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Analytical Method Validation Report for Quantitative Estimation of Diflubenzuron by HPLC Method

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Abstract: ***A simple, selective, precise and accurate high performance liquid chromatographic method for the analysis of Diflubenzuron in its formulations was developed and validated in the present study. The mobile phase consists of a mixture of acetonitrile, water and 1:4 dioxane in the proportion 60: 40: 0.03 respectively (v/v/v/). This was found to give sharp peak of Diflubenzuron at a run time of 20 min. HPLC analysis of Diflubenzuron was carried out at a wave length of 260 nm with a flow rate of 1.3mL/min. The linear regression analysis data for the calibration curve showed a good linear relationship with a regression coefficient 0.999 in the concentration range of 50% to 150%. The linear regression equation was y= 1856x+304.2. The developed method was employed with a high degree of precision and accuracy for the analysis of Diflubenzuron. The method was validated for accuracy, precision, robustness, ruggedness and specificity. The precision, accuracy, sensitivity, short retention time and composition of the mobile phase indicated that this method is useful for the quantification of Diflubenzuron.***

Keywords: Diflubenzuron, HPLC Method, Development and Validation.

1. Introduction

Diflubenzuron is chemically N-[(4-Chlorophenyl) carbamoyl]-2, 6-difluorobenzamide in (fig: 1), Empirical formula: $C_{14}H_9ClF_2N_2O_2$ and Molecular Weight: 310.68 g/mol^{-1}. Diflubenzuron is an odourless, white, crystalline solid. It is almost insoluble in water and poorly soluble in a polar organic solvent. In polar to very polar solvents, the solubility is moderate to good, e.g., in acetone 6.5 g/liter at 20°C. Diflubenzuron is highly soluble in N-methylpyrolidone, dimethylsulfoxide, and dimethylformamide. It controls insect pests, particularly forest tent caterpillar moths, boll weevils, gypsy moths, and other types of moths.

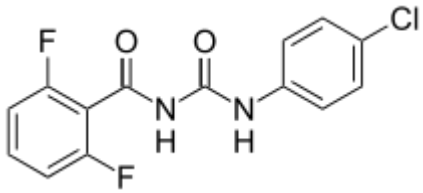


Figure 1: Chemical Structure of Diflubenzuron

Diflubenzuron is chitin synthesis inhibitors that act as an anti-moulting agent, leading to eath of larvae and pupae [1], one of the components of the insect's cuticle; therefore, the alformed cuticle cannot tolerate the internal pressure during ecdysis and is unable to give support to the muscles involved in the process and are thus incapacitated of discharging exuvia, eventually leading to death [2]. Diflubenzuron is also used pesticides in Brazilian aquaculture, mainly for the control of crustacean ectoparasites such as lerneae sp and arugulus sp [3].

Literature survey indicates that Diflubenzuron has been analysed by various analytical instrumental techniques like GLC [4]. Diflubenzuron determination and some metabolites in fish tissues have been previously described in the methods [5&6], forestry substrates [7]. cabbage under sub-tropical con- ditions [8], a range of environmental samples including soil, sediment, agricultural crops, milk, eggs and animal tissues [9]. HPLC methods [10 & 11,] SFC [12]. The analytical method for Diflubenzuron, recommended by CIPAC [13], is HPLC.

In this study, an analytical method was developed and validated for the determination of Diflubenzuron in its formulations. To the best of author knowledge, there is no one can report the determination of Diflubenzuron in its formulations. An attempt has been made to develop and validate to ensure their accuracy, precision and other analytical method validation parameters as mentioned in various gradients for pesticide formulation, the proposed method is suitable for their analysis with virtually no interference of the usual additives presented in pesticide formulations.

Instruments and Chemical

Instruments and Chemical used for the validation of Diflubenzuron we used High performance liquid chromatography, with UV / PDA detector, HPLC Analytical column of C18- Inertsil-3, 250mm x 4.6mm x 5µ and Analytical weighing balance - Mettler Toledo B204S, Millipore Nylon 0.2µm and Laboratory accessories. Diflubenzuron working Standard, SHARDA Diflubenzuron 480 SC, Acetonitrile – AR**,** 1:4 dioxane - AR and Millipore Water [14].

2. Analytical Method

The quantitative determination is carried out by HPLC system equipped with UV/VIS detector.

Chromatographic conditions

Column	C18- Inertsil-3, 250mm x 4.6mm x 5µ
Mobile Phase	For isocratic system, prepare a mixture of Acetonitrile, water and 1:4 dioxane in the proportion 60: 40: 0.03(v/v/v) respectively. Mix well. Filter through 0.2 µ Nylon membrane filter paper and degas prior to use.
Wavelength	260 nm
Flow Rate	1.3 mL / minute
Injection volume	20 µl
Run time	20 minutes
Blank solution	Use diluent as blank
Diluent	Use mobile phase as diluent

Preparation of Diflubenzuron Standard Solution
Weigh accurately about 50 mg of Diflubenzuron working Standard and transfer to a 20 ml volumetric flask. Add 10 ml of diluent and sonicate to dissolve. Dilute to volume with diluent and mix. Transfer 1.0 ml of solution into a 10 ml of volumetric flask and dilute to volume with the diluent and mix. (Dilution scheme: 50mg → 50.0 ml → 1 ml /10.0 ml)

Preparation of Test Solution
Weigh accurately about 104 mg of sample and transfer to a 50 ml volumetric flask. Add 10 ml of diluent and sonicate to dissolve. Dilute to volume with diluent and mix. Transfer 1.0 ml of solution into a 10 ml of volumetric flask and dilute to volume with the diluent and mix. (Dilution scheme: 104mg → 50.0 ml → 1 ml /10.0 ml)

System Suitability Solution: Use Diflubenzuron Standard working solution as system suitability solution.

Procedure
Separately inject equal volumes of blank, five replicate injections of system suitability solution (Diflubenzuron Standard working solution). Then inject two injections of test solution and record the chromatograms. Disregard any peak due to blank in the test solution. Calculate % RSD of five replicate injections of system suitability solution (Diflubenzuron Standard working solution). Check tailing factor and theoretical plates of the peak in the chromatogram obtained with 5^{th} injection of system suitability solution (Diflubenzuron Standard working solution). The limits are as below,
1) Theoretical plates should be not less than 2000.
2) Tailing factor should be less than 2.0.
3) % RSD should be not more than 2.0%.

Validation Parameters [14]

Specificity / Selectivity:
Selectivity was performed by injecting the diluent blank solution, excipient blend, system suitability solution, test solution. Acceptance criteria: The Diflubenzuron peak should be well resolved from any other peak and from each other. The diluent blank solution, excipient blend solution should not show any peak at the retention time of the Diflubenzuron. The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method.

Table 1: System suitability - Selectivity

Sr. No.	Area of Diflubenzuron
1	1994.67
2	1986.85
3	1978.57
4	1957.98
5	1979.95
Mean	1979.61
Standard Deviation (±)	13.68
(%) Relative Standard Deviation	0.69

All the injections were processed at the wavelength provided in the method. There was no interference observed from diluent blank solution, excipient blend solution with Diflubenzuron peak.

Linearity and Range for sample
For the linearity study five standard solutions of Diflubenzuron were prepared from the range starting from 50% to 150% of the theoretical concentration of assay preparation. The system suitability solution and the linearity solutions were injected as per the protocol. The linearity graph of concentration against peak response was plotted and the correlation coefficient was determined. Acceptance criteria: Correlation coefficient should be greater than or equal to 0.999. The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method. (Refer to Table-5 for system suitability results).

Table 5: System suitability - Linearity of standard

Sr. No.	Area of Diflubenzuron
1	2041.36
2	2018.04
3	2083.17
4	2034.28
5	2042.89
Mean	2043.95
Standard Deviation (±)	24.04
(%) Relative Standard Deviation	1.18

The average peak area of Diflubenzuron peak at each concentration level was determined and the linearity graph was plotted against the sample concentration in percentage. The results of linearity study are as given in Table - 6.

Table 6: Results of linearity of sample

Linearity Level	Sample Concentration (in %)	Sample Concentration (in ppm)	Peak Area	Correlation Coefficient
Level – 1	50	50	1240.68	0.999
Level – 2	75	75	1702.28	
Level – 3	100	100	2127.62	
Level – 4	125	125	2639.74	
Level – 5	150	150	3092.22	

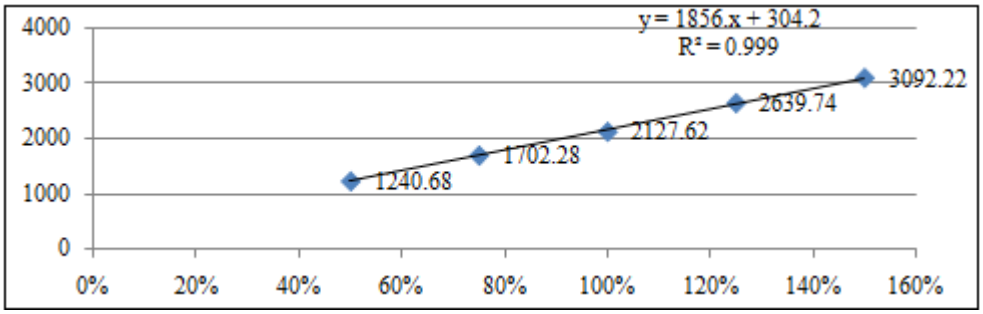
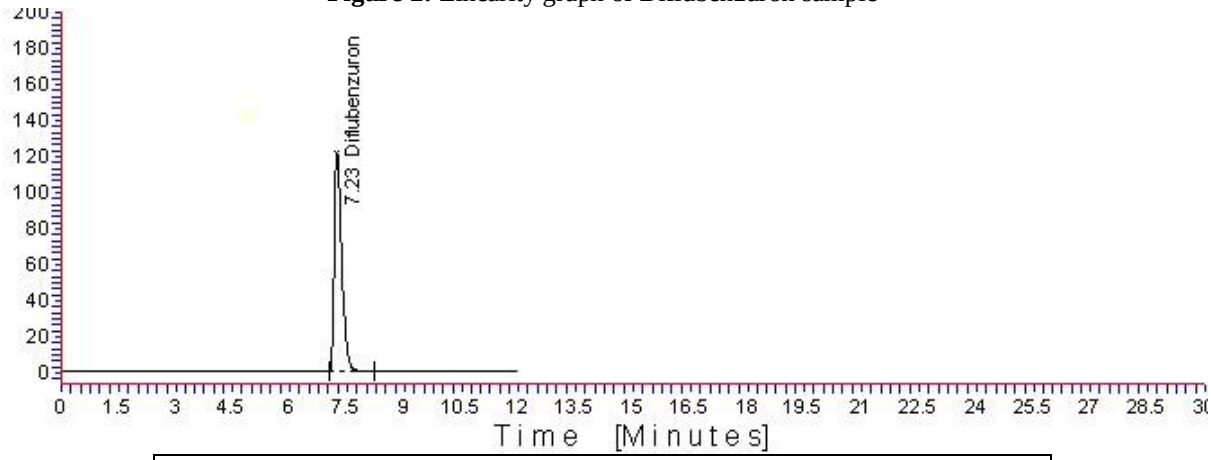


Figure 2: Linearity graph of Diflubenzuron sample



Result- Table						
Peak No	Retn.Time	Area	Height	Area %	Height %	Width@50%
1	7.23	1702.281	122.767	100	100	0.233

Figure 3: Chromatogram of Diflubenzuron sample

Forced Degradation

The forced degradation studies are performed to establish the stability indicating nature of the assay method and to observe any degraded compounds. Diflubenzuron WS and Sample (SHARDA Fungicide) are subjected to stress with 5N HCl, 5N NaOH, Thermal degradation and UV degradation. All the above solutions are chromatographed and recorded the chromatograms. The following stress conditions are followed for degradation

Table 2: System suitability – Forced Degradation

Sr. No.	Area of Diflubenzuron
1	2045.716
2	2028.956
3	2029.522
4	2024.265
5	2044.323
Mean	2034.56
Standard Deviation (±)	9.78
(%) Relative Standard Deviation	0.48

Table 4: Conditions – Forced Degradation

Sample stress condition	Description of stress condition
Acid degradation	5N HCl heated at about 60°C for 10 min on a water bath.
Alkali degradation	5N NaOH heated at about 60°C for 10 min on a water bath.
Thermal degradation	105ºC for 12 hours
UV degradation	expose to UV-radiation for 7 days

Table 4: % of degradation by applying different conditions

Acid Stress	**% Degradation**
Standard	0.018
Sample	0.023
Alkali Stress	**% Degradation**
Standard	0.002
Sample	0.032
Thermal Stress	**% Degradation**
Standard	0.010
Sample	0.002
UV Stress	**% Degradation**
Standard	0.245
Sample	0.014

Acceptance Criteria: The degradation peaks should be well separated from each other. The peak purity for Diflubenzuron peak should pass. There is no interference between the peaks obtained for the chromatograms of degradation preparations. The degradation peaks under forced degradation are well separated from each other. The peak purity for Diflubenzuron peak is passing. Hence, the method is very precise, selective and specific to the estimation of Assay of SHARDA Diflubenzuron 480 SC by HPLC and the same method is stability indicating, as the degraded products are well separated from Diflubenzuron and as well from each adjacent peaks.

Precision:

System Precision:

Procedure:

The system precision was performed by injecting 10 replicate injections of system suitability solution and the

chromatograms are reviewed for the system suitability criteria. Acceptance criteria: % RSD of peak areas of ten replicate injections of system suitability solution should not be more than 2.0% and system suitability criteria should pass as per analytical method. The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method.

Table 7: System precision

Sr. No.	Area of Diflubenzuron
1	2064.14
2	2060.56
3	2044.84
4	2080.54
5	2037.14
6	2041.57
7	2049.17
8	2044.28
9	2056.57
10	2048.98
Mean	**2052.78**
Standard Deviation (±)	12.94
(%) Relative Standard Deviation	0.63

Method Precision

Procedure:

Six test solutions of Diflubenzuron in SHARDA Diflubenzuron 480 SC and were prepared as per the analytical method. The % RSD of % assay of six test solutions was calculated. Acceptance criteria: % RSD of the results of six test solutions should not be more than 2.0%. The system suitability criterion was found to meet the pre-established acceptance criteria as per the analytical method. The results of assay obtained from six test solutions preparations are presented in Table - 9

Table 8: System suitability - Method precision
Analyst – 1 HPLC No.: EH/R&D/HPLC-024

Sr. No.	Area of Diflubenzuron
1	2057.94
2	2054.75
3	2054.36
4	2064.66
5	2050.69
Mean	2056.48
Standard Deviation (±)	5.25
(%) Relative Standard Deviation	0.26

Table 9: Results of method precision

Test Solution	% Assay of Diflubenzuron
1	99.45
2	99.03
3	98.73
4	99.08
5	99.68
6	99.02
Mean	99.17
Standard Deviation (±)	0.34
(%) Relative Standard Deviation	0.34

Intermediate Precision:

Procedure:

Six test solutions of SHARDA Diflubenzuron 480 SC was prepared as per the analytical method on different day. These test solutions were analyzed by a different analyst using different HPLC column of same make but having different serial number and different HPLC system. The % RSD of % assay results of twelve test solutions (six samples from method precision and six samples from intermediate precision) was calculated. Acceptance criteria: % RSD of the results of twelve test solutions (six of method precision and six of intermediate precision) should not be more than 2.0%. The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method. (Refer to Table -10 for system suitability results). The results of assay obtained from six test solutions are presented in Table - 11. % RSD of assay results from method precision and intermediate precision (12 results) are presented in Table - 12.

Table 10: System suitability - Intermediate precision
Analyst – 2 HPLC No.: EH/R&D/HPLC-023

Sr. No.	Area of Diflubenzuron
1	1881.33
2	1873.69
3	1861.66
4	1888.08
5	1861.40
Mean	1873.23
Standard Deviation (±)	11.83
(%) Relative Standard Deviation	0.63

Table 11: Results of Intermediate precision

Test Solution	% Assay of Diflubenzuron
1	99.12
2	97.32
3	99.44
4	98.77
5	99.66
6	100.26
Mean	99.10
Standard Deviation (±)	1.01
(%) Relative Standard Deviation	1.01

Table 12: Results of Twelve Test Solutions of Diflubenzuron in SHARDA Diflubenzuron 480 SC (six of method precision & six of intermediate precision)

Analysis performed during method precision study By Analyst 1 on system 1 and on column 1 on day 1	
Same column	% Assay of Diflubenzuron
1	99.45
2	99.03
3	98.73
4	99.08
5	99.68
6	99.02
Analysis performed during intermediate precision study By Analyst 2 on system 2 and on column 2 on day 2	
Column sr. no.	015337030136 01
Test Solution	% Assay of Diflubenzuron
7	99.12
8	97.32
9	99.44
10	98.77
11	99.66
12	100.26
Mean of twelve samples	99.13
Standard Deviation (±)	0.72
(%) Relative Standard Deviation	0.72

Robustness:

Procedure

Prepare two test solutions of the same lot (as used in 7.0.a and 7.0.b) of Diflubenzuron in SHARDA Diflubenzuron 480 SC as per analytical method. Inject this solution along with diluent blank solution and system suitability solution along different chromatographic conditions as shown below:

Change in flow rate (± 0.2 ml/minute)

Change in wavelength (± 2 nm)

Change in composition of mobile phase (± 20ml)

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

(Normal Experimental Condition: 1.3ml/minute)

The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method. (Refer to Table - 13 for system suitability results).

Table 13: System suitability - Robustness with change in flow rate

Sr. No.	Area of Diflubenzuron	
	1.1mL/ minute	1.5 mL/minute
1	2009.66	2006.89
2	2012.61	2003.39
Mean	2011.13	2005.14
Standard Deviation (±)	2.09	2.47
(%) Relative Standard Deviation	0.10	0.12

The assay results obtained with different flow rate conditions are as given in Table 14.

Table 14: Results for change in flow rate

Flow rate →	1.1mL/ minute	1.5 mL/minute
Sample	% Assay	
Test solution	100.27	100.41
Average assay result from method precision	99.17	99.17
Mean	99.72	99.79
Standard Deviation (±)	0.78	0.88
(%) Relative Standard Deviation	0.78	0.88

Change in Wavelength (± 2 nm):

(Normal Experimental Condition: 260nm)

The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method. (Refer to Table - 15 for system suitability results).

Table 15: System suitability - Robustness with change in wavelength

Sr. No.	Area of Diflubenzuron	
	258nm	262nm
1	2069.05	2083.49
2	2064.61	2077.08
Mean	2066.83	2080.28
Standard Deviation (±)	3.14	4.54
(%) Relative Standard Deviation	0.15	0.22

The assay results obtained with different wavelength conditions are as given in Table - 16.

Table 16: Results for change in wavelength

Wavelength →	258nm	262nm
Sample	% Assay	
Test solution	100.43	100.27
Average assay result from method precision	99.17	99.17
Mean	99.80	99.72
Standard Deviation (±)	0.89	0.78
(%) Relative Standard Deviation	0.89	0.78

Change in composition of Mobile Phase (± 20ml):

(Normal Experimental Condition: Acetonitrile: water: 1.4 dioxane = 60ml: 40ml: 0.03)

The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method (Refer to Table - 17 for system suitability results).

Table 17: System suitability - Robustness with change in composition of mobile phase

Sr. No.	Area of Diflubenzuron	
	58:42:0.03	62:38:0.03
1	2081.04	2069.16
2	2092.90	2075.03
Mean	2086.97	2072.10
Standard Deviation (±)	8.38	4.15
(%) Relative Standard Deviation	0.40	0.20

The assay results obtained with change in composition of mobile phase are as given in Table - 18.

Table 18: Results for change in composition of mobile phase

Composition of methanol & water	58:42:0.03	62:38:0.03
Sample	% Assay	
Test solution	99.43	99.97
Average assay result from method precision	99.17	99.17
Mean	99.30	99.57
Standard Deviation (±)	0.18	0.57
(%) Relative Standard Deviation	0.19	0.57

Stability of Analytical Solution:

Procedure:

System suitability solution and test solution of SHARDA Diflubenzuron 480 SC were prepared on 0^{th}, 12^{th}, 24^{th}, 36^{th} and 48^{th} hour of experiment and stored these solutions at room temperature for every time interval up to 48 hrs and analyzed these solutions on 48 hrs with freshly prepared test solution. The system suitability solution was prepared freshly at the time of analysis. The assay of SHARDA Diflubenzuron 480 SC in the sample was calculated. Acceptance criteria: The analyte is considered stable if there is no significant change in % assay. The assay results obtained during solution stability experiment are as given in Table- 20.

Table 20: Results for solution stability

% Assay results calculated against the freshly prepared system suitability sample	
Sample	% Assay of Diflubenzuron
0^{th} hr	100.09
12^{th} hr	99.31
24 hr	100.58
36 hr	99.29
48 hr	99.69
Mean	99.79
Standard Deviation (±)	0.55
(%) Relative Standard Deviation	0.55

3. Results and Discussion

System selectivity:
All the injections were processed at the wavelength provided in the Method. There was no interference observed from diluents blank solution, excipients blend solution with Diflubenzuron peak. The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical Method, hence this Method is selective**.**

Forced degradation:
There is no interference between the peaks obtained for the chromatograms of degradation preparations. The degradation peaks under forced degradation are well separated from each other. The peak purity for Diflubenzuron peak is passing. Hence, the Method is very precise, selective and specific to the estimation of Assay of Diflubenzuron in test solution of SHARDA Diflubenzuron 480 SC Fungicide as a Diflubenzuron 99% by HPLC and the same method is stability indicating, as the degraded products are well separated from Diflubenzuron and as well from each adjacent peak.

Linearity:
Linearity graph of the average area at each level against the concentration in Acetonitrile, water and 1:4 dioxane in the proportion 60: 40: 0.03 (v/v/v) is plotted and is found to be a straight line graph. The correlation coefficient is found to be more than 0.999. Hence it is concluded that the method is found to be linear in the range of 50% to 150% of the working concentration.

Precision:
The analysis was carried out on six test solutions of the SHARDA Diflubenzuron 480 SC Fungicide and by two different analysts using two different equipments within the same laboratory using two different columns of the same make but having different serial numbers on two different days. The % RSD of the twelve assay results which six of method precision and six from intermediate precision is found to be less than 2.0%. Thus, the method is found to be rugged and precise.
System precision=%RSD=0.63
Method precision=%RSD=00.26
Intermediate precision=%RSD=1.01

Robustness:
The analysis of the SHARDA Diflubenzuron 480 SC Fungicide 99% was carried out at different conditions of column lot, flow rate, wavelength, and change in composition of mobile phase., The % RSD between results obtained with changed condition and average result of Method precision is not more than 2.0%.The analytical Method meets the reestablished acceptance criteria for robustness study.
Thus, the Method is robust.

Stability of Analytical Solution:
The %RSD between assay results obtained for freshly prepared test solution and the stored test solutions is less than 2.0%there is no significant change in assay level observed up to 48 hours for test solution at room temperature. The system suitability was found to meet the pre-established criteria and it can be concluded that the solution is stable up to 48 hours at room temperature

4. Summary and Conclusion

The above summary and the validation data summarized in this paper show the analytical method of assay of SHARDA Diflubenzuron 480 SC Fungicide 99% by HPLC is found to be suitable, selective, specific, precise, linear, accurate and robust. The analytical solution is found to be stable up to 48 hours at room temperature. Hence, it is concluded that the analytical method is validated and can be used for routine analysis and for stability study.

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