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Development and Validation of Pyraclostrobin in its Formulations by HPLC Technique

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ABSTRACT - A simple, selective, precise and accurate high performance liquid chromatographic method for the analysis of Pyraclostrobin in its formulations was developed and validated in the present study. The mobile phase consists of a mixture of acetonitrile and 0.1% Formic acid in the proportion 70: 30 (v/v). This was found to give sharp peak of Pyraclostrobin at a run time of 20 min. HPLC analysis of Pyraclostrobin was carried out at a wave length of 230 nm with a flow rate of 1.0mL/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=3385x-120.7. The developed method was employed with a high degree of precision and accuracy for the analysis of Pyraclostrobin. 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 Pyraclostrobin.

Keywords: Pyraclostrobin, HPLC Method, Development and Validation.

I. INTRODUCTION

Pyraclostrobin is Chemically Methyl N-(2-(1-(4-chlorophenyl)-1H-pyrazol-3-yloxymethyl) phenyl)-(N-methoxy) carbamate**,** chemical formula is $C_{19}H_{18}ClN_3O_4$ and Molecular Weight: 387.82 g mol^{-1}. Pyraclostrobin is a fungicide used to control major plant pathogens in cereals and other crops. Pyraclostrobin is a white to crystals free from visible extraneous matter and added modifying agents. Pyraclostrobin soluble in water 1.9 mg/l (20 °C), in organic solvents like methanol, n-heptane, acetone (20 °C).

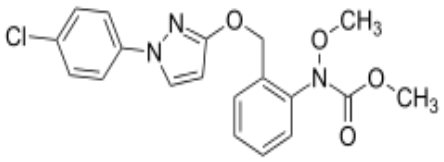

Fig-1 Chemical structure of Pyraclostrobin

Litaracher survey revels that Pyraclostrobin has been determined in different products by gas chromatography [1], gas chromatography with mass spectrometry [2]. Pyraclostrobin residues had been analyzed in vine utilizing liquid chromatography with mass spectrometry [3], in grapes using GC-MS [4-5], in peanut using a modified Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS)-LC-MS/MS method [6]. Some multiresidue analyses had been studied, such as 14 pesticides (including Pyraclostrobin) residues had been analyzed in mangoes using solid-phase micro extraction (SPME) followed by GC–MS [7] and 30 pesticides (including Pyraclostrobin) residues had been analyzed in grapes using GC–MS [8].

In this present investigation analytical method validation study is intended to summarize the validation results obtained during the validation of HPLC method for the assay of Pyraclostrobin in CABRIO Fungicide. To the best of author knowledge, there is no one can report the determination of Pyraclostrobin in CABRIO Fungicide 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 CHEMICALS REQUIRED

Instruments and Chemical used for the validation of Pyraclostrobin we used High performance liquid chromatography, with UV / PDA detector, HPLC Analytical column of RP-18, 250mm x 4.6mm x 5µ and Analytical weighing balance - Mettler Toledo B204S, Millipore Nylon 0.2µm and Laboratory accessories**.** Chemicals are Pyraclostrobin working standard, CABRIO Fungicide, Acetonitrile – AR**,** Formic acid – AR and Millipore Water [9].

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ANALYTICAL METHOD:

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

Chromatographic conditions:

Column	:	RP-18, 250mm x 4.6mm x 5µ
Mobile Phase	:	For isocratic system, prepare a mixture of Acetonitrile and 0.1% Formic acid in the proportion 70: 30(v/v) respectively. Mix well. Filter through 0.2 µ Nylon membrane filter paper and degas prior to use.
Wavelength	:	230 nm
Flow Rate	:	1.0 ml / minute
Injection volume	:	20 µl
Run time	:	20 minutes
Blank solution	:	Use Mobile phase as blank
Diluent	:	Use Mobile phase as diluent

Preparation of Pyraclostrobin Standard Solution:

Weigh accurately about 50 mg of Pyraclostrobin working Standard and transfer to a 20 ml volumetric flask. Add 10 ml of diluent and sonicated 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 200 mg of sample and transfer to a 50 ml volumetric flask. Add 10 ml of diluent and sonicated 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: 200mg → 50.0 ml → 1 ml /10.0 ml)

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

Procedure:

Separately inject equal volumes of blank, five replicate injections of system suitability solution (Pyraclostrobin 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 (Pyraclostrobin Standard working solution). Check tailing factor and theoretical plates of the peak in the chromatogram obtained with 5th injection of system suitability solution (Pyraclostrobin 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

The HPLC method is evaluated for following validation parameters.

Specificity / Selectivity:

Selectivity was performed by injecting the diluent blank solution, excipient blend, system suitability solution, test solution. Acceptance criteria: The Pyraclostrobin 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 Pyraclostrobin.

Table - 1: System suitability - Selectivity

S. No.	Area of Pyraclostrobin
1	3030.86
2	3089.40
3	3075.71
4	3051.52
5	3086.13
Mean	**3066.73**
Standard Deviation (±)	**24.94**
(%) Relative Standard Deviation	**0.81**

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 Pyraclostrobin peak.

Linearity and Range for sample

For the linearity study five sample solutions of Pyraclostrobin 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 ICH guidelines. 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, results are given in table-2.

Table -2: System suitability - Linearity of sample

S. No.	Area of Pyraclostrobin
1	3127.95
2	3110.68
3	3144.53
4	3123.24
5	3158.74
Mean	**3133.03**
Standard Deviation (±)	**18.80**
(%) Relative Standard Deviation	**0.60**

The average peak area of Pyraclostrobin 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-3.

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Table- 3: Results of linearity of sample

Linearity Level	Sample Concentration(in %)	Sample Concentration(in ppm)	Peak Area	Correlation Coefficient
Level –	50	50	1560.52	0.999
Level –	75	75	2462.94	
Level –	100	100	3229.87	
Level –	125	125	4092.46	
Level –	150	150	4977.45	

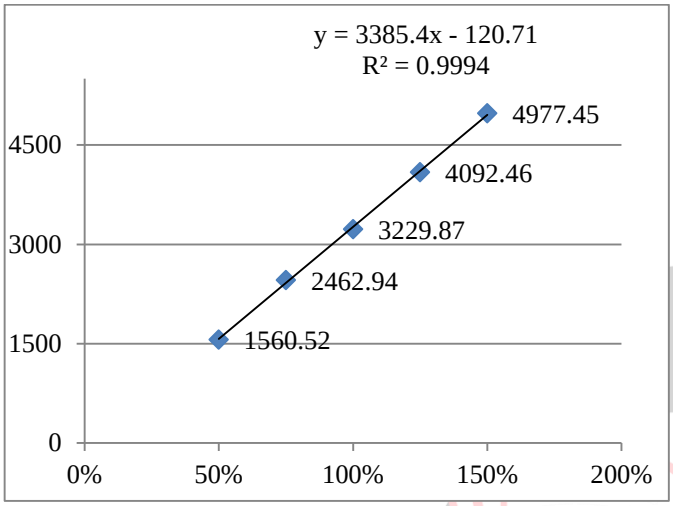
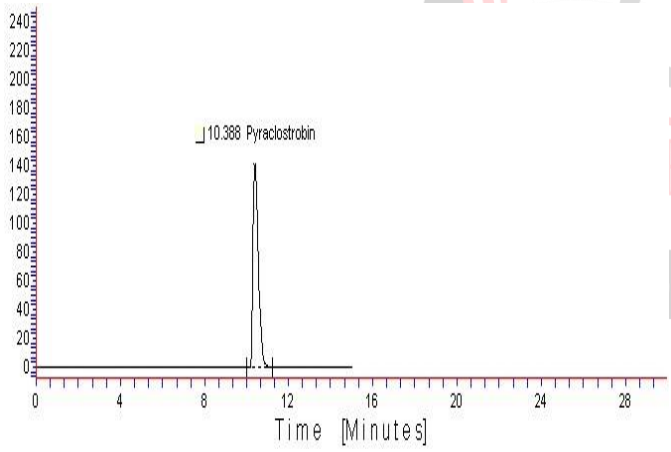


Figure 2: Linearity graph of Pyraclostrobin sample



Result-A Table						
S.No	Retn.Time	Area	Height	Area %	Height %	Width@50%
1	10.389	2462.943	141.522	100	100	0.283

Figure 3: Chromatogram of Pyraclostrobin 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. Pyraclostrobin WS and Sample (CABRIO 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 – 4: System suitability – Forced Degradation

S. No.	Area of Pyraclostrobin
1	3199.11
2	3193.30
3	3191.62
4	3115.00
5	3133.51
Mean	**3166.51**
Standard Deviation (±)	**39.22**
(%) Relative Standard Deviation	**1.24**

Table – 5: 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-6: % of degradation by applying different conditions

Acid Stress	% Degradation
Standard	0.021
Sample	0.053
Alkali Stress	**% Degradation**
Standard	0.016
Sample	0.021
Thermal Stress	**% Degradation**
Standard	0.506
Sample	0.247
UV Stress	**% Degradation**
Standard	0.051
Sample	0.025

Acceptance Criteria:

The degradation peaks should be well separated from each other. The peak purity for Pyraclostrobin peak should pass.

Conclusion:

There is no interference between the peaks obtained for the chromatograms of degradation preparations. The degradation peaks under forced degradation are well

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separated from each other. The peak purity for Pyraclostrobin peak is passing. Hence, the method is very precise, selective and specific to the estimation of assay of Pyraclostrobin in CABRIO Fungicide by HPLC and the same method is stability indicating, as the degraded products are well separated from Pyraclostrobin 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 results are present in table-7.

Table -7: System precision

Sr. No.	Area of Pyraclostrobin
1	3199.95
2	3160.12
3	3182.74
4	3198.13
5	3181.51
6	3159.19
7	3154.72
8	3128.07
9	3120.38
10	3140.47
Mean	**3162.53**
Standard Deviation (±)	**27.82**
(%) Relative Standard Deviation	**0.88**

Method Precision:

Procedure:

Six test solutions of Pyraclostrobin in CABRIO Fungicide 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 Tables-8 & 9.

Table - 8: System suitability - Method precision

Analyst – 1 HPLC No.: EH/R&D/HPLC-024

S. No.	Area of Pyraclostrobin
1	3240.01
2	3216.33
3	3265.56
4	3244.65
5	3232.37
Mean	**3239.78**
Standard Deviation (±)	**17.98**
(%) Relative Standard Deviation	**0.56**

Table - 9: Results of method precision

Test Solution	% Assay of Pyraclostrobin
1	99.33
2	100.81
3	102.50
4	100.33
5	101.99
6	100.93
Mean	**100.98**
Standard Deviation (±)	**1.14**
(%)Relative Standard Deviation	**1.13**

Intermediate Precision:

Procedure:

Six test solutions of CABRIO Fungicide were 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.

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

S. No.	Area of Pyraclostrobin
1	2910.91
2	2912.05
3	2920.83
4	2929.39
5	2951.67
Mean	**2924.97**
Standard Deviation (±)	**16.69**
(%) Relative Standard Deviation	**0.57**

Table - 11: Results of Intermediate precision

Test Solution	% Assay of Pyraclostrobin
1	99.63
2	99.19
3	99.84
4	98.98
5	100.24
6	99.56
Mean	**99.57**
Standard Deviation (±)	**0.45**
(%) Relative Standard Deviation	**0.45**

Table - 12: Results of twelve test solutions of Pyraclostrobin in CABRIO Fungicide (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 Pyraclostrobin**
1	99.33
2	100.81
3	102.50
4	100.33
5	101.99
6	100.93
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 Pyraclostrobin**
7	99.63
8	99.19
9	99.84
10	98.98
11	100.24
12	99.56
Mean of twelve samples	**100.28**
Standard Deviation (±)	**1.11**
(%) Relative Standard Deviation	**1.10**

ROBUSTNESS:

Procedure:

Prepare two test solutions of the same lot (as used in 7.0.a and 7.0.b) of Pyraclostrobin in CABRIO Fungicide 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: 1.0ml/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

S. No.	Area of Pyraclostrobin	
	0.8mL/minute	**1.2 mL/minute**
1	3115.00	3169.45
2	3133.51	3160.88
Mean	**3124.25**	**3165.16**
Standard Deviation (±)	**13.09**	**6.06**
(%) Relative Standard Deviation	**0.42**	**0.19**

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 →	**0.8mL/minute**	**1.2 mL/minute**
Sample	**% Assay**	
Test solution	99.53	100.43
Average assay result from method precision	100.98	100.98
Mean	**100.26**	**100.71**
Standard Deviation (±)	**1.03**	**0.39**
(%) Relative Standard Deviation	**1.02**	**0.39**

Change in Wavelength (± 2 nm):

(Normal Experimental Condition: 230nm)

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 Pyraclostrobin	
	228nm	**232nm**
1	3128.07	3108.42
2	3154.72	3110.69

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Mean	**3141.40**	**3109.55**
Standard Deviation (±)	**18.84**	**1.60**
(%) Relative Standard Deviation	**0.60**	**0.05**

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

Table - 16: Results for change in wavelength

Wavelength →	**228nm**	**232nm**
Sample	**% Assay**	
Test solution	99.21	99.62
Average assay result from method precision	100.98	100.98
Mean	**100.10**	**100.30**
Standard Deviation (±)	**1.25**	**0.96**
(%) Relative Standard Deviation	**1.25**	**0.96**

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

(Normal Experimental Condition: Acetonitrile: Formic Acid= 70ml: 30ml)

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 Pyraclostrobin**	
	ACN-68:FA-32	**ACN-72:FA-28**
1	2977.06	2984.56
2	2960.67	2997.25
Mean	**2968.86**	**2990.90**
Standard Deviation (±)	**11.59**	**8.97**
(%) Relative Standard Deviation	**0.39**	**0.30**

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	**ACN-68:FA-32**	**ACN-72:FA-28**
Sample	**% Assay**	
Test solution	99.83	99.42
Average assay result from method precision	100.98	100.98
Mean	**100.41**	**100.20**
Standard Deviation (±)	**0.81**	**1.10**
(%) Relative Standard Deviation	**0.81**	**1.10**

The analysis of the same lot of CABRIO Fungicide was carried out at different conditions of flow rate, wavelength, and change in composition of mobile phase. The system suitability was found to meet the pre-established criteria at all the conditions and 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 pre-established acceptance criteria for robustness study as per protocol. Thus, the method is robust.

Stability of Analytical Solution:

Procedure:

System suitability solution and test solution of CABRIO Fungicide 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 CABRIO Fungicide 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-19.

Table - 19: Results for solution stability

% Assay results calculated against the freshly prepared system suitability standard	
Sample	**% Assay of Pyraclostrobin**
0^{th} hr	99.88
12^{th} hr	100.03
24 hr	98.92
36 hr	100.07
48 hr	98.70
Mean	**99.52**
Standard Deviation (±)	**0.66**
(%) Relative Standard Deviation	**0.66**

The system suitability was found to meet the pre-established criteria and 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 48Hrs for test solution at room temperature. Thus, it can be concluded that the solution is stable up to 48Hrs at room temperature.

II. 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 Pyraclostrobin peak. The system suitability criteria were found to meet the pre-established acceptance

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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 Pyraclostrobin peak is passing. Hence, the Method is very precise, selective and specific to the estimation of Assay of Pyraclostrobin in test solution of CABRIO Fungicide as a Pyraclostrobin 99% by HPLC and the same method is stability indicating, as the degraded products are well separated from Pyraclostrobin and as well from each adjacent peak.

Linearity:

Linearity graph of the average area at each level against the concentration in acetonitrile and 0.1% Formic acid in the proportion 70: 30 (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 Pyraclostrobin in CABRIO 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 = 0.88

Method precision=%RSD = 1.13

Intermediate precision=%RSD = 0.57

Robustness:

The analysis of the Pyraclostrobin in CABRIO 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

III. SUMMARY AND CONCLUSION

The above summary and the validation data summarized in this paper show the analytical method of assay of Pyraclostrobin in CABRIO 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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