A method to assess glyphosate, glufosinate and aminomethylphosphonic acid in soil and earthworms

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17

18   **Abstract**

19   A new sensitive and selective analytical methodology to quantify glyphosate (GLY),

20   aminomethylphosphonic acid (AMPA), and glufosinate (GLU) in both soil and earthworms

21   (*Allolobophora chlorotica*) was developed. The extraction and purification methods were

22   optimized. The samples were extracted with various aqueous solutions (HNO_3, H_2O, KOH

23   and borate buffer) and derivatized with 9-Fluorenylmethyl chloroformate (FMOC-Cl). To

24   optimize the extraction step, a method to remove the excess FMOC-Cl was applied based on

25   liquid-liquid extraction with diethyl ether. The purification of derivatized extracts was carried

26   out using XLB solid phase extraction (SPE) cartridges before internal standard quantification

27   by liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS). The elution

28   step was optimized to obtain the best recoveries possible, which was with acidic methanol

29   (1% formic acid) (67 % for GLY, 70 % for GLU and 65 % for AMPA). The extraction and

30   purification method followed by analysis of the two herbicides and AMPA in soils using

31   LC/MS/MS determined limit of quantification (LOQ) values of $0.030\ \mu g\ g^{-1}$ for GLY,

32     $0.025\ \mu g\ g^{-1}$ for AMPA and $0.020\ \mu g\ g^{-1}$ for GLU . For earthworms, LOQ were $0.23\ \mu g\ g^{-1}$

33   for GLY, $0.20\ \mu g\ g^{-1}$ for AMPA and $0.12\ \mu g\ g^{-1}$ for GLU. .

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

35   earthworms.

36

37   **Keywords:** herbicides, soil organisms, liquid chromatography-tandem mass spectrometry,

38   solid-phase extraction, derivatization.

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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

67 [[12], [13]]. These soil organisms are used as models in ecotoxicology and several studies

68 have assessed the impact of GLY, AMPA or GLU on them [[14], [15]]. It has been

69 recognized that although commercial formulations containing GLU and GLY generally have

70 no effects on mortality, they may negatively impact earthworm enzyme activities, body

71 weight, reproduction, behavior (avoidance, foraging) and activity (surface casting) [[16],

72 [17]]. For instance, GLY has been reported to modify earthworm feeding behaviour and thus

73 to alter ecological interactions between earthworms, mycorrhizal fungi, and aboveground

74 plants [18]. However, bioaccumulation of these compounds in earthworms has never been

75 assessed even though it could give new insights into their potential ecotoxic effects on these

76 organisms and the consequences on soil functioning.

77   Detection of the potential presence of these herbicides in the environment has required the

78 development of specific analytical procedures because measurement of their residues is

79 challenging [19]. Indeed, the analysis of these molecules in environmental matrices remains

80 difficult with conventional detectors such as UV and fluorescence due to the lack of adequate

81 chemical groups in GLY, its metabolite AMPA and GLU molecules (i.e. chromophores or

82 fluorophores). In addition, their ionic character, complex formation with metals [[20], [21],

83 sorption to glassware [22], low volatility and insolubility in organic solvents associated with

84 their low molecular mass has increased the analytical difficulties, in particular the low

85 quantification limits required for water quality criteria.

86   Many analytical procedures have been developed in the last decades for the quantification

87 of GLY, AMPA and GLU including gas chromatography after a derivatization step [[23],

88 [24]], ion chromatography coupled with conductimetry [25] or inductively coupled plasma

89 spectrometry [[26], [27]] and liquid chromatography coupled to fluorescence and/or mass

90 spectrometry [[28], [29], [30], [31], [32]]. FMOC- Cl is the most common derivatization

91 agent used, as it allows, when associated with LC/MS/MS, a better detection and

92 quantification than non-derivatized herbicides. Indeed, it results in improved chromatographic

93 separation from the matrix, as well as superior selectivity and sensitivity [33]. However, even

94 if LC/MS/MS is a reliable method for quantifying GLY, GLU and AMPA at low detection

95 levels, their extraction from soils and living organisms is complicated by the matrix. It is

96 known that, in soil, these herbicides show high sorption to soil clays and organic matter [21].

97 Several extraction methods from soil have been reported [[4], [24], [34]] but in many cases,

98 the extraction method was specific for one type of soil [31], and to our knowledge no

99 extraction methods have been reported for earthworms.

100 In the present study, a new sensitive analytical methodology to quantify GLY, GLU and

101 AMPA residues in both soils and earthworms (*Allolobophora chlorotica*) using SPE – LC –

102 ESI – MSMS was developed. As the quantification of GLY, GLU and AMPA residues in

103 earthworms is original as never published elsewhere in our knowledge, for soils a particular

104 attention was made, regarding previous works, to optimize the extraction and purification

105 methods, especially by testing various aqueous solutions for extraction and elution mixtures

106 after purification by SPE. This fully characterized method was applied on soil and

107 earthworms samples.

108

109 **2. Material and methods**

110 *2.1. Chemicals and solutions*

111 HPLC grade quality solvents (acetonitrile (ACN), ethanol, diethyl ether, methanol

112 (MeOH), n-hexane), potassium hydroxide (38 %) Normapur (KOH), disodium tetraborate

113 decahydrate (borate), ammonium hydroxide solution (NH_4OH) and formic acid solution

114 (HCOOH) were purchased from VWR Prolabo (Paris, France). Formic acid, ACN and water

115 for LC/MS were purchased from Sigma Aldrich (LPCR, France). The ultra-pure water was

116 obtained through a Milli-Q system (18 MΩ cm) from Merck, Germany. FMOC-Cl and

117 dimethyl-dichloride silane (DMDCS) were purchased from Fluka and Aldrich, respectively

118 (l'Isle d'Abeau, France).

119   High purity pesticide standards (>98%) were supplied by Cluzeau Info Labo (Sainte-Foy-

120 la-Grande, France) for GLU (ammonium 2-amino-4-(hydroxymethylphosphinyl)butyrate;

121 CAS number: 77182-82-2) and by Sigma Aldrich (l'Isle d'Abeau, France) for GLY (N-

122 (phosphonomethyl)glycine; CAS number: 1071-83-6), AMPA (aminomethylphosphonic acid;

123 CAS number: 1066-51-9) and internal standards: APPA (1- aminopropyl phosphonic acid;

124 CAS number: 14047-23-5) and AMPPA (1- amino-2-methylpropyl phosphonic acid; CAS

125 number: 66254-55-5).

126   Stock solutions of each pesticide at 1 g L^{-1} and calibration standard solutions were

127 prepared in ultra-pure water and stored in silanised glassware or plastic flasks. A saturated

128 solution of 50 g L^{-1} borate buffer (pH 9) in ultra-pure water and a solution containing 10 g L^{-1}

129 of FMOC-Cl in ACN were used for the derivatization step prior to LC/MS/MS analyses.

130   All glassware in contact with GLY, GLU and AMPA was silanized. The solution for

131 glassware silanization was prepared by diluting 5% DMDCS in n-hexane. After 10 min of

132 contact, glass containers were rinsed twice with hexane then with MeOH before being dried in

133 a fume hood.

134

135 *2.2. Earthworm and soil sampling*

136 The earthworm *Allolobophora chlorotica* (green morph) was chosen as a model organism.

137 This earthworm species is common in temperate European regions and was chosen because it

138 lives close to the soil surface. It is thus potentially highly exposed to and impacted by

139 pesticides [35]. For method development, characterization and matrix-matched calibration

140 curves, pesticide-free earthworms were collected by hand from a fallow in Versailles, France

141 (48°48′31″N, 2°05′26″E). The fallow had not been treated with pesticides for more than 20

6

142   years. The individuals were used as the blank matrix and first analyzed to confirm the absence

143   of contamination with the targeted pesticide residues.

144   For method application, *A. chlorotica* individuals were manually collected in Spring 2016 by

145   superficially digging the soil in winter wheat fields located in the Long-Term Socio-

146   Ecological Site Zone Atelier Plaine & Val de Sèvre (ZA-PVS;

147   **http://www.za.plainevalsevre.cnrs.fr/**) [36]. Earthworms were then stored for 48 h in Petri

148   dishes on damp filter paper to void gut contents and then frozen at − 80 °C until analysis.

149   Soil cores were also sampled in the same wheat fields as the earthworms using a 5 cm Ø

150   soil auger at a 0–5 cm depth. The soils were frozen at -20°C before being analyzed. One part

151   of some soil samples was used for method development. For this, they were extracted with

152   water, in order to remove potential traces of herbicides, and dried at 50°C overnight in an

153   oven. They were again extracted with pure water and analyzed for GLY, GLU and AMPA

154   content. If none of these molecules were present, the soil samples were used for method

155   development.

156

157   *2.3. Soil extraction*

158   Soil samples were defrosted and 30 g collected and removed from roots and small stone

159   debris. Each sample was then homogenized by slight crushing and 15 g were put in a plastic

160   container (Figure 1) and spiked with the internal standards (IS) APPA and AMPPA (40 µL of

161   each at 10 mg L^{-1}). To allow the sorption of the IS onto the soil structure, the mixtures were

162   left in the dark for one hour (sufficient time for a total sorption) before starting the extraction

163   procedure.

164   After this delay, 20 mL of the extraction solution (10 mL borate buffer + 10 mL H_2O) was

165   added and the sample was stirred for one hour at room temp in the dark on a magnetic stirrer,

166   followed by centrifugation at 1 252 *g* for 30 minutes. The supernatant (extract 1) was

167   collected and the soil sample was re-extracted with 10 mL solution (5 mL borate buffer + 5

168   mL pure water) following the same procedure (extract 2). Both extracts (1+2) were combined

169   and 5 mL of FMOC- Cl (10 g L^{-1}) and 5 mL ACN were added. The samples were then

170   derivatized for 1 hour at room temperature in the dark while stirring. The samples were then

171   left for 2 hours at room temperature in the dark without stirring before to remove the excess

172   FMOC-Cl by liquid-liquid extraction (LLE) with diethyl ether (7.5 mL), through vortex

173   agitation for 1 minute. This LLE was repeated twice and this extraction allow to optimize the

174   analyse, thereby keeping the ionization chamber of the mass spectrometer clean. The aqueous

175   fraction was collected and adjusted to 250 mL with pure water. The pH was adjusted to 3 with

176   formic acid before the SPE procedure.

177   SPE was carried out using 6 mL Chromabond® XLB cartridges (Macherey-Nägel, France)

178   containing 200 mg of the phase and an autotrace® 280 (ThermoScientific, France). The

179   cartridge was first conditioned by successive addition of 5 mL MeOH, 5 mL pure water and 5

180   mL formic acid solution (pH 3) at 5 mL min^{-1}, then the 250 mL of the sample solution was

181   deposited at 10 mL min^{-1}. The cartridge was dried under nitrogen for 20 minutes and the

182   elution was carried out with 2×2 mL of MeOH containing 1 % formic acid at 5 mL min^{-1}.

183

184   *2.4. Earthworm extraction*

185   Earthworms (1 g) were cut into small pieces using Inox scissors and inserted into 15 mL

186   centrifugation tubes (Figure 2). The tubes were weighed and 40 µL of a mixture of IS at 10

187   mg L^{-1} was added and vortexed. The earthworms were then digested for 20 minutes at 50°C

188   with 2 mL KOH solution (pH 12) in order to solubilize all proteins and other molecules [37].

189   After centrifugation, the supernatant was derivatized in a plastic flask with 5 mL borate (50 g

190   L^{-1}), 1.5 mL ACN and 1.5 mL FMOC- Cl (10 g L^{-1}) at room temperature in the dark while

191   stirring for 1 hour. The volume of the derivatized extract was then adjusted to 50 mL with

8

192 pure water and adjusted to pH 3 with formic acid, followed by centrifugation for 15 minutes

193 at 1,252 g. The supernatant was extracted by LLE with diethyl ether (10 mL) through vortex

194 agitation for 1 minute. This LLE was repeated twice. The aqueous fraction was collected and

195 adjusted to 250 mL with pure water before SPE. The SPE procedure was identical to that used

196 for soil.

197

198 *2.5. LC/MS/MS Analysis*

199 A Thermo Scientific TSQ Quantum Access Triple Quadrupole Mass Spectrometer coupled

200 with a Surveyor pump and an Accela autosampler operating in heated positive electrospray

201 ionization mode (HESI+) was used. The sampler is equipped with a 20 μL injection loop and

202 the samples were kept at a temperature of 15°C. The analysis was performed on a Nucleodur

203 C_{18} Pyramid column (150 mm × 3 mm, 3 μm) at 25°C. Samples were analyzed using a mobile

204 phase water/ACN both containing 0.1% formic acid, at a flow rate of 0.5 mL min^{-1}. The

205 composition of the mobile phase was kept at 60:40 for 2 min, then held to 5:95 (v/v) in 8 min

206 (2 min hold), then 60:40 (v/v) in 2 min for 3 min.

207 Detection and quantitation of GLY, GLU and AMPA were performed using multiple

208 reactions monitoring (MRM). The ion source was operated in positive ion mode with a spray

209 voltage of 4,500 V and a vaporizer and capillary temperature of 300°C each. Nitrogen was

210 used for sheath and auxiliary gas pressure (20 and 10 arbitrary units) while argon was used for

211 collision pressure (1.5 arbitrary unit). Two precursor product ion transitions for each analyte

212 and each internal standard were used for quantitation. *Q* for quantification transition, *q* for

213 qualification transition and Ec for collision energy (V)) were used from precursor ion as

214 follows: glyphosate (*Q*) *m/z* 392/179 (42 V) and (*q*) *m/z* 392/88 (18V); AMPA (*Q*) *m/z*

215 334/179 (23 V) and (*q*) *m/z* 334/156 (69 V); glufosinate (*Q*) *m/z* 404/182 (13 V) and (*q*) *m/z*

216 404/136 (20 V); APPA (*Q*) *m/z* 362/179 (25 V) and (*q*) *m/z* 362/140 (5 V) and AMPPA (*Q*)

217   *m/z* 376/179 (25 V) and *m/z* (*q*) 376/156 (5 V). Data were acquired and processed using

218   Excalibur software.

219   Both ion transitions had mean accuracies between 70 and 120% and a precision of 20%

220   relative standard deviation (RSD), based on at least five replicates.

221

222   *2.6. Calibration, limits of detection and quantification*

223   For soils, a calibration step was performed by obtaining curves with pure water spiked with

224   increasing amounts of GLY, GLU and AMPA. Linearity of the method was evaluated

225   analyzing six standard solutions by triplicate in the range 0.010-1.0 mg L^{-1}. The concentration

226   of the internal standards was the same as those used for soil sample extraction. APPA was

227   used for the calibration while AMPPA was used to evaluate the effectiveness of the

228   derivatization step. . The procedure was the same as for derivatization of the soil extract. For

229   earthworms, a calibration step was carried out on the matrix by spiking blank small cut pieces

230   of earthworm with increasing concentrations of the three analytes , at six concentration levels

231   by triplicate in the range 0.010-1.0 mg L^{-1}. APPA and AMPPA were used as IS to evaluate

232   the calibration and extraction/derivatization steps, respectively. The blank earthworms were

233   analysed in the laboratory to ensure the absence of pesticide contamination. The spiked

234   earthworms were processed using the same procedure as the experimental samples.

235   The limits of quantification (LOQ) and detection (LOD) for soil and earthworms were

236   determined by the signal to noise ratio (S/N) with: S/N = 10 for LOQ and S/N= 3 for LOD.

237   For soil samples, the repeatability was determined by analyzing, on the same day, five sub-

238   samples (15 g) from a sample of soil spiked with 40 µL of a mixture of herbicides at 10 mg L^{-}

239   ^{1} each, using the same analytical method. The reproducibility was determined by analyzing

240   three sub-samples (15 g) from the same sample of soil used to determine the repeatability.

241   The sub-samples were analyzed using the same analytical method with a one-week interval

242   between analyses. The composition and the characteristics of soil sample used to validate the

243   method were soil texture (sand (18%); silt (39%) and clay (43%)), pH (H2O) of 8.11 and a

244   value of 4% for organic C.

245   For earthworms, the repeatability was determined by analyzing five blank earthworm

246   replicates with a mixture of GLY, GLU and AMPA at 0.05 mg L^{-1} and the reproducibility was

247   determined by analyzing three replicates.

248

249   **3. Results and Discussion**

250

251   *3.1. Column selection*

252   GLY, GLU, AMPA and both IS were separated on a Nucleodur C_{18} pyramid (150 mm × 3

253   mm diameter, 3 µm particle size) column. The retention times (RT) with the conditions used

254   as described in the Materials and Methods section were 6.45 min for GLU, 7.57 min for GLY,

255   8.79 min for AMPA, 9.74 min for APPA and 10.35 min for AMPPA.

256   Another column, the Nucleoshell RP_{18} (100 mm × 2 mm diameter, 2.7 µm particle size)

257   was tested, as lower dimensions were expected to increase the resolution and improve the

258   limits of detection. Macherey-Nägel suggests this column as a good solution for GLY, GLU

259   and AMPA separation (MN Appl. No.126110). The resolution was, as expected, better than

260   that obtained using the Nucleodur Pyramid and the S/N ratio was higher. However, the use of

261   ammonium acetate buffer at 50 mmol L^{-1} led to an increase in pressure above 500 bars which,

262   due to standard HPLC pump capacities, required frequent cleaning of the stationary phase and

263   the frits. In addition, the retention time of all the analytes was very short (less than 1 minute)

264   and not consistent with the Macherey-Nägel application note under the same conditions (same

265   flow rate and gradient).

266   Due to these limitations, even if the Nucleoshell allowed better resolution and lower

267   detection limits, it was decided to perform all analyses with the Nucleodur Pyramid column.

268

269   *3.2. Choice of internal standards (IS)*

270   Previous analytical methods developed to measure GLY, GLU and AMPA in diverse

271   matrices [[32], [34], [38], [39]] used isotope-labeled internal standards (i.e. (1,2-

272    $^{13}C^{15}N$)Glyphosate, (D$_2$$^{13}C^{15}N$)AMPA, D$_3$glufosinate) for efficient quantification and

273   derivatization. However, even if these IS appeared to be performing, problems of sensitivity

274   and stability over time were encountered. For this reason, it was decided to select other

275   internal standards such as APPA and AMPPA. These two molecules have a chemical

276   structure very close to the herbicides, are not used in agriculture, are very stable over time and

277   allow sensitive detection (Figure 3).

278

279   *3.3. Extraction optimization*

280   Previously, soil was generally extracted by stirring in diverse buffered or basic aqueous

281   solutions such as: 40 mM Na-tetraborate [40], 0.1 M KH_2PO_4 [41], water [[31], [42]], 0.6 M

282   KOH [[7], [43]] or mixture of sodium phosphate 0.03 M and trisodium citrate 0.01M [44].

283   These methods used stirring for different lengths of time, generally at room temperature, and

284   centrifugation to separate the soil from the extracting solution. In this context, different

285   extraction solutions were tested in the present study (borate buffer (pH 10), water (pH 6),

286   KOH (pH 13) and HNO_3 (pH 3)), using 15 g of soil spiked with 5 µg of AMPA, GLU and

287   GLY, and 2 µg of each IS. The protocol described in figure 1 was applied without the SPE

288   step. Results are presented in figure 4a and show the best recoveries were obtained with

289   borate buffer (51 % for AMPA, 53 % for GLY and 55 % for GLU). Also of note was that the

290   recoveries obtained with water were significantly better than those previously obtained by

291 Druart et al. [31] for GLY (37 %), equivalent for AMPA (38 %) and lower for GLU (40 %).

292 The composition of the soil and particularly its organic matter or clay content could explain

293 these differences [[40], [45]]. Moreover, with borate buffer for extraction, it was not

294 necessary to adjust the pH to achieve the derivatization phase [30] unlike with other solvents.

295 Indeed, if KOH is used, a supplementary step to adjust the pH from 13 to 9 before

296 derivatization is necessary. Using borate buffer this step is not required and the extract is in

297 the same solvent as that used for derivatization.

298 The extraction time was optimized using borate as the extraction solvent, and it was found

299 that an increase in the extraction time above 1 h did not increase recoveries (Figure 4b). In

300 contrast, recoveries decreased, probably due to re-adsorption of the herbicides on soil

301 particles during longer stirring times.

302

303 *3.4. SPE Optimization*

304 HLB cartridges are commonly used for a SPE pre-concentration step of FMOC-glyphosate,

305 FMOC-glufosinate and FMOC-AMPA [[29], [34], [45]]. The SPE procedure used in this

306 study was derived from that of Ghanem et al. [29] but the conditioning step was modified by

307 replacing the phosphate buffer (pH 3) with formic acid at pH 3 and the flow rate of the sample

308 was reduced to 10 mL min^{-1} to ensure better adsorption of the molecules onto the phase.

309 Differences between phosphate buffer and formic acid were not significant and formic acid

310 was used to make the SPE protocol easier. Elution with MeOH gave recoveries of 59 % for

311 AMPA, 63 % for GLY and 61 % for GLU. In order to increase these recoveries, different

312 solvents were tested. Recoveries obtained with these solvents are presented in figure 5.

313 Acidification of MeOH with 1 % formic acid increased the recoveries. Thus, 2×2 mL MeOH

314 (1 % formic acid) was chosen as the elution solution to pre-concentrate the soil and

315 earthworm samples.

316

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 $\geq 0.050\ \mu g\ g^{-1}$ 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 $\mu g\ g^{-1}$ 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

14

341   the quantity of soil extracted, which was 15 g in our case but 5 g or less in most other studies

342   (except Sun et al., 2017 [44], which used 10 g).

343

344   **4. Application to environmental samples**

345   The developed method was applied to analyze six soil samples and six earthworms collected

346   in the same soils. In parallel, blank samples were analyzed to confirm the absence of cross

347   contamination. As observed in figure 6, the analytes and internal standards were clearly

348   defined and, due to the SPE purification, the noise signal remained low.

349   GLU was rarely detected, and was only quantified in one soil sample and observed in trace

350   amounts in another one (table 3). However, GLY was found at quantifiable levels in all the

351   soil samples, and AMPA was detected in all except one. The concentrations ranged between

352   0.18 and 0.069 µg g^{-1} for GLY, ie largely above the LOQ. In contrast, for AMPA the

353   concentrations were closer to the LOQ and ranged from 0.073 to 0.025 µg g^{-1}. All the

354   measurements were confirmed by the qualification transition, with a deviation of the Q_1/Q_3

355   ratio within the accepted tolerance (in all case < 20%).

356   Comparing the observed concentrations, GLY and its AMPA metabolite appeared to be far

357   below the concentrations observed in the US and Argentina for example [[9], [47], [48]],

358   where glyphosate usage rates and occurrence are higher. However, they were within the range

359   usually observed in Europe, particularly in cereal crops [[7], [42], [46]]. In soils, the AMPA

360   concentration was commonly described as higher than that of GLY [[9], [42], [48]] but it was

361   not systematically the case and some soil properties were found to favor equivalent

362   concentrations, or even GLY > AMPA [46].

363   In earthworms, GLY was only quantified in two of the field-collected earthworms, even if

364   GLU, GLY and AMPA were regularly detected below LOQ. This relatively rare measurement

365   could be associated with low GLY bioavailability in soil [49]. Furthermore, GLY is

366   considered to show a low potential for bioconcentration, with a Bioconcentration Factor

367   (BCF) for fish calculated at 0.5 [5]. The two measurements in the soil samples and

368   earthworms in this study were used to calculate a BCF of 0.251/0.179= 1.4 and 0.230/0.130=

369   1.8, respectively. Both are within the range of 1.4-5.9, found by Contardo-Jara et al. [50] for

370   *Lumbriculus variegatus* in water.

371

5. Conclusion

373   A sensitive and selective analytical method was developed to quantify GLY, AMPA and GLU

374   in soils and earthworms, enabling the effective analyses of these compounds in field-collected

375   samples. After the extraction step, the extracts were derivatized with FMOC-Cl and purified

376   on SPE cartridges before internal standard quantification using LC/MS/MS in HESI+ mode.

377   The extraction and purification methods were optimized. For extraction, the best recoveries

378   were obtained with borate buffer and reached a maximum after 1h incubation. In order to

379   increase the performance of the SPE, various solvents were tested and the acidification of

380   MeOH with 1 % formic acid increased the recoveries, so elution with 2×2 mL MeOH (1 %

381   formic acid) was selected to pre-concentrate the soil and earthworm samples.

382   The method developed achieves a good linearity for the calibration responses in soil, and

383   high correlation coefficients were observed for earthworm samples using a quadratic

384   regression model. The LOD and LOQ values measured with this method were among the

385   lowest range of values reported for soil in the literature. The method also allowed sensitive

386   detection and quantification in a complex animal matrix such as earthworms, with a LOD of

387    $0.070\,\mu g\ g^{-1}$, $0.065\,\mu g\ g^{-1}$ and $0.040\,\mu g\ g^{-1}$ for GLY, AMPA and GLU, respectively.

388   Accordingly, the LOQ were $0.23\,\mu g\ g^{-1}$, $0.20\,\mu g\ g^{-1}$ and $0.12\,\mu g\ g^{-1}$ in earthworm samples

389   for GLY, AMPA and GLU, respectively.

390 The method was successfully applied to analyse residues in natural soils and earthworms

391 collected in cereal crop fields, with quantification of the three compounds in these field

392 samples. All measurements were confirmed by the use of two MS/MS transitions.

393 This optimized method for analyzing GLY, AMPA and GLU in soil and animal matrices

394 represents a promising analytical tool with regards to the current needs for monitoring

395 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 ($\mu g\ g^{-1}$)	0.009	0.007	0.006
	LOQ ($\mu g\ g^{-1}$)	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 ($\mu g\ g^{-1}$)	0.070	0.065	0.040
	LOQ ($\mu g\ g^{-1}$)	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);
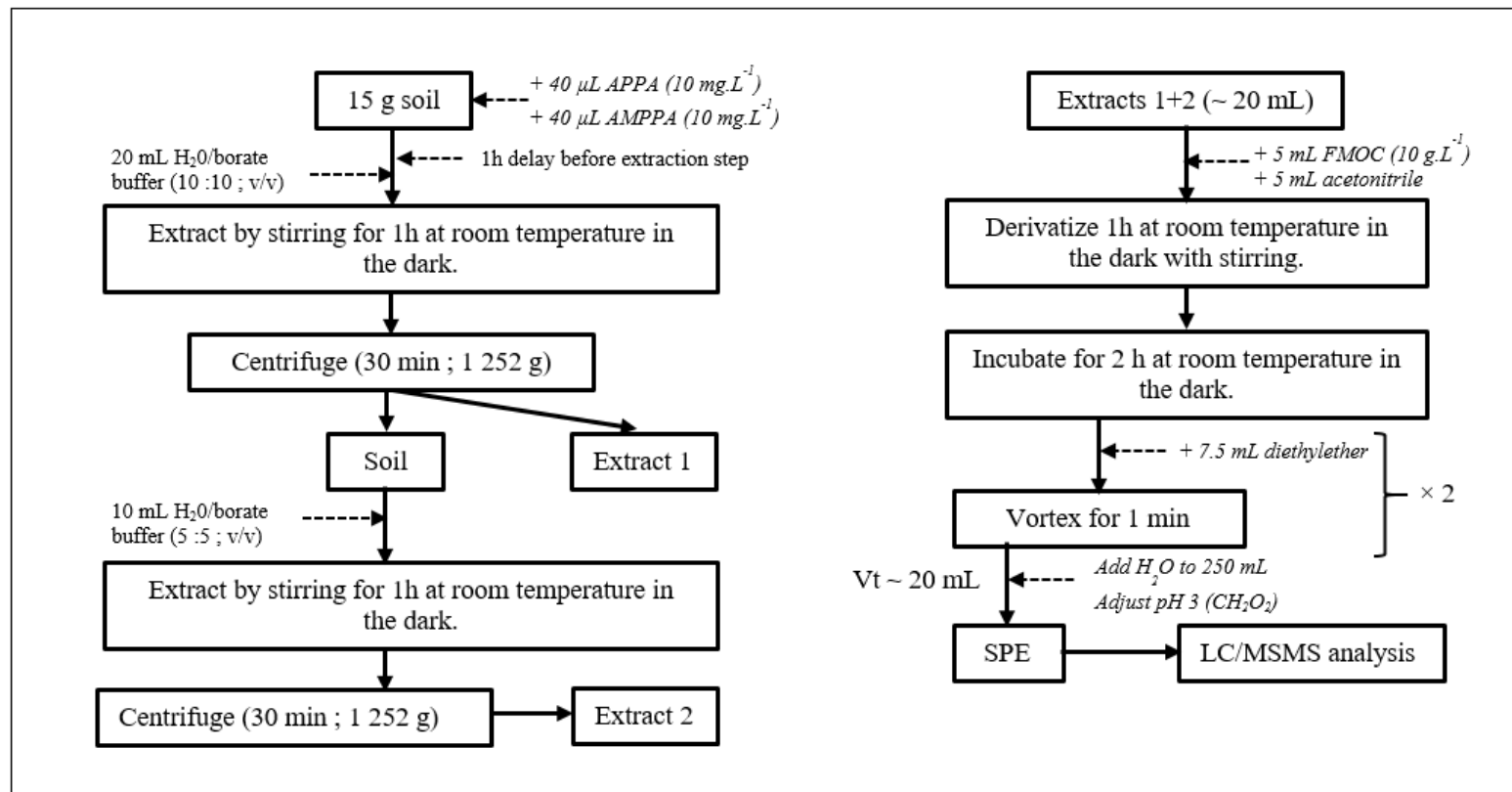** < LOQ: between limits of detection and quantification

Figure 1. Summary of the Analytical method used for extracting soils
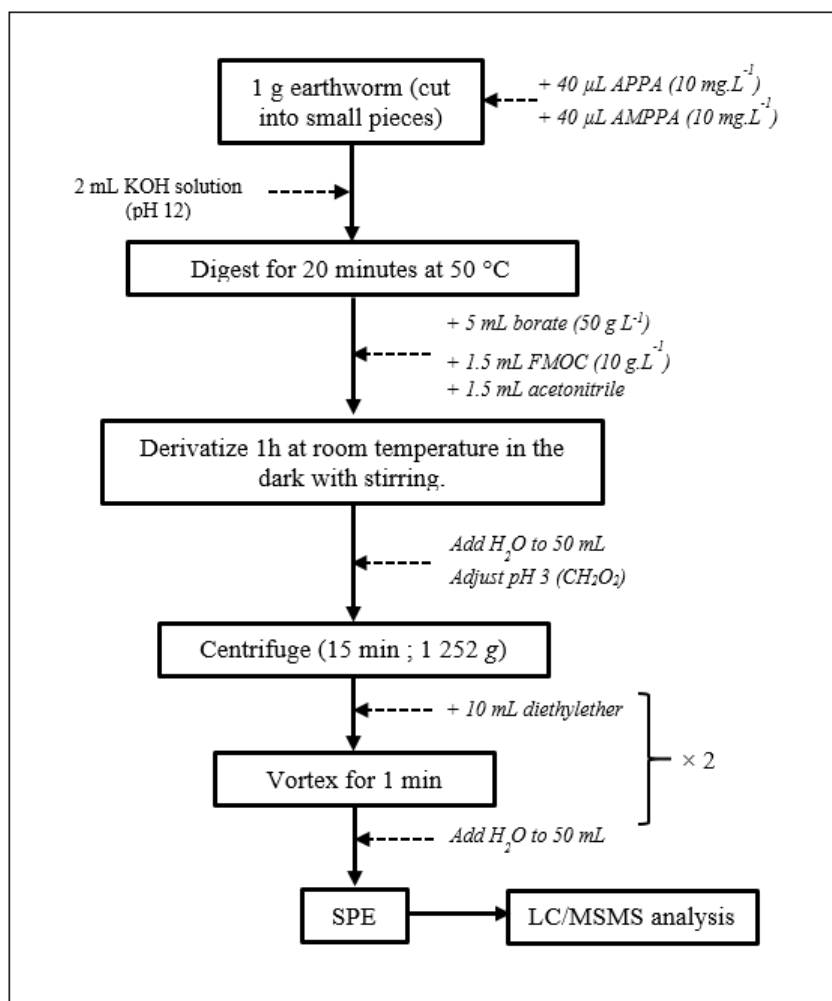
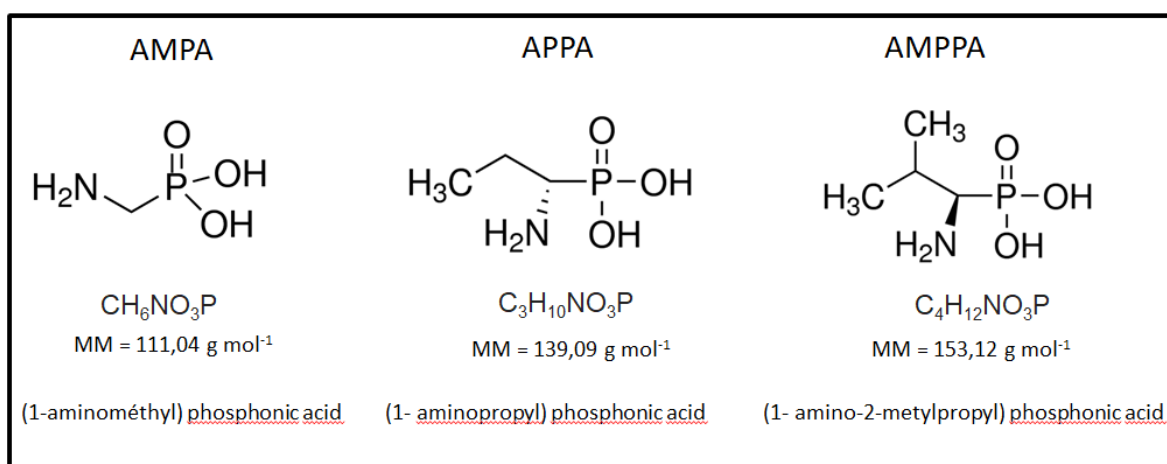Figure 2. Summary of the analytical method used for earthworms
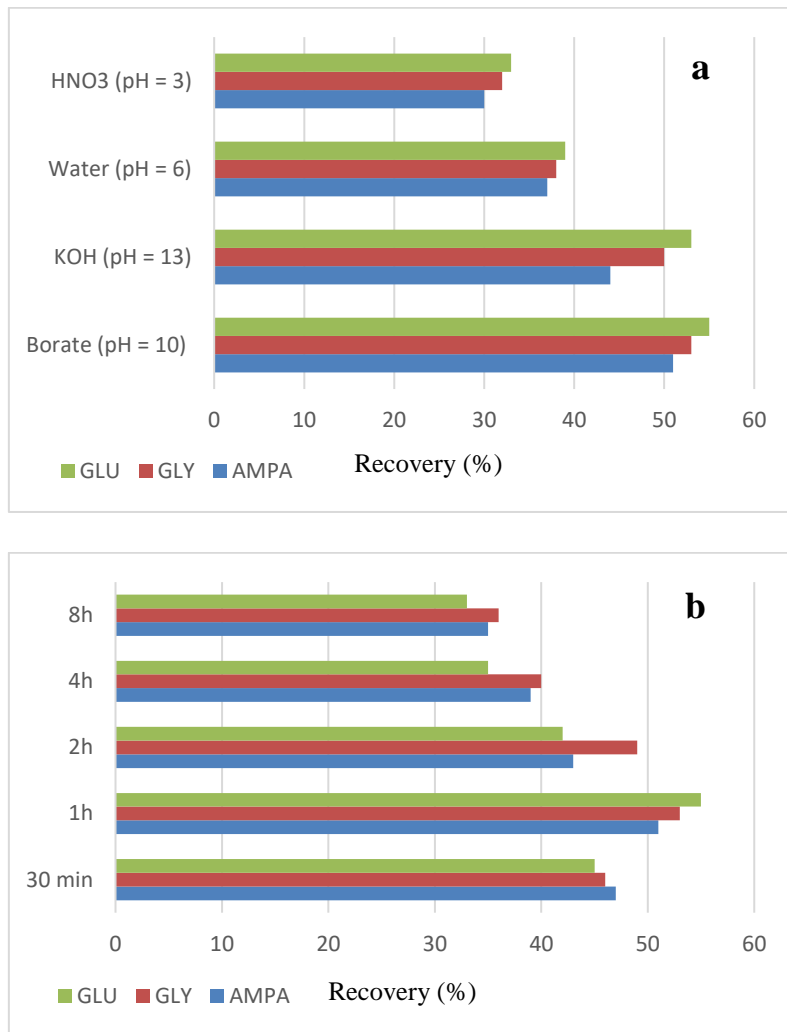

Figure 3. Structures of AMPA, APPA and AMPPA

Figure 4. Recoveries of Glu, Gly and AMPA obtained during the optimization steps to test extraction solvents (a) and extraction times (b).
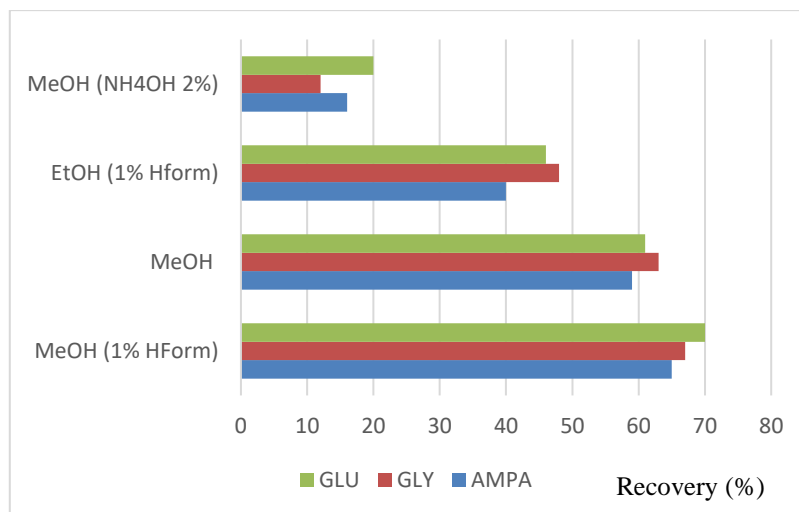
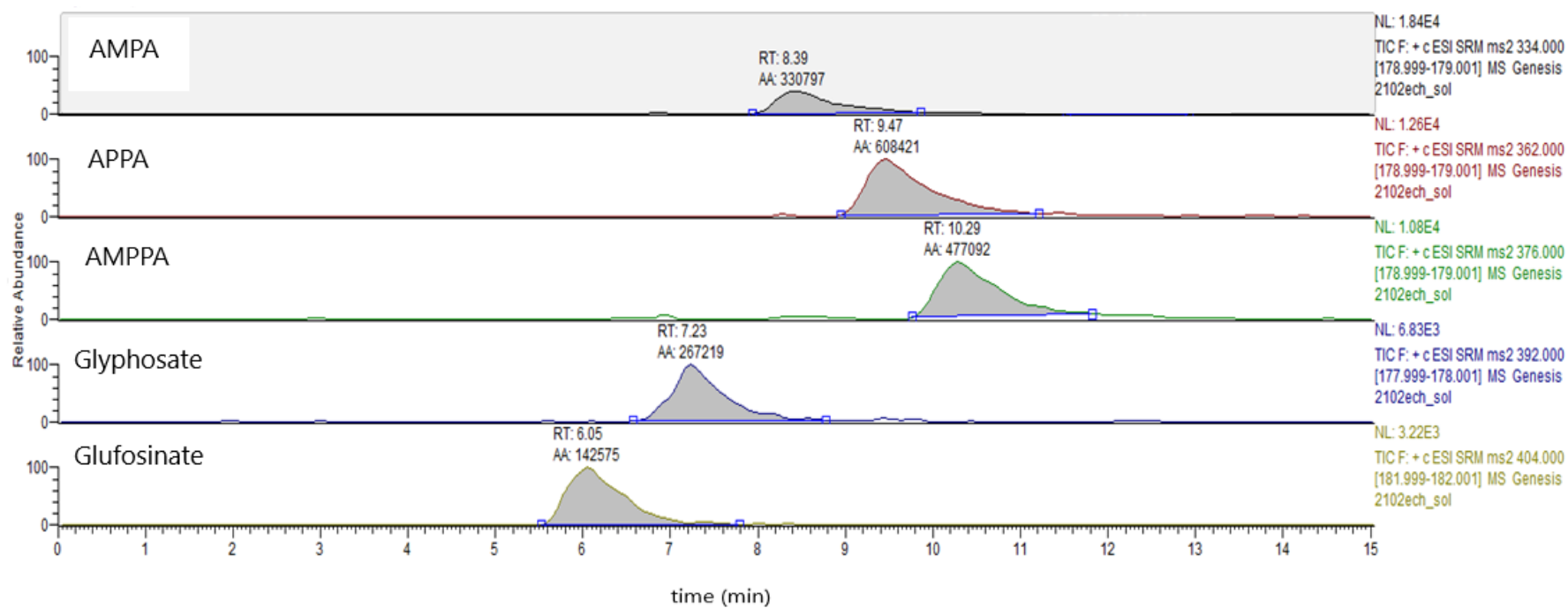Figure 5. Glu, Gly and AMPA recoveries from SPE

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}).