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Comparison of a Direct ELISA and an HPLC Method for Glyphosate Determinations in Water

FERNANDO RUBIO,[‡] LINDA J. VELDHUIS,[†] B. STEPHEN CLEGG,[§] JAMES R. FLEEKER,[#] AND J. CHRISTOPHER HALL*,[†]

Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada, N1G 2W1, ABRAXIS LLC, Northampton Business Center, 54 Steamwhistle Drive, Warminster, Pennsylvania 18974, Laboratory Services Division, University of Guelph, Guelph, Ontario, Canada, N1H 8J7, and Biochemistry Department, North Dakota State University, Fargo, North Dakota 58105

A competitive direct enzyme-linked immunosorbent assay (ELISA) and high-pressure liquid chromatographic (HPLC) methods were compared in terms of accuracy and precision for the detection and quantification of glyphosate-spiked Nanopure, tap, and river waters. The ELISA had a detection limit of 0.6 ng mL^{-1} and a linear working range of 1−25 ng mL^{-1}, whereas the HPLC method had a detection limit of 50 ng mL^{-1} and a linear working range of 100−10000 ng mL^{-1}. No statistically significant differences (95% confidence interval) were found between the ELISA and HPLC analysis of the three water matrixes. The coefficients of variation obtained with the ELISA in tap water were between 10 and 19%, whereas the coefficients of variation for the HPLC analysis were between 7 and 15%. The use of ELISA for the analysis of glyphosate in water is a cost-effective and reliable method capable of meeting water quality guidelines established for Europe and North America.

KEYWORDS: Glyphosate; enzyme-linked immunosorbent assays; high-pressure liquid chromatography

INTRODUCTION

Glyphosate (*N*-(phosphonomethyl) glycine), commonly sold under the trade name Roundup, is a nonselective herbicide that inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSP) in the shikimic acid pathway. Inhibition of this enzyme results in depletion of aromatic amino acids such as phenylalanine, tyrosine, and tryptophan (*1*). Glyphosate translocates readily in the plant, making it effective for controlling perennial weeds and overwintering rhizomes and tubers. It is registered for preplant or postharvest treatment in crops and on noncrop land (*2*). Glyphosate is extremely safe in the environment since it binds to soil colloids and is rapidly degraded by soil microbes.

The use of glyphosate by agriculturalists has grown over the years with the introduction of transgenic crops such as Roundup-Ready Soybeans which offer benefits such as broad spectrum weed control and low-cost weed control. However, the continued use of glyphosate raises the potential for residue accumulation in water and crop commodities. The maximum residue limit (MRL) concentration for food crops has been set at 0.1 μg mL^{-1} by both the Canadian Food and Drug Act as well as the U.S. Food and Drug Regulations (*3*). In the United States, glyphosate concentration for most crops is set at $\leq$ 0.2 μg g^{-1} (*4*). The Canadian Drinking Water Guideline is recommended at a maximum level of 0.28 μg mL^{-1}, whereas the level determined for freshwater aquatic life is 0.065 μg mL^{-1}. The maximum contaminant level for drinking water in the United States is 0.70 μg mL^{-1} (*5*). In the European Union, the maximum admissible level for glyphosate in drinking water is 0.1 ng mL^{-1} (*6*).

Glyphosate is persistent in soil with a half-life of 47 days (*7*) and strongly adsorbs to suspended organic and mineral particles in water (*8*, *9*). Glyphosate analysis in environmental matrixes is problematic because it is a small molecule and has structural similarity to many naturally occurring plant materials such as amino acids and secondary plant compounds. It is highly soluble in water thereby making its extraction with solvents difficult. Therefore, glyphosate isolation and quantitation poses a challenge to the analytical chemist due to the necessity of removing matrix effects before analysis. Plant and soil matrixes contain co-contaminants that render analysis more costly and time-consuming. Seiber et al. (*10*) used anion and cation exchange column chromatography and gel permeation chromatography for glyphosate analysis in soil. Analysis in water is typically done using conventional methods, such as high-presssure liquid chromatography (HPLC) with precolumn (*11*) and postcolumn derivatization (*9*, *12*, *13*) or by gas chromatography using electron capture (*14*), flame photometric (*15*), mass spectrometric (*16*, *17*), or nitrogen−phosphorus detectors (*18*). The detection limits achieved using these methods are generally higher than those typically obtained using enzyme-linked immunosorbent assays for most herbicides.

[†] Department of Environmental Biology, University of Guelph.
[‡] ABRAXIS LLC.
[§] Laboratory Services Division, University of Guelph.
[#] North Dakota State University.
* Corresponding author: J. Christopher Hall, Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1, Tel. (519) 824-4120 x2740, Fax. (519) 837-0442, email: jchall@evb.uoguelph.ca.

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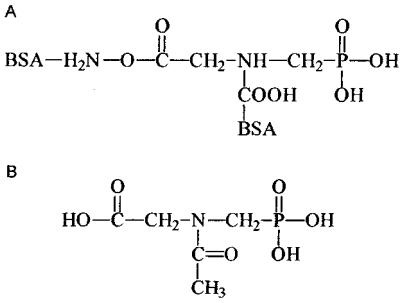

Figure 1. Structure of glyphosate bound to BSA (immunogen) (A) and acetylated glyphosate (B).

The enzyme-linked immunosorbent assay (ELISA) is a valuable tool in residue analysis and complements conventional analytical methods (*19*, *20*). ELISAs have been routinely used for the quantitative analysis of numerous pesticides in water with little or no matrix effects (*21*, *22*). An indirect immunosorbent assay for the detection and quantitation of glyphosate at levels as low as 7.6 μg mL^{-1} has been described by Clegg et al. (*13*). In this paper, we describe a direct ELISA for glyphosate that has a limit of detection (LOD) 500-fold lower than HPLC methods (*13*). The limit of detection was obtained by derivatizing the sample before ELISA lowering the detection and quantitation to the parts per trillion level (ng L^{-1}) in water (**Figure 1**). In contrast to time-consuming high-pressure-liquid chromatographic and gas chromatographic methods, enzyme-linked immunosorbent assays provide a sensitive, cost-effective, and efficient method for the analysis of environmental water samples potentially contaminated with glyphosate.

MATERIALS AND METHODS

Reagents and Instrumentation. Glyphosate, glyphosine, and glufosinate analytical standards were obtained from Chem Service (West Chester, PA). Rabbit anti-glyphosate serum (J. C. Hall laboratory) was prepared as described previously (*13*). Other reagents used were as follows: glyphosate-horseradish peroxidase (glyphosate-HRP) conjugate (Abraxis LLC, Warminster, PA); goat anti-rabbit antiserum, glutaraldehyde, (aminomethyl)phosphonic acid (AMPA) (Sigma-Aldrich, Milwaukee, WI); 3,3′,5,5′-tetramethylbenzidine, peroxidase substrate (BioFX Labs, Owing Mills, MD); amine-terminated superparamagnetic particles (Bangs Labs, Fishers, IN); bovine serum albumin (BSA; Serologicals, Kankakee, IL). All other chemicals were of reagent grade and obtained commercially. The photometric analyzer consisted of a microprocessor-based discrete wavelength photometer designed to measure absorbance through standard 12 × 75-mm test tubes with data transformation capabilities (Abraxis LLC, Warminster, PA). The magnetic separator consisted of a test tube holder that fits over a magnetic separation rack containing permanent rare earth magnets (Abraxis LLC, Warminster, PA).

Anti-Glyphosate Magnetic Particle Preparation. Antibody-coupled magnetic particles were prepared as described by Weston & Avrameas (*23*) and Rubio et al. (*21*) by activating the magnetic particles with glutaraldehyde and using the following modifications to these methods: goat-anti-rabbit antiserum was diluted to 5 mg of protein/mL in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, as estimated by absorbance at 280 nm. Diluted antiserum was mixed with magnetically concentrated and activated particles at a ratio of 1:1. Activated magnetic particles were reacted with goat anti-rabbit antiserum overnight at room temperature while the mixture was shaking. The covalently coupled anti-rabbit particles were washed and diluted to a working concentration with water containing 0.15 mol L^{-1} Tris/0.15 mol L^{-1} NaCl/0.1% bovine serum albumin (BSA)/0.001 mol L^{-1} ethylenediaminetetraacetic acid adjusted to pH 7.4. Rabbit anti-glyphosate antibody was added to the diluted goat anti-rabbit magnetic particles at a dilution of 1:25000 and incubated for 2 h.

Standard Curve and Sample Analysis. Calibration standards and water samples containing glyphosate and various organic and inorganic compounds used in the various studies were prepared by acetylation with acetic anhydride using the method of Cailla et al. (*24*) prior to ELISA. Derivatized samples were analyzed (assay procedure 1) by mixing 250 μL of sample, 250 μL of enzyme conjugate, and 500 μL of diluted anti-glyphosate particles in test tubes, and incubating at room temperature for 30 min. The reaction mixture was separated using the magnetic separator and washed twice with 1 mL of water. The color reaction was allowed to develop for 20 min at room temperature following addition of 500 μL of TBM substrate/chromogen solution, and quenched by the addition of 0.5 mL of 0.5% sulfuric acid.

To increase assay sensitivity, the protocol was modified (assay procedure 2) as follows: 500 μL of diluted anti-glyphosate particles were added to a test tube, the particles were pulled to the sides of the tube using the magnetic separator, and the supernatant was decanted. A total of 750 μL of the derivatized sample and 250 μL of the enzyme conjugate were added and incubated at room temperature for 30 min. The rest of the assay procedure was performed as described above. Photometric analysis of the final colored reaction was performed at a wavelength of 450 nm. Concentrations of glyphosate in the samples were determined by comparing the observed absorbance to a linear regression log−linear standard curve prepared from calibration standards containing known concentrations of glyphosate (0, 1.0, 5.0, and 25.0 ng mL^{-1}).

Cross-Reactivity. Cross-reactivity of the ELISA to a variety of agrochemicals was tested. Glyphosine [*N*,*N*-bis(phosphonomethyl)-glycine] and glufosinate 4-[hydroxy(methyl)phosphinoyl]-DL-homoalanine, two related herbicides, and AMPA, a metabolite of glyphosate, were a few of the tested compounds. Serial dilution of each agrochemical was prepared in distilled water starting at 1000 μg mL^{-1} and tested in the assay. The 50% absorbance inhibition (50% B/B_o), cross-reactivity (%), and least detectable dose (LDD) for each of the compounds were determined.

Testing for Interference. To study the effect of ions or other compounds found in surface waters on the ELISA, 0.1 ng mL^{-1} to 10 000 ng mL^{-1} of the following solutions were prepared in distilled water: calcium, copper, magnesium, nitrate, sodium, fluoride, phosphate, sulfate, humic acid, sodium chloride, and hydrochloric acid. The effect of methanol and acetone on assay performance was examined. Samples were tested neat and with the addition of 2.5 ng mL^{-1} of glyphosate.

Study for Variability. To test the variability between the beginning and end of an assay, two samples were run in two assays with 52 replicates, for a total assay batch of 60 tubes (including standards). The glyphosate concentration of the samples were chosen to cover the range of the assay. One of the samples had a nondetectable concentration, while another sample contained a 25 ng mL^{-1} concentration of glyphosate.

HPLC Study. Glyphosate was extracted from water using a cation exchange analytical column and hydrolyzed in a reactor coil at 36 °C (*25*) with sodium hypochlorite to form glycine. The glycine was then reacted with *o*-phthalaldehyde (OPA) in the presence of thiofluor in a second coil (55 °C) to produce a fluorescent isoindole, which was detected fluorometrically (λex = 330 nm, λem = 465 nm). All glyphosate standards were prepared in distilled water (dH_2O) and serially diluted in potassium dihydrogen phosphate buffer (K200) to the concentration range required for analysis. Water samples were filtered through 0.45 μm nylon filters and stored at 4 °C until analyzed. A volume of 30 μL of standard and/or sample was injected into the HPLC, which had a mobile phase of K200 and a flow rate at 0.4 mL min^{-1}. The mobile phase was isocratic for 17 min, followed by column regenerant for 2 min finishing the chromatographic run with an 11 min continuation of K200 mobile phase. Peak areas of the standards were plotted against the concentration of glyphosate, and the resulting standard curve was used to estimate glyphosate concentrations present in the water samples.

Correlation Study. Spiked water samples were prepared using serial dilutions of a 1000 μg mL^{-1} glyphosate standard. The samples used for the HPLC were 50, 100, 150, 200, 250, and 1000 ng mL^{-1} prepared in the three water types (Nanopure, tap and river). The enzyme-linked

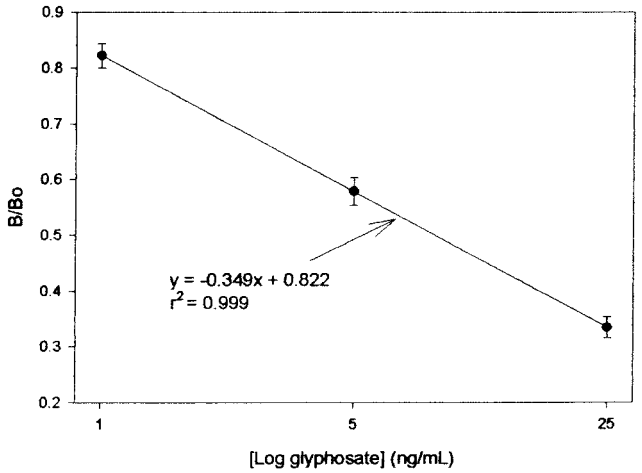

Figure 2. Standard curve for derivatized glyphosate. Each point represents the mean of 24 determinations. Vertical bars indicate ± 1 SD about the mean.

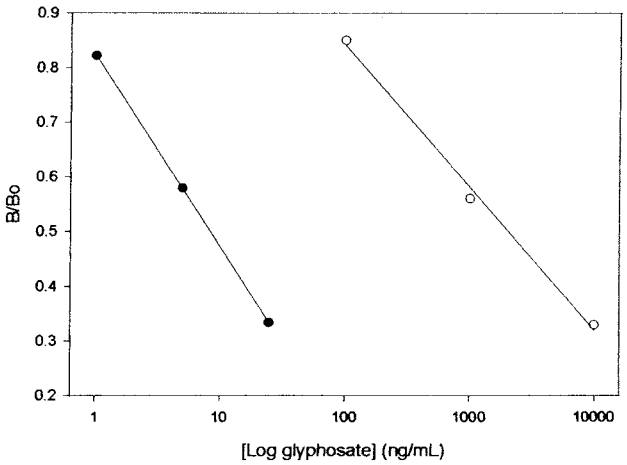

Figure 3. Effect of chemical derivatization on glyphosate assay sensitivity. Immunoassay performed following derivatization with (●) acetic anhydride or without (○) chemical derivatization of calibrators.

immunosorbent-assay-spiked samples were serially diluted to 0, 0.5, 1, 2, and 5 ng mL^{-1} from the standards used for the HPLC analysis.

RESULTS AND DISCUSSION

Standard Curve and Sensitivity. Dose−response data for glyphosate calibrators were collected over 30 days and represent 23 replicates; the mean standard curve is shown in **Figure 2**. At 1.0 ng mL^{-1} significant inhibition (18%, i.e., $1 - B/B_o$) by glyphosate of antiserum binding was observed. The limit of detection (LOD) was 1 ng mL^{-1} and least detectable dose (LDD) was estimated as 0.6 ng mL^{-1} at 90% B/B_o (mean absorbance value for the standard divided by the mean absorbance value for the zero standard) (*26*). The LOD was defined as the lowest glyphosate standard to have a B/B_o value of 10 standard deviation units greater than B_o. The LDD was defined as the lowest glyphosate standard to have a B/B_o 3 times greater than the standard deviation of B_o (*27*).

Low molecular weight compounds such as glyphosate are not immunogenic and do not elicit an immune response. To obtain antibodies to such compounds, one must couple them to a larger molecular weight molecule (carrier) such as bovine serum albumin (**Figure 1**). The binding site of antibodies raised against the low molecular weight compound (hapten) conjugated to the larger molecular weight carrier may also include recognition of the chemical linker. One way to increase the affinity of an antibody for a low molecular weight compound is to convert the hapten in the sample into a derivative that mimics the immunogen by the chemical addition of a reagent identical or similar to the spacer or chemical linker (*28*). Derivatization of analytes has been shown to increase immunoassay sensitivity (*24*, *28*−*30*). Derivatization of samples with acetic anhydride allowed us to increase the sensitivity of the assay by about 100 fold, from 100 ng mL^{-1} (**Figure 3**) to 1.0 ng mL^{-1}. The sensitivity of the method is consistent with the National Primary Drinking Water Regulation Maximum Contaminant Level Goal (MCLG) of 700 ng mL^{-1} (*4*), and also the concentration of 280 ng mL^{-1} recommended by Health and Welfare Canada in the *Canadian Water Quality Guidelines*. With a slight modification of the assay procedure (assay procedure 2), an LOD of 0.1 ng mL^{-1} was obtained, which is consistent with the European Union maximum admissible concentration (MAC) of 0.1 ng mL^{-1}.

Precision. The results from the precision study in which tap water samples were spiked with glyphosate at four concentrations and each measured five times in duplicate on five different days are shown in **Table 1**. The within- and between-day variations were estimated by the method of Bookbinder and Panosian (*31*). The coefficients of variation were between 1 and 20%.

Table 1. Precision of Glyphosate Concentration Estimates in Water as Determined with a Direct ELISA

	ng mL^{-1}			
sample[a]	1.25	2.50	5.0	20.0
replicates/day	5	5	5	5
days	5	5	5	5
N	25	25	25	25
mean (ppb)	1.45	2.90	5.75	17.05
%CV (within assay)	19.0	14.1	12.4	10.2
%CV (between assay)	15.3	11.5	1.2	9.7

[a] Glyphosate fortified tap water samples. Samples were assayed in replicates of 5 assays per day over 5 days.

Table 2. Accuracy of Glyphosate Concentration Estimates in Water using ELISA[a]

amount of glyphosate added (ng mL^{-1})	mean (ng mL^{-1})	SD (ng mL^{-1})	recovery (%)
2.50	2.30	0.15	92
5.00	5.10	0.75	102
10.0	10.20	0.75	102
20.0	18.30	1.65	92
mean			97 ± 1

[a] Water samples individually fortified at the above concentrations were assayed three times in duplicate using the immunoassay. All unfortified samples assayed had glyphosate concentrations less than the detection limit of the assay.

Accuracy. Known amounts of glyphosate were added to five water samples obtained locally. The samples included a municipal water source, drinking water from a well, and samples from a pond, a small creek, and a river. The accuracy was assessed by analyzing the samples before and after the addition of glyphosate, and then subtracting the estimated concentration of glyphosate obtained before spiking. The five water samples tested were free of glyphosate. On average, 97% of added glyphosate was recovered (**Table 2**). There was no statistical difference between the water types. The datum presented is the mean obtained from all water samples.

Specificity. In determining the specificity of the assay, it is important to examine the potential for structurally related

Table 3. Cross-Reactivity of the ELISA to Various Agrochemicals

chemical	LDD[a] (ng mL^{-1})	50% B/B_0 (ng mL^{-1})	cross-reactivity (%)
glyphosate	0.12	1.65	100
glyphosine	350	20000	<0.01
glufosinate	9500	250000	<0.01
AMPA	440000	1000000	<0.01
glycine	NR[b]	NR	<0.01
aldicarb	NR	NR	<0.01
acetochlor	NR	NR	<0.01
alachlor	NR	NR	<0.01
atrazine	NR	NR	<0.01
ametryn	NR	NR	<0.01
benomyl	NR	NR	<0.01
butylate	NR	NR	<0.01
captan	NR	NR	<0.01
carbaryl	NR	NR	<0.01
carbendazim	NR	NR	<0.01
carbofuran	NR	NR	<0.01
cyanazine	NR	NR	<0.01
2,4-D	NR	NR	<0.01
1,3-dichloropropene	NR	NR	<0.01
dinoseb	NR	NR	<0.01
MCPA	NR	NR	<0.01
metolachlor	NR	NR	<0.01
metribuzin	NR	NR	<0.01
pentachlorophenol	NR	NR	<0.01
picloram	NR	NR	<0.01
propazine	NR	NR	<0.01
sarcosine	NR	NR	<0.01
simazine	NR	NR	<0.01
terbufos	NR	NR	<0.01
thiabendazole	NR	NR	<0.01
thiopanate-methyl	NR	NR	<0.01

[a] Least detectable dose (90% B/B_0). [b] NR = no response.

compounds, metabolites, and/or other agrochemicals to inhibit glyphosate antiserum binding. Studies of glyphosate degradation have shown that degradation is primarily by microbial action, and there is only one significant soil metabolite (i.e., AMPA; *8*). The AMPA metabolite is considered to be nonpersistent (*18*) because AMPA levels initially increase as the concentration of glyphosate decreases; following this initial increase of AMPA its concentration decreases. AMPA and two structurally related herbicides, glyphosine and glufosinate, were tested for cross-reactivity. The cross-reactivity data are summarized in **Table 3**. The assay is specific for glyphosate with virtually no cross-reactivity (<0.001%) against the "phosphonomethyl" compounds and other agrochemicals tested.

Matrix Effects. The effects on the immunoassay of various inorganic and organic contaminants often found in water were determined by adding them to distilled water (**Table 4**). The assay is not affected by methanol or acetone at concentrations as high as 10%. The presence of calcium, copper, magnesium, nitrate, sodium fluoride at 10 000 μg mL^{-1}, phosphate and sulfate at 100 μg mL^{-1}, and humic acid at 1 μg mL^{-1} also had no effect on the glyphosate immunoassay. Hydrochloric acid above 0.25 N caused some interference, indicating that samples preserved with acid should be neutralized prior to evaluation of samples using the ELISA.

Study of Variability. Immunoassays are usually run in a batch format that consists of a set of standards, controls, and the samples. The amount of time needed to complete all pipetting steps depends on the number of samples being analyzed. Due to the time saved for pipetting all the samples at once, differences among data obtained may occur at the beginning versus the end of a sample que. With this in mind, a well-optimized immunoassay should exhibit minimal variation

Table 4. Effect of Various Ions and Organic Compounds on the ELISA for Glyphosate

compound	concentration[a] (ng mL^{-1})	recovery (%)
calcium chloride	10000	97
calcium sulfate	100	97
copper chloride	5000	104
humic acid	1000	98
magnesium chloride	10000	115
magnesium sulfate	100	91
sodium chloride	10000	89
sodium fluoride	10000	91
sodium nitrate	10000	87
sodium phosphate	100	97
acetone	<10%	103
methanol	<10%	99
hydrochloric acid	0.5 N	82

[a] Highest compound concentration (ng mL^{-1}) not causing assay interference. Interference is defined as recovery outside 100 ± 20%.

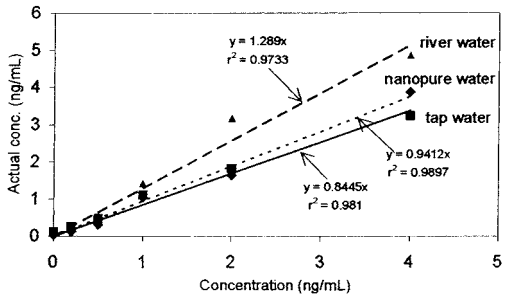

Figure 4. Comparison of the three spiked water matrixes (Nanopure water (◆), tap water (■) and river water (▲)) using the glyphosate CI-ELISA.

in analyte concentration from the beginning to the end of assay. The data obtained suggest minimal drift in the ELISA, since the sample with nondetectable concentration (0 ng mL^{-1}) was not detected at any point during the assay. Furthermore, a slope of 0.026 was obtained with the higher concentration of sample (25 ng mL^{-1}), which means that on a 60-tube assay the difference (ng mL^{-1}) between beginning and end of the assay would be a maximum increase of 6.2%.

Comparison Study. Three different water types were compared using the ELISA. The concentration range used (0, 0.5, 1.0, 2.0, 4.0 ng mL^{-1}) is within the range of the assay (**Figure 4**). The statistical comparison of spiked tap and Nanopure water showed no significant differences between estimations in these types of water samples. Furthermore, there was no difference among estimates made in the three water types up to 1.0 ng mL^{-1}. However, at the two higher concentrations, 2.0 and 4.0 ng mL^{-1} there were differences between estimates made in river water and those made in tap or Nanopure water as determined using the Tukey test ($P \leq 0.05$). This difference may be the result of matrix effects in river water.

A comparison of two different analytical techniques, HPLC and ELISA, demonstrates the usefulness of the glyphosate immunoassay (**Figure 5**). Regardless of the water type, there was no difference between the two methods as determined using the Tukey's test ($P \leq 0.05$). To compare the results of the two different analytical methods (ELISA vs HPLC) at different concentration ranges, the ELISA concentration values were corrected to fall within the detection range of the HPLC method.

CONCLUSIONS

The development of a competitive direct enzyme-linked immunoassay for glyphosate is a useful and effective tool that

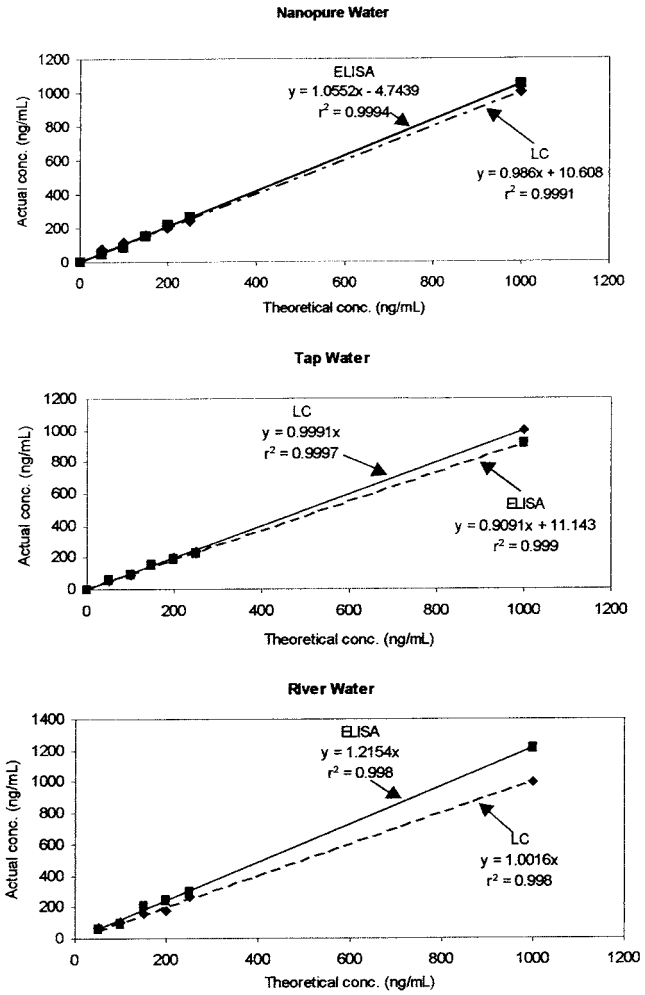

Figure 5. Glyphosate-spiked water samples (Nanopure, tap, river) were analyzed using CI-ELISA (■) and HPLC (◆).

will enable the analysis of glyphosate in water. The accepted allowable residue levels of glyphosate as specified by the water quality guidelines in Europe and North America can be detected with this cost-effective and efficient ELISA method. The ELISA has been shown to be as effective as HPLC but to have a lower limit of detection. Furthermore, potential matrix effects from river water had little or no effect on the ELISA results when compared to the HPLC method.

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