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Separation and estimation of azoxystrobin and difenoconazole formulation by reverse phase high performance liquid chromatography

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ABSTRACT

The Azoxystrobin and Difenoconazol molecules are being used alone and combinations system as a fungicide in the cultivation industry. Such an important combination molecule have to be analysed to estimate the active content exactly with less time and cost of analysis. A simple analytical method developed to analyse these two molecules within 15 minutes of analysis time. The separation was achieved through the Shimadzu BDS C18 (250 × 4.6 mm, 5µm) column with the flow rate as 1.0 ml/min with the detection at 230 nm. These method parameters were loaded in the Shimadzu HPLC (model: LC-2030) with acetonitrile and water (HPLC grade) at 60:40 ratio as the mobile phase. Validated shimadzu Liquid Chromatography solution software was used for all the calculations in this analytical method validation analysis. 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 and difenoconazol, HPLC analysis, Validated method, SANCO 3030/99 Rev.4, ICH guideline*

1. INTRODUCTION

The difenoconazol molecule has a wide activity in the cultivation industry and this molecule being very active on the fungi kingdom. The active functional groups in the Difenoconazol were chloride, ether; triazole and dioxolan are effectively defusing the spores' multiplication in the fungicides. These active functional groups are not only preventing the spores production and also forbidden the growth of the fungus on the cultivation. Azoxystrobin molecule alone is effectively used to prevent the fungal growth in a single site and multisite action due to its active cyanide, ether, keto, carboxylate and its pyrimidin groups. This molecule Azoxystrobin also combines with other fungicides viz; Thiram, Metalaxyl, Difenoconazol, Chlorothalonil, Tebuconazole and other fungicides also acting effectively against the fungal growth in the crops. Has a wide activity by penetration externally and internally in the plant parts and hence this molecule being very active on the fungi kingdom. The active functional groups in the Difenoconazol were chloride, ether; triazole and dioxolan are effectively defusing the spores multiplication in the fungicides. These active functional groups are not only preventing the spores production and also forbidden the growth of the fungus on the cultivation crops by internal and external actions.

The concentrations of these two molecules as a pesticide consider as an important combination against fungicide in the pest management programs. Therefore it is important to understand the active content of these molecules with a single analysis.

2.MATERIALS AND METHOD

2.1 Reagents and chemicals used

All the analytical grade solvents and water were used in this analytical method development. All the class A glass wear used in this research analytical method development.

2.2 Instrument

A calibrated chromatography HPLC instrument was used to develop this analytical method development for Azoxystrobin, Difenoconazol. The instrument parameters were given as:

Name of the instrument	:	High Performance Liquid Chromatography (HPLC)
Calibration Method	:	External Standard Method
Make	:	Shimadzu
Model	:	LC 2030

Detector	:	UV-Visible
Wavelength (λ)	:	230 nm
Column Temperature	:	40ºC
Column	:	Shimadzu BDS C18 (250 x 4.6 mm, 5µ)
Mobile Phase	:	Acetonitrile: Water; ratio of 60:40 (v/v)
Flow rate	:	1.0 ml/min
Injection volume	:	20 µl
Retention time (Approximately)	:	Difenoconazole– 11.3 minutes
	:	Azoxystrobin – 7.1 minutes

2.3 Preparation of Mobile phase

A volume of 60% Acetonitrile and 40% were mixed well, sonicated and used for analysis.

3. ANALYTICAL METHOD VALIDATION

3.1 Specificity

3.1.1 Preparation of standard stock solutions: An amount of 10.43 mg Difenoconazol reference standard with purity 95.9% and 10.11 mg Azoxystrobin reference standard with purity 99.0% were weighed accurately into a clean and dry 10 mL volumetric flask separately, dissolved with mobile phase and made up to the mark with mobile phase. This solution was equivalent to 1000.24 mg/L and 1000.89 mg/L. From this, an aliquot of each 2.5ml solution was mixed 25 mL volumetric flask, diluted with mobile phase. This solution was equivalent to 100 mg/L and analyzed to determine specificity.

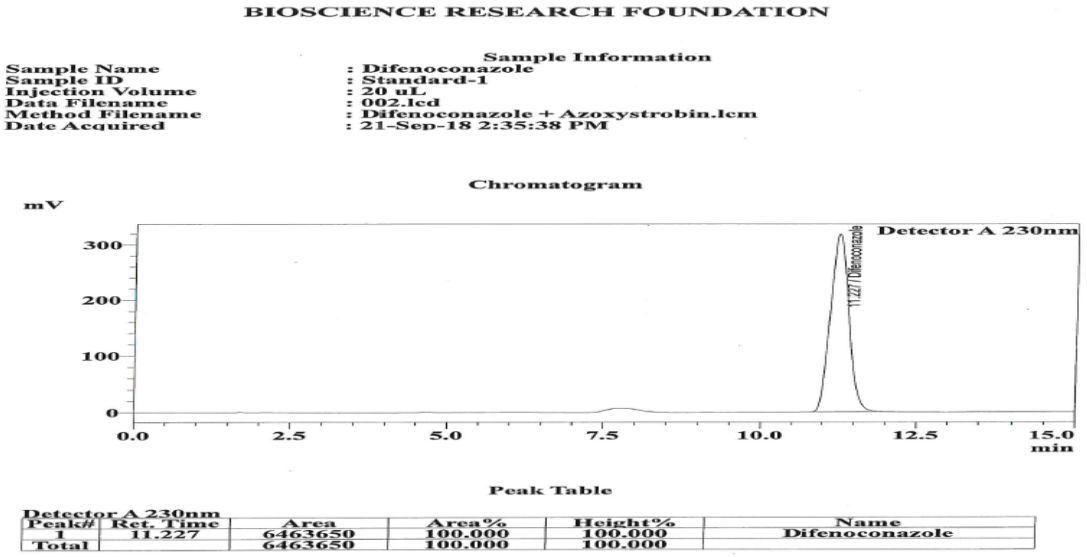

Fig. 1: Typical chromatogram for Difenoconazole

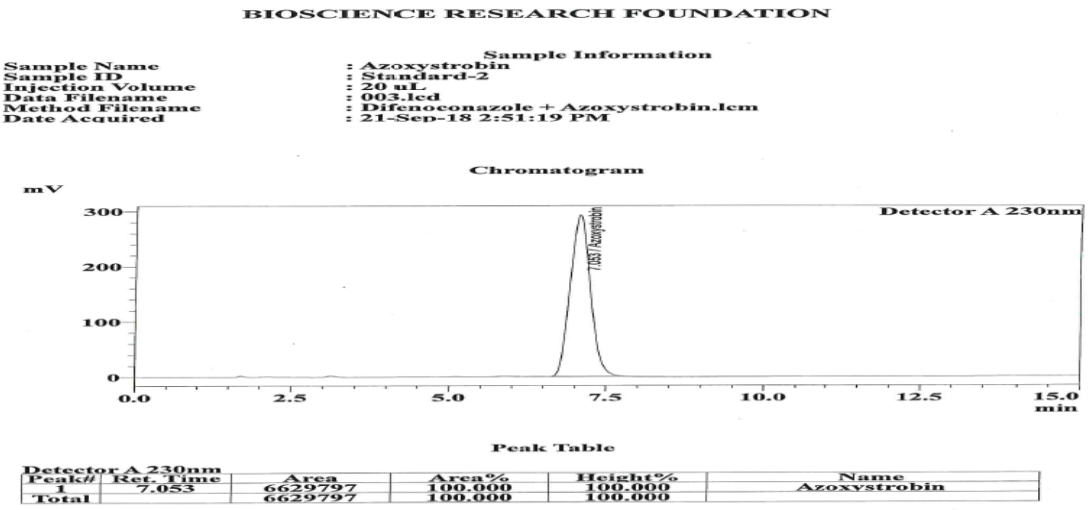

Fig. 2: Typical chromatogram for Azoxystrobin

3.1.2 Preparation of sample solution: An amount of 10.0 mg test substance was weighed accurately into a clean and dry 100 mL volumetric flask and dissolved in mobile phase and made up to the mark with the mobile phase. This solution was equivalent to 100 mg/L and used for determination of Specificity.

The specificity of HPLC method for Difenoconazol and Azoxystrobin was determined by injecting the Standard and Sample solutions along with blank (mobile phase) and observed that there was no interference found with the main peak of interest. Hence, this method was considered to be specific for the analysis of the test substance

3.2 Linearity

3.2.1 Preparation of standard stock solution and working standard: An amount of 10.0 mg of the standard was weighed into a 100 ml standard flask and this concentration (100 mg/L) was used to prepare further dilutions to get the 0.1, 1, 10, 30, 50 and 100 mg/L separately. The dilution details are presented in table 1.

Table 1: Dilutions (Azoxystrobin and difenoconazol reference standard)

Standard Code	Stock Concentration (mg/L)	Dilution Volume (ml)	Final Volume (ml)	Final Concentration (mg/L)
STD-1	100	0.01	10	0.1
STD-2	100	0.1	10	1
STD-3	100	1.0	10	10
STD-4	100	3.0	10	30
STD-5	100	5.0	10	50

The prepared standard solutions were injected by an auto sampler into the 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 2 and 3.

Table 2: Linearity of difenoconazol reference standard

Std. Code	Concentration (mg/L)	Replication	Ref. Std. Area	Mean Std. Area
1	0.1	Replication 1	4421	4428
		Replication 2	4435	
2	1	Replication 1	42246	42295
		Replication 2	42344	
3	10	Replication 1	317356	317795
		Replication 2	318234	
4	30	Replication 1	919241	921245
		Replication 2	923248	
5	50	Replication 1	1514509	1513281
		Replication 2	1512053	
6	100	Replication 1	3261029	3257396
		Replication 2	3253762	

Table 3: Linearity of Azoxystrobin reference standard

Std. Code	Concentration (mg/L)	Replication	Ref. Std. Area	Mean Std. Area
1	0.1	Replication 1	4784	4777
		Replication 2	4770	
2	1	Replication 1	51639	51563
		Replication 2	51487	
3	10	Replication 1	412253	413082
		Replication 2	413910	
4	30	Replication 1	1206197	1206897
		Replication 2	1207597	
5	50	Replication 1	1980103	1980904
		Replication 2	1981704	
6	100	Replication 1	4095523	4094448
		Replication 2	4093373	

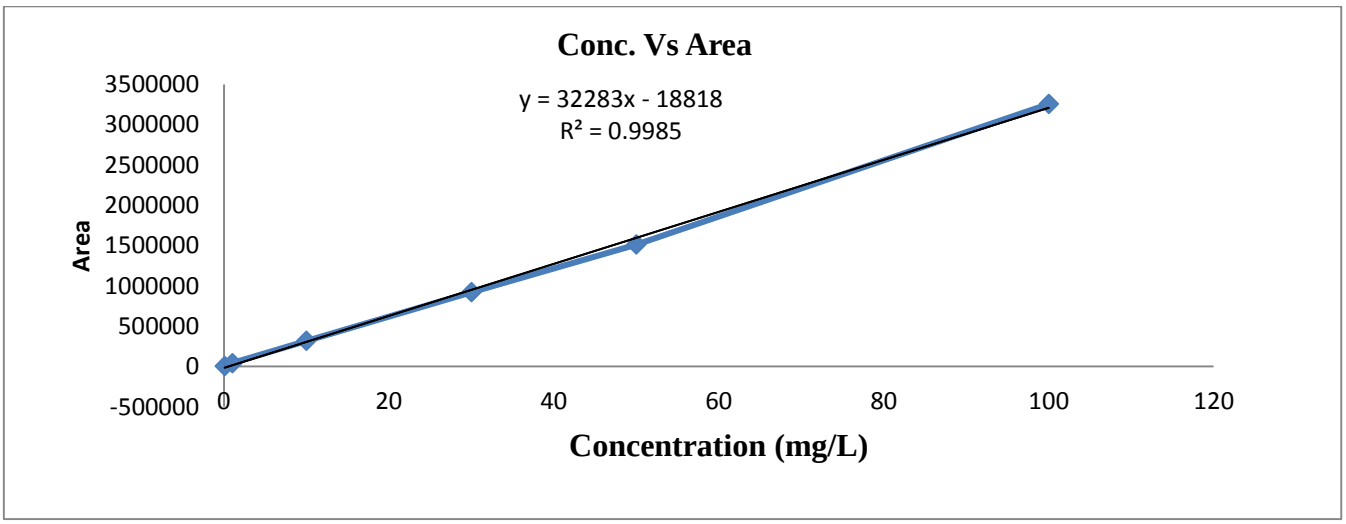

Fig. 1: Linearity curve for difenoconazol

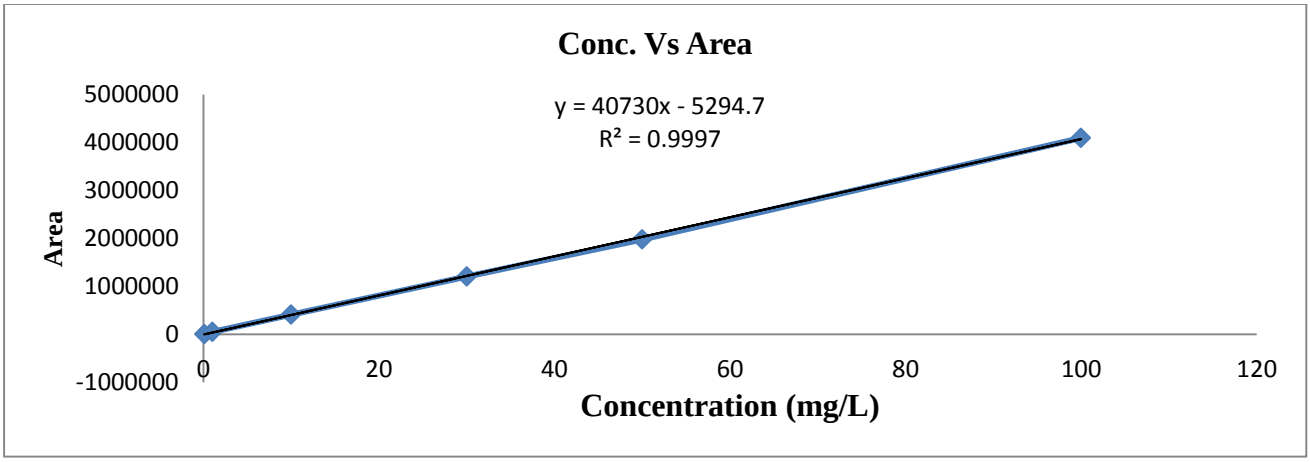

Fig. 2: Linearity curve for azoxystrobin

4.PRECISION

4.1 Preparation of standard solution

The Linearity standard solution 30 mg/L was prepared and used for the precision determination.

4.2 Preparation of sample solution

An amount of 14.25, 14.27, 14.28, 14.29 and 14.28 mg of azoxystrobin + difenoconazol Formulation were weighed in clean and dry 1000 ml volumetric flask separately, dissolved the contents with mobile phase and made upto the mark with the mobile phase. This solutions are equivalent 142.5, 142.7, 142.8, 142.9 and 142.8 mg/L. The prepared solutions were injected into HPLC and % RSD was calculated and the results are presented in TABLE 4.

Table 4: Precision (Difenoconazol)

Sample ID	Std. Conc. (mg/L)	Std./ Sample Area	Average Std. Area	Sample Conc. (mg/L)	Purity (P) %	A.I. Content (%)
Std-R1		319269				-
P1		600792		142.50		12.65
P2		600636		142.70		12.63
P3	10.0	600031	319533.0	142.80	95.9	12.61
P4		600490		142.90		12.61
P5		600447		142.80		12.62
Std-R2		319797				-
					MEAN	12.63
					SD	0.018
					% RSD	0.141

Table 5: Precision (Azoxystrobin)

Sample ID	Std. Conc. (mg/L)	Std./Sample Area	Average Std. Area	Sample Conc. (mg/L)	Purity (P) %	A.I. Content (%)
Std-R1		415089				-
P1		1207475		142.50		20.22
P2		1207865		142.70		20.20
P3	10.0	1208996	414851.0	142.80	99.0	20.20
P4		1210791		142.90		20.22
P5		1209020		142.80		20.20
Std-R2		414613				-
					MEAN	20.21
					SD	0.010
					% RSD	0.050

Formula

$$\text{A. I. Content (\%)} = \frac{\text{Sample Area} \times \text{Std. Conc. (mg/L)}}{\text{Average Std. Area} \times \text{Sample Conc. (mg/L)}} \times \text{Purity (P) \%}$$

Example calculation: P1 (Azoxystrobin)

$$\text{A. I. Content (\%)} = \frac{1207475 \times 10.0}{141851.0 \times 142.50} \times 99.0 = 20.22\%$$

The % RSD is within limit according to the modified Horwitz equation (Acceptable Limit <1.3 RSD for 100% active analyse as per SANCO/3030/99 Rev.4)

5. ACCURACY (% RECOVERY)

The recovery processes and the recovery determination was validated with two fortification level of processes.

5.1 Preparation of standard solution

The standard solution prepared for linearity (5 mg/L) was used as a standard in percent recovery determination.

5.2 Preparation of fortification level 1 (0.15 mg/L)

An amount of 2.82 mg of Azoxystrobin and Difenoconazol reference standard with purity 99.23 % was weighed accurately into a clean and dry 100 mL volumetric flask and dissolved in mobile phase and made up to the mark with the mobile phase. This solution was equivalent to 0.15 mg/L.

5.3 Preparation of fortification level 2 (1.5 mg/L)

An amount of 5.64 mg of Azoxystrobin and Difenoconazol reference standard with purity 99.23 % was weighed accurately into a clean and dry 100 mL volumetric flask and dissolved in mobile phase and made up to the mark with the mobile phase. This solution was equivalent to 1.5 mg/L.

The above preparations were analyzed under HPLC and checked for recovery (%). The results are presented in following table 6 and 7.

Table 6: Accuracy (Level-1 and 2 Recovery %) of Difenoconazol

Fortification Level	Std. Conc. (mg/L)	Std. / Sample area	Mean Std. Area	Recovery Conc. (mg/L)	Fortified Conc. (mg/L)	Recovery (%)	Avg. Recovery (%)
Std-R1	10.0	318737	318851.0	-	29.00	-	-
T1R1		925036		29.0115		100.04	99.96
T1R2		921780		28.9094		99.69	
T1R3		924487		28.9943		99.98	
T1R4		925028		29.0113		100.04	
T1R5		925279		29.0192		100.07	
T2R1		1506822		47.2579	48.0	98.45	98.50
T2R2		1504947		47.1991		98.33	
T2R3		1507640		47.2835		98.51	
T2R4		1510372		47.3692		98.69	
T2R5		1508068		47.2970		98.54	
Std-R2		318965		-		-	

Table 7: Accuracy (Level-1 and 2 Recovery %) of Azoxystrobin

Fortification Level	Std. Conc. (mg/L)	Std. / Sample area	Mean Std. Area	Recovery Conc. (mg/L)	Fortified Conc. (mg/L)	Recovery (%)	Avg. Recovery (%)
Std-R1	10.0	412998	414370.5	-	29.00	-	-
T1R1		1205981		29.1039		100.36	100.32
T1R2		1204307		29.0635		100.22	
T1R3		1205056		29.0816		100.28	
T1R4		1204473		29.0675		100.23	
T1R5		1208039		29.1536		100.53	
T2R1		1974404		47.6483	48.0	99.27	99.13
T2R2		1967610		47.4843		98.93	
T2R3		1972109		47.5929		99.15	
T2R4		1973271		47.6209		99.21	
T2R5		1971456		47.5771		99.12	
Std-R2		415743		-		-	

Example Calculation: **Recovery (Azoxystrobin) - T2R5**

$$\text{Recovery Conc. (mg/L)} = \frac{\text{Std. Conc. (mg/L)} \times \text{Sample Area}}{\text{Mean Std. Area}} = \frac{10 \times 1971456}{414370.5} = 47.5771 \text{ mg/L}$$

$$\text{Recovery (\%)} = \frac{\text{Recovery Conc. (mg/L)}}{\text{Fortified Conc. (mg/L)}} \times 100 = \frac{47.5771}{48.0} \times 100 = 99.12\%$$

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

From the Linearity Standard Solution concentration of 10 mg/L was used in these LOD & LOQ determinations. From this solution, 1 mg/L solution was prepared and further diluted to get the 0.01 & 0.1 mg/L concentration solutions were prepared. The dilution details were given in table 7, and the results are presented in following table 7 and 8.

Table 7: Dilutions (LOD & LOQ)

Stock Concentration (mg/L)	Dilution Volume (ml)	Final Volume (ml)	Final Concentration (mg/L)
1.0	1	10	0.1
0.1	1	10	0.01

Formula

$$LOD = \text{Average} + (3 \times \text{Standard Deviation})$$
$$LOQ = \text{Average} + (10 \times \text{Standard Deviation})$$

Table 8: Limit of Detection (LOD) and Limit of Quantification (LOQ) of Difenoconazol

Sample ID	Std. Conc. (mg/L)	Std./ Sample Area	Average Std. Area	Detected Conc.(mg/L)
STD-1	10.0	318746	319319.5	-
R1		403		0.0126
R2		432		0.0135
R3		384		0.0120
STD-2		319893		-
			MEAN	0.0127
			SD	0.0008
			LOD	0.0150

Sample ID	Std. Conc. (mg/L)	Std./ Sample Area	Average Std. Area	Detected Conc.(mg/L)
STD-1	10.0	318746	319319.5	-
R1		4391		0.138
R2		4287		0.134
R3		4457		0.140
STD-2		319893		-
			MEAN	0.137
			SD	0.0027
			LOQ	0.164

Table 9: Limit of Detection (LOD) and Limit of Quantification (LOQ) of Azoxystrobin

Sample ID	Std. Conc. (mg/L)	Std./ Sample Area	Average Std. Area	Detected Conc.(mg/L)
STD-1	10.0	413827	414646.0	-
R1		412		0.010
R2		483		0.012
R3		412		0.010
STD-2		415465		-
			MEAN	0.0105
			SD	0.0010
			LOD	0.0135

Sample ID	Std. Conc. (mg/L)	Std./ Sample Area	Average Std. Area	Detected Conc.(mg/L)
STD-1	10.0	413827	414646.0	-
R1		4753		0.115
R2		4805		0.116
R3		4579		0.110
STD-2		415465		-
			MEAN	0.114
			SD	0.0029
			LOQ	0.142

Example calculation: (LOD and LOQ)

Limit of Detection (Azoxystrobin) - R1

$$\text{Detected Conc. (mg/L)} = \frac{\text{Std. Conc. (mg/L)} \times \text{Sample Area}}{\text{Average Std. Area}} = \frac{10 \times 412}{414646.0} = 0.010 \text{ mg/L}$$

$$LOD = Mean\,Value + (3 \times SD)$$
$$LOD = 0.0105 + (3 \times 0.0010) = 0.0135$$

Limit Of Quantification (Difenoconazol) - R1

$$\text{Detected Conc. (mg/L)} = \frac{\text{Std. Conc. (mg/L)} \times \text{Sample Area}}{\text{Average Std. Area}} = \frac{10 \times 4391}{319319.5} = 0.138 \text{ mg/L}$$

$$LOQ = Mean\,Value + (10 \times SD)$$
$$LOQ = 0.137 + (10 \times 0.0027) = 1.064$$
$$LOQ = Mean\,Value + (3 \times SD)$$
$$LOQ = Mean\,Value + (10 \times SD)$$
$$LOQ = 0.117 + (10 \times 0.00311) = 0.15$$

7. ACTIVE CONTENT ANALYSIS OF AZOXYSTROBIN AND DIFENOCONAZOL

7.1 Preparation of Standard solution

An amount of 5 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

The received test solutions (10 mg/L) was prepared and dissolved by sonication and diluted appropriately and injected into HPLC.

$$\text{Azoxystrobin/Difenoconazol (mg/L)} = \frac{Concentration\ of\ standard\ (mg/L) \times\ Area\ of\ sample\ solution\ x\ Dilution\ Factor}{\text{Area of standard solution}}$$

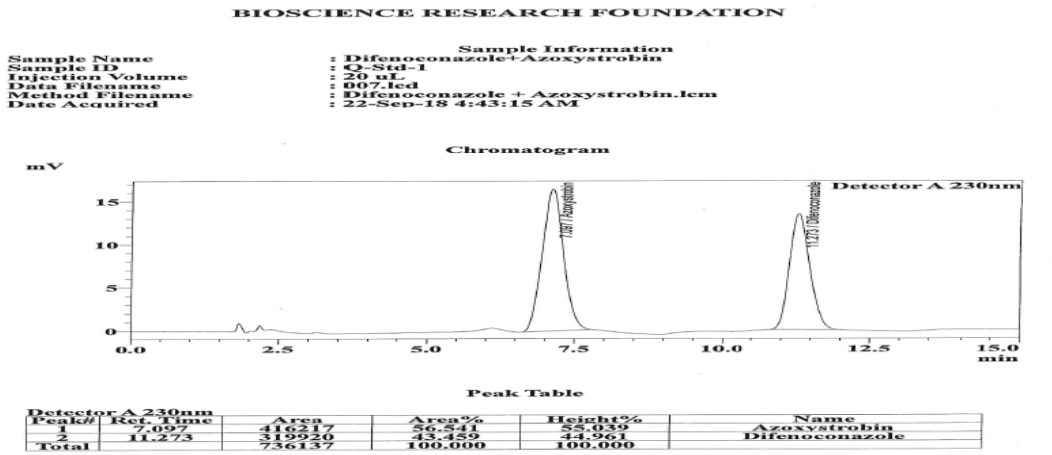

Fig. 3: A typical chromatogram for Sample analysis

8. CONCLUSION

8.1 Specificity

The blank, standard and the sample peaks did not interfere each other, hence the specificity were achieved as per the guideline SANCO 3030/99 Rev.4 requirement.

8.2 Linearity

The Linearity correlation co-efficient is achieved NLT 0.99 as per (SANCO 3030/99 Rev.4

8.3 System precision

The system precision is achieved as the % RDS for 5 replicates observed as 0.1% for Azoxystrobin and Difenoconazol, 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, 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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