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

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

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16

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<sub>3</sub>, H<sub>2</sub>O, 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  $0.030 \mu\text{g g}^{-1}$  for GLY,  
32  $0.025 \mu\text{g g}^{-1}$  for AMPA and  $0.020 \mu\text{g g}^{-1}$  for GLU . For earthworms, LOQ were  $0.23 \mu\text{g g}^{-1}$   
33 for GLY,  $0.20 \mu\text{g g}^{-1}$  for AMPA and  $0.12 \mu\text{g g}^{-1}$  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.

39

40

41

## 42 **1. Introduction**

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

52 In the environment, GLY is rapidly degraded into aminomethylphosphonic acid, its major  
53 metabolite. In water, degradation mainly results from photodegradation [3], and in soil from  
54 microbial biodegradation [4]. GLU and GLY are considered to be non-persistent field half-  
55 lives ( $DT_{50}$ ) of 7 and 24 days, respectively, whereas AMPA is persistent, with a field  $DT_{50}$  of  
56 419 days [5]. However, some evidence suggests that GLY may be more persistent than  
57 expected, with detection in runoff following spraying and rainfall several months after  
58 application. There are also reports that glyphosate-based herbicides have the potential to  
59 persist in the environment for up to 197 days after a single application [1]. Thus, intensive use  
60 of GLY and GLU-based herbicides can strongly disperse the active ingredient in the  
61 environment and has the potential to contaminate the environmental compartments i.e., water  
62 [6], soil [7], air [8] and organisms. GLY and AMPA, in particular, have both been frequently  
63 found in surface waters [[2], [9]].

64 The accumulation of GLY, GLU and AMPA in living soil organisms has so far only been  
65 assessed in snails [10]. Earthworms are prey for numerous predators [11] and key soil  
66 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<sub>4</sub>OH) 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<sup>-1</sup> 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<sup>-1</sup> borate buffer (pH 9) in ultra-pure water and a solution containing 10 g L<sup>-1</sup>  
129 of FMOCCl 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

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  $-80\text{ }^{\circ}\text{C}$  until analysis.

149 Soil cores were also sampled in the same wheat fields as the earthworms using a 5 cm  $\varnothing$   
150 soil auger at a 0–5 cm depth. The soils were frozen at  $-20\text{ }^{\circ}\text{C}$  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  $50\text{ }^{\circ}\text{C}$  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  $\mu\text{L}$  of  
161 each at  $10\text{ mg L}^{-1}$ ). 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  $\text{H}_2\text{O}$ ) 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  $1\ 252\text{ g}$  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 ( $10 \text{ g L}^{-1}$ ) 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  $5 \text{ mL min}^{-1}$ , then the 250 mL of the sample solution was  
181 deposited at  $10 \text{ mL min}^{-1}$ . The cartridge was dried under nitrogen for 20 minutes and the  
182 elution was carried out with  $2 \times 2 \text{ mL}$  of MeOH containing 1 % formic acid at  $5 \text{ mL min}^{-1}$ .

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  $40 \mu\text{L}$  of a mixture of IS at  $10$   
187  $\text{mg L}^{-1}$  was added and vortexed. The earthworms were then digested for 20 minutes at  $50^\circ\text{C}$   
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 ( $50 \text{ g}$   
190  $\text{L}^{-1}$ ), 1.5 mL ACN and 1.5 mL FMOC- Cl ( $10 \text{ g L}^{-1}$ ) 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

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  $\mu$ L injection loop and  
202 the samples were kept at a temperature of 15°C. The analysis was performed on a Nucleodur  
203 C<sub>18</sub> Pyramid column (150 mm  $\times$  3 mm, 3  $\mu$ m) 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<sup>-1</sup>. 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  $E_c$  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<sup>-1</sup>. 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<sup>-1</sup>. 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  $\mu$ L of a mixture of herbicides at 10 mg L<sup>-1</sup>  
239 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 (H<sub>2</sub>O) 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<sup>-1</sup> 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<sub>18</sub> 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<sub>18</sub> (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<sup>-1</sup> 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 <sup>13</sup>C<sup>15</sup>N)Glyphosate, (D<sub>2</sub><sup>13</sup>C<sup>15</sup>N)AMPA, D<sub>3</sub>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<sub>2</sub>PO<sub>4</sub> [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<sub>3</sub> (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<sup>-1</sup> 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

### 317 3.5. Calibration

318 The LC/MS/MS method was internally calibrated to quantify GLY, GLU and AMPA. For  
319 soil samples, a good linearity was observed for the responses with correlation coefficients  
320 showing values of 0.996 for GLY, 0.992 for GLU and 0.989 for AMPA, using the linear  
321 regression model. The deviation from linearity in responses for the earthworm samples was  
322 probably due to a matrix effect. Therefore, we used a quadratic regression model to minimize  
323 this deviation. The correlation coefficients were then 0.982 for GLY, 0.976 for GLU and  
324 0.985 for AMPA. For the earthworm samples, the calibration step was carried out in the  
325 matrix unlike soil samples. Indeed, there was no significant difference between the angular  
326 coefficients of the calibration curve in solution and in matrix.

327

### 328 3.6. Method performance criteria

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

332 Several previous studies proposed methods for determining GLY and AMPA in soils, and  
333 only a few of them included GLU [[34], [4]]. Most of these reported higher LOQ, with values  
334  $\geq 0.050 \mu\text{g g}^{-1}$  for GLY [[34], [43], [45], [46]] (table 2). However, Rampazzo Todorovic et al.  
335 [40] showed that all criteria including LOQ were highly dependent on the soil type, as they  
336 found that the LOQ for GLY ranged from 0.014 to 0.14  $\mu\text{g g}^{-1}$  in their tested soils. The  
337 present study led to comparable LOQ for GLY, GLU and AMPA, with the order  $\text{GLY} >$   
338  $\text{AMPA} > \text{GLU}$ . The same order (or equivalence between compounds) was also found by most  
339 other authors. In terms of repeatability and reproducibility, the present study is in the same  
340 range or better than most of the other studies. The main differences with other reports were

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  $\mu\text{g g}^{-1}$  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  $\mu\text{g g}^{-1}$ . 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  $\text{GLY} > \text{AMPA}$  [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

## 372 **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  $2 \times 2$  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  $0.070 \mu\text{g g}^{-1}$ ,  $0.065 \mu\text{g g}^{-1}$  and  $0.040 \mu\text{g g}^{-1}$  for GLY, AMPA and GLU, respectively.  
388 Accordingly, the LOQ were  $0.23 \mu\text{g g}^{-1}$ ,  $0.20 \mu\text{g g}^{-1}$  and  $0.12 \mu\text{g g}^{-1}$  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.

396

397

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406

407

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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.

		<b>Glyphosate</b>	<b>AMPA</b>	<b>Glufosinate</b>
<b>Soil</b> (m = 15 g)	LOD ( $\mu\text{g g}^{-1}$ )	0.009	0.007	0.006
	LOQ ( $\mu\text{g g}^{-1}$ )	0.030	0.025	0.020
	repeatability* (%)	5.0	7.4	7.0
	reproducibility* (%)	5.7	7.9	6.5
<b>Earthworm</b> (m = 1 g)	LOD ( $\mu\text{g g}^{-1}$ )	0.070	0.065	0.040
	LOQ ( $\mu\text{g g}^{-1}$ )	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<sup>-1</sup>, AMPA = 0.075 mg L<sup>-1</sup> and GLU = 0.050 mg L<sup>-1</sup>)

\*\* : mixture of GLY, GLU and AMPA at 0.050 mg L<sup>-1</sup>

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

	LOD ( $\mu\text{g g}^{-1}$ )	LOQ ( $\mu\text{g g}^{-1}$ )	Mass soil (g)	reference
<b>Glyphosate</b>	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
<b>AMPA</b>	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
<b>Glufosinate</b>	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 ( $\mu\text{g g}^{-1}$ )			Earthworms ( $\mu\text{g g}^{-1}$ )		
<b>Glyphosate</b>	<b>AMPA</b>	<b>Glufosinate</b>	<b>Glyphosate</b>	<b>AMPA</b>	<b>Glufosinate</b>
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



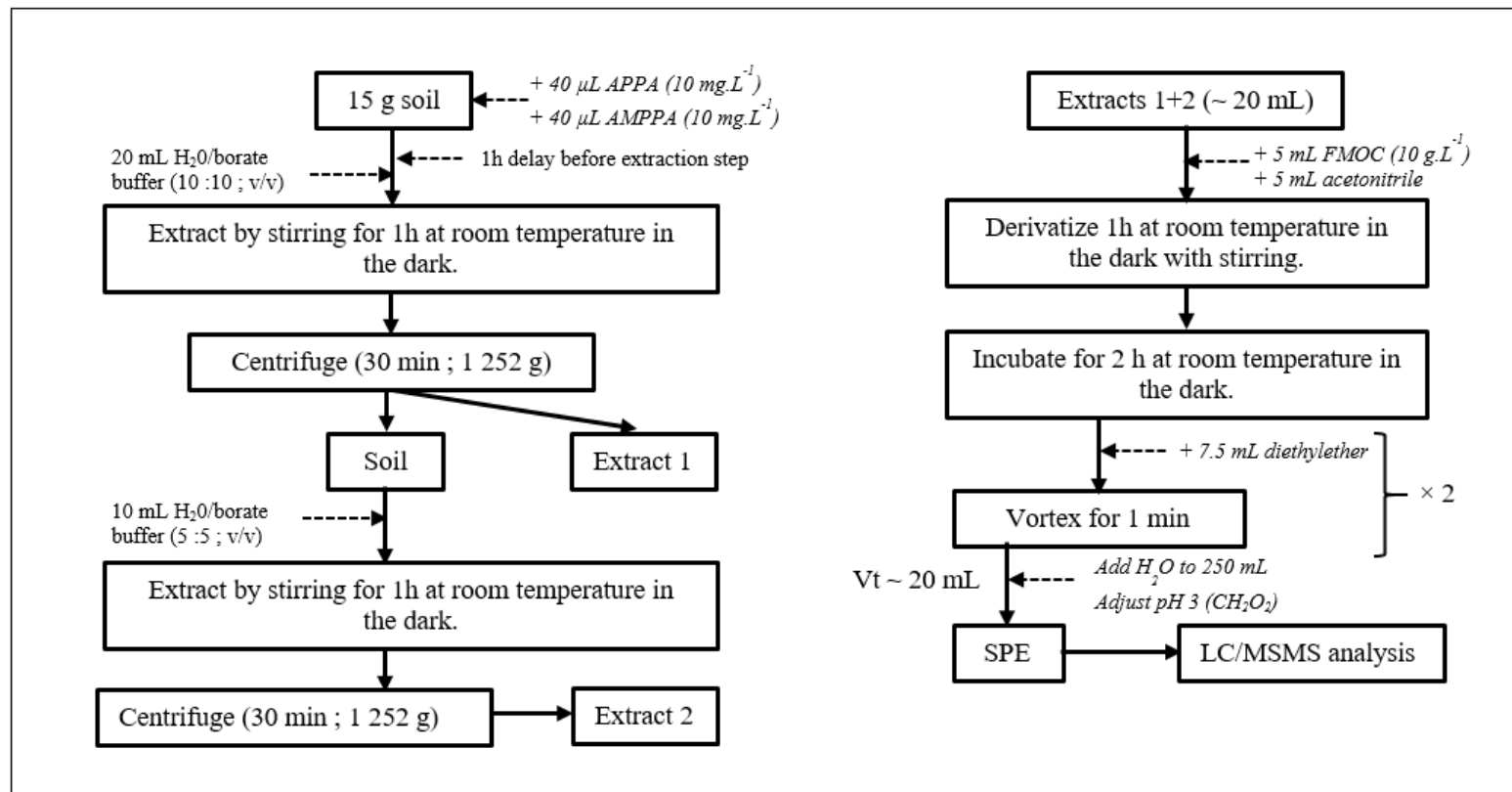


Figure 1. Summary of the Analytical method used for extracting soils

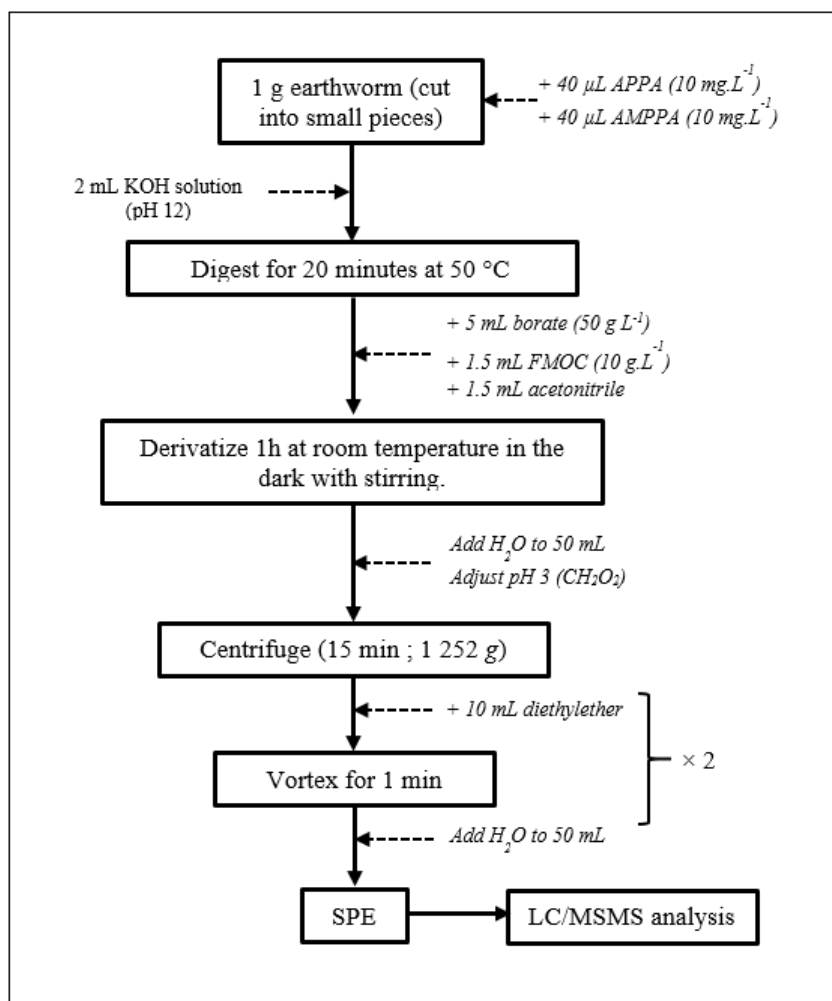


Figure 2. Summary of the analytical method used for earthworms

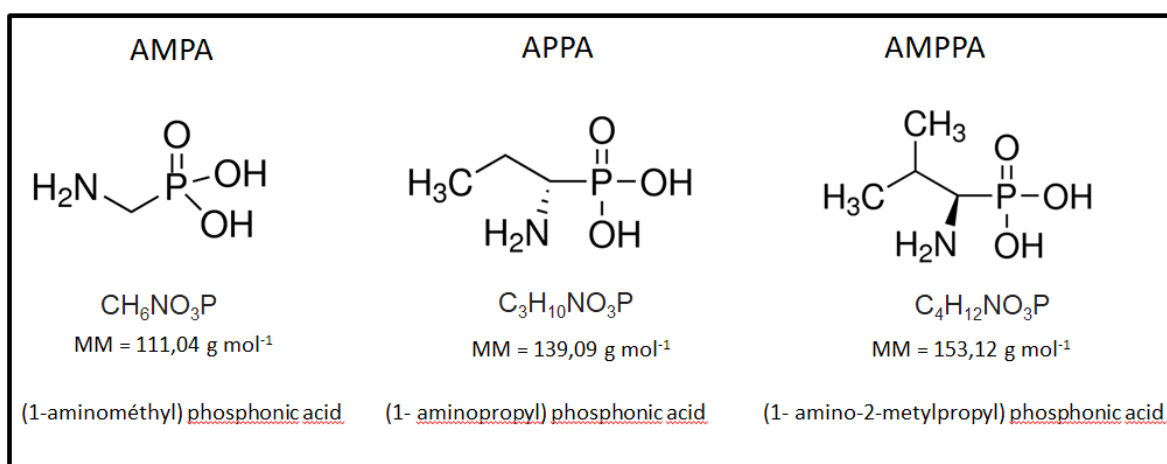


Figure 3. Structures of AMPA, APPA and AMPPA

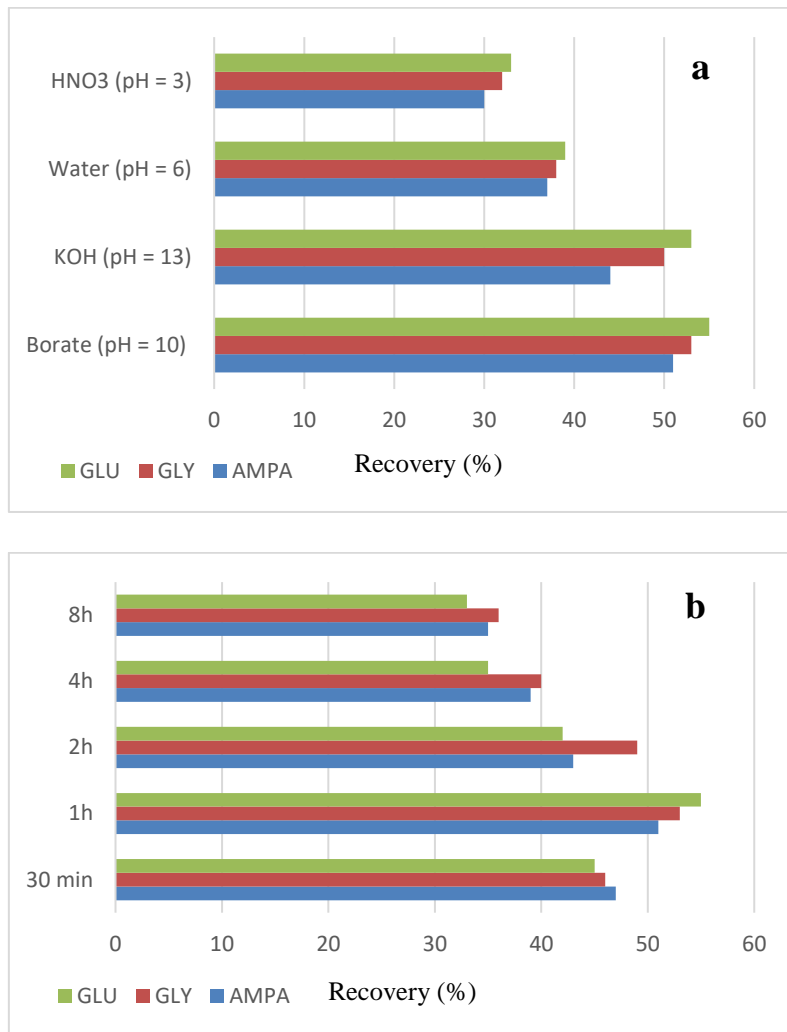


Figure 4. Recoveries of Glu, Gly and AMPA obtained during the optimization steps to test extraction solvents (a) and extraction times (b).

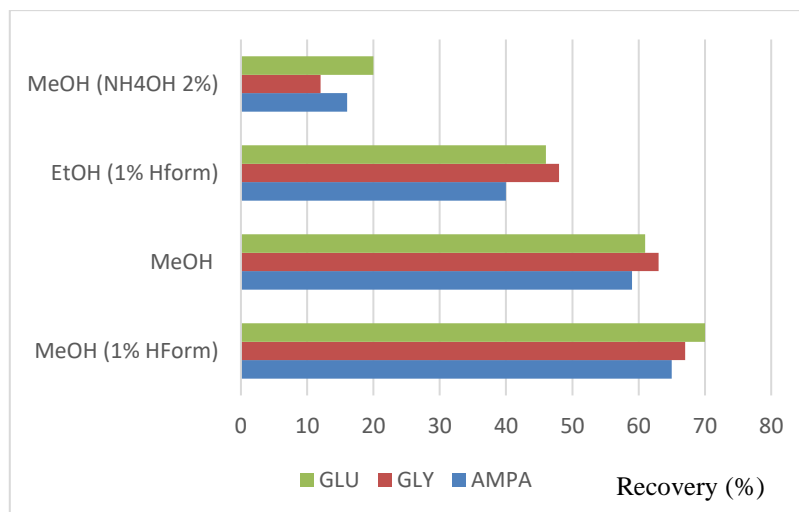


Figure 5. Glu, Gly and AMPA recoveries from SPE

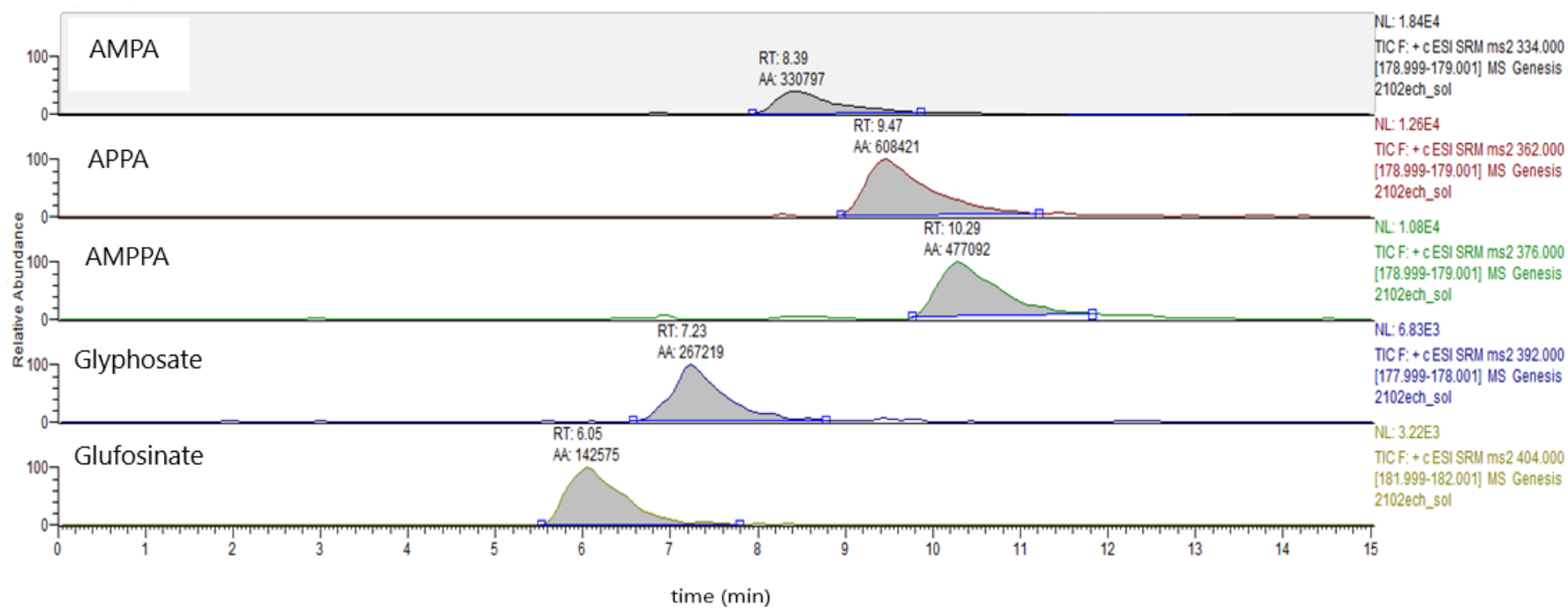


Figure 6. Chromatograms on the quantitation transitions of a soil sample where the compounds were determined as 0.070, 0.069 and 0.041  $\mu\text{g g}^{-1}$  respectively for AMPA, GLY and GLU (the internal standards APPA and AMPPA being at 0.1  $\text{mg L}^{-1}$ ).