Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Gas chromatographic method for the determination of hexaconazole residues in black tea*

Chinnachamy KARTHIKA†, Paul James SACHIN

(*United Planter's Association of South India (UPASI) Tea Research Foundation, Tea Research Institute, Nirar Dam Branch Post Office (BPO), Valparai 642127, Coimbatore District, Tamil Nadu, India*)

†E-mail: kartheecm@rediffmail.com

Received Nov. 24, 2007; revision accepted Dec. 24, 2007; published online Jan. 16, 2008

Abstract: A highly reliable, quantitative and sensitive analytical method for determining the residues of the fungicide, hexaconazole in black tea is described. The proposed method is based on liquid-liquid extraction followed by gas chromatographic determination, using nitrogen phosphorus detector (GC-NPD) for the identification and quantitation of hexaconazole. The most appropriate solvent mixture for extracting hexaconazole residues from black tea was *n*-hexane:acetone at 1:1 (v/v). The extract was cleaned up by adsorption column chromatography using activated florisil. Performance of the method was assessed by evaluating quality parameters such as recovery value, repeatability, reproducibility, linearity and limits of detection and quantitation. When the method was assessed for repeatability, the percentage of recovery ranged between 86% and 96% while the relative standard deviation was between 0.30% and 2.35%. In studies on reproducibility the recovery ranged from 81% to 85% and relative standard deviation from 1.68% to 5.13%, implying that the method was reliable. A field trial was conducted to verify the application of this method with real samples. Results prove that the validated method was suitable for extracting hexaconazole residues.

Key words: Hexaconazole, Residues, Black tea, Florisil, Gas chromatography-nitrogen phosphorus detector (GP-NPD)
doi:10.1631/jzus.B0720262 **Document code:** A **CLC number:** O657.7

INTRODUCTION

Hexaconazole [(R,S)-2-(2,4-dichlorophenyl)-1-(1H-1,2,4-triazole-1-yl-hexane-2-ol] is a systemic fungicide which is being used widely in India for controlling the blister blight (*Exobasidium vexans*) disease of tea (Premkumar and Baby, 2005).

Several multi-residue methods are available for the determination of residues of different triazoles in various food products, such as processed fruits, vegetables, grapes, must, wine and strawberries (Garland *et al*., 1999; Sannino, 2004; Zambonin *et al*., 2002) involving intensive sample preparation such as solid-phase extraction which is time consuming and labor intensive. The residues of these fungicides are analyzed by gas-liquid chromatography with nitrogen phosphorus detector (NPD) and electron capture detector (ECD), or by techniques such as liquid chromatography-tandem mass spectrometry for confirmation and quantitation (Bernal *et al*., 1997; Otero *et al*., 2003; Schermerhorn and Golden, 2005; Trosken *et al*., 2005). Data are available on the hexaconazole residues, but there is only limited information on the validation of the analytical method for its residues in black tea (Kumar *et al*., 2004; Manikandan *et al*., 2006). The objective of the present study is to give a detailed report on the simple and sensitive gas chromatography-nitrogen phosphorus detector (GC-NPD) method developed for the quantitation of hexaconazole residues in black tea, incorporating the validation parameters such as repeatability and reproducibility. Quality parameters such as precision, linearity and detection limits were also evaluated.

* Project supported by the National Tea Research Foundation, Kolkata, India

MATERIALS AND METHODS

Chemicals, solvents and reagents

Analytical standard of hexaconazole (purity 99.5%) was obtained from Dr. Ehrenstorfer (Augsburg, Germany) and the formulated product, Contaf 5EC (hexaconazole 5EC) from Rallis India Ltd., India. Dichloromethane, *n*-hexane, acetone and ethyl acetate required for liquid and gas chromatography were from Merck (Darmstadt, Germany), while other reagents such as sodium chloride and anhydrous sodium sulfate were from SD Fine Chemicals (India). Alumina (acidic, basic and neutral), florisil (60~100 mesh) and silica gel used as sorbents for purification through column chromatography were from M/s. Sigma-Aldrich, USA.

Stock standard solutions

A stock standard solution of hexaconazole (1000 mg/L) was prepared in acetone by weighing 0.025 g of the analyte into a 25 ml volumetric flask. Various concentrations of hexaconazole at 0.05, 0.1, 0.2, 0.4, 0.8 and 1.0 mg/L were prepared by serial dilutions from the stock solution for linearity study and to prepare calibration curve. The standard and various dilutions were stored in the dark at 4 °C.

Chromatographic columns

Glass columns (60 cm length×1.1 cm i.d.) with teflon stopcocks were packed from the bottom with a glass wool plug, and 10 g of various activated adsorbent materials were packed between two layers of anhydrous sodium sulfate.

Field trial for black tea preparation

A field trial was carried out during the wet season at United Planter's Association of South India's experimental farm at Valparai, Coimbatore District (1150 m above mean sea level), India, during the year 2006. There were 3 treatments each replicated in 6 plots (for harvest on 6 different days), each plot with 100 bushes. The plots were separated by guard rows. Hexaconazole formulation (Contaf 5EC) was sprayed at 200 ml/ha (the recommended dosage) and 400 ml/ha (double the recommended dose) through a hand operated knapsack sprayer with the recommended spray volume of 175 L water/ha. Untreated block was kept as control. About 2 kg of the green shoots (three leaves and a bud) were harvested from each plot and brought to the laboratory on the same day (3 h after spraying) and on Days 3, 5, 7, 10 and 14 after imposing treatments. The harvested shoots were separately manufactured as described below.

The leaves were processed in the Institute's miniature CTC (Crush, Tear and Curl) tea manufacturing unit as per standard practice (Ramasamy, 1996). The shoots were spread out in a withering trough and allowed to wither for 16~18 h. Withered leaves were then passed six times through a rotorvane for crushing and mixing leaves with the exuded juice which was then passed through a CTC machine. The cut "dhool" was then allowed to undergo fermentation for one hour by spreading it in a tray and maintaining a relative humidity of 90%~95%. The fermented "dhool" was dried in a mini-fluid bed drier to attain a final moisture content of 2%~3%. The drier mouth black tea samples were used for analysis.

Extraction and purification

Ten grams of control black tea sample was weighed in a 250 ml conical flask and 100 ml of *n*-hexane:acetone (1:1, v/v) was added, and then tightly sealed. The mixture was shaken on a mechanical shaker for 2 h. Supernatant was filtered through Whatman No. 1 filter paper and the contents of the flask were washed twice with 25 ml of the extracting mixture. The filtrate and the washings were pooled and transferred to a 500 ml separatory funnel. One hundred ml of saturated sodium chloride was added to the funnel and was vigorously shaken. The lower aqueous layer was discarded and the upper organic layer was once again extracted with 50 ml of saturated sodium chloride. Upper hexane layer was allowed to pass through a layer of anhydrous sodium sulfate to remove moisture into a 500 ml round bottom flask. The extracted residue was then concentrated to dryness using a rotary vacuum evaporator at 60 °C. The residue obtained was dissolved in 10 ml hexane.

Purification of the extract was performed by column chromatography using preactivated adsorbent florisil. Before use, the columns were conditioned with 50 ml of *n*-hexane without allowing them to dry out. The column was loaded with 10 ml hexane extract and eluted with 200 ml of 10% ethyl acetate in dichloromethane. Eluate was evaporated to dryness at 60 °C on the rotary evaporator. The residue was fi-

nally redissolved in 5 ml of acetone and filtered through a 22 μm nylon filter, prior to chromatographic analysis.

Instrumentation and operating conditions

Gas chromatographic analysis was carried out on a Hewlett Packard GC (5890 series II) and HP (3396 series III) integrator equipped with DB-5 column (phenyl methyl siloxane wide bore capillary column; 30 m length×0.53 mm i.d.×0.25 μm film thickness), coupled with NPD. The GC was operated at the oven temperature 225 °C, injector temperature 220 °C, detector temperature 250 °C and carrier flow (nitrogen) 5 ml/min.

RESULTS AND DISCUSSION

Gas chromatography-nitrogen phosphorus detector (GP-NPD) performance

The hexaconazole residue to be analyzed by GC-NPD was dissolved in 10 ml of acetone and the injection volume was 0.5 μl. The peak resolution and appearance were poor in such cases. Therefore, the final dilution volume was fixed at 5 ml and the injection volume raised to 1 μl.

Sample extraction performance

Black tea sample from the untreated control plot was spiked with hexaconazole at 0.5, 0.1, 1.0, 2.0 and 4.0 mg/kg. The contents were allowed to equilibrate for about 15 min so that the fungicide spread uniformly within the matrix. Subsequently, the spiked black tea sample was extracted with 150 ml of *n*-hexane:acetone (1:1, v/v) by shaking it for 2 h on a mechanical shaker. The contents were filtered through Whatman No. 1 filter paper and transferred to a 500 ml separating funnel. One hundred ml of saturated sodium chloride was added to the filtrate, shaken vigorously and allowed to settle. The aqueous phase was removed and the organic phase was again partitioned with 50 ml of saturated sodium chloride. After partitioning, the hexane layer was passed through an anhydrous sodium sulfate layer and collected in a 500 ml round bottomed flask. The extract was evaporated to dryness on a rotary vacuum evaporator at 60 °C, and the residue was dissolved in 10 ml hexane. Each sample was replicated three times.

Solvents such as hexane, acetone, acetone-dichloromethane and acetone-hexane are known to be used for extracting several commonly used fungicides. However, for hexaconazole there was a need to change the solvent by taking into consideration its solubility in solvents such as ethyl acetate and dichloromethane. The amount of solvent used and, if a mixture was chosen, the ratio between the components needed to be fixed. Based on the solubility of hexaconazole, the extraction efficiency of solvents such as dichloromethane and acetone was studied. The efficiency of two individual solvents in extracting hexaconazole from a black tea matrix was less significant and the recovery was very low. Therefore, mixtures of organic solvents such as acetone:dichloromethane at 1:1 and 3:1 (v/v) and methanol:water at 2:1 (v/v) were evaluated. The recovery percentage was 36%, 48% and 54%, respectively, when the above combinations were used. But a combination of 200 ml of acetone and *n*-hexane (1:1, v/v) was found to be appropriate for extraction of hexaconazole from the tea matrix.

Extract purification performance

Organic solvent extracts from black tea contain many interfering compounds from the matrix. To remove matrix interferences, purification efficiency of alumina, silica and florisil was tested. Purification by alumina (acidic, basic and neutral) and silica was not efficient for hexaconazole due to low retention. The eluate obtained with such adsorbing materials was less transparent and matrix interferences were also noted during gas chromatographic analysis. However, activated florisil was found to be effective in purifying the sample since the eluate was more transparent. The chromatograms had less peak interferences from the matrix.

Sample clean up performance

After choosing a suitable adsorbing material, the next step was to select a suitable eluant or a mixture of solvents which could successfully elute the target fungicide from florisil. Since the solubility of hexaconazole was greater in solvents such as dichloromethane, ethyl acetate, acetone and *n*-hexane, combinations of solvents such as *n*-hexane:dichloromethane (4:6, v/v), 5% ethyl acetate in hexane (150 ml), 5% diethyl ether in hexane (150 ml), ace-

tone:dichloromethane (1:3, v/v) and 5% ethyl acetate in dichloromethane (200 ml) were tested. Among these, the last combination was found to be the most efficient since the recovery percentage was >80% for all the fortified samples.

Method performance

Evaluation of quality parameters such as the recovery percentage, repeatability, reproducibility, linearity and limits of detection and quantitation (Zanella *et al*., 2000) is essential to assess the method performance. Calibration curve for all chromatographic analysis was prepared by plotting detector response (y) and analyte concentration (x) expressed by the equation: $y=c+mx$. The regressed linear equation obtained by the selected chromatographic conditions for hexaconazole was

$$y=-4513.8+3979.1x \ (r=0.999).$$

This equation was obtained by injecting a series of hexaconazole dilutions of 0.05~1.0 mg/L prepared as described in the section of stock standard solutions mentioned above. The correlation coefficient value indicated perfect linearity.

The limit of quantitation (LOQ) of hexaconazole was established by fortifying control black tea samples to the lowest level of the analyte from which the residues could be extracted with acceptable precision and accuracy under the established experimental conditions. The instrumental limit of detection of hexaconazole was 0.05 mg/kg and the amount of hexaconazole that can be extracted with acceptable precision from the black tea matrix is 0.1 mg/kg (LOQ).

The repeatability of the developed analytical method was determined by adding hexaconazole in five different concentrations to control black tea. The repeatability of spiked hexaconazole in black tea at the levels of 0.1, 0.5, 1.0, 2.0 and 4.0 mg/kg are summarized in Table 1.

The reproducibility of hexaconazole was determined by analyzing the fortified control black tea samples with hexaconazole over three consecutive days and by two different analysts as presented in Table 2.

Verification of method

Black tea samples prepared were subjected to residual analysis for hexaconazole. Results (Table 3) indicate that hexaconazole residues could be identified up to a level of 0.06 mg/kg, which proved that the newly developed method was suitable for quantifying the hexaconazole residues even when present in trace amounts in the black tea matrix. Dissipation of hexaconazole at 200 and 400 ml/ha followed first order kinetics. The correlation coefficient values and regression equations are presented in Table 4. The chromatograms are presented in Fig.1.

Table 1 Repeatability for the fungicide hexaconazole in black tea spiked at five levels*

Hexaconazole concentration (mg/kg)	Found (mg/kg) (mean±*SD*)	Recovery (%)	RSD_r (%)
0.1	0.0869±0.002	86.86	2.35
0.5	0.4678±0.009	93.55	1.91
1.0	0.9357±0.017	93.57	1.83
2.0	1.9198±0.006	95.99	0.29
4.0	3.8387±0.012	95.98	0.30

* Number of replicates at each level (n)=6 (three extractions with two injections each); All made under the same conditions on the same day; RSD_r: Relative standard deviation for repeatability

Table 2 Reproducibility for the fungicide hexaconazole in black tea spiked at five levels*

Hexaconazole concentration (mg/kg)	Found (mg/kg) (mean±*SD*)	Recovery (%)	RSD_R (%)
0.1	0.0817±0.004	81.71	5.13
0.5	0.4177±0.014	83.53	3.28
1.0	0.8588±0.026	85.88	3.06
2.0	1.7281±0.051	86.41	2.96
4.0	3.4159±0.057	85.40	1.68

* Number of replicates at each level (n)=6 (three extractions with two injections each); Each extraction and injection series was accomplished on 3 consecutive days; RSD_R: Relative standard deviation for reproducibility

Table 3 Residues of hexaconazole in black tea at different harvest intervals

Day	Residues of hexaconazole (mg/kg)		
	Control	200 ml/ha	400 ml/ha
0	BDL	1.30	2.19
3	BDL	0.78	1.37
5	BDL	0.43	0.58
7	BDL	0.22	0.27
10	BDL	0.06	0.19
14	BDL	BDL	BDL

BDL: Below detectable limit (<0.05 mg/kg)

Table 4 Kinetics of dissipation of hexaconazole in tea

Season	Dosage (ml/ha)	Regression equation*	Correlation coefficient (r)
Wet	200	$\log y=0.222-0.134x$	−0.982
	400	$\log y=0.369-0.115x$	−0.977

*Log $y=a+bx$, where y is the residue level in $\times10^{-6}$, x is the harvest interval in days, and a and b are regression constants

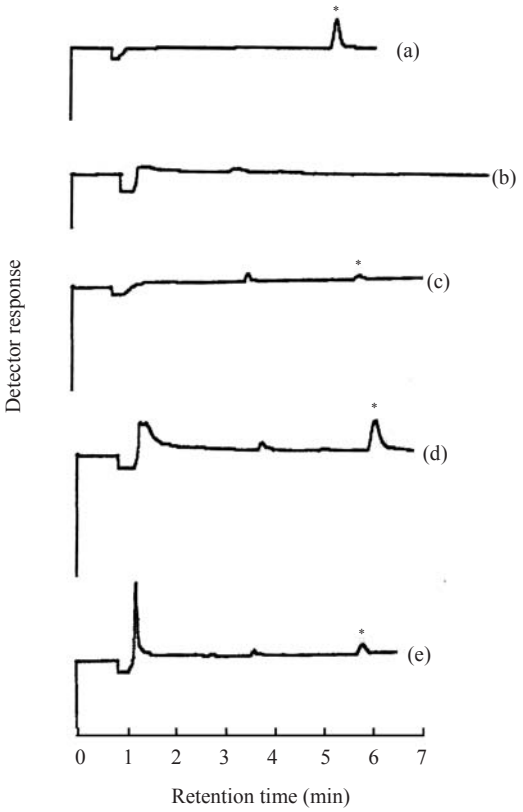

Fig.1 GC-NPD chromatogram of hexaconazole residues in black tea samples

* represents hexaconazole peak. (a) Standard hexaconazole; (b) Control black tea; (c) Fortified black tea; (d) Black tea on Day 1; (e) Black tea on Day 5

CONCLUSION

A simple GC-NPD method for the determination of the triazole fungicide hexaconazole was developed and applied for the analysis of its residues in black tea samples. The method is simple and highly reliable, and is exclusively applied for the determination of hexaconazole residues in black tea. The test results for calibration, linearity, repeatability and reproducibility show that this was a rapid and efficient method for the quantification of hexaconazole in black tea.

ACKNOWLEDGEMENT

Thanks are due to Dr. N. Muraleedharan, Director, United Planter's Association of South India Tea Research Foundation for going through the manuscript and offering valuable suggestions.

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