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Determination of glyphosate and its derivative, aminomethylphosphonic acid, in human urine by gas chromatography coupled to tandem mass spectrometry and isotope pattern deconvolution

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ABSTRACT

An analytical method for the simultaneous determination of glyphosate (GLY) and its main derivative, aminomethyl-phosphonic acid (AMPA), in human urine has been developed using gas chromatography coupled to tandem mass spectrometry (MS/MS) operated in multiple reaction monitoring mode (MRM). Sample preparation involved dilution of urine with water and derivatization with a mixture of trifluoroacetic acid anhydride and trifluoroethanol. Derivatization conditions such as reaction time and temperature, derivative stability, injection solvent, MS ionization mode and MS-MS transitions, among others, were studied to obtain the highest method sensitivity. The target compounds were initially quantified by the isotope dilution method using isotopically labelled analogs of GLY and AMPA as internal standards. However, due to spectral overlap between GLY and labelled GLY in the selected quantitative transition, a quantification method based on isotope pattern deconvolution (IPD) has been developed. The instrumental limits of detection were 0.05 ng mL^{-1} for both compounds, while the method detection limits were 0.39 and 0.25 ng mL^{-1}, for AMPA and GLY, respectively. The mean recoveries from urine and water spiked at different concentrations were 77 and 69% for AMPA and 90 and 102% for GLY, respectively, with mean relative standard deviations of 8–10% (urine samples, $n = 12$) and 3.6–4% (water samples, $n = 6$). Once validated, the feasibility of the method was tested by determination of AMPA and GLY in human urine samples from people living close to agricultural areas. The developed method affords the determination of these compounds at trace concentrations in complex matrices such as urine, avoiding elaborate handling and cleanup steps. Isotope pattern deconvolution has proven to be a successful alternative to calibration curve for GLY overriding the method uncertainties associated to spectral overlapping.

1. Introduction

Glyphosate (N-(phosphonomethyl)glycine)) (GLY) is a broad-spectrum herbicide widely used in agriculture, horticulture, gardening and infrastructure maintenance. This compound is the most frequently used herbicide worldwide especially after the introduction of genetically-modified glyphosate-resistant crops in 1996. The global GLY market size was over 850×10^3 Tons in 2015, with the European marked accounting for above 15% of the total volume, despite the limited used of these genetically modified crops in the EU (Global Market insights, 2018) [1].

The main degradation product of GLY is aminomethylphosphonic acid (AMPA), although this compound can also be formed by breakdown of organic phosphonates in detergents [2]. Despite GLY and AMPA are strongly absorbed to soils [3], they have been found at concentrations of μg L^{-1} in surface water and groundwater [4,5], although the occurrence of AMPA in water has often been attributed to detergent degradation rather than GLY transformation [6].

The identification of more than 20 glyphosate-resistant weed species have been described in different world areas [7], which have required the application of increasing GLY amounts. Recent studies on GLY content in top soils from different European regions have shown that 21% of the studied samples contained GLY, while AMPA was found in 42% of them [8].

The International Agency for Research on Cancer classified GLY as “probable carcinogenic for humans" [9,10]. A significant association between occupational exposure to GLY and cancer was reported in one study in 2014 [11], which raised a lot of concern because of its widespread use. Warnings about potential contamination of plants, soils and water bodies that could represent a risk for human health have

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been recurrently expressed. However, no evidence of human cancer risk increase has been found in non-occupational exposure [12]. In addition, the WHO and FAO Joint committee on pesticide residues reported that the use of GLY does not constitute a health risk for humans (WHO, 2016) [13] and the European Chemicals Agency (ECHA) did not find evidence of carcinogenic, mutagenic or teratogenic effects associated to GLY [14]. The toxic effects of GLY are still pending to be fully elucidated [15].

Human exposure to GLY is generally attributed to the ingestion of foods and feeds that contains residues of this herbicide. However, considering the widespread agricultural and non-agricultural GLY uses, direct exposure during and after application of the GLY formulations may also represent a significant contribution. Urine has been used in many studies to investigate human exposure to ambient contaminants, since it can be obtained in high amounts by noninvasive methods, and is adequate for determination of water-soluble compounds such as GLY and AMPA. Data on the occurrence of GLY and AMPA in human urine has been reported [12,16-21], showing higher levels in USA than in Europe. This difference agrees with the reported uses of GLY-based herbicides in these areas [17]. However, increasing urine GLY concentrations in Europe are being reported [19,22].

These reports highlight the need to develop further biomonitoring, epidemiological and toxicological studies for assessment of the health risks of GLY [23], which require simple, fast and sensitive methods. The analysis of this compound and its metabolite, particularly in polar matrices, is difficult due to their amphoteric character, low volatility, high aqueous solubility and absence of UV chromophores in the molecule. A variety of analytical methods for the determination of AMPA and GLY has been reported [24,25], including direct analysis by HPLC-MS-MS [18,26-30]. They usually include strong acidic mobile phases or hybrid-phase columns [31], which compromise routine analysis [32], therefore most of the reported methods usually involve a derivatization step. The most commonly derivatizing reagent for HPLC analysis is 9-fluorenylmethlychloroformate (FMOC) [32,33], while trifluoroacetic anhydride (TFAA) combined with trifluoroethanol (TFE) or 2,2,3,3,4,4,4-heptafluoro-1-butanol (HFB) [19,34,35] and different alkylsilyl derivatization reagents [36–38] have also been used for gas chromatographic determination.

The maximum concentration of GLY in drinking water set by the EU is 0.1 μg L^{-1} and the urine concentrations of this herbicide range between 0.5–1 μg L^{-1} [19]. Unfortunately, many reported methodologies cannot reach these LODs required for environmental or human biomonitoring studies. Sensitive and selective techniques based on MS-MS detection for the analysis of GLY and AMPA in water have been developed in the last years, either coupled to liquid [4,32,33,39], or gas chromatography [40], which can reach lower LODs. However, the high amounts of salts, urea, and other minor components such as proteins or hormones make urine a tricky matrix for organic compound analysis at trace concentrations. Few studies have developed MS/MS methods for human samples such as breastmilk [41] or urine [18,19,30,42-44].

The addition of the isotopically labelled target analyte standards to the sample and the construction of a calibration curve by measuring the ratio of natural/labelled compounds at different analyte concentrations provides lower LODs than external calibration or standard addition. This Isotope Dilution Mass Spectrometry (IDMS) method compensates for losses during sample pretreatment as well as matrix effects or instrumental instabilities. However, mass overlap between the natural and labelled compounds at their reference masses will result in non-linear calibration curves, therefore, for organic compounds with 7 to 10 Carbon atoms, mass differences of at least 3 mass units between the labelled and natural compounds is required. Unfortunately, increasing the degree of labeling of the internal standard could involve differences in physical chemical properties and/or changes in chromatographic retention times that could modify the behavior of the added standard in comparison with the target compound, mainly when using deuterium derivatives [45]. Alternatively, isotope pattern deconvolution (IPD) provides a good alternative to the conventional isotope dilution quantification [46]. IPD involves the calculation of fractional abundances of the reference mass ions measured in the sample spiked by known amounts of the labelled compound. These abundances can be expressed as a linear combination of the pure isotope pattern of the natural and labelled compounds and their corresponding molar fractions [46], providing the concentration of the analyte in each sample after deconvolution by multiple linear regression. The method does not require a calibration curve and can be applied with minimal labeling [47]. IPD has been successfully applied for the quantification of several organic contaminants in biological and environmental samples [47–49].

In this study, a highly selective and sensitive GC–MS-MS method for the simultaneous analysis of GLY and its derivative (AMPA) in human urine after derivatisation with TFAA and TFE has been developed. Specific attention has been paid to sample clean-up, derivatization conditions, compound stability, solvent for GC injection, MS ionization mode, and MS-MS parameters as well as the quantification procedure to achieve the low limits of detection required for determination of these analytes in urine samples. Isotopically labelled standards of GLY and AMPA were used for quantitation by isotope dilution and isotope pattern deconvolution. The described methodology is simple and fast, avoiding complex extraction and clean up procedures, which usually compromise method efficiency.

2. Material and methods

2.1. Chemicals and standards

The solvents for residue analysis, acetonitrile (ACN), ethyl acetate (EtAc), acetone and isopropanol, anhydrous sodium sulfate, silica gel 60 (0.063 – 0.200 mm), aluminum oxide 90 active basic (0.063–0.200 mm) and potassium carbonate were from Merck (Darmstadt, Germany). The syringe filters with PTFE membrane (0.45 μm and 13 mm D) were purchased from Teknokroma (Barcelona, Spain). Synthetic urine, mimicking regular human urine, was obtained from DYNA-TEK Industries (Lenexa, KS, USA). Water for HPLC Plus, citral (mixture of E and Z-isomers of 3,7-dimethyl-2,6-octadienal, 95%), trifluoroacetic anhydride (TFAA) (99%), 2,2,2-trifluoroethanol (TFE) (99.5%), and 2,2,3,3,4,4,4-heptafluoro-1-butanol (HFE) (98%) were from Sigma-Aldrich (St. Louis, MO, USA). Glyphosate (99.5% purity) and aminomethylphosphonic acid (99.8% purity) reference standards were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Supelco (Bellefonte, PA, USA), respectively. The isotope labelled 1,2–$^{13}C_2$ ^{15}N-glyphosate (98% chemical purity, >99% isotopic purity for ^{13}C and ^{15}N) and ^{13}C ^{15}N-AMPA (99% chemical purity, 99.2% isotopic purity) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Silica gel was cleaned in an ultrasonic bath (3 × 10 min) with EtAc. Then, it was dried and stored at 100ºC until use.

2.2. Standard solutions

Stock solution containing both analytes, GLY and AMPA, (1000 ng mL^{-1}) and an internal standard solution composed of 1,2–$^{13}C_2$ ^{15}N-GLY and ^{13}C ^{15}N-AMPA (1000 ng mL^{-1}) were prepared in HPLC water. Mixed AMPA and GLY solutions of intermediate concentrations (100 and 10 ng mL^{-1}) and isotope labelled compound solutions (100 ng mL^{-1} for each compound separately) were obtained by dilution of the stock solution with HPLC water. Working calibration standard solutions were prepared by adding the corresponding volume of the internal standard (final concentration 10 ng mL^{-1} for ^{13}C ^{15}N-AMPA and 5 ng mL^{-1} for 1,2–$^{13}C_2$ ^{15}N-GLY) to serial dilutions of these intermediate standard solutions to cover a concentration range between 0.05 to 10 ng mL^{-1}. Working calibration standards were obtained from synthetic urine, which was derivatized following the methodology for real samples.

2.3. Sample preparation and derivatization

Urine samples from volunteers living close to an intensive agricultural area were introduced into ultra-clean polypropylene containers with polyethylene leak proof screw caps of 150 ml (57×73 mm; Deltalab, Barcelona, Catalonia, Spain). The samples were filtered through 0.45 μm PTFE filters and stored in a freezer at −23 °C until further analysis in the laboratory (more information about participants and urine sampling can be found in [50]). Urine sample was thawed at room temperature and stirred in a Vortex-mixer (1 min). Samples were then diluted to 1:10 with HPLC water and 300 μL (representing 30 μL of the original urine) were introduced into 10 mL borosilicate glass round bottom culture tubes with screw cap (Pyrex, Stoke on Trent, United Kingdom). At this step, internal standard solutions containing 1,2–$^{13}C_2$ ^{15}N-glyphosate and ^{13}C ^{15}N-AMPA were added to obtain final concentrations between 5 and 10 ng mL^{-1}, respectively. Then, 100 μL of acetone and 1 mL of ACN were added. After vigorous stirring in a Vortex-mixer (1 min), the sample was reduced to dryness under a gentle stream of nitrogen at 40 °C.

The derivatization method was based on a previous analytical procedure [34] with slight modifications. Briefly, dry urine samples were treated with 0.5 mL of TFE or HBE and 1 mL of TFAA, the second at freezing cold temperature (−40ºC) because the reaction is extremely exothermic. The mixture was stirred in a Vortex-mixer (1 min), sonicated in an ultrasonic bath (10 min) and heated at 90ºC for one hour in a screw-capped tube. After cooling, the tube was uncapped and the sample was reduced to dryness under a very gentle stream of nitrogen at 80–85ºC (aprox 30 min). Once cooled, the extract was dissolved in 1 mL of EtAc and stirred in a vortex mixer (1 min).

We investigated the influence of temperature and reaction time on analyte recoveries. For this purpose, a standard solution of 2.5 ng mL^{-1} of AMPA and GLY in synthetic urine was submitted to the derivatization reaction at 80 °C, 90 °C and 100 °C and during 30 min, 1 h, 2 h, 3 h and 4 h.

Residues of reagent or byproducts formed during derivatization led to significant capillary column degradation. Several methods were tested to remove these residues, including longer drying times at 85 °C or 40 °C, drying under vacuum using a speedvac concentrator (Pierce Reacti-therm III & Reacti-vap III, Savant/Pacisa, Thermo Fisher Scientific, Waltham, MA, USA), and a post-derivatization cleanup by adsorption column chromatography with silica gel or aluminum oxide, using 1 g of the sorbent previously washed with 1 mL of EtAc. The analytes were eluted with 1.5 mL of EtAc. Finally, the oily residue was evaporated under a gentle stream of nitrogen and transferred to vials using 300 μL of the injection solvent for GC–MS-MS analysis.

2.4. Selection of the injection solvent

Significant adsorption of AMPA and GLY in the injection port was observed even after derivatization, resulting in poor peak shape and low response. Different solvents such as ACN, isopropanol, EtAc, and a combination of citral and EtAc (1:500, v/v) were evaluated to solve these problems considering the requirements of analyte polarities, injection volume, and solvent boiling temperatures.

2.5. Gas chromatography coupled to tandem mass spectrometry (GC–MS-MS)

AMPA and GLY derivatives were determined using an Agilent 7890B GC System (Agilent Technologies, Palo Alto, CA, USA) coupled to a 7000C triple quadrupole mass spectrometer (Agilent, CA, USA) in electron impact (EI) (+70 eV) and negative ion chemical ionization (NICI) with ammonia or methane as reagent gas. 1 μL of extracts were injected in split/splitless mode, keeping the split valve closed for 2 min, into a DB-5–625 capillary column (30 m x 0.25 mm, 0.25 um i.d., Agilent Technologies), using helium as carrier gas at a constant flow of 1.1 mL min^{-1}. A fused silica deactivated column (0.32 mm x 2 m, Agilent Technologies) was used as guard column. Injector temperature was 280 °C. The oven temperature was programmed from 75 °C (holding time 1.5 min) to 150 °C at 10 °C min^{-1}, and to 300 °C at 50 °C min^{-1}, holding time 5 min. Transfer line temperature was 270 °C and ion source temperatures were 250 °C and 280 °C, for NICI and EI, respectively. Nitrogen was used as collision gas at 1.5 mL min^{-1}. Analyte determination was performed in multiple reaction monitoring mode (MRM). Individual MRM conditions were experimentally established for each compound determining precursor and product ions and collision energies (Tables S1 and S2) that provided the highest selectivity and sensitivity.

2.6. Method validation

2.6.1. Linearity and limit of detection

The linearity of the method was tested using derivatized AMPA and GLY standard solutions and their corresponding isotope labelled compounds as internal standards, to construct a calibration curve in synthetic urine with nine concentration levels ranging from 0.05 ng mL^{-1} to 10 ng mL^{-1} (10 ng mL^{-1} and 5 ng mL^{-1} for AMPA and GLY internal standards, respectively).

The limit of detection was evaluated by three different methods: (1) from the lowest concentration level which produces a signal to noise ratio (S/N) higher than 3 for both ion transitions, with a relative standard deviation lower than 10%; (2) from the linear regression fit of the calibration curve as b + 3 STD (being b the y-intercept and STD the standard deviation of b calculated by the linear regression model); and (3) from the blank samples, as the mean + 3 SDT, based on at least 5 replicates.

2.6.2. Recovery, repeatability and reproducibility

The recoveries were assessed by spiking real samples with AMPA and GLY at three concentration levels, 0.5 ng mL^{-1}, 1 ng mL^{-1} and 5 ng mL^{-1}. The method reproducibility was determined by replicate analysis (n = 4) of water, synthetic urine and real urine samples spiked with AMPA and GLY. In addition, MS-MS analysis repeatability and reproducibility were evaluated by consecutive injections (n = 5, intra-day precision) or repeated injections at different days (n = 5, inter-day precision) of the above mentioned AMPA and GLY spiked samples. All determinations were performed by the isotope dilution method. The results of the recovery studies, repeatability and reproducibility were checked for compliance with the AOAC International guidelines [51].

2.6.3. Stability of standard solutions and samples

A set of spiked samples and calibration standards of different concentrations was analysed, stored at 4 °C and reinjected after approximately one month to determine the stability of standards and samples after derivatization. In addition, the stability of samples at different conditions during 7–10 days was also investigated. Two standard solutions of 10 ng mL^{-1} and two human urine samples spiked at the same level were prepared, stored under different conditions, and injected several times during this period (n = 6). One was left in the GC autosampler at room temperature and the other was stored at 4 °C between each injection.

2.6.4. Matrix effect

The influence of the sample matrix on the intensities of the MRM transitions of the AMPA and GLY derivatives was evaluated by comparing the relative MS-MS responses between standards prepared in synthetic and real human urine with those observed in purified water samples.

2.7. Quantitative determination of GLY by isotope pattern deconvolution (IPD)

In isotope pattern deconvolution (IPD), the experimental isotope abundances measured in the sample after spiking with labelled GLY are

expressed as a linear combination of the isotope abundances of the natural (A^1_{GLY} to A^i_{GLY}) and labelled GLY (A^1_{GLYlab} to A^i_{GLYlab}) as described in Eq. (1) [47]:

$$\begin{bmatrix} A^1_{GLY} & A^1_{GLY\,lab} \\ A^2_{GLY} & A^2_{GLY\,lab} \\ \vdots & \vdots \\ A^i_{GLY} & A^i_{GLY\,lab} \end{bmatrix} \times \begin{bmatrix} X_{GLY} \\ X_{GLY\,lab} \end{bmatrix} + \begin{bmatrix} e^1 \\ e^2 \\ \vdots \\ e^i \end{bmatrix} = \begin{bmatrix} A^1_M \\ A^2_M \\ \vdots \\ A^i_M \end{bmatrix} \quad (1)$$

Where A^1_M to A^i_M are the isotope abundances of each MRM transition in the mixture, X_{GLY} and $X_{GLY\,lab}$ correspond to the molar fractions of natural and labelled GLY in the mixture, respectively, and e^1 to e^i are the error vector. The best values of X_{GLY} and $X_{GLY\,lab}$ are calculated by least square minimization of the error vector. Calculation of the molar fractions provides the amounts (moles) of natural GLY, since the amounts (moles) of labelled GLY added to the mixture is already known

$$\frac{GLY\ (mol)}{GLYlab(mol)} = \frac{X_{GLY}}{X_{GLY\ lab}} \Rightarrow GLY\ (mol) = \frac{X_{GLY} \times GLYlab(mol)}{X_{GLY\ lab}} \quad (2)$$

The application of IPD requires accurate knowledge of the isotope composition of the natural and labelled GLY, and the exact concentration of the isotopically labelled GLY standard solution. These requirements involve measuring the spectral purity of the selected cluster ions to determine the abundances of M and M-1, which are needed to calculate the isotope composition of labelled GLY that usually differ from the information given by the manufacturer.

Cluster purity of the selected ion (370) was calculated by analysing standard solutions of GLY and labelled GLY by GC–MS NICI in selected ion monitoring (SIM) mode. The following ion masses were monitored: 369 (M-1), 370 (M), 371 (M + 1), 372 (M + 2), 373 (M + 3) and 374 (M + 4) with a 10 ms of dwell time. Five injections of different concentrations (1, 10 and 100 ng mL^{-1}) were performed. The experimental isotopic pattern for natural GLY was calculated by dividing the peak areas of each mass by the sum of all measured peak areas. These values were compared with the theoretical pattern of the M and M-1 cluster using a multiple linear regression method [52]. Once the M-1 contribution is determined, the experimental isotope distribution of the labelled GLY was compared with the theoretical distributions within a range of the ^{15}N-enrichment provided by the manufacturer by linear regression analysis using the Visual Basic macro developed for Excel [52].

Identification of the best MRM transitions for IPD determination from the unit mass resolution spectra in both quadrupoles (Table S1) allowed to measuring the fractional abundances of pure natural and labelled GLY for use in Eq. (1).

2.8. Quality control and quality assurance

The target compounds were positively identified by their retention times and the ratio of the two MRM transitions, which had to fall within ±20% of the average ratio obtained from standard solutions. Quantitative analysis was performed by the isotope dilution method using labelled AMPA and GLY standards. Additionally, GLY was also determined by IPD to evaluate the influence of spectral overlap between the native and labelled GLY transitions in the measured concentrations.

Blank samples (synthetic urine or purified water) were analysed within each batch of samples. AMPA and GLY concentrations reported in urine were blank-subtracted. Selected samples were spiked and analysed in triplicate to ensure that recovery and precision (calculated as the relative standard deviation) were in the range of those determined in the validation study. In view of the results of the stability study, samples and standard solutions were derivatized, and analysed within 10 days at the latest.

3. Results and discussion

A flowchart indicating the different conditions tested in each step of the analytical procedure and those finally selected is summarized in Fig. S1.

3.1. Derivatization

GLY and AMPA were derivatized prior to GC–MS-MS analysis. The acid functional groups were transformed into their corresponding ester derivatives and the amine group was acetylated [34] (Fig. S2). This derivatization step is essential for increasing their volatility and solvent solubility for GC analysis, and to prevent their sorption to glass and metallic surfaces. Two esterification reagents were considered, trifluoroethanol (TFE) and 2,2,3,3,4,4,4-heptafluoro-1-butanol (HFB). The latter led to higher mass fragments in GC–MS improving selectivity (Tables S1 and S2, Fig. S3 and S4) and, in the case of NICI determinations also sensitivity. However, whereas this derivative generated high responses for GLY, the highest response for AMPA was obtained with TFE. Furthermore, complete derivatization with HFB was difficult to achieve and the ester derivatives with this reagent were less stable than those with TFE. Thus, TFE was finally selected.

Investigation of the influence of temperature and reaction time on analyte recoveries showed no differences in derivatization yields at 80 °C, 90 °C and 100 °C (data not shown), so 90 °C was finally selected. Regarding reaction time, Fig. 1 shows the AMPA and GLY derivatization yields measured from the area of the quantitative transition for each reaction time. The abundance of the transition corresponding to derivatized AMPA did not differ between the studied reaction times. However, the best reaction time for GLY was 1 h, longer periods resulted in significant yield decrease. These results were consistent with previous studies on derivatization of different pesticides with phosphoric and amino acid groups in their molecules [53].

The use of TFE or HFB for AMPA and GLY derivatization prior to GC analysis has been described [19,34,35,40,53]. However, a comprehensive study on the influence of derivatization reagents, temperature, reaction time and derivative stability was still missing.

3.2. Extraction of AMPA and GLY from urine and extract clean-up

The derivatization procedure is very sensitive to the presence of water in the medium, even in very small quantities. This condition limited the initial amount of urine for analysis to 50–100 µL, since it must be entirely dried before adding the derivatizing reagents. Obviously, this requirement has a direct impact on the method detection limit. Different sample pre-treatments were investigated in order to increase sample amount. Several extraction and cleanup procedures have been reported for AMPA and GLY analysis in aqueous samples, commonly using anionic [36,40,54] and cationic [26,34,41] exchange sorbents. We tested

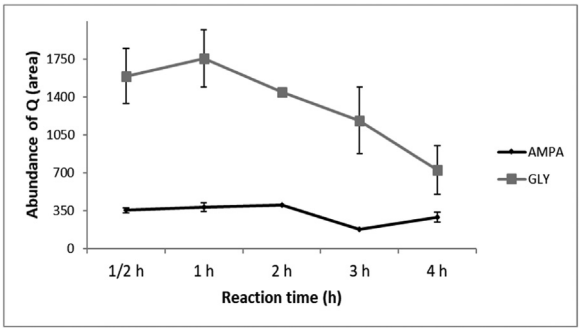

Fig. 1. Abundance of the quantitative transition (Q) of AMPA and GLY derivatives at different derivatization times.

Table 1
Percentage recoveries of AMPA and GLY derivatives using different treatments to remove derivatization residues. Otherwise indicated, the results correspond to duplicate experiments.

	2 h85 °C	3 h85 °C	4 h85 °C	Overnight 40 °C	Speed Vac 25 min	Aluminum oxide	Silica (n = 5)
AMPA	76–80	75–78	55–65	53–98	2.0–2.5	n.d.	91–135
GLY	89–93	89–95	73–82	67–85	3.1–4.6	n.d.	92–120

n.d., below detection limit.

the feasibility of several extraction/cleanup procedures prior to derivatization, including cation exchange using Isolute HAX [36], Strata XC [26], and anion exchange with Strata-SAX [54]. In contrast to published results, repeated experiments using cation and anion exchange cleanup procedures showed insufficient AMPA and GLY recoveries (40% at the most for GLY and 10% for AMPA). The analysis of untreated urine samples led to a rapid degradation of chromatographic performance, which required frequent injection port maintenance and pre-column change. We also compared the results obtained with 50 µL of untreated urine and with those diluting the sample with water at different ratios (1:5) and (1:10). For this purpose, AMPA and GLY standards prepared in synthetic urine or added to real urine samples at two levels (5 ng mL^{-1} and 15 ng mL^{-1}) were used. The best results in terms of analyte recoveries and GC performance were obtained by dilution of urine samples with water at a ratio of 1:10. This procedure was therefore adopted for the following experiments.

Residues of reagent or byproducts formed during derivatization led to significant capillary column degradation. Several methods were tested to remove these residues as described in the Materials and Method section. The highest and more reproducible recoveries for AMPA and GLY derivatives were obtained by column chromatography with silica gel (Table 1). Acceptable recoveries were also observed drying the sample during 2 h at 85 °C, although this time period was not enough to remove all derivatization residues. Higher drying times resulted in a significant loss of derivatized AMPA, likely due to its higher volatility, while drying overnight at milder conditions (40 °C) led to recovery values of high variability.

3.3. Selection of the injection solvent

Significant adsorption of AMPA and GLY in the injection port was observed even after derivatization, resulting in poor peak shape and low response. Initially, standards and samples were diluted in ACN as this solvent was reported to be the most suitable for gas chromatographic analysis of pesticide mixtures of a wide range of polarities [55]. However, significant adsorption of AMPA and GLY derivatives was observed after few injections, likely in the injection port and/or the guard column, therefore other solvents were evaluated. As shown in Fig.S5, a slightly higher response of AMPA and GLY derivative was found using EtAc; however, fast degradation of chromatographic peaks was also observed with this solvent after injection of derivatized AMPA and GLY extracted from real urine samples (Fig. S6).

Higher response of both analytes was obtained with the combination of citral and EtAc (1:500, v/v) (Fig. S5), which is consistent with previous studies reporting the use of citral to minimize GLY adsorption in GC [34,40]. Moreover, significant improvements in peak shape were observed from the single use of citral/EtAC as injection solvent after the injection of real samples, without any other change in the chromatographic system (Fig. S7). To minimize injection port maintenance, the amount of citral added to EtAc was lowered to the minimum level, which warranted good chromatographic performance (1:500, v/v).

In addition to poor peak shape, GLY derivative adsorption on the gas chromatographic system can produce a carryover effect. This effect was evaluated by analyzing a blank run with the respective pure solvent after the highest concentration standard or spiked samples. No detection of AMPA and GLY derivatives (including both transitions) were observed in any of these cases.

3.4. Optimization of GC–MS-MS parameters for the determination of AMPA and glyphosate derivatives

Response of GLY and AMPA derivatives in EI and NICI with methane and ammonia was compared. The full scan mass spectrum of a standard mixture of GLY and AMPA derivatives in both ionization modes is shown in Fig. S3. Mass spectra and fragmentation were consistent with those reported for these compounds [34,35] (Figs. S2 and S3). Higher sensitivity was observed using NICI in both cases. However, the MS spectra of derivatized GLY in NICI showed a unique dominant fragment at m/z 370 (Fig. S3). This single fragment could represent a limitation for analyte confirmation in the MS-MS method.

A larger number of mass fragments of high m/z values was obtained by EI for GLY derivatives, which provided higher selectivity and confirmation possibilities. Accordingly, GC–MS-MS with EI mode was optimized and evaluated for determination of these compounds. Precursor ions of m/z 411 and 302 for GLY and AMPA derivatives were initially selected, respectively, since higher m/z ions usually result in higher selectivity. The precursor and product ions selected for derivatized AMPA, GLY and their corresponding isotope labelled compounds, as well as the optimized collision energy providing the higher response for each transition, are shown in Table S1. Unfortunately, a significant sensitivity decrease was observed in comparison with the results of ions m/z 238 and 126 for GLY and AMPA, respectively. Moreover, blank samples did not show any interference at AMPA and GLY derivative retention times using these ions. The limits of detection (LOD) obtained at the optimized GC–MS-MS EI conditions are summarized in Table 2. As it can be observed, the LODs were insufficient to determine these compounds at the required levels; therefore, the performance of MS-MS NICI for the analysis of GLY and AMPA derivatives was investigated.

The influence of reagent gas (ammonia and methane), ion source temperature and reagent gas flow on the response of derivatized AMPA and GLY upon MS-MS NICI analysis are summarized in Table 3. The best results with ammonia were observed at 250 °C ion source temperature and 35% flow rate, while for methane the highest signals were obtained at 250 °C ion source temperature and 45% flow.

The precursor and product ions selected for each compound at the optimized MS parameters are shown in Table S1. The MS fragmentation of the trifluoro derivatives of AMPA and GLY corresponding to the precursor and product ions are shown in Fig. S2. Ion m/z 383 was the dominant mass fragment of the AMPA derivative (m/z 385 of the corresponding labelled AMPA), both with ammonia and methane as reagent gas. This mass fragment has a m/z value higher than the molecular weight

Table 2
Limit of detection (LOD) (ng mL^{-1}) of AMPA and GLY at different GC–MS-MS ionization techniques, electron impact (EI) and negative ion chemical ionization (NICI).

	EI	NICI (CH_4)	NICI (NH_3)
AMPA	0.50	0.10	0.05
GLY	50	0.90	0.05

Table 3
Optimization of ion source temperature and reagent gas pressure (methane or ammonia) in negative ion chemical ionization mass spectrometry (NICI-MS) The values expressed as the relative abundance of the parent ion in full scan mode.

MS conditions		AMPA m/z = 383	$AMPA_{lab}$ m/z = 385	GLY m/z = 370	GLY_{lab} m/z = 371
Ion source T (˚C)	100	0.47	na	0.44	0.40
35% NH_3	195	0.78	na	0.94	0.84
	210	0.95	na	0.86	na
	250	1	na	1	1
NH_3 (%)	20	0.48	0.46	0.59	0.49
Ion source T 195 ˚C	35	0.78	na	0.94	0.94
	40	0.98	na	0.88	na
	45	1	1	1	1
NH_3 (%)	35	1	na	1	na
Ion source T 250 ˚C	40	0.57	na	0.50	na
Ion source T (˚C)	130	0.59	0.50	0.44	0.37
35 (%) CH_4	190	0.83	na	0.67	na
	250	1	1	1	1
CH4 (%)	20	0.40	0.36	0.36	0.41
Ion source T 250 oC	35	0.71	0.75	0.84	0.87
	45	1	1	1	1

na, not analyzed.

of derivatized AMPA, whose formation mechanism has not been established yet. Fragmentation of this ion in both AMPA and labelled AMPA produces a unique peak at m/z 165. In the analyses of derivatized AMPA by GC–MS in NICI, this fragment is not formed and the mass spectrum is dominated by m/z 351 corresponding to $[M\text{-}HF]^-$, indicating that it is specific of the MS-MS system. However, low intensity of 351->245 transition was observed at concentrations below 0.75 ng mL^{-1} increasing significantly the LOD of AMPA if both transitions are considered. In view of these results, the 383->165 and 351->268 transitions were selected for quantitative and qualitative analysis of AMPA, respectively, whereas 351->245 was used for further confirmation of compound identification at concentration > 1 ng mL^{-1}.

The LODs for each compound at the optimized conditions are shown in Table 2. They correspond to the first concentration at which the Q (quantitative) and q (qualitative) transitions produce a signal to noise ratio of 3. Similar results were observed between methane and ammonia for AMPA, while higher LOD was obtained for GLY using methane as reagent gas. Other parameters such as repeatability or reproducibility did not show significant differences between both gasses. Finally, MS-MS NICI with ammonia was selected for the determination of AMPA and GLY in urine samples, as this reagent gas provided lower LODs for both compounds.

3.5. Method validation

3.5.1. Linearity and limit of detection

Both compounds showed a linear response in the calibration range from 0.05 ng mL^{-1} to 10 ng mL^{-1} (10 ng mL^{-1} and 5 ng mL^{-1} for AMPA and GLY labelled standards, respectively), with coefficients of determination values higher than 0.99.

As described in Section 2.6.1, the limit of detection was evaluated by three different methods and the results are summarized in Table 4. The lowest LOD values were obtained from the S/N ratio, 0.05 ng mL^{-1} for both analytes. The LODs calculated from blank samples were higher, 0.39 (AMPA) and 0.25 ng mL^{-1} (GLY), and they indicated the lowest concentration at which target compounds can be effectively determined in urine samples.

Direct comparison of these LODs with those reported in the literature is difficult, because of differences in units or the methods used, which are not always clearly specified. In addition, matrix effects have a significant impact in the LOD values, so they can vary between matrices using the same methodology. Focusing on those studies that measured AMPA and GLY in urine, our LODs are three orders of magnitude lower than others using liquid chromatography coupled to inductively coupled mass spectrometry [28,56] (Table S5). They are also lower than those using immunoassay techniques (ELISA) [16,57]. Methods based on GC–MS [10,17,24,31] have also LODs higher than those found in the present study, in the range of 1 to 10 ng mL^{-1}. However, similar instrumental LODs, 0.05 ng mL^{-1}, for both compounds, as in the present study have recently been reported with GC–MS-MS [37]. Finally, the LODs found in this study are higher than those reported using direct analysis with LC-MS-MS (between 0.01 and 0.05) (Table S5) [16,30,43].

Table 4
Limit of detection (LOD) of AMPA and GLY derivatives determined by GC–MS-MS in negative ion chemical ionization with ammonia (values in ng mL^{-1}).

	Urine		
	LOD[(a)]	LOD[(b)]	LOD[(c)]
AMPA	0.05	0.46	0.39
GLY	0.05	0.35	0.25
GLY (IPD)*			0.25

[(a)] From the first concentration level at which Q and q transitions produce a signal with a S/N ratio higher than 3.
[(b)] From the linear regression equation of the calibration curve as b + 3std (with b y-intercept and std the standard error of b).
[(c)] Mean blank value+3 std (n = 6).

3.5.2. Recovery, repeatability and reproducibility

As shown in Table 5, the method shows good repeatability and reproducibility with values between 0.54 to 5.5% and 1.1 to 6.9%, respectively, considering both compounds and the concentrations tested. These results are clearly below 20%, which is the value accepted for concentrations of 1 ng mL^{-1} [51].

Satisfactory recoveries of the target compounds have been observed (Table 6), ranging between 60 and 99% for AMPA and 71 to 102% for GLY, with relative standard deviations between 9 and 19% for spiked levels as low as 0.5 ng mL^{-1}. These values are within the range of those accepted for the AOAC guidelines [51] (the recovery should be in the range of 40–120% with an associated RSD lower than or equal to 30% for concentration levels of 1 ng mL^{-1}).

3.5.3. Stability of standard solutions and samples

The study of AMPA and GLY derivative stability showed high variability with good results with responses varying less than 10% between

Table 5
Repeatability and reproducibility for GC–MS-MS in negative ion chemical ionization with ammonia determined with standards solutions.

ng mL^{-1}	Repeatability (n = 5)			Reproducibility (n = 5)		
	1	5	10	1	5	10
Urine						
AMPA	5.5%	3.0%	1.2%	2.2%	3.6%	1.1%
GLY	2.3%	0.90%	0.54%	2.7%	1.3%	6.9%
GLY (IPD)*		1.8%		1.3%	3.3%	0.56%

* n = 3.

Table 6
Percentage recoveries (average ± standard error) and method reproducibility (% std) calculated from real biological and water samples at different spike levels.

	Urine (n = 4)			Water (n = 3)	
	0.5 ng mL^{-1}	1 ng mL^{-1}	5 ng mL^{-1}	1 ng mL^{-1}	2.5 ng mL^{-1}
AMPA	60±5.4(9.0%)	72±7.1(10%)	99±6.5 (6.5%)	70±3 (4.3%)	68±2(2.9%)
GLY	71±15(19%)	96±10(10%)	102±1.0 (1%)	106±4 (3.7%)	98±4(4.1%)

injections in some experiments, whereas no detection of the analytes was found in others. Concentration levels or sample type (standard solution *vs* spiked samples) did not explain these discrepant results. The stability of samples stored at different conditions (room temperature and at 4 °C) during 7–10 days was also investigated. Higher variability was found for those left at room temperature with variation coefficients of 5.4% and 7.4% for AMPA and GLY derivatives, respectively, for standard solutions and 3.9% and 2.9% in the case of human urine samples. These coefficients varied between 1.1% to 4.0% for derivatized AMPA and from 3.1 to 6.9% for derivatized GLY in the standard and human urine samples when stored at 4 °C. Nevertheless, the mean values of the two samples were not statistically different (t-Student, $p < 0.05$) with a relative standard deviation between replicate injections <10% in both cases. Quantification without taking into account the IS gave rise to higher variability, with variation coefficients between 16 and 19%, but again with no differences between samples stored at room temperature and at 4 °C. Moreover, decreases in analyte responses or changes in the peak shapes were not observed, which could have indicated a degradation of the AMPA and GLY derivatives. Accordingly, the processed samples were stable for at least 10 days, independently of the storage conditions. Samples and standard solutions were therefore prepared and analyzed within 7–10 days.

3.5.4. Matrix effect

No influence of the sample matrix on the intensities of the MRM transitions was observed at the working conditions. The relative responses measured from the slopes of the calibration curves in human urine relative to purified water were 0.72 and 0.99 for AMPA and GLY, respectively, while these ratios were 1.02 and 0.96 when comparing calibration curves prepared in synthetic urine and purified water. However, the calibration curves prepared from real or synthetic urine without any dilution step showed significant decreases in the slopes with relative ratios to water values of 0.43 for AMPA and 0.36 for GLY, indicating that undiluted urine contains compounds that interfere in the GC–MS-MS response.

3.6. Application to human urine samples

The developed GC–MS-MS method was applied to the analysis of human urine samples from people living close to intensive agricultural area where GLY is used (Table 7). AMPA was detected at concentrations between 0.14 and 1.7 ng mL^{-1}, while GLY levels varied between 0.10 and 1.84 ng mL^{-1}. Although these data correspond to few samples and do not represent a comprehensive survey, comparison with levels reported in other studies in USA and Europe [15,17,18], shows consistent

Table 7
AMPA and GLY concentrations (blank-subtracted) measured in human urine by GC–MS-MS in negative ion chemical ionization mode using isotope dilution quantification. The values are expressed as ng mL^{-1}. The GLY levels measured by IPD are included for comparison.

Urine sample	AMPA	GLY	IPD	%Differences
U1	1.13	0.49	0.46 ± 0.00	5.3
U2	1.40	1.84	1.75 ± 0.04	5.2
U3	0.22	0.25	0.22 ± 0.00	14
U4	0.14	0.32	0.36 ± 0.02	11
U5	0.32	0.17	0.19 ± 0.01	9.7
U6	0.64	0.17	0.26 ± 0.02	56
U7	0.61	0.25	0.32 ± 0.01	28
U8	0.30	0.10	0.14 ± 0.00	43
U9	0.29	0.32	0.38 ± 0.04	21
U10	0.33	0.25	0.21 ± 0.04	14
U11	1.74	NA	NA	–
Range	***0.14–1.74***	***0.10–1.84***	***0.14–1.75***	

NA, not quantified due to an interfering coelution.

results with the literature values that varied between 1.8 and 9.5 ng mL^{-1} mean GLY concentrations in urine from farmers and their families and between <LOD (0.1 ng mL^{-1}) and 1.78 ng mL^{-1} in the case of the general population.

Chromatograms corresponding to a standard solution and a human urine sample that contained AMPA and GLY at concentrations of 0.22 and 0.57 ng mL^{-1}, respectively, are shown in Fig. 2 as example.

3.7. GLY determination by isotope pattern deconvolution (IPD)

AMPA and GLY concentrations were determined by isotope dilution using isotopic labeling of the target compounds with ^{13}C and ^{15}N. This technique provides more precise and accurate results in comparison to more traditional quantitative methods such as external calibration or standard addition. This approach was fundamental to get reproducible responses in the determination of AMPA and GLY by GC–MS-MS. However, although there is a molecular mass difference of 3 units between the native and labelled compounds, the main fragment in the NICI spectra of GLY and labelled GLY only differs in one unit (Table S1 and Fig. S2). This small difference involves a spectral overlap resulting in cross contributions in peak areas between the native and labelled compound transitions (Fig. 3), which is negligible in the case of AMPA, but not for GLY. In this case, the determination of GLY concentrations by isotope pattern deconvolution could represent a better choice, since it has to be applied in combination with minimal labeling of the isotopically en-

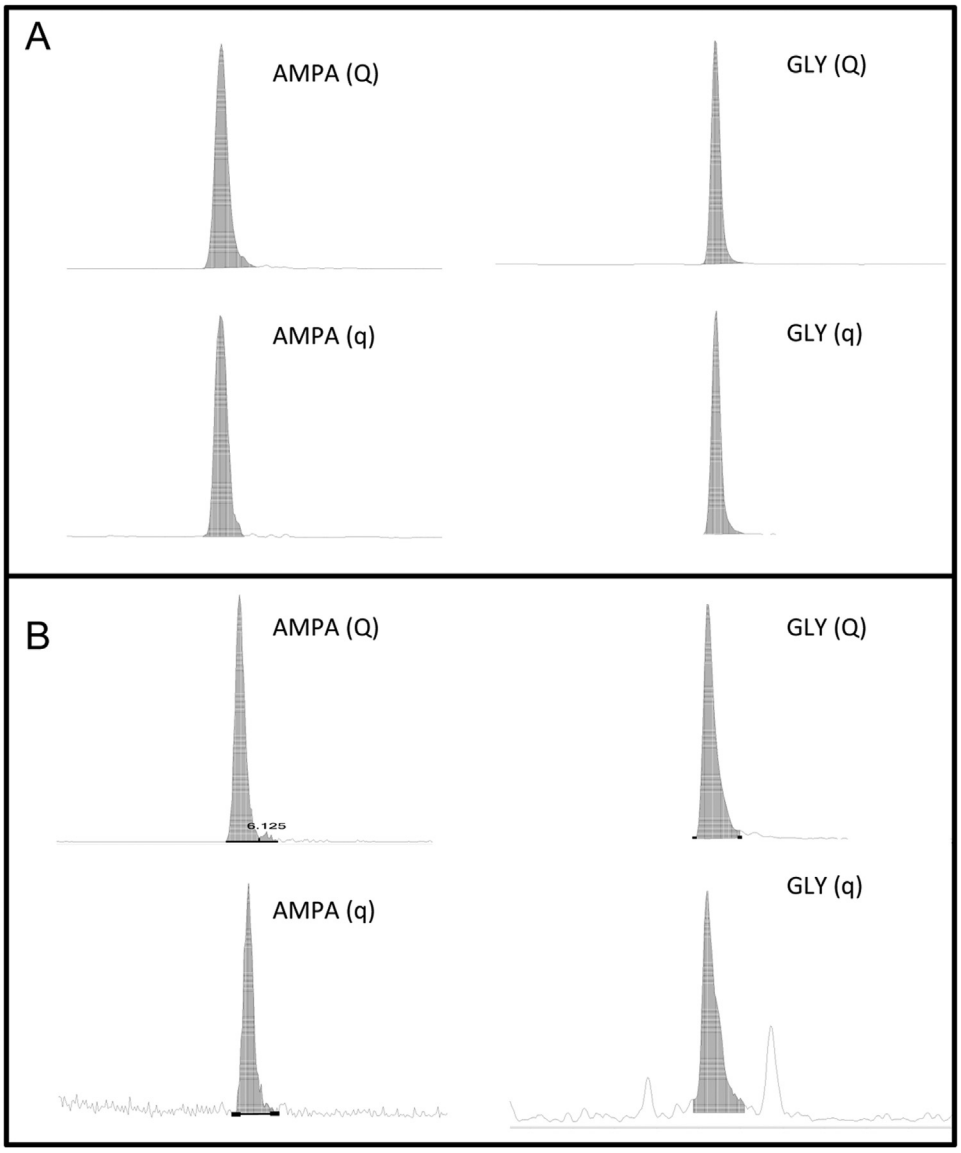

Fig. 2. GC–MS-MS chromatograms corresponding to (A) standard solution of AMPA and GLY derivatives in synthetic urine at the concentration level of 0.5 ng mL^{-1}, (B) human urine sample containing AMPA and GLY at concentrations of 0.22 ng mL^{-1} and 0.57 ng mL^{-1}, respectively. (Q), quantitative transition, (q), qualitative transition.

riched molecule (one unit mass difference) to avoid isotopic effects. In addition, it does not require a calibration curve construction as every single injection provides the concentration of the target compound in the sample.

As it has been mentioned above, IPD requires the knowledge of the isotope composition of both, natural abundance and labelled GLY. This implies the evaluation of the spectral purity of the cluster ions used for the quantification of GLY (*m/z* 370). Therefore, the mass range *m/z* 370–375 was measured in SIM mode at different concentration levels, both for GLY and labelled GLY. The isotope distribution was expressed as fractional abundances calculated by dividing the peak area of each mass by the sum of all peak areas measured for the cluster ion. Five replicate injections were performed to calculate the experimental uncertainty (Table S3). From these data, the contribution of M-1 was calculated by multiple linear regression [52]. Once the M-1 contribution was determined, this information was used to calculate the ^{15}N-enrichment of labelled GLY that gives the minimum in the square sum of residuals, when comparing with the theoretical distributions by linear regression. We found a M-1 contribution of 0.58% with an uncertainty of 0.20% and a ^{15}N-enrichment of labelled GLY of 98.10%. With this information, the theoretical isotope fractional abundances of GLY and labelled GLY in the three selected MRM transitions for IPD determination were calculated [58] and compared with the experimental fractional abundances obtained by the MS-MS analysis of standard solutions of labelled and natural GLY derivatives. Experimental fractional abundances were calculated as the area of the signal in each transition divided by the sum of the areas of all transitions measured. As it can be observed in Table S4, comparison of the theoretical and experimental values shows good agreement for GLY and labelled GLY.

For sample analysis, weighed urine samples were mixed with a weighed aliquot of the labelled GLY standard solution of known concentration, derivatized following the developed methodology and analyzed by duplicate by GC–MS-MS NICI to calculate the molar fractions of GLY and labelled GLY from the fractional abundances of the selected MRM transitions of the mixture (Eq. (1)) and, subsequently, the GLY concentrations in the samples (Eq. (2)).

Comparison of the analytical figures of merit provided by the IPD method with those obtained by isotope dilution (Tables 4 and 5) showed good repeatability and reproducibility for the IPD determinations, while the LOD based on blank values was similar to that measured with the isotope dilution method. As no reference certified material of GLY in human urine is available, method accuracy was assessed by measuring GLY concentrations of standard solutions prepared in synthetic urine ranging from 0.05 to 10 ng mL^{-1}. The GLY concentrations determined

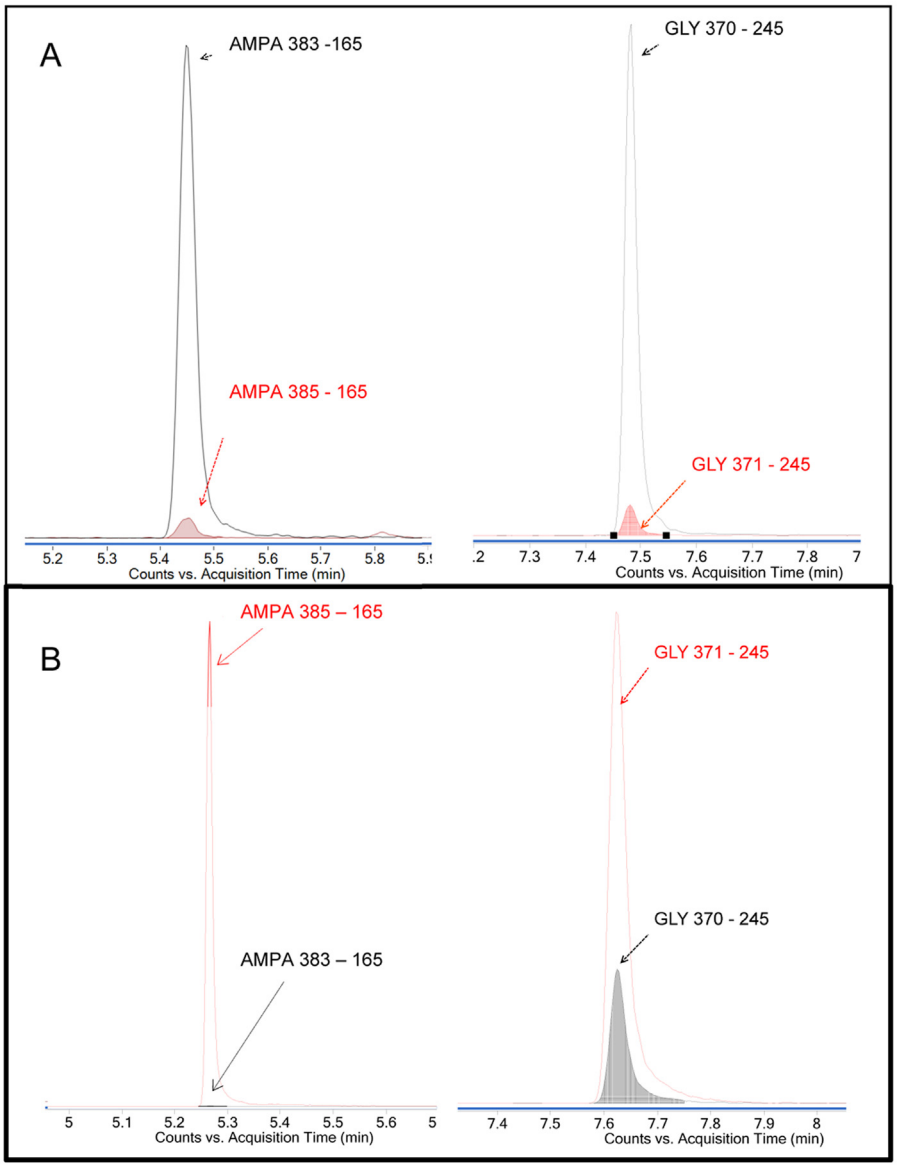

Fig. 3. Cross contributions in MS-MS transitions between native (black) and isotope labelled (red) compounds. (A) Standard solution of AMPA and GLY derivatives of 5 ng mL^{-1}. (B) Standard solution of isotope labelled AMPA and GLY derivatives of 5 ng mL^{-1}.

Table 8
Calibration curve standard solutions. Comparison between theoretical concentrations of GLY and those determined by IPD ($n = 3$).

[GLY] ng mL^{-1}	0.60	0.87	1.15	2.80	5.4	13
[GLY] IPD	0.68±0.01	0.94±0.00	1.29±0.01	3.01±0.02	6.1 ± 0.21	14±0.10
% Accuracy	13	7.8	12	11	13	13

by IPD were slightly higher than the theoretical values, but all them fall within 15% of the expected values (Table 8).

The influence of the spectral overlap between natural and labelled derivatives in the GLY concentrations measured in human urine samples was assessed by comparison of the values obtained using the isotope dilution and IPD methods in the analyzed samples (Table 7). In general, higher levels were obtained with IPD. Relative differences varied between 5.2 and 56%, with higher values not related to concentration levels or observed interferences in the sample. Although these differences are not very high taking into account the method reproducibility, they are statistically significant in most of the samples; therefore, the IPD method would be the best option to get reliable levels of GLY in urine samples.

4. Conclusions

A simple, fast and reliable method for the analysis of AMPA and GLY at low ng mL^{-1} level in human urine has been optimized. The method does not require complex sample handling or clean-up, only urine filtration and dilution with water, followed by AMPA and GLY derivatization to obtain less polar and more volatile compounds amenable to GC analysis. The derivatization with TFAA and TFE is simpler and faster than the more commonly used FMOC derivatization that requires careful pH control, preparation of reagent solutions and several cleanup steps to remove excess FMOC, which can interfere with the AMPA quantitation and may also react with any primary and secondary amines present in the sample.

MS-MS detection in NICI and the use of isotope labelled standards provided the high selectivity and sensitivity necessary to get low LOD, overcoming any possible matrix effects. In these conditions, LODs of 0.05 ng mL^{-1} were achieved with good reproducibility (between 1.1 to 6.9%) and extraction efficiency.

The use of isotope pattern deconvolution method for the quantification of GLY was proven a good alternative to the conventional isotope dilution method, overcoming quantitative errors due to the spectral overlap observed between natural and labelled GLY in the selected MRM transitions. Although IPD implementation requires some previous work before it can be applied, it is suitable for routine analysis since no calibration curve is needed and every single injection provides the concentration of GLY in the sample.

Thorough investigation of the results with synthetic and real samples showed that the method is simple and efficient enough to be implemented for AMPA and GLY analysis in human urine biomonitoring to assess health risks related to GLY exposure.

CRediT authorship contribution statement

EJ: Analysis, Formal analysis, Review and editing. **PF:** Supervision, Conceptualization, Writing original draft And Funding acquisition. **IF:** Analysis, Review and editing. **JG:** Writing – review and editing, Conceptualization, and Funding acquisition.

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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcoa.2023.100087.

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