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Analytical Method Development and Validation Analysis for Quantitative Assessment of Thifluzamide by HPLC Procedure

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ABSTRACT

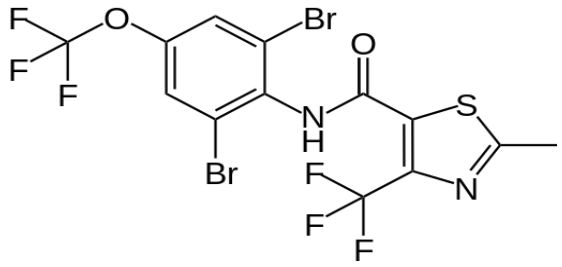
The precise, systematic, explicit, particular, linear, exact and robust scientific method was developed and validated for the assay of Thifluzamide in THIFLUZAMIDE 24% SC (CILPYROX) fungicide. Presently utilized Thifluzamide as a working standard having limit for assay of Thifluzamide in THIFLUZAMIDE 24% SC (CILPYROX) fungicide are not less than 95.0%. Acetonitrile, water and Phosphoric acid in the ratio (60:40:0.1 v/v/v) used as mobile phase and flow rate 1.0 ml / min. with 10 minutes run time. The detection was carried at 230 nm with column c18 - 250mm x 4.6mm x 5µ and ambient column temperature was maintained. The linearity of this method was found to be linear with a coefficient of regression at 0.999 in the concentration range of 50% to 150%. The linear regression equation was y=2174x-135.8. The present developed HPLC method is detected to be suitable. The analytical solution was detected to be stable up to 48 Hrs at room temperature.

Keywords: Thifluzamide , Robust, Precision, Linearity and Stability.

INTRODUCTION

Thifluzamide acts as inhibiting succinate dehydrogenase in the trycarboxylic acid cycle after being absorbed by the roots and leaves of the plants, which restrain with succinate ubiquinone reductase in the mitochondrial electron transport chain of fungi (Ravichandra, 2018), normally used as a solidfied drench or a foliar spray (Walter et al., 2011), it safeguards potato, strawberry, rice , coffee and maize in opposition to Rizoctonia solani which causes to sheath blight. It regulates rice sheat blight, maize sheath blight, tarspot disease and strawberry sheath blight, and it was invented by Monsanto(Adhikari et al., 1989). Thifluzamide is effective as foliar, soil and seed curative treatment adverse to basidiomycetes fungal pathogens. It is in white colored thick liquid. It could be used as preventive or prior to development of sheath blight disease approximately 45 days after transplanting Noriko T3 and Parizox T2 in paddy rice. It is an aromatic amide obtained by formal condensation of the carboxy group of 2-methyl-4-(trifluoromethyl)thiazole-5-carboxylic acid with the amino group of 2,6-dibromo-4-(trifluoromethoxy)aniline (Golden et al., 1998), furthermore it was tested for the efficacy against rice sheath blight during 2006 and 2009. Among the several test concenrtrations, Thifluzamide 24% SC (CILPIROX) at a proportion of 90 and 105g ai/ha was determined as effective in decreasing the disease intensity and increasing the crop yield. Its solubility in water is 20mg/L and forms emulsion with water. It is used as seed and foliar fungicide on a wide range of crops and a turfgrass with tradename as Greatam. This fungicide found as effective both curative and preventive activity without any symptoms such as phytotoxicity like necrosis, veinclearing, hyponasty, epynasty which involving in rice plants. The extensive utilization of Thifluzamide fungicide then its toxic nature impact on non-target species apart from target organisms in soil, the biomarkers response of earthworms concerning the stress induced by Thifluzamide with different concentrations ranges from 0 to 10mg/kg,

and also detected this fungicide instigates DNA damage, inhibited actions of GST, POS and SOD enzymes in the body of earth worms (Malik et al., 2022). The structure of Thifluzamide was as follows.



Structure of Thifluzamide

Chemical name: 2′,6′-dibromo-2-methyl-4′-(trifluoromethoxy)-4-(trifluoromethyl)thiazole-5-carboxanilide. **Molecular formula** is $C_{13}H_6Br_2F_6N_2O_2S$ and **Molecular weight** is 528.062 g/mol (Debra Edwards et al., 2011).

Early investigations expels that, there was accurate and reliable HPLC method has developed for using stability indicating method for the determination of Thifluzamide spontaneous deferments(Kumar et al., 2015). Subsequential literature survey, found antibacterial composition of Thifluzamide and Chlorothalonil, this combination of bactericidal (Xingkhai Cheng et al., 2020) leads to preventing and regulating contamination of diseases on fruit trees, cereals and edible crops, has greater effect of synergetic, postpones an overcomes the drug resistance of pathogenic bacteria, has the benefit of high bactericidal speed and long duration time, low cost usage (Kavitha et al., 2020). Thifluzamide represents an endothermic peak at 175-180^{0}c when measure by dissimilar scanning calorimeter analysis and having no other peak at less than that temperature(Hayakwa et al., 1998). Many researchers were delved on highly sensitive and more effective chromatographic procedures. The HPLC-MC/MS methods represents maximum residue in rice grain was too low and only 7 to 11% quotient of severe risk involved with thifluzamide in daily dietary intake in Chinese population utilizing rice (Guo et al., 2001).

The limits for Assay of Thifluzamide is not less than 95.0%. This analytical method verification report is intended to summarize the results obtained during the verification of HPLC method for the assay of Thifluzamide in THIFLUZAMIDE 24% SC (CILPYROX). A High Performance Liquid Chromatography-UV Detection (HPLC- UV/PDA) method for the quantitative determination of analytical method of assay of Thifluzamide in THIFLUZAMIDE 24% SC (CILPYROX) 20ml was developed and validated in the present study. The validation parameters such as Specificity or Selectivity, linearity, Method of precision, Intermediate Precision, Robustness and stability were studied according to the International Conference on Harmonization Guidelines with numbers: Q2A & Q2B of CPMP / ICH / 281 / 95 and non-pharmacopoeial method and developed in-house (Majors et al., 1980).

MATERIALS AND METHODS

Chemicals and reagents

Thifluzamide working standard and Thifluzamide , 10ml was received from reputed local chemical company. In the present study entire chemicals and reagents were utilized with high quality and purity and obtained from various sources. Acetonitrile-AR, Phosphoric Acid-AR, were purchased from Merck. Millipore water (HPLC- Grade) were procured from SD Fine chemicals, India. All the materials used were within the expiry date and stored at recommended storage conditions.

Preparation of Thifluzamide Standard Solution

Weigh accurately about 20 mg of Thifluzamide working Standard and transfer to a 50 ml volumetric flask. Add 20 ml of diluent and sonicate to deliquesce. Dilute to volume with diluent and mix. 1.0 ml of this solution transfer into a 10 ml of volumetric flask and then diluted to volume with the diluent and mix.

(Scheme of Dilution : 20mg → 50.0 ml → 1 ml /10.0 ml)

Preparation of sample Solution

Take 84mg weight of sample and then transfer into 50 ml volumetric flask. To dissolve, sonicate and augment 20ml of diluent(European agency, 1995). Dilute to volume with diluent and mix. In 10ml of volumetric flask 1.0 ml of this solution is transfer and diluted to volume with the diluent and then mix.

(Scheme of Dilution: 84mg → 50.0 ml → 1 ml /10.0 ml)

System Suitability Solution Preparation

Used Thifluzamide working standard solution as system suitability solution.

Procedure

Separately inject equal volumes of blank, five replicate injections of system suitability solution (Thifluzamide working standard solution). Subsequently inject two injections of test solution and record the chromatograms. Ignore any peak due to blank in the test solution. Calculate % RSD of five replicate injections of system suitability solution (Thifluzamide standard working solution). Check tailing factor and theoretical plates of the peak in the chromatogram obtained with 5th injection of system suitability solution (Thifluzamide working standard solution).

The limits are as below,

1. Theoretical plates should be greater than or equal to 2000.
2. Tailing factor should be not more than 2.0. and
3. % RSD should be below 2.0%.

No options while fixing limits mention 2 or 3 not 2 and 3. 2 is enough. Everything in same manner.

Instrumentation and Chromatographic conditions

For the current analysis, the HPLC - Agilent 1100 Series and HPLC- Waters - Alliance 510 pump with UV/ VIS detector was used. The Chromeleon software and Data Ace softwares were utilized for data acquirement. Sample injection was done by auto injector which was coupled with instrument itself. System was equipped with HPLC Analytical column C_{18} - (250mm x 4.6mm x 5µ dimensions) and column was maintained at ambient temperatures for quantification. Mettler Toledo-B204S as analytical weighing balance was employed for weighing the working substances (Popp et al., 2002).

Mobile phase preparation

Prepare a mixture of Acetonitrile, water and Phosphoric acid in the ratio 60:40:0.1 respectively used as diluent which was blank sample. Mix well. The rate of flow has been 1.0 ml / min. with 10 minutes run time and uses the 20 µl injection volume for testing sample quantity. The detection was carried at 230 nm with ambient chromatographic conditions. Then Filter through 0.2 µm Nylon membrane filter paper and degas prior to use.

HPLC Method validation

According to USP – non pharmacopoeial method and the International Conference on Harmonization Guidelines, the method was validated in terms of Specificity or selectivity, linearity, method of precision, intermediate precision, robustness and stability studies of the samples.

RESULTS AND DISCUSSION

Specificity /Selectivity:

In accordance of the analytical method the system suitability criteria were detected to converge with the pre-established acceptance criteria. The results of system suitability corresponding selectivity were shown in the Table 1 and standard chromatogram was given in the following Figure 1.

Table 1: System suitability - Selectivity

Sr. No.	Area of Thifluzamide
1	2141.18
2	2132.34
3	2158.00
4	2179.37
5	2189.70
Mean	**2160.12**
Standard Deviation(±)	**24.39**
(%) Relative Standard Deviation	**1.13**

Entire injections were processed at the wavelength furnished in the method. There was no interference observed from diluent blank solution, placebo with Thifluzamide peak. From the Table 1, it was evident that the % of Relative standard lesser than 2.0 percent (1.13).

Result: The method is selective.

Linearity:

In the theoretical concentration of preparation of assay, the linearity evaluation of five standard blends of Thifluzamide were developed in the span of initiating from 50% to 150%. The linearity solutions and the system suitability solutions were injected accordance with the protocol. The linearity graph of concentration in respect of peak performances was plotted and the correlation coefficient was detected. The average peak area of Thifluzamide peak at each concentration level was identified and the linearity graph was plotted against the sample concentration in percentage. The outcomes of linearity study are as given in Table 3. Below Figure 2 interprets, observation of a linearity graph of the average area at every level against the concentration (%) was plotted and was detected to be a straight line graph.

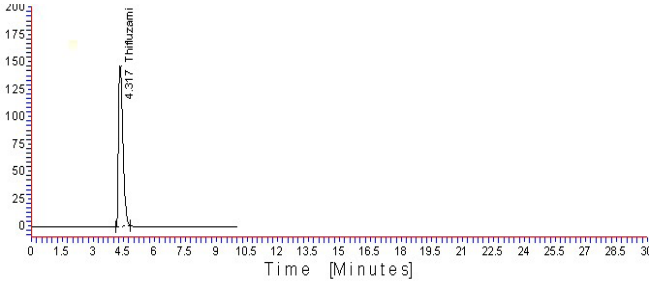


Figure 1: Standard chromatogram of Thifluzamide

Result-A Table

Peak No	Retn. Time	Area	Height	Area %	Height %
1	4.317	2179.367	146.169	100	100
Total		**2179.367**	**146.169**	**100**	**100**

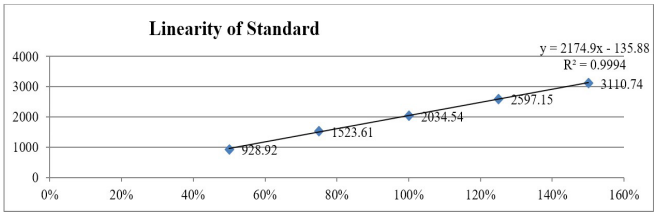


Figure 2: Linearity graph of Thifluzamide Standard

Table 2: System suitability - Linearity standard of Thifluzamide

Sr. No.	Area of Thifluzamide
1	2056.21
2	2036.21
3	2004.97
4	1980.83
5	1985.82
Mean	**2012.81**
Standard Deviation(±)	**32.57**
(%)Relative Standard Deviation	**1.62**

Results :

a) A linearity graph of the average area at each level against the concentration (%) is plotted and is found to be a straight line graph.

b) The correlation coefficient is detected to be greater than 0.999.

c) Hence it is concluded that, the method is found to be linear in the range of 50% to 150% of the working concentration.

d) The range for the analytical method is 50 ppm to 150 ppm.

Table 3: Results of linearity of standard

Linearity Level	Sample Concentration (in %)	Sample Concentration (in ppm)	Peak Area	Correlation Coefficient
Level – 1	20	20	928.92	0.999
Level – 2	30	30	1523.61	
Level – 3	40	40	2034.54	
Level – 4	50	50	2597.15	
Level – 5	60	60	3110.74	

Precision:

Method Precision:

Six test solutions of Thifluzamide in THIFLUZAMIDE 24% SC (CILPYROX) and were prepared as per the analytical method. The percentages of RSD and assay of six test solutions was calculated. % RSD concludes, with the results of six test solutions should be accept only less than 2.0%. By the inference of analytical method the system suitability criterion was detected to coincide the pre-established acceptance criteria. The outcomes of assay obtained from six test solutions preparations are presented in Table - 5.

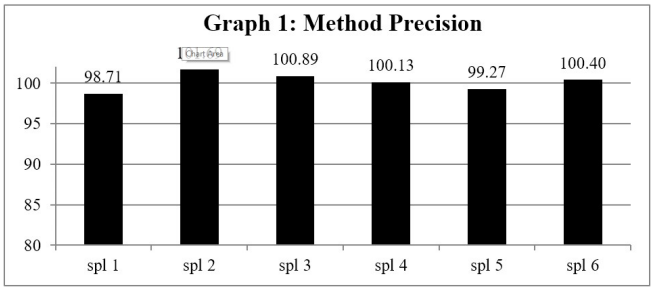
Table 4: System suitability - Method precision

Analyst – 1 HPLC No.: EH/R&D/HPLC-024

Sr. No.	Area of Thifluzamide
1	2199.71
2	2201.05
3	2211.95
4	2193.12
5	2120.85
Mean	**2185.34**
Standard Deviation (±)	**36.68**
(%) Relative Standard Deviation	**1.68**

Table 5: Results of method precision

Test Solution	% Assay of Thifluzamide
1	98.71
2	101.69
3	100.89
4	100.13
5	99.27
6	100.40
Mean	**100.18**
Standard Deviation (±)	**1.08**
(%) Relative Standard Deviation	**1.08**



Graphical representation of six sample values of Method Precision

Result : The % RSD of the six assay results is detected less than 2.0% and coincide the pre-established acceptance criteria. Hence, it is inferred that the method is precise.

Intermediate Precision:

Six test solutions of Thifluzamide in THIFLUZAMIDE 24% SC (CILPYROX) was prepared as per the analytical method on different day. These test solutions were analyzed by a distinct analyst using distinct HPLC column of same preparation but having distinct serial number and distinct HPLC system. The percentage of RSD of % assay outcomes of twelve test solutions (each of six samples from method precision and intermediate precision) was calculated. % RSD of the results of twelve test solutions (each of six samples from method precision and intermediate precision) should not be more than 2.0%.

Table - 6: System suitability - Intermediate precision

Analyst – 2 HPLC No.: EH/R&D/HPLC-023

Sr. No.	Area of Thifluzamide
1	2121.20
2	2095.28

3	2095.28
4	2105.86
5	2075.10
Mean	**2098.54**
Standard Deviation (±)	**16.87**
(%) Relative Standard Deviation	**0.80**

Table 7: Results of intermediate precision

Sample Solution	% Assay of Thifluzamide
1	99.57
2	101.09
3	101.38
4	100.40
5	102.30
6	101.53
Mean	**101.05**
Standard Deviation (±)	**0.95**
(%) Relative Standard Deviation	**0.94**

The system suitability criteria were detected to coincide the pre-established acceptance criteria as per the analytical method. (system suitability results are in Table 7). The results of assay obtained from six test solutions are presented in Table - 8 and the chromotogram of intermediate precision shown in Figure 3. % RSD of assay results from method precision and intermediate precision (12 results) are presented in Table - 8.

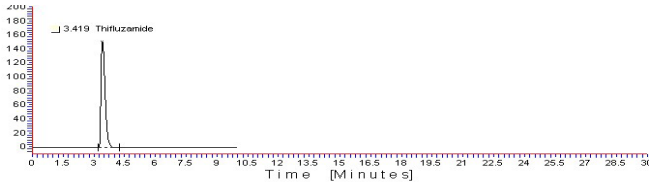


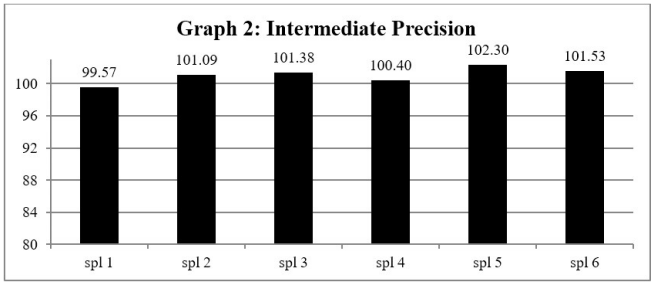
Figure : 3 Chromotogram of Thifluzamide/intermidiate Precision

Result-A Table					
Peak No	**Retn.Time**	**Area**	**Height**	**Area %**	**Height %**
1	3.419	2120.54	153.436	100	100
Total		**2120.54**	**153.436**	**100**	**100**

Table 8: Results of twelve test solutions of Thifluzamide in (each of six samples from method precision & intermediate precision)

Analysis performed during method precision study By first Analyst on system 1 and on column 1 on day 1	
Same column	**% Assay of Thifluzamide**
1	98.71
2	101.69
3	100.89
4	100.13
5	99.27
6	100.40

Analysis performed during intermediate precision study By second Analyst on system 2 and on column 2 on day 2	
Column sr. no.	015132560136 02
Test Solution	**% Assay of Thifluzamide**
7	99.57
8	101.09
9	101.38
10	100.40
11	102.30
12	101.53
Mean of twelve samples	**100.61**
Standard Deviation (±)	**1.07**
(%) Relative Standard Deviation	**1.06**



Graphical representation of six sample values of Intermediate Precision

Result:

The analysis was carried out on six test solutions of the same lot of the fungicide product by two distinct analysts with two separate equipments within the same laboratory using two distinct columns of the same preparation but having distinct serial numbers on two distinct days. The % RSD of the twelve assay results (six samples from each of method precision and intermediate precision) is identified to be less than 2.0%.

Thus, the method is determined to be rugged and precise.

Robustness:

Change in Column Lot

(Experimental Condition: c18 - 250mm x 4.6mm x 5µ**)**

Table 9: System suitability of Assay - Robustness with change in Column

Sr. No.	Area of Thifluzamide	
	Same column	**Different column**
1	2199.71	2078.16
2	2201.05	2102.85
Mean	2200.38	2090.50
Standard Deviation(±)	0.95	17.46
(%) RSD	0.04	0.84

The assay results were obtained with different flow rate conditions are as given in Table 10.

Table 10: Results of change column Lot

Flow rate →	Same column	Different column
Sample	**% Assay**	
Test solution	98.71	99.39
Average assay result from method precision	100.18	100.18
Mean	99.45	99.79
Standard Deviation (±)	1.04	0.56
(%) Relative Standard Deviation	1.05	0.56

The analytical method represents that the system suitability criteria were detected to coincide the pre-established acceptance criteria. Change in Column Lot results represents in above Table 10.

Change in Flow Rate (± 0.2 mL/minute):

(Normal Experimental Condition: 1.0ml/minute)

The analytical method represents that system suitability criteria were detected to coincide the pre-established acceptance criteria.

Table 11: System suitability - Robustness along with change in flow rate

Sr. No.	Area of Thifluzamide	
	0.8 mL/minute	1.2mL/minute
1	2043.29	2021.71
2	2036.50	2008.05
Mean	2039.90	2014.88
SD(±)	4.80	9.65
(%) RSD	0.24	0.48

The assay results obtained with different flow rate conditions are as given in Table 12.

Table 12: Results of change in flow rate

Flow rate →	0.8 mL/ minute	1.2mL/ minute
Sample	**% Assay**	
Test solution	100.09	98.38
Average assay result from method precision	100.18	100.18
Mean	100.14	99.28
Standard Deviation (±)	0.06	1.27
(%) Relative Standard Deviation	0.06	1.28

Change in Wavelength (± 2 nm):

(Normal Experimental Condition: 230nm)

The analytical method represents that the system suitability criteria were detected to coincide the pre-established acceptance criteria.

Table 13: System suitability - Robustness with change in wavelength

Sr. No.	Area of Thifluzamide	
	228 nm	232 nm
1	2038.84	2090.11
2	2044.27	2094.06
Mean	2041.55	2092.09
Standard Deviation (±)	3.84	2.80
(%) Relative Standard Deviation	0.19	0.13

The assay results obtained with different wavelength conditions are given in Table 14.

Table 14: Results of change in wavelength

Wavelength →	228 nm	232 nm
Sample	**% Assay**	
Test solution	99.23	98.94
Average assay result from method precision	100.18	100.18
Mean	99.71	99.56
Standard Deviation (±)	0.67	0.88
(%) Relative Standard Deviation	0.67	0.88

Change in composition of mobile phase (± 20ml):

(Normal Experimental Condition: Acetonitrile: water : Phosporic Acid = 600ml:400ml:1ml)

Table 15: System suitability - Robustness with change in mobile phase composition

Sr. No.	Area of Thifluzamide	
58ACN:42W:0.1P	**62ACN:**	**38W:0.1P**
1	2108.04	2104.25
2	2081.25	2095.63
Mean	**2094.64**	**2099.94**
Standard Deviation (±)	**18.94**	**6.10**
(%) RSD	**0.90**	**0.29**

The system suitability criteria were detected to coincide the pre-established acceptance criteria as per the analytical method.

The assay results obtained with change in mobile phase composition are as given in Table 16.

Table 16: Results change in composition of mobile phase

Mobile phase composition	58ACN:42W:0.1P	62ACN:38W:0.1P
Sample	**% Assay**	
Test solution	100.53	99.52
Average assay result from method precision	100.18	100.18
Mean	100.36	99.85

Standard Deviation (±)	0.25	0.47
(%) RSD	0.25	0.47

Result and Discussion

a) The analysis of the same lot of Thifluzamide in THIFLUZAMIDE 24% SC (CILPYROX) was carried out at different conditions of column lot, flow rate, wave length and change in composition of mobile phase.

b) The system suitability was detected to coincide the pre-established criteria at all the stipulations and the %RSD is not more than 2.0% in between results obtained with modified stipulation and average result of Method precision.

c) The analytical Method meets the pre-established acceptance criteria for robustness study as per protocol. Thus, the Method is robust.

Stability of Analytical Solution:

System suitability solution and test solution of Thifluzamide in THIFLUZAMIDE 24% SC (CILPYROX) brought to developed on session 0^{th}, 12^{th}, 24^{th}, 36^{th} and 48^{th} hour of experiment and stored these solutions at normal storage temperature for every time period up to 48 hrs and analyzed these solutions on 48 hrs with newly prepared test solution.

Results for Solution Stability shown in the below Table 17. During the analysis the system suitability solution was prepared afreshly. The assay of Thifluzamide in THIFLUZAMIDE 24% SC (CILPYROX) in the sample was calculated.

Table 17: Results for Solution Stability

% Assay results computed against the newly prepared system suitability standard	
Sample	**% Assay of Thifluzamide**
0^{th} hr	99.39
12^{th} hr	98.72
24 hr	97.41
36 hr	96.49
48 hr	100.46
Mean	**98.49**
Standard Deviation (±)	**1.57**
(%) Relative Standard Deviation	**1.60**

Result and Discussion

The system suitability was detected to coincide the pre-established criteria and the % RSD between assay results obtained for afreshly prepared test solution and the stored test solutions is less than 2.0%. The Assay level observes there is no significant change up to 48Hrs of test solution at room temperature. Hence, consequently it can be concluded that the solution is stable up to 48Hrs at room temperature.

CONCLUSION

The HPLC-UV/PDA method for determination of Thifluzamide for was completely validated by using specificity or selectivity, linearity, method of precision, intermediate precision, robustness and stability parameters. The approach was validated in accordance with ICH and non pharmacopeia standards. A simple economic HPLC method has been developed for the quantitative estimation of Thifluzamide injection with good precision, linearity, and robust. The prepared method was detected to be specific and accurate for the assay of Thifluzamide . A system suitability test was established and recorded for the Thifluzamide injection. The analyte was considered stable if there is no significant change in % assay. Hence the solution was found to be stable up to 48 Hours at room temperature. For these reasons, hence, it is concluded that the analytical method was validated, can be used for routine analysis and for stability study. Consequently, the suggested method can be easily used for the quantitative quality control in agro industries, and future research also.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest among the authors regarding publication of this article.

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