Development and Validation of RP-HPLC Method for the Analysis of Carboxin in its formulations

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Abstract:

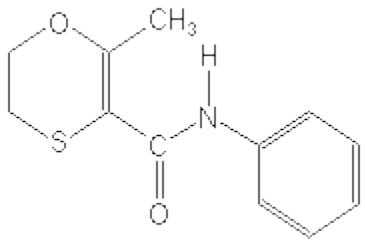
A simple, economic, selective, precise and accurate High Performance liquid Chromatographic method used for the analysis of Carboxin in its Formulations. Formulations was developed and validated in the present study. The mobile phase consists of Mixed Acetonitrile and water in the proportion 25:75 respectively. And this was found to give a sharp peak of Carboxin at a retention time of 10.27 min. HPLC analysis of Carboxin was carried out at a wavelength of 205 nm, With a flow rate of 0.8ml min^{-1} linear regression analysis data for the Calibration curve showed a good linear relationship with regression coefficient 0.999 in the concentration range of 50 ppm to 150 ppm. The linear regression equation was Y=5188×-364 the developed method was employed with a high degree of precision and accuracy for the analysis of Carboxin. The method was validated for accuracy, precision, robustness, detection and quantification limits as for ICH guidelines. The wide linearity range, accuracy, sensitivity, short retention time and composition of the mobile phase indicate that this method is better for the quantification of Carboxin

Key Words: Carboxin, **HPLC, Validation**.

Introduction:

Carboxin (2, 3-dihydro-6-methyl-oxathiin-5-carboxanilide, vitavax) is one of the several systemic fungicide (Figure 1) used in agriculture to control pathogenic fungi. Pesticides are widely used to protect the crops from a variety of pest. Pesticides comprise a large number of substances that belong to many different chemical classes. Fungicides as bitertanol, flutriafol, triadimefon and tebuconazole (triazoles), carboxin (anilide) and pyrimethanil (pyridine) are intensively applied to grapes at various stages of cultivation and during post-harvest storage to provide protection against rotting [1, 2]. Triazines, anilines and pyridines are important classes of fungicides with a wide range of useful activities. Many are systemic and they are highly active with as little as 60 g ha−1 being required (compared to the 250 g ha−1 for other fungicides as dithiocarbamates). They act by interfering with the synthesis of sterols, which are

essential for the construction of normal cell membrane [3–5]. Carboxin is anilide fungicide and intensively applied at various stages of cultivation and during post harvest storage to provide protection against rotting [6]. Although it has low mammalian toxicity, fungicide residues levels in food stuffs are generally legislated to minimize the exposure of consumers to the harmful or unnecessary intake of pesticides [6].



($C_{12}H_{13}NO_2S$)

Figure.1

The analysis of fungicides has been widely described in the recent literature and usually utilises the established multiresidue methods (MRM) of analysis [7, 8]. These methods involve solvent extraction and partitioning followed by solid-phase or gel permeation cleanup to achieve removal of co-extractives present in the sample extract. Most analytical methods developed in the literature are modification and variations that can improve these extraction and cleanup methods through changes in technologies to reduce the analysis time because sample preparation is still the bottleneck in the analytical laboratory, occupying more than 60% of the analyst's time [8].Advances could make by simplifying clean-up [9–12], improving extraction and miniaturization [9,12], increasing the use of liquid chromatography (LC) [11,13–18],intensifying automation [9], and introducing mass spectrometry(MS) detection [14–22].

A valid alternative is the enrichment on solid-phases cartridges, glass columns or disks packed with C18 [9, 13, 14], mixed cation exchange [10, 11], hydrophilic/lipophilic balance phases [10] or polymeric resins [22]. Detection limits attained ranged from 0.1to 180_g kg−1 depending on the compound and the determination. For the analysis of pesticides not amenable to gas chromatography several conventional LCmethods have been developed [23–25]. To analyze the large number of samples whose pesticide treatment history is usually unknown, the Food and Consumer Product Safety Authority (VWA) uses analytical methods capable of simultaneously determining a large number of pesticide residues. These multi-residue methods can determine about 450pesticides and their metabolites with MRL tolerances. In laboratory, traditionally gas chromatography in combination with mass spectrometric detection (GC–MS) and element-selective detection techniques have been used for the routine analysis of pesticides in foodstuff [26]. The determination of pesticides applied

in soya cultivation by using C8 co-column and subsequent chromatographic analysis by HPLC-DAD was developed. It proved that good recuperation for carboxin in soya cultivation [27]. Carboxin and oxycarboxin undergo photolytic reactions in the presence of organic and inorganic soil components. Humic and fulvic acids in aqueous solution lead to enhanced photo degradation of carboxin [28]. The extraction of carboxin from cabbage samples using florisil sorbent solid phase extraction following with HPLC-UV analysis has been used as a reliable tool in residue analysis. The carboxin residues found in the cabbage sample with the safety label are likely to be lower level than those in the sample without safety label [29].

The author has developed RP-HPLC method for the determination of Carboxin in its formulations based on the use of symmetry column, without use of any internal standard. An attempt has been made to develop and validate all methods to ensure their accuracy, precision, repeatability, reproducibility and other analytical method validation parameters as mentioned in the various guidelines.

CARBOXIN ASSAY BY HPLC-METHOD VALIDATION

Analysis of Carboxin has mainly been accomplished by different methods such as infrared spectroscopy, GC, HPLC methods were more frequently employed for the analysis of Carboxin in different environmental samples. However no reported RP-HPLC method for the analysis of Carboxin in its technical grade and its formulations. This chapter describes a validated RP-HPLC method for the quantitative determination of Carboxin. The author has developed RP-HPLC method based on the use of Waters symmetry C18 column, without use of any internal standard. An attempt has been made to develop and validate all methods to ensure their accuracy, precision, repeatability, reproducibility and other analytical method validation parameters as mentioned in the various guidelines.

PHYSICAL PROPERTIES AND GUIDELINES

Carboxin is a colorless crystal. Carboxin is slightly toxic. Symptoms of poisoning can include vomiting and headache. Recovery is very rapid if the exposed individual is treated quickly.

Table-1: Physical Properties of Carboxin

S.No.	Property	Description
1	Molecular Weight	235.3gm/mole
2	Appearance	colorless crystal
3	Density	1.36g/cm^3
4	Melting Point	93-95 degrees C
5	Boiling Point	Decomposes before boiling
6	Solubility in Water	195 mg/l at 25 ^{O}C
7	Octanol-water partition coefficient at pH 7, 20^{o}C Log P	2.3
8	Hazards LD_{50}	3820mg/kg
9	Henry's law constant at 25^{o}C (Pa m^3mol^{-1})	3.20 X 10^{-05}
10	CAS #Number	5234-68-4
11	Vapor Pressure @25^{0}C(mPa)	0.025
12	Specific gravity	1.36
13	Partition Coefficient	2. 1703.
14	Adsorption Coefficient	260 ml/gm
15	Maximum UV-vis absorption L mol^{-1} cm^{-1}	205nm = 17443, 295nm = 6585

Applicators and handlers of Carboxin should wear protective/impervious clothing and equipment to prevent skin contact.

Solubility:

Table-1.1: Solubility Properties of Carboxin

S.No.	Solvent	Solubility
1	Water	0.195 g/l at 25 ^{0}C
2	Acetone	177g/kg
3	Acetic acid	92.5g/L
4	Benzene	150 g/kg
5	Methanol	210 g/kg
6	Dichloromethane	353g/L
7	Ethanol	110g/kg

Chemical Class: Carboxanilide Fungicide.

INSTRUMENTS / EQUIPMENTS USED

a) High performance liquid chromatography, with UV / PDA detector
b) HPLC Analytical column of Nucleosil - C18, 100mm x 4.6mm x 5µm
c) Analytical weighing balance - Mettler Toledo B204S
d) Millipore Nylon 0.2µm
e) Laboratory accessories

CHEMICALS USED

a. Carboxin working standard
b. VITAVAX - 3F Fungicide
c. Acetonitrile -AR
d. Methanol-AR
e. Water-HPLC

REFERENCES:

Table-1.2: ICH Guide lines

S.No	Name	W.S No.	Purity on Dried Basis	LOD
1	Carboxin	WS-125	99.9%	0.10%

ICH Guideline number: Q2A & Q2B of CPMP / ICH / 281 / 95.

ANALYTICAL METHOD:

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

Chromatographic conditions:

Column	:	Nucleosil - C18, 100mm x 4.6mm x 5µm
Mobile Phase	:	Mixed Acetonitrile and water in the proportion 25:75 respectively
Wavelength	:	205nm
Flow Rate	:	0.8 ml / minute
Injection volume	:	10 µl
Run time	:	25 minutes
Blank solution	:	Use Methanol as blank
Diluent	:	Use Methanol as diluent

Preparation of Carboxin Standard Solution: Weighed accurately about 50 mg of Carboxin working standard and transferred into a 50 ml volumetric flask. Added 10 ml of diluent and sonicated to dissolve. Diluted to volume with diluent and mixed.

Transferred 1.0 ml of solution into a 10 ml of volumetric flask and diluted to volume with the diluent and mixed.(Dilution scheme: 50mg → 50.0 ml → 1 ml /10.0 ml)

Preparation of Test Solution: Weighed accurately about 147 mg of sample and transferred into a 50 ml volumetric flask. Added 10 ml of diluent and sonicated to dissolve. Diluted to volume with diluent and mixed. Transferred 1.0 ml of solution into a 10 ml of volumetric flask and diluted to volume with the diluent and mixed.(Dilution scheme: 147mg → 50.0 ml → 1 ml /10.0 ml)

System Suitability Solution:

Carboxin standard working solution is used as system suitability solution.

Procedure:

Equal volumes of blank and five replicate injections of system suitability solution were injected separately (Carboxin standard working solution). Then injected two injections of test solution and recorded the chromatograms. Any peak due to blank in the test solution was disregarded. % RSD of five replicate injections of system suitability solution (Carboxin standard working solution) was calculated. Checked tailing factor and theoretical plates of the peak in the chromatogram obtained with 5th injection of system suitability solution (Carboxin standard working solution).

The limits are as below,

1) Theoretical plates should be not less than 3000.
2) Tailing factor should be less than 2.0.
3) % RSD should be not more than 2.0%.

Injection scheme:

Table-1.3: Injection scheme

Sr. No.	Solutions to be injected	
01	Diluent Blank solution	1
02	System suitability solution (Carboxin standard solution)	5
03	Test Solution	2

Calculations:

$$\% \text{ Assay} = \frac{AT}{AS} \times \frac{WS}{50} \times \frac{1}{10} \times \frac{10}{1} \times \frac{50}{WT} \times \frac{L.C}{100} \times P$$

AT Average Peak area of Carboxin in test solution

AS Mean peak area of Carboxin in system suitability solution

WS	Weight of Carboxin working standard taken in mg
WT	Weight of Carboxin sample taken in mg
P	Assay of Carboxin working standard in % on as is basis
L.C	Label Claim

Express the results up to two decimals.

VALIDATION PARAMETERS

The HPLC method is evaluated for following validation parameters followed by ICH guideline Quality topics Q2A & Q2B of CPMP / ICH / 281 / 95.

Table-1.4: Validation Parameters

S. No.	**Validation Parameter**	**Assay**
1	Specificity / Selectivity	+
2	Linearity & Range of Carboxin Std from 50% to 150%	+
3	Precision i) System precision ii) Method precision iii) Intermediate precision (Ruggedness)	+
4	LOD & LOQ	+
5	Stability of analytical solutions	+

VALIDATION RESULTS:

The system suitability parameters were monitored throughout the validation study and are recorded in the validation report. The validation data is summarized in table-15.

Specificity / Selectivity:

Selectivity was performed by injecting the diluent blank solution, system suitability solution, test solution.

Acceptance criteria:

The Carboxin peak should be well resolved from any other peak and from each other.

The diluent blank solution should not show any peak at the retention time of the Carboxin.

Results: The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method. All the injections were processed at the

wavelength provided in the method. There was no interference observed from diluent blank solution with Carboxin peak.

Linearity:

Linearity and Range for standard:

For the linearity study five standard solutions of Carboxin 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. 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:

The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method. (Refer to Table-3.8 for system suitability results).

The average peak area of Carboxin 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 Tables-1.6&1.7. The linearity graph as shown in figure-1.

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.

Results:

The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method data is summarized in table-1.8.

Method Precision:

Procedure:

Six test solutions of Carboxin in VITAVAX - 3F 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%.

Results:

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-1.9&2.0**.**

Intermediate Precision:

Procedure:

Six test solutions of VITAVAX - 3F 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%.

Results:

The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method. (Refer to Table -2.1 for system suitability results). The results of assay obtained from six test solutions are presented in Tables-2.2. % RSD of assay results from method precision and intermediate precision (12 results) are presented in Table – 2.3. The analysis was carried out on six test solutions of the same lot of the drug product 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 (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 data is summarized in table-2.3.The graphical representations of intermediate precision and method precision as shown in figures -2&3.

LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ)

Observation: Limit of detection and Quantitation is established by injecting six times very low concentration of Carboxin standard preparation i.e. 0.5ppm & 1.0ppm. The relative standard deviation for the peak response of Carboxin obtained for six replicate injections is 0.54%.

Conclusion: Hence the described method could be used to detect and quantify minimum of 5 ppm of Carboxin for any given samples including in the samples collected for cleaning validation.

Stability of Analytical Solution:

Procedure:

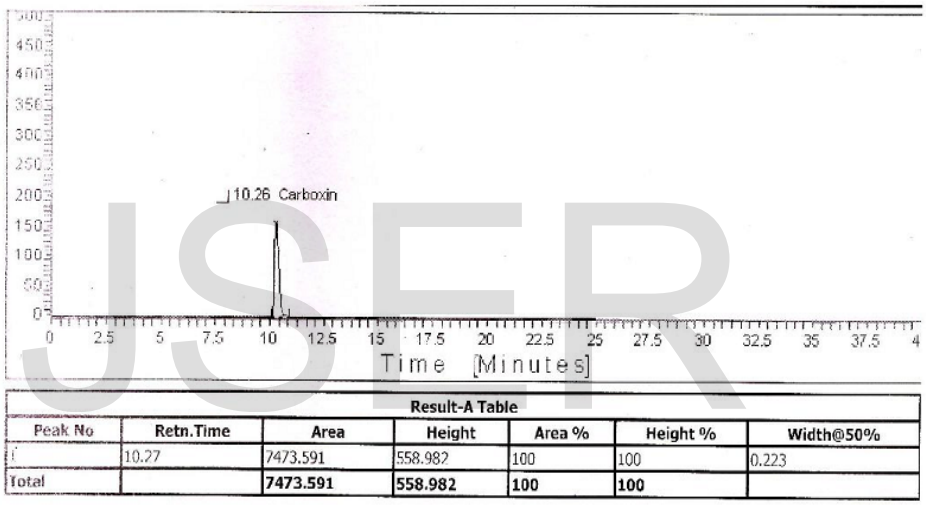
System suitability solution and test solution of VITAVAX - 3F Fungicide were prepared on 0^{th},12^{th}, 24^{th}, 36^{th} and 48^{th} hour of experiment and stored these solutions at room temperature for every time interval up to 48 hrs and analyzed these solutions on 48 hrs with freshly prepared test solution. The system suitability solution was prepared freshly at the time of analysis. The assay of VITAVAX - 3F Fungicide in the sample was calculated.

Acceptance criteria:

The analyte is considered stable if there is no significant change in % assay.

Results:

The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method (Refer to Tables -2.4&2.5 for system suitability results).



Result-A Table

Peak No	Retn.Time	Area	Height	Area %	Height %	Width@50%
1	10.27	7473.591	558.982	100	100	0.223
Total		**7473.591**	**558.982**	**100**	**100**	

Figure-1: chromatogram of Carboxin

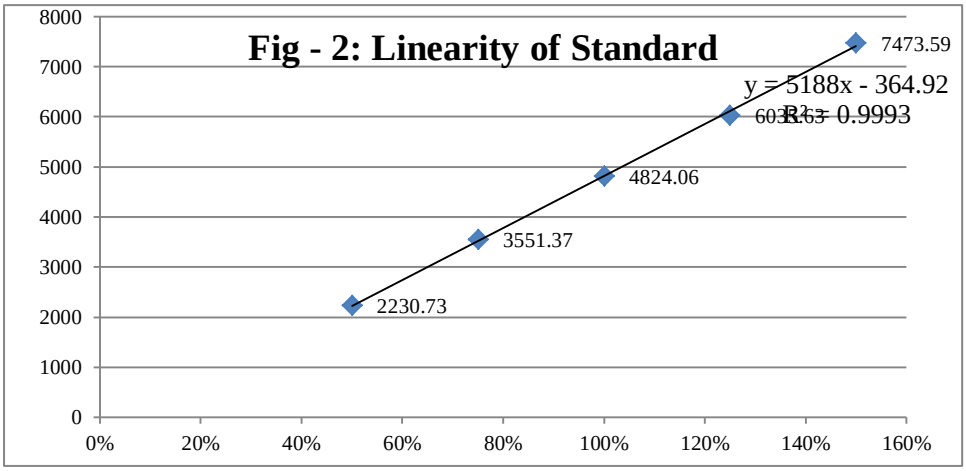
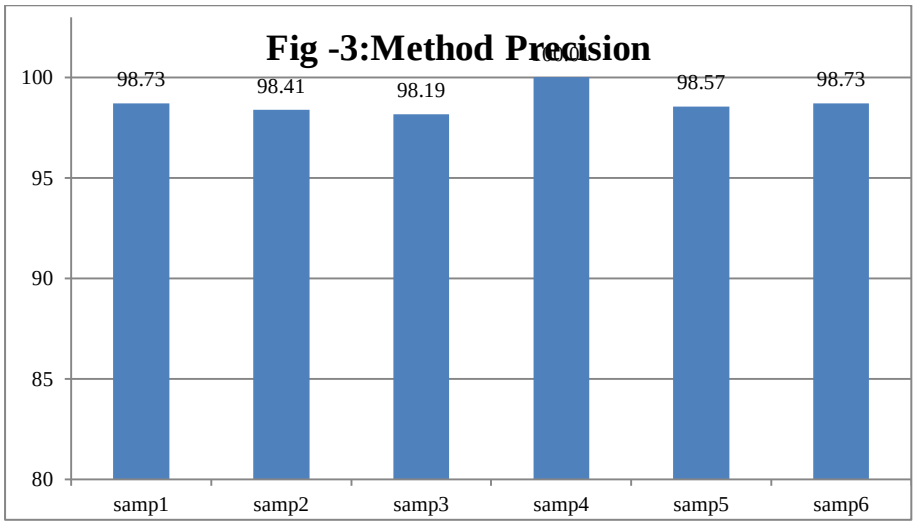


Figure-2: Linearity graph of Carboxin standard

Analyst – 1 HPLC No.: EH/R&D/HPLC-024



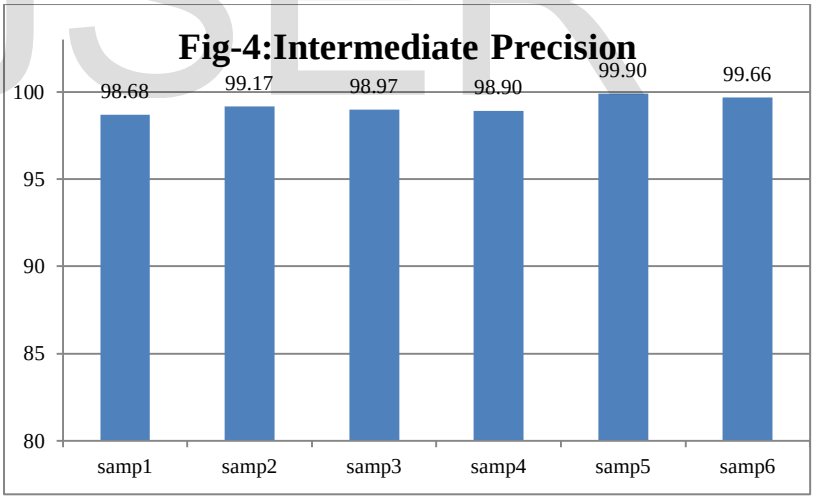


Table-1.5: System suitability - Selectivity

Sr. No.	Area of Carboxin
1	4573.12
2	4539.24
3	4580.60
4	4586.47
5	4557.91
Mean	**4567.47**
Standard Deviation (±)	**19.06**
(%) Relative Standard	**0.42**

Table-1.6: System suitability - Linearity of standard

Sr. No.	Area of Carboxin
1	4553.73
2	4589.04
3	4543.12
4	4549.53
5	4540.32
Mean	**4555.15**
Standard Deviation (±)	**19.66**
(%) Relative Standard	**0.43**

Table-1.7: Results of linearity of standard

Linearity Level	SampleConcentration (in%)	Sample Concentration	Peak Area	Correlation Coefficient
Level – 1	50	50	2230.73	**0.999**
Level – 2	75	75	3551.37	
Level – 3	100	100	4824.06	
Level – 4	125	125	6035.63	
Level – 5	150	150	7473.59	

The linearity plot of peak area of Carboxin Vs. standard concentration in percentage is presented in figure-1.

Table-1.8: System precision

Sr. No.	Area of Carboxin
1	4735.86
2	4766.43
3	4760.83
4	4778.49
5	4780.09
6	4791.91
7	4766.83
8	4777.59
9	4772.38
10	4771.53
Mean	**4770.19**
Standard Deviation (±)	**14.88**
(%) Relative Standard Deviation	**0.31**

Table-1.9: System suitability - Method precision

Sr. No.	Area of Carboxin
1	4832.20
2	4827.91
3	4841.97
4	4807.55
5	4785.99
Mean	**4819.13**
Standard Deviation (±)	**22.37**
(%) Relative Standard Deviation	**0.46**

Table-2.0: Results of method precision

Test Solution	% Assay of Carboxin
1	98.73
2	98.41
3	98.19
4	100.01
5	98.57
6	98.73
Mean	**98.77**
Standard Deviation (±)	**0.64**
(%) Relative Standard Deviation	**0.65**

Analyst – 2 HPLC No.: EH/R&D/HPLC-023

Table-2.1: System suitability - Intermediate precision

Sr. No.	Area of Carboxin
1	4677.68
2	4720.55
3	4722.77
4	4708.86
5	4699.26
Mean	**4705.82**
Standard Deviation (±)	**18.36**
(%) Relative Standard Deviation	**0.39**

Table-2.2: Results of intermediate precision

Test Solution	% Assay of Carboxin
1	98.68
2	99.17
3	98.97
4	98.90
5	99.90
6	99.66
Mean	**99.21**
Standard Deviation (±)	**0.47**
(%) Relative Standard Deviation	**0.48**

Table-2.3: Results of twelve test solutions of Carboxin in VITAVAX - 3F 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 Carboxin**
1	98.73
2	98.41
3	98.19
4	100.01
5	98.57
6	98.73
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 Carboxin**
7	98.68
8	99.17
9	98.97
10	98.90
11	99.90
12	99.66
Mean of twelve samples	**98.99**
Standard Deviation (±)	**0.58**
(%) Relative Standard Deviation	**0.59**

Table-2.4: System suitability – LOD & LOQ

Sr. No.	Area of Carboxin
1	4460.63
2	4408.42
3	4418.87
4	4457.60
5	4452.29
Mean	**4439.56**
Standard Deviation (±)	**24.13**
(%) Relative Standard Deviation	**0.54**

Table-2.5: Results for LOD & LOQ

Injection No.	Peak Response of LOD	Peak Response of LOQ
1	124.35	246.38
2	128.09	269.44
3	129.10	248.88
4	129.08	253.76
5	139.14	262.02
6	137.79	255.43
Average	**131.26**	**255.99**
Standard	**5.86**	**8.55**
%RSD	**4.47**	**3.34**

Table-2.6: System suitability - Solution stability

Time	Std. Area	Avg. std. area	Spl. area	Avg. Spl. area
	4614.55		4634.65	
0^{th} hr	4629.24	4621.89	4642.76	4634.65
	4615.71		4657.24	
12^{th} hr	4601.11	4608.41	4642.77	4657.24
	4702.49		4686.97	
24 hr	4718.05	4710.27	4682.74	4686.97
	4624.99		4610.85	
36 hr	4627.93	4626.46	4638.4	4610.85
	4760.36		4767.23	
48 hr	4756.73	4758.55	4763.65	4767.23
Mean	**4665.11**	**4665.11**	**4672.72**	**4671.38**
Standard Deviation	**62.39**	**65.85**	**53.74**	**60.5**
(%) Relative Standard Deviation	**1.33**	**1.41**	**1.15**	**1.29**

Table-2.7: Results for solution stability

% Assay results calculated against the freshly prepared system suitability standard	
Sample	**% Assay of Carboxin**
0^{th} hr	98.79
12^{th} hr	99.32
24 hr	97.9
36 hr	98.39
48 hr	98.57
Mean	**98.59**
Standard Deviation (□)	**0.52**
(%) Relative Standard Deviation	**0.53**

SUMMARY AND CONCLUSION:

Table-2.8: Summary and Conclusion

Sr. No.	Parameter	Result	Acceptance Criteria
1	**Specificity:** **Selectivity**	The Carboxin peak in test solution was found to be well resolved from peaks due to diluent blank solution. The diluent blank did not show any peak at the retention time of the Carboxin.	The Carboxin peak all should be well resolved from any other peak and from each other. The diluent blank solution should not show any peak at the retention time of the Carboxin.
2	**Linearity and Range of Standard**	Correlation coefficient = 0.999 Range = 50 ppm to 150 ppm	Correlation coefficient should be greater than or equal to 0.999.
3	**System precision**	% RSD = 0.31	% 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.
4	**Method precision**	% RSD = 0.65	% RSD of the results of six test solutions should not be more than 2.0%.
5	**Intermediate precision**	% RSD = 0.48	% RSD of the results of twelve test solutions (six of Method Precision and six of Intermediate Precision) should not be more than 2.0%.
6	**LOD**	% RSD = 4.47	% RSD of the results of six test solutions should not be more than 10.0%.
7	**LOQ**	% RSD =3.34	% RSD of the results of twelve test solutions (six of Method Precision and six of Intermediate Precision) should not be more than 5.0%.

8	**Stability of analytical solution**	No significant change was observed in the % assay upto 48 Hrs. Hence the solution is found to be stable up to 48 Hours at room temperature.	The analyte was considered stable if there is no significant change in % assay.

The above summary and the validation data summarized in this document shows that the analytical method of assay of Carboxin in VITAVAX - 3F Fungicide by HPLC is found to be suitable, selective, specific, precise, linear and robust. The analytical solution is found to be stable up to 48 Hrs at room temperature.

Hence, it is concluded that the analytical method is validated and can be used for routine analysis and for stability study.

CONCLUSION:

The method was found to be accurate and precise, as indicated by recovery studies close to 100 and % RSD is not more than 2.The summary of validation parameters of proposed HPLC method is given in tables. The simple, accurate and precise RP-HPLC method for the determination of Carboxin as Technical and formulation has been developed. The method may be recommended for routine and environmental analysis the investigated drug in formulations. The analytical solution hence, it is concluded that the analytical method is validated and can be used for routine analysis.

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