Development of an Enzyme-Linked Immunosorbent Assay for the Detection of the Organophosphorus Insecticide Acephate

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A competitive indirect enzyme-linked immunosorbent assay (ciELISA) for the organophosphorus insecticide acephate, *O,S*-dimethyl acetylphosphoramidothioate, was developed using a polyclonal antibody. Five different haptens mimicking the analyte were synthesized and conjugated with the carrier proteins bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) by the *N*-hydroxysuccinimide active ester and diazotization methods. Polyclonal antibodies raised against hapten−KLH conjugates in rabbits and hapten−BSA conjugates as coating antigens were screened and selected for the assay in the homologous and heterologous ELISA systems. The effects of various assay conditions such as detergent, organic solvents, pH, and preincubation of the mixture of the polyclonal antibody and the analyte on the sensitivity were evaluated. The IC_{50} value for acephate was 25 ng/mL in an optimized heterologous system using hapten-**4**−BSA as a coating antigen and a polyclonal antibody no. 8377 against hapten-**1**−KLH, showing the detection range of 5−140 ng/ mL and the lowest detection limit of 2 ng/mL. The cross-reactivities of the structurally related organophosphorus insecticides, including the major metabolite of the analyte, methamidophos, were less than 1%. Recoveries from the analyte-fortified tap water, mulberry leaves, and lettuce samples in the assay were in the range of 72−121% by simple extraction, concentration, and dilution. These results indicate that the ELISA could be a convenient and supplemental analytical tool for monitoring acephate residues in environmental and agricultural samples.

KEYWORDS: Acephate; organophosphorus insecticide; ELISA; polyclonal antibody; residue analysis

INTRODUCTION

Acephate, *O,S*-dimethyl acetylphosphoramidothioate, is a systemic organophosphorus insecticide, which was introduced by Chevron Chemical in 1971 (*1*, *2*). It was synthesized by *N*-acetylation of methamidophos, which is a cholinesterase inhibitor (*1*). Basically similar to methamidophos in activity, acephate is of low mammalian toxicity. It is used to control a wide range of biting and sucking insects, including aphids, thrips, lepidopterous larvae, leaf miners, leafhoppers, and cutworms in various crops (*3*).

Because the mammalian toxicity of acephate is much lower than that of methamidophos, the use of acephate has increased rapidly (*4−8*), while other organophosphorus insecticides such as dichlorvos and chlorpyrifos have been shown to decrease gradually (*9*, *10*).

For the conventional analysis of the acephate residue in crops, samples are extracted with ethyl acetate in the presence of a large amount of anhydrous sodium sulfate, cleaned up by silica gel chromatography, and determined by gas chromatography (GC) with nitrogen phosphorus detection (NPD) or flame photometric detection (FPD) (*11*, *12*). If necessary, an additional cleanup step for high-fat matrixes can be carried out by acetonitrile-*n*-hexane partitioning. Because of its very polar characteristic, acephate is not extracted from aqueous samples using the common liquid−liquid extraction (LLE) or solid phase extraction (SPE) procedures (*13*, *14*). Therefore, the known analytical methods for acephate residues in water are limited (*14*). Recently, sophisticated instrumental methods such as GC or liquid chromatography (LC) coupled with mass spectrometry (MS) have been reported for the determination of acephate residues in aqueous and agricultural samples (*14−16*).

Although these conventional GC and sophisticated instrumental methods are accurate, they require large amounts of organic solvents, a lot of time for sample preparation, and an analyst experienced with sample preparation, instrumental operation, and interpretation. In contrast, immunoassay and enzyme inhibition assays are rapid and cost effective and allow high sample throughput. Enzyme assays, in particular, have been used for the rapid screening of organophosphorus and carbamate

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insecticides in agricultural samples, but these assays lack selectivity to the insecticides inhibiting acetylcholinesterase activity. The immunoassay is commonly used for monitoring a target analyte in environmental and agricultural samples based on the specific antigen−antibody interaction. The purpose of this investigation, therefore, is to develop an enzyme-linked immunosorbent assay (ELISA) for the detection of acephate residues in agricultural and environmental samples, using polyclonal antibodies raised against various immunogens mimicking the analyte.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), goat antirabbit IgG peroxidase conjugate as the second antibody, and Freund's complete and incomplete adjuvants were all purchased from Sigma Chemical Co. (St. Louis, MO). Organic starting materials for hapten syntheses were purchased from Aldrich Chemical Co. (Milwaukee, WI) and Fisher Scientific (Pittsburgh, PA). Thin-layer chromatography (TLC) utilized 0.2 mm precoated silica gel 60 F_{254} on glass plates from E. Merck (Darmstadt, Germany), and detection was by ultraviolet light or iodine vapor stain. Flash chromatographic separations were carried out using Baker silica gel (40 μm average particle size) using the indicated solvents where the → notation denotes a stepwise concentration gradient. For the cross-reactivity study, the organophosphorus insecticides methamidophos, dichlorvos, phenthoate, and chlorpyrifos were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

Instrument. ^{1}H Nuclear magnetic resonance (NMR) spectra of compounds synthesized were obtained on a 300 MHz NMR spectrometer, DPX300 (Bruker, Germany), and a General Electric QE-300 spectrometer (Bruker NMR, Billerica, ME) using tetramethylsilane as an internal standard. Fast atom bombardment mass spectra (FAB-MS) using 3-nitrobenzyl alcohol as a matrix were obtained on a JEOL four sector tandem mass spectrometer, JMS-HX/HX110A (JEOL, Japan) at Korea Basic Science Institute and a ZAB-HS-2F mass spectrometer (VG Analytical, Wythenshawe, U.K.) at UC Davis. R_f values refer to TLC on silica gel 60 F_{254}, precoated plates (Merck) with visualization under exposure to either ultraviolet light (254 nm) or iodine vapor. ELISA was performed on 96 well microtiter plates (Nunc-Immuno plate, MaxiSorp surface, Roskilde, Denmark) and read spectrophotometrically with a microplate reader, Bio-Rad model 550 (Hercules, CA).

Buffer Solutions. Normal strength phosphate-buffered saline [1 × PBS; 8 g/L of sodium chloride (NaCl), 0.2 g/L of potassium phosphate, monobasic (KH$_2$PO$_4$), 1.2 g/L of sodium phosphate dibasic anhydrous (Na$_2$HPO$_4$), and 0.2 g/L of potassium chloride (KCl), pH 7.5], PBST [1 × PBS containing 0.05% (v/v) Tween 20, pH 7.5], 0.1 × PBST (0.1 × PBS containing 0.05% Tween 20), carbonate buffer [1.59 g/L sodium carbonate (Na$_2$CO$_3$), 2.93 g/L sodium hydrogen carbonate (NaHCO$_3$), pH 9.6], and 0.05 M borate buffer (19.1 g/L Na$_2$B$_4$O$_7$·10H$_2$O, pH 8) were used for immunoassay.

Hapten Synthesis and Verification. Because acephate does not have any suitable functional group to be conjugated with carrier proteins, four acephate haptens with carboxylic groups were synthesized and used for the immunogens and plate-coating antigens. Two different types of haptens were designed for the production of polyclonal antibodies specific to the target analyte acephate. Hapten-**1** (**Figure 1**, **5**) was prepared by replacing a methyl group adjacent to the sulfur atom of acephate with a six carbon spacer arm of a carboxylic group, and hapten-**2**, -**3**, and -**4** were prepared by replacing one of the hydrogens in the amine group of methamidophos with three or six carbon spacer arms of a carboxylic group. Hapten-**5**, the insecticide methamidophos, has no spacer arm.

Hapten-1, 6-(*N*-Acetyl-*O*-methylphosphonamidothioyl)hexanoic Acid (5). Ammonia (NH$_3$) gas was passed into an ice-chilled and stirred solution of dimethyl chlorothiophosphate (21.595 g, 134 mmol, **1**) in benzene for 2 h. The resulting product was warmed to 10−15 °C to remove the remaining ammonia gas, and then, the solvent was stripped off under vacuum to obtain compound **2** (*O,O*-dimethyl phosphoramidothioate). TLC (benzene/methylene chloride (7:3, v/v)): R_f, 0.19. ^{1}H
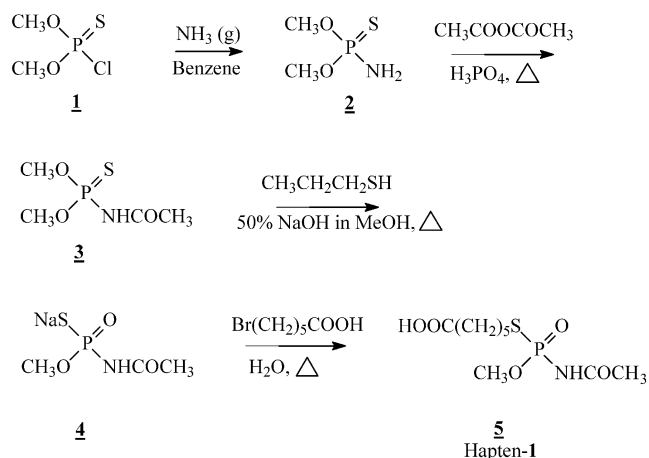

Figure 1. Procedure for the synthesis of hapten-1 (**5**).

NMR (CDCl$_3$): δ 3.74 (d, J = 13.8 Hz, 6H, 2OCH$_3$), 3.91 (d, J = 16.1 Hz, 2H, NH$_2$).

Phosphoric acid (0.3 mL) was added to a mixture of **2** (17.5 g, 0.12 mol) and acetic anhydride (12.25 g, 0.12 mol). The mixture was reacted at 80 °C for 1 h to obtain **3** (*O,O*-dimethyl *N*-acetylphosphoramidothioate).

A 50% NaOH solution in methanol was added to a mixture of **3** and 1-propanethiol (0.1676 g, 2.1 mmol). The mixture was refluxed for 3 h and kept overnight at 55 °C to obtain **4** (*O*-methyl *S*-sodio *N*-acetylphosphoramidothioate). TLC (0.5% acetic acid in methanol/methylene chloride (1:9, v/v)): R_f, 0.08. ^{1}H NMR (CDCl$_3$): δ 2.07 (d, J = 1.3 Hz, 3H, CH$_3$CO), 3.63 (d, J = 13.14 Hz, 3H, CH$_3$O).

A mixture of **4** (191.8 mg, 1 mmol) and 6-bromohexanoic acid (195.9 mg, 1 mmol) was dissolved in water (5 mL). The solution was reacted at 70 °C for 3 h, and then, the solvent was stripped off under vacuum. The residue was flash-chromatographed on silica gel eluting with 3 → 15% methanol in methylene chloride containing 1.5% acetic acid to obtain hapten-**1**. TLC (0.5% acetic acid in methanol/methylene chloride (1:9, v/v)): R_f, 0.5. ^{1}H NMR (CDCl$_3$): δ 1.46 (m, 2H, CH$_2$), 1.65 (q, J = 7.7 Hz, 2H, CH$_2$), 1.73 (q, J = 7.6 Hz, 2H, CH$_2$), 2.17 (d, J = 1.3 Hz, 3H, CH$_3$CO), 2.34 (t, J = 7.3 Hz, 2H, CH$_2$) 2.93 (ddt, J = 2.7 Hz, 2H, CH$_2$,) 3.85 (d, J = 13.1 Hz, 3H, CH$_3$O), ∼9.21 (b, 1H, COOH). FAB-MS: *m/z* 284 [M + H]$^+$.

Hapten-2, 6-(*O,S*-Dimethylthiophosphoramido)hexanoic Acid (Figure 2, 12). A solution of 6-aminohexanoic acid (6.56 g, 50 mmol, **6**) in water (15 mL) was stirred vigorously with ice cooling as benzyloxycarbonyl chloride (8 mL) and 5 N NaOH solution (10 mL) were added in portions, keeping the reaction mixture at pH 10. After it was stirred for an additional 1.5 h, the mixture was extracted with ether to remove neutral material and then acidified with dilute HCl solution. The mixture was extracted twice with methylene chloride. The extracts were dried over anhydrous magnesium sulfate and stripped of solvent to a white solid. This was recrystallized from ether at −70 °C to give in two crops 11.8 g (89%) of **7** (*N*-benzyloxycarbonyl-6-aminohexanoic acid); mp 52−55 °C. ^{1}H NMR (CDCl$_3$): δ 1.37 (m, 2H, CH$_2$), 1.52 (q, J = 7.2 Hz, 2H, CH$_2$), 1.64 (q, J = 7.3 Hz, 2H, CH$_2$), 2.34 (t, J = 7.3 Hz, 2H, CH$_2$), 3.19 (q, J = 6.4 Hz, 2H, CH$_2$), 4.8 (bs, 1H, NH), 5.09 (s, 2H, CH$_2$Ar), 7.3−7.4 (m, 5H, Ar).

Isobutylene gas (40 g) was passed slowly into an ice−acetone chilled and stirred solution of the acid **7** (11.8 g, 44.5 mmol) in methylene chloride (85 mL). Concentrated sulfuric acid (0.5 mL) was added, and the mixture was stirred while warming over 3 h to ambient temperature. After excess isobutylene had distilled out, the flask was closed with a stopper and kept at ambient temperature for 4 days. The mixture was diluted with some *n*-hexane to help break emulsions and extracted with sodium bicarbonate solution. The neutral organic phase was dried over anhydrous MgSO$_4$ and stripped of solvent under high vacuum to yield 12.3 g (86%) of **8** (*tert*-butyl 6-(benzyloxycarbonylamino)hexanoate) as a colorless oil. TLC (ether): R_f, 0.68. ^{1}H NMR (CDCl$_3$): δ 1.3− 1.65 (m, 6H, CH$_2$), 1.43 (s, 9H, CH$_3$), 2.20 (t, J = 7.4 Hz, 2H, CH$_2$), 3.19 (q, J = 6.6 Hz, 2H, CH$_2$), 4.78 (bs, 1H, NH), 5.09 (s, 2H, CH$_2$-Ar), 7.3−7.36 (m, 5H, Ar).
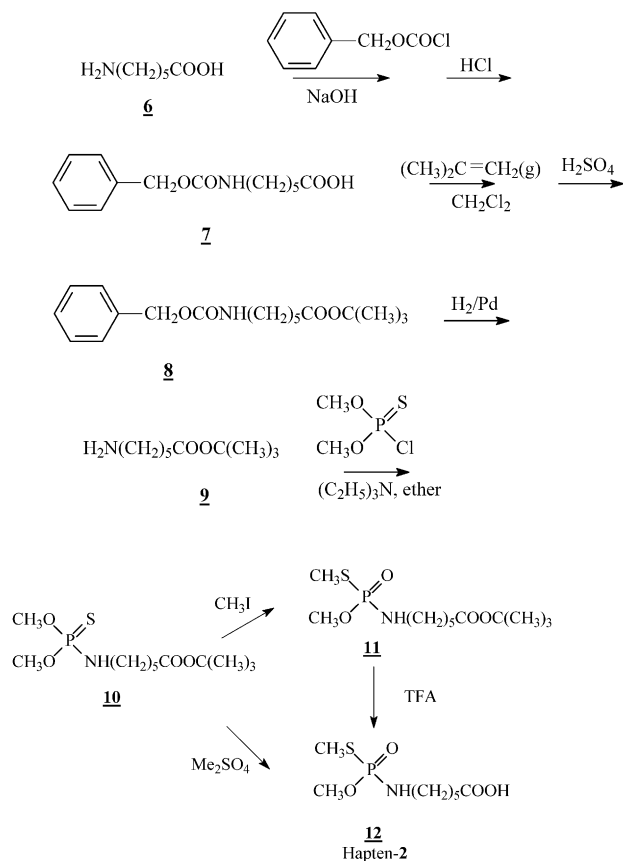
ELISA for Acephate

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Figure 2. Procedure for the synthesis of hapten-**2** (**12**).

Figure 3. Procedure for the synthesis of hapten-**3** (**18**) and hapten-**4** (**19**).

A solution of the ester **8** (2.08 g, 6.47 mmol) in ethanol (10 mL) and a 10% palladium on carbon catalyst (35 mg) were stirred under an atmosphere of hydrogen at ambient pressure for 24 h. The mixture was filtered, and the filtrate was stripped of solvent. The residue was redissolved in ethyl acetate and again stripped under high vacuum to yield 1.28 g of **9** (*tert*-butyl 6-aminohexanoate) as a colorless oil. ^{1}H NMR (CDCl$_3$): δ 1.36 (m, 2H, CH$_2$), 1.44 (s, 9H, CH$_3$), 1.4−1.5 (m, 2H, CH$_2$), 1.60 (m, 2H, CH$_2$), 1.65 (s, 2H, NH$_2$), 2.21 (t, J = 7.4 Hz, 2H, CH$_2$), 2.67 (t, J = 6.9 Hz, 2H, CH$_2$). The 1.65 peak was removed by treatment with D$_2$O.

A solution of O,O-dimethyl chlorothiophosphate (1.15 g, 7.2 mmol) in ether (5 mL) was added in portions to a stirred and cooled (10−15 °C) solution of the ester **9** and triethylamine (0.757 g, 7.5 mmol) in ether (3 mL). The reaction was mildly exothermic. After 10 min, the mixture was warmed briefly to reflux, cooled, washed with water and sodium bicarbonate solution, dried over anhydrous MgSO$_4$, and stripped to a colorless oil. This was flash-chromatographed on silica gel (25 g), eluting with 20 → 100% methylene chloride in *n*-hexane followed by 1 → 2% ether in methylene chloride. Fractions containing the pure product by TLC (ether/methylene chloride (1:9, v/v)) were stripped under high vacuum to yield 1.38 g (65%) of **10** (*tert*-butyl 6-(O,O-dimethylthiophosphoramido)hexanoate) as a colorless oil. TLC (ether/methylene chloride (1:9, v/v)): R_f, 0.63. ^{1}H NMR (CDCl$_3$): δ 1.35 (m, 2H, CH$_2$), 1.44 (s, 9H, CH$_3$), 1.4−1.6 (m, 4H, CH$_2$), 2.21 (t, J = 7.4 Hz, 2H, CH$_2$), 2.93 (m, 2H, CH$_2$), 3.68 (d, J = 13.7 Hz, 6H, CH$_3$O).

A sample of the ester **10** (100 mg, 0.32 mmol) was treated with dimethyl sulfate (3 μL, ~10 mol %), and the mixture was heated in an oil bath at 75 °C for 2 h. The resulting product was flash-chromatographed on silica gel (5 g) eluting with 1% isopropyl alcohol in methylene chloride followed by 1.5% acetic acid in 1 → 5% isopropyl alcohol in methylene chloride. Fractions containing the pure product by TLC were stripped under high vacuum to yield 52 mg (63%) of hapten-**2** as a colorless oil. TLC (methanol/methylene chloride/acetic acid (1:9:0.15, v/v/v)): R_f, 0.45. ^{1}H NMR (CDCl$_3$): δ 1.40 (m, 2H, CH$_2$), 1.55 (m, 2H, CH$_2$), 1.65 (m, 2H, CH$_2$), 2.25 (d, J = 14.7 Hz,
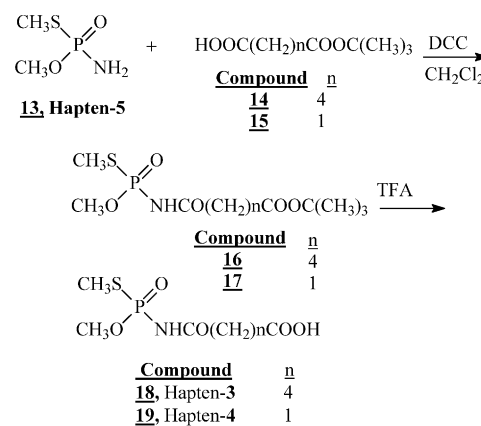
3H, CH$_3$S), 2.34 (t, J = 7.3 Hz, 2H, CH$_2$), 2.96 (m, 2H, CH$_2$), 3.76 (d, J = 12.5 Hz, 3H, CH$_3$O). The NH and COOH were not detected in CDCl$_3$.

A sample of the ester **10** (230 mg, 0.739 mmol) and methyl iodide (3 mL) in a 10 mL glass pressure vessel was warmed in an oil bath until reflux began, and then, the cap was tightened and the mixture was heated at 65 °C for 20 h. The resulting product was flash-chromatographed on silica gel (15 g) and eluted with methylene chloride (30 mL) followed by 1 → 5% isopropyl alcohol in methylene chloride. Fractions containing pure product by TLC were stripped under high vacuum to yield 218 mg (94%) of the intermediate ester **11** [*t*-butyl 6-(O,S-dimethylthiophosphoramido)hexanoate] as a viscous oil. TLC (isopropyl alcohol/methylene chloride (1:19, v/v)): R_f, 0.37. ^{1}H NMR (CDCl$_3$): δ 1.36 (m, 2H, CH$_2$), 1.44 (s, 9H, CH$_3$), 1.52 (m, 2H, CH$_2$), 1.58 (m, 2H, CH$_2$), 2.21 (t, J = 7.3 Hz, 2H, CH$_2$), 2.25 (d, J = 14.7 Hz, 3H, CH$_3$S), 2.99 (m, 2H, CH$_2$), 3.75 (d, J = 12.4 Hz, 3H, CH$_3$O).

Trifluoroacetic acid (TFA) (0.5 mL) was added to the ester **11**, and the mixture was allowed to stand at ambient temperature for 5 min. The TFA was stripped off under vacuum, and ethyl acetate was added twice and stripped to remove residual TFA. The residue was immediately flash-chromatographed on silica gel (6 g) eluting with 3 → 15% methanol in methylene chloride containing 1.5% acetic acid. Fractions containing pure product by TLC were stripped, and residual acetic acid was removed by the successive addition and removal of toluene (2 × 20 mL), ethyl acetate (20 mL), and methylene chloride (20 mL). Stripping the residue under high vacuum gave 150 mg of hapten-**2** as a viscous oil. TLC (methanol/methylene chloride (1:9, v/v)): R_f, 0.457. ^{1}H NMR (CDCl$_3$): δ 1.40 (m, 2H, CH$_2$), 1.55 (m, 2H, CH$_2$), 1.65 (m, 2H, CH$_2$), 2.25 (d, J = 14.7 Hz, 3H, CH$_3$S), 2.34 (t, J = 7.3 Hz, 2H, CH$_2$), 2.96 (m, 2H, CH$_2$), 3.76 (d, J = 12.5 Hz, 3H, CH$_3$O).

Hapten-3,6-(O,S-Dimethylthiophosphoramido)-6-oxohexanoic Acid (Figure 3, 17) and Hapten-4,3-(O,S-Dimethylthiophosphoramido)-3-oxopropanoic Acid (Figure 3, 18). For the synthesis of hapten-**3**, 1,3-dicyclohexylcarbodiimide (DCC, 336 mg, 1.63 mmol) was added to an ice-chilled solution of O,S-dimethyl phosphoramidothioate (methamidophos) (200 mg, 1.42 mmol), adipic acid mono-*tert*-butyl ester (300 mg, 1.48 mmol, **14**), and 4-(dimethylamino)pyridine (DMAP, 10 mg) in methylene chloride (4 mL). After 10 min, the mixture was kept at ambient temperature for 6 days. The mixture was filtered to remove dicyclohexylurea (DCU), and the filtrate was concentrated and flash-chromatographed on silica gel (25 g) eluting 10 → 30% ether in methylene chloride followed by 2 → 10% isopropyl alcohol in methylene chloride. Fractions containing pure product by TLC were stripped of solvent under high vacuum to yield 300 mg (65%) of compund **16** (*tert*-butyl 6-(O,S-dimethylthiophosphoramido)-6-oxohexanoate) as a viscous oil. ^{1}H NMR (CDCl$_3$): δ 1.44 (s, 9H, CH$_3$), 1.65 (m, 4H, CH$_2$), 2.24 (t, J = 7.0 Hz, 2H, CH$_2$), 2.39 (d, J = 16.1 Hz, 3H, CH$_3$S), 2.40 (t, J = 6.7 Hz, 2H, CH$_2$), 3.85 (d, J = 13.2 Hz, 3H, CH$_3$O), 8.44 (bd, 1H, NH).

This ester was readily converted to 115 mg (92%) of the acid, hapten-**3** (**18**) as oil, by treatment with TFA as described above for **11**.

TLC (methanol/methylene chloride (1:9, v/v; 1.5% acetic acid)): R_f, 0.40. ^{1}H NMR (CDCl$_3$): δ 1.71 (m, 4H, CH$_2$), 2.39 (m, 4H, CH$_2$), 2.38 (d, J = 16.4 Hz, 3H, CH$_3$S), 3.86 (d, J = 13.3 Hz, 3H, CH$_3$O), 9.14 (bd, 1H, NH). ^{13}C NMR (CDCl$_3$): δ 12.0 (d, J = 3.6 Hz), 23.88, 23.93, 33.4, 36.2 (d, J = 6.9 Hz), 53.7 (d, J = 6.6 Hz), 174.7, 177.6. The product was soluble in water. A 5 mg sample treated with NaHCO$_3$ solution evolved gas. Acidification and extraction with ether made it return to the acid showing no change on TLC.

For the synthesis of hapten-**4** (**19**) 1454.4 mg (7.05 mmol) of DCC in 10 mL of methylene chloride was added to a stirred solution of 665 mg (4.7 mmol) of methamidophos, **13**, and 828.08 mg (5.17 mmol) of mono-*tert*-butyl malonate, **15**, in 15 mL of methylene chloride chilled in an ice bath. A white precipitate formed almost immediately. Stirring was continued at 0 °C for 1 h and at room temperature overnight. The urea precipitate was filtered and washed with 10 mL of methylene chloride. The filtrate was washed once with water, and the organic layer was evaporated. The resulting yellow mixture was extracted with reagent grade acetone, and the acetone solution was evaporated. The concentrate was purified by silica gel flash chromatography using 100 mL of *n*-hexane, 100 mL of *n*-hexane/ethyl acetate/acetic acid (2:1: 0.1, v/v/v), and 100 mL of *n*-hexane/ethyl acetate/acetic acid (5:5:0.1, v/v/v). Fractions containing pure product were stripped, and the residual acetic acid was removed by the successive addition and removal of 40 mL of toluene, 20 mL of ethyl acetate, and 20 mL of methylene chloride. Stripping the residue under high vacuum gave 300 mg of the oily ester **17** (*tert*-butyl 3-(*O*,*S*-dimethylthiophosphoramido)-3-oxopropanoate). TLC (*n*-hexane/ethyl acetate/acetic acid (2:1:0.1, v/v/v)): R_f, 0.46 (twice developed). ^{1}H NMR (CDCl$_3$): δ 9.15 (bd, 1H, NH), 3.90 (d, J = 13.3 Hz, 3H, CH$_3$O), 3.32 (s, 2H, COCH$_2$) 2.40 (d, J = 16.1 Hz, 3H, CH$_3$S), 1.40 (s, 9H, CH$_3$).

This ester was readily converted to 200 mg of the oily acid, hapten-**4** (**19**), by treatment with TFA as described above for **11**. TLC (methanol/methylene chloride/acetic acid (1:9:1.5, v/v/v)): R_f, 0.7 (three times developed). ^{1}H NMR (CDCl$_3$): δ 9.15 (bd, 1H, NH), 3.90 (d, J = 13.3 Hz, 3H, CH$_3$O), 3.41 (s, 2H, COCH$_2$), 2.40 (d, J = 16.1 Hz, 3H, CH$_3$S). Low FAB(+)-MS: *m/z* 227.9 [M + H]$^+$.

Conjugation of Haptens with Carrier Proteins. The four haptens, hapten-**1**, -**2**, -**3**, and -**4**, were conjugated with proteins (BSA and KLH) by the active ester method (*17*), and hapten-**5** (**13**), methamidophos, was conjugated with a protein (BSA) by the diazotization method (*18*).

N-Hydroxysuccinimide (NHS) Active Ester Method. The haptens, except for hapten-**5**, were coupled covalently with the lysine moieties of the carrier proteins such as KLH and BSA according to the activated ester method. That is, each hapten (0.04 mmol) was dissolved in 0.2 mL of dry *N*,*N*-dimethylformamide (DMF) with equimolar NHS and a 10% molar excess of DCC. After the mixture was stirred at 22 °C for 5 h, the precipitated DCU was removed by filtration, and about 0.2 mL of the active ester was added slowly to a solution of the protein (10 mg of protein in 1 mL of 0.05 M borate buffer at pH 8) with vigorous stirring. The reaction mixture was stirred gently at 4 °C for 24 h to complete the conjugation and then dialyzed against normal strength PBS, which was changed with fresh one twice a day for 5 days. Finally, the conjugates were dispensed in 2 mL cryogenic vials and stored at −80 °C.

Diazotization Method. All steps of the preparation of the methamidophos−BSA conjugate were performed at 0 °C with stirring. A 1% aqueous solution of sodium nitrite (NaNO$_2$) was added dropwise to a solution of methamidophos (200 mg in 15 mL of 0.1 N HCl) until a positive starch iodide test was confirmed. After 30 min of additional stirring, 5 mL of the diazotization mixture was added slowly to BSA (5 mg/mL in the borate buffer, pH 9.0). The pH was maintained by addition of 5 N NaOH. The mixture was stirred for 2 h and then dialyzed as described above.

Immunization. Female New Zealand white rabbits weighing 3 kg were used for raising polyclonal antibodies. Routinely, 100 μg (protein equivalent) of each immunogen (hapten−KLH conjugate) in 0.5 mL of 0.85% saline was thoroughly emulsified with an equal volume of Freund's adjuvant. The emulsion was subcutaneously injected at five different sites on the neck and back of a rabbit. Three rabbits were used for each immunogen. Freund's complete adjuvant was used in the first injection, and Freund's incomplete adjuvant was used for

subsequent boost injections. Boosts were given every 3 weeks in the same manner. On the seventh day after each boost, about 10 mL of blood sample was taken from an ear vein to check the titer of the polyclonal antibody. The blood sample was allowed to coagulate for about 2 h at room temperature and then stored overnight in a refrigerator. The serum was decanted and centrifuged (800*g*), and the supernatant was stored in conveniently sized aliquots at −80 °C. Boosts were given six times.

Checkerboard Titration. Titers of the eight antisera were evaluated. Rabbit nos. 8377, 8390, and 8391 were immunized against hapten-**1**− KLH, nos. 301 and 302 were immunized against hapten-**2**−KLH, and nos. 11425 and 11427 were immunized against hapten-**3**−KLH, respectively. To check the titers of the antisera by the homologous indirect ELISA, each antiserum was diluted 256 000-fold. A checkerboard titration (*19*) was performed with the antisera collected from each rabbit. The checkerboard assay selected the combination of antiserum dilution and coating antigen concentrations (hapten−BSA conjugate) that demonstrated the greatest binding. The tested ELISA for acephate used a coating antigen concentration between 0.01 and 1 μg/mL and an antiserum dilution between 16 000 and 256 000.

Indirect ELISA, Competitive Inhibition ELISA, and Cross-Reactivities. Indirect ELISA and competitive indirect ELISA were performed according to the method of Voller et al. (*20*) as modified by Harrison et al. (*21*). For the checkerboard titration, an indirect ELISA was conducted. That is, 96 well microtiter plates were coated with 100 μL/well of the hapten−BSA conjugate in a carbonate buffer and allowed to stand overnight at 4 °C. On the following day, the plates were washed five times with 0.1 × PBST and thoroughly tapped dry. Sites not coated with the conjugate were blocked with 200 μL/well of 3% (w/v) skim milk in 1 × PBS. After they were incubated at 37 °C for 1 h, the plates were washed as described above. Antiacephate antiserum diluted with 1 × PBS (100 μL/well) was added, and the plates were incubated at room temperature for 1 h. After the plate was washed, 100 μL/well of a secondary antibody, goat antirabbit IgG conjugated with horseradish peroxidase diluted 1:10 000 with 1 × PBST, was added and the plates were incubated for 1 h at room temperature. The plate was washed, and 100 μL/well of a substrate solution (0.1 mL of 1% hydrogen peroxide and 0.4 mL of 0.6% 3,3′,5,5′-tetramethylbenzidine in dimethyl sulfoxide (DMSO) added to 25 mL of citrate−acetate buffer, pH 5.5) was added to each well. After 15 min at room temperature, the reaction was stopped by adding 50 μL/well of 4 N sulfuric acid. The yellow-colored plate was read spectrophotometrically in a dual wavelength mode at 450 nm using a reference wavelength of 655 nm. The amount of the enzyme bound, as indicated by the change of the colorless substrate to blue-colored product, is directly related to the amount of the rabbit antihapten antibody bound to the plate-coating antigen.

A competitive inhibition ELISA was used to assess the specificity of the antibody to free acephate and the cross-reactivities of structurally related compounds to the antibody. For competition, 50 μL of standards in assay buffer or diluted samples was placed in the wells and 50 μL of the antiacephate antiserum diluted with assay buffer was added to it. After it was mixed for 30 s and incubated at room temperature for 1 h, the plate was washed. The subsequent procedure was followed as described for the indirect ELISA. With the inhibition ELISA format, analytes that do not react with the antibody would produce absorbances near 100% of the zero analyte control; conversely, analytes that do react with the antibody would decrease the absorbance. Standard curves were calculated by mathematically fitting experimental points to a four parameter logistic equation (*22*) using a commercial software package (Origin, Microcal).

Because the cross-reactivity is defined as the ability of compounds, structurally related to the target analyte, to bind to the specific antibody raised against the analyte, some organophosphorus insecticides and a major metabolite of acephate were tested for selectivity of the ELISA by determining their respective IC$_{50}$ values in the competitive assays. Cross-reactivity values were calculated as the ratio of the IC$_{50}$ of the acephate standard to the IC$_{50}$ of the test compounds and expressed as a percentage.

Analyses of Some Acephate-Fortified Samples. *Tap Water.* For the recovery test, five concentrations (2, 5, 25, 50, and 100 ng/mL) of acephate in tap water were prepared with the acephate stock solution

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Table 1. Screening of the Antisera from Rabbits and Coating Antigens in a Competitive Indirect ELISA by Percent Inhibition of Color Development

		coating antigen									
		concentration of acephate (μg/mL)									
		hapten-**1**–BSA[a]		hapten-**2**–BSA		hapten-**3**–BSA		hapten-**4**–BSA		hapten-**5**–BSA	
immunogen	rabbit no.	10	100	10	100	10	100	10	100	10	100
hapten-**1**–KLH	8377	0	0	54[b]	66	0	0	94	97	0	0
	8390	6	72	36	59	34	35	6	6	5	5
	8391	0	0	16	25	5	3	14	16	3	15
hapten-**2**–KLH	301	0	0	0	0	0	0	6	11	7	11
	302	0	0	0	0	0	0	8	31	5	16
hapten-**3**–KLH	11425	0	0	0	0	0	0	0	13	10	11
	11426	0	0	0	0	0	0	0	0	0	0
	11427	0	0	0	0	0	0	0	7	0	0

[a] The data shown were determined at a coating antigen concentration of 1.0 μg/mL and an antibody dilution of 1:16 000. [b] No titer was observed using BSA alone as a coating antigen. [c] % Inhibition = $[(A - B)/A] \times 100$, where A is the absorbance of negative control and B is the absorbance of acephate. [d] The possibility of detecting more than 100 μg/mL of acephate in target samples would be very scarce.

in ethyl acetate. The sample was extracted with ethyl acetate according to the method of Ingelse et al. (*14*). That is, 25 mL of ethyl acetate was added to 2 mL of tap water treated with acephate. The mixture was shaken vigorously and filtered through a glass filter loaded with 10 g of anhydrous sodium sulfate, and the filtrate was evaporated to dryness under reduced pressure using a rotary evaporator at 50 °C. The residue was redissolved in 1 mL of 1 × PBS (pH 7.5), and the mixture was filtered through a 0.45 μm filter to remove suspended solids. The sample was extracted with ethyl acetate and concentrated twice for the immunoassay. Each analysis was done in triplicate.

Mulberry Leaves and Lettuce. The mulberry leaves and lettuce were obtained from the field where the insecticide was not applied. The sample was chopped in a Waring Blendor and stored at −20 °C until analysis. For the recovery test, five concentrations (50, 100, 500, 1000, and 3000 ng/mL for mulberry leaves and 300, 500, 1000, 2500, and 5000 ng/mL for lettuce) of acephate in the samples were prepared with the acephate stock solution in ethyl acetate. Acephate in the agricultural samples was extracted with acetone according to the established methods (*23, 24*). That is, 5 g of chopped samples was extracted with 50 mL of acetone using an ultrasonicator for 15 min. The mixture was filtered through filter paper (Whatman No. 2), and the filtrate was concentrated under reduced pressure using a rotary evaporator at 50 °C until the organic solvent was removed. The concentrate was made up to 25 mL with the buffer, and an aliquot of 25 mL was diluted twice for mulberry leaves and 10 times for lettuce; final dilution ratios were 1 to 10 for mulberry leaves and 1 to 50 for lettuce for the immunoassay. Each analysis was done in triplicate.

RESULTS AND DISCUSSION

Synthesis of Haptens and Their Conjugation to Carrier Proteins. The insecticide acephate in itself cannot be used as an immunogen because of its low molecular weight (MW = 183.2). Therefore, haptens mimicking the analyte insecticide and containing reactive groups for conjugation to carrier proteins must be synthesized to develop an ELISA for it. In the present investigation, five analogues of the target analyte were synthesized and used as immunogens and plate-coating antigens. To raise antibodies capable of recognizing the analyte acephate, the immunizing haptens were designed as almost perfect mimics of the target molecule in chemical structure in terms of electronic and hydrophobic properties. The syntheses of the haptens were carried out as outlined in **Figures 1**−**3**. The synthesis of hapten-**1** began by *N*-acetylation (*25*) of *O,O*-dimethylthiophosphoramide (*26*), **2**, to give *N*-acetyl-*O,O*-dimethylthiophosphoramide, **3**. The subsequent reaction with sodium propanethiolate (*25*) gave the sodium salt of *N*-acetyl-*O*-methylthiophosphoramide, **4**. Attempts to react this salt with several bromo acids in several common solvents including methanol, DMSO,
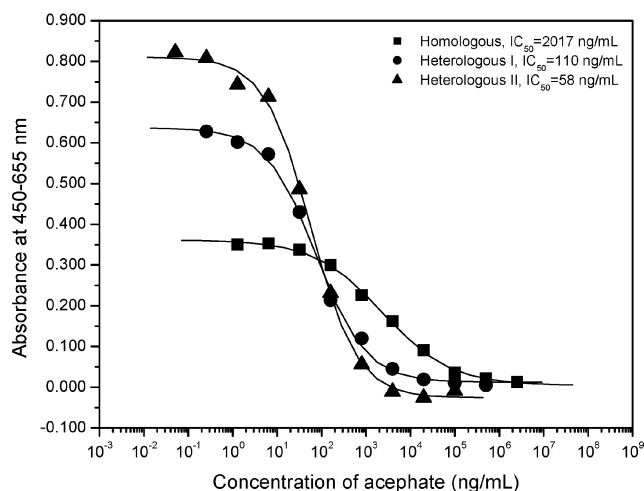

Figure 4. Screening of the coating antigen and the antiserum by the homologous and heterologous ELISAs. Homologous ELISA was made by the combination of antiserum no. 8390 raised against the immunogen hapten-**1**–KLH and the coating antigen hapten-**1**–BSA, and the heterologous ELISAs I and II were made by the combinations of antiserum no. 8377 raised against the immunogen hapten-**1**–KLH and the coating antigens hapten-**2**–BSA and hapten-**4**–BSA, respectively.

and DMF were unsuccessful, giving only complex mixtures of polar products by TLC. However, reaction with 6-bromohexanoic acid using water as the solvent gave a relatively low yield of the hapten-**1**, which was isolated and purified by chromatography. These results suggest that the salt, **4**, exists as a tight ion pair. If this is the case, in any future syntheses, the use of the potassium or cesium salt might facilitate the reaction by greater charge separation.

The synthesis of hapten-**2** utilized the intermediate *tert*-butyl 6-aminohexanoate **9**, which was prepared via the carbobenzoxy-protected esters **7** and **8** by analogous literature procedures (*29, 30*). This was converted to the thionophosphoramide ester **10**. Treatment of **10** with dimethyl sulfate not only isomerized it but also removed the *tert*-butyl group to give the thiolophosphoramide hapten-**2** in one step. Alternatively, using methyl iodide, the thionophosphoramide ester **10** can be isomerized to the thiolophosphoramide ester **11** in excellent yield. Subsequent treatment of **11** with TFA cleanly gives hapten-**2**. NMR spectra were consistent with all structures including the final haptens.

Hapten-**3** and -**4** were prepared via their *tert*-butyl ester, prepared by acylation (*27*) of the insecticide methamidophos with the mono-*tert*-butyl esters **14** and **15** of adipic acid or

Table 2. Effect of Various Factors on the Sensitivity of the ELISA[a]

factor	A_{max} (A)	slope (B)	IC_{50} (ng/mL) (C)	A_{min} (D)
	organic solvent contained in the assay buffer			
methanol (%)				
0	0.577	0.87	35.3 ± 4.10	0.041
10	0.545	0.77	49.5 ± 2.65	0.033
20	0.558	0.77	58.3 ± 2.95	0.054
40	0.493	1.04	86.9 ± 1.50	0.088
60	0.400	0.96	243.6 ± 13.95	0.060
80	0.572	0.84	807.2 ± 38.55	0.052
acetone (%)				
0	0.629	0.87	34.6 ± 4.50	−0.007
5	0.333	1.04	60.1 ± 7.06	−0.005
10	0.225	0.95	58.6 ± 3.82	−0.016
20	0.090	1.28	47.5 ± 4.45	0.013
	detergent in the assay buffer			
Tween 20 (%)				
0	0.570	0.95	34.9 ± 3.53	0.043
0.05	0.583	1.51	54.9 ± 4.67	0.016
	pH of the assay buffer			
pH				
5.5	0.789	0.85	60.9 ± 3.54	−0.016
6.5	0.768	0.91	55.6 ± 2.54	−0.013
7.5	0.637	0.93	38.6 ± 1.60	−0.010
8.5	0.593	0.86	32.1 ± 4.07	−0.015
	preincubation of the antibody and the analyte on the mixing plate			
time (h)				
0	0.585	0.97	16.7 ± 1.28	−0.015
1	0.375	0.97	28.7 ± 0.94	−0.027

[a] Assay conditions: coating antigen, hapten-4−BSA (1 μg/mL); blocking with 3% skim milk in 1 × PBS at 37 °C; antiserum, rabbit no. 8377 diluted by 1:16 000 in 1 × PBS; standard series of acephate were dissolved in the buffer; goat antirabbit IgG-HRP (1:10 000). Data are the means of quadruplicates.

malonic acid using DCC as a coupling reagent (*28*). Treatment of the resulting esters **16** and **17** with TFA removed the *tert*-butyl group to give the acid hapten-**3** and hapten-**4**, respectively, in excellent yield. The four haptens, hapten-**1**, -**2**, -**3**, and -**4**, with a carboxyl group were conjugated to carrier proteins by the active ester method, and methamidophos (hapten-**5**) with an amino group was conjugated to the proteins by the diazo-tization method.

Heterology is commonly used to eliminate problems arising from poor inhibition by the target compound, associated with the strong affinity of the antibodies for the bridging groups. It usually brings about somewhat weaker recognition of plate-coating antigens than that of the target analyte. Thus, lower analyte concentrations in the heterologous system can compete with these reagents under equilibrium conditions, which result in better assay sensitivity than in the homologous one (*31*, *32*). For the heterologous acephate ELISA of this investigation, heterology included hapten heterology using different hapten structures, site heterology, linker heterology using spacer arms with different lengths (*18*, *33*), and carrier heterology. All of the five haptens containing spacer arms of different lengths were conjugated to BSA for use as coating antigens to compare homologous and heterologous ELISA formats.

Screening and Selection of Coating Antigens and Antisera. To establish a sensitive ELISA, all combinations between coating antigens and antisera were screened via the inhibition by two different concentrations (10 and 100 μg/mL) of the analyte dissolved in the assay buffer, using the homologous or heterologous competitive indirect ELISA system. The inhibition ratio was expressed as a percentage of the difference between
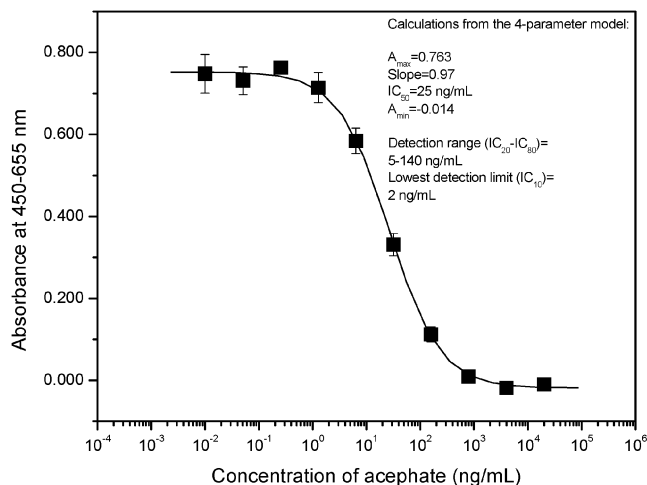

Figure 5. Standard curve obtained under the optimized condition of the ELISA. The error bars represent the standard deviation calculated from replicate calibration curves, which were obtained with the same set of standards ($n = 4$).

the absorbance of the analyte-free buffer and the absorbance of the analyte-containing buffer, divided by the former. The two polyclonal antibodies raised against hapten-**1** with a six carbon spacer arm adjacent to the sulfur atom of acephate were more sensitive to the target analyte in the homologous and heterologous ELISAs than those raised against other haptens, hapten-**2** and -**3**, with carbon spacer arms adjacent to the −NH− and −NHCO− moieties of acephate, respectively. There were relatively very high inhibitions in one homologous and two heterologous ELISAs using the combinations of hapten-**1**, -**3**, and -**4** as coating antigens with the antisera nos. 8377 and 8390 raised against the immunogen hapten-**1**−KLH (**Table 1**).

As shown in **Figure 4**, in three ELISAs conducted at 10 concentrations of acephate, the IC_{50} values in the homologous ELISA with the combination of the rabbit antiserum no. 8390 raised against the hapten-**1**−KLH immunogen and hapten-**1**−BSA as a coating antigen were 2017 ng/mL and those in the heterologous ELISAs I and II with the combination of the antisera no. 8377 raised against the hapten-**1**−KLH and hapten-**3**−BSA and hapten-**4**−BSA as coating antigens were 110 and 58 ng/mL, respectively. The most sensitive heterologous ELISA II was used to optimize the buffer-related factors for the assay.

Optimization of the ELISA. To determine the acephate residues in agricultural and environmental samples, it is essential to develop an ELISA with optimum sensitivity. For that purpose, the effects of the assay buffer-related factors such as detergent, organic solvent, pH, and preincubation of the mixture of the polyclonal antibody and the analyte on the sensitivity were evaluated.

Effect of Organic Solvent and Detergent. The effects of water miscible organic solvents, methanol or acetone, and detergent on the ELISA performance were evaluated. As seen in **Table 2**, the assay was more sensitive in the buffer without organic solvents. The IC_{50} value in the assay buffer containing methanol or acetone was higher than in the assay buffer without any organic solvent, indicating that an increase in the amount of organic solvent lowers the assay sensitivity in the acephate ELISA. Because acephate is very soluble in water (*3*), the use of organic solvents in assay buffer will not be necessary for the ELISA.

Tween 20 is a nonionic detergent and has been used in immunoassays to reduce nonspecific binding and improve sensitivity (*34*). However, in this investigation as shown in

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Table 3. Cross-Reactivity of Some Structurally Related Organophosphorus Insecticides to the Rabbit No. 8377 Antiserum in the ELISA[a]

compound	chemical structure	IC$_{50}$ (ng/mL)	CR[b] (%)
acephate	CH$_3$S, O, P, CH$_3$O, NHCOCH$_3$	30 ± 12	100
methamidophos (hapten-**5**)	CH$_3$S, O, P, CH$_3$O, NH$_2$	4442 ± 9	0.68
dichlorvos	CH$_3$O, O, P, CH$_3$O, OCH=CCl$_2$	>20 000	<0.02
phenthoate	CH$_3$O, S, P, CH$_3$O, SCHCOOCH$_2$CH$_3$	>20 000	<0.02
chlorpyrifos	CH$_3$CH$_2$O, S, P, CH$_3$CH$_2$O, O, N, Cl, Cl, Cl	4124 ± 17	0.73

a The heterologous ELISA was a combination of the antiserum no. 8377 raised against the immunogen hapten-**1**–KLH and the plate-coating antigen hapten-**4**–BSA. *b* % Cross-reactivity (CR) = (IC$_{50}$ of acephate/IC$_{50}$ of test compound) × 100.

Table 2, the IC$_{50}$ value in the buffer to which 0.05% (v/v) Tween 20 was added was about twice higher than that without the detergent, indicating that the addition of Tween 20 to the assay buffer does not enhance the sensitivity of the ELISA for the detection of acephate. Manclús and Montoya (*35*) also reported that Tween 20 had no effect on the sensitivity of an ELISA for a highly polar compound such as TCP (3,5,6-trichloro-2-pyridinol) and relatively polar sulfonylurea insecticides (*36–38*). This is probably due to the fact that the assay buffer without Tween 20 does not prevent the analyte and polyclonal antibody from binding; thus, the assay was more sensitive in the buffer without the detergent.

Effect of the Assay Buffer pH and Preincubation of the Analyte and Antiserum Mixture. To determine the effect of pH on the assay, the phosphate buffer was used in the range of pH 5.5–8.5. As shown in **Table 2**, the ELISA was more sensitive under neutral and slightly alkaline conditions than under slightly acidic ones. This means that the interaction between the antibody and the target analyte was most favored at pH 7.5–8.5. Because acephate is most stable to slightly acidic and neutral conditions (pH 5–7), the optimum pH range of the assay buffer was set at 7.5 for the ELISA.

The preincubation of the analyte and the antiserum no. 8377 on the mixing plate prior to the addition to the coated plates was tested to check the effect on the assay sensitivity of acephate. As seen in **Table 2**, preincubation did not enhance the sensitivity but affected the binding affinity of the antibody for the analyte, decreasing the absorbance.

On the basis of these results, the optimal conditions for the acephate ELISA are summarized as follows: A quantity of 1 μg/mL of the hapten-**4**–BSA conjugate as a coating antigen was coated onto the plate and placed at 4 °C overnight, and
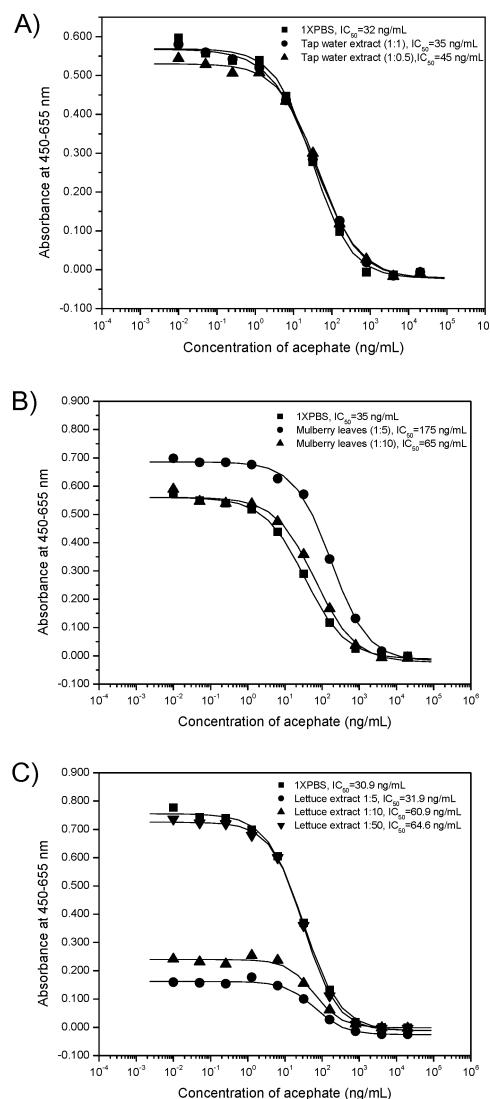

Figure 6. Matrix effect of some samples. (**A**) Extracts of tap water, (**B**) extracts of mulberry leaves, and (**C**) extracts of lettuce. The extractant is 1 × PBS.

then, the plate was blocked with 3% skim milk at 37 °C for 1 h. The antiserum no. 8377 raised against the hapten-**1**–KLH conjugate as an immunogen was diluted 16 000-fold with the assay buffer (pH 7.5) without detergent or organic solvent and competed with the target analyte dissolved in 1 × PBS (pH 7.5). In the heterologous assay optimized under these conditions, the IC$_{50}$ value of the analyte was 25 ng/mL, showing the detection range (IC$_{20-80}$) of 5–140 ng/mL and the lowest detection limit (LOD, IC$_{10}$) of 2 ng/mL (**Figure 5**).

Cross-Reactivities. In an assay that is a heterologous combination of hapten-**1**–KLH as an immunogen and hapten-**4**–BSA as a coating antigen, the antiserum no. 8377 did not recognize some structurally related organophosphorus insecticides, including the major metabolite of acephate, methamidophos. On the basis of the cross-reactivity (**Table 3**), the main antigenic determinant is the acetyl (–COCH$_3$) moiety in the structure.

Matrix Effect. Because of the matrixes such as inorganic and organic substances contained in some samples, the test samples used in ELISA should be diluted or purified by other methods prior to the determination of acephate residues. The extracts of tap water samples were concentrated twice as much as the originally fortified concentration. Standard curves of the

Table 4. Recovery by the ELISA of the Analyte Acephate from Fortified Samples

sample	fortified to sample (ng/mL)	theoretical concn in the ELISA[a](ng/mL)	detected (ng/mL)	mean recovery (%, $n = 3$)	coefficient of variation (%)[b]	remark
tap water	0	0	<LOD[c]		6	
	2	4	3.86 ± 0.56	97	1	
	5	10	11.76 ± 1.36	118	2	
	25	50	36.27 ± 0.42	73	1	
	50	100	71.52 ± 1.25	72	3	
	100	200	154.86 ± 4.00	77	9	
mulberry leaves	0	0	<LOD		3	
	50	5	4.64 ± 0.54	93	1	
	100	10	8.10 ± 0.47	81	1	
	500	50	48.28 ± 0.49	85	1	
	1000	100	81.03 ± .47	81	1	
	3000	300	307.05 ± 5.89	102	10	
lettuce	0	0	<LOD		2	
	300	6	6.05 ± 2.92	101	5	MRL[d]
	500	10	11.60 ± 2.68	116	4	3000
	1000	20	24.16 ± 0.70	121	1	ng/mL
	2500	50	53.89 ± 4.45	110	7	
	5000	100	115.4 ± 4.02	116	6	

[a] The extract of tap water was concentrated by half for immunoassay, and the extracts of mulberry leaves and lettuce were diluted 10 and 50 times with 1 × PBS, respectively. [b] Coefficient of variation is defined as the standard deviation divided by the mean, expressed as a percentage. [c] LOD means the lowest detection limit in the immunoassay. LODs are 2 ng/mL for tap water, 3 ng/mL for mulberry leaves, and 3 ng/mL for lettuce. [d] The value in lettuce was set by the Korea Food and Drug Administration.

water samples extracted were prepared with each concentrated water sample, as compared to the calibration curve prepared with the phosphate buffer as the control. As shown in **Figure 6**, the matrix effect of tap water samples extracted twice showed that their standard curves were close to the control curve. The matrix effects of the extracts of mulberry leaves and lettuce were reduced to a minimal level by 10- and 50-fold dilutions with the optimized assay buffer, respectively. These results indicate that the ELISA can determine acephate residues in water samples, mulberry leaves, and lettuce at the ppb level.

Recovery of Acephate Residues from Some Fortified Environmental and Agricultural Samples. Samples of tap water, mulberry leaves, and lettuce, which were fortified with five different levels of the analyte, were analyzed by the optimized ELISA. The extraction of acephate from the samples was carried out according to known methods. The recoveries of acephate from water samples by this ELISA were in the range of 72−118%, whereas those obtained from the same samples by GC−FPD were 60−120% (*14*). Recoveries of acephate from agricultural samples such as mulberry leaves and lettuce were very good, being in the range of 81−121% (**Table 4**).

This ELISA will be especially useful for the analysis of acephate residues in water samples, since acephate is very polar and nonextractable from water by the common LLE or SPE and requires more sophisticated instrumental methods (*14*). In addition, this technique could be a fast, cost effective, and selective assay for monitoring acephate residues within the maximum residue limits (MRLs) of agricultural samples. Therefore, this ELISA could be used as an alternative and supplemental analytical method to the conventional instrumental methods for monitoring acephate residues in environmental and agricultural samples.

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