Analytical method development of the combination pesticide Azoxystrobin and Epoxiconazole by R- HPLC

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ABSTRACT

A simple reverse phase liquid chromatographic method has been developed and subsequently validated for the Azoxystrobin and Epoxiconazole are a fungicide molecule; which are applicable for the treatment of the vegetable and fruits fungal decease. These Azoxystrobin and Epoxiconazole molecules were separated through a mobile phase consisting of the mixture of acetonitrile, methanol, and water in the ration of 10:30:60. All these solvents are HPLC grade. Column: Qualisil BDS C18 (250 x 4, 5µ); Flow rate: 1.0 ml/min; Detector: UV-Vis. Absorption (λ) at 230 nm of Shimadzu HPLC (model: LC-2030). The LC solution software was used for the analytical method, data integrations, and calculations in this analysis. There are two molecules were analyzed for separation and quantification. The results of the study showed that the proposed RP-HPLC method is simple, rapid, precise and accurate, which is useful for the identification and quantifications of these molecules interims of validation parameters viz., separation, system suitability, System Precision and linearity in a simple HPLC analysis.

Keywords: Azoxystrobin, Epoxiconazole, HPLC analysis, Validated Method, SANCO 3030/99 Rev.4, ICH Guideline.

1. INTRODUCTION

The Azoxystrobin and Epoxiconazole molecules were used for the treatment of fungicide of the crops separately as well as a combination. The Azoxystrobin pesticide is less toxic to human and other mammals, birds, insects, and earthworm too; but is has the power of the penetrating to soil and a very good in controls of fungal growth. The Epoxiconazole molecule was belonging to the azoles class. This molecule controls the metabolism of the fungal cells and hence controlled the fungal growth.

The combination product was used worldwide to control the fungal growth on the crops. Since the molecule chemically different and hence functions also different. The action of the controlling the fungus in a different mode; hence the wide range of fungus were controlled. This combination product is successful in the plant cultivation area. The entire pesticide molecule has to be analyzed for its purity, stability and other raw material, in-processes and solvent impurities for better understanding. Any analytical method has to be simple and reproducible and cost-effective during its analysis.

A simple and wide used instrument in the analytical era is HPLC and this instrument is used for qualitative and quantitative analysis effectively with respect to cost, time and simplicity. Moreover, this method is reproducible and can be used in Quality control and Research and Development area.

In this study, the mixture of the Azoxystrobin and Epoxiconazole pesticide was analyzed for with regulatory required validation method viz., precision, linearity, repeatability, and recovery by a simple HPLC method.

2. MATERIALS AND METHOD

2.1 Reagents and chemicals used: Methanol HPLC grade from Final, Acetonitrile HPLC grade from Rankem; Water HPLC grade was obtained from double distilled water purification system. In this study, reference standards were received form authorized supplier for Azoxystrobin and Epoxiconazole. All the glassware (standard flask / volumetric flasks) of class A grade were used in this analysis.

2.2 Instrument: Reverse phase chromatographic separation was achieved with HPLC (Shimadzu LC-2030, Prominence i series), equipped with a Uv-Vis. a detector coupled with auto sampler. The data collection and peak/analysis processed by an LC solution

(HPLC) software. Mobile phase mixture (Acetonitrile (10): Methanol (60): Water 30%) were used. The injection volume was 20 µl and the analysis was performed at ambient (25°C) temperature. The HPLC column was used by Phenomenex, Luna C18 (2), Length 250mm x 4.60mm, 100µ particle size and the detection wavelength is 230 nm.

3. ANALYTICAL METHOD VALIDATION

3.1 Specificity
3.1.1 Preparation of standard stock solutions:
An amount of 10.13 mg of Epoxiconazole reference standard with purity 98.8 % and 10.11 mg of Azoxystrobin reference standard with purity 99 % was weighed accurately in to a clean and dry 10 mL volumetric flask and dissolved in 10 mL of mobile phase and made up to the mark with the mobile phase. This was equivalent to 1000 mg/L. From these above standard solution 100 mg/L was prepared and analyzed as standard to determine specificity by HPLC method.

3.1.2 Preparation of Sample Solution
An amount of 13.5 mg of the test substance was weighed in to a 100 mL volumetric flask and dissolved and made up to the mark with the mobile phase. This was equivalent to 135 mg/l. From this, 1 ml solution was diluted with 10ml mobile phase. The concentration of the solution was equivalent to 13.5 mg/L.

3.2 Linearity
3.2.1 Preparation of Standard Stock Solution
The standard stock solution prepared for Specificity was used for Linearity determination. This solution was further diluted to 100 mg/L and used for the preparation of calibration solutions.

3.2.2 Preparation of Calibration Solutions
From the prepared Linearity standard solution, the serial dilutions were made to prepare further concentrations such as 1, 5, 10, 15, 20 and 25 mg/L separately. The dilution details are presented in table No.1

TABLE-1: Dilutions (Epoxiconazole & Azoxystrobin)

| Standard Code	Stock Conc. (mg/L)	Dilution Volume (ml)	Final Volume (ml)	Final Conc. (mg/L)
STD-1	100	0.1	10	1
STD-2	100	0.5	10	5
STD-3	100	1.0	10	10
STD-4	100	1.5	10	15
STD-5	100	2.0	10	20
STD-6	100	2.5	10	25

These standard solutions were injected into HPLC system and a linear curve was plotted for the concentration of standard versus observed peak area and the correlation coefficient was determined respectively. The results are presented in table No. 2 and 3.

TABLE-2: Linearity of Azoxystrobin reference standard.

Code	Conc. (mg/L)	Replication	Area	Mean Area
Std-1	1	R1	936972	936993.0
		R2	937014	
Std-2	5	R1	1778930	1779656.5
		R2	1780383	
Std-3	10	R1	2677529	2678531.0
		R2	2679533	
Std-4	15	R1	3510028	3517600.0
		R2	3525172	
Std-5	20	R1	4344815	4344658.0
		R2	4344501	
Std-6	25	R1	5246854	5244159.5
		R2	5241465	
			Intercept	845640
			Slope	176681
			Correlation Coefficient	0.999

TABLE-3: Linearity of Epoxiconazole reference standard

Conc. (mg/L)	Replication	Area	Mean Area
1	R1	337780	337625.0
	R2	337470	
5	R1	641024	641455.5
	R2	641887	
10	R1	921587	922427.0
	R2	923267	
15	R1	1217869	1218752.5
	R2	1219636	
20	R1	1551835	1551289.0
	R2	1550743	
25	R1	1897652	1897317.5
	R2	1896983	
		Intercept	288735.01
		Slope	63637.5851
		Correlation Coefficient	0.999
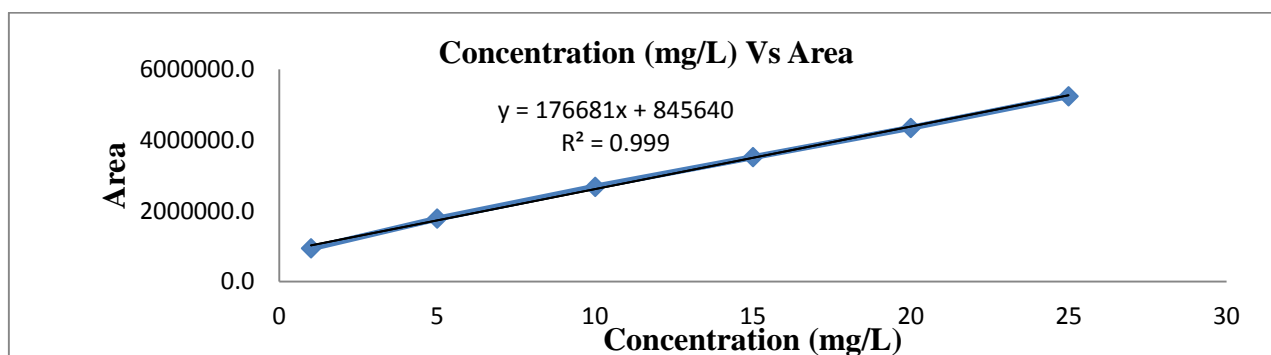
Figure-1: Linearity curve for Azoxystrobin
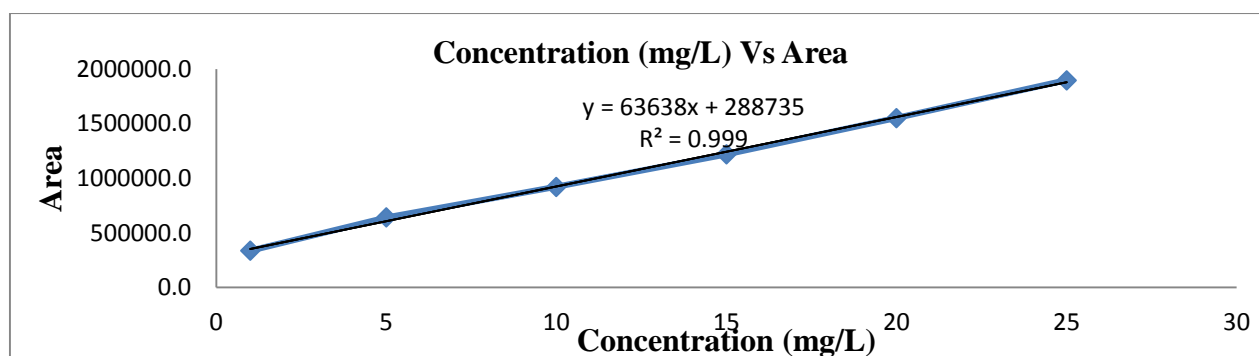
Figure-2: Linearity curve for Epoxiconazole

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Sample Name   : Azoxystrobin + Epoxyconazole
Sample ID   : L-Std-1-R1
Injection Volume : 20 uL
Data Filename   : 03.lcd
Method Filename : Azoxystrobin + Epoxyconazole.lcm
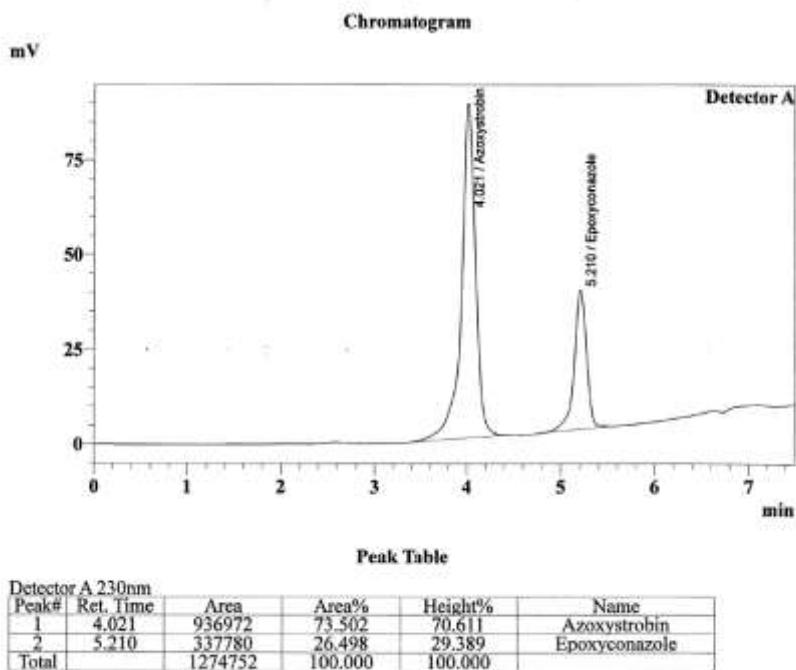Date Acquired   : 26-Feb-17 11:57:29 AM

Peak Table

Detector A 230nm

Peak#	Ret. Time	Area	Area%	Height%	Name
1	4.021	936972	73.502	70.611	Azoxystrobin
2	5.210	337780	26.498	29.389	Epoxyconazole
Total		1274752	100.000	100.000	

Figure-3: A typical HPLC chromatogram for linearity

4. PRECISION

4.1 Preparation of Standard Solution
The Linearity standard solution 1 mg/L was used for the precision determination.
4.2 Preparation of Sample Solution
The Specificity solution (13.5 mg/L) was used for the precision determination. This sample was injected 5 times and % RSD was calculated and the results are presented in following table No.4 and 5.

TABLE-4: Precision (Azoxystrobin)

Sample ID	Std. Conc. (mg/L)	Std. Area	Average Area	Sample Area	Sample Wt. (W) mg	Purity %	A.I. Content (%)
STD-R1	1	931283		-	-	-	-
STD-R2	1	930085		-	-	-	-
P1	-	-		2667734	13.6	99	20.87
P2	-	-	930684	2667505	13.6	99	20.86
P3	-	-		2667424	13.6	99	20.86
P4	-	-		2661689	13.6	99	20.82
P5	-	-		2664684	13.6	99	20.84
						Mean	20.85
						SD	0.02
						RSD	0.10

Table-5: Precision (Epoxiconazole)

Sample ID	Std. Conc. (mg/L)	Std. Area	Average Area	Sample Area	Sample Weight (W) mg	Purity (P) %	A.I. Content (%)
STD-R1	1	338454		-	-	-	-
STD-R2	1	338514		-	-	-	-
P1	-	-		936848	13.6	98.8	20.11
P2	-	-	338484	936755	13.6	98.8	20.11
P3	-	-		936636	13.6	98.8	20.10
P4	-	-		936865	13.6	98.8	20.11
P5	-	-		936878	13.6	98.8	20.11
						Mean	20.11
						SD	0.00
						RSD	0.01

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Sample Name   : Azoxystrobin + Epoxyconazole
Sample ID   : P1
Injection Volume : 20 uL
Data Filename : 05.lcd
Method Filename : Azoxystrobin + Epoxyconazole.lcm
Date Acquired : 26-Feb-17 4:05:40 PM

Peak Table

Detector A 230nm
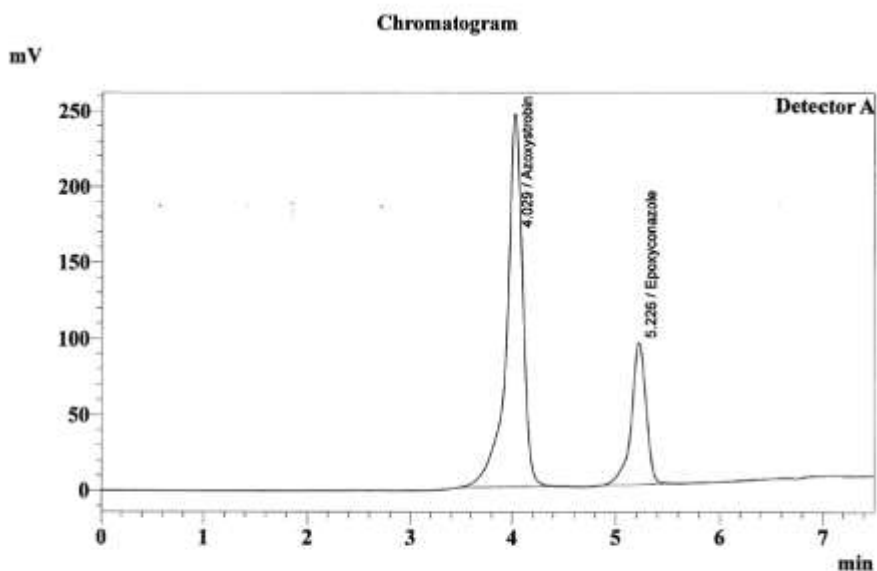
Peak#	Ret. Time	Area	Area%	Height%	Name
1	4.029	2667734	74.010	72.391	Azoxystrobin
2	5.226	936848	25.990	27.609	Epoxyconazole
Total		3604581	100.000	100.000	

Figure-4: A typical HPLC chromatogram for precision

5. ACCURACY (% RECOVERY)

The analytical method was validated for the recovery of the standard at two fortification levels.
5.1 Preparation of Standard Solution
The standard solution prepared for linearity (1 mg/L) was used as a standard in percent recovery determination.

5.2 Preparation of Fortification Level 1 –4 mg/L (Azoxystrobin & Epoxiconazole)
0.4 ml of Linearity standard solution (Epoxiconazole and Azoxystrobin) 100 mg/L was transferred into 10 ml volumetric flask and made up to the mark with the distilled water. The concentration of the prepared solution was equivalent to 4 mg/L of Azoxystrobin & Epoxiconazole respectively. These prepared solutions were used for recovery determination.

5.3 Preparation of Fortification Level 2 – 6 mg/L (Azoxystrobin & Epoxiconazole)
0.6 ml of Linearity standard solution (Epoxiconazole and Azoxystrobin) 100 mg/L was transferred into 10 ml volumetric flask and made up to the mark with the distilled water. The concentration of prepared solutions was equivalent to 6 mg/L of Epoxiconazole and Azoxystrobin respectively. These prepared solutions were used for recovery determination.

The above preparations were analyzed under HPLC and checked for recovery (%). The results are presented in following table No. 6 and 7.

Table-6: accuracy (Level-1 & 2 recovery %)-Azoxystrobin

Level	Std. Conc. (mg/L)	Std. Area	Avg. Std. Area	Sample Area	Recovery Conc. (mg/L)	Fortified Conc. (mg/L)	Recovery (%)
Level-1	1	931336	930921	3626657	3.90	4	97.39
		930505		3628115	3.90		97.43
		-		3628185	3.90		97.44
		-		3625810	3.89		97.37
		-		3628139	3.90		97.43
Level-2		-		5388507	5.79	6	96.47
		-		5385409	5.79		96.42
		-		5389408	5.79		96.49
		-		5386908	5.79		96.44
		-		5388573	5.79		96.47

Table-7: Accuracy (Level-1 & 2 recovery%)-Epoxiconazole

Level	Std. Conc. (mg/L)	Std. Area	Avg. Std. Area	Sample Area	Recovery Conc. (mg/L)	Fortified Conc. (mg/L)	Recovery (%)
Level-1	1	338257	338528	1260759	3.72	4	93.11
		338799		1261101	3.73		93.13
		-		1262103	3.73		93.21
		-		1263416	3.73		93.30
		-		1264562	3.74		93.39
Level-2		-		1952194	5.77	6	96.11
		-		1952170	5.77		96.11
		-		1953595	5.77		96.18
		-		1956059	5.78		96.30
		-		1955997	5.78		96.30

6. LIMIT of DETECTION (LOD) & LIMIT of QUANTIFICATION (LOQ)

From the Linearity Standard Solution (20 mg/L), 0.1 mg/L Standard solution was prepared. From the prepared Standard Solution (0.1 mg/L), required lowest 0.01 and 0.001 mg/L concentration of standard solutions were injected into HPLC to calculate the LOD & LOQ. The results are presented in following table No. 8-11.

Table-8 Limit of detection (LOD) Azoxystrobin

Code	Std. Conc. (mg/L)	Sample Area	Std. Area	Avg. Std. Area	Detected concentration (mg/L)
STD-R1	1	-	932388	931477	-
STD-R2	1	-	930566		-
T1R1	-	913	-	-	0.001
T1R2	-	902	-	-	0.001
T1R3	-	929	-	-	0.001
			MEAN		0
			SD		0
			LOD		0.001

Table 9: Limit of detection (LOD) Epoxiconazole

Code	Std. Conc. (mg/L)	Sample Area	Std. Area	Avg. Std. Area	Detected concentration (mg/L)
STD-R1	1	-	338670	338413	-
STD-R2	1	-	338156		-
T1R1	-	506	-	-	0.0015
T1R2	-	500	-	-	0.0015
T1R3	-	543	-	-	0.0016
			MEAN		0
			SD		0
			LOD		0.0016

Table 10: Limit of quantification (LOQ) Azoxystrobin

Code	Std. Conc. (mg/L)	Sample Area	Std. Area	Avg. Std. Area	Detected concentration (mg/L)
STD-R1	1	-	932388	931477	-
STD-R2	1	-	930566		-
T1R1	-	9056	-	-	0.0097
T1R2	-	9201	-	-	0.0099
T1R3	-	9307	-	-	0.01
			MEAN		0.01
			SD		0
			LOQ		0.0112

Table 11: Limit of quantification (LOQ) Epoxiconazole

Code	Std. Conc. (mg/L)	Sample Area	Std. Area	Avg. Std. Area	Detected concentration (mg/L)
STD-R1	1	-	338670	338413	-
STD-R2	1	-	338156		-
T1R1	-	4778	-	-	0.0141
T1R2	-	4992	-	-	0.0148
T1R3	-	5024	-	-	0.0148
			MEAN		0.01
			SD		0
			LOQ		0.019

Formula:

LOD   =   Average + (3 x Standard Deviation).
LOQ   =   Average + (10 x Standard Deviation)

7. ACTIVE CONTENT ANALYSIS OF EPOXICONAZOLE & AZOXYSTROBIN

7.1 Preparation of Standard solution
An amount of 10 mg of the standard was dissolved in 100 ml of mobile phase and diluted to get 10 mg/L was used as a standard in concentration analysis.

7.2 Preparation of Sample Solutions
An amount of 10 mg of the sample was dissolved in 100 ml of mobile phase and diluted to get 10 mg/L was used to determination of the active content

$$\text{Azoxystrobin \& Epoxiconazole (mg/L)} = \frac{A \times B \times DF}{C}$$

Where,

A   -   Concentration of standard (ppm)
B   -   Area of the sample solution
C   -   Area of standard solution
DF   -   Dilution Factor

8. CONCLUSION

8.1 Separation: The separation between the two peaks is achieved as NLT 1.5 min. The minimum requirement of the regulatory requirement as per (SANCO 3030/99 Rev.4 was achieved

8.2 Linearity: The Linearity correlation co-efficient is achieved NLT 0.999. The minimum requirement of the as per (SANCO 3030/99 Rev.4 was achieved

8.3 System Precision: The system precision is achieved as the % RDS for 5 replicates observed as 0.1% for azoxystrobin and 0.01% for Epoxyconazole, hence the minimum requirement of the (SANCO 3030/99 Rev.4 was NMT 15% RSD was achieved

8.4 System Recovery: The system recovery 92% to 101 % were achieved for azoxystrobin and Epoxyconazole, hence the minimum requirement of the (SANCO 3030/99 Rev.4.

8.5 Details of the Laboratory work were carried out.
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