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Development of Monoclonal Antibodies against Pirimiphos-methyl and Their Application to IC-ELISA

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To detect the organophosphorus (OP) pesticide pirimiphos-methyl in grain samples, a monoclonal antibody-based indirect competitive enzyme-linked immunosorbent assay (IC-ELISA) was developed and optimized. By the active esters method, pirimiphos-methyl hapten A was conjugated to keyhole limpet hemocyanin to be used as the immunogen for the production of monoclonal antibodies, and pirimiphos-methyl hapten B was conjugated to ovalbumin to be used as coating antigen. By using the monoclonal antibody and the coating antigen, an IC-ELISA has been developed. Under the established optimized conditions, the IC-ELISA showed an IC_{50} of 4.2 ng/mL with a detection limit of 0.07 ng/mL. The IC-ELISA showed negligible cross-reactivity with other OP pesticides except with pirimiphos-ethyl. Recoveries of pirimiphos-methyl from spiked grain samples ranged from 83 to 96%.

KEYWORDS: Pirimiphos-methyl; pesticide; monoclonal antibody; immunoassay; ELISA

INTRODUCTION

The use of pesticides in agriculture has continuously increased since World War II, which has led to increased world food production. However, there are problems associated with the use of pesticides in terms of health to humans and other organisms living in the environment as a whole (*1*, *2*). Organophosphorus (OP) pesticides are widely applied in agriculture. The utilization of this class of pesticides is favored over that of their more persistent organochlorine counterparts because of their potency and ability to degrade more readily in the environment (*3*). Despite being susceptible to rapid degradation, OP pesticide contaminant residues have been reported in food (*4*) and environmental samples (*5*). Toxicity resulting from exposure to OP pesticides has been recognized as a particular problem, and neurotoxic syndromes resulting from exposure to these compounds have been well described (*6*).

Pirimiphos-methyl (*O*-2-diethylamino-6-methylpyrimidin-4-yl *O*,*O*-dimethylphosphorothioate) is a powerful OP pesticide with a range of activity toward many pests (*7*); it is active by contact, ingestion, and vapor action. Pirimiphos-methyl is mainly used during the storage of grain products such as corn, rice, durum wheat, and sorghum, during which it displays excellent persistence, as reported in the IPCS-INTOX databank of the World Health Organization (WHO) (*8*). The toxicologically relevant effect after administration of pirimiphos-methyl is the inhibition of acetylcholinesterase activity; that is, pirimiphos-methyl can overstimulate the nervous system, causing nausea, dizziness, confusion, and, at very high exposures (such as accidents or major spills), respiratory paralysis and death (*7*). The European Union regulates the maximum residue limit (MLR) in stored wheat, barley, and oat grain at 5 mg/kg and in animal-origin fat at 0.01 mg/kg (*9*), so there is a need to monitor the pirimiphos-methyl content in grain and some other foods destined for human consumption.

The current methods for the detection of pirimiphos-methyl residues in grains and grain products are gas chromatography (*10*, *11*) or liquid chromatography (*12*, *13*), sometimes associated with detection by cholinesterase enzyme inhibition (*14*). Although these analytical methods are sensitive and reliable, they are somewhat expensive and time-consuming and need much effort for sample preparation before analysis. Therefore, there is a demand for more rapid and economical methods for the routine determination of pirimiphos-methyl. Immunoassays such as enzyme-linked immunosorbent assays (ELISA) have recently emerged as an alternative to the traditional methods that can meet such demands. Immunoassays are analytical methods based on the interaction of analyte with antibody that recognizes it with high affinity and specificity. They are simple and cost-effective, do not require sophisticated instrumentation, and are able to analyze many samples simultaneously (*15*). These features convert ELISAs into very powerful tools for pesticide residue analysis. Skerritt et al. (*16*) described the development of competitive ELISA based on polyclonal antibody for quantitation of pirimiphos-methyl in wheat grain and flour-milling fractions; the resulting ELISA had a detection limit of 0.02 ng/mL and showed 320% cross-reactivity with pirimiphos-ethyl.

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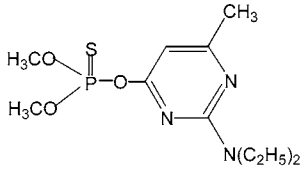
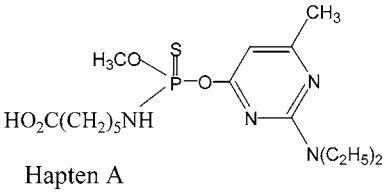
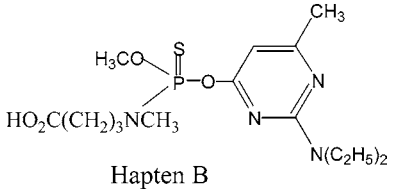


Pirimiphos-methyl



Hapten A



Hapten B

Figure 1. Chemical structures of pirimiphos-methyl and haptens used for the preparation of the immunogen and coating antigen.

The purpose of this investigation is to develop an indirect competitive (IC) ELISA using monoclonal antibodies for pirimiphos-methyl detection and quantification in grain samples.

MATERIALS AND METHODS

Chemicals, Immunoreagents, and Instruments. Analytical grade pirimiphos-methyl was obtained from Wako Chemicals (Tokyo, Japan). Other pesticides listed in **Table 3** used for cross-reactivity studies were obtained from either Dr. Ehrenstorfer (Augsburg, Germany) or Chem Service (West Chester, PA). Pirimiphos-methyl haptens A and B (**Figure 1**) were kindly provided by Professor Yong Tae Lee from Yeungnam University (Kyongsan, South Korea). Complete and incomplete Freund's adjuvants and *N*,*N*-dicyclohexylcarbodiimide (DCC) were supplied by Aldrich Chemical Co. (Milwaukee, WI). Keyhole limpet hemocyanin (KLH), ovalbumin (OVA), and *N*-hydroxysuccinimide (NHS) were purchased from Sigma Chemical Co. (St. Louis, MO). Polyethylene glycol (PEG) 1500 was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Dialysis membrane (Spectra/Por; MW cutoff 6000−8000) was obtained from Spectrum Laboratories Inc. (Rancho Dominguez, CA). The P3-X63-Ag8.653 murine myeloma cell line was obtained from Microbiology Laboratory, Medical College, Gyeongsang National University (Chinju, South Korea). Dulbecco's modified Eagle medium (DMEM), fetal calf serum, and supplements were obtained from Gibco BRL (Paisley, Scotland). Microculture plates (96 and 24 wells) were obtained from Falcon Co. (Lincoln Park, NJ). All other chemicals and organic solvents were of reagent grade or higher.

Standard solutions of pirimiphos-methyl and cross-reactants were prepared by dilution of stock solutions of these compounds (1 mg/mL) with methanol (MeOH).

Measurements of optical density for 96-well microtiter plates were performed on a Bio-Rad model 550 microplate reader (Bio-Rad Laboratories, Richmond, CA). MPM software (version 4.0, Bio-Rad) was used for data processing.

Preparation of Immunogen and Plate Coating Antigen. Hapten A was covalently attached to KLH for use as the immunogen, and hapten B was attached to OVA to be used as the coating antigen for competitive assays. The immunogen and plate coating antigen were conjugated to carrier molecules using modified active esters method (*17*). One hundred micromoles of each hapten was dissolved in 2 mL of absolute dimethylformamide (DMF) with equimolar NHS and DCC. The reaction was carried out at room temperature for 4 h with constant stirring, and then the mixture was centrifuged at 1750*g* for 10 min to remove the precipitated dicyclohexylurea. Afterward, the solution of activated compound was added drop by drop to the protein (KLH and OVA) solution (50 mg of protein in 5 mL of 0.2 M borate buffer, pH 9.0, and 1 mL of DMF). The reaction mixture was stirred gently at room temperature for 3 h, followed by overnight incubation at 4 °C. The formed conjugate was purified by dialysis in 0.05 M phosphate-buffered saline (PBS).

Immunization. Seven-week-old BALB/C female mice were immunized with hapten A−KLH conjugate. The first dose consisted of 30 μg of conjugate intraperitoneally injected as an emulsion of PBS and complete Freund's adjuvant. Two subsequent injections were given at 2-week intervals emulsified in incomplete Freund's adjuvant. One week after the last boost, serum was collected from the caudal vein of each mouse, and antisera titers were determined by a noncompetitive indirect ELISA. Three days before cell fusion, the mouse that produced antisera with high titer was given a final soluble intraperitoneal injection of 100 μg of conjugate in PBS without adjuvant.

Cell Fusion. Immunized mouse spleen cells (6×10^7) were fused with murine myeloma cells (P3-X63-Ag8.653) according to standard procedures (*18*). Briefly, mouse spleen lymphocytes were fused with myeloma cells at a 10:1 ratio using PEG 1500 as the fusing reagent. The fused cells were distributed in 96-well culture plates at a density of $\approx 10^5-10^6$ cells/well in 50 μL of DMEM supplemented with 25 μg/mL gentamicin and 20% fetal bovine serum (referred to as s-DMEM). One day after plating, 50 μL of HAT selection medium (s-DMEM supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine) was added to each well. Half of the medium in the wells was replaced by fresh HAT medium every other day. The HAT media were changed to HT media with no aminopterin when most of the nonfused cells were eliminated.

Hybridoma Selection and Cloning. Eight to 11 days after cell fusion, when the hybridoma cells had grown to ≈30−40% confluence in the well, culture supernatants were screened for the presence of antibodies that recognized pirimiphos-methyl. The screening consisted of the simultaneous performance of a noncompetitive and a competitive indirect ELISA to test the ability of antibodies to bind the hapten B−OVA conjugate and to recognize pirimiphos-methyl, respectively. For each culture supernatant, the signal obtained in noncompetitive conditions was compared with the competitive one, and the ratio of both absorbances was used as the criterion for selecting high-affinity antibody-secreting clones. Selected hybridomas were cloned by limiting dilution using HT medium. Stable antibody-producing clones were expanded and stored in liquid nitrogen.

Purification and Characterization of Anti-pirimiphos-methyl MAb. Female BALB/c mice, not less than 6 weeks old, were injected intraperitoneally with 500 μL of pristane, 10 days prior to interperitoneal injection of $\approx 5 \times 10^6$ hybridoma cells that had been resuspended in phosphate-buffered saline. Ascites fluid was collected from a single tapping with a 22-gauge needle within 14 days. Fluid was clarified by centrifugation at 500*g* for 10 min. Monoclonal antibodies (MAbs) were purified from mice ascites by ammonium sulfate precipitation followed by affinity chromatography on a protein G column (Bioprogen, South Korea). The protein concentration of the purified MAb was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA). The isotype of cloned MAb was determined with mouse monoclonal antibody isotyping kit (Roche Applied Science) according to the instructions of the manufacturer.

IC-ELISA. Checkerboard assays were used to select the most suitable anti-pirimiphos-methyl MAb and also used to determine the optimum amount of MAb and coating antigen for the competitive assays. In these checkerboard assays, several dilutions of MAbs were titrated against various amounts of the coating antigen. The checker-

board assays were performed as follows. Microtiter plates were coated with 100 μL/well of coating antigen hapten B−OVA (0, 125, 250, 500, 1000, and 2000 ng/mL) in carbonate−bicarbonate buffer (50 mM, pH 9.6) by overnight incubation at 4 °C. The plates were washed three times with PBST (phosphate-buffered saline containing 0.05% Tween 20) and were blocked by incubation with 1% skim milk in PBS (200 μL/well) at 37 °C for 1 h. After another washing step, 100 μL/well of MAb previously diluted with PBS (1/10, 1/100, 1/500, and 1/1000) was added. After incubation for 1 h, the plates were washed four times with PBST, and 100 μL/well of goat anti-mouse peroxidase-conjugated IgG (1/5000 dilution in PBS) was added. After incubation at 37 °C for 1 h, the plates were washed five times with PBST. Then, 100 μL/well of ABTS [2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)] solution containing 0.03% H_2O_2 was added and incubated for 30 min in the dark at room temperature. The absorbance was measured at 405 nm using a Bio-Rad model 550 microplate reader.

To minimize the background and maximize signal-to-noise ratios in the assay, plate blocking conditions were optimized: 1% BSA, 1% gelatin, 1% skim milk, and 1% casein were tested for their effects on the sensitivity of IC-ELISA.

Using the most suitable combination of the amount of coating antigen and MAb, the tolerance of ELISA to various water-miscible organic solvents used to dissolve pesticides was tested for assay optimization. Three solvents, methanol, acetone, and acetonitrile, were tested for their effects on Ab−Ag interactions. Briefly, each solvent was diluted to 5, 10, 20, and 40% in PBS. Then, a series of pirimiphos-methyl concentrations (1000, 100, 10, 1, 0.1, 0.01, and 0.001 ng/mL) were prepared in each diluted solvent and used in an IC-ELISA, respectively.

The procedure of the competitive assay was as follows. To microtiter plates coated and blocked as described above was added 50 μL/well of serially diluted primiphos-methyl standard solutions (1000, 100, 10, 1, 0.1, 0.01, and 0.001 ng/mL in 10% MeOH−PBS) or appropriately diluted pirimiphos-methyl extract (in 10% MeOH−PBS), followed by 50 μL/well of MAb (1/100 dilution in PBS). After incubation at 37 °C for 1 h, antibody binding was assessed as described above. Competitive curves were obtained by plotting absorbance against the logarithm of analyte concentration.

Determination of Cross-Reactivity. The cross-reactivity for several compounds structurally related to pirimiphos-methyl was tested using the indirect competitive ELISA procedure described above. The cross-reactivity values were calculated as % CR = (IC_{50} of pirimiphos-methyl/IC_{50} of analyte) × 100.

Analysis of Spiked Samples. For recovery studies, pirimiphos-methyl was spiked into durum wheat and barley; recoveries were determined by the IC-ELISA. Each sample was ground into fine pieces. Five grams of each sample was spiked with different concentrations of pirimiphos-methyl (100, 500, and 1000 ng/g) in a 50 mL conical tube. After the closed conical tubes had been kept in the dark at room temperature for 48 h, the solvent in the spiked samples was removed by evaporation at room temperature for 1 h. Each spiked sample was extracted with 25 mL of methanol for 15 min with shaker. After filtration through Whatman paper (no. 4), a 100 μL aliquot was added to PBS (final volume = 1 mL) without any further treatment, resulting in 10% MeOH concentration (v/v), and a sample dilution factor of 1:50. The extract was analyzed by IC-ELISA, and recovery was determined using the standard curve obtained from the standards in 10% MeOH−PBS.

RESULTS AND DISCUSSION

Production of Antibodies to Pirimiphos-methyl. Pirimiphos-methyl, like most pesticides, is a small and simple organic molecule that is nonimmunogenic by itself and lacks a functional group for coupling to proteins. Therefore, the synthesis of hapten and conjugation to carrier proteins are necessary and critical steps in the preparation of desirable immunoreagents. In this study, two pirimiphos-methyl derivatives (**Figure 1**) were used for conjugation with carrier proteins. In theory, immunogens with longer spacers between hapten and carrier protein (five to seven CH_2 groups) are preferable to those with shorter spacers

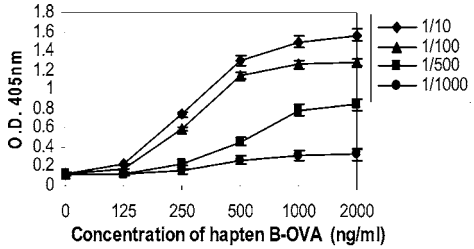


Figure 2. Results for ELISA checkerboard assay. Each point is the average of three determinations. Different lines represent different MAb 4D1 dilutions.

(*19*). Therefore, hapten A was conjugated with KLH to be used as an immunogen for the production of monoclonal antibodies, and hapten B was conjugated with OVA to be used as plate coating antigen. The haptens used in this study were similar but not identical as previously reported (*16*). As immunogen, the space between hapten and carrier protein in the previous study was two CH_2 groups, whereas in our study the space was five CH_2 groups.

Among the mice previously immunized with hapten A−KLH, the one with the highest polyclonal antibody titer was selected for cell fusion. In the screening of culture supernatants, only wells showing a signal inhibition of >80% in the presence of 1 μg/mL pirimiphos-methyl with respect to that obtained in the absence of analyte were considered to contain high-affinity antibody-secreting clones. Of the 384 wells of fusion plates, only 3 wells fulfilled the above-mentioned criterion, so they were accordingly expanded and clones expressing MAbs were selected.

About 10−15 days after hybridoma cell injection, ascites fluid was obtained from the intraperitoneal cavity of inoculated mice. MAbs were purified from mice ascites by ammonium sulfate precipitation followed by affinity chromatography on a protein G column. The protein concentration of the purified MAbs was determined to be ≈1.5 mg/mL using the Bio-Rad protein assay kit. An isotyping experiment revealed that the isotype of all MAbs was IgG1 with kappa light chain.

IC-ELISA. Prior to the competitive experiments, the ability of three MAbs (named 2F6, 4D1, and 4F6) to recognize hapten B−OVA was evaluated by checkerboard titration. All MAbs showed good binding with hapten B−OVA; titers were 1/250, 1/1000, and 1/500 for MAb 2F6, MAb 4D1, and MAb 4F6, respectively, with 500 ng/mL hapten B−OVA-coated microtiter plates. MAb 4D1 had the best titer for binding hapten B−OVA and, therefore, was selected as the most suitable one for ELISA.

The combination of the concentrations of MAb 4D1 and hapten B−OVA was also investigated and optimized by checkerboard assays. As shown in **Figure 2**, at 1/100 dilution of MAb in PBS, increasing the concentration of hapten B−OVA from 500 to 1000 ng/mL did not significantly increase the absorbance, indicating that 500 ng/mL of hapten B−OVA almost saturated the plate well surface. In addition, at the level of 500 ng/mL of hapten B−OVA, dilution of MAb from 1/100 to 1/500 resulted in the absorbance decreasing from 1.144 to 0.458. Therefore, the results indicated that the optimal coating concentration is 500 ng/mL and that the dilution of MAb 4D1 should be 1/100.

To minimize the background and maximize signal-to-noise ratios in the assay, blocking agents were evaluated to hinder nonspecific sorption of an antibody onto the less coated plate. Various blocking agents have been tested in this study. As shown in **Table 1**, all of the tested blockers enhanced the assay

Table 1. Effect of Blocking Agents on IC-ELISA with the Use of MAb 4D1[a]

blocking agent	Abs_{max}	Abs_{min}	IC_{50} (ng/mL)
none	1.693 ± 0.045	0.357 ± 0.028	14.5 ± 0.22
1% BSA	1.348 ± 0.013	0.283 ± 0.026	9.4 ± 0.21
1% gelatin	1.532 ± 0.024	0.321 ± 0.017	12.6 ± 0.19
1% skim milk	1.287 ± 0.018	0.087 ± 0.009	4.8 ± 0.14
1% casein	1.247 ± 0.025	0.218 ± 0.011	7.3 ± 0.17

[a] Data are the means of three determinations and expressed as mean ± SD.

Table 2. Effect of Organic Solvents on IC-ELISA with the Use of MAb 4D1[a]

solvent	concn (%)	Abs_{max}[b]	Abs_{min}	IC_{50} (ng/mL)
methanol	5	1.146 ± 0.022	0.084 ± 0.011	4.3 ± 0.16
	10	1.257 ± 0.018	0.082 ± 0.008	4.5 ± 0.14
	20	1.248 ± 0.032	0.089 ± 0.014	8.6 ± 0.27
	40	1.234 ± 0.025	0.086 ± 0.017	15.8 ± 0.32
acetone	5	1.013 ± 0.036	0.075 ± 0.013	16.2 ± 0.36
	10	0.654 ± 0.015	0.087 ± 0.009	24.6 ± 0.23
	20	0.235 ± 0.011	0.089 ± 0.016	37.8 ± 0.29
	40[c]			
acetonitrile	5	1.154 ± 0.028	0.086 ± 0.012	14.6 ± 0.25
	10	0.877 ± 0.031	0.092 ± 0.021	27.4 ± 0.38
	20	0.314 ± 0.021	0.098 ± 0.019	35.8 ± 0.25
	40[c]			

[a] Assay conditions are described in **Figure 3**. [b] Data are the means of three determinations and expressed as mean ± SD. [c] No data available due to poor color development.

sensitivity compared to "no blocking". Skim milk as a blocker showed a lower background signal, a higher ratio of maximum absorbance to minimum absorbance, and a higher sensitivity for the ELISA than other blockers. Therefore, 1% of skim milk was selected as the optimized blocking condition.

Because of the low solubility of pirimiphos-methyl in aqueous solution, such as in the water or PBS, organic solvents are commonly used to deliver the pirimiphos-methyl residues from food and environmental samples, so it is desirable to assess the effect of organic solvents on ELISA performance. The effects of the organic solvents (MeOH, acetone, and acetonitrile) on the ELISA system were evaluated by preparing standard curves in buffers containing various amounts of organic solvents (5, 10, 20, and 40% in PBS). The results are presented in **Table 2**. For acetone and acetonitrile, Abs_{max} decreased significantly as their solvent levels increased, indicating that these solvents suppress antibody−coating antigen binding. These results are consistent with the previous results of other researchers (*20*, *21*), who also found that solvents could significantly influence the signals. On the contrary, the MeOH concentration did not inhibit the color development even at the highest concentration tested (40%) as compared to that at 5% MeOH. Because IC_{50} values in the presence of acetone and acetonitrile were considerably higher than those in the presence of MeOH, we selected MeOH as the most suitable solvent for IC-ELISA. Assay sensitivity was highest at 5% MeOH. However, 10% MeOH was selected as the optimum concentration, because the IC_{50} value at 10% (4.5 ng/mL) was close to that at 5% (4.3 ng/mL) and Abs_{max} is higher at 10% MeOH.

The representative standard curve for the detection of primiphos-methyl in 10% MeOH/PBS by the competitive ELISA at the established conditions is shown in **Figure 3**. The IC_{50} value, the concentration causing 50% inhibition of the color,

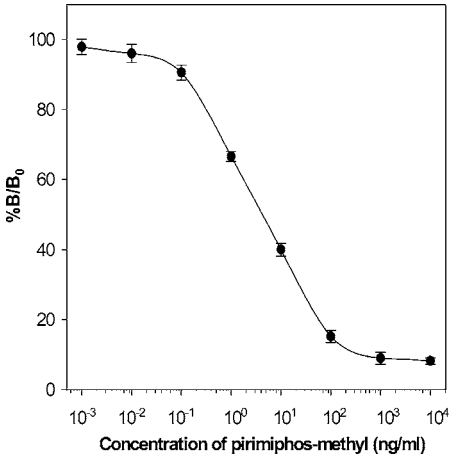


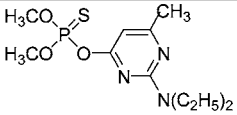
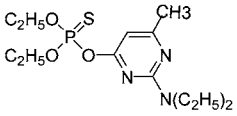
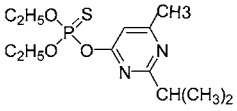
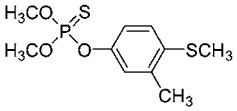
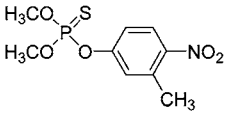
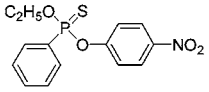
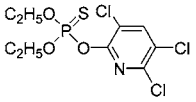
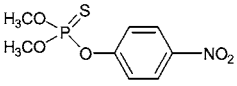
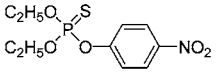
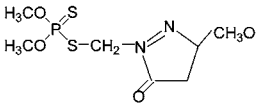
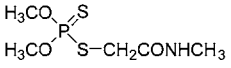
Figure 3. ELISA inhibition curves for pirimiphos-methyl by IC-ELISA. Assay conditions: primary antibody dilution 1/100; plate blocking with 1% of skim milk; coating antigen (hapten B–OVA) at 500 ng/mL; goat anti-mouse IgG–HRP (secondary antibody) dilution 1/5000; assay solution containing 10% MeOH in PBS, pH 7.4. Each point of the curve represents the mean ± SD of six assays. $\%B/B_0$ = (A_{405} with competing pirimiphos-methyl/A_{405} without pirimiphos-methyl) × 100.

was 4.2 ng/mL, and the detection limit was 0.07 ng/mL (10% inhibition). The sensitivity of this assay was somewhat lower than that observed in previously developed polyclonal antibody-based pirimiphos-methyl competitive ELISA (IC_{50} = 0.21ng/mL) (*16*).

Cross-Reactivity Studies. The cross-reactivity for several compounds structurally related to pirimiphos-methyl was tested (**Table 3**). Pirimiphos-ethyl, the diethyl ester analogue of pirimiphos-methyl, was characterized by the highest percent of cross-reactivity (38%). Some cross-reactivity observed with diazinon (≈0.5%) is understandable, because of its similar aromatic structure to pirimiphos-methyl. For other OP pesticides, cross-reactivity was negligible (<0.1%). The specificity of this assay is higher than that observed in the previously developed polyclonal antibody-based ELISA by Skerritt et al. (*16*). In their study, pirimiphos-ethyl showed 320% cross-reactivity with pirimiphos-methyl, whereas in our study, pirimiphos-ethyl showed 38.1% cross-reactivity with pirimiphos-methyl (**Table 3**).

Recovery Studies. Durum wheat and barley were spiked with pirimiphos-methyl (100, 500, and 1000 ng/g), and recoveries of pirimiphos-methyl from the grains were determined by IC-ELISA. It is generally known that antigen−antibody interaction in immunoassays can be affected by various substances existing in complex matrices. To reduce matrix interferences, two common approaches could be used. The first approach is sample cleanup, which is laborious and time-consuming and may affect assay reproducibility and recovery. The second approach is dilution of the extract. One of the major advantages of immunoassay techniques is their simplicity, so the second approach was used. In this study, samples for the recovery studies were prepared by extraction of 5 g of spiked grains with MeOH, followed by the dilution of the extract in PBS without any further treatment, resulting in 10% MeOH concentration (v/v). The results are presented in **Table 4**. Spike recoveries ranged from 83 to 96%. This is comparable with the recoveries of pirimiphos-methyl by the gas chromatographic (GC) method described by Hiskia et al. (*22*). In their study, the mean recoveries of pirimiphos-methyl by GC assay ranged from 79 to 87%. No false-positive results for blank (unspiked) samples were observed. The results were in a good agreement with the

Table 3. Cross-Reactivity of MAb to Pirimiphos-methyl and Related Compounds by IC-ELISA[a]

Compound	Structure	IC_{50} (ng/ml)	Cross-reactivity, %
Pirimiphos methyl		4.2	100
Pirimiphos ethyl		11	38.1
Diazinon		840	0.5
Fenthion		>5000	<0.1
Fenitrothion		>5000	<0.1
EPN		ni[b]	0
Chlorpyrifos		>5000	<0.1
Parathion methyl		ni	0
Parathion ethyl		ni	0
Methidathion		>5000	<0.1
Dimethoate		ni	0

[a] Assay conditions were the same as those described in **Figure 3**. [b] No inhibition.

Table 4. Recovery of Pirimiphos-methyl from Spiked Grain Samples

sample	spiked conn (ppb)	recovery (%)	CV (%)
durum wheat ($n = 5$)	100	94	12.3
	500	85	7.9
	1000	87	9.1
barley ($n = 5$)	100	96	11.4
	500	92	8.4
	1000	83	5.6

amounts spiked, demonstrating the applicability of the developed assay to grain samples. ELISA was characterized by good reproducibility; interassay CVs ranged from 5.6 to 12.3%. The maximum residue level (MRL) for pirimiphos-methyl on grains is 5 ppm (*9*), so the IC-ELISA can be applied for pirimiphos-methyl determination in grains in the concentration range mentioned above.

In conclusion, a monoclonal antibody-based IC-ELISA was developed for the detection of the OP insecticide pirimiphos-methyl in grain samples. The IC_{50} value of the optimized IC-ELISA was 4.2 ng/mL. The assay showed negligible cross-reactivity with other OP pesticides tested in this study except pirimiphos-ethyl. Compared to the previously developed ELISA for pirimiphos-methyl (*16*), the sensitivity of our IC-ELISA in terms of IC_{50} was somewhat lower. However, the specificity in

terms of cross-reactivity was considerably higher. Without the sample cleanup, methanol extracts of grains were diluted in PBS and subsequently applied to ELISA. Recoveries for spiked grain samples were reasonable. Therefore, this assay would be very useful for the rapid screening of pirimiphos-methyl-containing grain samples.

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LITERATURE CITED

(1) World Health Organisation. Informal consultation on planning strategy for the prevention of pesticide poisoning; WHO: Geneva, Switzerland, 1986; WHO/86.926.

(2) Jeyaratnam, J. Pesticide poisoning: a major global health problem. *World Health Status Q.* **1990**, *43*, 139−144.

(3) Eneji, I. S.; Buncel, E.; Vanlooon, G. W. Degradation and sorption of pirimiphos-methyl in two Nigerian soils. *J. Agric. Food Chem*. **2002**, *50*, 5634−5639.

(4) U.S. Food and Drug Administration. The Food and Drug Administration's (FDA) pesticide residue monitoring program−1993. *J. AOAC Int*. **1994**, *77*, 161A−185A.

(5) Hallberg, G. R. Pesticide pollution of groundwater in the humid United States. *Agric. Ecosyst. Environ.* **1989**, *26*, 299−367.

(6) Maroni, M.; Fait, A. Health effects in man from long-term exposure to pesticides. *Toxicology* **1993**, *78*, 1−80.

(7) U.S. EPA. Pirimiphos-methyl IRED facts; 2003; http://www.epa.gov/oppsrrd1/REDs/factsheets/pirimiphosmethyl_ired_fs.htm.

(8) WHO. *Data Sheet on Pesticides 49: Pirimiphos-methyl*; World Health Organization: Geneva, Switzerland, 1983; http://www.intox.org/databank/documents/chemical/pirimet/pest49 e.htm.

(9) European Food Safety Authority. Conclusion regarding the peer review of the pesticide risk assessment of the active substance pirimiphos-methyl. *EFSA Sci. Rep.* **2005**, *44*, 1−53.

(10) Rastrelli, L.; Totaro, K.; Simone, F. D. Determination of organophosphorus pesticide residues in Cilento (Campania, Italy) virgin olive oil by capillary gas chromatography. *Food Chem*. **2002**, *79*, 303−305.

(11) Bottomley, P.; Baker, P. G. Multi-residue determination of organochlorine, organophosphorus and synthetic pyrethroid pesticides in grain by gas−liquid and high-performance liquid chromatography. *Analyst* **1984**, *109*, 85−90.

(12) Herrmann, J. M.; Guillard, C.; Arguello, M.; Agüera, A.; Tejedor, A.; Piedra, L.; Fernández-Alba, A. Photocatalytic degradation of pesticide pirimiphos-methyl: determination of the reaction pathway and identification of intermediate products by various analytical methods. *Catal. Today* **1999**, *54*, 353−367.

(13) Al-Makkawy, H. K.; Madbouly, M. D. Persistence and accumulation of some organic insecticides in Nile water and fish. *Resour. Conserv. Recycl*. **1999**, *27*, 105−115.

(14) Carlo, M. D.; Pepe, A.; Gregorio, M. M. D.; Compagnone, A. V. D. Determining pirimiphos-methyl in durum wheat samples using an acetylcholinesterase inhibition assay. *Anal. Bioanal. Chem.* **2005**, *381*, 1367−1372.

(15) Moreno, M. J.; Abad, A.; Montoya, A. Production of monoclonal antibodies to the *N*-methylcarbamate pesticide propoxur. *J. Agric. Food Chem.* **2001**, *49*, 72−78.

(16) Skerritt, J. H.; Hill, A. S.; Beasley, H. L.; Edward, S. L.; Mcadam, D. P. Enzyme-linked immunosorbent assay for quantitation of organophosphate pesticides: fenitrothioin, chlorpyrifos-methyl, and pirimiphos-methyl in wheat grain and flour-milling fractions. *J. AOAC Int.* **1992**, *75*, 519−528.

(17) Langone, J. J.; Van Vunakis, H. Radioimmunoassay for dieldrin and aldrin. *Res. Commun. Chem. Pharmacol.* **1975**, *10*, 163−171.

(18) Galfre, G.; Howe, S. C.; Milstein, C.; Butcher, G. W.; Howard, J. C. Antibodies to major histocompatability antigens produced by hybrid cell lines. *Nature* **1977**, *266*, 550−552.

(19) Goodrow, M. H.; Hammock, B. D. Hapten design for conpound-selective antibodies: ELISA for environmentally deleterious small molecules. *Anal. Chim. Acta* **1998**, *376*, 83−91.

(20) Wang, X.; Chen, F.; Wan, P. J.; Huang, G. Development of monoclonal antibody-based enzyme-linked immunosorbent assay for gossypol analysis in cottonseed meals. *J. Agric. Food Chem.* **2004**, *52*, 7793−7797.

(21) Cho, Y. A.; Kim, Y. J.; Hammock, B. D.; Lee, Y. T.; Lee, H. S. Development of a microtiter plate ELISA and a dipstick ELISA for the determination of the organophosphorous insecticide fenthion. *J. Agric. Food Chem.* **2003**, *51*, 7854−7860.

(22) Hiskia, A. E.; Atmajidou, M. E.; Tsipi, D. F. Determination of organophosphorus pesticide residues in Greek virgin olive oil by capillary gas chromatography. *J. Agric. Food Chem*. **1998**, *46*, 570−574.

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