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Development of an enzyme immunoassay for detection of fipronil in environmental samples

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Abstract

An indirect competitive enzyme-linked immunosorbent assay (ELISA) was developed for the quantitative detection of fipronil in some environmental samples. Polyclonal fipronil antibodies were produced from rabbits immunized by hapten (fipronil) protein conjugate. The conjugate was prepared by activating the carboxylic groups of proteins; keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) with 1-ethyl-3- (3-diaminopropyl) carbodiimide hydrochloride (EDC) followed by directly coupled to the amino group of fipronil. The influence of some factors such as concentrations of organic solvent, ionic strength and pH of buffer on the immunoassay was studied. Under optimized assay conditions, the ELISA having limit of detection (LOD) of 0.026 ng/ml and IC_{50} value of 0.325 µg/ml. Performance of the immunoassay was demonstrated by recovery study for spiked soil, tomato and potato samples giving recovery values in the range of 91-102 % for different concentrations in different samples. Good correlation was achieved between ELISA and conventional high pressure liquid chromatography (HPLC) analysis. The ELISA has the sensitivity to measure fipronil in soil, tomato and potato at level relevant for exposure monitoring. This study demonstrates that this proposed ELISA turned out to be a powerful tool for monitoring the residues of fipronil in environmental samples at trace levels.

Key words: *Fipronil; ELISA; immunogen; environmental samples; HPLC; conjugate; recovery.*

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1. Introduction

Pesticides are used globally for enhancing crops yield and quality. However, the widespread uses of pesticides not only contaminate water, soil, and air, but also cause their accumulation in crops (e.g., fruit and vegetables). Pesticides are transported mainly by rain and wind from their points of application to neighboring crops and land, where their presence may be undesirable or harmful. Fipronil, a broad spectrum systemic phenyl-pyrazole insecticide can be used for control many pests on animals (EPA, 1996). It is also used on a variety of foliar and soil insects including corn rootworm, colorado potato beetle, rice stem borer, tomato fruit worm and cotton leaf worm that attack a variety of crops such as corn, sunflower, rice, vegetables and fruits, as well as insects resistant to pyrethroid, organophosphate and carbamate insecticides (Bobe *et al.*, 1997). The presence of pesticide residues in food and environment has posed a serious threat to human health and caused a great concern. In order to keep human from being affected, analytical and monitoring system of pesticides residue in food and environment must be developed (Fenik *et al.*, 2011).

Detection of fipronil residues in environmental samples and body fluids is usually performed by well established analytical techniques such as high performance liquid chromatography (HPLC) or gas chromatography (GC) coupled to sensitive and highly selective detectors (Hadjmohammadi *et al.*, 2006, Cheng *et al.*, 2014 and Saini *et al.*, 2014 b). Despite the advantage of being highly sensitive and selective, instrumental methods require extensive sample preparation and cleanup procedures that become laborious, time consuming and expensive when a large number of samples have to be analyzed in the monitoring studies.

Over the past few years, enzyme-linked immunosorbent assay (ELISA) methods have been developed as attractive options for the identification or quantification of a variety of agrochemicals in water, soil and agricultural products. These methods have been proven to be quantitative, relatively inexpensive, high throughput methods. In the literature there are few publications on the development of immunoassay of fipronil (Liu *et al.*, 2007 and Vasylieva *et al.*, 2015).

The aim of this study was to develop, optimize and characterize an indirect competitive immunoassay based on polyclonal antibody for fipronil. Using spiked environmental samples, ELISA assay performance was validated by HPLC. Also the developed immunoassay was applied to analysis fipronil residues in different environmental samples (soil and vegetables; tomato and potato).

2. Materials and methods

2.1. Chemicals and instruments

The analytical standard of fipronil (purity 96 %) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The compounds; 1-ethyl-3-(3-diamino-propyl) carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (Sulfo-NHS), keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), skim milk powder, goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate and 3,3′,5,5′-tetramethylbenzidine (TMB), analytical grade dimethyl formamide (DMF), methanol, ethyl acetate and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Co. Primary secondary amine (PSA) sorbent-Bondesil 40 µm was purchased from Agilent Technologies (USA). All other reagents were analytical grade and purchased from standard chemical companies.

96-well flat bottom polystyrene microtiter plates (Nunc, no. 442404 Denmark) were purchased from Sigma-Aldrich Co, ELISA microplate reader (STAT FAX - 2100), Ultraviolet visible spectrophotometer (T-80 $^{+}$ UV / VIS spectrometer PG Instrument Ltd) and High pressure liquid chromatography (HPLC) (Agilent Technologies HP-1200, USA were used in this study

2.2. Buffers and solutions

Phosphate buffered saline (PBS) (100, 50 and 10 mM, pH 7.4) , coating buffer (50 mM carbonate buffer, pH 9.6), washing buffer PBST (10 mM PBS containing 0.05 % Tween-20), blocking solution (5 % skim milk in PBS) and substrate buffer (100 mM phosphate citrate buffer, pH 5.4). Substrate (TMB) solution (400 µl of 0.6 % TMB-DMSO and 100 µl of 1 % H_2O_2 in 25 ml of substrate buffer) and stopping solution (2 M H_2SO_4).

2.3. Preparation of hapten-protein conjugates:

The hapten (fipronil) contains an amino group was coupled to carrier proteins; KLH or BSA via active ester method using EDC and Sulfo-NHS according to Staros *et al.*, (1986).

50 mM (10 mg) of EDC and 5 mM (1.25 mg) of Sulfo-NHS were added to 10 mg of KLH or BSA dissolved in 1 ml of 100 mM PBS, pH 7.4 and stirred for 10-15 min. 10 mg of fipronil (20 mM) was dissolved in 1 ml of DMF then added to the protein solution. The reaction mixture was stirred for 3 h at room temperature (RT) and then overnight at 4 ºC to complete the conjugation between hapten and protein. The conjugates (KLH-fipronil and BSA-fipronil) were dialyzed against 10 mM PBS, pH 7.4 for 48 h at 4 °C. During dialysis the buffer was changed six times. Conjugate formation was confirmed spectrophotometrically by the UV scan for fipronil, proteins (KLH or BSA) and their conjugates in the range of 200-300 nm. UV-Vis spectral data were used to ensure the successful coupling between the hapten and carrier proteins. The conjugates were applied as an immunogen (hapten-KLH) or a coating antigen (hapten-BSA).

2.4. Antibody production

Three male New Zealand white rabbits weighing 2.4 ± 0.1 kg (obtained from Poultry Department, Faculty of Agriculture, Alexandria University, Egypt) were immunized with immunogen to prepare polyclonal antibody according Lee *et al.*, (2003). The experimental protocols were approved by the Ethical Committee for the use and care of laboratory animals.

For each rabbit, 10 mg of hapten-KLH was dissolved in 0.5 ml of sterile saline solution (0.9 %) and emulsified with 0.5 ml of complete Freund's adjuvant (CFA). The emulsion was then injected

subcutaneously at multiple different sites on the neck and back of each rabbit. This was followed by four secondary boosters of the same dose of immunogen emulsified with incomplete Freund's adjuvant (IFA) at 2 weeks intervals. The rabbits were bled from ear vein 7 days after each booster and then antibody titers were determined by ELISA as described below. After the confirmation of good titer (the animals' serum contain a high level of antibody), the blood were collected. To obtain antiserum, blood samples were left to coagulate for 2 h at RT followed by centrifugation at 4000 g for 15 min at 4 °C. The supernatant (serum) was carefully collected and stored at -20 ºC. Purification of the polyclonal antibody (pAb) was achieved by 80 % saturated ammonium sulfate precipitation (Hebert, 1974). Pre-immune sera were collected prior immunization to provide control sera having no related humoral immune response.

2.5. Antibody titer

Enzyme – Linked immunosorbent assay (ELISA) has been used in this study to determine the antibody concentrations (titer) of the serum from each animal. The titer of the antibody was screened by measuring the binding of serial dilutions of the antibody to microtiter plates coated with hapten-BSA using non competitive indirect ELISA protocol according to Gee *et al.*, (1994).

96 wells polystyrene microtiter plates were coated with 100 µl / well of the antigen (100 µg/ml coating buffer) and incubated overnight at 4ºC. The plate was washed three times with washing buffer (PBST). Unbound sites of the plates were blocked with 300 µl/well of blocking buffer (5 % skim milk solution in PBS) and incubated at RT for 2 h. The plate was washed three times with washing buffer. 100 µl/well of serial dilutions (1/1 – 1/1000, 000) of primary antibody were added and incubated for 2 h at RT. The plate was washed again four times with PBST. Subsequently, 100 µl/well of a diluted (1:3000) anti-rabbit IgG-horseradish peroxidase (secondary antibody) was added and incubated for 2 h at RT. After another washing step, 100 µl/well of a substrate (TMB) solution was added and left 10 min at RT to develop color. The reaction was stopped by adding 50 µl/well of 2 M H_2SO_4. The absorbance was recorded at 450 nm using a microtiter plate reader (STAT FAX -2100).The antibody titer is expressed as the greatest dilution of antibody that give positive response.

2.6. Immunoassay optimization

Antigen coating and antibody concentrations for the non competitive assay were optimized by two - dimensional titration with checkerboard procedure (Zeng *et al.*, 2007). Briefly, the procedure was as above except, microplate was coated with different concentrations of antigen; hapten-BSA conjugate (25-1.56 µg/ml) and serial dilutions of antibody (1:500-1:16000) were used. Optimal antigen concentrations and antibody dilutions which produce absorbance around 0.7 - 1.0 unit were chosen. Then serial concentrations of fipronil (0.0001-100 µg/ml) were applied to select the best concentration of antigen and antibody dilution. Also, the influence of organic solvent concentrations (1, 2.5, 5 or 10 % methanol), ionic strength of buffer (10, 20, 40 or 50 mM PBS, pH 7.4) and the pH of buffer (5.5, 7.4 or 8.5) on the assay sensitivity was evaluated using the indirect competitive ELISA of fipronil under these conditions as follows:

Microtiter plate was coated with 100 µl/well of a suitable concentration of antigen (3.125 µg/ml) in coating buffer, incubated overnight at 4 ºC, washed, blocked the unbounded sites and washed as described above. 50 µl/well of fipronil standard (0.0001-100 µg/ml) or sample was added, immediately followed by 50 µl/well of the desired antibody dilution (1:2000). In this way, a competition was established between immobilized hapten and free analyte for the antibody binding sites. Plate was incubated for 2 h at RT, washed, then the steps were completed as indicated above and the absorbance was measured with an ELISA plate reader at 450 nm. Repeat the same procedure by using the different concentrations of methanol, ionic strength and pH of PBS. All experiments were conducted in triplicate.

2.7. Standard curve

Standard inhibition curve of fipronil by indirect competitive ELISA was carried out as previous procedure using serial concentrations of fipronil (0.00001-100 µg/ml) in 50 mM PBS, pH 7.4 containing 1% methanol, coating antigen (3.125 µg/ml) and antibody dilution (1:2000). The absorbance at 450 nm is inversely proportional to the concentration of fipronil in the standards and samples. The standard curve was constructed by plotting the A/A_o values against the log values of the fipronil concentrations, where A: absorbance at a given concentration of fipronil and A_o: absorbance at zero concentration of fipronil. From the standard curve it can be determine the IC_{50} which was defined as the concentration of inhibitor required to inhibit color development by 50 % and limit of detection (LOD) which defined as least detectable amount of inhibitor that quantitively assayed that yield 90 % A/A_o as well as the fipronil concentrations in the environmental samples.

2.8. Determination of fipronil in the environmental samples

The immunoassay can be used to analyze fipronil in some environmental samples (soil, potato and tomato). The potato; cara variety and tomato; roma variety were planted in February 6th 2016 and April 24th 2016, respectively according to the recommended agronomic practices at Agriculture Research station, Alexandria University, Egypt. There were three replicates for each plant (plot). At the fruitful stage of each plant (three months after plantation), the tested compound (fipronil) was applied as a commercial formulation (coach 20% SC) at the recommended dose (25 ml/100 L water) using CP3 sprayer. The soil under crop was of clay loam texture. The other relevant properties of the soil were organic carbon = 36.0%, pH = 8.2, sand 20.9 %, silt = 33.0 %, clay = 46.1% and EC = 3.38 dsm^{-1}.

2.8.1. Sample collection

Soil samples (1 kg) were collected randomly from five sites of each replicate/ plant after different time intervals; 0 (1 h), 1, 3, 5, 7 and 10 days after fipronil application at a depth 0-10 cm. Also, five fresh samples (1 kg/each) from each replicate for each vegetable were collected at the same time intervals. The collected samples were placed into polyethylene bags and transported to the laboratory as fast as possible in ice box. The samples were stored at -20 ^{o}C until analysis. Control samples were obtained from the same field before fipronil application.

2.8.2. Sample preparation

The soil samples were air-dried, ground and passed through 2 mm sieve. The potato and tomato samples were chopped and homogenized using a commercial blender for 2 min. The samples were extracted as following:

2.8.2.1. Extraction for ELISA analysis

An aliquot (10 g) of the homogenized sample was added to 10 ml of methanol for soil or ethyl acetate for potato and tomato. The mixture was vigorously shaken for 3 min using a vortex mixer and centrifuged at 3000 g for 10 min. Ethyl acetate supernatants of vegetable samples were evaporated to dryness under mild nitrogen stream. The residue was re-dissolved with 1 ml methanol. While, in soil sample the methanol supernatant was concentrated to 1 ml. Then all the extracts were diluted with 50 mM PBS, pH 7.4 (1:100). Finally, the samples were analyzed via ELISA.

2.8.2.2. Extraction and clean up for HPLC analysis

Extraction and clean up of fipronil residues have been done by using QuEChERS with some modifications according to Anastassiades *et al.*,(2003). An aliquot (10 g) of the homogenized sample was added to 10 ml of methanol for soil or ethyl acetate for potato and tomato then vigorously shaken for 1 min using a vortex mixer. Four grams of anhydrous $MgSO_4$ and 1 g of NaCl were added and vortexed again intensively for 2 min and then centrifuged for 10 min at 3000 g. One ml of supernatant was cleaned up using dispersive – solid phase extraction (d-SPE) which containing 50 mg of primary secondary amine (PSA), 150 mg of magnesium sulfate and 10 mg active charcoal (for tomato). Then the mixture was vortexed again for 2 min and centrifuged for 5 min at 3000 g. Finally, 0.5 ml of the supernatant was taken to HPLC analysis as described below.

Experiment with HPLC was performed on an Agilent HP 1200 liquid chromatographic system, consisting of a quaternary delivery pump, an auto-sampler with a 100-mL loop, a thermostatic column compartment and a DAD detector. A personal computer equipped with an Agilent ChemStation program was used to process the chromatographic data. The analytical column was Eclipse XDB-C18 column (150 x 4.6 mm ID; 5 µm).The sample injection volume was 100 µl and the absorbance was monitored at 205 nm. The mobile phase was isocratic prepared from methanol 70 %. The constant flow rate was 1.0 ml/min. Under these optimum conditions, the spiked samples were well separated.

2.9. Recovery studies

Recovery experiments were carried out to establish the reliability and validity of analytical method. Blank soil samples were collected from untreated area as pesticide free soil. The samples were air dried and passed through 2 mm sieve. 10 gm of the blank samples were spiked with appropriate concentrations (0.01-1 µg/g).

Pesticide free potato and tomato samples obtained from local grocery stores have been used as the blank samples. The samples were crushed using a blender and 10 gm of the samples were fortified with a standard solution at the final concentrations of 0.1, 1 and 10 µg/g. The mixture of the blank samples (soil, potato and tomato) with each level of fipronil allowed standing for 2 h at RT to distribute the pesticide evenly and to promote a greater interaction between the pesticide and the sample before initiating the extraction procedure. The spiked samples were extracted as above and then the

recoveries were determined by ELISA and compared with HPLC.

2.10. Statistical analysis

Data were expressed as mean ± SE. Statistical significance of data was determined with two-ways analysis of variance (ANOVA) by comparing means using Student Newman-Keuls Test with CoStat Program Version 6.2 (2002). Differences were considered at p value of less than or equal 0.05.

3. Result and Discussion

3.1. Confirmation of hapten-protein conjugates

The initial and critical step in the development of effective immunoassay for low molecular weight chemicals such as pesticides lies in the selection of appropriate hapten which can elicit the production of antibodies demonstrating maximum specificity and sensitivity for the target molecule (Kim *et al.*, 2003). Fipronil itself cannot be used as an immunogen because of its low molecular weight therefore; it must be conjugated to a carrier protein of high molecular weight (KLH or BSA) to develop an ELISA for it. Classical conjugation of a hapten to a protein is via a free primary amine or carboxylic acid forming a peptide bond. Szurdoki, *et al.,* (1995) mentioned that, the site of coupling hapten to the carrier, can be of major importance for the sensitively and selectively of the resulting antibody. The hapten (fipronil) used in this study contain an amino group and was conjugated covalently to proteins by activating the carboxylic groups of proteins with 1-ethyl-3-(3-diamino-propyl) carbodi-imide hydrochloride (EDC) and N-hydroxysulfo-succinimide (Sulfo-NHS) (Staros *et al.,* 1986) followed by their coupling to the amino group of hapten. In this method the conjugate was formed by linkage to the carrier protein predominantly via the glutamic and aspartic acid residues. Fipronil was coupled to two carrier proteins; KLH to prepare immunogenic conjugate and BSA to prepare coating conjugate.

The nature of conjugates was characterized by UV spectroscopy. Fig (1; a & b) shows qualitative differences between fipronil, carrier proteins (KLH & BSA) and their conjugates in the region of absorbance of protein (200 – 300 nm). The confirmation of conjugates using UV-vis spectrum is simplest and effective method. The shape of three curves was distinct and there were obvious differences in the absorbance patterns of the conjugates compared to those of the corresponding carriers. It was observed that, the conjugates; (fipronil-KLH) and (fipronil-BSA) had a peak at 270 and 275 nm, respectively. Their values were different from those of fipronil, BSA and KLH. This differential UV spectroscopic behavior indicated that nature of conjugate, fipronil and carrier proteins is qualitatively different. In conjugates, the shifting of the peak to a different position as compared with fipronil and proteins confirmed the successful conjugation. However, the antibody of high titer from this antigen could sufficiently support the hapten - protein conjugation. These data are in agreement with Zhu *et al.,* (2006) and Liu *et al.,* (2007) who used UV-vis spectra to ensure the successful coupling between hapten and carrier proteins.

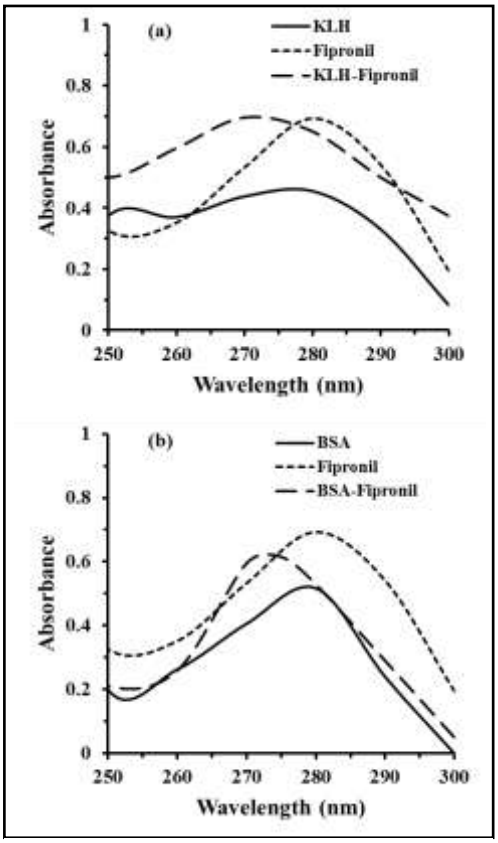

Fig. 1: UV spectra of protein-hapten conjugates. (a) KLH, fipronil and KLH-fipronil, (b) BSA, fipronil and BSA-fipronil.

3.2. Antibody titer

The titer of purified antibody was determined by measuring the binding of serial dilutions of the antibody to the corresponding coating antigen (hapten-BSA) using non competitive indirect ELISA protocol. Antibody titer is expressed as the greatest dilution ratio of antibodies that still gives a positive response. The antibody showed high level of polyclonal antibodies with the titer reached to 1:128,000 for the tested hapten. Liu *et al.,* (2007)

found that the polyclonal antibody titer of antiserum of rabbit immunized with fipronil-BSA against hapten-OVA was 1:25600. Morozova *et al.,* (2005) reported that, the higher antibody titer (1:10,000 – 1:100,000) required only a small amount of antiserum for performing ELISA.

3.3. Optimization of competitive ELISA

Checkerboard assay was carried out using different concentrations of coating antigen and serial dilutions of antibody to select the most proper concentrations of coating antigen and antibody that yielded absorbance around 0.7 - 1.0 units. Combinations of coating antigen concentrations and antibody dilutions were further tested with competitive inhibition experiments to fipronil (0.0001 - 100 µg/ml). The optimal competitive ELISA was performed with 3.125 µg/ml of coating antigen and a serum dilution of 1:2000 to achieve maximum sensitivity. These concentrations were used in all following experiments. Liu *et al.,* (2007) found that the two-dimensional titration of the antiserum of fipronil (fipronil-BSA) against hapten-OVA revealed that the absorbance was around 1.0 when the dilution of antiserum was 1:1600 and the concentration of the coating antigen was 2 µg/ml. On the other hand, Vasylieva *et al.,* (2015) concluded that, the optimum concentration of antigen (fipronil-Con) was 1 µg/ml and the dilution antibody (fipronil-Thy) was 1:6000 to achieve maximum sensitivity.

Many parameters can influence the binding of the antibody to the hapten causing differences between expected and observed ELISA results and poor correlation with instrumental analysis. Among these parameters were the organic solvent concentrations, pH and ionic strength of the assay buffer during the competition step. Most pesticides are not readily soluble in water (fipronil has moderate solubility in the aqueous solutions; ≈ 2 mg/L) therefore; an organic solvent is needed to prepare the assay buffer for ELISA. The organic solvent in assay buffer is necessary to keep hydrophobic analytes in solution and prevent their non specific binding on the plastic containers. Methanol is often used in sample preparation due to its being less volatile, commonly used to extract analytes from various matrixes or elute analytes from solid phase extraction (SPE) cartridge (Zhang *et al.,* 2016). So, the effect of methanol at different concentrations (1, 2.5, 5 and 10%) in PBS buffer was tested. The results indicated that the methanol concentrations strongly interfere with the assay sensitivity and the maximum absorbance (Fig 2a). The assay sensitivity was decreased by increasing methanol concentration. Methanol had the least effect at concentration up to 5 % (v/v), while 1 % methanol gave the best sensitivity. Based on the IC_{50} value obtained from standard curve (IC_{50} = 0.3 µg/ml), 1% methanol concentration was selected for subsequent experiments. This data was agreed with Watanabe *et al.,* (2002) and Vesylieva *et al.,* (2015) who reported that the assay sensitivity of ELISA was decreased by increasing methanol concentration. Also, Székács *et al.,* (2003) found that the presence of small amounts of methanol (0.5-1 % v/v) as co-solvent in the assay buffer significantly improved assay sensitivities and standard curve slops when optimized an enzyme immunoassay for the insect growth regulator fenoxycarb. On the other hand, Li and Li (2000) evaluated the effect of four common water miscible organic solvents (DMSO, methanol, acetone and acetonitrile) on the ELISA assay performance and they found that when these solvents were less than 2 % (v/v) in the ELISA, their effects on the antibodies were negligible. The sensitivity of the assay decreased with the high concentration of the organic solvent may be due to protein denaturation, decrease the affinity between antibody and antigen, destroy the Van der Waals force, the hydrophobic interaction in the antibody - antigen conjugation or even make the complex substance break down.

In contrast, an increase in ionic strength of buffer resulted in improvement of assay sensitivity (decrease IC_{50}) (Fig. 2b). These results suggest that the binding interaction between antibody and analyte/coating antigen is increased in solutions with high ionic strength. So, 50 mM of PBS buffer which was commonly used is the best salt concentration to improve assay performance of ELISA. Cho *et al.,* (2004) reported that increasing the concentration of phosphate ion in assay solution caused a continuous increase in assay sensitivity. While, Zeng *et al.,* (2006) reported that a higher salt concentration of PBS in the assay system resulted in higher IC_{50} because it can affect the antibody binding. Li *et al.,* (2007) found that the indirect competitive ELISA for imidacloprid was affected obviously by organic solvents and the buffer ionic strength. They observed that lower concentration of organic solvents (< 5 %) and the ionic strength (< 0.5 mol/ml) were little affected the assay, even advantageous to increasing the detecting sensitivity. Research on these factors can be helpful to optimize the condition of analysis. It is important in terms of the practice.

Figure 2c shows the influence of varying pH of the assay buffer (50 mM PBS) during the competition step in the presence of the analyte. There was change in sensitivity upon pH change in the range of 5.5 - 8.5. IC_{50} value was remarkably increased at basic pH, that is, the sensitivity of the antibody was lower than at neutral or acidic pH. The highest affinity of the antibody showed at pH 7.4 and selected as the optimum pH to carry out for the

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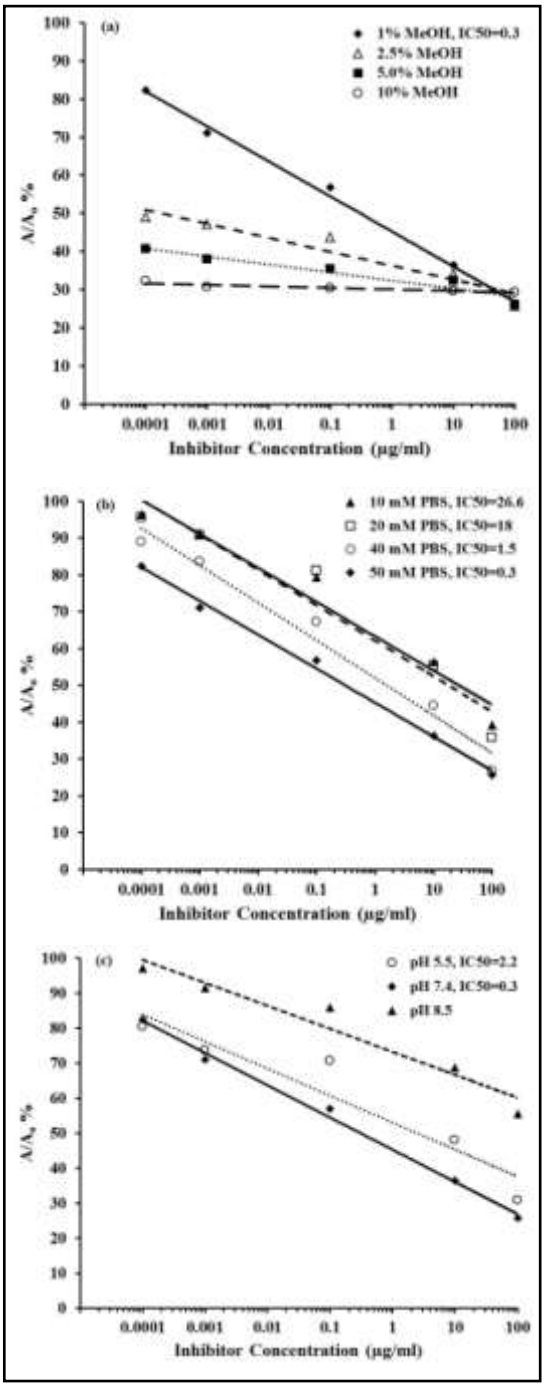

Fig. 2. ELISA competitive curves of fipronil prepared in PBS buffer containing: (a) various concentrations of methanol, (b) various concentrations of ionic strength and (c) various pH values. Absorbances are expressed as percent of the control absorbance to standardize the data sets. Data points are the mean values of triplicates. Assay conditions: coating antigen (BSA-fipronil) 3.125 µg/mL; antibody dilution 1:2000 final dilution in wells.

following experiments. This data is parallel with that of Vasylieva *et al.*, (2015) who found that the lowest IC_{50} value of fipronil was at pH 7.5. Many immunoassays are equally sensitive over a wide range of pH values. However, the function groups in the analyte are potentially susceptible to ionization resulting from pH changes (Watanabe *et al.*, 2002). The ionic strength and pH dependence of sensitivity of ELISAs for pesticides has been reported by many authors (Li *et al.*, 2015 and Xu *et al.*, 2011).

So, the assay optimization revealed that methanol at 1% and assay buffer with ionic strength; 50 mM at pH 7.4 resulted in the most sensitive assay.

3.4. Calibration curve and sensitivity

The assay optimization revealed that coating antigen of 3.125 µg/ml, antibody dilution was 1:2000 in 50 mM PBS, pH 7.4 containing 1 % methanol. There is not a general agreement for the calculation of assay sensitivity and working range of competitive immunoassays (Sherry, 1992). The IC_{50} value in the standard curve (Fig. 3) was 0.325 µg/ml and LOD was 0.026 ng/ml. All these data showed that the polyclonal antibody with high specificity was produced. Liu *et al.*, (2007) developed poly and monoclonal antibody using homologous hapten for preparation of the immunogen and coating antigen. Their assay had lower sensitivity (IC_{50}=17.95 µg/ml) compared to those described in the present study (0.325 µg/ml). While, Vasylieva *et al.*, (2015) found that the IC_{50}=0.018 µg/ml.

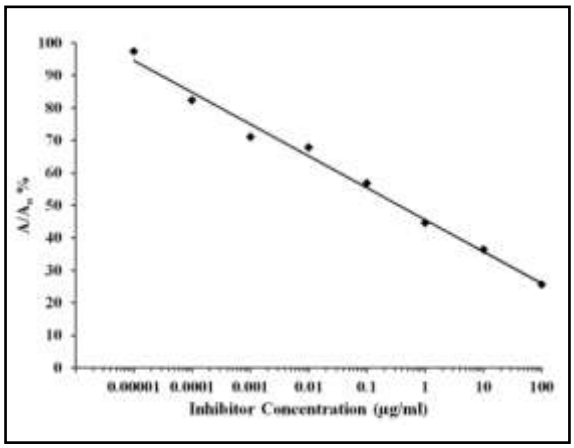

Fig. 3. Standard inhibition curve of fipronil using optimized ELISA (3.125 µg/ml of antigen, 1:2000 of antibody dilution and 50 mM PBS pH 7.4 containing 1% methanol).

3.5. Recoveries of fipronil from the spiked samples

Recovery experiments were carried out to check the validity of ELISA method in different matrixes (soil, tomato and potato) by adding known amount of fipronil to portions of blank samples and analyzing by the method detailed. The mean analytical recovery was calculated as the ratio between the

fipronil concentrations founded and the concentrations added, and expressed as percentage. In ELISA technique the recovery assays were performed with soil, tomato and potato samples spiked with fipronil at three concentration levels of 0.01, 0.1 and 1.0 µg/g for soil and 0.1, 1.0 and 10 µg/g for tomato and potato. Analysis was carried out in triplicate. Interestingly, good correlation between spiked and ELISA - measured fipronil was obtained. The recoveries of fipronil in soil, tomato and potato were in the range of 91-102 %, 96.6-100 % and 98-100.8 %, respectively (Table 1). The recoveries of fipronil from all tested matrixes were over 90 % which means that its extraction efficiency is very satisfactory. In order to verify fipronil levels determined by ELISA in spiked samples, fipronil content was also detected in the same samples using an instrumental technique, HPLC. Recoveries were found to be in the range of 90 - 97.3 %, 95.0 - 98.4 % and 98.6 - 99.7 % in soil, tomato and potato samples, respectively. As seen in Table (1), there was relatively good agreement between ELISA and HPLC data (r = 0.992 for soil, 0.999 for tomato and 0.995 for potato). These recovery values demonstrated that the method's accuracy of both techniques are sufficient for residue analysis.

The results demonstrated that this immunoassay was suitable for the quantitative detection of fipronil at trace levels in soil, tomato and potato without any addition sample preparation, thus reducing analysis time, especially in the case of large screening samples, and reducing the cost of analysis.

3.6. Determination of fipronil in environmental samples

Tomato and potato are susceptible to pests and diseases, which results in the use of large amounts of pesticides throughout its growing cycle. Among the main pesticides used in these vegetables, fipronil is the most cited due to its high efficiency of pest control. When improperly applied, residues from this pesticide can remain in the product or soil, generating an important pathway to human exposure and environmental contamination. Considering this, several studies have been concluded to verify the presence of pesticides residues in vegetables and soil as well as their adsorption and dissipation in soil. The effectiveness and applicability of the immunoassay technique established above was initially applied to detect fipronil residues in soil, tomato and potato samples under field conditions collected from Agriculture Research Station, Alexandria University, Egypt. The environmental samples were taken one day prior treatment and 0 (1 h), 1, 3, 5, 7 and 10 days after application. Table (2) demonstrated that the fipronil concentrations

Table 1: Recovery of fipronil in spiked samples of soil, tomato and potato measured by ELISA and

Fortified sample (µg/g)	ELISA (Mean ± SD) Recovery (%)	HPLC (Mean ± SD) Recovery (%)
Soil		
0.01	91.0 ± 1.15	90.1 ± 4.00
0.1	102.0 ± 1.78	97.3 ± 2.80
1	97.0 ± 0.86	94.8 ± 3.20
Tomato		
0.1	97.0 ± 0.90	97.5 ± 3.50
1	96.6 ± 1.03	95.0 ± 1.85
10	100.0 ± 0.38	98.4 ± 2.30
Potato		
0.1	99.9 ± 1.06	98.6 ± 2.00
1	98.0 ± 0.55	99.7 ± 2.50
10	100.8 ± 2.26	99.5 ± 1.35

Data are the average of triplicate samples ± standard

determined by ELISA were 52.5, 47.5, 40, 28, 16.87 and 13.75 µg/kg soil at depth of 1-10 cm at 0 time (1 h) ,1 , 3, 5, 7 and 10 days, respectively after fipronil application with the recommended rate (25 cm^3/100 L water/ fed). The data showed a gradual and continuous dissipation of fipronil residues in soil. The residues of fipronil degraded from 52.5 to 13.75 µg/kg over the experimental period of 10 days, with a dissipation rate of 73.81 %. The calculated half-life of fipronil was 5.2 days. The present results was paralleled with Saini *et al.,* (2014 a) who found that the half-life of fipronil ranged from 5-5.5 days after applying fipronil (regent 5 SC) at two doses in soil under chilli crop. Also, Hadjmohammadi *et al.,* (2006) observed that the residue of fipronil was declined from 32 to 9 µg/kg of soil cultivated with rice during 14 days of treatment at a rate of 40 gm ai/ha. Cheng *et al.,* (2014) observed a gradual and continuous dissipation of fipronil residues in soil. The residues of fipronil in soil degraded from 85.7 to 5.2 µg/kg over the experimental period of 60 days, with a dissipation rate of 93.9 %. Wang *et al.,* (2014) investigated the dissipation trends of fipronil in soil under field conditions at two different locations. The initial residues of fipronil were found to be 0.046 and 0.035 mg/kg in soil collected on 0 day (2 h) after the application at 25 gm ai/ha with half-life ($t_{1/2}$) period of 9.90 and 10.34 days. Similar result of the insecticide has been reported by Saini *et al.,* (2014 b) who investigated degradation of fipronil in sandy loam soil under field conditions by applying fipronil (regent 5 % SC) at 50 and 100 gm ai/ha in field. Samples were drawn periodically in triplicate on 0 (1 h), 1, 3, 7, 10, 15, 30, 60, and 90 days after treatments.

Table 2: Fipronil concentrations analyzed by ELISA in soil, tomato and potato

Time (Days)	Fipronil concentrations (µg/kg) Mean ± SD		
	Soil	Tomato	Potato
Before application			
	N.D	N.D	N.D
After application			
0	52.5 ± 1.6	9.5 ± 0.5	N.D
1	47.5 ± 2.1	6.8 ± 0.2	N.D
3	40.0 ± 2.8	5.6 ± 0.3	N.D
5	28.0 ± 2.4	4.3 ± 0.5	1.3 ± 0.03
7	16.9 ± 3.4	3.8 ± 0.7	3.0 ± 0.01
10	13.8 ± 1.5	2.2 ± 0.1	6.5 ± 0.14

N.D not detectable

Data are the average of triplicate samples ± standard deviation

The residues of fipronil in both the doses dissipated in the range of 93.33 - 100 % in 90 days with half-life period of 10.81 days for fipronil in soil. In soil samples, fipronil residues observed on 0 day (2 h) were found to be 28 µg/kg. Residues of fipronil reduced with passage of time. After 1, 3, 5, 7, 10, 15, 20 and 30 days of spray, the corresponding level of fipronil residues were found to be 22, 17, 15, 13, 11, 10, 8 and 5 µg/kg. On reaching 30 days of application, the fipronil residues were found to be dissipated by 82.1 %. Half-life of fipronil was observed to be 5.55 days (Duhan *et al.*, 2015).The concentrations of fipronil in tomato samples were gradually decreased over a period of experiment (10 days). The initial concentration of fipronil was 9.5 µg/kg and declined to 2.2 µg/kg after 10 days, with a dissipation rate of 76.84 % and the half-life of 4.7 days (Table 2). Dutta *et al.*, (2008) observed that 76.4 % of fipronil residues dissipated in cabbage on 15^{th} day with half-life 7.5 days. Saini (2012) observed that the half-life period of fipronil in chili was 3.19 days. This may be due to the smooth surface of chili which prevented the inside movement of insecticide. On the other hand, cauliflower has its surface rough to facilitate the inside movement insecticide and resulting in longer persistence of residues. Mohapatra *et al.*, (2010) reported that residues of fipronil in grapes leaves and berries degraded with half-life of 9.6 and 18.3 days at recommended dose, respectively. In contrast, fipronil concentration in potato tubers samples was not detectable until 3^{rd} day of fipronil spraying. The fipronil concentration was observed at the 5^{th} day of application and the concentration was increased from 1.3 µg/kg (at the 5^{th} day) to 6.5 µg/kg (at the 10^{th} day) (Table 2). Residues of fipronil detected in potato tubers at the 5^{th} day of fipronil was applied on vegetative parts of potato plants are evidence that penetrate, movement and translocation fipronil through plant tissues to tubers.

Some investigators had studies the absorption and translocation of pesticides through plant tissues after application (Bonmatin *et al.*, 2005 and Fossen, 2006). The present results were coincided with the data of Shokr, (2006) who observed that no residues of carbosulfan or imidacloprid were detected in potato tubers at 1 h and 1 day after spraying and it was found slight amount of carbosulfan residue (0.07 ppm) or imidacloprid (0.1 ppm) after 2 days of spraying, and increased to 0.14 ppm with 3 days of application. Therefore, it can be concluded that the dissipation of fipronil applied at different doses in different vegetables crops and fruits varied from each other. The degradation and disappearance of pesticides may be due to many factors, including plants, pesticides and environments, which affected the dissipation of pesticides on crops. Christensen, (2004) reported that the decline of pesticides may be due to biological, chemical or physical processes or if still in the field due to dilution by growth of crop (Walgenbach *et al.*,1991).

The present assay allow the detection of free fipronil dissolved in 50 mM PBS, pH 7.4 with 1 % methanol with an IC_{50} of 0.325 µg/ml and a LOD of 0.026 ng/ml. This limit of detection was not very low but would be sufficient for fipronil detection in vegetables in the range of the maximum residue level (MRL); 10 µg/kg in potato and 5µg/kg in tomato (EFSA, 2012).

4. Conclusion

This research was approached to prepare hapten-protein conjugate which elicit the immune system of rabbit to produce specific antibodies of fipronil. These locally produced polyclonal antibodies against fipronil showed high sensitivity and specificity, and the developed ELISA with antibody could detect 0.325 µg/ml of fipronil as IC_{50} and the lowest limit was 0.026 ng/ml. The immunoassay can be affected by many factors and optimization process is necessary to improve the sensitivity, accuracy and reproducibility. The application of the ELISA to the determination of fipronil in environmental samples showed good characteristics with simple operation, high sensitivity, good specificity and celerity. Moreover, ELISA offers other considerable advantages such as easy handling, small sample volumes needed, omission of clean up and concentration steps, high sample turnover in addition to acceptable costs especially for its capability of high throughput screening analysis. The proposed method (ELISA) was efficient in the determination of fipronil in environmental samples. This study

therefore, suggested that the use of fipronil at the recommended dose can be considered quite safe from the point of view of soil and vegetables health hazards as the residues left in soil and vegetables at the tested dose in 10 days studies. Finally, it was concluded that the monitoring of pesticides in food must be performed periodically to ensure that product quality and the safety of consumer's health.

Conflict of interest:

There are no conflicts of interest to declare.

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