Development and validation of stability-indicating RP-HPLC method for the estimation of azoxystrobin in its formulations

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The current work established and validated a simple, selective, precise, and accurate High Performance Liquid Chromatographic technique (HPLC) for the analysis of Azoxystrobin in its formulations. The mobile phase is made up of a combination of mobile phases comprising Acetonitrile and water in proportion, 80:20 (v/v). At a run duration of 15 minutes, this was found to yield a sharp peak of Azoxystrobin. Azoxystrobin was analysed using HPLC at a wavelength of 255 nm at a flow rate of 1.0 mL/min. The calibration curve’s linear regression analysis results revealed a satisfactory linear connection with a regression coefficient of 0.999 in the concentration range of 50% to 150%. The linear regression equation was y = 2025x +123.2. The proposed approach was used to analyze Azoxystrobin with a high degree of precision and accuracy. The method was validated for precision, accuracy, specificity, ruggedness and robustness. This method is useful for the quantification of Azoxystrobin because of its precision, accuracy, short retention duration, sensitivity, and mobile phase composition.

1. Introduction

Azoxystrobin is a broad-spectrum fungicide with pyrimidine rings that is used in agriculture to protect crops against fungal infections. It was initially released in 1998 as a new fungicide with a unique biochemical method of action. It is used on grape vines, cereals, potatoes, apples, bananas, citrus, tomatoes, and other crops to prevent spore germination. Rusts, Downey and powdery mildew, rice blast, and apple scab are among the diseases it combats. The Azoxystrobin pesticide is less toxic to humans, other mammals, birds, insects, and earthworms, but it has the ability to penetrate soil and control fungal growth very effectively. The azoles class included the Epoxiconazole chemical. This chemical regulates the metabolism of fungal cells, which in turn regulates fungal growth. The combo product was used to reduce fungus development on crops all over the world. Because the molecules are chemically distinct, their functions are likewise distinct. The action of regulating the fungus in a different way resulted in the control of a wide spectrum of fungus. In the field of plant culture, this combination product has proven to be effective. For a better understanding, the full pesticide molecule must be examined for purity, stability, and other raw material, in-process, and solvent impurities. During the analysis, any analytical methods must be simple, repeatable, and cost-effective. HPLC is a simple and widely used analytical device that is used for qualitative and quantitative analysis efficiently in terms of cost, time, and simplicity. Furthermore, this process is repeatable and may be applied to quality control as well as research and development in the field of agriculture.
WU Ying-xuan et al.¹ used High Performance Liquid Chromatography and Electrospray Ionization Tandem Mass Spectrometry to concurrently identify Azoxystrobin residues in legumes. At four spiking concentration levels of 0.05, 0.1, 0.2, and 0.5 mg/kg, the devised technique was verified. The linear ranges were 2.5 to 50 g/L, with average recoveries ranging from 89 to 99 percent and relative standard deviations ranging from 2.2 to 8.5 percent. Ehab M.H. Abdelraheem et al.² used HPLC-UV to validate a technique for extracting and quantifying Azoxystrobin residues in green beans and peas, and the results were verified by GC–MS. For green beans and peas, mean recoveries varied from 83.69 % to 91.58 % and 81.99 % to 107.85 %, respectively, in HPLC-UV analysis. In GC–MS analysis, mean recoveries varied from 76.29 % to 94.56 % and 80.77 % to 100.91 %, respectively. The approach has been shown to be effective for extracting and determining Azoxystrobin residues in green beans and peas based on these findings.

P.Marczewska et al.³ used high performance liquid chromatography with diode array detector (HPLC-DAD) in suspension concentrate pesticide formulations to create a technique for the simultaneous qualitative and quantitative measurement of Azoxystrobin and its related impurity (Z)-azoxystrobin. Individual recovery rates for azoxystrobin and (Z)-azoxystrobin were 97–103 % and 90–110 %, respectively. The impurity ((Z)-azoxystrobin) had a limit of quantification (LOQ) of 0.3 µg mL⁻¹, which was acceptable because it was less than the maximum permissible level under the regulations.

Monica et al.⁴ described a unique and sensitive technique for extracting, preconcentrating, and determining azoxystrobin and chlorothalonil, two extensively used fungicides. Solid-phase extraction (SPE) using a polymeric substance functionalized with gold nanoparticles (AuNPs) as sorbent is followed by high-performance liquid chromatography (HPLC) with a diode array detector in the proposed process (DAD). When applied to drinking and ambient water samples, the suggested approach enabled for the identification of fungicides as low as 0.05 µg L⁻¹ and provided good recoveries (75–95%).

For the detection of isopyrazam (IZM) and azoxystrobin (AZT) in cucumbers, Dan Hu et al.⁵ suggested a quick and sensitive analytical approach based on high-performance liquid chromatography–tandem mass spectrometry. At fortification doses of 1, 20, and 500 µg kg⁻¹ (n = 3), the suggested technique resulted in excellent recovery of IZM and AZT (91.48 to 114.62 %) and relative standard deviations of less than 13.1 %. IZM and AZT quantification limits were 0.498 and 0.499 µg kg⁻¹, respectively, substantially below the maximum residue level (0.5 mg kg⁻¹) specified for this kind of material.

SHI Feng et al.⁶ established a technique for determining azoxystrobin residue in Citrus Shatangju. The following were the HPLC conditions: The mobile phase is V(acetonitrile)/V( H2O) = 70/30, with a flow rate of 1.0 mL/min, an injection volume of 10 mL, and a detection wavelength of 257 nm. The average recovery of azoxystrobin was 85.23 %–92.04 %, and the lowest detection concentration was 0.01 g/g, respectively, which might be in line with pesticide residue standards. G. P. Balayiannis and colleagues⁷ devised and validated a technique for determining the active ingredients (a.s.) azoxystrobin, topramezone, acetamiprid, fluometuron, and folpet in commercially available formulations. All individual chemicals were recovered in the range of 97.8%–100.9 %.

![Chemical structure of Azoxystrobin](image)

**Fig. 1:** Chemical structure of Azoxystrobin

1. **Chemical name:** Methyl (2E)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy] phenyl}-3-methoxycrolylate.
2. **Empirical formula:** C₁₂H₁₇N₃O₅.
3. **Molecular weight:** 403.388 g/mol.

### 1.1. Instruments / equipments used

Here, we used High performance liquid chromatography, with UV / PDA detector, HPLC Analytical column of ODS2 - 250mm x 4.6mm x 5µ, Analytical weighing balance — Mettler Toledo B204S, Millipore Nylon 0.2µm and Laboratory accessories.

### 2. Chemicals Used

Here, we used Azoxystrobin working Standard, Amistar Fungicide, Methanol- AR, Sodium Hydroxide — AR, Hydrochloric Acid — AR, Acetonitrile and Millipore Water.

### 3. Preparation of Azoxystrobin Standard Solution

Weigh accurately about 50 mg of Azoxystrobin working Standard and transfer to a 50 mL volumetric flask. Add 20 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)
Table 1: System suitability — Selectivity

| Chromatographic conditions |       |
|-----------------------------|-------|
| **Column:**                 | ODS2 - 250mm x 4.6mm x 5μ |
| **Mobile Phase:**           | Prepare an 80:20 combination of acetonitrile and water for the isocratic system. Mix thoroughly. Before using, filter through 0.2μ Nylon membrane filter paper and degas. |
| **Wavelength:**             | 255 nm |
| **Flow Rate:**              | 1.0 ml / minute |
| **Injection volume:**       | 20 μl |
| **Run time:**               | 15 minutes |
| **Blank solution:**         | Use Mobilephase as blank |
| **Diluent:**                | Use Mobile phase as diluent |

3.1. Preparation of test solution

Weigh accurately about 200 mg of sample and transfer to a 50 ml volumetric flask. Add 20 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: 200mg 50.0 ml 1 ml /10.0 ml)

3.2. System suitability solution

Use Azoxystrobin Standard working solution as system suitability solution.

3.2.1. Procedure

Separately inject five replicate injections of the system suitability solution, each with equal volumes of blank (Azoxystrobin Standard working solution). After that, administer two injections of the test solution and record the chromatograms. Any peaks in the test solution created by a blank should be overlooked. Calculate the % RSD of five replicate system suitability injections (Azoxystrobin Standard working solution). In the chromatogram produced with the 5th injection of system suitability solution, check tailing factor and theoretical plates of the peak (Azoxystrobin 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%.

3.3. Validation parameters

3.3.1. Selectivity

The diluent blank solution, excipient mix, system suitability solution, and test solution were all injected to achieve selectivity. Criteria for acceptance: The Azoxystrobin peak should be easily distinguishable from other peaks and from each other. At the Azoxystrobin retention period, the diluent blank solution and excipient blend solution should not display any peak. According to the analytical procedure, the system suitability requirements fulfilled the pre-established acceptance criteria.

Table 2: System suitability - Selectivity

| Sr. No. | Area of Azoxystrobin |
|---------|-----------------------|
| 1       | 2091.60               |
| 2       | 2094.06               |
| 3       | 2090.11               |
| 4       | 2093.20               |
| 5       | 2090.29               |
| Mean    | 2091.85               |
| Standard Deviation (±) | 1.75          |
| (%) Relative Standard Deviation | 0.08          |

The wavelength specified in the technique was used to process all of the injections. This approach is selective since there was no interference from the diluent blank solution or the excipient blend solution with the Azoxystrobin peak.

3.4. Forced degradation

The forced degradation experiments are carried out to determine the stability indicating nature of assay method and to look for any deteriorated compounds. Azoxystrobin WS and the sample (AMISTAR FUNGICIDE) are exposed to 5N HCl, 5N NaOH, thermal degradation, and UV degradation. All of the aforesaid solutions were chromatographed and the chromatograms were recorded. For degradation, the following stress conditions are used.

Table 3: System suitability – forced degradation

| Sr. No. | Area of azoxystrobin |
|---------|-----------------------|
| 1       | 2012.30               |
| 2       | 2001.38               |
| 3       | 2008.47               |
| 4       | 2003.08               |
| 5       | 2023.58               |
| Mean    | 2009.76               |
| Standard Deviation (±) | 8.86          |
| (%) Relative Standard Deviation | 0.44          |

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     | 105°C for 12 hours               |
| UV degradation          | expose to UV-radiation for 7 days |
3.5. Acceptance criteria

The degradation peaks should be well separated from each other. Azoxystrobin peak purity should be acceptable.

3.6. Linearity

3.6.1. Linearity and range for azoxystrobin sample

Five standard solutions of Azoxystrobin were prepared for the linearity study, ranging from 50% to 150% of the theoretical concentration of the assay preparation. The linearity and system suitability solutions were injected according to the procedure. The correlation coefficient was calculated after plotting the linearity graph of concentration against peak response.

3.7. Acceptance criteria

Correlation coefficient should be greater than or equal to 0.999. According to the analytical procedure, the system suitability requirements fulfilled the pre-established acceptance criteria. (Refer to Table 5 for system suitability results).

Table 6: System suitability - Linearity of sample

| Sr. No. | Area of Azoxystrobin |
|---------|-----------------------|
| 1       | 5 2120.54             |
| 2       | 2113.57               |
| 3       | 2111.26               |
| 4       | 2125.79               |
| 5       | 2121.20               |
| Mean    | 2118.47               |
| Standard Deviation (±) | 5.94  |
| (%) Relative Standard Deviation | 0.28 |
Table 7: Results of linearity of sample

| Linearity Level | Sample Concentration (in %) | Sample Concentration (in ppm) | Peak Area | Correlation Coefficient |
|-----------------|-----------------------------|-------------------------------|-----------|-------------------------|
| Level – 1       | 50                          | 50                            | 1149.52   | 0.999                   |
| Level – 2       | 75                          | 75                            | 1636.57   |                         |
| Level – 3       | 100                         | 100                           | 2130.60   |                         |
| Level – 4       | 125                         | 125                           | 2651.12   |                         |
| Level – 5       | 150                         | 150                           | 3173.48   |                         |

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

3.8. Precision

3.8.1. System precision

3.8.1.1. Procedure. The system precision was determined by injecting ten replicate injections of the system suitability solution and examining the chromatograms for system suitability criteria.

3.9. Acceptance criteria

The % RSD of peak regions of ten replicate injections of the system suitability solution shall not exceed 2.0%, and the system suitability criterion should pass as per analytical procedure. According to the analytical procedure, the system suitability requirements fulfilled the pre-established acceptance criteria.

3.10. Method precision

3.10.1. Procedure

Six Azoxystrobin test solutions in AMISTAR FUNGICIDE were prepared according to the analytical procedure. Six test solutions were used to obtain the % RSD of % assay.

3.11. Acceptance criteria

The % RSD of the outcomes of six test solutions must not exceed 2.0%. According to the analytical procedure, the system suitability criterion fulfilled the pre-established acceptance requirements. Table 8 shows the results of the assay obtained from six test solution preparations.

The % RSD of the six test findings is less than 2.0 % and meets the pre-determined acceptability standards. As a result, it is concluded that the method is precise.

3.12. Intermediate precision

3.12.1. Procedure

Six test solutions of AMISTAR FUNGICIDE were prepared according to the analytical procedure on different day. These test solutions were analysed by a different analyst using different HPLC column of same make but with a different serial number and different HPLC system. Calculated the % RSD of % assay findings for twelve test solutions (six samples from technique precision and six samples from intermediate precision).

3.13. Acceptance criteria

% RSD of the results of twelve test solutions (six of method precision and six of intermediate precision) must not exceed 2.0%. The system suitability requirements fulfilled 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 8. % RSD of assay results from method precision and intermediate precision (11 results) are presented in Table 12.

The analysis was carried out on six test solutions of the same lot of the drug product by two different analysts using 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 findings (six procedure precision and
**Table 8: System precision**

| Sr. No. | Area of Azoxystrobin |
|---------|----------------------|
| 1       | 2159.07              |
| 2       | 2138.62              |
| 3       | 2128.63              |
| 4       | 2102.96              |
| 5       | 2133.52              |
| 6       | 2139.33              |
| 7       | 2125.47              |
| 8       | 2148.58              |
| 9       | 2114.36              |
| 10      | 2116.94              |

**Mean**: 2130.75  
**Standard Deviation (±)**: 16.76  
**(%) Relative Standard Deviation**: 0.79

**Table 9: System suitability - Method precision Analyst – 1 HPLC No.: EH/R&D/HPLC-024**

| Sr. No. | Area of Azoxystrobin |
|---------|----------------------|
| 1       | 2108.60              |
| 2       | 2108.05              |
| 3       | 2103.50              |
| 4       | 2105.86              |
| 5       | 2104.25              |

**Mean**: 2106.05  
**Standard Deviation (±)**: 2.25  
**(%) Relative Standard Deviation**: 0.11

**Table 10: Results of method precision**

| Test Solution | % Assay of Azoxystrobin |
|---------------|--------------------------|
| 1             | 100.44                   |
| 2             | 100.12                   |
| 3             | 100.47                   |
| 4             | 100.94                   |
| 5             | 99.58                    |
| 6             | 101.08                   |

**Mean**: 100.44  
**Standard Deviation (%)**: 0.55  
**(%) Relative Standard Deviation**: 0.55

**Table 11: System suitability — Intermediate precision Analyst — 2 HPLC No: EH/R&D/HPLC-023**

| Sr. No. | Area of Azoxystrobin |
|---------|----------------------|
| 1       | 2277.98              |
| 2       | 2233.53              |
| 3       | 2245.69              |
| 4       | 2272.05              |
| 5       | 2248.51              |

**Mean**: 2255.55  
**Standard Deviation (±)**: 18.76  
**(%) Relative Standard Deviation**: 0.83
Table 12: Results of intermediate precision

| Test Solution | % Assay of Azoxystrobin |
|---------------|-------------------------|
| 1             | 98.42                   |
| 2             | 99.47                   |
| 3             | 99.34                   |
| 4             | 98.34                   |
| 5             | 100.64                  |
| 6             | 98.26                   |
| Mean          | 99.08                   |
| Standard Deviation (%) | 0.93               |
| (%) Relative Standard Deviation | 0.94            |

Table 13: Results of twelve test solutions of Azoxystrobin in AMISTAR 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 Azoxystrobin |
|-------------|-------------------------|
| 1           | 100.44                  |
| 2           | 100.12                  |
| 3           | 100.47                  |
| 4           | 100.94                  |
| 5           | 99.58                   |
| 6           | 101.08                  |

Analysis performed during intermediate precision study By Analyst 2 on system 2 and on column 2 on day 2

| Column sr. no. | % Assay of Azoxystrobin |
|----------------|-------------------------|
| 01533703013602 | 99.76                   |
| Mean of twelve samples | 99.76               |
| Standard Deviation (%) | 1.02               |
| (%) Relative Standard Deviation | 1.02            |

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

4. Change in Flow Rate (+ 0.2 mL/minute : (Normal Experimental Condition: 1.0 mL/minute)

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).

The assay results obtained with different wavelength conditions are as given in Table 18.

3.14. Robustness

Prepare two test solutions of Azoxystrobin in AMISTAR FUNGICIDE according to the analytical procedure using the same lot (as used in 7.0.a and 7.0.b). Inject this solution along with diluent blank solution and system suitability solution under various chromatographic conditions as shown below:

1. Change in Column Lot
2. Change in flow rate (+0.2 ml/minute)
3. Change in wavelength (± 2 nm)
4. Change in composition of mobile phase (± 20 ml)

1. Change in Column Lot
   (Normal Experimental Condition: ODS2 - 250 mm x 4.6 mm x 5 μ)
   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).

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

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).

The assay results obtained with different wavelength conditions are as given in Table 18.
Table 14: System suitability - Robustness with change in Column

| Sr. No. | Same column | Different column |
|---------|-------------|------------------|
|         | Area of     |                  |
|         | Sr. No.     | 2125.26          | 2104.55          |
|         | 2           | 2106.05          | 2105.89          |
| Mean    | 2115.65     | 2105.22          |
| Standard Deviation (±) | 13.58 | 0.94 |
| (%) Relative Standard Deviation | 0.64 | 0.04 |

Table 15: Results for change in column

| Flow rate | Same column | Different column |
|-----------|-------------|------------------|
| Sample    | % Assay     |                  |
| Test solution | 101.01 | 101.29          |
| Average assay result from method precision | 100.44 | 100.44 |
| Mean | 100.73 | 100.87 |
| Standard Deviation (%) | 0.40 | 0.60 |
| (%) Relative Standard Deviation | 0.40 | 0.60 |

Table 16: System suitability - Robustness with change in flow rate

| Sr. No. | Area of Azoxystrobin | 0.8mL/minute | 1.2 mL/minute |
|---------|-----------------------|--------------|---------------|
| 1       | 2120.64               | 2125.79      |
| 2       | 2127.91               | 2117.51      |
| Mean    | 2124.28               | 2121.65      |
| Standard Deviation (±) | 5.14 | 5.86 |
| (%) Relative Standard Deviation | 0.24 | 0.28 |

Table 17: Results for change in flow rate

| Flow rate | 0.8mL/minute | 1.2 mL/minute |
|-----------|--------------|---------------|
| Sample    | % Assay      |               |
| Test solution | 99.89 | 99.75          |
| Average assay result from Method precision | 100.44 | 100.44 |
| Mean | 100.17 | 100.10 |
| Standard Deviation (+) | 0.39 | 0.49 |
| (%) Relative Standard Deviation | 0.39 | 0.49 |

Table 18: System suitability - Robustness with change in wavelength

| Sr. No. | Area of Azoxystrobin | 253 nm | 257 nm |
|---------|-----------------------|--------|--------|
| 253     | 2063.30               | 2055.62|
| 1       | 2074.39               | 2051.58|
| Mean    | 2068.84               | 2053.60|
| Standard Deviation (+) | 7.84 | 2.85 |
| (%) Relative Standard Deviation | 0.38 | 0.14 |

Table 19: Results for change in wavelength

| Wavelength | 253 nm | 257 nm |
|------------|--------|--------|
| Sample     | % Assay |        |
| Test solution | 99.96 | 99.84 |
| Average assay result from Method precision | 100.44 | 100.44 |
| Mean | 100.20 | 100.14 |
| Standard Deviation (+) | 0.34 | 0.42 |
| (%) Relative Standard Deviation | 0.34 | 0.42 |
Change in composition of Mobile Phase (± 20ml): (Normal Experimental Condition: Acetonitrile: water = 800ml: 200ml) The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical Method (Refer to Table 19 for system suitability results).

The assay results obtained with change in composition of mobile phase are as given in Table 20.

The same lot of AMISTAR FUNGICIDE was analyzed under various circumstances, including column lot, flow rate, wavelength, and change in mobile phase composition. The system suitability was determined to match the pre-established parameters under all settings, with a % RSD of less than 2.0 % between results obtained under different conditions and the average result of Method precision. As per protocol, the analytical Method satisfies the pre-established approval criteria for the robustness study. As a result, the Method is robust.

3.15. Stability of the sample solution

3.15.1. Procedure

System suitability solution and test solution of AMISTAR FUNGICIDE were prepared on 0\(^{th}\), 12\(^{th}\), 24\(^{th}\), 36\(^{th}\) and 48\(^{th}\) hour of experiment and maintained at room temperature for every time interval up to 48 hours, and these solutions were evaluated on the 48\(^{th}\) hour with newly prepared test solution. The system suitability solution was prepared freshly at the time of analysis. The assay of AMISTAR FUNGICIDE in the sample was calculated.

3.16. 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 21.

The system suitability was found to meet the pre-established criteria, with % RSD of less than 2.0% between assay results obtained for newly prepared test solution and stored test solutions. For test solution at room temperature, no significant change in assay level has been detected up to 48 hours. Thus, it can concluded that the solution remains stable at room temperature for up to 48 hours.
4. Summary and Conclusion

The validation data presented in this study reveals that the analytical method of assaying Azoxystrobin in AMISTAR FUNGICIDE by HPLC is determined to be suitable, selective, specific, precise, linear, accurate, and robust. At room temperature, the analytical solution is determined to be stable for up to 48 hours. Hence, it is concluded that the analytical method has been validated and may be used for regular analysis and stability testing.

5. Source of Funding

None.

6. Conflict of Interest

None.

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