Asian Journal of Chemistry; Vol. 27, No. 10 (2015), 3647-3650

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2015.18901

Development of Monoclonal Antibody Based Enzyme-Linked Immunosorbent Assay for Neonicotinoid Insecticides Thiamethoxam Residue in Environmental Water Samples

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Received: 4 December 2014; *Accepted*: 15 April 2015; *Published online*: 22 June 2015; AJC-17318

This work describes the analytical performance of newly developed method-based enzyme-linked immunosorbent assay (ELISA) for neonicotinoid insecticide thiamethoxam to effectively exploit as a rapid and simple detection technology for pesticide residue on the scenes of the environmental water monitoring samples. The enzyme-linked immunosorbent assay represents the satisfactory analytical characteristics (IC_{50} value, 0.0255 mg L^{-1}; limit of detection, 0.001 mg L^{-1}) to detect thiamethoxam at the maximum residue limits (MRL) or there about in samples. For the enzyme-linked immunosorbent assay analysis, acetonitrile showed the lowest influence on the assay performance was selected as the best extraction and the final concentration in the well could be up to 5 % (v/v) without any negative influence on the enzyme-linked immunosorbent assay. Dilution of sample extracts with water was effective in eliminating matrix interference. Average recoveries from thiamethoxam-spiked environmental water samples were > 75 % using a rapid and simple extraction method with hand shaking for 5 min. Analytical results obtained from the enzyme-linked immunosorbent assay were comparable to those obtained from the conventional HPLC method. These findings strongly indicate that the proposed method-based enzyme-linked immunosorbent assay for determination of thiamethoxam residue in environmental water may be routinely employed as a rapid, simple and quantitative preliminary screening method for the monitoring of safety of environmental water.

Keywords: Neonicotinoid insecticides thiamethoxam, Enzyme-linked immunosorbent assay, Screening analysis, Sample matrix.

INTRODUCTION

Pesticide residues have been studied since the last half of the twentieth century, mainly because of problems associated with environmental and food contamination. Neonicotinoid, major classes of pesticides, have been widely used for controlling the pests of various crops[1]. Since the launch of imidacloprid in the early 1990s, neonicotinoid insecticides have represented the fastest-growing class of insecticides introduced to the worldwide insecticide market. Worldwide annual sales of neonicotinoids, with the largest sales volume among all insecticides, are approximately $1 billion, accounting for 11-15 % of the total insecticide market[2].

Thiamethoxam [(EZ)-3-(2-chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazinan-4-ylidene(nitro)amine], is a novel nitromethylene derived neonicotinoid belonging to sub class of the nicotinic compounds and it represents the first example of second generation neonicotinoids with a unique structure and outstanding broad spectrum insecticidal activity, neonicotinoid interfere with the nicotinic acetylcholine receptor (nAchRs) and therefore, have specific activity against insect nervous system[3]. Thiamethoxam has high water solubility (4.1 g/L at 25 °C), low octanol water partitioning coefficient (0.74) and low vapour pressure (6.6×10^{-9} Pa at 25 °C). Over the past decade, thiamethoxam have been widely used for controlling aphids, whitefly, plant hoppers, thrips, mearly bugs, beetles, *etc.* and is applied to various agricultural products by soil, seed and foliar treatments[4]. As a consequence, their residues may occur in agricultural products, such as fruits and vegetables and in the environmental water, therefore, they pose a potential hazard for mankind. Monitoring of pesticide residues is crucial for proper assessment of human exposure to pesticides through drinking water. Modern instrumental methods, such as HPLC/MS or GC/MS have shown excellent sensitivity and selectivity that enable analyses of neonicotinoid insecticides in diverse samples at trace levels. They also provide solid evidence to confirm both the identity and quantity of the residues detected. In spite of the high sensitivity and selectivity of the instrumental techniques, they are still not effective enough to directly determine trace amounts of pesticides in drinking water when taking into account the complicated pretreatment procedures and costs. As is well known, enzyme-linked immunosorbent assay (ELISA) could be proven to be rapid and simple preliminary screening methods to detect the

target pesticide in food or environmental samples and its character of high sample throughput can fulfill the requirements for monitoring pesticides quickly and cost-effectively. Consequently, ELISA is becoming one of the most powerful tools for analyzing pesticides in diverse food or environmental samples[5,6].

Up to now, several ELISAs have been developed for neonicotinoids, including imidacloprid, acetamiprid[7,8], thiamethoxam[9], dinotefuran[10] and imidaclothiz[11], but thiamethoxam residue in environmental water ELISA has not been reported. In this paper, the analytical performance and reliability of the newly developed ELISA for the determination of thiamethoxam in environmental water was detected by ELISAs.

EXPERIMENTAL

Pesticide-grade thiamethoxam (purity of 99.7 % by HPLC) and structurally related neonicotinoid insecticides of thiamethoxam used for the cross-reactivity studies and IgGRaM-HRP used for Thiamethoxam residue ELISAs were purchased from Sigma Chemical Co., Ltd. (Shanghai, China). Pesticide-grade organic solvents and other chemicals were from A Johnson Matthey Company and Sinopharm Chemical Reagent Beijing Co., Ltd. (Beijing, China) and all reagents and solvents were analytical grade. The monoclonal antibody against thiamethoxam was obtained from immunized female Balb/c white mice and stored at Henan Higher Education Engineering Technology Research Center for Animal Diseases Control and Residues Supervision.

ELISA analysis: Environmental water samples were selected obtained respective to tap water, lake water and river water and these 10 mL water samples of each were fortified by adding aliquots of standard solutions of thiamethoxam in methanol and allowed to set at room temperature for about 0.5 h prior to extraction.

The samples were mixed with methanol (20 mL) in a 50 mL disposable conical flask and this was vigorously shaken with a vortex mixer for 3 min. After filtration (PVDF membrane filter, 0.22 μm), the extracts were vacuum-concentrated to dry and dissolved with 1 mL phosphatic buffer solution (PBS, 0.01 mol/L, pH = 7.8) and then the diluted sample solutions were analyzed with the ELISA.

ELISA: Well wash 4 MK2 plate washer and the absorbance was measured with a Varioskan Flash multifunctional microplate reader (Thermo Fisher Scientific, USA).

ELISA determination: Several working standard solutions used for calibration (0.512, 0.256, 0.128, 0.064, 0.032, 0.016, 0.008, 0.004 and 0.002 mg/L) were prepared in phosphatic buffer solution/methanol (9:1). Working standard solutions and diluted sample extracts were analyzed according to the following procedure: 100 μL of either working standard solution or sample extract was added to the ELISA plate pre-coated with the thiamethoxam coating antigen followed by 50 μL of an anti-thiamethoxam monoclonal antibody in suitable concentration. After 15 min at ambient temperature, 50 μL of the well-mixed solutions was added to the ELISA plate pre-coated with IgGRaM-HRP solution, reacting 0.5 h at 37 °C. Then 50 μL/well of the enzyme substrate was added in the well-mixed solutions. After 10 min at 37 °C, 2 moL/L sulfuric acid as reagents for terminating and colouring was added in wells of the plate and the absorbance at 450 nm was measured by microplate reader.

Sample concentrations were calculated by the calibration curve. Assay sensitivity was estimated as the concentration of analyte affording a 20 % inhibition (IC_{20} value). The limit of detection (LOD) and the dynamic range was defined as IC_{10} values and as the range of concentrations between IC_{20} and IC_{80} values[12].

Sample pretreatment for HPLC analysis: Water samples (10 mL) fortified with thiamethoxam were vigorously shaken with 100 mL of methanol for 10 min and then transferred into a 10 mL PTFE centrifuge tube containing 150 mg anhydrous magnesium sulfate, 50 mg C_{18} PSA and 50 mg C_{18} in advance. Then the tubes were well capped and shaken for 3 min. The tubes were then centrifuged at 3000 rpm for 5 min. An about 0.5 mL aliquot of extract solution was filtered with a PTFE membrane syringe-driven filter unit and transferred into sample vials for the HPLC analysis.

Chromatographic analysis: The HPLC system consisted of an Agilent (Palo Alto, CA, USA) 1260 series equipped with a quaternary analytical pump, an autosampler, a column oven and a Variable wavelength ultraviolet detector. Analytical separation of thiamethoxam was performed on a Extend-C_{18} (250 mm × 4.6 mm, 5 μm particle size, Agilent technologies, USA) reversed-phase column used in conjunction with a guard column (20 mm × 4.6 mm, 5 μm particle size). The column oven temperature was kept at 40 °C and the sample injection volume was 10 μL. The mobile phase was methanol/water (20/80, v/v) at 1.0 mL/min and the concentrations of thiamethoxam were determined by external calibration using peak area measurements at 254 nm.

RESULTS AND DISCUSSION

Assay performance: The standard curve for thiamethoxam based on triplicate determinations is given in Fig. 1. The limit of detection and the sensitivity of the ELISA were 0.0022 and 0.025 mg/L respectively and the dynamic range of the ELISA was 0.002 to 0.289 mg/L.

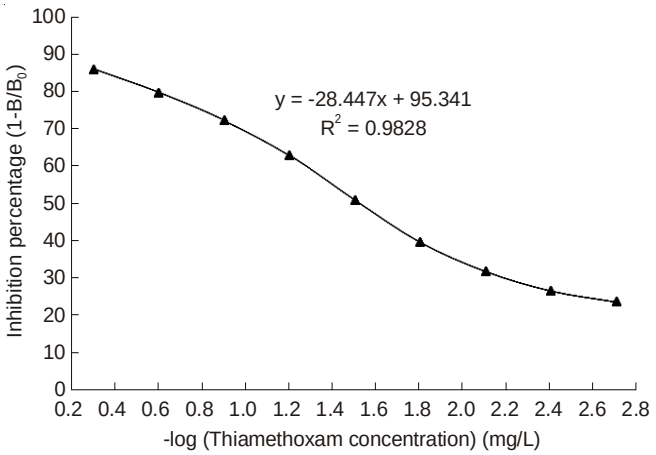
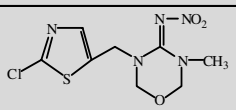
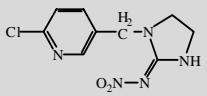
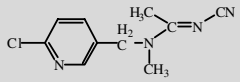
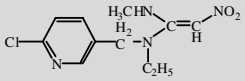
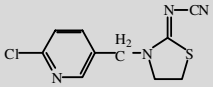
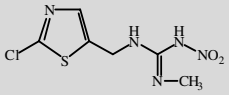


Fig. 1. ELISA standard curve for thiamethoxam obtained using the self-made standard solutions prepared in phosphatic buffer solution/methanol (9:1). The final concentration of methanol (in each well) is 5 %. Each point is the average of triplicate determinations

Selectivity: As shown in Table-1, cross-reactivities were estimated as the percentage obtained by calculating the ratio of the IC_{50} value of thiamethoxam to that of the given analogue. The results showed that although these insecticides were all belong to neoticotinoids analogue, the monoclonal antibody was highly selective for thiamethoxam.

TABLE-1
CROSS-REACTIVITY OF THE ANTI-THIAMETHOXAM MONOCLONAL ANTIBODY AGAINST SEVERAL STRUCTURALLY RELATED NEONICOTINOID ANALOGS

Analogue	Chemical structure	IC_{50} (mg/L)	Cross-reactivity (%)
Thiamethoxam		2.55×10^{-2}	100
Imidacloprid		1.50×10^{-2}	0.017
Acetamiprid		1.88×10^{-2}	< 0.01
Nitenpyram		7.81×10^{-2}	< 0.01
Thiacloprid		2.11×10^{-1}	< 0.01
Clothianidin		2.27×10^{-4}	9.31

Note: Cross-reactivity (%) = (IC_{50} value of thiamethoxam/IC_{50} value of other analogue) × 100

Accuracy and precision of ELISA: Several thiamethoxam-spiked environmental water samples were determined based on the process of "ELISA determination", for which no significant matrix interference was observed. Although the average recoveries of thiamethoxam at three levels of concentration, 0.015, 0.5 and 5 mg/mL in all water samples except lake water samples appeared to tend toward over estimations, thiamethoxam was accurately detected in each sample at all spiked levels with satisfactory recoveries (84.0-104.0 %) (Table-2). Intra-assay was tested by conducting four replicates analyses and inter-assay was done by analyzing four replicates on three separate days. The detected dates of intra-assay repeatability and inter-assay reproducibility told us that the average intra-assay relative standard deviation (RSDs) ranged from 3.3 to 6.9 % for all samples.

Result of comparative verification by HPLC: Several thiamethoxam-spiked environmental water samples were determined based on the process in sample pretreatment for HPLC analysis and the HPLC conditions in Instruments used for chromatographic analysis. The facts which the similar results were presented in Table-3 indicated us the developed ELISA of thiamethoxam residue in environmental water should be considered to satisfactorily and reliably determine thiamethoxam residue in environmental water and should be acted as a useful method to judge what excessive levels of thiamethoxam in environmental water.

TABLE-2
ANALYSIS OF THREE KINDS OF WATER SAMPLES SPIKED WITH THIAMETHOXAM AT DIFFERENT CONCENTRATIONS

Substrate	Spiked level (mg/L)	Average detected concentration (n = 6)	Relative standard deviation (%)	Recovery (%)
Intra-assay precision				
Tap water	0.01	0.0092	3.7	92.0
	0.50	0.4862	5.3	97.2
	5.00	4.8772	4.1	97.5
Lake water	0.01	0.0084	6.6	84.0
	0.50	0.4423	5.4	88.5
	5.00	4.5372	6.9	90.7
River water	0.01	0.0087	5.2	87.0
	0.50	0.4524	3.3	90.5
	5.00	4.6452	4.2	92.9
Inter-assay precision (seven separate days)				
Tap water	0.01	0.0094	5.7	94.0
	0.50	0.4662	4.3	93.2
	5.00	4.7772	3.5	95.5
Lake water	0.01	0.0104	6.8	104.0
	0.50	0.4314	5.3	86.3
	5.00	4.5017	5.1	90.0
River water	0.01	0.0084	5.1	84.0
	0.50	0.4501	4.3	90.0
	5.00	4.5854	3.1	91.7

TABLE-3
ANALYSIS OF THREE KINDS OF WATER SAMPLES SPIKED WITH THIAMETHOXAM AT DIFFERENT CONCENTRATION LEVELS

Substrate	Spiked level (mg/L)	Average detected concentration (n = 6)	Relative standard deviation (%)	Recovery (%)
Tap water	0.01	0.0095	4.7	95.0
	0.50	0.4897	6.3	97.9
	5.00	4.9320	7.1	98.6
Lake water	0.01	0.0088	9.4	88.0
	0.50	0.4781	6.1	95.6
	5.00	4.8317	5.4	96.6
River water	0.01	0.0097	6.8	97.0
	0.50	0.4762	5.3	95.2
	5.00	4.8438	4.4	96.9

Conclusion

This paper describes the performance of the newly developed kit-based monoclonal ELISA for the determination of the neonicotinoid insecticide thiamethoxam residue in environmental water. The assessed ELISA has enough sensitivity for the detection at the MRL levels of the insecticide for the tested samples. Furthermore, except clothianidin, although it showed slight cross-reactivity against imidacloprid, acetamiprid, nitenpyram and thiacloprid at the MRL levels could be determined without incurring a possible false-positive due to them. Finally, these results highlight the usefulness of the ELISA to screen the thiamethoxam residue in tap water, lake water or river water, because of the puny matrix inter-ference, samples can be conceivably analyzed after rapid and simple extraction with methanol and dilution of methanolic extracts prepared in minutes without the need for complicated sample clean-up and concentration procedures. These research findings suggest

that the proposed kit-based ELISA for thiamethoxam is not only rapid and simple analytical method, but also sufficiently accurate compared to the conven-tional chromatographic analyses. Therefore, it will greatly contribute to rapid and smooth distribution of safer agricultural products as an exceptional screening tool and it should also be considered as a confirmation means of the quantitative determination which was thiamethoxam residue in environ-mental water by HPLC or GC methods.

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