Scholars Academic Journal of Biosciences (SAJB) **ISSN 2321-6883 (Online)**
Sch. Acad. J. Biosci., 2016; 4(10B):911-916 **ISSN 2347-9515 (Print)**

(An International Publisher for Academic and Scientific Resources)
www.saspublishers.com DOI : 10.36347/sajb.2016.v04i10.020

Original Research Article

A New Gas Chromatographic Method for Determination of Difenoconazole Residues in Pomegranate Fruits

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Abstract: A simple and inexpensive method was developed using solid-phase extraction, together with gas chromatographic method for determination of difenoconazole residues in pomegranate fruits. The evaluated parameters include the extracts by deactivated acidic alumina chromatographic column using hexane + di ethyl ether (9:1 and 8:2, v/v) mixture, hexane and acetonitrile solvents. The method was validated using pomegranate fruit samples spiked with difenoconazole at different fortification levels (0.01 and 0.1 µg/g). Average recoveries (using each concentration six replicates) ranged 83-95%, with relative standard deviations less than 2%, calibration solutions concentration in the range 0.005-1.0 µg/mL and limit of detection (LOD) and limit of quantification (LOQ) were 0.005µg/g and 0.01µg/g respectively. Finally the pomegranate fruit residue samples were analyzed by GC.
Keywords: GC, Difenoconazole, pomegranate fruits, LOD and LOQ

INTRODUCTION

Difenoconazole was a triazole fungicide with proven bio-efficacy which leads to morphological and functional changes in the fungal cell membrane [1]. The chemical name of this product is 1-[2-[2-chloro-4-(4-chlorophenoxy)phenyl]-4-methyl-1,3-dioxolan-2-ylmethyl]-1H-1,2,4-triazole [2]. For original drug, it is white solid and solubility in water is 3.3mg/L.As well as that, this product is soluble in organic solvents. The mode of action is systemic poisoning and it also has protective and therapeutic effect [3]. As a product with high-level of safety, this product can be extensively used in fruit trees, vegetables and other crops [4, 5]. And the mechanism is to prevent the growth of fungi by inhibiting sterol biosynthesis of cell wall. In the present study, the determination of difenoconazole residues in pomegranate fruits followed by solid phase extraction and new validated GC method.

Various methods have been described for the determination of these residues, using solid-phase micro extraction (SPME) Supercritical fluid extraction (SFE) and liquid – liquid extraction [6]. However, none of the published researches to date have reported the residue analysis of difenoconazole in pomegranate fruit.

MATERIALS AND METHODS

Standards, Reagents and samples

The analytical standard of difenoconazole (99.2%) was obtained from Sigma Aldrich. HPLC grade acetonitrile and water was purchased from rankem, analytical grade solvents i.e., hexane, diethyl ether, acetone and acidic alumina were supplied from Merck Limited and pomegranate fruits were purchased from local market.

Standard stock solutions

The difenoconazole stock solutions was individually prepared in acetonitrile at a concentration level 500 µg/g and stored in a freezer at -18°C. The stock standard solutions were used for up to 3 months. Suitable concentrations of working standards were prepared from the stock solutions by dilution using acetonitrile, immediately prior to sample preparation.

Sample preparation

Representative 100.0 gram portions of pomegranate fruit fortified with 0.1 mL of working standard stock solution. The sample was allowed to stand at room temperature for one hour, before it was kept at refrigerator condition, until analysis.

Extraction for pomegranate fruit

The representative sample 25g of pomegranate fruit and 10 g of grains was taken in a 500ml stoppered conical flask and extracted with 100 ml acetonitrile using an end-over-end shaker for about 30 minutes and filtered. Extraction was repeated twice. The collected

filtrate was concentrated to 50ml at 50°C using rotary vacuum evaporator.

Partition

To the concentrated extract (50ml) 10ml of hexane was added. Shaken vigorously for 1 minute, discarded the hexane layer, concentrated acetonitrile to near dryness and recovered the residue in 3-5ml of hexane.

Extraction for pomegranate juice

25 ml of acetonitrile and 10 ml of hexane were added to the 10 ml of pomegranate juice. Shaken vigorously for 1 minute, discarded the hexane layer, concentrated acetonitrile to near dryness and recovered the residue in 3-5ml of hexane.

Clean up

A chromatographic column was prepared by using 10g of deactivated acidic alumina in hexane. The material in hexane was transferred into the column and washed the column with 10ml of hexane + diethyl ether (9:1) solvent mixture. Eluted the compound with 50ml of a mixture of hexane – ether (8:2 v/v). The eluate was concentrated to dryness, recovered in acetone and analysed by GC-ECD method.

Instrumentation

GC-ECD Separation parameters

Instrument	:Shimadzu GC-2014 gas chromatograph system connected with GC Solution software system.
Detector	:Electron capture detector (ECD)
Column used	:DB-5- capillary column length 30 meters, I.D – 0.53mm and 1.0µm film thickness.
Gas flow rate Nitrogen	:30 ml/minute
Split injection mode	:Split/purge ratio – 1:3; Makeup gas – 35 ml/min.
Injected Volume	:1.0 µl
Temperature conditions	
Oven	:310 °C
Injector	:305 °C
Detector	:320°C
Retention time (Approximately)	
Difenoconazole	:4.1 minutes

Method validation

Method validation ensures analysis credibility. In this study, the parameters accuracy, precision, linearity and limits of detection (LOD) and quantification (LOQ) were considered [7]. The accuracy of the method was determined by recovery tests, using samples spiked at concentration levels of 0.01 and 0.1 µg/g. Linearity was determined by different known concentrations (0.005, 0.01, 0.05, 0.1, 0.5 and 1.0 µg/mL) were prepared by diluting the stock solution. The limit of detection (LOD µg/g) was determined as the lowest concentration giving a response of 3 times the baseline noise defined from the analysis of control (untreated) sample. The limit of quantification (LOQ µg/g) was determined as the lowest concentration of a given fungicide giving a response of 10 times the baseline noise.

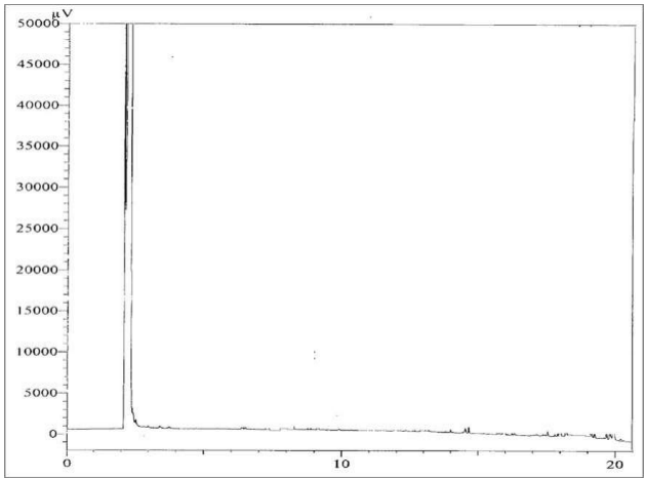

Fig-1: Representative Chromatogram at pomegranate fruit control

RESULTS AND DISCUSSION

Specificity

Aliquots of difenoconazole, control sample solution, extracted solvents and mobile phase solvents were assayed to check the specificity. There were no matrix peaks in the chromatograms to interfere with the analysis of residues shown in (Figure 1 and 2). Furthermore, the retention time of difenoconazole was 5.5 min (Approximately).

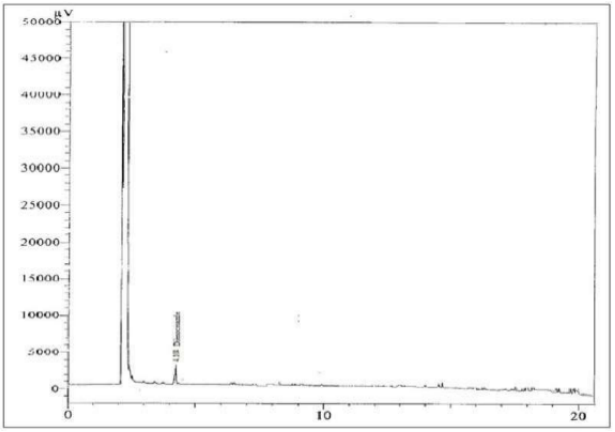

Fig-2: Representative Chromatogram at fortification level of 0.01 µg/g

Linearity

50.40 mg of difenoconazole reference standard was taken into 50 mL volumetric flask and dissolved in acetonitrile, sonicated and made upto the mark with the same solvent. The concentration of the stock solution was 1000 µg/mL. From this stock solution prepared by different known concentrations of standard solutions (0.005, 0.01, 0.05, 0.1, 0.5 and 1.0 µg/mL) were prepared into different 10 mL volumetric flasks and made upto the mark with acetone. The serial dilution details were presented in Table 1. These standard solutions were directly injected into a GC. A calibration curve has been plotted of concentration of the standards injected versus area observed and the linearity of method was evaluated by analyzing six solutions. The calibration details were given in Table 2. The peak areas obtained from different concentrations of standards were used to calculate linear regression equation. This was Y=105221X + 17.65 with correlation coefficient of 1.0000 respectively. A calibration curve showed in (Figure 3).

Table 1. Serial dilutions of linearity standard solutions

Stock solution concentration (µg/mL)	Volume taken from stock solution (mL)	Final make up volume (mL)	Obtained concentration (µg/mL)
1000	0.100	10	10.0
10	1.000	10	1.0
10	0.500	10	0.5
10	0.100	10	0.1
1	0.5	10	0.05
1	0.1	10	0.01
0.1	0.5	10	0.005

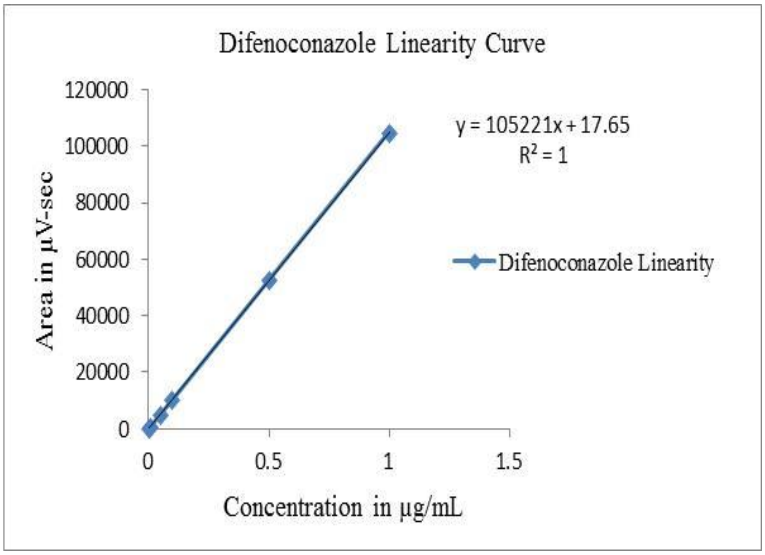

Fig-3: Representative Calibration curve of difenoconazole

Accuracy and Precision

Recovery studies were carried out at 0.01 and 0.1 µg/g fortification levels for difenoconazole in pomegranate fruit. The recovery data and relative standard deviation values obtained by this method are summarized in Table 2.

Table 2: Recoveries of the difenoconazole from fortified pomegranate fruit control sample (n=6)

Fortification Concentration in µg/g	Replication	Recovery (%)
0.01	R1	81
	R2	83
	R3	84
	R4	85
	R5	84
	R6	82
	Mean	**83.17**
	STDEV	**1.47**
	RSD in %	**1.77**
0.1	R1	92
	R2	94
	R3	93
	R4	94
	R5	95
	R6	94
	Mean	**93.67**
	STDEV	**1.03**
	RSD in %	**1.10**

These numbers were calculated from four (6) replicate analyses of given sample (difenoconazole) made by a single analyst on one day. The repeatability of method satisfactory (RSDs<2 %).

Detection and Quantification Limits

The limit of quantification was determined to be 0.01 µg/g. The quantitation limit was defined as the lowest fortification level evaluated at which acceptable average recoveries (83-94%, RSD<2%) were achieved. This quantitation limit also reflects the fortification level at which an analyte peak is consistently generated at approximately 10 times the baseline noise in the chromatogram [8, 9]. The limit of detection was determined to be 0.01 µg/g at a level of approximately three times the back ground of control injection around the retention time of the peak of interest.

Storage Stability

A storage stability study was conducted at refrigerator condition (5 ± 3°C) and Ambient temperature (25 ± 5°C) of 0.1 µg/g level fortified fruit samples were stored for a period of 30 days at this temperature. Analysed for the content of difenoconazole before storing and at the end of storage period. The percentage dissipation observed for the above storage period was only less than 3% for difenoconazole showing no significant loss of residues on storage. The results are presented in Table 3 and 4.

Table 3: Storage stability Details at refrigerator condition (5 ± 3°C)

Fortification Concentration in µg/g	Storage Period in Days	Recovery in %
0.1	0	97
		95
		95
		94
		95
		94
	Average	**95.00**
	STDEV	**1.10**
	RSD in %	**1.15**
	30	93
		92
		93
		93
		92
		93
	Average	**92.67**
	STDEV	**0.52**
	RSD in %	**0.56**

Table 4: Storage stability Details at ambient Temperature (25 ± 2°C)

Fortification Concentration in µg/g	Storage Period in Days	Recovery in %
0.1	0	95
		94
		92
		91
		93
		94
	Average	93.17
	STDEV	1.47
	RSD in %	1.58
	30	91
		90
		91
		93
		92
		93
	Average	91.67
	STDEV	1.21
	RSD in %	1.32

CALCULATIONS

The concentration of acetaminophen in the samples analyzed by HPLC was determined directly from the standard curve.

$$Y = mx + c$$

Where,
Y = peak area of standard (mAU*sec)
m = the slope of the line from the calibration curve
x = concentration of injected sample (mg/L)
c = 'y' intercept of the calibration curve

The recovered concentration or Dose concentration was calculated by using the formula:

$$\text{Recovered concentration or Dose concentration} = \frac{(x-c) \text{ X } D \text{ X } 100}{m \text{ X } P}$$

Where,
m = the slope of the line from the calibration curve
x = sample area of injected sample (mAU*sec)
c = 'y' intercept of the calibration curve
D = Dilution Factor
P = Purity of Test item

$$\% \text{ Recovery} = \frac{\text{Recovered Concentration}}{\text{Fortified Concentration}} \times 100$$

CONCLUSIONS

This paper describes a fast, simple sensitive analytical method based on GC-ECD to determine the difenoconazole residues in pomegranate fruit. The SPE extraction procedure is very simple and inexpensive method for determination of difenoconazole residues in pomegranate fruit. Satisfactory validation parameters such as linearity, recovery, precision and LOQ were established by following South African National Civic Organization (SANCO) guidelines [10]. Therefore, the proposed analytical procedure could be useful for regular monitoring, residue labs and research scholars to determine the difenoconazole residues in different commodities (fruit, juice, seed, oil, and water and soil samples).

ACKNOWLEDGEMENT

The authors are thankful to the Dr. Gowtham Prasad, S.V.V University, Hyderabad for his keen interest and help.

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