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Development and Validation of Stability Indicating UPLC method for simultaneous Quantification of Thiophanate-methyl, Metalaxyl and Captan 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 Thiophanate-methyl, Metalaxyl and Captan in pesticide formulation (DS), using Poroshell 120 EC-C18 (100 mm × 4.6 mm, 2.7µm) column. Mixture of 0.1% ortho-phosphoric acid: Acetonitrile (50:50 v/v) was used as mobile phase. The flow rate was kept 0.7 ml/min and detection was carried out at 205 nm. The limit of detection was 0.0002 mg/ml, 0.0002 mg/ml and 0.001 mg/ml for Thiophanate-methyl, Metalaxyl and Captan respectively.The limit of quantitation values was 0.0004 mg/ml, 0.0004 mg/ml and 0.0020 mg/ml for Thiophanate-methyl, Metalaxyl and Captan respectively. The linearity of proposed method was investigated in the range of 0.0004-0.297 mg/ml (r^2=0.9998), 0.0004-0.153 mg/ml (r^2=0.9993) and 0.0020-0.742 mg/ml (r^2=0.9996) for Thiophanate-methyl, Metalaxyl and Captan respectively. The percentage recovery found to be in range from 99.7-100.8 %,100.2-100.9% and 99.2-100.7% for Thiophanate-methyl, Metalaxyl and Captan respectively. The % RSD values for intraday precision study and inter-day precision study were < 1.90, < 2.10 and < 1.65 for Thiophanate-methyl, Metalaxyl and Captan respectively as per modified Horwitz equation as requirements by CIPAC.The developed method was found to be specific, linear, precise, accurate and robust.

KEYWORDS: Thiophanate-methyl; Metalaxyl; Captan; Stability indicating; Validation; Horwitz equation; DS-Dry powder for Seed treatment, CIPAC

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INTRODUCTION

Thiophanate-methyl, is dimethyl 4, 4′-(*o*-phenylene) bis (3-thioallophanate). Thiophanate-methyl is Systemic Fungicide with protective and curative action, absorbed by the leaves and roots of plants / crops , effective against a wide range of fungal pathogens including eyespot and other disease of cereals. Also used additionally as a wound protectant for pruning cuts of trees. **Metalaxyl** is methyl *N*-(methoxyacetyl)-*N*-(2, 6-xylyl)-DL-alaninate; methyl 2-{[(2, 6-dimethylphenyl) methoxyacetyl] amino} propionate. Metalaxyl is Systemic fungicide with protective and curative action, taken up by leaves, stems and roots. **Captan** is *N*-(trichloromethylthio) cyclohex-4-ene-1, 2-dicarboximide. Captan is non-systemic fungicide with protective and curative action. Structures of compounds shown in figure 1-3.

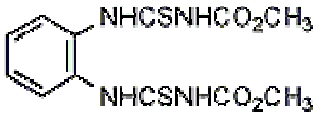

Figure 1. Structure of Thiophanate-methyl

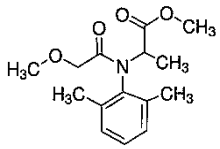

Figure 2. Structure of Metalaxyl

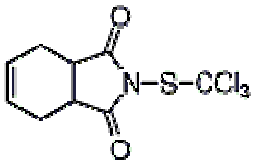

Figure 3. Structure of Captan

Various publications are available regarding determination method of Thiophanate-methyl, Metalaxyl and Captan but most of the methods are applicable either to Thiophanate-methyl or Metalaxyl or Captan in various pesticide formulations or in foods or water or biological samples. UPLC MS/MS method was reported for quantification of Thiophanate-methyl[1] also spectrophotometric method using iodine-azide reaction was reported for determination of Thiophnate-methyl[2]. GC method for quantification of Metalaxyl and its formulated products [3]. RP-HPLC [4] and UHPLC method [5] for determination of Metalaxyl residues, UPLC-MS method for Metalaxyl in well water [6], GC-MS method for determination Metalaxyl and identification of adjuvants in wettable powder formulation [7]. RP-HPLC method for determination of Captan in drinking water [8] and in technical sample by HPLC and GC [9], LC-MS/MS method for determination Captan metabolite in human plasma and urine [10] and in fruits and vegetable samples [11], UPLC-MS method Captan residues in food matrices [12], GC-MS/MS method Captan residues in vegetables and food samples [13, 14] and in kaki fruits [15]. Spectrophotometric method for determination of Captan in commercial formulations [16].

RP-HPLC method for simultaneous determination of Captan and Metalaxyl residues in grapes [17] and RP-HPLC method for simultaneous determination of Thiophanate-methyl and Captan residue in IPA solution used by orchard workers [18]

To the best of our knowledge, there is no reported UPLC method for simultaneous quantification of Thiophanate-methyl, Metalaxyl and Captan in pesticide formulations. Thus, efforts were made to develop fast, selective and sensitive stability indicating method for quantification of Thiophanate-methyl, Metalaxyl and Captan 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. The method has been applied to the simultaneous quantification of Thiophanate-methyl, Metalaxyl and Captan in technical and pesticide formulations.

EXPERIMENTAL

Materials:Certified Reference materials (CRM) of Thiophanate-methyl, Metalaxyl and Captan 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 Thiophanate-methyl (98.3%), Metalaxyl (98.6%) and Captan (98.8%). These standards used for further analysis. Sample of Pesticide formulation for seed treatment (DS) containing Thiophanate-methyl 10% w/w, Metalaxyl 5% w/w and Captan 25% w/w was prepared in laboratory. HPLC grade acetonitrile was purchased from Fischer Scientific, Mumbai (India). Mili-Q (Millipore India Pvt. Ltd) system used to obtain HPLC grade water. Analytical grade Ortho-phosphoric acid (88%), Hydrochloric acid (35%), Sodium Hydroxide pellets and 30% v/v Hydrogen Peroxide solution were obtained from SD Fine Chemicals Ltd, Mumbai (India).

Instrumentation:Agilent Infinity-II UPLC system is used for the development and validation of method, which is comprised of a quaternary solvent pump, Photo Diode array detector and auto sampler with Open-Lab software.

Mobile phase preparation:The mobile phase consists of 0.1 % Ortho-phosphoric acid and Acetonitrile in 50:50 (v/v) ratio. Buffer was prepared by adding 1.0 ml of Ortho-phosphoric acid in 1000 ml HPLC grade water and filtered through a 0.45 µm nylon membrane (Millipore Pvt. Ltd, Bengaluru, India) and degassed in an ultrasonic bath.

Diluent preparation: Mobile phase used as diluent.

Standard Preparation:The Standard stock solution prepared in 50 ml volumetric flask by dissolving 102.3 mg of Thiophante-methyl (98.3%), 50.85 mg of Metalaxyl (98.6%) and 250.17 mg of Captan (98.8%) standard in 20 ml of Acetonitrile. 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 0.2011 mg/ml of Thiophanate-methyl, 0.100 mg/ml of Metalaxyl and 0.494 mg/ml of Captan.

Sample Preparation: Sample solution was prepared by taking about 100 mg of sample in 50 ml volumetric flask and 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:

Column	:	Poroshell 120 EC-C18 (Agilent Technologies) (100 mm x 4.6 mm, 2.7 µm)
Mobile phase	:	Mobile Phase-A: Mobile Phase-B 0.1 % OPA: Acetonitrile (50:50 v/v)
Flow	:	0.70 ml/min
Injection Volume	:	1 µl
Column Temperature	:	30°C
Wavelength	:	205 nm
Run Time	:	12 minutes

Initial analysis of sample: Sample was analyzedand results were tabulated in Table 1.

Table 1: Results of initial analysis

Sr. No	Ingredients	Active Ingredient content (A.I) % w/w
1	Thiophanate-methyl	9.55
2	Metalaxyl	5.11
3	Captan	25.94

Calculation:

Active content (%w/w) forThiophanate-methyl/ Metalaxyl / Captan

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

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 Thiophanate-methyl, Metalaxyl and Captan in pesticide formulation. The analytical condition was selected, keeping in mind the different chemical nature of these three actives. 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. Mobile phase is selected on the basis of the chemical structure of three actives. The acidic pH range of mobile phase was found suitable for solubility, resolution, stability and peak shape of three components. Considerably good results were obtained with 0.1 % Ortho-phosphoric acid solutions as buffer. Acetonitrile was chosen as organic constituents to reduce the longer retention time and better peak shape. Finally the mobile phase composition consisting of 0.1% OPA and Acetonitrile in 50:50 ratio (v/v) was fixed. Optimized proportion of mobile phase has shown good resolution between Thiophanate-methyl, Metalaxyl and Captan and also the degradation product which generated during forced degradation study. Wavelength selection and PDA scan graph are given in figure 4.

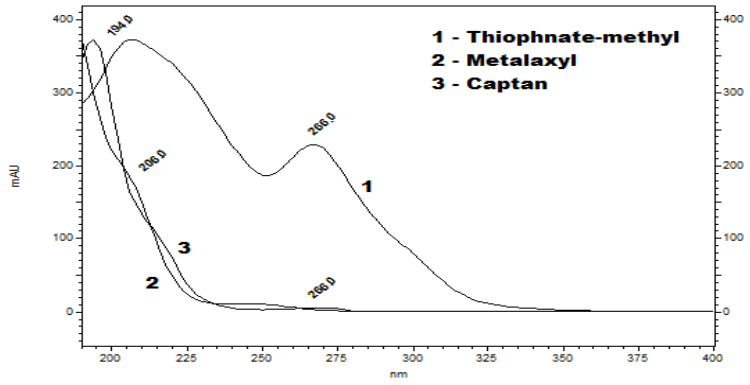

Figure 4. Wavelength Scan Overlay of Standard Preparation

Forced degration study (Stress study) and stability indicating test

In order to determine the stability indicating power of analytical method for quantification of Thiophanate-methyl, Metalaxyl and Captan, the various stressed conditions to be conducted for forced degradation studies as per ICH guidelines[19, 20]. The used forced degradation conditions, stress agent concentration and times of stress, were found to affect degradation, preferably 1% to 20% and

not complete degradation of active materials. The discovery such conditions was based on trial and error. Refer Table 2 for % degradation (%w/w) 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 15 minutes at room temperature. After 15 minutes sample was neutralized with 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 15 minutes at room temperature. After 15 minutes sample was neutralized with 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 15 minutes at room temperature. After 15 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, 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 flask 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)					
	Thiophanate-methyl		Metalaxyl		Captan	
		Degradation		Degradation		Degradation
Initial	9.55	---	5.11	---	25.94	---
Acidic	8.50	1.05	4.90	0.21	22.73	3.21
Alkaline	6.03	3.52	4.46	0.65	20.59	5.35
Oxidative	7.04	2.51	3.86	1.25	24.63	1.31
Thermal	9.53	0.02	5.02	0.09	24.00	0.94
Photolytic	9.50	0.05	3.90	1.21	25.39	0.55

Method validation

The method validation was carried out as per ICH guidelines[21] and SANCO guidelines [22].Various method validation parameters were performed.

Specificity: Specificity of the method was determined by injecting mobile phase blank, formulation blank (placebo), Thiophanate-methyl standard, Metalaxyl standard, Captan 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. Also peak purity was found satisfactory. Refer figure 5-8.

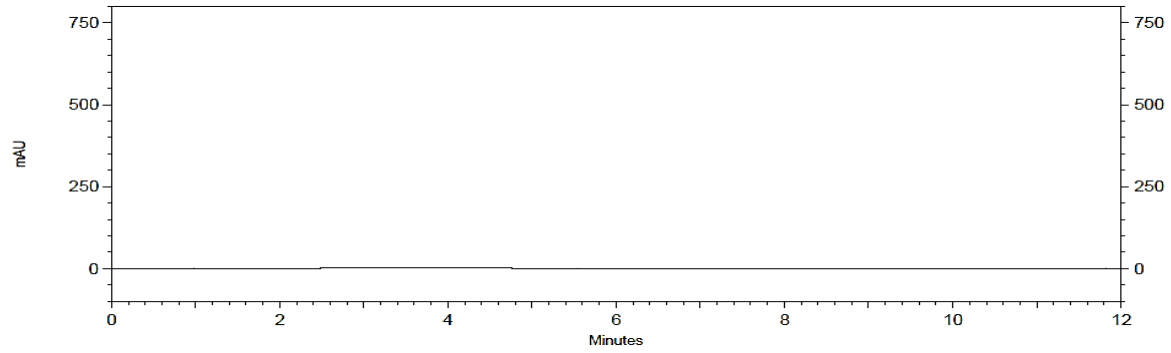

Figure 5. Chromatogram of Blank

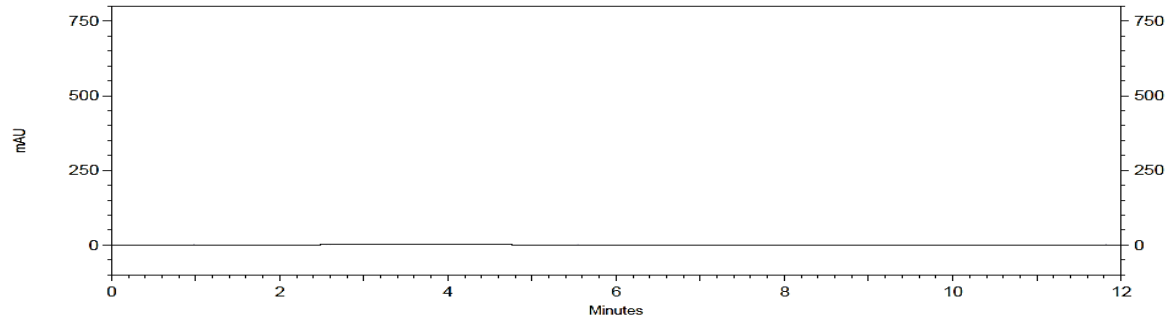

Figure 6. Chromatogram of Formulation Blank (placebo)

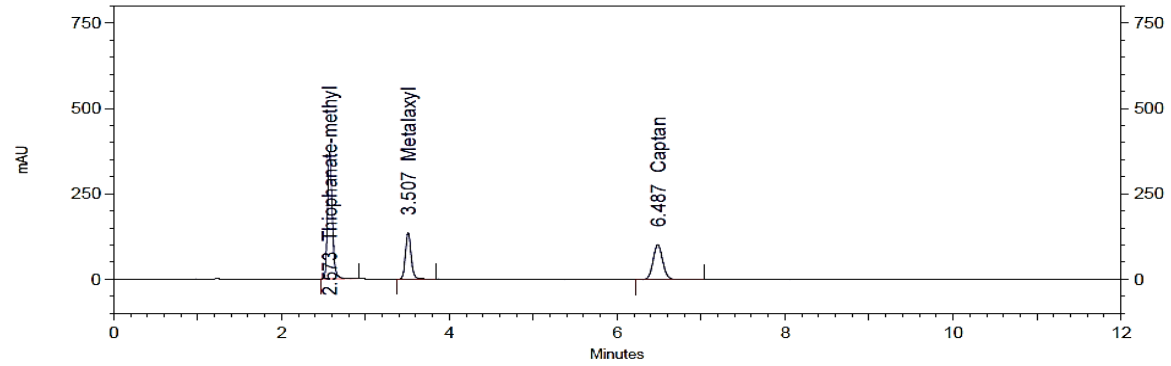

Figure 7. Chromatogram of Standard Preparation

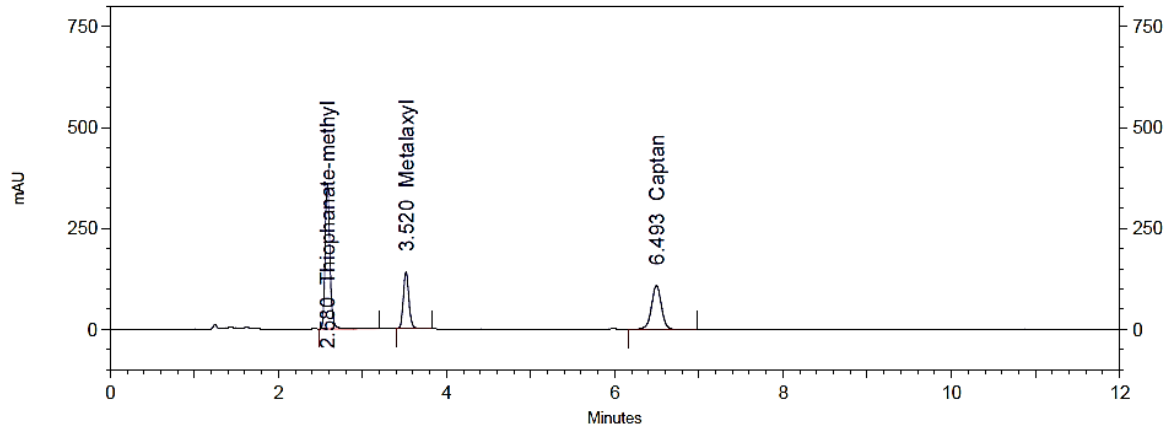

Figure 8. Chromatogram of Sample Preparation

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
	Thiophanate-methyl	Metalaxyl	Captan	
% RSD of retention time	0.11	0.01	0.06	< 1.0 %
% RSD of peak area	0.54	0.49	0.68	< 1.0 %

Precision: The Precision was evaluated at three levels, repeatability and reproducibility (intraday) and intermediate precision (inter-day). Each level of precision was investigated by six replicate injections of concentration 0.201 mg/ml, 0.10 mg/ml and 0.50 mg/ml of Thiophanate-methyl, Metalaxyl and Captan respectively. Table 4 showing acceptable % RSD values calculated by modified Horwitz equation

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

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

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

Sr. no.	Compound	% Analyte (w/w)	Analyte Ratio (C)	% RSD (calc.)
1	Thiophanate-methyl	10	0.10	1.90
2	Metalaxyl	5	0.05	2.10
3	Captan	25	0.25	1.65

Table 5: Results of Precision studies

	Thiophanate-methyl (% w/w)		Metalaxyl (% w/w)		Captan (% w/w)	
	Intraday	Inter-day	Intraday	Inter-day	Intraday	Inter-day
Mean (% w/w)	9.52	9.48	5.16	5.11	26.00	25.99
% RSD	0.83	0.34	0.80	0.22	0.40	0.60

Limit of Detection (LOD) and Limit of Quantitation (LOQ): The limit of detection and limit of quantitation were evaluated by serial dilution of Thiophanate methyl, Metalaxyl and Captan

from system suitability standard solution. The solution was injected 6 times and % RSD calculated. If % RSD was less than 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

	Thiophanate-methyl (mg/ml)	Metalaxyl (mg/ml)	Captan (mg/ml)
Limit of Detection	0.00020	0.00020	0.0010
Limit of Quantitation	0.00040	0.00040	0.0020

Linearity: The linearity was evaluated by measuring 6 different concentration levels from LOQ, 50%, 80%, 100%, 120 % and 150% of standard solution of Thiophanate-methyl, Metalaxyl and Captan. 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

	Thiophanate-methyl (mg/ml)	Metalaxyl (mg/ml)	Captan(mg/ml)
Linearity Range	0.0004-0.297	0.0004-0.153	0.002-0.742
Correlation Coefficient (R^2)	0.9998	0.9993	0.9994
Slope (m)	988776258.17	867786917.34	220740984.90
Y-intercept (C)	-707442.87	559956.74	1098760.22

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
Thiophanate-methyl	LOQ	0.00043	0.00043	99.7	0.15
	80%	0.16113	0.16169	100.3	0.92
	100%	0.20142	0.20259	100.6	0.95
	120%	0.24170	0.24357	100.8	0.22
Metalaxyl	LOQ	0.00048	0.00048	100.2	0.52
	80%	0.08338	0.08417	100.9	0.29
	100%	0.10422	0.10445	100.2	0.39
	120%	0.12506	0.12614	100.9	0.23
Captan	LOQ	0.00200	0.00198	99.2	0.31
	80%	0.39550	0.39541	100.0	0.28
	100%	0.49438	0.49767	100.7	0.91
	120%	0.59325	0.59439	100.2	0.09

* Each value corresponds to the mean of three determinations

Stability of solutions: The stability of standard solution and sample solution was test for an interval 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 samplesolutions were found stable up to 72 hours at ambient temperature.

Robustness: The robustness of method was studied by performing small, deliberate changes in flow, mobile phase composition and column temperature. The quantification values of sample solutions were unaffected and in accordance with that of initial.

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. Refer Table 9.

Table 9: Calculated Combined and Expanded Uncertainty

Components	Mean Value (% w/w) (n=20)	Combined Relative Uncertainty (U_c)	Expanded Uncertainty (U) (% w/w)
Thiophanate-methyl	9.50	0.00559	± 0.10
Metalaxyl	5.16	0.00481	± 0.05
Captan	26.02	0.00567	± 0.29

CONCLUSION

A simple, specific, rapid, sensitive and reliable UPLC method has been developed for quantification of Thiophanate-methyl, Metalaxyl and Captan in their pesticide formulation. Stress study showed that all degradation products were well separated from Thiophanate-methyl, Metalaxyl and Captan 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 Thiophanate-methyl, Metalaxyl and Captan in their single or combination formulated products with different strengths and different formulation types. Hence developed method can be adopted to regular quality control analysis of production samples and stability samples.

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