# Development of an Enzyme-Linked Immunosorbent Assay for the Detection of Glyphosate

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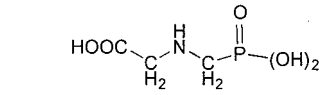
A competitive indirect enzyme-linked immunosorbent assay (CI-ELISA) was developed to quantitate the herbicide glyphosate [*N*-(phosphonomethyl)glycine] in water. The ELISA has a detection limit of 7.6 $\mu$g mL$^{-1}$ and a linear working range of 10−1000 $\mu$g mL$^{-1}$ with an $IC_{50}$ value of 154 $\mu$g mL$^{-1}$. The glyphosate polyclonal antisera did not cross-react with a number of other herbicides tested but did cross-react with the glyphosate metabolite aminomethylphosphonic acid and a structurally related herbicide, glyphosine [(*N*,*N*-bis(phosphonomethyl)glycine]. The assay was used to estimate, quantitatively with accuracy and precision, glyphosate concentrations in water samples. Water samples were analyzed directly, and no sample preparation was required. To improve detection limits, water samples were concentrated prior to analysis, resulting in the increase of the detection limits by 100-fold. After the sample preconcentration step, the detection limit improved to 0.076 $\mu$g mL$^{-1}$ with an $IC_{50}$ value of 1.54 $\mu$g mL$^{-1}$, and a linear working range was 0.1−10 $\mu$g mL$^{-1}$. Glyphosate concentrations determined by ELISA correlated well with those determined by high-pressure liquid chromatography ($r^2 = 0.99$). This assay contributes to reducing the costs associated with conventional residue analysis techniques for the quantitation of glyphosate in water.

**Keywords:** *Glyphosate; ELISA; phosphonomethylglycine; AMPA; herbicide; residue; HPLC; postcolumn derivatization; analysis; immunoassay*

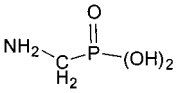
## INTRODUCTION

Glyphosate [*N*-(phosphonomethyl)glycine, Figure 1] is the active ingredient in a number of commercial herbicides produced by Monsanto, Cheminova, and Zeneca Corp. In Ontario, Canada, various formulations are scheduled for use that include products with the trade names Clear It, Expedite, Eject, Glyfos, Laredo, Renegade, Roundup, Touchdown 480, Vision, and Wrangler. Glyphosate is a nonselective herbicide used for weed control in a variety of agricultural crops, preplant or postharvest with no cropping restrictions (Ontario Ministry of Agriculture, Food and Rural Affairs, 1998). Over the past 15 years, the use of the herbicide glyphosate in the Province of Ontario has increased dramatically. For example, in the years 1978, 1988, and 1993, glyphosate usage has been 0.5, 3.1, and 10%, respectively, of the total kilograms of all herbicides used in Ontario (Ontario Ministry of Agriculture, Food and Rural Affairs, 1978, 1988, 1993). Glyphosate is a member of the amino acid herbicide family, and its mode of action is through inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSP), an enzyme of the shikimic acid pathway. This enzyme is important in the biosynthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan. A blockage of the shikimic acid pathway leads to a depletion of the free pool of aromatic amino acids in higher plants (Duke, 1988).

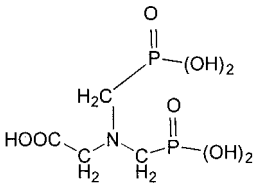
Over the past decade, glyphosate has become increasingly important in the area of "herbicide tolerant" transgenic crops. In 1997, glyphosate tolerant soybeans were the most common transgenic crop grown on large acreages in North America including other herbicide tolerant crops such as maize, cotton, and canola (James, 1997). Some benefits associated with the use of these crops include less herbicide requirements, better weed control, increased crop yield, better soil moisture conservation, more flexibility in agronomic management, and no carry-over of herbicide residues. As the use of transgenic plants increases, there will be interest in potential glyphosate residues. Although initial application rates of glyphosate in these crops are lower, the




Glyphosate (N-phosphonomethylglycine)




AMPA (aminomethylphosphonic acid)




Glyphosine (N,N bis-phosphonomethylglycine)

**Figure 1.** Structures of glyphosate, AMPA (metabolite), and glyphosine used in the synthesis of immunogens and cross-reactivity studies.

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increase in overall glyphosate use may contribute residues in crop commodities.

Currently, glyphosate is registered under the Pesticide Control Products Act (PCP) in Canada as a preplant or postharvest treatment for the control of annual and perennial weeds in crops and on noncrop land. Under the PCP Act, it is registered as a preharvest treatment of selected crops, for control of perennial grasses, and for crop desiccation. As a result, maximum residue limits (MRLs) have been established under the Canadian Food and Drug Regulations for barley, soybeans, peas, wheat, lentils, flax, and barley and wheat milling fractions excluding flour. The maximum residue permitted on other crops is considered to be negligible at 0.1 ppm (Food and Drugs Act, 1993). Glyphosate is moderately persistent in soil (Dibyendu et al., 1989) with an average half-life of 47 days and is readily adsorbed to most soils (Wauchaupe et al., 1992). In water, primary degradation is by microbes and glyphosate is strongly adsorbed to suspended organic and mineral matter (Rueppel et al., 1977; Thompson et al., 1989). The Canadian Drinking Water standard has been established at 0.28 $\mu$g mL$^{-1}$ (ppm), whereas the maximum contaminant level (MCL) for safe drinking water in the United States is 0.70 $\mu$g mL$^{-1}$ (ppm).

A number of approaches have been taken with respect to the analysis of glyphosate and its major metabolite, aminomethylphosphonic acid (AMPA). It has been demonstrated that AMPA is the only major metabolite found in plants, water, and soil (Sprankle et al., 1978). Both the parent herbicide and its AMPA metabolite are polar and very water soluble, and as a result have limited solubility in organic solvents. Glyphosate and AMPA are also structurally similar to many naturally occurring plant materials such as amino acids and small sugars; hence, it is difficult to extract and isolate them from any complex plant or soil matrices. The challenge for the analytical chemist involves overcoming these problems and ensuring the accuracy of the analytical method with an emphasis on keeping the method as simple as possible.

Original methods for detection of glyphosate involved analysis by thin-layer chromatography (TLC) (Sprankle et al., 1978; Young et al., 1977), whereas later methods involved gas chromatographic (GC) techniques after chemical derivatization (Guinivan et al., 1982). These methods are very tedious, time-consuming, and difficult, with poor and inconsistent recoveries. The extraction and isolation of residues from plant or soil matrices are difficult and often involve anion and cation exchange column chromatography and gel permeation chromatography (GPC) (Seiber et al., 1984). Multiple derivatization techniques were often used, and this tended to make the methods more difficult and time-consuming. Derivatization techniques are often adapted to facilitate determination and quantitation of glyphosate and its (AMPA) metabolite using selective detectors including flame photometric (Moye and Deyrup, 1984), electron capture (Eberbach and Douglas, 1991), mass spectrometric (Deyrup et al., 1985; Mogadati et al., 1996), and nitrogen−phosphorus detectors (Roy and Konar, 1989).

As an alternative to determination by gas−liquid chromatography (GLC), a number of methods have been published using high-pressure liquid chromatography (HPLC). The HPLC methods involve use of precolumn derivatization (Lundgren, 1986) or postcolumn derivatization techniques (Thompson et al., 1989; Cowell et al., 1986). These methods are also difficult and require extensive sample preparation and expensive or elaborate analytical equipment. An HPLC method (postcolumn fluorescence detection) for the analysis of glyphosate and its (AMPA) metabolite in waters has been collaboratively studied (Oppenhuizen and Cowell, 1991). More recently, an automated on-line HPLC fluorescence method for the analysis of glyphosate and AMPA was demonstrated (Papadopoulou et al., 1998). Another method involving postcolumn fluorescence detection was presented for various crops (Wigfield and Lanouette, 1991), and still another method showed the robustness of analysis of glyphosate in seeds and water samples using negative ion electrospray LC/MS (Startin and Hird, 1998).

The enzyme-linked immunosorbent assay (ELISA) is recognized as a valuable tool in residue analysis and compliments conventional analytical methods (Johnson and Hall, 1996; Parnell and Hall, 1998). ELISA provides rapid sample testing and accurate results and is more cost-effective than conventional chromatographic analysis (Hall et al., 1990). The sensitivity and specificity of the technique have made it useful in a variety of situations such as fate and persistence studies, environmental residue analysis, worker exposure studies, spray-tank rinse checks, and plant hygiene studies. ELISAs have been used successfully for the quantitative analysis of numerous pesticides in water matrices with little or no matrix interference (Rubio et al., 1991; Lawruk et al., 1994).

Currently, there is no immunochemical analytical method for the detection and quantitation of glyphosate. In contrast to expensive and time-consuming HPLC and GC methods, ELISA may provide a sensitive, cost-effective, and efficient method for analyzing environmental samples containing glyphosate. In this paper, we report the quantitative performance of an indirect ELISA for glyphosate detection and quantitation in water.

## MATERIALS AND METHODS

**Chemicals and Instrumentation.** The analytical standard of glyphosate (Figure 1) was obtained from Monsanto Co. (St. Louis, MO), and $^{14}$C-radiolabeled glyphosate with a specific activity of 316 $\mu$Ci mg$^{-1}$ (54 mCi mmol$^{-1}$) was purchased from Amersham Life Science (Buckinghamshire, U.K.). Freund's incomplete adjuvant, 2,2-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid diammonium (ABTS) substrate tablets, *N*,*N*′-dicyclohexylcarbodiimide (DCC), and urea hydrogen peroxide were obtained from Sigma Chemical Co. (St. Louis, MO). Goat anti-rabbit horseradish peroxidase (GARHRP) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide−HCl (EDAC) was purchased from Pierce Immunochemicals (Rockford, IL). Immulon 4 flat-bottom microtiter plates were purchased from Fisher Scientific (Don Mills, ON, Canada). ELISA plates were analyzed using a Model 3550-UV microplate reader from Bio-Rad Laboratories (Richmond, CA). The HPLC system consisted of a Varian 9010 dual solvent delivery pump with a Varian 9095 auto-sampler. A Pickering PCX 5000 postcolumn reaction system was used coupled to a Varian 9070 fluorescence detector. The analytical and guard columns were cation exchange columns of 4-mm i.d. × 150 mm, 8 $\mu$m $K^+/H^+$, form and 3 mm i.d. × 20 mm, 8 $\mu$m $K^+/H^+$, respectively. All other chemicals were of reagent grade and obtained commercially.

**Synthesis of Immunogens and Coating Conjugates.** The limited solubility of glyphosate in organic solvents (e.g., 1,4-dioxane) made it impossible to synthesize the immunogens and coating conjugates using the Fleeker method (1987); therefore, an alternate synthetic route was devised. Briefly, the glyphosate−bovine serum albumin (BSA) immunogen was

prepared by dissolving the acid form of glyphosate (0.019 mM) in 7.5 mL of 0.1 M 4-morpholineethanesulfonic acid (MES) buffer and, similarly, 200 mg of BSA was dissolved in 5.0 mL of 0.1 M MES buffer. The two solutions were added to 39 mg (0.019 mM) of EDAC and allowed to react for 2 h at room temperature. The solution containing the glyphosate−BSA (gly−BSA) conjugate was dialyzed (Spectrapor 1; 6000−8000 MW cutoff) for 24 h at 4 °C against four 1 L changes of distilled water. It was determined by use of [$^{14}$C]glyphosate that a total of 15 glyphosate molecules were bound to each molecule of BSA. The solution containing the conjugate was frozen in 500-$\mu$L aliquots. The other immunogen [gly−keyhole limpet hemocyanin (KLH)] was prepared according to the method of Yao and Mahoney (1989). A suspension of KLH was centrifuged at 1400*g* for 5 min. The supernatant was removed and the pellet transferred to a dialysis tube and dialyzed overnight against 3 × 1 L of PBS. To the solution was added gluteraldehyde to a final concentration of 0.2% (v/v). The KLH gluteraldehyde was dialyzed overnight against 3 × 1 L of PBS, and then glyphosate was added and the reaction was allowed to continue for 2 h. The glyphosate KLH solution was dialyzed overnight against 3 × 1 L of PBS, and it was determined by use of [$^{14}$C]-glyphosate that 54 molecules of glyphosate were bound to each molecule of KLH. Two coating conjugates of glyphosate (gly−PSA; gly−OVA) were synthesized by conjugating glyphosate to porcine serum albumin (PSA) and ovalbumin (OVA), respectively. The synthetic route was similar to that used for the gly−BSA immunogen described previously.

**Polyclonal Antisera Production.** For each of the two immunogens, gly−BSA and gly−KLH, a pair of New Zealand white rabbits were injected, intramuscularly, with 500 $\mu$g of immunogen in 1.0 mL of a 10 mM phosphate-buffered 15 mM NaC1 (PBS) pH 7.5/Freund's incomplete adjuvant (1:1 v/v) emulsion. The primary immunizations were repeated at weekly intervals for 3 weeks, and following a 4-week rest period, secondary immunizations (boosts) of immunogen (200 $\mu$g) in 1.0 mL of PBS and Freund's incomplete adjuvant (1:1, v/v) were injected intramuscularly every 3 weeks. The rabbits were bled monthly and the sera tested for anti-glyphosate antibody 1 week after each secondary boost.

**Antisera Titer Determination.** Glyphosate-specific antisera titers were monitored as described by Campbell (1984) and Gee et al. (1988). Checkerboard binding studies between glyphosate antiserum and glyphosate coating conjugates were used to determine the optimal dilution of each. The optimal dilutions chosen were those that provided an absorbance of 1.0−1.2 at 405 nm, following an incubation of 30 min at room temperature (22 °C).

**Standard Curve and Sample Analysis.** The ELISAs were performed as follows. Immulon 4 flat-bottomed plates were coated with 100 $\mu$L of coating conjugate per well (original coating conjugate of 0.2 mg mL$^{-1}$ diluted 1/500000 in PBS ) and allowed to incubate overnight at 4 °C. The plates were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS−Tween) and patted dry on paper towels. Sites not containing coating conjugate were blocked with 200 $\mu$L well$^{-1}$ of 0.01% gelatin in water (w/v). After a 20 min incubation, the plates were washed and dried as previously described. Anti-glyphosate antibody was diluted 1/5000 with PBS (optimal predilution). Following this dilution, glyphosate standards or samples containing glyphosate were prepared in PBS. The antisera and sample or standard solutions were mixed 1:1 (v/v) and allowed to incubate in test tubes for 60 min. The final antiserum dilution (optimal dilution) was 1/10000. The pre-incubated mixtures were transferred to the plates (100 $\mu$L per well). The plates were incubated for another 60 min at 22 °C in the dark before being washed with PBS−Tween and dried. The next step involved the addition of the secondary antibody, GARHRP. The optimal dilution was 1/5000 in PBS, and 100 $\mu$L was added to each well and allowed to incubate at 22° C for 60 min. The wells were then washed as described earlier. The next step was the addition of 100 $\mu$L of substrate [1 mg L$^{-1}$, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium] and 1.0 mg mL$^{-1}$ urea hydrogen peroxide in 10 mM citric acid/10 mM sodium phosphate (citrate buffer, pH 9.0). The reaction proceeded for 30 min and was stopped with 0.5 M citric acid (100 $\mu$L well$^{-1}$).

**Standard Curve.** The absorbance at 405 nm is inversely proportional to the concentration of glyphosate in the standards and samples. To normalize the absorbance, the background absorbance was subtracted from each value followed by division of each value by the positive control (0 ng mL$^{-1}$ glyphosate). The standard curves were constructed by plotting the normalized absorbance values ($A/A_0$) against the log values of the glyphosate concentration. A commercial computerized graphing program (SigmaPlot) was used for data analysis and presentation. Glyphosate concentrations in the water samples were interpolated from the standard curve.

**Cross-Reactivity.** A variety of agrochemicals were tested for cross-reactivity to the glyphosate antisera. Glyphosine [*N*,*N*-bis(phosphonomethyl)glycine], a related herbicide, and AMPA, a metabolite of glyphosate, were also tested for cross-reactivity. A number of other structurally related smaller molecules were also tested for their cross-reactivity. A 1000 $\mu$g mL$^{-1}$ (ppm) standard of each agrochemical was prepared using distilled water and tested against the glyphosate antibodies. The $IC_{50}$, percent cross-reactivity, and least detectable dose for each of the compounds were determined.

**High-Pressure Liquid Chromatography (HPLC).** Glyphosate and AMPA were separated and detected by HPLC. After HPLC separation using a cation exchange analytical column, both compounds were hydrolyzed in a reactor coil at 36 °C 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 highly fluorescent isoindole, which was detected fluorometrically ($\lambda_{ex}$ = 330 nm, $\lambda_{em}$ = 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-$\mu$m nylon filters and stored refrigerated until analyzed. A volume of 30 $\mu$L of standard and/or sample was injected into the liquid chromatograph, which had a mobile phase of K200 with a flow rate set at 0.4 mL min$^{-1}$. The total chromatographic run was 30 min.

The mobile phase of K200 was isocratic for 17 min, column regenerant (potassium hydroxide: RG019) 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 interpolate glyphosate concentrations in the water samples.

**Water Concentration.** All water samples (100 mL) were filtered through 0.45-$\mu$m nylon filters prior to being concentrated to dryness on a rotary evaporator with a water bath at 50 °C. The samples were reconstituted in 1.0 mL of PBS. This represented a 100× concentration for each sample (100 mL of sample in a 1.0 mL final volume). Due to the sensitivity of the HPLC method, sample extracts were diluted prior to analysis.

## RESULTS AND DISCUSSION

**Conjugation of Glyphosate.** Glyphosate is considered to be a small molecule (MW = 169.08 amu) and as such is not immunogenic. To render small molecules such as this immunogenic, it is normal practice to conjugate them to larger protein carriers such as BSA and KLH. Classical conjugation of a hapten to a protein is via a free primary amine or carboxylic acid forming a peptide bond. Glyphosate has a free carboxylic acid and a secondary amine in addition to a phosphonomethyl substituent that are available for conjugation to a protein. A search of the literature was unable to provide a suitable conjugation technique for use on the phosphonomethyl terminal end. Conjugation through the secondary amine to the protein was not successful because monitoring the conjugation reaction with radiolabeled [$^{14}$C]glyphosate revealed that sufficient co-

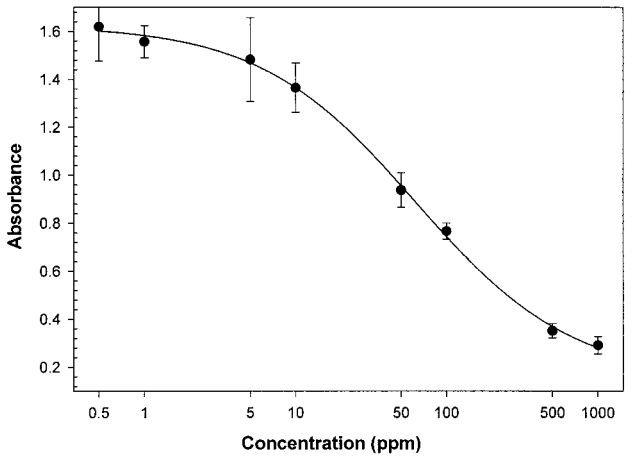


**Figure 2.** Plot of glyphosate concentration in parts per million versus the normalized absorbance. The line presented is fit to a logistic parameter curve, $r^2 = 0.9988$ with a coating conjugate (CC) dilution of 1/500000.

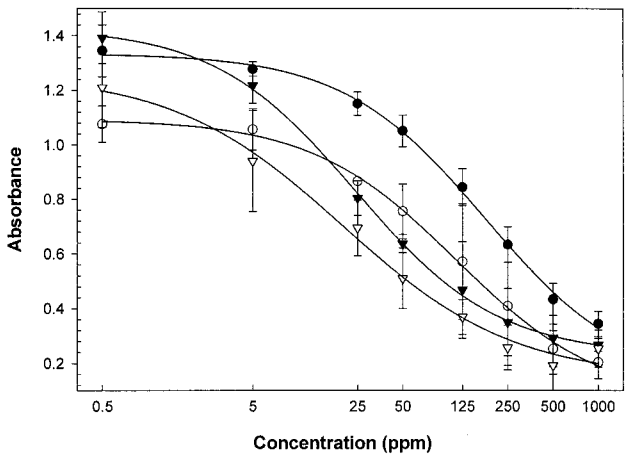


**Figure 3.** Effect of coating conjugate (CC) dilutions on the sensitivity of inhibition of the glyphosate ELISA. Corresponding concentrations and $r^2$ are given [(▼) CC 1/500000, $r^2 = 0.9992$; (●) CC 1/100000, $r^2 = 0.9982$; (▽) CC 1/1000000, $r^2 = 0.9912$; (○) CC 1/200000, $r^2 = 0.9997$] for each curve.

valent attachment was not achieved. Hence, the conjugation of glyphosate to the protein was directed through the carboxylic functional group. The use of [$^{14}$C]glyphosate in these conjugation reactions showed covalent attachments (epitope densities) of 14 and 54 glyphosate molecules to BSA and KLH, respectively.

**ELISA Optimization.** A representative inhibition curve for glyphosate derived from checkerboard binding studies is given in Figure 2. Initially, the checkerboards indicated that dilutions of 1/10000 and 1/50000 were optimal for serum and coating conjugates, respectively. However, competitive inhibition studies using these concentrations failed to provide significant inhibition with standard solutions of glyphosate, and inhibition of glyphosate was observed in the 500−10000 ppm range. The lack of sensitivity of this assay is not surprising, and often assays involving very small molecules are sensitive only in the parts per million range. A previous assay for glyphosate (Hammock et al., 1989) had sensitivities at higher levels in the range of 10−1000 M (1690−169000 ppm), and the assay was also very sensitive to pH. As observed in immunoassays for a variety of other pesticides (Goh et al., 1990; Koppatschek, 1990; Kido, 1997), this assay for glyphosate was sensitive to matrix effects such as ionic strength, pH, and solvent concentration of the final sample solutions. The optimum pH for the assay was determined to be 7.7, and the assay was found to tolerate solvent (methanol) concentrations as high as 10%. By proper monitoring of solvent concentration in the final sample solution, especially after a concentration step, the sensitivity of the assay was improved. The assay was found to be more sensitive when the final sample solvent (methanol) concentration was held to a maximum of 5%. It was found during further optimization of the assay that serial dilutions of the coating conjugate (gly−OVA) in PBS or distilled water dramatically increased the sensitivity of the assay. The sensitivity of the assay was found to be maximized when the coating conjugate was diluted further from 1/100000 to 1/1000000 (Figure 3). It was anticipated that the original 1/100000 dilution of coating conjugate could be stored at 4 °C and used to prepare the next dilutions; however, the 1/100000 solution was found to be unstable during short storage times (Figure 4). The sensitivity of the assay decreased dramatically when the solution was stored for short periods of time ranging from 18 to

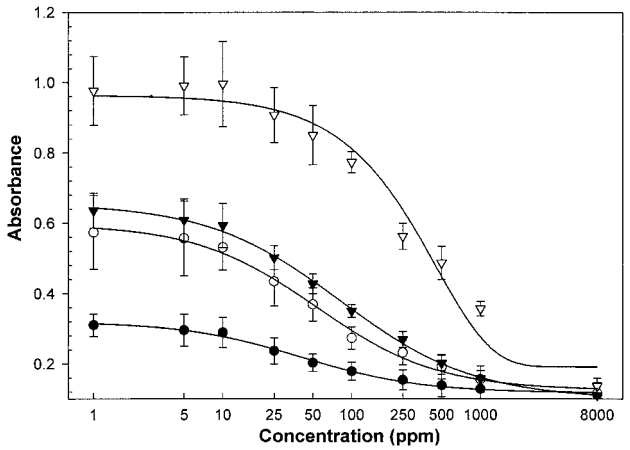


**Figure 4.** Stability of the 1/100000 dilution of coating conjugate versus time. The 95% confidence interval is indicated for four replicate analyses. The number of days are (▽) 0, (▼) 18, (○) 21, and (●) 31.

31 days. Therefore, serial dilutions of coating conjugates were prepared each time plates were coated.

The relatively low sensitivity of this assay can be attributed to a number of factors, which include the type of antibodies and the small size of the parent molecule. In assay development, it is recognized in most situations that those developed with monoclonal antibodies are often more sensitive than those developed with polyclonal antibodies. Two indirect immunoassays for the herbicide picloram were compared, and the linear working range for poly- and monoclonal assays were 5−5000 and 1−200 ng mL$^{-1}$, respectively. The $IC_{50}$ values were 140 and 10 ng mL$^{-1}$ for the two respective assays (Deschamps et al., 1990). A similar improvement in the sensitivity of this assay may be possible if a monoclonal assay or recombinant antibody were developed.

The overall immunoassay performance can be altered by factors that include hapten structure, addition of immunogen spacer group, type of coating conjugate, and assay format (Wie and Hammock, 1984; Deschamps and Hall, 1991). The introduction of phage display technology along with the development of recombinant-antibody technology has resulted in assays being developed for environmental contaminants such as diuron (Karu et al.,1994), atrazine (Ward et al., 1993), parathion

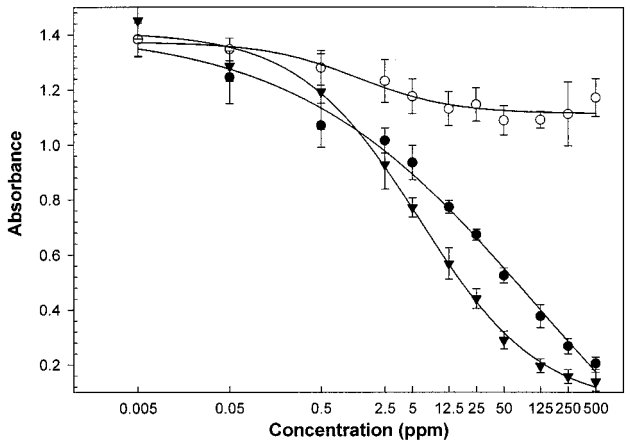


**Figure 5.** Inhibition curves for the cross-reactivity of (●) glyphosate, (○) AMPA, and (▼) glyphosine with polyclonal glyphosate serum.

(Garrett et al., 1997), *s*-triazines (Kramer and Hock, 1996), cyclohexanediones (Webb et al., 1997), and dioxin (Lee et al., 1998). The difficulties involved in the development of an assay that is extremely sensitive to glyphosate may be overcome by use of these technologies. Recently, these "third-generation" antibodies have been shown to be useful for the development of assays for the herbicide picloram; this assay was demonstrated to be as sensitive as assays derived from original monoclonal antibodies (Yau et al., 1998). Charlton et al. (1998) estimate that sensitivities of detection of the immunochemical assays may be increased by 10−100-fold if recombinant antibodies are used.

**Cross-Reactivity.** In determining the selectivity of the assay, it is important to examine the potential for inhibition of glyphosate antibodies by structurally related compounds, metabolites, and/or other agrochemicals. Degradation studies of glyphosate have shown that degradation is primarily by microbial action and only one significant soil metabolite (AMPA) is present (Rueppel et al., 1977). The AMPA metabolite is considered to be nonpersistent (Roy et al., 1989) because AMPA levels initially increased then decreased as the concentration of glyphosate decreased. AMPA and a structurally related herbicide, glyphosine [*N*,*N*-bis(phosphonomethyl)glycine], were tested for cross-reactivity. The antibodies produced were specific for glyphosate and showed a greater affinity for glyphosine (more cross-reactive) with limited cross-reactivity to the AMPA metabolite (Figure 5). There was no cross-reactivity (<0.1%) observed for a wide variety of other agrochemicals tested, which included alachlor, metolachlor, metribuzin, cyanazine, tebuthiuron, simazine, prometryn, bromacil, fusilade, 2,4-D, 2,4-DP, 2,4-DB, dicamba, PCP, MCPP, MCPA, picloram, 2,4,5-TP, 2,3,6-TBA, monuron, chloroxuron, triclopyr, and nitrofen. The cross-reactivity to glyphosine was determined to be of no consequence in the analysis of samples because the production of this particular herbicide was for use as a ripener in sugar cane plantations and its production was discontinued in 1984. However, because of the cross-reactivity toward glyphosine, further cross-reactivity studies were conducted with structures similar to glyphosate (Figure 6). None of these compounds were found to show any cross-reactivity toward the glyphosate serum, providing further confirmation of the selectivity of the serum. It is hypothesized that the antibodies in the serum are capable of identifying the "phosphonomethyl" or "phosphonate" moiety when it is associated with the secondary "amino" substituent group. Successive addition of carbons adjacent to the phosphonomethyl group did not facilitate cross-reactivity in the absence of amine functional groups. The addition of two carbon atoms to the amino group (3-aminopropylphosphonic acid) did not show any cross-reactivity. Although the phosphonate moiety was present in the compounds tested for cross-reactivity, there may still be other "phosphonates" present in natural products that may be cross-reactive; therefore, any positive results need to be confirmed by HPLC. Finally, the herbicide glufosinate ammonium was tested and showed no cross-reactivity.

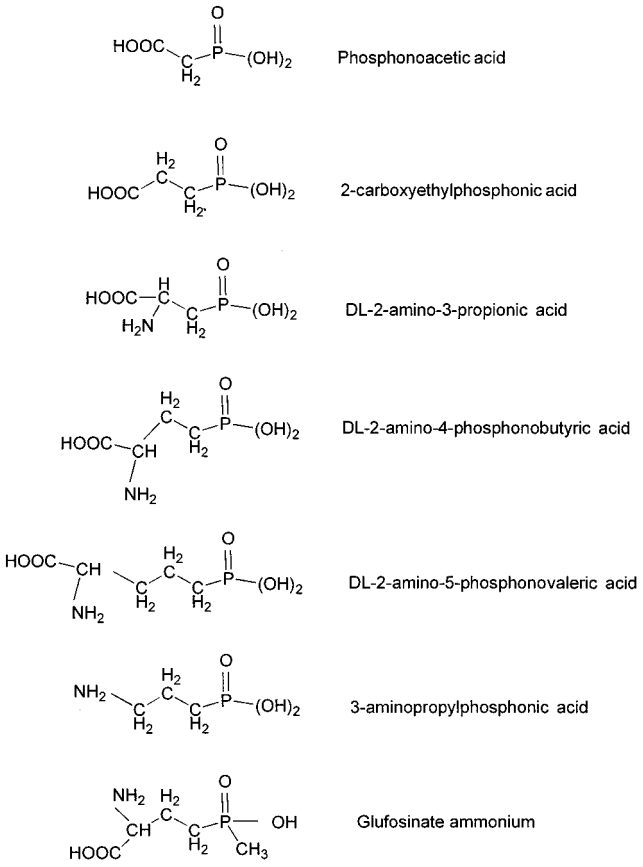


**Figure 6.** Chemical compounds structurally related to glyphosate tested for cross-reactivity in the glyphosate ELISA.

**Fortified Water Samples.** The linear working range of the ELISA was 10−1000 $\mu$g mL$^{-1}$ (Figure 2). The utility of the glyphosate ELISA was determined by a comparison and validation analytical results obtained by HPLC. The recovery of glyphosate from fortified water samples determined by ELISA and HPLC are presented in Table 1. The HPLC method was suitable for the detection of glyphosate in water samples at concentrations ranging from 0.01 to 10000 $\mu$g mL$^{-1}$, and recoveries determined from fortified samples ranged from 104 to 127%. The correlation coefficient for these fortified samples was $r^2 = 0.999$. Because the linear working range of the glyphosate ELISA assay was 10−1000 $\mu$g mL$^{-1}$, a concentration step was required to achieve detection limits similar to that of the HPLC method. With the concentration step, glyphosate residues could be determined at or below quantities specified (0.28 $\mu$g mL$^{-1}$) by Health and Welfare Canada in the *Canadian Water Quality Guidelines*. A 100-mL sample of water and/or fortified water was concentrated by evaporation. The final sample volume was 1.0 mL,

**Table 1. Recovery of Glyphosate from Fortified Water Samples Determined by Enzyme Immunoassay Using Polyclonal-Based ELISA and HPLC**

| glyphosate added ($\mu$g mL$^{-1}$) | mean ($\mu$g mL$^{-1}$) | SD ($\mu$g mL$^{-1}$) | CV[a] (%) (replicates) |
|---|---|---|---|
| | ELISA | | |
| 0.01 | 0.0317 | 0.01 | 31.5 (4) |
| 0.10 | 0.332 | 0.104 | 31.3 (4) |
| 1.0 | 2.06 | 0.315 | 15 (4) |
| 10.0 | 17.3 | 3.10 | 17.9 (4) |
| | HPLC | | |
| 0.01 | 0.0104 | 0.00179 | 17.2 (6) |
| 0.05 | 0.0528 | 0.0058 | 11 (6) |
| 0.10 | 0.122 | 0.0127 | 10.4 (3) |
| 0.50 | 0.553 | 0.044 | 7.9 (3) |
| 1.0 | 1.195 | 0.139 | 11.6 (3) |
| 5.0 | 5.85 | 0.152 | 2.6 (3) |
| 10.0 | 10.6 | 0.376 | 3.8 (6) |

[a] Coefficient of variation.

representing a 100× concentration factor for each sample (100-mL sample in a 1.0 mL final volume). Correlation of HPLC and ELISA estimates was $r^2 = 0.99$, and these results indicate the good agreement between the two analytical methods. The slope of the line (1.4) was not equal to 1, indicating that glyphosate values determined by ELISA were greater than those obtained by the HPLC method. One possible explanation for this may be a matrix effect introduced by concentration of the water.

The limit of detection (LOD) of the ELISA was determined to be 7.6 $\mu$g mL$^{-1}$ and was calculated according to methods used by the American Chemical Society (ACS). The LOD was calculated by taking the absorbance of the positive control and subtracting 3 times the standard deviation (SD) of the control blank. The limit of quantitation (LOQ) was calculated in a similar fashion using 10 times the SD and was determined to be 22.7 $\mu$g mL$^{-1}$. A reliable detection limit (RDL) was determined to be 11.8 $\mu$g mL$^{-1}$ using 6 times the SD. The $IC_{50}$, or the concentration of glyphosate required for 50% inhibition of the absorbance of the positive control, was 154 $\mu$g mL$^{-1}$. Subsequent fortification of samples at levels as low as 0.01 ppm (data not shown) indicated the assay was suitable for determining glyphosate at these lower levels. The matrix effect (data not shown) can be eliminated or at least compensated for if unknown samples are run simultaneously with a series of standards (standard curve) in the sample matrix rather than in PBS. A variety of techniques are available that would be useful in improving the detection level and/or the linear working range of the immunoassay. Immunoaffinity chromatography is a technique that has been used favorably in some applications to increase sensitivity and has been shown to be useful for improving the sensitivity of monoclonal-based assays. It is quite likely that this technique may be useful here and would maximize the sensitivity of the assay. The ELISA assay was more rapid than the HPLC method in that multiple samples could be analyzed on a single plate. As many as 40 samples (in duplicate) can be analyzed simultaneously in a few hours, whereas each single HPLC analysis required a minimum of 30 min.

**Conclusions.** The glyphosate competitive indirect ELISA is suitable for the accurate determination of glyphosate concentrations in the range of 10−1000 $\mu$g mL$^{-1}$ (ppm) without sample concentration. A simple concentration step improved the assay and allowed the concentration range to be decreased to 0.1−10 $\mu$g mL$^{-1}$ (ppm). The assay was sensitive to buffer concentration, pH, and organic solvents, and thus each of these conditions must be carefully controlled. The assay was found to be cross-reactive to only one compound, glyphosine [*N*,*N*-bis(phosphonomethyl)glycine]. All other agrochemicals and small structurally related molecules tested in the assay showed no cross-reactivity. The AMPA metabolite of glyphosate showed limited cross-reactivity in this assay. The immunoassay showed a good correlation with conventional HPLC with an $r^2 = 0.99$ and a slope of 1.4. The assay provides rapid sample analysis, thereby decreasing laboratory costs associated with sample preparation, solvent use, and instrument costs.

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