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RESEARCH ARTICLE

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Targeted ultra-performance liquid chromatography/tandem mass spectrometric quantification of methylated amines and selected amino acids in biofluids

Tim Roggensack | Benedikt Merz | Niels Dick | Achim Bub | Ralf Krüger

Department of Physiology and Biochemistry of Nutrition, Max Rubner-Institut, Karlsruhe, Germany

Correspondence
R. Krüger, Department of Physiology and Biochemistry of Nutrition, Max Rubner-Institut, Haid-und-Neu-Str. 9, 76131 Karlsruhe, Germany.
Email: ralf.krueger@mri.bund.de

Present address
Tim Roggensack, Department of Safety and Quality of Milk and Fish Products, Max Rubner-Institut, Kiel, Germany.

Rationale: Methylated amino compounds and basic amino acids are important analyte classes with high relevance in nutrition, physical activity and physiology. Reliable and easy quantification methods covering a variety of metabolites in body fluids are a prerequisite for efficient investigations in the field of food and nutrition.

Methods: Targeted ultra-performance liquid chromatography/tandem mass spectrometric (UHPLC/MS) analysis was performed using HILIC separation and timed ESI-MRM detection, combined with a short sample preparation. Calibration in urine and blood plasma was achieved by matrix-matched standards, isotope-labelled internal standards and standard addition. The method was fully validated and the performance was evaluated using a subset from the Karlsruhe Metabolomics and Nutrition (KarMeN) study.

Results: Within this method, a total of 30 compounds could be quantified simultaneously in a short run of 9 min in both body fluids. This covers a variety of free amino compounds which are present in very different concentrations. The method is easy, precise and robust, and has a broad working range. As a proof of principle, literature-based associations of certain metabolites with dietary intake of respective foods were clearly confirmed in the KarMeN subset.

Conclusions: Overall, the method turned out to be well suited for application in nutrition studies, as shown for the example of food intake biomarkers in KarMeN. Application to a variety of questions such as food-related effects or physical activity will support future studies in the context of nutrition and health.

1 | INTRODUCTION

Accurate and specific quantification of metabolites in body fluids is a key prerequisite for studies investigating for example the nutritional and physiological aspects of food consumption as well as physical activity. Besides emerging untargeted metabolomic approaches[1-3] there is still an urgent need for reliable targeted approaches with focus on specific compounds or compound classes. For instance, compounds with low mass and high polarity, such as methylated amino compounds, may not always be covered by generalized metabolite profiling methods. One approach to overcome poor retention in conventional reversed-phase (RP) chromatography is the use of hydrophilic interaction chromatography (HILIC) for targeted multi-component liquid chromatography/mass spectrometry (LC/MS) analysis.[4,5]

Several methylamines were found to be associated with the consumption of specific foods, including trigonelline for coffee,[6] stachydrine (proline-betaine) for citrus fruits[7], trimethylamine-*N*-oxide

(TMAO) for fish,[8-12] or histidine-containing compounds (*N*-methylhistidine isomers, dipeptides anserine and carnosine) for meat.[11,13-17] In the context of physical activity, it has been reported that certain compounds are increased after heavy exercise, such as β-aminoisobutyric acid,[18] or correlate inversely with fitness (e.g. asymmetric *N,N*-dimethylarginine, ADMA).[19] It is also known from epidemiological studies that specific methylated amines are associated with the risk for cardiovascular diseases, for instance ADMA[20,21] or TMAO.[22-25] TMAO is also a striking example of the recently recognized importance of the gut microbiota for food-related health issues.[26,27] In summary, this compound class is of high relevance and an interesting target for all studies related to nutrition, physical activity, and health.

A variety of methods for targeted amine analysis has been reported, mostly applying LC/MS. For instance, the analysis of betaines was described previously, e.g. fruit-derived proline-betaines.[7,28] Glycine-betaine is often analyzed in combination with other nitrogen-containing compounds such as choline, *N,N*-dimethylglycine (DMG), trimethylamine (TMA) or TMAO.[29-34] Quantification of methylated arginine compounds such as ADMA is established in a clinical context.[35-38] Dipeptides and methylhistidines were described recently as putative markers of meat intake.[13-15,39-41] However, methods integrating a high number of these compounds into one validated quantitative method are scarce.

Here, we report the evaluation and validation of an easy and robust quantitative ultra-performance liquid chromatography/tandem mass spectrometric (UPLC/MS/MS) method covering 30 methylated amines and basic amino acids present in human urine and blood plasma. Suitability for the method was proved by analyzing a subset of urine samples from the Karlsruhe Metabolomics and Nutrition (KarMeN) study.[42] Based on the above-mentioned literature, we choose the food groups coffee, citrus fruits, and meat for a proof of principle for the quantified metabolites. The validated method enables reliable quantification of methylated amines and is currently being applied in several human studies.

2 | EXPERIMENTAL

2.1 | Materials and standards

Acetonitrile (LCMS grade) was obtained from VWR (Darmstadt, Germany). Ammonium formate (HPLC grade) and formic acid (LC/MS grade) and most amine standards were from Sigma-Aldrich (Steinheim, Germany). Deuterated internal standards (ISTDs) were either from Eurisо-Top (Saarbrücken, Germany), EQ Laboratories (Augsburg, Germany) or TRC (Toronto, Canada), supplied by Biozol (Eching, Germany).

2.2 | LC/MS instrumentation and method

For compound quantification, an Acquity UPLC H-Class system coupled to a Xevo TQD triple quadrupole mass spectrometer (Waters, Eschborn, Germany) was used. A previously described HILIC/ESI-MRM method comprising six methylated amines[33,34,43] was extended to cover 30 amine compounds in one short run, and was adapted to the body fluids, urine and blood plasma.

A polar HILIC column (Acquity BEH Amide, Waters) with the following dimensions was used for separation: 100 × 2.1 mm, 1.7 μm particle size. The column oven was maintained at 30°C and the autosampler at 12°C. The injection volume was 1 μL at a flow rate of 0.6 mL/min using a solvent delay of 1 min. Eluent A was a 1:1 mixture of acetonitrile and aqueous 50 mM ammonium formate, which was adjusted to pH 3.2 with formic acid (FA). Eluent B was acetonitrile with 0.05% FA. The following inverse acetonitrile gradient was used: from initial 12% A to 51.5% A in 3 min, then isocratic until 4 min, then to 85% A at 5.9 min, and back to 12% A. The duty cycle was 9 min.

Mass spectrometric detection was performed with electrospray ionization (ESI) in positive ion mode using a capillary voltage of 2.0 kV. The desolvation temperature and gas flow rate were set to 500°C and 1000 L/h. The source temperature and cone gas flow rate were 150°C and 50 L/h. Analyte-specific quantification was performed by multiple reaction monitoring (MRM) with time windows and two transitions per analyte, where possible. Isotope-labelled compounds were used as internal standards. Details of the MRM method are provided in Table 1. TargetLynx (Waters) and Microsoft Excel were used for data processing using the Apex Track algorithm for peak integration. Peaks were smoothed by mean (1–2 times, smoothing width 2) and the calibration was quadratic without weighting.

2.3 | Sample preparation and matrix calibration

Stock solutions of standards and their isotope-labelled counterparts were prepared at 1 mg/mL in 50% acetonitrile, and 90% acetonitrile was used for further dilution. The analytes were sorted into four groups with different calibration range, adapted to the expected concentration range in the respective matrix. Samples were prepared by protein precipitation and dilution with eluent A, either by a factor 1:10 for plasma or a factor 1:25 for urine. The ISTD-mixture contained 17 compounds (dimethylamine (DMA)-d6, TMAO-d9, sarcosine-d3, α-aminoisobutyric acid-d3, DMG-d6, choline-d9, creatinine-d3, betaine-d3, proline-d7, creatine-d3, trigonelline-d3, histidine-^{13}C6, carnitine-d9, π-methylhistidine-d3, citrulline-d7, arginine-^{13}C6-^{15}N4, ADMA-d6) at three different levels (50/100, 250 or 500 μM depending on the calibration range of the respective compound) and was spiked directly to the precipitation solution.

Matrix-matched calibrators (6 levels) and controls (3 levels) were prepared by adding 10 μL of each of the four analyte mixtures with different concentration range directly to the human matrix sample, and subsequent dilution as described above. Aliquots of the identical sample were used as calibrators and controls and spiked accordingly. Standard addition was performed to quantify the original analyte concentrations of this specific matrix sample. This calculation was performed for each analysis sequence to account for variations in

TABLE 1 Compound table for urine and plasma analysis

	Compound	RT	ISTD	Precursor ion	Products		Area ratio	Cone	Collision energy		Calibration group[§]	
					1	2			1	2	Urine	Plasma
		[min]	#	[*m/z*]	[*m/z*]	[*m/z*]	2/1	[V]	[eV]	[eV]	#	#
1	Dimethylamine	1.74	2	46.0	30.0	31.0	0.83	45	30	28	B	C
2	Dimethylamine-d6	1.76	n.d.	52.1	34.0			45	30		I2	I3
3	Trimethylamine	1.59	2	60.0	44.0	45.0	1.14	40	20	16	D	C
4	TMAO	1.51	5	76.0	58.1	59.1	0.98	35	15	15	A	C
5	TMAO-d9	1.53	n.d.	85.1	68.1			15	15		I1	I3
6	Sarcosine	3.29	7	90.0	44.0			18	10		C	D
7	Sarcosine-d3	3.29	n.d.	93.0	47.1			18	10		I3	I3
8	*N,N*-Dimethylglycine	2.92	14	104.0	58.1			28	12		C	C
9	α-Aminobutyric acid	3.20	13	104.0	58.0			20	10		D	B
10	Choline	1.59	15	104.0	44.9	60.0	1.75	42	16	16	C	C
11	β-Aminoisobutyric acid	2.71	13	104.1	30.1	86.0	0.92	32	10	10	C	C
12	γ-Aminobutyric acid	2.63	13	104.2	69.1	87.0	8.42	25	11	11	D	D
13	β-Aminoisobutyric acid-d3	2.76	n.d.	107.1	29.9			32	10		I2	I2
14	Dimethylglycine-d6	2.94	n.d.	110.0	64.1			28	12		I3	I3
15	Choline-d9	1.58	n.d.	113.1	69.1			42	16		I3	I3
16	Creatinine*	1.50	18	114.1	44.1	86.1	5.50	40	12	12	None*	A
17	Proline	3.00	21	116.0	70.2			32	13		C	A
18	Creatinine-d3*	1.49	n.d.	117.0	47.0			40	12		None*	I1
19	Betaine	2.60	20	118.0	58.1	59.1	2.92	38	16	16	C	B
20	Betaine-d3	2.62	n.d.	121.0	62.2			38	16		I3	I2
21	Proline-d7	3.02	n.d.	122.9	76.9			32	13		I3	I1
22	*N*-Methylproline	2.66	21	130.2	84.1			32	14		D	B
23	Hydroxyproline	3.52	21	132.1	67.9	86.1	1.94	40	16	14	C	B
24	Creatine	3.16	25	132.2	44.2	90.1	1.01	32	18	14	B	B
25	Creatine-d3	3.19	n.d.	135.1	92.9			32	14		I2	I2
26	Trigonelline	2.78	27	138.2	78.0	92.1	2.93	32	26	24	B	D
27	Trigonelline-d3	2.79	n.d.	141.1	97.0			32	24		I2	I3
28	Stachydrine	2.57	27	144.1	58.1	84.0	0.79	40	24	20	B	D
29	γ-Butyrobetaine	1.92	36	146.1	60.0	87.1	1.57	45	12	16	D	C
30	Histidine	4.64	32	156.0	94.8	109.9	15.48	25	14	12	A	A
31	Betonicine	2.94	27	160.1	58.1			40	24		D	D
32	Histidine-^{13}C6	4.65	n.d.	162.0	115.0			33			I1	I1
33	Carnitine	2.41	36	162.0	60.0	102.8	1.13	36	16	16	B	B
34	π-Methylhistidine	4.60	37	170.1	95.9	108.9	1.11	32	14	14	B	B
35	τ-Methylhistidine	4.26	37	170.1	108.9	124.2	4.45	32	14	14	B	C
36	Carnitine-d9	2.41	n.d.	171.1	102.8			36	16		I2	I2
37	τ-Methylhistidine-d3	4.27	n.d.	173.0	127.2			32	14		I2	I2
38	Arginine	4.60	41	175.1	70.0	115.9	0.34	32	18	14	C	A
39	Citrulline	4.06	40	176.2	70.0	112.9	0.58	20	16	12	C	B
40	Citrulline-d7	4.06	n.d.	183.0	120.1			20	12		I2	I2
41	Arginine-^{13}C6-^{15}N4	4.62	n.d.	185.1	75.1			32	14		I3	I2
42	*N*-Methylarginine	4.28	41	189.2	70.1	116.2	0.27	32	19	15	D	C
43	ADMA	4.13	45	203.0	88.1	116.1	0.91	32	16	14	B	D

(Continues)

TABLE 1 (Continued)

	Compound	RT	ISTD	Precursor ion	Products		Area ratio	Cone	Collision energy		Calibration group[§]	
					1	2			1	2	Urine	Plasma
		[min]	#	[*m/z*]	[*m/z*]	[*m/z*]	2/1	[V]	[eV]	[eV]	#	#
44	SDMA	4.08	45	203.0	88.1	116.1	2.48	32	16	14	B	D
45	ADMA-d6	4.09	n.d.	209.2	94.2			32	14		I2	I3
46	Carnosine	4.72	32	227.1	110.0	156.1	0.51	32	14	16	C	D
47	Anserine	4.67	32	241.1	109.0	170.1	0.42	40	18	16	B	D

[*]Quantified in plasma only, intensity in urine too high.
[§]Spike range/maximum spike of calibration standards [μM]: Urine: 5000 (A), 1000 (B), 200 (C), 40 (D); Plasma = 500 (A), 200 (B), 100 (C), 40 (D); Internal STD: 50/100 (I1), 250 (I2), 500 (I3).
Time windows were at least 0.7 min per compound. Dwell times were calculated automatically by the instrument software using the following conditions: peak width 4 s; required points per peak 12. Both transitions were used for calculation of quantitative values, if applicable.

sample preparation and instrument response. With this information, a second data processing run was performed, now using the real concentrations of the calibrators (matrix background + spike). These calibration curves were then used for subsequent quantification of unknown samples.

2.4 | Method evaluation

Precision and accuracy (bias with respect to the calculated value) were tested using spiked matrix samples as described in section 2.3 (3 levels, each n = 3). Within-day precision was determined batch-wise (n = 12), and controls of regular sample sequences were used to determine day-to-day precision.

The recovery and matrix effect were estimated in one experiment (2 levels, each n = 3; ISTDs 1 level, n = 3). Matrix samples were either spiked before or after sample preparation to calculate recoveries. These matrix samples were run together with matrix-free standards of identical nominal concentration to calculate matrix effects. Thus, matrix effects were determined by comparing normal LC/MS runs with and without matrix, rather than by infusion experiments. The recovery and matrix effects were determined for standards and internal standards separately at different concentrations. In the case of non-labelled standards, a matrix blank was used for background subtraction.

Since analyte-free samples were not accessible, working ranges were assessed by using surrogate matrices and subsequent dilutions (n = 3). The surrogate urine matrix was prepared as follows: 20 mM ammonium formate, 208 mM urea, 1.5 mM ureic acid, 25 mM potassium hydrogen phosphate and 75 mM sodium chloride.[44] Surrogate blood plasma was prepared by dissolving 7% bovine serum albumin in PBS buffer. Lower limits of quantification (LLOQs) and limits of detection (LODs) were also estimated from these linearity experiments using the following criteria: LLOQ: signal-to-noise ration (S/N) >10, coefficient of variance (CV) <30%, deviation from linear regression curve <30%; LOD: S/N >3. The sum of matrix background plus highest spike (= real concentration of highest matrix calibrator) was defined as the upper limit of quantification (ULOQ).

Stability was determined by storing control samples (3 levels, n = 3 each) at various conditions. The following storage conditions were tested: 2 months at −20°C, 1 week at 6–8°C, 6 h at room temperature (RT), and after 3 freeze–thaw cycles (−20°C and RT).

2.5 | Human study samples

A detailed description of the KarMeN study can be found elsewhere.[42] The study is registered at the German Clinical Trials Register (number DRKS00004890). Briefly, 301 female and male participants from age 18 to 80 were included. The aim of the study was to investigate associations of the human metabolome (plasma/serum and urine) with age, sex, diet, body composition and physical activity. During the study, numerous parameters were assessed, for instance current food consumption, body composition, medical history, physical activity and cardiorespiratory fitness. Several urine and one blood samples were collected at the study center and analyzed by clinical biochemistry and a variety of metabolomics methods, including ^{1}H-NMR, LC/MS and GC×GC/MS.[43] Kidney function was estimated by measuring glomerular filtration rate (GFR, mL/min/1.73 m^2) based on 24-h urine creatinine clearance. Urinary metabolite excretion was expressed in mmol/24 h.

2.6 | Dietary assessment

Trained study personnel assessed the food intake of each individual (in g/day) in a personal interview using a 24-h dietary recall with the software EPIC-Soft.[45,46] Participants used standard units (such as slice of bread, soup bowl), household measurements (such as tablespoon) and a picture booklet providing photographs of portion sizes for various foods to indicate the consumed amount per meal. Based on

the literature, we selected a subset of 69 participants from this study group with either a high consumption of coffee, citrus fruits, fish and seafood, or total meat (including sausages and meat products), also stratified into white and red meat[11,13-17] or a negligible consumption of the respective foods. Thus, a comparison of metabolite concentrations between consumers and non-consumers or high and low consumption, respectively, could be performed for selected foods. General characteristics and dietary intake of the KarMeN subset are given in File S1, supporting information.

2.7 | Statistical evaluation

As a proof of principle, partial Spearman correlation analysis adjusted for GFR was used to investigate the associations between urinary metabolite concentrations and the food groups of interest. All statistical analyses were performed using SAS software version 9.4 (SAS Institute, Cary, NC, USA) with p-values <0.05 considered as statistically significant. To correct for multiple testing, the false discovery rate (FDR) was controlled at 0.05 using the Benjamini-Hochberg method.[47]

3 | RESULTS AND DISCUSSION

3.1 | Method development

In a first step, more than 40 amino compounds of interest in the context of nutrition and physiology were chosen and tested for possible integration into the new method. Non-methylated and partially N-methylated analogues of fully N-methylated amines were included wherever possible, e.g. in the case of the amino acids (hydroxy)proline, histidine or arginine. Moreover, aminobutyric acids were included as interesting targets with similar properties (e.g. polarity, basicity). Electrospray source conditions as well as fragmentation parameters were optimized by syringe infusion and a short, previously established acetonitrile gradient on the HILIC column.[33,34,43] Most compounds showed strong ESI signals and efficient fragmentation. However, a few compounds gave low signal intensity and/or were not stable under the selected HILIC conditions without derivatization, for instance phosphorylated compounds (phosphocholine, phosphocreatine), polyamines (putrescine, spermine), ornithine and taurine. However, none of the affected analytes was essential for our studies, and the goal was to make the method as easy as possible. In the end, a panel of about 30 analytes with the highest priority was selected. In principle, this panel could be further extended, since a number of additional analytes with good sensitivity was identified, including acetylcarnitine (as an example of the acylcarnitines), spermidine (as an example for amines) and additional amino acids such as valine, serine or tryptophane. A list of included analytes is available in Table 1.

Two MRM transitions were applied wherever possible and optimized, except for the few compounds which showed only one intense product ion. Both transitions were used for quantification in order to maximize peak intensities. Nevertheless, transition intensity ratios (see Table 1) may still be used to monitor peak purity and to account for possible interferences, as in the classical quantifier/qualifier approach. Therefore, we have investigated ratio variance in urine and plasma and compared it with ratios in pure standards. Overall, we found consistent intensity ratios, irrespective of matrix or concentration. The ratio variation was usually below 15%, and often below 5%. Only a few compounds showed higher ratio variance especially at very low concentrations (e.g. hydroxyproline, γ-aminobutyric acid, TMA, anserine), pointing to minor interferences for those compounds.

Since we had also isomers and compounds with identical precursor mass and very similar fragmentation, the gradient had to be optimized to separate critical pairs, for instance the methylhistidines (m/z 170), the aminobutyric acids and dimethylglycine (m/z 104), and the dimethylarginines (m/z 203). Resulting separations using the final gradient are shown in Figure 1 and File S2, supporting information.

It is known that for HILIC somewhat longer equilibration times are necessary than for RP chromatography. However, we found that 2.5 min equilibration time was sufficient to guarantee stable retention times for the next injection, leading to a total duty cycle of 9 min between two injections. Based on this final gradient, an MRM method with time windows was established, in order to optimize detection efficiency. The parameters were set to give at least 12 points per peak with 4 s peak width, leading to dwell times between 8 and 12 ms.

In the next step, we tested the influence of the matrix (urine, plasma) on analytical performance. Samples were diluted with organic solvent, which eventually leads to protein precipitation especially in the case of plasma samples. After centrifugation, samples could be directly injected without the need for further sample cleanup. However, it turned out that it was crucial to dissolve the samples in a solvent resembling the starting conditions of the gradient as closely as possible to maintain stable retention times and peak shape. The effect was more pronounced in the case of urine, obviously caused by the higher salt content and pH variations. In contrast, plasma matrix is always quite similar and its pH is highly regulated.

In further experiments, possible interferences from matrix as well from concomitant standard compounds were tested, including the isotope-labelled internal standards. No severe interferences were found at the respective retention times of the analytes. For internal calibration, up to 16 isotope-labelled standards were used. However, isotope-labelled analogues were lacking for about half the analytes, which may limit accuracy and specificity for these compounds. To minimize inaccuracy as far as possible, each analyte was assigned to a surrogate ISTD with close structural similarity and retention time. Different dilution factors were used depending on the matrix, a factor 25 for urine and a factor 10 for plasma. Moreover, analytes were sorted into four groups with different calibration ranges, depending on the expected concentrations and the matrix: urine or plasma.

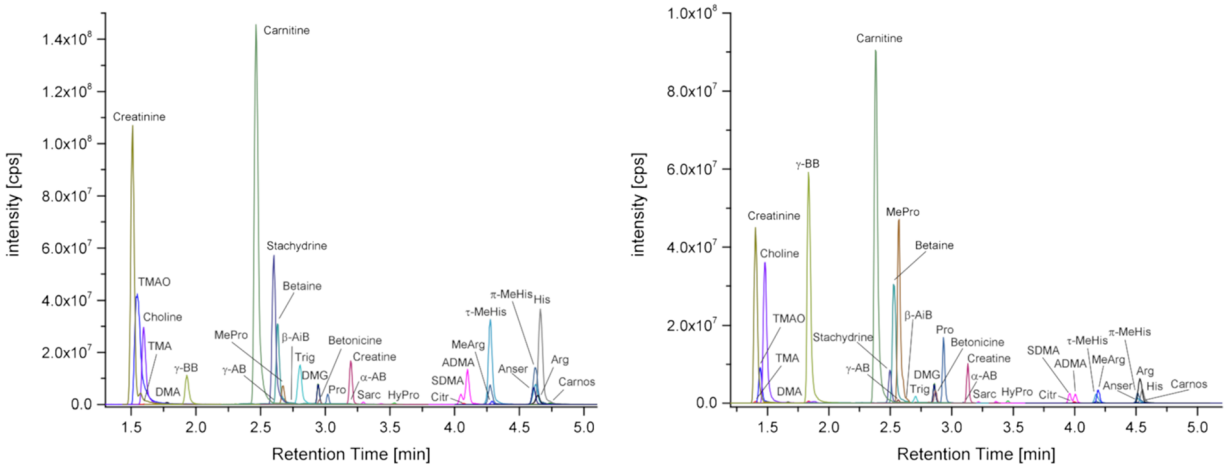

FIGURE 1 LC/MS chromatograms of spiked matrix samples; left panel: Urine*; right panel: Blood plasma (*note: urine without creatinine spike, to avoid peak saturation). α-AB = α-aminobutyric acid; β-AiB = β-aminoisobutyric acid; γ-AB = γ-aminobutyric acid; γ-BB = γ-butyrobetaine; π-MeHis = π-methylhistidine; τ-MeHis = τ-methylhistidine; ADMA = asymmetric dimethylarginine; Anser = anserine; Arg = arginine; Carnos = carnosine; Citr = citrulline; DMA = dimethylamine; DMG = *N,N*-dimethylglycine; His = histidine; HyPro = hydroxyproline; MeArg = *N*-methylarginine; MePro = *N*-methylproline; Pro = proline; Sarc = sarcosine; SDMA = symmetric dimethylarginine; TMA = trimethylamine; TMAO = trimethylamine-*N*-oxide; Trig = trigonelline [Color figure can be viewed at wileyonlinelibrary.com]

3.2 | Method validation

A summary of the validation data is shown in File S3, supporting information.

Precision and accuracy were tested batch-wise and in series using spiked control samples. For some analytes, values for the lowest control were below the LLOQ; consequently, these were not considered for validation. Generally, the precision was good with CV <8% for most analytes, in many cases <5%. Only a few analytes had CV >15%, primarily in the very low concentration range, e.g. citrulline, *N*-methylarginine or the butyric acids (urine), or choline, β-aminoisobutyric acid, betonicine and DMA (plasma). Accuracy was checked by analyzing the bias versus a calculated reference value, including the amount present in the sample before spiking. Since the latter had to be estimated from standard addition, this reference can only be an approximation to the true value. Consequently, no accuracy values are provided for the lowest control samples, since for those the calculation error for any reference value would be in the same order as the analytical error. Nevertheless, the bias of higher levels was small and did not exceed 15% in the case of urine, except for a few analytes with slightly higher bias in the low concentration range, e.g. the dimethylarginines, *N*-methylproline or the butyric acids. The observed deviations may be in part artificial and are probably related to the applied background subtraction procedure, since no bias >10% was observed for isotope-labelled compounds, for which no background subtraction was necessary. Moreover, a recent method comparison with ^{1}H-NMR demonstrated excellent correlation in urine, with R^2 >0.87 for those compounds which were accessible with both methods.[48] For plasma, the estimated accuracy was as good as for urine, at least for the majority of compounds. However, some compounds showed a somewhat lower accuracy, especially those with histidine or arginine moieties. Probably this was due to a certain plasma protein binding, reflected also by comparably low recoveries (see below). Note that precision for these compounds was still quite high and not compromised.

Most LLOQs were in the low μM range, depending on the calibration range applied. The method has a broad working range, the factor between LLOQ and ULOQ was between 20 and >400, depending on the compound. This is important to cover the expected large variations, for instance after current food consumption. Taking into account S/N only, the covered concentration range is even higher, at least a factor 100 and up to four orders of magnitude for a number of compounds. LOD values (S/N 3:1) are mostly in the nM range.

In the case of urine, the recovery was excellent with values between 71 and 109%. The matrix effects were a bit more pronounced; however, the signal intensities of spiked matrix samples were still within 75 and 125% of those of the matrix-free standards for most compounds. Matrix suppression with signal intensities <75% was the exception, e.g. in the case of citrulline or *N*-methylarginine. Moreover, matrix enhancement up to 163% was observed for very few compounds, e.g. *N*-methylproline or TMA. These results are in accordance with the simple sample preparation protocol: without extensive purification, analyte losses are unlikely, whereas matrix compounds are still present and may compromise sensitivity. However, validation data show clearly that both analyte loss and matrix effects are in a tolerable range and do not compromise the performance of the method. In plasma, the effects of recovery and matrix were a bit more pronounced than in urine, but still tolerable and efficiently compensated for by ISTD addition. Matrix suppression up to 60% was observed for very few compounds only, e.g. for β-aminoisobutyric acid or trigonelline. Matrix enhancement up to 70% was found e.g. for τ-*N*-methylhistidine, ADMA or sarcosine. In principle, good recoveries were also obtained, similar to urine.

Nevertheless, some analytes showed a remarkably lower recovery, especially arginine and histidine compounds, but also citrulline and hydroxyproline. This finding is consistent with a certain affinity to plasma proteins, which seems to be more relevant for compounds bearing more than one basic nitrogen. For instance, it is known that ADMA shows low hemodialysis efficiency, pointing to the significant plasma binding which was estimated to 30–90%.[49,50] However, one has to keep in mind that the current protocol is intended for analysis of the accessible fraction rather than the tightly-bound analyte pool, thus covering mainly free amino compounds. Access to the complete tightly bound analyte pool would require significantly altered sample preparation, which was beyond the scope of the present method. Consequently, estimates of true control values have to be taken with caution, considering that a certain fraction of the spike may be tightly bound. Nevertheless, this is rather a problem of correct calculation of reference values than of the analysis accuracy itself. Moreover, all affected analytes show high precision, albeit at the lower concentration of the accessible analyte fraction.

Stability was tested using different conditions, as depicted in section 2. Most compounds are quite stable, and the observed deviations were usually <15% and rather reflect the uncertainty of the method than the instability of the analyte.

3.3 | Analysis of KarMeN study samples and associations with current food consumption

In order to prove the suitability of the method for real-life study samples, urine samples from KarMeN were analyzed. Results of partial Spearman's correlation analyses are given in File S1, supporting information. The main results for the respective food groups are shown in Figure 2.

One of the most frequently reported biomarkers for food consumption is trigonelline, which is highly associated with coffee consumption.[6] This association was clearly confirmed by our data ($\rho = 0.76$; $p < 0.0001$).

A moderate association was also observed between stachydrine (proline-betaine) and consumption of citrus fruits ($\rho = 0.56$; $p < 0.0001$) or citrus juice ($\rho = 0.58$; $p < 0.0001$). This is also in accordance with the literature.[7] A weak association was also found with the analogous compound betonicine (hydroxyproline-betaine). Moreover, associations with citrus fruits and juices were found for *N*-methylproline and (less pronounced) for proline, but not for hydroxyproline. These results are in accordance with the fact that hydroxyproline is a degradation-product of collagen,[51,52] whereas the other proline compounds are mainly present in varying amounts in different fruits.

Another significant association was found between TMAO and consumption of fish and seafood ($\rho = 0.30$; $p = 0.0217$). Interestingly, association of fish consumption with the related compounds DMA and TMA was negligible; it was not significant in our small study group. Indeed, TMAO was proposed as a biomarker for fish consumption previously.[9-12] However, there is strong evidence that the specificity of this marker is limited since TMAO is produced from many sources, and food ingestion explains only a small part of TMAO variation.[34] Figure 2E shows the associations of various food groups with urinary TMAO level. Fish clearly had the strongest association. The influence of other food groups (including meat) was negligible,

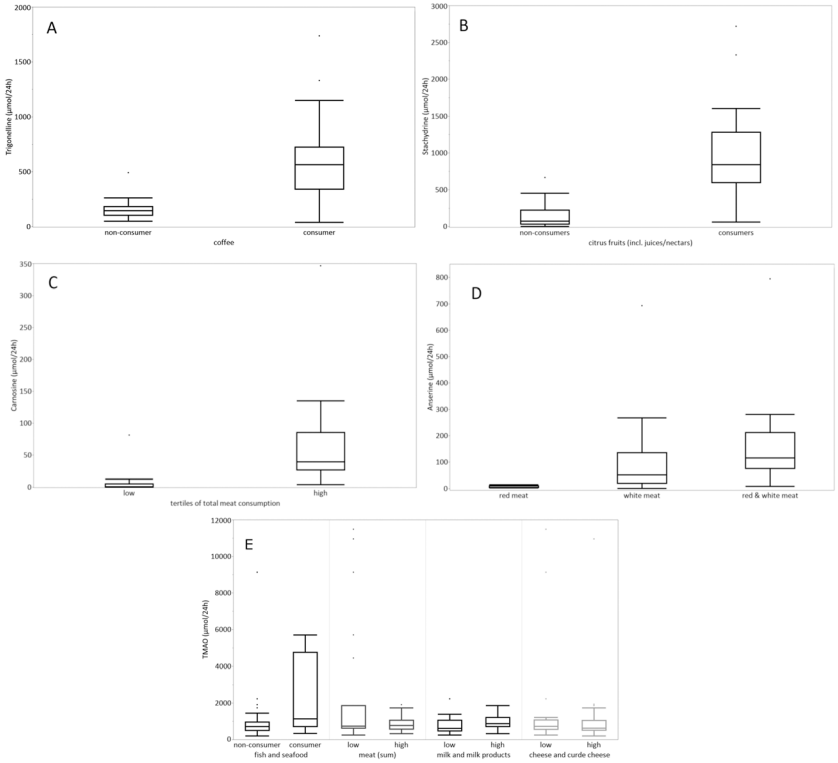

FIGURE 2 Box plots of specific dietary intake (percentiles) and urinary concentrations of related metabolites: A, Coffee and trigonelline; B, citrus fruits and stachydrine; C, meat and carnosine; D, red or white meat and anserine; E, fish, meat or dairy and TMAO

with one exception: the association with milk was as strong as with fish ($\rho = 0.31$; $p = 0.0192$), albeit with high variation. Interestingly, this was observed for milk only, but not for cheese. Such differences between various milk products have been discussed before, but results were not clear.[53-55] The present finding is in accordance with recently published results from intervention studies with different milk products.[56-58]

A number of analytes in our method have been related to consumption of meat, some of them being specific for consumption of red or white meat. In our subgroup, strong associations with total meat consumption (meat and meat products including sausages) were found especially for the dipeptide carnosine ($\rho = 0.76$; $p < 0.0001$) and for τ-*N*-methylhistidine ($\rho = 0.66$; $p < 0.0001$). Interestingly, associations with the analogous compounds anserine and π-*N*-methylhistidine were much less pronounced. In contrast, anserine ($\rho = 0.63$; $p < 0.0001$) and π-*N*-methylhistidine ($\rho = 0.54$; $p < 0.0001$) were both highly specific for consumption of white meat, e.g. chicken, whereas carnosine and τ-*N*-methylhistidine showed no specificity. This is in accordance with the previous findings.[11,13-17] Moreover, a number of further analytes showed moderate associations with total meat consumption, especially γ-butyrobetaine ($\rho = 0.45$; $p = 0.0004$), but also carnitine. However, none of these compounds showed specificity for red or white meat (File S1, supporting information).

It has to be noted that the KarMeN subset used for the present investigation was not representative, which means that the strength of the association cannot be generalized. However, the expected associations between selected foods and certain analytes were clearly detected with significant power. In summary, analysis of the KarMeN urine samples showed that the method is suitable for use in human studies, especially in the context of nutrition.

4 | CONCLUSIONS

The presented HILIC/MRM method is reasonably easy to apply and allows monitoring of several amine compounds in biological fluids, all of them being relevant in the context of nutrition, physical activity, or health. Validation proved clearly that the method is suited for the intended purpose. The precision, working range and LLOQ are appropriate for using the method in nutrition studies, as exemplified in a KarMeN subset. Already established associations of food consumption with respective metabolites could be confirmed with high confidence. The method has already been applied to a recent study investigating the effect of high intensity training on the metabolome[48] and to two dairy intervention studies.[56-58] Thus, the method is fit-for-purpose, and will be used in upcoming studies.

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ORCID

Benedikt Merz https://orcid.org/0000-0002-6856-3390
Achim Bub https://orcid.org/0000-0001-6907-5519
Ralf Krüger https://orcid.org/0000-0003-1904-8621

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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