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ORIGINAL PAPER

Determination of glyphosate in groundwater samples using an ultrasensitive immunoassay and confirmation by on-line solid-phase extraction followed by liquid chromatography coupled to tandem mass spectrometry

Josep Sanchís · Lina Kantiani · Marta Llorca · Fernando Rubio · Antoni Ginebreda · Josep Fraile · Teresa Garrido · Marinella Farré

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Abstract Despite having been the focus of much attention from the scientific community during recent years, glyphosate is still a challenging compound from an analytical point of view because of its physicochemical properties: relatively low molecular weight, high polarity, high water solubility, low organic solvent solubility, amphoteric behaviour and ease to form metal complexes. Large efforts have been directed towards developing suitable, sensitive and robust methods for the routine analysis of this widely used herbicide. In the present work, a magnetic particle immunoassay (IA) has been evaluated for fast, reliable and accurate part-per-trillion monitoring of glyphosate in water matrixes, in combination with a new analytical method based on solid-phase extraction (SPE), followed by liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS), for the confirmatory analysis of positive samples. The magnetic particle IA has been applied to the analysis of about 140 samples of groundwater from Catalonia (NE Spain) collected during four sampling campaigns. Glyphosate was present above limit of quantification levels in 41% of the samples with concentrations as high as 2.5 μg/L and a mean concentration of 200 ng/L. Good agreement was obtained when comparing the results from IA and on-line SPE-LC-MS/MS analyses. In addition, no false negatives were obtained by the use of the rapid IA. This is one of the few works related to the analysis of glyphosate in real groundwater samples and the presented data confirm that, although it has low mobility in soils, glyphosate is capable of reaching groundwater.

Keywords Glyphosate · Groundwater · ELISA · Immunoassay · On-line SPE · LC-MS/MS

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J. Sanchís · L. Kantiani · M. Llorca · A. Ginebreda · M. Farré (✉)
Institute of Environmental Assessment and Water Research (IDAEA-CSIC),
C/Jordi Girona, 18-26,
08034 Barcelona, Spain
e-mail: mfuqam@cid.csic.es

F. Rubio
Abraxis LLC,
54 Steam Whistle Drive,
Warminster, PA 18974, USA

J. Fraile · T. Garrido
Catalan Water Agency,
Provença 204-208,
08036 Barcelona, Spain

Introduction

Glyphosate [(*N*-phosphonomethyl)glycine, CAS no. 1071-83-6] is an organophosphorus broad-spectrum herbicide used for weed and vegetation control. The active molecule was developed in 1970s and marketed as a product called Roundup in 1973, which found great usage and became the most widely used herbicide around the world [1].

Glyphosate is rather small in size, and the three polar functional groups (carboxyl, amino and phosphonate) present in its structure make it to be strongly retained on soil mineral components [2]. It also has relatively long half-

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lives of about 47 and 49–70 days in soil and in water, respectively, making it fairly persistent in the environment, thus it can still be detected long after application and to some distance from the application site. Its high solubility in water (12,000 mg/L) aids in the transport of glyphosate from terrestrial to aquatic environments. Such molecules can be transported to surface and ground waters, either in solution or in suspension when bound to sediments. Even though groundwater samples have not been extensively investigated within the scientific community, Torstensson et al. reported in 2005 glyphosate concentrations in groundwater samples above the European maximum limit of 0.1 μg/L (Directive 2006/118/EC) [3].

Glyphosate presents a low acute toxicity to animals, because its biochemical mode of action affects the shikimic acid pathway, which is present in plants but it does not exist in animals [4]. However, various studies in the last decade have shown possible toxicological effects linked to its use. It has been reported to shorten the development rate in insects (*Chrysoperla externa*) by lengthening the period prior to reproduction and reducing fertility [4]. Also, glyphosate may toxically threaten amphibian species [5–8], with 96–100% decrease in larval population and 68–86% juvenile amphibians on treatment with 3.8 mg/L of glyphosate [5]. Moreover, long exposure to glyphosate can cause endocrine effects on mammals [9]. Because of these concerns glyphosate (as well as its metabolite aminomethyl phosphonic acid (AMPA)) was included in the annex III of the 2008/105/EC Directive as a *substance subject to review for possible identification as priority substance or priority hazard substance*. It is therefore essential to incorporate efficient control of glyphosate into the existing organic pollutant monitoring schemes of water supplies, especially when considering the increased rate of this compound's use around the world.

Detection of glyphosate at trace levels in environmental samples is difficult due to its zwitterionic behaviour and complexation with metal ions. Existing analytical methods for the detection of this herbicide in waters and other matrices like soils are based on chromatographic techniques, usually coupled to mass spectrometric detection systems. Generally, derivatization of the sample is required prior analysis in gas chromatography (GC) in order to convert the polar glyphosate to a less polar more volatile derivative and also in liquid chromatography (LC), making the analysis of this compound quite challenging. Derivatisation of the sample prior to GC analysis were achieved employing trifluoroacetic acid–trifluoroacetic anhydride–trimethylorthoacetate reagent [10], isopropyl chloroformate and diazomethane (CH_2N_2) [11] and trifluoroacetic anhydride and 2,2,3,3,4,4,4 heptafluoro-1-butanol [12] among others. However, during the last decade, LC coupled to tandem mass spectrometry (LC-MS/MS) is the technique of choice for the analysis of glyphosate due to its high selectivity and sensitivity [13, 14]. Hanke et al. achieved limits of detection in the nanogramme-per-litre range for glyphosate in natural waters by a LC-MS/MS method based on a derivatization with 9-fluorenyl methyl chloro formate (FMOC-Cl), which is the most common pre-column derivatisation reagent, and solid-phase extraction (SPE) [13]. A method based on high-performance ion chromatography coupled to inductively coupled plasma dynamic reaction cell mass spectrometry was developed for detection of glyphosate and its main metabolite, AMPA, in surface and wastewaters [15]. This method, although yielded good recovery values of 103% and 104% for glyphosate and AMPA, respectively, it failed to reach the required detection limits without further clean-up. On the other hand, immunoassays have been established as rapid, robust, accurate and cost-efficient analytical techniques in the determination of organic pollutants in environmental samples. The analysis of glyphosate has been reported by means of several enzyme-linked immunosorbent assays (ELISA) [16–18]. A commercially available glyphosate IA from Abraxis LLC was evaluated by Byer et al. [19]. The present study has been carried out using a new IA kit from Abraxis LLC, which presents an improved limit of quantification (LOQ) and the analytical range is between 75 and 4,000 ng/L.

The objectives of this work were: first, to assess the good performance of this IA for rapid monitoring of glyphosate in groundwater, second to develop an on-line SPE-LC-MS/MS for confirmation and quantification of glyphosate in groundwater, and test the good applicability of the proposed methods by the evaluation of the glyphosate presence in real groundwater samples in Catalonia (Spain) during four sampling campaigns using a combined strategy using a rapid screening with the magnetic particle IA and confirmation using LC-MS/MS. In this study, 139 samples collected during four sampling campaigns (2007–2010) in different locations of Catalonia were evaluated using an IA based on paramagnetic particles attached with antibodies specific to glyphosate. The results illustrate the presence of glyphosate in groundwater from Catalonia, establishing the levels of this persistent herbicide in one of the main sources of drinking water in sampled locations. To the authors knowledge, this is one of the first studies reporting glyphosate concentrations levels in groundwater in Europe.

Materials and methods

Sample collection Groundwater samples were collected by the Catalan Water Agency between May and September in 2007, 2008, 2009 and 2010. The samples were collected in 500-mL amber glass bottles. Then, 20-mL aliquot of each

sample were separated and frozen during the transport to the laboratory and analysed immediately after sampling by the IA. The rest of the samples were frozen and stored in the dark in order to inhibit the degradation mechanism [19].

A total of 139 samples from 69 wells located in 11 different sampling sites (water bodies) in Catalonia (Spain) were analysed. Figure 1 displays the geographic location of the sampling sites. The number of samples varied between different campaigns: 18 samples from five different areas, 19 samples from eight areas, 37 samples from eight areas and 55 samples from ten different areas were collected during 2007, 2008, 2009 and 2010, respectively. The main characteristics of the sampling areas are summarised in Table 1. With the exception of one, all the areas studied presented a high impact from intensive agriculture and they were qualified as of high risk areas.

Chemicals Analytical standards of glyphosate (reference 45521) and glyphosate-2-^{13}C (99% isotopic purity and reference 606502) were purchased from Sigma-Aldrich (Steinheim, Germany). The derivatisation agent FMOC-Cl (≥99.0% purity and reference 23814) and auxiliary reagents ethylenediaminetetraacetic acid (EDTA; 99.4–100.6% purity and reference E9884), sodium tetraborate ($Na_2B_4O_7$; 99% purity and reference 221732) and potassium hydroxide (KOH pellets, ≥85% purity and reference 221473) were also purchased from Sigma-Aldrich. HPLC-grade methanol, acetonitrile (ACN), ultra-pure water, dimethyl sulfoxide (DMSO) and formic acid and hydrochloric acid for analysis (25%) were supplied by Merck (Darmstadt, Germany). FMOC-Cl stock solution of 650 μM was prepared by dilution of 0.0168 g of FMOC-Cl in 100 mL of ACN. Tetraborate buffer was prepared by diluting 4 g of $Na_2B_4O_7$ in 500 mL of ultra-pure water. EDTA oversaturated solution was prepared by diluting 41.6 g of EDTA in 100 mL of ultra-pure water. All stock solutions were prepared weekly and stored at 4 °C, with exception of FMOC-Cl stock solution, which was prepared daily.

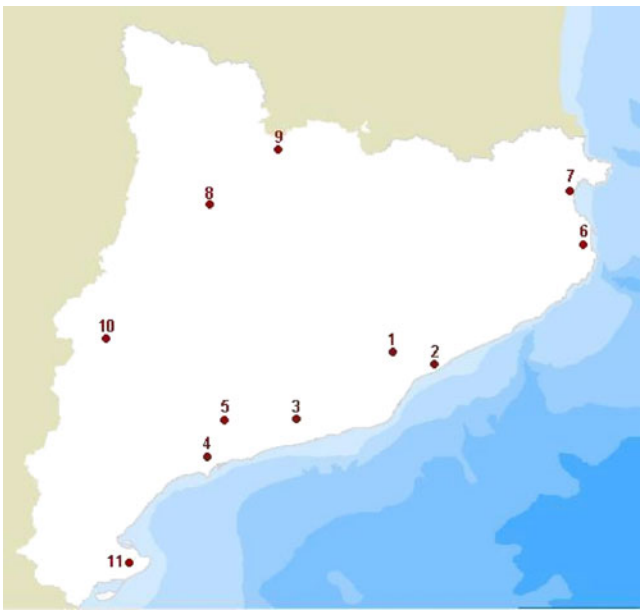

Fig. 1 Sampling areas

Magnetic particle immunoassay The glyphosate IA was developed and supplied by Abraxis LLC. This IA is based on polyclonal antibodies attached to paramagnetic particles, and the competitive reaction between derivatized glyphosate and derivatized enzyme labelled glyphosate for the antibody binding sites on the magnetic particles. The analysis procedure was performed in accordance with the operating manual accompanying the glyphosate kit. Very briefly, an aliquot of 250 μL of each sample was thoroughly mixed with 100 μL of diluted DMSO that served as derivatisation agent and incubated at room temperature for 10 min. After this period, 300 μL of derivatised sample and 500 μL suspended glyphosate antibody-coupled paramagnetic particles were mixed in a glass test tube and incubated for 30 additional minutes at room temperature. Incubation of another 30 min at room temperature followed after the addition of 250 μL of glyphosate enzyme conjugate. A magnetic field separator was then applied in order to separate any reagents unbound to the magnetic particles and keep hold of the bound reagents. Decanting of unwanted material took place after three washing cycles with deionised water; 500 μL of colour solution, containing the enzyme substrate (hydrogen peroxide) and the chromogen (3,3′,5,5′-tetramethylbenzidine), were added to the particles, and the mixture was incubated for 20 min at room temperature. The colour development reaction was stopped and stabilised by the addition of 500 μL of 2% sulphuric acid solution, and absorbance was then read at 450 nm using a photometer *Photometric Analyzer II* (Abraxis LLD, Warminster, PA) within 15 min after adding the stopping solution. Colour development was inversely proportional to glyphosate concentration. Standard calibration curves were prepared testing nine levels of increasing concentrations of glyphosate from 0.1 to 5 μg/L. The standard sigmoidal curves were fitted to a four-parameter equation according to the following formula:

$$A = B + \frac{T - B}{1 + 10^{(\mathrm{LogEC_{50}} - \mathrm{LogC}) \times \mathrm{HS}}}$$

Where A is absorbance, T is the maximum absorbance value, B is the minimum absorbance value, EC_{50} is the concentration producing 50% of the maximum absorbance, C is the concentration and HS is the slope at the inflection

Table 1 General characteristics of sampling areas

Sampling site	Dominant lithology	Total surface (km^2)	Multilayer	Permeability (m/day)	Transmissivity (m^2/day)	Dependency with surface waters	Intensive agriculture risk	Monitoring campaigns
1	Alluvial	165	No	40–300	100–4,000	Yes	Moderate	2009 and 2010
2	Granite and Palaeozoic	444	No	0.1–4 (granite); 10–20 (quaternaries)	20 (granite); 100–400 (quaternaries)	Yes	High	2010
3	Detritus not alluvial	72	Yes	No data	90–360	No	Nule	2008, 2009 and 2010
4	Detritus not alluvial	179	Yes	100–2,500	10–50 (clay); 2,000–3,000 (gravels)	Yes	High	2008, 2009 and 2010
5	Detritus not alluvial	265	Yes	100–2,500	10–50 (argyles); 2,000–3,000 (graves)	Yes	High	2008, 2009 and 2010
6	Alluvial	184	Yes	No data	100–1,500 (deep layers); 200–30,000 (surface layers)	Yes	High	2007 and 2008
7	Alluvial	165	Yes	100−1,000	2,500−11,000	Yes	High	2007, 2008, 2009 and 2010
8	Alluvial	18	No	No data	No data	Yes	High	2007, 2008, 2009 and 2010
9	Alluvial	191	No	No data	No data	Yes	High	2007, 2008, 2009 and 2010
10	Alluvial	275	No	350−4,200	No data	Yes	High	2007, 2008 and 2010
11	Alluvial	328	No	No data	500	Yes	High	2009 and 2010

point of the sigmoid curve. A standard curve was prepared with each set of samples analysed and two-matrix blank samples were analysed along with each sample set to determine possible interferences. No interferences were detected above the LOQ during the samples analysis. The average of at least three replicates was calculated and presented in this work.

Immunoassay evaluation The recoveries and the matrix effects on the IA were previously studied and reported [18, 20, 21]. Nevertheless, the matrix interference can be quite variable depending on the different types of water. For this reason, the first step of this work was to evaluate the suitability of the IA for the different types of ground water and river water selected in this study. Therefore, the different types of water as well as ultra-pure water, and tap water, free on glyphosate were fortified with glyphosate in a wide range of concentrations covering from 25 to 10 μg/L, were assayed after derivatization using the IA procedure described above, and the standard curves were fitted for the different types of water.

In a previous work [18], the possible interference of structurally related compounds was evaluated. In the present work, this study was extended and the possible cross reactivity of other organic pollutants commonly found in groundwater from these sampling areas was studied. The compounds included here were triazine compounds (atrazine, desethyl atrazine and terbuthylazine), phenylurea compounds (diuron and linuron) and organophosphates (fenitrothion, diazinon, malathion and dimethoate) and measured with the IA. The cross-reactivity values were calculated according to the equation:

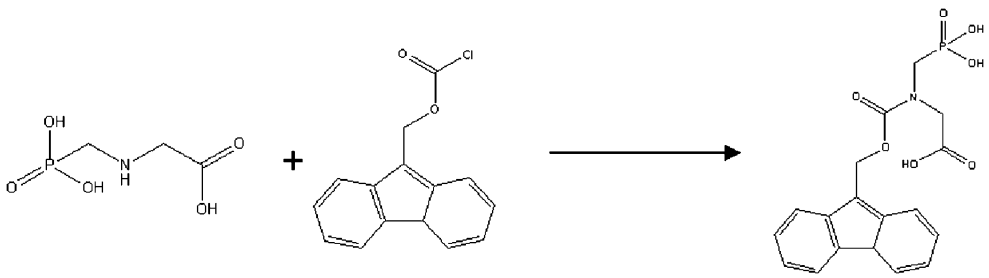

Fig. 2 Chemical reaction between glyphosate and FMOC-Cl

Table 2 Optimal instrumental parameters of the on-line SPE extraction

	Solvent	Volume (μL)	Flow (μL/min)
Activation	MeOH	2,000	2,000
Equilibration	ACN (0.1% formic acid)	2,000	2,000
Sample loading	ACN (0.1%formic acid)	1,000	2,000
Wash	H_2O	500	1,000
Elution	90% $AcNH_4$ 2.5 mM (pH=9.0)−10% MeOH		

Immunoreactivity equivalents

$$= (IC_{50}\ \text{glyphosate}/IC_{50}\ \text{tested compounds}) \times 100$$

In addition, 30 blind prepared samples in assay buffer and 30 blind prepared samples in groundwater free of glyphosate were evaluated in triplicates, in order to assess the accuracy, precision and possible false negative and positive detected by the IA.

Sample preparation for the instrumental analysis Four millilitres of water samples were placed in an amber vials, were spiked with ^{13}C-glyphosate subrogate standard and were acidified with HCl 6 M to pH=1.0. The acidified samples were stirred during 1 h in order to break the metal-glyphosate complexes that may happen under real environmental conditions. After this time, the presence of glyphosate is assumed to be in free form and the samples were neutralised with KOH 6 M. Derivatisation of the samples was performed according to the method previously described by Hanke et al. [13]. Very briefly, 1 mL of FMOC-Cl 650 μM in ACN and borate buffer (1:1) were added to the samples, and the mixture was stirred during 2 h at room temperature. Then the samples were acidified to pH 3 with formic acid, and 0.5 mL of aqueous EDTA (1.1 M) was added in order to prevent further metal complexation of glyphosate. The derivatised glyphosate (gly-FMOC) incorporates a fluorenylmethyloxycarbonyl group bounded to the glyphosate's amine group (Fig. 2). The stability of gly-FMOC stored at 4 °C during 12 h was proved. However, drastic loses of signal were detected when derivatized samples were stored overnight. Therefore, instrumental analysis was always carried out within the 12 h after derivatization.

Table 3 SRM transitions

Compound	m/z>m/z	DP (V)	CE (eV)	EP (eV)	CXP (eV)
Gly-FMOC	390>168	40	15	12	8
	390>150	40	18	12	8
^{13}C-gly-FMOC	391>169	40	15	12	8
	391>151	40	18	12	8
Glyphosate	168>150	40	30	12	8
	168>124	40	20	12	8
^{13}C-labellerd glyphosate	169>151	40	30	12	8
	169>125	40	20	12	8

SRM simple reaction monitoring

Table 4 Instrumental mass spectrometric parameters

Curtain gas	40
High ion spray(V)	4,500
Source temperature (°C)	390
Ion source gas 1	60
Ion source gas 2	50

On-line extraction procedure Derivatised water samples were loaded onto C18EC (Spark Holland, Emmen, The Netherlands) SPE cartridges previously conditioned with 2 mL of methanol and equilibrated with 1 mL of water at 2 mL/min. Derivatised samples (2 mL) were loaded at a slower flow rate (2 mL/min) with 1 mL ACN (0.1% formic acid) as transfer solvent. SPE cartridges were then washed

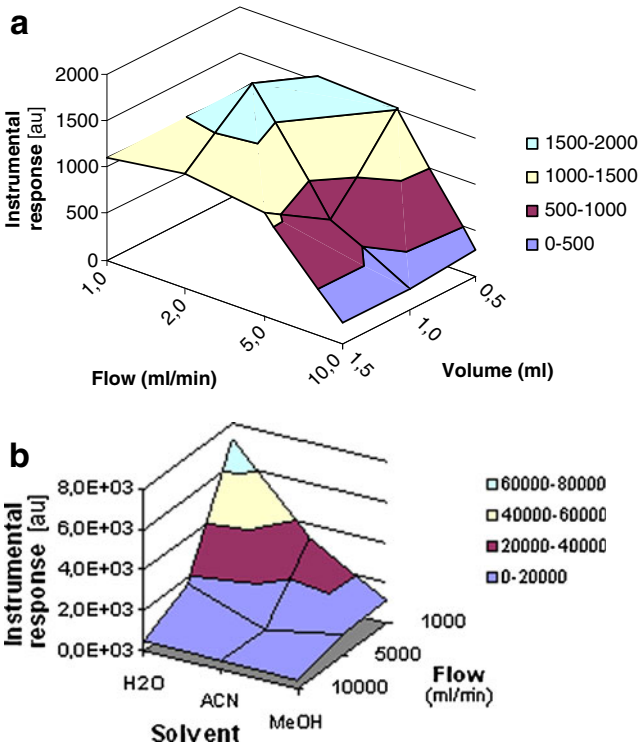

Fig. 3 Instrumental signals (in arbitrary units) obtained during the optimization of the on-line extraction: (**a**) extraction step with three volumes of ACN with formic acid at four different flow rates; (**b**) washing step with three solvents at three different flow rates

with 0.5 mL of water at 1 mL/min flow rate. Elution was carried out using the mobile phase solvents. Following the elution step, and in order to avoid sample carry over, multiple valve and clamp washes were carried out with water. The optimal instrumental parameters for the on-line SPE extraction are summarised in Table 2.

Liquid chromatography coupled to tandem mass spectrometry LC was performed using the Symbiosis Pico system (Spark Holland, Emmen, The Netherlands) equipped with a 5-mL sample loop. The chromatographic separation was achieved with a LC column Synergy 4 μ Hydro-RP 50×2.0 mm, 4 μm (Phenomenex, reference 00B-4375-B0). Mobile phase composition consisted of (A) ammonium acetate (2.5 mM, pH=9.0) and (B) methanol. The elution gradient conditions for the LC mobile phase started with 10% eluent B, maintained isocratic during 1 min, increasing to 90% of eluent B in 1 min and holding for 1 min more. Initial conditions were reached in 1 min and re-equilibration was achieved in 2 min. The flow rate was kept at 0.2 mL/min through the total chromatographic run. As pointed elsewhere [13], the presence of ammonium acetate and pH=9 are needed in order to obtain a good chromatographic shape of gly-FMOC although high concentrations of the modifier decreased the S/N ratio.

The Symbiosis Pico LC system was coupled to a 4000QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer equipped with a Turbo Ion Spray source from Applied Biosystems-Sciex (Foster City, California, USA), employed in the negative electrospray ionisation mode (ESI (−)).

Simple reaction monitoring was used in order to obtain the required quantification points for confirmation. Quantification was performed with the Analyst software version 1.5 (Applied Biosystems).

Optimal instrumental were set as follows: curtain gas (CUR)=40; collision gas (CAD): high; ion spray (IS)=−4,500 V; source temperature (TEM): 390; ion source gas 1 (GS1): 60; ion source gas 2 (GS2): 50. All the instrumental parameters are summarised in Tables 3 and 4.

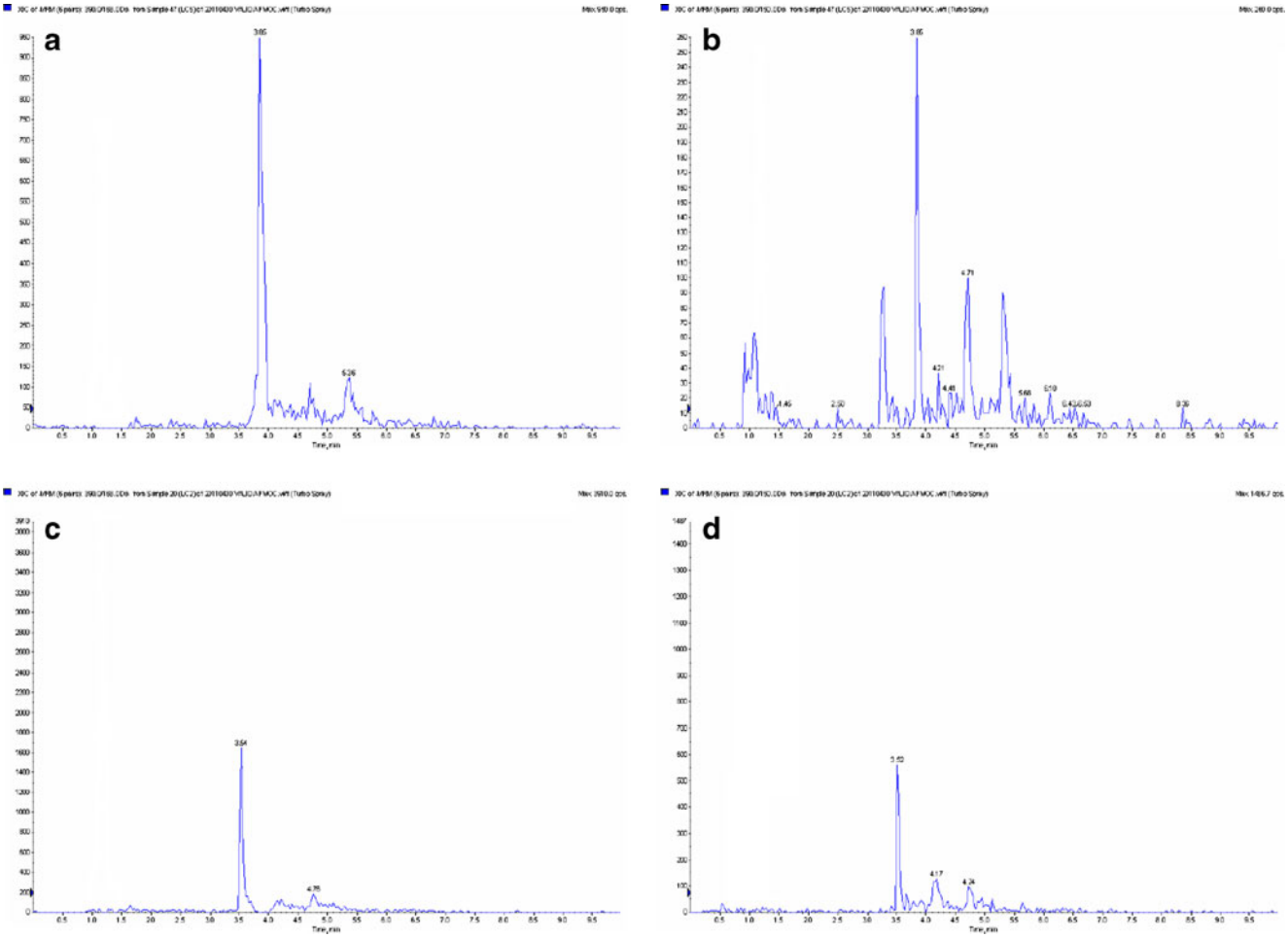

Fig. 4 Chromatogram of blank groundwater samples spiked at 5 ng/L ((**a**) quantification and (**b**) confirmation transitions) and 10 ng/L ((**c**) quantification and (**d**) confirmation transitions)

Results and discussion

Optimisation of LC-MS/MS Due to the previous experience in our group, a Synergy Hydro-RP (50×2 mm, 4 μm) analytical column was selected. For the mobile phase, different compositions and solvents were tested including water, methanol, acetonitrile and ammonium acetate (2.5 mM, pH=9.0). Solvents used for the mobile phase were methanol and ammonium acetate, and the elution gradient was optimised by varying the percentage of organic solvent throughout the run. The optimised gradient was selected in order to obtain the best signal-to-noise ratio. The use of ammonium acetate was crucial for the Gly-FMOC peak shape and retention time.

For the optimization of MS/MS conditions, a solution of Gly-FMOC at a concentration of 1 mg/L was infused in order to select the two most relevant transitions of product ions. Once identification of the most abundant fragment ions was achieved, as well as the ionisation parameters for each transition, full-scan chromatograms were obtained, indicating the retention of derivatised glyphosate. Flow injection analysis was then used, in order to optimise the ion source conditions in the mass spectrometer, namely the ion source TEM, IS voltage, CUR, GS1 and GS2 and CAD. Final MS/MS conditions, as well as precursor ion and product ions, selected for the identification and quantification of each compound, are summarised in Tables 3 and 4.

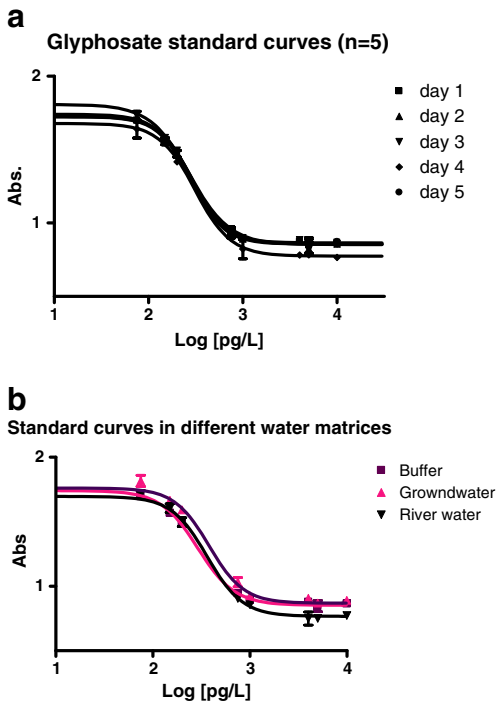

Fig. 5 Glyphosate standard curves. (**a**) Inter-day repeatability. (**b**) Matrix effects study

Table 5 Specificity studies

Compound	CR %
Glyphosate	100
Glyphosine	0.1
Gluphosinate	0.025
AMPA	<0.001
Glycine	<0.001
Atrazine	<0.001
Desethyl atrazine	<0.001
terbuthylazine	<0.001
Diuron	<0.001
Linuron	<0.001
Fenitrothion	<0.001
Diazinon	<0.001
Malathion	<0.001
Dimethoate	<0.001

Cross-reactivity studies observed with glyphosate commercial immunoassay

Optimization of on-line SPE The type of sorbent, injection volume, sample loading and wash solvent were investigated in order to improve the on-line extraction process. Different sorbent types were studied; C_{18}EC, C_{18}HD, HLB, Hysphere Resin GP and Varian polymer phase PLRPs. Best recovery was achieved with C_{18}EC with a mean value of 89% being slightly better than C18-HP cartridges (mean value, 68%), and Resin GP cartridges (mean value, 62%).

Injection volume tests were performed with partial injections on a 5-mL sample loop in order to check for breakthrough in the range of 20–2,500 μL. No breakthrough volume was found at 2,500 μL, which was the maximum admitted amount using partial loop injection. Therefore, 2.5 mL was set as injection volume.

Cartridge activation, sample loading and cartridge washing steps were also optimised. Different volumes and

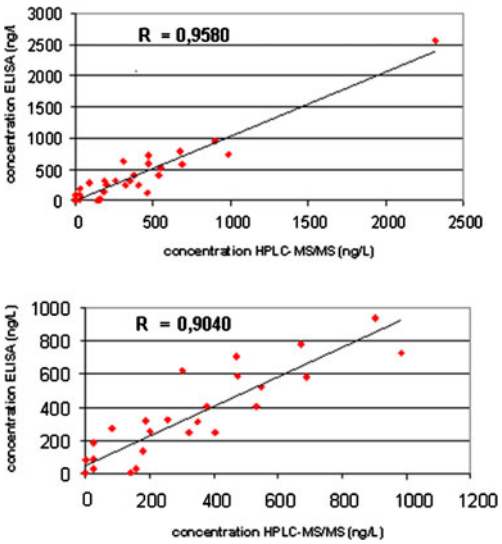

Fig. 6 Correlation between data obtained with ELISA kit and HPLC-MS/MS method

flow rates of methanol were tested to optimise cartridge activation and final conditions were 2 mL of methanol at 2 mL/min flow rate. Six different solvents methanol, ACN, water, ammonium acetate 2.5 mM at pH=9.0, ACN (0.1% formic acid) and water (0.1% formic acid) were tested in order to select the optimal elution solvent. Different volumes of ACN (0.1% formic acid) were evaluated at different flow rates. As can be seen in Fig. 3a, the highest signal was obtained when the transfer solvent was 2 mL of acidified ACN at 2 mL/min followed by 1 mL of ACN at 2 mL/min for equilibration. Finally, the washing step was also optimised using different solvents and flow rates, obtaining the maximum instrumental response using 0.5 mL of water at a flow rate of 1 mL/min. Finally, cartridge elution was performed by the gradient elution. The recovery of Gly-FMOC was calculated from the peak area obtained for the most intense transition.

On-line SPE-LC-MS/MS method validation The method was validated according to the EU Decision 2002/657/EC. Blank groundwater was spiked at three concentrations levels: 80.0, 200 and 400 ng/L. Six replicates of each concentration were analysed at each concentration levels. The intraday reproducibility was calculated resulting in 15%, 12% and 8%, respectively.

Criteria for the LOQ was established as the lowest concentration fulfilling all of the following criteria: (1) bias from the calibration curve less than 25%, (2) relative standard deviation of four replicates below 19%, (3) peak shapes acceptable and (4) signal-to-noise ratio at least 10. Method limit of detection and method limit of quantification (MLOQ) were found to be 3.2 and 9.6 ng/L, respectively.

The decision limit (CCα) was defined as the lowest concentration level at which the method is able to discriminate the gly-FMOC presence, with a statistical certainty of 99%. By analysing 20 blanks, CCα was estimated as 1.6 ng/L. The detection capability (CCβ) was defined as the smallest concentration of gly-FMOC that may be detected, identified and/or quantified in a sample with an error probability of β. By analysing 20 samples spiked at CCα, CCβ was established as 3.1 ng/L.

Linearity was assessed by constructing a seven-point calibration curve (ranging between 50 and 500 ng/L) in triplicate. Least-square linear regression analysis was performed by plotting the peak area of the analyte over the analyte concentration. R^2 of 0.99925 was achieved.

In order to assess the possible carryover of the method blank samples were analysed after analysis of groundwater samples fortified at 5 μg/L. In all these cases, blank samples showed values for glyphosate under the LOQ. Therefore, carryover could be considered negligible (Fig. 4).

Table 6 Summary of glyphosate concentrations in groundwater samples analysed during four sampling campaigns

Sampling site	Number of analysed samples (no samples over MLOQ)					Median (ng/L)					Average (ng/L)					Range (ng/L)				
	2007	2008	2009	2010	Total	2007	2008	2009	2010	Total	2007	2008	2009	2010	Total	2007	2008	2009	2010	Total
1	0	0	6 (2)	7 (5)	13 (7)	–	–	<MLOQ	154	126	–	–	102	581	360	–	–	<MLOQ-384	<MLOQ-2560	<MLOQ-2560
2	0	0	0	7 (5)	7 (6)	–	–	–	186	186	–	–	–	212	212	–	–	–	<MLOQ-524	<MLOQ-524
3	0	2 (0)	6 (1)	5 (1)	13 (2)	–	<MLOQ	<MLOQ	<MLOQ	<MLOQ	–	<MLOQ	77	145	97	–	<MLOQ	<MLOQ-284	<MLOQ-624	<MLOQ-624
4	0	1 (0)	4 (2)	4 (2)	9 (4)	–	<MLOQ	66	137	<MLOQ	–	<MLOQ	76	143	100	–	<MLOQ	<MLOQ-148	<MLOQ-273	<MLOQ-273
5	0	2 (2)	7 (2)	6 (1)	15 (5)	–	204	<MLOQ	<MLOQ	<MLOQ	–	204	109	97	117	–	198-209	<MLOQ-534	<MLOQ-458	<MLOQ-534
6	3 (0)	3 (3)	0	0	6 (3)	<MLOQ	620	–	–	285	<MLOQ	603	–	–	314	<MLOQ	544-646	–	–	<MLOQ-646
7	6 (2)	3 (1)	8 (5)	9 (5)	26 (13)	<MLOQ	<MLOQ	146	126	76	124	292	189	146	171	<MLOQ-345	<MLOQ-827	<MLOQ-492	<MLOQ-404	<MLOQ-827
8	1 (0)	1 (1)	1 (0)	1 (1)	4 (2)	<MLOQ	480	<MLOQ	107	66	<MLOQ	480	<MLOQ	107	159	<MLOQ	480	<MLOQ	107	<MLOQ-480
9	3 (1)	3 (3)	4 (2)	5 (3)	15 (9)	<MLOQ	749	72	243	243	122	741	137	286	304	<MLOQ-315	717-756	<MLOQ-379	<MLOQ-729	<MLOQ-756
10	5 (1)	4 (0)	0	9 (7)	18 (8)	<MLOQ	<MLOQ	–	242	<MLOQ	47	<MLOQ	–	304	171	<MLOQ-137	<MLOQ	–	<MLOQ-939	<MLOQ-939
11	0	0	1 (1)	2 (2)	3 (3)	–	–	366	431	366	–	–	366	431	409	–	–	366	80-781	80-781
Total	18 (4)	19 (10)	37 (15)	55 (32)	129 (61)	<MLOQ	198	<MLOQ	126	<MLOQ	80	314	125	252	202	<MLOQ-345	<MLOQ-827	<MLOQ-534	<MLOQ-2560	<MLOQ-2560

Immunoassay performance and specificity The IA intra-assay precision was evaluated by determining the variation (CV%) between replicates assayed at various concentrations on the standard curves (Fig. 5); as can be seen, good precision was shown by the IA with CV% of 13.4. Figure 5 presents some examples of standard curves performed in assay buffer, blank river water and different blank grown water are presented. As can be seen, good agreement was found between fortified blank natural waters and the standard curve prepared in assay buffer and no significant changes on slopes were found. The recovery percentages range from 93% to 105% and 92% to 102% for groundwater and river water, respectively.

Specificity studies are summarised in Table 5. Very low cross reactivity was found for glyphosine and glufosinate, and no cross reactivity was found with other related compounds such as AMPA, in agreement with previous studies. No interference was found with other organic pollutants studied here, including other organophosphate compounds.

Sixty blind samples were prepared spiking glyphosate concentrations in the range between 0 and 4 μg/L. Thirty of these samples were prepared in assay buffer, and 30 samples more were prepared in a real groundwater samples free in glyphosate. The samples were analysed by magnetic particle immunoassay. The results of this test showed that no false negatives or false positives were obtained by the IA, very good correlation was obtained between the results obtained using the IA and the concentrations of fortification with coefficient of correlation R^2=0.9907 in assay buffer and R^2=0.9816 in groundwater. .In addition, slight tendency to overestimation was observed in groundwater

Finally, all the samples of the last sampling campaign were analysed in parallel by means of the magnetic particle IA and on-line SPE-LC-MS/MS. The average relative error between the IA analyses and the confirmation method was lower than 12%. In Fig. 6, both series of analysis are plotted and a correlation index of R^2=0.9580 was found.

Applicability of the method Glyphosate was investigated in 139 samples, and it was detected at quantifiable levels in 61 samples (47%). Table 6 summarises the median concentration, average and range of concentrations along the different campaigns. In addition, a summary of all results are presented in Table S1 in the Electronic supplementary material. All samples were analysed using the magnetic particle immunoassay, and positive samples were confirmed by instrumental analysis. No false negatives were found using the immunoassay. The concentrations of glyphosate range from MLOQ to 2.6 μg/L, and the average was 202 ng/L (samples under limit of quantification were computed as half the MLOQ for the average calculation). Mean concentrations of glyphosate are presented in Fig. 7. In general, in terms of average concentrations, slight differences were obtained along the sampling campaigns, which range from 97 ng/L for the cleanest site to 409 ng/L. As it was expected, more contaminated areas (sites 6, 9 and 11) were found in those regions of thriving agriculture activity. However, the higher value was achieved in 2010, in site no. 1, which corresponds to an area with moderate agricultural activity. In addition, a significant difference was obtained compared with the same site during 2009 campaign. In this case, the presence of glyphosate can be related to their increasing use as herbicide for non-agricultural applications, such as, the control of weeds on margins or streams and drains, around buildings, railways, roads and industrial areas.

All sampling campaigns were carried out during the application season but, in some of the sampling areas (1, 3, 4 and 11), an increasing trend was observed along the different campaigns, and in others, such as, 5, 7, 8 and 9, the higher average concentrations were obtained during the first sampling campaign in 2008. In this sense, it should be mentioned that the degradation of glyphosate is highly variable according to the environmental conditions. The degradation of glyphosate in surface water has been reported to be very fast. Whereas, in groundwater glyphosate is rapidly adsorbed to organic matter, precipitated and then can be retained in the soil where half-life can be longer than 2 years [22]. In

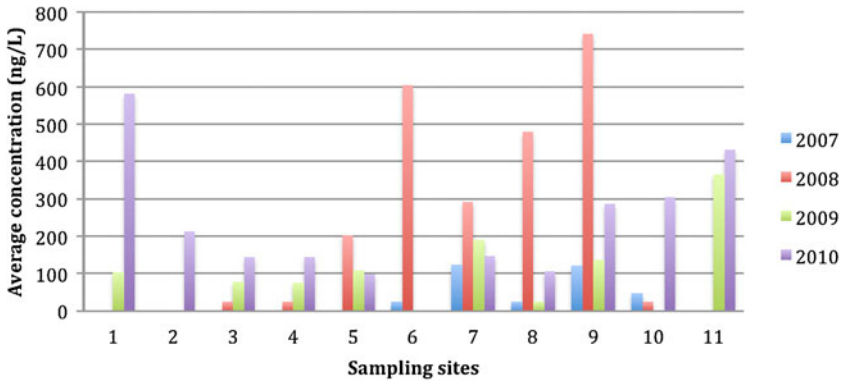

Fig. 7 Average concentrations of the sampled areas during four sampling campaigns

addition, the mobility and leaching capability of glyphosate also depend on the type of soil. Borggaard et al. [2] reported that the different glyphosate forms can be moved by leaching through uniform gravelly soils and in structured soils with macro-pores, being determinant other factors such as rain precipitations, timing, tillage and vegetation. Therefore, the results showing the higher concentrations can be associated to sites where the sampling was carried out immediately after glyphosate application in the area. In addition, glyphosate can be accumulated in soil leaching by precipitation [23]. This fact can partially explain high concentrations in some areas during 2008, such as sites 5 and 7, which coincides with the onset of spring rains in 2008 after 3 years of heavy drought [24] that could have favoured the dissolution of glyphosate retained in the soil. After these high levels in the 2008 campaign, during the 2009 and 2010, campaigns registered a progressive decrease.

The presence of glyphosate in groundwater has been exiguously reported, and very few works have been carried out to study this presence. In most of previous studies, no quantifiable levels of glyphosate were found in groundwater, even in areas where surface water is found to contain the herbicide [13, 25]. However, it should be pointed out that these studies were carried out with analytical methods presenting LOQ in the range of micrograms per litre, and the present study use a, IA capable to detect glyphosate at pictogram-per-litre range without sample pre-treatment, just derivatisation, and an on-line SPE-LC-MS/MS method for confirmation of the glyphosate at nanogram-per-litre range. Second, in this study the sampling campaigns were carried out during the peak season of glyphosate application in those areas, in order to investigate main areas susceptible of glyphosate accumulation in soils. These areas should be determined and controlled in order to follow the behaviour and dissolution of this herbicide under certain environmental conditions as after rains.

Conclusions

The magnetic particle IA for glyphosate analysis from Abraxis LLC was proved to be a suitable, sensitive and cost-effective method for the fast ultra-trace screening analysis of a large number of real groundwater samples. The here presented IA is the most sensitive in the literature for the analysis of glyphosate. In addition, a new methods based on on-line SPE-LC-MS/MS was developed and validated as rapid confirmatory analytical method for glyphosate analysis at ultra-trace level.

The good performance of these analytical approaches, as well as, the applicability of the combined methodology for the analysis of glyphosate in groundwater has been proved using the approach for the analysis of groundwater from 11 different areas in Catalonia. The results showed a 41% of the samples presenting quantifiable concentrations of glyphosate when were sampled.

In addition, the results of this study corroborate the hypothesis of previous studies pointing that glyphosate may exhibit certain grade of mobility in soils. This is the first that experimental data about glyphosate reaching groundwater provided. Despite the tendency of glyphosate of being immobilised in soils, aquifer contamination with glyphosate has been demonstrated to happen because of its intensive use. Higher concentrations for 2008 were registered and it was linked to 2008 spring precipitations finishing with a 3-year drought period.

Since the environmental source of glyphosate is certainly related to agricultural practices, runoff to surface waters is very likely to occur. Therefore, the potential ecological impact of this contamination should be taken in consideration in a more global view. Although the levels reported in this work are relatively low, their variability is significant through space and time, and an increase tendency has been observed in some sampling points, underpinning the importance of further analysis of glyphosate and their degradation products in groundwater samples.

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