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Entomology

Determination and Uncertainty Analysis of Imidacloprid Residue in Flue Cured Leaf Matrix of *Nicotiana Tabacum* L.

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Abstract

Presence of pesticide residues in tobacco (*Nicotiana tabacum* L.) leaf matrix increases health risk of the consumer, and hence, determination of pesticide residues in tobacco is an issue of serious concern around the world. Present study details the sample preparation and analysis of imidacloprid residues in the Flue Cured Virginia (FCV) tobacco by high-performance liquid chromatography (HPLC) coupled with ultra-violet (UV) detector. The extraction method involves mechanical agitation of tobacco leaf matrix with acetone (1:10) for 45 min at 200 rpm, followed by sequential liquid-liquid-partitioning and clean-up with florisil. The method provided 83.7-97.5% recovery with precision relative standard deviation (RSD) less than 10%. Matrix induced signal suppression was recorded at lower level of pesticide spike (mention concentration to specify what do mean by lower level). The method provided acceptable intra-laboratory precision (HorRat ratio mostly < 0.5) and global uncertainty (11.72% at guidance residue level (GRL) of 5 µg g^{-1} for imidacloprid in tobacco), which complies with the international regulatory specifications. By considering efficiency of method, economics of analysis and analyst's safety, the present method can be adopted by laboratories to monitor imidacloprid residue in FCV tobacco leaf matrix for consumers' safety.

Highlights

- A method for extraction and analysis (by HPLC) of imidacloprid residue in tobacco was standardized.
- Tobacco matrix showed peak suppression (upto ~16%) of imidacloprid.
- Recovery efficiency of the method was 83.7-97.5% with RSD <10%.
- Uncertainty and economics of analysis were calculated.

Keywords: *Nicotiana tabacum* L., Imidacloprid residue, Method validation, Uncertainty analysis

Imidacloprid [1-(6-chloro-3-pyridylmethyl)-N-nitro-2-imidazolidinimine], a neonicotinoid insecticide with low dosage and systemic activity, is being widely used to control aphids, thrips and whiteflies in various agricultural crops, including cash crops like tobacco (*Nicotiana tabacum* L.) (Sreedhar *et al.*, 2011). Tobacco is one of the world's leading non-edible cash crops, and grown extensively in countries such as India, China, Brazil, USA, etc. for the production of tobacco leaf (economic part). Inspite of several awareness campaigns about the imminent potential

health problems associated with tobacco, millions of people, particularly in lower and middle-income countries, still indulge in cigarette smoking (WHO, 2011), and presence of pesticide residues, further, increases smokers' health risk. Clark *et al.*,1998) reported presence of imidacloprid in the cigarette butt, main-stream smoke and side-stream smoke of the cigarettes made with tobacco leaves harvested from imidacloprid treated crop. Recent studies in the field of medical research have postulated that the neonicotinoid insecticides namely, imidacloprid and acetamiprid, may adversely affect human health, especially the developing brain (Kimura-Kuroda *et al.,* 2012). Therefore, while considering consumers' safety, maximum permissible/ acceptable residue level for imidacloprid in tobacco has been adopted by various countries around the world. India has a guidance residue level (GRL) of 5 µg g^{-1} for imidacloprid in tobacco as per Coresta 2013, whereas, some European countries follow a maximum residue limit of 5 to 50 µg g^{-1} for tobacco and tobacco products (Liu *et al.,* 2005). Thus, determination of imidacloprid residues in tobacco leaf is an important issue of serious concern from consumers' safety point of view. Determination of imidacloprid residues in crops/ food products (Chin-Chen *et al.,* 2009; Kapoor *et al.,* 2013; Hendawi *et al.,* 2013) and environmental components (Mahapatra *et al.,* 2011; Samnani *et al*., 2011; Srivastava, 2012) have been reported in literature. However, little information is available in this context for tobacco which results in hurdles for identification and quantification of imidacloprid residues, owing to matrix interference (Cao *et al.,* 2001). Some attempts have been made to determine imidacloprid residues in tobacco leaf (Placke and Weber, 1993; Liu *et al.,* 2005). However, none of these studies focused on the matrix effect, intra-laboratory precision and most importantly, uncertainty associated with the method. The objective of the present study was to formulate an effective, rapid and sensitive method for determination of imidacloprid residue in Flue Cured tobacco leaf matrix by HPLC. The matrix effect, intra-laboratory precision and uncertainty associated with the method were investigated. The method was used to analyze field samples for imidacloprid residues, and the economics of analysis was also estimated.

Materials and Methods

Chemicals and Materials

Analytical grade certified reference standard of imidacloprid ($C_9H_{10}ClN_5O_2$, molecular weight: 255.66) with purity 99.4 % was procured from Accustandard® (New Haven, USA). HPLC grade solvents namely, acetone, n-hexane, dichloromethane, acetonitrile and water, were purchased from Merck India Limited (Mumbai). The adsorbent, florisil, was received from Sisco Research Laboratories (Mumbai, India). The other reagents like sodium chloride (NaCl) and anhydrous sodium sulphate (Na_2SO_4) were of analytical grade and collected from SD Fine Chemicals (Mumbai, India). The FCV tobacco leaf matrix was collected from the research farm of Central Tobacco Research Institute, Rajahmundry, Andhra Pradesh, which did not receive any application of imidacloprid. Leaves were oven dried at 60°C for 2 h. The dried leaves (after removing mid rib) were powdered, homogenized, sieved (through 1 mm) and stored in plastic bags, until analysis.

Standard Solution Preparation

The stock solution of imidacloprid (200 µg mL^{-1}) was prepared in a volumetric flask (certified "A" class) by dissolving 5 (± 0.1) mg of reference standard in 25 mL of acteonitrile, and subsequently, the working solutions of desired imidacloprid concentrations (0.05-7.5 µg mL^{-1}) were prepared by serial dilution technique.

Sample Preparation

To extract the imidacloprid residue, 5 g of tobacco matrix with 50 mL of acetone was agitated at 200 rpm for 45 minutes over an orbital shaker at room temperature (32 ± 2 °C) and vacuum filtered. The leachates, thus collected, were reduced to 2 mL under vacuum evaporation at 22 °C. Then 50 mL of 10% NaCl solution was added to the reduced acetone extract and liquid-liquid partitioning (L-L-P) was performed by adding 60 mL of n-hexane, thrice (20 mL in each time). The hexane fractions were discarded, and the aqueous layer was again partitioned (L-L-P) with a total 100 mL of dichloromethane, thrice (50 mL + 30 mL + 20 mL). The organic fractions were collected through anhydrous Na_2SO_4 layer and evaporated to dryness under vacuum. The residuum was re-dissolved in 10 mL of acetonitrile and loaded onto a column bed prepared with 1: 4: 1 parts of anhydrous Na_2SO_4: florisil: anhydrous Na_2SO_4 for clean-up purpose. The column was eluted with 50 mL of acetonitrile and the leachate was collected. Finally, the leachate was reduced to 25 mL and analysed by HPLC.

Instrumentation

A Shimadzu LC-8A series HPLC system, coupled with an UV-detector, was used in the present study. The stationary phase, a RP-18 column, was maintained at 30 °C, and the

isocratic mobile phase, composed of acetonitrile: water (30:70, v/v), was eluted at a flow rate of 0.7 ml min^{-1}. The UV detector was set at 270 nm wavelength of maximum absorbance (λ_{max}). The HPLC analysis was performed by injecting 20 ìL of sample through a rheodyne injector.

Method Performance, Validation and Statistics

The analytical method was validated as per the Single Laboratory Validation approach (European Commission Decision 2002; Thompson *et al.,* 2002). The calibration range, linearity and sensitivity were tested at nine different concentrations of imidacloprid (0.05 to 7.5 µg mL^{-1}) for solvent standard and tobacco matrix matched standard.

The matrix effect was assessed from matrix matched standards prepared in the similar fashion as that for solvent standards using a matrix extract of the untreated (no imidacloprid) tobacco. The matrix effect (ME %) was calculated as:

ME % = (peak area of post extraction spike*100/peak area of solvent standard) (1)

Recovery of imidacloprid from tobacco matrix was studied at three different fortification levels of imidacloprid [0.5 times of GRL (2.5 µg g^{-1}), at GRL (5 µg g^{-1}) and 1.5 times of GRL (7.5 µg g^{-1})]. To estimate the accuracy in recovery, the experiments were carried out by two different analysts on three different days. The per cent recovery was calculated as:

% Recovery = (peak area of pre-extraction spike*100/peak area of post extraction spike) (2)

The precision in terms of repeatability (two different analysts prepared six samples each on a single day) and intermediate precision (two different analysts prepared six samples each on three different days) were determined separately at three different spike levels of imidacloprid [0.5 times of GRL (2.5 µg g^{-1}), at GRL (5 µg g^{-1}) and 1.5 times of GRL (7.5 µg g^{-1})]. The intra-laboratory precision which indicates the acceptability of a method with respect to precision was determined by calculating the Horwitz ratio (HorRat) (Horwitz and Albert, 2006) for studied imidacloprid concentrations as:

HorRat =RSD/PRSD (3)

where, PRSD is the predicted relative standard deviation = $2C^{-0.15}$ and where C is the concentration expressed as a mass fraction (for example, 2.5 µg g^{-1} = $2.5*10^{-6}$).

Measurement of Uncertainty

The uncertainty associated with the method was investigated at three different fortification levels of imidacloprid (2.5, 5 and 7.5 µg g^{-1}, which were 0.5, 1 and 1.5 times of GRL) as per the statistical procedure of the EURACHEM/CITAC Guide CG 4 (EURACHEM/CITAC, 2000). Five uncertainty sources namely, uncertainty associated with the calibration graph (U_1), day-wise uncertainty associated with precision (U_2), analyst-wise uncertainty associated with precision (U_3), day-wise uncertainty associated with accuracy/bias (U_4), and analyst-wise uncertainty associated with accuracy/bias (U_5), were evaluated. The respective uncertainty sources can be calculated as follows:

$$U_1 = (s/b_1)[(1/p) + (1/n) + \{(C_0 - `C)^2/s_{xx}\}]^{1/2} \quad (4)$$

where, s is the standard deviation of the residuals of the calibration curve, b_1 is the slope of the calibration curve, p is the number of measurements of the unknown, n is the number of points used to form the calibration curve, C_0 is the calculated concentration of the analyte from the calibration curve, ‘C is the average of all of the standards used to make the calibration curve, and s_{xx} is calculated as:

$$s_{xx} = \Sigma (C_j - `C)^2 \quad (5)$$

where, j = 1, 2, ..., n. C_j is the concentration of each calibration standard used to build up the calibration curve.

$$U_2 = s_1/n^{1/2} \quad (6)$$

where, s_1 is the standard deviation of the results obtained from a single analyst on different days and n is the number of assays.

$$U_3 = s_2/n^{1/2} \quad (7)$$

where, s_2 is the standard deviation of the results obtained from different analysts on a particular day and n is the number of assays.

$$U_4 = s_1(\eta)/n^{1/2} \quad (8)$$

where, $s_1(\eta)$ is the standard deviation of the percentage recoveries obtained from a single analyst on different days and n is the number of assays.

$$U_5 = s_2(\eta)/n^{1/2} \quad (9)$$

where, $s_2(\eta)$ is the standard deviation of the percentage recoveries obtained from different analysts on a particular day and n is the number of assays.

The global uncertainty (U) was calculated as:

$$U = (U_1^2 + U_2^2 + U_3^2 + U_4^2 + U_5^2)^{1/2} \quad (10)$$

The expanded uncertainty was reported as twice the value of the global uncertainty.

Results and Discussion

Method performance and validation

The calibration curves of imidacloprid in pure solvent and tobacco matrix were obtained by plotting the peak area against the corresponding concentrations of imidacloprid standards with linearity ranging between 0.1 to 7.5 µg mL^1 (Fig. 1). The Figure 1 clearly shows the linearity of both calibration curves, with coefficient of determination > 0.99, within the studied range of imidacloprid concentrations. The sensitivity of the instrument for imidacloprid was 0.05 µg mL^{-1} and that of the method was 0.1 µg mL^{-1}. The Figure 1 depicts that though both calibrations are linear, but the matrix matched calibration curve (slope= 76572) has lower slope value than that of the solvent calibration curve (slope= 83079). It indicates the peak suppression effect of tobacco matrix which could be because of presence of co-eluents originated from matrix co-extractives. The effect of matrix for a particular pesticide varies with varying the crop/ plant materials and thus, it will be inappropriate to infer about the tobacco matrix effect from the information reported on other crops, in literatures. Similarly, imidacloprid peak suppression was reported in mango matrix by Banerjee *et al.*, (2009). To minimize the matrix influence, sequential liquid-liquid-partitioning with brine solution and n-hexane were performed to remove water and lipid soluble impurities, respectively. Then, the sample was cleaned-up with florisil column to reduce matrix influence, and analyzed by HPLC-UV. The Figure 2 shows imidacloprid peak with a retention time (R_t) of 12.2 min in spiked tobacco matrix. The Table 1 indicates that tobacco matrix has 2.5-16% signal suppression (indicated by "-ve" sign) effect within the studied fortification range of 2.5 to 7.5 µg g^{-1}, and the suppression effect was high at lower fortification level of 2.5 µg g^{-1}. The Table 1 showed that recoveries varied from 83.7-92.2% at 2.5, 87.9-95.4% at 5 and 89-97.5% at 7.5 µg g^{-1} fortification level with RSD values < 20%, which complied with the international criteria of 70-120% recovery (EURACHEM/CITAC, 2000). The recovery (83.7-97.5%) of the present method is also comparable with earlier reports of Liu *et al.*, (2005) (89.8-95.4% recovery) and Placke and Weber (1993) (71-97% recovery). The Table 1 depicts that the HorRat ratios pertaining to intra-laboratory precision are mostly less than 0.5, which indicate that this method is quite rugged (Banerjee *et al.*, 2009).

Table 1: Recovery, HorRat ratio and matrix effect for the analysis of imidacloprid

	Analyst 1								
	Day 1			Day 2			Day3		
Fortification level (µg g^{-1})	7.5	5	2.5	7.5	5	2.5	7.5	5	2.5
% Recovery	97.53	93.18	92.08	89	89.64	91.64	92.24	95.43	91.56
SD[a]	6.02	3.95	6.26	4.7	5.05	6.69	3.87	3.63	6.01
RSD[b]	6.17	4.24	6.8	5.31	5.63	7.31	4.2	3.81	6.55
HorRat	0.53	0.34	0.49	0.45	0.45	0.53	0.36	0.30	0.47
ME (%)[c]	-2.47[d]	-6.82	-7.92	-11.01	-10.36	-8.36	-7.76	-4.57	-8.44
	Analyst 1								
	Day 1			Day 2			Day3		
Fortification level (µg g^{-1})	7.5	5	2.5	7.5	5	2.5	7.5	5	2.5
% Recovery	94.7	89.55	83.76	91.05	87.94	85.06	91.76	88.72	83.97
SD	2.85	4.85	4.14	3.76	3.43	2.66	5.16	3.16	5.22
RSD	3.01	5.42	4.94	4.13	3.90	3.13	5.62	3.56	6.22
HorRat	0.26	0.43	0.36	0.35	0.31	0.23	0.47	0.28	0.45
ME (%)	-5.3	-10.4	-16.23	-8.95	-12.06	-14.94	-8.24	-11.28	-16.03

[a]SD = standard deviation, [b]RSD = relative standard deviation, [c] ME (%)= matrix effect, [d] "-" indicates signal suppression.

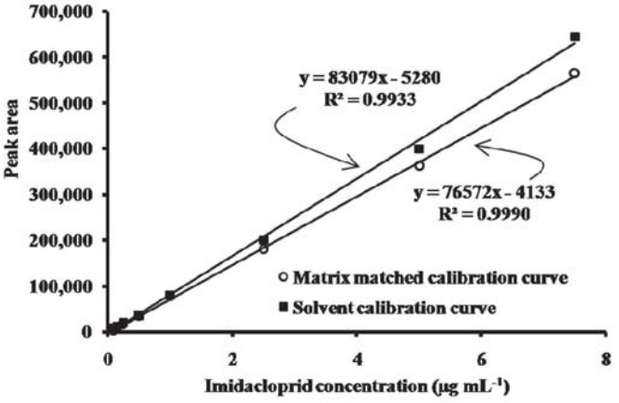

Fig. 1: Solvent and tobacco matrix matched calibration curves of imidacloprid.

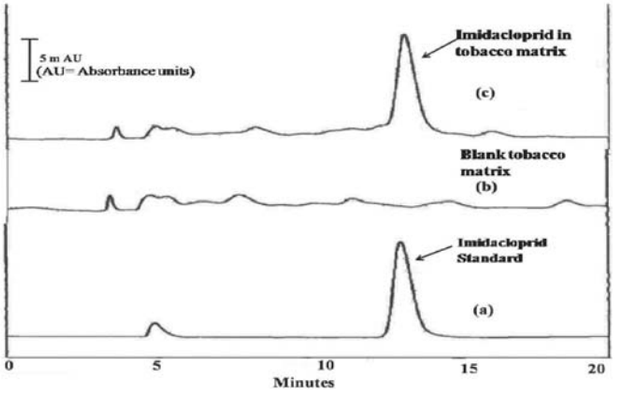

Fig. 2: HPLC chromatogram of (a) imidacloprid solvent standard (5 µg ml^{-1}), (b) blank tobacco matrix and (c) tobacco matrix spiked with imidacloprid (5 µg g^{-1}).

Uncertainty Analysis

The global uncertainty associated with the method was also analyzed (Table 2). It varied upto 13.39% which was much below the internationally accepted 50% level of uncertainty (EURACHEM/CITAC, 2000). It is clear from the Table 2 that the method has lower uncertainty associated with precision (<0.7% for both U_2 and U_3) and accuracy/ bias (<9.73% for both U_4 and U_5) which might be due to good recoveries with low RSD. These findings indicated that the method provided repeatable and reliable results, with acceptable recovery.

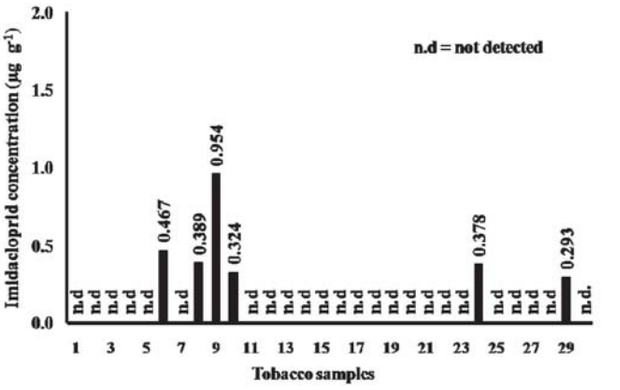

Fig. 3: Imidacloprid residues in flue cured tobacco leaf samples.

Table 2: Uncertainty components (expressed as relative measures) analysis for imidacloprid

Fortification level (µg g^{-1})	Calibration curve (U_1)	Precision		Accuracy/ bias		Global uncertainty (U)	Expanded uncertainty (2U)
		U_2	U_3	U_4	U_5		
2.5	0.062	0.023	0.026	0.927	0.963	1.339	2.79
5	0.066	0.055	0.039	0.875	0.773	1.172	2.34
7.5	0.075	0.073	0.063	0.973	0.837	1.289	2.57

Field samples analysis

The developed method was successfully employed to analyze thirty FCV tobacco leaf samples collected from local farmers' field (Fig. 3). Imidaclprid residues were detected in six samples, and the concentrations ranged between 0.293 to 0.954 µg g^{-1}, which were below the GRL (5 µg g^{-1}) of imidacloprid as per CORESTA (2013).

Cost of analysis and advantages over earlier reported methods

The economics of analysis was calculated by considering the cost of inputs (solvents and reagents, only). The cost was found to be INR 216 per sample (where, 61.52 INR = 1 US Dollar) for the present method, as compared to the INR 970 for Placke and Weber's (1993) method. As per

our estimate, by this method one laboratory chemist could process around 9 samples per day up to the stage of ready-to-inject condition for HPLC analysis as compared to nearly 3-4 samples/person/day by the Placke and Weber's (1993) method. If cost of analysis is considered, as per our estimate, the present method (INR 216 per sample) is similar to that of the method (INR 214 per sample) reported by Liu *et al.,* (2005). But, the present method has an advantage over the method reported by Liu *et al.,* (2005) because it does not involve any usage of methanol, which is a known human poison (Kute *et al.,* 2012; Desai *et al.,* 2013) and its use, even in research institutes, requires legal permission from the government customs departments. Thus, after considering the cost of analysis and safety of analyst, the present method is more acceptable than earlier reports.

Conclusion

In this study, we are reporting a method for detecting imidacloprid residue in tobacco leaf matrix with 83.7-97.5% recovery (RSD <10%). The method holds good linearity (R_2 > 0.99) for the range of 0.1 and 7.5 µg mL^{-1}. The current method which complies with the international specifications, along with comparable cost of analysis, has potential to increase the overall work efficiency of a pesticide residue testing laboratory dealing specifically the monitoring of imidacloprid residue in tobacco leaf.

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