Immunoaffinity Purification of Chlorimuron-ethyl from Soil Extracts Prior to Quantitation by Enzyme-Linked Immunosorbent Assay

Claudia Sheedy and J. Christopher Hall*

Department of Environmental Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

A competitive-indirect enzyme-linked immunosorbent assay (CI-ELISA) was developed to quantify chlorimuron-ethyl in soil. The linear working range of the assay was from 1 to 1000 ng mL^{-1}. The assay had an I_{50} value of 54 ng mL^{-1}, with a limit of detection of 2 ng mL^{-1} and a limit of quantification of 27 ng mL^{-1}. Three soils were extracted using a carbonate buffer (pH 9.0) and the extracts spiked with chlorimuron-ethyl. Because of the effects of coextractants (matrix effects) from soil on the accuracy and precision of the ELISA, immunoaffinity chromatography (IAC) was used to purify chlorimuron-ethyl from soil extracts prior to analysis. The immunoaffinity columns, which had a total binding capacity of 1350 ng of chlorimuron-ethyl mL^{-1} of immunosorbent, were prepared by binding anti-chlorimuron-ethyl antibodies to protein G Sepharose 4B. Although the matrix effects were largely removed using the affinity column, they could be completely removed by first passing the extract through a column containing epoxy-coupled 1,6-diaminohexane (EAH) Sepharose 4B to remove organic acids prior to IAC. Assay sensitivity was increased 100-fold using IAC to purify and simultaneously concentrate chlorimuron-ethyl from soil extracts. The purification strategy (EAH followed by IAC chromatography) removed matrix effects from all three soils and allowed for the accurate quantitation of chlorimuron-ethyl in soil extracts.

Keywords: *Immunoaffinity chromatography; ELISA; residue; soil; sulfonylureas*

INTRODUCTION

Chlorimuron-ethyl (ethyl 2-[[[[(4-chloro-6-methoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]benzoate) is a sulfonylurea herbicide used on soybeans to control many annual broadleaf weeds such as jimsonweed, ragweed, redroot pigweed, and smartweed (*1*). Chlorimuron-ethyl is a low-use-rate herbicide, with application doses typically <40 g of active ingredient (ai) ha^{-1}. This herbicide has been reported to be detrimental to some subsequent rotational crops, due to its persistence in certain types of soils (*2*). Several immunoassays have been developed to monitor the presence of sulfonylurea and closely related triazolopyrimidine herbicide residues in the environment (*3*−*5*). Although sensitive, most of these immunoassays are affected by the presence of co-contaminants (matrix effects) and are unable to provide accurate determination of the herbicide residues in many matrices. Co-contaminants affect the binding of antibodies to the herbicide, causing a shift or a distortion of the standard curve, thereby leading to improper quantitation of the herbicide concentration.

Immunoaffinity chromatography (IAC) has been examined as a possible technique to overcome matrix effects and has proved to be successful in many cases, particularly for the analysis of aflatoxins (*6*), cyclopiazonic acid (*7*), triazines (*8*), phenylureas (*9*, *10*), and polycyclic aromatic hydrocarbons (*11*, *12*). IAC presents several advantages over traditional sample preparation techniques, which include (i) a highly specific method to extract and purify pesticides present in environmental samples at concentrations as low as parts per trillion and parts per billion levels (*13*); (ii) the ability to purify single or multiple analytes by differential elution of the various analytes from the column, depending on the characteristics and cross-reactivities of the antibodies used to build the column (*14*); and (iii) rapid and effective sample pretreatment resulting in a clean sample that can be quantified by high-performance liquid chromatography (HPLC), gas chromatography−mass spectrometry (GC-MS), liquid chromatography−mass spectrometry (LC-MS), or enzyme-linked immunosorbent assay (ELISA) (*15*).

The objectives of the present study were to develop and evaluate a purification strategy to remove matrix effects prior to quantification of chlorimuron-ethyl in soil by a competitive-indirect immunoassay. The purification strategy consisted of epoxy-coupled 1,6-diaminohexane (EAH) chromatography, which removed organic matter from soil extracts, followed by IAC, which extracted and concentrated chlorimuron-ethyl prior to quantification. Polyclonal antibodies for chlorimuron-ethyl were conjugated to protein G Sepharose 4B gel. Similar to other studies with other biologically active chemicals, our findings show that the chlorimuron-ethyl immunoaffinity column effectively removed soil co-contaminants. Details of ELISA development, column preparation, chromatographic behavior, and conditions for regeneration of the column as well as protocols for application of IAC prior to ELISA are presented.

MATERIALS AND METHODS

Chemicals and Materials. [*pyrimidine*-2-^{14}C]Chlorimuron-ethyl (ethyl 2-[[[[(4-chloro-6-methoxy-2-pyrimidinyl)amino]-

* Author to whom correspondence should be addressed [telephone (519) 824-4120, ext. 2740; fax (519) 837-0442; e-mail jchall@evbhort.uoguelph.ca].

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carbonyl]amino]sulfonyl]benzoate (specific activity = 513.38 MBq mmol^{-1}); [*phenyl*(U)-^{14}C]ethametsulfuron-methyl ((methyl 2-[[[[[4-ethoxy-6-(methylamino)-1,3,5-triazin-2-yl]amino]carbonyl]amino]sulfonyl]benzoate) (specific activity = 605.72 MBq mmol^{-1}); [*phenyl*-^{14}C]chlorsulfuron (2-chloro-*N*-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]benzenesulfonamide) (specific activity = 2.15 MBq mmol^{-1}), and metsulfuron-methyl (methyl 2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl] benzoate) (specific activity = 3.29 MBq mmol^{-1}) were obtained from E. I. Du Pont De Nemours and Co. Inc. (Wilmington, DE). [3,4-^{14}C]Glufosinate ammonium ((2-amino-4-(hydroxymethylphosphonyl)butanoic acid); specific activity = 206.48 MBq mmol^{-1}) was provided by Hoechst (Frankfurt, Germany). [2,6-^{14}C]Picloram ((4-amino-3,5,6-trichloropicolinic acid); specific activity = 1328 MBq mmol^{-1}) was provided by Dow AgroSciences (Indianapolis, IN). [*ring*(U)-^{14}C]-2,4-Dichlorophenoxyacetic acid (specific activity = 580.90 MBq mmol^{-1}) was obtained from Sigma Chemical Co. (St. Louis, MO). Bakerbond spe cartridges (J. T. Baker Inc., Phillipsburg, NJ) were purchased from Fisher Scientific Inc. (Mississauga, ON). EAH Sepharose 4B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). The immunogen (chlorimuron−bovine serum albumin) and enzyme conjugate (chlorimuron−ovalbumin) were prepared according to the method of Fleeker (16). The polyclonal antisera were obtained from Dr. J. Christopher Hall's laboratory and produced as described by Parnell and Hall (*3*).

Competitive Indirect (CI)-ELISA Protocol. The protocol was essentially as previously described (*17*). Briefly, microwells of an ELISA plate were each coated with chlorimuron−ovalbumin (OVA) coating conjugate (100 μL) diluted in phosphate-buffered saline (PBS) (3 μL in 12 mL of PBS) and incubated at 4 °C overnight. Wells were emptied and washed three times with PBS containing 0.05% (v/v) Tween 20 (PBST), and then PBS (200 μL) containing 3% (w/v) nonfat milk powder (Bio-Rad Laboratory Ltd., Hercules, CA) was added to each well and incubated for 1 h at 25 °C to block any unoccupied sites on the plate. Polyclonal antibodies were preincubated for 1 h at 25 °C with the same volume of standard solutions containing known concentrations of chlorimuron-ethyl. This mixture (100 μL) was added to individual wells on the microtiter plate after the blocking solution was removed, and the microtiter plate was washed three times with PBST. The plate was incubated for 1 h at 25 °C and washed three times with PBST. An aliquot (100 μL) of diluted (5000-fold in PBS) goat anti-rabbit immunoglobulin G (IgG) antibody conjugated to horseradish peroxidase (Pierce Chemicals Co., Rockford, IL) was added to the plate, incubated for 1 h at 25 °C, and washed three times with PBST. 2,2′-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABST) diammonium (Sigma Chemicals Co., St. Louis, MO) (100 μL of 0.4 mg/mL in 50 mM citrate buffer, pH 4.0) containing 0.005% H_2O_2 was added and incubated for 30 min. Absorbance (*A*) was measured at 405 nm using a microtiter plate reader (model 3550-UV, Bio-Rad). Relative absorbance was calculated using the formula A/A_0, where A_0 is the absorbance of the well in which the antibody was not challenged with free chlorimuron-ethyl. Relative absorbance was plotted against log concentration of free chlorimuron-ethyl to generate a standard curve.

EAH Sepharose 4B Column. EAH Sepharose 4B gel was washed according to the manufacturer's instructions (Pharmacia Fine Chemicals). Briefly, gel slurry (2 mL) was washed on a sintered glass funnel with 80 mL of 0.5 M NaCl. The slurry was transferred to an empty disposable Bakerbond spe cartridge, and the gel was allowed to settle. A polyethylene frit was placed on top of the settled beads, and the column washed with 15 mL of PBS.

The EAH Sepharose 4B column was allowed to equilibrate at 25 °C and washed with 10 mL of PBS before use. ^{14}C-Radiolabeled chlorimuron-ethyl (1 mL of 0.5 μg/mL in PBS) was applied to the column and the effluent collected. Air was flushed through the column to ensure all effluent was collected. The effluent was analyzed by liquid scintillation using a Beckman LS6K-SC scintillation counter (Beckman Instruments Inc., Fullerton, CA) with Ecolite (+) (ICN Biomedicals Inc. Irvine, CA) as the scintillant. This experiment was also conducted with ^{14}C-radiolabeled picloram, 2,4-D, metsulfuron, ethametsulfuron-methyl, and glufosinate ammonium to determine if herbicides with structures different from that of chlorimuron-ethyl would bind to the EAH matrix. After each use, the column was washed with 5 mL of PBS and stored at 4 °C in PBS containing 0.02% thimerosal.

Preparation of the Chlorimuron-ethyl-Specific Affinity Column. Chlorimuron-ethyl-specific immunosorbent was prepared by coupling chlorimuron-ethyl-specific polyclonal antibodies to protein G Sepharose 4B. The gel was washed according to the manufacturer's instructions (Pharmacia Fine Chemicals), and the coupling procedure was adapted from that of Harlow and Lane (*18*). Briefly, the matrix (2 mL) was washed with PBS (10 mL) followed by 0.2 M sodium borate buffer pH 9.0 (20 mL) on a sintered glass funnel. The slurry was transferred to a centrifuge tube and centrifuged for 5 min at 3000*g*. Buffer was removed, and the hydrated sorbent was added to anti-chlorimuron-ethyl serum (5 mL, undiluted) and gently rocked for 1 h at 25 °C. The gel was washed twice with 10 mL of sodium borate (0.2 M, pH 9.0), centrifuged for 5 min at 3000*g*, and then resuspended in 10 mL of sodium borate (0.2 M, pH 9.0) containing 20 mmol of dimethylpimelimidate (Sigma Chemical Co.). The slurry was mixed for 30 min at 25 °C on a rocking table. The coupling reaction was stopped by washing the gel once with 0.2 M ethanolamine, pH 8.0 (10 mL). The gel was incubated for 2 h at 25 °C in 0.2 M ethanolamine, pH 8.0, with gentle rocking, washed with PBS by centrifugation (1 min at 3000*g*), resuspended in PBS containing 0.02% thimerosal, and stored at 4 °C until required for use. The coupling efficiency was verified by SDS-PAGE of the beads before and after treatment with dimethylpimelimidate (*19*). Briefly, samples of beads taken before and after covalent coupling were boiled in Laemmli sample buffer, analyzed on a 10% SDS−polyacrylamide gel, and stained with Coomassie blue. Good coupling is indicated by the presence of antibody bands in the "before" sample, but not in the "after" sample because the antibodies were covalently bound to the beads and did not enter the gel (*18*). Following verification of covalent linkage of the antibodies to the beads, the affinity matrix (0.25 mL) was packed into an empty disposable Bakerbond spe cartridge, and the immunosorbent was held in place between two porous polyethylene frits. The column was filled with PBS containing 0.02% thimerosal and stored at 4 °C until required for use.

Determination of the Immunoaffinity Column Capacity. The capacity of the chlorimuron-ethyl immunoaffinity matrix was determined by applying known amounts of [^{14}C]chlorimuron-ethyl in PBS (1 μg mL^{-1}; 0.240 mmol) to the immunosorbent. To ensure maximum binding, the effluent was applied to the column twice and washed with PBS (25 mL). Bound [^{14}C]-chlorimuron-ethyl was eluted with methanol (3 mL). Eluates were collected directly into scintillation vials, and radioactivity was determined by liquid scintillation spectroscopy. The column was immediately regenerated by washing with PBS (10 mL).

Evaluation of Affinity Column Specificity and Cross-Reactivity with Other Sulfonylureas. To evaluate how specific the binding of chlorimuron-ethyl to the immunoaffinity column was, radiolabeled chlorimuron-ethyl was applied to the column and eluted either with non-radiolabeled chlorimuron-ethyl or chlorsulfuron. The immunoaffinity column was washed with PBS (5 mL). One milliliter of PBS containing [^{14}C]chlorimuron-ethyl (2.40 mM; 1 μg mL^{-1}) was applied to the column, which was washed with PBS (10 mL) followed by Nanopure water (10 mL) until no radioactivity was detected in the effluent. One milliliter of non-radiolabeled chlorimuron-ethyl solution (2.40 mM; 1 μg mL^{-1} PBS) was applied to the column to elute the radiolabeled chlorimuron-ethyl. The effluent was collected and monitored by liquid scintillation spectroscopy.

To evaluate the cross-reactivity of the immunoaffinity column with sulfonylurea herbicides other than chlorimuron-ethyl, binding of these other sulfonylurea herbicides to the affinity column was investigated. The immunoaffinity column was washed with PBS (5 mL), and 1 mL of [^{14}C]chlorimuron-

Table 1. Composition and Physical Characteristics of Soils

	composition, %			physical characteristics	
soil type	clay, %	silt, %	sand, %	organic matter, %	pH[a]
sandy loam[b]	6.9	16.6	76.4	2.2	6.9
silty clay[b]	40.9	47.9	11.1	3.3	7.8
silty clay loam[c]	30.0	51.0	18.0	12.0	5.2

[a] 1:2 soil to water. [b] Obtained from J. A. Ferguson, Elora Research Station, ON, Canada. [c] Obtained from Dr. R. Protz, Department of Land Resource Sciences, University of Guelph, ON, Canada.

ethyl solution (2.40 mM; 1 μg mL^{-1} PBS) was added to the column. The column was washed with PBS (10 mL) followed by Nanopure water (10 mL). The bound herbicide was eluted with methanol (3 mL), collected directly into a scintillation vial, and analyzed by liquid scintillation spectroscopy. The experiment was also repeated using metsulfuron-methyl and chlorsulfuron.

Evaluation of IAC as a Purification Strategy Prior to a CI-ELISA of Chlorimuron-ethyl in Soil. *Soil Extraction.* Elora sandy loam (Elora, ON), Hunter silty clay (Huron County, ON), and Lincoln silty clay loam (Cayuga, ON) soils (Table 1) were extracted with 0.1 M sodium carbonate buffer (pH 9.0), according to the method of Young (*20*). Extraction buffer (20 mL) was added to soil (5 g), and the mixture was agitated vigorously for 45 min on a soil shaker. The mixture was centrifuged (8000*g*) for 10 min, and the supernatant was decanted and filtered through Whatman filter paper No. 1 (Fisher Scientific Inc., Mississauga, ON). Nanopure water (10 mL) was added to the remaining soil, and the mixture was agitated for 15 min on a soil shaker and centrifuged for 10 min at 15000*g*. The supernatant was decanted, filtered through Whatman filter paper No. 1, and pooled with the carbonate supernatant previously collected. The soil extract was mixed thoroughly, neutralized with 1 N HCl, and stored at −20 °C until use. The soil extracts were spiked with chlorimuron-ethyl (0, 25, 50, and 100 ng/mL) prior to purification.

Purification of Chlorimuron-ethyl from the Soil Extracts. The EAH Sepharose 4B column was allowed to equilibrate at 25 °C and was washed with PBS (10 mL) prior to application of the soil extract (1 mL). The column was then washed with 1 mL of PBS, and the effluent (2 mL) was collected in a single test tube. EAH-purified soil extracts (1 mL) were applied to the immunoaffinity column previously equilibrated with PBS (10 mL). The column was then washed with PBS (5 mL) followed by Nanopure water (5 mL). Chlorimuron-ethyl was eluted from the column with methanol (3 mL). The methanol eluate was evaporated to dryness under a stream of nitrogen at 50 °C and reconstituted in PBS (1 mL). The solution was filtered through a 0.22 μm filter (MSI, Westboro, MA) and analyzed by CI-ELISA.

RESULTS AND DISCUSSION

Assay Performance. The linear working range of the competitive-indirect immunoassay was 1−1000 ng mL^{-1} (Figure 1). The mean I_{50} value was 54 ng mL^{-1}, with a limit of quantification (LOQ) of 27 ng mL^{-1} and a limit of detection (LOD) of 2.0 ng mL^{-1}. The LOQ was defined as the lowest chlorimuron-ethyl standard to have a mean absorbance value separated from the mean blank absorbance value (A_0) by 3 times the standard deviation of A_0 (*16*). The LOD was defined as the lowest chlorimuron-ethyl standard to have a mean absorbance value separated from A_0 by 10 standard deviations of A_0 (*16*). The interwell variability of absorbance values was determined on eight separate wells for each chlorimuron-ethyl standard (Table 2). The interwell coefficient of variation (CV) ranged from 5.7 to 11.4% over the standard curve, with a mean interwell CV of 8.2%. The interwell CV for background binding absorbance values was 11%. The interassay variability of the chlorimuron standard A/A_0 values was determined by repeating the assay seven times ($n = 8$). Interassay CV ranged from 3.5 to 8.4% over the standard curve, with a mean CV of 6.3% (Table 3). CV values increased with an increase in chlorimuron-ethyl concentration due to decreasing A/A_0 values. These results are similar to those obtained by Deschamps and Hall (*21*) for both their polyclonal and monoclonal immunoassays for picloram.

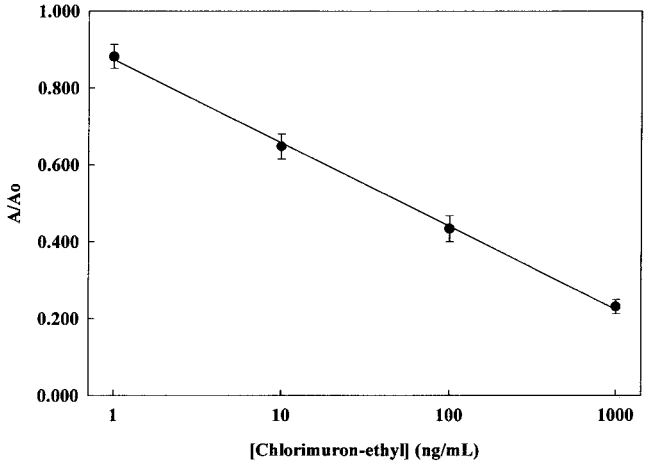

Figure 1. Standard curve of the polyclonal antibody-based indirect enzyme immunoassay for determination of chlorimuron-ethyl. Bars represent the standard errors of the means; equation of the line $y = -0.2167(\log x) + 0.8738$ ($r^2 = 0.99$).

Table 2. Interwell Variability of CI-ELISA Absorbance Values

chlorimuron-ethyl, ng/mL	mean absorbance ± SD[a]	CV, %
0	1.083 ± 0.062	5.7
1	0.967 ± 0.071	7.4
10	0.743 ± 0.059	7.9
100	0.580 ± 0.054	9.3
1000	0.351 ± 0.026	7.4
background	0.125 ± 0.014	11.4

[a] Mean of eight replicates, i.e., eight wells.

Table 3. Interassay Variability of CI-ELISA Standard Curve A/A_0 Values

	relative absorbance, A/A_0[a]		
chlorimuron-ethyl, ng/mL	mean[b]	SD	CV, %
1	0.882	0.031	3.5
10	0.648	0.033	5.2
100	0.434	0.034	7.9
1000	0.231	0.019	8.4

[a] A/A_0 = (abs std − abs background)/(max abs − abs background). [b] Mean of eight separate assays, i.e., eight replicate ELISA plates.

Binding of Organic Matter and Herbicides to EAH Sepharose 4B Column. In our preliminary experiments using IAC, it was determined that binding of chlorimuron-ethyl was affected by the presence of cocontaminants, that is, organic matter in the soil extracts, and that EAH Sepharose 4B effectively bound organic matter. Furthermore, the binding of these cocontaminants to the Sepharose matrix of the immunoaffinity column significantly limited the repeated use of the column. On the basis of these results, an EAH Sepharose 4B column was used to eliminate humic acids prior to IAC. A new EAH column was prepared for every soil extract to avoid contamination. Most of the brown-yellow components of the soil extracts were retained by

Table 4. Herbicide Recovery from the EAH Sepharose 4B Matrix

herbicide	dpm added[a] ± SD	dpm recovered[b] ± SD	recovery, %
chlorimuron-ethyl	14908[b] ± 629	14380 ± 412	96
2,4-D	12890 ± 212	13300 ± 256	104
ethametsulfuron-methyl	3390 ± 93	3620 ± 124	107
glufosinate-ammonium	3622 ± 49	3820 ± 113	105
metsulfuron-methyl	4000 ± 195	4300 ± 151	108
picloram	3773 ± 110	3636 ± 44	96

[a] Mean of at least three replicates. [b] Mean of six replicates.

the EAH Sepharose column, and a clear effluent was collected. This effluent was decontaminated to the point it could be added directly to the immunoaffinity column. EAH purification allowed the preservation of the immunoaffinity columns, extending the number of uses from several to 50 cycles without loss in efficiency. In contrast, the anti-triasulfuron affinity columns used by Ghildyal and Kariofillis (*2*) to purify triasulfuron from soils could be regenerated only 5 times because no prior purification was performed. However, the EAH-purified soil extract could not be analyzed directly by ELISA because some unknown contaminant(s), which interfered with the detection of chlorimuron-ethyl, was (were) still present. In other experiments, it was determined that chlorimuron-ethyl and several other weak acid herbicides including 2,4-D, ethametsulfuron-methyl, glufosinate-ammonium, metsulfuron-methyl, and picloram did not bind to the EAH Sepharose matrix (Table 4).

The mechanism by which organic co-contaminants adsorb to the EAH Sepharose 4B matrix is unknown. We speculate that ionic interactions occur at pH 9.0 between the anionic humic acids and the free amino group of the EAH Sepharose 4B. Therefore, both chlorimuron-ethyl and the organic acids may be expected to bind to the EAH Sepharose 4B matrix. However, chlorimuron-ethyl and other weak acid herbicides readily passed through the column, whereas most yellow and brown soil components associated with humic and fulvic acids were trapped on the EAH column. The higher charge-to-mass ratio of organic matter compared to that of weak acid herbicides may explain this phenomenon. Although the soil extracts after EAH chromatography were colorless, they still contained contaminants that significantly interfered with the immunoassay. These contaminants originated from the soils, as evidenced by similar experiments conducted with chlorimuron-ethyl dissolved in PBS that did not show any matrix effects following EAH chromatography (results not shown). In addition, multiple passages through the EAH resin did not improve chlorimuron-ethyl determination by CI-ELISA if IAC was not used.

Anti-chlorimuron-ethyl Immunoaffinity Column. *Antibody Coupling.* The efficiency of the anti-chlorimuron-ethyl polyclonal antibodies coupling to the protein G Sepharose 4B matrix was measured using SDS-PAGE and confirmed by immunoassay. There were no protein bands detected after the antibodies were covalently bound to protein G Sepharose 4B (Figure 2, lanes 2 and 4). Bands were present in lanes where antibodies were not covalently bound to protein G Sepharose 4B (Figure 2, lanes 3 and 5). These results were confirmed by immunoassay. The immunoassay conducted with serum prior to coupling with protein G Sepharose 4B gave results similar to those obtained using the standard ELISA procedure for chlorimuron-ethyl. The ELISA for chlorimuron-ethyl with the serum collected after coupling to protein G Sepharose 4B did not develop any color, demonstrating the absence of the chlorimuron-ethyl-specific antibodies due to their linkage to protein G Sepharose 4B.

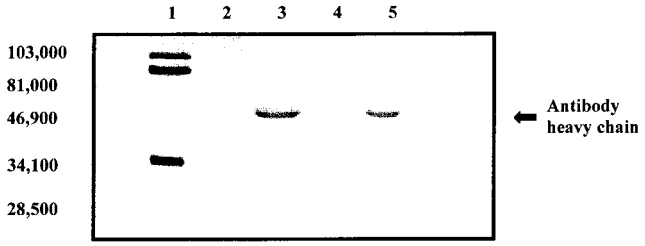

Figure 2. 10% SDS-PAGE of protein G Sepharose 4B beads before and after covalent coupling to the anti-chlorimuron-ethyl antibodies: (lane 1) molecular weight markers (MW indicated on left side of the gel, in kDa); (lanes 2 and 4) beads after covalent coupling (9 and 1 μL, respectively); (lanes 3 and 5) beads before covalent coupling (9 and 1 μL, respectively).

Capacity. To determine the capacity of the immunoaffinity column to bind chlorimuron-ethyl, excess [^{14}C]-chlorimuron-ethyl was applied to the column repeatedly to ensure complete binding. Bound [^{14}C]chlorimuron-ethyl was removed with methanol and determined by liquid scintillation spectroscopy. No [^{14}C]chlorimuron-ethyl remained on the column following methanol elution (data not presented). Results from three anti-chlorimuron-ethyl immunoaffinity columns (0.250 mL bed volume) showed that each column was able to bind ~0.815 nmol (338 ng) of chlorimuron-ethyl. Therefore, the total capacity of a column was 3.25 nmol (1350 ng) of chlorimuron-ethy/mL of immunoaffinity gel. This capacity is not as high as those obtained for phenylureas and cyclopiazonic acid affinity columns mentioned by Pichon et al. (*14*) and Yu et al. (*7*), respectively. The use of polyclonal antibodies may be responsible for this lower binding capacity, because the concentration of chlorimuron-ethyl-specific antibodies in the serum is diluted among the other nonspecific antibodies. Both Pichon et al. (*14*) and Yu et al. (*7*) used monoclonal antibodies for their affinity columns.

Elution Conditions. Mild eluting agents were first tried to remove bound chlorimuron-ethyl from the immunoaffinity column. No chlorimuron-ethyl was eluted with low-pH (glycine−HCl, pH 2.7) or high-pH (0.1 M carbonate, pH 10; triethylamine, pH 11.5) buffers, whereas small quantities (<10%) of chlorimuron-ethyl were eluted with high-salt buffers (5 M LiCl, 3.5 M MgCl, and 1 M ammonium sulfate) and denaturing agents (2 M urea and 1 M guanidine−HCl). Because antibody−antigen interactions are generally disrupted by organic solvents (*15*), methanol and other organic solvents were evaluated as eluants. As the concentration of methanol in aqueous solutions was increased from 50 to 90%, more chlorimuron-ethyl was eluted. However, pure methanol was required to completely elute all bound chlorimuron-ethyl from the affinity matrix. Other solvents such as 1,4-dioxane and ethanol did not completely remove bound chlorimuron-ethyl from the column. Although methanol is considered to be damaging to the tertiary structure of antibodies, many researchers have also found that this solvent is often the only effective eluant to remove pesticides from many different types of immunoaffinity columns (*2*, *22*). Regardless of the effect of methanol on the tertiary structure of the antibody, each immunoaffinity column could be used 50 times when regenerated immediately after use.

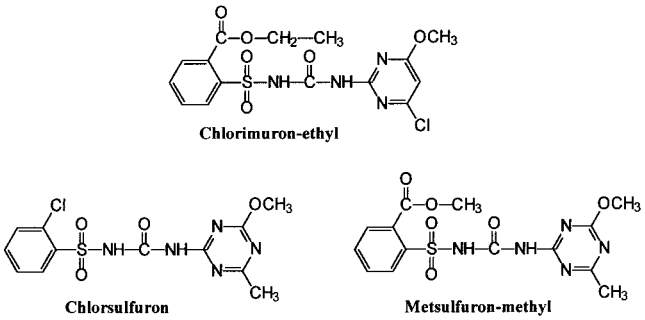

Figure 3. Structures of chlorimuron-ethyl, chlorsulfuron, and metsulfuron-methyl.

Specificity and Cross-Reactivity. The specificity of the immunoaffinity column was determined by saturating it with [^{14}C]chlorimuron-ethyl followed by the addition of non-radiolabeled chlorimuron-ethyl to displace it. All of the [^{14}C]chlorimuron-ethyl bound to the affinity column was eluted with the addition of non-radiolabeled chlorimuron-ethyl (2.40 mM; 1 μg mL^{-1} PBS). A similar experiment in which chlorsulfuron (Figure 3) was used resulted in no displacement of bound [^{14}C]chlorimuron-ethyl. Immunoaffinity column cross-reactivity was evaluated by applying (in two separate experiments) two sulfonylurea herbicides ([^{14}C]chlorsulfuron and [^{14}C]-metsulfuron-methyl (Figure 3)), with chemical structures similar to that of chlorimuron-ethyl, to the column. The column behavior was determined by monitoring the presence of the ^{14}C-labeled herbicides in the column effluent by liquid scintillation spectroscopy. Approximately 11 and 35% of total [^{14}C]chlorsulfuron and [^{14}C]-metsulfuron-methyl added to the anti-chlorimuron-ethyl immunoaffinity column remained bound.

Immunoaffinity Chromatography for the Isolation of Chlorimuron-ethyl from Soil Extracts. Extracts from chlorimuron-ethyl-free sandy loam, silty clay, and silty clay loam were spiked with different amounts of chlorimuron-ethyl prior to EAH and IAC and analysis by CI-ELISA. Chlorimuron-ethyl concentrations were estimated from a standard curve of known concentrations of chlorimuron-ethyl in PBS (Figure 1). Soils were not spiked directly, to avoid the influence of extraction efficiency on chlorimuron-ethyl recovery during the purification strategy. However, in other experiments, chlorimuron-ethyl extraction efficiency from soil was >80%, which agrees with soil extraction efficiencies obtained using other sulfonylurea herbicides (*20*). Furthermore, due to the extent of matrix effects present in the crude soil extracts, losses of chlorimuron-ethyl during extraction could not have been detected by CI-ELISA.

Results of chlorimuron-ethyl determination in crude, EAH-purified, and both EAH-purified and IAC-purified concentrated sandy loam, silty clay, and silty clay loam soil extracts are presented in Figures 4, 5, and 6, respectively. Generally, chlorimuron-ethyl concentrations in both crude and EAH-purified soil extracts were overestimated, although to a significantly lesser extent in the latter extract, except in the case of the silty clay loam soil extract, for which results agreed with PBS spiked values (Figure 6).

The immunoaffinity column removed matrix effects present in the EAH-purified soil extracts and allowed accurate and precise quantification of chlorimuron-ethyl by CI-ELISA. Curves obtained following both EAH and IAC were very similar to the PBS standard curve, by

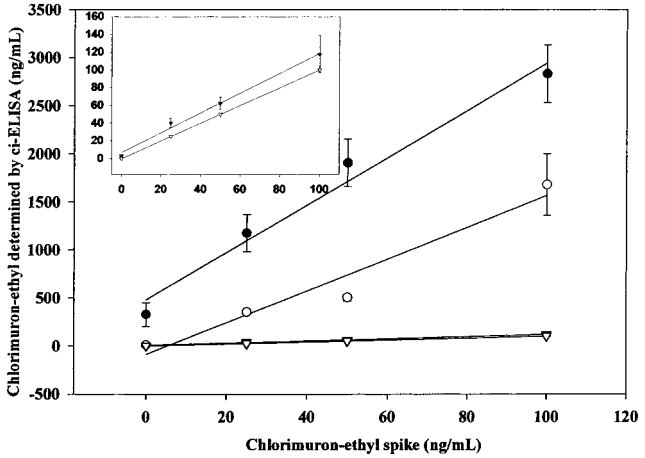

Figure 4. Determination, by CI-ELISA, of chlorimuron-ethyl concentrations in spiked sandy loam soil extracts. Soil extracts were analyzed crude (●; $y = 24.65x + 481.49$; $r^2 = 0.98$), after EAH chromatography (○; $y = 16.55x - 87.35$; $r^2 = 0.95$), or after both EAH and IAC (▼; $y = 1.12x + 6.65$; $r^2 = 0.99$). Controls consisted of PBS spiked with chlorimuron-ethyl (▽; $y = x$). (Inset) EAH−IAC compared to PBS curve at a smaller scale. Bars represent the standard errors of the means. Where bars are not visible, they are smaller than the symbol.

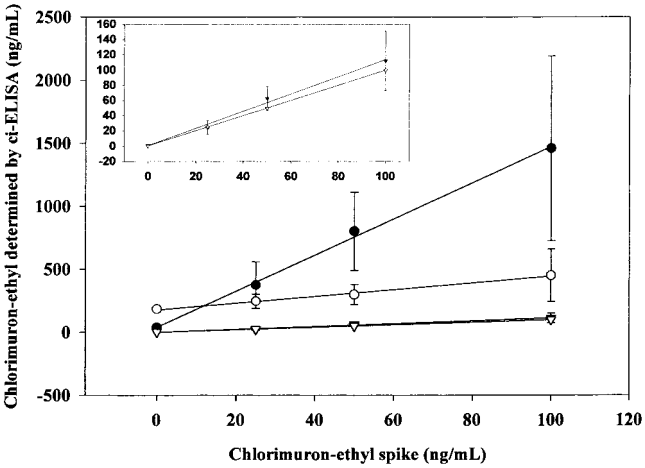

Figure 5. Determination, by CI-ELISA, of chlorimuron-ethyl concentrations in spiked silty clay soil extracts. Soil extracts were analyzed crude (●; $y = 14.34x + 40.93$; $r^2 = 0.99$), after EAH chromatography (○; $y = 2.68x + 177.85$; $r^2 = 0.99$), or after both EAH and IAC (▼; $y = 1.13x + 0.49$; $r^2 = 0.99$). Controls consisted of PBS spiked with chlorimuron-ethyl (▽; $y = x$). (Inset) EAH−IAC compared to PBS curve at a smaller scale. Bars represent the standard errors of the means. Where bars are not visible, they are smaller than the symbol.

which the quantity of chlorimuron-ethyl measured by CI-ELISA was the same as the concentration of herbicide added to the soil extract. The results were consistent and gave CV values similar to those obtained for the standard curve of chlorimuron-ethyl using PBS (Tables 2 and 3).

Chlorimuron-ethyl Recovery. Different volumes of silty clay soil extract (1, 10, and 100 mL) were spiked with the same quantity (25 ng) of chlorimuron-ethyl to evaluate the efficiency of IAC in concentrating and recovering chlorimuron-ethyl from the soil extract. The immunoaffinity column effectively bound chlorimuron-ethyl, even at concentrations as low as 0.25 ng mL^{-1} (0.25 ppb). The high recoveries (>93%) observed at the lowest concentration (0.25 ppb in 100 mL) using IAC allowed chlorimuron-ethyl to be concentrated 100-fold prior to detection by CI-ELISA (Table 5). Concomitantly,

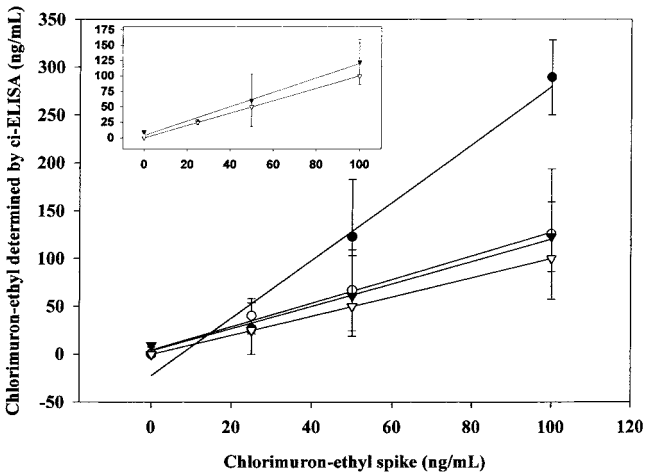

Figure 6. Determination, by CI-ELISA, of chlorimuron-ethyl concentrations in spiked silty clay loam soil extracts. Soil extracts were analyzed crude (●; $y = 3.0x - 22.44$; $r^2 = 0.97$), after EAH chromatography (○; $y = 1.23x + 4.61$; $r^2 = 0.99$), or after both EAH and IAC (▼; $y = 1.17x + 3.72$; $r^2 = 0.99$). Controls consisted of PBS spiked with chlorimuron-ethyl (▽; $y = x$). (Inset) EAH−IAC compared to PBS curve at a smaller scale. Bars represent the standard errors of the means. Where bars are not visible, they are smaller than the symbol.

Table 5. Chlorimuron-ethyl Recovery after both EAH and Affinity Chromatography

	chlorimuron-ethyl added		recovery		
vol (mL)	dpm	concn, ng/mL	mean ± SD[a]	CV,%	%[b]
1	4800	25	4780 ± 25	0.5	99.5
10	4800	2.5	4703 ± 170	3.6	98
100	4800	0.25	4458 ± 49	1.1	93

[a] Mean of three replicates. [b] Percent recovery (mean/total dpm added).

the LOD and LOQ of the chlorimuron-ethyl immunoassay were lowered by a similar factor (0.02 versus 0.2 ng mL^{-1} for LOD and 0.27 versus 27 ng mL^{-1} for LOQ). Although it was suggested by Yu et al. (*7*) that analyte recoveries were higher when columns underwent longer regeneration times (>14 h), the anti-chlorimuron-ethyl affinity column was regenerated with PBS (5 mL) in 5 min with minimal losses in efficiency (data not shown).

Compared to traditional analytical techniques used to determine sulfonylurea herbicides in water and soil, IAC extraction combined with ELISA detection is simple, rapid, and specific (*23*). Although the present assay had an LOQ higher than those seen with LC-MS, sample concentration by IAC allowed quantification of chlorimuron at the sub-parts per billion level. IAC can be used to concentrate the herbicide 100-fold without any additional step. Moreover, IAC-ELISA led to higher recoveries (>93%) than those observed with traditional analytical techniques combined with solid-phase extraction: 80% with LC-MS (*24*), 60% with HPLC-MS (*25*), and 90% by capillary electrophoresis (*26*). The higher recoveries obtained with IAC are probably due to the high binding specificity and capacity of the affinity column for the herbicide compared to solid-phase extraction cartridges, which have demonstrated limited capacities and low specificity for polar analytes such as the sulfonylurea herbicides (*15*). Moreover, traditional techniques involve extensive purification prior to herbicide quantification, increasing the time and costs of sample analysis (*27*). The purification strategy developed in the present study consists of only two steps, EAH and IAC, thereby limiting herbicide losses during purification. Finally, EAH and IAC could be coupled, very easily, to any classical analytical method, in both on-line and off-line modes.

The purification/detection strategy developed in the present research is one of the most sensitive analytical methods to monitor sulfonylurea herbicide residues in the environment. It also compares favorably with any other known method in terms of herbicide recovery and has shown excellent specificity as well. Results obtained with soil, a highly complex matrix, indicate a great potential for IAC in environmental monitoring and demonstrated the usefulness of antibodies in analytical chemistry.

Conclusion. The purification strategy developed consists of EAH chromatography, which removed organic matter from soil extracts, followed by IAC, which extracted and concentrated chlorimuron-ethyl prior to quantification by a competitive-indirect immunoassay. Polyclonal antibodies for chlorimuron-ethyl were conjugated to protein G Sepharose 4B gel. Similar to other studies with different biologically active chemicals, our findings show that the chlorimuron-ethyl immunoaffinity column effectively removed additional soil cocontaminants. This strategy was successfully used to remove matrix effects from three distinctly different soils and allowed for the accurate determination of chlorimuron-ethyl in soil extracts.

Compared to conventional analytical techniques used to determine sulfonylurea herbicides in environmental samples, the purification strategy developed in this study is simple and specific. This strategy necessitates only two columns, without any sample pretreatment such as organic partitioning (*26*). By using antibodies specific for chlorimuron-ethyl, IAC provides a very effective purification method that isolates chlorimuron-ethyl and eliminates co-contaminants prior to ELISA. IAC also gives higher recoveries than most extraction methods used in analytical chemistry (*28*).

ABBREVIATIONS USED

ABST, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) substrate tablets; ai, active ingredient; CI-ELISA, competitive-indirect enzyme-linked immunosorbent assay; CV, coefficient of variation; 2,4-D, (2,4-dichlorophenoxy)acetic acid; dpm, disintegrations per minute; EAH, epoxy-coupled 1,6-diaminohexane; I_{50}, amount of chlorimuron-ethyl required to inhibit color development by 50%; GC, gas chromatography; HPLC-MS, high-performance liquid chromatography−mass spectrometry; IAC, immunoaffinity chromatography; LC-MS, liquid chromatography−mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; OVA, ovalbumin; PBS, phosphate-buffered saline; PBST; phosphate-buffered saline−Tween; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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