

Research Article

Monitoring of fungicide residues in betel leaf collected from different locations of Bangladesh

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ABSTRACT

An analytical method using Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) extraction technique and Gas Chromatography (GC) coupled with Electron Capture Detector (ECD) was used for the determination of 2 fungicide (difenoconazole and thiram) residues in betel leaf samples collected from different locations (Barishal, Gazipur and Rajshahi) of Bangladesh. The analytical method used in this study was validated by evaluating the accuracy, precision, limit of detection (LOD), limit of quantification (LOQ) and linearity. The accuracy was found acceptable, ranging from 91.33% - 99.16% with an acceptable precision ($\leq 6.58\%$). A total of 61 real samples were analyzed in this study. Among the analyzed samples of betel leaf, 5 were found to contain residues of difenoconazole and thiram, ranged from 0.110 mg/kg to 0.648 mg/kg. The level of detected thiram residues (0.110-0.210 mg/kg) were above maximum residue limits (MRLs) and the level of difenoconazole residues (0.360-0.648 mg/kg) were below MRLs. This study provides an overview of fungicide residues contamination in betel leaf samples collected from different locations across Bangladesh.

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INTRODUCTION

The betel plant is a perennial evergreen vine with shiny, heart-shaped leaves and white catkin flowers. It is originated in central and eastern Malaysia and gradually spread across tropical Asia, eventually reaching Madagascar and East Africa. In India, it is commonly grown in states such as Tamil Nadu, Madhya Pradesh, West Bengal, Maharashtra, and Uttar Pradesh. Today, betel cultivation is especially widespread in India, Bangladesh, and Sri Lanka (DAWN 2002). Betel leaves are commonly used as a stimulant, antiseptic and natural breath freshener. They are also valued for their medicinal benefits and are traditionally used to help treat conditions such as difficulty or reduced urination,

headaches, constipation, sore throats, and issues related to breast milk secretion (Palaniappan *et al.*, 2012).

The major disease of betel leaf is foot rot, leaf rot and anthracnose. Foot and leaf rot is the most prevalent disease affecting betel leaf cultivation (DAWN 2002). To minimize crop losses and preserve leaf quality, farmers frequently apply chemical fungicides. However, many farmers do not adhere to the recommended Pre-Harvest Interval (PHI), as a result, fungicide residues often remain on the harvested leaves. The presence of fungicide residues in betel leaf is a major concern for the consumer's as the leaves are chewing directly without any household process even without any wash with clean water. In order to protect consumer's health and also fulfill the consumer's demand of safe food,

fungicides should be used following Good Agricultural Practice (GAP). Monitoring of fungicide residues is the essential tool to ensure GAP. To monitor fungicide residues nationally in the betel leaves, an accurate and efficient analytical method is required. In this study, 2 fungicides (difenoconazole and thiram) were selected.

To determine pesticide residues in betel leaf and other matrices Gas Chromatography (GC), Gas Chromatography associated with Mass Spectrometry (GCMS), High Performance Liquid Chromatography (HPLC), and Liquid Chromatography associated with Mass Spectrometry (LC-MS) are the most commonly used techniques. In Bangladesh, for the analysis of pesticide residues researchers mostly depends on Gas Chromatography (Hoque *et al.*, 2022, Hossain *et al.*, 2014, Islam *et al.*, 2019, 2019a), and in a very few cases the researchers using LC-MS/MS for the analysis of pesticide residues (Prodhan *et al.*, 2022).

In Bangladesh, determination of fungicide residues in different matrices has scarcely been investigated. Several research work has been conducted with mostly organophosphorus insecticides (Aktar *et al.*, 2017, Tasnim *et al.*, 2022, Parven *et al.*, 2021, Parvin *et al.*, 2021, Hasan *et al.*, 2017, Habib *et al.*, 2021, Prodhan *et al.*, 2018) and synthetic pyrethroid insecticides (Islam *et al.*, 2014, Prodhan *et al.*, 2021) in a wide variety of matrices. There are also several research work was found with organochlorine insecticides (Prodhan *et al.*, 2018a) and carbamate insecticides residues (Kabir *et al.*, 2007; 2008). To the best of our knowledge in Bangladesh, none of the research work has been conducted for the determination of fungicide residues in betel leaf. Therefore, the present study was initiated to develop an analytical method for the determination of fungicide residues in betel leaf using QuEChERS extraction and Gas Chromatography.

MATERIALS AND METHODS

Preparation of fungicide standard solution

Pesticide standard stock solutions of difenoconazole and thiram were prepared separately in acetonitrile (ACN) at a concentration of 1000 mg/L and stored at -20°C until use. A mixed standard solution of 50 mg/L in ACN containing all the aforementioned fungicides was prepared by adding the appropriate volume of each individual stock solution in a 50 ml volumetric flask and made to volume by addition of ACN. An intermediate mixed standard solution of 10 mg/L in ACN was prepared from the mixed standard solution of 50 mg/L. Then working standard solutions of 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, and 5.0 mg/L in ACN were prepared by transferring the appropriate amount from 10 mg/L intermediate mixed standard solution into seven separate 10-mL volumetric flasks. All the standard solutions were kept in a freezer at -20°C until use.

Extraction and clean up

QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction technique is widely used extraction and clean up technique for fungicide residue analysis in food matrices. This technique was first introduced by Anastassiades *et al.* (2003), which is gaining popularity day by day compared to the other existing technique.

In this study, this extraction and cleanup technique was slightly modified based on the previous study (Prodhan *et al.*, 2015; 2016). The chopped samples were grounded thoroughly with the fruit blender. A representative 10 g portion of thoroughly homogenized sample was weighted in a 50 mL polypropylene centrifuge tube. Then 10 mL of acetonitrile (ACN) was added into the centrifuge tube. The centrifuge tube was closed properly and shaken vigorously for 30 s by the use of a vortex mixer. Then, 4 g of anhydrous magnesium sulfate (MgSO₄) and 1 g of sodium chloride (NaCl) were added into the centrifuge tube, and it was shaken immediately by the vortex mixer for 1 minute to prevent the formation of magnesium sulfate aggregates. Afterwards, the extract was centrifuged for 5 min at 5000 rpm. An aliquot of 3 mL of the ACN layer was transferred into a 15 mL micro centrifuge tube containing 600 mg anhydrous MgSO₄, 150 mg charcoal activated powder, and 120 mg Primary Secondary Amine (PSA). Then it was thoroughly mixed by vortex for 30 s and centrifuged for 5 minutes at 4000 rpm. (Laboratory Centrifuges, Sigma-3K30, Germany). After centrifugation, a 1 mL supernatant was filtered by a 0.2 µm PTFE filter, and then it was taken in a clean GC vial for injection.

Detection and quantification of pesticide residue in samples

The concentrated extracts were subjected to analysis by GC-2010 (Shimadzu) with Electron Capture Detector (ECD). The separation was done by RTX-CL capillary column (30 m long, 0.25 mm i.d and 0.25 µm film thicknesses), nitrogen was used as carrier (column flow 1.92 mL/min.) and make up gas as well. The temperature for the injector and the detector was set to 280°C and 300°C, respectively, and for the column oven the temperature was programmed, the detailed programming of column oven temperature are presented in Table 1 and 2. Split mode was used for the injection of samples (1 µL). The total run time for difenoconazole and thiram was 9 minutes. Identification was performed by comparing the retention time of the prepared matrix matched standard and the quantification was done using the calibration curve prepared with matrix matched calibration standard.

Table 1: Conditions of column oven temperature for difenoconazole determination

Column oven	Rate	Temperature (°C)	Hold time (min)
Initial	-	180	0
temperature: 180°C	10	210	1
	5	230	1

Table 2: Conditions of column oven temperature for thiram determination

Column oven	Rate	Temperature (°C)	Hold time (min)
Initial	-	220	0
temperature: 220°C	5.0	250	3

RESULTS AND DISCUSSION

Quality assurance

The validation was done following the European Commission guidelines: SANTE/11312/2021 (EC 2021). During this study, recovery tests were conducted on betel leaf samples. The fungicides free collected samples were cut into small pieces and stored at -20°C until homogenization. Homogenization was done by fruit blender. A 10 g homogenized sample was spiked prior to the determination procedure by the addition of a mixed pesticide standard working solution to reach the final fortification levels of 0.10 mg/kg. After the addition of each concentration in the matrix, the mixture was equilibrated by shaking and the samples were allowed to settle for 30 minutes prior to extraction in order to ensure the sufficient contact of the analytes with the whole matrix. Then the samples were prepared according to the method which was described earlier. Precision in case of Repeatability (RSD_r) was determined at the same fortification levels of 0.10 mg/kg on the same day.

The average recoveries and the relative standard deviations are presented in Table 3. A very good accuracy and precision was found for both of the selected pesticides. The accuracy was 99.16% for difenoconazole, and it was 91.33% for thiram. The precision was 6.58% and 4.93% for difenoconazole and thiram, respectively. The limit of quantification (LOQ) was set following the EC guidelines (SANTE/11312/2021), which was the lowest level of fortification for every fungicide providing satisfactory accuracy (average recoveries for individual analyte ranged from 70 to 120%) and precision ($\text{RSD}_r \leq 20\%$).

Table 3: Accuracy (%) and RSD (%) of the selected fungicides in betel leaf.

Pesticides	Accuracy (%)	Precision (% RSD)	LOD (mg/kg)	LOQ (mg/kg)	Linearity
Difenoconazole	99.16	6.58	0.003	0.01	0.997
Thiram	91.33	4.93	0.003	0.01	0.998

Monitoring of fungicide residues in real samples

The concentrated extracts of betel leaf samples collected from different markets were analyzed by GC-2010 (Shimadzu) with Electron Capture Detector (ECD) with the pre-set parameters. The level of detected fungicide residues

found in the analyzed samples and their maximum residue levels are outlined in Table 4. A representative chromatogram of few positive samples is presented in **Figure 1&2**.

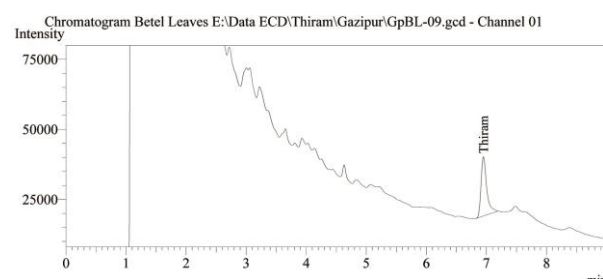


Figure 1: Chromatogram of thiram found in one of the contaminated sample of betel leaf (BtLGp-9) collected from Gazipur.

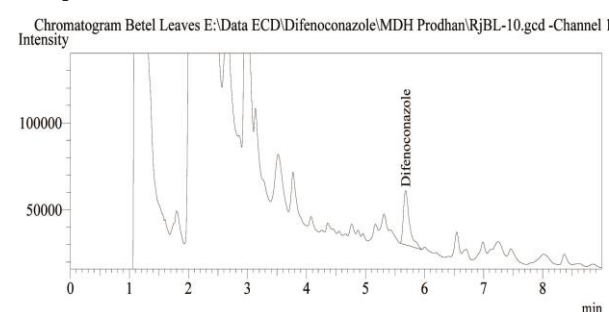


Figure 2: Chromatogram of difenoconazole found in one of the contaminated sample of betel leaf (RjBL-10) collected from Rajshahi.

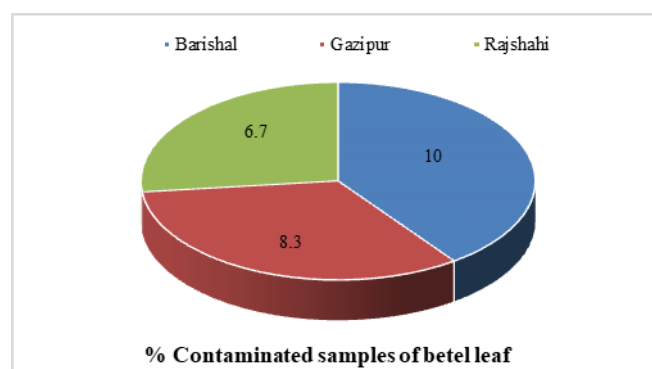


Figure 3: Comparison of contamination (%) in betel leaf samples collected from different areas of Bangladesh.

Table 4: Fungicide residue levels (mg/Kg) found in betel leaf samples collected from different market places in Bangladesh.

Area of collection	No. of analyzed samples	No. of contaminated samples	% contaminated sample	Detected fungicide	Range of residue levels (mg/Kg)	EU MRLs (mg/Kg)
Barisal	10	1	10.0	Difenoconazole	0.648	3.0
Gazipur	36	1	8.3	Difenoconazole	0.360	3.0
		2		Thiram	0.110-0.210	0.05
Rajshahi	15	1	6.7	Difenoconazole	0.580	3.0
Total	61	5	8.2			

A total of 61 samples were analyzed. Out of 61 samples, 5 (8.2 % of the total number of samples) contained detectable difenoconazole and thiram residues and 56 samples (91.8 % of the total number of samples) contained no detectable residues of the sought fungicides. The range of

difenoconazole residues were 0.360 to 0.648 mg/kg and the range of thiram residues were 0.110 to 0.210 mg/kg (Table 4). The detected difenoconazole residues were below MRLs set by European Union (EC 2021a) and the detected thiram residues were above the EU-MRLs. The findings of the

present study indicates that the growers of betel leaves are using difenoconazole and thiram for the control of different diseases of betel leaf.

The mean percentage of contaminated samples was 8.2% (Table 4). The findings of the present study are in a good agreement with [Prodhan et al. 2023](#) and they found that 10.9% samples of betel leaf were contaminated by different insecticides. The results of the present study are also supported by the findings of [Prodhan et al. 2021a](#). They found that 7.8% guava and mango samples were contaminated by different insecticides. It is good indications that in case of betel leaf, the percentage of contaminated samples is much more lower than the others crops grown commercially in Bangladesh. In case of yard long bean, the contamination was found 20% for yard long bean ([Tasnim et al., 2022a](#)), it was 17% for cauliflower ([Alam et al., 2023](#)), 22.5% for hyacinth bean ([Rahman et al., 2021](#)), 14% for tomato ([Nahar et al., 2020](#)), 13% for eggplant ([Nisha et al., 2021](#)), 11 % for pointed gourd ([Islam et al., 2021](#)), and 10% for cucumber ([Hasan et al., 2021](#)).

Based on area of collection, the highest contaminated samples (10% of the total number of analyzed samples) were collected from Barishal followed by Gazipur (8.3% of the total number of analyzed samples) and Rajshahi (6.7% of the total number of analyzed samples) (Figure 3). Among the 5 contaminated samples of betel leaves, 1 was collected from Barishal, 3 were from Gazipur and 1 was from Rajshahi (Table 4). A total of 10 samples were collected from Barishal. Out of 10 samples, 1 was contaminated with difenoconazole residue. The level of detected difenoconazole residue was 0.648 mg/kg, which was below MRLs. In case of Gazipur, 3 samples were contaminated with difenoconazole and thiram residues among the 10 analyzed samples. One sample was contaminated with difenoconazole residue and the level of detected difenoconazole residue was 0.360 mg/kg. Two samples were contaminated with thiram residues and the level of detected thiram residues were 0.110 and 0.210 mg/kg. In case of Rajshahi, 1 sample was contaminated with difenoconazole residue among the 10 analyzed samples. The level of detected difenoconazole residue was 0.580 mg/kg. From the above findings, it can be concluded that the farmers of Barishal district are using higher amount of fungicides than the others area of Bangladesh.

Food safety has become a major concern for the consumers, in case of betel leaves, it is more crucial as betel leaves are consumed directly without any processing. Therefore, these findings indicates that more attention should be given by the policymakers along with relevant stakeholders to take appropriate measures to regulate and reduce the indiscriminate use of chemical fungicides in betel leaf cultivation for disease management. Furthermore, the use of bio-fungicides is strongly recommended as a safer alternative to harmful chemical fungicides for controlling major diseases of betel leaves.

CONCLUSION

An efficient analytical method for the quantification of difenoconazole and thiram residues has been developed using GC coupled with Electron Capture Detector (ECD) and validated by evaluating the accuracy, precision, linearity, limit of detection (LOD) and limit of quantification (LOQ).

A very good accuracy (average recoveries ranged from 91.33% to 99.16%) and precision ($RSD_r \leq 6.58\%$) was found for both of the analytes. This method was applied successfully to analyze selected fungicide residues in 61 betel leaf samples. Among the analyzed samples of betel leaf, 5 (8.2 % of the total number of samples) contained detectable difenoconazole and thiram residues and 56 samples (91.8 % of the total number of samples) contained no detectable residues of the sought pesticides. The range of difenoconazole residues were 0.360 to 0.648 mg/kg and the range of thiram residues were 0.110 to 0.210 mg/kg. The detected difenoconazole residue was below MRLs set by European Union ([EC, 2021](#)) and the detected thiram residues were above the EU-MRLs. Therefore, the proposed method described in this study can be used satisfactorily to analyze fungicide residues in betel leaf. The findings of this study will contribute to enhancing public awareness and assist policymakers in adopting appropriate measures to reduce pesticide residue levels in betel leaves. Furthermore, the proposed method will be highly beneficial for scientists and analysts who are interested in the determination of pesticide residues in betel leaf and similar leafy commodities.

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