Development of an ELISA for the Detection of the Residues of the Insecticide Imidacloprid in Agricultural and Environmental Samples

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A competitive enzyme-linked immunosorbent assay (ELISA) for the chloronicotinyl insecticide imidacloprid was developed using a polyclonal antibody produced against a hapten conjugated through the imidazolidine to keyhole limpet hemocyanin. In the standard curve of imidacloprid, an IC_{50} of 17.3 ng/mL was obtained using a competitive heterologous system at pH 10. Very low cross-reactivity was found for some structurally related compounds including the insecticide thiacloprid. The high cross-reactivity with a metabolite containing the carbonyl group in the imidazolidine moiety suggests the involvement of its polarity and stereochemical fitness in forming the antibody−antigen complex. The effects of various assay conditions, including organic solvents, detergent content, salt concentration, and pH on the sensitivity were evaluated. High-performance liquid chromatography was run for comparison to validate the ELISA with fortified water samples, the correlation being 0.997−0.998 (n = 15) with a slope of 1.10−1.38. The ELISA turned out to be a convenient tool for monitoring imidacloprid residues in agricultural and environmental samples.

Keywords: *Imidacloprid; ELISA; polyclonal; cross-reactivity; agricultural/environmental; monitoring*

INTRODUCTION

Imidacloprid [1-(6-chloro-3-pyridinyl)methyl-4,5-dihydro-*N*-nitro-1*H*-imidazole-2-amine] belongs to a new class of substances with a great affinity for insect nicotinergic acetylcholine receptors. Due to its broad efficacy as a systemic insecticide, imidacloprid has been widely used in rice paddies to control sucking insects, soil insects, and some chewing insects and in apple orchards to control various insect pests in Korea.

Imidacloprid also has major worldwide use in many crops. In this connection, some concern arises about the residues that may remain in agricultural products and in the environment. Current methods for detecting imidacloprid residues include high-performance liquid chromatography (HPLC) (*1*−*5*) and gas chromatography−mass spectrometry (GC-MS) (*6*−*8*). These instrumental methods are accurate but expensive and time-consuming, requiring lengthy sample extraction and cleanup procedures. In contrast, immunoassays generally are more sensitive, simpler, faster, and much less expensive. This investigation was carried out to develop an immunological method for monitoring and quantifying efficiently imidacloprid residues in agricultural products and environmental samples. The assay also could be used for product stewardship to improve its application for control of insect pests. As discussed later, this assay will complement the assays recently reported by Omoda et al. (*9*) and Li and Li (*10*).

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MATERIALS AND METHODS

Chemicals. Imidacloprid of analytical and working grade was a gift from Bayer AG. Bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), goat anti-rabbit IgG peroxidase conjugate, and Freund's complete and incomplete adjuvants were all obtained from Sigma Chemical Co. (St. Louis, MO).

Instrument. ELISAs were carried out 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).

Hapten Synthesis and Verification. *Hapten **1***. To a solution of 6.15 g of imidacloprid (24 mmol) in 100 mL of aqueous ethanol in an Erlenmeyer flask were added iron powder and ammonium chloride. The mixture was refluxed for 39 h and filtered. The filtrate was concentrated, and the residue was recrystallized to afford 3.27 g of the desired product **1** (60.5%, mp 210−212 °C): ^{1}H NMR (300 MHz, D_2O) δ 8.51 (m, 1 H), 8.45 (br s, 1 H), 7.89 (br s, 1 H), 7.50 (br s, 1 H), 4.75 (s, 2 H), 3.64−3.59 (m, 4 H); IR (ν_{max}, KBr) 3143, 1667, 1564, 1285, 1112.

To a solution of 113 mg (0.5 mmol) of compound **1** in 6 mL of pyridine was added 510 mg (5 mmol) of succinic anhydride. The mixture was stirred at room temperature for 12 h, and 10% HCl solution was added to it. The mixture was concentrated in vacuo to give the crude product, which was chromatographed on silica gel using methanol/ethyl acetate (1:2, v/v) to afford the desired product **2** (115 mg, 70.6%, mp 249−253 °C): ^{1}H NMR (300 MHz, D_2O) δ 8.84−8.82 (m, 1 H), 8.38 (br s, 1 H), 8.12−8.08 (m,1 H), 7.90−7.87 (m, 1 H), 7.58−7.56 (m, 1 H), 4.91 (s, 2 H), 3.91 (t, J = 8.48 Hz, 2 H), 3.74 (t, J =

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9.15 Hz, 2 H), 2.90 (t, J = 6.08 Hz, 2 H), 2.65 (t, J = 5.66 Hz, 2 H); ^{13}C NMR (300 MHz, D_2O) δ 183.39, 178.78, 153.12, 150.98, 129.01, 127.67, 125.08, 64.64, 53.11, 50.21, 48.33, 35.15, 34.85; IR (ν_{max}, KBr) 3400, 1742, 1442, 1382; FAB-MS, m/z 326.1 $[M + H]^+$.

Hapten ***2***. To a solution of 3.84 g of imidacloprid (15 mmol) in 81 mL of chloroform was added the solution of methyl succinyl chloride in 81 mL of chloroform (31.5 mmol) and 3.64 g of triethylamine. The resulting solution was stirred at 0 °C for 24 h and concentrated. To the residue was added 5 mL of 10% HCl solution. The resulting mixture was extracted three times with chloroform. The combined extracts were washed with brine, dried over anhydrous magnesium sulfate, filtered, and concentrated to afford 1.690 g of methyl ester **3** (30.1%, mp 121−124 °C): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.36 (s, 1 H), 7.75−7.72 (m, 1 H), 7.39−7.36 (m, 1 H), 4.61 (s, 2 H), 4.21 (t, J = 8.47 Hz, 2 H), 3.71−3.66 (m, 2 H), 3.66 (s, 3 H), 2.90 (t, J = 6.27 Hz, 2 H), 2.67 (deformed t, J = 8.44 Hz, J = 3.36 Hz, 2 H); ^{13}C NMR (300 MHz, $CDCl_3$) δ 173.28, 171.51, 156.01, 152.35 149.88, 139.59, 128.80, 125.34, 52.42, 47.41, 45.31, 44.66, 31.50, 29.01; IR (ν_{max}, KBr) 2953, 1728, 1567, 1460, 1259.

To a solution of 489 mg (1.32 mmol) of compound **3** in 16 mL of chloroform was added 166 mg (3.96 mmol) of $LiOH\cdot H_2O$ in 2 mL of water. The resulting solution was stirred at 5−10 °C for 4 h and diluted with 3.96 mL of 1 N HCl solution. The mixture was condensed in vacuo to give the crude product, which was chromatographed on silica gel using methanol/ethyl acetate (1:2, v/v) to afford the desired product **4** (357 mg, 83.0%, mp 229−231 °C). It was designated 4-{2-(azanitromethylene)-3[(6-chloro-3-pyridyl)methyl]imidazolidinyl}-4-oxobutanoic acid: ^{1}H NMR (300 MHz, D_2O) δ 8.30−8.26 (m, 1 H), 7.81−7.75 (m, 1 H), 7.51−7.48 (m, 1 H), 4.64 (s, 2 H), 3.52 (br s, 2 H), 3.41 (br s, 2 H), 2.47−2.41 (m, 4 H); ^{13}C NMR (300 MHz, D_2O) δ 182.12, 178.01, 157.29, 153.41, 151.16, 144.45, 130.23, 128.00, 64.66, 51.83, 49.26, 48.60, 35.31, 33.62; IR (ν_{max}, KBr) 3419, 2931, 1717, 1636, 1386, 1248; FAB-MS, m/z 355.9 $[M + H]^+$.

Conjugation of Carboxylic Acid Haptens to Carrier Proteins. Compounds **2** (hapten **1**) and **4** (hapten **2**) were coupled covalently to carrier proteins by using the *N*-hydroxysuccinimide ester method (*11*). That is, the carboxylic acid hapten (0.20 mmol) was dissolved in 1.0 mL of dry *N*,*N*-dimethylformamide (DMF) with equimolar *N*-hydroxysuccinimide and a 10% molar excess of dicyclohexylcarbodiimide. After 3.5 h of stirring at 22 °C, the precipitated dicyclohexylurea was removed by centrifugation, and the DMF supernatant was added to protein solutions.

Proteins (50 mg each of BSA and KLH) were dissolved in 5.00 mL of H_2O, and 1.05 mL of DMF was added slowly to each tube with vigorous stirring. One-fourth of the DMF supernatant was added to each protein solution (0.05 mmol per tube), bringing the final DMF concentration to 20%. The reaction mixtures were stirred gently at 4 °C for 22 h to complete the conjugation and then dialyzed exhaustively against normal strength phosphate-buffered saline (1× PBS; 8 g of NaCl, 0.2 g of KH_2PO_4, 1.2 g of Na_2HPO_4, and 0.2 g of KCl). Precipitates in the conjugates of hapten **1** and hapten **2** were partially redissolved by adjusting the pH to 10−11 during mixing, followed by a gradual return to pH 7.3. All conjugates dissolved easily at the lower concentrations and higher pH values used for ELISA plate coating (<10 μg/mL, pH 9.6).

Protein Determination. The protein contents of the hapten−protein conjugates were determined according to the Bio-Rad protein assay based on the method of Bradford (*12*). That is, the standard protein concentrations were 0, 6.25, 12.5, 25, 50, 75, and 100 μg/mL. The serial sample dilutions were 1:50, 1:100, 1:200, and 1:500. The Bio-Rad reagent was prepared by mixing 3 mL of Bio-Rad reagent with 12 mL of distilled water. For protein determination, 60 μL of each sample or standard was pipetted into each of three wells, and 240 μL of diluted Bio-Rad reagent was added to each well. After incubation at room temperature for 10 min, the plate was read at 595 nm. The resulting protein contents of the hapten-protein conjugates of hapten **1**−BSA, hapten **1**−KLH, hapten **2**−BSA, and hapten **2**−KLH were 5.1, 6.0, 4.2, and 4.8 mg/mL, respectively.

Determination of the Coupling Density. The free amino groups of the hapten−protein complex were determined with a modification of the trinitrobenzenesulfonic acid (TNBSA) method (*13*). That is, 0.5 mg/mL of protein or 0.5 mg/mL of hapten−protein conjugate solution in 1× PBS, 1.0 mL of carbonate buffer (1.59 g/L Na_2CO_3, 2.93 g/L $NaHCO_3$, pH 9.6), and 1.0 mL of 0.1% TNBSA were reacted at 40 °C for 2 h. After that, 1.0 mL of 10% sodium dodecyl sulfate (SDS) was added, and the reaction was terminated with 0.5 mL of 1 M HCl. After thorough mixing, the absorbance was measured at 335 nm. The coupling density was estimated by comparing the absorbance with the corresponding values of hapten-free proteins. From the available amino groups, 68% of hapten **1** and 34% of hapten **2** were conjugated in the hapten−KLH conjugates and 27% of hapten **1** and 20% of hapten **2** were conjugated in the hapten−BSA conjugates, respectively.

Immunization. Female New Zealand white rabbits weighing 3 kg were used for raising antibodies. Routinely, 100 μg of immunizing antigen dissolved in PBS was emulsified with Freund's complete adjuvant (1:2, v/v) and injected intradermally at multiple sites on the back. After 26 days, the animal was boosted with an additional 100 μg of antigen and bled 7−10 days later. The second and third boostings were performed every 3 weeks in the same way as above.

Checkerboard Titration. A checkerboard titration (*14*) was performed with each of the bleeds collected from different rabbits. The checkerboard assay selected the combination of antiserum dilution and coating antigen concentration (hapten−BSA) that would provide the greatest sensitivity in ELISAs. The optimized ELISA for imidacloprid used a coating antigen concentration between 1 and 1000 ng/well and an antiserum dilution between 16000 and 256000.

Coated Antigen Format. 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 with PBS plus 0.05% Tween 20 (PBST). Sites not coated with the conjugate were blocked with 200 μL/well of 3% (w/v) skim milk in 1× PBS. After 1 h of incubation at room temperature, the plates were washed as described previously. Standards (or samples diluted) were mixed with equal volumes of diluted anti-imidacloprid antibody for 30 s on the mixing plate and preincubated for 2 h at room temperature. The preincubated mixture was transferred to the wells (100 μL/well) and incubated for 1 h at room temperature for competition. To the washed plate was added a quantity of 100 μL/well of a goat anti-rabbit−horseradish peroxidase conjugate. After 2 h, the plate was washed, and 100 μL of a substrate solution [0.1 mL 1% H_2O_2 and 0.4 mL 0.6% 3,3′,5,5′-tetramethylbenzidine in dimethyl sulfoxide (DMSO) were added to 25 mL of citrate−acetate buffer, pH 5.5] was added to each well. After 20 min, the plates were read spectrophotometrically in a dual wavelength mode at 450 nm minus 650 nm. The development of a yellow color was inversely proportional to the amount of imidacloprid present.

ELISA, Competitive Inhibition ELISA, and Cross-Reactivity Studies. Enzyme immunoassay and competitive inhibition enzyme immunoassay were performed according to the method of Voller et al. (*15*) as modified by Harrison et al. (*16*). That is, hapten−protein (BSA) conjugates (plate-coating antigens) were immobilized in the wells of 96-well microplates, and the wells were added sequentially with rabbit anti-hapten antibody, enzyme-labeled goat anti-rabbit antibody, and enzyme substrate. The amount of enzyme bound, as indicated by the change of colorless substrate to blue product, is directly related to the amount of rabbit anti-hapten antibody bound to the plate-coating antigen. Thus, ELISA is used to measure the amount of a specific antibody in a sample or for the comparison of the relative binding of an antibody to different conjugates immobilized on the solid phase. Competitive inhibition ELISA was used to measure free analytes, such as imidacloprid, or for the comparison of the relative binding of soluble inhibitors to an antibody to determine antibody specificity. The analytes of analytical grade were dissolved in

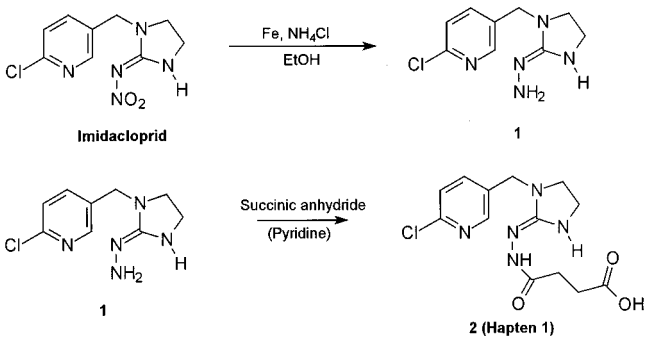


Figure 1. Schematic representation of the synthesis of hapten **1**.

methanol (stock solution = 10 mg/mL) and used to examine the antibody cross-reactivities of the ELISA, being diluted sequentially from 25000 to 0.0128 ng/mL. With the inhibition ELISA format, analytes that do not react with the antibody would produce absorbances near 100%; conversely, analytes that do react with antibody would decrease in percentage of absorbance. Standard curves were calculated from the raw data using a four-parameter (sigmoidal) equation (*17*). The optimized ELISA for imidacloprid was used to examine the cross-reactivity of the antibody.

Analysis of Imidacloprid Residues in Spiked Samples by ELISA. *Apples.* To 10 grams of apples cleaned, sliced and crushed were added 20 mL of methanol in a 50 mL-beaker and the mixture was homogenized for 5 min. The homogenized samples were filtered through Whatman No. 2 filter paper by suction. The glassware container and the residues were washed with methanol (20 mL), filtered and combined with the previous filtrate. The methanol was removed in vacuo at 60 °C. The concentrate was transferred to a 10-mL volumetric flask and made to the volume with 1× PBS (pH 10). For the recovery test, four levels of imidacloprid concentration, 16, 80, 400, and 2000 ng/mL, in apple extracts were prepared with the stock methanol solution of imidacloprid (100 μg/mL). No aged samples were analyzed in this study.

Environmental Samples. Imidacloprid-free water collected from an isolated pond for the irrigation of rice paddy fields was used for the surface water samples, after the suspended matter was removed. As ground water samples, water collected from a depth of >100 m was used without filtration. Four concentrations of imidacloprid in water samples, 16, 80, 400, and 2000 ng/mL, were prepared with the imidacloprid stock solution in methanol. For ELISA of imidacloprid, the aliquots of each sample were diluted 50 times with PBS of pH 10. Each analysis was done in triplicate.

Solid-Phase Extraction (SPE) of Imidacloprid from Water Samples. For the extraction of imidacloprid in pond water, an SPE method described by Baskaran et al. (*4*) was slightly modified. Briefly, the C_{18} SPE tube (3 mL tube, Alltech) was preconditioned with 2 mL of methanol and 2 mL of deionized water. Twenty-milliliter water samples in the glass bottles were loaded on the tube joined to a 20-mL reservoir and eluted at a 3−5 mL/min flow rate. The bottles and the reservoirs were washed with 20 mL of deionized water (5 mL × 4), and the washings were applied onto the tube. After the water had passed through, the tube was dried by suction for 15 min and eluted with 5 mL of *n*-hexane, and the *n*-hexane washings were discarded. Finally, the tube was eluted with 3 mL of ethyl acetate, and the eluate was evaporated to dryness under N_2. The residue was redissolved in 1 mL of PBS of pH 10, and an aliquot diluted 50 times with the buffer was analyzed by the optimized ELISA.

RESULTS AND DISCUSSION

Hapten Synthesis. The structures of the haptenic compounds are shown in Figures 1 and 2. For hapten **1**, the nitro group of imidacloprid was reduced to an amino group, over an exceedingly long reaction time.

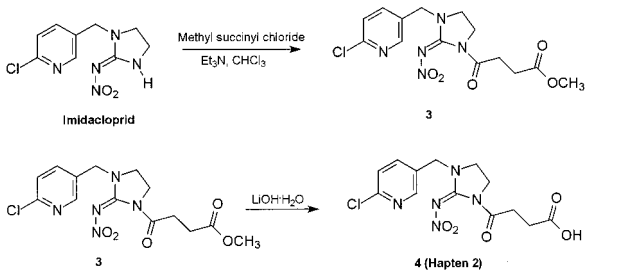


Figure 2. Schematic representation of the synthesis of hapten **2**.

Table 1. Screening of Antisera from Test Rabbits by Competitive Indirect ELISA

		concn of imidacloprid (ng/mL)			
antiserum		coated with hapten **1**−BSA[a]		coated with hapten **2**−BSA	
immunogen	rabbit	5000	625000	5000	625000
hapten **1**−KLH	A	−[b]	−	−	6.3[c]
	B	−	−	−	−
	C	−	−	−	−
hapten **2**−KLH	D	78.0	97.3	52.3	91.6
	E	23.5	74.9	8.8	46.8
	F	45.3	51.6	−	41.5

[a] No titer was observed using BSA alone as coating antigen. [b] −, no inhibition. [c] % inhibition = [1 - ($A_{imidacloprid}/A_{control}$)] × 100.

The resulting NH_2 group was reacted with succinic anhydride at room temperature to give the carboxylic hapten **1**. For hapten **2**, the proton in the imidazolidine ring ($K_a = 1.2 \times 10^{-7}$ at 25 °C) was readily reacted with the chlorine of methyl succinyl chloride to remove hydrogen chloride in the presence of triethylamine. The resulting methyl ester was readily hydrolyzed with $LiOH \cdot H_2O$ to give the desired carboxylic acid hapten **2**. This hapten proved to be a useful immunogen as seen in Table 1. Omoda et al. (*9*) made a hapten by adding a linker to the pyridine ring by replacing chlorine. Meanwhile, Li and Li (*10*) synthesized two different haptens by introducing a linker to the imidazolidine ring and the pyridine ring of imidacloprid, respectively.

Titration of Antisera. Hapten **1**−KLH and hapten **2**−KLH conjugates were injected five times into each rabbit as immunogens, respectively. The antisera collected after each boosting were subjected to titration by the indirect ELISA. All of the antisera showed the highest titer after the final boosting as seen in Figure 3, which shows the antisera collected from rabbit D. Antisera were tested against both the homologous and heterologous haptens coupled to BSA. No antisera showed significant cross-reactivity against BSA alone.

Screening and Selection of Antisera. To select the antisera suitable for the ELISA, each antiserum produced by the six rabbits (three for each immunogen) was screened for the inhibition by the two concentrations of the analyte imidacloprid dissolved in the assay buffer, using the homologous and heterologous systems. The inhibition ratio was calculated by the difference in absorbance between the buffer containing imidacloprid and that without the analyte. As seen in Table 1, the antisera produced by the three rabbits injected with the hapten **1**−KLH conjugate showed almost no or very low inhibition ratios by the analyte imidacloprid. This result suggested that the antisera exhibited affinity against the hapten **1**−KLH, but the analyte imidacloprid could not displace the coating antigen. This means that no

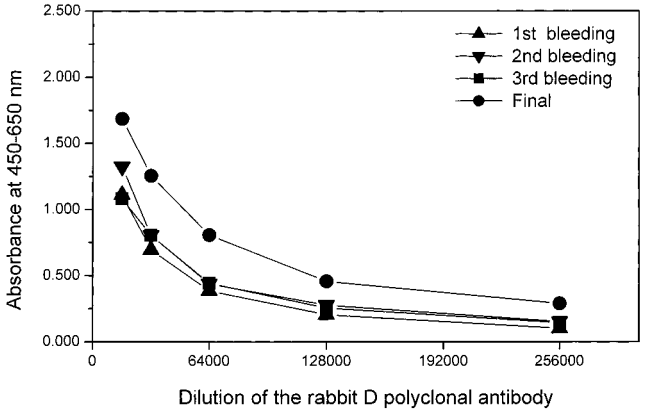


Figure 3. Titration of the antiserum from rabbit D by homologous indirect ELISA.

antibody specific to imidacloprid was produced, which could be detected with the current assay format. Antibodies raised against hapten **1** may be useful in future assays for imidacloprid using alternate reporter haptens or for assays for imidacloprid degradation products. In contrast, all three antisera raised by the hapten **2**−KLH conjugate exhibited an inhibition by imidacloprid. Because the antiserum raised by rabbit D showed the highest inhibition ratio of the three, it was used for the subsequent optimization and characterization of the ELISA.

Optimization of the ELISA. In the sigmoidal curve used for ELISA, the upper and lower asymptotes of the four-parameter fit represent the absorbances corresponding to the minimum and maximum concentrations of the analyte imidacloprid, respectively. The linear portion of the curve involving the central point, IC_{50}, is the analytical range. The sensitivity of the ELISA is represented by the IC_{50} value, which is the concentration of the analyte imidacloprid inhibiting the absorbance of the control by 50%. Therefore, the smaller the IC_{50}, the higher the resulting sensitivity of an ELISA for imidacloprid detection. To determine qualitatively and quantitatively the imidacloprid residues in the environmental samples, it is essential to develop an ELISA of optimum sensitivity. The sensitivity of a competitive ELISA is influenced by the amounts of both the primary antibody and the coating antigen (*18*). Therefore, the amounts of both the anti-imidacloprid antibody (rabbit D antiserum) and the coating antigen (hapten **1**−BSA) were varied in an effort to determine the optimum conditions for the assay. Besides, to enhance the analytical sensitivity, the effects of preincubation time of the mixture of the analyte and the antiserum on the mixing plate and the assay buffer-related factors such as solvents, detergents, ionic strength, and pH were examined.

Optimal Concentrations of Coating Antigen and Antiserum. As shown in Table 2, the optimal concentration and dilution ratio of the coating antigen and antiserum for this ELISA were 5 μg/mL and 1:16000, respectively.

Solvent Effects. Most pesticides are not readily soluble in water; therefore, some polar solvents are used to prepare the assay buffer for ELISA. Accordingly, the effect of solvents on the specific interaction between the analyte and the antibody should be elucidated. As seen in Figure 4, the absorbances increased compared with the control as the content of methanol in the final reaction increased, probably by affecting the affinity between the coating antigen and the antibody. In

Table 2. Effect of Concentrations of Coating Antigen and Antiserum on the Sensitivity of Imidacloprid ELISA[a]

coating antigen (μg/mL)	antiserum (dilution)	A_{max} (*A*)	slope (*B*)	IC_{50} (ng/mL) (*C*)	A_{min} (*D*)	*A*/*D*
1	1:32000	0.491	0.855	59.0	0.012	40.9
5	1:16000	1.201	0.880	57.5	0.011	109.2
10	1:16000	0.896	0.929	58.9	−0.002	448.0

[a] ELISA conditions: coating antigen, hapten **1**−BSA (5 μg/mL); antiserum from rabbit D (1:16000) in 1× PBS; standard series of imidacloprid were dissolved in 1× PBS; preincubation of the analyte and antiserum for 2 h in the mixing plate; goat anti-rabbit IgG-HRP (1:5000). Data are the means of quadruplicate.

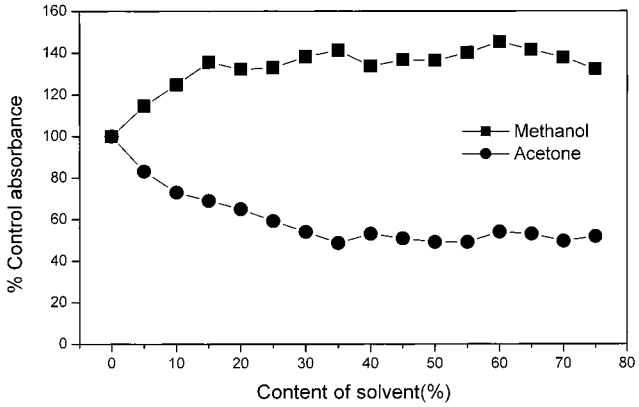


Figure 4. Inhibition of ELISA color development as a function of the concentration of methanol (■) or acetone (●). Control represents color development in the absence of the corresponding organic solvent. Data shown are the means of quadruplicate.

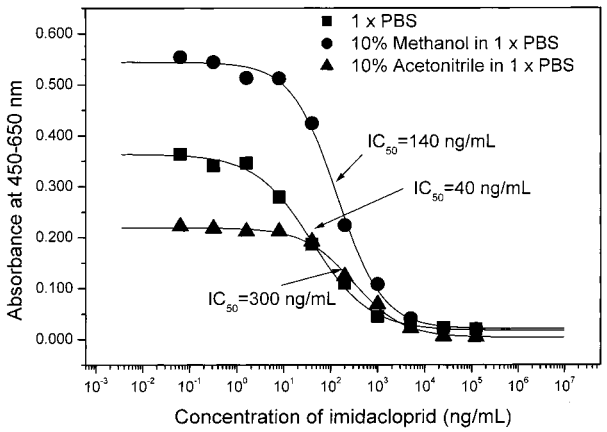


Figure 5. Effect of organic solvent on the ELISA for the determination of the analyte imidacloprid residues, as performed under unoptimized conditions. The standard curves were obtained in phosphate buffer (pH 7.5) with and/or without organic solvent.

contrast, addition of acetone decreased the absorbances. On the basis of this result, the polar solvents methanol and acetonitrile were used to examine their effects on ELISA. The assay buffers (1× PBS) containing 10% methanol and 10% acetonitrile exhibited the increased IC_{50} values of 140 and 300 ng/mL, respectively, compared with 40 ng/mL in the control (1× PBS), as seen in Figure 5. Also, IC_{50} values of imidacloprid were significantly decreased in the presence of DMSO and DMF (data not shown), indicating that the organic solvents suppress antibody−hapten binding. This result indicates that it is very important to use the assay buffer containing no or very little organic solvents in the ELISA for monitoring the trace amounts of imida-

Table 3. Effect of Preincubation of the Analyte and Antiserum Mixture on the Sensitivity of the Imidacloprid ELISA[a]

time of pre-incubation (h)	A_{max} (*A*)	slope (*B*)	IC_{50} (ng/mL) (*C*)	A_{min} (*D*)	*A*/*D*
0	1.201	0.881	57.5	0.011	109.2
1	1.007	0.834	32.9	0.007	143.9
2	0.770	0.809	29.5	0.002	385.0
4	0.201	0.412	842	0.006	33.5

[a] ELISA conditions: coating antigen, hapten **1**−BSA (5 μg/mL); antiserum from rabbit D (1:16000) in 1× PBS buffer; standard series of imidacloprid were dissolved in 1× PBS buffer; goat anti-rabbit IgG-HRP (1:5000). Data are the means of quadruplicate.

cloprid residues in agricultural and environmental samples. Nevertheless, 2−4% methanol would have no detectable effect. Taking into account the water solubility of imidacloprid (520 μg/mL), it will not be necessary to use any organic solvents that decrease the sensitivity of the ELISA because much smaller amounts of imidacloprid are practically applied in the field. Therefore, in the subsequent experiments for the optimization of the ELISA, the assay buffer containing no organic solvents was used.

Preincubation of the Analyte and Antiserum Mixture on the Mixing Plate. To characterize the effect of the preincubation of the mixture of the analyte and the rabbit D antiserum prior to the addition to the coated plates on the analytical sensitivity of the imidacloprid ELISA, four different incubation times (0−4 h) on the mixing plates were tested. As shown in Table 3, the 2 h preincubation showed the lowest IC_{50} value. The 2 h incubation time was selected for further work because of the lowest IC_{50} value and the highest *A*/*D* ratio.

Detergent Effects. Tween 20 is a nonionic detergent commonly used in immunoassays to reduce nonspecific binding and improve sensitivity (*19*). However, there are quite a few papers showing that Tween 20 decreased the sensitivity due to nonspecific hydrophobic interactions between the detergent and nonpolar small analytes in an aqueous environment, thereby interfering with the specific analyte−antibody interaction (*20*−*24*). Shan et al. (*25*) also reported that Tween 20 (0.05%) significantly affected the binding between antibody and hapten, and hence the IC_{50} with Tween 20 was ∼20 times higher than that without the detergent in their immunoassay study on the pyrethroid insecticide esfenvalerate.

Likewise, in our investigation the IC_{50} measured in the assay buffer without Tween 20 was lower than that with the detergent, as seen in Figure 6 or Table 4. This result may be also due to the nonspecific hydrophobic interactions between the detergent and the analyte imidacloprid, even if it has a relatively high water solubility (0.61 g/L, at 20 °C). Manclús and Montoya (*26*) indicated that a highly polar compound such as 3,5,6-trichloro-2-pyridinol (TCP) was not affected by Tween 20 with regard to nonspecific interactions.

Gelatin and BSA each added to the assay buffer instead of the detergent Tween 20 failed to enhance the sensitivity of the imidacloprid ELISA or to lower the background (data not shown).

Salt Concentration Effects. As seen in Figure 7, the increased salt concentration in the assay buffer did not greatly enhance the assay sensitivity (IC_{50}), only decreasing the absorbance by reducing the interaction between the antibody and the coating antigen. For an optimal ELISA of imidacloprid, the assay buffer containing 1× PBS without Tween 20 or organic solvents was suitable for the competition between the antibody and the target analyte or the immobilized coating antigen.

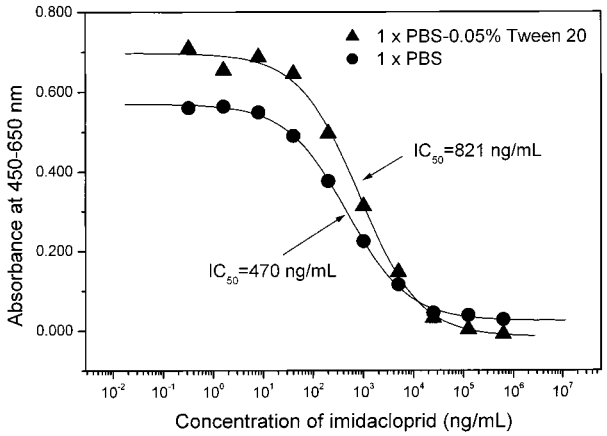


Figure 6. Effect of Tween 20 added to the assay buffer on the characteristics of the imidacloprid standard curve. The standard curves were obtained in phosphate buffer (pH 7.5) with and/or without Tween 20.

Table 4. Effect of the Detergent Tween 20 on the Sensitivity of the Imidacloprid ELISA[a]

Tween 20[b] (%)	A_{max} (*A*)	slope (*B*)	IC_{50} (ng/mL) (*C*)	A_{min} (*D*)	*A*/*D*
0	0.820	0.784	26.7	0.006	136.7
0.05	1.013	0.938	173	0.020	50.7

[a] ELISA conditions: coating antigen, hapten **1**−BSA (5 μg/mL); antiserum, rabbit D (1:16000); preincubation of the analyte and antiserum for 2 h on the mixing plate; goat anti-rabbit IgG-HRP (1:5000). [b] Concentration of Tween 20 in 1× PBS. Data are the means of quadruplicate.

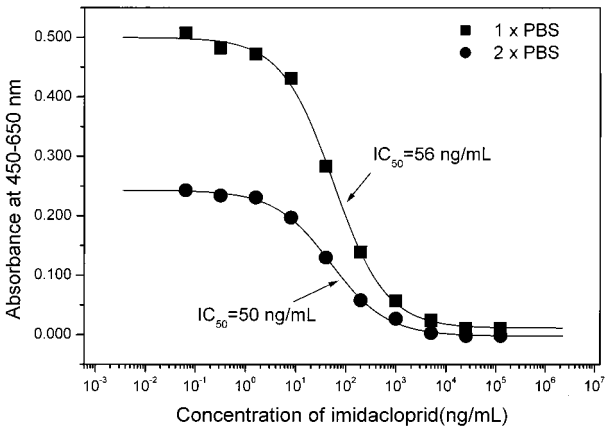


Figure 7. Effect of the salt concentration (1× and 2× PBS) of the assay buffer on the characteristics of the imidacloprid competitive standard curve. The standard curves were obtained in phosphate buffer (pH 7.5) with different ionic strengths.

Effect of pH on the Sensitivity of Imidacloprid ELISA. The influence of the pH on the assay was examined (Figure 8). Many immunoassays are equally sensitive over a wide range of pH values. However, the assay for imidacloprid was pH-sensitive. The affinity of rabbit D antiserum toward imidacloprid and coating antigen was very pH-dependent as shown in Table 5. The pH probably affects both the analyte and the antibody.

The rabbit D antiserum on the plate coated with hapten **1**−BSA exhibited better reactivities and hence

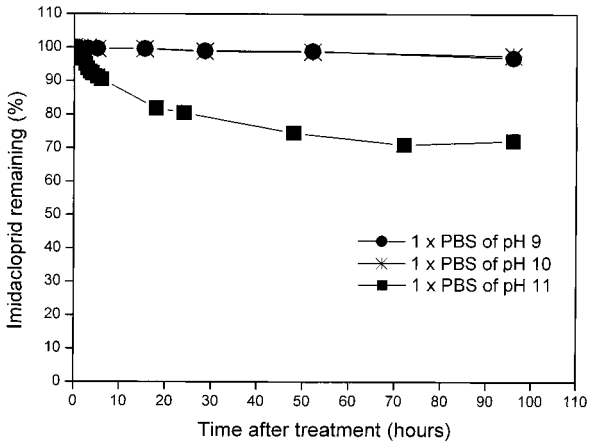


Figure 8. Hydrolysis of imidacloprid in 1× PBS of different pH values at room temperature. Initial concentration of imidacloprid was 1 ppm.

Table 5. Effect of pH on the Sensitivity of the Imidacloprid ELISA[a]

pH	A_{max} (*A*)	slope (*B*)	IC_{50} (ng/mL) (*C*)	A_{min} (*D*)	*A*/*D*
4	0.822	0.634	61.4	−0.035	23.5
6	0.814	0.702	41.7	−0.009	90.4
7.5	0.799	0.619	27.1	−0.016	49.9
8	0.807	0.759	21.7	0.002	403.5
9	0.779	0.756	19.7	0.005	155.8
10	0.742	0.644	17.3	0.005	148.4
11	0.522	0.734	8.1	0.003	174.0

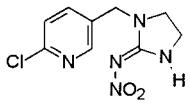
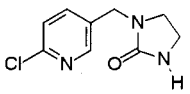
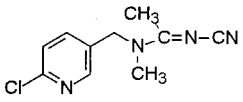
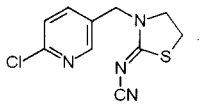
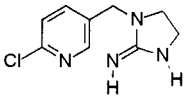
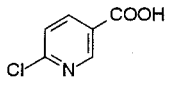
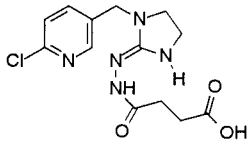
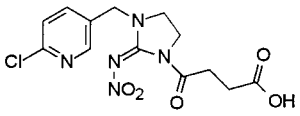
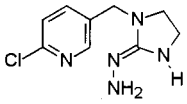
[a] ELISA conditions: coating antigen, hapten **1**−BSA (5 μg/mL); antiserum, rabbit D (1:16000); preincubation of the analyte and the antiserum for 2 h on the mixing plate; goat anti-rabbit IgG-HRP (1:5000). Analyte and antiserum were diluted in 1× PBS of different pH values. Data are the means of quadruplicate.

IC_{50} values under alkaline conditions were decreased relative to those under acidic or neutral conditions, suggesting that the affinity of the antibody raised against imidacloprid increased with an increase in pH. Although the higher the pH (up to pH 11), the lower the IC_{50} value was as seen in Table 5, the assay using the buffer of pH 11 was not adopted to prevent the destruction of the antibody and the hydrolysis of imidacloprid under such a strongly alkaline condition.

Cross-Reactivities by Related Compounds. Cross-reactivity values were calculated as the ratio of the IC_{50} of the imidacloprid standard to the IC_{50} of the test compounds and expressed as a percentage. The three major metabolites of imidacloprid, the insecticides acetamiprid and thiacloprid containing the same (6-chloro-3-pyridinyl)methyl moiety in its chemical structure as imidacloprid, the amino analogue of imidacloprid (compound **1**), and the two haptens were tested, compared with imidacloprid. As can be seen in Table 6, the 6-chloro-3-pyridinyl moiety common to all of the test compounds including imidacloprid in isolation did not contribute to the formation of the antibody−antigen complex, as verified by the metabolite **3**, 6-chloronicotinic acid. Either other moieties or several determinants spread out over the whole molecule appeared to be necessary for significant cross-reactivity. This argument that multiple functionalities are needed is illustrated by the low cross-reactivity of acetamiprid and thiacloprid. These materials may bind to the antibody, but their affinity is not high enough to displace the hapten in a competitive assay.

Furthermore, the very high cross-reactivity (152%) of the metabolite **1** containing the imidazolidin-2-one moiety and the very low cross-reactivity (<0.8%) of metabolite **3**, 6-chloronicotinic acid, suggest that the antibody recognizes a moiety containing the methylene and imidazolidine. Especially, the fact that metabolite **1** and hapten **2**, both containing one or two carbonyl groups, showed 1.5- and 111-fold higher cross-reactivities, respectively, indicates that the polar carbonyl group interacts well with the antibody-binding sites, favoring the electronic interactions between the analyte and the antibody. Hapten **2** would be expected to show exceptionally high cross-reactivity because it was the hapten used for immunization. The carbonyl of metabolite **1** is anticipated to mimic the doubly bonded nitrogen in imidacloprid. Compound **1** would not be expected to cross-react because the nitro group was chemically reduced. In particular, the reason hapten **1** containing two carbonyl groups showed relatively low cross-reactivity (9.3%) could be due to a steric unfitness to combine with the antibody. The data suggest that most of the molecule is needed before significant binding occurs. The high level of cross-reactivity of metabolite **1** (152%) and the low cross-reactivity of compound **1** (3.8%) are very interesting. There are several possibilities to explain this observation. One possibility is that hapten **1** broke down to the ketone during the process of raising antibodies. Thus, there may be a mixture of antibodies to both the parent and the breakdown product corresponding to metabolite **1** used in our assay. The other possibility is that when we use antibodies to hapten **2** in conjunction with a coating antigen prepared from hapten **1**, the assay will not distinguish well between imidacloprid and the corresponding ketone. We will test this hypothesis in future studies using a homologous assay based on a second hapten to the imidazolidine ring. Li and Li (*10*) reported that the antibodies obtained from the immunogen having a linker on the pyridine ring of imidacloprid were more specific to imidacloprid than those obtained from the other immunogen having a linker attached to the imidazolidine ring in the cross-reactivity study with some related metabolites. In this respect, they mentioned that the antibodies thus produced mainly recognize the imidazolidinyl-associated moiety at the far end. In our case, however, the antibodies did not well recognize the far pyridine ring. Instead, the polar carbonyl group on the imidazolidine ring was even more specific than the $=N-NO_2$ moiety in imidacloprid. This will be due to the polarity of the carbonyl group that can interact well with the antibody. The big difference in the cross-reactivities between imidacloprid and compound **1** indicates the importance of the polarity in the antigen−antibody interaction. The cross-reactivity of more compounds needs to be run before one can assign the cross-reactivity due to individual functionalities. For example, Newsome (*27*) reported that 4-chlorophenol, 1,2,4-triazole, iprodione (fungicide), and imazalil (fungicide) did not exhibit significant inhibition as the related compounds in an ELISA of the triazole fungicide triadimefon. This result also suggests that neither the chlorine-substituted phenyl, the triazole, nor the imidazole moiety alone binds sufficiently well to the antibody to compete it off the coating antigen. For example, in studies with the pyrethroid allethrin, Wing et al. (*28*) reported that neither the alcohol nor the acid moiety of the pyrethroid

Table 6. Cross-Reactivity of Some Structurally Related Compounds to the Rabbit D Antiserum in the ELISA

compound	chemical structure	cross-reactivity[a] (%)
imidacloprid		100
metabolite **1**, 1-[(6-chloro-3-pyridinyl)-methyl]-imidazolidin-2-one		152
acetamiprid		7.1
thiacloprid		1.5
metabolite **2**, 1-[(6-chloro-3-pyridinyl)-methyl]-imidazolidin-2-imine		5.3
metabolite **3**, 6-chloronicotinic acid		<0.8
hapten **1**, 4-(2-1-[(6-chloropyridin-3-yl)-methyl]imidazolidin-2-ylidene-hydrazine)-4-oxobutanoic acid		9.3
hapten **2**, 4-2-(azanitromethylene)-3[(6-chloro (3-pyridyl))methyl]imidazo-lidinyl-4-oxobutanoic acid		11126
compound **1** 1-(6-chloro-3-pyridylmethyl)-N-aminoimidazolidin-2-ylideneamine		3.8

[a] % Cross-reactivity=(IC_{50} imidacloprid/IC_{50} other compound)×100.

[b] The assay used antibodies raised to hapten **2** with a coating antigen to hapten **1**.

bound well to the antibody, even though the combined ester bound tightly.

The current ELISA will detect both imidacloprid and one of its major degradation products. To make the assay specific, one would have to separate the two materials before analysis or develop a second ELISA with different specificity for the two compounds so that an algorithm could be used to distinguish the compounds.

Triazoles are known to be of low natural immunogenicity (*27*), and the aminotriazole herbicide amitrole was found to be a poor immune epitope (*29*, *30*). Meanwhile, Forlani et al. (*31*) reported an ELISA for triazole fungicides with the hemisuccinate of 2-2,4-dichlorophenyl-3-(1*H*-1,2,4-triazol-1-yl)propanol (DTP) as the immunogen. They pointed out the role of the substituents present on the chain connecting the 1,2,4-triazole and the 2,4-dichlorophenyl group in explaining the cross-reactivity with the structurally related compounds in terms of the antibody−antigen complex formation.

In the study of an ELISA for the triazole fungicide myclobutanil, Székács and Hammock (*32*) disclosed that the amide and carboxyl derivatives of myclobutanil, which contains a cyano group in its structure, had 3.5- and 2-fold lower IC_{50} values, respectively, than myclobutanil. However, the commercial triazole fungicides containing the same 1*H*-1,2,4-triazole moiety as myclobutanil displayed low cross-reactivities. On the basis of these results, they concluded that the triazole moiety is not large enough or characteristic in electronic properties to be solely recognized by B-lymphocytes.

Comparison between Homologous and Heterologous Assays. For the trace amounts of pesticide residues in agricultural and environmental samples, the heterologous assay has been widely used. As seen in Figure 9, in the imidacloprid competitive standard

Table 7. Optimized Conditions for the Imidacloprid ELISA

coating antigen[a] (μg/mL)	rabbit D antiserum[b] (dilution)	preincubation time of the analyte and the antiserum (h)	pH of 1× PBS for the preincubation	A_{max} (*A*)	slope (*B*)	IC_{50} (ng/mL) (*C*)	A_{min} (*D*)	*A*/*D*	detection range (ng/mL) (IC_{20}–IC_{80})
5	1:16000	2	10	0.742	0.644	17.3	0.005	148.4	5–125

[a] Coating antigen: hapten **1**–BSA. [b] Antibodies raised against hapten **2**–KLH.

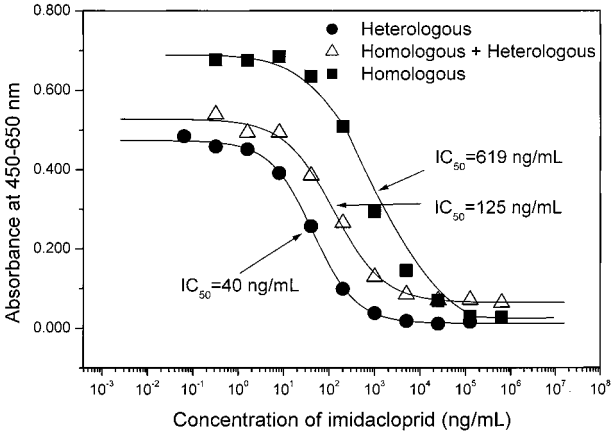


Figure 9. Comparison between homologous ELISA and heterologous ELISA in the imidacloprid competitive standard curve.

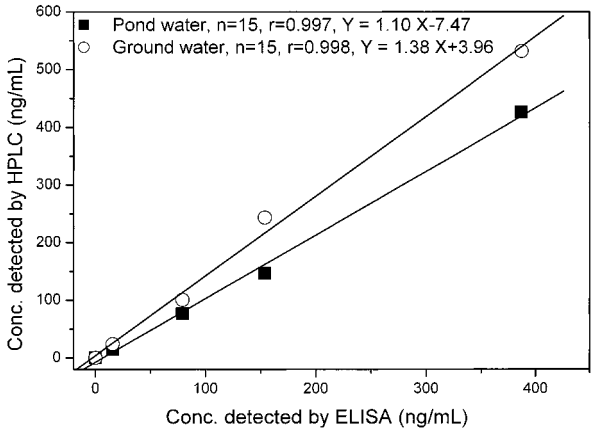


Figure 10. Correlation between the result from ELISA and that from HPLC for water samples.

Table 8. Recovery of the Analyte Imidacloprid Fortified to Agricultural and Environmental Samples by the ELISA

sample	imidacloprid fortified (ng/mL)	imidacloprid detected (ng/mL)	mean recovery (%, *n* = 3)	% CV
apple extract	0	ND[a]		4
	16	34	213	3
	80	119	149	6
	400	395	99	5
	2000	1869	94	9
pond water for irrigation	0	ND	-	6
	16	34	213	1
	80	163	204	5
	400	430	108	4
	2000	2372	119	6
ground water from 100-m depth	0	ND		5
	16	25	156	7
	80	106	133	8
	400	413	103	6
	2000	1836	92	10
pond water for irrigation (with C_{18} SPE)	0	ND		3
	16	15	94	5
	80	76	95	6
	160	147	92	12
	400	426	107	9
ground water (with C_{18} SPE)	0	ND		2
	16	24	150	2
	80	100	125	4
	160	243	152	11
	400	532	133	9

[a] ND, not detected.

curve, the IC_{50} value by the homologous assay was 619 ng/mL, whereas in the heterologous assay it was 40 ng/mL, which is some 15 times more sensitive than in the homologous system.

On the basis of these results, the optimal conditions for the imidacloprid ELISA are summarized in Table 7.

Recoveries of Imidacloprid from the Spiked Samples. As seen in Table 8, the recoveries of imidacloprid from apple samples by the above ELISA were in the range of 92–213%. However, because it has been reported that methanol tends to solubilize protein and lipid coextractives, which could interfere with imidacloprid determination (*33*), the recoveries could be increased by removing the coextractives. Meanwhile, the recoveries from water samples that were spiked with low concentrations of imidacloprid showed unstable results.

Considering the fact that the water samples were cleaned up using C_{18} SPE (Supelclean LC-18, 3 mL tubes, Supelco, Bellefonte, PA), some constituents therein would affect the reaction of the antibody. Nevertheless, all of the spiked samples exhibited positive reactions indicating the presence of imidacloprid, as compared with the control without imidacloprid. As shown in Figure 10, HPLC was run for comparison to validate the ELISA with fortified water samples, the correlation being 0.997–0.998 ($n = 15$) with a slope of 1.10–1.38. Therefore, this ELISA could be used as an analytical method for monitoring imidacloprid residues in agricultural and environmental samples. Almost at the same time as our research, Omoda et al. (*9*) reported on an ELISA using monoclonal antibodies raised against a hapten synthesized by adding a linker to the 6-position of the pyridine ring by replacing the chlorine atom. They reported that the competitive direct ELISA showed the determining range of 0.5–10 ng/mL. Our assays are complementary because they will have vastly different specificities. Most immunoassays show the greatest recognition for the parts of the hapten farthest from the point of conjugation. Thus, the Omoda assay and this one could be used to confirm each other or in an array format. Meanwhile, Li and Li (*10*) reported an IC_{50} of 35 ng/mL with a set of antibodies obtained with a linker introduced to the pyridine ring of imidacloprid. Because the hapten design in the development of an ELISA is very important, further modifications of the haptens used as coating and/or immunizing antigens may enhance the sensitivity and specificity of this and the two previously published ELISAs (*9*, *10*).

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