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Optimization and Validation of HPLC Based Analytical Method for Simultaneous Determination of Thiamethoxm Residues

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In present study, RP-HPLC-DAD protocol was optimized and calibrated over 0-50 µg/mL of standard thiamethoxam and subsequently applied to determine its residues in selected fruits (guava and citrus) and vegetables (cauliflower, tomato and okra) after 0, 1, 7, 14, 21 and 28 days of foliar spray (0.5 mg/plant). The results regarding method validation indicated that optimized HPLC-DAD method was linear over broad range of thiamethoxam concentration (0-60 µg/mL) with recovery rates 79.64-89.30 %. Furthermore, the incidence level of thiamethoxam in selected fruits (guava and citrus) and vegetables (tomato, cauliflower and okra) was found to be higher (0.3-0.89 µg/g) than maximum residue limit (MRL) established by Codex Alimentarius Commission (0.5 µg/g) after 0 and 1 day of foliar spray. While thiamethoxam residues observed after 7, 14 and 21 of foliar application were found to be within permissible limits. The persistence of thiamethoxam in selected fruits and vegetables decreased in the following order: okra, citrus, guava, cauliflower and least in tomato. Overall, it was speculated that optimized method might be quick, easy, cheap, effective, rugged and safe (QuEChERS) choice for thiamethoxam analysis of food commodities.

Keywords: RP-HPLC-DAD, Thiamethaxam, Validation and Optimization, LOD/LOQ, Fruits and vegetables.

INTRODUCTION

High performance liquid chromatography (HPLC) has become a versatile and state of the art tool for the characterization and quantification of valuable food ingredients *i.e.*, phenolics[1,2], organic acids[3], toxins[4,5] and insecticides[6,7]. Optimization and validation of analytical method are the key elements to demonstrate the scientific soundness of developed protocol. The appropriate selection of organic solvent for analyte extraction is very important for competent analytical protocol to remove potential interferences from the samples[8,9]. The extraction solvent must be compatible with the nature (polar or non-polar) and structure of analyte[10].

Thiamethaoxam (TMX): (EZ)-3-(2-chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazinan-4-ylidene(nitro)amine (Fig. 1) is a recently developed nitro-substituted neonicotinoid that acts as agonistically on insect nicotinic acetylcholine receptors (nAChR) to paralyze central nervous system (CNS) of sucking and chewing pests and being more selective towards insects and commercialized all over the world[8,11,12]. Although pesticides play a significant role in crop protection and quality preservation of food commodity at various stages of cultivation

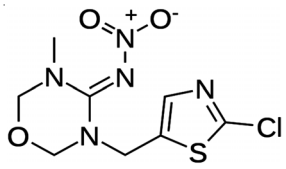

Fig. 1. Generic representation of thiamethoxam

but their environmental residues especially in water and air execute chronic disorders on aquatic biota and aves[13]. This condition as well as other hidden hazards regarding insecticide occurrence imposed unbearable economic losses to manufacturers, handlers and producers of food and feed products. Therefore, it would be of practical approach to detect and quantify toxic neonicotinoid (NEO) insecticide residues in fruits and vegetables, which would ultimately require an easy, economic and reliable analytical protocol[12]. The present study was aimed to optimize and validate reverse phase high performance liquid chromatography (RP-HPLC) based protocol for quick, easy, cheap, effective, rugged and safe (QuEChERS) detection and quantification of thiamethaxom residues in selected fruits and vegetable.

EXPERIMENTAL

Thiamethoxam a neonicotioid insecticide marketed under the trade name ACTARA (Syngenta, Pakistan) was selected for the present study. Fruit bearing trees (citrus and guava) were chosen from Nuclear Institute for Agriculture and Biology (NIAB) and Postgraduate Agriculture Research of Sciences (PARS), University of Agriculture, Faisalabad. Vegetables including tomato, cauliflower and okra were grown in the agricultural farms of Nuclear Institute for Agriculture and Biology.

Thiamethoxam standard was purchased from Fluke (ST. Louis, USA) whereas Merck (Germany) supplied HPLC grade solvents including methanol, acetonitrile, acetic acid and ethyl acetate. Ultra-pure deionized water used for HPLC solvent was of Victor lines diagnostics. Nylon Filters (pore size, 0.45 μm) Millipore (USA), clean up column and cartridges used were provided by Supelco Park, Bellefonte, USA.

Preparation of mobile phase: Acetonitrile and water (in variable ratios) mixed with 50 μL of 0.1 M phosphoric acid (pH 3) was used as mobile phase. The pH of mobile phase was as adjusted to 3.0 with 10 % glacial acetic acid before mixing with acetonitrile. The resultant mobile phase was filtered through 0.45 μm nylon filters (Millipore, USA) fitted in filtration assembly and degassed by sonication (Ulltech, USA) before using in the HPLC system.

Preparation of standard solutions: The stock solution of standard thiamethoxam (1 mg/mL) was prepared in acetonitrile and water (70:30 v/v). The working standards of different concentrations (2.5, 5, 10, 25 and 50 μg/g) were prepared periodically by diluting the stock solutions with HPLC solvent.

HPLC method validation: The standard solutions thiamethoxam were run under different column conditions with isocratic solvent (acetonitrile and water of variable composition) until well-resolved peak of the standard were obtained.

Linearity: The linearity of the method was accomplished within the concentration range of 0-50 μg/mL of thiamethoxam. Five different concentrations 2.5, 5, 10, 25 and 50 μg/g of standard thiamethaoxam were injected in triplicate and plotted against HPLC detector response.

Limits of detection and limits of quantification: Limit of detection (LOD) was calculated when the signal to noise ratio was 3:1. The limit of quantification (LOQ) with acceptable accuracy was determined with a signal to noise 10:1.

Spiking of fruits and vegetables with insecticide: To determine the recovery rates, precision and accuracy, 10 g fresh and healthy fruits and vegetable were spiked with 0.1 and 1 μg/g of standard thiamethoxam. The spiked samples were kept at room temperature for 0.5 h and then processed for further analysis.

Extraction and cleanup of thiamethoxam residue: For the extraction of thiamethoxam, a reported procedure by Wang *et al.*[10] with some modifications was adopted. Briefly, 10 g homogenized plant material were mixed with 50 mL of acetonitrile containing 1 g anhydrous sodium sulphate and 0.5 g sodium chloride for salting out specific analyte from food matrix. The mixture was shaken in an orbital shaker for 45 min and residues were filtered through 0.45 μm filter paper (Millipore). The filtrates containing residues were further cleaned up using florisil column and activated charcoal. The final solution was evaporated in rotary evaporator (EYELA N-N series, Japan) under reduced pressure and concentrated with help of N_2 stream. The residues were re-dissolved in acetonitrile before injection into HPLC system.

Accuracy: The accuracy of the method was evaluated by spiking known amounts of selected insecticides. The obtained results were compared with the theoretical concentration. For this purpose, 0.1 and 1 μg/g of thiamethoxam was injected before extraction. Each concentration was made in triplicate.

Precision: Precision of the proposed method was expressed in terms of % RSD. The within day precision was based upon the results of five replicate analysis of three different concentration of analytes on a single day. The between-day precision was determined from the same samples analyzed for five consecutive days.

Selectivity: The selectivity of the proposed method was checked by making mixture of selected insecticide and excipients. The mixture was shaken well with 70 mL mobile phase and then 1 mL of this filtrate was transferred into 25 mL volumetric flask and mobile phase was then added to volume to obtain a final solution containing 10 μg/g which was injected to HPLC under developed conditions.

HPLC conditions for determination of neonicotinoid residues: The samples containing insecticide residues were finally injected to HPLC system, LC-10A (Shimadzu, Japan) through syringe (Injection Loop = 20 μL) for their qualitative and quantitative characterization under following pre-optimized HPLC conditions. Thiamethoxam was eluted using analytical column C18, (Discovery, Supelco) having dimensions 250 × 4.6 mm, 5 μm, with mobile phase CH_3CN: H_2O (30:70) slightly acidified (50 μL of 0.1 M H_3PO_4). The column was maintained at 30 °C and mobile phase was programmed flow rate 1.4 mL/min at 145 Kg/cm^2 pressure. The eluted thiamethoxam was detected at 280 nm and quantified using HPLC data acquisition Software CLASS LC–10A.

Spraying of vegetables and fruits: Healthy fruits (guava and citrus) and vegetables (tomato, cauliflower and okra) were firstly tagged and then sprayed with recommended dose (0.5 mg/plant) of thiamethoxam. For this purpose, a commercially available insecticide formulation (ACTARA, Syngenta, Pakistan) was sprayed on selected fruits and vegetables.

Collection of fruit and vegetable samples: Random samples of fruits (guava and citrus) and vegetables (cauliflower, tomato and okra) were collected at different time intervals (0, 1, 7, 14, 21 and 28 days) after spraying to assess the fate and the residual concentrations of thiamethoxam under field conditions.

Preparation of samples for thiamethoxam contamination: Harvested samples of fruits and vegetables were brought to the Food Toxicology Lab, Nuclear Institute for Agriculture and Biology. Skin and pulp portion of fruits and vegetables were separated and preserved at -4 °C in airtight polythene bags for further processing. Extraction of thiamethoxam and analysis was carried out under optimized conditions as described above.

Statistical analysis: Triplicate runs were made for each experiment to report data as mean ± SD. A probability level (*p*

< 0.05) was used to denote the statistically significant variation in thiamethoxam level in different selected fruits and vegetables[14]. All statistical tested were conducted using Minitab 16 statistical software and Microsoft Excel 2010.

RESULTS AND DISCUSSION

Analytical method validation is a prerequisite of any reliable chromatographic study. Validation of analytical protocol should be regulated by any international or national standards, synchronized procedure like IUPAC/AOAC/ISO and may be successively implemented as official international methods for international collaborative study. The analytical parameters recommended for method validation include instrumental precision, linearity of the calibration curve, selectivity and sensitivity of solute detection, inter-day and intra-day reproducibility, limit of detection, limit of quantification, recovery percentage, robustness and ruggedness[15]. The data obtained regarding validation of undertaken chromatographic technique has been assembled in Table-1 and elaborated in the subsequent sections.

HPLC method development and optimization: The HPLC conditions for analysis of thiamethoxam were optimized to establish separation using single C18 column in isocratic mode (concentration of solvent to elute analyte remains constant). Different mobile phases under various column conditions (temperature, pH, flow rates) and detection wavelengths were checked to analyze standard thiamethoxam for suitable run time. Initially variety of mobile phases and stationery phases were tested to obtain the best separation and resolution. Method development was started with less polar mobile phase (50 % acetonitrile). However no peak was obtained at acceptable retention time. The polarity of the mobile phase was then enhanced by the addition of water and 1 M phosphoric acid. Finally, acetonitrile and water in the ratio of 70:30 (v/v) with 50 µL of 0.1 M phosphoric acid at flow rate of 1.4 mL/min and pH 3 produced quit sharp peak of selected thiamethoxam with C18 column (Supelco Discovery, USA).

Validation of HPLC method: The developed chromatographic based method for the simultaneous determination of thiamethoxam was validated following IUPAC/AOAC/ISO regulations and recommendations. For this purpose, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision selectivity and specificity were investigated.

Linearity: The linearity of developed method was checked over concentration range 0-50 µg/mL of standard insecticide. The results observed in this context have been interpreted in Fig. 2. Thiamethoxam concentration was injected in triplicate (2.5, 5, 10, 25 and 50 µg/mL) plotted against observed HPLC-DAD response (peak area mV).

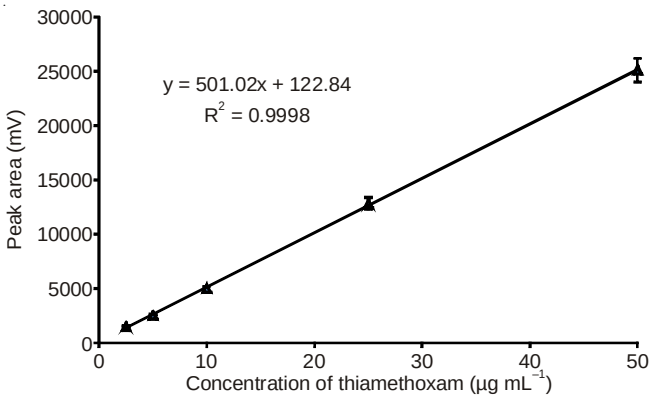

Fig. 2. HPLC-DAD calibration curve of thiamethoxam for concentration 0-50 ppm

Detector response of thiamethoxam expressed in Fig. 2, has been represented with linear regression equation Y = 501.02x + 122.84. The co-efficient of determination observed in this regard was 0.9998, which revealed that there exist good linear relationship between the concentration of analyte injected and detector response (peak area = mV) observed.

Hence, the data regarding calibration of developed method inferred that adopted chromatographic method has been efficiently applied to determine thiamethoxam residues in the concentration range of 0-50 (µg/mL). The published reports indicate that no data available regarding HPLC analysis of thiamethoxam while Ko *et al.*[7] determined that imidacloprid and its metabolite 6 chloro nicotinic acid were to be linear over wide range of concentration (0-50 mg/mL) but its regression coefficient (R^2) of 0.9991 was smaller as compared to those observed during present work (0.9998).

Limit of detection and limit of quantitation: The analytical instrument was calibrated daily with mixture solutions of thiamethoxam and limit of detection (LOD) and limit of quantification (LOQ) for thiamethoxam determined at signal-to-noise ratio (S/N) of 3:1 and 10:1 were found to be 0.02 and 0.06 ppm, respectively.

Accuracy and precision: To determine accuracy and precision of the developed methodology between day (inter-day) and within day (intra-day) analysis of the thiamethoxam samples were conducted in duplicate for five days and results obtained have been incorporated in Table-2. The values of repeatability (RSD_r) and reproducibility (RSD_R) were in the range of 3-5. These reported values were concordant with other neonicotinoid insecticides determined by Dankyi *et al.*[16].

Selectivity: The selectivity of the developed method was checked by making mixtures of thiamethoxam with some contaminants and injected to HPLC. The obtained chromatogram when compared with the standard one of thiamethoxam was not showed any kind of interference or co-eluting peaks. More than 85 % of the recovery authenticated that extraction solvent

TABLE-1
ANALYTICAL PARAMETERS EXECUTED FOR RP-HPLC-DAD DETERMINATION OF THIAMETHOXAM

Neonicotinoid insecticide	Conc. (µg/mL)	Calibration curve	R^2	LOD (µg/mL)	LOQ (µg/mL)	Precision (%)	
						RSD_r	RSD_R
Thiamethoxam	0-50	y = 501.02x + 122.84	0.9990	0. 02	0.06	3	5

RSD = Relative standard deviation, r = Repeatability, R = Reproducibility

TABLE-2
MEAN RECOVERIES, INTRA-DAY ASSAY (RSD_r), REPRODUCIBILITY (RSD_R) OF THIAMETHOXAM (TMX) AT DIFFERENT SPIKING LEVELS OF SELECTED FRUITS AND VEGETABLES USING RP-HPLC-DAD

Sample		Mean	RSD_r	RSD_R
NEO	Spiked level (µg/mL)			
		Tomato		
TMX	0.10	89.30	6.6	5.9
	1.00	84.66	3.5	4.2
		Okra		
TMX	0.10	78.96	8.6	8.9
	1.00	79.26	5.1	6.1
		Cauliflower		
TMX	0.10	83.47	7.9	3.4
	1.00	81.08	3.2	6.2
		Citrus		
TMX	0.10	80.75	8.3	2.5
	1.00	79.64	4.6	7.1
		Guava		
TMX	0.10	86.17	5.1	3.5
	1.00	83.10	2.1	2.2

Where mean denotes recovery of thiamethoxam from spiked samples.

and mobile phase was proved most compatible choice for the effective detection and quantification of thiamethoxam residues in agricultural resources.

Extraction efficiencies: Extraction is a critical sample preparation step, which decisively affect the efficiency of intended chromatographic method. The appropriate selection of organic solvent in sample pretreatment procedure to extract the analyte of interest, is most favourable process to recover efficiency and remove potential interferences from the samples[10,17]. The extraction method always depend on structure of analyte as well as elution of neonicotinoids from biological matrix and necessitate polar organic solvent (acetonitrile) for extraction purposes due to polar nature of these pesticides.

Thiamethoxam spiked fruits and vegetables were extracted with acetonitrile and water (70:30 v/v) containing anhydrous sodium sulphate and sodium chloride for salting out specific analyte from food matrix. The data obtained regarding thiamethoxam recovery from spiked fruits and vegetables samples has been elaborated in Table-2. The results indicated that overall recovery percentage of thiamethoxam when spiked at level of 0.1-1.00 µg/mL was in the range of 78.96-89.30 % with relative standard deviation (RSD_r) 1.20-0.076. It was observed that recovery rate of thiamethoxam was higher (89.30 %) for tomato and lower in okra (78.96 %) at spiking level of 0.1 µg/mL. Thiamethoxam recoveries 78.96-89.30 % with inter-day assay (RSD_r) and reproducibity (RSD_R) values were observed as 5.1-8.6 and 2.5-8.9 at 0.1 (µg/g) spiking level, respectively. While at spiking of 1 (µg/g) of sample, RSD_r and RSD_R values were in the range of 2.1-5.1 and 2.2-7.1, respectively indicated appreciable recovery and precision as recommended by DG SANCO guidelines[18]. Furthermore, data obtained regarding recovery of thiamethoxam (Table-2) revealed that selected insecticide was not affected by interfering compounds present in fruit and vegetable and inferred excellent extraction efficiency of solvent (acetonitrile and water) to trap neonicotinoid insecticide.

RP-HPLC-DAD determination of thiamethoxam contamination: The fruits and vegetables samples, randomly harvested after 0, 1, 7, 14, 21 and 28 days after foliar spray of thiamethoxam (0.5 mg/plant) were extracted using above mentioned solvent system, cleaned-up and injected to RP-HPLC-DAD system under optimized conditions. Typical chromatograms produced (Fig. 3) in this context was shown a sharp and reproducible peaks at retention time of 3.8 min. The results of triplicate analysis (mean ± SD) as assembled in Table-3 revealed a significant decline in thiamethoxam residues with delay of harvesting period.

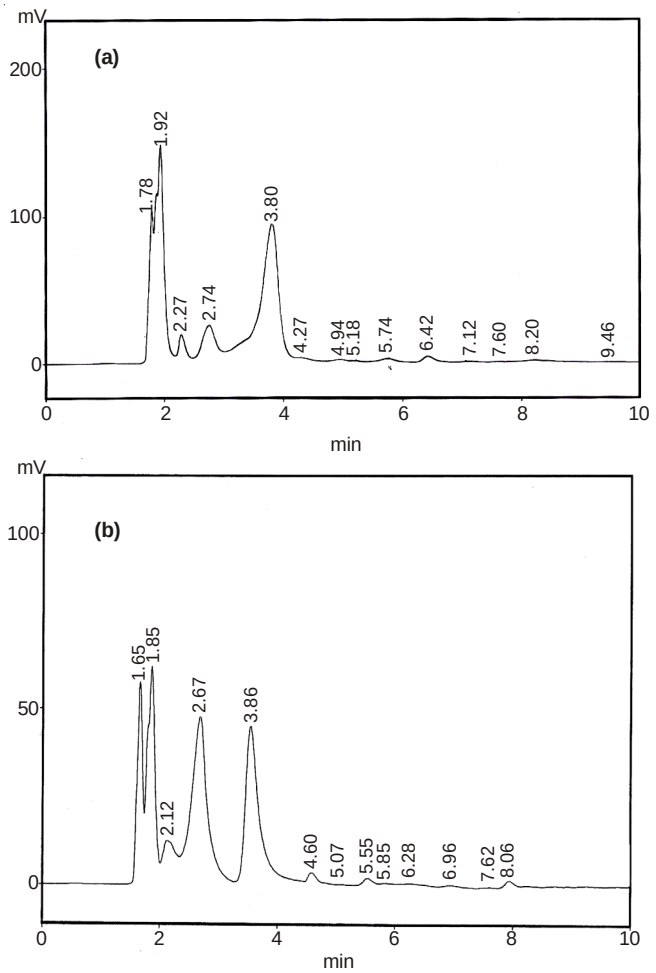

Fig. 3. Typical chromatogram of thiamethoxam in citrus (a) and tomato (b) at Rt 3.86 min

It is evident from the data that thiamethoxam was deposited initially (0 days) in tomato, okra, cauliflower, guava and citrus at levels 0.63 ± 0.02, 0.89 ± 0.03, 0.85 ± 0.03, 0.78 ± 0.03 and 0.71 ± 0.02 (µg/g), respectively. After 1 day of foliar spray the observed thiamethoxam residues in tomato, okra, cauliflower, guava and citrus were found to be 0.30 ± 0.01, 0.55 ± 0.02, 0.63 ± 0.02, 0.45 ± 0.02, 0.58 ± 0.02 (µg/g), quite higher than Codex Alimentarius Commission[19] permissible limits (0.5 µg/g) while fruits and vegetables harvested after 7 days of foliar application were regarded as safe with thiamethoxam level of 0.28 ± 0.01, 0.35 ± 0.01, 0.30 ± 0.01, 0.03 ± 0.01, 0.36 ± 0.01 and 0.42 ± 0.01 (µg/g) for tomato, okra, cauliflower, guava and citrus, respectively.

TABLE-3
PERSISTENCE OF THIAMETHOXAM (SPRAYED CONCENTRATION 0.5 mg/plant) IN SELECTED FRUITS AND VEGETABLES

Harvest time (days)	Tomato (µg/g)	Okra (µg/g)	Cauliflower (µg/g)	Guava (µg/g)	Citrus (µg/g)
0 (1 h)	$0.63 \pm 0.02^{d}_{a}$	$0.89 \pm 0.03^{d}_{d}$	$0.85 \pm 0.03^{d}_{c}$	$0.78 \pm 0.03^{d}_{c}$	$0.71 \pm 0.02^{d}_{b}$
1	$0.30 \pm 0.01^{b}_{a}$ (51.61)	$0.55 \pm 0.02^{c}_{d}$ (38.02)	$0.63 \pm 0.02^{c}_{d}$ (25.88)	$0.45 \pm 0.02^{c}_{b}$ (42.3)	$0.58 \pm 0.02^{c}_{c}$ (18.3)
7	$0.28 \pm 0.01^{b}_{a}$ (54.83)	$0.35 \pm 0.01^{b}_{d}$ (60.67)	$0.3 \pm 0.01^{b}_{a}$ (64.7)	$0.36 \pm 0.01^{c}_{b}$ (53.84)	$0.42 \pm 0.01^{b}_{c}$ (40.84)
14	$0.12 \pm 0^{a}_{a}$ (82.25)	$0.25 \pm 0.01^{a}_{d}$ (71.91)	$0.28 \pm 0.01^{b}_{d}$ (67.05)	$0.29 \pm 0.01^{b}_{d}$ (62.82)	$0.11 \pm 0.01^{a}_{a}$ (84.5)
21	$0.07 \pm 0^{a}_{a}$ (88.7)	$0.04 \pm 0.01^{a}_{a}$ (94.38)	$0.11 \pm 0.01^{a}_{d}$ (88.23)	$0.02 \pm 0.01^{a}_{d}$ (98.71)	$0.02 \pm 0.01^{a}_{d}$ (98.59)
28	$0.03 \pm 0.01^{a}_{d}$ (96.77)	< LOD	< LOD	< LOD	< LOD

Values are mean ± SD of triplicate experiments. Alphabets superscripted in a column and superscripted in row indicate significant variation in observed pesticide concentration at different time intervals and among selected fruits and vegetables, respectively. Values in parenthesis indicate percentage dissipation.

Maximum dissipation level (51.61-96.77 %) was observed in tomato followed by guava (42.3-100 %), okra (38.02-100 %), cauliflower, (25.88-100 %) and citrus (18.3-100 %) as presented in Table-3.At the end of harvesting interval, only tomato contained 96.77 % dissipation while all other fruits and vegetables showed 100 % as no pesticide residues were detected below limit of detection for thiamethoxam *i.e.* 0.5 (µg/g)[19]. When compared with previously reported studies, the observed values of thiamethoxam residues during present work were comparable with those investigated by Singh and Kulshrestha[20] in study of okra such as 0.47 ± 0.07, 0.30 ± 0.02, 0.023 ± 0.01 (µg/g) at 0, 1 and 7 days intervals, respectively.

Conclusion

In present work, RP-HPLC-DAD protocol was optimized, validated and applied to selected fruits and vegetables for the determination of thiamethoxam. The observed results speculated that thiamethoxam residues in selected fruits and vegetable during initial harvest intervals (0 and 1 day) were higher than Codex Alimentarius Commission. However, fruits and vegetables harvested after 7 days of foliar spray were found to be relatively safe from health point of view. Furthermore, the optimized RP-HPLC-DAD based method might be regarded as QuEChERS choice to analyze thiamethoxam residues in agriculture and food commodities.

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