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Research Article

Development and validation of QuEChERS method for neonicotinoids in cotton

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Abstract

Simple and efficient analytical method was standardized and validated for the estimation of residues of three neonicotinoids viz. imidacloprid, thiacloprid and thiamethoxam on cotton leaves, flowers, nectariferous tissue and pollen. The samples were spiked with the standards at 0.01, 0.25 and 0.50 mg/kg levels. Acetonitrile was used for the extraction of the pesticides from the respective commodity and QuEChERS method involving the use of dispersive-solid phase extraction (DSPE) was employed for the sample clean-up. The clear extract in acetonitrile obtained was further used for the quantification of all the three pesticides with High Performance Liquid Chromatography (HPLC) equipped with PDA detector and C18 column. HPLC grade acetonitrile, water (70:30, v/v) was used as a mobile phase at 0.30 ml/min. Distinct peaks at retention time of 16.07, 22.24 and 13.06 min were obtained for imidacloprid, thiacloprid and thiamethoxam, respectively. The consistent recoveries above 80 percent were obtained for all the substrates at different spiking levels. Selectivity, linearity, lower limit of quantitation, precision and accuracy were also evaluated during the validation of the methodology.

Keywords cotton, HPLC, neonicotinoids, QuEChERS, validation

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Introduction

Neonicotinoids are novel class of systemic insecticides that affects the central nervous system of sucking and piercing insects, resulting in paralysis and death of the insect [1]. In present agriculture regime, they are dominating the market because of their high systemicity, broad spectrum of action and reduced dose requirement [2]. The class is also playing a crucial role for the maintenance of cotton crops fighting against the various insects degrading the quality and quantity of the crop yield. The only problem that questioned their use is their effect on pollinators [3-4]. Iwasa reported that the accumulation of neonicotinoid residues in flower (pollens and nectar) leads to decrease in populations of pollinators especially honeybees and it has raised a serious concern all over the world [5]. To validate the fact, it has become necessary to measure how much residue of neonicotinoids is actually persisting in the crop that is said to be affecting the population of these pollinators.

Several studies have been employed to quantify and detect the presence of neonicotinoids in different vegetables [6] and other food commodities [7]. Simple and modified QuEChERS method were developed as well as validated for sample preparation of various neonicotinoids in honey samples [8]. The estimation of neonicotinoids in cotton seed, cotton field soil and pollen [9-10] was also reported but little data

is available [11-12] for estimation of all the three selected neonicotinoids in cotton leaves, flowers, nectariferrous tissue and pollen using a single and simple QuEChERS technique. The present study was designed with the main aim of standardizing the precise method of estimation, detection and confirmation of three widely used neonicotinoids- imidacloprid, thiacloprid and thiamethoxam in cotton (leaves, flowers, nectariferrous tissue and pollen) using the QuEChERS technique that can be used for the quantitative analysis of the residue by HPLC in all the four selected matrices of cotton.

Methodology

Standards and reagents

The technical grade analytical standard of Imidacloprid (purity 99.9%), Thiacloprid (purity 99.9 %) and Thiamethoxam (purity 99.0 %) was obtained from M/S Sigma-Aldrich. Solvents like water (HPLC grade), acetonitrile (HPLC grade) and reagents like sodium chloride (NaCl), anhydrous sodium sulfate (Na_2SO_4) were obtained from SRL Pvt Limited, New Mumbai, India. Anhydrous magnesium sulphate ($MgSO_4$), Primary Secondary Amine (PSA) bondesil 40 µm, and Carbon SPE bulk sorbent was obtained from Agilent Technologies, USA. Before use, anhydrous sodium sulphate (Na_2SO_4) and anhydrous magnesium sulphate ($MgSO_4$) were purified with acetone and baked for 4 h at 600°C in muffle furnace to remove possible phthalate impurities. All common solvents were redistilled in glass apparatus before use. The suitability of the solvents and other chemicals was ensured by running reagent blanks before actual analysis.

High-performance liquid chromatograph (HPLC)

The reversed phase HPLC (Model SPA-M20A) equipped with C_{18} column and photodiode array (PDA) detector, dual pump was used for the estimation of neonicotinoids on different matrices. The HPLC column, a Luna 5 µm C_{18} column (250 × 4.6 mm size) was obtained from Agilent Technologies (India). HPLC was equipped with LC-20AT pump and CBM-20A system controller. For instrument control, data acquisition and processing, LC Solution software was used.

Standard solution

The standard stock solutions of all the three test pesticides (1 mg/mL) were prepared in HPLC grade acetonitrile. Serial dilutions were further made from the stock solutions to get the concentrations viz. 0.01, 0.05, 0.10, 0.25, 0.50 and 1.00 µg/mL with HPLC grade acetonitrile required for preparing the calibration curves. All standard solutions were stored at –4°C before use.

Sample collection and QuEChERS sample preparation

Leaves, flowers, nectariferrous tissues and pollens of cotton crop collected from the experimental fields of Punjab Agricultural University, Ludhiana, Punjab, India were used as substrates for standardization of the methodology for estimation of neonicotinoids. The samples were chopped and blended in warring blender and prepared by following the slightly modified QuEChERS method as per the suitability for the commodity.

Leaf samples

A sample of 10 g chopped and macerated leaves were weighed into a 50 mL centrifuge tube and spiked at different test levels (0.5, 0.25 and 0.01 µg/ml). The tubes were kept for 2 hours and then mixed with 20 mL acetonitrile and homogenized for 2 minutes at 10,000 rpm. Anhydrous sodium chloride 5 ± 0.1 g was added to the centrifuge tube and mixture was shaken for 10 min at 50 rpm on rotospin (Tarson®). The contents were centrifuged at 2500 rpm for 5 min. An aliquot of 10 mL acetonitrile was transferred over 10 ± 0.1 g sodium sulfate in a test tube and shaken well to absorb moisture from the sample if any. The acetonitrile extract was then subjected to cleanup by dispersive solid phase extraction (DSPE). An aliquot of 6 mL acetonitrile was taken in a tube containing 0.15 ± 0.01 g PSA sorbent, 0.90 ± 0.01 g anhydrous $MgSO_4$ and 0.05 ± 0.01 g graphitized carbon black and the mixture was thoroughly mixed on vortex spinix (Tarson®).

Table 1. Recovery and repeatability of Imidacloprid in different commodities of cotton

Matrix	Recovery level (µg/g)	Mean (%)	S.D. (%)	RSDr(%)
Leaves	0.50	85.88	2.17	2.52
	0.25	97.64	4.29	4.39
	0.01	88.99	4.21	4.73
Flowers	0.50	88.91	3.69	4.15
	0.25	92.36	4.39	4.75
	0.01	85.33	4.22	4.94
Nectariferous Tissue	0.50	99.69	4.83	4.84
	0.25	90.57	3.85	4.25
	0.01	86.64	3.05	3.52
Pollen	0.50	95.55	3.16	3.30
	0.25	91.27	3.18	3.48
	0.01	87.66	4.89	5.58

Table 2. Reproducibility of Imidacloprid recovery at 0.01 µg/g level

Matrix	Day	Recovery (%)	RSDr(%)	RSDR (%)
Leaves	1	86.57	2.81	3.05
	2	88.05	2.62	
	3	85.88	2.52	
Flowers	1	87.97	2.31	3.91
	2	86.85	3.86	
	3	88.91	4.15	
Nectariferous Tissue	1	92.45	2.89	3.46
	2	87.12	1.92	
	3	99.69	4.84	
Pollen	1	86.96	3.70	3.15
	2	93.89	1.70	
	3	95.55	3.30	

Table 3. Recovery and repeatability of Thiacloprid in different commodities of cotton

Matrix	Recovery level (µg/g)	Mean (%)	S.D. (%)	RSDr(%)
Leaves	0.50	96.59	2.98	3.08
	0.25	98.31	3.34	3.39
	0.01	86.33	4.27	4.94
Flowers	0.50	87.21	3.01	3.45
	0.25	92.76	4.95	5.34
	0.01	84.91	4.74	5.58
Nectariferous Tissue	0.50	90.59	4.14	4.57
	0.25	91.91	3.14	3.42
	0.01	93.91	4.08	4.34
Pollen	0.50	98.74	2.39	2.42
	0.25	90.64	3.57	3.94
	0.01	88.15	3.96	4.49

Again, the contents were centrifuged at 2500 rpm for 3 min and a 4 mL aliquot of acetonitrile extract was collected and kept as such in a centrifuge tube for residue analysis.

Flower samples

Around 5 g chopped and macerated sample of flowers were weighed into a 50 mL centrifuge tube and spiked at different test levels followed by addition of 20 mL acetonitrile. The same procedure as explained for leaf samples was repeated to collect the 4 mL aliquot of acetonitrile extract which was further used for residue analysis.

Nectar samples

Around 5 g chopped and macerated calyx were weighed in 50 mL centrifuge tube, spiked at the three test levels (0.5, 0.25 and 0.01 µg/ml) and mixed with 20 mL acetonitrile. The same procedure as explained for leaf samples was repeated to collect the 4 mL aliquot of acetonitrile extract that was analyzed for residue.

Pollen samples

A 2 g macerated pollen samples were weighed in 10 mL centrifuge tube and spiked at the three test levels. 5 mL of acetonitrile was added to each tube and mixture was homogenised. 2 ± 0.1 g of anhydrous sodium chloride was added to the centrifuge tube and mixture was shaken for 5 min over vortex mixture. The contents were centrifuged at 2500 rpm for 3 min. The acetonitrile extract was directly then subjected to cleanup by dispersive solid phase extraction (DSPE). An aliquot of 5 mL acetonitrile was taken in a tube containing 0.10 ± 0.01 g PSA sorbent and 0.60 ± 0.01 g anhydrous $MgSO_4$. The content was thoroughly mixed on vortex spinix (Tarson®). Tubes were centrifuged at 2500 rpm for 3 min and a 2 mL aliquot of acetonitrile extract was kept as such for residue analysis.

Estimation of residues

The residues of imidacloprid, thiacloprid and thiamethoxam were analysed on HPLC equipped with PDA detector at wavelength 272, 244 and 229 nm, respectively and HPLC grade water, acetonitrile (70:30, v/v) was used as mobile phase at flow rate of 0.30 mL/min. The injection volume was fixed to 20 µL. Under these operating conditions the retention times of imidacloprid, thiacloprid and thiamethoxam were found to be 16.07, 22.24 and 13.06 min, respectively. Recoveries were estimated by comparison of peak height/peak area of the standards with that of the spiked samples run under identical conditions.

Method validation

The validation of the method was made on the basis of SANCO guidelines [13] by concentrating on the factors viz. selectivity, linearity, limit of detection, limit of quantification, accuracy, precision (Repeatability and reproducibility).

Results and Discussion

HPLC chromatograms

Reversed-phase HPLC, with PDA detection, has proven to be good method for the determination of neonicotinoids because no derivatization step is needed. Chromatographic separation in C_{18} columns provided good results. The detection at 272, 244 and 229 nm offered suitable chromatograms for the quantification of imidacloprid, thiacloprid and thiamethoxam in real samples allowing a complete separation of its signal from those of foreign substances present in the samples (Figure 1).

Method validation

The analysis of HPLC chromatograms were made to evaluate the selected parameters and following results were compiled

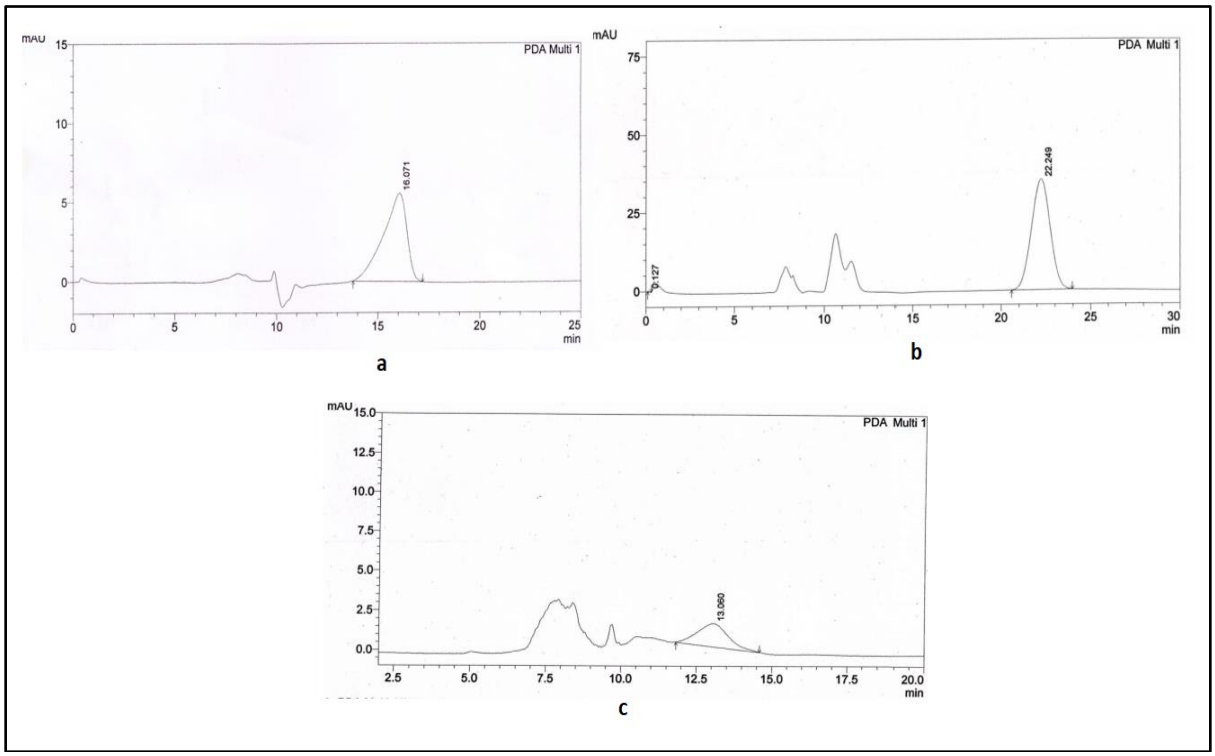


Figure 1. Chromatograms of Standards (a) Imidacloprid, (b)Thiacloprid, (c)Thiamethoxam

Selectivity

Selectivity of the method was assessed by comparing the HPLC chromatogram of a set of different blank samples with that of 3 sets of samples (Leaves, flowers, nectariferrous tissue and pollens) spiked at the LOQ level (0.01 µg/g for HPLC detection) [12]. The peaks in the control samples did not interfere the peak of spiked samples at the identified retention times of all the test samples (Figure 2).

Linearity

The calibration curves with respect to imidacloprid, thiacloprid and thiamethoxam produce a linear relationship between detector response (y) and analyte concentration (x = 0.2, 0.4, 0.6, 0.8 and 1.0 µg/ml). The parameters obtained by the selected chromatographic conditions for the three pesticides give calibration shown in corresponding graphs (Figure 3). The correlation coefficient $R^2 \geq 0.999$ indicated the linearity of the obtained results according to SANCO guidelines [13].

Limit of detection and limit of quantitation

The limit of detection (LOD in mg/kg) of each analyte was determined from the analysis of 3 control samples and worked out to be 0.003 µg/g. Limit of quantification (LOQ) for the compound was worked out on the basis of the response of the nanogram of standard working solution injected as well as the sample weight in mg injected so that the base line of the instrument remains stable and no noise is observed. As 0.2 ng of standard neonicotinoids produced 10% cent deflection and the instrument remained stable even after injection of 20 µL of each substrates of cotton, the LOQ for all the three test neonicotinoids was found to be 0.01 µg/g i.e. less than MRL.

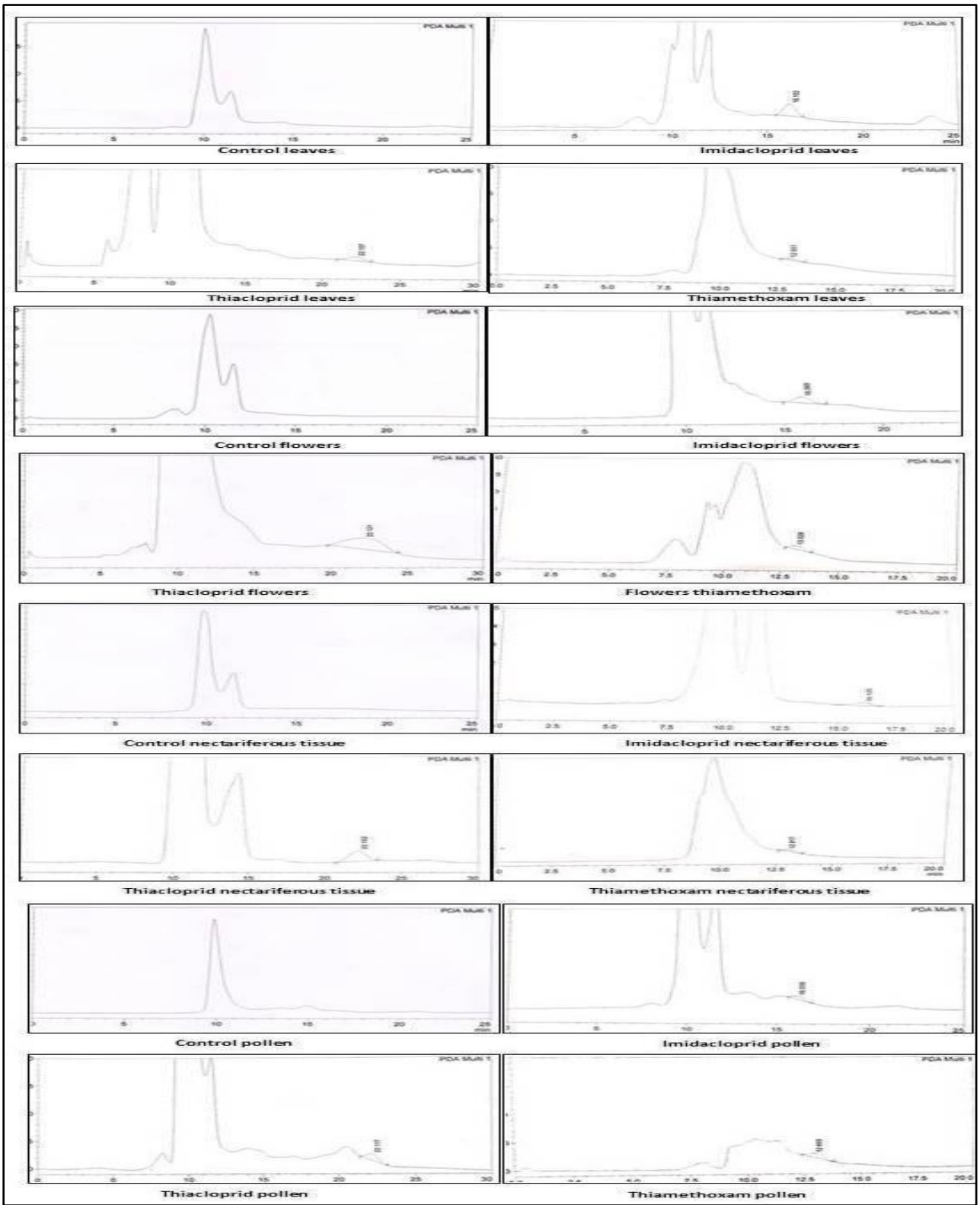
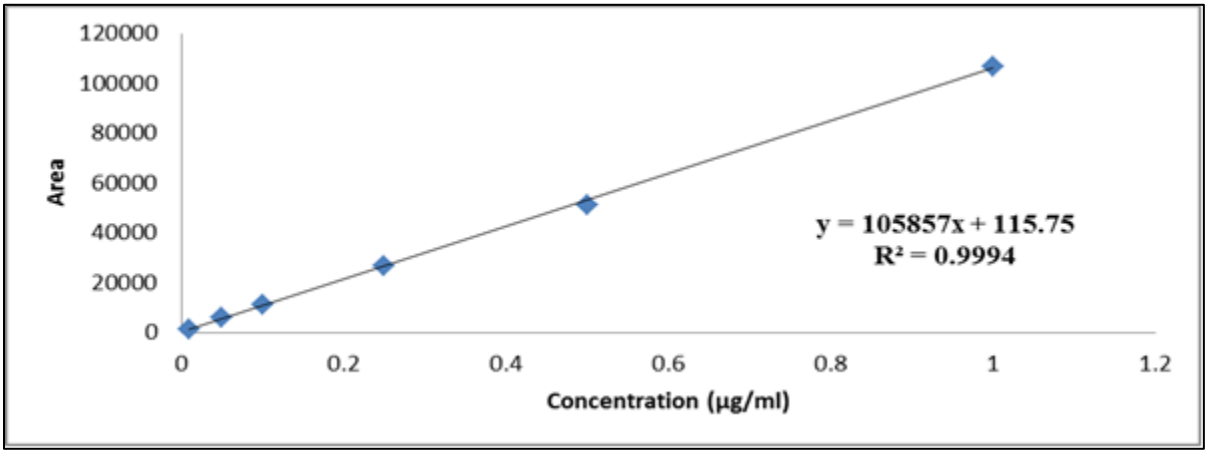


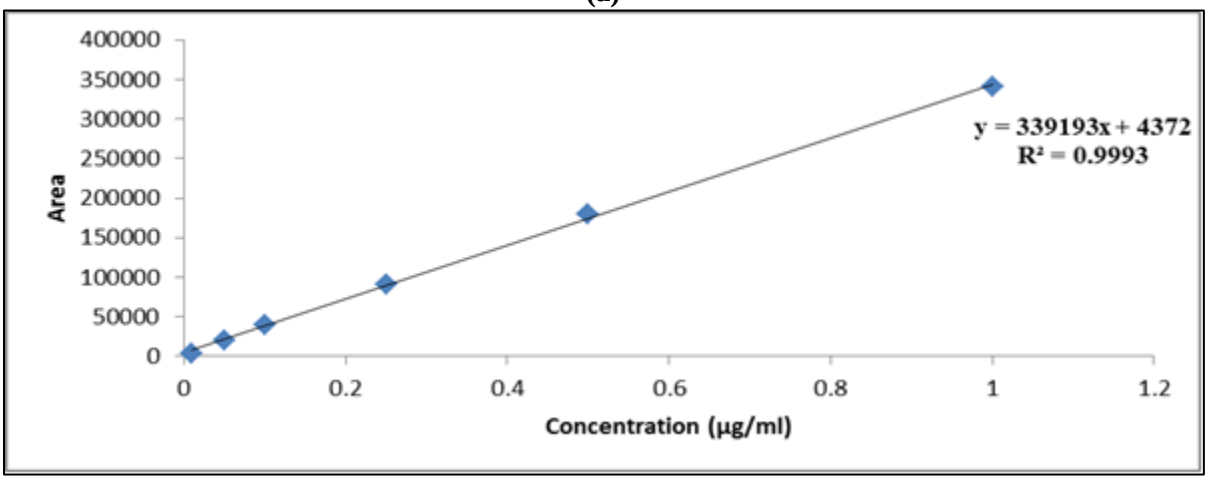
Figure 2. Chromatograms indicating selectivity of the molecules in spiked substrates

Precision (Repeatability)

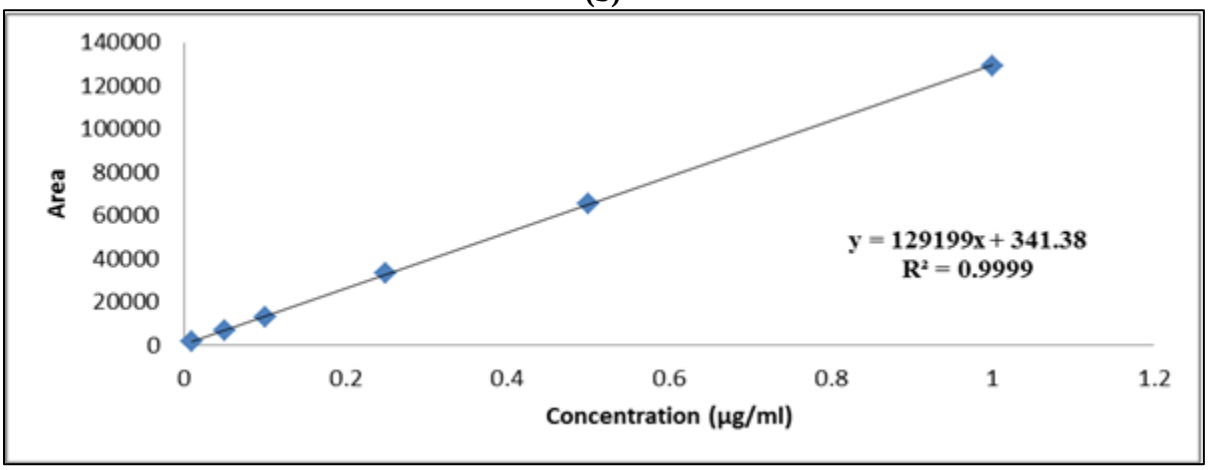
Repeatability (RSDr) of the developed analysis method was determined by spiking imidacloprid, thiacloprid and thiamethoxam in different concentrations to different substrates of cotton. The within-batch recovery and repeatability (RSDr) of spiked imidacloprid, thiacloprid and thiamethoxam in samples at the levels of



(a)



(b)



(c)

Figure 3. Calibration curve for different concentrations of (a) Imidacloprid (b) Thiacloprid (c) Thiamethoxam

Table 4. Reproducibility of Thiacloprid recovery at 0.01 µg/g level

Matrix	Day	Recovery (%)	RSDr(%)	RSDR (%)
Leaves	1	88.54	5.86	3.94
	2	92.56	2.01	
	3	96.59	3.08	
Flowers	1	95.23	4.56	3.79
	2	88.49	2.25	
	3	87.21	3.45	
Nectariferous Tissue	1	91.15	3.52	5.30
	2	92.16	6.44	
	3	90.59	4.57	
Pollen	1	91.25	3.70	2.54
	2	91.38	1.04	
	3	98.74	2.42	

Table 5. Recovery and repeatability of Thiamethoxam in different commodities of cotton

Matrix	Recovery level (µg/g)	Mean (%)	S.D. (%)	RSDr (%)
Leaves	0.50	97.56	1.56	1.60
	0.25	89.48	2.87	3.20
	0.01	98.10	1.22	1.24
Flowers	0.50	97.70	1.41	1.44
	0.25	89.10	2.67	2.99
	0.01	82.57	0.90	1.09
Nectariferous Tissue	0.50	84.80	1.25	1.47
	0.25	94.60	0.71	0.75
	0.01	82.17	0.65	0.79
Pollen	0.50	87.30	0.50	0.57
	0.25	97.30	0.46	0.47
	0.01	92.37	0.81	0.87

Table 6. Reproducibility of Thiamethoxam recovery at 0.01 µg/g level

Matrix	Day	Recovery (%)	RSDr(%)	RSDR (%)
Leaves	1	88.33	4.46	2.95
	2	94.45	2.22	
	3	97.56	1.60	
Flowers	1	93.60	4.33	2.82
	2	90.34	2.18	
	3	97.70	1.44	
Nectariferous Tissue	1	89.08	3.34	4.25
	2	92.45	6.51	
	3	84.80	1.47	
Pollen	1	89.11	4.89	3.40
	2	87.52	3.53	
	3	87.30	0.57	

0.01, 0.25, and 0.50 µg/g are summarized in Table 1, 3 and 5. The precision (repeatability) in different substrates spiked with different pesticides were less than 6%. The results were found to be satisfactory for all the concentration levels investigated and within specified range according to the SANCO criterion (≤20%) [13].

Precision (Reproducibility)

The reproducibility (RSD_R) of this analytical method was determined by analyzing spiked samples under various test conditions (different analysts and different days). The between-batch recoveries and reproducibility (RSD_R) investigated at several levels are given in Table 2, 4 and 6. The precision (reproducibility) of neonicotinoids in different substrates less than 6% and all measurements are within 15% at all concentrations. The SANCO criterion for the same is ≤20%.

Accuracy

The accuracy of an analytical method is the pact amid the true value of analyte in the sample and the value acquired by analysis. Accuracy is generally explained as the recovery by using the consistent procedure and known added amounts of analyte. The recovery tests were carried out on 6 replicates at each spike level. The average recoveries achieved for neonicotinoids at all concentrations and conditions explored were determined as above 85% in all the samples (Table 1, 3 and 5). According to SANCO guidelines, the mean recovery must be in the acceptable range (70-120%). Therefore, the results are indicating that the method was sensitive and suitable for determination of these neonicotinoids.

Conclusion

Validation of the developed QuEChERS method favors its use for the estimation of the neonicotinoids (Imidacloprid, Thiacloprid and Thiamethoxam) residue in leaves, flowers, nectariferous tissue and pollen of the cotton crop, demonstrating the great versatility of this method. The proposed analytical method using liquid chromatography techniques for the determination of neonicotinoids in different cotton commodities has been demonstrated to be sensitive, fast, precise, accurate and robust and can be used to monitor neonicotinoids residues in cotton. Use of simple/highly-available analytical instrumentation, i.e. HPLC/PDA for quantitative analysis instead of expensive tandem mass spectrometers may add another advantage of this proposed methodology.

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Conflict of Interests

The authors declare that there is no conflict of interests.

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