JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of an Enzyme-Linked Immunosorbent Assay for the Organophosphorus Insecticide Bromophos-ethyl

KWANG-OK KIM,† YOO JUNG KIM,‡ YONG TAE LEE,‡ BRUCE D. HAMMOCK,§ AND HYE-SUNG LEE*,†

Department of Food Science and Nutrition, Kyungpook National University, Daegu 702-701, Korea; Department of Biochemistry and Institute of Natural Sciences, Yeungnam University, Kyongsan 712-749, Korea; and Department of Entomology and Cancer Research Center, University of California, Davis, California 95616

A competitive enzyme-linked immunosorbent assay (ELISA) was developed for the quantitative detection of the organophosphorus insecticide bromophos-ethyl. Four bromophos-ethyl derivatives (haptens) were synthesized and were coupled to carrier proteins through the pesticide thiophosphate group to use as an immunogen or as a coating antigen. Rabbits were immunized with either one of two haptens coupled to bovine serum albumin for production of polyclonal antibodies, and the sera were screened against one of the haptens coupled to ovalbumin. Using the serum with highest titer, an antigen-coated ELISA was developed, which showed an IC_{50} of 3.9 ng/mL with a detection limit of 0.3 ng/mL (20% inhibition). An antibody-coated ELISA using an enzyme tracer was also developed, which showed an IC_{50} of 6.5 ng/mL with a detection limit of 1.0 ng/mL (20% inhibition). The antibodies showed negligible cross-reactivity with other organophosphorus pesticides except with the insecticides bromophos-methyl and chlorpyrifos in the antibody-coated assay only. Recoveries of bromophos-ethyl from fortified crop and water samples ranged from 82 to 128% and from 95 to 127%, respectively.

KEYWORDS: Bromophos-ethyl; insecticide; immunoassay; ELISA

INTRODUCTION

Due to the widespread use of pesticides, there is increasing concern over food and environmental contamination caused by their use. The current methods such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been used successfully, with high sensitivity and reliability, for analysis of many pesticides (*1*). However, these classical methods require a high capital expenditure and skilled analysts and involve time-consuming sample preparation steps. Therefore, there is a growing demand for more rapid and economical methods for determining pesticide residues. Immunoassays have recently emerged as an alternative to the traditional methods that can meet such demands. Immunochemical techniques that have been used extensively in clinical laboratories began recently to gain acceptance as a fast, sensitive, and cost-effective tools for detecting trace amounts of chemicals such as pesticides (*2*).

Bromophos-ethyl [*O*,*O*-diethyl *O*-(4-bromo-2,5-dichlorophenyl)phosphorothioate] is an organophosphorus insecticide and acaricide, which is effective against a wide range of insects (*3*). The most sensitive and toxicologically relevant effect after administration of bromophos-ethyl is inhibition of acetylcholinesterase activity (*4*). Analysis of bromophos-ethyl is carried out by multiresidue methods using gas−liquid chromatography (*5*, *6*). An enzyme-linked immunosorbent assay (ELISA) for this pesticde has not yet been reported.

The development of an immunoassay requires the production of antibodies to the analyte. Because pesticides are small molecules, pesticide derivatives, namely haptens, must be synthesized and coupled to carrier proteins to induce antibody production. One type of hapten for organophosphorus pesticides is the one with an amino carboxylic acid bridge at thiophosphate group, which has been used successfully in the development of ELISA for several organophosphorus pesticides (*7*−*12*). We have developed a novel method for the synthesis of such haptens, which is much easier than the previous one (*7*). This paper describes the application of this method to the synthesis of haptens for bromophos-ethyl from which specific antibodies to bromophos-ethyl were obtained. Using the antibodies, sensitive and selective ELISAs for bromophos-ethyl were developed.

MATERIALS AND METHODS

Chemicals and Instruments. Organophosphorus pesticides including bromophos-ethyl were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Ethyl dichlorothiophosphate, 4-aminobutyric acid, 5-aminovaleric acid, 6-aminocaproic acid, 3-(methylamino)butyric acid hydrochloride, *N*-hydroxysuccinimide, 1,3-dicylohexylcarbodiimide, 4-(dimethylamino)pyridine, chlorform-*d*, silica gel (60−230 mesh), and polyoxyethylene (20) sorbitan monolaurate (Tween 20) were obtained from Aldrich (Milwaukee, WI). Bovine serum albumin (BSA), ovalbumin (OVA), peroxidase-labeled goat anti-rabbit IgG, Freund's

† Kyungpook National University.
‡ Yeungnam University.
§ University of California.

10.1021/jf025703+ CCC: $22.00
Published on Web 10/04/2002

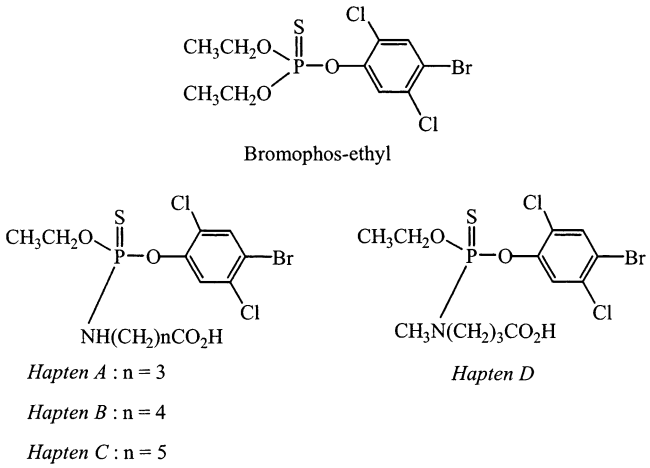


Figure 1. Structures of the haptens for bromophos-ethyl used for immunization and antigen coating.

complete and incomplete adjuvants, and Sephadex G-25 were purchased from Sigma (St. Louis, MO). Tetramethylbenzidine was obtained from Boehringer Mannheim (Mannheim, Germany). Analytical (silica gel F254) and preparative thin-layer chromatography (TLC) plates (silica gel, 1 mm) were purchased from Merck (Darmstadt, Germany). The dialysis membrane [molecular weight (MW) cutoff 12000−14000] was obtained from Spectrum Laboratories (Rancho Dominguez, CA). Microtiter plates (Maxisorp 442404) were purchased from Nunc (Roskilde, Denmark). ELISA plates were washed with a model 1575 ImmunoWash, and well absorbances were read with a model 550 plate reader, both from Bio-Rad (Hercules, CA). UV−vis spectra were recorded on a Varian (Palo Alto, CA) Cary 3 spectrophotometer. NMR spectra were obtained with a Bruker (Rheinstetten, Germany) ARX spectrometer (300 MHz). Chemical shift values are given relative to internal tetramethylsilane. Coupling constants are expressed in hertz, and the abbreviations d, t, q, qn, sx, m, and ar represent doublet, triplet, quartet, quintet, sextet, multiplet, and aromatic, respectively.

Synthesis of Haptens. The haptens used for immunization and antigen coating are presented in **Figure 1**. The synthetic routes for the haptens are illustrated in **Figure 2**. All of the haptens were purified by column chromatography to give a single spot on TLC plates. The procedure for the synthesis of hapten A was as follows.

III. The starting material 4-bromo-2,5-dichlorophenol (**II**) was synthesized according to a published procedure (*13*). A solution of **II** (0.92 g, 1.9 mmol) in 5 mL of acetonitrile was added dropwise to a stirred mixture of 3.2 g (19.2 mmol) of ethyl dichlorothiophosphate (**I**), 10 g of finely ground K_2CO_3, and 10 mL of acetonitrile. After 1 h of stirring at room temperature (RT), the mixture was filtered through Celite, and the solvent was removed under reduced pressure. The residue was subjected to column chromatography [silica gel, hexane/benzene (6:1)] to give 0.64 g (44%) of the product (**III**) as a colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.73 (1H, d, J = 1.3, ar), 7.61 (1H, d, J = 2.1, ar), 4.47 (2H, qxd, J = 11.1 and 7.1, $C\underline{H}_2CH_3$), 1.49 (3H, txd, J = 7.1 and 1.0, $CH_2C\underline{H}_3$).

Hapten A. To a stirred solution of 100 mg (0.26 mmol) of **III** in 0.5 mL of methanol cooled in an ice−water bath was added dropwise a solution of 38 mg (0.68 mmol) of KOH and 32 mg (0.31 mmol) of 4-aminobutyric acid in 0.25 mL of methanol. After stirring for 5 min, the reaction mixture was filtered and extracted with 1 N HCl−chloroform. The extract was dried over $MgSO_4$, and the solvent was evaporated. Column chromatography [silica gel, chloroform/ethyl acetate/acetic acid (19:9:1)] of the residue gave 72 mg (33%) of a white solid: ^{1}H NMR ($CDCl_3$) δ 7.67 (1H, d, J = 0.7, ar), 7.64 (1H, d, J = 1.6, ar), 4.21 (2H, dxqxd, J = 9.2, 7.1, and 1.8, $C\underline{H}_2CH_3$), 3.39 (1H, qn, J = 7.1, NH), 3.19 (2H, dxq, J = 12 and 6.8, $NHC\underline{H}_2$), 2.46 (2H, t, J = 7.2, $C\underline{H}_2CO_2H$), 1.88 (2H, qn, J = 7.0, $CH_2C\underline{H}_2CH_2$), 1.36 (3H, t, J = 7.1, $C\underline{H}_2C\underline{H}_3$).

Haptens B, C, and D were synthesized according to the same procedure as for *hapten A*, using 5-aminovaleric acid, 6-aminocaproic acid, and 4-(*N*-methylamino)butyric acid, respectively.

Hapten B. The yield was 40%: ^{1}H NMR ($CDCl_3$) δ 7.67 (1H, d, J = 0.6, ar), 7.65 (1H, d, J = 1.1, ar), 4.21 (2H, m, $C\underline{H}_2CH_3$), 3.37 (1H, m, NH), 3.13 (2H, m, J = 6.7, $NHC\underline{H}_2$), 2.36 (2H, t, J = 7.2, $C\underline{H}_2CO_2H$), 1.69 (2H, qn, J = 7.1, $NHCH_2C\underline{H}_2$), 1.60 (2H, qn, J = 6.9, $C\underline{H}_2CH_2CO_2H$), 1.37 (3H, t, J = 7.2, $CH_2C\underline{H}_3$).

Hapten C. The yield was 35%: ^{1}H NMR ($CDCl_3$) δ 7.66 (1H, s, ar), 7.66 (1H, s, ar), 4.21 (2H, dxqxd, J = 9.5, 7.2, and 2.1, $C\underline{H}_2CH_3$), 3.27 (1H, m, NH), 3.11 (2H, dxq, J = 11 and 6.9, $NHC\underline{H}_2$), 2.36 (2H, t, J = 7.3, $C\underline{H}_2CO_2H$), 1.71−1.42 [6H, m, $CH_2(C\underline{H}_2)_3C\underline{H}_2$], 1.37 (3H, txd, J = 7.2 and 0.51, $CH_2C\underline{H}_3$).

Hapten D. The yield was 69%: ^{1}H NMR ($CDCl_3$) δ 7.66 (1H, s, ar), 7.55 (1H, d, J = 1.1, ar), 4.21 (2H, m, J = 7.2, $C\underline{H}_2CH_3$), 3.34 (2H, m, $NC\underline{H}_2$), 2.87 (3H, d, J = 8.4, NCH_3), 2.43 (2H, t, J = 7.2, $C\underline{H}_2CO_2H$), 1.92 (2H, qn, J = 6.7, $CH_2C\underline{H}_2CH_2$), 1.35 (3H, t, J = 7.1, $C\underline{H}_2CH_3$).

Preparation of Hapten—Protein Conjugates. *Haptens A and C* were covalently attached to BSA to be used as immunogens. *Haptens B and D* were attached to OVA to be used as the coating antigens for serum screening and competitive assay, respectively. *Haptens A−D* were conjugated to horseradish peroxidase (HRP) to be used as enzyme tracers. The method of conjugation used was the active ester method (*11*). The procedure for the synthesis of this ester is described below. Other active esters were synthesized by using the same procedure. Hapten A (97 mg, 0.22 mmol) was dissolved in dichloromethane (5 mL) to which *N*-hydroxysuccinimide (28 mg, 0.24 mmol), *N*,*N*-dicyclohexylcarbodiimide (63 mg, 0.24 mmol), and 4-(dimethylamino)-pyridine (2.7 mg, 0.022 mmol) were added. The mixture was stirred for 3 h and filtered, and the solvent was removed. Chromatography of the resultant oil on silica gel using chlorform/ethyl acetate/acetic acid (30:9:1) followed by preparative TLC using the same eluent gave the active ester as a syrup (42 mg, 43%): ^{1}H NMR ($CDCl_3$) δ 7.67 (1H, s, ar), 7.63 (1H, d, J = 1.6, ar), 4.21 [2H, dxqxd, J = 9, 7, and 1 (approximate values, peaks were not well resolved), $C\underline{H}_2CH_3$], 3.56 (1H, m, N$\underline{H}$), 3.25 (2H, dxq, J = 13 and 6.8, $NHC\underline{H}_2$), 2.85 (4H, s, succinyl), 2.73 (2H, t, J = 7.2, $C\underline{H}_2CO_2H$), 1.98 (2H, qn, J = 7.0, $CH_2C\underline{H}_2CH_2$), 1.37 (3H, t, J = 7.1, $CH_2C\underline{H}_3$).

Active Ester of Hapten B. Yield 39%; ^{1}H NMR ($CDCl_3$) δ 7.67 (1H, s, ar), 7.65 (1H, d, J = 0.93, ar), 4.21 (2H, m, $C\underline{H}_2CH_3$), 3.45 (1H, m, NH), 3.16 (2H, dxq, J = 12 and 6.8, $NHC\underline{H}_2$), 2.85 (4H, s, succinyl), 2.65 (2H, t, J = 7.2, $C\underline{H}_2CO_2H$), 1.82 (2H, qn, $NHCH_2C\underline{H}_2$), 1.66 (2H, qn, J = 7.1, $C\underline{H}_2CH_2CO_2H$), 1.37 (3H, t, J = 7.1, $CH_2C\underline{H}_3$).

Active Este of Hapten C. Yield 41%; ^{1}H NMR ($CDCl_3$) δ 7.67 (1H, s, ar), 7.65 (1H, d, J = 0.93, ar), 4.21 (2H, dxqxd, J = 4.7 and 2.2, $C\underline{H}_2CH_3$), 3.57 (1H, m, NH), 3.26 (2H, dxq, J = 13 and 6.8, $NHC\underline{H}_2$), 2.85 (4H, s, succinyl), 2.73 (2H, t, J = 7.2, $C\underline{H}_2CO_2H$), 1.37 (3H, t, J = 7.1, $CH_2C\underline{H}_3$).

Active Ester of Hapten D. Yield 37%; ^{1}H NMR($CDCl_3$) δ 7.66 (1H, d, J = 0.8, ar), 7.55 (1H, d, J = 1.5, ar), 4.16 (2H, dxqxd, J = ~10, 7.1, and 2.4, $C\underline{H}_2CH_3$), 3.39 (2H, m, $NC\underline{H}_2$), 2.89 (3H, d, J = 11, $C\underline{H}_3N$), 2.85 (4H, s, succinyl), 2.69 (2H, t, J = 7.5, $C\underline{H}_2CO_2H$), 2.04 (2H, qn, J = 7.3, $CH_2C\underline{H}_2CH_2$), 1.36 (3H, txd, J = 7.1 and 0.6, $CH_2C\underline{H}_3$).

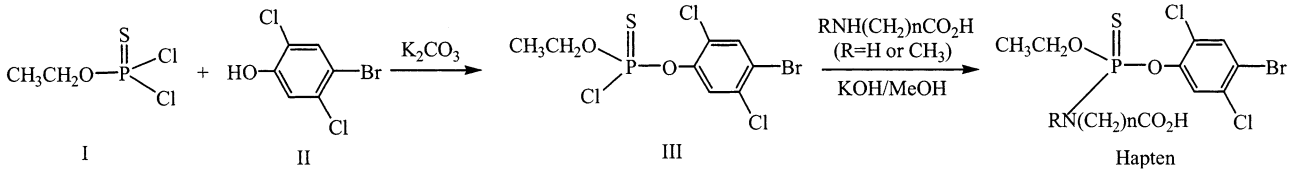


Figure 2. Synthetic route for haptens.

The procedures for coupling haptens to the carrier proteins were as follows. To prepare hapten−BSA conjugates (immunogens), BSA (20 mg) was dissolved in 2 mL of borate buffer (0.2 M, pH 8.7) to which 0.4 mL of DMF was added. A solution of an active ester (16 mg, 0.03 mmol) dissolved in 0.1 mL of DMF was then added to the stirred protein solution, and stirring was continued overnight at 4 °C. Hapten−OVA conjugates (coating antigens) were prepared by using the same procedure. Hapten−HRP conjugates (enzyme tracer) were prepared according to the same procedure except that two hapten/protein molar ratios (10 and 50) were employed. The conjugates were separated from the uncoupled haptens by gel filtration (Sephadex G-25) using PBS (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4). Finally, the eluates were dialyzed in water overnight and then freeze-dried.

Immunization of Rabbits. Female New Zealand white rabbits were immunized with hapten A− or hapten C−BSA. Routinely, 500 μg of the conjugate dissolved in 500 μL of PBS was emulsified with Freund's complete adjuvant (1:1 volume ratio) and injected intradermally at multiple sites on the back of each rabbit. After 2 weeks, each animal was boosted with an additional 500 μg of the conjugate emulsified with Freund's incomplete adjuvant and bled 7−10 days later. After this, boosting and bleeding were continued on a monthly basis. Serum was separated from blood cells by centrifugation, and sodium azide was added as a preservative at a final concentration of 0.02%. Serum was then aliquotted and stored at −70 °C.

Screening of Antisera. Several dilutions of each serum were titrated against the coating antigen (hapten B−OVA, 1000 ng/well) to measure the reactivity of antibodies. The procedure was similar to that for checkerboard assays described below.

Competitive Indirect ELISA. Checkerboard assays, in which several dilutions of sera were titrated against various amounts of the coating antigen, were used to select the most suitable antiserum and to have a rough estimate of appropriate antigen coating and antibody concentrations for competitive assays. The checkerboard assays were performed as follows. All incubations except that for antigen coating were carried out at RT. Microtiter plates were coated with hapten D−OVA (125−2000 μg/mL, 100 μL/well) in PBS (10 mM, pH 7.4) by overnight incubation at 4 °C. The plates were washed five times with PBST (10 mM PBS containing 0.05% Tween 20, pH 7.4) and were blocked by incubation with 1% gelatin in PBS (200 μL/well) for 1 h. After another washing step, 100 μL/well of antiserum previously diluted with PBST (1/1250−1/10000) was added. After incubation for 1 h, the plates were washed and 100 μL/well of a diluted (1/3000) goat anti-rabbit IgG−HRP was added. The mixture was allowed to incubate for 1 h, and after another washing step, 100 μL/well of a TMB solution (400 μL of 0.6% TMB−DMSO and 100 μL of 1% H_2O_2 diluted with 25 mL of citrate−acetate buffer, pH 5.5) was added. The reaction was stopped after 10 min by adding 50 μL of 2 M H_2SO_4, and absorbance was read at 450 nm.

From the results of the checkerboard assays, an antiserum raised against hapten A−BSA was selected as the most suitable one. Then, the concentrations of the antibodies and the coating antigen (hapten D−OVA) for competitive indirect assays were optimized. Additionally, the tolerance of ELISA to various water-miscible organic solvents used to dissolve pesticides was tested for assay optimization. For this test, standard pesticide solutions were prepared in various concentrations of acetone, acetonitrile, or methanol (10, 20, 40, and 80% in PBS, which became 5, 10, 20, and 40%, respectively, after combination with equal volumes of diluted antisera). The effect of buffering capacity of assay solution on ELISA performance was also studied using different concentrations of phosphate in 20% methanol−PBS to dissolve the pesticide (10, 90, 190, and 390 mM phosphate, which became 10, 50, 100, and 200 mM, respectively, after combining with equal volumes of the antisera diluted with 10 mM PBST). The influence of pH of the assay solution, that is, 50 mM PBS with 20% methanol, was also studied.

The procedure of the competition assay was as follows. To microtiter plates coated and blocked as described above was added 50 μL/well of serial dilutions of the analyte in methanol−PBS, followed by 50 μL/well of a previously determined antiserum dilution. After incubation at RT for 1 h, antibody binding was assessed as described above. Competition curves were obtained by plotting absorbance against the logarithm of analyte concentration. Sigmoidal curves were fitted to a four-parameter logistic equation (*14*), from which IC_{50} values (concentration at which binding of the antibody to the coating antigen is inhibited by 50%) were determined.

Competitive Direct Assay. Checkerboard assays, in which various dilutions of sera were titrated against various amounts of enzyme tracers (hapten A, C, or D conjugated to HRP), were used to select the most suitable antiserum and enzyme tracer and to have a rough estimate of their appropriate concentrations for competitive assays. The procedure for the checkerboard assays was the same as that for competitive assays (see below) except that only solvent instead of pesticide solution was added at the competition step. After the most suitable antiserum and enzyme tracer had been selected from the checkerboard assays, their quantities for the competitive direct assays were optimized. The tolerance of ELISA to various water-miscible organic solvents at the competition step was tested by using the same procedure as that for the indirect assay. The influence of phosphate ion concentration and pH of assay buffer (20% methanol−PBS) on ELISA performance was also studied using the same procedure as for the indirect assay. The direct assays were performed as follows. All incubations except that for precoating the wells with protein A were carried out at RT. Microtiter plates were coated with protein A (5 μg/mL, 100 μL/well) in carbonate−bicarbonate buffer (50 mM, pH 9.6) by overnight incubation at 4 °C. The plates were washed five times with PBST and were blocked by incubation with 100 μL/well of 1% gelatin in PBS for 1 h. Then, the plates were washed and coated with 100 μL/well of the antiserum dilutions in PBST for 1 h. After another washing step, serial dilutions of the analyte in 10% MeOH−PBS were added (50 μL/well) followed by 50 μL/well of an enzyme tracer previously diluted with PBS (77 ng/mL). After incubation for 1 h and another washing step, 100 μL/well of a TMB solution was added. The reaction was stopped after an appropriate time by adding 50 μL of 2 M H_2SO_4, and absorbance was read at 450 nm. Competition curves were obtained by using the same procedure as for the indirect assays.

Determination of Cross-Reactivities. Several organophosphorus pesticides and the metabolite of bromophos-ethyl (phenol) were tested for cross-reactivity using both the indirect and direct ELISA procedures described above. The cross-reactivity values were calculated as follows:

$$(IC_{50} \text{ of bromophos-ethyl}/IC_{50} \text{ of compound}) \times 100$$

Analysis of Spiked Samples. Solutions of bromophos-ethyl in methanol for the fortification of vegetable samples were prepared at 10, 50, 100, 500, and 1000 ng/mL. To 1 g of the finely chopped leaves of the vegetables grown pesticide-free was added 1 mL of a fortification solution. After being set aside for 24 h, the vegetable leaves were incubated in 5 mL of methanol for 10 min with four vigorous shakes and then filtered through Whatman no. 1 filter paper. The container and the residues were rinsed with 5 mL of methanol and filtered, and the filtrate was combined with the previous filtrate. Methanol was evaporated to dryness under reduced pressure, and the residue was resuspended in 10 mL of 20% methanol−PBS (50 or 10 mM for indirect and direct assay, respectively). The extract was analyzed by both the indirect and direct ELISAs.

Water samples from two different sources (tap water and pond water) were spiked with bromophos-ethyl by adding 1 mL of a fortification solution to 1 mL of membrane-filtered (0.25 μm) water samples. After evaporation of the sample under a slow stream of nitrogen, the residue was treated the same way as for vegetable samples.

RESULTS AND DISCUSSION

Hapten Selection and Synthesis. A suitable hapten for immunization should preserve the structure of the target compound as much as possible. The phosphorothioate organophosphorus (OP) pesticides such as bromophos-ethyl have a thiophosphate group in common and differ only in the structure of the aromatic rings. Therefore, to achieve a high selectivity in the bromophos-ethyl ELISA, it was desirable to synthesize

haptens having a bridge at the thiophosphate group preserving the substitute groups on aromatic ring unique to bromophos-ethyl. Haptens in this study have such structural features. Heldman et al. (*15*) were the first to synthesize a hapten for an OP pesticide with a spacer arm at the thiophosphate group. However, a generic method was developed later by McAdam et al. (*16*, *17*). This method was applied to the synthesis of haptens for the development of ELISAs of several OP pesticides (*7*−*12*). This method requires a synthetic route involving seven steps including protection and deprotection of both amino and carboxyl groups. In an effort to simplify the synthetic process for this class of haptens we developed a simpler generic method that requires only two steps (*7*). It involves the reaction of *O*-methyl (ethyl) dichlorothiophosphate with a phenol and K_2CO_3 in acetonitrile and the reaction of the substitution product with an amino carboxylic acid (not protected) and KOH in methanol (see **Figure 2** for an example). Secondary as well as primary amino acids could also be attached as the spacer arm. This method was successfully applied to the synthesis of four haptens for bromophos-ethyl (**Figure 1**). The reactions proceeded facilely with relatively high yields: 55 and 33−77% in the first and second reactions, respectively. The reaction time was relatively short: 1 h and a few minutes for the first and second reactions, respectively. All of the haptens purified by column chromatography were in a quite pure form, giving a single spot on the TLC plate. This method was successfully applied in this laboratory for the synthesis of haptens of several other OP pesticides such as chlorpyrifos (*7*), chlorpyrifos-methyl, diazinon, fenitrothion, parathion-methyl, and isofenphos (*18*), and we applied for a patent. All of the carboxylic acid haptens could be converted to the succinimide esters, active esters for coupling haptens to carrier proteins.

Competitive Indirect ELISA. The checkerboard assays, in which several dilutions of the sera were titrated against various amounts of the coating antigen (hapten D−OVA) were used to select the most suitable antiserum and to optimize antigen coating and antibody concentrations. Hapten D was used as a coating hapten because it is heterologous to the immunizing haptens in the structure of the bridge group. Hapten heterology is commonly used to eliminate problems associated with the strong affinity of the antibodies to the spacer arm that leads to no or poor inhibition by the target compound. Heterology usually results in weaker recognition of plate-coating antigens compared with recognition of the target compound, allowing analyte at low concentrations to compete with coating antigens. The antiserum from hapten A was selected as the most suitable one on the basis of its titer value which is the highest. The optimum condition selected was the combination of the serum from hapten A−BSA (third boost) diluted 1/1000 and the coating antigen in 150 ng/well.

The use of organic solvents for extraction and/or solid phase cleanup is very common in the analysis of nonpolar pesticide residues in food and environmental samples, so it is desirable to assess the effect of organic solvents on ELISA performance. The effects of solvents (acetone, acetonitrile, and methanol) on the ELISA system were evaluated by preparing standard curves in buffers containing various amounts of organic solvent (5, 10, 20, and 40% in PBS). The results are presented in **Table 1**. These solvents significantly influenced assay performance. The speed of color development (estimated from A_{max}) in the presence of acetone or acetonitrile was much slower compared with that in the presence of methanol. It is interesting to note that although the maximum absorbance was enhanced by increasing the concentration of methanol, an opposite trend was observed with acetone and acetonitrile. IC_{50} values in the presence of acetone and acetonitrile were much higher than those in the presence of methanol. Accordingly, we selected methanol as the most suitable cosolvent. Several other workers reached the same conclusion as ours in that methanol caused the least negative effect on the performance of these assays. However, their assays showed diverse direction and magnitude of response to increasing concentration of methanol (*19*−*22*). **Table 1** shows that increasing the concentration of methanol causes an initial increase followed by a decrease in sensitivity showing the lowest IC_{50} value at 20% methanol. **Table 1** also presents the effect of the phosphate ion concentration at the competition step on ELISA characteristics. Increasing the concentration of phosphate ions caused an initial improvement followed by a decline in assay sensitivity. The optimum concentration selected was 50 mM phosphate, showing the lowest IC_{50} value. **Table 1** also presents the effect of pH of assay solution on ELISA. The physiological pH, pH 7.4, was selected as the optimum for the assay.

Table 1. Influence of Organic Cosolvent, Phosphate Ion, and pH of the Assay Solution on Assay Parameters of Indirect ELISA[a]

variable	concn	Abs_{max}	slope	IC_{50} (ng/mL)
acetone	5%	0.434	0.497	31
	10%	0.434	0.433	47
	20%	0.448	0.384	22
	40%	0.183	0.383	2.7
acetonitrile	5%	0.562	0.318	33
	10%	0.479	0.348	124
	20%	0.309	0.250	168
	40%[b]			
methanol	5%	0.746	0.470	3.4
	10%	0.810	0.387	1.9
	20%	0.863	0.417	1.9
	40%	0.913	0.366	2.1
phosphate ions[c]	10 mM	0.835	0.389	9.1
	50 mM	0.823	0.360	7.1
	100 mM	0.670	0.390	16
	200 mM	0.645	0.374	14
pH	6.0	0.667	0.253	12
	6.5	0.714	0.246	28
	7.0	0.841	0.299	15
	7.4	0.861	0.331	12
	8.0	0.838	0.266	17
	8.5	0.694	0.275	21

[a] Assay conditions: antiserum to hapten A–BSA, diluted 1/1000 with 10 mM PBST; coating antigen, hapten D–OVA, 150 ng/well; goat anti-rabbit IgG–HRP diluted 1/2000. Data were obtained from the four-parameter sigmoidal fitting. Data are the means of triplicates. [b] Data fitting was impossible due to poor color development. [c] Final concentration of phosphate ions of the competition buffer containing 138 mM NaCl and 2.7 mM KCl.

Figure 3 shows a typical inhibition curve obtained under these optimized conditions. The IC_{50} value of the assay was 3.9 ng/mL with a detection limit of 0.3 ng/mL (20% inhibition). The statistical detection limit estimated as the concentration that correspond to the $\%B/B_0$ of the control (zero dose) minus 2 times the standard deviation of the control was 0.2 ng/mL. The sensitivity of the indirect ELISA in terms of detection limit is considerably higher than that of the GC method, which shows a routine detection limit of 10 ng/mL for bromophos-ethyl (*6*).

Direct Competitive Assay. The checkerboard assays, in which several dilutions of the sera were titrated against various amounts of the enzyme tracers, were used to select the most suitable antiserum and enzyme tracer. The final decision on selecting the best combination was made from the results of

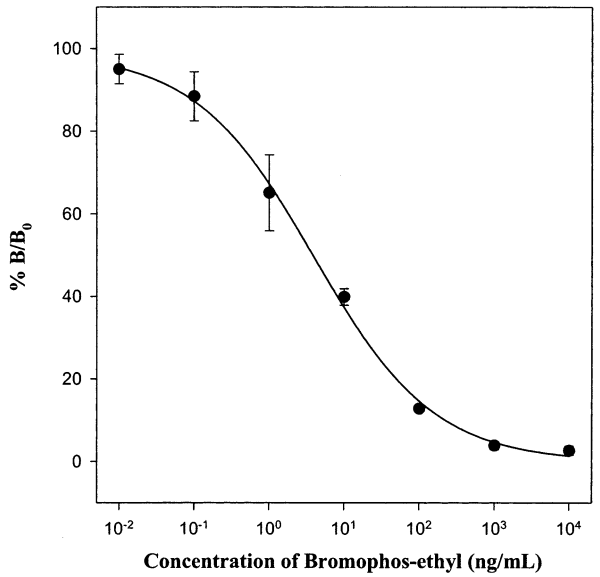


Figure 3. ELISA competition curves of bromophos-ethyl by indirect competitive assay. Reagent concentrations: antiserum (against hapten A–BSA), 1/1000; coating antigen (hapten D–OVA), 150 ng/well; goat anti-rabbit IgG–HRP, 1/3000. Each point represents the mean of 16 determinations. Vertical bars indicate ±SD about the mean.

competitive assays for the various combinations, based on the sensitivity of the assays (IC_{50} value and slope of the calibration curve). The optimum combination selected was the antiserum from hapten A−BSA (third boost) and the tracer hapten A−HRP prepared at a 10:1 hapten/protein molar ratio. The antibody-coated ELISA using this combination was optimized with regard to the dilution of antiserum and enzyme tracer.

The effects of solvents (acetone, acetonitrile, and methanol) on the ELISA system were evaluated by preparing standard curves in buffers containing various amounts of solvent (5, 10, 20, and 40% in PBS). The results are presented in **Table 2**. As observed in the indirect assay, the speed of color development at the competition step decreased with increasing concentration of acetone and acetonitrile, resulting in >50% retardation of color development at 20 and 40% concentrations. Such an effect was not observed with methanol. IC_{50} values in the presence of acetone and acetonitrile were much higher than those in the presence of methanol (**Table 2**). Therefore, methanol was the most suitable cosolvent for the assay, in agreement with the results of several other studies (*23*−*25*). **Table 2** shows that assay sensitivity continues to improve with increasing concentration of methanol. However, 20% methanol was selected as the optimum concentration, because the IC_{50} value at 20% methanol (2.1 ng/mL) is close to that at 40% methanol (1.6 ng/mL) and the slope of the calibration curve is much sharper at 20% methanol. **Table 2** also presents the effect of the concentration of the phosphate buffer in the competition solution on ELISA characteristics. Increasing the concentration of the phosphate caused initial improvement followed by a decline in assay sensitivity. The optimum concentration selected was 10 mM phosphate, which showed the lowest IC_{50} value. **Table 2** also presents the effect of pH of assay solution on ELISA. The physiological pH, pH 7.4, was selected as the best one.

Figure 4 shows a typical inhibition curve obtained under the optimized condition. The IC_{50} value of the assay was 6.5 ng/mL with a detection limit of 1 ng/mL (20% inhibition). The statistical detection limit (concentration that correspond to the $\%B/B_0$ of the control minus 2 times the standard deviation) was 1.0 ng/mL. As observed in the indirect assay, the sensitivity of the direct ELISA in terms of detection limit is considerably higher than that of the GC method, which shows a routine detection limit of 10 ng/mL for bromophos-ethyl (*6*).

Table 2. Influence of Organic Cosolvent, Phosphate Ion, and pH of the Assay Solution on Assay Parameters of Direct ELISA[a]

variable	concn	Abs_{max}	slope	IC_{50} (ng/mL)
acetone	5%	1.032	0.878	11
	10%	0.889	0.770	12
	20%	0.464	0.665	8.1
	40%	0.348	0.921	9.4
acetonitrile	5%	1.088	0.896	20
	10%	0.804	0.794	53
	20%	0.316	0.751	54
	40%	0.124	0.861	28
methanol	5%	0.874	0.402	3.9
	10%	0.944	0.576	5.4
	20%	1.125	1.029	2.1
	40%	0.989	0.500	1.6
phosphate ions[b]	10 mM	0.964	0.981	11
	50 mM	0.835	0.928	28
	100 mM	0.831	0.974	25
	200 mM	0.885	0.885	16
pH	6.0	0.685	1.118	32
	6.5	0.798	1.114	20
	7.0	0.881	1.286	23
	7.4	0.880	1.173	17
	8.0	0.873	1.123	24
	8.5	0.954	1.157	23

[a] Assay conditions: precoating with protein A (0.5 μg/well); blocking with 1% gelatin; antiserum to hapten A–BSA, diluted 1/2000 with 10 mM PBST; enzyme tracer, hapten A–HRP, 3.8 ng/well. Data were obtained from the four-parameter sigmoidal fitting. Data are the means of triplicates. [b] Final concentration of phosphate ions of the competition buffer containing 138 mM NaCl and 2.7 mM KCl.

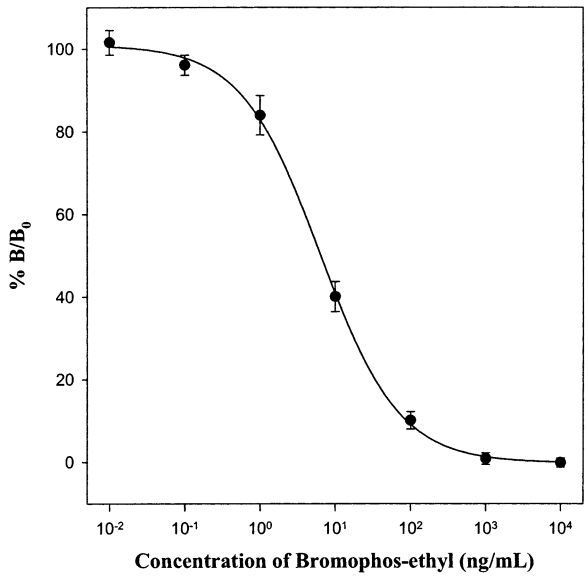
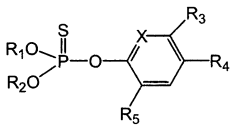
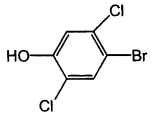
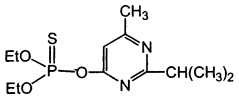
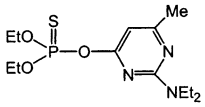
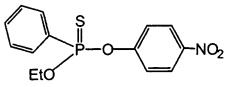


Figure 4. ELISA inhibition curves for bromophos-ethyl by direct competitive assay. Reagent concentrations: precoating agent protein A, 0.5 μg/well; antiserum (against hapten A–BSA), 1/2000; enzyme tracer (hapten A–HRP), 3.8 ng/well. Each point represents the average of 16 determinations. Vertical bars indicate ±SD about the mean.

Cross-Reactivity Studies. Several organophosphorus pesticides as well as the metabolite of bromophos-ethyl (phenol) were tested for cross-reactivities. **Table 3** shows the cross-reactivity that was found by both the indirect and direct assays, expressed as a percentage of the IC_{50} of bromophos-ethyl. The

Table 3. Cross-Reactivity of Compounds Structurally Related to Bromophos-ethyl Determined by Indirect and Direct Competitive ELISAs[a]



Compound	Structure						Indirect ELISA		Direct ELISA	
	R_1	R_2	X	R_3	R_4	R_5	IC_{50} (μg/mL)[b]	CR(%)[c]	IC_{50} (μg/mL)[b]	CR(%)[c]
Bromophos-ethyl	Et	Et	C	Cl	Br	Cl	0.015	100	0.008	100
Bromophos-methyl	Me	Me	C	Cl	Br	Cl	0.15	10	0.014	58.7
Chlorpyrifos	Et	Et	N	Cl	Cl	Cl	0.16	9.4	0.021	38
Chlorpyrifos-methyl	Me	Me	N	Cl	Cl	Cl	9.7	0.15	0.147	5.4
Parathion	Et	Et	C	H	NO_2	H	> 100	< 0.03	> 100	< 0.03
Parathion-methyl	Me	Me	C	H	NO_2	H	> 100	< 0.03	> 100	< 0.03
Fenitrothion	Me	Me	C	Me	NO_2	H	> 100	< 0.03	> 100	< 0.03
4-Bromo-2,5-dichlorophenol							> 100	< 0.03	> 100	< 0.03
Diazinon							> 100	< 0.03	> 100	< 0.03
Pirimiphos-ethyl							> 100	< 0.03	> 100	< 0.03
EPN							> 100	< 0.03	> 100	< 0.03

[a] Assay conditions were the same as those described in **Table 1** (indirect ELISA) or **2** (direct ELISA). The standards were prepared in 20% MeOH–PBS (90 mM) or 10% MeOH–PBS (10 mM), respectively, and were combined with antisera diluted with 10 mM PBST. [b] IC_{50} values of the pesticides below chlorpyrifos-methyl could not be determined accurately due to the limited solubility at high concentrations; however, it was clear that inhibition was <50% at 100 μg/mL. [c] Cross-reactivity (%) = (IC_{50} of bromophos-ethyl/IC_{50} of other compound) × 100.

interference with the assays was negligible except with bromophos-methyl and chlorpyrifos in the direct assay. The appreciable cross-reactivities of antibodies for these pesticides are understandable, because they have the same or similar aromatic structure as bromophos-ethyl. It is interesting to note that when the same antiserum was used, there was significant cross-reactivity to bromophos-methyl and chlorpyrifos when the direct assay was used, whereas the cross-reactivity to these two pesticides was much lower when the indirect assay was used. The result can be rationalized by assuming that recognition of the antibodies to the aromatic ring structure of the antigen relative to the thiophosphate structure is higher in the direct assay, which would allow bromophos-methyl and chlorpyrifos to compete with the competitor (enzyme tracer) more effectively in the direct assay. It may be concluded that the competitive ELISAs that were developed are suitable for the sensitive and selective detection of bromophos-ethyl, with the exception of bromophos-methyl and chlorpyrifos in the direct assay.

Recovery Studies. Three kinds of vegetables and waters from two different sources were spiked with bromophos-ethyl and analyzed by both indirect and direct ELISAs. Recoveries of the pesticide from spiked vegetable samples are presented in **Table 4**. Of six combinations of the kind of vegetables and assay format, four showed recoveries ranging from 89 to 112% (sesame/indirect and kale/indirect were exceptions). The range of recovery for all combinations was between 82 and 128%. The plot of the recovered pesticide against the spiked pesticide for all combinations gave regression lines with slopes and intercepts very close to 1 and 0 ng/mL, respectively. Fortification of vegetable samples was carried out by incubating the chopped vegetable leaves in fortification solutions for 24 h. Thus, pesticide may have penetrated quite deeply into the interior of the leaves during the incubation period. However, the recovery data suggest that pesticide can be recovered to near perfection by using the extraction procedure adopted in this study. Recoveries from tap water and pond water determined by both indirect and direct ELISAs are presented also in **Table 4**. The range of recovery was between 95 and 127%. The plot of the recovered pesticide against the spiked pesticide gave regression lines with slopes and intercepts very close to 1 and 0 ng/mL, respectively. Despite the simple pretreatment taken for sample preparation (evaporation of liquid and dissolution of the residue in buffer), no significant matrix effect was observed. Overall, the ELISAs developed in this study can accurately determine bromophos-ethyl residues in vegetable and water samples after the simple and rapid extraction procedure used in this study.

Comparison of the Indirect and Direct Assays. The sensitivity of the indirect assay in terms of IC_{50} value is a bit

Table 4. Recovery of Bromophos-ethyl Spiked into Vegetable and Water Samples[a]

sample	fortified concn (ng/mL)	recoveries by indirect ELISA	recoveries by direct ELISA
lettuce	10	101	107
	50	104	105
	100	91	108
	500	99	98
	1000	102	99
kale	10	113	89
	50	100	111
	100	128	107
	500	117	109
	1000	103	98
sesame	10	97	100
	50	101	99
	100	96	108
	500	98	92
	1000	82	111
pond water	10	99	102
	50	99	101
	100	97	106
	500	96	107
	1000	96	115
tap water	10	102	99
	50	103	127
	100	110	100
	500	112	95
	1000	112	104

[a] Assay conditions were the same as those described in **Table 3**. Data are the means of triplicates. The slopes of the least-squares plot of recovered versus spiked concentration (log–log), in respective order, were 0.999, 0.980, 0.995, 1.022, 0.972, 1.007, 0.959, 1.147, 1.123 and 1.026.

better than that of the direct assay (IC_{50} values of 3.9 vs 6.5 ng/mL). However, the direct assay gives much sharper standard curves (slopes of 0.4 vs 0.9). As far as selectivity is concerned, the indirect assay is better, that is, lower cross-reactivity of the antibodies. Therefore, if sensitivity is more important than selectivity in an analysis, then the direct assay should be chosen. If samples are suspected of containing multiple organophosphorus pesticides, then the direct assay would be the better one to use.

ABBREVIATIONS USED

Abs_{max}, maximum absorbance; BSA, bovine serum albumin; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; IC_{50}, concentration of analyte giving 50% inhibition of the maximum absorbance; IgG, immunoglobulin G; NMR, nuclear magnetic resonance; OVA, ovalbumin; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline−0.05% Tween 20; TLC, thin-layer chromatography; TMB, 3,3′,5,5′-tetramethylbenzidine.

LITERATURE CITED

(1) AOAC International. *Official Methods of Analysis*, 18th ed.; Association of Official Analytical Chemists: Arlington, VA, 1995; Section 970.52.

(2) Hammock, B. D.; Gee, S. J.; Harrison, R. O.; Jung, F.; Goodrow, M. H.; Li, Q. X.; Lucas, A.; Szekacs, A.; Sundaram, K. M. S. Immunochemical technology in environmental analysis: addressing critical problems. In *Immunochemical Methods for Environmental Analysis*; Van Emon, J., Mumma, R., Eds.; ACS Symposium Series 442; American Chemical Society: Washington, DC, 1990; pp 112−139.

(3) Worthing, C. R. *The Pesticide Manual: A World Compendium*, 8th ed.; The British Crop Protection Council: Farnham, Surrey, U.K., 1987; Entry 01390.

(4) Ware, G. W. *Pesticides, Theory and Application*; Freeman: New York, 1983; p 41.

(5) Weeren, R. D.; Eichler, D. Bromophos-ethyl. *Anal. Methods Pestic. Plant Growth Regul.* **1978**, *10*, 41−43.

(6) Their, H.-P., Zeumer, H., Eds. *Manual of Pesticide Residue Analysis*; VCH: Weinheim, Germany, 1987; Vol. 1, pp 282−295.

(7) Cho, Y. A.; Lee, H.-S.; Park, E. Y.; Lee, Y. T.; Hammock, B. D.; Ahn, K. C.; Lee, J. K. Development of an ELISA for the organophosphorus insecticide chlorpyrifos. *Bull. Korean Chem. Soc.* **2002**, *23*, 481−487.

(8) Edward, S. L.; Skerritt, J. H.; Hill, A. S.; McAdam, D. P. An improved immunoassay for chlorpyrifos-methyl (Reldan) in grain. *Food Agric. Immunol.* **1993**, *5*, 129−144.

(9) Hill, A. S.; Skerritt, J. H.; Bushway, R. J.; Perkins, L. B.; Larkin, K.; Thomas, M.; Korth, W.; Bowmer, K. H. Development and application of laboratory and filed immunoassays for chlorpyrifos in water and soil matrices. *J. Agric. Food Chem.* **1994**, *42*, 2051−2058.

(10) Manclús, J. J.; Primo, J.; Montoya, A. Development of enzyme-linked immunosorbent assay for the insecticide chlorpyrifos. 1. Monoclonal antibody production and immunoassay design. *J. Agric. Food Chem.* **1996**, *44*, 4052−4062.

(11) McAdam, D. P.; Hill, A. S.; Beasley, H. L.; Skerritt, J. H. Mono- and polyclonal antibodies to the organophosphate fenitrothion. 1. Approaches to hapten−protein conjugation. *J. Agric. Food Chem.* **1992**, *40*, 1466−1470.

(12) Skerritt, J. H.; Hill, A. S.; Thwaites, H. L.; Edward, S. L.; McAdam, D. P. Enzyme-immunoassay for quantification of the organophosphate pesticides, fenitrothion, chlorpyrifos-methyl and pirimiphos-methyl in wheat grain and flour milling fractions. *J. AOAC Int.* **1992**, *75*, 519−528.

(13) Ross, F.; Park, V., III. Process for preparing 2,5-dichloro-4-bromophenol. U.S. Patent 3,728,403, 1973.

(14) Raab, G. M. Comparison of an logistic and a mass-action curve for radioimmunoassay data. *Clin. Chem.* **1983**, *29*, 1757−1761.

(15) Heldman, E.; Balan, A.; Horowitz, O.; Ben-Zion, S.; Torten, M. A novel immunoassay with direct relevance to protection against organophosphate poisoning. *FEBS Lett.* **1985**, *180*, 243−248.

(16) McAdam, D. P.; Skerritt, J. H. Synthesis of organothiophosphate antigens for the development of specific immunoassays. *Aust. J. Chem.* **1993**, *46*, 959−967.

(17) Skerritt, J. H.; Lee, N. J. Approaches to the synthesis of haptens for immunoassay of organophosphate and synthetic pyrethroid insecticides. In *Residue Analysis in Food Safety: Applications of Immunoassay Methods*; Beier, R. C., Stanker, L. H., Eds.; ACS Symposium Series 621; American Chemical Society: Washington, DC, 1996; pp 124−149.

(18) Park, H. J.; Park, W. C.; Jung, T. W.; Ra, C. S.; Lee, Y. T. Development of an ELISA for the organophosphorus insecticide isofenphos. *Bull. Korean Chem. Soc.* **2002**, *23*, 481−487.

(19) Krämer, P. M.; Marco, M.-P.; Hammock, B. D. Development of a selective enzyme-linked immunosorbent assay for 1-naphthol—the major metabolite of carbaryl (1-naphthyl *N*-methylcarbamate). *J. Agric. Food Chem.* **1994**, *42*, 934−943.

(20) Marco, M.-P.; Gee, S. J.; Cheng, H. M.; Liang, Z. Y.; Hammock, B. D. Development of an enzyme-linked immunosorbent assay for carbaryl. *J. Agric. Food Chem.* **1993**, *41*, 423−430.

(21) Shan, G.; Leeman, W. R.; Stoutamire, D. W.; Gee, S. J.; Chang, D. P. Y.; Hammock, B. D. Enzyme-linked immunosorbent assay for the pyrethroid permethrin. *J. Agric. Food Chem.* **2000**, *48*, 4032−4040.

(22) Wengatz, I.; Stoutamire, D. W.; Gee, S. J.; Hammock, B. D. Development of an enzyme-linked immunosorbent assay for the detection of the pyrethroid insecticide fenpropathrin. *J. Agric. Food Chem*. **1998**, *46*, 2211−2221.

(23) Hill, A. S.; McAdam, D. P.; Edward, S. L.; Skerritt, J. H. Quantitation of bioresmethrin, a synthetic pyrethroid grain protectant, by enzyme immunoassay. *J. Agric. Food Chem*. **1993**, *41*, 2011−2018.

(24) Lee, J. K.; Ahn, K. C.; Park, O. S.; Kang, S. Y.; Hammock, B. D. Development of an ELISA for the detection of the residues of the insecticide imidacloprid in agricultural and environmental samples. *J. Agric. Food Chem*. **2001**, *49*, 2159−2167.

(25) Matsuura, S.; Hamano, Y.; Kita H.; Takagaki, Y. Preparation of mouse monoclonal antibodies to okadaic acid and their binding activity in organic solvents. *J. Biochem. (Tokyo)* **1993**, *114*, 273−278.

Received for review May 29, 2002. Revised manuscript received August 25, 2002. Accepted August 29, 2002. This work was supported by Korea Research Foundation Grant KRF-2000-GA0023. Additional support from U.S. NIEHS Superfund Basic Research Program P42 ES04699 and U.S. NIEHS Environmental Health Center P30 ES05707 is also acknowledged.

JF025703+