© 2018 IJSRST | Volume 4 | Issue 5| Print ISSN: 2395-6011 | Online ISSN: 2395-602X
Themed Section: Science and Technology

Development and Validation of Stability Indicating Ultra Performance Liquid Chromatography method for simultaneous Quantification of Thiophanate-methyl, Fipronil and Pyraclostrobin in Pesticide Formulation

Dilip K. Patel*, Babu Lal Swami, T.L. Rajawat
School of Basic and Applied Science, Raffles University, Neemrana-Alwar, Rajasthan, India

ABSTRACT

A novel stability-indicating ultra-performance liquid chromatography (UPLC) method has been developed and validated for quantification of Thiophanate-methyl, Fipronil and Pyraclostrobin in pesticide formulation (FS), using Poroshell 120 EC-C18 (100 mm × 4.6 mm, 2.7µm) column. Mixture of 0.1% ortho-phosphoric acid: acetonitrile (40:60 v/v) was used as mobile phase. The flow rate was kept 0.75 ml/min and detection was carried out at 275 nm. The limit of detection was 0.00017 mg/ml, 0.0010 mg/ml and 0.00022 mg/ml for Thiophanate-methyl, Fipronil and Pyraclostrobin respectively. The limit of quantitation values was 0.00035mg/ml, 0.0020mg/ml and 0.00035mg/ml for Thiophanate-methyl, Fipronil and Pyraclostrobin respectively. The linearity of proposed method was investigated in the range of 0.00038-0.661mg/ml (r^2=0.9993), 0.00202-0.743mg/ml (r^2=0.9997) and 0.0004-0.091mg/ml (r^2=0.9996) for Thiophanate-methyl, Fipronil and Pyraclostrobin respectively. The percentage recovery found to be in range from 98.4-100.0 %, 98.4-99.1% and 98.5-99.3% for Thiophanate-methyl, Fipronil and Pyraclostrobin respectively. The % RSD values for intraday precision study and inter-day precision study were <1.65, <1.68 and <2.33 for Thiophanate-methyl, Fipronil and Pyraclostrobin respectively as per modified Horwitz equation as requirements by CIPAC. The developed method was found to be specific, linear, precise, accurate and robust. This method is also useful for quantification of Thiophanate-methyl, Fipronil and Pyraclostrobin in their single or combination formulated products, environmental samples (soil, water), and agricultural products for pesticide residue analysis.

Keywords: Thiophante-methyl; Fipronil; Pyraclostrobin; Stability indicating; Validation; Horwitz equation; FS-Flow-able concentrate for Seed treatment, CIPAC - Collaborative International PesticidesAnalytical council, Uncertainty in measurements.

I. INTRODUCTION

Thiophanete-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, 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. **Fipronil,** is (±)-5-amino-1-(2,6-dichloro-α,α,α-trifluoro-p-tolyl)-4 trifluoromethylsulfinylpyrazole-3-carbonitrile. Fipronil in broad-spectrum insecticide which acts as blocker of the GABA-regulated chloride channels, fipronil is toxic by contact and ingestion. Used for control of rootworms, wireworms, termites, plant bugs, moths, beetle etc. **Pyraclostrobin** is methyl N-{2-[1-(4-chlorophenyl) pyrazol-3-yloxymethyl] phenyl} (N-methoxy)carbamate, which inhibits mitochondrial respiration by blocking electron transfer at the cytochrome bc 1 complex. Pyraclostrobin is fungicide with protectant, curative and translaminar properties to control of major plant pathogens [1]. Structures of compounds shown in figure 1-3.

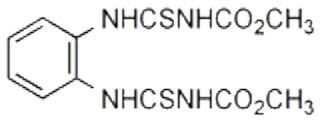

Figure 1. Structure of Thiophanate-methyl

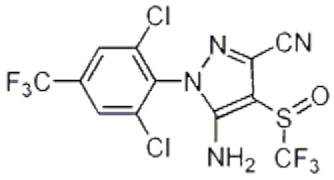

Figure 2. Structure of Fipronil

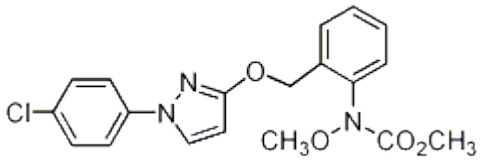

Figure 3 Structure of Pyraclostrobin

Various publications are available regarding determination method of Thiophanate-methyl, Fipronil and Pyraclostrobin but most of the methods are applicable either to Thiophante-methyl or Fipronil or Pyraclostrobin in various pesticide formulations or in foods or water samples. UPLC MS/MS method was reported for quantification of Thiophanate-methyl [2] also spectrophotometric method using iodine-azide reaction was reported for determination of Thiophanate-methyl [3]. Gas chromatographic (GC-FPD/ GC-NPD) methods for Fipronil residues in agricultural products [4] .GC-ECD method for Fipronil residue in honey and pollen plasma and also in surface water [5, 6]. GC-MS method for Fipronil residues in foods, water samples and agricultural products [7, 8, 9], ELISA methods for fipronil residues in humanserum and urine matrices [10], HPLC method for fipronil in bovine plasma and parakill [11,12] and Fipronil in its technical [13].UPLC-MS method for Pyraclostrobin residues in food and also in drinking water [14, 15] also HPLC methods are reported for the determination of Pyraclostrobin in its technical and residues in grapes and tomatoes [16, 17, 18]. Simultaneous determination of Thiophanate-methyl and Fipronil residues in herbal teas by UPLC-MS-MS [19] and simultaneous determination of Fipronil and Pyraclostrobin in food sample by UPLC-MS-MS [20] were reported.

To the best of our knowledge, there is no reported UPLC method for simultaneous quantification of Thiophanate-methyl, Fipronil and Pyraclostrobin in pesticide formulations. Thus, efforts were made to develop fast, selective and sensitive stability indicating method for simultaneous quantification of Thiophanate-methyl, Fipronil and Pyraclostrobin in their combined pesticide formulation using ultra performance liquid chromatographic method. 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. Uncertainty in measurements were also calculated for each active ingredients. The method has been applied to the simultaneous estimation of Thiophanate-methyl, Fipronil and Pyraclostrobin in technical and pesticide formulations.

II. EXPERIMENTAL

2.1 Materials: Certified Reference materials (CRM) of Thiophante-methyl, Fipronil and Pyraclostrobin was 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 werequalified against CRMs and calculated purity found as for Thiophnate-methyl - 98.3%, Fipronil - 98.6% and

Pyraclostrobin - 99.0%. These standards used for further analysis. Sample of Pesticide formulation for seed treatment (FS) containing Thiophante-methyl 225 g/l, fipronil 250g/l and Pyraclostrobin 50g/l 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).

2.2 Instrumentation: The UPLC 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.

2.3 Mobile phase preparation: The mobile phase consist of Mobile phase A - 0.1 % Ortho-phosphoric acid and Mobile phase B – Acetonitrile in 40:60 (v/v) ratio. Mobile phase- A 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.

2.4 Diluent preparation: Mobile phase used as diluent.

2.5 Standard Preparation: The Standard stock solution prepared in 50 ml volumetric
flask by dissolving 225.54 mg of Thiophante-methyl (98.3%), 255.74 mg of Fipronil (98.6%) and 27.90 mg of Pyraclostrobin (99%) 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 0.443 mg/ml of Thiophante-methyl, 0.504 mg/ml of Fipronil and 0.0552mg/ml of Pyraclostrobin.

2.6 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.

2.7 Chromatographic condition: Method involves use of Poroshell 120 EC-C18 (Agilent Tech) column with length of100 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 compositions of mobile phase tried in isocratic mode. Mobile Phase-A: Mobile Phase-B 0.1 % OPA: Acetonitrile (40:60 v/v) was selected which gave good resolution. The flow rate was maintained at 0.75 ml/min and detection at 275 nm was carried out with injection volume of 1µl.

2.8 Initial analysis of sample: Sample was analyzedin accordance with section 2.5-2.7 and 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	Thiophanate-methyl	232.8	23.28	1.223
2	Fipronil	245.8	24.58	
3	Pyraclostrobin	26.1	2.61	

2.9 Calculation:

Active content (%m/v) for Thiophante-methyl/ Fipronil / Pyraclostrobin

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

III. RESULTS AND DISCUSSION

3.1 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, Fipronil and Pyraclostrobin in pesticide formulation. The analytical condition were selected, keeping in mind the different chemical nature of Thiophanate-methyl, Fipronil and Pyraclostrobin [21]. 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. The acidic pH range 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 mobile phase-A. For the selection of organic constituents of mobile phase-B, acetonitrile 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 (0.1% OPA): Mobile phase-B (Acetonitrile) in 40:60 v/v ratio. Optimized proportion of mobile phase has shown good resolution between Thiophanate-methyl, Fipronil and Pyraclostrobin and also the degradation product which generated during forced degradation study. Wavelength selection and PDA scan graph are given in figure 4.

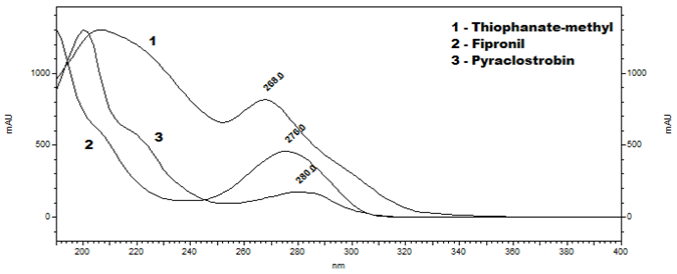

Figure 4. Wavelength scan overlay of standard preparation

4. Forced degradation study (Stress Study) and stability indicating test

In order to determine the stability indicating power of analytical method for quantification of Thiophanate-methyl, Fipronil and Pyraclostrobin, the various stressed conditions to be conducted for forced degradation studies as per ICH guidelines [22, 23]. 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 (%m/v) in each stress conditions.

4.1 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 2 hours at room temperature. After 2 hours sample was neutralized with 0.1N NaOH, diluted with diluent and filtered through 0.45µ nylon syringe filter and injected.

4.2 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 2hours at room temperature. After 2 hours sample was neutralized with 0.1N HCl, diluted with diluent and filtered through 0.45µ nylon syringe filter and injected.

4.3Oxidative condition: Oxidative degradation study was performed by taking about 100 mg of sample in 50 volumetric flask and added 5 ml of 5% H2O2 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.

4.4 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.

4.5Photolytic 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)					
	Thiophanate-methyl		Fipronil		Pyraclostrobin	
		Degradation		Degradation		Degradation
Initial	23.28	---	24.58	---	2.61	---
Acidic	21.23	2.05	22.00	2.58	2.34	0.27
Alkaline	14.58	8.70	21.86	2.72	2.33	0.28
Oxidative	20.49	2.79	22.92	1.66	2.45	0.16
Thermal	23.25	0.03	24.59	-0.01	2.67	-0.06
Photolytic	23.42	-0.14	24.45	0.13	2.13	0.48

5. Method validation

The method validation was carried out as per ICH guidelines [24] and SANCO guidelines [25]. Various method validation parameters were performed [26].

5.1 Specificity: Specificity of the method was determined by injecting mobile phase blank, formulation blank, Thiophanate-methyl standard, Fipronil standard, Pyraclostrobin standardand 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.

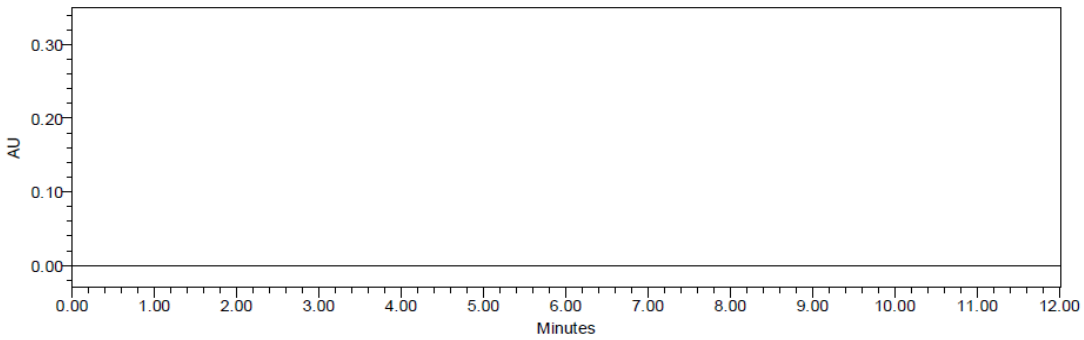

Figure 5. Chromatogram of blank

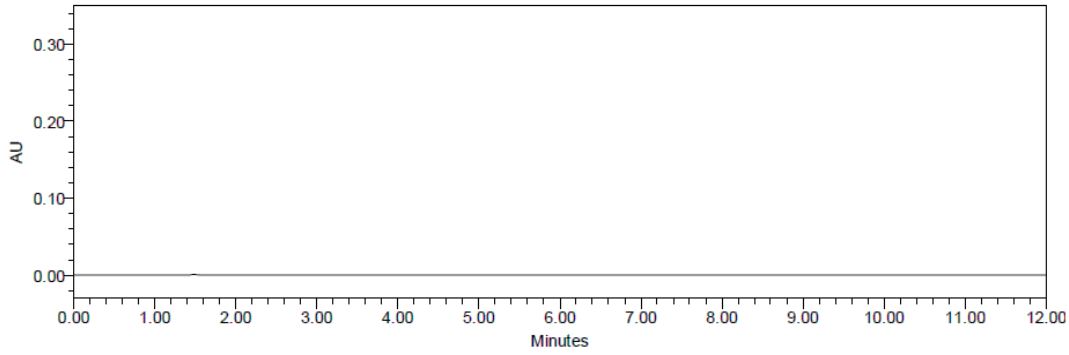

Figure 6. Chromatogram of placebo

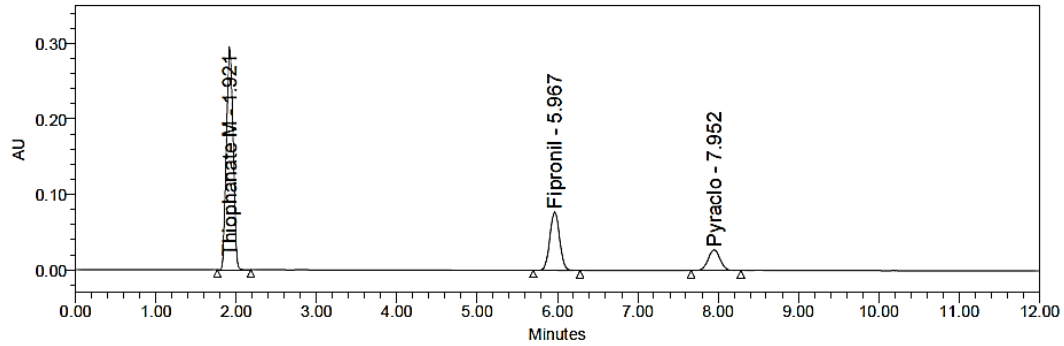

Figure 7. Chromatogram of standard preparation

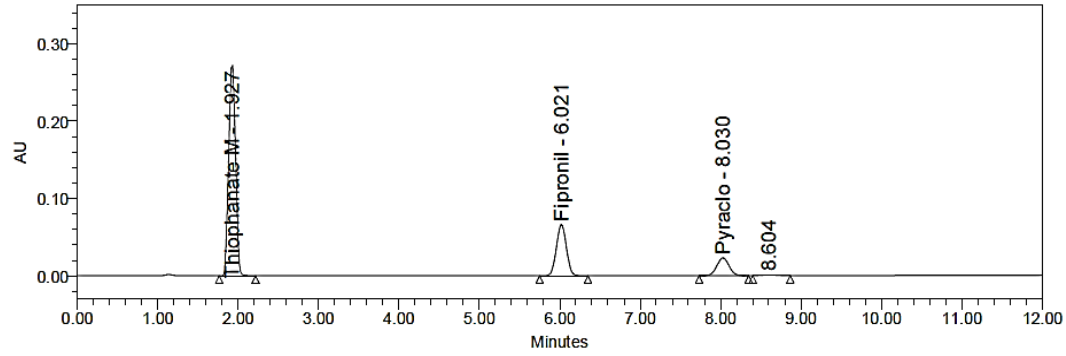

Figure 8. Chromatogram of sample preparation

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

Table 3. System Suitability of standard solution

Parameters	Results			Limits
	Thiophanate-methyl	Fipronil	Pyraclostrobin	
% RSD of retention time	0.27	0.18	0.23	< 1.0
% RSD of peak area	0.28	0.26	0.31	< 1.0

5.3 Precision: The Precision was evaluated by repeatability (intraday) and intermediate precision (inter-day). Each level of precision was investigated by six replicate injections of standard solution of Thiophanate-methyl, Fipronil and Pyraclostrobin with concentration about 225 mg/ml (22.5% mv), 250 mg/ml (25.0 % m/v), 25 mg/ml (2.5% m/v) respectively and 6 different preparations of same sample. Table 4 showing acceptable % RSD values calculated by modified Horwitz equation.

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

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

Sr. no.	Compound	% Analyte(m/v)	Analyte Ratio (C)	% RSD (calc.)
1	Thiophanate-methyl	25	0.25	1.65
2	Fipronil	22.5	0.225	1.68
3	Pyraclostrobin	2.5	0.025	2.33

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

Table 5. Results of Precision studies

	Thiophanate-methyl		Fipronil		Pyraclostrobin	
	Intraday	Inter-day	Intraday	Inter-day	Intraday	Inter-day
Mean (% m/v)	23.35	23.44	24.96	24.97	2.64	2.69
% RSD	0.70	0.43	0.90	0.42	0.83	0.86

5.4 Limit of Detection (LOD) and Limit of Quantitation (LOQ): The limit of detection and limit of quantitation were evaluated by serial dilutions of Thiophanate methyl, Fipronil and Pyraclostrobin from standard stock 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)	Fipronil (mg/ml)	Pyraclostrobin (mg/ml)
Limit of Detection	0.00017	0.00101	0.00022
Limit of Quantitation	0.00035	0.00202	0.00035

5.5 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, Fipronil and Pyraclostrobin. 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	Fipronil	Pyraclostrobin
Linearity Range (mg/ml)	0.00038-0.661	0.00202-0.743	0.0004-0.091
Correlation Coefficient (R^2)	0.9993	0.9997	0.9996
Slope (m)	3821287.33	1280229.84	5024705.09
Y-intercept (C)	16664.94	-341.53	82.78

5.6 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
Thiophante-methyl	LOQ	0.000418	0.00041	98.9	1.58
	80%	0.35514	0.35512	100.0	0.08
	100%	0.44392	0.43979	99.1	0.84
	120%	0.53271	0.52445	98.4	0.32
Fipronil	LOQ	0.001996	0.00196	98.4	0.69
	80%	0.39604	0.39327	99.3	0.05
	100%	0.49505	0.49167	99.3	0.30
	120%	0.59406	0.58871	99.1	0.27
Pyraclostrobin	LOQ	0.000441	0.00043	98.5	1.18
	80%	0.04839	0.04805	99.3	0.07
	100%	0.06049	0.05998	99.2	0.12
	120%	0.07259	0.07183	99.0	0.24

* Each value corresponds to the mean of three determinations.

5.7 Stability of solutions: The stability of standard solution and sample solution was test for an 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.

5.8 Robustness: The robustness of the method was studied by determining effects of small variation of flow rate (0.75 ± 0.05 ml/min),mobile phase composition 0.1% OPA: Acetonitrile (40±5: 60 ±5) and column temperature (30°C ± 5°C) were performed. It was found that % m/v values were unaffected after these small variations.

6 Uncertainty in 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 [27]. Refer 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)
Thiophanate-methyl	23.37	0.006604	± 0.30
Fipronil	24.84	0.007669	± 0.37
Pyraclostrobin	2.67	0.007143	± 0.04

IV. CONCLUSION

A simple, specific and reliable UPLC method has been developed for quantification of Thiophanate-methyl, Fipronil and Pyraclostrobin in their pesticide formulation. Stress study showed that all degradation products were well separated from Thiophnate-methyl, Fipronil and Pyraclostrobin peaks confirming its stability indicating power. Method validation study showed that the method is specific, linear, accurate, robust and easily reproducible. This method is also useful for quantification of Thiophanate-methyl, Fipronil and Pyraclostrobin 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 adopted to regular quality control analysis of production samples and stability samples, environmental samples.

V. ACKNOWLEDGMENT

The authors are thankful to School of Basic and Applied Science, Raffles University, Rajasthan, India for encouragement and permission for publication.

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