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Development of a Monoclonal Antibody-Based Enzyme-Linked Immunosorbent Assay for the Herbicide Chlorimuron-ethyl

JING ZHAO,† GUO-XIANG YI,† SU-PING HE,† BAO-MIN WANG,*,† CAI-XIA YU,† GANG LI,† ZHI-XI ZHAI,† ZHAO-HU LI,† AND QING X. LI‡

College of Agronomy and Biotechnology, China Agricultural University, Beijing 100094, China, and Department of Molecular Biosciences and Bioengineering, University of Hawaii, Honolulu, Hawaii 96822

Hybridomas secreting a monoclonal antibody (mAb) against the herbicide chlorimuron-ethyl (CE) were produced by fusing the mouse myeloma cell line (SP2/0) with splenocytes from a mouse immunized against the conjugate of the sulfonamide moiety of CE and bovine serum albumin (BSA). The mAb, designated 1F5C5A10, had very weak affinity with metsulfuron, ethametsulfuron, pyrazosulfuron, bensulfuron, and chlorsulfuron. Two mAb-based indirect competitive enzyme-linked immunosorbent assays (icELISA) were developed. A conventional icELISA (icELISA-I) showed a concentration of half-maximum inhibition (IC_{50}) of 11.6 ng/mL with a dynamic range of 1.6−84 ng/mL. A simplified icELISA (icELISA-II) had an IC_{50} of 28.7 ng/mL and a dynamic range of 2.2−372 ng/mL. The two assays were tested on spiked water and soil samples. CE (1−500 ng/mL) fortified in water samples could be analyzed directly without any sample preparation by both immunoassays with an average recovery between 74 and 114%. icELISA-II, but not icELISA-I, was able to accurately analyze the herbicide residues in the crude soil extracts with recoveries between 99 and 129% without obvious matrix effects due to its lesser amount of sample used. In contrast to icELISA-I, icELISA-II is more convenient, whereas it consumes more reagents of coating antigen and goat anti-mouse IgG−peroxidase.

KEYWORDS: Chlorimuron-ethyl; sulfonylurea herbicide; enzyme-linked immunosorbent assay; ELISA; immunoassay; monoclonal antibody

INTRODUCTION

Chlorimuron-ethyl (CE) is a highly effective postemergence sulfonylurea herbicide used to control many annual broadleaf weeds in soybean fields, manufactured by DuPont under the trade name Classic. Because an important feature of sulfonylurea herbicides is their low application rate (10−40 g/ha) (*1*), the residues apparently are often extremely low. Therefore, it is a challenge for their determination in environmental samples. At present, methodologies reported for the detection of sulfonylurea herbicides include gas chromatography (GC) (*2*, *3*), capillary electrophoresis (*4*, *5*), liquid chromatography (LC) (*1*, *4*, *6*−*11*), LC−mass spectrometry (*12*, *13*), immunoassay (*8*, *14*−*20*), immunosensor (*21*), and bioassay (*8*, *22*). Among these methods, enzyme immunoassay is commonly used because it is rapid, selective, sensitive, and cost-effective. Several enzyme immunoassays using either polyclonal or monoclonal antibodies (pAb or mAb) have been described for the analysis of the sulfonylurea residues in the environment (*8*, *14*, *15*, *17*).

The frequently used methods for chlorimuron-ethyl analysis include LC (*6*, *7*), bioassay (*22*), and pAb-based enzyme-linked immunosorbent assay (ELISA) (*16*, *20*). Although traditional instrumental methods have the sensitivity necessary for CE residue analysis, they are expensive and time-consuming and need rigorous sample preparation. The bioassay is relatively simple to carry out, but it is not sufficiently specific. These disadvantages may be avoided by using immunoassays as an alternative approach to chlorimuron-ethyl analysis. The ELISAs reported for CE were all based on pAbs, of which one was specific to chlorimuron-ethyl (*16*) and the other was class-specific to sulfonylurea herbicides (*20*). We report the production of a highly specific mAb against CE and the development of an indirect competitive ELISA (icELISA) based on the mAb. Two assay formats (icELISA-II and icELISA-I) for CE were optimized and tested with spiked water and soil samples.

MATERIALS AND METHODS

Reagents. Chlorimuron-ethyl, ethametsulfuron, metsulfuron, chlorsulfuron, ethyl 2-(aminosulfonyl) benzoate, and pyrazosulfuron were purchased from Beijing Chemical Reagents Co. (Beijing, China). Cell culture media (Dulbecco's modified Eagle's medium, DMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL (Paisley, Scotland).

* Corresponding author (telephone +86 10 6273 1305; fax +86 10 6273 2567; e-mail wbaomin@263.net).
† China Agricultural University.
‡ University of Hawaii.

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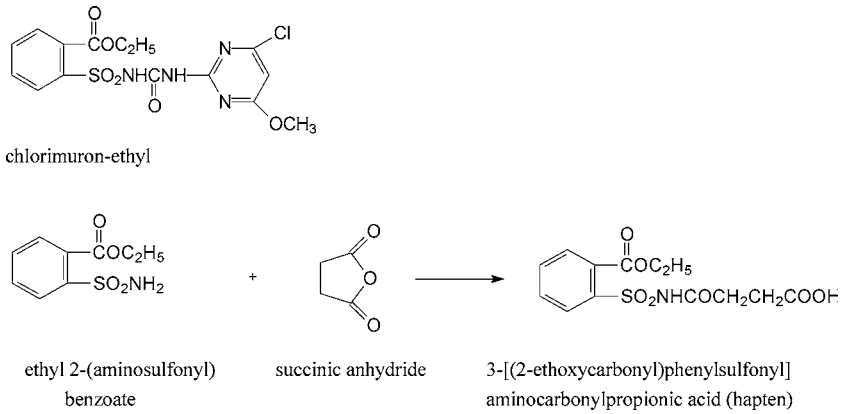


Figure 1. Structure of chlorimuron-ethyl and synthesis of hapten.

Reagents purchased from Sigma (St. Louis, MO) were cell freezing medium DMSO (serum free); hypoxanthine, aminopterin, and thymidine (HAT); hypoxanthine and thymidine (HT) medium supplements; l-glutamine, penicillin, streptomycin, and goat anti-mouse IgG conjugated with horseradish peroxidase (GAM-HRP); complete and incomplete Freund's adjuvant, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), bovine serum albumin (BSA), ovalbumin (OVA), and *o*-phenylenediamine (OPD). Polyethlene glycol 2000 was purchased from Fluka (Buchs, Switzerland). Mouse antibody isotyping kit and a protein-A-CL Sepharose 4B affinity column were obtained from Pierce (Rockford, IL). All other chemicals used were of analytical grade.

Apparatus. Cell culture plates and 96-well polystyrene microtiter plates were purchased form Costar (Corning, NY). An automated plate washer (Wellwash 4 MK2), a microplate reader (Multiskan MK3), and a direct heat CO_2 incubator were purchased from Thermo (Vantaa, Finland). An electric heating constant-temperature incubator was purchased from Tianjin Zhonghuan Experiment Electric Stove Co. Ltd. (Tianjin, China). Syringe filters (25 mm, 0.2 and 0.45 μm pore sizes, Acrodisc) and filter unit (Acrocap) were purchased from Pall (Ann Arbor, MI).

Medium. DMEM containing 10−20% (v/v) FBS was supplemented with 0.2 M glutamine, 50000 units/L penicillin, and 50 mg/L streptomycin. The standard medium was used for growing of myeloma and hybridoma cells.

Buffers and Solutions. The following solutions were used: (i) coating buffer (0.05 M carbonate buffer, pH 9.6); (ii) phosphate-buffered saline (PBS) (0.1 M phosphate buffer containing 0.9% NaCl, pH 7.5); (iii) PBS with 0.1% (v/v) Tween-20 (PBST); (iv) PBST containing 0.5% (w/v) gelatin (PBSTG); (v) citrate−phosphate buffer (0.01 M citric acid and 0.03 M Na_2HPO_4, pH 5.5); (vi) substrate solution (4 μL of 30% H_2O_2 added to 10 mL of citrate−phosphate buffer containing 2 mg/mL OPD); and (vii) a stop solution (2 M H_2SO_4).

Myeloma Cell Line. The HAT-sensitive Balb/c mouse myeloma cell line SP2/0-Ag14 obtained from the China Institute of Veterinary Drug Control (Beijing, China) was used in fusion experiments.

Synthesis of Protein Conjugates. The hapten was the ethoxycarbonyl phenylsulfonamide moiety of chlorimuron-ethyl with a succinic acid spacer (**Figure 1**). The procedure was performed as described earlier **(*23*)**. Briefly, 1.5 g of ethyl 2-(aminosulfonyl) benzoate and 0.6 g of succinic anhydride were dissolved in 15 mL of dioxane followed by the addition of 4.6 mL of DBU dropwise within 10 min. The solution was stirred for 2 h at 22 °C and then acidified with 2 M HCl added slowly. The solution was removed by rotary evaporation. The residue was reconstituted in 25 mL of ethyl acetate and washed with distilled water three times. After the ethyl acetate extract had been dried over anhydrous Na_2SO_4, the solvent was evaporated. The hapten product was recrystallized in ethanol. The conjugates of hapten−BSA and hapten−OVA were synthesized as immunogen and coating antigen, respectively. The hapten (14.4 mg), NHS (18.2 mg), and DCC (32 mg) were dissolved in 0.4 mL of DMF. The activation reaction was carried out for 1 h at 22 °C and for 18 h at 4 °C. The mixture was centrifuged, and 250 μL of supernatant was added dropwise to BSA or OVA solution (50 mg of BSA or OVA in 5 mL of 0.1 M, pH 9.0, borate buffer). The solution was stirred for 4 h at 4 °C. The reaction mixture was dialyzed against five changes of PBS for 5 days and stored at −20 °C after lyophilization.

Immunization Protocol. Five Balb/c female mice (7−8 weeks old, numbered from 1 to 5) were injected subcutaneously with 0.1 mg of hapten−BSA conjugate dissolved in 0.1 mL of PBS mixed with 0.1 mL of Freund's complete adjuvant. Four subsequent injections were carried out at 2-week intervals using Freund's incomplete adjuvant. One week after the three injections, mice were eye-bled, and sera were tested for antihapten antibody titer and for CE recognition properties in icELISA. Three days after the last injection in adjuvant, mice selected to be spleen donors for hybridoma production were boosted intraperitoneally with 0.1 mg of hapten−BSA conjugate in 0.1 mL of PBS.

Fusion Protocol. The mouse that had the highest titer and best specificity was selected for the fusion. Three days after the booster injection, spleen cells that were collected from the mouse were fused with the SP2/0 cell line using PEG-2000 at a ratio of 10:1 of spleen to myeloma cells; the SP2/0 myeloma cells were precultured in standard medium supplemented with 15% FBS. After fusion, cells were resuspended in standard medium supplemented with 20% FBS and 1% (v/v) HAT medium supplement and distributed in 96-well culture plates at an approximate density of $(3-7) \times 10^5$ cells/well. The plates were incubated at 37 °C in a CO_2 incubator containing 5% CO_2 in the air. Selective growth of the hybrid cells was carried out in the DMEM supplemented with HAT. Four days after fusion, cell culture plates were resupplied with HAT medium by replacing 100 μL/well of supernatant with fresh medium. Seven days after fusion, the wells were replaced with the standard medium supplemented with 1% (v/v) HT medium supplement. The supernatant was tested by icELISA. Positive hybridomas were cloned by limiting dilution, and clones were further selected by icELISA.

Establishment of Conventional icELISA (icELISA-I, Figure 2a). In all of the procedures, microtiter plates were washed on an automated plate washer with 250 μL of PBS or PBST per well four times. A microtiter plate was first coated with 200 μL of hapten−OVA in coating buffer per well for 3 h at 37 °C. After four washes with PBS, each well was blocked with 200 μL of 3% nonfat dry milk in PBS for 30 min at 37 °C. After the plate had been washed with PBST, 100 μL of various concentrations of the standard in PBSTG were pipetted into each well followed by the addition of 100 μL of sera, supernatant, or purified mAb solution diluted in PBSTG. The plate was tapped gently and then incubated for 0.5 h at 37 °C. The unbound antibody was removed by washing the plates four times with PBST, and then 200 μL of goat anti-mouse IgG−peroxidase conjugate in PBSTG was added to each well followed by incubation at 37 °C for 0.5 h. After the plate had been washed with PBST again, 200 μL of substrate solution per well was added. The reaction was stopped by adding 100 μL of 2 M H_2SO_4 per well. Absorbance was read at 492 nm in the microplate reader.

Establishment of Simplified icELISA (icELISA-II, Figure 2b). The washing step and the buffers used in icELISA-II were the same as those of icELISA-I. Briefly, a microtiter plate was first coated with

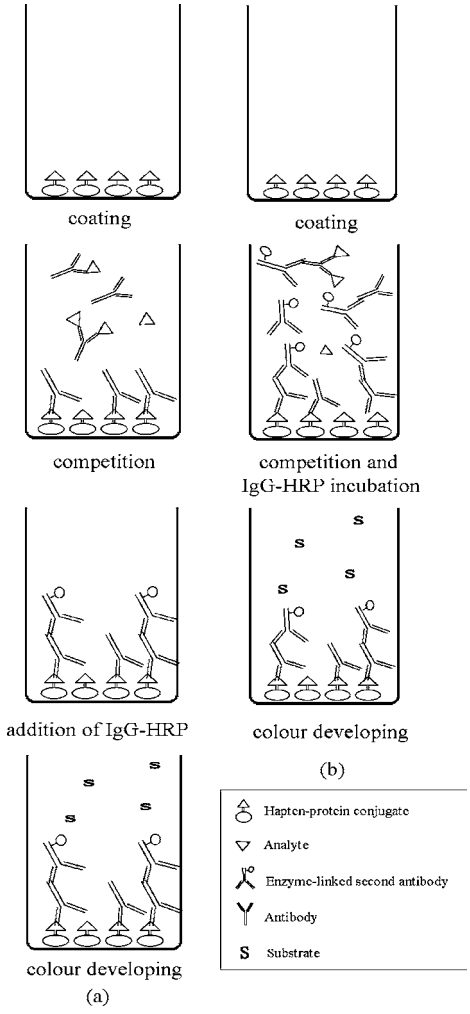


Figure 2. Schematic presentation of (**a**) icELISA-I format and (**b**) icELISA-II format.

200 μL of hapten−OVA per well for 3 h at 37 °C. Each well was blocked with 200 μL of 3% nonfat dry milk in PBS for 0.5 h at 37 °C. After the plate had been washed with PBST, 20 μL of the standard in PBSTG was pipetted into each well followed by the addition of 90 μL of goat anti-mouse IgG−peroxidase conjugate in PBSTG, and then 90 μL of purified mAb solution in PBSTG was added. The plate was tapped gently and then incubated for 0.5 h at 37 °C. The substrate solution was added at 200 μL per well followed by the addition of 2 M H_2SO_4 to stop the reaction. Absorbance was read at 492 nm on the microplate reader.

Evaluation of Sera and Supernatants by Indirect ELISA. As soon as the hybrid cells grew semiconfluently, the culture supernatant was assayed for titers and antibody affinity with chlorimuron-ethyl in the icELISA-I format. The ones having the high titer and good sensitivity were selected for subsequent tests.

Determination of Antibody Characteristics. The clone, designated 1F5C5A10, having a high antibody titer and good sensitivity in the culture supernatant was expanded in mice pretreated with 0.3 mL of mineral oil for production of mAb in ascites. The antibody was purified by centrifugation, ammonium sulfate precipitation, and protein-A-CL Sepharose 4B affinity chromatography. The immunoglobulin isotype was determined with a mouse antibody isotyping kit. The assay cross-reactivity with sulfonylurea herbicides commonly used in China was determined in icELISA-I format. The affinity of mAb 1F5C5A10 to CE was tested in icELISA-I and icELISA-II.

Spiking Test. Soil and river water samples collected from Beijing were used in the spiking tests. The stock solution was 10 mg/mL of CE in methanol. A 0.1 mg/mL standard in PBSTG/glycerol (1:1) was made from the stock solution. The river water samples (pH 7.8) were spiked with the 0.1 mg/mL solution at concentrations of 1, 5, 10, 50, 100, and 500 ng/mL of CE and mixed thoroughly. The solution was analyzed directly with icELISA-I and icELISA-II. A total of six plates with three well replicates were done at different days for six sets of separate fortified samples.

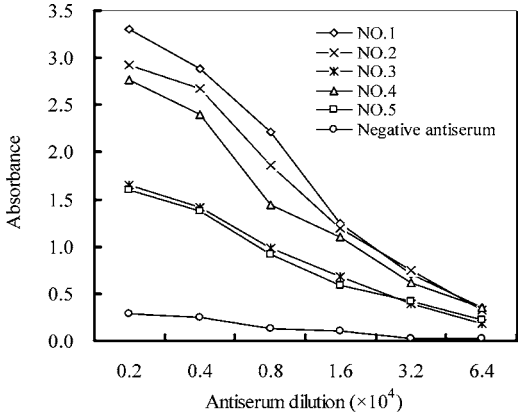


Figure 3. Antisera titer determined in icELISA-I format.

The soil sample with a pH of 9.1 (in water) and an organic matter content of 0.62% consisted of 68.6% sand, 17.4% silt, and 14.1% clay. It contained 21.9% moisture when it was collected. After it had been air-dried in the dark for 1 week, the soil sample was spiked at concentrations of 0.02, 0.04, 0.08, 0.16, 0.31, 0.63, and 1.25 μg/g of chlorimuron-ethyl. CE stock solution was diluted in PBSTG and gradually added to the dry soil with vigorous mixing to achieve a final soil moisture content of 10% (w/w). The mixture was allowed to stand at 4 °C overnight prior to extraction. The soil sample was extracted with 0.15 M $NaHCO_3$ for 2 h at room temperature with shaking at intervals and centrifuged at 3000 rpm. The supernatant was collected and analyzed directly with icELISA-I and icELISA-II. Six separate fortifications and extractions were made for the soil sample, and six replicate ELISAs with three well replicates were conducted. Average recoveries of chlorimuron-ethyl from water and soil samples determined by the two icELISAs were calculated and compared.

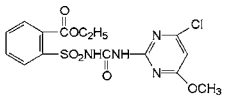
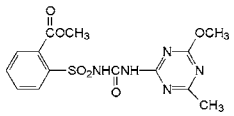
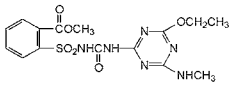
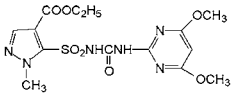
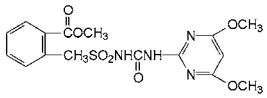
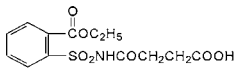
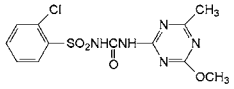
RESULTS AND DISCUSSION

Antisera Screening. After three injections, the titers of antisera were estimated in icELISA-I format using hapten−OVA conjugate as coating antigen (0.25 μg/mL). The titer was defined as the serum dilution that gave an absorbance of 1.0 in the noncompetitive assay conditions. The results showed that the titer of antisera was (0.4−1.6) × 10^4 (**Figure 3**). The ability of the sera to recognize chlorimuron-ethyl was subsequently tested in icELISA-I. The sera from mice 1, 2, 3, 4, and 5 bound competitively to CE and produced IC_{50} values of 36.8, 35.0, 42.3, 38.2, and 44.2 ng/mL, respectively. Mouse 2, whose sera had the lowest IC_{50} for CE, was selected in a subsequent cell fusion experiment.

Characteristics of mAb against Chlorimuron-ethyl. One week after fusion, growing clones were observed in 472 of 480 wells (98% fusion efficiency). Following two subclonings by limiting dilution, two hybridomas producing CE-specific mAbs were considered for expansion and mAb production. The mAbs in ascites produced with two hybridomas were tested with icELISA-I. Considering the titer and affinity to chlorimuron-ethyl, the clone 1F5C5A10 was selected for the further studies. The antibody was the IgG1 isotype with κ light chain and had a high affinity constant of 3.27 × 10^{-9} M^{-1} estimated by indirect ELISA (*24*).

Cross-reactivities were expressed as a percentage of the IC_{50} of CE. None of the tested compounds were significantly recognized by the mAb. The cross-reactivities were 2.2, 2.1, and 1.1% for metsulfuron, ethametsulfuron, and pyrazosulfuron,

Table 1. Cross-Reactivity of Chlorimuron-ethyl and Related Analytes[a]

analytes	structure	IC_{50} (ng/mL)	cross-reactivity (%)
chlorimuron-ethyl		11.6	100
Metsulfuron		551	2.2
ethametsulfuron		341	2.1
pyrazosulfuron		1100	1.1
Bensulfuron		9163	0.13
3-[(2-ethoxycarbonyl)phenylsulfonyl]aminocarbonylpropionic acid (hapten)		11625	0.10
chlorsulfuron		13324	0.09

[a] Cross-reactivities are expressed as a percentage of the IC_{50} of chlorimuron-ethyl. The assay cross-reactivity with sulfonylurea herbicides commonly used in China was determined in the icELISA-I format.

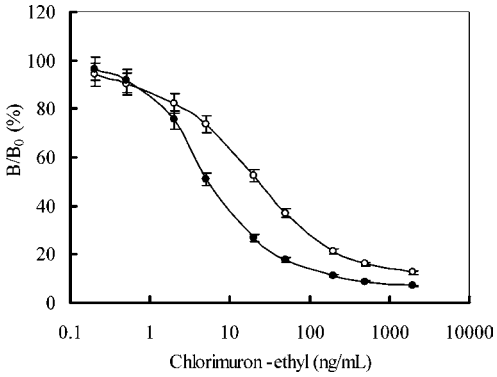


Figure 4. Inhibition curves of mAb 1F5C5A10 for chlorimuron-ethyl in icELISA-I (●) and icELISA-II (○). B_0 and B are OD in the absence and presence of chlorimuron-ethyl, respectively.

respectively. The other analytes showed <0.5% of cross-reactivities (**Table 1**).

Optimization of icELISA-I and icELISA-II. The two icELISA formats were optimized. The coating antigen (0.5 mg/mL), purified mAb (2.4 mg/mL), and anti-mouse IgG−peroxidase conjugate (1 mg/mL) were at dilution ratios of 1:2000, 1:16000, and 1:2000 for icELISA-I and 1:1000, 1:16000, and 1:500 for icELISA-II, respectively. The inhibition curves were established (**Figure 4**). Under optimized conditions, icELISA-I had an IC_{50} value of 11.6 ng/mL and a working range of 1.6−84 ng/mL (the lowest and highest quantification limits were defined as about 80 and 20% B/B_0, respectively). The IC_{50} value was lower than that of icELISA based on the sera of the mouse used for fusion. For icELISA-II, an IC_{50} value of 28.7 ng/mL and a working range of 2.2−372 ng/mL were obtained. The IC_{50} value of the two ELISAs was significantly decreased compared with the chemical-specific pAb-based ELISA (54 ng/mL) (*16*) and was higher than that of the class-specific pAb-based ELISA (0.2 ng/mL) (*20*). The concentrations and total amounts of immunoreagents used, for example, 0.25 μg/L of CA concentration in icELISA-I versus 0.5 μg/L in icELISA-II, are likely responsible for the assay sensitivity (the position of the standard curve). icELISA-I, with 100 μL of sample, had a lower IC_{50} than icELISA-II, with 20 μL of sample (**Figure 4**). The larger volume ratios of extracts in icELISA-I had apparently larger matrix effects as compared with the smaller volume ratios of extracts in icELISA-II (**Table 3**). Compared to icELISA-I, icELISA-II can shorten the assay time by at least 0.5 h and eliminate one washing step. However, the amount of coating antigen and goat anti-mouse IgG−peroxidase conjugate used in icELISA-II was about 2-fold and 1.8-fold those in icELISA-I, respectively.

In the two icELISAs, PBS rather than the usual PBST was used to wash plates before the blocking step. Our routine experiments showed that PBS instead of PBST as washing buffer in this step can obviously decrease the background because the residue of Tween-20 in the plate well may prevent

Table 2. Comparison of Recoveries of Chlorimuron-ethyl from Spiked River Water Samples Determined by icELISA-I and icELISA-II ($n = 6$)

spiked concn (ng/mL)	av recovery (%) ± SD[a] icELISA-I	icELISA-II
500	74 ± 5	91 ± 10
100	111 ±13	85 ± 12
50	114 ± 17	84 ± 8
10	89 ± 9	90 ± 10
5	77 ± 8	90 ± 12
1	75 ± 4	102 ± 10

[a] The standard deviations meant six sample preparations.

Table 3. Comparison of Recoveries of Chlorimuron-ethyl from Spiked Soil Samples Determined by icELISA-I and icELISA-II ($n = 6$)

spiked concn (μg/g)	av recovery (%) ± SD[a] icELISA-I	icELISA-II
1.25	326 ± 52	106 ± 14
0.63	256 ± 36	109 ± 15
0.31	218 ± 22	99 ± 10
0.16	220 ± 24	100 ± 13
0.08	216 ± 9	111 ± 12
0.04	229 ± 14	129 ± 18
0.02	325 ± 16	129 ± 12

[a] The standard deviations meant six sample preparations.

absorption of the milk protein onto the plate and affect the blocking effect.

Recovery of Chlorimuron-ethyl from Water and Soil Samples. Table 2 shows the analysis results of CE in water samples determined by icELISA-I and icELISA-II. The river water samples had minimal matrix effect on the analysis of CE in either icELISA-I or icELISA-II. The two icELISAs accurately measured chlorimuron-ethyl residues in the river water in a range of 1−500 ng/mL. The recoveries of CE from the river water samples determined by icELISA-I ranged from 74 to 114% with an average of 90%. For icELISA-II, the recoveries of CE from the water samples ranged from 84 to 102% with an average of 90%.

The soil was fortified with chlorimuron-ethyl in a concentration range of 0.02−1.25 μg/g. The recoveries of CE from the soil sample with the two icELISAs differed greatly (**Table 3**). The recovery by icELISA-I averaged 256%, ranging from 216% to 326%. For icELISA-II, the recovery was from 99 to 129% with an average of 112%. Apparently, icELISA-II gave better results than icELISA-I. Although the IC_{50} value of icELISA-I was lower than that of icELISA-II, there was serious interference when icELISA-I was used to analyze the crude soil extraction. The lower ratio of sample volume in the plate wells in icELISA-II may result in the low interference. The pH of the mixture was 9.0 when the soil sample was added in the wells of icELISA-I, whereas it was 7.5 in the wells of icELISA-II. When the crude soil extract was diluted with PBSTG at a ratio of 1:5 and analyzed by icELISA-I, the recovery was the same as that by icELISA-II. Thus, the pH change and matrix effects were the main cause of excessively high recoveries of CE from the soil samples as determined by icELISA-I.

In conclusion, new mAb-based ELISAs (icELISA-I and icELISA-II) were developed for the detection of chlorimuron-ethyl in river water samples, although icELISA-II needs greater amounts of reagents. icELISA-II is a time-saving procedure, and CE in the crude soil extracts can be analyzed directly without obvious matrix effect. Compared to other analytical techniques used to determine sulfonylurea herbicides in environmental samples, the pretreatment employed in this study was simple and convenient.

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