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Residue determination of glyphosate, glufosinate and aminomethylphosphonic acid in water and soil samples by liquid chromatography coupled to electrospray tandem mass spectrometry

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Abstract

This paper describes a method for the sensitive and selective determination of glyphosate, glufosinate and aminomethylphosphonic acid (AMPA) residues in water and soil samples. The method involves a derivatization step with 9-fluorenylmethylchloroformate (FMOC) in borate buffer and detection based on liquid chromatography coupled to electrospray tandem mass spectrometry (LC–ESI-MS/MS). In the case of water samples a volume of 10 mL was derivatized and then 4.3 mL of the derivatized mixture was directly injected in an on-line solid phase extraction (SPE)–LC–MS/MS system using an OASIS HLB cartridge column and a Discovery chromatographic column. Soil samples were firstly extracted with potassium hydroxide. After that, the aqueous extract was 10-fold diluted with water and 2 mL were derivatized. Then, 50 μL of the derivatized 10-fold diluted extract were injected into the LC–MS/MS system without pre-concentration into the SPE cartridge. The method has been validated in both ground and surface water by recovery studies with samples spiked at 50 and 500 ng/L, and also in soil samples, spiked at 0.05 and 0.5 mg/kg. In water samples, the mean recovery values ranged from 89 to 106% for glyphosate (RSD < 9%), from 97 to 116% for AMPA (RSD < 10%), and from 72 to 88% in the case of glufosinate (RSD < 12%). Regarding soil samples, the mean recovery values ranged from 90 to 92% for glyphosate (RSD < 7%), from 88 to 89% for AMPA (RSD < 5%) and from 83 to 86% for glufosinate (RSD < 6%). Limits of quantification for all the three compounds were 50 ng/L and 0.05 mg/kg in water and soil, respectively, with limits of detection as low as 5 ng/L, in water, and 5 μg/kg, in soil. The use of labelled glyphosate as internal standard allowed improving the recovery and precision for glyphosate and AMPA, while it was not efficient for glufosinate, that was quantified by external standards calibration. The method developed has been applied to the determination of these compounds in real water and soil samples from different areas. All the detections were confirmed by acquiring two transitions for each compound.
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Keywords: Glyphosate; Glufosinate; AMPA; Water; Soil; Liquid chromatography; Electrospray interface; Tandem mass spectrometry; Derivatization

1. Introduction

Glyphosate [*N*-(phosphonomethyl)glycine] and glufosinate [ammonium DL-homoalanin-4-(methyl) phosphinate] are broad spectrum, nonselective, post-emergence herbicides extensively used in various applications for weed control in aquatic systems and vegetation control in non-crop areas. Aminomethylphosphonic acid (AMPA) is the major degradation product of glyphosate found in plants, water and soil [1]. Chemical structures of these phosphorus-containing herbicides are given in Fig. 1.

Due to the extensive worldwide use of these compounds and the restrictive regulations for water in the European Union, very sensitive methods for the determination of pesticide residues are required. However, the determination of these two herbicides at the sub μg/L level is difficult due to their ionic character, low volatility, low mass and lack of chemical groups that could facilitate their detection. Even more difficult can result the residue determination in soil at low concentration levels (e.g. below 0.1 mg/kg), due to the complexity of this matrix sample. Most methods developed

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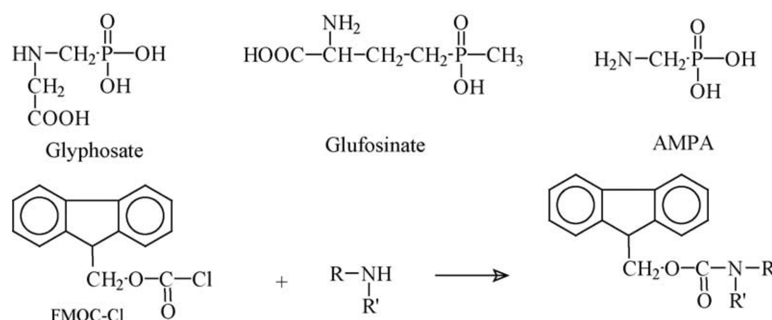

Fig. 1. Chemical structures of glyphosate, AMPA and glufosinate, and derivatization reaction with FMOC. R: H or alkyl group.

until now require derivatization procedures to enable analysis by gas chromatography (GC) or high-performance liquid chromatography (HPLC). GC/MS methods involved derivatization with different reagents [2–8] to confer volatility to the analytes. Normally, there is quite a lot of sample manipulation, and the methods are time-consuming and tedious.

Physicochemical characteristics of these compounds fit better with LC analysis, although the lack of adequate chemical groups (e.g. chromophores, UV absorption, fluorogenics) hamper their measurement by conventional detectors. For these reasons, both pre-column and post-column derivatization procedures have been employed. Pre-column procedures are based mainly on derivatization with 9-fluorenylmethyl chloroformate (FMOC) [9–15] to form fluorescent derivatives (improve detection) and/or to reduce the polar character of the analytes facilitating the chromatographic retention. In post-column procedures, the most common reaction is with o-phthalaldehyde (OPA) and mercaptoethanol [16] or with OPA and *N*,*N*-dimethyl-2-mercaptoethylamine [17]. Normally, HPLC has been used in combination with fluorescence detection after derivatization [11–17], although in a few cases glyphosate has been determined directly by ion chromatography (IC) with UV detection [18] or suppressed conductivity detection [19], but with limited sensitivity. The potential of capillary electrophoresis combined with mass spectrometry [20] and with indirect fluorescence detection [21] has also been explored, although the lack of sensitivity and/or selectivity of these techniques together with the difficulty for preconcentrating the analytes, limited their application in the field of residues.

In our research group, we have developed efficient and selective methods based on the use of coupled-column liquid chromatography (LC–LC), which was proved to be an excellent way of minimizing sample treatment and improving sensitivity in a variety of sample matrices, as water, soil, fruit and vegetables [11,13–15,22]. However, the use of conventional fluorescent detection limited the sensitivity required in pesticide residue analysis, and also hampered the unequivocal confirmation of the residues detected, which nowadays is widely accepted that has to be reached by MS techniques. Searching a method that could satisfy the requirements of sensitivity and selectivity, and unequivocal confirmation of glyphosate in water, the use of MS spectrometric techniques in combination with LC has been investigated by several groups. Thus, IC has been applied, due the ionic character of this analyte, coupled to MS with electrospray interface [23], while RPLC has been used in combination with ICP-MS with P detection [24]. However, the sensitivity reached with these techniques was not sufficient. Lee et al [9] obtained better results with the combination LC–MS. In this case, the molecular ions of the derivatized glyphosate, AMPA and glufosinate, as well as a fragment ion of each compound, were monitored in negative ionisation mode obtaining detection limits around 0.1 μg/L. The use of isotope-labelled glyphosate as internal standard minimised derivatization variations and matrix effects. However, although MS based methods could be considered as highly selective methods, the occurrence of false positives might be still possible mainly in the analysis of relatively dirty samples, as some interferences can share the same MS properties as the analyte. This may also occur in water sample analysis as it has been reported in some papers, producing constructive discussions on this subject [25].

The improved sensitivity and selectivity of tandem MS make this technique ideal for the trace level determination of polar and/or ionic pesticides in water by LC–MS/MS methods, as it has been proved in our laboratory [26–27]. This technique was also applied several years ago to the determination of glyphosate and AMPA in water [10], although considerable variation was observed caused by irreproducibility in derivatization and fragmentation. 4-mL volume was passed through the SPE cartridge, claiming detection limits for glyphosate and AMPA around 0.03 μg/L.

When dealing with more complex matrices, such as soil samples, an important loss in the sensitivity can occur a consequence of the ionisation suppression from the co-extracted components of the matrix, hampering correct quantification. This matrix-effect depends on the analyte-sample combination. Different approaches have been used either to minimize or to correct the matrix effect, such as increasing the sample pretreatment, performing matrix-matched calibration, using an isotope labelled standard or simply diluting the sample [28]. Thus, the labeled glyphosate has been used as internal standard for the LC–MS determination of this herbicide [9].

Confirmation of the identity of residues in unknown samples is of utmost importance in order to avoid reporting false

positives. Recently, the European Union has adopted the concept of identification points (IPs) as quality criterium for the confirmation of contaminant residues [29]. For compounds with an established MRL, a minimum of three IPs is required for satisfactory confirmation of the compound identity. When LC–MS/MS technique is used, the monitoring of two MS/MS transitions, e.g. using one precursor ion and two product ions, allows to earn four IPs, fulfilling the requirements of this criterium [25].

The aim of this paper is to develop a rapid and robust method for the determination of low concentrations of glyphosate, its principal degradation product, AMPA, and glufosinate in water and soil by SPE–LC–ESI-MS/MS, that fulfil the requirements of excellent sensitivity and unequivocal confirmation of the residues detected according to the European Union guidelines. Following the most widely accepted criteria, four IPs will be achieved, thus avoiding the possibility of reporting false positives.

2. Experimental

2.1. Chemicals

Glyphosate (98%), glufosinate (99%) and AMPA (99%) reference standards were purchased from Dr Ehrenstorfer (Augsburg, Germany), Riedel-de-Häen (Seelze, Germany) and Sigma (St Louis, MO, USA), respectively. Isotope-labeled glyphosate (1,2-^{13}C, ^{15}N), used as surrogate internal standard (IS), was purchased from Dr Ehrenstorfer. Analytical reagent-grade disodium tetraborate decahydrate was obtained from Scharlab (Barcelona, Spain) and 9-fluorenylmethylchloroformate (FMOC-Cl) was purchased from Sigma. Reagent-grade hydrochloric acid, formic acid, potassium hydroxide (KOH), acetic acid (HAc) and ammonium acetate (NH_4Ac) as well as LC-grade acetonitrile were purchased from Scharlab. LC-grade water was obtained by purifying demineralised water in a Nanopure II system (Barnstead Newton, MA, USA).

Standard stock solutions were prepared dissolving approximately 50 mg powder, accurately weighted, in 100 mL of water obtaining a final concentration of approximately 500 mg/L. A 50-mg/L composite standard was prepared in water by mixing and diluting the individual standard stock solutions. Standard working solutions for the LC–MS/MS analysis and for fortification of samples were prepared by dilution of the 50-mg/L composite standard with water. All standard solutions were stored in nonsilanized glass.

The isotope-labeled glyphosate was purchased as 1.1 mL of 100-μg/mL stock solution in water. A 11-μg/mL standard solution was prepared by dissolving 1.1 mL of the stock solution in 10 mL of water. Standard working solutions were prepared by diluting the intermediate standard solution with water.

Solutions of 5% borate buffer (pH approximately 9) in HPLC-grade water and solutions containing 12,000 mg/L of FMOC-Cl in acetonitrile were used for the derivatization step prior to the analysis.

2.2. Instrumentation

For the analysis of water samples, the mass spectrometer was interfaced to a LC system based on a 233XL autosampler with a loop of 4.3 mL (Gilson, Villiers-le-Bel, France) and 2 pumps: an Agilent 1100 (Agilent, Waldbron, Germany) binary pump used to condition and wash the cartridge (P-1) and a Waters Alliance 2695 (Waters, Milford, MA, USA) quaternary pump used for the chomatographic separation (P-2), as can be seen elsewhere [24]. The SPE preconcentration was performed using an Oasis HLB cartridge, 20 mm × 2.1 mm i.d. (Waters), as C-1. For the LC separation, a Discovery column C_{18}, 5 μm 50 × 2.0 mm i.d. (Supelco, Bellefonte, PA, USA), was used as C-2. Mobile phase consisted of water pH 2.5 (adjusted with formic acid) in P-1, and mixtures of aqueous 5 mM acetic acid/ammonium acetate (pH 4.8) water and acetonitrile in P-2.

For the analysis of soil samples, the mass spectrometer was directly interfaced to the Waters Alliance 2695 (Waters) quaternary pump. The mobile phases and the column used were the same as in the case of water samples.

A Quattro LC (quadrupole-hexapole-quadrupole) mass spectrometer (Micromass, Manchester, UK) with an orthogonal Z-spray-electrospray interface was used. Drying gas as well as nebulising gas was nitrogen, generated from pressurized air in a NG-7 nitrogen generator (Aquilo, Etten-Leur, NL). The nebuliser gas flow was set to approximately 80 L/h and the desolvation gas flow to 800–900 L/h. Datastation operating software was MassLynx v4.0.

For operation in MS/MS mode, collision gas was Argon 99.995% (Carburos Metalicos, Valencia, Spain) with a pressure of approximately 1×10^{-3} mbar in the collision cell. Capillary voltage of 3.5 kV was used in positive ionization mode. The interface temperature was set to 350 °C and the source temperature to 120 °C. Dwell times of 0.17 s/scan were chosen.

2.3. SPE procedure

The conditioning of the Oasis cartridge was performed with LC-grade water at pH 2.5 at a flow-rate of 1 mL/min for 7 min. An aliquot of 4.3 mL of water sample was preconcentrated (1 mL/min) into the cartridge and washed with acidified LC-grade water during 4 min. After washing, the sample was transferred in backflush mode to the C-2 column and a gradient in P-2 started.

2.4. LC procedure

To perform the chromatographic separation, the gradient used in P-2 was water 5 mM HAc/NH_4Ac (pH 4.8)–acetonitrile, where the percentage of organic modifier was changed as follows: 0 min, 10%; 5 min, 10%; 5.1 min,

90%; 9 min, 90%; 9.1 min, 10%; 14 min, 10%. The chromatographic separations were completed within 20 min.

2.5. Sample procedure

The derivatization procedure was based on Sancho et al. [14,15] (see Fig. 1), with slight modifications.

2.5.1. Water samples

Ground and surface water samples were collected in plastic bottles from different sites of the Valencian Mediterranean region and stored in a freezer at −18 °C until analysis. Ten millilitre of water sample was introduced into a glass tube together with 100 μL of isotope-labeled glyphosate standard (110 μg/L). Samples were derivatised by adding 0.6 mL of 5% borate buffer (pH 9) followed by 0.6 mL of FMOC-Cl reagent (12000 mg/L), and allowing the reaction to take place overnight at room temperature. After that, samples were filtered through a 0.45 μm syringe filter and acidified with hydrochloric acid until pH 1.5. Finally, 4.3 mL of the acidified derivatized samples were directly injected into the SPE–LC–ESI-MS/MS system.

Fortification of surface or ground waters for recovery experiments was performed by adding 1 mL of 5 or 50 ng/mL mixture solutions to 100 mL of blank water sample in order to yield fortification levels of 50 or 500 ng/L, respectively.

2.5.2. Soil samples

Soil samples was collected from a public garden, suspected to have been contaminated by glyphosate. Air-dried soil samples were homogenized and 5.0 g subsamples were transferred to centrifuge tubes (50 mL). Samples were extracted by shaking with 0.6 M KOH (10 mL) on a mechanical shaker for 30 min, and then centrifuged at 3500 rpm for 30 min. The alkaline sample extracted was separated and neutralized by adding drops of HCl 6 M and 0.6 M until pH 7, approximately. After that, the neutralized supernatant was 10-fold diluted with HPLC-grade water. The derivatization step was performed as follows: 2-mL of the 10-fold diluted supernatant was pipetted into a glass tube together with 120 μL of the labelled internal standard (1.10 mg/L), 120 μL of 5% borate buffer (pH 9) and 120 μL of FMOC-Cl reagent (12000 mg/L). The tube was swirled and left overnight at room temperature. After that, samples were filtered through a 0.45 μm syringe filter and acidified with hydrochloric acid until pH 1.5. Finally, 50 μL of the acidified derivatized extract was directly injected into the LC–ESI-MS/MS system.

Fortification of soil samples for recovery experiments was performed by adding 1 mL of 250 ng/mL or 2500 ng/mL mixture solutions to 5.0 g of blank soil sample in order to yield fortification levels of 0.05 mg/kg or 0.5 mg/kg, respectively. Samples were equilibrated for 1 h prior to extraction.

AMPA and glyphosate were quantified using isotope labelled glyphosate as internal standard, in both water and soil samples. In the case of glufosinate, quantification was performed with external calibration.

2.6. Validation study

Linearity of the method was evaluated analysing eight standard solutions by duplicate, in the range 25–5000 ng/L for water samples, and in the range 1–500 μg/L for soil extracts.

Precision (repeatability, expressed as relative standard deviation, in %) and recoveries were determined within day by analysing fortified blank samples in quintuplicate. This experiment was performed at two spiking levels: 50 and 500 ng/L in water, and 0.05 and 0.5 mg/kg in soil.

The limits of detection (LOD), defined as the lowest concentration that the analytical process can reliably differentiate from background levels, were obtained when the signal was three times the average of background noise in the chromatogram at the lowest analyte concentration assayed. The limits of quantification (LOQ) were established as the lowest concentration assayed and validated, which gave satisfactory recovery (70–120%) and precision (<15% RSD).

The specificity of the method was evaluated by analysing a blank procedure, a processed blank sample, and a blank sample spiked at the lowest fortification level assayed (LOQ), i.e. 50 ng/L in water and 0.05 mg/kg in soil. Under these conditions, the response obtained for both the blank procedure and the blank samples should not exceed 30% of the response corresponding to the LOQ.

2.7. Data evaluation

To ensure the quality of the analysis when processing real-world samples, blank samples fortified at the LOQ and 10 × LOQ concentration levels (50 and 500 ng/L for waters, and 0.05 and 0.5 mg/kg for soils) were used as quality controls (QC) distributed along the batch of samples every three-four injections. The quantification of the sample batch was considered satisfactory if the QC recoveries were in the range of 70–120%. The values found in real samples were confirmed by means of the two transitions selected for each compound. In this way, quantification was carried out independently with each transition (see MS Optimisation), accepting a deviation of ± 20% in the concentrations obtained with both transitions.

3. Results and discussion

3.1. MS optimisation

Full-scan MS spectra and product-ion MS/MS spectra of the FMOC derivatives of glyphosate, glufosinate and AMPA were recorded in both positive and negative ionisation modes.

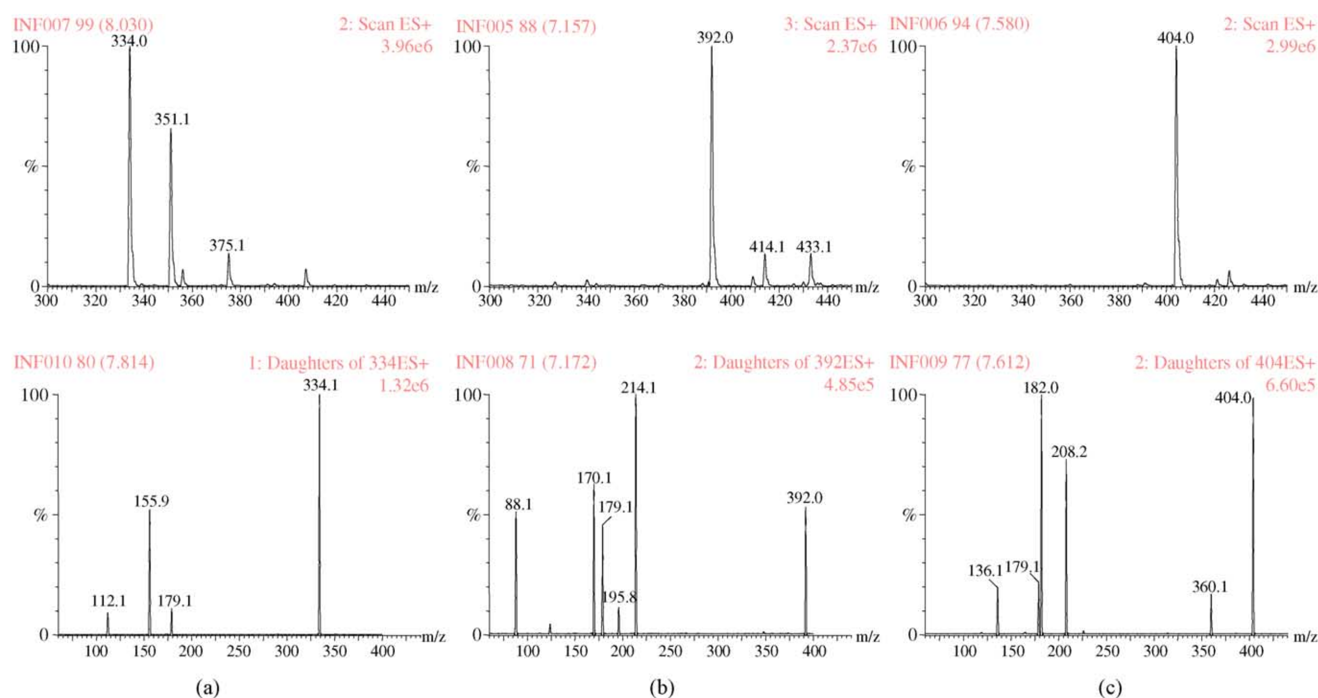

Fig. 2. The positive ion electrospray full scan mass spectrum (top) and product ion spectra (bottom) of (a) AMPA-FMOC, (b) glyphosate-FMOC and (c) glufosinate-FMOC, obtained from the chromatographic peak of 10 mg/L standard solution of each compound, previously derivatizated.

Spectra were obtained from the chromatographic peak of 10 mg/L standard solution of each compound, previously derivatized.

Although these compounds have been traditionally recorded in negative ion mode [9,10], in our work the sensitivity in positive ion mode was found to be approximately two times higher. Moreover, the product ions observed in negative ion mode were due to neutral unspecific losses of FMOC, or FMOC plus water. Thus, any isobaric compound that could have been derivatized with FMOC and also presented a water loss, would show the same product ions in its MS/MS spectra, being therefore not very selective. For all these reasons, positive ion mode was selected.

The positive-ion electrospray full scan spectrum of AMPA-FMOC at a cone of 30 V showed a base peak at *m*/*z* 334 corresponding to the protonated derivatized molecule $[M+H]^+$. The MS/MS spectra showed three abundant fragments at *m*/*z* 179, 156 and 112 (Fig. 2a). As can be seen in Fig. 3a, fragments at *m*/*z* 179, *m*/*z* 156 (M-178) and *m*/*z* 112 (M-222) would appear in any isobaric amine that could have been derivatized with FMOC. As there were not significant differences in the selectivity of these transitions, the criterium applied for their selection was the sensitivity, choosing the two most sensitive ones.

The positive-ion electrospray full scan spectrum of glyphosate-FMOC at a cone of 30 V showed a peak at *m*/*z* 392 corresponding to the protonated derivatized molecule $[M+H]^+$. The MS/MS spectra showed abundant fragments at *m*/*z* 214, 179, 170 and 88 (Fig. 2b). The fragments at *m*/*z* 179 and the fragments at *m*/*z* 214 (M-178) and *m*/*z* 170 (M-222) would appear in any isobaric amine that could have been derivatized with FMOC (Fig. 3a). Thus, the selected reaction monitoring (SRM) transitions chosen were 392 → 88 for quantification as the most selective (see Fig. 3b) and

Table 1
Optimised MS/MS parameters for the FMOC derivatives of glyphosate, AMPA, glufosinate and internal standard, selected for the residue analysis of water and soil

Compound	Cone voltage (V)	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)[a]	Collision energy (eV)
Glyphosate-FMOC	30	392.0	*Q* 88.1	20
			q 214.1	10
Glufosinate-FMOC	30	404.0	*Q* 136.1	25
			q 208.2	10
AMPA-FMOC	30	334.0	*Q* 179.1	20
			q 112.1	15
Isotope-labeled glyphosate-FMOC	30	395.0	*Q* 91.1	20
			q 217.1	10

[a] *Q*, Transition used for quantification; *q*: transition used for confirmation.

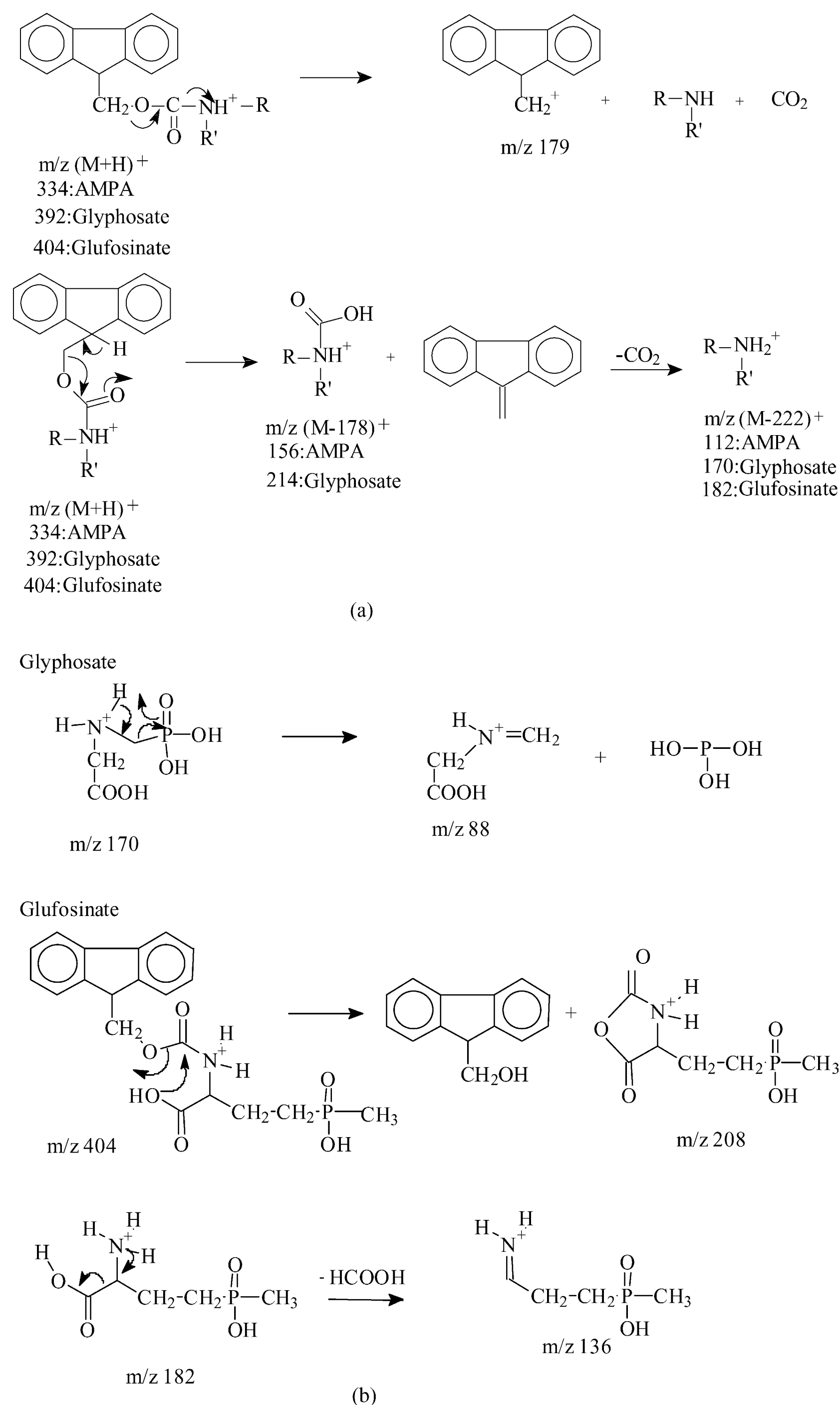

Fig. 3. (a) Common fragmentation pathway for the three derivatised compounds; (b) specific fragmentation pathway for glyphosate and glufosinate.

392 → 214 for confirmation as it was the most sensitive among the less selective.

In the case of glufosinate, the positive-ion electrospray full scan spectrum showed a peak at *m/z* 404 corresponding to the protonated molecule of glufosinate-FMOC. The MS/MS spectrum showed four abundant fragments at *m/z* 208, 182 (M-222), 179 and *m/z* 136 (Fig. 2c). We choose the most selective transitions: 404 → 208 and 404 → 136 (see Fig. 3b) despite their lower sensitivity.

The selected reaction monitoring (SRM) transitions chosen for the residue determination of the three compounds, as well as the optimised MS/MS parameters, are shown in Table 1.

3.2. Method optimisation

Firstly, several attempts were carried out in order to determine these compounds directly, i.e. without any previous

derivatization. For this purpose we checked Hydrophilic Interaction Chromatography using an Atlantis™ HILIC 5 μm Silica Column (100 mm × 2.1 mm i.d., Waters). This column offers superior retention for very polar compounds that are not well retained under reversed-phase conditions. Although the retention obtained with this column at acidic pH was satisfactory, we observed poor sensitivity, making necessary a preconcentration step. We did not try to perform such a preconcentration because this step is difficult for sub-ppb levels of glyphosate and forces one to a higher sample manipulation. Additionally, the conditions to obtain satisfactory retention and peak shape were very specific and changed drastically when changing either pH of the sample or modifier concentration in the mobile phase, decreasing the robustness of the method. For these reasons, a derivatisation procedure was carried out in order to increase the retention of analytes in the most common RPLC cartridges and to work under no so strict conditions.

Derivatization procedures with FMOC-Cl have already been reported in the literature [9–15]. Due to the low solubility and stability of FMOC-Cl in water, this reagent is usually prepared in acetonitrile. Normally the high concentration of FMOC required for the derivatization, makes that the derivatized sample presents a high percentage of acetonitrile. Thus, a dilution step with water is necessary to reduce the organic percentage [14], with the subsequent loss of sensitivity, to retain glyphosate, glufosinate and AMPA in the cartridge due to the high polar character of these compounds, even derivatized. In this paper, we decreased the volume of the FMOC solution used but increasing its concentration and also the volume of water sample derivatized with the aim of minimizing the dilution factor. The effect of adding different FMOC concentrations with different reaction times was studied. The best results for both, water and soil samples, were obtained after performing the reaction overnight with a FMOC concentration of 12,000 mg/L.

On the other hand, as the borate solution could not buffer properly the alkaline sample extract, a neutralizing step was necessary before the derivatization. Any attempt of fixing the volume of HCl necessary to neutralize the KOH excess failed due to the different nature of the soils. Therefore, this step was done manually adding drops of HCl 6 M and 0.6 M until pH around 7.

Once the derivatization reaction took place overnight, hydrochloric acid was added to stop the reaction, by lowering the pH.

In soil samples, after direct injection of 50 μL of the derivatized acidified extract, recoveries around 25% with RSD up to 80% were obtained for the three analytes, showing a severe matrix effect in both the MS instrument and/or the derivatization procedure. Among the solutions described to solve this problem (see Section 1), the increase of the sample treatment was not considered as the best strategy for monitoring programs where rapid methods are preferred. Moreover, the use of matrix-matched standards calibration is not a robust approach when environmental samples are analysed, due to their different origin and composition, making the selection of a blank matrix difficult. Thus, the use of internal standards (IS) was tested, but only isotope-labelled glyphosate was commercially available.

As expected, the use of this IS improved accuracy and precision for glyphosate as it compensated the matrix effects, due to the similar chemical behaviour of analyte and IS. However, still ionization inhibition occurred lowering the sensitivity of the overall analytical procedure. In the case of AMPA and glufosinate, although better recoveries were obtained (around 116–127%), the RSDs were still unacceptable (higher than 15%).

Therefore, the dilution of soil extracts with LC grade water was assayed as a fast and simple way to minimize matrix interferences. Thus, five soil samples of different origins were fortified at the 0.5 mg/kg and their extracts derivatized and, 10-fold and 20-fold diluted with water. According to our results (see Table 2), 10- and 20-fold dilution would be adequate for accurate quantification, even without internal standard. However, the use of internal standard improved the RSDs, especially for glyphosate. In the case of glufosinate, quantification with labelled glyphosate IS did not improve the results. A similar situation has been previously reported in literature, when using analogues IS, demonstrating the difficulty of selecting an adequate IS when the labelled analyte is not available [28]. Finally, glyphosate and AMPA were quantified using internal standard meanwhile glufosinate was quantified with external standard calibration. A 10-fold dilution of the extract was chosen as it led to the best LODs.

In regard to water samples, after injection of 4.3 mL of the derivatized sample into the SPE–LC–MS/MS, recoveries

Table 2
Effect of dilution of soil extracts previously to the derivatization step on the recovery and reproducibility of the method ($n = 5$)[a]

Compound	Without dilution		10-Fold dilution		20-Fold dilution	
	%Recovery[b] (%RSD)	%Recovery[c] (%RSD)	%Recovery[b] (%RSD)	%Recovery[c] (%RSD)	%Recovery[b] (%RSD)	%Recovery[c] (%RSD)
Glyphosate	25 (79)	97 (6)	83 (24)	98 (3)	83 (23)	91 (11)
AMPA	28 (46)	127 (27)	87 (9)	98 (11)	89 (8)	98 (10)
Glufosinate	27 (56)	116 (18)	94 (8)	118 (19)	92 (8)	107 (9)

[a] Five different soil samples, spiked at 0.5 mg/kg each.
[b] Quantification without internal standard.
[c] Quantification with internal standard.

Table 3
Validation of the developed LC–MS/MS procedure for the determination of FMOC derivatives of glyphosate, aminomethylphoshonic acid (AMPA) and glufosinate in water and soil samples

	Groundwater		Surface water		Soil samples		LOD	
	50 ng/L	500 ng/L	50 ng/L	500 ng/L	0.05 mg/kg	0.5 mg/kg	Water[a] (ng/L)	Soil[b] (μg/kg)
Glyphosate[c]	89 (9)	96 (3)	106 (3)	102 (2)	90 (7)	92 (4)	5	5
AMPA[c]	97 (10)	116 (9)	111 (8)	106 (9)	89 (5)	88 (1)	5	5
Glufosinate[d]	72 (7)	75 (12)	84 (9)	88 (7)	83 (6)	86 (5)	5	5

Detection limits, mean recoveries (%) and relative standard deviations (%) of the overall analytical procedure ($n = 5$).
[a] Estimated from a LC–MS/MS chromatogram corresponding to a 25 ng/L standard.
[b] Estimated from a LC–MS/MS chromatogram corresponding to a 1 μg/L standard.
[c] Relative recovery, using labeled glyphosate as IS.
[d] Absolute recovery.

around 60% were obtained for glyphosate and AMPA and around 75% for glufosinate, showing an important matrix effect. The use of IS improved the results for glyphosate and AMPA, while unsatisfactory recoveries were obtained (around 140%) for glufosinate. Thus, glyphosate and AMPA were quantified using IS, but not glufosinate, similarly to the analysis of soil. In this case, a dilution of the water sample was not assayed due to the high sensitivity required.

Phosphate and phosphonate compounds might be prone to adsorption onto nonsilanized glass especially in non-metal-free solvents. Therefore, a simple adsorption study was carried out in order to evaluate this effect for the three analytes, using standard solutions and fortified surface and

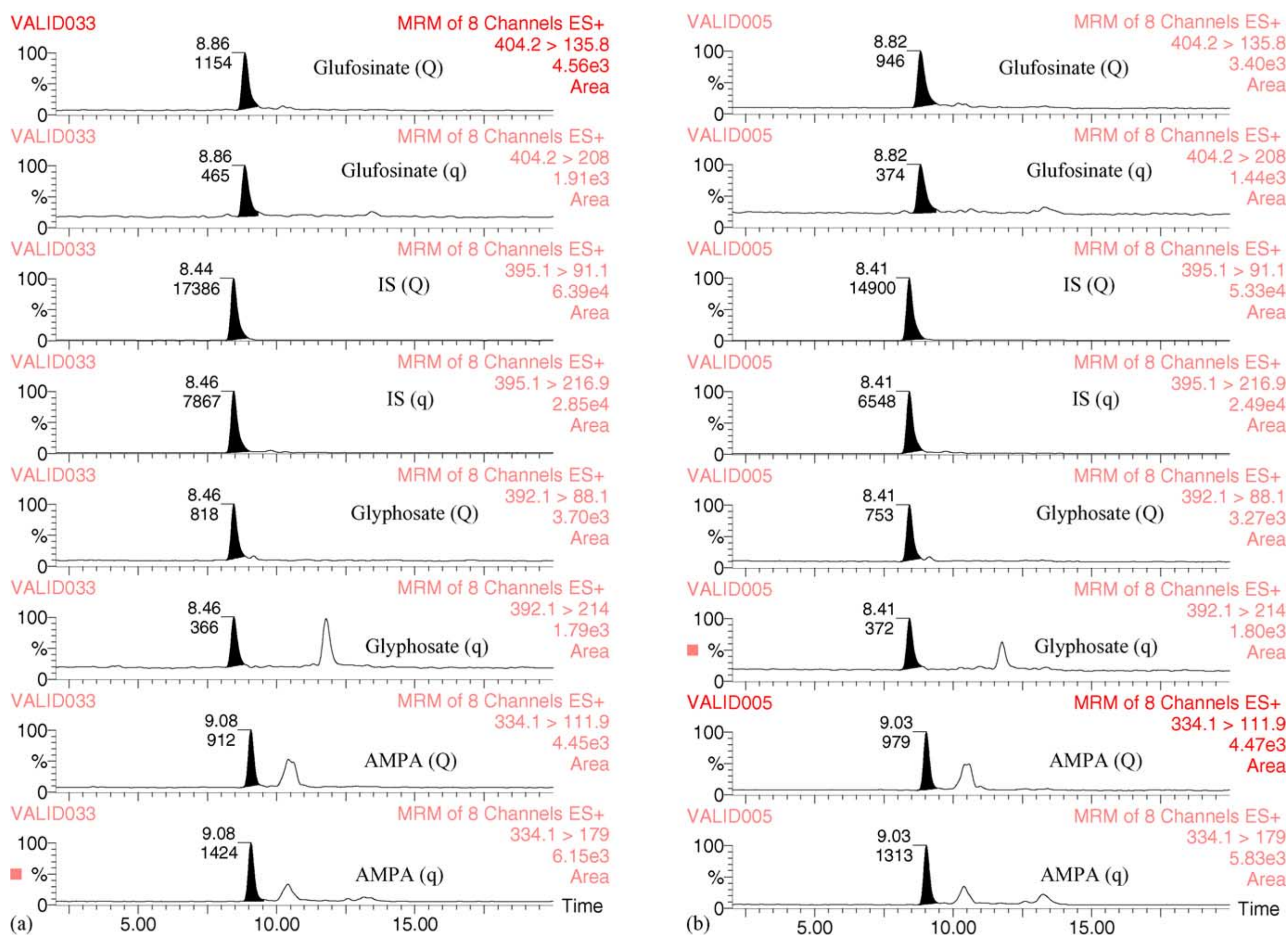

Fig. 4. LC–MS/MS chromatograms for (a) standard solution of glyphosate, glufosinate and AMPA (50 ng/L) and (b) groundwater sample spiked at 50 ng/L. (IS) Internal standard, (*Q*) quantitative transition, (*q*) qualitative transition.

groundwater samples. After letting stand for 24 h before derivatization, no significative adsorption was observed, as the recoveries were within the normal accepted values, i.e. 70–110%. Therefore, the unsatisfactory recoveries obtained in the preliminary experiments were found to be related to matrix effects rather than to adsorption processes onto the glass material.

3.3. Method validation

Quadratic calibration curves were obtained for all three compounds, in the range 25–5000 ng/L for water analysis, and in the range 1–500 μg/L for soil extracts, with correlation coefficients (r^2) higher than 0.995 in all cases. The method was found to be precise (RSD < 12%) and accurate, with satisfactory recoveries, between 72 and 116% in water, and between 83 to 92% in soil. The slightly higher recoveries for AMPA in water samples could be explained by a partial correction when using the labelled glyphosate. Notwithstanding, the results were considered satisfactory at the low concentration levels assayed.

Limits of quantification (LOQ) were taken as the lowest fortification level successfully validated, i.e. 50 ng/L for all compounds in water samples and 0.05 mg/kg in soil. Limits of detection were calculated from the most diluted standard analysed (25 ng/L for water samples and 1 μg/L for soil samples) and were estimated to be 5 ng/L for all compounds in water samples, and 5 μg/kg in soil. Table 3 summarizes all data obtained during method validation.

As an example of the excellent sensitivity and selectivity of the method, Fig. 4 shows typical SPE–LC–MS/MS chromatograms for a standard solution (50 ng/L) and a groundwater sample spiked at 50 ng/L. This figure also shows the benefit of selecting selective transitions. As can be seen, chromatograms corresponding to the less selective transitions, i.e. those chosen for AMPA (Q, q) and glyphosate (q), show the presence of additional peaks.

3.4. Analysis of real-world samples

The developed SPE–LC–MS/MS method was applied to the analysis of both ground and surface water (approximately 50 samples) collected in selected sites from the Spanish Mediterranean region, an important agricultural area where glyphosate is widely used. Moreover, the developed LC–MS/MS method was applied to the analysis of six soil samples.

Within each batch of 8–10 samples, a calibration curve at concentrations between 25 and 1000 ng/L, in the case of water, and between 1 and 500 μg/L, in the case of soil, was injected before and after the samples. Every three samples, QC prepared at the LOQ level and at the 10 × LOQ level were alternately inserted. Quality control (QC) consisted on blank groundwater, surface water or soil, which were spiked with the analytes. These blank samples were previously analysed to confirm the absence of the analytes. Satisfactory QC recoveries were obtained for all the compounds (between 70

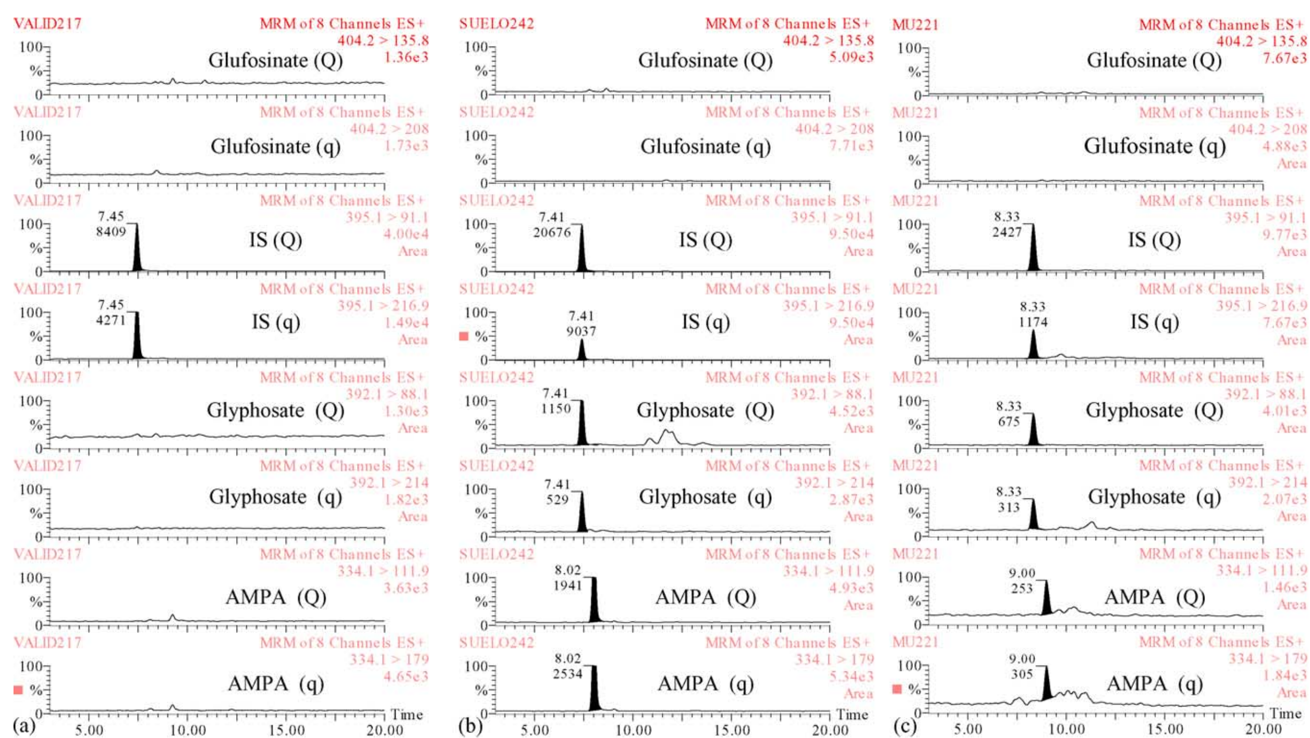

Fig. 5. LC–MS/MS chromatograms corresponding to (a) blank soil sample, (b) soil sample, containing glyphosate and AMPA at concentration level of 0.28 and 0.38 mg/kg, respectively and (c) groundwater sample, containing glyphosate and AMPA at concentration level of 304 and 87 ng/L, respectively. (IS) Internal standard, (Q) quantitative transition, (q) qualitative transition.

and 120%) demonstrating the robustness of the method along the period of time of the analysis.

Glufosinate was not detected in any of the samples analysed. However, glyphosate was found in 20% of the water samples, at concentration levels between 55 and 484 ng/L, whereas AMPA was detected in 38% of the water samples, at concentrations between 51 and 175 ng/L. Fig. 5b shows chromatograms corresponding to a groundwater sample, that contained glyphosate and AMPA (304 and 87 ng/L, respectively).

In relation to soil, glyphosate was detected in four of the six samples analysed, at concentration levels between 0.17 and 0.73 mg/kg, and AMPA was found in three of these samples, at concentrations between 0.04 and 5.61 mg/kg. Fig. 5c shows chromatograms corresponding to a soil sample, containing glyphosate and AMPA (0.28 and 0.38 mg/kg, respectively).

All the detections were confirmed by the qualification transition (q) selected, obtaining a deviation in the calculated concentration (using both Q and q transitions) within the accepted tolerance, in all cases $<\pm 20\%$.

4. Conclusion

The SPE–LC–ESI-MS/MS method developed in this work allows the rapid, accurate and selective determination of very low residue levels of glyphosate, AMPA and glufosinate in water and soil samples. All efforts to determine these compounds without any previous derivatization were not enough satisfactory, mainly due to problems in the preconcentration step. Therefore, a pre-derivatization with 9-fluorenylmethylchloroformate in borate buffer was carried out to decrease the polarity of analytes and to improve their retention time in the RPLC system employed. Although these compounds had been traditionally recorded in negative ion mode, positive ionisation has been selected in this paper, as the specificity and sensitivity of the selected transitions improved under this ionisation mode. The developed method achieves excellent LODs for both water (5 ng/L) and soil (5 μg/kg), and allows the correct quantification and confirmation of positive samples at 50 ng/L for water and 0.05 mg/kg for soil. As the method does not require off-line preconcentration of the sample, 10 mL of water are sufficient to perform the derivatization step. Thus, 4.3 mL of the derivatized mixture are sufficient to be preconcentrated on-line in the SPE–LC system in order to reach the required sensitivity, allowing an easy automation and rendering a high analytical throughput. The higher complexity of the soil samples leads to considerable matrix effects, inhibiting the ionisation of the analytes. A 10-fold dilution of the soil extract previously to the derivatization step, together with the use of isotopically labelled glyphosate as internal standard, is a simple way to minimize and compensate for this undesirable effect and to obtain satisfactory quantification. The developed methodology has been applied to the determination of glyphosate, glufosinate and AMPA in real-world water and soil samples. All the detections were confirmed by the use of two MS/MS transitions.

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