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Method Validation of LC-MS/MS Analysis of Neonicotinoid Insecticides in Soil

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A simple, sensitive and inexpensive analytical method was developed using solid-phase extraction for the simultaneous determination of five neonicotinoid insecticides in soil matrix using LC-MS/MS and validated. The samples were extracted with acetonitrile and subsequent cleanup was done by dispersive solid-phase extraction (QuEChERS method). The quantification was carried out by liquid chromatography-tandem mass spectrometry with electrospray ionization source (LC-ESI-MS/MS). After the optimization of the extraction parameters, the method was validated by evaluating, linearity, limits of detection and quantification, precision (repeatability) and accuracy (recovery). Validation was based on analysis at three fortification levels and showed satisfactory recoveries (77.03 to 115.08 %) and high precision (RSDs between 2.01 to 13.83%). Low limits of detection and quantification could be achieved for all the five analytes ranging from 0.0007 to 0.002 and 0.002 to 0.008 µg/g, respectively. The developed method was applied to the analyses of neonicotinoid residues in soil from sugarcane field and pot soil.

Key words: Neonicotinoid insecticides, Multi-residue analysis, Soil, LC/MS/MS

The use of neonicotinoid insecticides in crops has been quite significant particularly in vegetables, cotton and protected cultivation systems. The neonicotinoid insecticides act as agonists at the insect nicotinic acetylcholine receptor, which are highly toxic to sap-sucking insects and biting pest insects on a wide range of crops and fruit trees. (Kapoor *et al*., 2013; Zhang *et al*., 2010; Magalhaes *et al*. 2009 and Nauen *et al*. 2008). These compounds are most commonly used on vegetables, cotton and sugarcane in India (Ramasubramanian, 2013; Sharma and Singh, 2013 and Timmeren *et al*., 2012). Five neonicotinoids viz., imidacloprid, acetamiprid, thiacloprid, thiamethoxam and clothianidin are commercialized in India and generally used by farmers mainly to reduce the yield loss caused by sucking pests. However, intense vegetable farming, the frequency and intensity of usage coupled with the mode of application may result in large volumes of insecticides in the environment.

In Tamil Nadu, neonicotinoid pesticide use is increasing, but little information is available regarding their environmental impacts resulting from their use. Using neonicotinoid insecticides for seed treatments and soil application is a common practice in field crop production (Mane and Mohite, 2014 and Jeschke *et al*. (2011)). Neonicotinoid treated seeds are also widely used. Imidacloprid and Thimethoxam are used extensively in seed treatment. This necessitates the study of concentration and residues of neonicotinoids in the environment extremely important.

Residue analyses in soil are reported to be done by liquid-liquid extraction (LLE), matrix solid-phase dispersion (MSPD), solid-phase microextraction (SPME) and QuEChERS method (Xu *et al*., 2016, Schaafsma, 2015, Caldas *et al*., 2011, Durovic *et al*., 2010 and Anastassiades *et al*., 2003). The residues of neonicotinoids are mainly determined by HPLC and LC/MS/MS (Abdel-Ghany *et al*., 2016; Fernandez *et al*., 2015; Sahoo, 2015; Wang *et al*., 2012; Karmakar *et al*., 2012; Galeano *et al*., 2013 and Mohan *et al*., 2010). The present study aimed at developing a simple method for analysis of five neonicotinoid insecticides in soil and assessing the levels of neonicotinoid residues in soils from crop fields of Tamil Nadu.

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Material and Methods

Liquid chromatography was performed in a Waters Alliance 2695 Separations Module with all required accessories like autosampler, a membrane degasser, a quaternary pump and a data system. Mass spectrometry was performed in a Acquity TQD with an ESI interface. The LC separation was carried out in an XTerra analytical column C18, 5 µm (4.8 × 250 mm). Analytical instrument control, data acquisition and treatment were performed by software Mass lynx version 4.1, 2005 (Waters, Milford, USA).

Reagents and standards

High purity MS grade solvents (Acetonitrile) and reagents were used. Acetonitrile and formic acid were purchased from M/s. Merck. Magnesium sulfate and anhydrous sodium chloride (from Merck) were heated at 650°C for 4 h and kept in desiccators until use. Primary secondary amine (PSA) and Graphitized carbon black (GCB) was obtained from M/s. Agilent Technologies. Millipore water was used during the whole analysis. All 5 pesticide standards (acetamiprid,

imidacloprid, thiacloprid, thiamethoxam and clothianidin) were purchased from M/s. Sigma Aldrich, Bangalore, India and were of purity >90% (w/w).

Stock solutions were prepared using acetonitrile solvent. Spiking solutions for measuring method recovery were prepared from stock solutions. Calibration standards in at least five concentrations were also prepared from stock solutions and diluted in acetonitrile. The working standards were used to find out the retention time of these compounds and for quantitative determination of residues in samples. All stock, spiking and calibration standards were transferred to glass volumetric flasks after preparation and stored at 4°C.

Soil samples

Soil samples were collected in the study areas from March to April, 2016. The soil samples were collected using a shovel at a depth of 30 to 45 cm from several areas from the field and pooled to get a composite sample. The final sample was then collected by quartering method, air dried, sieved and refrigerated at about 4°C until use.

Sample extraction and clean-up

Analysis for pesticide residues is often carried out following different steps for pretreatment mainly including solvent extraction, clean-up, concentration and final determination. The soil sub samples were prepared by weighing 10 g in 50 mL tube. Standards were added at spiking concentrations and left for 30 minutes. These sample tubes were vortexed for 30 seconds after adding 20 mL of acetonitrile, in order to homogenize and fluidize the sample. In each tube a mixture of salts (4 g magnesium sulphate, 1 g sodium chloride) was added. The extract was stirred for 1 min. in vortex, in order to maximize the distribution of the analytes in the organic phase. The samples were centrifuged at 6000 rpm for 10 minutes and the supernatant was transferred to another tube containing 600 mg magnesium sulphate, 100 mg PSA and 10 mg GCB, vortexed and centrifuged at 3000 rpm for 10 minutes. The upper extract was filtered through a 0.2µm syringe filter. Finally, one ml of the extract taken in glass vials were analyzed by LC-MS/MS.

Optimization of instrument parameters

Optimization of MS/MS conditions includes the selection of the ionization mode, identification of the parent and product ions, and selection of the collision voltages. In the LC-MS/MS equipped with Xterra C18 column, an isocratic flow of binary solvents ie., 0.5% HCOOH in water (A) and 0.5% HCOOH in CH3CN (B) was employed. The mobile-phase was programmed as follows: 50% A + 50 % B for 12 min. The flow rate was 500 µL/min and column temperature set at 30°C. Mass spectral analyses were performed using an LC-TQD operating in the positive ion mode using an ESI interface. ESI in the negative mode did not give any signal for the five analytes tested. Preliminary tunings were carried out with continuous introduction of standard solutions with syringe pump infusion flow rate at 5 µL min^{-1}. MS/MS parameters were standardised by Intellistart software for tuning. The MS conditions were capillary voltage 1 KV; Desolvation gas 1100 lt hr^{-1} and 500° C; Cone gas 50 lt hr^{-1}; Source temperature 150° C and collision gas flow 0.18 ml min^{-1}. Two specific fragments of each neonicotinoid compound was monitored. The multiple reaction monitoring (MRM) transitions were used for confirmation. A summary of the precursor and product ions, collision energy and cone energy for each analyte is given in Table 1. The most intense peak of product ion was used for quantification (quantifier ion) and the second peak for confirmation (qualifier ion).

Method validation

The method was validated by the following parameters: linearity, specificity, limits of detection and quantification, recovery, precision and accuracy. The soil previously extracted using the same method and shown to be free of interfering peaks were only used as samples in the recovery study. All the analyses were carried out using the same blank soil sample.

Specificity

The specificity of the analytical method for neonicotinoids detection was confirmed by obtaining positive results from soil sample containing the analytes, coupled with negative results from samples which do not contain it (negative controls).

Linearity studies

Linearity was studied based on a five-point standard calibration graph by plotting the Mass detector response against concentration of the standards within the range 0.025 to 0.5µg ml-1 making three replicates for each concentration.

Detection and quantification limits

The quantification limit for pesticides reported in this study, was based upon the lowest concentration that could be consistently and/or reliably recovered (> 70%) in our laboratory from spiked samples. For this purpose, 7 independent analyses of soil sample spiked with pesticides at a level of 0.025 µg g-1 were performed. The LOD were calculated from the standard deviation associated with the measurement of the pesticides and student t test value. The limits of quantification (LOQs) were calculated by considering a value of 3.3 times the LOD.

Accuracy and precision

Method recovery studies were performed at five spiking concentration levels (0.025, 0.05, 0.01, 0.25, 0.5 µg g^{-1} making 7 replicates for each concentration. The spiked samples were equilibrated and processed by adopting the above said extraction and clean up procedure. Accuracy and precision of the method was determined from the measurements during recovery study carried out by spiked samples. Repeatability of the method was evaluated through the relative standard deviation (RSD %).

Results and Discussion

Soil is a complex matrix because of its organic and inorganic contents. It possesses many active sites that can retain pesticide residues. In this study, acetonitrile demonstrated to be the best solvent for extracting multi-class neonicotinoid residues from soil samples. The good sample clean-up effect was achieved by using a sorbent system of MgSO4, PSA and GCB. The main advantages of this dispersive solid phase method was less labor and organic solvent requirement and high recoveries for a wide range polarities of pesticides. Concentration was not done in the last step and the filtered sample was fed into the instrument. Similarly, the final extracts without concentration were used for LC/MS/MS determination of pesticide residues in rice by Shakouri et al., 2014. Whereas, Assalin et al. (2014) followed concentration of soil sample extracts and reconstituted it with solvent in the last step before injection. Good linearity was found for all the five pesticides and linear regression coefficients (r2) were higher than 0.990 (Table 2).

Table 1. MRM transitions for ions of neonicotinoids in LC-MS/MS

Pesticide	Retention Time(min.)	Ion Monitoring (m/z)		Cone (V)	Collision(V)
		Parent ion	Daughter ion		
Acetamiprid	6.73	223.16	126.115	26	16
		223.16	56.222	26	22
Thiacloprid	7.74	253.096	126.126	30	36
		253.096	90.23	30	20
Imidacloprid	6.65	256.132	209.146	24	19
		256.132	175.205	24	16
Thiamethoxam	5.61	292.168	211.109	24	23
		292.168	132.104	24	13
Clothianidin	6.30	250.10	169.11	16	16
		250.10	132.11	12	12

Table 2. Mixed standard solutions, linear range, variation coefficient and linear equation of five neonicotinoid residues

Pesticides	Linear range(µg /ml)	Variation coefficient	Linear equation
Thiamethoxam	0.025 – 0.5	0.9970	67.3054x +-445.016
Imidacloprid	0.025 – 0.5	0.9980	76.869x +-549.413
Thiacloprid	0.025 – 0.5	0.9991	440.44x +-1043.75
Acetamiprid	0.025 – 0.5	0.9989	350.487x + -817.951
Clothianidin	0.025 – 0.5	0.9972	111.492x +358.624

The mean per cent recoveries of five neonicotinoid compounds from soil samples at the fortification level of 0.025 to 0.5 µg g-1 were 77.03 to 115.08 % and RSDs of 2.01 to 13.83% were obtained. Limits of detection and quantification ranged from 0.0007 to 0.002 and 0.002 to 0.008 µg g-1, respectively. The results are shown in Table 3.

Table 3. Average recovery (%), RSDs, limits of detection (LOD) and limits of quantitation (LOQ) of five neonicotinoid pesticides in soil

Pesticide	Spiked level (mg L^{-1})										LOD	LOQ
	0.025		0.05		0.10		0.25		0.50			
	Recovery %	RSD	Recovery %	RSD	Recovery %	RSD	Recovery %	RSD	Recovery %	RSD		
Thiamethoxam	93.84	13.83	80.30	9.06	77.03	11.04	81.16	6.29	81.50	6.92	0.0015	0.005
Imidacloprid	115.08	7.66	91.11	7.16	99.96	5.19	95.53	4.27	91.19	3.29	0.0015	0.005
Thiacloprid	87.35	6.36	84.50	3.53	95.48	3.27	93.68	2.01	91.59	2.59	0.001	0.003
Acetamiprid	89.46	5.21	83.38	4.86	95.60	3.75	97.23	2.93	94.69	2.35	0.002	0.008
Clothianidin	89.14	10.96	96.72	10.32	108.03	8.45	105.95	5.76	102.10	5.21	0.0007	0.002

The validated method was then applied for analysis of real samples. The results obtained from the analysis of five soil samples from crop fields and glass house showed imidacloprid and clothainidin residues. One sample collected from sugarcane field contained detectable concentrations of clothianidin (0.079 µg g^{-1}) and the glass house soil sample showed 0.01885 µg g^{-1} imdacloprid residue. The farmer survey revealed that clothianidin was applied in sugarcane fields for the control of subterranean pests. Pesticides may reach the soil through direct application to the soil surface, incorporation in the top few inches of soil,

or during application to crops. The fate of pesticides in soil and water environments is influenced by the physico-chemical properties of the pesticide and the soil. Clothianidin residues of concentration 7.0 ng g^{-1} were reported in soils collected from the seeding zone of corn fields (Xu *et al.,* 2016). Rios *et al* (2016) studied neonicotinoid insecticide residues in soil in fields with a history of seed treatment use on crops and reported neonicotinoid residues to the tune of 4.36 ng g^{-1} and 59.86 ng g^{-1} for parent soil and surface dust, respectively.

The glass house soil sample analysed in this study had the history of seed treatment with imidacloprid which was done before two months of sampling. The detection of residues even after two months is in confirmation with the reports given by Tisler *et al.* (2009) and Krohn and Hellpointner (2002) who reported DT50 values of 130 and 160 days for imidacloprid in soil. The persistence and metabolism studies of imidacloprid in sugarcane field soil conducted by Sharma and Singh (2013) revealed detectable level of residues in soil till 90 days of application at both the test doses of 20 and 80 g a.i. ha^{-1}. But Sahoo (2015), reported that imidacloprid residues could not be detected in soil 60 days after sowing cotton seeds treated @ 3.5 and 14 g a.i. kg^{-1} respectively. Also, Mandal *et al* (2010) found higher dissipation of imidacloprid in soil under brinjal wherein the soil samples collected after 15 days did not reveal the presence of imidacloprid.

The combination of persistence (over months or years) and solubility of the neonicotinoid pesticides may lead to contamination and the potential for accumulation in soils, sediments, water bodies and even non applied field as reported by Goulson (2013).

Conclusion

The high extraction efficiency and low matrix effects achieved by the method, satisfactory validation parameters such as linearity, recovery, precision and LOQ finds the method workable for routine residue analysis/regular monitoring of neonicotinoid insecticides in soil matrix. This study was limited to samples collected from a small number of farmers in only one phase of the growing season. At the same time, the significance of the presence of neonicotinoids in soils should not be discounted. The presence of these compounds in the environment suggests that all kinds of non target organisms may be exposed to them. Further, residue studies in other agricultural areas of Tamil Nadu are needed in order to assess the levels of neonicotinoid pesticide residues in soils.

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