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Original Research Article

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING UPLC METHOD FOR SIMULTANEOUS QUANTIFICATION OF IMIDACLOPRID, THIRAM AND CARBOXIN IN PESTICIDE FORMULATION

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Abstract: A novel stability-indicating ultra-performance liquid chromatography (UPLC) method has been developed and validated for quantification of Imidacloprid, Thiram and carboxin in pesticide formulation (FS), using Poroshell 120 EC-C18 (100 mm × 4.6 mm, 2.7µm) column. Mixture of Water: Methanol (40:60 v/v) was used as mobile phase. The flow rate was kept 0.40 ml/min and detection was carried out at 250 nm. The limit of detection was 0.0006mg/ml, 0.0007mg/ml and 0.0007mg/ml for Imidacloprid, Thiram and Carboxin respectively. The limit of quantitation values was 0.0019mg/ml, 0.0015mg/ml and 0.0015mg/ml for Imidacloprid, Thiram and Carboxin respectively. The linearity of proposed method was investigated in the range of 0.0019-0.596mg/ml (r^2=0.9997), 0.0015-0.178mg/ml (r^2=0.9997) and 0.0015-0.175mg/ml (r^2=0.9997) for Imidacloprid, Thiram and Carboxin respectively. The percentage recovery found to be in range from 98.1-99.9 %, 98.3-100.7% and 98.6-99.6% for Imidacloprid, Thiram and Carboxin respectively. The % RSD values for intraday precision study and interday precision study were <1.66, <2.0 and <2.0 for Imidacloprid, Thiram and Carboxin respectively, as per modified Horwitz equation as requirements by CIPAC. The method was found to be specific, linear, precise, accurate and robust. This method is also useful for quantification of Imidacloprid, Thiram and Carboxin in their single or combination formulated products, with different strengths and different formulation types.

Keywords: Imidacloprid; Thiram; Carboxin; Stability indicating; Validation; Horwitz equation; FS-Flow-able concentrate for Seed treatment, CIPAC- Collaborative International Pesticides Analytical Council. Uncertainty in measurements

Introduction: Imidacloprid is (E)-1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine. Imidacloprid is Systemic insecticide with translaminar activity. Acts as an antagonist by binding to postsynaptic nicotinic receptors in the insect central nervous system. **Thiram** is bis (dimethylthiocarbamoyl) disulfide. Basic contact fungicide with protective action. Non-specific, multi-site fungicide which inhibits numerous enzymes in the fungus, resulting in subsequent inhibition of

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spore germination and mycelial growth. **Carboxin** is 5, 6-dihydro-2-methyl-1, 4-oxathi-ine-3-carboxanilide. Carboxin is Systemic fungicide which inhibits mitochondrial function by disrupting complex II (succinate dehydrogenase) in the respiratory electron transport chain. Structures of compounds[1] shown in figure 1-3

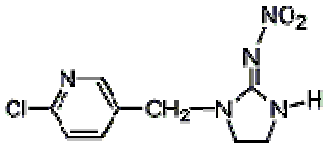

Fig. 1 Structure of Imidacloprid

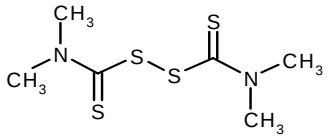

Fig. 2 Structure of Thiram

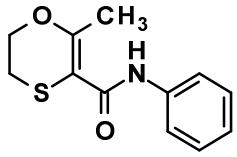

Fig.3 Structure of Carboxin

Various publications are available regarding determination method of Imidacloprid, Thiram and Carboxin but most of the methods are applicable either to Imidacloprid or Thiram or Carboxin in various pesticide formulations or in foods or water samples. UPLC MS/MS method was reported for quantification of Imidacloprid in paddy, vegetables, soil and water samples[2,3,4,5,6], by HPLC for formulation products [7,8] also by Chrono-potenitiometry in formulation and river water samples[9] also by Gas chromatography GC-NPD/ GC-ECD)[10] also by ELISA method [11] and voltametric method in potato samples[12].Normal phase HPLC method reported for Thiram[13]and also by LC/MS/MS [14].HPLC method for Carboxin was reported for formulation samples and in cabbage [15, 16], gas chromatographic method GC-AED [17] and GC-MS [18]reported and spectrophotometric method for formulation and environmental samples [19].Simultaneous determination of Imidacloprid and Carboxin residues in herbal teas and food sample by UPLC-MS-MS[20, 21] and simultaneous determination of Imidacloprid and Thiram in chilli sample by UPLC-MS[22]were reported.

To the best of our knowledge, there is no reported UPLC method for simultaneous quantification of Imidacloprid Thiram and Carboxin in pesticide formulations. Thus, efforts were made to develop fast, selective and sensitive stability indicating method for simulteneous quantification of Imidacloprid, Thiram and Carboxin in their combined pesticide formulation using ultra performance liquid chromatography. In the current work developed a simple, reliable and reproducible, stability indicating UPLC method which was duly validated by statistical parameters precision, accuracy-recovery, linearity, robustness, solution stability also uncertainty in measurement. The method has been applied to the simultaneous quantification of Imidacloprid, Thiram and Carboxin in technical and pesticide formulations.

Materials and Method:

Materials: Certified Reference materials (CRM) of Imidacloprid, Thiram and Carboxin were procured from Sigma Aldrich. The technical grade materials of above active ingredients were obtained from market. The analytical standards were prepared by purification of these technical grade materials. The analytical standards were qualified against CRMs and purity found as for Imidacloprid- 99.2%, Thiram - 99.0% and Carboxin - 99.5%.These standards used for further analysis. Sample of Pesticide formulation for seed treatment (FS) containing Imidacloprid 240 g/l, Thiram 70g/l and Carboxin 70g/l was prepared in laboratory. HPLC grade methanol was purchased from Fischer Scientific, Mumbai (India). Mili-Q (Millipore India Pvt. Ltd) system

used to obtain HPLC grade water. Analytical grade Hydrochloric acid (35%), Sodium Hydroxide pellets and 30% v/v Hydrogen Peroxide solution were obtained from SD Fine Chemicals Ltd, Mumbai (India).

Instrumentation: The Chromatographic system used to perform development and validation of this quantification method is of WATERS Acquity UPLC comprised of a binary solvent pump, Photo Diode array detector and auto sampler with Empower 2 software.

Mobile phase preparation: The mobile phase is consist of mixture Water and Methanol in 40:60 (v/v) proportion.

Diluent preparation: Mobile phase used as diluent.

Standard Preparation: The Standard stock solution prepared in 50 ml volumetric flask by dissolving 199.41 mg of Imidacloprid (99.2%), 59.63 mg of Thiram (99.0%) and 58.43 mg of Carboxin (99.5%) standard in 10 ml of diluent. This solution then sonicated for 10 minutes and diluted to volume with diluent. Further 5 ml of this solution is taken in 50 ml volumetric flask and made up to mark with the diluent. This standard solution contains 0.396 mg/ml of Imidacloprid, 0.118 mg/ml of Thiram and 0.116 mg/ml of Carboxin.

Sample Preparation: Sample solution was prepared by taking about 100 mg of sample in 50 ml volumetric flask, about 10 ml of diluent was added and sonicated for 10 minutes with intermittent shaking. The content was brought back to ambient temperature and diluted to volume with diluent. The sample was filtered through 0.45µm nylon syringe filter.

Chromatographic condition: Method involves use of Poroshell 120 EC-C18 (Agilent Technologies) column with length of 100 mm, internal diameter 4.6 mm and 2.7 µm particle size of stationary phase. The column oven temperature maintained at 30°C throughout the analysis. Different composition tried in isocratic mode. Mobile Phase-A Water: Mobile Phase-B Methanol (40:60 v/v) was selected which gave good resolution. The flow rate was maintained at 0.4 ml/min and detection at 250 nm was carried out with injection volume of 1 µl.

Initial analysis of sample: Sample was analyzed in accordance with above mentioned conditioned and calculated results were tabulated in table 1.

Table 1: Results of initial analysis

Sr. No	Ingredients	Active Ingredient content (A.I)		Specific Gravity (Sp.Gr.)
		g/L	% m/v	
1	Imidacloprid	243.8	24.38	1.148
2	Thiram	69.2	6.92	
3	Carboxin	75.2	7.52	

Calculation:

Active content (%m/v) for Imidacloprid/ Thiram / Carboxin

$$= \frac{\text{Mean sample Area}}{\text{Mean Standard Area}} \times \frac{\text{Standard Weight}}{50} \times \frac{5}{50} \times \frac{50}{\text{Sample Weight}} \times P \times \text{Sp.Gr}$$

Results and Discussion:

Development and optimization of UPLC Method: In the present work, an analytical method based on UPLC using PDA detector has been developed and validated for the quantification of Imidacloprid, Thiram and Carboxin in pesticide formulation. The analytical condition was selected, keeping in mind the different chemical nature of Imidacloprid, Thiram and Carboxin[23].The development trials were taken by using the degraded sample of each component was done, by keeping them in various extreme conditions. The column selection has been done on the basis of back pressure, resolution, peak shape and day to day reproducibility of retention time. After evaluating all these factors, Agilent make Poroshell 120 EC C18 (100 mm x 4.6 mm, 2.7 µm particle size) column was found to be giving satisfactory results. The selection of mobile phase is based on the chemical structure of three actives. Considerably good results were obtained

with Water as mobile phase-A. For the selection of organic constituents of mobile phase-B, Methanol was chosen to reduce the longer retention time and to attain good peak shape. Finally the mobile phase composition consisting of in Mobile phase-A (Water): Mobile phase-B (Methanol) in 40:60 v/v ratio is chosen. Optimized proportion of mobile phase has shown good resolution between Imidacloprid, Thiram and Carboxin and also the degradation product which generated during forced degradation study. Wavelength selection and PDA scan graph are given in figure 4.

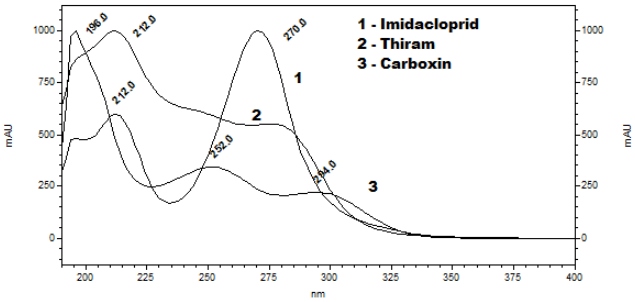

Figure 4: Wavelength scan overlay of standard preparation

Forced degradation study(Stress Study) and stability indicating test: In order to determine the stability indicating power of analytical method for quantification of Imidacloprid, Thiram and Carboxin, the various stressed conditions to be conducted for forced degradation studies as per ICH guidelines[24, 25]. The used forced degradation conditions, stress agent concentration and times of stress, were found to affect degradation, preferably1% to 30% and not complete degradation of active materials. The discovery such conditions was based on trial and error. Refer Table 2 for % degradation (%m/v) in each stress conditions.

Acidic condition: Acidic degradation study was performed by taking about 100 mg of sample in 50 volumetric flask and added 5 ml of 0.1N HCl and kept for 1hour at room temperature. After 1 hour sample was neutralized with 5 ml of 0.1N NaOH, diluted with diluent and filtered through 0.45µ nylon syringe filter and injected.

Alkaline condition: Alkaline degradation study was performed by taking about 100 mg of sample in 50 volumetric flask and added 5 ml of 0.1N NaOH and kept for 1hour at room temperature. After 1 hour sample was neutralized with 5 ml of 0.1N HCl, diluted with diluent and filtered through 0.45µ nylon syringe filter and injected.

Oxidative condition: Oxidative degradation study was performed by taking about 100 mg of sample in 50 volumetric flask and added 5 ml of 5% H_2O_2 and kept for 30 minutes at room temperature. After 30 minutes sample was diluted with diluent and filtered through 0.45µ nylon syringe filter and injected.

Thermal condition: Thermal degradation was performed by exposing formulation sample at 54°C for 14 days. This condition also known as Accelerated Heat Study (AHS).About 100 mg of sample taken in 50 volumetric flask diluted with diluent, sonicate and filtered through 0.45µ nylon syringe filter and injected.

Photolytic condition: Photolytic degradation study was performed by exposing formulation sample to sunlight for 14 days. About 100 mg of sample taken in 50 volumetric flasks diluted with diluent sonicate and filtered through 0.45µ nylon syringe filter and injected.

Table 2: Results of Forced degradation study

Condition	**Active Ingredient Content(A.I) (% m/v)**					
	Imidacloprid		Thiram		Carboxin	
		Degradation		Degradation		Degradation
Initial	24.38	---	6.92	---	7.52	---
Acidic	22.92	1.46	6.32	0.60	7.05	0.47
Alkaline	23.02	1.36	6.28	0.64	7.08	0.44
Oxidative	23.23	1.15	6.53	0.39	5.43	2.09
Thermal	24.23	0.15	6.98	-0.06	7.49	0.03
Photolytic	24.22	0.16	6.99	-0.07	7.56	-0.04

Method validation: The method validation was carried out as per ICH guidelines [26] and SANCO guidelines [27].Various method validation parameters were performed [28].

Specificity: Specificity of the method was determined by injecting mobile phase blank, formulation blank, Imidacloprid standard, Thiram standard and Carboxin Standard and sample solution. Since there was no interference between the peaks of active ingredients in standard, sample as well as in mobile phase blank and formulation blank (placebo). Also peak purity was found satisfactory. Refer figure 5-8.

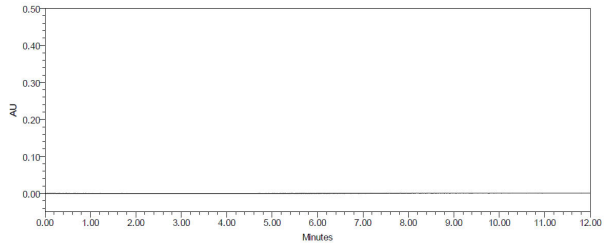

Fig. 5: Chromatogram of blank

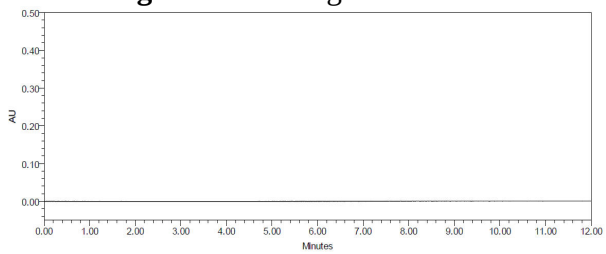

Fig. 6: Chromatogram of placebo

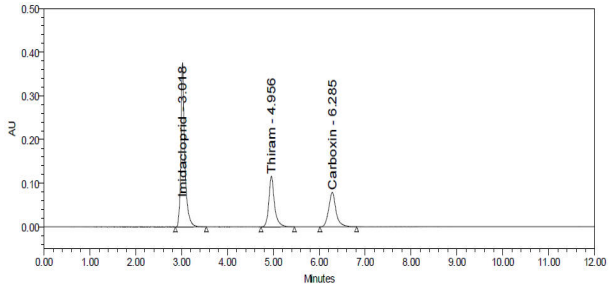

Fig. 7: Chromatogram of standard preparation

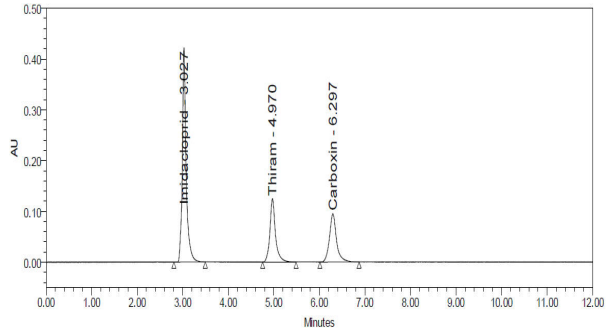

Fig. 8: Chromatogram of sample preparation

System Suitability: System suitability is integral part of method validation. % RSD of retention times and peak area of six replicate injection of standard solution were less than 1.0 %.(Refer Table 3)

Table 3: System Suitability of standard solution

Parameters	Results			Limits
	Imidacloprid	Thiram	Carboxin	
% RSD of retention time	0.04	0.08	0.09	< 1.0 %
% RSD of peak area	0.25	0.26	0.46	< 1.0 %

Precision: The Precision was evaluated at two levels, repeatability (intraday) and intermediate precision (interday). Repeatability precision was investigated by six replicate injections of Imidacloprid, Thiram and Carboxin with concentration 240 mg/ml (24.0% mv), 70 mg/ml (7.0% m/v) and 70 mg/ml (7.0% m/v) respectively and six different preparation of same sample, for intermediate precision proceed same as repeatability precision but performed on different day. Table 4 showing acceptable % RSD values calculated by modified Horwitz equation

$$\% \text{ RSD} = < 2^{(1-0.5 \log C)} \times 0.67$$

Table 4: Acceptable % RSD values calculated by modified Horwitz Equation

Sr. no.	Compound	% Analyte(m/v)	Analyte Ratio(C)	% RSD (calc.)
1	Imidacloprid	24.0	0.24	1.66
2	Thiram	7.0	0.07	2.00
3	Carboxin	7.0	0.07	2.00

The results of precision was expressed as % RSD and was tabulated in Table 5

Table 5: Results of Precision studies

	Imidacloprid		Thiram		Carboxin	
	Intra day	Inter day	Intra day	Inter day	Intra day	Inter day
Mean (% m/v)	24.44	24.46	6.95	6.97	7.71	7.60
% RSD	0.59	0.72	1.00	0.91	1.01	1.00

Limit of Detection (LOD) and Limit of Quantitation (LOQ): The limit of detection and limit of quantitation were evaluated by serial dilution of Imidacloprid, Thiram and Carboxinfrom standard stock solution. The solution was injected 6 times and % RSD calculated. If % RSD was ≤ 10%, then this level termed LOQ. If % RSD exceeds 10%, then this level termed LOD. Table 5 showing LOD and LOQ values. Refer Table 6

Table 6: Limit of Detection and Limit of Quantitation study

	Imidacloprid (mg/ml)	Thiram (mg/ml)	Carboxin (mg/ml)
Limit of Detection	0.0006	0.0007	0.0007
Limit of Quantitation	0.0019	0.0015	0.0015

Linearity: The linearity was evaluated by measuring 6 different concentration levels from LOQ, 50%, 80%, 100%, 120 % and 150% of standard solution of Imidaloprid, Thiram and Carboxin. The linearity curve plotted - concentration of standard (mg/ml) against mean peak areas and the correlation coefficient value was computed. The summary of the parameters shown in Table 7

Table 7: Linearity study

	Imidacloprid	Thiram	Carboxin
Linearity Range(mg/ml)	0.0019-0.596	0.0015-0.178	0.0015-0.175
Correlation Coefficient (R^2)	0.9997	0.9997	0.9997
Slope (m)	5771498.7	8704124.9	7860808.54
Y-intercept (C)	-3407.47	-3616.9	-3843.92

Accuracy and recovery: Accuracy (% Recovery) of analytical method was determined at four concentration levels by spiking known amount of pure actives in placebo i.e. LOQ, 80%, 100% and 120%. The accuracy was calculated as % of recovery. The mean recovery results were tabulated in Table 8.

Table 8: Results of accuracy study

Components	Level	Amount added*(mg/ml)	Amount found*(mg/ml)	% Mean Recovery	% RSD
Imidacloprid	LOQ	0.00219	0.00218	99.9	1.01
	80%	0.32149	0.31907	99.2	1.08
	100%	0.40186	0.40070	99.7	0.64
	120%	0.48223	0.47331	98.1	1.75
Thiram	LOQ	0.00149	0.00149	100.2	0.71
	80%	0.09702	0.09683	99.8	1.68
	100%	0.12128	0.12209	100.7	0.50
	120%	0.14553	0.14309	98.3	0.38
Carboxin	LOQ	0.01500	0.00149	99.3	1.55
	80%	0.09608	0.09499	98.9	1.54
	100%	0.12010	0.11959	99.6	0.56
	120%	0.14412	0.14211	98.6	1.83

**Each value corresponds to the mean of three determinations*

Stability of solutions: The stability of standard solution and sample solution was test for intervals 24 h, 48 h and 72 h. at ambient temperature. There were no any significant changes observed in peak areas and assay values. It was concluded that the standard and test preparation was found stable up to 72 hours at ambient temperature.

Robustness: The robustness of the method was unaffected when small, deliberate changes like, flow change, mobile phase composition and column temperature were performed. No significant impact observed on results due to change in flow rate, mobile phase composition and column oven temperature.

Uncertainty of measurement (U): Uncertainty of method was measured through the data of uncertainty due to Repeatability, Calibration uncertainty of equipment or glassware, Readability of equipment, CRM purity of concentration, Linearity of calibration curve and Recovery of the analyte. The Combined Relative Uncertainty (U_c) and Expanded Uncertainty (U) were calculated[29] and tabulated in Table 9.

Table 9: Calculated Combined and Expanded Uncertainty

Components	Mean Value (% m/v) (n=20)	Combined Relative Uncertainty (U_c)	Expanded Uncertainty (U) (% m/v)
Imidacloprid	24.48	0.004360	± 0.21
Thiram	6.95	0.007941	± 0.11
Carboxin	7.71	0.005060	± 0.08

Conclusion:

A simple, specific and reliable UPLC method has been developed for quantification of Imidacloprid, Thiram and carboxin in their pesticide formulation. Stress study showed that all degradation products were well separated from Imidacloprid, Thiram and Carboxin peaks confirming its stability indicating power. Method validation study showed that the method is specific, linear, accurate and easily reproducible. This method can also be used for quantification of Imidacloprid, Thiram and Carboxin in their single or combination formulated products with different strengths and different formulation types. This method can also useful for analysis of environmental samples (soil, water), agricultural products for pesticide residue analysis of same actives but required additional extraction procedure. Hence developed method can be adapted to regular quality control analysis of production samples and stability samples, environmental samples.

Acknowledgement

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