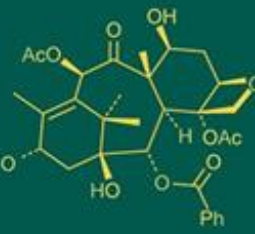
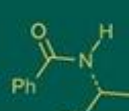
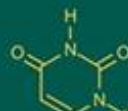
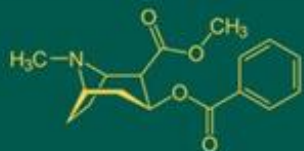


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Residue dynamics of azoxystrobin, tebuconazole and propiconazole in rice and its processed commodities

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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 residues in rice. Fungicides viz., azoxystrobin, tebuconazole and propiconazole were applied at twice on the aerial portions of the rice plants. Rice leaf and grain samples were collected after second spray (15 days before harvest). Rice leaf, grain, processes samples (brown rice, polished rice, rice bran and straw) were initially extracted using ethyl acetate 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. As per the SANTE guidelines, Targeted compounds were showed the Limits of Quantification (LOQ) 0.01 mg kg⁻¹ for all the fungicides. At the fortification level of 0.01-0.05 mg kg⁻¹, the average recoveries ranged from 70 to 118% with RSD <20% and correlation coefficient values (R²) were more than 0.98.

Keywords: Azoxystrobin, tebuconazole, propiconazole, fungicides, gc-ms/ms, rice, validation

Introduction

Rice as one of staple food in the world is one of the most important food product. Rice content a very important source of carbohydrates, protein and nutrients. In modern agriculture, agrichemical or pesticides are used globally in farming sector to control pest population (Onojoh *et al.*, 2013)^[1]. Pesticides are substances (natural or manmade) used to control pests, weeds, and diseases in plants in various agronomic practices (Dhaliwal *et al.*, 2015)^[2]. The use of pesticides in irrigated rice has intensified in recent years due to the higher incidence of foliar diseases and insect pests. (Telo *et al.*, 2017)^[8].

The presence of pesticide residues in food is a major public health concern, and identifying the presence of such residues in all types of food is important to guarantee food safety (Hou *et al.*, 2013)^[3]. The consumption of pesticide-contaminated food product may becomes potential health risk for human body. Recently, study of cumulative risk assessment the possibility of multiple pesticides exposure that could cause unanticipated adverse effects on human health is crucial issues in many areas (Chen *et al.*, 2009)^[4]. When applied on paddy crop, fungicide active ingredients can remain as residues on leaves, husks and grain; their concentrations depend on application rate and timing, chemical half-life, environmental conditions (sunlight, rain, microbial activity), and post-harvest processing (milling, washing, cooking), which can reduce but not always eliminate residues. Field dissipation studies commonly show residues fall to trace levels within days-weeks (Saha *et al.*, 2020)^[5].

Materials and Methods

Fungicides selected

Three fungicides were selected for the study *i.e.*, azoxystrobin, tebuconazole and propiconazole, all are recommended for use in rice by the Central insecticides board and registration committee (CIBRC). Rice samples from the control plots were used in the fortification procedure and as the matrix blanks for the matrix matched calibration standards.

Preparation of working standards

Required volume was pipette out from intermediate standard solution into a 10 mL volumetric flask to prepare working standards for serial dilutions (six concentrations viz., 0.05, 0.1, 0.25, 0.5, 0.75 and 1 g mL⁻¹). Then the volumetric flasks were made to the mark with Appropriate solvent. All the standards of different concentrations were stored in deep freezer at - 20 °C

Multi-residue method development for azoxystrobin, tebuconazole and propiconazole using GC-MS/MS

The multi residue method was developed for analysis of Azoxystrobin, Tebuconazole and Propiconazole using a Shimadzu Gas Chromatography System (GC-2030) equipped with a Triple Quadrupole Mass Spectrometer (8040 Nexes). Chromatographic separation was equipped in SH-Rxi-5SIL MS (30m×0.25m×0.25µm) capillary column. The initial temperature was set at 60 °C with a hold time of 1min, ramped at 40 °C, till 170 °C, later increased at 10 °C to 310 °C in a hold time of 3min. The autosampler used in the study is AOC-20s+ and the injection port is AOC-20i+. Helium was used as

Carrier Gas maintained at 1mL.min and Argon used for fragmentation of analytes as Collision Induced Dissociation (CID).

The inlet operating conditions were µL injection volume, 1.3 mL min⁻¹ flow rate, and GC oven temperatures programming as described in Table 3.3 where the samples were injected in a splitless mode and the run time was 20.75 min. The Electron impact (EI) mode was at (+70 eV); the monitoring was from m/z 50 to 500. The ion source, Injector, and Quadrupole analyzer temperatures were fixed at 230, 250, and 250 °C, respectively (Table-1).

Table 1: Parent, precursor ions, product ions and retention index and time for method development and validation of selected fungicides in GC-MS/MS

Compound	Azoxystrobin	Tebuconazole	Propiconazole
Retention Index -1	3086	2391	2344
Ret Time (min)	18.035	13.061	12.767
Ion -1	344.1>329.1	250.10>125.10	173.0>145.0
CE	16	22	16
Ref.1	388.1>345.1	125.10>89.00	173.0>109.0
CE	18	18	28
Ref.2	388.1>300.1	-	259.0>69.0
CE	20	-	14
Ref.3	344.1>183.1	-	259.0>173.0
CE	24	-	14

Method validation for azoxystrobin, tebuconazole, propiconazole

The developed analytical method for azoxystrobin, tebuconazole, propiconazole residue estimation was validated as per the Document N° SANTE/11312/2021 guidelines (a guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed). The rice commodities viz., rice leaf, rice grain, brown rice after dehulling, polished rice, rice bran and straw were extracted as per the AOAC official method of analysis 2007.01, 20th edition of 2016, chapter.

Recovery studies

The NLR 34449 rice samples (1 kg- leaves, kg- grains, 1 kg- straw) collected from untreated plots were brought to the laboratory and samples were processed into various commodities viz., polished rice, brown rice, bran, were homogenized using high volume homogenizer and each homogenized sample of 10 g was transferred into 50 mL centrifuge tubes. The required quantity of fungicides viz., azoxystrobin, tebuconazole and propiconazole intermediary standards prepared from CRMs were added to rice leaf, grain, bran, polished rice, brown rice, straw samples to get fortification levels of three concentrations and each replicated six times. These fortification levels were selected to know the suitability of the method to quantify insecticides (LOQ) and fungicides in rice samples below MRLs of Codex Alimentarius Commission (CAC).

To this sample 20mL of ethyl acetate for GC-MS/MS were added and homogenized the sample at 14000-15000 rpm for 3 minutes. To this, g of saturated sodium chloride were added and vortexed for 1 minute and centrifuged for 10 minutes at 4500 rpm to separate the organic layer. 9 mL of aliquot was transferred into 50 mL centrifuge tube containing 5g of sodium

sulphate and vortexed for 1 minute. 2 mL aliquot was transferred into 15 mL centrifuge tube containing 0.2g of Primary secondary amine (PSA) and 0.6g of Magnesium sulphate anhydrous and vortexed for 30 seconds and centrifuged for 5 minutes at 4500 rpm. A supernatant layer of 1 mL. was transferred into 15 mL tube and filtered it through PTFE (Polytetrafluoroethylene) filter % 0 % . 22 µm), and this 1 mL filtrate was taken into LC vial for analysis directly with GC-MS/MS.

$$\text{Recovery\%} = \frac{\text{Residue quantified in fortified sample}}{\text{Fortified level}} \times 100$$

Sequence of injections

- Injection of reagent blank
- Injection of reference standard of suitable concentrations within calibration range.
- Injection of samples
- Injection of suitable concentration within calibration range after every six samples.

Residue estimation under different chromatography conditions

For estimation of residues of dinotefuran, different operational parameters for UHPLC-PDA were presented in table-1. Residues were estimated by comparison of peak area of the standards with that of peak in the samples run under identical conditions.

Interpretation of data

$$\text{Residues (mg kg}^{-1}\text{)} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \frac{\text{Concentration of standard}}{\text{weight of the sample (g)} \times \text{final volume of extract}}$$

Results and discussion

Method validation, calibration, LOD and LOQ

Method validation for estimation of azoxystrobin, tebuconazole and propiconazole residues in different rice commodities using

GC-MS/MS system based chromatographic technique.

Based on the response of the detector (GC-MS-TQ) to different quantities (μg) of CRM standards injected, it was found that the LOD for azoxystrobin, tebuconazole and propiconazole was 0.003 and 0.01 $\mu\text{g mL}^{-1}$, respectively and the calibration graph for the concentrations of 0.01 to 0.15 $\mu\text{g mL}^{-1}$ is $y = 30475x - 253.14$, $y = 133152x - 563.01$ and $y = 174708x + 569.24$ with a coefficient of determination >0.99 for azoxystrobin, tebuconazole and propiconazole as given in (Table-2)

Table 2: Calibration studies for azoxystrobin, tebuconazole and propiconazole standard alone and in various rice commodities

S. No	Chemicals	Parameter	Regression Equation	R ²	LOD ($\mu\text{g mL}^{-1}$)	LOQ ($\mu\text{g mL}^{-1}$)
1	Azoxystrobin	Standard	$y = 30475x - 253.14$	0.998	0.003	0.01
		Rice leaf	$y = 76.659x + 534.69$	0.998		
		Rice grain	$y = 240.4x + 2035.$	0.990		
		Brown rice	$y = 119.2x - 324.8$	0.990		
		Polished rice	$y = 168.9x + 894.7$	0.990		
		Rice bran	$y = 46.62x - 272.6$	0.997		
		Straw	$y = 67.355x - 233.2$	0.998		
2	Tebuconazole	Standard	$y = 13315x - 563.0$	0.991	0.003	0.01
		Rice leaf	$y = 146.8x + 2164.$	0.990		
		Rice grain	$y = 716.1x + 1380.$	0.996		
		Brown rice	$y = 213.0x - 672.1$	0.972		
		Polished rice	$y = 137.3x - 1197$	0.933		
		Rice bran	$y = 60.70x - 584.6$	0.997		
		Straw	$y = 136.2x + 283.2$	0.998		
3	Propiconazole	Standard	$y = 17470x + 569.2$	0.994	0.003	0.01
		Rice leaf	$y = 166.0x + 1371.2$	0.995		
		Rice grain	$y = 467.5x + 545.2$	0.996		
		Brown rice	$y = 173.7x - 188.6$	0.977		
		Polished rice	$y = 112.1x + 279.4$	.993		
		Rice bran	$y = 104.3x + 446.0$	0.994		
		Straw	$y = 141.1x - 83.33$	0.998		

Matrix -match calibration studies

The blank matrix for various rice commodities viz., rice leaf, rice grain, brown rice, polished rice, bran and straw was extracted as per QuEChERS method of sample extraction. The blank matrices were spiked with three pesticides viz., azoxystrobin, tebuconazole and propiconazole in the linear concentrations (0.01 to 0.15 $\mu\text{g mL}^{-1}$). The analysis was performed in GC-MS/MS for azoxystrobin, tebuconazole and propiconazole. The calibration graphs were drawn between the concentrations vs. detector response in the form of peak area. The calibration equations and coefficient of determination were presented in the (Table-2). The calibration studies revealed that azoxystrobin, tebuconazole and propiconazole followed the constant linear response in all the sample matrices (rice leaf, rice grain, brown rice, polished rice, rice bran and straw) with a coefficient of determination of > 0.99 .

Matrix effect studies for azoxystrobin, tebuconazole, propiconazole, in different rice commodities

Matrix effect is the enhancement or suppression of the detector

response in the particular matrix. The studies were conducted in six rice commodities for all the fungicides. The responses were taken by spiking the pesticides individually in a blank matrix and analysed in the same method for validation to other parameters. Highest interference was noticed for rice straw. However, the interference was in the range of -20 to +20% for all the test fungicides in various matrices. Among the various rice commodities studied straw showed maximum interference (-12 to -16%) for all the 5 test pesticides viz., The rice leaf matrix enhancement for three fungicides viz., azoxystrobin, tebuconazole, propiconazole. (Table-3).

It was found that matrix effect in terms of suppression or enhancement of the detection system response is a common phenomenon in method development and validation procedures. It can be dealt with adopting suitable extraction and clean -up procedures, dilution of matrix, preparation of matrix-match calibration curves. Rice grain and polished rice matrices showed matrix enhancement for all the three fungicides, whereas rice bran and brown rice showed matrix interference for all the fungicides.

Table 3: Matrix Effect Studies

Insecticides / fungicides	Concentration ($\mu\text{g/mL}$)	Rice leaf	Grain	Polished rice	Rice bran	Brown rice	Straw
Azoxystrobin	0.01	-3.2	2.3	2.1	-2.4	-3.6	-12
Tebuconazole	0.01	7.5	6.8	2.5	-2.8	-3.5	-13
Propiconazole	0.01	5.8	2.6	5.8	-3.8	-5.8	-12

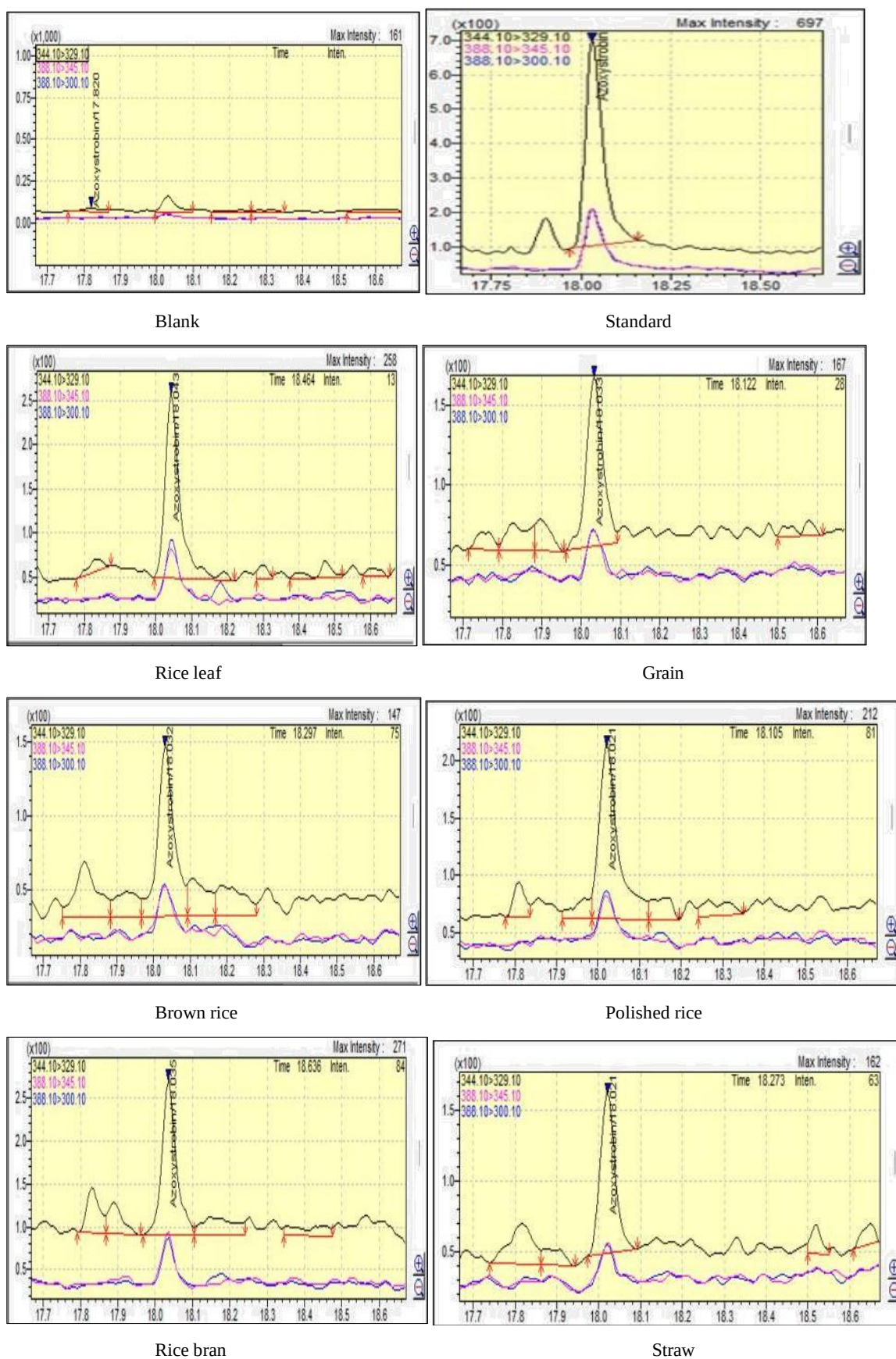


Fig 1: Chromatograms of Azoxystrobin in Rice Leaf, Grain, Brown rice, Polished rice, Rice bran and Straw Matrice

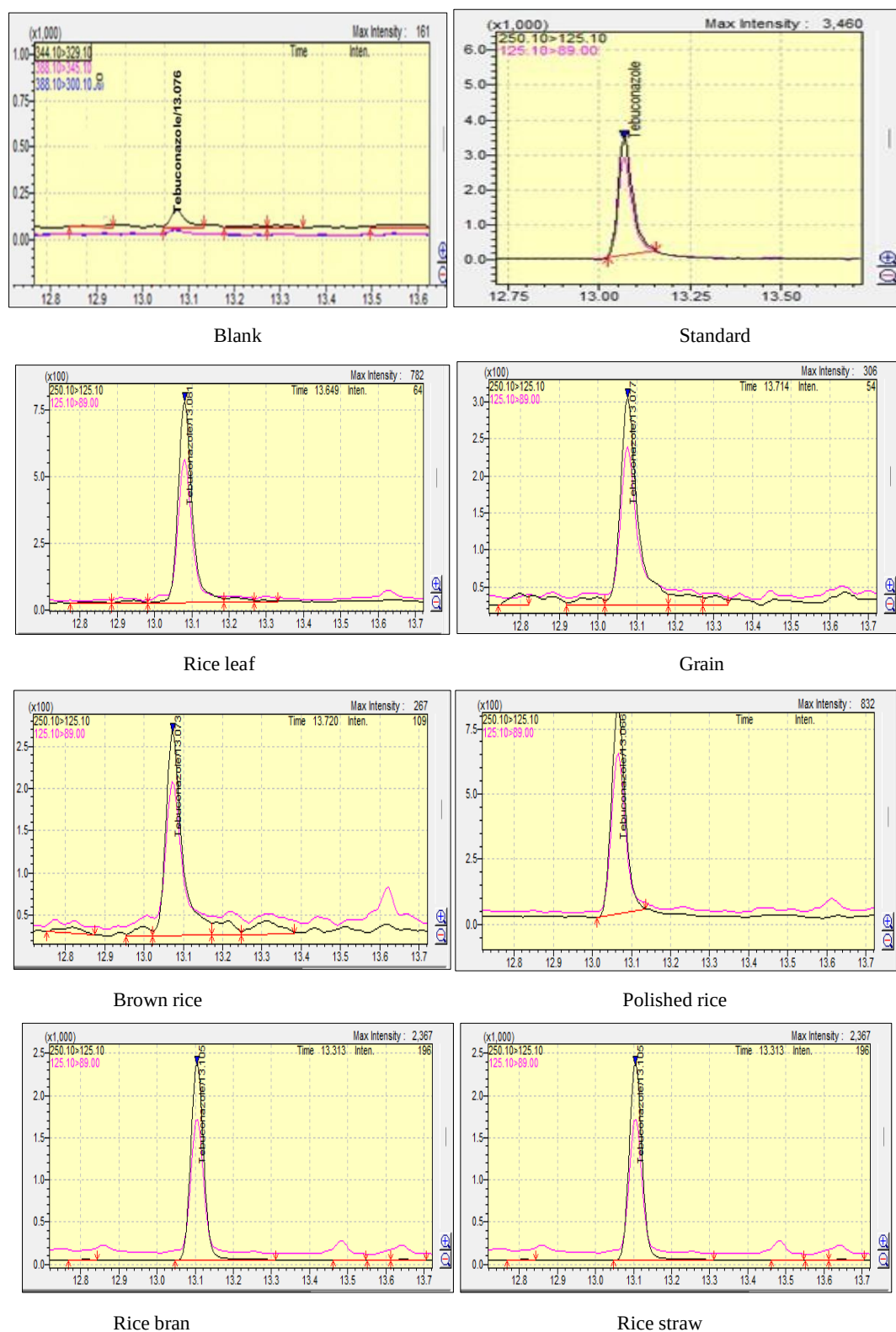


Fig 2: Chromatograms of tebuconazole in rice leaf, grain, brown rice, polished rice, rice bran and straw matrices

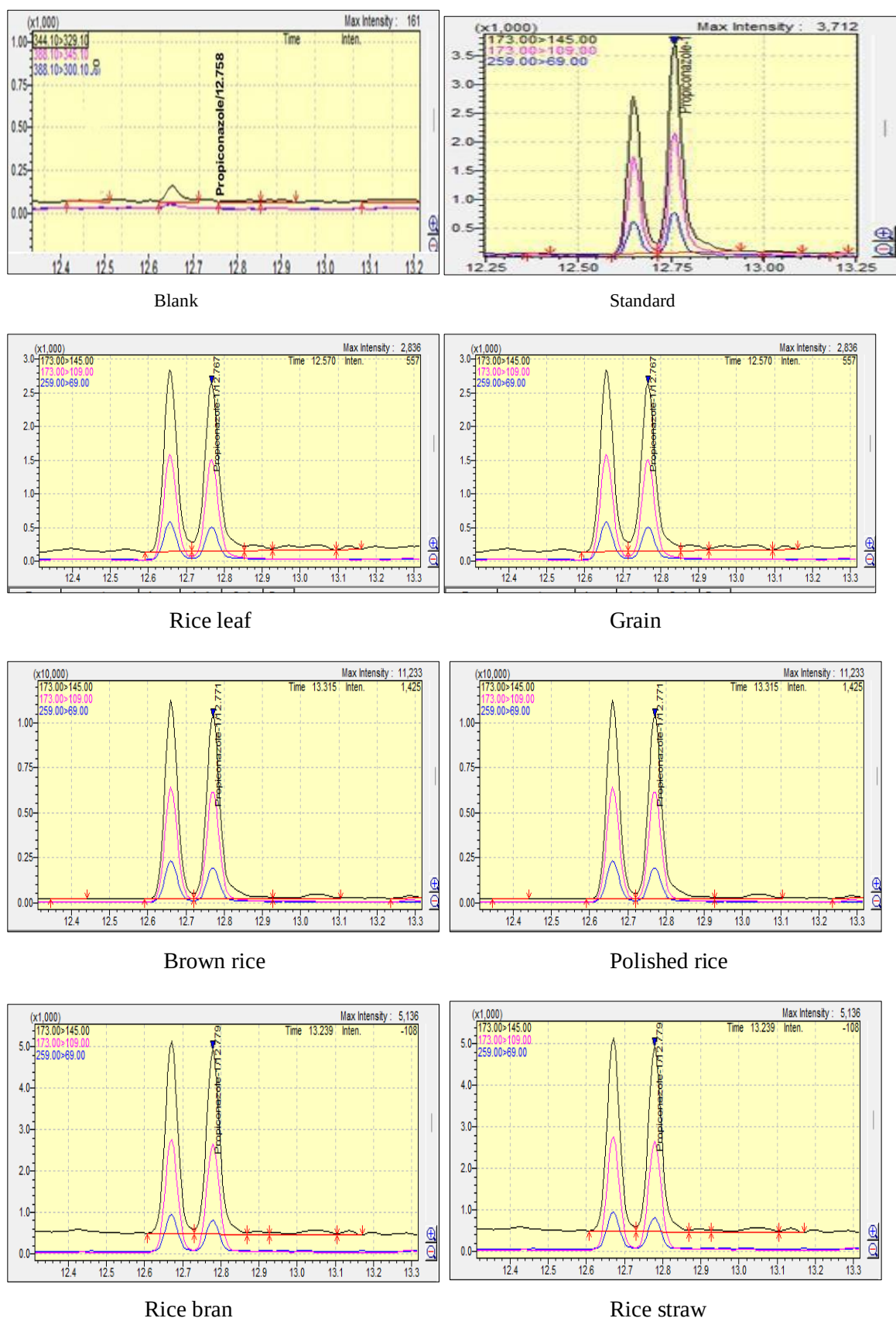


Fig 3: Chromatograms of propiconazole in rice leaf, grain, brown rice, polished rice, rice bran and straw matrices

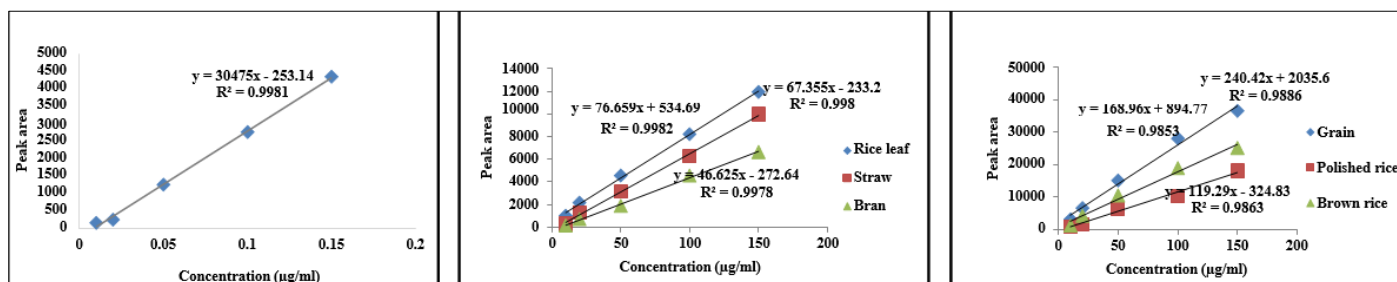


Fig 4: Calibration curve and matrix match calibration for azoxystrobin in various rice commodities

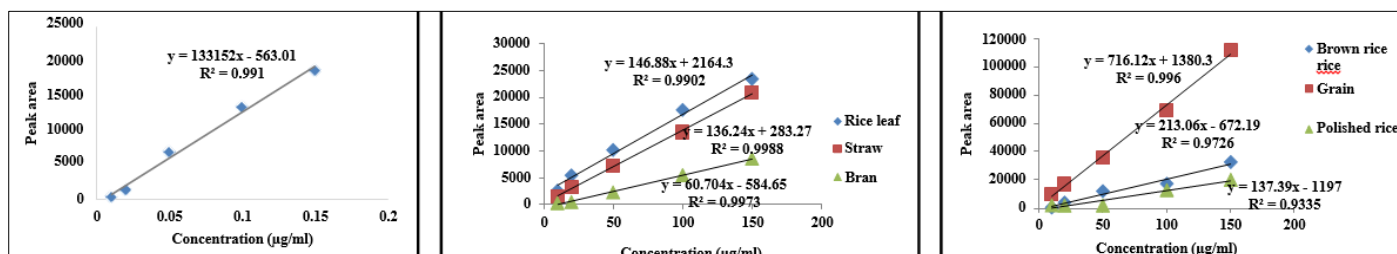


Fig 5: Calibration curve and matrix match calibration for tebuconazole in various rice commodities

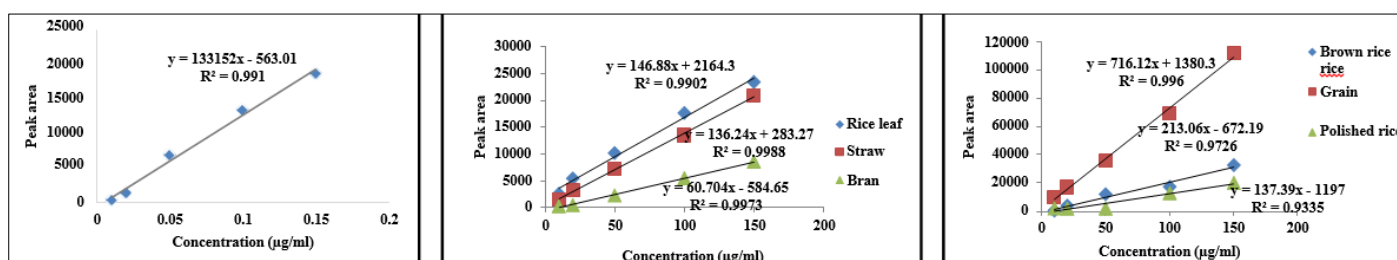


Fig 6: linearity and matrix-matched calibration curves of propiconazole in rice leaf, straw, bran, grain, brown rice, and polished rice

Recovery studies

The validation of method adopted for analysis of rice sample under study was done by performing the recovery experiments. Untreated rice commodity samples were fortified with standard solution of azoxystrobin, tebuconazole and propiconazole at LOQ ($0.01 \mu\text{g} \cdot \text{g}^{-1}$), 2 times of LOQ ($0.02 \mu\text{g} \cdot \text{g}^{-1}$) and 5 times of LOQ ($0.05 \mu\text{g} \cdot \text{g}^{-1}$) level.

The validation of method adopted for analysis of rice samples under study was done by performing the recovery experiments. Untreated rice commodities rice leaf, rice grain, brown rice, polished rice, bran and straw samples were fortified with standard solution of test fungicide at LOQ ($0.01 \mu\text{g} \cdot \text{g}^{-1}$), 2 times of LOQ ($0.02 \mu\text{g} \cdot \text{g}^{-1}$) and 5 times of LOQ ($0.05 \mu\text{g} \cdot \text{g}^{-1}$) level.

Azoxystrobin

Azoxystrobin fortified or spiked samples were extracted, cleaned up and analyzed. Rice samples viz., Rice leaf, grain, polished rice, brown rice, rice bran and straw fortified with azoxystrobin at 0.01 , 0.02 and $0.05 \mu\text{g} \cdot \text{g}^{-1}$ were analysed and the mean recoveries were 78, 78 and 72% for rice leaf; 73, 70 and 72% for grain; 92, 72 and 72% for polished rice; 75, 72 and 73% for brown rice; 72, 72 and 71% for rice bran and 87, 87 and 71% for straw respectively. (Table-4).

Tebuconazole

Tebuconazole fortified or spiked samples were extracted, cleaned up and analyzed. Rice samples viz., Rice leaf, grain, polished rice, brown rice, rice bran and straw fortified with tebuconazole at 0.01 , 0.02 and $0.05 \mu\text{g} \cdot \text{g}^{-1}$ were analysed and the mean recoveries were 91, 101 and 74% for rice leaf; 77, 70

and 74% for grain; 75, 113 and 113% for polished rice; 88, 72 and 73% for brown rice; 99, 95 and 118% for rice bran and 112, 76 and 73% for straw respectively. (Table-4).

Propiconazole

Propiconazole fortified or spiked samples were extracted, cleaned up and analyzed. Rice samples viz., Rice leaf, grain, polished rice, brown rice, rice bran and straw fortified with propiconazole at 0.01 , 0.02 and $0.05 \mu\text{g} \cdot \text{g}^{-1}$ were analysed and the mean recoveries were 81, 104 and 110% for rice leaf; 77, 82 and 87% for grain; 74, 84 and 89% for polished rice; 74, 78 and 80% for brown rice; 75, 85 and 97% for rice bran and 81, 94 and 105% for straw respectively. (Table-4).

The acceptance criteria for recovery were 70 to 120 %. In the present study the recovery studies fall in the range of 70-120% with the relative standard deviation (RSD) less than 20% as per the method validation guidelines of SANTE 2021.

Chauhan *et al.* (2018) [6] analyzed azoxystrobin samples on (GC-MS/MS) using MRM mode to achieve lower detection level. Targeted compounds were determined within 30.0 minutes showing the (LOQ) $0.005 \text{ mg} \cdot \text{kg}^{-1}$ for all the fungicides. At the fortification level of 0.01 - $0.1 \text{ mg} \cdot \text{kg}^{-1}$, the average recoveries ranged from 75.25 to 93.58%.

Telo *et al.* (2017) developed a Gas Chromatography method using Electron Capture Detector for multi residue analysis for 5 fungicides and insecticides viz., azoxystrobin, difenconazole, propiconazole, tebuconazole and trifloxystrobin, cypermethrin, lambdacyhalothrin, permethrin, and thiamethoxam and reported that the calibration curves obtained from the matrix matched standards of rice grains and rice hulls extracts presented good linearity with coefficients of determination greater than 0.995

for all studied pesticides. The method presented recovery values between 80.7 and 119.7% with good precision in terms of relative

standard deviation (RSD), with values ranging from 0.4 to 19.4% for the different processed grains. For the rice hull, recovery ranged from 80.1 to 111.8%, with RSD values from 1.2 to 18.7%. No interferences were observed.

Feng *et al.*, 2022^[7] investigated the dissipation and residues of

trifloxystrobin and tebuconazole in wheat under field conditions in China using high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS)^[7] after QuEChERS extraction. The recovery rates of trifloxystrobin, trifloxystrobin acid, and tebuconazole in wheat grains, wheat plants, and straw ranged from 82% to 109%. Based on this analysis, trifloxystrobin and tebuconazole residues in wheat grains would not affect the health of the general population in China.

Table 4: Recovery studies

Compound	Commodity	Level of fortification (µg/ml)	Mean Recovery %	SD	RSDr (%)
Azoxystrobin	Rice leaf	0.01	78	26.5	3.41
		0.02	78	4.45	5.69
		0.05	72	1.47	2.03
	Grain	0.01	73	2.24	3.06
		0.02	70	1.49	2.13
		0.05	72	1.31	1.82
	Polished rice	0.01	92	10.64	11.52
		0.02	72	2.22	3.09
		0.05	72	1.93	2.69
	Brown rice	0.01	75	3.31	4.40
		0.02	72	1.90	2.64
		0.05	73	1.21	1.66
	Rice bran	0.01	72	1.92	2.67
		0.02	72	0.58	0.81
		0.05	71	1.01	1.42
	Rice straw	0.01	87	8.03	9.19
		0.02	71	0.46	0.65
		0.05	71	0.51	0.71
Tebuconazole	Rice leaf	0.01	91	2.92	3.22
		0.02	101	0.58	0.58
		0.05	74	1.03	1.38
	Grain	0.01	77	5.31	6.93
		0.02	70	0.36	0.50
		0.05	74	0.59	0.80
	Polished rice	0.01	75	1.83	2.44
		0.02	113	6.45	5.73
		0.05	113	6.45	5.73
	Brown rice	0.01	88	4.15	4.73
		0.02	72	1.04	1.45
		0.05	73	0.62	0.84
	Rice bran	0.01	99	0.16	0.16
		0.02	95	1.64	1.73
		0.05	118	1.27	1.07
	Rice straw	0.01	112	2.46	2.20
		0.02	76	1.00	1.32
		0.05	73	2.38	3.25
Propiconazole	Rice leaf	0.01	81	2.33	2.85
		0.02	104	2.65	2.53
		0.05	110	2.56	2.33
	Grain	0.01	77	0.53	0.69
		0.02	82	0.47	0.58
		0.05	87	0.64	0.73
	Polished rice	0.01	74	1.51	2.02
		0.02	84	0.57	0.67
		0.05	89	0.92	1.03
	Brown rice	0.01	74	2.42	3.26
		0.02	78	0.62	0.79
		0.05	80	0.89	1.11
	Rice bran	0.01	75	1.30	1.72
		0.02	85	0.97	1.15
		0.05	97	1.47	1.51
	Rice straw	0.01	81	1.33	1.64
		0.02	94	1.26	1.34
		0.05	105	0.38	0.36

Conclusion

An analytical method was developed and validated for estimating fungicides residues in rice samples using Gas Chromatography Mass Spectrometry (GC-MS/MS). Calibration curves showed good linearity, with an R² value of 0.9951. The results of the method performance showed that the recovery ranges for rice matrices were between 70% and 120% and the RSD was within the accepted level (below 20%) for

residue determination given by SANTE/11312/2021 guidelines. Residue levels remained well below the established MRL of 1.5 mg kg⁻¹, indicating safe use under recommended conditions.

Authors' Contribution

Conceptualization of research:(LV*, DK, MKT, AP)

Designing of the experiments: (LV*, DK, MKT)

Contribution of experimental materials: (LV*, DK)

Execution of field/lab experiments and data collection: (LV*, DK, MKT)

Analysis of data and interpretation: (LV*)

Preparation of the manuscript: (LV*)

Declaration: All authors declare that they have no conflict of interest.

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