ARTICLE | Article in Press

Quadruple isotope dilution versus conventional calibration methods for accurate urinary hippuric acid quantification with a synthesized isotopically labelled material

Süleyman Bodur · Arda Atakol · Kumsal Eroğlu · Ömer Tahir Günkara · Sezgin Bakırdere

Received: 19 August 2026 / Accepted: 23 October 2026
© The Author(s) 2026

Abstract

Hippuric acid is a key biomarker in toxicological and metabolic investigations. This study presents an accurate UHPLC-MS/MS method for the quantification of hippuric acid in urine matrix. A quadruple isotope dilution (ID^4) strategy was utilized to compensate possible matrix effects and variations in calibration and increase accuracy. Hippuric acid–d_5 was synthesized in-house from glycine and benzoyl chloride–d_5, lowering material costs to easily affordable levels. The mass spectrometry and chromatographic parameters were optimized for improved sensitivity and separation. After the optimization experiments, the analytical performance of the UHPLC-MS/MS system was evaluated. The developed method exhibited a wide linear range from 55.80 ng/kg to 530.10 µg/kg, with limit of detection (LOD) of 15.6 ng/kg and limit of quantification (LOQ) of 52.1 ng/kg. The method was validated with a certified reference material and gradient spiked recovery runs in the same matrix, resulting in excellent agreement with the certified value and consistent recoveries. Results were put into comparison with more conventional techniques of external standard (ES) calibration and single–point isotopically labelled standard addition which demonstrated the superiority of the ID^4 method. The proposed method is intended as a candidate reference procedure for hippuric acid in urine, and it can be used to assign values to quality control and calibration materials for routine LC-MS/MS methods. In addition, the in-house synthesis of hippuric acid-d_5 makes this approach affordable for laboratories outside metrology institutes.

Keywords Hippuric acid · Quadruple isotope dilution · UHPLC-MS/MS · Urine

Introduction

Hippuric acid is commonly used as a urine biomarker in occupational toxicology, environmental exposure assessment, and metabolic profiling. It measures both exogenous toluene exposure and endogenous metabolism of dietary polyphenols, making it a useful indicator across different fields of study and clinical testing[1,2]. Urine concentrations of hippuric acid frequently exceed milligrams per liter levels; however, accurate trace-level

This Article in Press is shared early to give you faster access to new research. It is citable and carries a permanent DOI. The final edited version will replace it automatically.



https://doi.org/10.1038/s41598-026-78063-7

Published online: 26 October 2026

measurements are still required. In early exposure circumstances, modest increases in hippuric acid concentrations may occur before the clinical symptoms[3,4]. Moreover, falling levels must be recorded with high accuracy to confirm detoxification. Like any biomarker with both endogenous and exogenous originators, analytical methods that can resolve minor changes in concentration with high fidelity are essential in distinguishing the undesired exposure from naturally produced levels[5,6]. However, hippuric acid levels are strongly influenced by dietary intake[7], and such small differences can only be interpreted reliably when the results obtained in different laboratories. For this reason, routine methods should be traceable to accurate reference measurements.

Liquid chromatography coupled with tandem mass spectroscopy (LC-MS/MS) is a recurring instrument used for analysis of biomarkers including hippuric acid[8–10]. However, urine is a notoriously complicated matrix that can vastly differ in ionic strength, presence of metabolites, and pH levels. These may alter calibration sensitivities and interfere with ionization significantly[11,12]. The conventional method of external standard (ES) calibration is prone to being unreliable in terms of reproducibility and recovery. The isotopically labeled internal standard (IS) addition approach, often called isotope dilution mass spectrometry (IDMS) seems to be a well-established solution to this problem[13–15]. Nevertheless, a single-point IS addition can still perform poorly when dealing with concentration dependent suppression and elevation effects, as matrix factors are rarely constant across the calibration range[16,17]. This can lead to irreproducible results, which can be essentially vital for the likes of long-term studies and interlaboratory comparisons.

To overcome these challenges, the quadruple isotope dilution (ID^4) strategy has been employed in the present study. ID^4 is established as one of the most robust analytical methodologies and it demonstrates improved accuracy, matrix tolerance, and methodological ruggedness[18,19]. Traditional isotopically labelled material (ILM) based IS addition uses a single spike of a selected concentration of the IS to adjust for variabilities caused by the matrix and the analytical system[20,21]. Such an approach requires precise knowledge of the exact isotopic composition and purity of the ILM as well as an extremely careful weighing operation, since the quantification directly depends on comparison with the known concentration of the IS. The ID^4 strategy distinguishes from the traditional method since it employs one sample blend and three calibration blends to build a set of equations that mathematically isolates the analyte concentration. The ID^4 experimental design makes the final result independent of three important factors: the isotopic composition of the sample, the isotopic composition of the IS, and the mass fraction of the IS [22]. This indicates that once an ID^4 method is optimized chromatographically and spectroscopically, it is innately robust and can handle matrix effects, instrument drift, and even modest changes in ILM purity. This strength enables the technique to be quick and easy to operate, even more so than traditional standard addition (SA) procedures with the added benefits of excellent accuracy and robustness[23].

A major limitation of isotope dilution strategies lies in the cost and availability of certified ILMs. These compounds are often considered niche, are commercially sourced at very high prices and pose the challenges imposed by poor availability[24,25]. This limits the accessibility of reference grade quantification to well-funded laboratories and material certification bodies. On the contrary to specialized isotope labeled molecules, simple deuterated building blocks are widely available[26]. In this study, by synthesizing hippuric acid–d_5 in house from glycine and benzoyl chloride–d_5, the effective cost of the synthesized ILM was much lower than the commercial ones, considering catalog prices from the top suppliers and compared with the most affordable of the commercial standards at the time. It should be noted that this is a highly conservative estimate, as it does not factor in the significant logistical and financial overhead associated with purchasing and managing micro-quantities of commercial standards. Embracing the ID^4 strategy alongside the in-house synthesis approach creates a scalable, highly practical framework. This approach enables laboratories to obtain reference-level measurements for applications such as assigning values to in-house quality control materials, calibrators, and proficiency testing samples, while avoiding the high costs and limited availability often associated with commercial ILMs.

The method development focused on optimizing the chromatographic conditions, electrospray ionization efficiency, and multiple reaction monitoring (MRM) transitions for the analyte and its ILM to obtain higher sensitivity and lower detection limits. The method's real-life performance was demonstrated and validated through direct

analysis of the hippuric acid level in a certified reference material (CRM) of urine, yielding results in satisfactory agreement with the certified value. To further assess the recovery performance at different analyte concentrations, which can induce variable matrix effects, an extra validation step was performed using a series of spiked recovery runs in the same CRM matrix, leveraging its authenticity. This dual validation approach confirms the method's robustness, accuracy, and suitability for trace-level biomarker analysis in complex clinical samples. By integrating in-house synthesis with a mathematically resilient calibration strategy, this study establishes a cost-effective and analytically powerful framework for high-precision quantification of hippuric acid in urine samples.

Materials and methods

Chemicals and apparatus

Benzoyl chloride-d_5 (purity = 99%), glycine (purity ≥ 99%), formic acid (purity ≥ 98%), sodium hydroxide (purity ≥ 98%), carbon tetrachloride (purity ≥ 99.5%), hydrochloric acid (37%), and benzoyl chloride (purity ≥ 99%) were bought from Sigma-Aldrich (Darmstadt, Germany), while acetonitrile (purity ≥ 99.9%) was supplied from Supelco (Pennsylvania, United States). Urine certified reference material (CRM, UME CRM 1315)[27], which was employed during the validation of developed method, was supplied by the Scientific and Technological Research Council of Türkiye National Metrology Institute (TÜBİTAK UME). Elga Flex 3 (High Wycombe, United Kingdom) was employed to purify tap water in order to achieve ultrapure water with a resistivity of 18.2 MΩ.cm at 25 °C. Standard and CRM solutions were gravimetrically prepared using an analytical balance (OHAUS, Pioneer PX224/E model).

Instrumentation

An ultra-high performance liquid chromatography (UHPLC, Agilent Infinity II 1260) was integrated with a tandem mass spectrometer (MS/MS, Agilent Ultivo G6465B) to perform chromatographic separation and mass spectrometric detection. For achieving the chromatographic separation, an ACQUITY UPLC® BEH C18 analytical column (1.7 µm of particle size, 2.1 mm of internal diameter and 50 mm of length) was utilized. An isocratic elution consisting of 87.5% of mobile phase A (ultrapure water) and 12.5% mobile phase B (acetonitrile) was employed in this study. In addition, both mobile phases were supplemented with 0.10% (*v/v*) of formic acid to enhance ionization efficiency in positive ionization mode. Additionally, the positive ionization mode was chosen for the entire study to avoid potential urine matrix-related ion interferences, which have been reported in the negative ionization mode in a previous study[10].

A mixed standard solution containing hippuric acid (11.05 mg/kg) and hippuric acid-d_5 (10.64 mg/kg) was directly injected into the MS/MS system using the autosampler part of the LC system to find the optimum values of the MS/MS parameters. During the evaluation of MS/MS conditions, the parameters were systematically optimized to achieve a high signal to noise ratio (S/N) for the analyte and its ILM. The optimum values of fragmentor voltage, collision cell voltage, drying gas flow rate, nebulizer gas pressure, capillary voltage, drying gas temperature and ionization mode, in addition to precursor and product ions for hippuric acid and hippuric acid–d_5 are summarized in Table 1.

Synthesis and characterization of hippuric acid and hippuric acid-d_5

According to the literature, hippuric acid (N-benzoylglycine) was successfully synthesized and subsequently purified[28]. A solution of glycine (1.33 mol/L, 7.5 g) was prepared in 75 mL of 10% NaOH, after which benzoyl chloride (13.4 mL) was added dropwise onto the alkaline glycine solution. The reaction mixture was stirred for 2 h at room temperature to complete the reaction. After the completion of reaction, a small amount of crushed

Table 1 The optimized MS parameters for hippuric acid and hippuric acid-d_5.

Molecule	Precursor ion/product ion for qualitative determination, m/z	Precursor ion/product ion for quantitative determination, m/z	Fragmentor voltage, V	Collision cell voltage, V
Hippuric acid	180.1/105	–	85	16
	–	180.1/77	80	35
Hippuric acid-d_5	185.1/110	–	75	16
	–	185.1/82	80	38
Constant value for each precursor/product ion				
Drying gas flow rate, L/min	12.0			
Nebulizer gas pressure, psi	20.0			
Capillary voltage, V	4800			
Drying gas temperature, ˚C	300			
Ionization mode	Positive			
Dwell time	100 ms			

ice was added into the solution and then concentrated HCl was slowly poured in while stirring until the solution became acidic (pH = 2–3). Next, the precipitates were filtered and then washed stepwise with carbon tetrachloride and cold water to remove residual impurities. In the final step, the white solid product was dried and recrystallized in boiling ultrapure water. The synthesized hippuric acid, which was used in further experiments as a standard material, was characterized by NMR and MS/MS systems. The ^{1}H NMR spectrum (Electronic Supplementary Material (ESM) Figure S1) was recorded in CD_3OD at 500 MHz, showing δ values of 8.05–7.99 (m, 1 H, N-H), 7.87–7.84 (m, 2 H, Ar-H), 7.57–7.52 (m, 1 H, Ar-H), 7.48–7.45 (m, 2 H, Ar-H), 4.10 (s, 2 H, CH_2) ppm. According to the MS spectra, the precursor ion and product ions were monitored as 180.1 (*m/z*, ESM Figure S2 (A)) and 105/77/ (*m/z*, ESM Figure S2 (B)), respectively. The recorded analytical data verified the synthesis of hippuric acid.

In the synthesis of N-benzoyl-d_5-glycine, 7.5 mg (0.10 mmol) of glycine was dissolved in 75 µL of 10% NaOH solution, and then benzoyl chloride-d_5 (14 µL) was slowly added to the mixture. The reaction mixture was stirred on an orbital shaker at room temperature for 2 h. After completion, the resulting precipitate was filtered, yielding yellow crystals. The formation of yellow crystals showed that the obtained product contained impurities. To purify the small amount of product obtained, the crystals were carefully washed with about 3 mL of chloroform. In the final step, white crystals were obtained, which were employed to prepare an isotopically labelled standard solution of hippuric acid.

The synthesized isotopically labelled material was characterized by the MS/MS system. The precursor ion of the synthesized ILM was monitored as 185.1 (*m/z*, ESM Figure S2 (C)) while product ions were recorded as 110/82 (*m/z*, ESM Figure S2 (D)). The obtained mass spectra proved that the synthesis of hippuric acid-d_5 was successfully acquired.

ESM Figure S2 (A and C) shows that the precursor ions of hippuric acid and its ILM (*m/z* = 180.1 and 185.1) correspond to the protonated forms ($[M+H]^+$) while the fragments (*m/z* = 105.0 and 110.0) are generated by cleavage of amide bonds under the applied ESI voltage. In addition, ESM Figure S2 (B and D) presents the fragments generated from the precursor ions of hippuric acid and hippuric acid–d_5 under the collision cell energy and in the presence of nitrogen gas.

Preparation of CRM

A vial of CRM was prepared according to the guideline given by TÜBİTAK National Metrology Institute (UME)[27]. Regarding the guideline, the vial with its inner cap was placed on an analytical balance (4-digit), and then the tare function was applied. Afterward, 2.9 mL of ultrapure water was carefully added onto the lyophilized CRM, and the vial was slightly shaken to complete the dissolution process. In the final step, the vial was weighed nine times

to determine the mean mass of the added ultrapure water with its standard deviation (2.9254 ± 0.0002 g). In addition, a correction formula provided in the guideline ($Certified\ value = \frac{m_{avg}}{m}$, where m_{avg} is the mean value of added water as determined by TÜBİTAK UME and m presents the mass of ultrapure water added in this study) was implemented to adjust the measured values. The prepared CRM solution was diluted 50.9-fold with the mobile phase for use in the application of the standard addition (SA) calibration method and ID^4 strategy. Furthermore, the dilution process for the prepared CRM solution was applied 49.3-fold using the mobile phase for the implementation in both ES and IS calibration methods.

CRM solution used in the ES and IS calibration methods

In the ES and IS calibration methods, 0.25 g of the CRM solution (49.3-fold dilution) was weighed into a vial. After that, 0.03 g of hippuric acid-d_5 (5.33 mg/kg, in the mobile phase) was added to the CRM solution. In the final step, the total amount of the solution was adjusted to 1.0 g by adding the mobile phase. The prepared CRM solution (approximately 200-fold dilution) was analyzed in sextuplicate using the UHPLC-MS/MS system.

CRM solutions employed in the SA calibration method

The CRM solution (50.9-fold dilution) was weighed as 0.25 g into separate vials, and then suitable amounts of hippuric acid standard solution (5.12 mg/kg, prepared in the mobile phase) were added to all vials except the first one. Subsequently, the solutions, which were measured in triplicate (except for the first vial) using the UHPLC-MS/MS system, were made up to 1.0 g with ultrapure water. In addition, the first vial, which was the CRM solution without any standard addition, was analyzed six times by the UHPLC-MS/MS system.

CRM solutions used in the ID^4 approach

In the ID^4 strategy, 2.5 g of the CRM solution (50.9-fold dilution) was weighed into a centrifuge tube and then filled up to 10.0 g with ultrapure water. For the CRM solutions with standard additions, 0.64 g of the CRM solutions was weighed into two separate centrifuge tubes and then proper amounts of hippuric acid standard solutions (10.71 mg/kg) were added for the recovery experiments. Detailed preparations of all CRM solutions and calibration/sample blends are provided in ESM Table S1, S2 and S3.

Design of the quadruple isotope dilution (ID^4) blends

During the implementation of the ID^4 strategy, three different design options can be employed: (1) two calibration and two sample blends, (2) one calibration blend and three sample blends, or three calibration blends and one sample blend. In our study, three calibration blends with different isotope ratios and one sample blend were prepared to apply the ID^4 strategy. The content of hippuric acid in the CRM and spiked CRM solutions was calculated via the equation described by Pagliano et al.[18]. The formula applied in this study (Eq. 1) is presented below and was implemented using Microsoft Excel (Office 365). Additionally, Eqs. 2, 3, 4, and 5 should be substituted into the corresponding parts of Eq. 1 to obtain the complete formula prior to its application.

$$w_X = -\frac{m_{X^{*}-2} \cdot m_{X^{*}-3} \cdot a_1 + m_{X^{*}-1} \cdot m_{X^{*}-3} \cdot a_2 + m_{X^{*}-1} \cdot m_{X^{*}-2} \cdot a_3}{m_{X^{*}-1} \cdot a_1 + m_{X^{*}-2} \cdot a_2 + m_{X^{*}-3} \cdot a_3} \cdot \frac{m_Y\,(XY),\ aq}{m_X\,(XY),\ aq} \tag{1}$$

$$m_{X^{*}-\alpha} = w_{X^{*}-\alpha} \frac{m_{X^{*}\pi(X^{*}Y-\alpha),\,aq}}{m_{Y^{*}\pi(X^{*}Y-\alpha),\,aq}} (\alpha : 1, 2or3) \tag{2}$$

$$a_1 = (r_{XY} - r_{X^{*}Y-1}) \cdot (r_{X^{*}Y-2} - r_{X^{*}Y-3}) \tag{3}$$

$$a_2 = (r_{XY} - r_{X^*Y-2}) \cdot (r_{X^*Y-3} - r_{X^*Y-1}) \tag{4}$$

$$a_3 = (r_{XY} - r_3) \cdot (r_{X^*Y-1} - r_2) \tag{5}$$

Each prepared blend was injected twice into the UHPLC-MS/MS system, and the mean peak area ratio obtained from these duplicate injections was used in the ID^4 strategy. In addition, a bootstrap-like resampling approach was applied to determine the mean and standard deviation values of hippuric acid content in the CRM and spiked CRM solutions. For this calculation, all 81 possible combinations were considered, which arise from the ID^4 design comprising three calibration blends with different isotope ratios and one sample blend which were prepared in triplicate. A graphical representation about the application of ID^4 is given in Fig. 1.

where,

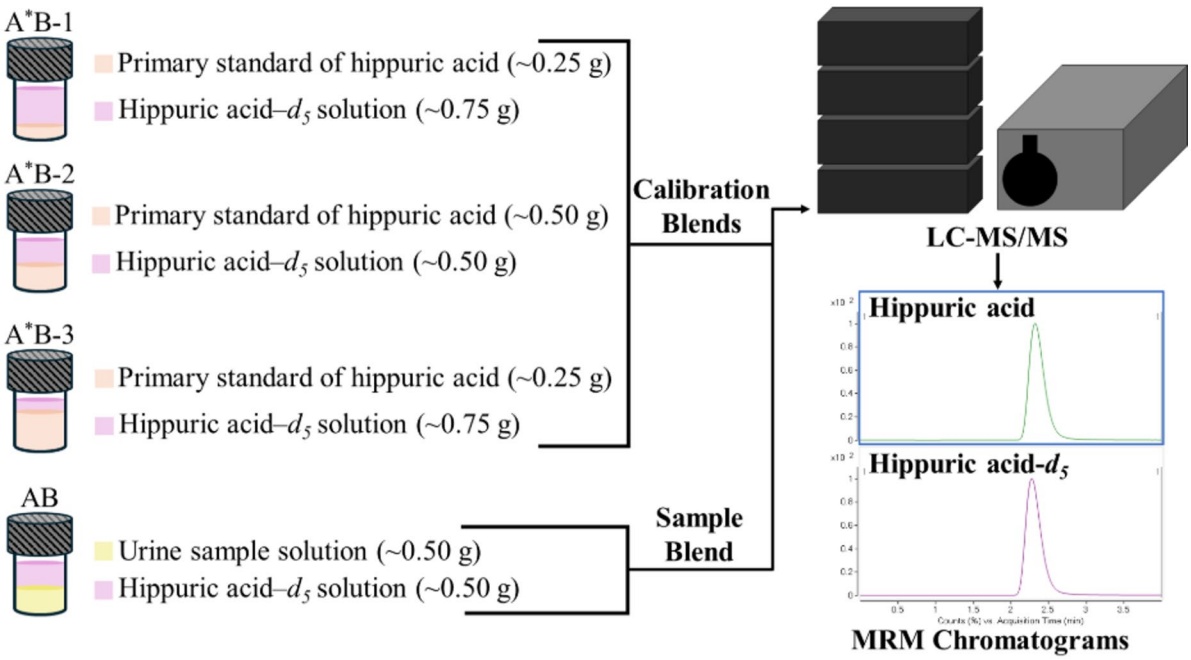


Fig. 1 Graphical representation about the application of ID^4 strategy.

X* Standard solution corresponding to an analyte.

X Analyte in a sample solution.

Y ILM belonging to the analyte.

XY Sample blend consisting of both the analyte and its ILM.

X*Y Calibration blend consisting of the standard solution of the analyte and its ILM.

w_X Mass fraction of the analyte in an aqueous sample solution.

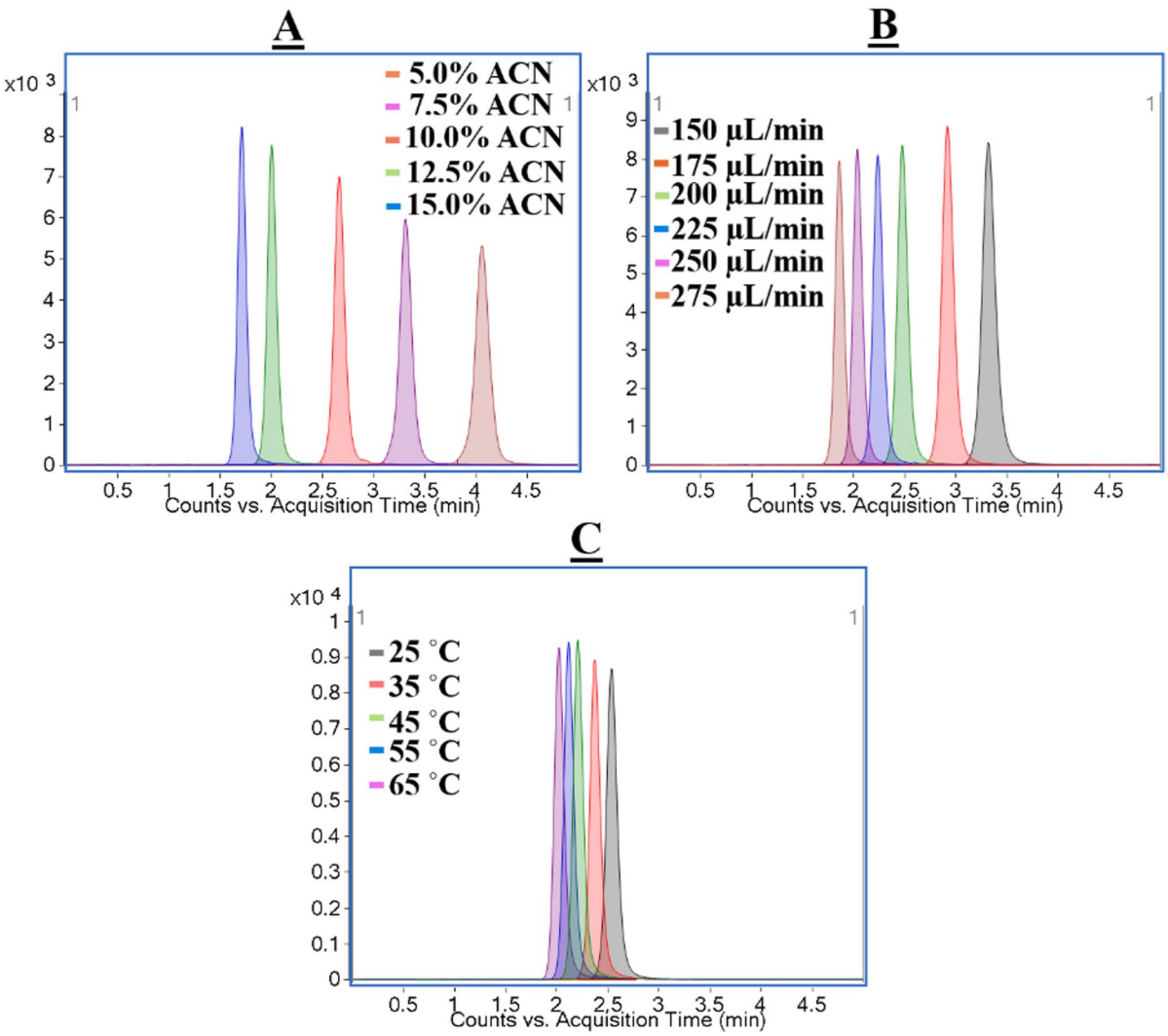


Fig. 2 (**A**) Effect of mobile phase composition (Conditions: 111.64 µg/kg of hippuric acid, 250 µL/min of mobile phase flow rate, 25 °C of column temperature and 10 µL of injection volume), (**B**) Effect of mobile phase flow rate (Conditions: 111.64 µg/kg of hippuric acid, 12.5% of acetonitrile as mobile phase, 25 °C of column temperature and 10 µL of injection volume) and (**C**) Effect of column temperature (Conditions: 111.64 µg/kg of hippuric acid, 12.5% of acetonitrile as mobile phase, 200 µL/min of mobile phase flow rate and 10 µL of injection volume).

$\mathbf{m_x}$ Mass of the aqueous solution used in the preparation of a blend containing either the standard solution (X^*) or the sample solution (X) together with the ILM (Y).

$r_{\propto}$: Peak area ratio of analyte and its ILM ($\propto$: XY or X^*Y).

Results

Optimization of chromatographic conditions

In a chromatographic system, mobile phase composition is one of the most important parameters for achieving suitable retention and selectivity[29]. Generally, acetonitrile based aqueous solutions have been used to perform elution process of hippuric acid from C18 analytical columns in the literature[30,31]. For these reasons, 5.0%, 7.5%, 10.0%, 12.5% and 15% acetonitrile (all mobile phase solutions contained 0.10% (*v/v*) formic acid) were compared to find the optimum mobile phase composition. As shown in Fig. 2 (A), faster elution and an increase in signal intensities were monitored, along with a decrease in peak widths, as the proportion of the organic additive in the mobile phase increased. Although the highest analytical signals were obtained with 15% acetonitrile, 12.5% acetonitrile, which provided signals farther than dead time and produced almost perfectly symmetrical peak shapes, was selected as the optimum mobile phase composition.

It is well-known that mobile phase flow rate is another crucial parameter to eluate analyte(s) within a short analysis period and obtain symmetrical analytical signals[32]. For this reason, six different mobile phase flow rates were tested. Mobile phase flow rates (150, 175, and 200 µL/min) gave almost the same peaks in terms of signal intensity, yet broader peaks were monitored from 150 to 175 µL/min of mobile phase flow rates compared to 200 µL/min. Furthermore, a decrement in signal intensities was observed after 200 µL/min of flow rate (Fig. 2 (B)). The reason for these decreases might be explained by the escape of the formed ions from the cone region in the MS/MS system due to the high flow rates. As a result of this optimization, 200 µL/min of mobile phase flow rate was used in further studies.

Temperature has a significant effect on the viscosity of the mobile phase; therefore, it can influence peak resolution in addition to reducing column backpressure at a constant mobile phase flow rate[33]. To investigate the temperature effects on the peak shape and signal intensity, 25 °C, 35 °C, 45 °C, 55 °C and 65 °C of column temperatures were tried. As a result of this optimization, 45 °C of column temperature was chosen as the optimum one.

Injection volume is one of the most critical parameters in liquid chromatographic methods because it majorly influences signal-to-noise ratio (S/N), peak symmetry, and peak resolution as well as the mass load in the column[34]. For this reason, injection volumes varying from 2.5 µL to 100 µL were tested to identify an efficient injection volume in terms of peak shapes and S/N ratio. As expected, increasing the injection volumes resulted in broader peaks and increments in the S/N, without causing any asymmetry in the peaks (ESM Figure S3). Furthermore, the analytical column used has a total empty volume of approximately 170 µL, and the manufacturer recommends that the injection volume should not exceed 3% of the column volume (approximately 5 µL) for isocratic methods[35]. Based on this technical information, the injection volume should be limited to a maximum of 5 µL to prevent sample overloading. However, injection volumes of 5 µL and 10 µL gave nearly identical peak symmetry and width in our experiments. Therefore, an injection volume of 10 µL was selected as the optimum value, as it provided sufficient S/N for achieving low detection and quantitation limits while also preventing sample overloading and minimizing sample consumption.

Table 2 Recovery results and CRM analysis for hippuric acid determination.

Used approach	Spiked Concentration, µg/kg	%Recovery ± SD	
SA Calibration	59.81	93.5%±2.7*	
	78.41	104.1%±0.6*	
ID^4	148.2	99.5%±1.6**	
	311.2	100.8%±1.1**	
Used approach	Certificated value for hippuric acid, mg/kg	Calculated value for hippuric acid, mg/kg	Statistical evaluation
ES calibration	31.2 ± 1.1	34.09 ± 0.79***	Statistically significant difference was observed between the experimental and certified values.
IS calibration		33.43 ± 0.25***	
SA calibration		30.57 ± 0.46***	
ID^4		31.54 ± 0.57**	No statistically significant difference was observed between the experimental and certified values.

*The arithmetic mean of the concentration or recovery result and its standard deviation were determined from triplicate measurements.

**The arithmetic mean of the concentration or recovery result and its standard deviation were calculated using the bootstrap–like resampling approach (81-combination).

***The arithmetic mean of the concentration and its standard deviation were obtained from sextuplicate measurements.

Evaluation of the analytical performance of the developed method

To evaluate the dynamic range, limit of detection (LOD) and limit of quantitation (LOQ) of the developed LC-MS/MS method, fourteen different hippuric acid standard solutions in the range of 28.09 ng/kg–530.10 µg/kg which are prepared using the optimum mobile phase were injected into the analytical system. A wide dynamic range was observed between 55.80 ng/kg and 530.10 µg/kg (ESM Figure S4 (A)) with a favorable linearity (coefficient of determination ($R^2 = 0.9995$)). While 28.09 ng/kg of hippuric acid standard solution gave the LOD-like signal ($S/N \approx 5$), the LOQ signal (ESM Figure S4 (B)) was recorded from 55.80 ng/kg of hippuric acid standard solution. Additionally, the LOD and LOQ values were estimated as 15.64 ng/kg and 52.14 ng/kg, respectively, using the formulas; $LOD = 3 \cdot \frac{s}{m}$ and $LOQ = 10 \cdot \frac{s}{m}$, where s is the standard deviation of 55.80 ng/kg hippuric acid standard solution ($n = 6$) and m is the slope of the calibration plot shown in ESM Figure S4 (A).

Evaluation of the equilibration period for achieving a stable isotopic ratio

Prior to the implementation of the ID^4 strategy, the equilibration period between the analyte and the corresponding ILM should be optimized to determine the duration over which the isotope ratio remains stable[36,37]. For this purpose, a blend containing 55.16 µg/kg of hippuric acid and 57.46 µg/kg of hippuric acid–d_5 was analyzed using the UHPLC-MS/MS system over a period ranging from 9.5 to 304.5 min, with consecutive injections at 5.0 min intervals. In addition, Tukey's post–hoc test (performed using JASP software version 0.19.0.3) was used to assess the results at a 95% confidence level. The results presented in ESM Figure S5 revealed that there is no need to keep the prepared blends for an extended period prior to analysis.

Recovery experiments

To carry out the spiked recovery experiments using the exact matrix-matching calibration method, both unspiked and spiked CRM solutions were prepared according to the procedure described in Sect. 2.4.2. A peak area correction was implemented to draw the exact matrix–matching calibration plot. The corrected peak areas were calculated by subtracting the mean peak area values of unspiked CRM solutions from the mean peak area values of spiked CRM solutions. For the spiked CRM solutions (59.81 µg/kg and 78.41 µg/kg), the recoveries were determined to be 93.5%±2.7% and 104.1%±0.6%, respectively, based on the exact matrix–matching calibration plot presented in ESM Figure S6.

The preparation of spiked CRM solutions and blends is detailed in Tables S2 and S3. All blends prepared were introduced to the UHPLC-MS/MS system to determine the isotopic ratio using the peak areas of hippuric acid and hippuric acid–d_5. In addition, blends were prepared using the unspiked CRM solution and 0.32/0.42 mg/kg of hippuric acid–d_5 to obtain corrected peak area ratios to accurately calculate the recovery results. The experimental spiked concentrations were calculated via the formula presented in Eq. 1, and the recovery results for the spiked CRM solutions (148.20 µg/kg and 311.20 µg/kg) were found to be 99.5%±1.6 and 100.8%±1.1, respectively.

If the exact matrix–matching calibration method and the ID^4 strategy was compared using the recovery results, the ID^4 strategy presented more accurate analytical results than the other calibration method, as anticipated. Considering the satisfactory recovery results attained from the ID^4 strategy, the developed UHPLC-ID^4-MS/MS method has demonstrated its suitability as a highly reliable alternative for biomonitoring.

CRM analysis

UME CRM 1315 was analyzed with the developed UHPLC-LC-MS/MS to validate its accuracy and applicability for real urine samples. During the analysis of the CRM, the ES, IS, and SA calibration methods, as well as the ID^4 strategy, were compared to determine the most accurate and applicable approach. The calibration plots for the ES, IS, and SA methods used in the CRM analysis are presented in ESM Figure S7 (A, B, and C). All experimental results are summarized in Table 2. All sample dilutions were taken into account when calculating hippuric acid content in the CRM for each approach to determine the final concentration. Furthermore, statistical evaluation was performed using the formula "$t_{experimental} = \frac{\bar{x}-x_t}{\frac{s}{\sqrt{N}}}$, where $\bar{x}$ is the mean of experimental value, x_t is the certified value, $t_{experimental}$ is the experimental t-value at 95% of confidence level, s is the standard deviation of experimental value and N is number of replicates". Additionally, $t_{critical}$ is 2.57 for sextuplicate measurements at 95% of confidence level[38]. In line with the recorded analytical results (Table 2), the ID^4 strategy not only yielded the most accurate result based on the statistical evaluation, but it also demonstrates that highly reliable and remarkable analytical accuracy is attainable for standard clinical and research laboratories without the need for exhaustive cleanup procedures or prohibitively expensive commercial standards.

The ES (34.09 mg/kg), IS (33.43 mg/kg), and SA (30.57 mg/kg) calibration methods gave results that deviated significantly from the certified value, whereas the ID^4 strategy yielded a result (31.54 mg/kg) statistically consistent with the certified value (31.2 mg/kg) without requiring extensive sample cleanup. These deviations may be partly related to matrix-induced signal suppression or enhancement in the complex urine matrix, but the present data do not allow this effect to be distinguished from other possible contributing factors. The agreement obtained with ID^4 demonstrates that this approach provides accurate results for this sample under the applied conditions.

Discussion

Comparison of the developed analytical methodology with the current literature

Damokhi et al. conducted a study on the determination of hippuric acid and methyl hippuric acid in urine samples using a deep eutectic solvent–based dispersive liquid–liquid microextraction (DES-DLLME), coupled with an HPLC system. In the reported study, a multivariate optimization approach was employed to evaluate the optimum conditions for the DES-DLLME procedure. After establishing optimum conditions, analytical performance tests were carried out, and the linear range, LOD, and LOQ values were reported as 24 µg/L–20 mg/L with an R^2 value of 0.9986, 8.0 µg/L, 24 µg/L for hippuric acid, respectively. Additionally, recovery experiments were performed to assess the applicability of the DES-DLLME method, and the recovery results for hippuric acid were found to be between 97% and 103.8% [30].

In another study, a magnetic dispersive micro solid phase extraction (M-DμSPE) method based on Fe_3O_4-SiO_2-EN@Zn-Al-LDH nano sorbent was developed and combined with an HPLC-UV system for the quantification of hippuric acid in urine samples. In this study, the dynamic range was determined to be between 15 µg/L and 500 mg/L, while the LOD and LOQ values were calculated as 5 µg/L and 14 µg/L, respectively[39].

Sarani et al. developed a vortex-assisted dispersive liquid–liquid microextraction (VADLLME) – HPLC–diode array detector (DAD) method for the determination of hippuric acid in urine samples to monitor toluene exposure. A Box-Behnken experimental design was implemented to statistically assess the optimal conditions of the VADLLME method. The method exhibited a dynamic range of 1 µg/L to 900 µg/L, and LOD/LOQ values were calculated as 0.15/0.5 µg/L. Furthermore, recovery results were reported in the range of 93.80%–100.60% [4].

In another study found in the literature, a single-drop liquid–liquid–liquid microextraction method was developed for the preconcentration of hippuric acid from urine and serum samples prior to HPLC-UV analysis. Under the optimized experimental conditions, LOD, LOQ, and linear range were determined as 0.3 µg/L, 1.0 µg/L and 1–400 µg/L, respectively. Moreover, acceptable recovery results, which were between 91.4% and 99.3% for urine and serum samples, were obtained in the result of recovery experiments[40].

When the developed UHPLC-ID^4-MS/MS method was compared with previously reported studies, it exhibited lower LOD and LOQ values. Furthermore, we achieved excellent recovery results for the spiked urine CRM samples, cleanly surpassing those reported in the literature. These recovery results also demonstrated the accuracy and precision of the quantification by compensating possible matrix effects, instrumental fluctuations and sample preparation losses. Moreover, the proposed UHPLC-ID^4-MS/MS method does not require additional sample preparation steps such as preconcentration. Consequently, these findings suggest that the developed method can be regarded as a highly dependable approach that provides near-reference level accuracy for the quantification of hippuric acid in urine samples.

Conclusion

This research demonstrated the integration of the UHPLC-MS/MS system with the ID^4 strategy to formulate a reliable alternative methodology for the quantification of hippuric acid in urine samples with high precision and accuracy. To the best of our knowledge, this study represents the first application of the UHPLC-MS/MS system in combination with the ID^4 strategy, providing a highly accurate and precise approach for the quantification of hippuric acid. All critical UHPLC and MS/MS parameters were elaborately optimized to enhance S/N and achieve low LOD and LOQ values for hippuric acid. Subsequently, the analytical performance of the system was evaluated using aqueous standard solutions of hippuric acid, and the LOD and LOQ values were determined to be 15.64 ng/kg and 52.14 ng/kg, respectively. Furthermore, a conveniently wide dynamic range was observed between 55.80 ng/kg and 530.10 µg/kg with a satisfactory linearity ($R^2 = 0.9995$). Afterward, recovery experiments were carried out using the spiked urine CRM solutions, and the spiked concentrations were calculated employing the exact matrix-matching calibration method and the ID^4 strategy. The exact matrix-matching calibration method yielded acceptable recovery results (93.5%±2.7 and 104.1%±0.6), whereas excellent recovery results (99.5%±1.6 and 100.8%±1.1) were achieved with the ID^4 strategy. Additionally, the analysis of UME CRM 1315 was performed using the ES, IS, and SA calibration methods, as well as the ID^4 strategy. Among these approaches, only the ID^4 strategy prevailed in producing a result (31.54 ± 0.57 mg/kg) statistically consistent with the certified value (31.2 ± 1.1 mg/kg). While the direct analysis of UME CRM 1315 showcased this excellent accuracy, conducting future multi-laboratory validation studies on diverse, uncharacterized matrices will be the logical next step to fully establish the ID^4 approach as a standardized procedure. In light of the current results, the developed UHPLC-ID^4-MS/MS method demonstrates strong potential to serve as a highly accurate alternative and a robust candidate for future reference measurement procedures for quantifying hippuric acid in urine samples. Moreover, the combination of the UHPLC-MS/MS system and the ID^4 strategy can be extended to accurately and precisely quantify other biomarkers in biological fluids following the implementation of appropriate

sample preparation procedures. Furthermore, the starting materials of the ILMs corresponding to the biomarkers to be quantified can be purchased, enabling the in-house synthesis of the ILMs under suitable conditions at a lower cost. This approach allows a reduction in the cost of analyzing most chemicals while achieving high accuracy and precision.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-026-78063-7.

Acknowledgements -.

Author contributions Süleyman Bodur: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft.Arda Atakol: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – original draft.Kumsal Eroğlu: Investigation, Validation.Ömer Tahir Günkara: Investigation, Validation.Sezgin Bakırdere: Conceptualization, Investigation, Methodology, Validation, Supervision, Writing–review & editing.

Funding Not applicable.

Data availability Data will be available upon reasonable request.

Code availability Not applicable.

Declarations

Competing interests The authors declare no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by-nc-nd/4.0/.

References

1. Dehghani, F., Yousefinejad, S., Mobaraki, N., Nekoeinia, M. & Hemmateenejad, B. Rapid determination of hippuric acid as an exposure biomarker of toluene using a colorimetric assay and comparison with high-performance liquid chromatography. *Sci. Rep.* **14**, 9935 (2024).
2. Pérez-Jiménez, J. et al. Urinary metabolites as biomarkers of polyphenol intake in humans: A systematic review. *Am. J. Clin. Nutr.* **92**, 801–809 (2010).
3. Hu, C., Yang, Z., Yan, F. & Sun, B. Extraction of the toluene exposure biomarkers hippuric acid and methylhippuric acid using a magnetic molecularly imprinted polymer, and their quantitation by LC-MS/MS. *Microchim. Acta*. **186**, 135 (2019).
4. Sarani, B., Rahmani, M. & Abbasian, A. R. Estimation of micro-extraction process hippuric acid for toluene exposure monitoring in urine samples. *Sci. Rep.* **15**, 29585 (2025).
5. Boogaard, P. J. & Van Sittert, N. J. Biological monitoring of exposure to benzene: A comparison between S-phenylmercapturic acid, trans,trans-muconic acid, and phenol. *Occup. Environ. Med.* **52**, 611–620 (1995).
6. Ticinesi, A., Guerra, A., Nouvenne, A., Meschi, T. & Maggi, S. Disentangling the Complexity of Nutrition, Frailty and Gut Microbial Pathways during Aging: A Focus on Hippuric Acid. *Nutrients* **15**, 1138 (2023).

7. Penczynski, K. J. et al. Relative validation of 24-h urinary hippuric acid excretion as a biomarker for dietary flavonoid intake from fruit and vegetables in healthy adolescents. *Eur. J. Nutr.* **56**, 757–766 (2017).
8. Chiu, C. H. et al. Use of urinary hippuric acid and o-/p-/m-methyl hippuric acid to evaluate surgical smoke exposure in operating room healthcare personnel. *Ecotoxicol. Environ. Saf.* **217**, 112231 (2021).
9. Ohashi, Y. et al. Simultaneous determination of urinary hippuric acid, o-, m- and p-methylhippuric acids, mandelic acid and phenylglyoxylic acid for biomonitoring of volatile organic compounds by gas chromatography-mass spectrometry. *Anal. Chim. Acta*. **566**, 167–171 (2006).
10. Penner, N., Ramanathan, R., Zgoda Pols, J. & Chowdhury, S. Quantitative determination of hippuric and benzoic acids in urine by LC-MS/MS using surrogate standards. *J. Pharm. Biomed. Anal.* **52**, 534–543 (2010).
11. Bowman, B. A., Ejzak, E. A., Reese, C. M., Blount, B. C. & Bhandari, D. Mitigating Matrix Effects in LC–ESI–MS-MS Analysis of a Urinary Biomarker of Xylenes Exposure. *J. Anal. Toxicol.* **47**, 129–135 (2023).
12. Hopsort, G., Latapie, L., Serrano, G., Loubière, K., Tzedakis, T. & K. & Deciphering the human urine matrix: a new approach to simultaneously quantify the main ions and organic compounds by ion chromatography/mass spectrometry (IC-MS). *Anal. Bioanal Chem.* **415**, 5337–5352 (2023).
13. Chambers, E., Wagrowski-Diehl, D. M., Lu, Z. & Mazzeo, J. R. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *J. Chromatogr. B Analyt Technol. Biomed. Life Sci.* **852**, 22–34 (2007).
14. Meija, J. & Mester, Z. Paradigms in isotope dilution mass spectrometry for elemental speciation analysis. *Anal. Chim. Acta*. **607**, 115–125 (2008).
15. Trufelli, H., Palma, P., Famiglini, G. & Cappiello, A. An overview of matrix effects in liquid chromatography–mass spectrometry. *Mass. Spectrom. Rev.* **30**, 491–509 (2011).
16. Fu, Y., Li, W. & Picard, F. Assessment of matrix effect in quantitative LC-MS bioanalysis. *Bioanalysis* **16**, 631–634 (2024).
17. Matuszewski, B. K., Constanzer, M. L. & Chavez-Eng, C. M. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal. Chem.* **75**, 3019–3030 (2003).
18. Pagliano, E., Mester, Z. & Meija, J. Reduction of measurement uncertainty by experimental design in high-order (Double, triple, and quadruple) isotope dilution mass spectrometry: Application to GC-MS measurement of bromide. *Anal. Bioanal Chem.* **405**, 2879–2887 (2013).
19. Yazıcı, E., Bodur, S., Erarpat, S., Arslan, U. E. & Bakırdere, S. Assessment of external calibration, internal standard calibration and quadruple isotope dilution strategies for the determination of acrylamide in wastewater samples after LC-MS/MS quantification. *Microchem. J.* **190**, 108741 (2023).
20. Wieling, J. LC-MS-MS experiences with internal standards. *Chromatographia* **55**, S107–S113 (2002).
21. Tsikas, D. & Perspectives of Quantitative, G. C. M. S. LC-MS, and ICP-MS in the Clinical Medicine Science—The Role of Analytical Chemistry. *J. Clin. Med.* **13**, 7276 (2024).
22. Pagliano, E., Mester, Z. & Meija, J. Calibration graphs in isotope dilution mass spectrometry. *Anal. Chim. Acta*. **896**, 63–67 (2015).
23. Ular Çağatay, N., Bodur, S., Bodur, S. E., Maviş, M. E. & Bakırdere, S. Combination of quadruple isotope dilution strategy and dispersive solid phase extraction method for accurate quantification of selected steroid hormones. *Anal. Methods*. https://doi.org/10.1039/d4ay01545b (2024).
24. Jaber, M. A. et al. Advantages of using biologically generated 13 C-labelled multiple internal standards for stable isotope-assisted LC-MS-based lipidomics. *Anal. Methods*. **15**, 2925–2934 (2023).
25. Panuwet, P. et al. Biological Matrix Effects in Quantitative Tandem Mass Spectrometry-Based Analytical Methods: Advancing Biomonitoring. *Crit. Rev. Anal. Chem.* **46**, 93–105 (2016).
26. Atzrodt, J., Derdau, V., Kerr, W. J. & Reid, M. Deuterium- and Tritium-Labelled Compounds: Applications in the Life Sciences. *Angew Chem. Int. Ed.* **57**, 1758–1784 (2018).
27. TÜBİTAK National Metrology Institute. (2021). https://rm.ume.tubitak.gov.tr/sertifika/ume_crm_1315_sertifika.pdf?rnd=9/28/2025 5:57:34PM
28. Liu, X. Q. et al. Synthesis and insecticidal evaluation of tetrahydroimidazo[1,2-a]pyridin-5(1H)-one derivatives. *Chin. Chem. Lett.* **27**, 7–10 (2016).
29. Boyes, B. E. & Dong, M. W. Modern trends and best practices in mobile-phase selection in reversed-phase chromatography. *LC-GC Europe*. **31**, 572–583 (2018).
30. Damokhi, A., Yousefinejad, S., Jafari, S., Soleimani, E. & Dehghani, F. Deep eutectic solvent-based dispersive liquid-liquid microextraction coupled with HPLC and central composite design for biological monitoring of hippuric acid and methyl hippuric acid in urine. *J. Mol. Liq.* **386**, 122506 (2023).
31. Yadav, A., Basu, A. & Chakarbarti, A. Method for estimation of hippuric acid as a biomarker of toluene exposure in urine by high-performance liquid chromatography after extraction with ethyl acetate. *Environ. Dis.* **4**, 17 (2019).
32. Guillarme, D., Heinisch, S. & Rocca, J. L. Effect of temperature in reversed phase liquid chromatography. *J. Chromatogr. A*. **1052**, 39–51 (2004).
33. Guillarme, D., Ruta, J., Rudaz, S. & Veuthey, J. L. New trends in fast and high-resolution liquid chromatography: A critical comparison of existing approaches. *Anal. Bioanal Chem.* **397**, 1069–1082 (2010).

34. Werres, T., Schmidt, T. C. & Teutenberg, T. The influence of injection volume on efficiency of microbore liquid chromatography columns for gradient and isocratic elution. *J. Chromatogr. A*. **1641**, 461965 (2021).
35. What is the. maximum injection I can use with the ACQUITY UPLC BEH C18 1.7µm 2.1x100mm column (186002352) without overloading it? - WKB106988 - Waters. https://support.waters.com/KB_Chem/Columns/WKB106988_What_is_the_maximum_injection_volume_I_can_make_on_SKU_186002352_-_ACQUITY_UPLC_BEH_C18_17m_21x100mm_Column_without_overloading_the_column
36. Bates, J., Bahadoor, A., Tittlemier, S. A. & Melanson, J. E. Comparison of calibration strategies for accurate quantitation by isotope dilution mass spectrometry: a case study of ochratoxin A in flour. *Anal. Bioanal Chem.* **416**, 487–496 (2024).
37. Bodur, S. et al. Dispersive solid phase extraction and quadruple isotope dilution–mass spectrometry combination for the accurate and sensitive quantification of capsaicin in pepper, domestic wastewater and human saliva samples by GC–MS system. *Microchem. J.* **208**, 112246 (2025).
38. Skoog, D. A., West, D. M., Holler, F. J. & Crouch, S. R. *Fundamentals of Analytical Chemistry* (Cengage Learning, 2021).
39. Firoozichahak, A., Rahmani, A., Kazemi, M. & Rahimpoor, R. Magnetic layered double hydroxides for the sensitive dispersive solid phase microextraction of hippuric acid in urine samples prior to HPLC-UV analysis. *J. Chromatogr. B Analyt Technol. Biomed. Life Sci.* **1224**, 123761 (2023).
40. Toulabi, P., Daneshfar, A. & Sahrai, R. Determination of hippuric acid in biological fluids using single drop liquid-liquid-liquid microextraction. *Anal. Methods*. **2**, 564–569 (2010).

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Authors and Affiliations

Süleyman Bodur[1,2,3] · Arda Atakol[4] · Kumsal Eroğlu[1] · Ömer Tahir Günkara[1] · Sezgin Bakırdere[1,5]

✉ Sezgin Bakırdere
bsezgin23@yahoo.com

[1] Faculty of Art and Science, Department of Chemistry, Yıldız Technical University, 34220 İstanbul, Türkiye

[2] Faculty of Pharmacy, Department of Analytical Chemistry, İstinye University, 34010 İstanbul, Türkiye

[3] İstinye University, Scientific and Technological Research Application and Research Center, 34010 İstanbul, Türkiye

[4] Faculty of Pharmacy, Department of Analytical Chemistry, İstanbul Kent University, 34406 İstanbul, Türkiye

[5] Turkish Academy of Sciences (TÜBA), Vedat Dalokay Street, No: 112, 06670 Çankaya, Ankara, Türkiye