116/68.130. The method was assessed for linearity, lower limits of detection and quantitation, and reproducibility. A selected cohort of patients with systolic heart failure (SHF; n=43) and healthy controls (n=42) were measured to verify the assay is suitable for the analysis of clinical samples.

Results: Quantitative analysis of TMAO using LC-ToF-MRM enabled linearity to be established between 0.1 and 75 μmol\L, with a lower limit of detection of 0.05 μmol\L. Relative standard deviations reported an inter-day variation of ≤ 20.8% and intra-day variation at ≤ 11.4% with intra-study quality control variation of 2.7%. Run times were 2.5 min. Clinical application of the method reported that TMAO in SHF was elevated compared to healthy controls (p<0.0005).

Conclusion: LC-ToF-MRM offers a highly selective method for accurate mass measurement of TMAO with rapid and reproducible results. Applicability of the method was shown in a selected cohort of patient samples.

Keywords: TMAO; high resolution mass spectrometry; multiple reaction monitoring; time of flight; heart failure; LC-MS/MS

Introduction

Trimethylamine *N*-oxide (TMAO) is a downstream metabolite of carnitine and choline, driven by intestinal microbiota, which has engendered considerable research effort as a new biomarker for a variety of cardiovascular diseases [1-3]. It is also being investigated in other diseases where the aetiology is thought to be related to a perturbance of the gut microbiome, including colorectal cancer [4], diabetes [5] and renal disease [6] as well as being a putative central mediator in cholesterol balance [7]. Described methods for detecting TMAO are centred on the use of liquid chromatography-tandem mass spectrometry with multiple reaction monitoring (LC-MS/MS-MRM), often utilising a stable isotopically labelled standard for quantitation [e.g. 8-10].

Clinical laboratories are increasingly reliant on LC-MS platforms which offer sensitive and robust solutions to analysis [11-13]. The measurement of small molecules and peptides is primarily conducted using triple quadrupole mass spectrometers because of the levels of sensitivity achieved, wide dynamic range (6-7 orders of magnitude) and the relatively high throughput [14-16]. LC-MS/MS-MRM on triple quadrupoles is increasingly being seen as a gold standard for measurement of molecules in clinical laboratories [17]. Whilst limitations exist with these approaches [18], the selectivity and wide dynamic range leads to a high level of sensitivity which has benefitted many different fields. For many analytes, sufficient selectivity is delivered by triple quadrupole instruments. However, in certain circumstances improvements in selectivity are necessary. For example, recent developments in the analysis of common clinical analytes, such as the metabolites of vitamin D, demonstrate that improved levels of selectivity would be advantageous [19].

[Orthogonal acceleration] Time of flight ([oa]ToF) mass spectrometers are able to measure target ions with high mass resolution. Instruments such as the Waters Synapt series are able to increase sensitivities through the utilisation of a 100 % ToF duty cycle, a process known as target enhancement. Instead of using a continuous beam of ions, as would be typical in ToF configurations, a travelling waveform is used in order to establish a series of packets which each undergo a short ToF

separation as they travel through an ion guide, known as the transfer cell. The [oa]ToF pusher is then synchronised with the arrival of ion packets having the targeted *m/z* [20].

The assay described herein has incorporated hydrophilic interaction liquid chromatography (HILIC) which is particularly suitable for polar molecules [21]. An emphasis was put on having high throughput with an ambition of developing a rapid, reproducible method for clinical investigations. To our knowledge, there are currently no routine clinical methods for biomarker quantitation that utilise the high accuracy produced by LC-ToF analyses and thus, we sought to investigate whether LC-ToF-MRM could be used to establish a high throughput assay for TMAO measurement in clinical samples.

Materials and Methods

Materials

TMAO (98.9 % purity) was purchased from Sigma-Aldrich (Gillingham, UK) and its labelled isotope, D_9-TMAO (≥ 98 % purity, 99.9 % enrichment), was purchased from Cambridge Isotopes (Tewksbury, MA, USA). Water, acetonitrile, methanol (MeOH), formic acid (HCOOH) (all Optima™ LC-MS grade) and 25 % extra pure ammonium hydroxide (NH_4OH) in H_2O (Acros Organics) were purchased from Fisher Scientific (Loughborough, UK). Stripped plasma (4 x charcoal, EDTA pooled gender: product code HMPLEDTA2-STRPD-HEV-53432) was purchased from Seralab (Haywards Heath, UK).

Sample Preparation

Blood samples were collected from an antecubital vein into collection tubes containing ethylenediaminetetraacetic acid (Sarstedt, Nümbrecht, Germany). After collection, plasma was immediately separated by centrifugation at 1500 x g for 15 min, transferred to aliquots and stored at -80 °C until analysis. Sample preparation was done according to described methods [9] using stable-isotope dilution by mixing 20 µL of plasma with 80 µL of 10 µmol\L D_9-TMAO in MeOH. Protein precipitation was achieved by a 1 min vortex period followed by centrifugation at 16900 x g for 20 min. After centrifugation, the supernatant was removed and transferred to a vial for analysis. All blood samples used in this manuscript were drawn from individuals who had given written informed consent and, where applicable, study protocols complied with the declaration of Helsinki and were approved by the local ethics committee.

Sample Analysis

Samples were analysed by liquid chromatography-tandem mass spectrometry with multiple reaction monitoring using a ToF mass analyser. LC-ToF-MRM was performed in positive ion electrospray ionisation mode using an Acquity I-class UPLC (Waters Corp., Milford, MA, USA) coupled to a Synapt G2-S high resolution mass spectrometer (Waters Corp., Milford, MA, USA). Optimised mass spectrometer source settings can be found in Table 1. An Acquity UPLC BEH HILIC column (130 Å, 1.7 µm, 2.1 mm x 10 mm, Waters Corp., Milford, MA, USA) with an Acquity UPLC BEH HILIC VanGuard pre-column (130 Å, 1.7 µm, 2.1 mm x 5 mm, Waters Corp., Milford, MA, USA) was used. Buffer A was 0.025 % NH_4OH, 0.045 % HCOOH (pH 8.1), with buffer B as pure acetonitrile. An injection volume of 5 µL and a flow of 600 µL\min were used with a column temperature of 50 °C. The gradient started with 95 % B at 0 min linearly reducing to 4 % B at 0.8 min and returning to 95 % B at 1.9 min with a total analysis time of 2.5 min. MRM was performed by pre-filtering the precursor ions using the quadrupole mass analyser for *m/z* values of 76.1 (TMAO) and 85.1 (D_9-TMAO) and supplying a collision voltage ramp in the transfer cell of 10 to 20 V and 10 to 25 V, respectively. The ToF analyser is able to achieve greater specificity via high mass accuracy and therefore transition fragments of *m/z* 58.066 & 59.073 (TMAO) and 66.116 & 68.130 (D_9-TMAO) were monitored (Figure 1). Confirmation of analyte was achieved by mass spectra and retention time (Figure 2). Peak areas for the most abundant fragment were calculated using QuanLynx (Waters Corp., Milford, MA, USA) and results were reported as a response ratio of TMAO to D_9-TMAO and converted to concentration in µmol\L.

<INSERT TABLE 1 NEAR HERE>

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Calibration, Recovery and Matrix Interferences

Calibration experiments were created using 9 concentrations of TMAO ranging from 0 to 75 µmol\L. TMAO-free plasma was obtained by gel filtration (Sephadex® G-25, Sigma-Aldrich, Gillingham, UK) of charcoal stripped plasma. TMAO was spiked into the resultant eluent to form concentrations of 0, 0.05, 0.1, 0.5, 2, 10, 25, 50 and 75 µmol\L. Each of the neat calibration solutions was diluted with 4 parts D_9-TMAO in MeOH to simulate the extraction process, vortexed for 1 min, centrifuged and transferred to a LC vial for analysis. Injections were performed in triplicate using analysis methods as described. Lower limit of quantitation (LLOQ) was determined with an analyte response of at least 5 times the response compared to a blank and a reproducibility of ≤ 20 %, with lower limit of detection (LLOD) determined as a response of at least 3 times that of the blank with a reproducibility of > 20 % [22]. All samples were preceded by a blank injection (80 % MeOH) to ensure carryover was not present.

In order to assess inter-day variation, calibrations were performed once daily, on five consecutive days. Additionally, to assess intra-day variation, a total of five calibrations were completed within one 24 hour period. Response ratios were calculated for TMAO to D_9-TMAO, variations were assessed with relative standard deviations (RSDs) and a linear fit was calculated using least squares regression.

Recovery and matrix interferences were assessed by performing two additional calibration analyses according to guidelines by Matuszewski et al [23]. One set of calibrations was performed with TMAO initially dissolved in water, at the concentrations stated previously, and the other with TMAO spiked into the extracted solution of the TMAO-free plasma. Each one of these additional calibrations was completed alongside the main calibration, with set order rotation, over the five consecutive days.

Matrix effect (ME), recovery of the extraction procedure (RE) and overall process efficiency (PE) were calculated according to the equations detailed by Matuszewski et al [23]. Further standard addition experiments were performed to assess the recovery of spiked TMAO in to the pre-treated (stripped plasma without gel filtration) and untreated (unmodified plasma from a healthy volunteer) matrices. Triplicate analyses were performed on the neat matrix and a series of 100 mL aliquots of each matrix spiked with 1, 2, 3, 4 or 5 μL of 1 mmol\L TMAO stock solution. Each aliquot was mixed thoroughly and extracted using the described method. Signal variations were calculated by comparison of the reported and expected TMAO values.

Reproducibility of Extraction

In order to confirm that the extraction method is reproducible, plasma samples from ten individuals, a mixture of healthy and heart disease patients across the normal working range, were extracted using the above process. Each plasma sample was extracted three times into separate microcentrifuge tubes. All extractions were run in triplicate, in a randomised order, using the described method and RSDs were calculated for the measured response ratios of analyte to internal standard across all 9 injections for each individual.

Assay Imprecision

Assay imprecision was analysed following guidelines found in the NCCLS EP5-A document [24]. Three plasma samples previously observed to contain low (1.5 μmol\L), medium (5 μmol\L) and high (40 μmol\L) levels of TMAO were analysed in duplicate, twice daily for a total of twenty consecutive days. Estimates were calculated for within-run and total precision.

Clinical Application

With the aim of demonstrating the assay's applicability to measure clinical samples, eighty five samples were analysed from two groups of participants, one containing individuals diagnosed with systolic heart failure (SHF; n =43, 56 % male) and the other with apparently healthy controls free from cardiovascular disease (CON; n = 42, 52 % male). Group differences were analysed using the Mann-Whitney U test for non-parametric data. All statistical analyses were performed using IBM SPSS Statistics (v22.0.0.1, IBM). All data are expressed as mean ± standard deviation unless otherwise defined. Healthy participant quality control (QC) extracts ([TMAO] = 3.2 μmol\L) were injected after every eight duplicate sample analyses.

Results

Calibration, Recovery and Matrix Interferences

None of the blank injections showed peaks corresponding to TMAO or D_9-TMAO, which demonstrated zero carryover. All calibration experiments spiked in TMAO-free plasma produced a correlation coefficient (r^2) of ≥ 0.998. No TMAO was detected in the 0 μmol\L standard, confirming removal of TMAO through gel filtration. Although a weak signal for TMAO was obtained in the 0.05 μmol\L standard, it was deemed as below the limit of quantitation; all other concentrations reported a TMAO signal. Inter-day RSDs were ≤ 20.8 %, with intra-day RSDs of ≤ 11.4 %. Variation for multiple injections ranged from 2.4 to 21.3 % across concentrations, with 0.1 μmol\L determined as the LLOQ, and 0.05 μmol\L as the LLOD. A complete list of RSDs, with accompanying 95 % confidence intervals, is shown in Table 2.

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Comparison of the three sets of calibration experiments reported a mean ME of 98 %. Mean RE was calculated to be 107 % and mean PE was 103 %. Table 3 displays a summary of ME, RE and PE for each individual concentration point. Recovery of TMAO in the extraction of pre-treated and untreated matrices, as assessed by standard addition experiments, reported mean recovery values of 102 and 96 %, respectively (Table S1).

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Reproducibility of Extraction

Calculated TMAO plasma concentrations for the ten individuals ranged from 1.9 to 25.4 μmol\L. The RSDs for the triplicate extractions ranged from 1.4 to 6.6 %, demonstrating a reproducible extraction method across a range of plasma concentrations. A summary of results for all plasma samples can be seen in Table 4.

<INSERT TABLE 4 NEAR HERE>

Assay Imprecision

According to the calculations presented in the NCCLS EP5-A document [24], assay estimates for within-run precision were 6.6, 3.3 and 1.9 %, with total precision calculated at 7.2, 4.4 and 2.8 % for low, medium and high TMAO level plasma, respectively.

Clinical Samples

Samples of patients with SHF showed increased levels of TMAO in plasma compared to healthy controls ($p < 0.0005$), with the median (interquartile range) as 9.0 (4.2 – 14.4) and 4.0 (3.1 – 5.0) μmol\L, respectively (Figure 3). There were no differences in age between groups (72.7 ± 9.3 vs 72.9 ± 4.9, SHF v CON, $p = 0.83$), nullifying the possible increase in TMAO due to the previously reported positive association with aging [9]. The RSD for repeated QC injections was 2.7 % (95 % confidence intervals; 2.3 – 3.5 %) and the mean RSD for all sample replicate injections was 2.5 %.

<INSERT FIGURE 3 NEAR HERE>

Discussion

The measurement of TMAO using an isotopically labelled standard in conjunction with a LC-high mass accuracy ToF-MRM assay provides a suitably sensitive assay which is linear, precise and accurate. In the protocol described in the present study, target enhancement is utilised to improve the overall sensitivity. This capability enhances duty cycle by synchronising the timing of the product ions of interest with the ToF pusher. The assay greatly benefits by having a much enhanced signal with little detriment to noise.

To our knowledge, we describe the first example of a clinical assay utilising a Synapt generation ToF-MRM assay for a compound that can provide clinical prognostic value to a variety of diseases [1-3, 25]. The assay is linear over nearly 3 orders of magnitude, has a limit of detection of 0.05 μmol\L and has a total injection-to-injection run time of 3 min. We have shown the ability to measure TMAO in human clinical samples with excellent levels of precision and reproducibility (sub 3 %). Median inter- and intra-day reproducibility lay below 4 %. Importantly, we demonstrated a clinical measurement range for this molecule of between 2 and 55 μmol\L (see Figure 3), and within this range the assay reported RSDs of 2.5 – 5.0 and 1.0 – 3.5 % for inter- and intra-day variation, respectively. These data demonstrate the utility of the described assay for the analysis of clinical samples. The sample preparation is minimal, demonstrably repeatable, very amenable to automation and takes less than 30 min from thaw to injection. With ToF instruments there is a compromise between absolute sensitivity and resolution. This assay utilised a half-way setting which enabled good sensitivity at 20K resolution (full width height maximum). Thus, settings could be used that achieve either greater sensitivity (at the expense of resolution) or greater resolution (at the expense of sensitivity).

As clinical diagnostic services evolve, LC-MS platforms are envisioned to constitute a major part of the diagnostic provision. Next steps for this transition will include the need for high mass accuracy instrumentation to overcome interferences that can affect the accuracy of measurement with

some nominal mass MRM systems [18, 26-27]. Initial exploration of this technology has already been done in toxicology laboratories [28]. It is envisaged that, with the perpetuating interest in the gut microbiome and health, research interest in TMAO and other gut-related analytes will increase (e.g. betaine, carnitine and acylcarnitines [29]). ToF-MRM assays have the inherent capability to be multiplexed and thus have incorporated into a single analysis these related analytes and their respective isotopically labelled standards for parallel quantitative measurement. High level selectivity that may be required to delineate some species in multiplex assays, those displaying extremely similar retention time/mass-to-charge profiles, is benefited by employment of the high resolution and high mass accuracy that is afforded by new generation Q-ToF or Q-Orbitrap instruments [30-32]. Furthermore with the Synapt series of mass spectrometers, travelling wave ion mobility technology offers another method of separation which has been shown to afford clinical utility in a qualitative [33] and quantitative [34] manner.

As a proof of concept, we applied the method to a selected set of clinical samples in order to evaluate TMAO levels in patients with SHF, compared to healthy controls free from cardiovascular disease. The sample sets were age- and sex-matched. The results plotted in Figure 4 indicate that, although there is overlap in sample distribution, individuals suffering from systolic HF generally report higher levels of TMAO. The variation seen across the two populations may be present due to a diverse range of HF severity and potential confounding variables that could produce elevated levels in some healthy individuals (e.g. diet, renal function). When comparing with reported TMAO concentrations, healthy control participants showed similar median values to previous healthy cohorts [2,6]. Patients suffering from SHF reported elevated levels to other HF cohorts [2,3], but similar to those suffering from chronic kidney disease [6]. This result, although interesting, is derived from a limited patient population but demonstrates the clinical applicability of the described method.

ToF-MRM offers high levels of selectivity and is suitable for highly reproducible analysis of TMAO in clinical samples. The uptake of this technology within clinical laboratories is achievable due to its reliability, selectivity, high-throughput, reproducibility, mass accuracy, precision and sensitivity that can be achieved.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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Table 1. Optimised mass spectrometer source conditions for positive ion mode electrospray ionisation of trimethylamine *N*-oxide.

Source Settings	
Capillary Voltage (kV)	0.5
Cone Voltage (V)	15
Source Offset	40
Temperatures (°C)	
Source	150
Desolvation Gas	650
Gas Flows & Pressures	
Cone Gas (L\hr)	250
Desolvation Gas (L\hr)	900
Nebuliser Gas Pressure (bar)	7

Table 2. Relative standard deviations calculated from repeated calibration analyses detailing inter-day, intra-day and total variance at all calibration points.

	RSD (95 CI, %)		
[TMAO] (µmol\L)	**Inter-day**	**Intra-day**	**All Injections**
0.1	20.8 (13.5 - 32.1)	11.4 (7.4 - 17.6)	21.3 (17.6 - 22.5)
0.5	5.8 (3.8 - 9.0)	5.4 (3.5 - 8.2)	8.9 (7.3 - 9.4)
2	5.0 (3.2 - 7.6)	3.5 (2.2 - 5.3)	5.7 (4.7 - 6.0)
10	3.3 (2.1 - 5.1)	1.0 (0.7 - 1.6)	3.6 (3.0 - 3.8)
25	2.5 (1.6 - 3.9)	2.5 (1.6 - 3.9)	2.9 (2.4 - 3.0)
50	3.0 (1.9 - 4.6)	1.8 (1.2 - 2.8)	3.1 (2.6 - 3.3)
75	1.4 (0.9 - 2.2)	1.4 (0.9 - 2.2)	2.4 (2.0 - 2.5)

Inter-day: calculated for 5 experiments conducted over 5 consecutive days

Intra-day: calculated for 5 experiments conducted within a period of 24 hours

All injections: calculated using all 30 repeated injections taken from the inter- and intra-day experiments

Note: 95 CI = 95 % confidence intervals; TMAO = trimethylamine *N*-oxide; RSD = relative standard deviation

Table 3. Matrix effect (ME), recovery (RE) and process efficiency (PE) for each calibration point calculated using repeated experiments for TMAO spiked into water (Neat), plasma prior to extraction (Spike EX_{PRE}) and plasma after extraction (Spike EX_{POST}). Adapted from Matuszewski et al. (20).

	Mean Response Ratio (TMAO to D_9-TMAO)					
[TMAO] (µmol\L)	**Neat**	**Spike EX_{PRE}**	**Spike EX_{POST}**	**ME (%)**	**RE (%)**	**PE (%)**
0.1	0.0003	0.0004	0.0002	77%	167%	129%
0.5	0.0011	0.0013	0.0013	115%	100%	115%
2	0.0053	0.0052	0.0060	113%	87%	98%
10	0.0299	0.0277	0.0269	90%	103%	93%
25	0.0814	0.0779	0.0812	100%	96%	96%
50	0.1763	0.1677	0.1699	96%	99%	95%
75	0.2790	0.2585	0.2556	92%	101%	93%
			Mean	98%	107%	103%

Note: D_9-TMAO = deuterated trimethylamine *N*-oxide; TMAO = trimethylamine *N*-oxide

Table 4. Relative standard deviations calculated for triplicate extractions of ten plasma samples across a working range of TMAO concentrations.

Plasma Sample	Plasma [TMAO] (µmol/L)	Mean Response Ratio	SD	RSD (CI) (%)
A	1.9	0.0036	0.0002	5.9 (4.2 – 10.1)
B	2.5	0.0066	0.0001	1.4 (1.0 – 2.4)
C	3.2	0.0096	0.0003	3.4 (2.5 – 5.9)
D	5.6	0.0212	0.0009	3.9 (2.8 – 6.7)
E	6.9	0.0274	0.0007	2.5 (1.8 – 4.2)
F	7.2	0.0287	0.0020	6.6 (4.7 – 11.3)
G	11.1	0.0479	0.0019	3.7 (2.7 – 6.4)
H	17.0	0.0760	0.0021	2.5 (1.8 – 4.4)
I	18.5	0.0833	0.0036	4.0 (2.9 – 6.9)
J	25.4	0.1164	0.0027	2.2 (1.6 – 3.8)

Mean response ratio and SD were calculated using all 9 injections provided by 3 technical replicates in each of 3 biological replicates

Note: CI = 95 % confidence intervals; RSD = relative standard deviation; SD = standard deviation; TMAO = trimethylamine *N*-oxide

Figure Captions

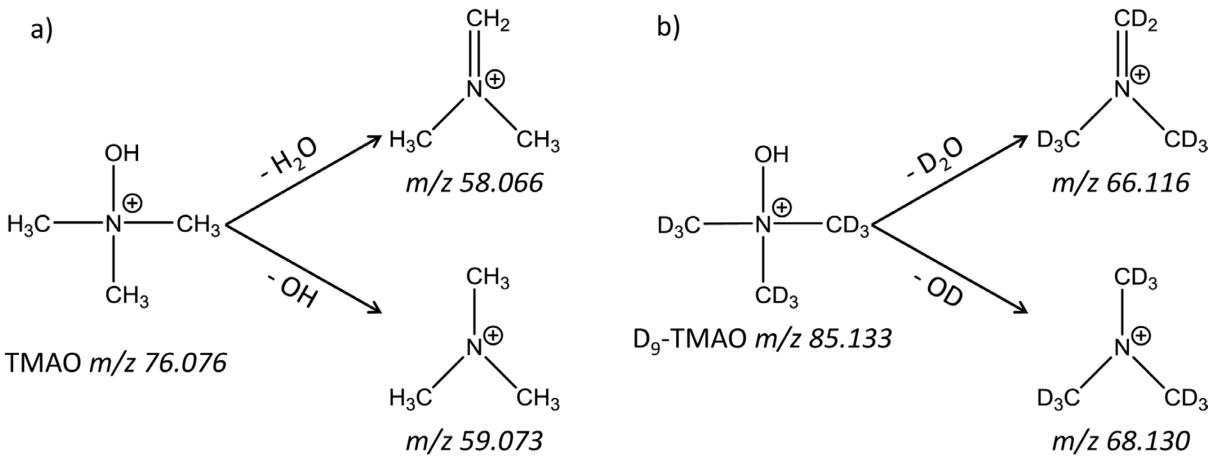
Fig. 1 Precursor and product ion formations for a) trimethylamine *N*-oxide (TMAO) and b) its deuterated stable isotope (D_9-TMAO) with corresponding mass-to-charge ratios (*m/z*)

Fig. 2 Example unsmoothed extracted ion chromatograms from stable-isotope dilution-liquid chromatography-time of flight mass spectrometry with multiple reaction monitoring of a) trimethylamine *N*-oxide and c) its deuterated stable isotope (D_9-TMAO) with corresponding peak mass spectra b) and d), respectively. Data obtained from a pooled plasma sample of heart failure patients with an estimated TMAO concentration of 7.7 μmol\L

Note: *m/z* denotes mass-to-charge ratio

Fig. 3 Individual value plots with median (dashed line) to show trimethylamine *N*-oxide concentrations in human plasma from a select cohort of patients suffering from systolic heart failure and healthy age-matched controls

Note: * denotes $p < 0.0005$



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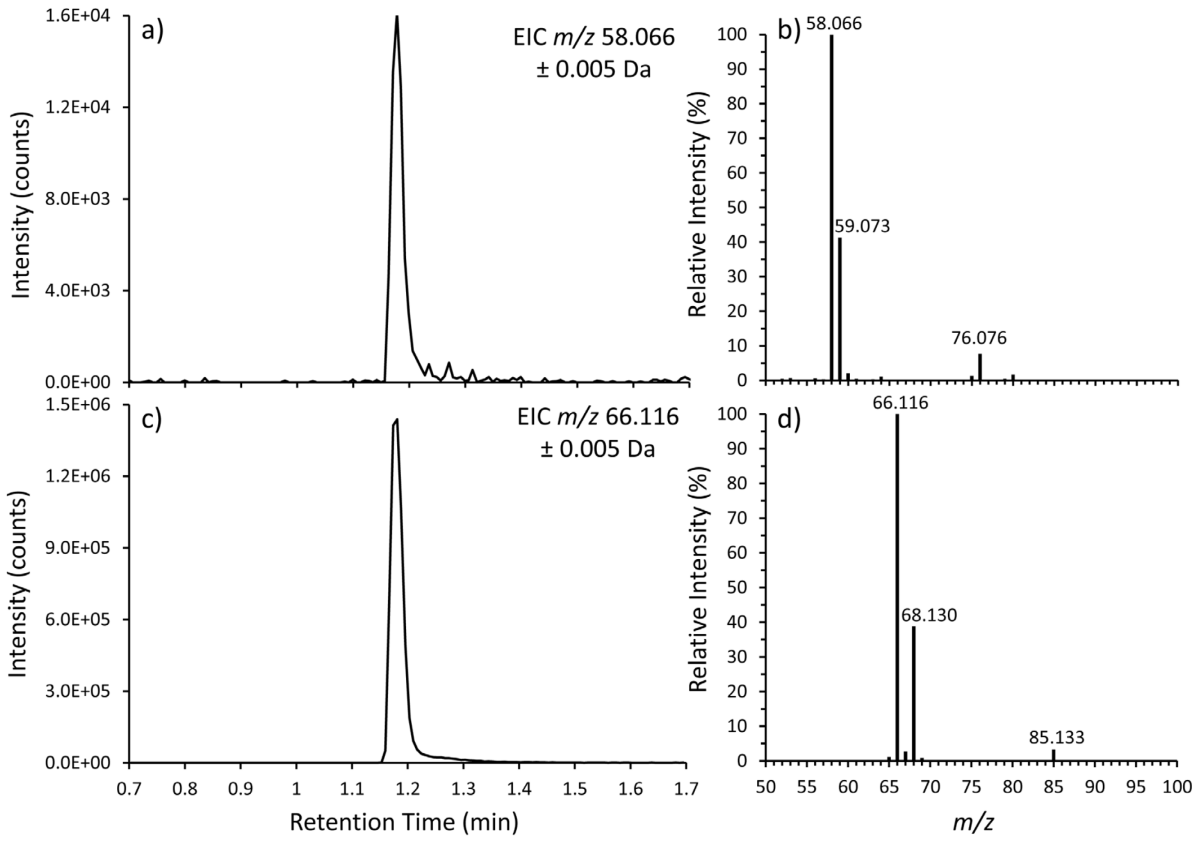

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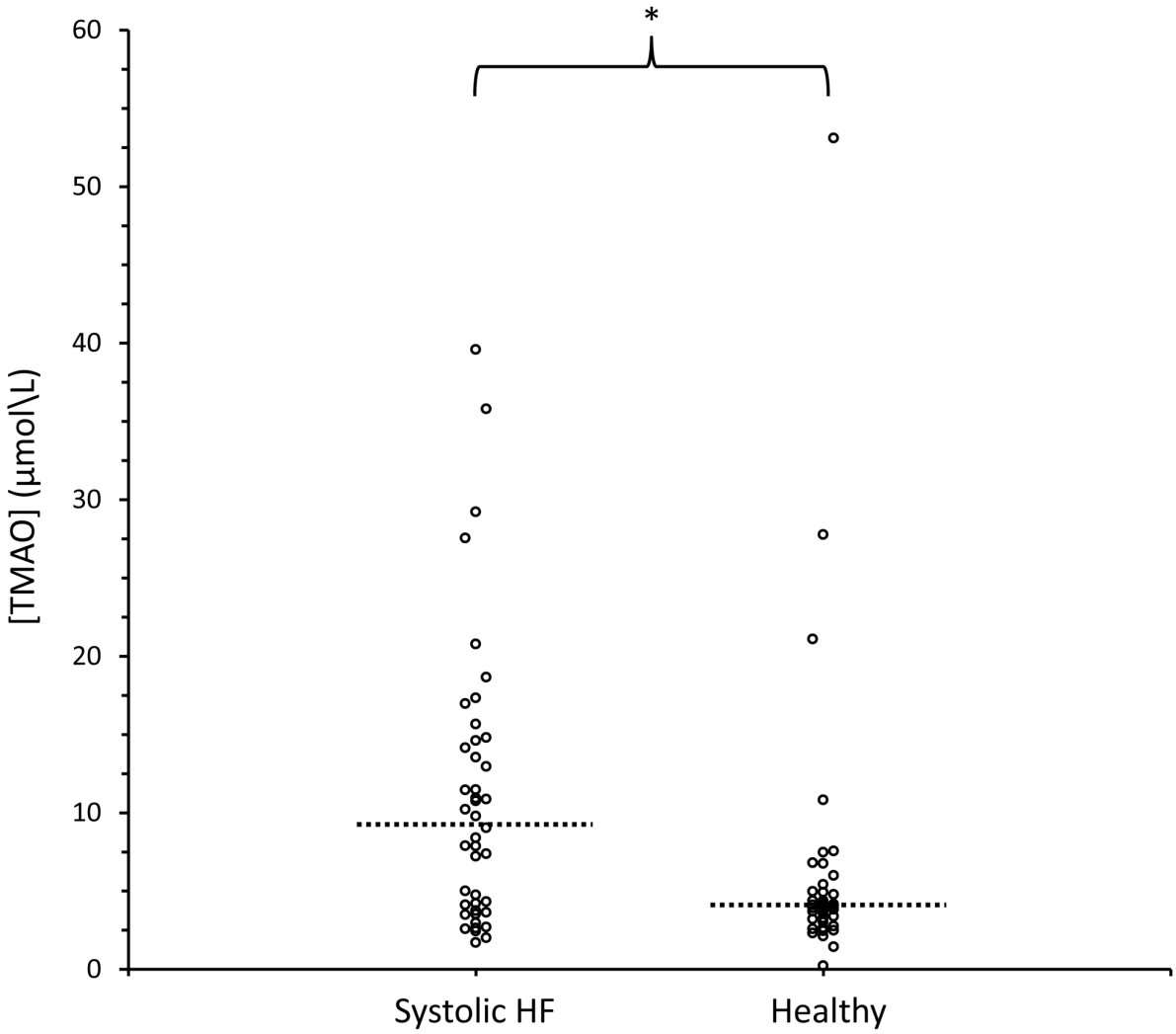

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