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Glyphosate Immunosensor. Application for Water and Soil Analysis

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A fully automated immunosensor for the herbicide glyphosate has been developed on the basis of the immunocomplex capture assay protocol. The sensor carries out on-line analyte derivatization prior to the assay and uses a selective anti-glyphosate serum, a glyphosate peroxidase enzyme tracer, and fluorescent detection. Under optimal conditions, the detection limit achieved is 0.021 μg/L with an analysis rate of 25 min per assay, autonomy of more than 48 h, and sensor reusability >500 analytical cycles. The immunosensor is able to discriminate structurally related molecules, such as aminomethylphosphonic acid, the main metabolite of glyphosate, and other related herbicides, such as glufosinate and glyphosine. Interferences from naturally occurring species (anions, cations, and humic substances) and their elimination were also studied. The immunosensor has been successfully applied to water and soil sample analysis, with good recoveries at levels lower than 1 μg/L. Results obtained with the immunosensor correlate well with data from a magnetic particle ELISA and LC/LC/MS chromatographic method.

In the past decade, the use of glyphosate [(*N*-phosphonomethyl)glycine] has grown steadily, and it has become the most frequently used herbicide all around the world.[1] It is used as a nonselective postemergence herbicide in applications for weed and vegetation control in agricultural, industrial, and domestic uses. The success of glyphosate comes from its effectiveness for controlling perennial weeds and overwintering rhizomes and tubers, as well as from its safety in the environment due to its ability to bind to soil colloids and to be degraded by soil microbes, but the main factor for the increased use of glyphosate has been the introduction of glyphosate-resistant transgenic crops.[2] However, the continued use of glyphosate raises the potential for residue accumulation in water and crop commodities. Although glyphosate has been considered as a "toxicologically harmless"[3] compound, recent studies suggest that glyphosate-based pesticides affect cell cycle regulation.[4] Glyphosate residues have been found in many samples, including vegetables.[5] The maximal permissible level of glyphosate in drinking water is 0.70 mg/L in the United States[6] and 0.1 μg/L in the European Union.[7] In crops, the allowed levels are different for each vegetable, ranging from 0.1 to 20 mg/kg in some cereals.[8,9]

Glyphosate determination in agricultural and environmental matrixes is problematic, because it is a very polar small molecule, so that its solubility in water is very high but it is insoluble in organic solvents. In addition, it has structural similarity to naturally occurring plant materials, such as amino acids, which can cause interferences. Furthermore, glyphosate adsorbs strongly to clay minerals[10] and organic or mineral particles in water[11,12] and shows high affinity to metal cations complexing them.[13] Such an analyte, therefore, is difficult to isolate and analyze.

Difficulties in glyphosate monitoring and control could raise an environmental problem, because this compound is more and more frequently applied and, therefore, detected. As an example, widespread contamination of groundwater has been recently found in Denmark.[14] According to Canadian researchers, glyphosate could enhance the growth of fungi that infest wheat crops.[14] Other glyphosate unknown effects could be perhaps detected if powerful and easy to run analytical methods were available. Furthermore, no general vision of the impact of glyphosate can be achieved due to the lack of sensitive, selective, rapid, and economical methods for the analysis of this compound.

Current analytical methods for glyphosate determination in water and other matrixes, for example, soils, are based on chromatographic techniques. In general, gas chromatography (GC) needs precolumn derivatization in order to improve analyte

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volatility, and although conventional GC detection, such as flame ionization,[15] flame photometry,[16] and electron capture,[17] has been employed, mass spectrometry (MS) in its different modes is the preferred detection system.[15,18,19] Sensitivity achieved by GC techniques, after sample treatment, leads to a limit of quantification values around 0.05−0.1 μg/L. A more suitable method for determining glyphosate is liquid chromatography (LC), and several approaches have been used, such as precolumn[20] and postcolumn[21,22] derivatization, ion chromatography with conductivity,[23] and electrochemical[24] and MS[25,26] detection, with sensitivities in the micrograms-per-liter level that can be improved down to 0.02 μg/L with sample preconcentration. Even capillary electrophoresis with indirect detection[27,28] has been employed for this analyte, although sensitivity reached was only in the micromolar range. Novel methodologies with high sensitivity that are based on the use of LC/LC/MS and LC/MS/MS are currently under development.[29] All these methodologies are expensive, highly specialized, time-consuming, and generally lack the sensitivity to be directly applied to environmental samples. The recommended EPA method for glyphosate in drinking water[30] uses high performance liquid chromatography (HPLC) with direct injection of the sample, postcolumn derivatization, and fluorescence detection and has a limit of detection of 9 μg/L without preconcentration.

In all cases, the analytical methodology is nearly exclusive for this analyte, since the working conditions cannot be applied to the determination of pesticides different from glyphosate, except for some organophosphorus, such as glufosinate and others, and this chemical cannot be determined by a multiresidue method. In this sense, the determination of glyphosate is carried out by a small number of well-equipped and -trained laboratories. For those reasons, the "glyphosate paradox"occurs, which means that the most widely used agrochemical in the world is one of the most rarely determined.

Unlike other pesticides, the application of immunoanalytical techniques for glyphosate determination has been troublesome, although recent improvements have been reported. The first work of Clegg et al.[31] produced a selective antibody for glyphosate with low affinity (I_{50} = 154 mg/L), and preconcentration was required so as to obtain good sensitivity. The properties of this immunoassay were further critically enhanced by means of a prior-to-assay analyte derivatization (acylation) reaction,[32] which led to an I_{50} as low as 1.65 μg/L. Analyte derivatization has been employed on some occasions in both clinical[33] and environmental[34] analysis with positive results, because the product of the reaction shows a structure nearly identical to that of the paratope (hapten and linker to carrier protein) that elicits the immune response. In fact, another highly sensitive enzyme immunoassay for glyphosate,[35] recently developed, makes use of an analyte derivatization, employing a similar concept. A different example has been recently published for glufosinate.[36]

Immunosensing covers a list of methodologies that use immunochemistry for the rapid, sensitive, reversible, and automatic determination of an analyte, and the development and application of immunosensors for organic pollutants has become popular. Although many approaches have been investigated for this purpose, maybe the best issue in terms of sensitivity, rapidity of response, and economy is the employment of the same principles as in batch immunoassay but working in an automated flow system.[37] This kind of device would be very interesting for the on-line monitoring and control of glyphosate in streams, as well as for all cases in which results are needed rapidly, but to our knowledge, a glyphosate immunosensor has not been reported.

In this work, the basis of a derivatization-assisted enzyme immunoassay is employed in the development of an automated immunosensor for the determination of glyphosate in water and soil samples. Attention was especially paid to analytical performance, on-line analyte derivatization, full automation, and interferences, as well as their removal. The immunosensor operates with an assay format based on the competition between the analyte and an enzyme-labeled hapten taking place in solution and the further capture of the immunocomplexes on a protein A/G-specific immunoglobulin-binding support. This assay format is well-established and has been employed successfully (high sensitivity and operational life, as well as application to real samples) in previous works, in both single-analyte[37] and multianalyte[38] modes. The advantages of immunosensing over conventional batch immunoassay are the rapidity of response; full automation, including sample conditioning; and sensitivity improvement. These performances are frequently necessary when monitoring the presence of pollutants in the environment.

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MATERIALS AND METHODS

Reagents and Equipment. Glyphosate analytical standards and structure-related compounds were obtained from Chem Service (West Chester, PA), Sigma-Aldrich (Steinehim, Germany), and Dr. Eherenstorfer (Augsburg, Germany). Lyophilized rabbit anti-glyphosate serum, enzyme tracer glyphosate−horseradish peroxidase conjugate, and glyphosate-derivatization reagent and diluent were described previously[32] and were obtained from Abraxis LLC (Warminster, PA). Ultralink immobilized protein A/G support was from Pierce (Rockford, IL). 3-(*p*-Hydroxyphenyl)-propanoic acid (HPPA) was from Sigma (St. Louis, MO). Humic acid sodium salt was from Aldrich (Madrid, Spain). All other reagents were of analytical grade.

TBS buffer (42 mM Tris (hydroxymethyl)aminomethane, 276 mM NaCl, 54 mM KCl, and HCl, pH 8.9) was used as washing buffer. The dissociation agent was 0.1 M glycine/HCl buffer, pH 2.0. Antibody solution was prepared in TBS containing 0.1% (m/v) bovine serum albumin (BSA, Boehringer, Mannheim, Germany); the tracer was dissolved in TBS containing 0.05% (v/v) Proclin 300 (Supelco, Bellefonte, PA). Enzyme substrates HPPA and H_2O_2 were prepared in TBS at pH 8.0. Derivatization solution was prepared by mixing the derivatization reagent and the diluent (DMSO) at 2.35% v/v. All the solutions employed were vacuum-filtered through a 0.45-μm Durapore membrane filter (Millipore, Madrid, Spain). The manifold employed has been described elsewhere.[39]

Immunoassay Format and Signal Treatment. The immunosensor system was designed so as to carry out automatically all the steps, including the derivatization reaction, which takes place simultaneously to other operations: First, the antibody (300 μL, 10 mg/L), tracer (300 μL, 1/17 500) and derivatized standard or sample (500 μL) solutions are mixed, and 1 mL of the mixture is injected through the protein A/G reactor (0.25 mL/min). After a washing step (TBS, 4 × 1 mL at 4 mL/min), HPPA (200 μL, 0.8 g/L) and H_2O_2 (0.012% v/v, 200 μL) are mixed, and 100 μL of the mixture is injected through the reactor, being incubated for 7 min. While this is taking place, the native standard or sample solution (989 μL) is mixed with the derivatization solution (80 μL), and the mixture is sent to a delay coil until the following assay cycle. The product of the enzymatic reaction is then transferred to the flow cell, and the fluorescence peak is registered. Reactor regeneration is then carried out by injecting the dissociation agent (2.5 mL at 0.5 mL/min), followed by TBS (3 × 1 mL at 4 mL/min). The total assay time is 25 min.

Peak heights are recorded by means of the Chrom-Card Manager software package (Fissons Instruments, Rodano, Italy). The analytical signals are normalized according to the equation

$$\text{normalized signal (\%)} = 100 \times (B - B_\infty)/(B_0 - B_\infty)$$

where B is the peak height for a standard or sample, B_0 is the peak height for a blank, and B_∞ is the peak height recorded in absence of enzyme tracer (background signal). In calibrations, the experimental dose−response points were fit to a four-parameter logistic equation, where the I_{50} parameter is indicative of the competition sensitivity. Sigmaplot 2.0 (Jandel Scientific, San Rafael, CA) was used for data treatment.

Sample Preparation. Natural underground water samples (see composition in Table 1a) were spiked with glyphosate and kept at 4 °C until use (less than a week). Prior to analysis, water samples were filtered through a 0.22-μm Durapore membrane filter and then mixed 9/1 v/v with 10-fold concentrated TBS, and the final pH was set at 8.9 with HCl.

Sixteen soil samples were collected from different orchards of Valencia (Spain) and treated and analyzed for habitual soil parameters (Table 1b). For glyphosate determination, soil samples were extracted according to the procedure described by Sancho et al.[40] Briefly, 10 g of homogenized dried soil was shaken with 20 mL of 0.6 M KOH for 30 min and centrifuged at 2500*g* for 20 min, and the supernatant was spiked with glyphosate and filtered through a 0.22-μm Durapore membrane filter. Prior to analysis, the extracts were diluted with TBS, and the pH was set to 8.9 with HCl.

ELISA and HPLC Analysis. Comparison analysis methods were magnetic particles ELISA performed according to Rubio et al[32] and a chromatographic highly sensitive method (LC/LC-FD) developed by Hidalgo et al.[29]

RESULTS AND DISCUSSION

Assay Optimization and Performances. Although capture format-based immunosensors have been developed[37] for several analytes (herbicides, insecticides, and algaecides), including multianalyte approaches,[38] the optimization of this glyphosate sensor required the study of many variables, since it shows some features that are different from those of a standard capture immunosensor format. First, the need for an analyte derivatization reaction introduces a new variable that influences others. In this sense, the sample buffering parameters had to be tested and optimized for maximal assay sensitivity. In previous experiments carried out in batch immunoassays,[32] it was stated that the phosphate ion is an important interfering agent of the glyphosate immunoassay, so this chemical could not be used as buffering agent; therefore, tris(hydroxymethyl)aminomethane, carbonate, and borate were studied. A clear competition curve was registered when employing tris(hydroxymethyl)aminomethane as the buffering substance, whereas carbonate and borate buffers led to nondefined curves. In further experiences, as stated below, carbonate and borate anions did also interfere in the analysis.

The pH of the reaction was also studied, since the derivatization reaction, the antibody and HRP activity, and the protonation degree of the analyte are all pH-dependent. As shown in Figure 1a, when testing the assay performance by varying the pH of the reaction, it was found that no signal was observed at pH below 8.5, and the I_{50} value was minimal (maximal sensitivity) at pH 8.9, growing slightly until pH 9.3 and sharply for higher pH values. It is worth mentioning that the pH dependence of the immunosensor for glyphosate is much more critical than in sensors previously developed, mainly due to the influence of analyte acid−base chemistry (pK_{a1} 0.8, pK_{a2} 3.0, pK_{a3} 6.0, pK_{a4} 11.0).[41]

The influence of ionic strength was also studied (Figure 1b), employing different salt concentrations ranging from 0.075 M to a maximum of 0.5 M and always keeping the same ratio of tris-

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Table 1. Chemical Parameters of Samples

(a) Water Samples (data in mg/L)

sample	pH	cond[a]	d.r.[b]	Cl^-	HCO_3^-	SO_4^{2-}	SiO_2	Ca^{2+}	Mg^{2+}	Na^+
W-1	6.5	25	27	0.7	10.4		11.2	2.1	0.3	2.5
W-2	7.4	394		7.8	158.3	15.2		56.5	12.2	4.9
W-3	7.6	237		6.9	135l	11.6		33.5	6.6	12.2
W-4	6.5	270		12.9	145.8	25.9		38.1	11.4	6.8
W-5	7.4	394	259	7.6	277.8	19.3	7.2	60.1	25.3	5.1
W-6	7.0	198		12.7	107	17.2		27	9.9	11.4
W-7	7.6	350	240	2.1	252.4	21.3	3.2	62.1	18.2	0.7
W-8	7.4	224		39.7	295.3	43.0	6.9	86.6	23.3	20.7
W-9	7.3	238		6.9	160	12.2		47.5	4.5	7.0
W-10	7.5	874		96.8	285.6	148		110.3	31.4	51.3

(b) Soil Samples

sample	origin	M.O.[c] g/kg	clay %	silt %	sand %	pH W[d]	pH $CaCl_2$	Na meq/L	Ca + Mg meq/L	CE 1:2.5[e] dS/m
S-1	Pobla de Vallbona	2.0	18	28	54	8.49	7.57	1.0	6.7	0.698
S-2	Villamarchante	2.2	5	57	38	8.30	7.51	1.6	13.0	1.109
S-3	Utiel	1.2	4	43	53	8.67	7.76	3.6	9.3	0.624
S-4	Valencia	1.8	14	20	66	8.29	7.75	2.4	35.0	1.520
S-5	Valencia	1.2	11	7	82	8.19	7.53		18.6	2.330
S-6	Cheste	43.4	30	5	65	7.78	7.63	66.3	40.0	8.630
S-7	Bronchales	2.9				6.21	5.54	8.6	20.0	0.669
S-8	Partida Alcores	13.4	20	45	35	8.46	7.69	0.1	5.6	0.694
S-9	Sagunto	15.8	20	40	40	8.25	7.68	4.9	24.2	3.037
S-10	Valencia	42.0	6	58	36	8.30	7.61	6.5	11.2	1.567
S-11	Villanueva Segura	19.3	4	80	16	8.87	7.98		6.5	0.800
S-12	Vallbona	9.7	25	58	17	8.53	7.72	0.4	5.0	0.238
S-13	Ribarroja	30.4	16	25	59	8.22	7.44		6.5	1.204
S-14	Montserrat	10.5	27	44	29	8.66	7.81	3.8		0.384
S-15	Catarroja	21.3	4	48	48	8.03	7.67	0.5	19.2	2.403
S-16	Casas Bajas	16.5	22	22	56	8.67	7.63	0.4	4.6	0.446

[a] Conductivity in μS/cm. [b] Dry residual (mg/L). [c] Organic matter. [d] Water. [e] 1:2.5 conductivity in the extract 1:2.5.

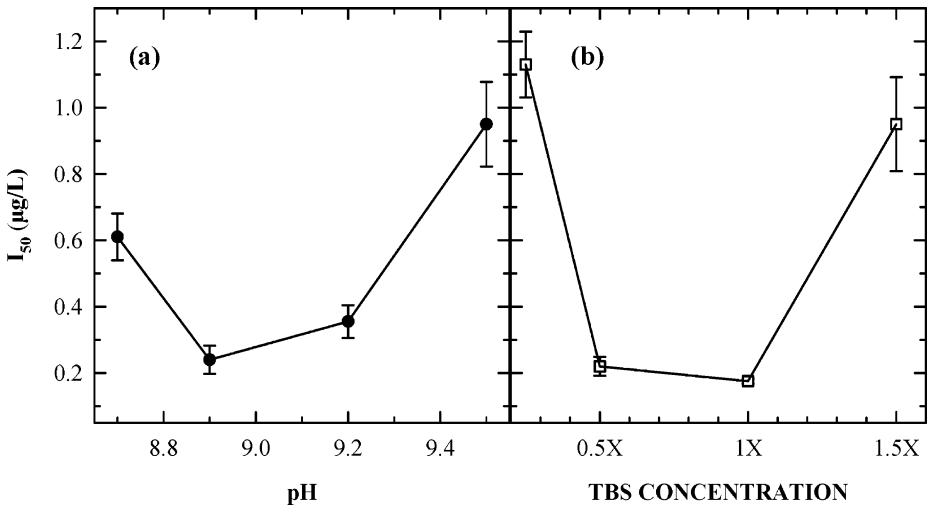

Figure 1. Immunosensor sensitivity optimization. (a) Effect of pH. (b) Effect of ionic strength, expressed as TBS concentration. Each point represents the mean ± SD of two assays.

(hydroxymethyl)aminomethane, sodium chloride, and potassium chloride. The optimal conditions corresponded to a saline concentration of 42 mM tris(hydroxymethyl)aminomethane, 276 mM NaCl and 54 mM KCl. This solution was used as the assay and washing buffer.

The optimized calibration curve for glyphosate standards, obtained with the conditions mentioned above, is shown in Figure 2. The sensitivity parameters from the curve are an I_{50} value of 0.177 μg/L, with a limit of detection (analyte concentration that generates a normalized signal of 90%) of 0.021 μg/L. The glyphosate concentrations that can be measured are between 0.050 and 1.0 μg/L. These values indicate that the glyphosate immunosensor is more sensitive than the ELISA employing the same immunoreagents[32] or other immunoassays for this analyte.[35] This improvement in sensitivity is supposed to be due to the different way of carrying out the competition reaction. Indeed, in batch heterogeneous immunoassays, the antibody is immobilized and the competition takes place on a surface, whereas in the capture immunosensor, this process is performed in the bulk of the solution, and this could result in an improvement of the sensitivity

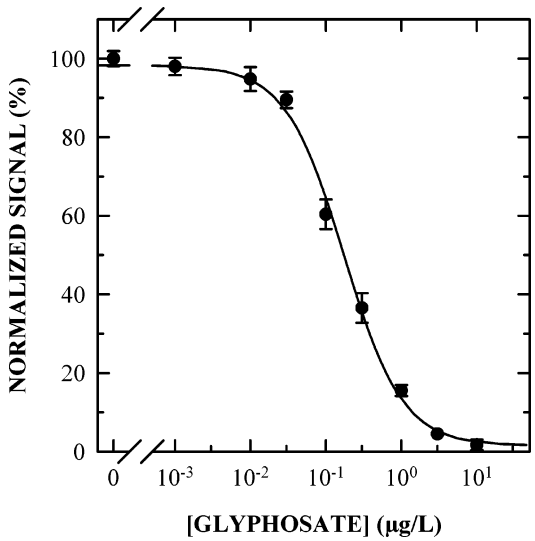

Figure 2. Competition curve for glyphosate in TBS, pH 8.9. Each point represents the mean ± SD of four replicates.

in the competition. This has been observed in the past for other analytes.[37] Compared to the published[29] detection limits of chromatographic methods after sample preconcentration (0.02 μg/L), the sensitivity of this immunosensor is very good. The extreme sensitivity of the glyphosate sensor allows its measurement in drinking water, according to the EU guidelines, without sample preconcentration.

Another quality parameter of the immunosensor is its autonomy and working life. Because all the basic operations—not taking into account the sample pretreatment—are carried out automatically, the autonomy of the immunosensor depends only on the stability of reagents. In this sense, it is helpful to employ stabilizers in the antibody and tracer solutions, since these reagents have biological activity and are prone to degradation. The employment of the protein BSA for the antibody dilution and Proclin 300 for the tracer has been shown to extend the working life of these reagents in solution, and they can be kept at 4 °C for more than 10 days without loss of properties (fully operative for more than 3 days when kept at room temperature). The reagent that limits the autonomy of the immunosensor system is the derivatization solution, since it is prone to hydrolysis caused by humidity. When preparing the derivatization reagent under argon atmosphere and if it is protected from moisture, its shelf life is longer than 48 h; therefore, this is considered to be the immunosensor autonomy. Regarding the working life of the protein A/G immunosurface, reactors could be used for at least 500 assay cycles. This agrees with the reusability of protein A/G surface observed in previous works,[38,39] since the regeneration conditions are always the same.

Assay precision was estimated by measuring replicates of spiked water and soil extract samples. W-1 water sample was spiked with glyphosate at 0.1 μg/L and analyzed 10 times, with a final result of 0.10 ± 0.03 μg/L. For a spiking level of 1.0 μg/L, the result of 10 replicates was 1.2 ± 0.2 μg/L. In soils, S-2 sample was analyzed as native by diluting the extract 1/24 v/v with TBS. The final result (12 replicates) indicated that no glyphosate was detected (percentage of normalized signal 100 ± 3). A different sample extract, from soil S-12, was spiked at 7.5 μg/L and analyzed (9 replicates) after 1/24 v/v dilution, giving as a result 7.9 ± 0.6 μg/L. The same extract was also spiked at 25 μg/L, diluted 1/99 and analyzed (12 replicates), obtaining a result of 19.5 ± 1.5. It can be appreciated from these results that precision is, in general, acceptable, with CV values lower than 20% in most cases, which is comparable to those obtained in published immunoassays for glyphosate,[32,35] although higher than data obtained using chromatography.[29]

Selectivity. Usually, the study of the selectivity in competitive immunoassays is performed by determining the cross-reactivity between the analyte and structurally related compounds, and this analytical property is considered "acceptable" or "good" when cross-reactivity values for molecules similar to the analyte are low (<10% and <1%, respectively). Table 2a shows the cross-reactivity for some phosphonomethyl herbicides, the glyphosate metabolites aminomethyl phosphonic acid (AMPA), sarcosine, and other phosphorus-containing compounds; amino acids; organophosphorus pesticides; and herbicides from other families that are used together with glyphosate in herbicide formulations. It is clear that the immunosensor is highly selective for glyphosate, since the cross-reactivity values are in all cases lower than 0.1%. The selectivity is due to the recognition capacity of the antibody for the derivatized glyphosate and also due to the derivatization reaction, which is very effective for glyphosate in the working conditions (pH around 9) but does not affect at all any molecule lacking the amine group or can be less effective for other chemicals in these conditions.

The absence of cross-reactivity does not mean that the immunosensor is free of other interferences, since a study of the matrix effect reveals the opposite. When testing the effect of naturally occurring chemicals such as anions, cations, humic acids or inorganic pollutants, it has been observed that some of these species can act as potential interfering agents (Table 2b) and can lead to a loss of immunosensor performance. Some of the substances tested, such as silicate, triphosphate, Ca^{2+}, arsenate, or borate, are not expected in water or soil extracts at the interfering levels, but the presence of the others can be troublesome. Furthermore, the effect of the interfering species is not only a decrease in assay sensitivity but also a signal decay, and this can result in false positive results for samples in the absence of analyte but containing those interfering agents. In this sense, an in-depth study of the interference and its possible occurrence is to be carried out.

The most critical case is the phosphate anion, whose interference at a concentration higher than 50 mg/L can be due to its similar structure to the phosphate ion at pH 8.9 (monohydrogenphosphate HPO_4^{2-}), and the phosphonic moiety of glyphosate at the same pH ($^-OOC-CH_2-NH-CH_2-PO_3H^-$). Depending on the country, drinking water may have a maximum phosphate content of 1 mg/L or below, whereas raw sewage water contains, on an average, ~30 mg/L. Therefore, levels of phosphate anions found in most waters are lower that the interfering value, and the interference of phosphate is negligible except from those of contaminated waters.

Another interfering anion is the hydrogencarbonate HCO_3^-, whose interference could be due to a collateral interaction between the nucleophilic N atom from glyphosate and the electrophilic C atom from HCO_3^-, thus inhibiting the derivatization reaction. In fact, it has been observed that the presence of HCO_3^- and SO_4^{2-},

Table 2. Interferences

(a) specific interferences

compd	I_{50} (μg/L)	CR[a] (%)
AMPA[b]	10 000	0.002
gluphosinate	>10 000	<0.002
glyphosine	1000	0.02
fosetylaluminum	5000	0.004
sarcosine	>30 000	<0.0006
CEPA[c]	600	0.03
phosphonoacetic acid	10 000	0.002
methylphosphonic acid	10 000	0.002
glycine	>100 000	<0.0002
lysine	>20 000	<0.001
aspartic acid	10 000	0.002
atrazine	>10 000	<0.002
simazine	>2000	<0.01
terbuthylazine	>800	<0.025
alachlor	>3000	<0.006
2,4-D	>10 000	<0.002
organophosphorus insecticides[d]	>5000	<0.004

(b) matrix effect

interfering agent	noninterfering concentration[e]	effect on assay performance[f]
phosphate (PO_4^{3-})	50	signal decay, loss of sensitivity[g]
sulfate (SO_4^{2-})	100	loss of precision
arsenate (AsO_4^{3-})	200	signal decay
borate ($B_2O_7^{4-}$)	200	signal increase, loss of sensitivity
silicate ($Si_3O_7^{2-}$)	200	signal decay, loss of sensitivity
hydrogencarbonate (HCO_3^-)	125	signal decay, loss of sensitivity
triphosphate ($P_3O_{10}^{5-}$)	1000	loss of sensitivity
aluminum(III)	10	signal increase, loss of sensitivity
copper(II)	0.1	signal decay, loss of sensitivity
calcium chloride ($CaCl_2$)	1000	signal decay, loss of sensitivity
humic acid	1	loss of sensitivity

[a] Cross-reactivity. [b] Aminomethylphosphonic acid. [c] 2-Carboxyethylphosphonic acid. [d] Diazinon, chlorpyrifos and fenthion individually assayed. [e] Concentrations in mg/L. [f] Using higher interfering agent concentration. [g] Loss of sensitivity means higher I_{50} value in competition.

as well as other anions and cations, alters the phytotoxic activity of glyphosate.[42,43] Because this anion can be found in water at levels around 200−300 mg/L and it interferes at concentrations higher than 125 mg/L, it should be eliminated or masked prior to analysis.

A study to eliminate HCO_3^- by means of adding HCl to the sample until pH 3−3.5; further degassing by sonication for 10 min; and finally, pH and ionic strength setting, was carried out. This procedure was effective for HCO_3^- concentration as high as 300 mg/L, which is enough for most natural waters, and did not affect the glyphosate content in the sample, since this chemical is very stable in acidic media.

The sulfate anion is also a potential interfering agent, since levels higher than 200 mg/L lead to signal variation. This interference could also be due to a misfunction in the derivatization reaction caused by the electrophilic S atom. Although it is not usual to find sulfate in drinking water at levels higher than 200 mg/L (see Table 1 a), concentrations as high as 300−400 mg/L can be found in natural waters that have been in contact with gypsum or other minerals containing sulfate. Trials for eliminating or masking sulfate were carried out. The most promising was precipitation with Ba^{2+}, Sr^{2+}, and Ca^{2+} added as chloride salts, but no satisfactory results were obtained. The addition of Ba^{2+} at concentrations higher than 100 mg/L also caused a marked interference; the use of Sr^{2+} also caused interference, most likely due to the very high amount of this cation (1000 mg/L) required to separate the sulfate.

Other species, such as Al(III)[44] and Cu(II)[45], are also studied because they cause interference at low concentrations, 10 and 0.1 mg/L, due to the binding of the analyte to these chemicals. Experiments were conducted to eliminate or minimize these interferences. We tried to mask Al(III) and Cu(II) by adding fluoride (0.1 M) and diethylenetriaminopentaacetic acid (0.01 M), respectively, but although the effect of Al(III) was lowered, the interferences were not removed at all.

Humic substances[46] are also a potential interference due to the low concentration that affects the assay (1 mg/L) and their occurrence in natural waters. Furthermore, humic acids bind to the analyte, but it can also bind the antibody.[47] In fact, humic acids are always a serious problem in glyphosate determination. Sample filtration through activated carbon (Norit SA Super, Norit, Amersfoort, The Netherlands) was performed to eliminate the influence of humic substances, but as was expected, this also led to a loss of ~40% of the analyte. The interference of humic acids was lowered using a Dionex Ion-Pac NG1 guard column, selected for its capacity to remove organic matter from aqueous samples, even soil extracts. The treatment consisted of injecting through the column 1.5 mL of sample at 1 mL/min, discarding the first 0.5 mL. This treatment allowed us to analyze samples with a final content of humic acid of 4 mg/ L.

In soil analysis, the original alkaline extract (20 mL per 10 g soil) has to be diluted prior to analysis in order to set the analyte concentration into a useful working range as well as to set the pH and ionic strength. This also dilutes the matrix so that if the dilution factor is high enough (1/24 to 1/99 v/v), the interferences from the chemicals above-mentioned are, thus, bypassed, taking into account that many of the interfering ions (phosphate, carbonate, sulfate, and copper) are precipitated in the alkaline medium employed in the extraction process. The only interference that could be troublesome could come from humic substances found in soils containing high amounts of organic matter, thus requiring a special cleanup step.

Application to Real Samples. The immunosensor for glyphosate was applied to a set of spiked and unspiked water and soil samples, treated as described in the Experimental Section.

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Table 3. Analysis of Spiked Water Samples; Recoveries

(a) sample W-1 at different levels

level added	level found[a]	recovery (%)
0	<LOD	
0.05	0.048 ± 0.010	96
0.10	0.110 ± 0.030	110
0.25	0.240 ± 0.040	96
0.50	0.610 ± 0.007	122
0.75	0.930 ± 0.130	124

(b) different water samples

sample	level added	level found[a]	recovery (%)
W-2	0.07	0.069 ± 0.007	99
	0.70	0.660 ± 0.070	94
W-3	0.07	0.073 ± 0.005	104
	0.70	0.601 ± 0.012	86
W-4	0.07	0.070 ± 0.020	100
	0.70	0.730 ± 0.110	104
W-5	0.07	0.075 ± 0.008	107
	0.70	0.794 ± 0.014	113
W-6	0.07	0.076 ± 0.012	109
	0.70	0.830 ± 0.020	119
W-7	0.07	0.080 ± 0.006	114
	0.70	0.763 ± 0.009	109
W-8	0.07	0.083 ± 0.008	119
	0.70	0.62 ± 0.05	89
W-9	0.07	0.080 ± 0.020	114
	0.70	0.690 ± 0.020	99
W-10	0.07	0.083 ± 0.008	119
	0.70	0.760 ± 0.070	109

[a] All concentrations in μg/L. LOD limit of detection.

The study started by spiking a water sample (W-1, see composition in Table 1) at different levels and analyzing each subsample in triplicate. As is shown in Table 3a, acceptable recoveries were obtained at all levels from 0.05 to 0.75 μg/L, which indicates the suitability of the immunosensor for the determination of glyphosate in water, provided that matrix interfering agents are absent. The recovery study for water was extended to all samples (W-2 to W-10), first unspiked and after spiking at two levels (0.07 and 0.7 μg/L). Analysis was performed after eliminating hydrogencarbonate from samples by degassing (see above). All unspiked samples gave analyte values lower than the limit of detection (0.023 μg/L). The absence of false positives indicates the suitability of the immunosensor for screening or automatic analysis.

On the other hand, results of analyses with spiked samples are shown in Table 3b. Recovery values are, in general, excellent for the low analyte levels determined, with all values between 80 and 120% and nearly all values between 90 and 115%. It should be pointed out that when these analyses were repeated on samples without eliminating HCO_3^- (data not shown), results from samples W-5, W-7, and W-8 were poor, with recoveries higher than 150%, which confirms the interfering effect of hydrogencarbonate in the sample and the suitability of the degassing method for its elimination.

In soils, recovery studies were carried out at different dilution factors of the extract to assess the influence of this variable. With this goal, 16 soil samples were extracted (see Experimental Section), and aliquots were spiked and then diluted 1/24 and 1/99 v/v prior to analysis. All extracts were analyzed as native and fortified at levels of 7.5 and 30 mg/L so that the analyte concentration after dilution was 0.3 μg/L. Results of triplicate analyses, expressed as the amount of glyphosate in the original soil, are shown in Table 4. It is remarkable that results obtained after 1/99 v/v dilution are, in general, much better than those obtained with a lower dilution factor, which is to be expected, since the soil matrix (humic substances, cations, anions, etc.) is also diluted, and its interferences are weaker. Furthermore, important interferences that could be carbonate and sulfate are negligible in practice because carbonates present in soils (whose presence can be deduced from the pH in water and $CaCl_2$ columns in Table 1b) and sulfate are insoluble in the alkaline medium employed in the extraction. On the other hand, samples with no analyte added and giving detectable amounts of glyphosate corresponded in nearly all cases to organic matter content higher than 10 g/kg, so these data could be the result of the presence of interfering humic substances in the extract, rather than a glyphosate residue in the native soil sample. Briefly, the application of a 1/99 v/v dilution leads to good recoveries in all cases, except for one sample (S-9). It is clear that the application of a higher dilution factor of the extract involves a loss of sensitivity in analyte determination in the original sample, but because of the good sensitivity of the method, it is able to measure glyphosate concentrations at the submicrogram-per-liter level, allowing us to employ a dilution factor as high as 1/99, v/v, and to measure the original glyphosate amounts in soil in the 10−100 μg/kg range. This can be considered very low due to the high quantities of this herbicide that are dispensed in weed treatments, since the dosages are in the range from 84 (standard) to 380 (massive) mg/m^2 soil,[48] which is equivalent to 0.3−1.5 mg/kg.

Finally, to assess the suitability of the method for practical applications, a comparison study was performed. Representative spring water samples as well as soils (all different from those employed in recovery studies and totally unknown) were analyzed (native and spiked) as blind samples by the immunosensor as well as by the magnetic particle immunoassay and HPLC, each methodology in a different laboratory. Prior to immunosensor analysis, all water samples were treated as described above so as to eliminate the hydrogencarbonate interference, while soil extracts were all diluted 1/99 v/v. The results of the determinations with the three techniques are shown in Table 5.

For water samples, acceptable correlation is found when comparing sensor data to the reference, ELISA, and HPLC values, except for the W-A sample, which shows remarkable deviations

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Table 4. Analysis of Spiked Soil Extracts; Recoveries[a]

sample	native (1)[b]	spiked (1)[c]	recovery (%)	native (2)[d]	spiked (2)[e]	recovery (%)
S-1	<LOD	16 ± 3	106.7	<LOD	61 ± 2	101.7
S-2	<LOD	8 ± 5	53.3	<LOD	63 ± 2	105.0
S-3	<LOD	4 ± 5	26.7	<LOD	68.7 ± 0.7	114.5
S-4	<LOD	9 ± 2	60.0	<LOD	66 ± 2	110.0
S-5	<LOD	6.5 ± 0.6	43.3	<LOD	69 ± 4	115.0
S-6	n.a.[f]	n.a.[f]		19 ± 9	67 ± 7	80.0
S-7	n.a.[f]	n.a.[f]		6 ± 2	56 ± 7	83.3
S-8	10.9 ± 0.2	24.9 ± 0.9	93.3	7 ± 3	57 ± 3	83.3
S-9	52 ± 18	200 ± 150	986.6	13 ± 2	98 ± 6	141.7
S-10	6.6 ± 0.4	23.0 ± 0.1	109.3	6 ± 4	65 ± 9	98.3
S-11	6.70 ± 0.08	19.6 ± 0.4	86.0	<LOD	77 ± 8	128.3
S-12	<LOD	15.6 ± 0.3	104	<LOD	58 ± 2	96.7
S-13	13.6 ± 0.8	45 ± 3	209.3	11 ± 2	79 ± 5	113.3
S-14	22 ± 8	640 ± 50	4120	4 ± 3	65 ± 14	101.7
S-15	<LOD	61 ± 8	406.7	<LOD	54 ± 4	90.0
S-16	13.2 ± 1.8	80 ± 8	445.3	6.8 ± 1.2	65.5 ± 0.6	97.8

[a] Results expressed as μg glyphosate per kg soil. All data are the mean ± SD of three replicates. [b] Unspiked extract diluted 1/24 v/v. LOD 3.6 μg/kg soil. [c] Extract spiked at 7.5 μg/L (equivalent to 15 μg/kg soil) and diluted 1/24 v/v. [d] Unspiked extract diluted 1/99 v/v. LOD 5.2 μg/kg soil. [e] Extract spiked at 30 μg/L (equivalent to 60 μg/kg soil) and diluted 1/99 v/v. [f] Not analyzed.

Table 5. Analysis of Blind Samples. Comparison with HPLC and ELISA

(a) water samples[a]					(b) native soil extracts[b]				(c) spiked soil extracts[b]				
sample	spiking level	MPIA[c]	sensor	HPLC	sample	MPIA[c]	sensor	HPLC	sample	spiking level	MPIA[c]	sensor	HPLC
W-A	1.00	0.58 ± 0.07	1.45 ± 0.09	0.41 ± 0.08	S-A	60 ± 5	44 ± 14	50 ± 6	S-A*	200	250 ± 30	240 ± 30	240 ± 20
W-B	0.04	0.10 ± 0.03	0.03 ± 0.02	0.04 ± 0.01	S-B	6.1 ± 0.7	<LOD	<LOD[d]	S-B*	8	10.3 ± 0.5	7.6 ± 1.8	<LOD[d]
W-C	0.20	0.28 ± 0.08	0.19 ± 0.06	0.20 ± 0.06	S-C	17 ± 2	<LOD	16.4 ± 0.7	S-C*	100	128 ± 8	130 ± 30	99 ± 8
W-D	0.00	<LOD	<LOD	<LOD	S-D	<LOD	<LOD	<LOD[d]	S-D*	40	35 ± 3	18 ± 4	37 ± 3
W-E	0.10	0.14 ± 0.02	0.09 ± 0.01	0.11 ± 0.03	S-E[e]	270 ± 20	320 ± 50	300 ± 30					
W-F	0.50	0.40 ± 0.05	0.59 ± 0.04	0.35 ± 0.04	S-F	<LOD	<LOD	15.6 ± 0.6	S-F*	20	28.2 ± 0.7	20 ± 9	34.4 ± 0.9

[a] Prior to analysis, water samples were brought to pH 3−3.5 with HCl and sonicated for 10 min for removal of HCO_3^-. Results expressed in μg/L for the mean ± SD of three replicates. [b] Prior to immunosensor and ELISA analysis, extracts were diluted 1/99 (v/v) with assay buffer. Results expressed in μg/kg soil for the mean ± SD of three replicates. [c] Magnetic particles immunoassay. [d] LOD for HPLC in soils 10 μg/kg. [e] Soil labeled S-E was found to be contaminated with glyphosate when being analyzed by the sensor, and the analysis was repeated (result listed in Table 5b is, therefore, the mean ± SD of six replicates). HPLC and ELISA analyses, also performed in duplicate (3 replicates each) confirmed the contamination. This extract was not spiked for further analysis.

between the three methods and from its spiking value. No explanation has been found for the behavior of sample W-A; maybe the presence of interfering species such as humic substances can initiate the difference in this sample result. All the methodologies have indicated that no glyphosate contamination was found in these water samples, since the values found during the analysis corresponded well to the spiked values.

In soil samples, the sensor is able to detect a low glyphosate contamination in the S-A sample (44 μg/kg) and a high one (300 μg/kg) in the S-E soil, both corroborated by HPLC and ELISA analyses. Other samples show no contamination in practice, and results from spiked samples are in agreement with added levels of analyte. In all cases, acceptable correlation between the sensor and both comparison methods is obtained. These results show the suitability of the immunosensor for monitoring glyphosate in medium to low organic matter content soils with minimum sample treatment.

Finally, is should be mentioned that the rapidity of response is optimal when using the immunosensor, since the result is achieved after 25 min of sample introduction, lower than batch immunoassay (more than 60 min) or HPLC (more than 45 min). The sampling throughput could be improved by using a multichannel device, and it has been estimated that 10 results/h can be achieved with a four-channel parallelized manifold. However, immunoassay seems to be best in terms of time per analysis when a large number of samples are to be processed, while chromatography is recommended for confirmation or when both glyphosate and its metabolite AMPA are to be monitored.

CONCLUSIONS

Immunosensing with capture format can be considered as a reliable tool for the rapid and highly sensitive determination of glyphosate in water and soil with minimum sample preparation. The derivatization reaction has been demonstrated to be highly effective at very low concentrations of glyphosate, with a high yield and in aqueous medium. The employment of protein A/G support in the immunocomplex capture format, as well as good-featured immunoreagents and analyte derivatization reaction, leads to a system with very high sensitivity and selectivity for glyphosate and that is able to measure automatically the presence of this herbicide at very low levels. Interferences that could be troublesome due to their occurrence can be reduced in some cases by means of a simple treatment. Analytical comparison on real samples between the immunosensor and other technologies, such as ELISA and HPLC, shows good correlations at very low analyte levels. For the first time, an instrumental technique alternative to

reference chromatographic methodology for glyphosate is available. The rapidity of response and the full automation of the system show the possibility of employing it in field analysis in an advantageous manner when compared to chromatographic methods.

The immunosensor could be very helpful in the rapid analysis of water and medium to low organic matter content soil samples for the control of glyphosate, since it could avoid the tedious and expensive chromatographic determination of this analyte in a large number of samples. This work is, to our knowledge, the first time that a sensor system for glyphosate is reported. The immediate result of this work is the application of the sensor to determine glyphosate in vegetables, especially those grown with no-till farming.

Another issue to be addressed in the future is the use of chemiluminescent signals, which can improve its sensitivity and portability even further, leading to a simpler manifold. In this sense, the whole equipment (fluidics, electronics, and detector) has already been built in a portable compact device.

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SUPPORTING INFORMATION AVAILABLE

The manifold scheme and signal/cylces graph are available as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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