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Hapten synthesis, generation of polyclonal antibodies and development of ELISA for determination of thiamethoxam residues in food and environmental samples

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An ELISA (enzyme-linked immunosorbent assay) method for the determination of thiamethoxam residues has been developed. The method involves the synthesis of hapten and generation of polyclonal antibodies in rabbit using the hapten-protein (bovine serum albumin) conjugate carrier. Recovery experiments show the accuracy of estimation of thiamethoxam residues that was within the linear working range of 1 to 60 ng mL^{-1} with the r^2=0.992. Influence of different parameters, like detergent concentration, solvent, *p*H, ionic strength, has been studied. Cross reactivity was tested using different analogs. The method tolerates the solvent methanol concentration up to 15%. However, other polar solvents influence negatively on the binding. The detergent Tween 20 has not made any impact on the absorbance. The optimized ELISA for thiamethoxam used 1-60 ng/well and an antiserum dilution between 10000 and 200000. Recoveries were above 98%. The method was successfully applied for the analysis of food and environmental samples.

Keywords: ELISA, residues, thaimethoxime, water and food samples, hapten, polyclonal antibodies
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Introduction

Most of the officially accepted methods of pesticide residue analysis deal with the instrumental methods, such as gas-liquid chromatography and liquid chromatography coupled with mass spectrometry. These methods rely on highly skilled technical expertise capable of isolating the pesticide components from complex matrixes and subsequently their detection at very low levels. Even though the techniques are highly accurate and meet the USFDA and other federal requirements, the procedures involved in this are labour intensive and laboratory oriented. The developments in the analytical science during the past three decades promoted a new technique, namely enzyme-linked immunosorbent assay (ELISA) on the principle of antigen-antibody interaction[1-4]. ELISA technique was recognized as a valuable tool in residue analysis and complements the conventional analytical methods[5-10]. ELISA technique provides accurate and rapid sample testing and the generation of the results is more cost-effective when compared with conventional chromatographic analysis.

In immunoassay, synthesis of a suitable hapten is a very important phase. The design and synthesis of an appropriate hapten influences the sensitivity and specificity of the immunoassay. The production of antibodies specific for pesticides relies on an animal's immune response to the administered pesticide-protein conjugate. Hence, the effectiveness of the immuno-analytical method for monitoring the environmental contaminants depends on the immunochemical properties of the antibody. The high sensitivity and specificity of the technique have made it useful in variety of studies, such as environmental fate, persistence, residue analysis and in risk assessment studies[11]. The literature clearly shows that the ELISA techniques are successfully used for the quantitative analysis of number of pesticides in water, soil, food and in other substrates with little or no matrix interferences[12-19].

Among the new class of pesticides Nicotinoids represent a novel and distinct class under the category of insecticides with remarkable chemical and biological properties. One such compound thiamethoxam[20,21] is the first commercially available second-generation neonicontinoid and belongs to the thionicotinyl sub-class. Different formulations of the product are marketed under the trademarks Actara® for foliar application and for soil treatment and as

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Cruiser® for seed treatment. Chemically thiamethoxam was named as 3-(2-chlorothiazol-5-ylmethyl)-5-methyl-4-nitroimino-1,3,5-oxadiazinane. The compound with broad-spectrum insecticidal properties offers excellent control of a wide variety of commercially important pests in many crops. Low application rates, flexible application methods, excellent efficacy and favourable safety profile make the new insecticide well suited in modern agricultural practices.

Currently, there is no immunochemical method for the detection and quantification of thiamethoxam residues. Such an assay may result in a sensitive and cost-effective means of analyzing environmental samples containing thiamethoxam when compared to other techniques. In this paper, we report a new ELISA method for the successful screening of thiamethoxam residues in environmental samples using polyclonal antibodies. The method was optimized under different conditions and the data was compared with conventional HPLC technique.

Materials and Methods

Reagents

Reference analytical standard of thiamethoxam from Supelco, USA was used. The other chemicals used in the synthesis of hapten were obtained from Aldrich, Milwaukee, WI, USA. Bovine serum albumin (BSA), Keyhole limpet heamocyanin (KLH), goat anti-rabbit peroxidase IgG conjugate and Freund's complete and incomplete adjuvant were all obtained through Sigma–Aldrich Corporation, Banglore, India. Analytical grade solvents used in the study were obtained from Merck, Darmstadt, Germany. All other reagents used in the studies were also of analytical grade. Polystyrene ELISA plates were obtained from Coaster, Cambridge, MA and read spectrophotometrically with a micro plate reader Bio-Rad model 550 (Hercules, CA).

Synthesis of Haptene

5.82 g of thiamethoxam (20 m*M*) was taken in an Erlenmeyer flask containing 100 mL aqueous methanol. To this 1 g of iron powder and 10 mL of ammonium chloride were added and the mixture was refluxed for 24 h. The contents were filtered using a Whatmann 41 filter paper. The filtrate was concentrated under a stream of nitrogen and the residue was re-crystallized to yield 3.66 g of the compound (**1**) (Fig.1). The compound was characterized by NMR, IR spectral studies and by elemental analysis. The spectral characteristics are; ^{1}H-NMR-(250MHz, d_6-DMSO) 7.63 (s, 1H), 4.99 (s, 2H), 5.06 (s, 2H), 4.75 (s, 2H), 3.22 (s, 2H), 2.81 (s, 3H). IR- (ν_{max}, KBr) 1653, 1552. Elemental analysis - Calculated $C_8H_{12}ClN_5OS$; C, 36.71; H, 4.62; Cl, 13.55; N, 26.76; S, 12.25. Found: C, 36.68; H, 4.60; Cl, 13.51; N, 26.79; S, 12.23.

130 mg (0.5 m*M*) of the compound was dissolved in 5 mL of pyridine. To this 510 mg (5 m*M*) of succinicanhydride was added, stirred for 12 h at room temperature using a magnetic stirrer and allowed to settle for 30 min. Then 0.5 mL of 10% HCl was added. The solution was concentrated under a stream of nitrogen, resulting into the hapten (intermediate), compound (**2**). The compound was characterized by spectral studies and elemental analysis. The ^{1}H-NMR studies show the characteristic signals at (250MHz, d_6-DMSO) 9.7 (s, 1H), δ 7.61 (s, 1H), 4.51 (s, 2H), 5.03 (s, 2H), 4.72 (s, 2H), 2.84 (s, 3H) 1.89 (m, 4H). IR-(ν_{max}, KBr) 1720, 1648, 1552, 1421 and 1215. Elemental analysis-Calculated $C_{12}H_{16}ClN_5O_4S$; C, 39.84; H, 4.46; Cl, 9.80; N, 19.36; S, 8.86. Found: C, 39.79; H, 4.40; Cl, 9.76; N, 19.41; S, 8.84.

Preparation of Protein Conjugates

Hapten was covalently attached to the BSA/KLH proteins. For this purpose, 0.20 m*M* of compound (**2**) was dissolved in 2 mL of *N,N*-dimethylformamide (DMF). To this equimolar quantity of *N*-hydroxy-succinimide and a 10% molar excess of dicyclohexyl-carbodimide (DCC) were added. After vigorous shaking, the mixture was centrifuged and supernatant was collected. To this BSA or KLH solution (100 mg), dissolved in 5 mL of H_2O and 1 mL of DMF, was added. Mixture was stirred gently at 4°C for 24 h to complete the conjugation. Subsequently, it was dialyzed with 6 L of distilled water and stored in a deep freezer at 5°C.

Polyclonal Antibody Production

A solution containing 200 µg of immunogen thiamethoxim in 1 mL of 10 m*M* phosphate buffered saline (PBS; *p*H 7.6) and 1mL Freund's complete adjuvant was prepared. 1 mL of the solution was injected intramuscularly into rabbits weighing around 3 kg. The immunization was repeated at 21 d interval for four times. The rabbits were bled monthly and the serum was tested for anti-thiamethoxam antibody titre.

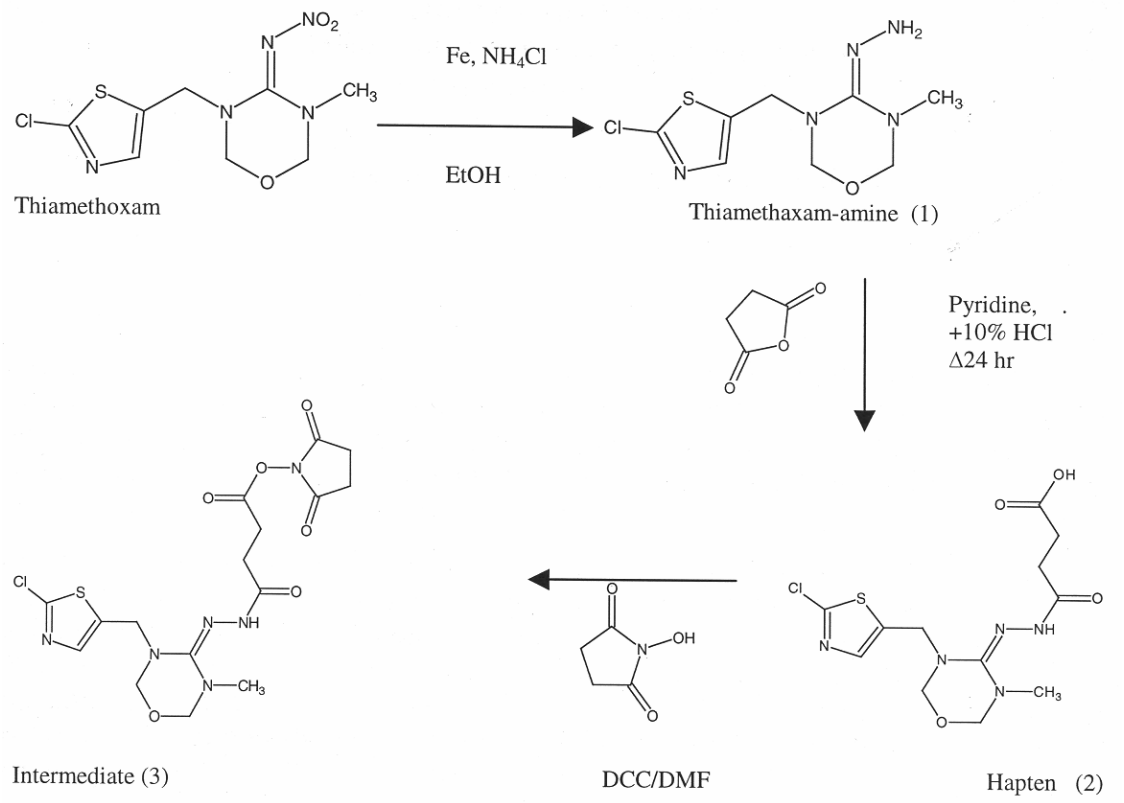


Fig. 1 — Scheme for synthesis of hapten

Antisera Titre Determination

Thiamethoxam specific antisera titres were performed with each of the bleeds collected from different rabbits. The Checkerboard assay[22] selected the combination of antiserum dilution and coating antigen conjugate concentration (Hapten-BSA) that would provide the greatest sensitivity in ELISA. The optimized ELISA for thiamethoxam used a coating antigen concentration between 1 and 60 ng/well and an antiserum dilution between 10000 and 200000.

ELISA

Ninety six-well micro titre plates were coated overnight by incubation at 4°C with 100 µL of the hapten–BSA conjugate in carbonated buffer solution. Sites not coated with the conjugate were blocked with a solution of 3% (w/v) skim milk and the *p*H of the blocking solution was adjusted to 7.6 using PBS. The plates were washed with PBS solution. After 2 h of incubation at room temperature the plates were rewashed. An aliquot of thiamethoxam analytical standard solution was mixed with diluted anti-thiamethoxam antibody on the mixing plate and incubated for 30 min at room temperature. The mixture was transferred into the wells (100 µL/well) and re-incubated for 2 h at room temperature for competition. To the washed plates, a quantity of 100 µL/well of a goat anti-rabbit-horseradish peroxidase IgG conjugate was added. After 30 min the plates were washed, and 100 µL of substrate solution (0.1 mL of 1% H_2O_2 and 0.4 mL of 0.6% 3,3',5,5'-tetramethylbenzidine) in dimehtylsulfoxide (DMSO) was added. To each well 25 mL of citrate-acetate buffer of *p*H 5.5 was added. After 20 min, the plates were read spectrophotometrically at 460 nm. The development of yellow colour was inversely proportional to the amount of thiamethoxam present. To identify the anitsera suitable for the ELISA, each antiserum produced by the four rabbits (two for each immunogen hapten-BSA/Hapten-KLH) were screened by the inhibition at two concentrations of the analyte thiamethoxam prepared in the assay buffer, using the homologous system. The inhibition ratio was calculated by the difference in absorbance between the buffer containing thiamethoxam and that without analyte. The antisera produced by the rabbits injected with the hapten–KLH conjugate showed almost no or very low inhibition ratios by the analyte thiamethoxam.

Results and Discussion

To determine thiamethoxam residues quantitatively and qualitatively in the environmental samples, it is essential to develop the ELISA with optimum sensitivity. The sensitivity of the ELISA is represented by the IC_{50} value, which is the concentration of the analyte thiamethoxam inhibiting the absorbance of the control by 50%. To optimize the method different parameters such as temperature, concentration of antibodies, conjugates, and their composition were tested. The amounts of both the anti-thiamethoxam antibody and the coating antigen (Hapten–BSA) were varied in an effort to determine the optimum conditions for the assay. Further to enhance the analytical sensitivity, the effect of pre-incubation time of the mixture of analyte and the antiserum on the mixing plate and the assay buffer related factors such as solvents, detergents, ionic strength and *p*H were also examined. The optimized ELISA for thiamethoxam used a coating antigen concentration between 1 and 60 ng/well and an antiserum dilution between 10000 and 200000 with the r^2=0.992. Effect of pre-incubation time on analyte and the antiserum makes no significant difference in the competitive assays. However, for experimental purpose a 30 min incubation time was selected.

Effect of *p*H

The *p*H dependence of the thiamethoxam ELISA method was studied in detail. Sodium phosphate buffer, Tris(hydroxy methyl amino methane)-HCl buffer and citrate buffer solutions were used over the range of *p*H 4.0-8.4. Results are summarized in Table 1. The affinity of antiserum towards the thiamethoxam and the coating antigen depended more on the *p*H. The IC_{50} values decreased more significantly in alkaline conditions with increase in *p*H. In view of the reported stability of the compound in acidic and neutral solutions and to prevent the destruction of antibody and hydrolysis of thiamethoxam, *p*H 7.6 was selected for further studies.

Table 1 — Effect of *p*H on the affinity of antiserum towards the thiamethoxam and the coating antigen

*p*H	A_{max} (A)	Slope (B)	IC_{50} (ng mL^{-1}) (C)	A_{min} (D)	A/C
4.4	0.810	0.630	60.8	-0.031	0.0133
5.6	0.802	0.690	43.2	-0.008	0.0186
6.4	0.794	0.615	25.6	-0.015	0.0310
7.6	0.799	0.706	20.7	0.004	0.0385
8.4	0.738	0.697	18.1	0.005	0.0407

ELISA conditions: Coating antigen, hapten-BSA (60 ng mL^{-1}); antiserum, rabbit (1:10000); preincubation of the analyte and the antiserum for 2 h on the mixing plate; goat anti-rabbit IgG (1:10000). Analyte and antiserum were doluted in 1×PBS of different *p*H values. Data are the means of the quadruplicate.

Effect of Different Solvents

Performance of ELISA in the presence of different polar solvents was tested. Solvents like methanol, ethanol, acetone, acetonitrile, 2-propanol, DMF and DMSO were studied for this purpose. The data is presented in Fig. 2. Methanol when used as solvent, the absorbance values increased with percentage of solvent. However, when tested with all other solvents there was clear decrease in absorbance. The decrease in IC_{50} values of thiamethoxam indicates that the binding capacity of antibody and the hapten is influenced by the organic solvents. For experimental purpose, studies were conducted with 15 % methanol.

Effect of Nonionic Detergent

Influence of the nonionic detergent Tween 20, which is generally used for the immunoassays to reduce the nonspecific binding and improve the sensitivity[23], was studied in detail. During the study, *p*H of the solution was maintained at 7.6. Data show that the increase in the concentration of nonionic detergent Tween 20 decreased the absorbance value. Hence, further studies were conducted without the addition of any nonionic detergent.

Influence of Salt Concentration

To establish the optimal conditions, the influence of ionic strength at the *p*H value 7.6 was studied. The data presented in Fig. 3 show that the increase in salt concentration in the assay brought a decrease in the absorbance value. Further, this did not increase the assay sensitivity $IC_{50.}$ For an optimal ELISA of

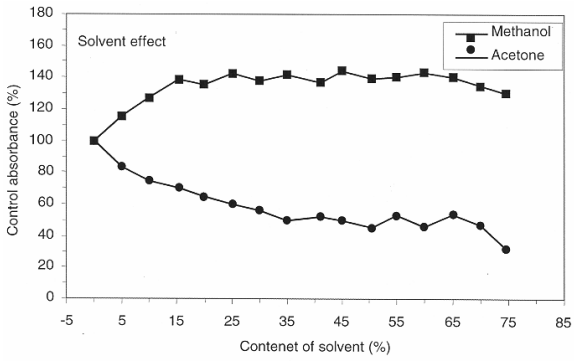


Fig. 2 — Effect of solvent methanol and acetone on binding capacity of hapten.

thiamethoxam, the assay buffer containing 0.5 *M* NaCl without Tween 20 and with an organic solvent methanol up to 15% was suitable for the competition between the antibody and the target analyte or the immobilized coating antigen.

Cross Reactivity

To establish the selectivity of the assay, the potential for inhibition of thiamethoxam antibodies by structurally related compounds was studied at different concentrations 1 to 25000 ng mL^{-1}. The optimized ELISA for thiamethoxam was used to examine the cross-reactivity of the antibody. Standard curves were plotted and data was fitted into four-parameter (sigmoidal) equation[24].

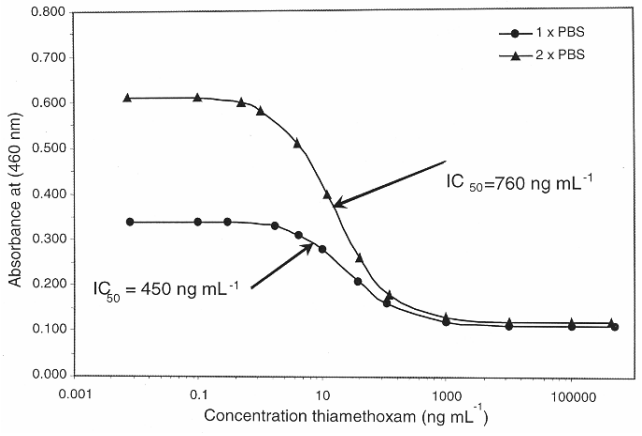


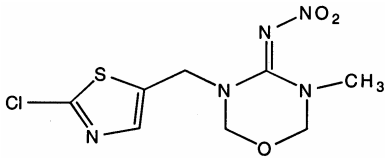
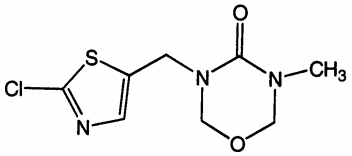
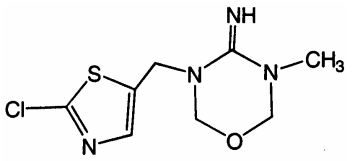
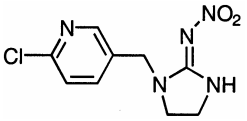
Fig. 3 — Influence of salt concentration on binding capacity of antibody and hapten.

The cross reactivity values were calculated as the ratio of the IC_{50} values of the thiamethoxam standard to the test compound in terms of percentage. The data presented in Table 2 show the percentage of cross reactivity of thiamethoxam with respect to various compounds. Apart from the above data, a series of experiments were conducted with structurally similar agrochemicals, mainly insecticides. No cross reactivity was found with respect to pyridinyl pesticides Acetamprid, Imidacloprid, Thiazopyr, thiazolyl pesticides Thiacloprid, Thiabendazole, Sulfonylurea herbicides Chlorimuron-methyl, Metsulfuron-methyl, Rimsulfuron, Tribenuron-methyl, and Bromacil, Carbendazim, Diazomet, Difenconazole and Tricyclazole.

Analysis of Thiamethoxam Residues in Spiked Samples by ELISA

Agricultural produce (tomato) and water samples were tested for the suitability of the ELISA method. 50 g of tomato sample was homogenized using 100

Table 2 — Cross-reactivity of some structurally related compounds to the rabbit D antiserum in the assay of thiamethoxam

Compound	Structure	CR[a] (%)
Thiamethoxam		100
Metabolite 1 3-(2-chlorothiazol-5y1methyl)-5-methyl-[1,3,5]oxadiazinan-4-one.		147
Metabolite 2 3-(2-chlorothiazol-5y1methyl)-5-methyl-[1,3,5]-oxadiazinan-4-ylidene-amine.		7.4
Metabolite 3 Imidacloprid		154

a = Percentage cross reactivity is determined by calculating the ratio of the amount of compound required to displace 50% of the enzyme conjugate to the amount of thiamethoxam

Table 3 — Analysis of tomato extract and ground water samples spiked with thiamethoxam

Substrate	Thiamethoxam		Mean	
Sample fortified	Spiked (ng mL^{-1})	Recovery (ng mL^{-1})	(%, n= 3)	(% of CV)
Tomtato extract	0	ND[a]	-	-
	2	2.02	101	3
	5	5.25	105	6
	10	10.11	114	4
	25	24.61	98	9
	40	38.75	97	4
	60	56.55	94	8
Ground water	0	ND	-	-
	2	2.08	104	2
	5	5.06	101	1
	10	9.86	99	6
	25	24.33	97	8
	40	38.69	97	9
	60	58.65	98	6

ND = Not detected

mL of methanol using a high speed homogenizer for 2 min. Residue was concentrated by removing excess of solvent and collected quantitively. Concentrate was transferred into 10 mL volumetric flask and made to the volume with 1×PBS (*p*H 7.6). For the recovery test, six levels of thiamethoxam concentrations 2, 5, 10, 20 25, 40 and 60 ng mL^{-1} were prepared and analyzed. An un-spiked control was also maintained for comparison.

As an environmental sample, natural water was collected from an isolated source and confirmed that it was free from pesticide contamination. It was filtered and spiked with different concentrations of analytical standard solution and diluted 10 times with PBS to *p*H 7.6 and subjected to ELISA. The recovery of thiamethoxam in water and tomato samples by ELISA was in the range of 92-98% and the results presented in Table 3 were comparable with HPLC analysis. 45 ground water samples and 25 tomato fruit samples collected from the local markets were analyzed by using the method developed. None of the samples showed the residues.

Conclusion

In this present study, a carboxylic hapten was synthesized by reducing the nitro group of thiamethoxam to an amino group. The resulting amino group was reacted with succinicanhydride at room temperature to give the carboxylic hapten. The hapten was proved to be a useful immunogen and no significant cross reactivity was observed when tested for several compounds. The method is sensitive and selective in determination of the residues of thiamethoxam up to 1 ng mL^{-1}. The method accurately estimates the residues of thiamethoxam within the linear working range 1 to 60 ng mL^{-1} with the r^2=0.992 and the detection limits are well down the USEPA requirement. Thus, the ELISA method developed successfully demonstrated its applicability in quantification of residues of thiamethoxam in environmental samples.

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