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Validation of a simple method for the determination of glyphosate and aminomethylphosphonic acid in human urine by UPLC-MS/MS

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ABSTRACT

Environmental pollutants such as pesticides can be detrimental to human health and/or to the environment. Their excessive use may produce toxicity through various mechanisms. Glyphosate is a broad-spectrum herbicide with a high worldwide distribution. Due to this, this chemical is classified as a 'Group 2A – probably carcinogenic to humans' by the International Agency for Research on Cancer. Human biomonitoring is considered the golden standard for exposure assessment and provides a very useful tool in public health. Therefore, it is important to develop methods to determine traces of this herbicide and its metabolite, aminomethyl phosphonic acid, in human biological samples. A new method for glyphosate and aminomethyl phosphonic acid determination in human urine is herein described and discussed. It is based on the derivatization procedure with Fluorenylmethoxycarbonyl chloride and quantification by ultra-performance liquid chromatography-tandem mass spectrometry. The method was optimized and suitably validated, with a linear range from 1 to 20 μg L^{-1} in the case of glyphosate and 0.5–20 μg L^{-1} for aminomethyl phosphonic acid. Limits of detection and quantification were 0.5 and 1 μg L^{-1} for glyphosate and 0.1 and 0.5 μg L^{-1} for aminomethyl phosphonic acid, respectively. Mean relative recoveries ranged 108–109% for glyphosate and 104–119% for aminomethyl phosphonic acid and intermediate precision values varied from 11.90 to 12.70% for glyphosate and 4.8–9% for aminomethyl phosphonic acid. The validated method has been applied in human urine from female farmers indirectly exposed to pesticides. This procedure can be used to monitor potential exposure of humans to glyphosate and aminomethyl phosphonic acid in epidemiological studies and for routine controls in public health.

1. Introduction

Human exposure to complex mixtures of environmental pollutants is a reality in society nowadays. Environmental pollutants refer to all of the exogenic, non-essential factors for humans, which, when released into the environment, can be detrimental to human health and/or to the environment [1]. Within this group of pollutants, some pesticides and metals stand out [2,3].

The use of pesticides in agriculture has allowed humans to obtain safer food by reducing vector-borne diseases. However, their excessive use may produce toxicity through various mechanisms [3]. General population is exposed to small concentrations of pesticides through diet and the environment throughout their lives, being more important this exposure on the population using them professionally [4].

Glyphosate (N-phosphonomethyl-glycine) (GLY) is a broad-spectrum herbicide with a high worldwide distribution, used in agricultural and forestry environments and also in non-agricultural areas such as water systems, parks, road verges, and gardens [5]. Concerning its toxici mechanism, it is thought that its true toxicity is caused by the surfactant that decouples elements of oxidative phosphorylation, which causes oxidative stress, although this is still controversial [6]. GLY has come under international debate since the International Agency for Research on Cancer (IARC) classified this chemical as a 'Group 2A – probably carcinogenic to humans' [7]. However, the United States Environmental Protection Agency has classified GLY in category IVpractically non-toxic and not an irritant [8]. In addition, in the European Union, GLY has been

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thoroughly assessed by Member States, the European Food Safety Authority (EFSA) and the European Chemicals Agency (ECHA), leading to its classification as non-carcinogenic to humans [9,10]. In 2017, the European Commission authorized the use of GLY for 5 years further [11].

Human biomonitoring (HBM), which involves the measurement of a chemical in biological substances such as blood, urine, hair, or milk is considered the golden standard for exposure assessment and provides a very useful tool in public health [12–14]. GLY is a non-persistent substance that tends to not be accumulated in the human body and consequently is quickly metabolized and excreted via urine within 4–72 h after exposure [15]. The aminomethyl phosphonic acid (AMPA) is its major metabolite [16]. The HBM of GLY and its metabolite in urine is a useful strategy in order to obtain valuable information about recent internal exposure to pesticides (1–3 days) [15]. Also, HBM allows identification and elimination of possible sources of exposure, studying relationships between pollutants and health effects, identifying groups of populations vulnerable to these pollutants and setting priorities in environmental and health research [17].

Both GLY and AMPA are polar and amphoteric compounds that are very soluble in water which hinder their analysis [18,19]. Thus, it is necessary to employ tedious and time-consuming cleaning procedures in order to facilitate their chromatographic separation by GC, GC–MS, LC or LC–MS [20] or detection by Capillary Electrophoresis [21]. Several methods for GLY and AMPA analysis in different matrices, mainly in environmental samples, are reported in the literature [18,22–29]. Most of these methods are tedious for including numerous steps for the purification and derivatization of the compound. Derivatization procedures can be made both pre-column and post-column. Pre-column procedures are based mainly on derivatization with 9-fluorenylmethyl chloroformate (FMOC) [22,23,26,30–33] to form fluorescent derivatives (improve detection) and/or to reduce the polar character of the analytes facilitating the chromatographic retention [34]. The development of new methods to reduce the steps in the analysis of these compounds is of interest for the scientific community [35].

HBM studies of these compounds are scarce and have not been widely developed yet, reflecting the problems associated with this analysis. Thus, Jensen et al. [36] validated a procedure with an additional clean up step before the direct analysis of GLY and AMPA in human milk and urine by LC-MS/MS, without involving real samples. Later, McGuire et al. [16] applied this method in real human milk and urine samples, showing that the sensitivity of this method was not good enough to detect these compounds in human milk, while only traces were detected in urine samples. Parvaez et al. [37] also developed a procedure, detecting only GLY in human urine samples without derivatization by LC-MS/MS. Furthermore, Connolly et al. [38] developed a new LC-MS/MS method to detect only GLY, not AMPA, in urine by including a previous solid phase extraction step which was applied to detect this compound in amenity horticulturalists first [38,39] and in the general young population later [40]. In view of these previous results, the aim of the present study was to develop a new validated method to detect both, GLY and AMPA, in human urine with a good sensitivity and without additional clean up steps. The optimization and validation of the proposed method was carried out according to the holistic approach [41,42]. The present procedure has been intended for routine determination in urine samples from farmers who handle GLY.

2. Material and methods

2.1. Chemicals and reagents

Glyphosate (GLY), Aminomethylphosphonic acid (AMPA), and Fluorenylmethoxycarbonyl chloride (FMOC-Cl) were supplied by SIGMA–Aldrich Chemie GmbH (Steinheim, Germany). Standard solutions of GLY and AMPA were prepared in Milli-Q water (100 mg mL^{-1}) and diluted as required for their use as working solutions (0.5 – 20 ng mL^{-1}).

Table 1
Detailed steps of the procedures under study.

	Option A	Option B
Sample volume	5 mL	1 mL
Condition of the sample	Without lyophilization	Lyophilized
Derivatization	1 mL borate buffer 5% 500 μL FMOC-Cl 1.5 g L^{-1}	2 mL borate buffer 5% 2 mL FMOC-Cl 8 g L^{-1}

All chemicals and reagents used in this study were analytical grade materials. Hydrochloric acid and acetonitrile were purchased from Merck (Darmstadt, Germany). Deionized water (418 MΩ/cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, USA).

2.2. Derivatization procedure

Several alternatives for derivatization of GLY and AMPA with FMOC-Cl were submitted to a preliminary evaluation, designing different procedures adapted to the aim of this work and based on the method from Demonte et al. [26], with different modifications that were adapted to our samples (human urine). Thus, different aspects were evaluated: volume and conditioning of the sample, concentration of the FMOC-Cl and buffer volume (Table 1). From the initial evaluation of the proposed approaches, the following method was finally selected for further optimization and validation: Blank urine samples (1 mL), previously homogenized, were spiked with different amounts of standards for calibration, between 0.5 and 20 μg L^{-1} GLY and AMPA. Each quality control (QC) sample was prepared using blank urine samples (1 mL) spiked with the same standard stocks and working solutions of GLY and AMPA used in the calibration studies. The concentrations of the different QC samples were as follows: low QC level—0.5 μg L^{-1} (GLY) and (AMPA); medium QC level—5 μg L^{-1} of each compound; and high QC level—20 μg L^{-1} of each compound. Once spiked, all the urine samples were submitted to lyophilization for 24 h. After that, the derivatization stage started with the addition of 5% borate buffer to achieve a pH 9, in which the derivatization reaction occurs, followed by the derivatization reagent, FMOC-Cl at an optimized concentration of 8 mg mL^{-1}. This reaction took place at a 60 °C for 30 min. Afterwards, 250 μL of HCl pH 1 was added to stop the reaction and the samples were centrifuged (8 min, 8000 rpm). Finally, samples were passed through a 0.2 μm filter before injection into the UPLC-MS/MS system.

2.3. Chromatographic conditions

Chromatographic separation was performed using an ultra high resolution liquid chromatograph (ACQUITY UPLC™, Waters, Milford, MA, USA) coupled to a Xevo TQ-S micro (Waters) consisting of a triple quadrupole mass spectrometer equipped with an electrospray ion source operated in positive mode. LC analyses were performed on an Acquity UPLC BEH® C18 column (particle size 1.7 μm, 2.1 × 100 mm). Injection volume was 10 μL and flow rate was 0.5 mL/min. Two different solvents were used as a mobile phase: Solvent A (Water 10 mM ammonium acetate) and solvent B (Acetonitrile:Water 95:5 10 mM ammonium acetate), the following gradient was used: 0.0–1.0 min 5% B, 1.0–7.0 min

Table 2
Precursor and fragment *m/z* values for each specific compound and their respective cone voltages and collision energy values.

Compound	Cone voltage (V)	Precursor ion (*m/z*)	Product ion (*m/z*)[a]	Collision energy (eV)
Glyphosate-FMOC	20	392.0	*Q* 87.8	30
			q 179.1	10
AMPA-FMOC	20	334.0	*Q* 155.8	20
			q 179.1	15

[a] *Q*: transition used for quantification; *q*: transition used for confirmation.

Figure 1.

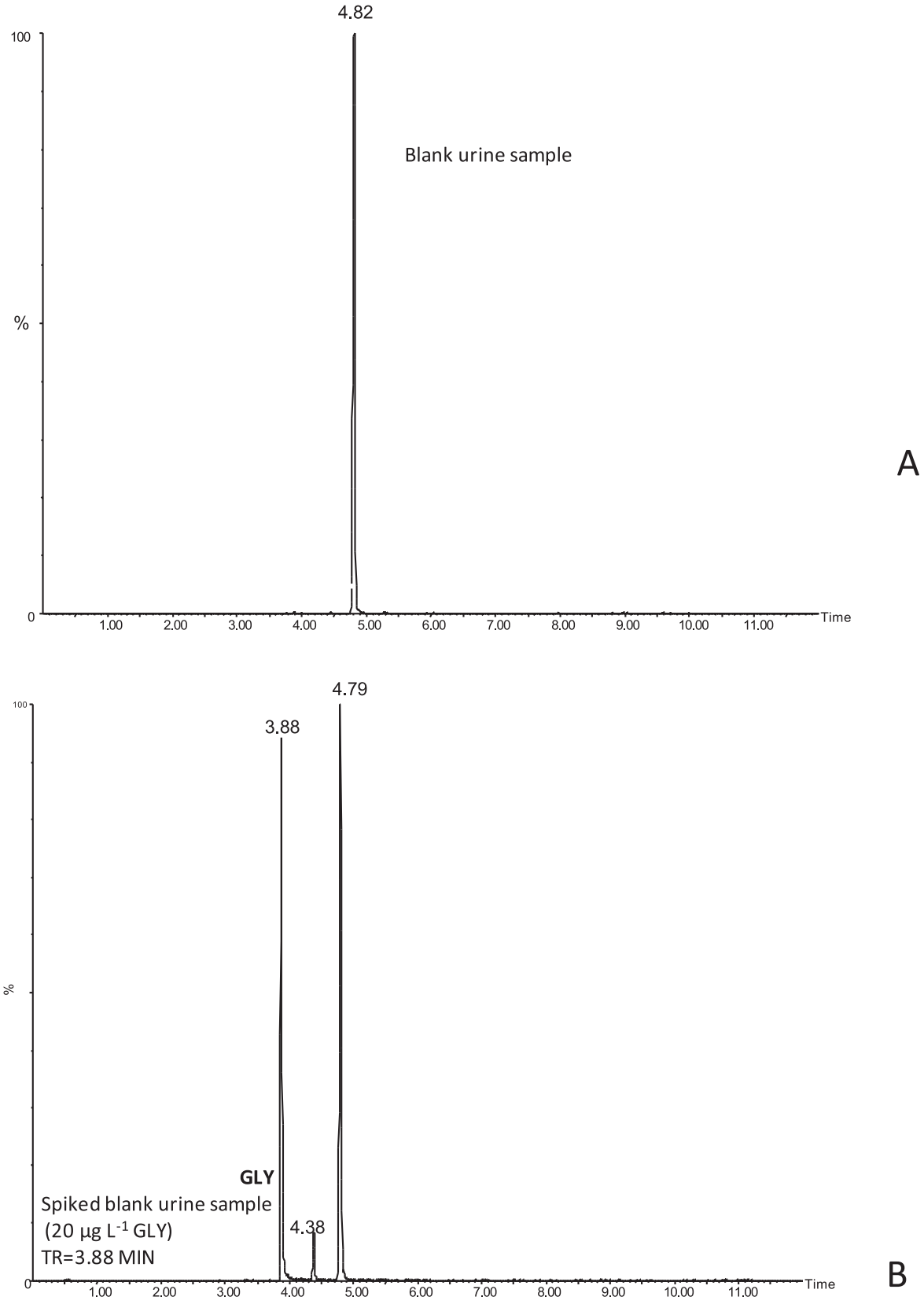

Fig. 1. UPLC-MS/MS chromatogram for A) urine blank and B) urine blank spiked with GLY and AMPA standard (20 µg L^{-1}).

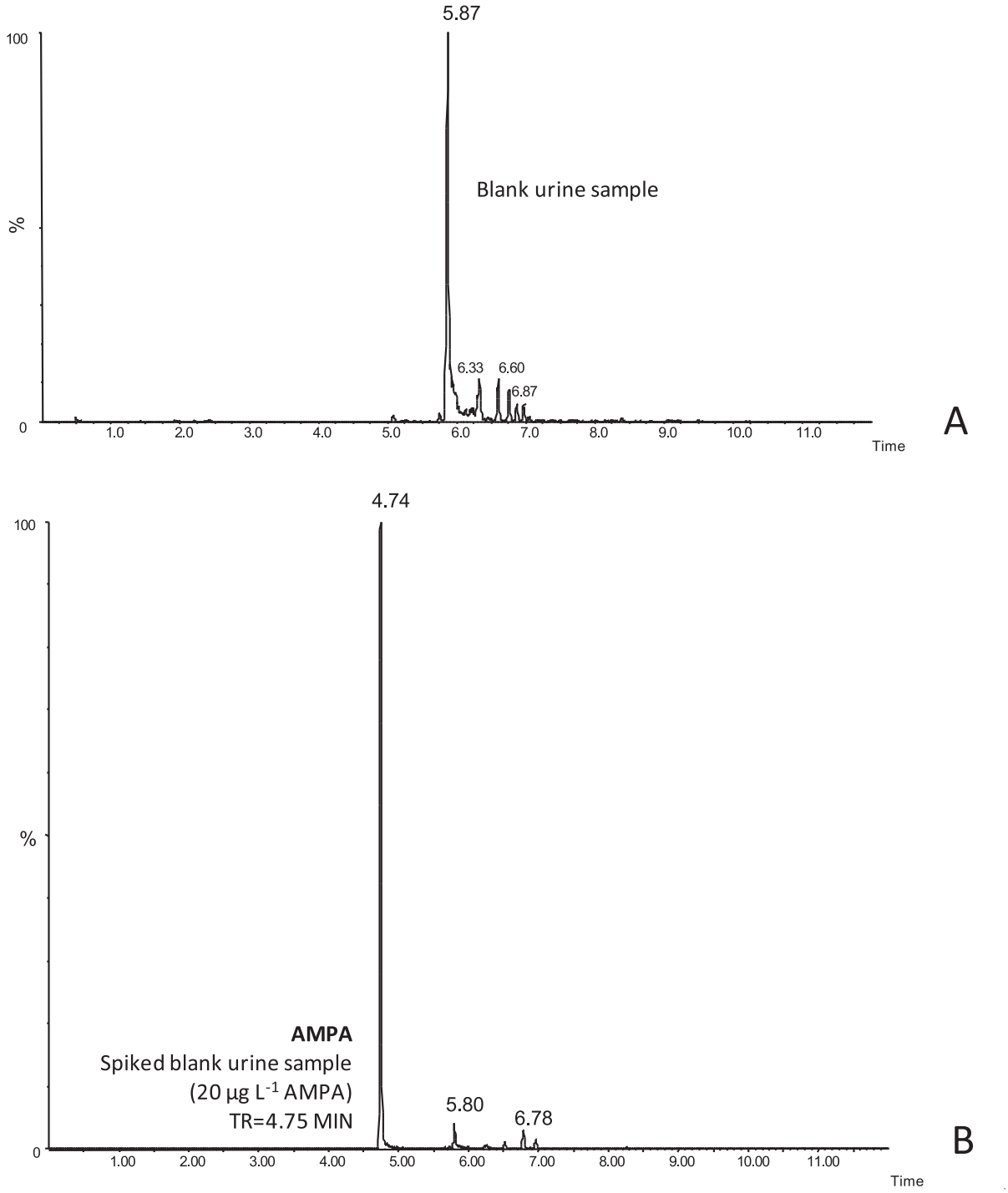

Fig. 1. (*continued*).

from 5% to 70% B, 7.0–9.0 min from 70% to 100% B, 9.0–10.0 min 100% B, then 5% B up to 12.0 min. Multiple Reaction Monitoring (MRM) was applied where the precursor ions and fragments ions were monitored at Q1 and Q3, respectively. The *m/z* values for the precursor and fragment for each specific compound along with their respective cone voltages and collision energy values are shown in Table 2. For UPLC-ESI-MS/MS analyses, the mass spectrometer was set to the following optimized tune parameters: capillary voltage: 2.90 kV, source temperature: 150 °C, desolvation temperature: 350 °C, source desolvation gas flow: 650 L h^{-1} and source cone gas flow: 50 L h^{-1}. Chromatographic and mass spectrometry data handling was performed using MassLynx software v 4.1 (Waters).

2.4. Statistical criteria for method validation

Once the method was developed, the next step was the validation to verify that it fulfills the requirements for its later application. Thus, the proposed method was validated taking into account the guidelines for the validation of analytical methods [43] regarding their linearity, sensitivity, precision and recovery parameters.

A calibration curve was prepared for each compound; blank urine samples were spiked with standards of GLY and AMPA in different

concentrations 0.5, 1, 2, 5, 10 and 20 μg L^{-1} and injected in triplicate. To assess the matrix effect, we developed calibration curves in urine free of GLY and AMPA.

Three validation standards covering the optimal working range were used, which were measured in triplicate for three different days. Three concentration levels (1, 5 and 20 μg L^{-1}) of GLY and (0.5, 5 and 20 μg L^{-1}) AMPA were spiked into urine samples in order to determine the repeatability and the precision through relative recovery assays.

2.5. Application of the validated method in human urine samples

2.5.1. Human subjects

All procedures used in this study were approved by the Coordinating Committee of Ethics of the Biomedical Investigation of Andalucía (protocol number: 0231-N-17), and informed consent was obtained from each subject. A total of 20 women farmers were recruited from Marinaleda, Sevilla (Spain), which was part of a larger investigation about the possible toxic effects of the indirect pesticides exposition in women who collect fruits and vegetables in the field. This geographic location was targeted because it is an eminently agricultural municipality, whose economy is based on agricultural production.

2.5.2. Urine collection and preservation

Urine collection was carried out by the participants themselves, who were provided with a 125 mL polypropylene urine container and the urine collection instructions. A first-morning urine sample was collected from each volunteer. Samples were transported to the laboratory where they were subsampled and stored at −80 °C prior to being lyophilized. As lyophilization increases the samples stability, once lyophilized, the samples were stored until their analysis.

3. Results and discussion

3.1. Pre-column derivatization with FMOC-Cl

The concentration of sodium tetraborate is known to be critical, as an excess may interfere with the solvents of the chromatographic system, while a low concentration may lead to insufficient buffering capacity to complete the derivatization [26]. For this reason, two different volumes of the borate buffer (5%) were evaluated in the present study (Table 1). The final concentrations of the buffering reagent tested with reference to the starting volume of the sample were 2.3 g L^{-1} for Option A and 6 g L^{-1} for option B. This concentration range is in agreement with others reported in literature [26,44]. In this case, the option B was the one that allowed to achieve the results obtained.

Both GLY and AMPA are very polar compounds that require a previous derivatization procedure to improve the sensibility and to detect trace levels by UPLC-MS/MS. FMOC-Cl is the most used compound to react at a room temperature with primary and secondary amines without needing a previous oxidation method. The required amount of this derivatizing reagent is also critical to ensure a complete and reproducible reaction, due to the presence of very high levels of amines on urine (in the forms of uric acid and urea). An excess of reagent must be used for the complete derivatization of the analytes present in the samples. This amount was experimentally determined in a range of concentrations between 1.5 and 8 g L^{-1} of FMOC-Cl and in a range of volume added between 0.5 and 2 mL referred to the initial volume of sample considered in each tested procedure (Table 1). The optimization of this excess of FMOC-Cl is important since the unreacted reagent becomes an undesirable impurity and the formation of by-products occurs during the reaction [26]. In our case, 2 mL of FMOC-Cl 8 g L^{-1} was used (method B). The selected method worked adequately, not introducing problems with the excess of reagent but reaching the optimum concentration to derivatize the GLY and AMPA present in urine samples and also the natural amines present in human urine.

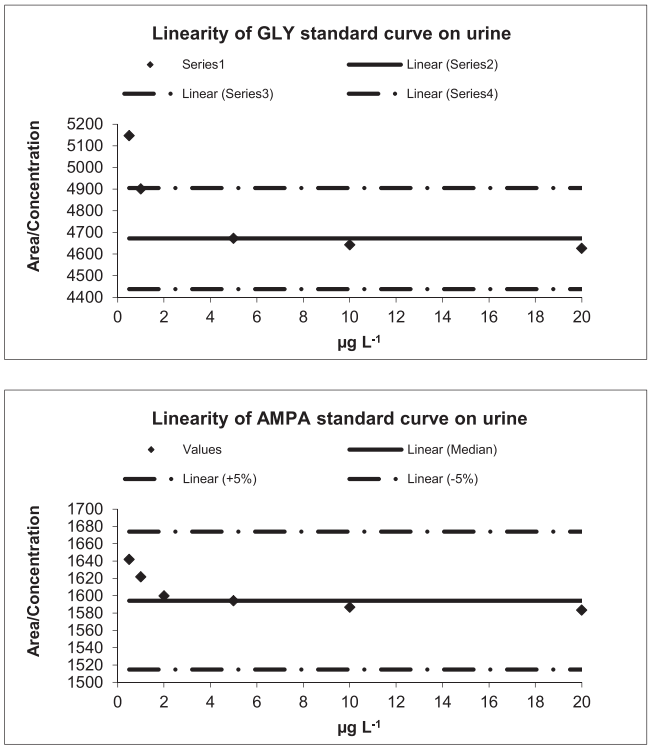

Fig. 2. Huber plot for assessing the linear range of GLY (a) and AMPA (b) in urine.

3.2. Matrix effects

The matrix effects were determined in urine samples following the expression: ME = 100 × [(slope matrix/slope solvent) − 1], expressed in percentage. The values obtained were 23.02 for GLY and −18.63 for AMPA, meaning mild matrix effect ($|10| < ME < |20|$) for AMPA and medium ($|20| < ME < |50|$) for GLY, so slight matrix effects were observed in the present work. Therefore, in order to obtain more accurate results matrix-matched calibration curves were employed for quantification and validation procedure.

3.3. Method validation

In order to develop the UPLC–MS/MS for the detection of GLY and AMPA, commercially available standard solutions of these compounds were assayed to acquire mass spectra and adjust mobile phase strength. The first experiment carried out was to select the optimal ESI-MS parameters and the appropriate ions analyzing individual solutions of GLY and AMPA derivatized with FMOC-Cl to monitor the MS intensity. Although these compounds are usually analyzed in negative ion mode [25,31,36] it has been reported the positive ion mode analyzing as well [20,26,45]. In this case case, GLY and AMPA were analyzed in both negative and positive ion modes, obtaining greater sensitivity in the positive mode and, hence, this positive ion mode was selected. A major ion for each compound was evident in the mass spectra of the compounds, *m/z* 392 for GLYP–FMOC and 334 for AMPA–FMOC, corresponding to the protonated molecular ions $[M + H]^+$. Multiple reactions monitoring (MRM) mode was used to obtain the maximum sensitivity for quantitative analysis (Table 2). In order to study the matrix effects on the ionization, both blank urine samples and blank urine samples spiked with GLY and AMPA solutions were prepared, and the peak areas of GLY and AMPA standard solutions (20 μg L^{-1}) prepared in blank urine samples were compared with the signal obtained in blank urine samples (Fig. 1A and B).

The concentration-dependent responses were calculated from GLY

Table 3
Estimations of within-condition repeatability (S_w), between-condition repeatability (S_B), intermediate precision (intra-laboratory reproducibility, S_{IP}) and its relative standard deviations (%RSD_{IP}), and recoveries of GLY and AMPA assayed in human urine, at three concentration levels, in three different days. Reference RSD values and recovery percentages by AOAC. Limits of detection (LOD) and quantitation (LOQ) and linearity for both compounds.

	GLY-AMPA VALIDATION					
	1 μg L^{-1}	0.5 μg L^{-1}	5 μg L^{-1}		20 μg L^{-1}	
	GLY	AMPA	GLY	AMPA	GLY	AMPA
S_W	0.13	0.03	0.81	0.15	3.05	0.79
S_B	0.06	0.08	0.28	0.39	1.19	2.40
S_{IP}	0.11	0.05	0.68	0.26	2.59	1.53
RSD_{IP}(%)	10.8	9	12.70	4.8	11.90	7.3
Relative Recovery (%) (n = 9)	107	119	108	107	109	104
RSD_{AOAC} (%)	21–30	21–30	21–30	21–30	15–21	15–21
Acceptable Recovery Range (%)	40–120	40–120	40–120	40–120	60–115	60–115
	GLY			**AMPA**		
LOD (μg L^{-1})	0.5			0.1		
LOQ (μg L^{-1})	1			0.5		
Linearity (μg L^{-1})	LOQ-20			LOQ-20		

and AMPA standards prepared in blank urine samples, and were measured by a 6-point calibration curve with a linear range within 0.5–20 μg L^{-1}. The regression equations obtained were y = 4611x + 302.64 (R^2 = 0.9998) and y = 1574.7x + 53.664 (R^2 = 0.9992) for GLY and AMPA, respectively.

3.3.1. Linearity

Six different concentrations of GLY and AMPA were spiked to blank human urine (0.5–20 μg L^{-1}), submitting them to the proposed method. The calibration plot (signal response/analyte concentration against their concentrations) was established according to Huber [46] by replicate analysis (n = 3) at all concentration levels (Fig. 2). The target line had zero slopes and the intercept represents the median of the response factors obtained in a fashion similar to the action limits of control charts. Both parallel horizontal lines on the graphic represent the 0.95 and 1.05 times the median value. As can be observed, no intersections with the lines were found in the case of AMPA, so the linear ranges of the methods apply to the full ranges studied. However, on the GLY representation, the 0.5 value cannot be adjusted to the lineal ranges, discarding this concentration as lineal for our method.

3.3.2. Sensitivity

For validation purposes, it is normally sufficient to provide an indication of the level at which detection becomes problematic and quantification is acceptable in terms of repeatability, precision and trueness. For this aim, the limits of detection (LOD) and quantitation (LOQ) were determined based on the standard deviation of the blank, by measuring 10 independent urine sample blanks once each, and were estimated according to the equation $Y_{LOD \text{ or } LOQ} = Y_{blank} + nS_{blank}$, where Y_{blank} and S_{blank} are the average value of the blank signals and its corresponding standard deviation. In these expressions, n = 3 in the case of LOD and n = 10 in the case of LOQ. Afterwards, Y_{LOD} and Y_{LOQ} values are converted into concentration units by using the calibration function. The LOD and LOQ obtained are 0.5 and 1 μg L^{-1} for GLY and 0.1 and 0.5 μg L^{-1} for AMPA, respectively (Table 3). Other authors obtained values of 1 μg L^{-1} for GLY LOD in urine using LC-MS [47], also 0.02 and 0.1 μg L^{-1} for GLY and 0.03 and 0.1 μg L^{-1} for AMPA for LOD and LOQ, respectively or 0.1 μg L^{-1} for GLY LOD by LC-MS/MS [16,37]. Our values are similar to those obtained by Connoly et al. [48] in human urine (GLY LOQ: 0.5 μg L^{-1}) by LC-MS/MS.

3.3.3. Precision

Precision refers to the closeness of agreement between independent test results obtained under stipulated conditions, and according to the International Conference on Harmonisation Guidelines [43]. Thus, precision may be considered at three levels: repeatability, intermediate precision and reproducibility. The first one expresses the precision evaluated under the same experimental conditions over a short time interval, and it is termed as intra-assay or within-run. On the other hand, intermediate precision applies to within-laboratory variations: different days, different analysts or equipments, and it is sometimes called between-run or inter-assay precision [42]. The third level, reproducibility, expresses the precision between-laboratories in collaborative studies, and it will not be considered in this work.

To assess the precision study, blank urine samples were spiked at three concentrations of GLY and AMPA standards (0.5, 2 and 20 μ L^{-1}), in triplicate (n = 3) within the same day, as recommended by the ICH guidelines, and over a period of three days. Afterward, they were subjected to the proposed method and results were obtained. Considering three different days as the main source of variation, an analysis of variance (ANOVA) was performed for each concentration, obtaining estimates within-days (S_w), also known as repeatability, and between-day (S_B). Also, the intra laboratory reproducibility or intermediate precision (S_{IP}) was obtained [41,42]. All these parameters are shown in Table 3. From these data, the corresponding relative standard deviations, RSD_{IP} are calculated, and were compared with the acceptable RSD percentages obtained from the AOAC Peer Verified Methods (PVM) program [42,46]. As a quick rule [42], the RSD_{IP} results should be compared with one-half the corresponding RSD values tabulated. Our results, at the three concentration levels considered, were lower or the same order than the one-half %RSD_{AOAC} tabulated, so the proposed method can be considered as precise (Table 3).

3.3.4. Trueness and recovery

The trueness of an analytical procedure expresses the closeness of agreement between the mean value obtained from a series of measurements and the value which is accepted, either a conventional value or an accepted reference value like validation standard [42]. It can be obtained from the same ANOVA results previously described for the intermediate precision, and it is normally expressed in terms of bias or recovery obtained for each validation standard considered [49]. These recoveries are defined as the ratio between the mean concentration of analyte measured in the fortified sample and the concentration of analyte added ("true" reference value, not determined by method) in the fortified sample, expressed as a percentage. The recoveries obtained for the three validation standards are shown in Table 3.

They have been checked for suitability by comparison with the published acceptable recovery range as a function of the analyte concentration [42]. In our case, two concentrations of the three validation standards ranged between 0.5 and 5 μg L^{-1}, the recovery range (%) could oscillate between 40 and 120% for the two of them (GLY and AMPA). The third one validation standard was 20 μg L^{-1} and the recovery range (%) could oscillate between 60 and 115% in both cases (GLY and AMPA). The recoveries obtained oscillated between these values 119% for 0.5 μg L^{-1} (AMPA), 108 and 107% for 5 μg L^{-1} (GLY and AMPA, respectively) and 109 and 104% for 20 μg L^{-1} (GLY and AMPA, respectively). Thus, the method can be considered as acceptable in terms of recoveries. These recoveries are higher than those obtained by Bernal et al. [20] in rat serum samples by liquid chromatography–fluorescence–mass spectrometry method. This may be due to the nature of the samples, as urine has less interference than plasma. However, recently, Lopez-Ruiz et al. [50] have optimized a method to determine polar pesticides in human blood by LC-MS, obtaining recoveries of the same order as ours. Also, similar recoveries were obtained in human samples (milk and urine) by Jensen et al. [36] when a direct

Table 4
Comparison of the developed method with recently published articles.

Determination method	Type of sample	Sample preparation	Compound analyzed	Compound detected	LOD/LOQ (μg L^{-1})	References
HPLC-ESI-MS	Human urine	Not showed	GLY	GLY	LOQ 2	[47]
LC-MS/MS	Human Milk	FMOC-Cl derivatization	GLY, AMPA	No detected	LOQ 0.5*	[51]
LC-MS/MS	Human Milk and urine	No derivatizationAdditional clean up	GLY, AMPA	GLY, AMPA	LOQ 0.1	[16,36]
LC-MS/MS	Human urine	Solid Phase extraction	GLY	GLY	LOD 0.5	[38–40]
LC-MS/MS	Human urine	No derivatization	GLY	GLY	LOQ 0.5	[37]
HRAM LC-MS	Human urine	EDTA and SPE pre-treatment FMOC-Cl derivatization	GLY, AMPA, glufosinate	Only AMPA	LOD 0.02	[52]
UPLC-MS/MS	Human urine	FMOC-Cl derivatization	GLY, AMPA	GLY, AMPA	LOQ 1 and 0.5	Present study

* μg g^{-1}

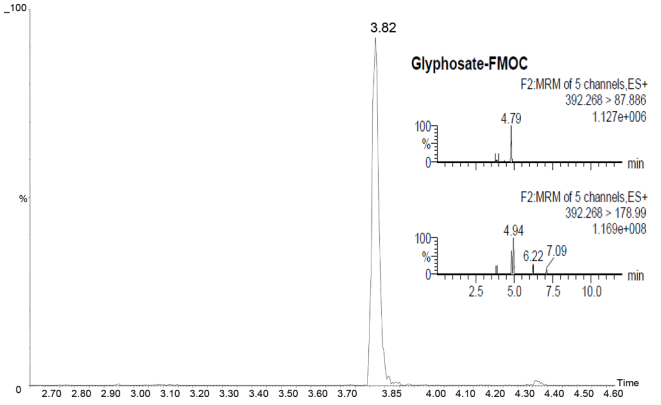

Fig. 3. UPLC-MS/MS chromatogram from one urine sample extract.

determination of GLY and AMPA by LC-MS/MS were applied.

Taking into account these considerations, the analytical procedure developed in this work can be considered suitably validated.

3.4. Comparison of the developed method with other methods

There are several published methods to detect GLY and AMPA in different matrices (biological samples such as milk or environmental as soils and waters) and with several techniques (GC–MS, LC–MS, Capillary Electrophoresis…) [32–34,51]. However, these methods cannot be used for urine routine analysis without a previous validation in this biological matrix. On the other hand, there are some authors that employed different sample treatments to detect these contaminants in biological samples. Thus, Jensen et al. [36] developed a reliable method for determining GLY and AMPA in human urine including an additional clean up step. Recently, Franke et al. [52] developed a new method to detect GLY, AMPA and glufosinato from human urine by HRAM LC–MS, however, their method in urine was suited only for AMPA analysis. Similarly, Parvaez et al. [37] and Connolly et al. [38] developed both LC–MS/MS method but only detecting GLY in human urine samples without derivatization in the first case and including a previous solid phase extraction step in the second one. Thus, it can be concluded that the method from the present study is faster than the other options, as it does not require a pre-cleanup, while it is good enough to detect and quantify not only AMPA, but also GLY in cross-sectional studies.

The effectiveness of the present method for the simultaneous extraction and determination of GLY and AMPA was compared with other recently published works. Table 4 summarizes the comparative results.

3.5. GLY and AMPA determination in women farmers

A total of 20 samples were analyzed using the proposed method. A blank sample and two quality controls were prepared and injected with the samples. Only one of the samples showed GLY positive results (Fig. 3) and all of them were negative in terms of AMPA detection (concentrations below LOQ). The GLY concentration detected was 2 μg L^{-1}. This result is similar to those detected previously in American farmers when urine was collected the same day of pesticides application (3.2 μg L^{-1}) [53]; after two days since application in French farmers (2 μg L^{-1}) [47] or post-application in Irish farmers (1.72 μg L^{-1}) [38].

4. Conclusions

In this study, a simple and fast UPLC–MS/MS method was developed and validated for the determination not only of GLY but also its metabolite AMPA in human urine, proving to be sensitive, reproducible, accurate and robust. The recoveries (108–109% GLY and 104–119% AMPA) and intermediate precisions obtained (11.90–12.70% GLY and 4.8–9% AMPA) allow its validation. Moreover, it has been possible to apply the present method for detection and quantification of both compounds in human urine from women farmers, showing the presence of GLY in urine from one of the participants. For this reason, we can conclude that the novelty of the present method is to achieve the determination of both GLY and AMPA in human urine with rapid sample preparation and derivatization without an additional cleaning step as is usual in most published methods. This procedure can be used to monitor potential exposure of humans to GLY and AMPA in epidemiological studies and for routine controls in public health.

CRediT authorship contribution statement

Jose Martin-Reina: Conceptualization, Investigation, Writing - original draft. **Bouchra Dahiri:** Conceptualization, Investigation. **Pilar Carbonero-Aguilar:** Resources, Methodology. **M. Eugenia Soria-Dıaz:** Methodology, Formal analysis. **A. Gustavo González:** Validation, Formal analysis. **Juan Bautista:** Supervision, Writing - review & editing. **Isabel Moreno:** Supervision, Writing - review & editing, 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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