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Gas Chromatography - Mass Spectrometry Electron Impact Ionization (GC-EI-MS) method for the Simultaneous Determination of Twelve Azole Fungicide Residues in Fish,

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Research Article

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Keywords: Soxhlet extraction, fungicides, gas chromatograph, electron impact ionization, Mass spectrometer

ABSTRACT

A sensitive multi-residual analytical method based on Gas Chromatography Electron impact Ionization Mass Spectrometry (GC-EI-MS) has been developed for the simultaneous determination of twelve azole group of fungicides (Tetraconazole, Penconazole, Tricyclazole, Paclobutrazole, Hexaconazole, Diniconazole, Propiconazole, Tebuconazole, Epoxyconazole, Etoxazole, Fluquinconazole, Difenconazole) in market fish samples. The composite samples were homogenized, freeze dried and ground to obtain powdered samples which were extracted by the Soxhlet extraction procedure using a hexane-acetonitrile mixture (v/v, 3:1). The extracts were cleaned using florisil and analysed for azoles using GC-MS. The method was validated in fish samples spiked with investigated compounds at different concentration levels (0.005 and 0.05 µg/g). The average recoveries (n=5) ranged between 85-105% with a relative standard deviation (RSD) of less than 5%. This method has the lowest limit of quantification (LOQ) at 0.005 µg/mL and the limit of detection (LOD) at 0.001µg/mL. The developed method was applied successfully for the determination of residues in market fish samples.

INTRODUCTION

Agricultural activities are prone to induce pesticide contamination in aquatic ecosystems [1,6]. A large group of fungicides have been introduced in agriculture for the prevention of diseases, as well as, for the quantity and quality control of agricultural products. Nowadays, research on contamination of surface waters or sediments has become a major preoccupation in environmental science. A consequence of this widespread contamination, in which pesticides are often combined in a mixture, is the bio accumulation in fish [4,7] or accumulation in edible tissue [3] which poses a potential risk to consumers [2,5]. In light of these concerns, evaluation of levels of pesticides in fish is an important objective for environmental and health sciences.

The fungicide group, demethylation inhibitors (DMI), which contain the azole fungicides, was introduced in the mid 1970s. Azoles are used on many different types of plants, including fields of crops, fruit trees, small fruit, vegetables and turf. These fungicides are highly effective against different fungal diseases, especially powdery mildews, rusts, and fungi/fungal including leaf-spotting. Fungicides can be toxic to humans and contaminated food is consumed by people. It is important to monitor the use of fungicides. Multi-residue approaches, with low quantification limits, are a rapid methodological answer. There are some researchers who have published papers related to fungicides' residues analysis in different substrates [9, 10]. The representative structures are presented in Figure-1.

To our knowledge, no analysis methods were developed for simultaneous determination of azoles in fish samples. The present studied azole group fungicides have lesser water solubility and a higher partition coefficient. In this view, there are chances for the residues to accumulate in edible parts. In this study, twelve azole-type fungicides (Tetraconazole, Penconazole, Tricyclazole, Paclobutrazole, Hexaconazole, Diniconazole, Propiconazole,

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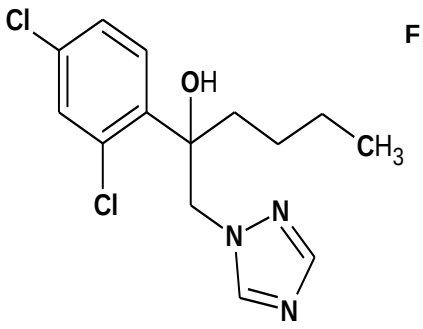
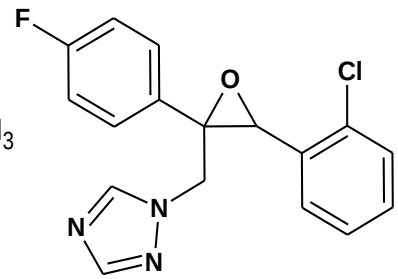
Tebuconazole, Epoxyconazole, Etoxazole, Fluquinconazole, Difenconazole) were simultaneously analyzed and quantified in fish samples. The aim of the present work was to develop and validate the simultaneous determination of azoles in fish samples using GC-EI-MS.

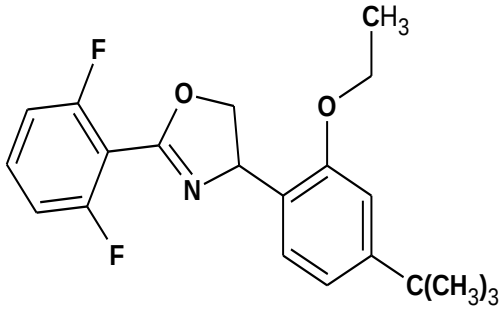
Figure.1 Molecular structures of multiresidual fungicides



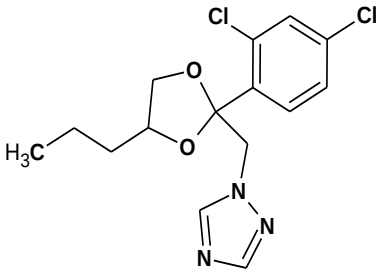
Hexaconazole



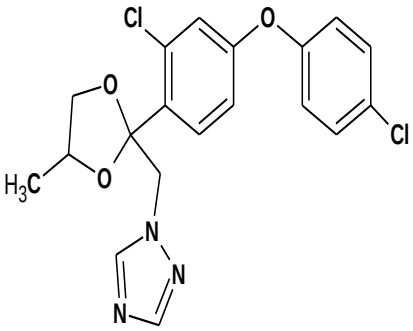
Epoxiconazole



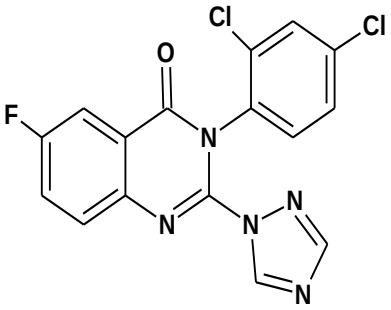
Etoxazole



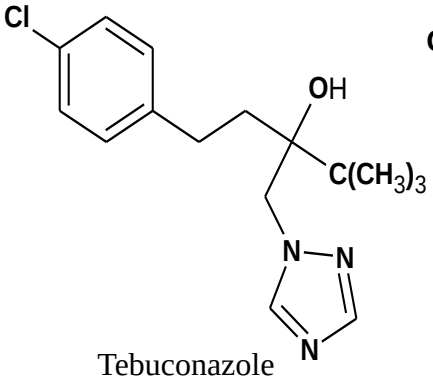
Propiconazol



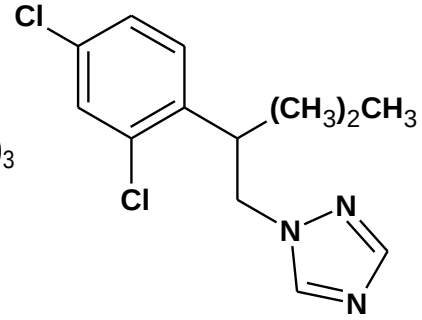
Difenoconazole



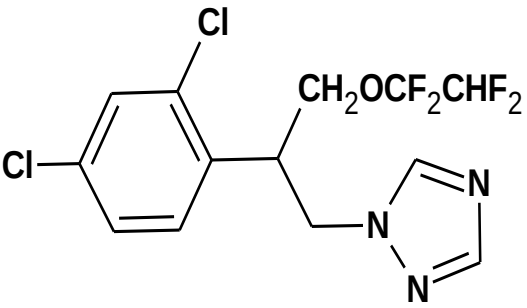
Fluquiniconazole



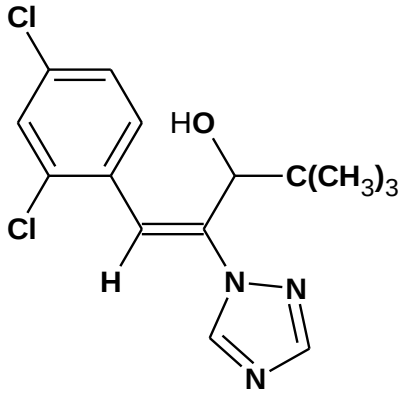
Tebuconazole



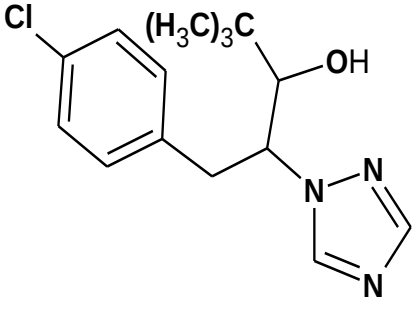
Penconazole



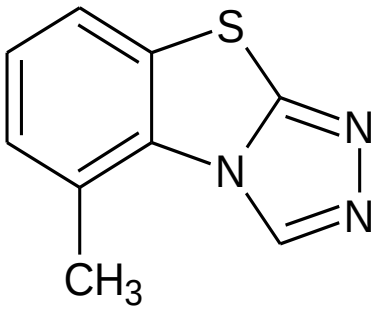
Tetraconazole



Diniconazole



Palcobutrazole



Tricyclazole

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MATERIALS AND METHODS

The azole group of fungicides analyzed in the current study included: Tetraconazole, Penconazole, Tricyclazole, Paclobutrazole, Hexaconazole, Diniconazole Propiconazole, Tebuconazole, Epoxyconazole, Etoxazole, Fluquinconazole and Difenconazole (Sigma Aldrich, USA). The purities were 97.5% - 99.8% as certified by manufacturer. Acetone, Hexane, Florisil, anhydrous sodium sulphate were purchased from Merck Chemicals Ltd, Mumbai. Fish samples (Common carp) were obtained from the local market at Padappai, Chennai.

Preparation of Calibration Solutions

Stock solutions were prepared separately by dissolving aliquots of reference analytical standards of the twelve triazoles fungicides in HPLC grade Acetone. Calibration solutions were prepared with acetone using appropriate volumes of each standard stock solution.

Soxhlet Extraction Procedure

The fish samples (common carp) were ground in a blender to obtain a homogeneous composite sample. A 10 g of the sample was weighed and wrapped in aluminum foil. Samples were kept in the freezer for one hour prior to freeze-drying. The samples were freeze dried using liquid nitrogen and ground using mortar and pestle and stored in pre-cleaned glass bottles for soxhlet extraction. About 5 g of homogenized fortified fish samples were wrapped in a filter paper. The wrapped fish samples were placed in a cellulose extraction thimble. The fish samples were extracted with 75mL of hexane: acetone (3:1) mixture for about 2 hours by soxhlet extraction. After extraction, the extract was concentrated to dryness on a Buchi rotary evaporator fitted to a vacuum pump. The residues were re-dissolved in 10 mL of hexane for column clean-up.

A glass column was packed with 1g of silica gel slurry prepared with n-Hexane in between two layers (1 cm) of anhydrous sodium sulphate. The concentrated extract was poured on the top of the column and eluted with 20 mL of n-Hexane. The collected eluate was concentrated and diluted with 1 mL of acetone and quantified with GC-MS.

Instrumentation

Gas chromatograph-Mass spectrometry

GC-MS analysis was performed using Shimadzu GC MS-QP5000 (Shimadzu, Japan). The HP-1 MS capillary column (30m x 0.25mm i.d with 0.1 µm film thickness) was used for separation. Injection was carried out in the split mode (5:1) at an injector temperature of 290°C. Helium gas was used as a carrier gas with a flow rate of 1.0 mL/min. The column temperature was maintained at 160°C for 13 min and then programmed at 10°C min-1 to 200°C for 5 min followed by a final ramp to 290°C at a rate of 50°C min-1, and held for 6 min. The ion source and transfer line temperature were 300°C respectively. An injection volume of 1.0 µL was used. All the samples were analyzed in Electron Impact Ionization (EI) mode.

RESULT AND DISCUSSION

The analytical method for the determination of azole group fungicides in fish should present high detectability to allow detection of low quantities of analytes. GC coupled with MS is fairly appropriated for azole group of fungicides. A simple and suitable gas chromatographic mass spectrometry method for the azole group fungicides was developed in fish samples. The analytical conditions were selected after testing the different parameters such as column types, injector, detector, column temperatures and other chromatographic and samples preparation conditions.

Method Validation

Within the laboratory, validation was performed to evaluate the analytical performances of GC-MS according to the following criteria: Specificity, Linearity, assay accuracy, limit of determination (LOD) and Limit of Quantification (LOQ) in spiked fish samples. For validation, the SANCO guideline was used SANCO/12495/2011, 2012 [8].

Specificity

Specificity was evaluated by analyzing fish samples to ensure that nothing interfered with the target analytes. Blank fish samples were extracted by soxhlet and the extracts submitted to chromatographic analysis. There were no significant matrix peaks observed in the retention times. A typical chromatogram of blank, under experimental conditions, is presented in Figure 2.

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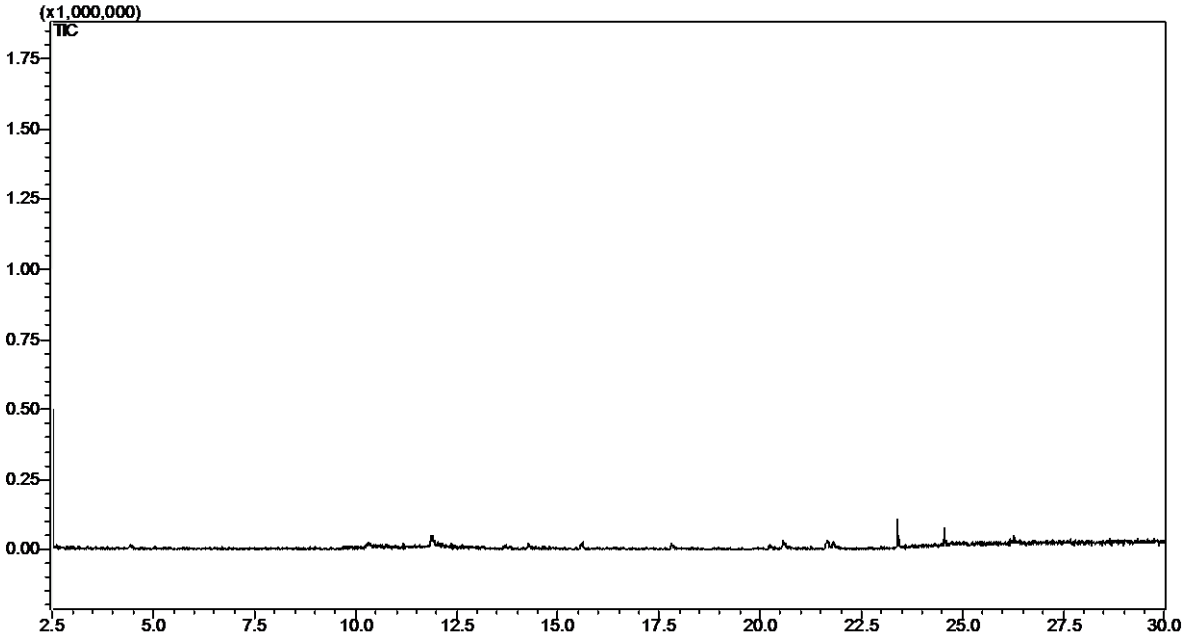
Figure : 2 Representaive Chromatograph - Blank



Linearity

Six calibration solutions (1.0, 0.5, 0.1, 0.01, 0.005 and 0.001 µg/mL) were prepared for construction of calibration curves by combining various volumes of the standard stock solution. Calibration curves were constructed by linear regression of the peak area versus concentration. The limit of determination (LOD) was determined based on the signal to noise ratio of 3:1. Linearity was investigated by the evaluation of the linear regression and it was expressed by the coefficient of correlation (r). Representative retention time and molecular mass are presented in Table 1. A typical chromatogram of standard is presented in Figure 3.

Table 1. The azole fungicides, retention times, Base Peaks and Fragments

S.No	Name of the Compound	Molecular Mass (m/z)	Retention Time	Base Peak (m/z)	Fragments (m/z)
1	Tetraconazole	372	10.3	336	101, 171
2	Penconazole	284	11.9	159	115,248
3	Tricyclazole	189	13.8	109	118,162
4	Paclobutrazole	293	14.3	336	101,167
5	Hexaconazole	314	15.6	83	111,214
6	Diniconazole	326	17.8	70	114,165
7	Propiconazole	342	20.3	69	111,173
8	Tebuconazole	307	20.6	125	103,250
9	Epoxyconazole	329	21.7	192	108,209
10	Etoxazole	359	23.4	141	115,161
11	Fluquinconazole	376	24.6	340	108,241
12	Difenconazole	342	26.3	205	139,230

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Figure : 3 Representaive Chromatograph of 12 Azole fungicides–Standard–0.01 µg/mL

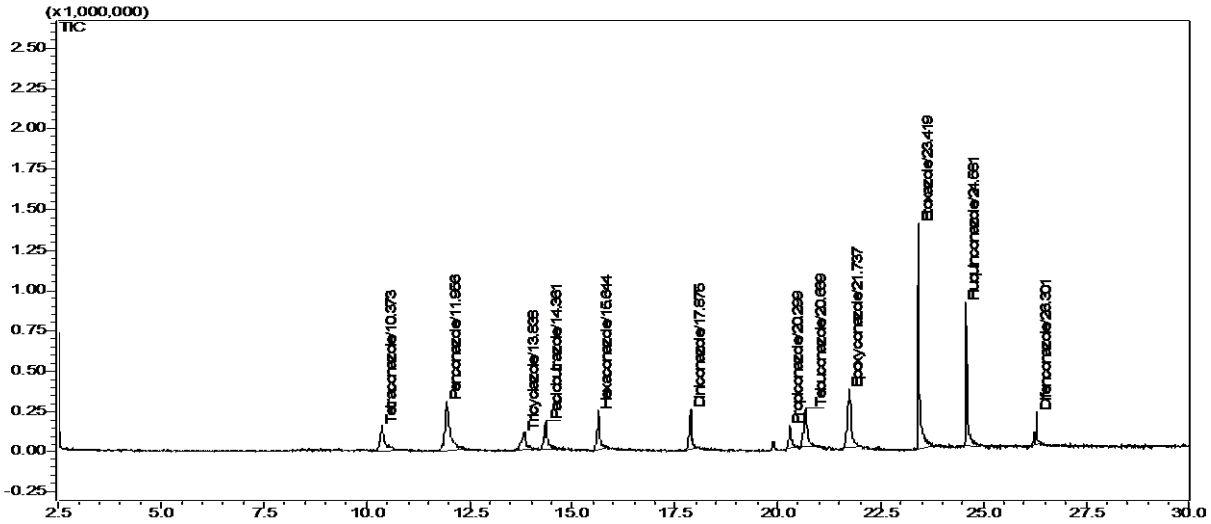

Table 2: The recovery and relative standard deviations (n=5) for spiked fish samples at two different concentration levels of fungicides from fish samples

Fungicides	Spiked (µg/g)	Fish RR (%)	Fish (n=5) (%)
Tetraconazole	0.005	86.1	3.1
	0.05	89.3	1.7
Penconazole	0.005	88.7	3.4
	0.05	91.7	2.7
Tricyclazole	0.005	86.7	2.4
	0.05	91.3	2.3
Paclobutrazole	0.005	88.0	2.3
	0.05	91.7	2.7
Hexaconazole	0.005	90.7	3.4
	0.05	91.7	2.7
Diniconazole	0.005	88.3	3.5
	0.05	93.1	1.9
Propiconazole	0.005	85.7	2.4
	0.05	92.3	2.7
Tebuconazole	0.005	87.3	2.4
	0.05	93.7	1.6
Epoxyconazole	0.005	88.7	2.3
	0.05	93.4	1.1
Etoxazole	0.005	84.3	2.5
	0.05	89.4	2.2
Fluquinconazole	0.005	86.7	2.9
	0.05	91.1	2.2
Difenconazole	0.005	87.8	3.3
	0.05	93.7	1.6

RR = Relative Recovery

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Limit of Quantification (LOQ)

The limit of quantification (LOQ) of the method was defined as the lowest concentration that could be determined with accuracy and precision below 10% over five analytical runs and it was obtained using fish samples.

Assay Accuracy and Precision

Recovery studies in fish samples were conducted by fortifying different concentrations of standard solutions (0.005 µg/g and 0.05 µg/g) of analytes. For the repeatability analysis, five replicated determinations were made at each concentration level. After fortification of standards, the samples were homogenized as per extraction procedure and analyzed GC-MS. The RSD% for each concentration was calculated. The recovery details and relative standard deviation details are presented in Table 2.

Real Sample Analysis

Three Batches of fish samples were collected from the local market at Padappai, Chennai. The samples were pretreated as described in the sample preparation, extracted using the soxhlet procedure and analyzed by GC-MS. The results are summarized in Table 3. It is revealed that the recommended method could be applied for the trace analysis of selected fungicides in real fish samples.

Table 3: Concentrations of twelve fungicides in three batches of fish in real samples

Fungicides Founded	Fish - (µg/g)		
	Batch-1	Batch-2	Batch-3
Tetraconazole	ND	ND	ND
Penconazole	ND	ND	ND
Tricyclazole	ND	ND	ND
Paclobutrazole	ND	ND	ND
Hexaconazole	ND	0.005	ND
Diniconazole	ND	ND	ND
Propiconazole	0.05	ND	ND
Tebuconazole	ND	ND	ND
Epoxyconazole	ND	ND	ND
Etoxazole	ND	ND	ND
Fluquinconazole	ND	ND	ND
Difenconazole	ND	ND	0.005

ND- Not Detected

CONCLUSION

A Soxhlet freeze – dried extraction, followed by gas chromatography and mass spectrometry with electron impact ionization (GC-EI-MS), was successfully developed and validated for the simultaneous 12 azole fungicides (Tetraconazole, Penconazole, Paclobutrazole, Hexaconazole, Diniconazole, Tricyclazole, Propiconazole, Tebuconazole, Epoxyconazole, Etoxazole, Fluquinconazole, Difenconazole) in fish. A complete and efficient cleanup step was developed to avoid carry-over and to ensure that the successive applications in real fish samples. Good analytical performances were attained for all studied pesticides, including an excellent linear dynamic range and a

suitable precision. The proposed method fits the requirement for the determination of selected fungicides in real fish samples.

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