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Development of Fluorescence Polarization Immunoassay for the Rapid Detection of 6-Chloronicotinic Acid: Main Metabolite of Neonicotinoid Insecticides

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A fluorescence polarization immunoassay (FPIA) for the quantitative determination of 6-chloronicotinic acid (6-CNA) using polyclonal antibody was developed. The 6-CNA−protein (bovine serum albumin and soybean trypsin inhibitor) conjugates and fluorescein-labeled 6-CNA derivative (tracer) were prepared and used as the immunogens and tracer, respectively. The synthesized tracer was purified by thin layer chromatography (TLC) and showed a good binding to antiserum (73/5) which was obtained from the immunized rabbit (No. 73) with 6-CNA−BSA conjugate. The detection limit (10% inhibition) of FPIA was 4 μg/mL, and IC_{50} value was 32 μg/mL. The FPIA showed a cross-reaction for 5-amino-2-chloropyridine (60%), but no cross-reaction for other pesticides was observed. Recoveries for spiked apple, urine, soil, and water samples (5, 50, and 500 ppm) averaging between 78.6 ± 8.8 and 114 ± 18% were reasonable and in good agreement with the amounts spiked. Although the developed FPIA possesses low sensitivity, this assay is more simple and quick than other analytical methods, such as high performance liquid chromatography and gas chromatography. Thus, the developed FPIA method could be a useful tool for express screening 6-CNA in agricultural, environmental, and biological samples.

KEYWORDS: 6-Chloronicotinic acid; neonicotinoid insecticides; fluorescence polarization immunoassay; polyclonal antibodies

INTRODUCTION

In recent years, modern agriculture has benefited from the use of agrochemicals including insecticides, herbicides and fungicides (*1*). However, the presence of these agrochemicals, parent compounds and their major metabolites in an agricultural product and the environment has become a serious problem (*2*). These chemicals could be worldwide found in soil, surface and groundwater. Consequently, they can contaminate drinking water and agricultural products presenting an important risk to human health (*3*).

Imidacloprid [1-6(chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine] is one of the neonicotinoid insecticides, which operates as a competitor to postsynaptic nicotinic receptors in a central nervous system of the insect (*4*). Because of its insecticidal effectiveness at low application rates and safety for humans and the environment, imidacloprid has been extensively used in agricultural areas of many countries (*5*). The metabolite of imidacloprid, 6-chloronicotinic acid (6-CNA), was produced by exposure of imidacloprid to mammals. Recently, this compound has been artificially produced and widely used as an intermediate for agrochemicals, feed additives, pharmaceuticals and animal food enrichments (*6*). Thus, there is a need for monitoring residue levels, and legal limits for imidacloprid have been set by U.S. EPA for crops (0.05−48 mg/kg) (*7*). However, no international legal limit has been established for 6-CNA until now.

Many analytical methods, such as high-performance liquid chromatography (HPLC) (*8*−*10*), liquid chromatography−mass spectrometry (LC−MS) (*11*−*13*), LC−MS/MS (*14*), and gas

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chromatography−mass spectrometry (GC−MS) (*10*, *15*) have been reported for the determination of imidacloprid and 6-CNA. Recently, an enzyme-linked immunosorbent assay (ELISA) (*16*−*18*) has been developed for the detection of imidacloprid, but no immunoassay for 6-CNA has been published.

The instrumental methods are accurate and reliable but expensive, laborious, and time-consuming, and often require complicated cleanup. For these reasons, more simple, rapid, and easy-to-use detection methods needed for screening a large number of samples among the scientists. Immunochemical methods, especially enzyme-linked immunosorbent assay (ELISA), are suitable tools for their requirement and very useful as on-site screening analytical methods for pesticide residues (*19*) because the methods allow rapid and sensitive analysis with high sample throughput (*20*). Besides, immunoassay methods are cost-effective and easy to perform and require a small sample volume (*21*). However, ELISA is a heterogeneous method and separation of free and antibody-bound analyte, as well as long reaction time (1−2 h), is needed; in addition, this method involves multiple washing steps. A very promising way for the simplification of immunoassays for routine applications is a shift from heterogeneous methods (with separation) to homogeneous assays (without separation) (*22*). Fluorescence polarization immunoassay (FPIA) is the most extensively used homogeneous technique, which meets the requirements of a simple, reliable, fast and cost-effective analysis. FPIA is a competitive immunoassay method based on increase in the fluorescence polarization of a small fluorescent-labeled hapten (tracer) when bound by a specific antibody. If the sample contains free (unlabeled) analyte, its molecules will compete with the tracer for antibody-binding sites and the polarization signal will decrease. Recently, the use of FPIA for the determination of pesticides (*23*, *24*), antibiotics (*25*−*27*), and mycotoxins (*22*, *28*) in agricultural products and environmental samples has been reported. However, as we described above, any immunoassays for 6-CNA detection have not been reported until nowadays.

In this paper, we first describe the development of a FPIA for 6-CNA based on a new rabbit polyclonal antibody and its application for detection of 6-CNA in the artificially spiked apple, urine, water, and soil samples.

MATERIALS AND METHODS

Materials and Apparatus. Analytical grade 6-chloronicotinic acid (6-CNA) and other related pesticides, bovine serum albumin (BSA), soybean trypsin inhibitor (STI), 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), *N*,*N*-dicyclohexylcarbodiimide (DCC), dimethyl formaldehyde (DMF), fluorescein isothiocyanate (FITC, Isomer I), complete and incomplete Freund's adjuvant, and salts were purchased from Sigma Chemical Co. (St. Louis, MO). Thin layer chromatography (TLC) plates (silica gel 60, fluorescent, 1 mm, 20 × 20 cm) were obtained from Merck Co. (Darmstadt, Germany). All chemicals and organic solvents were of reagent grade or higher. Water used in all experiments was purified with a NANO pure system (Barnstead International, Dubuque, IA). Sodium borate buffer (BB, 0.05 M, pH 9.0) was used for all FPIA experiments. Standard solutions of 6-CNA and cross-reactants were prepared by dilution of stock solutions of these compounds (10 mg/mL in methanol). Fluoresceinthiocarbamyl ethylenediamine (EDF) was synthesized from FITC and ethylenediamine as described previously with modifications (*29*).

Fluorescence polarization and intensity were measured by TDx/FLx analyzer (Abbott Laboratories, North Chicago, IL) in semiautomatic PhotoCheck mode. Glass cuvettes (up to 10 in one run) were loaded into the special carousel followed the measurement of polarization (mP value). The total assay time for 10 samples was ∼7 min.

Preparation of Immunogens. 6-CNA was covalently bound to carrier proteins (BSA and STI) according to the modified method described previously (*30*), and these conjugates were used as the immunogens. Briefly, 8 mg (50 μmol) of 6-CNA was dissolved in 1 mL of DMF. Then 7 mg (about 60 μmol) of NHS and 16 mg (about 80 μmol) of DCC was added to a DMF solution containing 6-CNA, mixed and kept at room temperature (RT) overnight in the dark. The next day, 30 mg of BSA (0.5 μmol) or 24 mg of STI (0.5 μmol) was dissolved in 2 mL of 50 mM carbonate buffer (pH 9.6) in five-milliliter glass vials, and then 100 μL of DMF was added. The solution was cooled in a refrigerator for 2 h. The activated ester was added to protein solution in the ratio 1:30. The mixture was shaken for 2 h at RT and left overnight in a refrigerator. The conjugates were purified by dialysis in PBS (pH 7.4) with 3 changes per day during 3 days. The protein concentrations were measured using protein assay kit (Bio-Rad Laboratories, Richmond, CA).

Synthesis of Fluorescein-Labeled Tracer. To synthesize a fluorescein-labeled 6-CNA (6-CNA−EDF), 23 mg (200 μmol) of NHS and 41 mg (200 μmol) of DCC were dissolved in 5 mL of DMF and mixed for 30 min. Five hundred microliters of this solution (containing 20 μmol of NHS and 20 μmol of DCC) was withdrawn and added to 3 mg (∼10 μmol) of 6-CNA. The reaction mixture was stirred overnight at RT in the dark. Then, 2.5 mg (5 μmol) of EDF was added to the mixture and mixed well, followed by incubation for 1 h at RT. Red EDF was dissolved in 5 min, and the solution color changed to yellow. Small portions of the reaction mixture were purified by TLC using chloroform/methanol (4:1, v/v) as eluent. The major yellow band was collected and eluted with methanol. An aliquot (100 μL) of the tracer solution was purified again on the new TLC plate using the same eluent. The main band at $R_f = 0.9$ was collected, eluted with 0.5 mL of methanol, and twice purified tracer was stored at 20 °C in the dark.

Immunization and Titration of Antiserum. To raise antibodies, four 30-week-old female New Zealand white rabbits (experimental rabbits: No. 76, 75, 74, and 73) were separated into 2 groups, where one group (No. 76 and 75 rabbits) was immunized with 6-CNA−STI conjugate and the other (No. 74 and 73 rabbits) with 6-CNA−BSA conjugate. For immunization, 100 μg of immunogen was dissolved in 0.1 mL of sterilized phosphate buffer saline (PBS, pH 7.4), emulsified with Freund's complete adjuvant (1:2, v/v) and injected hypodermically at multiple sites on the back of rabbits. After 1 month, the rabbits were boosted with an additional 100 μg of immunogen that emulsified with Freund's incomplete adjuvant (1:2, v/v) and bled 7 days later. The second to fifth boost injections and blood-gathering were performed every 1 month by the same way as the described above. Antisera were directly lyophilized and stored at −20 °C in the dark before use. Titration of antisera was determined by FPIA. Briefly, the antisera were diluted 100-fold with BB and an appropriate tracer was adjusted to a concentration of ∼20 μM in the final reaction (corresponding to ∼2000 intensity units). To 0.5 mL of tracer solution in the glass cuvettes was added 0.5 mL of antisera, mixed, and analyzed using fluorescence polarization of TDx/FLx analyzer.

FPIA. Fifty microliters of 6-CNA standard solution (or sample), 0.5 mL of tracer solution, and 0.5 mL of antiserum solution in optimal dilution were added consecutively to glass cuvette and mixed followed by measurement. Standard curves were plotted as mP versus logarithm of analyte concentration. To optimize the FPIA, two experimental parameters were investigated. First, the effects of various concentrations (10, 15, and 20 μM) of tracer on signal response were investigated. Second, the tolerance of FPIA to various water-miscible organic solvents used to dissolve pesticides was tested for assay optimization. For this experiment, three absolute solvents (acetone, acetonitrile, and methanol), BB, and water (deionized) were investigated for their effects on Ab−Ag interactions. Additionally, cross-reactivity (CR) for different compounds structurally related to 6-CNA was determined by performing competitive assay and comparing the analyte concentration giving half-maximal inhibition (IC_{50} value), and calculated as % CR = (IC_{50} of 6-CNA/IC_{50} of analyte) × 100.

Analysis of Artificially Spiked Samples. For recovery studies 6-CNA-free samples (apple, human urine, deionized water, and soil samples) were spiked with 6-CNA at levels of 5, 50, and 500 μg/mL (or μg/g) and recoveries were determined by FPIA. The sample

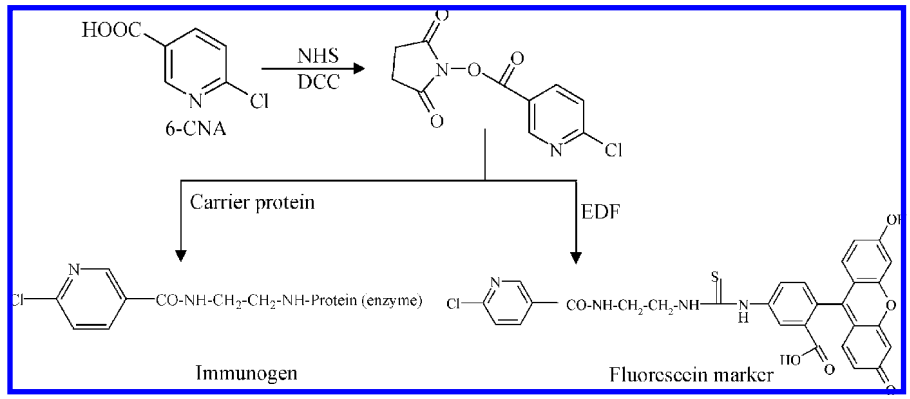


Figure 1. Schematic representation of the synthesis of 6-CNA−protein conjugate as immunogens and 6-CNA−EDF as a fluorescein tracer.

preparations of apple and soil for FPIA were performed according to the modified method described previously (*16*). Briefly, 5 g of soil and apple samples was transferred into a 50 mL conical tube and spiked with 6-CNA (5, 50, and 500 μg/g), and then had been kept in the dark at RT for 1 h. To 5 g of each spiked sample was added 25 mL of water, and then the mixture was vigorously shaken by hand for 5 min. After centrifugation at 5000 rpm for 5 min and filtration through filter paper (Whatman No. 1), the extracts were diluted 10-fold with water and applied to FPIA. Meanwhile, water samples spiked with 6-CNA (5, 50, and 500 μg/mL) were directly applied to FPIA after filtration and urine samples spiked with 6-CNA were filtered, diluted 10-fold with water and applied to FPIA. Blank samples were prepared as described above but not spiked with 6-CNA. 6-CNA concentration in spiked samples was calculated after fitting of the standard curve using the four-parameter logistic model.

RESULTS AND DISCUSSION

Production of Polyclonal Antibody. In common with the other immunochemical techniques FPIA requires the production and characterization of immunoreagents as well as the optimization and validation of an analytical system. The antibody and fluorescent-labeled hapten (tracer) are key components in the development of FPIA. 6-CNA, like most of the pesticides, is not immunogenic and should be therefore conjugated to a carrier protein to elicit the immune response. Thus, in our work, the 6-CNA molecule was conjugated via the carboxyl group to the carrier protein (BSA and STI) and also to the amino derivative of fluorescein (EDF) to be used as the immunogen and competitor (fluorescent tracer), respectively (**Figure 1**). To produce the antibodies, 6-CNA−BSA and 6-CNA−STI conjugate were injected into each rabbit as immunogens. We collected antiserum from four rabbits after each boosting, and these were subjected to titration by the FPIA. In total, 24 kinds of antisera were collected from 6 times bleeding and applied to FPIA. The antiserum from rabbits No. 75 and 73 showed a higher titration than the antiserum from rabbits No. 76 and 74 by FPIA. We selected four antisera, 75/5, 75/6, 73/5, and 73/6, which were collected from fifth and sixth bleeding, because these antisera showed a high titer and good sensitivity in FPIA performed with the fluorescence tracer. Although antiserum from No. 76 and 74 rabbits exhibited a good titer, these antisera showed low sensitivity in FPIA (data was not shown). We think the mice immunized with 6-CNA−protein could probably induce many kinds of antibodies against 6-CAN and protein. We thought that No. 76 and 74 rabbits induced antibodies to BSA and STI protein.

Selection of Tracer and Antiserum. The FPIA is a homogeneous assay technique based on differences in polarization of the fluorescence-labeled species of free and bound fractions; it involves the competition between free analyte and tracer for binding to a specific antibody. Three main yellow bands at R_f = 0.5, 0.8, and 0.9 on the TLC plate after tracer purification were extracted with methanol (0.5 mL) and checked for binding to antisera. The extract obtained from R_f = 0.9 showed an affinity to each antiserum, and we selected and used the extract as a tracer for the optimization of FPIA. To select an antiserum suitable for the FPIA, the four antisera (75/5, 75/6, 73/5, and 73/6) were tested for inhibition by several concentrations of 6-CNA dissolved in absolute methanol. The inhibitions by the presence of 6-CNA are shown in **Figure 2**. All antisera showed good sensitivity by FPIA using 6-CNA−EDF tracer (R_f = 0.9), and 73/5 antiserum obtained from the immunized rabbit with 6-CNA−BSA showed the best sensitivity (IC_{50} = 46 μg/mL) by FPIA. Therefore, 73/5 antiserum was selected and used for the optimization of FPIA.

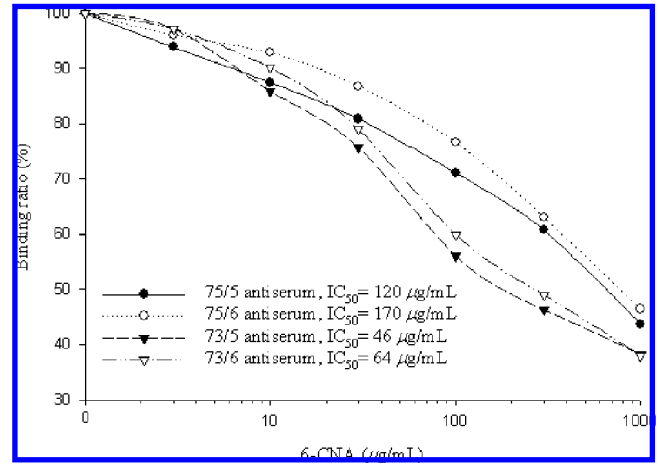


Figure 2. Comparison of competitive standard curves using different antisera.

Optimization of the FPIA. The main objective of the FPIA was the qualitative and quantitative detection of 6-CNA contamination in a short time. The appropriate antiserum and tracer for FPIA were investigated and selected in preliminary assessments. The tracer concentration is one of the key parameters for the development and optimization of the FPIA procedure, which have a high influence on assay sensitivity. The tracer determines the intensity of emitted polarized light and also contributes to the competition for antibody binding. Thus, the lowest possible tracer concentration, which permits the reliable detection of a label and produces the minimum effect on the competition, should be used to develop a sensitive assay (*22*). To optimize the FPIA, assay conditions were determined with various concentrations of tracer and working solutions. The standard curves of FPIA using three tracer concentrations (10, 15, and 20 μM) were shown in **Figure 3**. No significant

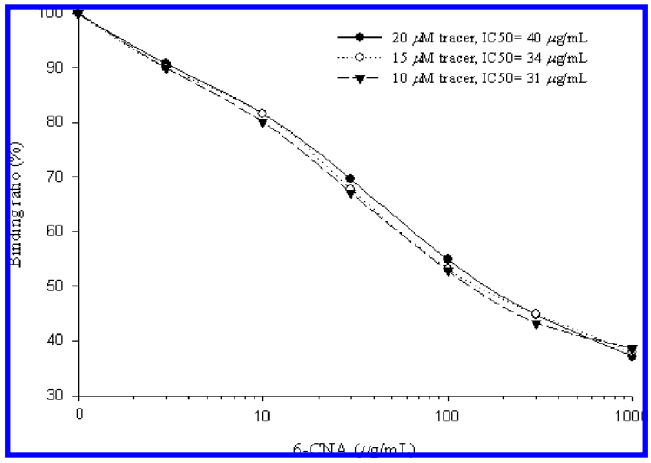


Figure 3. Influence of tracer concentrations on the FPIA standard curves for 6-CNA using the 73/5 antiserum.

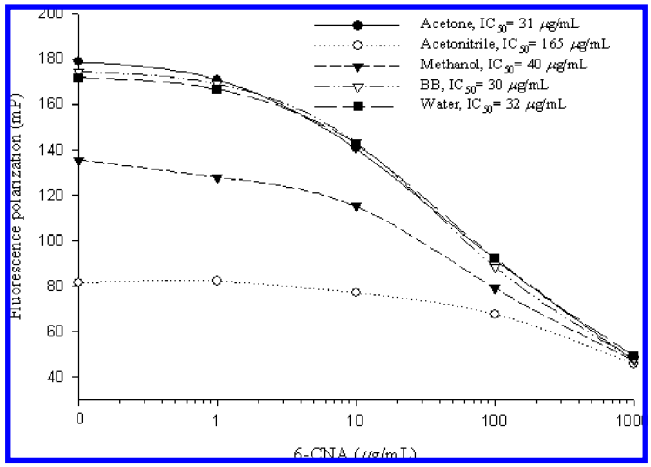


Figure 4. Effect of organic solvents on the FPIA of 6-CNA using the 73/5 antiserum.

differences were observed, and IC_{50} values and LOD of these curves were almost similar. However, in this result, 10 μM of tracer (IC_{50} = 31 μg/mL) showed higher sensitivity than the others (IC_{50} for 15 and 20 μM of tracer were 34 and 40 μg/mL, respectively). Thus, we selected 10 μM as the tracer concentration for the optimization of FPIA.

Organic solvent, methanol, is commonly used to extract the pesticide residues from food and environmental samples, so it is desirable to assess the effect of organic solvents on immunoassay performance (*31*). We also evaluated the effects of the organic solvents (methanol, acetone, and acetonitrile) which are commonly used to extract pesticide from samples and aqueous solution (water and BB). The results are presented in **Figure 4**. The mP values of acetone, water, and BB increased compared with the control as the content of methanol, probably by affecting the affinity between the coating antigen and the antibody. In contrast, the addition of acetonitrile decreased the mP values and showed low sensitivity (IC_{50} = 165 μg/mL). According to this result, acetone (IC_{50} = 31 μg/mL), water (IC_{50} = 32 μg/mL), and BB (IC_{50} = 30 μg/mL) showed an increase of sensitivity, compared with 40 μg/mL in control (methanol). Therefore, we selected water as an assay buffer because 6-CNA possesses good solubility in aqueous solution so that it will not be necessary to use any organic solvents and the sensitivity of standard curves using three solutions is similar. In summary, the optimized FPIA for 6-CNA was obtained using the various standard solutions in water, 10 μM of tracer concentration (6-CNA−EDF, R_f = 0.9) and 73/5 antiserum (1500-fold diluted

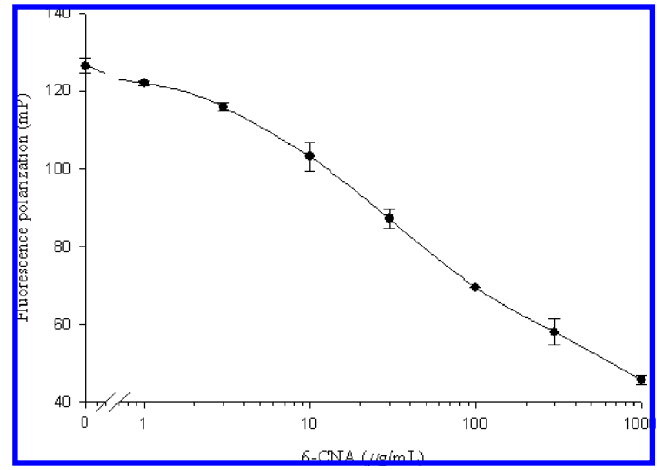
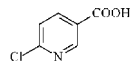
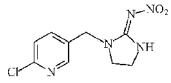
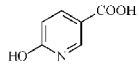
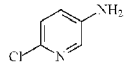
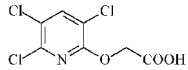
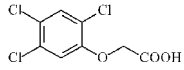
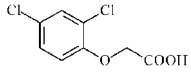
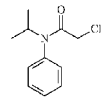
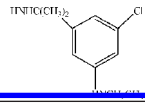


Figure 5. Optimized FPIA standard curve for 6-CNA detection using the 73/5 antiserum. The procedures are described in the text. Each point of the curve represents the mean ± SD (standard deviation) of n = 3 assays.

with BB). **Figure 5** shows a typical FPIA standard curve. The detection limit (10% inhibition) for 6-CNA was 4 μg/mL, and the IC_{50} value was 32 μg/mL. The cross-reactivity for several compounds structurally related to 6-CNA was tested and calculated as the ratio of the IC_{50} value of the 6-CNA standard to the IC_{50} of the tested compounds. Imidacloprid, 6-hydroxynicotinic acid, and 5-amino-2-chloropyridine (5A2Cpy) containing the same part (6-chloro) in their chemical structure as 6-CNA, and propachlor, triclopyr, atrazine, 2.4.5-T, and 2.4-D which are commonly used in agriculture, were tested and compared with 6-CNA. As shown in **Table 1**, cross-reactivity for 5A2Cpy (60%) was observed by FPIA, whereas no cross-reaction was observed for other pesticides. Monoclonal antibody (*16*, *17*) and polyclonal antibody (*5*) against imidacloprid have been reported, and these showed no cross-reaction to 6-CNA. This means that imidacloprid and 6-CNA possess a different epitope in their chemical structures. The cross-reactivity observed with 5A2Cpy is understandable, because of its similar structure to 6-CNA. Although the structure of 6-hydroxynicotinic acid is also similar to that of 6-CNA, no cross-reactivity to 6-hydroxynicotinic acid was observed.

Recovery Studies. Apple, urine, soils, and water samples spiked with 6-CNA (5, 50, and 500 ppm) were determined in triplicate by FPIA. Although it is generally known that immunoassays are used to simply and rapidly analyze pesticides, the antigen−antibody interaction can be affected by a variety of compounds (*32*). FPIA is susceptible to interference with different components existing in some matrices such as plant extracts and serum (*1*). To reduce matrix interferences, two common approaches could be used. The first approach is sample cleanup, which is laborious and time-consuming and may affect assay reproducibility and recovery. The second approach is dilution of the extract. One of the major advantages of immunoassay techniques is their simplicity, so the second approach was used in this study. In our study, we used water for the extraction of 6-CNA from spiked apple and soil samples because extraction by organic solvents will often elute protein and other components which interrupt the interaction between the antibody and free analyte (*6*). After extraction, the optimum dilution factor of the extracts of apple and soil samples using water was determined. After 10-fold dilution with water, the diluted extracts of apple and soil samples showed a similar mP value with it of water without 6-CNA (data was not shown). Meanwhile, the water and urine samples without extraction step

Table 1. Cross-Reactivity of 73/5 Antiserum to 6-CNA and Related Compounds by FPIA[a]

compound	structure	IC50 value (μg/mL)	cross-reactivity (%)
6-chloronicotinic acid		31	100
Imidacloprid		ni[b]	0
6-hydroxynicotinic acid		ni	0
5A2cpy		52	60
Triclopyr		ni	0
2,4,5-T		ni	0
2,4-D		ni	0
Propachlor		ni	0
Atrazine		ni	0

[a] Assay conditions were the same as those described in **Figure 5**. [b] No inhibition.

Table 2. Recovery of 6-CNA from Spiked Sample by FPIA

sample	6-CAN concn (ppm) spiked	detected	recovery ± SD[a] (%)
apple	5	4.2 ± 0.2	84 ± 4
	50	45.2 ± 3.6	90.4 ± 7.2
	500	505.2 ± 55.3	101 ± 11.1
urine	5	5.4 ± 0.7	108 ± 14
	50	56.2 ± 4.8	112.4 ± 9.6
	500	501.9 ± 95.8	100.4 ± 19.2
soil	5	5.7 ± 0.9	114 ± 18
	50	47.6 ± 2.6	95.2 ± 5.2
	500	459.5 ± 119.9	91.9 ± 23.9
water	5	4 ± 0.6	80 ± 12
	50	39.3 ± 4.4	78.6 ± 8.8
	500	561.5 ± 30.6	112.3 ± 6.1

[a] SD = standard deviation, $n = 3$.

were directly applied to FPIA after filtration and dilution, respectively. Spike recoveries averaged between 78.6 and 112.4%, as shown in **Table 2**. No false-positive results for blank (unspiked) samples were observed. The results were in good agreement with the amounts spiked, demonstrating the applicability of the developed FPIA to agricultural, environmental, and biological sample analysis.

In conclusion, the FPIA based on a polyclonal antibody for the detection of 6-CNA in spiked samples was developed and optimized. To produce antibodies, 6-CNA−BSA and 6-CNA−STI conjugates were used as immunogens, and 73/5 antiserum which showed a good titer and sensitivity was selected. After the synthesis and purification of tracer, three kinds of tracers were obtained and one (6-CNA−EDF tracer, $R_f = 0.9$) of them exhibited a good affinity to 73/5 antiserum and sensitivity in FPIA. The IC_{50} value of the optimized FPIA with water was 32 μg/mL, and limits of detection (LODs) of the assay were 4 μg of 6-CNA per 1 mL of water or 1 g of apple and 4.6 μg of 6-CNA per 1 mL of urine or 1 g of soil. The assay showed no cross-reactivity with other pesticides tested in this study except 5A2Cpy (60%). Compared to the previously developed HPLC (LOD: 80 ng/mL) and GC−MS (LOD: 16 pg/mL) for 6-CNA (*8*, *33*), their sensitivity was higher than that of FPIA. However, the FPIA appears to meet the performance criteria for 6-CNA residue testing of agricultural and environmental samples without complicated cleanup. Without the complicated sample cleanup, the extracts of apple and soils were diluted in BB and subsequently applied to FPIA. Recoveries (ranging from 78.6 to 112.4%) for spiked apple, urine, soil, and water samples were acceptable, and the method was characterized by good reproducibility; coefficients of variance (CVs) ranged from 0.03 to 5.8%. Therefore, this assay would be very useful for the rapid screening of 6-CNA in agricultural, environmental, and biological samples.

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