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# Method validation for determination of commonly used fungicides in rice and husk by gas liquid chromatography: Tandem mass spectrometry

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**Abstract**
The objective of this study is to establish and validate a modified quick, easy, cheap, effective, rugged and safe (QuEChERS) to analyze various fungicide (azoxystrobin, difenconazole, pencycuron, propiconazole, tebuconazole and trifloxystrobin) residues in rice. The six fungicides were initially extracted from rice samples using acetonitrile and water (2:1) followed by cleanup with the Primary Secondary Amine (PSA). Samples were concentrated and analyzed on gas chromatography–tandem mass spectrometry (GC–MS/MS) using MRM mode to achieve lower detection level. Targeted compounds were determined within 30.0 minutes showing the limits quantification (LOQ) 0.005 $mgkg^{-1}$ for all the fungicides except pencycuron (0.01 $mgkg^{-1}$) At the fortification level of 0.01–0.1 mg $kg^{-1}$, the average recoveries ranged from 75.25 to 93.58% with RSD <20% and correlation coefficient values ($R^2$) were more than 0.98.

**Keywords:** fungicides, GC-MSMS, rice, straw, validation

## Introduction
The most economically important staple food crop of eastern and southern parts of India is rice, which is a *Kharif* crop, requiring rainfall more than 100 cm and temperature of around 25 °C. It is estimated that approximately 114 million tonnes of extra milled rice need to be produced by 2035 to meet the global demand. For this purpose, more area is required for rice cultivation but the possibility of expanding the area in the near future is limited. Thus, the need of extra rice production has come from a productivity gain [1]. Attaining maximum productivity is possible when high yielding varieties are used for cultivation and a provision is made for the protection of crop against its enemies [2]. Amongst the various biotic factors, rice diseases are one of the most important factors affecting the production and productivity. More than 70% grain loss in India is due to various fungal diseases [3].

Fungicides from different groups have been widely used to control fungal diseases in rice and other cereals. Among them, members of the strobilurin, triazole, oximinoacetate strobilurin and phenylurea family provide good control of various fungal diseases. They all are broad-spectrum fungicide with systemic modes of action (absorbed through the leaves, stems, or roots) and only pencycuron is a non-systemic fungicide. However, due the use of these fungicides crop, products as well as the environment contaminate acutely [4] still these biocidal chemical compounds are used largely for treating various diseases like gray rot, downy and powdery mildews [5]. The presence of such fungicide residues in food products is of great concern for public health, thus, identifying the presence of such residues in all types of food (both fresh and industrialized) is an important issue to guarantee food safety [4, 6]. The earlier procedures for the analysis of traditional pesticides were tedious, labour-consuming and health hazardous, however, a very flexible new approach, *i.e.*, QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) for the determination of multi-residues was developed in 2003 by Anastassiades and his co-workers based on an extraction with acetonitrile followed by a partitioning step and then cleaning up by dispersive solid-phase extraction (d-SPE). Although it was initially designed for the analysis of pesticide residues in high moisture food matrices with low fat content [7] but later several modifications were made to work well for dry and fatty matrices [7, 8]. Few reports on the analysis of multi-pesticide residues in rice have appeared to date. Liu and his coworkers [9] in 2006 analyzed 40 pesticides in rice by using gas chromatography–mass spectrometry (GC–MS) after extracting with dichloromethane and

cleaned up with florisil solid-phase extraction column. Zhang *et al*. (2006) [10] reported 109 pesticides analysis method, in which, the sample was treated with gel permeation chromatography (GPC) and then analyzed by GC–MS.

In this study, an efficient, accurate and cost effective analytical method was developed for the extraction of commonly used fungicides (azoxystrobin, difenoconazole, pencycuron, propiconazole, tebuconazole and trifloxystrobin) alone or in mixed formulation residue in rice. The quantification was done by using highly sophisticated and latest chromatographic technique i.e., gas liquid chromatography tandem mass spectrometry. The method was designed to achieve good analytical results at lowest LOD for the targeted fungicides in the method validation.

## Material and Methods

### Fungicides Selected

Six fungicides were selected for the study *i.e*. azoxystrobin, difenoconazole, pencycuron, propiconazole, tebuconazole and trifloxystrobin, all are recommended for use in rice by the central insecticides board and registration committee (CIBRC). Rice purchased from local market was used in the fortification experiment and as the matrix blanks for the matrix matched calibration standards.

### Preparation of standard solution

A stock solution of each standard (100 ppm) was prepared in *n*-hexane and this stock solution was further used for the dilution with *n*-hexane serially (1.00, 0.50, 0.25, 0.125, and 0.05 ppm) for drawing a calibration curve (Fig.1a, b). These standard solutions were stored at 4 °C before use. Individual chromatographic behavior was used to determine and prepare the composition of the mixed standard solution for the multiple-pesticide residue analysis. Detail of individual fungicide like molecular formula, molecular weight, Retention time (Rt) and mass by charge (m/z) are listed in Table 1.

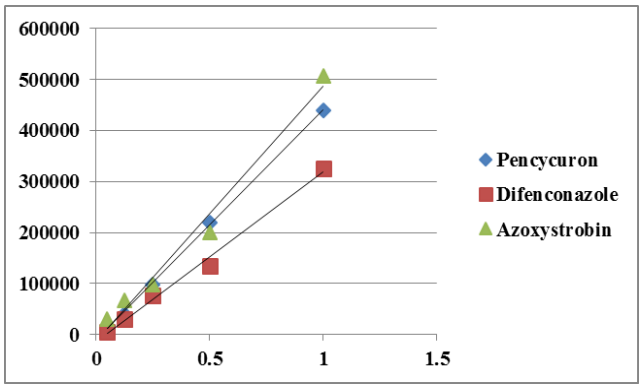



**Fig 1a:** Calibration plot of Pencycuron, Difenconazole and Azoxystrobin

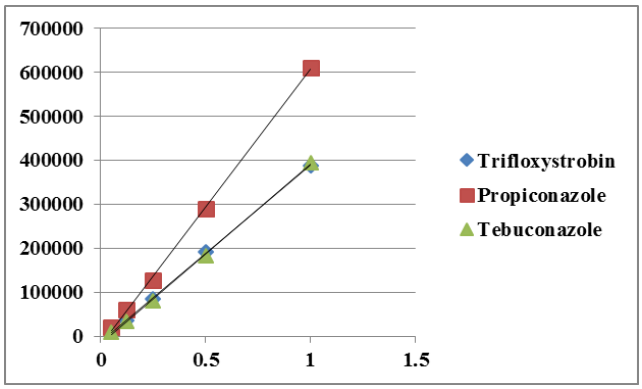



**Fig 1b:** Calibration plot of Trifloxystrobin, Propiconazole and Tebuconazole

**Table 1:** Fungicides, Molecular Formula, MW (g $mol^{-1}$), $R_t$ (min.), m/z

| Fungicides | Molecular formula | MW (g $mol^{-1}$) | $R_t$ (min.) | m/z |
|---|---|---|---|---|
| Azoxystrobin | $C_{22}H_{17}N_3O_5$ | 403.38 | 28.66 | 329,183,156 |
| Difenoconazole | $C_{19}H_{17}Cl_2N_3O_3$ | 406.26 | 27.75 | 265,202,209 |
| Propiconazole | $C_{15}H_{17}Cl_2N_3O_2$ | 342.22 | 18.52 | 191,173,69 |
| Tebuconazole | $C_{16}H_{22}ClN_3O$ | 307.82 | 19.00 | 125,153,70 |
| Trifloxystrobin | $C_{20}H_{19}F_3N_2O_4$ | 408.37 | 18.31 | 190,130,162 |
| Pencycuron | $C_{19}H_{21}ClN_2O$ | 328.80 | 9.53 | 180,125,127 |

### Reagents and Standards

Technical grade analytical standards [Certified Reference Material (CRM)] of six fungicides with ≥ 98% purity were procured from Sigma Aldrich, India. Sodium chloride with ≥ 99.9% purity from Merck, Darmstadt, Germany, AR grade anhydrous sodium sulphate from S.D. Fine Chemicals, Mumbai, anhydrous magnesium sulphate ($MgSO_4$) from ACROS Organic, New Jersey, USA and the sorbent primary secondary amine (PSA) from Agilent Technologies India Pvt. Ltd were used. The solvents like acetonitrile, *n*-hexane and ethyl acetate for fungicide residue analysis were purchased from Suprasolv (Merck) Germany. All common solvents were redistilled before use. The suitability of the solvents and other chemicals was investigated by running reagent blanks before actual analysis.

### Instrumentation

(a) Chromatographic separation for six fungicides was performed on a GC-MSMS System Shimadzu Model GCMSTQ-8040 supplied by Shimadzu Corporation, Kyoto, Japan, which is equipped with split/splitless injection port and autosampler (Shimadzu AOC 20S) coupled with triple-quadrupole (TQ) mass spectrometer. The GC separation was performed in SH-Rxi-5Sil MS (30 m × 0.25 mm × 0.25 µm film thickness) of 5% diphenyl and 95% dimethyl polysiloxane. It provides us high analysis efficiency, MRM performance and creates method itself that automatically sets the optimal measurement times for each component. Furthermore, at standby mode consumption of both carrier gas as well as power is greatly reduced which contributes to reduced running costs and lower the environmental load. Argon (99.9999%) was used as a carrier gas at an initial flow of 1.46 ml $min^{-1}$. Oven temperature was programmed as 80 °C for 2 min, 20 °C $min^{-1}$ to 180 °C for 0 min, 5 °C $min^{-1}$ to 300 °C for 3 min, injector port temperature 250 °C, ion source temperature 200 °C, interface temperature 250 °C and loop time 0.3 second. The flow rate of gas was 1.46 ml $min^{-1}$ through the column with split ratio 1:10. All the fungicides were eluted within 30.0 min as shown in Fig.2.

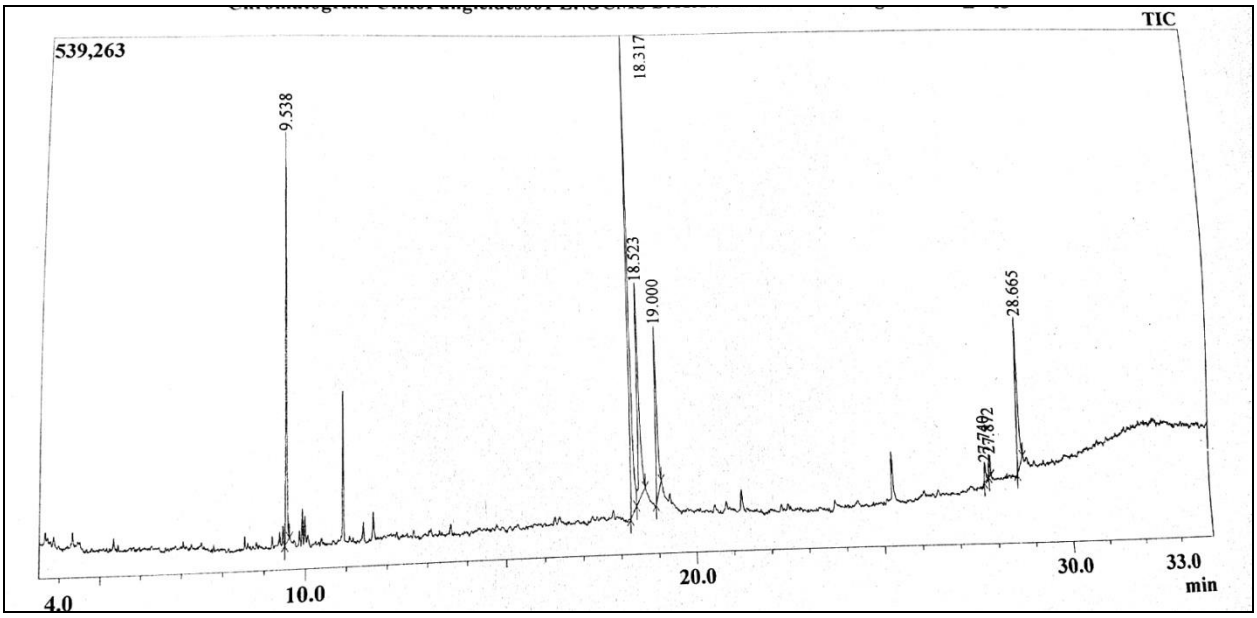



**Fig 2:** Chromatogram of six fungicides

**Sample preparation**

**(a) Rice:** Approximately 500 g of dehusked rice grain samples were grounded in a steel mixer to form a fine powder. Total 10 g rice samples with three replicates were weighed into 50 ml teflon centrifuge tube. The replicated samples were spiked with fungicide mixture standard at different fortification levels and allowed them to stand for at least 15 min for better absorbance of the fungicide in the matrix. Another set of five replicates, without spiking, was prepared to serve as the control. Distilled water (20 ml) was added in fine powdered rice mixture and mixed on a vortex for 1 min, let it stood for 10 min, then added 20 ml acetonitrile in it. Sample tubes were then kept in a deep freezer at -20 °C for 10 min. Homogenized them at 10,000-12,000 rpm until it became milky by using silent crusher to ensure that the solvent could have interacted well with the entire sample. Thereafter, 2 g sodium chloride was added to the sample in the centrifuge tube and shook vigorously for 1 min and then the extract was centrifuged for 2 min at 3000 rpm. Two easily distinguishable clear layers were formed upper layer was of organic solvent while lower layer was of aqueous. A volume of 10 ml collected from upper organic layer with the help of micropipette and the layer was transferred into another 25 ml centrifuge tube containing 10 g anhydrous sodium sulphate ($Na_2SO_4$) and then the sample was vortexed for 1 min. Thereafter, 5 ml aliquot was taken out from it, poured in cleanup tube already having 0.2 g PSA and 0.6 g anhydrous magnesium sulphate ($MgSO_4$) and centrifuged for 5 min at 3500 rpm. Thereafter, 4.0 ml of aliquot was taken out and concentrated to dryness. The final volume was made 4 ml, filtered by using 0.22 µm nylon syringe filter and then transferred into an auto-sampler vial for chromatography injection at GC-MS/MS.

**(b) Husk:** Residues in rice husk was extracted by using California Department of Food and Agriculture (CDFA) method. For this purpose, five replicates of husk (25 g) were weighed in a conical flask, along with the control. The replicated samples were fortified with the pesticide mixture standard at different fortification levels and allowed them to stand for some time for the better absorbance of the pesticide in the matrix. Now 75 ml acetonitrile was added in it and shaken for one and a half hour on mechanical shaker. The extract was filtered through a bed of anhydrous sodium sulphate and concentrated up to 5 ml. For cleaning up, the column was compactly packed with 1 g florisil keeping 0.5 g anhydrous sodium sulphate in between. The column was pre wetted with 10 ml of Hexane: Acetone (9:1 v/v). Then the sample was loaded, eluted with 10 ml of Hexane: Acetone concentrated up to 2 ml and analyzed on GC-MSMS.

**Results**

**Method validation**

**Calibration, LOD and LOQ:** Blank samples of rice were analyzed to verify the absence of interfering species at about the retention time of the analytes. The linearity of the method was studied by analyzing different matrix matched standard solutions in triplicate at five concentrations ranging from 0.05 to 1 $mgl^{-1}$ (Fig. 1a, b). The parameters like linear regression equations, correlation coefficient ($R^2$) and value limits of quantitation (LOQ) and limit of detection (LOD) were given in Table 2. The recovery experiment was conducted by spiking the sample at three different levels with three replicates and a non-spiked blank sample kept as a control.

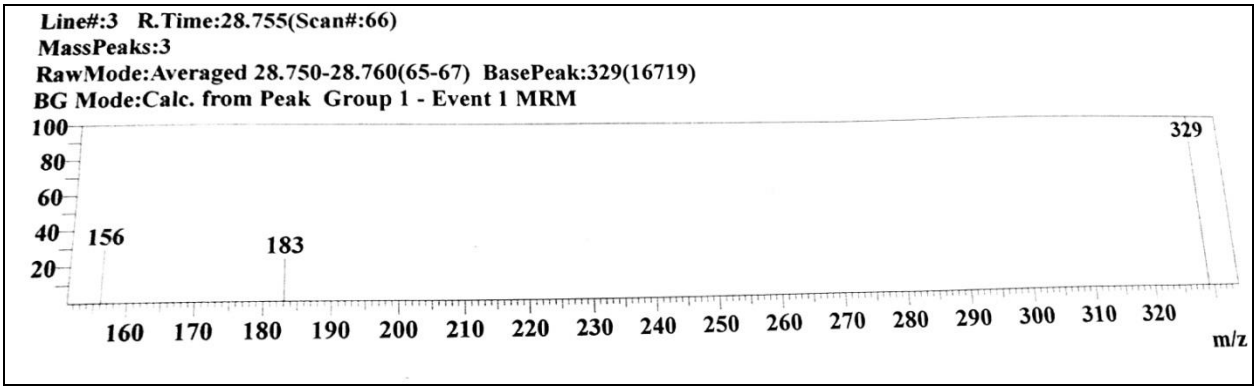
The matrix-dependent limits of quantitation (LOQ) and limit of detection (LOD) were calculated for the analytical methodology by using the blank and calibration standards of rice. The LODs of six insecticides were the concentrations that produced a signal to noise (peak-to-peak) ratio of three. The LOQs were defined based on signal-to-noise ratio of 10. Estimation of LOQs from the chromatogram corresponding to the lowest point used in matrix-matched calibration. Recoveries were determined for three replicates of the spiked samples at three different levels of each fungicide for rice with standard working solutions as per method (Table 3). Two MS–MS transitions were used in MRM mode quantification transition (Q) and confirmation transition (q). Under these conditions, fungicides peaks obtained within run time of 30.0 min allowing complete separation of its signal from those of foreign substance present in the sample (Fig.3). Triple quadruple GC-MS/MS in the multiple reactions monitoring (MRM) mode provides matchless sensitivity and selectivity for detection.

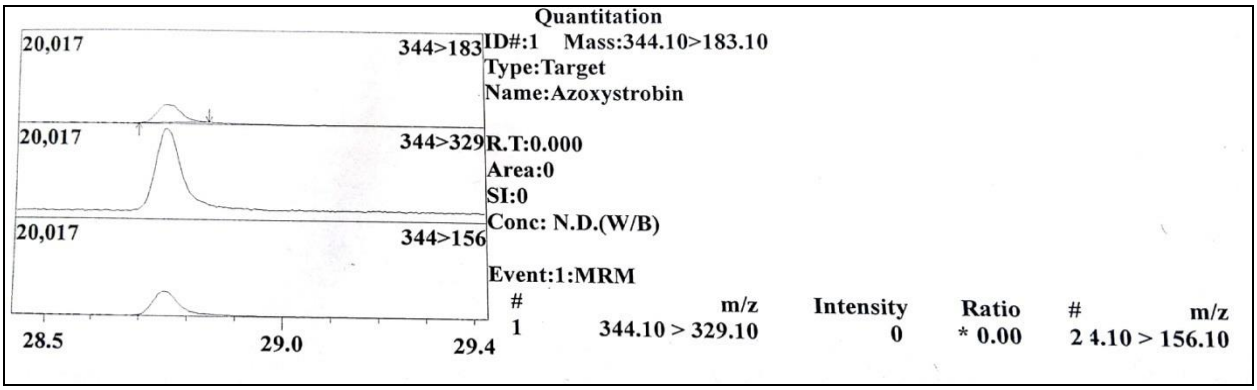
**Table 2:** Regression equation, $R^2$, LOD and LOQ ($mgkg^{-1}$)

| Compound | Matrix | Regression Equation | $R^2$ | LOD | LOQ |
|---|---|---|---|---|---|
| Azoxystrobin | Rice | Y= 49892x-12059 | 0.982 | 0.01 | 0.005 |
| Difenoconazole | Rice | Y= 24550x-11059 | 0.991 | 0.01 | 0.005 |
| Propiconazole | Rice | Y= 62802x-19848 | 0.999 | 0.01 | 0.005 |
| Tebuconazole | Rice | Y= 40901x-17520 | 0.999 | 0.01 | 0.005 |
| Trifloxystrobin | Rice | Y= 40162x-12727 | 0.999 | 0.01 | 0.005 |
| Pencycuron | Rice | Y= 45100x-10901 | 0.999 | 0.05 | 0.01 |

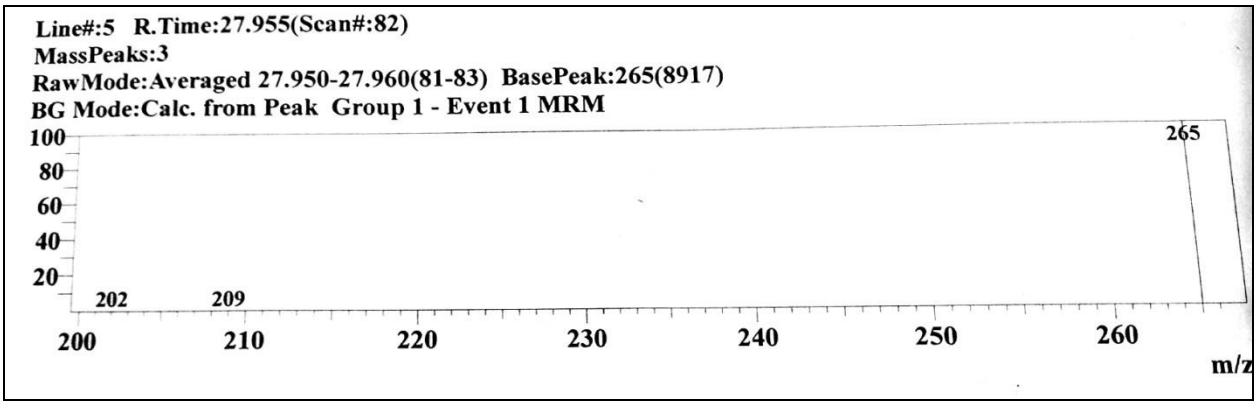
**Table 3:** Recovery and RSD values calculated from analyses of samples spiked with six in three spiked levels

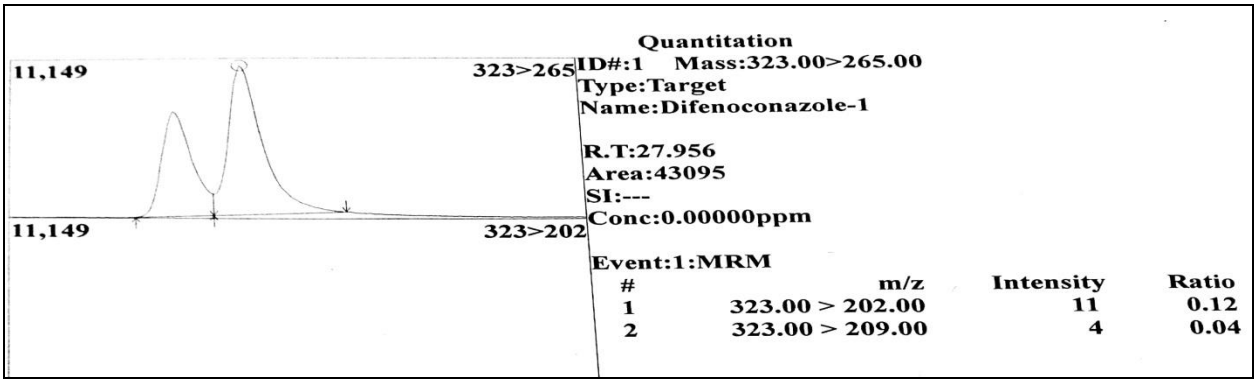
| Compound | Level of fortification (µg/ml) | Recovery% | SD | $RSD_r$ (%) |
|---|---|---|---|---|
| Azoxystrobin | 0.01 | 82.32 | 0.980 | 1.16 |
| | 0.05 | 85.64 | 0.339 | 0.89 |
| | 0.1 | 92.71 | 0.919 | 1.11 |
| Difenoconazole | 0.01 | 79.50 | 1.925 | 2.42 |
| | 0.05 | 81.25 | 0.893 | 1.10 |
| | 0.1 | 86.70 | 0.580 | 0.67 |
| Propiconazole | 0.01 | 75.25 | 0.786 | 1.04 |
| | 0.05 | 80.12 | 1.041 | 1.30 |
| | 0.1 | 83.55 | 0.836 | 1.00 |
| Tebuconazole | 0.01 | 82.35 | 0.980 | 1.19 |
| | 0.05 | 86.48 | 0.339 | 0.39 |
| | 0.1 | 90.15 | 0.919 | 1.02 |
| Trifloxystrobin | 0.01 | 81.75 | 0.918 | 1.12 |
| | 0.05 | 88.00 | 0.691 | 0.78 |
| | 0.1 | 93.58 | 0.467 | 0.50 |
| Pencycuron | 0.05 | 80.28 | 0.142 | 0.18 |
| | 0.1 | 83.80 | 0.630 | 0.75 |
| | 0.5 | 89.75 | 0.319 | 0.36 |



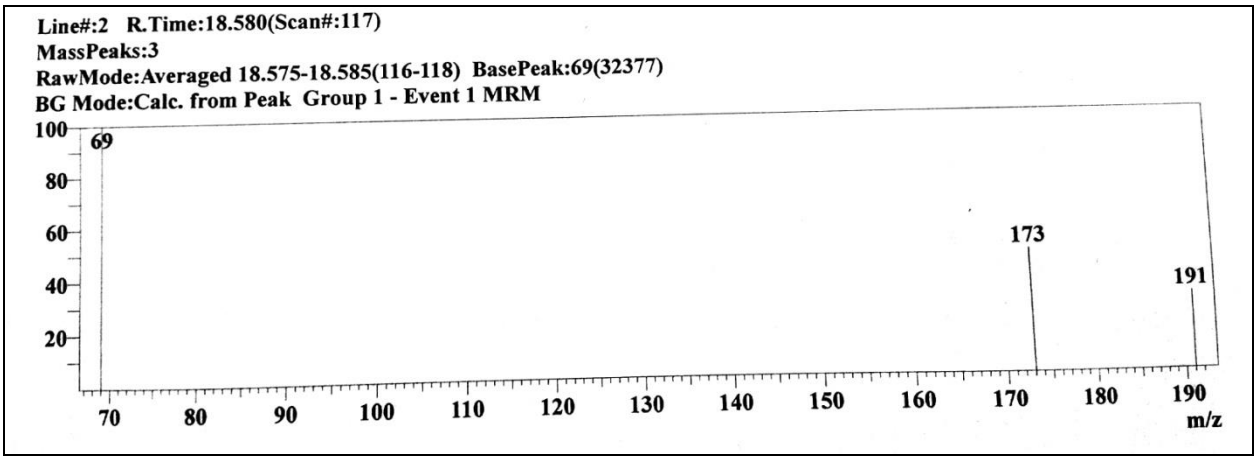



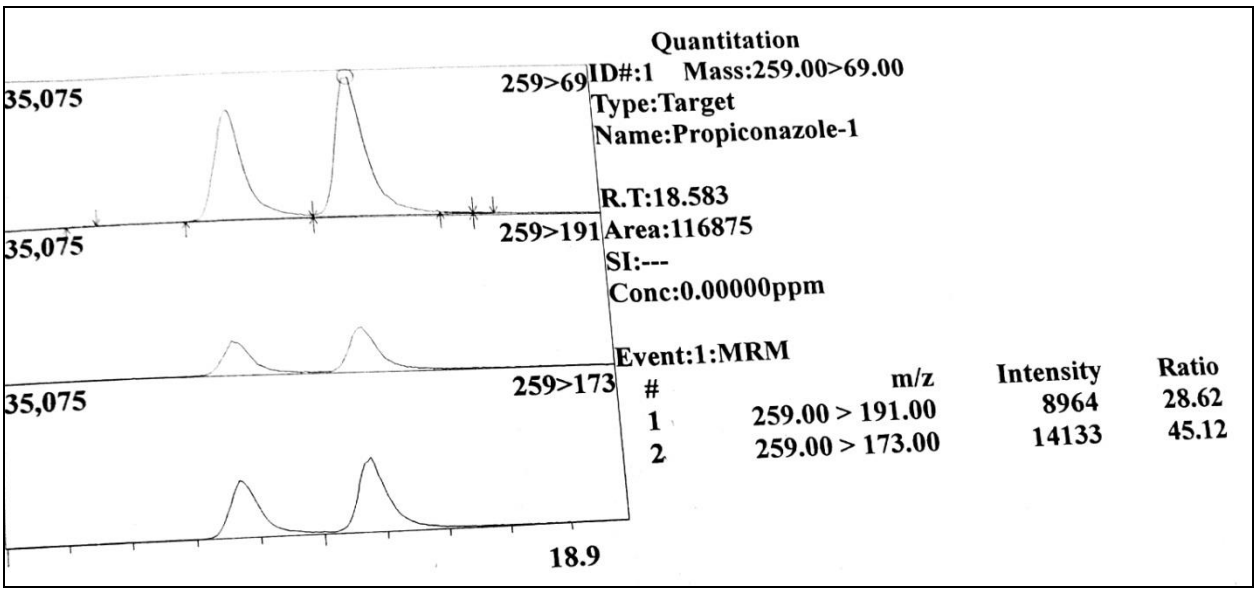

Azoxystrobin



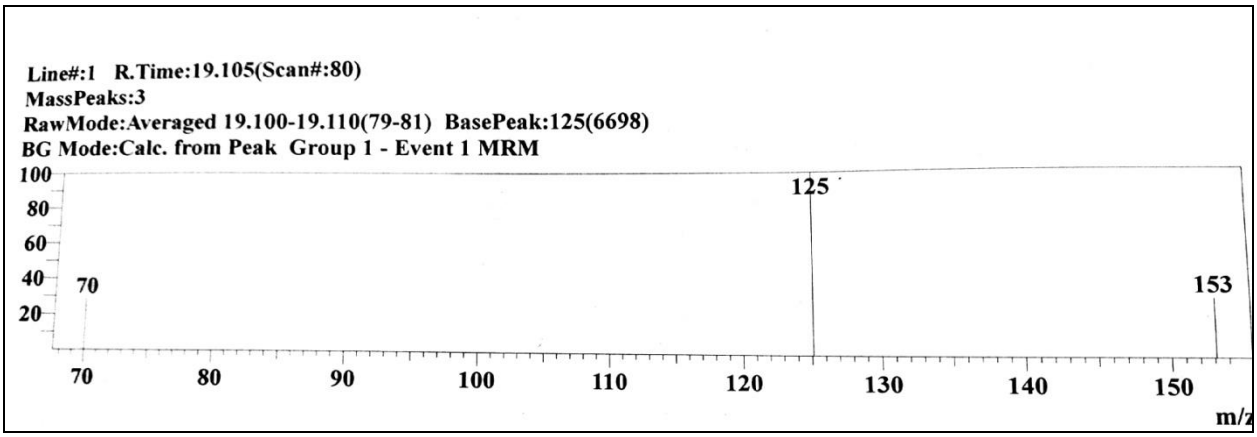



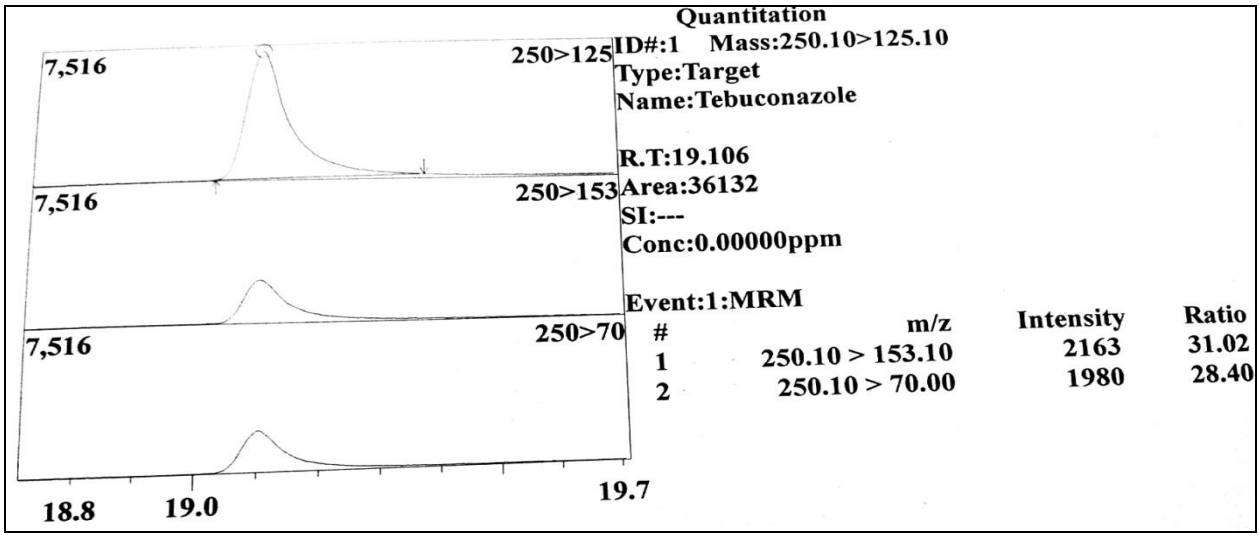

Difenoconazole



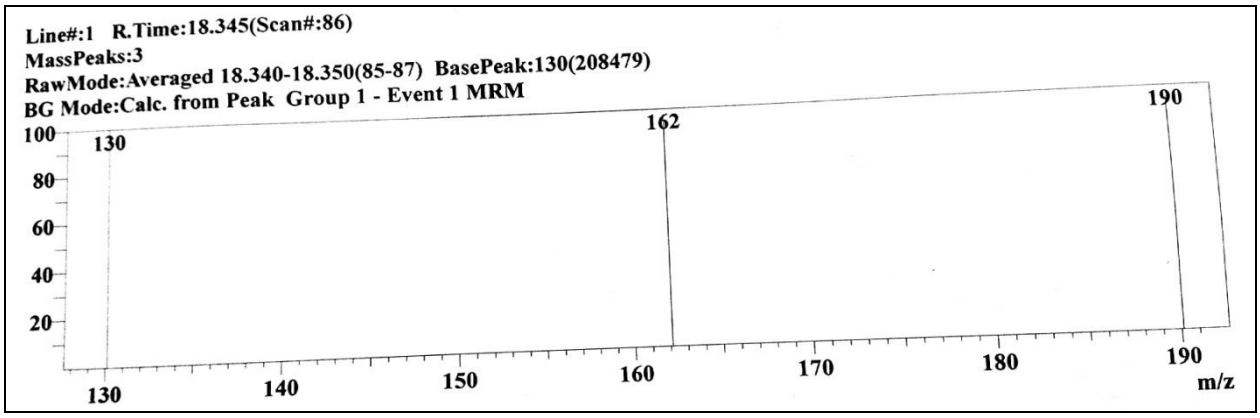



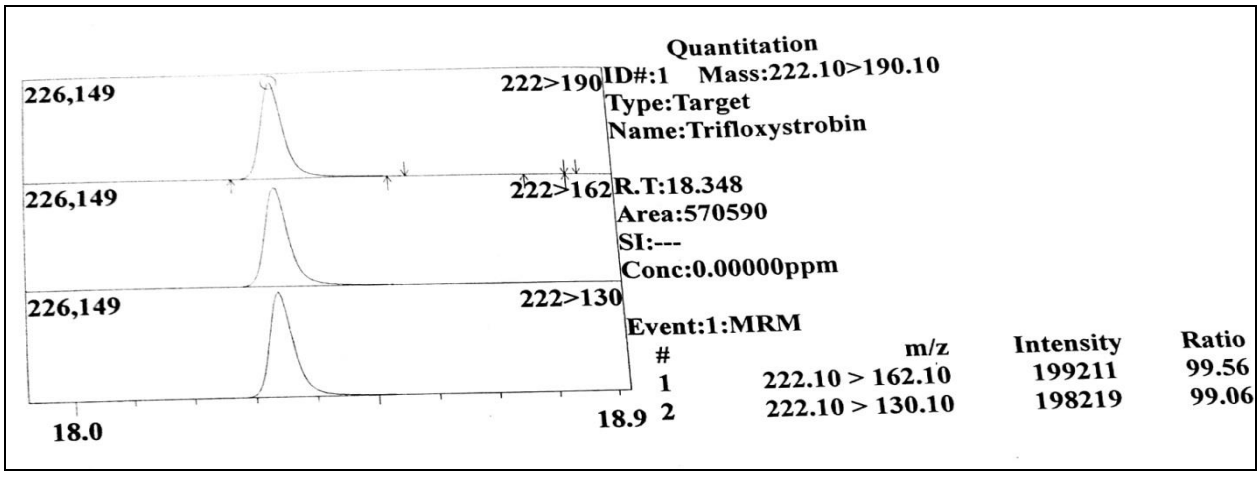

Propiconazole



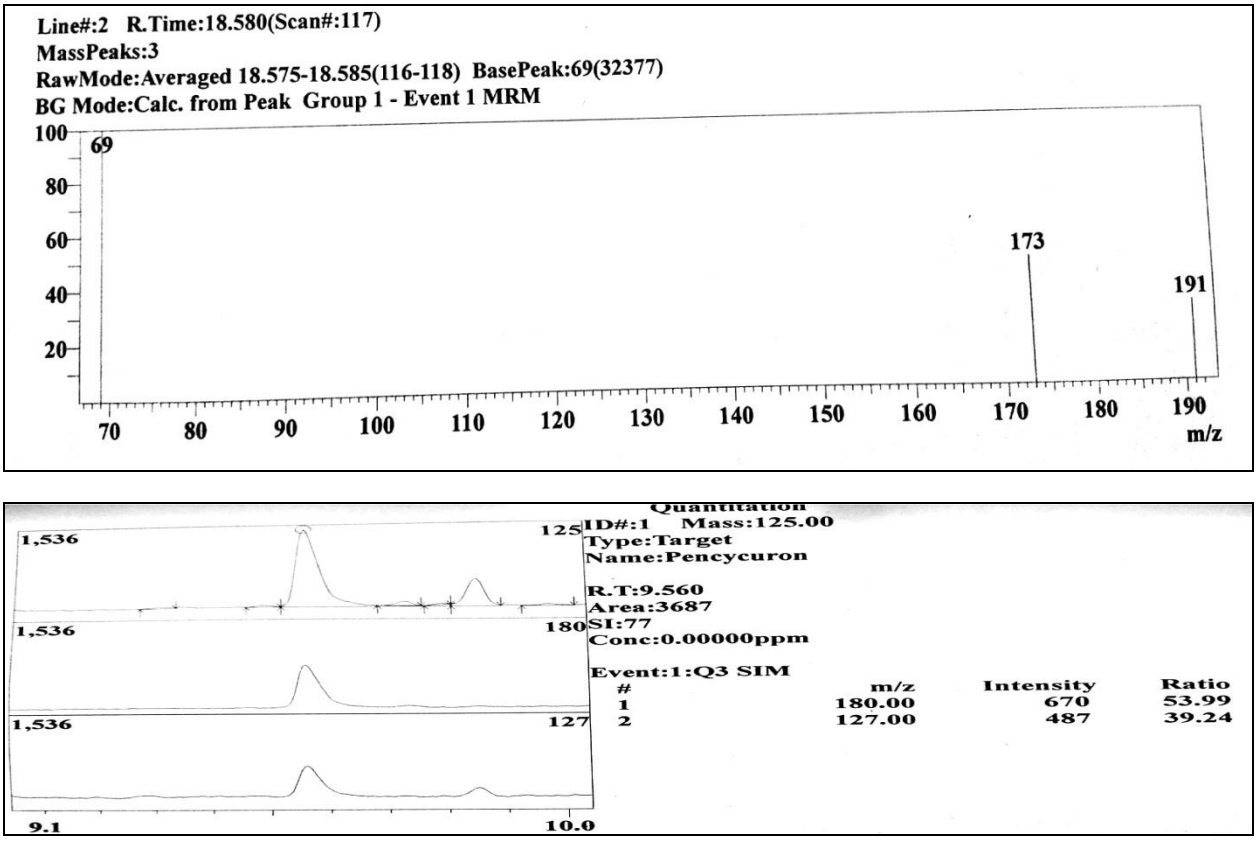




Tebuconazole




Trifloxystrobin




Pencycuron

**Fig 3:** GC-MS/MS chromatograms of six standard solution showing daughter ions

## Conclusion

The present study is a primary approach for determination of residue persistivity in fungicides. Due to good linearity, precision, recoveries of significant range and high sensitivity of GC-MS/MS, QuEChERS technique used in this method was suitable for determining fungicides under investigation in rice and husk.

## Acknowledgments

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