Evaluation of two extraction methods to determine glyphosate and AMPA in soil.

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ABSTRACT

Argentine agricultural production is fundamentally based on a technological package that combines direct seeding and glyphosate with transgenic crops (soybean, maize and cotton). Therefore, glyphosate is the most employed herbicide in the country, where 180 to 200 million liters are applied every year. Due to its widespread use, it is important to assess its impact on the environment. However, glyphosate´s unique physico-chemical characteristics difficult its determination at residue level, especially in soils with high organic matter content, such as the central eastern Argentine soils, where strong analytical interferences are normally observed. The aim of this work was to compare the efficiency of two extraction methods of glyphosate in different representative soils of Argentina. One method is based on the use of phosphate buffer as extracting solution and dichloromethane to minimize matrix organic content. The other method employs potassium hydroxide (KOH) for the soil extraction of analytes and involves a clean-up step using solid phase extraction (SPE) to minimize the interferences. Both methodologies involve a derivatization with 9-fluorenyl-methyl-chloroformate (FMOC) in borate buffer, the use of isotope labelled glyphosate as internal standard and detection based on ultra-high-pressure liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). Recoveries obtained for soil samples spiked at 0.1 and 1 mg kg^{-1} were satisfactory in both methods (70% – 120%). However, significant differences were observed in the matrix effect, being the SPE clean-up step insufficient to remove the interferences, whereas the dilution and the clean-up with dichloromethane were more effective minimizing the ionic suppression.

Key words: Glyphosate; AMPA; Soil; Ultra-performance Chromatography; Matrix effects

1. INTRODUCTION

Glyphosate (N-[phosphonomethyl] glycine) is a broad-spectrum herbicide, used in agriculture to control weeds. The main uses of glyphosate are in genetically modified glyphosate-resistant crops (i.e., soybean, corn, cotton) (Roberts et al., 1998) and during the fallow period in no-till practices. Nowadays, glyphosate-based herbicides are the most commonly used in Argentina representing 60% of the total sold pesticides (Contardo-Jara et al., 2009).

Once glyphosate reaches the soil, it is strongly sorbed to soil by binding to clay minerals, layer silicates, metal oxides, non-crystalline materials or organic matter (Vereecken, 2005; Borggaard and Gimsing, 2008). Sorption of glyphosate is a reversible process that regulates the half-life and mobility of the herbicide and the risk of contaminating courses of surface and groundwater. Whereas degradation of adsorbed glyphosate is notably slow (Newton et al., 1994), due to a dynamic process, free glyphosate can move into the soil solution where it is rapidly and completely degraded by soil microorganisms (Ia and Maggi, 2018). The primary metabolites are glyoxylate and aminomethylphosphonic acid (AMPA) which eventually degrade to water, carbon dioxide, ammonia and phosphate (Sviridov et al., 2015).

Despite the low mobility that glyphosate presents in soil and its microbiological degradation, both glyphosate and AMPA have been found in natural water courses (Peruzzo et al., 2008; Battaglin et al., 2009), where it is principally bound to the suspended particulate matter and deposited in the sediment (Aparicio et al., 2013). Transport of the glyphosate molecule strongly bound to soil colloids to other

environmental compartments is the result of runoff or leaching (Kjær et al., 2005; Scribner et al., 2007) or air pollution (Neary et al., 1993; Mendez et al., 2017).

A thorough assessment of the environmental occurrence of glyphosate, despite its low ecotoxicological potential, is necessary given to its worldwide application, especially in countries like Argentina, where large areas are dedicated to transgenic varieties of glyphosate tolerant soybean (Peruzzo et al., 2008). In addition, there is growing interest in monitoring glyphosate due to its recent classification as probably carcinogenic to humans (group 2A) by the International Agency for Research on Cancer (IARC) (Williams et al., 2016).

Due to the ionic character, high polarity, low volatility and low molecular weight of glyphosate (Stalikas and Konidari, 2001), it is difficult to develop simple methods for the extraction and determination of this compound at residue level in soil samples. Moreover, the analytical determination of glyphosate is particularly difficult in soils with high organic matter content, due to their higher complexity and likely presence of interfering compounds.

At present, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is the most used methodology, because the high sensitivity and selectivity allows the determination of glyphosate at residue level. However, pre-column derivatization with fluorenylmethyl chloroformate (FMOC) is usually required in order to reduce the polar character of the analytes, facilitating the chromatographic retention into the reversed-phase columns commonly used (Miles et al., 1986). There are several works that determined glyphosate and AMPA in soil using this technique (Sancho et al., 1996; Lee et al., 2002; Ibáñez et al., 2005). The principal inconvenience that presents LC-MS/MS in complex matrices, such as soil samples, is an important loss in the signal

intensity that can occur as a consequence of coeluting compounds with the ionization analyte (matrix effect).

The aim of this paper is to compare two methods for extraction and determination of glyphosate at low concentrations in samples of different representative soils of Argentina. The first analytical method (phosphate method) is based on the use of phosphate buffer as extracting solution and dichloromethane to minimize matrix organic content (Primost et al., 2017). The second method (alkaline method) employs potassium hydroxide (KOH) for the soil extraction of the analytes, and solid phase extraction (SPE) clean-up to minimize the interferences (Botero-Coy et al., 2013). Sensitivity, recoveries, matrix effects and robustness were evaluated for both methods in soils from Argentina.

2. MATERIALS AND METHODS

1. Chemicals

Glyphosate and AMPA (PESTANAL®, 99.9%) reference standards were purchased from Seasinglab (Tandil, Argentina). Isotope-labelled glyphosate (1, 2-^{13}C, ^{15}N), used as internal standard (IS), was purchased from Sigma (Bs. As., Argentina). Analytical reagent-grade disodium tetraborate decahydrate, ammonium acetate (NH_4Ac, reagent grade) and 9-fluorenmethylcholoroformate (FMOC-Cl) were supplied by Seasinglab. HPLC-grade methanol, HPLC-grade acetonitrile and dichloromethane (CH_2Cl_2) were purchased from Seasinglab. HPLC-grade water was obtained by purifying demineralized water in ELGA purelab ultra (Illinois, USA). OASIS HLB cartridges (60 mg) were purchased from D'Amico Sistemas (Bs. As., Argentina).

2. Instrumental analysis

Ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) analysis was performed using an ACQUITY UPLC™ system coupled to a Quattro Premier™ XE tandem quadrupole mass spectrometer (Waters).

For the chromatographic separation, an Acquity UPLC BEH C18 column (1.7 µm, 50 x 2.1 mm) (Waters) fitted with an Acquity VanGuard BEH C18 pre-column (1.7 µm, 5 x 2.1 mm) (Waters) was used. The flow rate for the mobile phase was 0.4 mL min^{-1}. Mobile phase was a time-programmed gradient using organic-free water modified with ammonium acetate 5 mM (phase A) and methanol modified with ammonium acetate 5 mM (phase B). The percentage of organic modifier (B) was changed linearly as follows: 0 min, 0%; 0.2 min, 0%; 2.5 min, 70%; 3.5 min, 100%; 4.5 min, 100%; 5.0 min, 0%; and 6 min, 0%. The column was kept at 60 °C and the sample manager was maintained at 8 °C. The injection volume was 20 µL. Drying as well as nebulizing gas was nitrogen, obtained from a nitrogen generator. The cone gas and desolvation gas flows were optimized at 2 L h^{-1} flow and 600 L h^{-1}, respectively. For operation in MS/MS mode, collision gas was Argon 99.995% with a pressure of 4.04×10^{-3} mbar in the T-Wave cell. Positive ionization mode was performed using capillary voltage of 3.0 kV. The desolvation gas temperature was set to 400 °C and the source temperature to 120 °C. Dwell times of 0.10 s/scan were chosen. Masslynx NT v 4.1 (Waters) software was used to process quantitative data obtained from calibration standards and from samples.

3. *Sampling area*

Eight representative soils were selected from different regions of Argentina, with no history of glyphosate application at least in the last 10 years, corresponding to different taxonomic orders: Marcos Juárez (Córdoba province), Santiago del Estero (Santiago del Estero province), Famaillá (Tucumán province), Pergamino (Buenos Aires province), Cerro Azul (Misiones province), Balcarce (Buenos Aires province), Alto Valle (Río Negro province) and Corrientes (Corrientes province).

The sampling depth was 0-5 cm deep. Samples were dried at constant temperature in an oven at 30°C, and then ground and sieved to a particle size of 2 mm. The physicochemical and granulometric characteristics of the studied soils are show in table 1.

4. *Analytical procedure*

Two extraction methods were evaluated in soils without history of application of glyphosate. For each method studied, precision (repeatability, in terms of % RSD) and accuracy (percentage recoveries) were estimated by recovery experiments in the selected soils, at two fortification levels each (100 and 1000 µg kg^{-1}), and analyzed in triplicate. In order to obtain glyphosate and AMPA concentrations in the "blank" samples, non-spiked soils were also analyzed in duplicate. Recoveries between 70%-120%, with RSD lower than 20%, were considered as satisfactory (guideline SANCO/12571/2013).

The procedure applied in the phosphate method was as follows (figure 1): 5.0 g fortified soil sample, previously dried at 30ºC and homogenized, was weighted into a 50-mL centrifuge tube. The sample was extracted with 25 mL of $KH_2PO_4/Na_2B_4O_7$ buffer

(0.1 M, pH=9) in an ultrasonic bath for 30 min. Then, it was centrifuged at 3500 rpm for 10 min, and 2 mL of the supernatant was spiked with 10 µL of isotope-labeled glyphosate (1,2-^{13}C,^{15}N) stock solution (10 mg L^{-1}) and derivatized with 2 mL of FMOC-Cl reagent in acetonitrile (1 mg mL^{-1}). The tube was shaken vigorously and left overnight at room temperature (between 12 and 15 h). After that, in order to eliminate the excess of FMOC, a liquid–liquid extraction with 5 ml of CH_2Cl_2 and centrifugation at 3000 rpm for 10 min was performed. Finally, the aqueous phase was filtered through a 0.22 µm nylon filter and 20 µL of the final extract was injected into the UPLC-ESI-MS/MS system.

The procedure applied in the alkaline method was as follows (figure 1): 2.0 g fortified soil sample was extracted with 10 mL 0.6 M KOH in an ultrasonic bath for 30 min and centrifugation at 3500 rpm for 10 min. Then, 1 mL of the supernatant was diluted with 1 mL HPLC-grade water. The soil extract was adjusted to pH 9 by adding HCl (6 M and 0.6 M) and it was loaded onto an OASIS HLB cartridge (60 mg), previously conditioned with 3 mL methanol and 3 mL water. The non-retained sample extract was collected, spiked with 10 µL of isotope-labeled glyphosate (1, 2-^{13}C,^{15}N) stock solution (10 mg L^{-1}) and then derivatized with 120 µL borate buffer and 120 µL of FMOC-Cl reagent. The tube was shaken vigorously and left overnight at room temperature (between 12 and 15 h). After that, the derivatized extracts were centrifugated and acidified with HCl (c) to pH 1.5 and let stand for 1 h. Then, the sample was filtered through a 0.22 µm nylon filter and 20 µL of the final extract was injected into the UPLC-ESI-MS/MS system.

The mass spectrometry parameters for targeted substances are presented in table 2. Confirmation of the identity of glyphosate and AMPA in samples was carried out by acquisition of three MS/MS available transitions. The most intensive product ion from

each precursor ion was selected for quantification (Q), whereas secondary and tertiary transitions (q_1 and q_2) were used for confirmation purposes. Positive findings were confirmed calculating at least the peak area ratios between Q and q_1 (Q/q) and comparing them with ion-ratios obtained from a reference standard. A finding was considered positive when the concentration ratio was in the range 0.8–1.2. The agreement in retention time between standards and samples was also required, with maximum deviation of 2.5%.

The linearity of the method was studied by performing a calibration curve standard solutions at concentrations of 1, 5, 10, 50, 100 and 1000 µg L^{-1}, each point by triplicate. Satisfactory linearity using weighed (1/X) least squares regression was assumed when the correlation coefficient (r^2) was higher than 0.99, based on analyte peak areas measurement, and the residuals lower than 30%. Standard solutions were spiked with 10 µL of isotope-labeled glyphosate stock solution (10 mg L^{-1}), equivalent amount that in the analyzed samples, in order to evaluate the matrix effect. After UHPLC–MS/MS analysis, responses obtained for the isotope-labeled glyphosate in the soil extract (Y) were compared with the responses obtained in standard solutions (Z). The ratio (Y/Z×100) was taken as absolute matrix effect (Marín et al., 2009).

The limit of detection (LOD), defined as the lowest concentration that the analytical process can reliably differentiate from background levels, was estimated for a signal to noise ratio of 3 from the chromatograms of samples spiked at the lowest analyte concentration assayed (0.1 mg kg^{-1}), making use of the quantification transition (Q). The limit of quantification (LOQ), defined as the smallest value of analyte that can be determined quantitatively, was estimated similarly to the LD but for a signal-to-noise ratio of 10.

205 *5. Statistical Analyses*

206 Analyses of variance were performed with SAS version 6.12 software (SAS
207 Institute, 1989-1996). The data were analyzed using a mixed linear model (PROC
208 MIXED). The random effect was repeated and the fixed effects were soil, method, and
209 fortification levels. Mean comparisons were evaluated with a significance level of 0.05
210 using LSMEANS.

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213 **3. RESULTS AND DISCUSSION**

214

215 *1. MS method*

216 The three selected reaction monitoring (SRM) transitions chosen for residue
217 determination of glyphosate and AMPA derivatives (glyphosate-FMOC and AMPA-
218 FMOC, respectively), and two available transitions for isotope-labeled glyphosate
219 derivative (IS-FMOC), as well as the optimized MS/MS parameters, are shown in table 2.
220 The response factors (analyte area/IS area ratio) for the different concentrations,
221 normalized by IS concentration, showed a good linearity in the range 1–1000 µg L^{-1} for
222 both compounds (figure 2) with correlation coefficients (r^2) greater than 0.99 and
223 residuals always below 30%.

224 LOQ and LOD were estimated from the SRM chromatograms of samples spiked
225 at the lowest tested level. In method 1, the LOQ level was 1.0 µg kg^{-1} for glyphosate and
226 1.5 µg kg^{-1} for AMPA, while the LOD was 0.2 µg kg^{-1} and 0.5 µg kg^{-1}, for glyphosate and

AMPA respectively. For method 2, the LOQ was 1.0 µg kg^{-1} for glyphosate and 2.0 µg kg^{-1} for AMPA, while the LOD was 0.3 µg kg^{-1} and 0.7 µg kg^{-1}, respectively.

2. Glyphosate and AMPA recoveries

The accuracy of a chromatographic method is usually characterized by recovery, defined as the fraction of the analyte determined after addition of a known amount of the analyte to a sample, and it can seriously be affected by sample treatment and quantification procedure. Recovery was calculated as:

$$R\ (\%) = \frac{(Csample - Cblank)}{Cfortification} x100$$

where C_{sample} is the concentration determined in fortified sample, C_{blank} is the concentration determined in unfortified sample and $C_{fortification}$ is the concentration of fortification.

Precision and accuracy of analytical procedures were evaluated by spiking the samples at two different concentration levels (100 and 1000 µg kg^{-1}), and analyzing them in triplicate.

As table 3 shows, results obtained were satisfactory for glyphosate in all studied soils. Glyphosate recoveries obtained for both fortification levels ranged between 74 and 99 % for phosphate method and from 73 to 118 % for method 2. RSDs were below 20% in all cases except for Marcos Juárez soil at the fortification level of 0.1 mg kg^{-1} extracted with alkaline method (24%). The results obtained shows that glyphosate recoveries are generally higher when alkaline method is applied ($p < 0.0001$). However, differences in both methods performance depend on soil type ($p = 0.0002$). The chemical and

248 granulometric characteristics of the soils studied (table 1), such as pH and clay, organic
249 matter, silt, and sand content, do not seem to have any influence in glyphosate
250 recoveries.

251 AMPA recoveries were satisfactory in all studied soils (table 3). Recoveries
252 ranged between 70 and 89% (0.1 mg kg^{-1}) and 73 to 87% (1.0 mg kg^{-1}) in phosphate
253 method, whereas recovery values between 77 to 115% (0.1 mg kg^{-1}) and 68 to 103%
254 (1.0 mg kg^{-1}) were obtained for alkaline method. RSDs were below 20% in all cases.
255 Only in Marcos Juárez`s soil, recovery using alkaline method was less than using
256 phosphate method. In the rest of the soils, alkaline method recovered equal or more
257 than phosphate method.

258 It is important to note that two of the soils employed in the experiments
259 (Pergamino and Cerro Azul) presented previous concentrations of glyphosate and
260 AMPA. Therefore, recovery calculation could not be satisfactorily calculated at the 0.1
261 mg kg^{-1} level. Altogether, taking all the soils at the two fortifications, alkaline method had
262 a higher glyphosate recovery than phosphate method (98 and 90%, respectively). This
263 result was not significant for AMPA recovery where values about 79 and 86% were
264 obtained for phosphate and alkaline method, respectively.

265 Aside from the eight soils used in this study, recoveries were also tested for the
266 same type of soils, but with a history of glyphosate application in the last years (table 4).
267 Recoveries obtained were not significantly different between the agricultural and
268 nonagricultural soils.

269

270

3. Matrix effects

UHPLC–MS/MS coupled with electrospray ionization (ESI) is relatively sensitive to interferent molecules (Antignac et al., 2005), where a greater amount of additives (from eluents or sample matrices) are produced in ESI droplets that may lower evaporation efficiency and the ability of analytes to reach the gas phase. As a result, there could be a competition between the analyte and a co-eluting matrix component during ionization that can decrease the analyte ionization (ion suppression) or increase its ionization (ion enhancement). This phenomenon has a remarkably negative effect on the accuracy of the analytical method when dealing with complex matrices, such as soil samples, where an important loss of sensitivity can occur and may lead to unreliable results. Several strategies have been suggested to minimize or to correct the matrix effect, such as increasing the sample pretreatment, performing matrix-matched calibration, simply diluting the sample, or the most currently applied, using an isotope labeled standard (Sancho et al., 2002; Hao et al., 2007). The sample clean-up step can help to reduce the presence of interfering components in the final extract, but it might be compromised with soil matrices, where a variety of interferences with different chemical properties are present and multiple extraction steps are usually necessary, with the consequent risk of analyte loss. On the other hand, sample dilution offers a fast, simple and effective way to minimize matrix interferences, so that fewer matrix components will be injected into the analytical system (Schuhmacher et al., 2003; Lee et al., 2007). It is important to improve chromatographic separation that allows the analytes to elute in an appropriate period of time, in order to avoid the co-elution with matrix components. Matrix effects were estimated for each studied soil by comparison of the isotope-labeled glyphosate responses in solvent and in soil extracts, after the extraction procedure described for each method.

296 The soil and method influence on the matrix effect (figure 3) ($p < 0.0001$). Matrix
297 effects observed in phosphate method for all the tested soils ranged between 3 and
298 32%, being more intense in soils of Cerro Azul and Balcarce. On the other hand, strong
299 signal suppression was observed for most soils studied in alkaline method. With
300 exceptions of Alto Valle and Corrientes soils, matrix effects observed with this method
301 were higher than 33%, reaching values up to 85 % (Cerro Azul soil). These results agree
302 with previous works that reported signal suppression higher than 70% in different
303 Colombian and Argentine soils (Botero-Coy et al., 2013). Balcarce and Cerro Azul soils
304 show the higher matrix effect. The higher matrix effect in Cerro Azul is likely due to its
305 higher Fe and Al hydroxides content, which are known to interact with glyphosate
306 (Gimsing and Borggaard, 2002), whereas in Balcarce soil is likely due to its higher OM
307 content, which is known to interact with glyphosate (Albers et al., 2009). Alkaline method
308 involved several procedures in order to minimize the strong matrix effects commonly
309 observed in South American soils (Botero-Coy et al., 2013), such as dilution of the
310 extract with water, modification of pH and application of SPE cleanup step. The SPE
311 cleanup was performed with OASIS cartridge HLB, which is expected to retain some
312 organic components of the matrix, while analytes of high polarity/ionic character flow
313 through. In this case, the high matrix effect observed could be explained by the presence
314 of interferences that are not removed by SPE. On the other hand, in phosphate method,
315 the lower matrix effect observed may be due to the dilution factor applied to the samples
316 in the extraction procedure. Despite the fact that dilution of soil with extract buffer has
317 shown good results to minimize matrix effect, the main disadvantage is the loss of
318 analytical sensitivity, becoming a commitment factor between sensitivity and peak shape
319 in the trace analysis of pesticides.

320 It is important to remark that the interferences remnants in the final extract in both
321 methods are different. In the case of dichloromethane clean up, it is mainly removed the
322 excess of FMOC (Peruzzo et al., 2008; Primost et al., 2017), whereas in the SPE step
323 the organic matter are eliminated (Botero et al., 2013). Both, FMOC and organic matter
324 are critical points in the determination of glyphosate and AMPA but in different way. In
325 some cases, it is important to eliminate the organic matter for the liberation of glyphosate
326 and AMPA, so the use of SPE before the derivatization step is important for a good
327 recovery performance. However, the SPE process does not eliminate the excess of
328 FMOC. And because of this, the performance of the partition is better to remove this
329 interference.

330 The results obtained in this work show the importance of matrix effect
331 compensation in the analysis of pesticide residues in soil samples. Due to the presence
332 of a great variety of interferences that could modify the quantification levels, the use of
333 correction factors to deal with matrix effects are extremely important, especially in cases
334 in which the results for each sample matrix are obtained according to a calibration curve
335 prepared in pure solvent. In this sense, the use of isotopically labelled glyphosate as
336 internal standard is a simple way, widely employed in glyphosate and AMPA
337 determination, to minimize and correct this undesirable effect and compensate for any
338 error occurrence during sample processing to obtain a satisfactory quantification.

339

340 **4. CONCLUSIONS**

341 The purpose of this study was to compare two methods of extraction of glyphosate
342 and AMPA in soil samples from Argentina. Both methods show satisfactory recoveries

for the different studied soils. However, there is a remarkable difference regarding the matrix effect. The method based on the use of phosphate buffer as extracting solution shows lower signal suppression, compared to the method that employs potassium hydroxide for extraction of analytes soil and solid phase extraction (SPE) clean-up. In addition, method based on the use of phosphate buffer involves fewer sample processing, which reduces the possibility of errors by loss of analyte or sample contamination, and it is also cheaper, which is an important factor in routine work.

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471 Fig. 1. Analytical procedures for the two studied methods.

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474 Fig. 2. UPLC-MS/MS chromatograms and calibration curves for glyphosate and AMPA.

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477 Fig. 3. Comparison of matrix effect between both methods.

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