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Olivier Delhomme[1,2], Anaïs Rodrigues[1], Ana Hernandez[1], Supansa Chimjarn[1], Colette Bertrand[3], Marjolaine Bourdat-Deschamps[4], Clémentine Fritsch[5], Céline Pelosi[6], Sylvie Nélieu[4], Maurice Millet[1]

[1] Université de Strasbourg, CNRS-UMR 7515, ICPEES, 67087, Strasbourg, France.

[2] Université de Lorraine, 57070, Metz, France.

[3] Université Paris-Saclay, INRAE, AgroParisTech, UMR ECOSYS, 78026, Versailles, France.

[4] Université Paris-Saclay, INRAE, AgroParisTech, UMR ECOSYS, 78850, Thiverval-Grignon, France.

[5] Laboratoire Chrono-environnement, UMR 6249 CNRS - Université de Franche-Comté Usc INRAE, 16 route de Gray 25030 Besançon cedex, France.

[6] INRAE, Avignon Université, UMR EMMAH, 84000, Avignon, France.

Abstract

A new sensitive and selective analytical methodology to quantify glyphosate (GLY), aminomethylphosphonic acid (AMPA), and glufosinate (GLU) in both soil and earthworms (*Allolobophora chlorotica*) was developed. The extraction and purification methods were optimized. The samples were extracted with various aqueous solutions (HNO_3, H_2O, KOH and borate buffer) and derivatized with 9-Fluorenylmethyl chloroformate (FMOC-Cl). To optimize the extraction step, a method to remove the excess FMOC-Cl was applied based on liquid-liquid extraction with diethyl ether. The purification of derivatized extracts was carried out using XLB solid phase extraction (SPE) cartridges before internal standard quantification by liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS). The elution step was optimized to obtain the best recoveries possible, which was with acidic methanol (1% formic acid) (67 % for GLY, 70 % for GLU and 65 % for AMPA). The extraction and purification method followed by analysis of the two herbicides and AMPA in soils using LC/MS/MS determined limit of quantification (LOQ) values of 0.030 μg g^{-1} for GLY, 0.025 μg g^{-1} for AMPA and 0.020 μg g^{-1} for GLU . For earthworms, LOQ were 0.23 μg g^{-1} for GLY, 0.20 μg g^{-1} for AMPA and 0.12 μg g^{-1} for GLU. .

The developed method was applied to determine these compounds in natural soils and earthworms.

Keywords: herbicides, soil organisms, liquid chromatography-tandem mass spectrometry, solid-phase extraction, derivatization.

1. Introduction

The non-selective herbicide glyphosate is currently the major organophosphate herbicide used worldwide [1]. Since its introduction as an active herbicide ingredient in 1971, the worldwide market for GLY has continuously increased, with a noticeable boost after 1990 due to the worldwide introduction of genetically modified crops [2]. Indeed, together with ammonium glufosinate , another broad-spectrum herbicide, they are extensively applied on a large variety of crops (e.g., cereals, vineyards, potatoes, peas, orchards) as well as in non-agricultural areas such as private gardens and industrial areas. One reason for this intensive use is their high efficacy against most weeds and affordable price compared to other herbicides.

In the environment, GLY is rapidly degraded into aminomethylphosphonic acid , its major metabolite. In water, degradation mainly results from photodegradation [3], and in soil from microbial biodegradation [4]. GLU and GLY are considered to be non-persistent field half-lives (DT_{50}) of 7 and 24 days, respectively, whereas AMPA is persistent, with a field DT_{50} of 419 days [5]. However, some evidence suggests that GLY may be more persistent than expected, with detection in runoff following spraying and rainfall several months after application. There are also reports that glyphosate-based herbicides have the potential to persist in the environment for up to 197 days after a single application [1]. Thus, intensive use of GLY and GLUbased herbicides can strongly disperse the active ingredient in the environment and has the potential to contaminate the environmental compartments i.e., water [6], soil [7], air [8] and organisms. GLY and AMPA, in particular, have both been frequently found in surface waters [[2], [9]].

The accumulation of GLY, GLU and AMPA in living soil organisms has so far only been assessed in snails [10]. Earthworms are prey for numerous predators [11] and key soil organisms as they influence soil structure, organic matter dynamics, and plant productivity

[[12], [13]]. These soil organisms are used as models in ecotoxicology and several studies have assessed the impact of GLY, AMPA or GLU on them [[14], [15]]. It has been recognized that although commercial formulations containing GLU and GLY generally have no effects on mortality, they may negatively impact earthworm enzyme activities, body weight, reproduction, behavior (avoidance, foraging) and activity (surface casting) [[16], [17]]. For instance, GLY has been reported to modify earthworm feeding behaviour and thus to alter ecological interactions between earthworms, mycorrhizal fungi, and aboveground plants [18]. However, bioaccumulation of these compounds in earthworms has never been assessed even though it could give new insights into their potential ecotoxic effects on these organisms and the consequences on soil functioning.

Detection of the potential presence of these herbicides in the environment has required the development of specific analytical procedures because measurement of their residues is challenging [19]. Indeed, the analysis of these molecules in environmental matrices remains difficult with conventional detectors such as UV and fluorescence due to the lack of adequate chemical groups in GLY, its metabolite AMPA and GLU molecules (i.e. chromophores or fluorophores). In addition, their ionic character, complex formation with metals [[20], [21], sorption to glassware [22], low volatility and insolubility in organic solvents associated with their low molecular mass has increased the analytical difficulties, in particular the low quantification limits required for water quality criteria.

Many analytical procedures have been developed in the last decades for the quantification of GLY, AMPA and GLU including gas chromatography after a derivatization step [[23], [24]], ion chromatography coupled with conductimetry [25] or inductively coupled plasma spectrometry [[26], [27]] and liquid chromatography coupled to fluorescence and/or mass spectrometry [[28], [29], [30], [31], [32]]. FMOC- Cl is the most common derivatization agent used, as it allows, when associated with LC/MS/MS, a better detection and quantification than non-derivatized herbicides. Indeed, it results in improved chromatographic separation from the matrix, as well as superior selectivity and sensitivity [33]. However, even if LC/MS/MS is a reliable method for quantifying GLY, GLU and AMPA at low detection levels, their extraction from soils and living organisms is complicated by the matrix. It is known that, in soil, these herbicides show high sorption to soil clays and organic matter [21]. Several extraction methods from soil have been reported [[4], [24], [34]] but in many cases, the extraction method was specific for one type of soil [31], and to our knowledge no extraction methods have been reported for earthworms.

In the present study, a new sensitive analytical methodology to quantify GLY, GLU and AMPA residues in both soils and earthworms (*Allolobophora chlorotica*) using SPE – LC – ESI – MSMS was developed. As the quantification of GLY, GLU and AMPA residues in earthworms is original as never published elsewhere in our knowledge, for soils a particular attention was made, regarding previous works, to optimize the extraction and purification methods, especially by testing various aqueous solutions for extraction and elution mixtures after purification by SPE. This fully characterized method was applied on soil and earthworms samples.

2. Material and methods

2.1. Chemicals and solutions

HPLC grade quality solvents (acetonitrile (ACN), ethanol, diethyl ether, methanol (MeOH), n-hexane), potassium hydroxide (38 %) Normapur (KOH), disodium tetraborate decahydrate (borate), ammonium hydroxide solution (NH_4OH) and formic acid solution (HCOOH) were purchased from VWR Prolabo (Paris, France). Formic acid, ACN and water for LC/MS were purchased from Sigma Aldrich (LPCR, France). The ultra-pure water was obtained through a Milli-Q system (18 MΩ cm) from Merck, Germany. FMOC-Cl and dimethyl-dichloride silane (DMDCS) were purchased from Fluka and Aldrich, respectively (l'Isle d'Abeau, France).

High purity pesticide standards (>98%) were supplied by Cluzeau Info Labo (Sainte-Foy-la-Grande, France) for GLU (ammonium 2-amino-4-(hydroxymethylphosphinyl)butyrate; CAS number: 77182-82-2) and by Sigma Aldrich (l'Isle d'Abeau, France) for GLY (N-(phosphonomethyl)glycine; CAS number: 1071-83-6), AMPA (aminomethylphosphonic acid; CAS number: 1066-51-9) and internal standards: APPA (1- aminopropyl phosphonic acid; CAS number: 14047-23-5) and AMPPA (1- amino-2-methylpropyl phosphonic acid; CAS number: 66254-55-5).

Stock solutions of each pesticide at 1 g L^{-1} and calibration standard solutions were prepared in ultra-pure water and stored in silanised glassware or plastic flasks. A saturated solution of 50 g L^{-1} borate buffer (pH 9) in ultra-pure water and a solution containing 10 g L^{-1} of FMOC-Cl in ACN were used for the derivatization step prior to LC/MS/MS analyses.

All glassware in contact with GLY, GLU and AMPA was silanized. The solution for glassware silanization was prepared by diluting 5% DMDCS in n-hexane. After 10 min of contact, glass containers were rinsed twice with hexane then with MeOH before being dried in a fume hood.

2.2. Earthworm and soil sampling

The earthworm *Allolobophora chlorotica* (green morph) was chosen as a model organism. This earthworm species is common in temperate European regions and was chosen because it lives close to the soil surface. It is thus potentially highly exposed to and impacted by pesticides [35]. For method development, characterization and matrix-matched calibration curves, pesticide-free earthworms were collected by hand from a fallow in Versailles, France (48°48′31″N, 2°05′26″E). The fallow had not been treated with pesticides for more than 20 years. The individuals were used as the blank matrix and first analyzed to confirm the absence of contamination with the targeted pesticide residues.

For method application, *A. chlorotica* individuals were manually collected in Spring 2016 by superficially digging the soil in winter wheat fields located in the Long-Term Socio-Ecological Site Zone Atelier Plaine & Val de Sèvre (ZA-PVS; **http://www.za.plainevalsevre.cnrs.fr/**) [36]. Earthworms were then stored for 48 h in Petri dishes on damp filter paper to void gut contents and then frozen at − 80 °C until analysis.

Soil cores were also sampled in the same wheat fields as the earthworms using a 5 cm Ø soil auger at a 0–5 cm depth. The soils were frozen at -20°C before being analyzed. One part of some soil samples was used for method development. For this, they were extracted with water, in order to remove potential traces of herbicides, and dried at 50°C overnight in an oven. They were again extracted with pure water and analyzed for GLY, GLU and AMPA content. If none of these molecules were present, the soil samples were used for method development.

2.3. Soil extraction

Soil samples were defrosted and 30 g collected and removed from roots and small stone debris. Each sample was then homogenized by slight crushing and 15 g were put in a plastic container (Figure 1) and spiked with the internal standards (IS) APPA and AMPPA (40 µL of each at 10 mg L^{-1}). To allow the sorption of the IS onto the soil structure, the mixtures were left in the dark for one hour (sufficient time for a total sorption) before starting the extraction procedure.

After this delay, 20 mL of the extraction solution (10 mL borate buffer + 10 mL H_2O) was added and the sample was stirred for one hour at room temp in the dark on a magnetic stirrer, followed by centrifugation at 1 252 *g* for 30 minutes. The supernatant (extract 1) was collected and the soil sample was re-extracted with 10 mL solution (5 mL borate buffer + 5 mL pure water) following the same procedure (extract 2). Both extracts (1+2) were combined and 5 mL of FMOC- Cl (10 g L^{-1}) and 5 mL ACN were added. The samples were then derivatized for 1 hour at room temperature in the dark while stirring. The samples were then left for 2 hours at room temperature in the dark without stirring before to remove the excess FMOC-Cl by liquid-liquid extraction (LLE) with diethyl ether (7.5 mL), through vortex agitation for 1 minute. This LLE was repeated twice and this extraction allow to optimize the analyse, thereby keeping the ionization chamber of the mass spectrometer clean. The aqueous fraction was collected and adjusted to 250 mL with pure water. The pH was adjusted to 3 with formic acid before the SPE procedure.

SPE was carried out using 6 mL Chromabond® XLB cartridges (Macherey-Nägel, France) containing 200 mg of the phase and an autotrace® 280 (ThermoScientific, France). The cartridge was first conditioned by successive addition of 5 mL MeOH, 5 mL pure water and 5 mL formic acid solution (pH 3) at 5 mL min^{-1}, then the 250 mL of the sample solution was deposited at 10 mL min^{-1}. The cartridge was dried under nitrogen for 20 minutes and the elution was carried out with 2×2 mL of MeOH containing 1 % formic acid at 5 mL min^{-1}.

2.4. Earthworm extraction

Earthworms (1 g) were cut into small pieces using Inox scissors and inserted into 15 mL centrifugation tubes (Figure 2). The tubes were weighed and 40 µL of a mixture of IS at 10 mg L^{-1} was added and vortexed. The earthworms were then digested for 20 minutes at 50°C with 2 mL KOH solution (pH 12) in order to solubilize all proteins and other molecules [37]. After centrifugation, the supernatant was derivatized in a plastic flask with 5 mL borate (50 g L^{-1}), 1.5 mL ACN and 1.5 mL FMOC- Cl (10 g L^{-1}) at room temperature in the dark while stirring for 1 hour. The volume of the derivatized extract was then adjusted to 50 mL with pure water and adjusted to pH 3 with formic acid, followed by centrifugation for 15 minutes at 1,252 *g*. The supernatant was extracted by LLE with diethyl ether (10 mL) through vortex agitation for 1 minute. This LLE was repeated twice. The aqueous fraction was collected and adjusted to 250 mL with pure water before SPE. The SPE procedure was identical to that used for soil.

2.5. LC/MS/MS Analysis

A Thermo Scientific TSQ Quantum Access Triple Quadrupole Mass Spectrometer coupled with a Surveyor pump and an Accela autosampler operating in heated positive electrospray ionization mode (HESI+) was used. The sampler is equipped with a 20 μL injection loop and the samples were kept at a temperature of 15°C. The analysis was performed on a Nucleodur C_{18} Pyramid column (150 mm × 3 mm, 3 μm) at 25°C. Samples were analyzed using a mobile phase water/ACN both containing 0.1% formic acid, at a flow rate of 0.5 mL min^{-1}. The composition of the mobile phase was kept at 60:40 for 2 min, then held to 5:95 (v/v) in 8 min (2 min hold), then 60:40 (v/v) in 2 min for 3 min.

Detection and quantitation of GLY, GLU and AMPA were performed using multiple reactions monitoring (MRM). The ion source was operated in positive ion mode with a spray voltage of 4,500 V and a vaporizer and capillary temperature of 300°C each. Nitrogen was used for sheath and auxiliary gas pressure (20 and 10 arbitrary units) while argon was used for collision pressure (1.5 arbitrary unit). Two precursor product ion transitions for each analyte and each internal standard were used for quantitation. *Q* for quantification transition, *q* for qualification transition and Ec for collision energy (V)) were used from precursor ion as follows: glyphosate (*Q*) *m/z* 392/179 (42 V) and (*q*) *m/z* 392/88 (18V); AMPA (*Q*) *m/z* 334/179 (23 V) and (*q*) *m/z* 334/156 (69 V); glufosinate (*Q*) *m/z* 404/182 (13 V) and (*q*) *m/z* 404/136 (20 V); APPA (*Q*) *m/z* 362/179 (25 V) and (*q*) *m/z* 362/140 (5 V) and AMPPA (*Q*) *m/z* 376/179 (25 V) and *m/z* (*q*) 376/156 (5 V). Data were acquired and processed using Excalibur software.

Both ion transitions had mean accuracies between 70 and 120% and a precision of 20% relative standard deviation (RSD), based on at least five replicates.

2.6. Calibration, limits of detection and quantification

For soils, a calibration step was performed by obtaining curves with pure water spiked with increasing amounts of GLY, GLU and AMPA. Linearity of the method was evaluated analyzing six standard solutions by triplicate in the range 0.010-1.0 mg L^{-1}. The concentration of the internal standards was the same as those used for soil sample extraction. APPA was used for the calibration while AMPPA was used to evaluate the effectiveness of the derivatization step. . The procedure was the same as for derivatization of the soil extract. For earthworms, a calibration step was carried out on the matrix by spiking blank small cut pieces of earthworm with increasing concentrations of the three analytes , at six concentration levels by triplicate in the range 0.010-1.0 mg L^{-1}. APPA and AMPPA were used as IS to evaluate the calibration and extraction/derivatization steps, respectively. The blank earthworms were analysed in the laboratory to ensure the absence of pesticide contamination. The spiked earthworms were processed using the same procedure as the experimental samples.

The limits of quantification (LOQ) and detection (LOD) for soil and earthworms were determined by the signal to noise ratio (S/N) with: S/N = 10 for LOQ and S/N= 3 for LOD. For soil samples, the repeatability was determined by analyzing, on the same day, five sub-samples (15 g) from a sample of soil spiked with 40 µL of a mixture of herbicides at 10 mg L^{-1} each, using the same analytical method. The reproducibility was determined by analyzing three sub-samples (15 g) from the same sample of soil used to determine the repeatability. The sub-samples were analyzed using the same analytical method with a one-week interval between analyses. The composition and the characteristics of soil sample used to validate the method were soil texture (sand (18%); silt (39%) and clay (43%)), pH (H2O) of 8.11 and a value of 4% for organic C.

For earthworms, the repeatability was determined by analyzing five blank earthworm replicates with a mixture of GLY, GLU and AMPA at 0.05 mg L^{-1} and the reproducibility was determined by analyzing three replicates.

3. Results and Discussion

3.1. Column selection

GLY, GLU, AMPA and both IS were separated on a Nucleodur C_{18} pyramid (150 mm × 3 mm diameter, 3 µm particle size) column. The retention times (RT) with the conditions used as described in the Materials and Methods section were 6.45 min for GLU, 7.57 min for GLY, 8.79 min for AMPA, 9.74 min for APPA and 10.35 min for AMPPA.

Another column, the Nucleoshell RP_{18} (100 mm × 2 mm diameter, 2.7 µm particle size) was tested, as lower dimensions were expected to increase the resolution and improve the limits of detection. Macherey-Nägel suggests this column as a good solution for GLY, GLU and AMPA separation (MN Appl. No.126110). The resolution was, as expected, better than that obtained using the Nucleodur Pyramid and the S/N ratio was higher. However, the use of ammonium acetate buffer at 50 mmol L^{-1} led to an increase in pressure above 500 bars which, due to standard HPLC pump capacities, required frequent cleaning of the stationary phase and the frits. In addition, the retention time of all the analytes was very short (less than 1 minute) and not consistent with the Macherey-Nägel application note under the same conditions (same flow rate and gradient).

Due to these limitations, even if the Nucleoshell allowed better resolution and lower detection limits, it was decided to perform all analyses with the Nucleodur Pyramid column.

3.2. Choice of internal standards (IS)

Previous analytical methods developed to measure GLY, GLU and AMPA in diverse matrices [[32], [34], [38], [39]] used isotope-labeled internal standards (i.e. (1,2-$^{13}C^{15}N$)Glyphosate, ($D_2{}^{13}C^{15}N$)AMPA, D_3glufosinate) for efficient quantification and derivatization. However, even if these IS appeared to be performing, problems of sensitivity and stability over time were encountered. For this reason, it was decided to select other internal standards such as APPA and AMPPA. These two molecules have a chemical structure very close to the herbicides, are not used in agriculture, are very stable over time and allow sensitive detection (Figure 3).

3.3. Extraction optimization

Previously, soil was generally extracted by stirring in diverse buffered or basic aqueous solutions such as: 40 mM Na-tetraborate [40], 0.1 M KH_2PO_4 [41], water [[31], [42]], 0.6 M KOH [[7], [43]] or mixture of sodium phosphate 0.03 M and trisodium citrate 0.01M [44]. These methods used stirring for different lengths of time, generally at room temperature, and centrifugation to separate the soil from the extracting solution. In this context, different extraction solutions were tested in the present study (borate buffer (pH 10), water (pH 6), KOH (pH 13) and HNO_3 (pH 3)), using 15 g of soil spiked with 5 µg of AMPA, GLU and GLY, and 2 µg of each IS. The protocol described in figure 1 was applied without the SPE step. Results are presented in figure 4a and show the best recoveries were obtained with borate buffer (51 % for AMPA, 53 % for GLY and 55 % for GLU). Also of note was that the recoveries obtained with water were significantly better than those previously obtained by

Druart et al. [31] for GLY (37 %), equivalent for AMPA (38 %) and lower for GLU (40 %). The composition of the soil and particularly its organic matter or clay content could explain these differences [[40], [45]]. Moreover, with borate buffer for extraction, it was not necessary to adjust the pH to achieve the derivatization phase [30] unlike with other solvents. Indeed, if KOH is used, a supplementary step to adjust the pH from 13 to 9 before derivatization is necessary. Using borate buffer this step is not required and the extract is in the same solvent as that used for derivatization.

The extraction time was optimized using borate as the extraction solvent, and it was found that an increase in the extraction time above 1 h did not increase recoveries (Figure 4b). In contrast, recoveries decreased, probably due to re-adsorption of the herbicides on soil particles during longer stirring times.

3.4. SPE Optimization

HLB cartridges are commonly used for a SPE pre-concentration step of FMOC-glyphosate, FMOC-glufosinate and FMOC-AMPA [[29], [34], [45]]. The SPE procedure used in this study was derived from that of Ghanem et al. [29] but the conditioning step was modified by replacing the phosphate buffer (pH 3) with formic acid at pH 3 and the flow rate of the sample was reduced to 10 mL min^{-1} to ensure better adsorption of the molecules onto the phase. Differences between phosphate buffer and formic acid were not significant and formic acid was used to make the SPE protocol easier. Elution with MeOH gave recoveries of 59 % for AMPA, 63 % for GLY and 61 % for GLU. In order to increase these recoveries, different solvents were tested. Recoveries obtained with these solvents are presented in figure 5. Acidification of MeOH with 1 % formic acid increased the recoveries. Thus, 2 × 2 mL MeOH (1 % formic acid) was chosen as the elution solution to pre-concentrate the soil and earthworm samples.

3.5. Calibration

The LC/MS/MS method was internally calibrated to quantify GLY, GLU and AMPA. For soil samples, a good linearity was observed for the responses with correlation coefficients showing values of 0.996 for GLY, 0.992 for GLU and 0.989 for AMPA, using the linear regression model. The deviation from linearity in responses for the earthworm samples was probably due to a matrix effect. Therefore, we used a quadratic regression model to minimize this deviation. The correlation coefficients were then 0.982 for GLY, 0.976 for GLU and 0.985 for AMPA. For the earthworm samples, the calibration step was carried out in the matrix unlike soil samples. Indeed, there was no significant difference between the angular coefficients of the calibration curve in solution and in matrix.

3.6. Method performance criteria

The method performance criteria were evaluated for soil and earthworms (table 1). To our knowledge, no previous scientific studies investigated the quantification of the compounds of interest in earthworms.

Several previous studies proposed methods for determining GLY and AMPA in soils, and only a few of them included GLU [[34], [4]]. Most of these reported higher LOQ, with values ≥ 0.050 µg g^{-1} for GLY [[34], [43], [45], [46]] (table 2). However, Rampazzo Todorovic et al. [40] showed that all criteria including LOQ were highly dependent on the soil type, as they found that the LOQ for GLY ranged from 0.014 to 0.14 µg g^{-1} in their tested soils. The present study led to comparable LOQ for GLY, GLU and AMPA, with the order GLY > AMPA > GLU. The same order (or equivalence between compounds) was also found by most other authors. In terms of repeatability and reproducibility, the present study is in the same range or better than most of the other studies. The main differences with other reports were the quantity of soil extracted, which was 15 g in our case but 5 g or less in most other studies (except Sun et al., 2017 [44], which used 10 g).

4. Application to environmental samples

The developed method was applied to analyze six soil samples and six earthworms collected in the same soils. In parallel, blank samples were analyzed to confirm the absence of cross contamination. As observed in figure 6, the analytes and internal standards were clearly defined and, due to the SPE purification, the noise signal remained low.

GLU was rarely detected, and was only quantified in one soil sample and observed in trace amounts in another one (table 3). However, GLY was found at quantifiable levels in all the soil samples, and AMPA was detected in all except one. The concentrations ranged between 0.18 and 0.069 µg g^{-1} for GLY, ie largely above the LOQ. In contrast, for AMPA the concentrations were closer to the LOQ and ranged from 0.073 to 0.025 µg g^{-1}. All the measurements were confirmed by the qualification transition, with a deviation of the Q_1/Q_3 ratio within the accepted tolerance (in all case < 20%).

Comparing the observed concentrations, GLY and its AMPA metabolite appeared to be far below the concentrations observed in the US and Argentina for example [[9], [47], [48]], where glyphosate usage rates and occurrence are higher. However, they were within the range usually observed in Europe, particularly in cereal crops [[7], [42], [46]]. In soils, the AMPA concentration was commonly described as higher than that of GLY [[9], [42], [48]] but it was not systematically the case and some soil properties were found to favor equivalent concentrations, or even GLY > AMPA [46].

In earthworms, GLY was only quantified in two of the field-collected earthworms, even if GLU, GLY and AMPA were regularly detected below LOQ. This relatively rare measurement could be associated with low GLY bioavailability in soil [49]. Furthermore, GLY is considered to show a low potential for bioconcentration, with a Bioconcentration Factor (BCF) for fish calculated at 0.5 [5]. The two measurements in the soil samples and earthworms in this study were used to calculate a BCF of 0.251/0.179= 1.4 and 0.230/0.130= 1.8, respectively. Both are within the range of 1.4-5.9, found by Contardo-Jara et al. [50] for *Lumbriculus variegatus* in water.

5. Conclusion

A sensitive and selective analytical method was developed to quantify GLY, AMPA and GLU in soils and earthworms, enabling the effective analyses of these compounds in field-collected samples. After the extraction step, the extracts were derivatized with FMOC-Cl and purified on SPE cartridges before internal standard quantification using LC/MS/MS in HESI+ mode. The extraction and purification methods were optimized. For extraction, the best recoveries were obtained with borate buffer and reached a maximum after 1h incubation. In order to increase the performance of the SPE, various solvents were tested and the acidification of MeOH with 1 % formic acid increased the recoveries, so elution with 2 × 2 mL MeOH (1 % formic acid) was selected to pre-concentrate the soil and earthworm samples.

The method developed achieves a good linearity for the calibration responses in soil, and high correlation coefficients were observed for earthworm samples using a quadratic regression model. The LOD and LOQ values measured with this method were among the lowest range of values reported for soil in the literature. The method also allowed sensitive detection and quantification in a complex animal matrix such as earthworms, with a LOD of 0.070 µg g^{-1}, 0.065 µg g^{-1} and 0.040 µg g^{-1} for GLY, AMPA and GLU, respectively. Accordingly, the LOQ were 0.23 µg g^{-1}, 0.20 µg g^{-1} and 0.12 µg g^{-1} in earthworm samples for GLY, AMPA and GLU, respectively.

The method was successfully applied to analyse residues in natural soils and earthworms collected in cereal crop fields, with quantification of the three compounds in these field samples. All measurements were confirmed by the use of two MS/MS transitions.

This optimized method for analyzing GLY, AMPA and GLU in soil and animal matrices represents a promising analytical tool with regards to the current needs for monitoring commonly used pesticides in the environment.

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Table 1: Performance criteria of the method developed for soil and earthworms: limits of detection (LOD) and quantification (LOQ), repeatability and reproducibility.

		Glyphosate	AMPA	Glufosinate
Soil (m = 15 g)	LOD (µg g^{-1})	0.009	0.007	0.006
	LOQ (µg g^{-1})	0.030	0.025	0.020
	repeatability* (%)	5.0	7.4	7.0
	reproducibility* (%)	5.7	7.9	6.5
Earthworm (m = 1 g)	LOD (µg g^{-1})	0.070	0.065	0.040
	LOQ (µg g^{-1})	0.23	0.20	0.12
	repeatability** (%)	7.8	8.3	7.2
	reproducibility** (%)	8.4	9.2	8.0

* : concentration of compounds in the soil sample (GLY = 0.10 mg L^{-1}, AMPA = 0.075 mg L^{-1} and GLU = 0.050 mg L^{-1})
** : mixture of GLY, GLU and AMPA at 0.050 mg L^{-1}

Table 2 : comparison of the method performance with those in the literature

	LOD (µg g^{-1})	LOQ (µg g^{-1})	Mass soil (g)	reference
Glyphosate	0.020	0.050	2	[7]
	0.010	nd	5	[24]
	0.005	0.050	5	[34]
	0.004 – 0.047	0.014 – 0.023	3	[40]
	0.010	0.040	10	[44]
	0.020	0.050	2	[45]
	nd	0.010	5	[46]
	0.009	0.030	15	This work
AMPA	0.030	0.050	2	[7]
	0.005	0.050	5	[34]
	0.025 – 0.12	0.084 – 0.089	3	[40]
	0.010	0.030	2	[45]
	nd	0.010	5	[46]
	0.007	0.025	15	This Work
Glufosinate	0.005	0.050	5	[34]
	0.006	0.020	15	This work

nd : not determined

Table 3: Concentrations measured in the six soil and earthworm samples

Soil (µg g^{-1})			Earthworms (µg g^{-1})		
Glyphosate	**AMPA**	**Glufosinate**	**Glyphosate**	**AMPA**	**Glufosinate**
0.069	0.070	0.041	< LOQ	< LOQ	nd
0.093	0.025	nd*	< LOQ	< LOQ	nd
0.095	nd	nd	nd	< LOQ	< LOQ
0.097	0.045	< LOQ**	nd	< LOQ	nd
0.18	0.048	nd	0.25	nd	< LOQ
0.13	0.073	nd	0.23	nd	nd

* nd: not detected (< limit of detection);
** < LOQ: between limits of detection and quantification

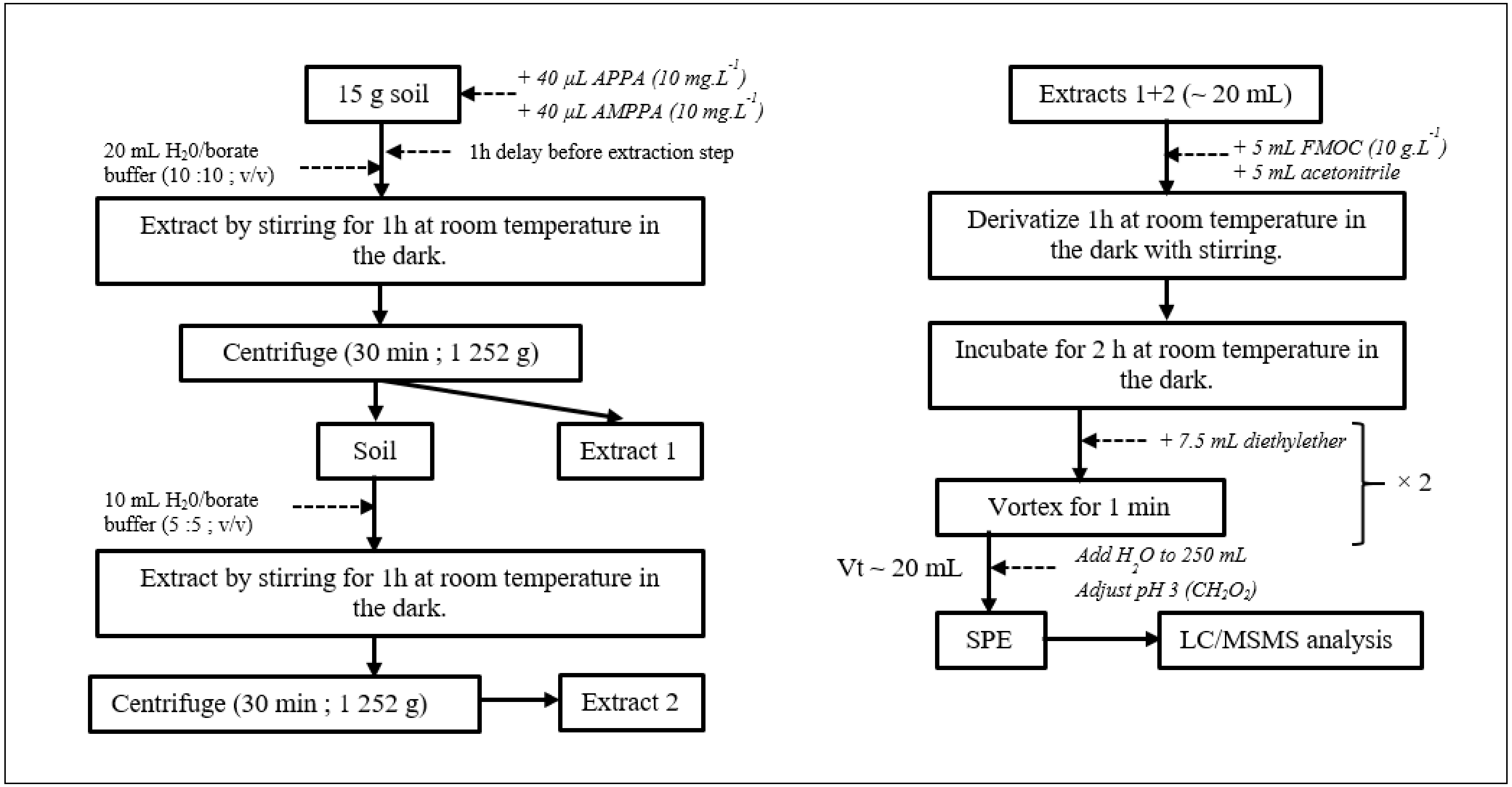

Figure 1. Summary of the Analytical method used for extracting soils

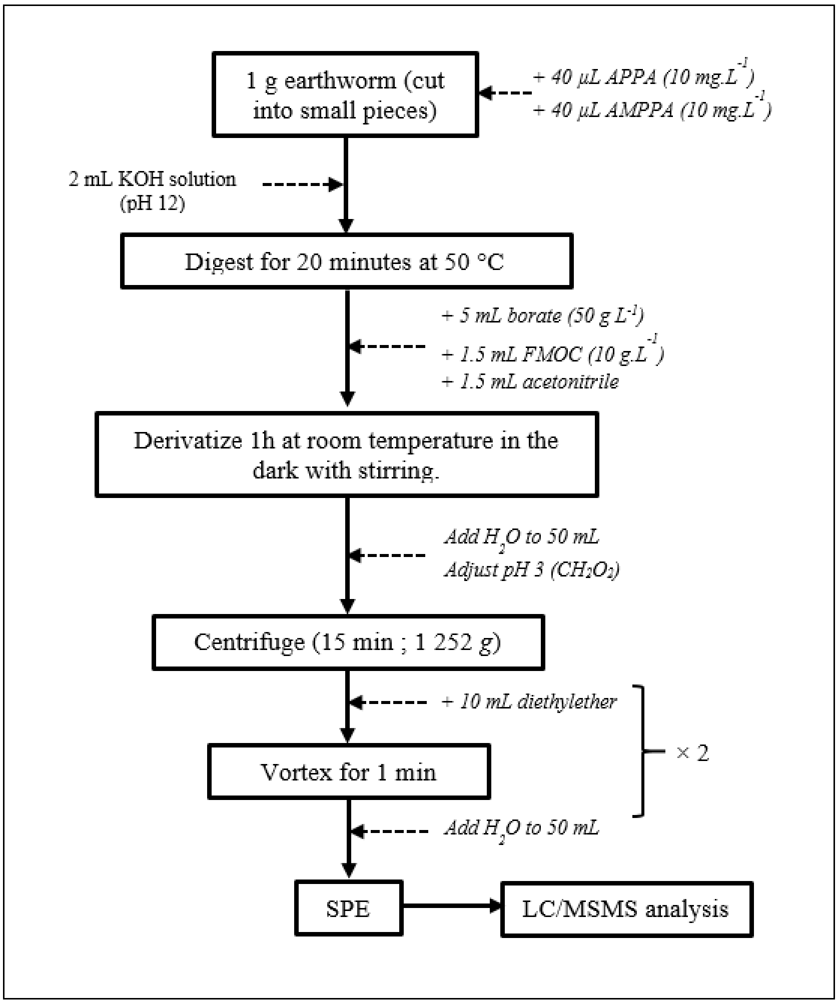

Figure 2. Summary of the analytical method used for earthworms

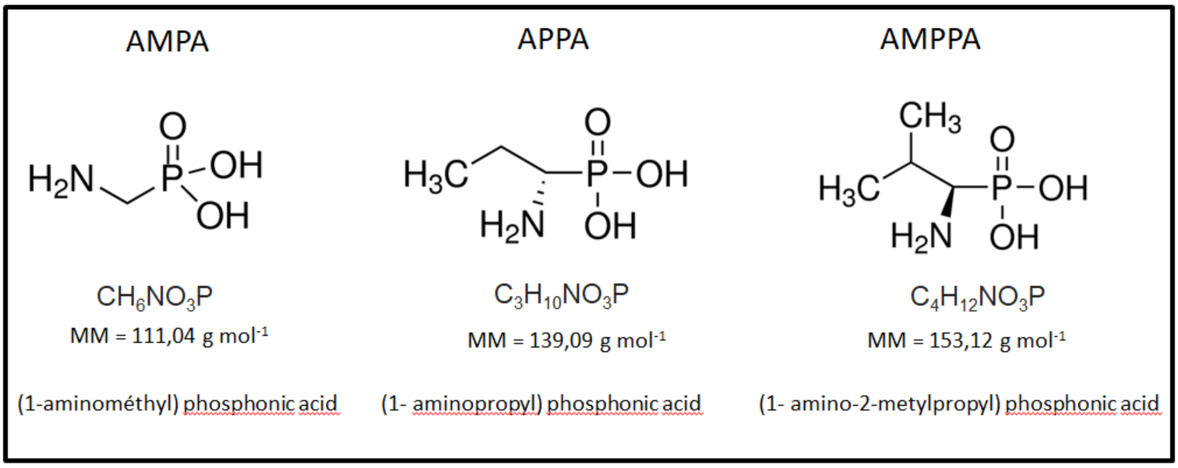

Figure 3. Structures of AMPA, APPA and AMPPA

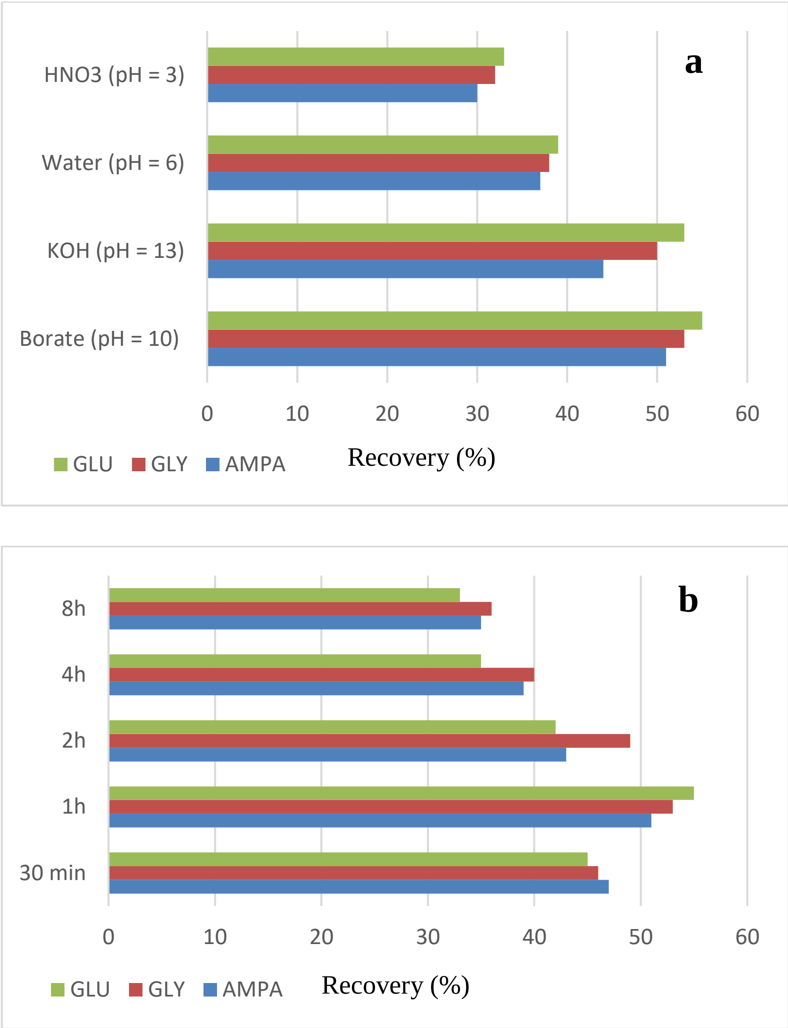

Figure 4. Recoveries of Glu, Gly and AMPA obtained during the optimization steps to test extraction solvents (a) and extraction times (b).

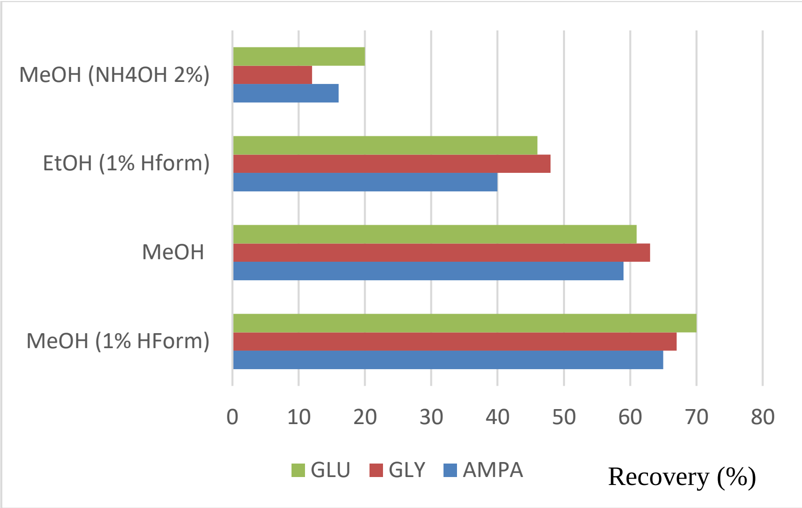

Figure 5. Glu, Gly and AMPA recoveries from SPE

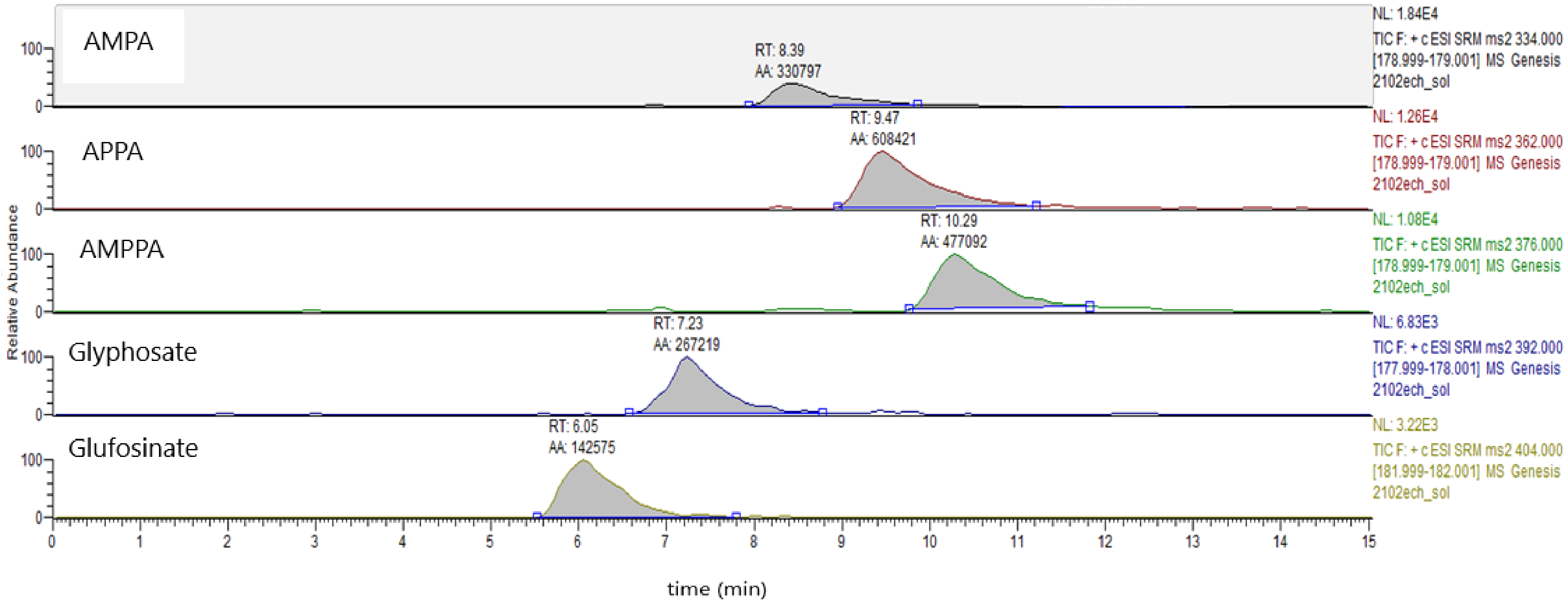

Figure 6. Chromatograms on the quantitation transitions of a soil sample where the compounds were determined as 0.070, 0.069 and 0.041 µg g^{-1} respectively for AMPA, GLY and GLU (the internal standards APPA and AMPPA being at 0.1 mg L^{-1}).