Development of an Enzyme-Linked Immunosorbent Assay for the Insecticide Imidacloprid

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Enzyme-linked immunosorbent assays (ELISAs) were developed for imidacloprid, a neonicotinoid insecticide. Haptens were designed in such ways that spacer arms were introduced on either the pyridinyl or the imidazolidinyl ring of imidacloprid. Two sets of polyclonal antibodies were raised from rabbits immunized with two different immunogens and were characterized with an indirect ELISA format. Cross-reactivities and effects of organic solvents on the assays were evaluated. One set of antibodies shows approximately equal cross-reactivities to imidacloprid and its major metabolites with half-maximum inhibition concentrations (I_{50}) of 73−88 ppb. Another is specific to imidacloprid with an I_{50} of 35 ppb. The assay was initially applied to the analysis of imidacloprid in fortified water, coffee cherry, and bean extracts.

Keywords: *ELISA; immunoassay; imidacloprid; metabolites*

INTRODUCTION

Imidacloprid, 1-[(6-chloro-3-pyridinyl)methyl]-*N*-nitro-2-imidazolidinimine, is a systemic insecticide for the control of sucking insects. It was first introduced to the United States in 1994 and is widely used on various crops with soil, seed, and foliar applications (U.S. EPA, 1994). It is especially systemic when used as a seed or soil treatment. Imidacloprid can block the nicotinergic receptor and result in insect paralysis and eventual death (Bai et al., 1991; Buckingham et al., 1997; Liu and Casida, 1993). Imidacloprid has relatively low mammalian toxicity (Mullins, 1993; Zwart et al., 1994) because it has a higher binding affinity to insect nerve receptors than to mammalian receptors.

Several methods have been developed for the analysis of imidacloprid. High-performance liquid chromatography (HPLC) is the method of choice since direct gas chromatographic (GC) analysis is not suitable because of the thermolability and polarity of the compound (Baskaran et al., 1997; Fernandez-Alba et al., 1996; Ishii et al., 1994; Macke, 1998; Placke and Weber, 1993). Imidacloprid can be analyzed by GC or GC/MS after alkaline hydrolysis of the parent compound to the imidazolidin-2-one (Vilchez et al., 1996). Derivatization to trifluoroacetyl or heptafluorobutyryl derivatives can be used prior to GC/MS analysis (MacDonald and Meyer, 1998; Rouchaud et al., 1994). Oxidation of imidacloprid and their metabolites to 6-chloronicotinic acid followed by GC/MS analysis is also a feasible method (Vilchez et al., 1996).

While GC, HPLC, and GC/MS methods are well-established for pesticide analyses, immunoassays have proven, as an alternative method, to provide advantages of being rapid, sensitive, and cost-effective. To date, many immunoassays have been developed for environmental monitoring of pesticides (U.S. EPA, 1996; Van Emon and Gerlach, 1995). Here, we report the development of an enzyme-linked immunosorbent assay (ELISA) for the insecticide imidacloprid using polyclonal antibodies.

MATERIALS AND METHODS

Reagents. All reagents were of analytical grade unless specified otherwise. Imidacloprid (96.9% purity) and the metabolite standards were obtained from Bayer Corp, Stillwell, KS. Ethyl 6-bromohexanoate and 3-mercaptopropionic acid were obtained from Lancaster Synthesis Inc. (Windham, NH). Chemicals purchased from Sigma (St. Louis, MO) were biotinylated goat anti-rabbit IgG (B-7389), goat anti-rabbit IgG-horseradishperoxidase (IgG-HRP) (A-6154), avidin-HRP (A-3151), bovine serum albumin (BSA) (A-7030), keyhole limpet hemocyanin (KLH) (H-2133), phosphate-citrate buffer capsule with sodium perborate (P-4922) and carbonate-bicarbonate buffer capsules (C-3401), *o*-phenylenediamine (OPD) (P-9029), Freund's complete and incomplete adjuvants. TiterMax Gold was obtained from CytRx Corp. (Norcross, GA). The ELISA was carried out in 96-well polystyrene microplates (MaxiSorp F96, Nalge Nunc International, Denmark).

Chemical Characterization. ^{1}H NMR spectra were obtained on a Nicolet NT-300 MHz instrument at 300 MHz for solutions in $CDCl_3$ and are described as multiplicity, coupling constant (J) in hertz (Hz), number of protons, and assignment. Chemical shifts (δ, ppm) are relative to internal tetramethylsilane (TMS). Both low-resolution and high-resolution mass spectra were obtained by University of California at Berkeley MS Center with a fast atom bombardment (FAB) ionization method.

Synthesis of 1-[(6-Chloro-3-pyridinyl)methyl]-3-carboxylpentyl-*N*-nitro-2-imidazolidinimine (Hapten I). To a stirred solution of imidacloprid (1.03 g, 4 mmol) in dimethyl formamide (DMF, 15 mL) was added sodium hydride (0.21 g of 50% oil dispersion, 4.3 mmol) at 0 °C. After 1 h, ethyl-6-bromohexanoate (2.23 g, 10 mmol) was added dropwise, and stirring was continued for 12 h at ambient temperature. The reaction mixture was poured into 80 mL of water, and the pH of the solution was adjusted to 6−7. The mixture was extracted with chloroform (2 × 80 mL), and the organic layer was washed with water and dried over anhydrous sodium sulfate. After

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the solvent was removed by a rotary evaporator under reduced pressure, the crude product was hydrolyzed in acidic aqueous solution (2 N HCl, 60−70 °C, 4 h). After the reaction mixture was adjusted to pH 6 using 1 N NaOH solution, it was extracted with ethyl acetate (2 × 50 mL). The solvent was removed with a rotary evaporator, and the residue was purified with silica gel chromatography (ethyl acetate:methanol, 9:1). Hapten I was obtained as a white solid (yield 35%). ^{1}H NMR ($CDCl_3$): δ 2.34 (t, J = 7.6, 2H), 3.30 (t, J = 7.3, 2H), 3.52 (t, J = 8.8, 2H), 3.71 (t, J = 7.3, 2H), 4.47 (s, 2H), 7.36 (d, J = 8.0, 1H), 7.71 (dd, J = 2.4, 8.3, 1H), 8.31 (d, J = 2.2, 1H). LRMS (FAB) m/z: 370 (MH^+, 100). HRMS (FAB) calcd $C_{15}H_{20}N_5O_4ClH^+$ 370.1282, found 370.1281.

Synthesis of 1-[(6-Carboxylethylthio-3-pyridinyl)methyl]-*N*-nitro-2-imidazolidinimine (Hapten II). To a solution of imidacloprid (1.03 g, 4 mmol) in dimethyl sulfoxide (DMSO, 15 mL) was added dropwise a DMSO solution (5 mL) containing 0.42 g of 3-mercaptopropionic acid (4 mmol) and 0.45 g of KOH (8 mmol). The reaction mixture was stirred while the temperature was gradually increased to 100 °C, and the temperature was maintained for 1 h. Water (50 mL) was added after the reaction mixture was cooled to room temperature. The solution was adjusted to pH 3 using 6 N HCl and extracted with ethyl acetate (100 mL). The organic extract was washed with water and dried over anhydrous sodium sulfate, and then ethyl acetate was evaporated with a rotary evaporator under reduced pressure. The residue was purified with silica gel chromatography (ethyl acetate:methanol, 9:1). Hapten II was obtained as white solid (yield 45%). ^{1}H NMR ($CDCl_3$): δ 2.86 (t, J = 7.1, 2H), 3.41 (t, J = 7.0, 2H), 3.52 (t, J = 8.9, 2H), 3.80 (t, J = 8.0, 2H), 4.50 (s, 2H), 7.20 (d, J = 8.8, 1H), 7.53 (dd, J = 2.2, 8.3, 1H), 8.34 (d, J = 2.2, 1H).

Immunogens. To 0.1 mmol of the hapten was added 1.0 mL of DMF solution containing 0.2 mmol of *N*-hydroxysuccinimide and 0.2 mmol of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (DEC). The mixture was stirred at room temperature for 3.5 h and then was centrifuged to remove precipitated urea. The resulting active ester (500 μL) was added slowly to BSA solution (150 mg BSA in 10 mL of 0.1 M borate buffer, pH 9.0) or KLH solution (30 mg KLH in 10 mL of 0.1 M borate buffer, pH 9.0). The reaction mixture was stirred at room temperature overnight. The content was dialyzed against distilled water (4 × 1.0 L) and followed by phosphate-buffered saline containing 0.05% Tween 20 (PBST) (1.0 L) at room temperature overnight.

Immunization. Three New Zealand white rabbits (2−4 kg) were immunized with I-KLH, and three others were immunized with II-KLH. Two of each group initially received 100 μg of immunogen emulsified in complete Freund's adjuvant, followed by two sequential booster injections of immunogen emulsified in incomplete Freund's adjuvant at 2−3-week intervals after the initial immunization. One of each group initially received 100 μg of immunogen emulsified in TiterMax Gold, followed by two sequential booster injections of immunogen emulsified in TiterMax Gold at 4-week intervals after the initial immunization. Injections were made intradermally and subcutaneously at multiple sites on back of the animals. The titers of the antisera were monitored using checkerboard titration. Seven days after the last injection, the rabbits were bled, and the antisera were collected and stored at −80 °C.

Indirect ELISA and Indirect Competitive ELISA. A microplate was coated with 0.25−2.0 ng of I-BSA or II-BSA (100 μL/well in 0.05 M carbonate−bicarbonate buffer, pH 9.6) by incubation at 37 °C overnight. The plate was washed 4 times with PBST and blocked with 0.3% BSA in PBST (PBST-BSA) (200 μL/well) by incubation at 37 °C for 1 h. The plate was washed and incubated with antiserum from a rabbit immunized with I-KLH or II-KLH (1:2000 to 1:60 000 in PBST-BSA) at 37 °C for 1 h. For this step of the competitive ELISA, the plate was incubated with a mixture of a constant concentration of antisera with various concentrations of analytes. The plate was washed and further incubated with goat anti-rabbit IgG-HRP (1:12000 in PBST-BSA, 100 μL/well) at 37 °C for 1 h. After another washing, 100 μL/well of OPD (1.0 mg/mL in 0.05 M citrate−phosphate with 0.03% sodium perborate, pH 5.0) was added. The reaction was stopped with sulfuric acid (2 M, 50 μL/well) after 15−30 min at room temperature. The absorbance at 490 nm was then read on a Vmax microplate reader, and the data were fitted using Softmax software (Molecular Devices, Sunnyvale, CA).

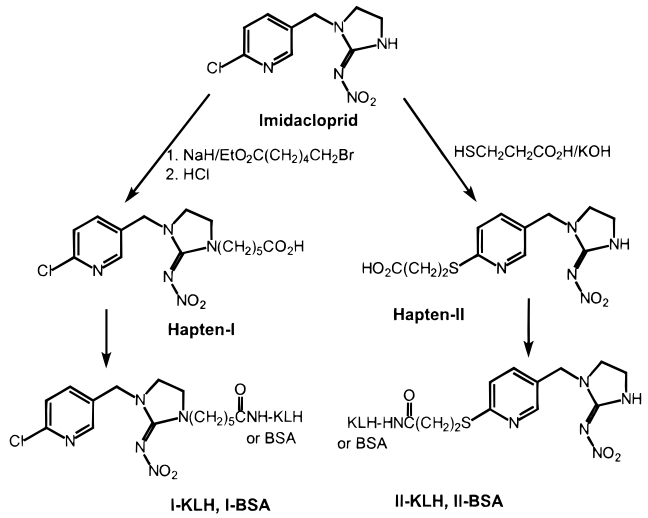

Figure 1. Synthetic scheme of imidacloprid haptens and hapten−protein conjugates.

Fortification of Imidacloprid in Water, Coffee Cherry, and Bean Extract Solution. Tap water was fortified with imidacloprid up to 3000 ppb and assayed directly by ELISA. Cherry and coffee bean crude extracts, prepared by homogenizing cherry and coffee bean (25 g) with 300 mL of methanol and 1% aqueous sulfuric acid (3:1, v:v) for 3 min, were fortified with imidacloprid up to 500 ppb. The fortified solutions (0.5 mL) were mixed with water (1 mL), and a gentle stream of nitrogen was used to evaporate methanol. The content was then extracted with ethyl acetate (1.0 mL). After removal of ethyl acetate under a gentle stream of nitrogen, the extracts were reconstituted in PBST (1.0 mL), and competitive ELISA was performed to quantify imidacloprid in the extracts.

RESULTS AND DISCUSSION

Synthesis of Haptens and Antigens. Imidacloprid haptens were synthesized by introducing a spacer on the imidazolidinyl ring and the pyridinyl ring of the parent compound (Figure 1). The two reactions were carried out in alkaline conditions. A problem encountered was the low stability of imidacloprid in alkaline conditions. Kagabu et al. (1998) synthesized various N-alkylated derivatives in the study of the solubility of imidacloprid analogues by reaction of imidacloprid with alkyl iodide using sodium hydride as a strong base. Therefore, ethyl 6-bromohexanoate was used to link with the imidazolidinyl ring. A slightly polar product was obtained after imidacloprid reacted with ethyl 6-bromohexanoate. Hydrolysis of the ethyl ester under acidic condition led to hapten I. An attempt to react imidacloprid with succinic anhydride under various conditions failed, although derivatization using trifluoroacetyl or heptafluorobutyryl anhydride was reported for GC analysis of imidacloprid (MacDonald and Meyer, 1998; Rouchaud et al., 1994).

Reaction of imidacloprid with 3-mercaptopropionic acid resulted in another hapten, II. The reaction condition is important, and use of excess alkali should be avoided because imidacloprid itself and the product can decompose under alkaline conditions. In an attempt, two decomposed products were isolated. However, none of them were expected products. The major one was a

Table 1. Summary of Titers of Antisera

coating antigen[b]	titer of antisera[a]					
	Ab-Ia[c]	Ab-Ib	Ab-Ic	Ab-IIa[c]	Ab-IIb	Ab-IIc
I-BSA	2 000	4 000	4 000	10 000	25 000	15 000
II-BSA	6 000	8 000	8 000	40 000	50 000	50 000

[a] The titer of antiserum is defined as the antiserum dilution that gave twice absorbance of the background. Ab-Ia, -Ib, and -Ic were from rabbits immunized with I-KLH. Ab-IIa, -IIb, and -IIc were from rabbits immunized with II-KLH. [b] The coating antigen was at 1.0 ng in 100 μL/well. [c] It was produced from a rabbit receiving the adjuvant TiterMax.

cyclic urea derived from imidacloprid due to loss of ═N—NO at alkaline conditions. The minor product was derived from the reaction of the cyclic urea with 3-mercaptopropionic acid and was not selected as a hapten candidate due to the different structural feature. Hapten II was formed under a carefully controlled reaction condition and was purified from the reaction mixture by silica gel chromatography. The haptens were conjugated to carrier proteins, BSA and KLH, via a carbodiimide method. The hapten densities in molar ratio were estimated by a UV method and were 2, 11, 233, and 397 for I-BSA, II-BSA, I-KLH, and II-KLH, respectively.

Immunization and Characterization of Antisera. I-KLH and II-KLH were used to immunize rabbits. A new adjuvant, TiterMax Gold, was used for comparison with the conventional Freund's adjuvant. TiterMax consists of a microparticulate stabilized water-in-oil emulsion of a metabolizable oil, squalene, with the adjuvant block copolymer CRL89-41. It is nontoxic and produces higher titers (Bennett et al., 1992). The rabbits receiving TiterMax produced remarkably higher titers only after primary immunization (1:30 000 for Ab-IIa). Final antisera were obtained after a total of three immunizations in a period of 3 months. The titers of antisera against immunogen II-KLH were much higher than those against immunogen I-KLH (Table 1). The final antisera from the three rabbits receiving the same immunogen were pooled together and stored at −80 °C for future use because their responses were similar (Table 1). However, the results reported here were from antiserum-Ia (Ab-Ia, against I-KLH) and antiserum-IIa (Ab-IIa, against II-KLH). They were used 1:6000 and 1:40 000 dilutions, respectively, in the subsequent works.

Inhibition. Competitive inhibition was performed to determine whether the polyclonal antibodies can recognize free imidacloprid. It was found that II-BSA is a better coating antigen than I-BSA. Figure 2 shows a typical standard curve of inhibition by imidacloprid using Ab-IIa and II-BSA as coating antigen in the indirect competitive ELISA. The half-maximum inhibition (I_{50}) was 35 and 73 ppb of imidacloprid for antisera IIa and Ia, respectively (Table 2) when II-BSA was used as coating antigen (0.5 ng/well) in an indirect competitive ELISA.

Cross-Reactivity. It is a common phenomenon that antibodies recognize compounds structurally similar to the immunizing haptens. Three major imidacloprid metabolites were used to examine the cross-reactivity of the antibodies (Figure 3). The antibodies from rabbits immunized with I-KLH and II-KLH showed different recognition patterns (Table 2). The antibodies against II-KLH are more specific to imidacloprid than those against I-KLH. This is probably due to the fact that the

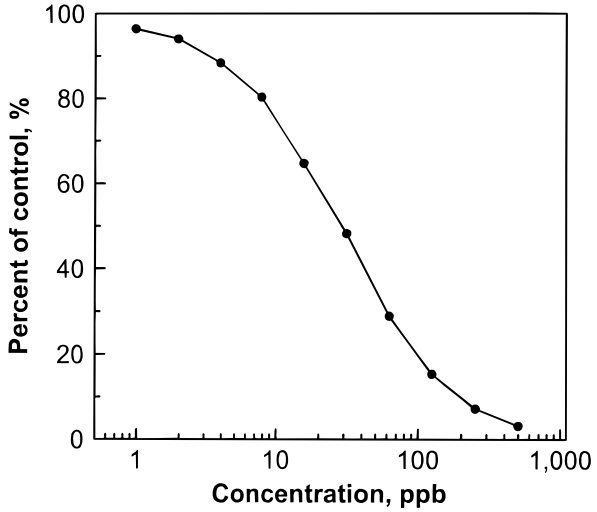

Figure 2. Standard curve of inhibition by imidacloprid using Ab-IIa and II-BSA. The plate was coated with 0.5 ng of II-BSA in 100 μL/well, and Ab-IIa was diluted 1:40000.

Table 2. Cross-Reactivity of Antibodies to Imidacloprid and Its Metabolites

inhibitor	antiserum-Ia[a]		antiserum-IIa[a]	
	I_{50}, ppb	cross-reactivity, %	I_{50}, ppb	cross-reactivity, %
imidacloprid	73	100	35	100
imidacloprid olefin	88	83	222	16
5-HO-imidacloprid	85	86	332	11
6-chloronictinic acid	>1000	<7	>1000	<4

[a] The plate was coated with 0.5 ng of II-BSA in 100 μL/well, and Ab-Ia and IIa were diluted 1:6000 and 1:40000, respectively.

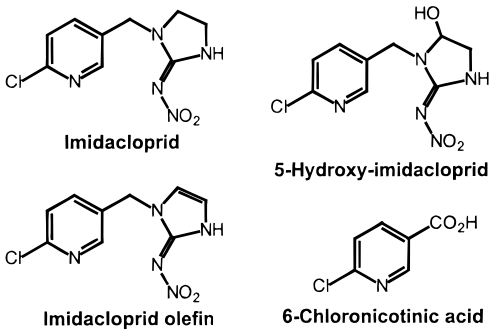

Figure 3. Chemical structures of imidacloprid and its major metabolites.

spacer arm in hapten II was attached to the pyridinyl ring. The antibodies thus produced (Ab-IIa, -IIb, and -IIc) mainly recognize the imidazolidinyl-associated moiety at the far end. The structures of the imidazolidinyl ring in 5-hydroxyimidacloprid and imidacloprid olefin are different from that in imidacloprid; therefore, the antibodies show low cross-reactivities (<20%) for these two metabolites. On the other hand, the spacer in I-KLH is attached to the N atom in the imidazolidinyl ring, and the antibodies produced mainly recognize the pyridinyl ring at the other end of the molecule. The antibodies (Ab-Ia, -Ib, and -Ic) showed approximately equal recognition with imidacloprid and the metabolites, 5-hydroxy imidacloprid and imidacloprid olefin. None of the antibodies significantly cross-reacted with 6-chloronictinic acid (Table 2).

Effect of Organic Solvents. Four common water-miscible organic solvents (DMSO, methanol, acetone, and acetonitrile) were used to evaluate their effects on

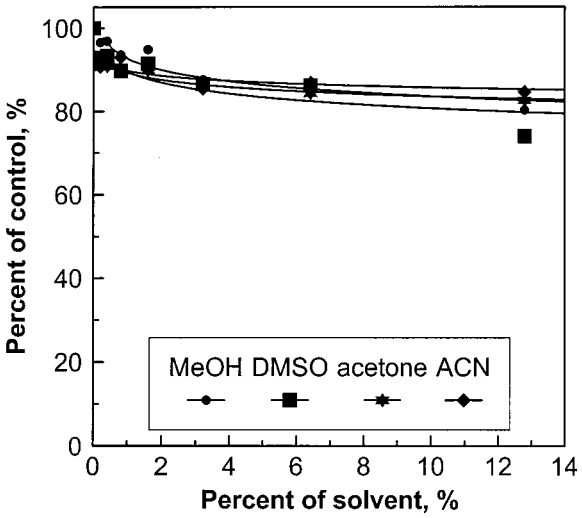

Figure 4. Effects of solvents on the assay performance (Ab-IIa and II-BSA). The plate was coated with 0.5 ng of II-BSA in 100 μL/well, and Ab-IIa was diluted 1:40000. The solvent percentage was a final concentration in the assay.

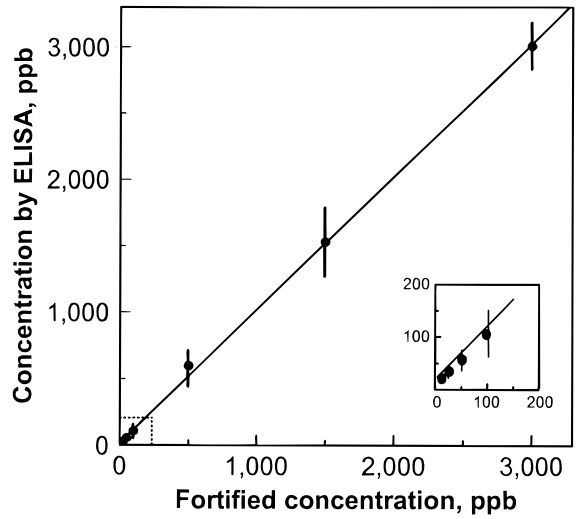

Figure 5. Correlation between imidacloprid concentrations determined by ELISA and those fortified in water. The plate was coated with 0.5 ng of II-BSA in 100 μL/well, and Ab-IIa was diluted 1:40000.

the assay performance. When these solvents were less than 2% (v/v) in the ELISA, their effects on the antibodies were negligible (Figure 4). The reason to choose these four organic solvents was that they are commonly used in ELISA procedures. Methanol and acetone are used to extract imidacloprid from coffee matrixes (unpublished). Acetonitrile is a common solvent for HPLC analysis of imidacloprid. DMSO is often used to prepare standard stock solution for ELISA runs. The results indicate that the presence of a small amount of solvents may not be a problem when the ELISA method is integrated with other procedures.

Imidacloprid-Fortified Water and Coffee Extracts. Tap water was fortified with imidacloprid at different levels up to 3000 ppb and assayed by the indirect ELISA. The concentrations of imidacloprid determined by ELISA correlated very well with the fortification values with a slope of 1.0 and a correlation coefficient of 1.00 (Figure 5). The results showed that ELISA can accurately measure the concentration of imidacloprid in water.

When methanol extracts of coffee cherries and beans were spiked with imidacloprid at a level of 500 ppb and the extracts were assayed by ELISA after methanol solvent was evaporated, extracted with ethyl acetate, and re-substituted in PBST buffer, the recoveries of imidacloprid were 108 ± 18 and 94 ± 13% for cherries and beans, respectively (data not shown). Ethyl acetate extraction was to minimize nonspecific interference in the crude methanol extracts. In real practices, the amount of coffee samples can be reduced.

CONCLUSION

Two ELISAs were developed for the analysis of the insecticide imidacloprid residues. Polyclonal antibodies were raised from rabbits immunized with two immunogens of which the haptens had a spacer at either the pyridinyl ring or the imidazolidinyl ring of imidacloprid. An assay was specific to imidacloprid with an I_{50} of 35 ppb and had minimal cross-reactivities with major imidacloprid metabolites. Another assay gave a broad cross-reactivity profile for imidacloprid and its major metabolites and thus is suitable for the quantification of total imidacloprid residues. The satisfactory recoveries and correlation between measured and fortified concentrations in water and coffee extracts suggest that the assays can be used for the determination of imidacloprid residues in the environment and biological matrixes.

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