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Development and Application of a Liquid Chromatography−Mass Spectrometry Method To Evaluate the Glyphosate and Aminomethylphosphonic Acid Dissipation in Maize Plants after Foliar Treatment

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ABSTRACT: A simple and fast method has been developed and validated to measure glyphosate (GLYP) and aminomethylphosphonic acid (AMPA), which were previously derivatized with 9-fluorenylmethylchloroformate (FMOC-Cl), in maize plants using liquid chromatography (LC) coupled to fluorescence (FLD) and electrospray ionization mass spectrometry (ESI-MS) detection. The method has shown to be consistent, reliable, precise, and efficient. Moreover, the limits of detection (LOD) and quantification (LOQ) reached with the proposed method for GLYP and AMPA are lower than the established maximum residue levels (MRLs). The validated method was applied to quantify GLYP and AMPA in genetically modified (GM) maize foliar treated with the herbicide. It has been found that the GLYP dissipation was mainly due to the progressive dilution effect after herbicide treatment. Finally, it was also observed that the GLYP residue dissipation trend in maize shoot (leaves and stem) tissue determined by LC−ESI-MS matched that determined by liquid scintillation.

KEYWORDS: *glyphosate, aminomethylphosphonic acid, dissipation, maize plants, LC−FLD-ESI-MS, liquid scintillation*

INTRODUCTION

Glyphosate (GLYP) [*N*-(phosphonomethyl)glycine] is a widely used broad spectrum foliar applied herbicide for vegetation control, introduced in the early 1970s. During the past years, the introduction of transgenic plants resistant to GLYP has increased its use in the agricultural practices in a way similar to the growing of the transgenic commerce.[1] The compound is absorbed into the leaves and readily translocated to underground parts. The first step in the degradation pathway is essentially the cleavage to glyoxylate being aminomethylphosphonic acid (AMPA), its primary metabolite. Although GLYP mainly targets 5-enolpyruvylshikimate-3-phosphatesynthetase (EPSPS), photosynthesis and respiration are also affected with deep changes in plant growing.

The physicochemical properties of GLYP make its analysis very difficult, and it is not surprising the large number of analytical methods proposed in the literature for its quantitative detection.[2−24] Among the methods proposed for the determination of GLYP in crops and animal tissues, the chromatographic ones, either gas and liquid chromatography,[2−21] are the most effective in facing the problems of interference compounds (naturally occurring amino-acids and amino-sugars) in the analyzed samples. In both techniques, a derivatization step (pre- or postcolumn) is required to make detectable the compounds. The compatibility of aqueous samples with the reversed-phase chromatographic separation system and the possibility of performing derivatization in aqueous solution joined to a less demanding sample pretreatment made liquid chromatography the preferred technique. From the literature devoted to GLYP determination in vegetables and food products, the most employed approach is the precolumn derivatization with 9-fluorenylmethylchloroformate (FMOC-Cl) coupled with fluorescence detection.[7] According to our knowledge, no studies are actually available on GLYP or AMPA determination by LC in maize shoots (leaves and stem).

The main goal of this work is to develop a new analytical method based on liquid chromatography coupled to fluorescence and electrospray ionization mass spectrometry (LC−FLD-ESI-MS) to determine GLYP and AMPA in maize plant samples to evaluate the dissipation of both compounds after foliar treatment. In a previous work, we developed a LC−FLD-ESI-MS methodology to study the toxicokinetics of GLYP and AMPA in rat plasma.[18] In the present study, the LC−FLD-ESI-MS conditions have been adapted to achieve the best separation with this new matrix (maize shoots). Moreover, we have proposed new sample treatment, derivatization, and validation procedures. The results obtained were also compared to earlier data and to existing maximum residue levels (MRLs) legislation[25−27] to check the suitability of the method. Once the first goal was achieved, the proposed method was successfully applied to analyze residues of GLYP and AMPA in maize shoot (leaves and stem from a GLYP-resistant maize variety (MON832)) samples following three different herbicide treatments (control, 0.8 kg acid equivalent/hectare (ae/ha),

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1.6 kg ae/ha) obtained at six different harvest times, with the aim of elucidating the dissipation trend of the active ingredient and its main metabolite. Another objective of this work was to investigate the influence of the GLYP content in the treatments applied, as well as the proliferation growth in untreated and treated (0.8 and 1.6 kg ae/ha) maize plants. Finally, LC−FLD-ESI-MS dissipation data were compared to the dissipation trends monitored by liquid scintillation after application of radiolabeled GLYP.

MATERIALS AND METHODS

Reagents and Materials. GLYP [*N*-(phosphonomethyl)glycine: 95% pure, w/w], AMPA (aminomethylphosphonic acid: 99% pure, w/w), and FMOC-Cl (9-fluorenylmethylchloroformate) were provided by SIGMA Aldrich Chemie Gbmh (Steinheim, Germany). Acetonitrile and methanol (LC grade) were purchased from Lab-scan Ltd. (Dublin, Ireland). Reagent grade boric acid, disodium tetraborate decahydrate, ammonium hydroxide, and ammonium formate were obtained from Scharlab (Barcelona, Spain). All other chemicals were of the highest quality grade and obtained from commercial sources. A drying oven from Selecta (Barcelona, Spain) was used to dry maize leave samples, which were grinded in a Moulinette chopper machine from Moulinex (Paris, France). A 5810R refrigerated benchtop Eppendorf centrifuge (Hamburg, Germany) and a Vibromatic mechanical shaker from Selecta (Barcelona, Spain) were also used, while pH values were measured on a Crison pH-meter (Barcelona, Spain). LC-grade water was obtained by purifying demineralized water in a Millipore Milli-RO plus system together with a Milli-Q system (Bedford, MA). A 200 mM disodium tetraborate buffer solution (pH 9) was prepared in LC-grade water, and a 20 mM FMOC-Cl solution was prepared in acetonitrile. Syringe cellulose filters (17 mm 0.45 μm) from Nalgene (Rochester, NY) were used.

Standards. Standard stock solutions were prepared by dissolving approximately 50 mg of GLYP or AMPA, accurately weighted, in 100 mL of LC-grade water, obtaining a final concentration of approximately 500 mg/L. These solutions were further diluted with LC-grade water for preparing the working solutions. Blank maize samples (0.1 g of dry powdered non treated maize shoot samples) were spiked with GLYP and AMPA at different concentrations, and then treated according to the extraction procedure described in this Article. Each quality control (QC) sample was prepared using blank non treated maize samples (0.1 g) spiked with different amounts of GLYP and AMPA. The concentrations of the different QC samples were as follows: low QC level, 0.15 mg/kg (GLYP) and 0.20 mg/kg (AMPA) for ESI-MS and 0.30 mg/kg (GLYP) and 0.70 mg/kg (AMPA) for FLD; medium QC level, 150 mg/kg for ESI-MS and FLD; high QC level, 800 mg/kg for ESI-MS and FLD. All standard (stock, working, and matrix based) solutions were stored in polypropylene containers and kept in the dark at 4 °C, and they were stable for over 1 month.

Chromatographic System. An Agilent Technologies (Palo Alto, CA) 1100 series LC-FLD-MS system was used, consisting of a vacuum degasser (model number G1322A), a quaternary pump (G1311A), a standard autosampler (G1313A), a thermostatted column compartment (G1316A), a fluorescence detector (FLD, G1321A), and a single quadrupole MS (G1946D) analyzer with an API electrospray (ESI) source (G1948A), all controlled by an Agilent Chemstation software. A Synergi 4 μm MAX-RP 80 Å (250 mm × 4.6 mm i.d.) was used as analytical column, and it was protected by a Synergi C_{12} security guard cartridge (4 mm × 3.0 mm i.d.), both from Phenomenex (Torrance, CA). The mobile phase selected was a mixture of ammonium formate 20 mM [pH 8.5] in water (A) and acetonitrile (B), applied at a flow rate of 1 mL/min in a gradient mode as follows: (i) 0 min (A−B, 86:14, v/v); (ii) 0−5 min (A−B, 80:20, v/v); (iii) 5−13 min (A−B, 76:24, v/v); (iv) 13−18 min (A−B, 0:100, v/v); (v) 18−21 min (A−B, 0:100, v/v); and (vi) 22−25 min (A−B, 86:14, v/v); with a postseparation time of 5 min. The injection volume was set at 30 μL (draw speed 50 μL/min), and the temperature selected was 45 °C. The detection wavelengths (λ) of the FMOC-derivatized analytes were 240 nm (excitation, λ_{ex}) and 320 nm (emission, λ_{em}) to quantify GLYP, and 250 nm (λ_{ex}) and 620 nm (λ_{em}) to quantify AMPA. The FLD was programmed to monitor exclusively from 0 to 7 min of the chromatographic run the selected λ_{ex} and λ_{em} for GLYP-FMOC, while from 7 min to the end of the chromatographic run, the only recorded were the optimal λ_{ex} and λ_{em} for AMPA-FMOC. The ESI interface was operated in positive mode having performed flow injection analysis (FIA) tests of the more relevant MS parameters. Full-scan LC−MS spectra were obtained by scanning from m/z 50 to 500. The most abundant ion of each compound was quantified in SIM mode.

GLYP and AMPA Accumulation/Dissipation in Treated GM Maize. Greenhouse experiments were carried out at the Department of Agroenvironmental Science and Technology (University of Bologna, Italy) with a GLYP-resistant maize variety (MON832). Plants were grown hydroponically in sterilized sand, fertilized, and irrigated as necessary for a vigorous growth in a greenhouse maintained at 27/22 °C day/night temperature with natural light supplemented by artificial light to provide a 12 h photoperiod. The experimental design was a randomized complete block (RCB) with three treatments (control, 0.8 kg ae/ha, 1.6 kg ae/ha), six harvest times (0, 7, 14, 28, 42, 56 days after GLYP treatment (DAT)), and three replicates of two plants (n = 6) for each treatment at each sampling time. According to Reddy et al.,[28] to minimize interference from different ingredients in the GLYP commercial formulation, technical grade GLYP acid (>97% purity, Sigma Aldrich, St. Louis, MO) and Tween 20 (Sigma Aldrich, St. Louis, MO) at 0.5% (v/v) were employed for the preparation of spray solutions at 0.8 and 1.6 kg ae/ha. Tween 20-treated plants were considered as control (or GLYP untreated). Maize plants were treated at 2-leaf growth stage with a portable sprayer equipped with a flat-fan nozzle delivering an output volume equivalent to 185 L/ha. The harvest time 0 (0 DAT) was set after 10 h from plant spraying, when according to preliminary investigations (data not shown) 70−80% of GLYP was absorbed into plant tissues. At each harvest time, plants were excised at the grown substrate surface, washed with running water, rinsed with methanol/distilled water (1:9, v/v) to remove GLYP unabsorbed on leaf surface, and blotted dry with paper towels, and afterward shoot fresh weights (FW) were recorded. Shoot samples were air-dried, and dry weights (DW) were recorded. The dry shoot samples were finely ground and conserved at −20 °C until the analysis of GLYP and AMPA content by liquid chromatography. The trial was repeated with the aim to investigate the total GLYP residues determined by liquid scintillation in shoot and root samples from GM maize treated with radiolabeled GLYP (GLYP-(phosphonomethyl-^{14}C), Syngenta, Basel, Switzerland) at 0.8 and 1.6 kg ae/ha. The general experimental conditions were as described in the above study,[28] except for preparation of spray solutions. For each single treated plant in the spray solutions at 0.8 and 1.6 kg ae/ha containing technical grade GLYP acid and Tween-20 at 0.5% (v/v) were, respectively, added 2.64 and 5.28 kilobecquerel (kBq) of [^{14}C]-GLYP, corresponding to 4% of [^{14}C]-GLYP on total ae applied per plant (96% of nonradioactive ae). Liquid scintillation analyses were carried out according to Dinelli et al.[29] At different sampling times (0, 7, 14, 28, 42, 56 DAT), the entire plants were harvested, leaf surfaces were washed with methanol/water (1:9, v/v), and unabsorbed radioactivity was subsequently quantified by liquid scintillation spectroscopy (LSS) (1409 Liquid Scintillation Analyzer; Wallac, U.S.). In view of investigating the relative GLYP total residues in different plant parts, plants were then dissected into two sections: shoot and roots. The different plant sections were weighed, frozen in liquid nitrogen, finely ground, and extracted with LC-grade water (1:4 g FW/mL). After centrifugation (12 000 rpm, 10 min), the supernatant was assayed for radioactivity by LSS. Plant debris contained in the centrifugation pellet was dried and combusted in a Packard 387 oxidizer (Packard Instrument Co., Downers Grove, IL). The nonextracted radioactivity was then quantified by LSS. Data were analyzed by analysis of variance appropriate to a randomized complete block design. Where there was evidence of an overall effect of treatment (as provided by the F-test for the treatment effect), individual treatment comparisons at each harvest time were carried out

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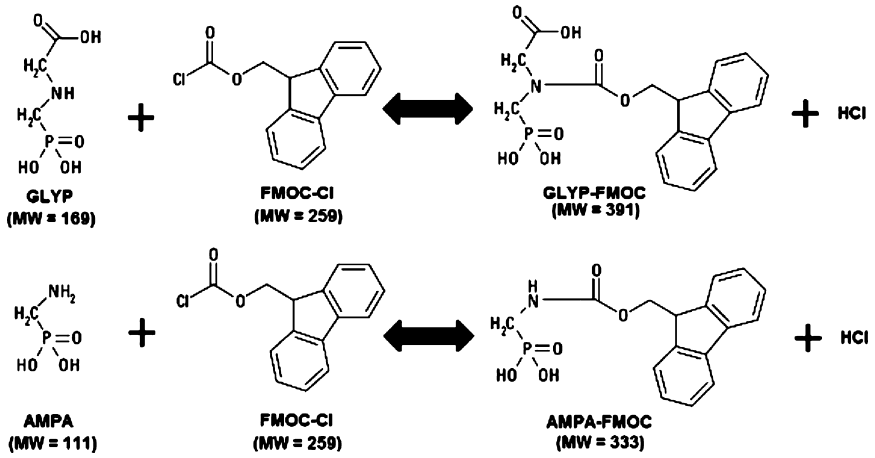

Figure 1. Chemical structures of GLYP and AMPA, and derivatization reaction with FMOC-Cl (MW: molecular weight).

using *t* tests. All statistical tests were carried out with CoStat software (CoHort Software, Monterey, CA). Nonlinear regression analysis was used to determine the GLYP half time dissipation (DT_{50}), which represents the time (d) after the treatment required to observe a reduction of GLYP content in maize plant tissues equal to 50% with respect to that observed at 0 DAT. A sigmoidal log−logistic model was used to relate GLYP concentration (Y) to time after the treatment (X) according to the following formula:

$$Y = a + \frac{b - a}{1 + e^{\{c[\ln(X) - \ln(DT_{50})]\}}}$$

where a denotes the lower GLYP concentration limit (lower asymptote), b denotes the upper GLYP concentration limit (upper asymptote), and c denotes the curve slope around DT_{50}. The regression parameters were computed using Bioassay97 software.[30]

Sample Treatment for LC−MS Analysis. A sub sample of 0.1 g of the powdered maize shoot sample was transferred to a 50 mL centrifuge tube, and then 20 mL of a mixture water−methanol (50:50 (v/v)) was added. After 50 min of mechanical shaking, the mixture was centrifuged at 10 000 rpm and 25 °C for 20 min. The supernatant was collected and transferred to a different tube, and it was evaporated under N_2 stream, and reconstituted with 1 mL of water. The extract was passed through a 0.45 μm pore size filter and then placed in the autosampler for derivatization and further injecting into the LC−FLD-ESI-MS system.

Derivatization. The derivatization procedure shown in Figure 1 was carried out using the control program of autosampler (See Table 1). It must be specified that the derivatization reaction was performed at 25 °C, and that a 200 mM solution of borate buffer (pH 9) and a 20 mM solution of FMOC-Cl were used for this process.

LC−MS Method Validation. Validation was carried out following different International guidelines.[31−33] The limits of detection (LOD) and quantification (LOQ) were determined by injecting a number of extracts from blank maize shoot samples (n = 6) and measuring the magnitude of the background response. LOD and LOQ were experimentally estimated as 3 and 10 times the signal-to-noise-ratio (S/N), respectively. To assess the selectivity of the method, extracts from blank and spiked maize shoot samples were assayed. The recovery of GLYP and AMPA was determined in six replicates at three concentrations (low, medium, and high QC levels), comparing the peak areas of both compounds from standard solutions with those from: (i) extracted blank maize shoot samples spiked with the same amounts of the compounds and then treated accordingly to the above-mentioned procedure for obtaining the recovery percentages (blank A); and (ii) extracted blank maize shoot samples treated as described above and afterward spiked with the same amounts of GLYP and AMPA, to check the possible effect of the matrix on the ESI ionization (blank B). Matrix matched standard calibration curves were employed for quantifying the analyte content in maize shoots. Blank shoot samples were spiked with variable amounts of GLYP and AMPA in an analytical range between 0.04 mg/kg (ESI-MS) or 0.13 mg/kg (FLD) and 1000 mg/kg for GLYP; and 0.06 mg/kg (ESI-MS) or 0.24 mg/kg (FLD) and 1000 mg/kg for AMPA. To assess the intraday precision and accuracy of the method, blank maize shoot samples were spiked at three concentrations of GLYP and AMPA (low, medium, high QC levels) on the same day. In each run, a calibration curve was done, and six replicates were analyzed. The interday precision and accuracy were evaluated by injecting six sample replicates at the three above-mentioned concentrations against a calibration curve on three consecutive days. Precision was defined at the percentage relative standard deviation (%RSD) at a given concentration for each QC sample; meanwhile, accuracy was calculated through the relative error (%RE).

Table 1. Derivatization Program

command	vial	speed/repetitions
draw 6 μL	sample or standard	200 μL/min
needle wash	water	two times
draw 12 μL	sodium borate 200 mM	200 μL/min
needle wash	water	two times
draw 6 μL	FMOC-Cl 20 mM	200 μL/min
needle wash	water	two times
mix 24 μL in seat	maximum speed	10 times
wait	10 min	
inject		

RESULTS

LC−MS Method Development. It was possible to obtain a good separation in less than 12 min with a slight variation of the gradient elution program presented in our previous work[18] (see Chromatographic System section). As it could be seen in Figures 2 and 3, GLYP-FMOC and AMPA-FMOC (FMOC-derivatized analytes) peaks were perfectly resolved. The optimized values for the other chromatographic parameters were analogous to those previously presented.[18]

Sample Treatment Optimization for LC Analysis. Maize shoot samples that were not treated with GLYP (blank samples) were dried and grinded; afterward, portions (0.5 g) were taken to which were added known amounts of GLYP and AMPA. The mixture was thoroughly shaken, and at this point, several solvents were assayed as extractants: water, methanol, ethanol, dichloromethane, chloroform, and a mixture water−methanol (1:1, v/v). The obtained results showed that the

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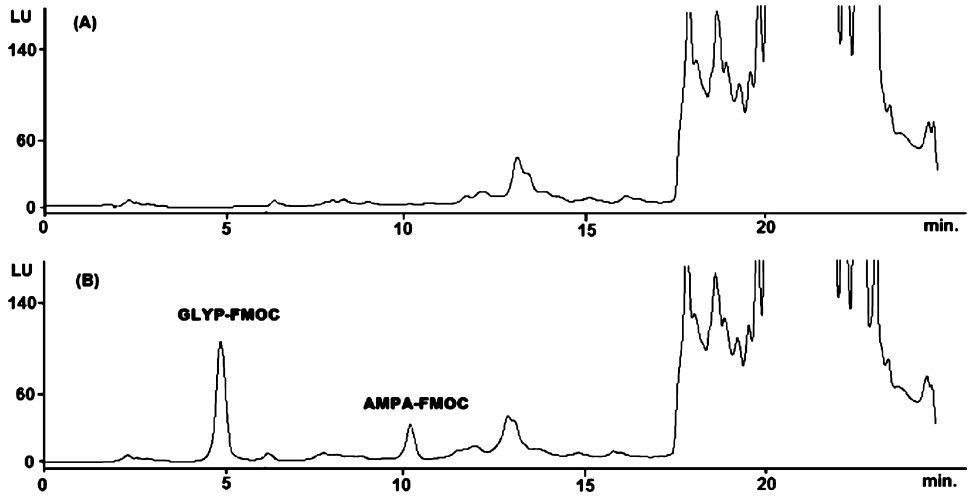

Figure 2. FLD chromatograms (λ_{ex} 240 nm and λ_{em} 320 nm for GLYP monitored from 0 to 7 min, and λ_{ex} 250 nm and λ_{em} 620 nm for AMPA monitored from 7 min) of (A) a blank maize leave sample and (B) a blank maize leave sample spiked with 50 mg/kg of GLYP and 10 mg/kg of AMPA. Chromatographic conditions are described in detail in the Chromatographic System section (LU: luminescence units).

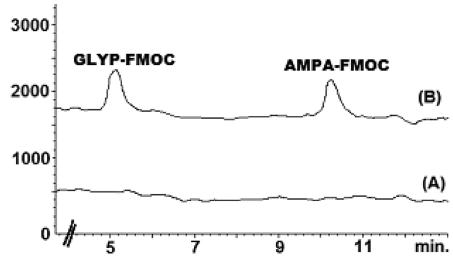

Figure 3. Representative LC−ESI-MS (SIM mode) chromatogram of (A) blank maize leave sample and (B) blank maize leave sample spiked with LOQ GLYP and AMPA (0.4 and 2 ng/mL, respectively). Chromatographic and mass spectrometry conditions are described in detail in the Chromatographic System and Mass Spectrometry Optimization sections, respectively.

mixture of water and methanol (1:1, v/v) provided the highest recoveries percentages (40% for GLYP and 30% for AMPA), and it was also beneficial to the later evaporation step that would be necessary to concentrate the samples. Different solvent volumes were employed (between 10 and 40 mL), and a slight increase was observed in the recoveries when using 20 mL (50% and 35% for GLYP and AMPA, respectively). Different quantities of maize dried shoots (0.1−2.0 g) were tested to study their influence on the recovery percentages. The best results were found for the lower amounts; in fact, for 0.1 g, the recoveries were 79% and 60% for GLYP and AMPA, respectively. Different extraction times were tested (20−80 min), and it was observed that at lower times, the recovery percentages were too low, and at times higher than 50 min, the improvement was not relevant. So, it was decided to employ 50 min of extraction time. Regarding the centrifugation parameters, different speeds (between 5000 and 10 000 rpm), times (between 10 and 45 min), and temperatures (from 15 to 25 °C) were assayed. After several experiments, the cleanest chromatograms were obtained when employing 10 000 rpm, 20 min, and 25 °C. The preconcentration step was done by using N_2 or by drying in a stove at 90 °C. The results obtained were good enough for both of them, although the evaporation with a N_2 stream was selected, as the time required for concentrating the sample was shorter. Finally and after performing several tests, 1 mL of water was employed to redissolve the residue.

Derivatization. First, several concentrations of FMOC-Cl (between 3 and 30 mM) were tested. It was found that with lower FMOC-Cl concentrations, the detection of the analytes was difficult, mainly due to matrix interferences, which affected the AMPA-FMOC determination. With higher concentrations of FMOC-Cl (>20 mM), the reagent excess generated interference peaks. For these reasons, 20 mM was finally selected. Regarding the concentration of borate buffer (500 mM), it was observed that the presence of acetonitrile, from the mobile phase, originated some salt precipitation and provoked problems inside the chromatographic column. After several tests were performed, a more diluted solution, 200 mM, was selected, which solved these problems. In relation to the volume ratios, the best results were obtained when using a 1:2:1 ratio of FMOC-Cl, borate buffer, and sample (v/v/v). The derivatization time was also studied (within 2 and 30 min). It was observed that the signal increased until 10 min, remaining constant for higher times. The derivatization procedure was carried out using the control program of autosampler (see Table 1) at 25 °C. In Figures 2 and 3, typical chromatograms were shown of maize shoot samples, where it can be observed that no matrix or derivatization reagent peaks affected the LC−FLD-ESI-MS analysis of GLYP-FMOC and AMPA-FMOC.

Mass Spectrometry Optimization. The optimization of the ESI-MS parameters and the selection of the appropriate ions were previously carried out by flow injection analysis (FIA) of the individual solutions of GLYP and AMPA derivatized with FMOC-Cl to monitor the MS intensity at which the conditions produced the greatest sensitivity for both compounds.[18] GLYP and AMPA can be ionized in both positive and negative ESI modes,[20] although they are usually analyzed in negative ion mode.[21] The greater sensitivity was obtained in the positive mode (as reported elsewhere[11,18]), so this ionization mode was selected. The optimal ESI-MS conditions operating in positive mode were set as follows: capillary voltage, 3500 V; drying gas (N_2) temperature 275 °C; drying gas (N_2) flow of 10 L/min; nebulizer pressure at 40 psi; fragmentor voltage at 70 V. Selected ion monitoring (SIM) mode was used to obtain the maximum sensitivity for quantitative analysis, and the following mass-to-charge (m/z) values were chosen for SIM analysis: 392 for quantification and 214, 170 confirmation of GLYP-FMOC, and 334 for

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quantification and 156, 112 for confirmation of AMPA-FMOC. To check how the matrix influenced the ionization, the peak areas of GLYP-FMOC and AMPA-FMOC in standard solutions were compared to those obtained in blank samples B. The recoveries of both compounds at the three concentrations assayed were close to 100% (Table 2). Hence, it was concluded that the matrix (maize) did not affect the electrospray ionization of the FMOC-derivatized analytes.

Table 2. Extraction Recoveries and Matrix Effect of GLYP and AMPA from Spiked Maize Shoot Samples (n = 6)

compound	concentration (mg/kg)	blank maize leaves A mean (%) ± SD	blank maize leaves B mean (%) ± SD
GLYP	0.30[a]	81 ± 3.8	96 ± 2.9
	0.15[b]	82 ± 4.5	98 ± 3.7
	150[a]	85 ± 5.1	97 ± 3.9
	150[b]	86 ± 5.9	101 ± 4.7
	800[a]	80 ± 4.0	95 ± 3.3
	800[b]	79 ± 4.9	97 ± 3.8
AMPA	0.70[a]	64 ± 6.0	99 ± 5.2
	0.20[b]	63 ± 6.6	100 ± 6.1
	150[a]	66 ± 5.8	98 ± 3.5
	150[b]	65 ± 6.8	97 ± 3.9
	800[a]	62 ± 3.7	96 ± 5.2
	800[b]	61 ± 4.6	99 ± 5.8

[a]FLD. [b]ESI-MS.

Validation of the Method. To assess the selectivity of the method, extracts from blank maize shoot samples were assayed, along with maize shoot samples spiked with 50 mg/kg of GLYP and 10 mg/kg of AMPA at (Figure 2) and those spiked at the LOQ levels (Figure 3). No matrix interference was evident in the FLD or ESI-MS chromatograms obtained. The LOD and LOQs values obtained for both compounds with the proposed method are listed in Table 3, and as it could be expected the LOD and LOQs values were better for ESI-MS. The recovery percentages are summarized in Table 2. It could be observed that the extraction efficacy of GLYP and AMPA with the proposed sample treatment at the three concentration levels ranged from 79% to 86% for GLYP and 61% to 67% of AMPA. Regarding the linearity studies, the graphs obtained were straight lines with an intercept not significantly ($p < 0.05$) different from zero, which confirmed the linearity through the range studied and the lack of bias; the determination coefficient values (R^2) were >0.99 (see Table 3). The intra- and interday precision and accuracy of GLYP and AMPA are presented in Table 3. The precision (%RSD) of GLYP and AMPA was for all of the cases lower than 9% for intra- and interday studies using both detectors, although it must be remarked that those %RSD were a bit higher for ESI-MS. The accuracy (%RE) of both compounds ranged from 2.1% to 6.4% for intraday and 1.2% to 9.4% for interday, respectively. Taking into account those good precision and accuracy results (%RSD and %RE values lower than 10%) obtained with the proposed method, the use of an internal standard was not required.

GLYP and AMPA Accumulation/Dissipation in Treated GM Maize Samples. The validated method was applied to quantify GLYP and AMPA in maize samples (Table 4), with it being found that the residue concentration in plant treated with 1.6 kg ae/ha was approximately twice as much as those treated with 0.8 kg ae/ha.

The time trend of fresh and dry plant weight in untreated and treated (0.8 and 1.6 kg ae/ha) GM maize is reported in Figures 4 and 5. At 7 and 14 DAT, plants treated with 0.8 and 1.6 kg ae/ha exhibited a FW significantly lower than untreated GM plants. However, 28 DAT no differences were observed between treated and untreated GM plants (Figure 4). As concerns plant DW, during the trial no significant difference of DW among treated and untreated plants was observed (Figure 5). The dissipation of GLYP in plant tissue of GM maize has been postulated due to biomass production after the treatment and the consequent progressive dilution of active ingredient in tissue.[28] With the aim to verify this postulation, a theoretical GLYP dissipation for both applied doses was calculated: the initial GLYP residue, detected at time 0, was progressively diluted according to the observed increase of plant biomass (Table 4; Figures 4 and 5). For both herbicide doses, the comparison of fitting curves calculated from observed and theoretical GLYP residues in plant tissue is reported in Figure

Table 3. Method Validation Parameters and Calibration Curve Data for GLYP and AMPA Determination in Maize Shoot Samples

		GLYP		AMPA	
validation parameter		FLD	ESI-MS	FLD	ESI-MS
intraday precision (%RSD)	low	5.8	6.6	4.8	5.8
	medium	3.9	5.4	6.6	7.2
	high	3.5	4.1	5.5	6.5
interday precision (%RSD)	low	4.7	7.5	6.7	7.1
	medium	4.4	6.7	5.3	6.2
	high	−5.3	−5.6	7.5	8.3
intraday accuracy (%RE)	low	2.1	2.9	4.3	5.1
	medium	−3.2	−4.1	5.8	6.4
	high	3.6	4.3	3.4	4.2
interday accuracy (%RE)	low	1.2	1.8	−8.6	−9.4
	medium	−4.0	−5.1	4.1	4.8
	high	2.8	4.1	−5.0	−5.7
LOD (mg/kg)		0.04	0.01	0.07	0.02
LOQ (mg/kg)		0.13	0.04	0.24	0.06
linear range (mg/kg)		0.13−1000	0.04−1000	0.24−1000	0.06−1000
correlation coefficient (R^2)		0.997	0.999	0.999	0.998

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Table 4. GLYP and AMPA Content (μg ae/kg dry weight ± standard deviation (SD)) Determined by LC−ESI-MS in Shoot Samples from GM Maize Treated with GLYP (0.8 and 1.6 kg ae/ha)[a]

	0.8 kg ae/ha			1.6 kg ae/ha		
DAT	GLYP	AMPA	ratio	GLYP	AMPA	ratio
0	4112 ± 699	73 ± 15	56	8910 ± 1247	155 ± 12	57
7	1624 ± 244	35 ± 3	46	2595 ± 130	40 ± 5	65
14	779 ± 93	315 ± 57	2	1099 ± 88	149 ± 27	7
28	77 ± 17	4 ± 1	19	81 ± 10	10 ± 2	8
42	12 ± 2	3 ± 1	4	50 ± 12	3 ± 1	17
56	<LOD	<LOD		9 ± 2	<LOD	

[a]DAT = days after treatment.

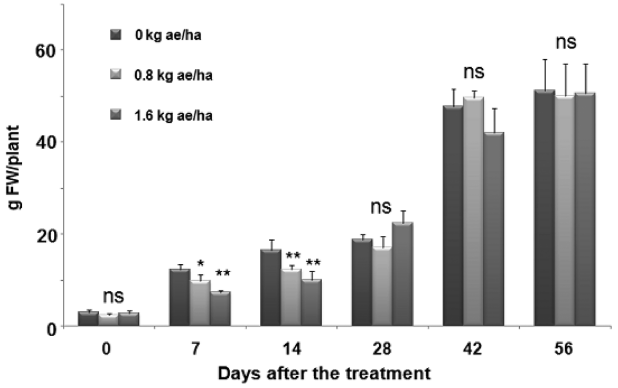

Figure 4. Shoot (leaves and stem) fresh weight (FW) of GM maize untreated and sprayed with 0.8 and 1.6 kg ae/ha as a function of the days after the treatment. Above the bars, the statistical difference of 0.8 and 1.6 kg ae/ha treated plants as compared to untreated control is reported (ns = not significant; * = significant different at $P < 0.05$; ** = significant different at $P < 0.01$).

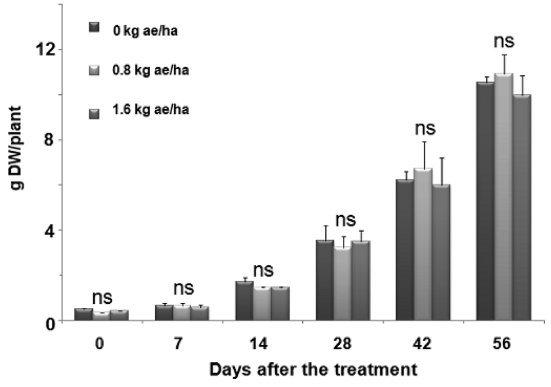

Figure 5. Shoot (leaves and stem) dry weight (DW) of GM maize untreated and sprayed with 0.8 and 1.6 kg ae/ha as a function of the days after the treatment. Above the bars, the statistical difference of 0.8 and 1.6 kg ae/ha treated plants as compared to untreated control is reported (ns = not significant; * = significant different at $P < 0.05$; ** = significant different at $P < 0.01$).

6. At both doses, the dissipation half time of GLYP, determined from theoretical values, was not significantly different from that determined from observed values: at 0.8 kg ae/ha, it was equal to 3.84 ± 1.51 days (at $P < 0.05$ upper and lower confidence limit of 6.03 and 1.56 days, respectively), while at 1.6 kg ae/ha, it was equal to 3.96 ± 0.78 days (at $P < 0.05$ upper and lower confidence limit of 6.36 and 1.65 days, respectively).

Finally, to validate the ESI-MS analytical method, in a parallel trial the total GLYP residues in shoot and root from GM maize

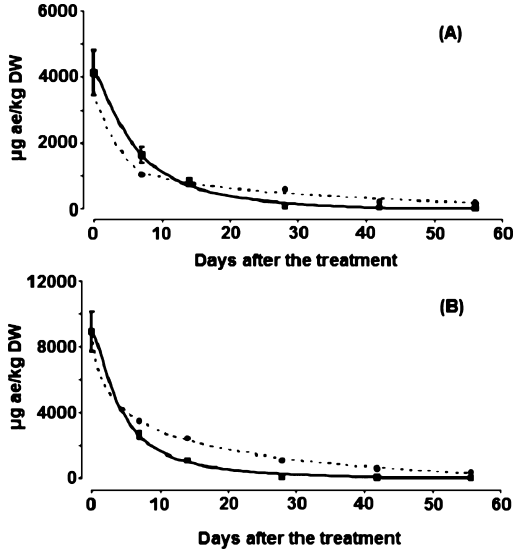

Figure 6. Observed (■) and theoretical (●) GLYP residues (mean ± SD) in shoots (leaves and stem) from (A) GM maize plants treated with 0.8 and (B) 1.6 kg ae/ha. The theoretical values were calculated from the observed GLYP concentration at 0 DAT by the progressive dilution according to the sole increase of plant biomass. On the basis of a symmetric logistic model, the fitting curves of observed and theoretical values are represented by solid and dotted lines, respectively.

treated with radiolabel herbicide (at the doses of 0.8 and 1.6 kg ae/ha) were determined by liquid scintillation (Table 5). Except for the determinations at 42 and 56 DAT with 0.8 kg ae/ha and those at 28 and 56 DAT with 1.6 kg ae/ha, no statistical difference was observed between ESI-MS chromatography and liquid scintillation quantification of total GLYP residues. In addition, the GLYP residue dissipation trend in maize shoot tissue determined by ESI-MS matched that determined by liquid scintillation (Tables 4 and 5).

■ DISCUSSION

In this work, a fast and simple LC−FLD-ESI-MS method has been developed to determine GLYP and AMPA, which were online derivatized with FMOC-Cl before passing through the column, in maize plants. As it has been previously commented, the chromatographic conditions were quite similar, but not equal, to those one employed in a previous work where those compounds were analyzed in rat plasma.[18] However, the derivatization step was different because of matrix, which was totally different from rat plasma, and derivatization reagent peaks that interfered with the detection of the FMOC-derivatized analytes. To solve these problems, the derivatization

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Table 5. Total GLYP Residues (μg ae/kg dry weight ± SD) Determined by Liquid Scintillation in Shoot (Leaves and Stem) and Root Samples from GM Maize Treated with Radiolabeled GLYP (0.8 and 1.6 kg ae/ha)[a]

	0.8 kg ae/ha		1.6 kg ae/ha	
DAT	shoot	root	shoot	root
0	4456 ± 356 (ns)	nd	9323 ± 746 (ns)	nd
7	1976 ± 246 (ns)	89 ± 13	2722 ± 327 (ns)	405 ± 53
14	800 ± 144 (ns)	124 ± 24	1164 ± 175 (ns)	978 ± 156
28	50 ± 10 (ns)	434 ± 100	223 ± 40 (**)	657 ± 125
42	45 ± 6 (**)	458 ± 160	75 ± 11 (ns)	455 ± 105
56	15 ± 4 (**)	377 ± 83	22 ± 5 (**)	356 ± 96

[a]In brackets is reported the statistical difference as compared to LC−ESI-MS determination (GLYP + AMPA residues) . ns = not significant; * = significantly different at $P < 0.05$; ** = significantly different at $P < 0.01$. DAT = days after treatment. nd = not detected.

step was optimized. A new sample treatment was developed and optimized. It must be pointed out that to our knowledge no studies are actually available on GLYP or AMPA determination by LC in maize shoots (leaves and stem). Taking into account the simple extraction procedure employed, the results were good enough. No relevant differences were found in the recovery percentages at the different concentrations. The LODs and LOQs of the analytes were low enough to measure dissipation of GLYP and its main metabolite AMPA in maize plant tissue samples. The FLD results were worst in comparison with ESI-MS, but they are good enough for making FLD an economic alternative for experiments in which such high sensitivity is not required. It must be also commented that the limits reached with both detectors are very useful if the MRL established by the U.S. Environmental Protection Agency is taken into account,[25] 13 mg/kg of GLYP and its metabolites; *codex alimentarius*,[26] 5 mg/kg of GLYP and AMPA; and SANCO (Santé et Consommateurs, Directorate General Health and Consumers; European Commission; Brussels, Belgium) pesticide document,[27] 1 mg/kg. Moreover, when comparing the LODs and LOQs with previous published methods, where GLYP was determined in cereals by LC−MS/MS,[21] LOD 0.02 mg/kg, or when GLYP and AMPA were determined in soybean employing again LC−MS/MS[20] with LODs and LOQs of 0.09 and 0.30 mg/kg for GLYP and 0.1 and 0.034 mg/kg for AMPA, respectively, the sensitivity obtained with the proposed method is higher for ESI-MS, and the results provided by FLD are slightly lower[20] or higher[21] than the MS/MS values. However, it is not possible to make a true comparison between those data as the matrices studied were different. The analytical characteristics of the proposed method confirmed the linearity through the range studied and the lack of bias; meanwhile, the accuracy, intra-, and interday precision results indicated that the proposed method had a good precision and accuracy. As it could be expected, those results were slightly better for FLD than for ESI-MS, but in both cases were satisfactory.

In relation to the analysis of the maize plant samples, it can be stated after examining the results showed in Table 4 that the content of GLYP in maize shoot samples from treated GM crops was dependent on the applied dose. However, the dissipation half time of GLYP was not significantly different as a function of herbicide dose: at 0.8 kg ae/ha, it was equal to 5.39 ± 0.51 days (at $P < 0.05$ upper and lower confidence limit of 7.63 and 3.16 days, respectively), while at 1.6 kg ae/ha, it was equal to 4.02 ± 0.48 days (at $P < 0.05$ upper and lower confidence limit of 6.12 and 1.92 days, respectively). Even if the comparison with available literature is not easily achievable due to different experimental conditions and plant material, the GLYP shoot residue level observed in this research was comparable with previous studies.[28] Seven days after the spray of 0.093 kg ae/ha, the mean content of GLYP in GLYP-resistant maize shoot was 308 ± 208 μg ae/kg DW;[28] in the present study, 7 DAT the observed GLYP level was 1 magnitude order higher in view of 10−20-fold higher spray doses (Table 4). The GLYP residue in the fodder from resistant maize was reported to be ranging between 1800 and 41000 μg ae/kg DW at 7 days after the treatment with 2.5 kg ae/ha (in the present investigation, 1624 and 2595 μg ae/kg DW 7 DAT with 0.8 and 1.6 kg ae/ha, respectively), while the GLYP residues in the maize forage were 50−520 μg ae/kg DW, respectively, 48−65 days after the treatment with 2.5 kg ae/ha (in the present investigation, 12 and 50 μg ae/kg DW 42 DAT with 0.8 and 1.6 kg ae/ha, respectively) (see Table 5).[34]

The data related to the time trend of fresh and dry plant weight in untreated and treated GM maize (Figures 4 and 5) suggested that the elongation growth of treated GM plants within 2 weeks after the treatment is reduced as compared to untreated GM plants, as evidenced by the reduced FW. However, this effect was transient as after 2 weeks the treated plants recovered. In contrast, the GLYP treatment did not affect the proliferation growth of treated GM plants, as evidenced by the trend of plant DW that was not statistically different between treated and untreated plants. It is intriguing that the significant reduction of FW was observed within 14 DAT, corresponding to the maximum content of AMPA residues in plant tissues (Table 4). The investigated GM maize is not affected by GLYP as possessing insensitive EPSPS enzyme. In contrast, AMPA is phytotoxic to both GM and no GM plants, and its mode of action is apparently different from that of GLYP.[35] Unexpectedly, at both doses, the contribution of AMPA to GLYP dissipation in maize shoot tissue appeared of limited extent (Table 4). The studied GM maize (MON832) contains CP4 EPSPS (from the soil bacterium *Agrobacterium* sp. strain CP4) and GLYP oxidoreductase (GOX, from *Achromobacter* sp. strain LBAA) genes.[36] The CP4 EPSPS protein expressed by the relative gene is highly tolerant to inhibition by GLYP, while the GOX enzyme accelerates the normal degradation of GLYP into AMPA.[36] In the present investigation, the GOX activity appeared independent from the herbicide dose (Table 4). At both doses, the AMPA content and GLYP/AMPA ratio followed a similar trend with a maximum GOX activity at 14 DAT. The irregular GLYP/AMPA ratio along the time after the treatment suggested an up-regulated GOX enzyme activity as a function of plant growth stage.

The analysis of the information reported in Figure 6 suggested that in maize plant tissues the GLYP dissipation is

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mainly due to the progressive dilution effect after herbicide treatment. However, a discrepancy in GLYP content between observed and theoretical fitting curves was observed between 28 and 56 DAT and between 14 and 56 DAT for the treatments at 0.8 and 1.6 kg ae/ha, respectively (Figure 6): the GLYP residues detected in maize plant tissue were lower than those expected according to the simple dilution effect. GLYP is an amphimobile herbicide, translocated into roots, from which it can be exuded into the soil:[36] in the present investigation, the ESI-MS chromatography determinations were carried out only on maize shoot tissue, and the dissipation contribution of root translocation/exudation could not be ascertained.

Finally, it could be postulated that the GLYP residue dissipation trend in maize shoot obtained using ESI-MS was comparable with those determined by LSS. However, it must be mentioned that the proposed LC−ESI-MS method permitted one to distinguish between GLYP and AMPA and the use of radiolabeled GLYP was not required; meanwhile, the LSS had the advantage of being more economic than the LC−ESI-MS system, and at the same time the consumption of reagents and solvents was lower.

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Notes

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ABBREVIATIONS USED

GLYP, glyphosate; AMPA, aminomethylphosphonic acid; FMOC-Cl, 9-fluorenylmethylchloroformate; GLYP-FMOC and AMPA-FMOC, FMOC-derivatized analytes; GM, genetically modified; LSS, liquid scintillation spectroscopy; DAT, days after treatment; FW, fresh weight; DW, dry weight; QC, quality control; SIM, selected ion monitoring; λ_{ex}, excitation wavelength; λ_{em}, emission wavelength; FIA, flow injection analysis; ae/ha, acid equivalent/hectare; GOX, GLYP oxidoreductase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthetase; DT_{50}, half time dissipation; kBq, kilobecquerel; RCB, randomized complete block; SANCO, Santé et Consommateurs; MRLs, maximum residue levels; MW, molecular weight; LU, luminescence units

REFERENCES

(1) Clive, J. *Global Status of Commercialized Biotech/GM Crops:2008.ISAAA brief No 39*; ISAAA: Ithaca, NY, 2008.

(2) Stalikas, C. D.; Konidari, C. N. Analytical methods to determine phosphonic and amino acid group-containing pesticides. *J. Chromatogr., A* **2001**, *907*, 1−19.

(3) Tadeo, J. L.; Sánchez-Brunete, C.; Pérez, R. A.; Fernández, M. D. Analysis of herbicide residues in cereals, fruits and vegetables. *J. Chromatogr., A* **2000**, *882*, 175−191.

(4) Alferness, P. L.; Iwata, Y. Determination of glyphosate and AMPA in soil, plant and animal matrices, and water by capillary gas chromatography with mass-selective detection. *J. Agric. Food Chem.* **1994**, *42*, 2751−2759.

(5) Royer, A.; Beguin, S.; Tabet, J. C.; Hulot, S.; Reding, M. A.; Communal, P. Y. Determination of glyphosate and aminomethylphosphonic acid residues in water by gas chromatography with tandem mass spectrometry after exchange ion resin purification and derivatization. Application on vegetable matrixes. *Anal. Chem.* **2000**, *72*, 3826−3832.

(6) Tekel, J.; Hatrík, S. Pesticide residue analyses in plant material by chromatographic methods: clean-up procedures and selective detectors. *J. Chromatogr., A* **1996**, *754*, 397−410.

(7) Roseboom, H.; Berkhoff, C. J. Determination of the herbicide glyphosate and its major metabolite aminomethylphosphonic acid by high-performance liquid chromatography after fluorescence labelling. *Anal. Chim. Acta* **1982**, *135*, 373−377.

(8) Hogendoom, E. A.; Ossendrijver, F. M.; Dijkman, E.; Baumann, R. A. Rapid determination of glyphosate in cereal samples by means of pre-column derivatisation with 9-fluorenylmethyl chloroformate and coupled-column liquid chromatography with fluorescence detection. *J. Chromatogr., A* **1999**, *833*, 67−73.

(9) Li, B.; Deng, X.; Guo, D.; Jin, S. Determination of glyphosate and aminomethylphosphonic acid residues in foods using high performance liquid chromatography-mass spectrometry/mass spectrometry. *Chin. J. Chromatogr.* **2007**, *25*, 486−490.

(10) Hernández, F.; Hidalgo, C.; Sancho, J. V. Determination of glyphosate residues in plants by precolumn derivatization and coupled-column liquid chromatography with fluorescence detection. *J. AOAC Int.* **2000**, *83*, 728−734.

(11) Ibáñez, M.; Pozo, O. J.; Sancho, J. V.; López, F. J.; Hernández, F. Residue determination of glyphosate, glufosinate and aminomethylphosphonic acid in water and soil samples by liquid chromatography coupled to electrospray tandem mass spectrometry. *J. Chromatogr., A* **2005**, *1081*, 145−155.

(12) Arregui, M. C.; Lenardón, A.; Sanchez, D.; Maitre, M. I.; Scotta, R.; Enrique, S. Monitoring glyphosate residues in transgenic glyphosate-resistant soybean. *Pest Manage. Sci.* **2004**, *60*, 163−166.

(13) García de Llasera, M. P.; Gómez-Almaraz, L.; Vera-Avila, L. E.; Peña-Alvarez, A. Matrix solid-phase dispersion extraction and determination by high-performance liquid chromatography with fluorescence detection of residues of glyphosate and aminomethylphosphonic acid in tomato fruit. *J. Chromatogr., A* **2005**, *1093*, 139−146.

(14) Guinivan, R. A.; Thompson, N. P.; Wheeler, W. B. Derivatization and cleanup improvements in determination of residues of glyphosate and aminomethylphosphonic acid in blueberries. *J. AOAC Int.* **1982**, *65*, 35−39.

(15) Khrolenko, M. V.; Wieczorek, P. P. Determination of glyphosate and its metabolite aminomethylphosphonic acid in fruit juices using supported-liquid membrane preconcentration method with high-performance liquid chromatography and UV detection after derivatization with p-toluenesulphonyl chloride. *J. Chromatogr., A* **2005**, *1093*, 111−117.

(16) Veiga, F.; Zapata, J. M.; Fernandez Marcos, M. L.; Alvarez, E. Dynamics of glyphosate and aminomethylphosphonic acid in a forest soil in Galicia, north-west Spain. *Sci. Total Environ.* **2001**, *271*, 135−144.

(17) Miles, C. J.; Wallace, L. R.; Moye, H. A. Determination of glyphosate herbicide and (aminomethyl)phosphonic acid in natural waters by liquid chromatography using pre-column fluorogenic labelling with 9-fluorenylmethyl chloroformate. *J. AOAC Int.* **1986**, *69*, 458−461.

(18) Bernal, J.; Bernal, J. L.; Martín, M. T.; Nozal, M. J.; Anadón, A.; Martínez-Larrañaga, M. R.; Martínez, M. A. Development and validation of a liquid chromatography−fluorescence−mass spectrometry method to measure glyphosate and aminomethylphosphonic acid in rat plasma. *J. Chromatogr., B* **2010**, *878*, 3290−3296.

(19) Druart, C.; Delhomme, O.; de Vaufleury, A.; Ntcho, E.; Millet, M. Optimization of extraction procedure and chromatographic separation of glyphosate, glufosinate and aminomethylphosphonic acid in soil. *Anal. Bioanal. Chem.* **2011**, *399*, 1725−1732.
(20) Martins-Júnior, H. A.; Lebre, D. T.; Wang, A. Y.; Pires, M. A. F.; Bustillos, O. V. An alternative and fast method for determination of glyphosate and aminomethylphosphonic acid (AMPA) residues in soybean using liquid chromatography coupled with tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 1029−1034.
(21) Granby, K.; Johannesen, S.; Vahl, M. Analysis of glyphosate residues in cereals using liquid chromatography-mass spectrometry. *Food Addit. Contam.* **2003**, *20*, 692−698.
(22) Chen, Z.; He, W.; Beer, M.; Megharaj, M.; Naidu, R. Speciation of glyphosate, phosphate and aminomethylphosphonic acid in soil extracts by ion chromatography with inductively coupled plasma mass spectrometry with an octopole reaction system. *Talanta* **2009**, *78*, 852−856.
(23) Yang, G.; Xu, Q.; Shen, M.; Wang, W.; Xu, L.; Chen, G.; Fu, F. Determination of organophosphorus pesticides by capillary electrophoresis inductively coupled plasma mass spectrometry with collective sample introduction technique. *Electrophoresis* **2009**, *30*, 1718−1723.
(24) Rasul Jan, M.; Shah, J.; Muhammad, M.; Ara, B. Glyphosate herbicide residue determination in samples of environmental importance using spectrophotometric method. *J. Hazard. Mater.* **2009**, *169*, 742−745.
(25) http://www.federalregister.gov/articles/2011/05/11/2011-11205/glyphosate-pesticide-tolerance (accessed: 3/23/2012).
(26) http://www.codexalimentarius.net/pestres/data/pesticides/details.html?id=158 (accessed: 3/23/2012).
(27) http://ec.europa.eu/sanco_pesticides/public/ (accessed 3/23/2012).
(28) Reddy, K. N.; Rimando, A. M.; Duke, S. O.; Nandula, V. K. Aminomethylphosphonic acid accumulation in plant species treated with glyphosate. *J. Agric. Food Chem.* **2008**, *56*, 2125−2130.
(29) Dinelli, G.; Marotti, I.; Bonetti, A.; Catizone, P.; Urbano, J. M.; Barnes, J. Physiological and molecular bases of glyphosate resistance in *Conyza bonariensis* biotypes from Spain. *Weed Res.* **2008**, *48*, 257−265.
(30) Onofri, A. Bioassay97: a new Excel VBA macro to perform statistical analyses on herbicide dose-response data. *Ital. J. Agrometeorol.* **2005**, *3*, 40−45.
(31) International Conference on Harmonization Tripartite Guideline, ICH Topic Q2, Validation of Analytical Procedures: Text and Methodology, Geneva (2005), http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html (accessed: 3/23/2012).
(32) Tompson, M.; Ellison, S. L.; Wood, R. Harmonized guidelines for single laboratory validation of methods of analysis (IUPAC Technical Report). *Pure Appl. Chem.* **2002**, *74*, 835−855.
(33) Document no. SANCO/825/00 rev.8.1 (16.11.2010), European Commission. Directorate General Health and Consumer Protection. Guidance document on residue analytical methods (accessed: 3/23/2012).
(34) George, C. Nature of glyphosate residues in corn plants which are tolerant to Roundup herbicide; Monsanto Co.: U.S., 1995; Report MSL-14018 (unpublished).
(35) Reddy, K. N.; Rimando, A. M.; Duke, S. O. Aminomethylphosphonic acid, a metabolite of glyphosate, causes injury in glyphosate-treated, glyphosate-resistant soybean. *J. Agric. Food Chem.* **2004**, *52*, 5139−5143.
(36) Kremer, R. J.; Means, N. E.; Kim, S. Glyphosate affects soybean root exudation and rhizosphere microorganisms. *Int. J. Environ. Anal. Chem.* **2005**, *85*, 1165−1174.