Microchemical Journal 210 (2025) 113063

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Internal standard strategies for amino acid and polyamine quantification in rat urine and plasma via chemical derivatization-assisted LC–MS/MS

Mingyu Kim [1], Sang-Hoon Song [1], Suji Kim, Ye Jin Jung, Sooyeun Lee [*]

College of Pharmacy, Keimyung University, 1095 Dalgubeol-daero, Dalseo-gu, Daegu 42601, Republic of Korea

ARTICLE INFO

Keywords:
Liquid chromatography–tandem mass spectrometry
Amino acids
Polyamines
Internal standard
Matrix effects
Drug addiction

ABSTRACT

Amino acids and polyamines play essential roles in physiological processes, such as protein synthesis, neurotransmission, and cell growth, and are emerging as potential biomarkers for diseases including cancer and diabetes. The accurate quantification of these compounds in biological sample is challenging, particularly due to matrix effects during liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis. This study aimed to mitigate the matrix effects by employing a mixed-mode internal standard (IS) strategy that uses both isotope-derivatized ISs (Method 1, with analyte standards derivatized using an isotopic reagent, BzCl-d_5) and isotopic standards (Method 2, where isotopic analyte standards are derivatized with BzCl). A novel method was developed for the simultaneous quantitative analysis of 21 amino acids and three polyamines in rat urine and plasma samples using LC–MS/MS with benzoyl chloride derivatization. To improve analytical accuracy, the two IS preparation techniques were explored and the initial results showed poor parallelism for several analytes using Method 1. However, significant improvements were observed with Method 2, highlighting the impact of the IS strategy on reducing the matrix effects and improving quantification accuracy. By combining both approaches, we successfully achieved accurate quantification of the target compounds in biological matrices. This methodology offers a powerful tool for investigating metabolic alterations in diseases, enhancing our understanding of disease pathology and aiding in biomarker identification.

1. Introduction

Amino acids serve as building blocks for protein synthesis and precursors for hormone synthesis, neurotransmitters, nucleic acids, and other biomolecules. Furthermore, they play a crucial role in various biochemical processes such as neurotransmission and energy metabolism [1,2]. The disturbance of amino acid metabolism has been reported in conditions such as cancer [3–6], diabetes [7], cardiovascular diseases [8], neurodegenerative diseases [9], and drug addiction [10,11]; thus, amino acid profiling has been increasingly applied to discover more efficient diagnosis and therapy options of the diseases. For example, glycine was proposed as a biomarker for brain tumors [3], while the clinical significance of d-serine was demonstrated in monitoring pathological conditions of kidney disease and Alzheimer's disease [12,13]. Furthermore, a biomarker panel of tyrosine and glutamine-leucine in human serum was capable of diagnosing early-stage colorectal cancer [14]. A decrease of the glutamine and glutamate levels in human posterior cingulate cortex was reported as an early biomarker for neurodegeneration [15]. Moreover, dynamic changes in the biosynthesis/metabolism of amino acids, including the phenylalanine, tyrosine, and tryptophan biosynthesis and the valine, leucine, and isoleucine biosynthesis were observed in rat plasma as warning signs of drug dependence [10]. Furthermore, polyamines, the decarboxylated products of ornithine or amino acids, were also investigated in biological fluid samples. The alteration of polyamine metabolism monitored in human plasma was proposed as a biomarker for Parkinson's disease [16], and polyamine profiles in human urine were proposed as diagnostic tools for head, neck, colorectal, and pancreatic cancer [17–19]. Amino acids and polyamines have been presented as key biomarkers for the diagnosis of many diseases, therefore, the relative comparison between normal and pathological conditions as well as their accurate quantification in biological samples is essential.

Profiling of amino acids and polyamines in biological samples has been performed via chromatography coupled with mass spectrometry (MS) or nuclear magnetic resonance spectroscopy [20–25]. Recently, liquid chromatography–tandem mass spectrometry (LC–MS/MS) is

* Corresponding author.
E-mail address: sylee21@kmu.ac.kr (S. Lee).
[1] These authors contributed equally to this study.

https://doi.org/10.1016/j.microc.2025.113063
Received 24 November 2024; Received in revised form 13 February 2025; Accepted 14 February 2025
Available online 15 February 2025

extensively applied to metabolic profiling, owing to its superior sensitivity, wide coverage of polarity of metabolites, and rapid and simple sample preparation [26–28]. However, the accurate quantification of endogenous amino acids is still challenging, due to their physicochemical properties, such as low molecular weight, high polarity and hydrophilicity, the poor separation and low ionization efficiency of LC-MS/MS, as well as the absence of a blank matrix and resultant unpredictable matrix effects. To overcome such issues, various chemical derivatization methods have been developed using dansyl chloride [29,30], 9-fluorenylmethyl chloroformate [31], butanolic HCl [32], 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate [33], propyl chloroformate [23], *o*-phthaldialdehyde (OPA) [34], or benzoyl chloride (BzCl) [35–38] to improve analytical sensitivity and selectivity, as well as increase retention times in reverse-phase LC and enhance ionization efficiency in MS. Among the different reagents, dansyl chloride, OPA, and BzCl are preferred for amino acid derivatization. However, dansyl chloride has certain limitations; Song et al. reported that monoamines labeled with the dansyl group exhibit low collision-activated dissociation efficiency on a triple quadrupole mass spectrometer, making multiple reaction monitoring (MRM) unsuitable for high-sensitivity analysis [39]. Additionally, isotope-coded dansyl chloride is not commercially available. Even though OPA reacts quickly with biological amines it is only specific to primary amines, which limits its applicability for detecting compounds such as spermidine and spermine. Furthermore, it produces unstable derivatives [40]. In contrast, BzCl can benzoylate primary and secondary amines as well as alcohol groups into amides and esters, respectively, via a base-catalyzed Schotten-Baumann reaction [41,42]. BzCl is considered a preferred derivatizing reagent for LC-MS/MS-based multi-target analysis owing to its short reaction times (seconds at room temperature) and high stability (one week at room temperature) [39]. Moreover, the availability of its commercially stable isotope-labeled forms (e.g., BzCl-d_5) enhances its suitability for isotope-coded labeling in the preparation of internal standards (ISs) for various analytes [36–38,43]. Consequently, BzCl is regarded as the most preferred derivatizing reagent for LC-MS/MS-based multi-target analysis, targeting both amino acids and polyamines.

Incorrect quantification of analytes in biological samples can result in errors in data interpretation and subsequent decision-making. In particular, unexpected matrix effects pose a challenge to accurate quantification, emphasizing the need for thorough validation of the analytical methods. The selection of appropriate ISs can correct the inaccurate quantitative results caused by matrix effects in LC–MS/MS [44]. During LC–MS/MS method development and validation, ISs are chosen based on their similarities with the physicochemical characteristics of analytes, mainly in chromatographic conditions, and the differentiation from the analytes in mass fragmentation (e.g., no crosstalk between MRM transitions from stable isotope labelled and non-labelled chemicals) [45,46]. For the quantification of endogenous compounds in biological samples, surrogate matrices, such as artificial fluids, neat solutions, and stripped matrices, are often used to prepare calibrators; thus, the identification of matrix interferences in authentic samples is not straightforward, while the parallelism between the surrogate and authentic matrices is questionable. The use of stable isotopically labeled ISs is a competent approach to remove matrix effects, compared with other approaches such as changing the sample preparation process and modifying the LC or MS conditions [47,48].

In chemical derivatization-assisted LC–MS/MS analyses, ISs can be prepared via two different methods: either derivatizing the analyte standards with an isotope-labeled reagent (e.g., BzCl-d_5) or derivatizing the isotopic analyte standards with a regular reagent (e.g. BzCl) [49]. The former method offers significant time and costs savings for the simultaneous quantification of multiple metabolites [48]. However, the potential discrepancies between the analytes and ISs within the matrix are challenging, because ISs are derivatized separately from the target compounds in the biological sample. This issue becomes more problematic when analyzing endogenous analytes without matrix-matched calibrators, leading to quantitative errors. Therefore, during the development and validation of methods that use stable isotope-coded derivatizing reagents, it is crucial to identify and address the discrepancies between the target compounds and ISs due to derivatization, whether within the matrix or not.

The aim of this study was to develop chemical derivatization-assisted LC–MS/MS methods for the accurate and reproducible quantitative analysis of 21 amino acids and three polyamines in rat urine and blood samples. With this aim, this study focused on overcoming the matrix effects by utilizing isotope-derivatized ISs prepared using both an isotope reagent and isotope standards. Water was used to prepare calibrators, and the validity of this approach was fully demonstrated, via comparisons of the two different IS preparation methods: (1) the analyte standards were derivatized with an isotopic reagent [BzCl-d_5] (IS A) or (2) the isotopic analyte standards were derivatized with BzCl (IS B).

2. Materials and methods

2.1. Chemicals and reagents

All solvents were of high-performance LC grade. Alanine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, leucine, lysine, methionine, ornithine mono hydrochloride, phenylalanine, proline, putrescine, serine, spermidine, threonine, tryptophan, tyrosine, valine, arginine, gamma-aminobutyric acid (GABA), and BzCl were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenylalanine-d_5 was purchased from CDN Isotopes Inc. (Pointe-Claire, Canada). Arginine-d_7, aspartic acid-$^{13}C_4D_3^{15}N$, cysteine-$^{13}C_3^{15}N$, histidine-$^{13}C_6^{15}N_3$, methionine-$^{13}C_5$, tryptophan-$^{13}C_{11}$, tyrosine-d_7, and BzCl-d_5 were purchased from Cambridge Isotope Laboratories (Tewkesbury, MA, USA). All analytical stock solutions (1 mg/mL) were prepared in water, except for tryptophan and tyrosine, which were prepared in methanol and stored at −80 °C. The working standard solutions and working IS solutions (ISs A and B) used for the analysis of rat urine and plasma were prepared in water and stored at −80 °C until analysis. The concentrations of each analyte and IS are presented in Method S1.

2.2. Sample preparation

Rat urine and plasma samples were prepared as previously described [37,50,51] with minor modifications, and the procedure for sample preparation is presented in Fig. 1. The urine samples were centrifugated at 20,800 g for 10 min at 4 °C to remove any particles, and the supernatant was filtered using a 0.45-μm polyvinylidene fluoride (PVDF) microporous membrane. The filtered urine sample was diluted 5-fold with water and 20 μL of diluted rat urine was sequentially mixed with 10 μL of water and 10 μL of working IS B solution. Then, 10 μL of 100 mM sodium carbonate and 10 μL of a BzCl solution (2% (v/v) in acetonitrile) were sequentially added and the mixture was vortexed for 1 min for derivatizing the target compounds and stable isotope labeled compounds (IS B, used for Method 2).

20 μL of the three-fold diluted plasma sample was mixed with 10 μL of water and 10 μL of the working IS B solution, prepared via protein precipitation using 80 μL of ice-cold methanol, followed by centrifugation at 20,800 g for 10 min at 4 °C. Afterwards, 10 μL of 100 mM sodium carbonate and the BzCl solution (2 % (v/v) in acetonitrile) were sequentially added to 20 μL of the supernatant, followed by vortexing for 1 min for the derivatization of the analytes and stable isotope-labeled compounds (IS B, used for Method 2).

In Method 1, the working IS A solution was derivatized with BzCl-d_5 (2 % (v/v) in acetonitrile). The procedure involved a 1:1:1 reaction of the working IS A solution, 100 mM sodium carbonate, and BzCl-d_5 solution (2% (v/v) in acetonitrile), and the reaction was terminated using formic acid. Then, 10 μL of the IS A solution derivatized with BzCl-d_5 (2 % (v/v) in acetonitrile) was mixed with the sample prepared via BzCl (2% (v/v) in acetonitrile) derivatization. All the experimental

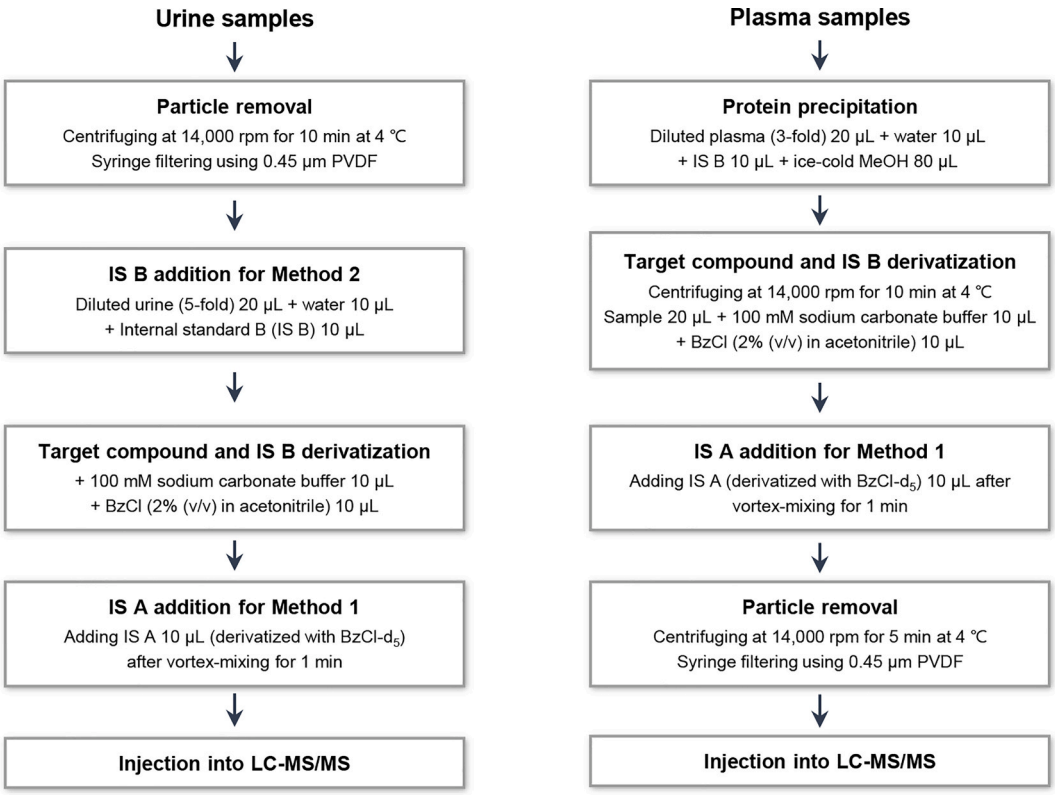

Fig. 1. Sample preparation procedure.

procedures were performed in an ice bath.

2.3. LC–MS/MS analysis

LC–MS/MS analysis was conducted using a Nexera X2 LC-30AD and LCMS-8050 system (SHIMADZU Corporation, Kyoto, Japan) coupled with an LC-30AD pump degasser (DGU-205R) and a SIL-30AD auto-sampler (SHIMADZU Corporation). Data were processed using LabSolutions (SHIMADZU Corporation). The column and guard column used for the analysis of rat urine were Acquity UPLC HSS T3 (2.1 mm × 100 mm, 1.8 μm, Waters, Milford, MA, USA) and Acquity UPLC HSS T3 VanGuard Pre-Column (2.1 mm x 5 mm, 1.8 μm, Waters), respectively, and those for the analysis of rat plasma were Atlantis T3 (2.1 mm × 100 mm, 3 μm; Waters) and Atlantis T3 VanGuard (2.1 mm × 5 mm, 3 μm; Waters), respectively. The temperature was set at 40 °C for both analyses. The mobile phase consisted of 2 mM ammonium formate and 0.1% formic acid in water (mobile phase A) and 100 % acetonitrile (mobile phase B) at a flow rate of 300 μL/min. The gradient elution was as follows: 0–0.5 min, 5% B; 0.5–7 min, 5–85% B; 7–7.1 min, 85–95% B; 7–9 min, 95% B; 9–9.1 min, 95–5% B; 9–14 min, 5% B. The sample injection volumes for urine and plasma were 1 and 5 μL, respectively, and the auto-sampler temperature was maintained at 4 °C.

The MS/MS system was operated at positive electrospray ionization. The optimal conditions were: nebulizing gas flow, 3 L/min; drying gas flow, 10 L/min; heating gas flow, 10 L/min; interface voltage, 4 kV; interface temperature, 300 °C; desolvation line temperature, 526 °C; heat block temperature, 400 °C. The analytes and ISs were identified and quantified by their MRM, the details of which are presented in Tables S1 and S2.

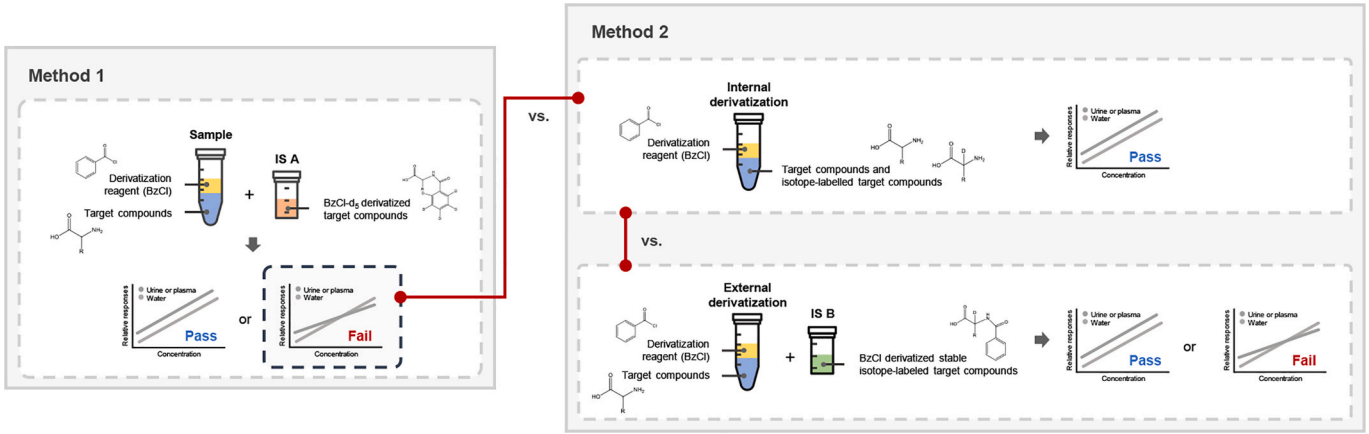

Fig. 2. Schematic of the investigation of internal standard strategies.

2.4. Comparison of two different IS preparation methods (Methods 1 and 2) by parallelism evaluation between water and rat urine or plasma

Fig. 2 presents the internal standard selection strategy employed in this study. In Method 1, all analyte standards were derivatized with BzCl-d_5 separately from the sample to prepare the derivatized IS. This derivatized IS was then mixed with the sample immediately before LC-MS/MS analysis. ISs that satisfied the acceptance criteria of the parallelism evaluation of Method 1 were designated as IS A. For analytes that failed to meet the acceptance criteria of Method 1, Method 2 was applied and then compared to Method 1. In Method 2, the commercially available isotopic analyte standards (arginine-d_7, aspartic acid-$^{13}C_4d_3^{15}N$, cysteine-$^{13}C_3^{15}N$, histidine-$^{13}C_6^{15}N_3$, methionine-$^{13}C_5$, phenylalanine-d_5, tryptophan-$^{13}C_{11}$, and tyrosine-d_7), along with the analyte standards (arginine, aspartic acid, cysteine, histidine, methionine, phenylalanine, tryptophan, and tyrosine), were derivatized with BzCl during the sample preparation process. ISs that satisfied the acceptance criteria of Method 2 were designated as IS B. Furthermore, in Method 2, the matrix effect of the derivatized IS was investigated by comparing the internal (matrix-mixed derivatization) and external (matrix-separated derivatization) derivatization approaches.

2.5. Validation study

The validation parameters including the matrix effect, selectivity, sensitivity, linearity, accuracy, precision, parallelism, and stability were evaluated according to previous studies [52–55]. Since the analytes are endogenous compounds, water was used as a blank matrix after evaluating parallelism. The slopes of the calibration curves prepared from rat plasma or urine were compared with those prepared from water, and the coefficient of variation (CV) of the slopes was calculated. Additionally, the endogenous concentrations of the analytes, estimated by extrapolation to the negative *x*-intercept from standard addition, were compared with those interpolated from calibration curves derived from water. The relative error (RE) was then calculated for the comparison. The sensitivity, linearity, accuracy, and precision were assessed using water, while selectivity, stability, and matrix effect were evaluated using water, rat urine, or plasma. The quality control (QC) samples were prepared at low, medium, and high concentrations (LQC, MQC, and HQC, respectively) in the range of the calibration curve of each analyte. The in-process stability, autosampler stability, freeze and thaw stability, and sample storage stability were evaluated for 30 d at –80 °C using both spiked water samples at LQC and HQC levels, as well as pooled urine or plasma samples collected from six different rats. The detailed experimental conditions and accepted criteria for the validation parameters were the same as those described in the previous studies [55–57].

3. Results and discussion

3.1. Optimization of derivatization conditions

An advantage of LC-MS/MS analysis is its ability to detect target compounds with high sensitivity and specificity without the need for derivatization. Nevertheless, many studies employ derivatization to enhance ionization efficiency and optimize retention times [58–63]. The derivatization efficiency was assessed across five BzCl concentration levels (1%, 2%, 4%, 8%, and 10%) in acetonitrile, using rat urine and plasma, as well as their surrogate matrix (water). The concentrations of BzCl had a more pronounced effect in rat urine compared to plasma. As shown in Fig. S1A, lower percentages of BzCl (1% or 2%) yielded higher results, whereas increasing the concentration beyond this point led to similar or reduced yields in rat urine. Notably, the peak areas of polyamines, such as putrescine, spermidine, and spermine, declined sharply at higher BzCl concentrations. Specifically, the spermine levels decreased by approximately 2.93-fold (LQC), 2.55-fold (MQC), and 2.61-fold (HQC) at 8% BzCl compared with 1% BzCl. Conversely, the tyrosine levels increased by 1.35-fold (LQC) and 1.22-fold (MQC) at 2% BzCl compared with 1% BzCl in rat urine, while polyamines showed no significant differences (within ±20%); hence, 2% BzCl was selected for further analysis.

In rat plasma samples, the variations in peak areas of most target compounds remained within ±20% across the tested BzCl concentration levels, except for asparagine (MQC), histidine (LQC and MQC), ornithine (MQC), spermidine (MQC), tryptophan (MQC), and tyrosine (LQC). The maximum derivatization yield for histidine occurred at 2% BzCl, yielding a 2.39-fold increase (MQC) compared with 1% BzCl. The derivatization time was also examined at reaction intervals of 1, 5, and 10 min. As shown in Fig. S2, a reaction time of 1 min was sufficient to achieve the maximum derivatization yield. For polyamines, extending the reaction time beyond 10 min led to significant degradation, with specific reductions as follows: spermidine showed a −1.52-fold decrease (LQC in water), while spermine exhibited decreases of −3.05-fold (LQC in water), −1.74-fold (MQC in water), −1.74-fold (HQC in water), −1.23-fold (LQC in urine), −1.30-fold (MQC in urine), and −1.41-fold (HQC in urine) compared with the results obtained after 1 min (Fig. S2A). Therefore, the derivatization time was set at 1 min.

3.2. Comparison of two different IS preparation methods (Methods 1 and 2)

The absolute quantification of multiple endogenous metabolites is not straightforward due to the absence of analyte-free matrix-matched calibrators and unavoidable or unexpected matrix interference, posing a significant hurdle in method validation. An effective method for addressing such issues is the use of stable isotope-labeled standards for the target compounds. However, when simultaneously analyzing multiple targets, using individual ISs for each compound is often impractical due to their lack of commercial availability. Previous studies employed, strategies with isotope derivatization reagents to overcome this problem [48,49,64]. The limitations of this approach lie in the variations in the labeling efficiencies of the derivatization reagents depending on the target compounds, primarily due to diverse matrix interferences in biological samples. Consequently, meeting one condition for the selection of ISs for certain analytes may preclude the satisfaction of the requirements of the other analytes. In this study, we addressed this challenge by implementing a mixed-mode IS strategy, involving the use of both isotope-derivatized ISs with an isotopic reagent, and isotopic standards as ISs.

At the initial step of the method development, the parallelism between rat urine or plasma and water as a surrogate matrix was investigated. Cysteine was included as an analyte only in rat plasma due to its presence at trace levels in rat urine Furthermore, the addition IS A before sample extraction (mixing with BzCl) in Method 1 led to the derivatization of the endogenous target compounds by BzCl-d_5, despite using formic acid for reaction termination. Thus, IS A was added just before LC–MS/MS analysis. All target compounds, except arginine, aspartic acid, histidine, methionine, phenylalanine, tryptophan, and tyrosine in urine samples and aspartic acid, cysteine, methionine, and tyrosine in plasma samples showed acceptable parallelism using IS A (Method 1); the CV of the slopes was less than 15%, and the RE between the extrapolated mean concentrations from the negative *x*-intercept from standard addition in pooled rat urine or plasma and the interpolated mean concentrations using water as a surrogate matrix was less than 15% (Figs. S3 and S4, Tables S3 and S4). Regarding the analytes where parallelisms were problematic when applying Method 1 (marked with an asterisk in Tables S3 and S4), Method 2 was applied, and the parallelisms were significantly improved as shown in Figs. 3 and 4 (Method 1 vs. Internal derivatization, Method 2) as well as Tables 1 and 2 (Method 1 vs. Internal derivatization, Method 2). The CV between the slopes of rat urine and water decreased notably for tyrosine (from 48.2% to 3.0%). The RE between the mean concentrations derived from the standard addition and the surrogate matrix approach also showed

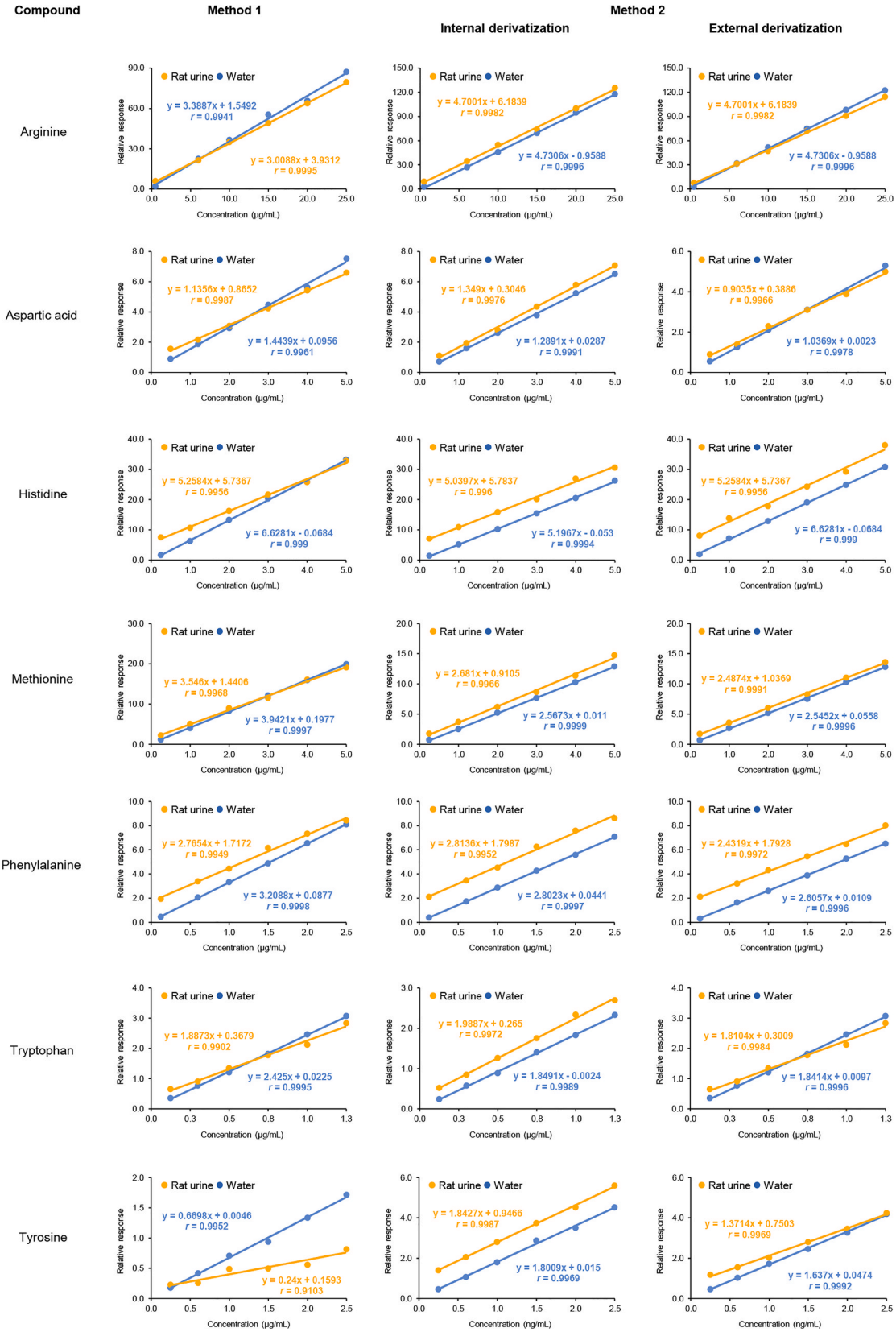

Fig. 3. Calibration curves for seven amino acids using pooled rat urine (authentic matrix, $n = 3$) and water (surrogate matrix, $n = 3$).

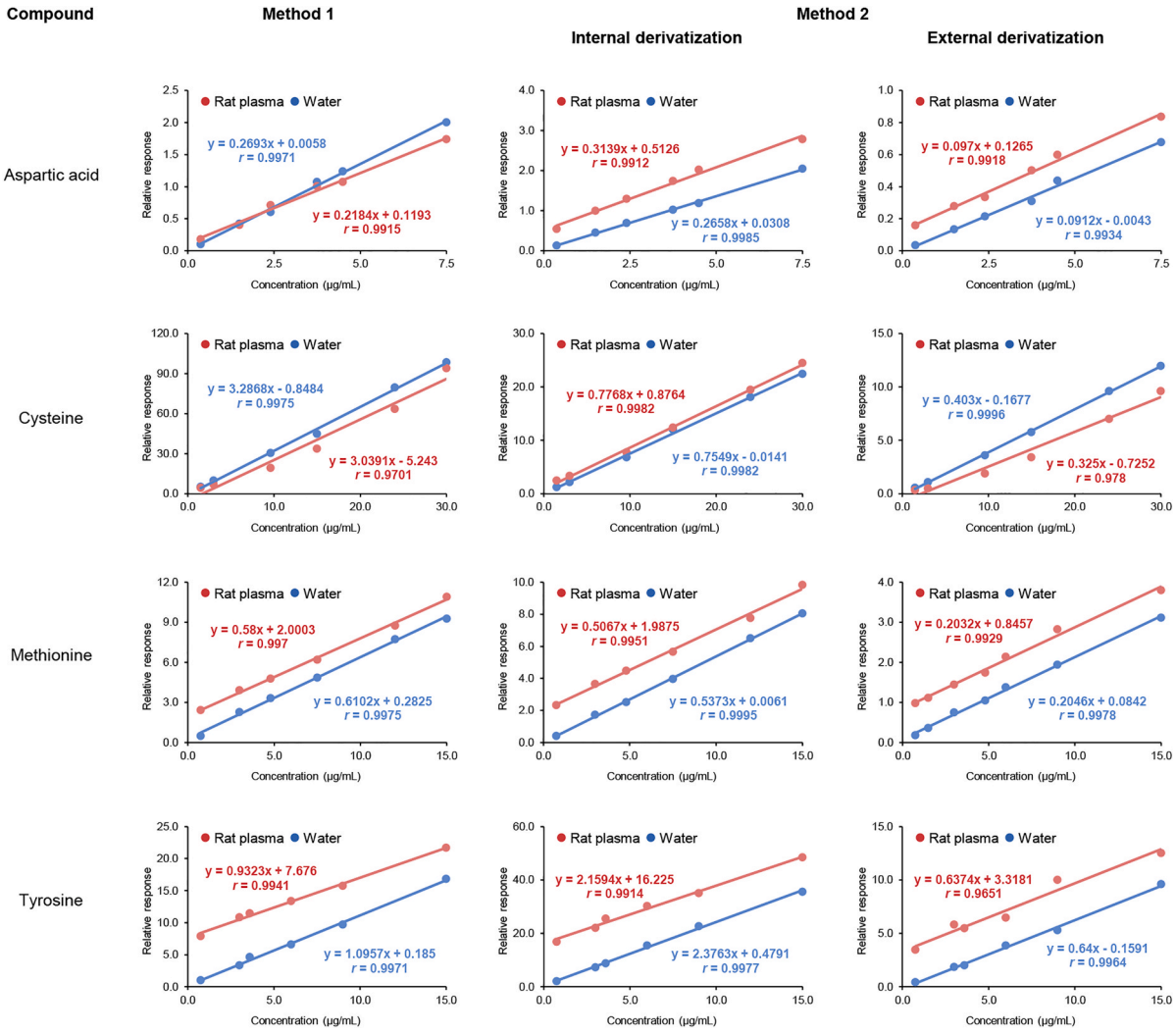

Fig. 4. Calibration curves for four amino acids using pooled rat plasma (authentic matrix, $n = 3$) and water (surrogate matrix, $n = 3$).

significant improvements; in urine, arginine improved from 46.3% to −14.4%, aspartic acid from 30.1% to 5.31%, histidine from 19.6% to 2.61%, methionine from 22.4% to −2.94%, phenylalanine from 17.7% to 2.03%, tryptophan from 27.2% to −9.02%, and tyrosine from NC to −0.584% (Table 1). Similarly, for plasma, the RE of aspartic acid improved from 22.9% to −11.0%, cysteine changed from NC to −4.42%, methionine improved from 18.3% to 5.87%, and tyrosine improved from 16.9% to 11.7% (Table 2). The retention times of the ISs for the analytes in Methods 1 and 2 were mostly similar, as shown in Tables S1 and S2. Such results imply that in chromatography, the selection of ISs for different analytes depends on various factors beyond the similarities in elution characteristics, even within the same sample matrix.

The matrix effects were compared between Method 1 and 2 for the analytes where parallelisms were not satisfied in Method 1 (Tables S5 and S6). Method 2 improved the CV of the matrix effect for phenylalanine (from 27.0% to 12.1% at LQC) and tyrosine (from 15.3% to 10.9% at LQC) in rat urine. Similarly, the CV of tyrosine in plasma significantly improved, decreasing from 75.9% to 11.8% at the LQC level and from 31.9% to 9.7% at the HQC level. Furthermore, the ion suppression decreased considerably from 36.9% to 77.3% at LQC and from 56.7% to 69.0% at HQC. Moreover, the CV of cysteine notably decreased from 42.9% to 10.0% at LQC, and ion suppression improved from 46.4% to 82.6% at LQC and from 72.5% to 90.3% at HQC using Method 2. These findings demonstrate an enhancement in reproducibility by employing distinct isotopic analyte standards for the analytical challenges posed by matrix effects even when utilizing isotope derivatization reagents as ISs. Furthermore, when using a surrogate matrix for the analysis of endogenous substances, the assessment of parallelism with the authentic matrix should be considered together with the matrix effects.

It was previously reported that the efficiency of dansylation labeling of amino acids was affected to varying degrees depending on the coexisting constituents within the sample matrix [65]. Moreover, mixing analyte standards coded with an isotopic reagent such as ISs with the sample immediately before LC-MS/MS analysis (Method 1 in present study), is considered a major disadvantage, because the recovery of the analytes is not corrected [48]. Despite the commercial availability and cost-effectiveness of the isotopic reagents, compared with isotopic analyte standards, these disadvantages may lead to unsatisfactory experimental outcomes, as shown in Tables 1 and 2. Thus, we further investigated whether these results could be attributed to differences in labeling efficiencies resulting from the separate derivatization of authentic matrices, such as urine or plasma. For this purpose, we compared the parallelisms between rat urine or plasma and water using the internal and external derivatization of Method 2 to investigate whether the failure of Method 1 was due to the separate derivatization of the matrices. As shown in Figs. 3 and 4, as well as Tables 1 and 2, external derivatization revealed that aspartic acid, histidine, methionine, phenylalanine, and tryptophan in rat urine and/or plasma exhibited acceptable parallelism (i.e., $< \pm 15\%$, RE and $<15\%$, CV). However, arginine (in rat urine), cysteine (in rat plasma), and tyrosine

Table 1
Parallelism comparison between Methods 1 and 2 in rat urine.

Compound name	Method 1					Method 2								
						Internal derivatization					External derivatization			
	IS A	Negative *x*-intercept from standard addition	Surrogate matrix approach	RE (%)	Slope difference	IS B	Negative *x*-intercept from standard addition	Surrogate matrix approach	RE (%)	Slope difference	Negative *x*-intercept from standard addition	Surrogate matrix approach	RE (%)	Slope difference
		Extrapolated mean concentration (μg/mL)	Interpolated mean concentration (μg/mL)		CV (%)		Extrapolated mean concentration (μg/mL)	Interpolated mean concentration (μg/mL)		CV (%)	Extrapolated mean concentration (μg/mL)	Interpolated mean concentration (μg/mL)		CV (%)
Arginine	Bz-d_5-Arginine-	1.31	0.703	46.3	7.13	Bz-Arginine-d_7	1.32	1.51	−14.4	3.56	1.14	0.670	41.2	8.66
Aspartic acid	Bz-d_5-Aspartic acid	0.762	0.533	30.1	14.6	Bz-Aspartic acid-$^{13}C_4d_3^{15}N$	0.214	0.226	5.31	5.72	0.430	0.373	13.3	10.7
Histidine	Bz-d_5-Histidine	1.09	0.876	19.6	13.4	Bz-Histidine-$^{13}C_6^{15}N_3$	1.15	1.12	2.61	5.72	1.11	0.78	12.6	5.89
Methionine	Bz-d_5-Methionine	0.406	0.315	22.4	6.85	Bz-Methionine-$^{13}C_5$	0.340	0.350	−2.94	3.81	0.417	0.385	7.67	2.53
Phenylalanine	Bz-d_5-Phenylalanine	0.621	0.508	17.7	10.2	Bz-Phenylalanine-d_5	0.639	0.626	2.03	3.65	0.737	0.684	7.19	5.06
Tryptophan	Bz-d_5-Tryptophan	0.195	0.142	27.2	14.6	Bz-Tryptophan-$^{13}C_{11}$	0.133	0.145	−9.02	7.68	0.166	0.158	4.82	2.25
Tyrosine	Bz-d_5-Tyrosine	NC	NC	NC	48.2	Bz-Tyrosine-d_7	0.514	0.517	−0.584	2.96	0.547	0.429	21.6	8.76

IS, internal standard; Bz, benzoyl; NC, not calculated; CV, coefficient of variation; RE, relative error.
Interpolated concentrations were calculated by interpolation from surrogate matrix calibration curves. Extrapolated concentration was calculated by extrapolating to the negative *x*-intercept from the standard addition calibration curve. RE (%) is {(extrapolated concentration − interpolated concentration)/extrapolated concentration} * 100.

Table 2
Parallelism comparison between Methods 1 and 2 in rat plasma.

Compound name	Method 1				Method 2								
					Internal derivatization				External derivatization				
	IS A	Negative *x*-intercept from standard addition	Surrogate matrix approach	RE (%)	Slope difference	IS B	Negative *x*-intercept from standard addition	Surrogate matrix approach	RE (%)	Slope difference	Negative *x*-intercept from standard addition	Surrogate matrix approach	RE (%)	Slope difference
		Extrapolated mean concentration (μg/mL)	Interpolated mean concentration (μg/mL)		CV (%)		Extrapolated mean concentration (μg/mL)	Interpolated mean concentration (μg/mL)		CV (%)	Extrapolated mean concentration (μg/mL)	Interpolated mean concentration (μg/mL)		CV (%)
Aspartic acid	Bz-d_5-Aspartic acid	0.546	0.421	22.9	13.4	Bz-Aspartic acid-$^{13}C_4d_3^{15}N$	1.63	1.81	−11.0	10.8	1.30	1.43	−10.0	8.75
Cysteine	Bz-d_5-Cysteine	NC	NC	NC	5.63	Bz-Cysteine-$^{13}C_6^{15}N$	1.13	1.18	−4.42	7.22	NC	NC	NC	13.6
Methionine	Bz-d_5-Methionine	3.45	2.82	18.3	10.2	Bz-Methionine-$^{13}C_5$	3.92	3.69	5.87	7.87	4.16	3.72	10.6	6.72
Tyrosine	Bz-d_5-Tyrosine	8.23	6.84	16.9	10.7	Bz-Tyrosine-d_7	7.51	6.63	11.7	6.96	NC	NC	NC	21.1

IS, internal standard; Bz, benzoyl; NC, not calculated; CV, coefficient of variation; RE, relative error.
Interpolated concentrations were calculated by interpolation from surrogate matrix calibration curves. Extrapolated concentration was calculated by extrapolating to the negative *x*-intercept from the standard addition calibration curve. RE (%) is {(extrapolated concentration − interpolated concentration)/extrapolated concentration} * 100.

(in rat urine and plasma) did not meet the acceptance criteria. The extraction recovery of ISs could be different between Method 2 with internal derivatization and external derivatization or between Method 2 with internal derivatization and Method 1. The difference in the extraction recovery levels may also vary depending on the concentrations of the analytes. Such results imply that the matrix-separated derivatization for the preparation of IS A could not fully correct the effect of matrix interference during sample preparation. Additionally, previous studies have reported that isotopic effects originating from deuterated compounds can also lead to unexpected matrix effects in reverse-phase LC [48,66]. Therefore, when using isotope-labeled derivatization reagents, particularly for the quantification of endogenous compounds lacking matrix-matched calibrators, it is crucial to assess the compatibility between the target compounds and IS to ensure accurate quantification.

3.3. Method validation

Based on the results of the comparison between Methods 1 and 2, the optimized methods with a mixed-mode IS strategy were fully validated for rat urine and plasma. Figs. S5 and S6 shows representative chromatograms of the target compounds in water as a blank matrix (A), water fortified with the LLOQ levels of each analyte (B), and rat urine or plasma (C) samples. No signals interfering with the signal of the analytes and ISs were observed in water. The endogenous concentrations of all the target compounds were clearly detected in the authentic rat urine or plasma, however, only endogenous cysteine was not detected in rat urine. The sensitivity, linearity, matrix effect, method precision and accuracy, and stability of the LC-MS/MS method for rat urine samples are summarized in Table S7. The LOD and LLOQ values were between 0.01 μg/mL (putrescine) and 0.25 μg/mL (serine) and between 0.05 μg/mL (spermine) and 10 μg/mL (glycine), respectively. The mean regression coefficients (*r*) for each analyte were between 0.9958 (glycine) and 0.9988 (glutamine). Moderate ion suppression or enhancement was observed, with the mean values of the matrix effects for each analyte ranging from 62.5% (putrescine) to 126.9% (spermine), and their CVs ranging from 2.5% (valine) to 14.7% (valine). The CVs for the repeatability and intermediate precision ranged from 3.0% (phenylalanine) to 14.8% (histidine), and the accuracy was between 0.1% (valine) and −8.0% (tyrosine). The analytes in urine were stable under all the tested conditions. The results of the validation parameters for the LC-MS/MS method in rat plasma are summarized in Table S8. The LOD values ranged from 0.0009 (putrescine) to 0.750 (glutamine) μg/mL, and the LLOQ values ranged from 0.0075 (spermine) to 90.0 (glutamine) μg/mL. The mean of the *r* value for each analyte ranged from 0.9904 (glutamic acid) to 0.9990 (tyrosine). The mean values of the matrix effect of each analyte ranged from 69.0% (tyrosine) to 130.7% (lysine). The CVs of the matrix effects were from 6.1% (phenylalanine) to 14.7% (arginine). The CVs for repeatability and intermediate precision ranged from 6.3% (cysteine) to 18.1% (GABA), while the accuracy of the method ranged from a bias of 0.1% (alanine) to 10.3% (glycine). Stability experiments revealed that glycine was unstable [118.5% (plasma) and 138.7% (HQC in water)] in samples stored on ice at room temperature for up to 4 h. Therefore, the entire plasma analysis was performed under the current optimized conditions. The implemented mixed-mode IS strategy met the criteria of all the validation parameters and demonstrated superior method accuracy and reproducibility.

3.4. Quantification of amino acids and polyamines in rat urine and plasma

Table 3 shows the quantitative results of the endogenous amino acids and polyamines measured in urine and plasma samples collected from healthy rats in this study (LC-MS/MS methods with the mixed-mode IS strategy) and previously reported ones [2,67–71]. Our results show the wide range of the mean concentrations of amino acids and polyamines in

Table 3
Endogenous concentrations of amino acids and polyamines in rat urine and plasma samples.

Compound name	Rat urine						Rat plasma		
	Experimental (μg/mL)	Reported (μg/mL)			Experimental (μg/mg$_{creatinine}$)	Reported (μg/mg$_{creatinine}$)	Experimental (μg/mL)	Reported (μg/mL)	
Amino acids									
	Quantified (n = 6)	[67] (n = 3)	[68] (n = 10)	[2] (n = 6)	Quantified (n = 6)	[69] (n = 6)	Quantified (n = 5)	[67] (n = 6)	[2] (n = 6)
Alanine	3.4 ± 0.3	132.7	19.3	1.4 ± 0.2	8.3 ± 1.1	15.4 ± 3.0	19.2 ± 4.9	32.2	15.6 ± 2.3
Arginine	4.1 ± 0.7	2.5	14.7	0.9 ± 0.1	10.1 ± 2.2	NA	14.9 ± 2.5	14.9	12.6 ± 1.1
Asparagine	2.7 ± 0.2	2.3	17.6	2.3 ± 0.3	6.6 ± 0.9	12.7 ± 2.1	3.7 ± 0.5	7.1	7.8 ± 0.9
Aspartic acid	0.9 ± 0.2	7.2	2.7	3.7 ± 0.3	2.1 ± 0.4	9.7 ± 0.9	< LLOQ	0.7	2.7 ± 0.4
Cysteine	NA	NA	NA	2.4 ± 0.2	NA	NA	1.8 ± 0.5	NA	4.1 ± 0.6
GABA	1.7 ± 0.4	0.6	NA	NA	4.2 ± 0.8	4.1 ± 0.8	ND	ND	NA
Glutamine	7.9 ± 0.7	0.7	34.1	12.6 ± 1.2	19.6 ± 2.9	45.1 ± 18.1	43.2 ± 8.1	68.8	26.5 ± 3.6
Glutamic acid	3.3 ± 1.1	23.1	8.4	3.0 ± 0.4	8.1 ± 2.1	11.7 ± 5.6	6.8 ± 0.7	10.8	6.6 ± 0.9
Glycine	182.0 ± 8.9	126.2	6.7	4.2 ± 0.6	457.1 ± 102.5	29.3 ± 1.6	8.8 ± 1.6	19.5	7.4 ± 1.1
Histidine	2.3 ± 0.4	1.6	9.6	1.7 ± 0.2	5.8 ± 1.4	16.9 ± 1.0	3.5 ± 0.7	9.4	3.8 ± 0.5
Leucine + Isoleucine	2.3 ± 0.4	0.8	4.0	NA	5.7 ± 1.6	7.4 ± 0.3	12.5 ± 5.3	9.2	NA
Lysine	5.8 ± 1.3	3.5	16.8	1.5 ± 0.1	14.3 ± 3.0	26.4 ± 6.1	29.0 ± 4.5	26.8	6.2 ± 0.8
Methionine	0.9 ± 0.1	0.5	3.4	0.9 ± 0.1	2.3 ± 0.5	4.5 ± 3.4	2.5 ± 0.5	6.0	4.5 ± 0.6
Ornithine	1.8 ± 0.5	2.2	NA	NA	4.4 ± 1.6	7.6 ± 2.5	2.6 ± 0.5	3.6	NA
Phenylalanine	1.2 ± 0.2	1.1	4.1	1.3 ± 0.2	2.8 ± 0.5	11.2 ± 1.6	3.4 ± 0.9	7.4	11.4 ± 1.0
Proline	2.6 ± 0.5	8.9	7.8	1.2 ± 0.1	6.3 ± 1.0	10.3 ± 1.4	6.8 ± 0.9	14.6	1.8 ± 0.3
Serine	3.0 ± 0.4	2.2	14.1	17.2 ± 2.2	7.3 ± 1.1	9.3 ± 1.8	9.5 ± 1.4	20.3	29.6 ± 3.6
Threonine	5.1 ± 0.7	1.8	NA	2.9 ± 0.4	12.3 ± 2.6	6.1 ± 0.3	8.4 ± 1.2	13.7	43.2 ± 6.2
Tryptophan	0.3 ± 0.1	0.3	<LLOQ	0.8 ± 0.1	0.8 ± 0.2	12.0 ± 1.2	5.9 ± 0.5	10.0	16.5 ± 1.8
Tyrosine	1.2 ± 0.3	1.0	4.6	2.1 ± 0.2	2.8 ± 0.5	57.1 ± 11.1	4.9 ± 0.8	8.8	2.4 ± 0.2
Valine	1.3 ± 0.2	0.9	4.7	7.7 ± 1.1	3.1 ± 0.8	10.7 ± 0.9	5.4 ± 1.5	11.6	23.6 ± 3.4
Polyamines									
	Quantified (n = 6)				Quantified (n = 6)	[70] (n = 6)	Quantified (n = 5)	[71] (n = 19)	
Putrescine	12.2 ± 0.7	NR			30.4 ± 5.1	50.5 ± 13.7	0.06 ± 0.01	0.09 ± 0.005	
Spermidine	3.4 ± 1.5	NR			8.3 ± 2.8	48.8 ± 15.1	0.21 ± 0.09	0.4 ± 0.03	
Spermine	0.75 ± 0.5	NR			1.9 ± 1.7	0.6 ± 0.1	0.03 ± 0.01	0.09 ± 0.01	

Data represent mean ± SEM; NA, not available; ND, not detected; NR, not reported; LLOQ, low limit of quantification. Experimental concentrations were measured by the LC-MS/MS methods with the mixed-mode IS strategy. Reported concentrations are expressed with the changed unit from the concentrations previously reported.

urine samples; the concentrations of amino acids ranged from 0.3 μg/mL (0.8 μg/mg$_{creatinine}$, tryptophan) to 182 μg/mL (457.1 μg/mg$_{creatinine}$, glycine) and those of polyamines were 12.2 μg/mL (30.4 μg/mg$_{creatinine}$, putrecine), 3.4 μg/mL (8.3 μg/mg$_{creatinine}$, spemidine), and 0.75 μg/mL (1.9 μg/mg$_{creatinine}$, spermine). In plasma, the mean concentrations of amino acids ranged from 1.8 μg/mL (cysteine) to 43.2 μg/mL (glutamine), except for aspartic acid and GABA, whose concentrations were below the LLOQ value. The concentrations of polyamines were 0.06 μg/mL (putrescine), 0.21 μg/mL (spermidine), and 0.03 μg/mL (spermine). Several previous studies also reported the mean concentrations of amino acids in rat urine ranging as follows: 0.3 μg/mL (tryptophan) to 132.7 μg/mL (alanine) [67]; <LLOQ (tryptophan) to 34.1 μg/mL (glutamine) [68]; 0.8 μg/mL (tryptophan) to 17.2 μg/mL (serine) [2]; 4.1 μg/mL$_{creatinine}$ (GABA) to 57.1 μg/mL$_{creatinine}$ (tyrosine) [69]. The variation in the reported concentrations of the same amino acid across the different studies can be attributed to biological and/or analytical methodological differences. Factors like the timing and conditions of urine collection, as well as variables such as water intake, urine volume, and flow rate [72], can contribute to variations in urinary metabolite concentrations. Furthermore, despite the use of a surrogate matrix (e.g., water), the parallelism between the surrogate matrix and rat urine was not evaluated in the previous studies [68]. This oversight could lead to inaccuracies in the quantitative results. However, the comparison between the control and treatment groups should remain unaffected. Therefore, it is essential to consider these factors when planning and interpreting data from urinary metabolomics studies in animals.

According to previous studies, the mean concentrations of amino acids in rat plasma ranged from ND (GABA) to 68.8 μg/mL (glutamine) [67] and from 1.8 μg/mL (proline) to 43.2 μg/mL (tryptophan) [2]. In addition to potential technical variations, normal plasma amino acid concentrations can vary due to factors such as diet, stress, and age [73,74]. Cysteine is a semi-essential amino acid necessary for protein synthesis, being the precursor of glutathione, a major antioxidant molecule in the body [75,76]. However, previous studies on amino acid profiling have not included quantification of cysteine. This is likely due to its potential degradation caused by the oxidation–reduction activity during the analytical process. Therefore, previous studies often employed chemical derivatization to protect the thiol group of cysteine [77,78]. According to a previous study, the concentration of non-derivatized cysteine consistently decreased during LC-MS/MS analysis [78]. In this study, benzoylation of the thiol group using BzCl could ensure both the stability and accurate quantification of cysteine.

Our results indicated that the concentrations of most amino acids (except for glycine and three polyamines) were higher in plasma than in urine. It is well known that the urinary metabolite profile reflects the final metabolic processes within the body and often exhibits higher concentrations compared to those found in the blood [79]. However, the reabsorption of amino acids in the kidney proximal tubule could produce urine amino acid profiles with lower concentrations than those in plasma [80,81]. This emphasizes the need for additional considerations when using amino acids and polyamines for diagnosing diseases in both normal and pathological states.

4. Conclusions

The use of analyte standards derivatized using an isotopic reagent as ISs remains prevalent in metabolic profiling due to its methodological simplicity and cost-effectiveness. Despite the several advantages, the use

of isotope-coded derivatization reagents in chemical derivatization-assisted LC-MS/MS analysis can lead to unexpected matrix effects. This study rigorously validated the feasibility of using the mixed-mode IS approach to mitigate such matrix effects. The enhanced parallelism between the authentic and surrogate matrices, along with the improved ion suppression of matrix effects confirmed the validation of the presented method. The developed method is both sensitive and accurate for quantifying amino acids and polyamines in rat urine and plasma samples. Consequently, this approach could be immensely useful for future studies on metabolic disruptions caused by diseases or abnormal conditions *in vivo*.

CRediT authorship contribution statement

Mingyu Kim: Writing – original draft, Visualization, Methodology, Formal analysis. **Sang-Hoon Song:** Writing – original draft, Visualization, Methodology, Formal analysis. **Suji Kim:** Investigation, Formal analysis. **Ye Jin Jung:** Investigation, Formal analysis. **Sooyeun Lee:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (RS-2023-00242428 and RS-2024-00459985).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2025.113063.

Data availability

Data will be made available on request.

References

[1] M. Nakatsukasa, C. Sotozono, K. Shimbo, N. Ono, H. Miyano, A. Okano, J. Hamuro, S. Kinoshita, Amino acid profiles in human tear fluids analyzed by high-performance liquid chromatography and electrospray ionization tandem mass spectrometry, 799–808 e1, Am. J. Ophthalmol. 151 (2011), https://doi.org/10.1016/j.ajo.2010.11.003.

[2] Y. Wen, X. Yuan, F. Qin, L. Zhao, Z. Xiong, Development and validation of a hydrophilic interaction ultra-high-performance liquid chromatography–tandem mass spectrometry method for rapid simultaneous determination of 19 free amino acids in rat plasma and urine, Biomed. Chromatogr. 33 (2019), https://doi.org/10.1002/bmc.4387.

[3] V. Righi, O.C. Andronesi, D. Mintzopoulos, P.M. Black, A.A. Tzika, High-resolution magic angle spinning magnetic resonance spectroscopy detects glycine as a biomarker in brain tumors, Int. J. Oncol. 36 (2010) 301–306, https://doi.org/10.3892/ijo_00000500.

[4] P. Gao, C. Zhou, L. Zhao, G. Zhang, Y. Zhang, Tissue amino acid profile could be used to differentiate advanced adenoma from colorectal cancer, J. Pharm. Biomed. Anal. 118 (2016) 349–355, https://doi.org/10.1016/j.jpba.2015.11.007.

[5] A. Synakiewicz, A. Stanislawska-Sachadyn, M. Sawicka-Zukowska, G. Galezowska, J. Ratajczyk, A. Owczarzak, M. Skuza, L. Wolska, T. Stachowicz-Stencel, Plasma free amino acid profiling as metabolomic diagnostic and prognostic biomarker in paediatric cancer patients: a follow-up study, Amino Acids 53 (2021) 133–138, https://doi.org/10.1007/s00726-020-02910-8.

[6] J. Zhang, X. Wen, Y. Li, J. Zhang, X. Li, C. Qian, Y. Tian, R. Ling, Y. Duan, Diagnostic approach to thyroid cancer based on amino acid metabolomics in saliva by ultra-performance liquid chromatography with high resolution mass spectrometry, Talanta 235 (2021), https://doi.org/10.1016/j.talanta.2021.122729.

[7] T.J. Wang, M.G. Larson, R.S. Vasan, S. Cheng, E.P. Rhee, E. McCabe, G.D. Lewis, C. S. Fox, P.F. Jacques, C. Fernandez, C.J. O'Donnell, S.A. Carr, V.K. Mootha, J. C. Florez, A. Souza, O. Melander, C.B. Clish, R.E. Gerszten, Metabolite profiles and the risk of developing diabetes, Nat. Med. 17 (2011) 448–453, https://doi.org/10.1038/nm.2307.

[8] M. Magnusson, G.D. Lewis, U. Ericson, M. Orho-Melander, B. Hedblad, G. Engström, G. Östling, C. Clish, T.J. Wang, R.E. Gerszten, O. Melander, A diabetes-predictive amino acid score and future cardiovascular disease, Eur. Heart J. 34 (2013) 1982–1989, https://doi.org/10.1093/eurheartj/ehs424.

[9] E. Socha, M. Koba, P. Kośliński, Amino acid profiling as a method of discovering biomarkers for diagnosis of neurodegenerative diseases, Amino Acids 51 (2019) 367–371, https://doi.org/10.1007/s00726-019-02705-6.

[10] S. Kim, W.-J. Jang, H. Yu, I.S. Ryu, C.-H. Jeong, S. Lee, Integrated non-targeted and targeted metabolomics uncovers dynamic metabolic effects during short-term abstinence in methamphetamine self-administering rats, J. Proteome Res. 18 (2019) 3913–3925, https://doi.org/10.1021/acs.jproteome.9b00363.

[11] M.J. Seo, S.-H. Song, S. Kim, W.J. Jang, C.-H. Jeong, S. Lee, Mass spectrometry-based metabolomics in hair from current and former patients with methamphetamine use disorder, Arch. Pharm. Res. 44 (2021) 890–901, https://doi.org/10.1007/s12272-021-01353-3.

[12] C. Madeira, M.V. Lourenco, C. Vargas-Lopes, C.K. Suemoto, C.O. Brandão, T. Reis, R.E. Leite, J. Laks, W. Jacob-Filho, C.A. Pasqualucci, L.T. Grinberg, S.T. Ferreira, R. Panizzutti, d-serine levels in Alzheimer's disease: implications for novel biomarker development, Transl. Psychiatry 5 (2015) e561–e, https://doi.org/10.1038/tp.2015.52.

[13] T. Kimura, A. Hesaka, Y. Isaka, Utility of d-serine monitoring in kidney disease, Biochim. Biophys. Acta Proteins Proteom. 1868 (2020), https://doi.org/10.1016/j.bbapap.2020.140449.

[14] J. Li, J. Li, H. Wang, L.-W. Qi, Y. Zhu, M. Lai, Tyrosine and glutamine-leucine are metabolic markers of early-stage colorectal cancers, 257–259 e5, Gastroenterology 157 (2019), https://doi.org/10.1053/j.gastro.2019.03.020.

[15] B. Zeydan, K. Kantarci, Decreased glutamine and glutamate: an early biomarker of neurodegeneration, Int. Psychogeriatr. 33 (2021) 1–2, https://doi.org/10.1017/S1041610219001807.

[16] S. Saiki, Y. Sasazawa, M. Fujimaki, K. Kamagata, N. Kaga, H. Taka, Y. Li, S. Souma, T. Hatano, Y. Imamichi, N. Furuya, A. Mori, Y. Oji, S.I. Ueno, S. Nojiri, Y. Miura, T. Ueno, M. Funayama, S. Aoki, N. Hattori, A metabolic profile of polyamines in Parkinson disease: a promising biomarker, Ann. Neurol. 86 (2019) 251–263, https://doi.org/10.1002/ana.25516.

[17] M.K. Venäläinen, A.N. Roine, M.R. Häkkinen, J.J. Vepsäläinen, P.S. Kumpulainen, M.S. Kiviniemi, T. Lehtimäki, N.K. Oksala, T.K. Rantanen, Altered polyamine profiles in colorectal cancer, Anticancer Res. 38 (2018) 3601–3607, https://doi.org/10.21873/anticanres.12634.

[18] S.I. Nissinen, M. Venäläinen, P. Kumpulainen, A. Roine, M.R. Häkkinen, J. Vepsäläinen, N. Oksala, T. Rantanen, Discrimination between pancreatic cancer, pancreatitis and healthy controls using urinary polyamine panel, Cancer Control 28 (2021), https://doi.org/10.1177/10732748211039762.

[19] B.C. DeFelice, O. Fiehn, P. Belafsky, C. Ditterich, M. Moore, M. Abouyared, A. M. Beliveau, D.G. Farwell, A.F. Bewley, S.M. Clayton, J.A. Archard, J. Pavlic, S. Rao, M. Kuhn, P. Deng, J. Halmai, K.D. Fink, A.C. Birkeland, J.D. Anderson, Polyamine metabolites as biomarkers in head and neck cancer biofluids, Diagnostics (Basel). 12 (2022) 797, https://doi.org/10.3390/diagnostics12040797.

[20] N. Shanaiah, M.A. Desilva, G.A. Nagana Gowda, M.A. Raftery, B.E. Hainline, D. Raftery, Class selection of amino acid metabolites in body fluids using chemical derivatization and their enhanced 13C NMR, Proc. Natl Acad. Sci. U. S. A. 104 (2007) 11540–11544. https://doi.org/10.1073/pnas.0704449104.

[21] M.P. Lorenzo, D. Dudzik, E. Varas, M. Gibellini, M. Skotnicki, M. Zorawski, W. Zarzycki, F. Pellati, A. García, Optimization and validation of a chiral GC–MS method for the determination of free d-amino acids ratio in human urine: application to a Gestational diabetes mellitus study, J. Pharm. Biomed. Anal. 107 (2015) 480–487, https://doi.org/10.1016/j.jpba.2015.01.015.

[22] T. Sakamoto, Z. Qiu, M. Inagaki, K. Fujimoto, Simultaneous amino acid analysis based on 19F NMR using a modified OPA-derivatization method, Anal. Chem. 92 (2020) 1669–1673, https://doi.org/10.1021/acs.analchem.9b05311.

[23] M.-Z. Peng, Y.-N. Cai, Y.-X. Shao, L. Zhao, M.-Y. Jiang, Y.-T. Lin, X. Yin, H.-Y. Sheng, L. Liu, Simultaneous quantification of 48 plasma amino acids by liquid chromatography-tandem mass spectrometry to investigate urea cycle disorders, Clin. Chim. Acta 495 (2019) 406–416, https://doi.org/10.1016/j.cca.2019.05.011.

[24] E. Hanff, S. Ruben, M. Kreuzer, A. Bollenbach, A.A. Kayacelebi, A.M. Das, F. von Versen-Höynck, C. von Kaisenberg, D. Haffner, S. Ückert, D. Tsikas, Development and validation of GC–MS methods for the comprehensive analysis of amino acids in plasma and urine and applications to the HELLP syndrome and pediatric kidney transplantation: evidence of altered methylation, transamidination, and arginase activity, Amino Acids 51 (2019) 529–547, https://doi.org/10.1007/s00726-018-02688-w.

[25] A. Desmons, E. Thioulouse, J.-Y. Hautem, A. Saintier, B. Baudin, A. Lamazière, C. Netter, F. Moussa, Direct liquid chromatography tandem mass spectrometry analysis of amino acids in human plasma, J. Chromatogr. A 1622 (2020), https://doi.org/10.1016/j.chroma.2020.461135.

[26] Z. Liu, M.-J. Tu, C. Zhang, J.L. Jilek, Q.-Y. Zhang, A.-M. Yu, A reliable LC-MS/MS method for the quantification of natural amino acids in mouse plasma: method validation and application to a study on amino acid dynamics during hepatocellular carcinoma progression, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 1124 (2019) 72–81, https://doi.org/10.1016/j.jchromb.2019.05.039.

[27] R.S. Carling, K. John, R. Churchus, C. Turner, R.N. Dalton, Validation of a rapid, comprehensive and clinically relevant amino acid profile by underivatised liquid chromatography tandem mass spectrometry, Clin. Chem. Lab. Med. 58 (2020) 758–768, https://doi.org/10.1515/cclm-2019-0604.

[28] M. Mierzchała-Pasierb, M. Lipińska-Gediga, M.G. Fleszar, Ł. Lewandowski, P. Serek, S. Płaczkowska, M. Krzystek-Korpacka, An analysis of urine and serum amino acids in critically ill patients upon admission by means of targeted LC–MS/MS: a preliminary study, Sci. Rep. 11 (2021) 19977, https://doi.org/10.1038/s41598-021-99482-8.
[29] K. Guo, L. Li, Differential 12C-/13C-isotope dansylation labeling and fast liquid chromatography/mass spectrometry for absolute and relative quantification of the metabolome, Anal. Chem. 81 (2009) 3919–3932, https://doi.org/10.1021/ac900166a.
[30] H.-L. Cai, R.-H. Zhu, H.-D. Li, Determination of dansylated monoamine and amino acid neurotransmitters and their metabolites in human plasma by liquid chromatography–electrospray ionization tandem mass spectrometry, Anal. Biochem. 396 (2010) 103–111, https://doi.org/10.1016/j.ab.2009.09.015.
[31] P. Uutela, R.A. Ketola, P. Piepponen, R. Kostiainen, Comparison of different amino acid derivatives and analysis of rat brain microdialysates by liquid chromatography tandem mass spectrometry, Anal. Chim. Acta. 633 (2009) 223–231, https://doi.org/10.1016/j.aca.2008.11.055.
[32] U. Harder, B. Koletzko, W. Peissner, Quantification of 22 plasma amino acids combining derivatization and ion-pair LC–MS/MS, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 879 (2011) 495–504, https://doi.org/10.1016/j.jchromb.2011.01.010.
[33] M. Casado, C. Sierra, M. Batllori, R. Artuch, A. Ormazabal, A targeted metabolomic procedure for amino acid analysis in different biological specimens by ultra-high-performance liquid chromatography–tandem mass spectrometry, Metabolomics 14 (2018) 76, https://doi.org/10.1007/s11306-018-1374-4.
[34] A. Önal, S.E.K. Tekkeli, C. Önal, A review of the liquid chromatographic methods for the determination of biogenic amines in foods, Food Chem. 138 (2013) 509–515, https://doi.org/10.1016/j.foodchem.2012.10.056.
[35] P.A. Malec, M. Oteri, V. Inferrera, F. Cacciola, L. Mondello, R.T. Kennedy, Determination of amines and phenolic acids in wine with benzoyl chloride derivatization and liquid chromatography–mass spectrometry, J. Chromatogr. A 1523 (2017) 248–256, https://doi.org/10.1016/j.chroma.2017.07.061.
[36] X. Meng, H. Bai, Q. Ma, P. Zhang, H. Ma, Y. Deng, Broad targeted analysis of neurochemicals in rat serum using liquid chromatography tandem mass spectrometry with chemical derivatization, J. Sep. Sci. 43 (2020) 4006–4017, https://doi.org/10.1002/jssc.202000709.
[37] J.-M.T. Wong, P.A. Malec, O.S. Mabrouk, J. Ro, M. Dus, R.T. Kennedy, Benzoyl chloride derivatization with liquid chromatography–mass spectrometry for targeted metabolomics of neurochemicals in biological samples, J. Chromatogr. A 1446 (2016) 78–90, https://doi.org/10.1016/j.chroma.2016.04.006.
[38] B. Widner, M.C. Kido Soule, F.X. Ferrer-González, M.A. Moran, E.B. Kujawinski, Quantification of amine- and alcohol-containing metabolites in saline samples using pre-extraction benzoyl chloride derivatization and ultrahigh performance liquid chromatography tandem mass spectrometry (UHPLC MS/MS), Anal. Chem. 93 (2021) 4809–4817, https://doi.org/10.1021/acs.analchem.0c03769.
[39] P. Song, O.S. Mabrouk, N.D. Hershey, R.T. Kennedy, In vivo neurochemical monitoring using benzoyl chloride derivatization and liquid chromatography-mass spectrometry, Anal. Chem. 84 (2012) 412–419, https://doi.org/10.1021/ac202794q.
[40] O. Özdestan, A. Üren, A method for benzoyl chloride derivatization of biogenic amines for high performance liquid chromatography, Talanta 78 (2009) 1321–1326, https://doi.org/10.1016/j.talanta.2009.02.001.
[41] J.M. Cox, J.P. Butler, B.S. Lutzke, B.A. Jones, J.E. Buckholz, R. Biondolillo, J. A. Talbot, E. Chernet, K.A. Svensson, B.L. Ackermann, A validated LC-MS/MS method for neurotransmitter metabolite analysis in human cerebrospinal fluid using benzoyl chloride derivatization, Bioanalysis 7 (2015) 2461–2475, https://doi.org/10.4155/bio.15.170.
[42] T.-Y. Zhang, S. Li, Q.-F. Zhu, Q. Wang, D. Hussain, Y.-Q. Feng, Derivatization for liquid chromatography-electrospray ionization-mass spectrometry analysis of small-molecular weight compounds, TrAC Trends Anal. Chem. 119 (2019), https://doi.org/10.1016/j.trac.2019.07.019.
[43] A. Kovac, Z. Somikova, N. Zilka, M. Novak, Liquid chromatography-tandem mass spectrometry method for determination of panel of neurotransmitters in cerebrospinal fluid from the rat model for tauopathy, Talanta 119 (2014) 284–290, https://doi.org/10.1016/j.talanta.2013.10.027.
[44] A.K. Hewavitharana, N.S. Abu Kassim, P.N. Shaw, Standard addition with internal standardisation as an alternative to using stable isotope labelled internal standards to correct for matrix effects-Comparison and validation using liquid chromatography- tandem mass spectrometric assay of vitamin D, J. Chromatogr. A 1553 (2018) 101–107, https://doi.org/10.1016/j.chroma.2018.04.026.
[45] A. Tan, S. Hussain, A. Musuku, R. Massé, Internal standard response variations during incurred sample analysis by LC-MS/MS: case by case trouble-shooting, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877 (2009) 3201–3209, https://doi.org/10.1016/j.jchromb.2009.08.019.
[46] A.K. Hewavitharana, Matrix matching in liquid chromatography-mass spectrometry with stable isotope labelled internal standards–is it necessary? J. Chromatogr. A 1218 (2011) 359–361, https://doi.org/10.1016/j.chroma.2010.11.047.
[47] A. De Nicolò, M. Cantù, A. D'Avolio, Matrix effect management in liquid chromatography mass spectrometry: the internal standard normalized matrix effect, Bioanalysis 9 (2017) 1093–1105, https://doi.org/10.4155/bio-2017-0059.
[48] M.H. El-Maghrabey, N. Kishikawa, N. Kuroda, Current trends in isotope-coded derivatization liquid chromatographic-mass spectrometric analyses with special emphasis on their biomedical application, Biomed. Chromatogr. 34 (2020), https://doi.org/10.1002/bmc.4756.
[49] P. Bruheim, H.F.N. Kvitvang, S.G. Villas-Boas, Stable isotope coded derivatizing reagents as internal standards in metabolite profiling, J. Chromatogr. A 1296 (2013) 196–203, https://doi.org/10.1016/j.chroma.2013.03.072.
[50] Y. Fu, Z. Zhou, Y. Li, X. Lu, C. Zhao, G. Xu, High-sensitivity detection of biogenic amines with multiple reaction monitoring in fish based on benzoyl chloride derivatization, J. Chromatogr. A 1465 (2016) 30–37, https://doi.org/10.1016/j.chroma.2016.08.067.
[51] T. Li, R. Wang, P. Wang, The development of an ultra-performance liquid chromatography-tandem mass spectrometry method for biogenic amines in fish samples, Molecules 28 (2022) 184, https://doi.org/10.3390/molecules28010184.
[52] F.T. Peters, O.H. Drummer, F. Musshoff, Validation of new methods, Forensic Sci. Int. 165 (2007) 216–224, https://doi.org/10.1016/j.forsciint.2006.05.021.
[53] B.R. Jones, G.A. Schultz, J.A. Eckstein, B.L. Ackermann, Surrogate matrix and surrogate analyte approaches for definitive quantitation of endogenous biomolecules, Bioanalysis 4 (2012) 2343–2356, https://doi.org/10.4155/bio.12.200.
[54] C. Hess, K. Sydow, T. Kueting, M. Kraemer, A. Maas, Considerations regarding the validation of chromatographic mass spectrometric methods for the quantification of endogenous substances in forensics, Forensic Sci. Int. 283 (2018) 150–155, https://doi.org/10.1016/j.forsciint.2017.12.019.
[55] M.M. Khamis, D.J. Adamko, A. El-Aneed, Strategies and challenges in method development and validation for the absolute quantification of endogenous biomarker metabolites using liquid chromatography-tandem mass spectrometry, Mass Spectrom. Rev. 40 (2021) 31–52, https://doi.org/10.1002/mas.21607.
[56] S. Kim, M.S. Lee, M. Kim, B.J. Ko, H.S. Lee, S. Lee, Derivatization-assisted LC-MS/MS method for simultaneous quantification of endogenous gamma-hydroxybutyric acid and its metabolic precursors and products in human urine, Anal. Chim. Acta 1194 (2022), https://doi.org/10.1016/j.aca.2021.339401.
[57] M. Kim, S. Oh, S. Kim, M. Ji, B. Choi, J.W. Bae, Y.S. Lee, M.J. Paik, S. Lee, Alcohol perturbed locomotor behavior, metabolism, and pharmacokinetics of gamma-hydroxybutyric acid in rats, Biomed. Pharmacother. 164 (2023), https://doi.org/10.1016/j.biopha.2023.114992.
[58] M. Niwa, M. Watanabe, N. Watanabe, Chemical derivatization in LC-MS bioanalysis: current & future challenges, Bioanalysis 7 (2015) 2443–2449, https://doi.org/10.4155/bio.15.177.
[59] S. Zhao, L. Li, Chemical derivatization in LC-MS-based metabolomics study, TrAC Trends Anal. Chem. 131 (2020), https://doi.org/10.1016/j.trac.2020.115988.
[60] R. Jiang, Y. Jiao, F. Xu, Chemical derivatization-based LC–MS for metabolomics: advantages and challenges, Bioanalysis 8 (2016) 1881–1883, https://doi.org/10.4155/bio-2016-0192.
[61] B.-L. Qi, P. Liu, Q.-Y. Wang, W.-J. Cai, B.-F. Yuan, Y.-Q. Feng, Derivatization for liquid chromatography-mass spectrometry, TrAC Trends Anal. Chem. 59 (2014) 121–132, https://doi.org/10.1016/j.trac.2014.03.013.
[62] Y. Zhu, P. Deng, D. Zhong, Derivatization methods for LC–MS analysis of endogenous compounds, Bioanalysis 7 (2015) 2557–2581, https://doi.org/10.4155/bio.15.183.
[63] P. Deng, Y. Zhan, X. Chen, D. Zhong, Derivatization methods for quantitative bioanalysis by LC-MS/MS, Bioanalysis 4 (2012) 49–69, https://doi.org/10.4155/bio.11.298.
[64] T. Toyo'oka, LC-MS determination of bioactive molecules based upon stable isotope-coded derivatization method, J. Pharm. Biomed. Anal. 69 (2012) 174–184, https://doi.org/10.1016/j.jpba.2012.04.018.
[65] W. Han, L. Li, Matrix effect on chemical isotope labeling and its implication in metabolomic sample preparation for quantitative metabolomics, Metabolomics 11 (2015) 1733–1742, https://doi.org/10.1007/s11306-015-0826-3.
[66] S.S. Iyer, Z.P. Zhang, G.E. Kellogg, H.T. Karnes, Evaluation of deuterium isotope effects in normal-phase LC-MS-MS separations using a molecular modeling approach, J. Chromatogr. Sci. 42 (2004) 383–387, https://doi.org/10.1093/chromsci/42.7.383.
[67] E. Takach, T. O'Shea, H. Liu, High-throughput quantitation of amino acids in rat and mouse biological matrices using stable isotope labeling and UPLC-MS/MS analysis, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 964 (2014) 180–190, https://doi.org/10.1016/j.jchromb.2014.04.043.
[68] N. Gray, R.S. Plumb, I.D. Wilson, J.K. Nicholson, A validated UPLC-MS/MS assay for the quantification of amino acids and biogenic amines in rat urine, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 1106–1107 (2019) 50–57, https://doi.org/10.1016/j.jchromb.2018.12.028.
[69] C. Seo, M. Na, J. Jang, M. Park, B. Choi, S. Lee, M.J. Paik, Monitoring of altered amino acid metabolic pattern in rat urine following intraperitoneal injection with γ-hydroxybutyric acid, Metabolomics 14 (2018) 111, https://doi.org/10.1007/s11306-018-1409-x.
[70] H.S. Lee, C. Seo, Y.A. Kim, M. Park, B. Choi, M. Ji, S. Lee, M.J. Paik, Metabolomic study of polyamines in rat urine following intraperitoneal injection of γ-hydroxybutyric acid, Metabolomics 15 (2019) 58, https://doi.org/10.1007/s11306-019-1517-2.
[71] N. de Vera, L. Camón, E. Martínez, Blood polyamines in the rat, J. Pharm. Pharmacol. 47 (1995) 204–205, https://doi.org/10.1111/j.2042-7158.1995.tb05779.x.
[72] M.A. Fernández-Peralbo, M.D. Luque de Castro, Preparation of urine samples prior to targeted or untargeted metabolomics mass-spectrometry analysis, TrAC Trends Anal. Chem. 41 (2012) 75–85, https://doi.org/10.1016/j.trac.2012.08.011.
[73] C. Obled, M. Arnal, Age-related changes in whole-body amino acid kinetics and protein turnover in rats, J. Nutr. 121 (1991) 1990–1998, https://doi.org/10.1093/jn/121.12.1990.
[74] G. Ventura, S. Le Plenier, N. Neveux, G. Sarfati, L. Cynober, A. Raynaud-Simon, J. P. De Bandt, Effect of age, stress and protein supply on plasma amino acids during

continuous enteral nutrition; a pragmatic study in rats, Clin. Nutr. 40 (2021) 3931–3939, https://doi.org/10.1016/j.clnu.2021.04.045.
[75] R. Dalangin, A. Kim, R.E. Campbell, The role of amino acids in neurotransmission and fluorescent tools for their detection, Int. J. Mol. Sci. 21 (2020) 6197, https://doi.org/10.3390/ijms21176197.
[76] A. Muthuraman, M. Ramesh, S.A. Shaikh, S. Aswinprakash, D. Jagadeesh, Physiological and pathophysiological role of cysteine metabolism in human metabolic syndrome, Drug Metab. Lett. 14 (2021) 177–192, https://doi.org/10.2174/1872312814666211210111820.
[77] G. Chwatko, E. Bald, Determination of cysteine in human plasma by high-performance liquid chromatography and ultraviolet detection after pre-column derivatization with 2-chloro-1-methylpyridinium iodide, Talanta 52 (2000) 509–515, https://doi.org/10.1016/s0039-9140(00)00394-5.
[78] Y. Kawano, M. Shiroyama, K. Kanazawa, Y.A. Suzuki, I. Ohtsu, Development of high-throughput quantitative analytical method for L-cysteine-containing dipeptides by LC-MS/MS toward its fermentative production, AMB Express 9 (2019) 91, https://doi.org/10.1186/s13568-019-0817-2.
[79] B.M. Kapur, K. Aleksa, What the lab can and cannot do: clinical interpretation of drug testing results, Crit. Rev. Clin. Lab. Sci. 57 (2020) 548–585, https://doi.org/10.1080/10408363.2020.1774493.
[80] R. Rossi, S. Danzebrink, K. Linnenbürger, D. Hillebrand, M. Grüneberg, V. Sablitzky, T. Deufel, K. Ullrich, E. Harms, Assessment of tubular reabsorption of sodium, glucose, phosphate and amino acids based on spot urine samples, Acta Paediatr. 83 (1994) 1282–1286, https://doi.org/10.1111/j.1651-2227.1994.tb13017.x.
[81] F. Verrey, D. Singer, T. Ramadan, R.N. Vuille-dit-Bille, L. Mariotta, S.M. R. Camargo, Kidney amino acid transport, Pflugers Arch. 458 (2009) 53–60, https://doi.org/10.1007/s00424-009-0638-2.