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Rapid Analysis of Tetraconazole Residues in Fruits and Vegetables using Ethyl Acetate Extraction and Gas Chromatography-tandem Mass Spectrometry

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A method based on ethyl acetate extraction and gas chromatography with tandem mass spectrometry was developed for determining tetraconazole residues in fruits and vegetables. A 10 g homogenized sample was mixed with 10 mL ethyl acetate, shaken vigorously for 3 min, stored at −20 °C for 15 min, and then vortexed vigorously for 1 min; 1 g NaCl and 4 g anhydrous $MgSO_4$ were added. The clean-up was carried out by applying dispersive solid-phase with 150 mg $MgSO_4$ and 50 mg primary secondary amine. Three precursor product ion transitions for tetraconazole were measured and evaluated to provide the maximum degree of confidence. Average recoveries in fruits and vegetables at three levels (0.005, 0.05 and 0.5 mg/kg) ranged from 85.53% to 110.66% with relative standard deviations (RSD_r) from 1.3% to 17.5%. The LODs ranged from 0.002 to 0.004 μg/kg, and LOQs ranged from 0.006 to 0.012 μg/kg. This method was also applied to determine tetraconazole residue in cucumber dissipation experiment under field conditions. The half-lives of tetraconazole in cucumber were in the range of 2.1-3.1 days.

Key Words : Tetraconazole, Dispersive solid phase extraction, Gas chromatography with tandem mass spectrometry, Fruit, Vegetable

Introduction

Tetraconazole, ((±)-1-[2-(2,4-dichlorophenyl)-3-(1,1,2,2-tetrafluoroethoxy)propyl]-1*H*-1,2,4-triazole, is a new systematic triazole fungicide, which has been registered in China for use in strawberry, while its registered in cucumber and melon is pending. This fungicide is a steroid demethylation inhibitor, acting mainly on the vegetative stages of fungi by blocking the mycelial growth either inside or outside the host plant. Tetraconazole (Figure 1) is effective against a broad spectrum of diseases such as powdery mildew, brown rust, *Septoria*, and *Rhynchosporium* on cereals, powdery mildew and scab on apples, powdery mildew on vegetables and sugar beets.[1]

Use of agrochemicals at various stages of cultivation plays an important role in crop protection and quality preservation. Maximum residue limits (MRLs) in foodstuffs have been set by Government agencies to guarantee consumer safety and to regulate international trade. The range of MRLs for tetraconazole in apple, tomato, cucumber, melon, strawberry and watermelon is from 0.05 mg/kg to 2 mg/kg settled by European Union and Japan.[2-5] Tetraconazole has low acute toxicity via the oral, dermal and inhalation routes, but it poses toxicity to rat liver.[6]

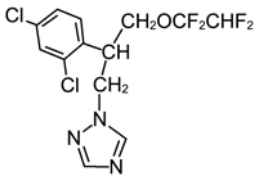

Figure 1. Chemical structure of tetraconazole.

Therefore, through monitoring of tetraconazole residues is crucial for proper assessment of human exposure to pesticides through foods. Liquid-liquid extraction (LLE) followed by multiple operation steps have been used as a conventional sample preparation method for analysis of tetraconazole in food.[7-9] However, these methods are generally time consuming and a large amount of solvents are often required. Tetraconazole was in the registration period for use on cucumber and melon in China at present, so it is timely and important to establish a rapid, simple and effective analytical method of tetraconazole residue detection in fruits and vegetables.

An important extraction technique for vegetables and fruits is the QuEChERS (quick, easy, cheap, effective, rugged and safe) method recently introduced.[10] The merits of this method include decreased costs of experimental apparatus and solvents as well as high sample throughout. To our knowledge, there was no report with this QuEChERS extraction technique for determining tetraconazole residue in vegetables and fruits. In this paper, a simple, inexpensive and rapid method based on modified QuEChERS method for determining tetraconazole is described, in which ethyl acetate is used as the solvent for the sample and, after mixing, centrifuging, and cleaning up, the sample extract is analyzed by gas chromatography-tandem mass spectrometry (GC-MS/MS). The entire method is very simple. Toxic solvents, such as acetonitrile, benzene, and dichloromethane are avoided. Eight samples can easily be analyzed by one analyst in 2 h at minimal cost. The reliability of the develop-

ed analytical method was confirmed with field-treated samples.

Experimental

Chemical and Reagents. Tetraconazole standard (purity ≥ 98.47%) and 4% tetraconazole aqueous emulsion were obtained from Italy Isagro (China) Co., Ltd. LC grade ethyl acetate, acetonitrile and acetone were purchased from Fisher Chemicals (Fair Lawn, NJ, USA); NaCl and $MgSO_4$ were analytical grade purchased from Beihua fine-chemicals Co. (Beijing, PRC). Ultra-pure water was obtained from a Milli-Q system (Bedford, MA, USA). Primary secondary amine (PSA) and graphitized carbon (GCB) were purchased from Agela (Beijing, China).

Instruments. A Varian 4500 GC system with a 1177 Series split-splitless auto-injector, a 8400 Series autosampler and a 300 triple quadrupole MSD. A Varian FactorFour Capillary Column VF-5 ms analytical column (30 × 0.25 mm i.d. × 0.25 mm film thickness), was used for GC separation, with helium (99.9999%) as the carrier gas at a constant flow rate of 1.0 mL/min. The column temperature was initially at 160 °C (hold for 3 min), increased to 280 °C (hold for 3 min) at the rate of 20 °C/min. The temperature of the injector port was 250 °C and a volume of 1 μL was injected in splitless mode. The total running time was 12.0 min, the retention time of tetraconazole is 7.72 min.

The mass spectrometer was operated in electron ionization (EI) mode at 70 eV. The temperatures of the transfer line, manifold and ionization source were set at 280, 40, and 250 °C, respectively. The electron multiplier (EM) voltage was set at 1100 V when performing multiple reaction monitoring, and solvent delay was set to 5.0 min. The multiple reaction monitoring was selected to be scan mode. The scan time was 0.6 s. The precursor ion was *m/z* 336 and its product quantitative ion was *m/z* 204 and qualitative ions were *m/z* 155 and *m/z* 218, when the collision energy was set at 35 V.

Preparation of Standards and Calibration Curve. The stock solution of tetraconazole (100 mg/L) was prepared in acetone and serially diluted to produce working solutions of 0.005, 0.05, 0.5, 1.0 and 2.0 mg/L. All solutions were protected against light with aluminum foil and stored in a refrigerator at 4 °C. A calibration curve was generated by plotting peak area versus the concentration of the tetraconazole.

Sample Extraction. A 2 kg of fruit and vegetable samples (cucumber, tomato, apple, melon, strawberry and watermelon) were chopped and homogenized. In this study, two initial extraction steps were used.

Ethyl Acetate as the Extraction Solvent: Ten grams of representative portion of homogenized samples was weighed into a 50 mL centrifuge tube. The samples were fortified with a suitable volume of working standard solution for the recovery experiment, well mixed and equilibrated for 1 h. Then 10 mL ethyl acetate was added and the cap was screwed. The sample was shaken vigorously for 3 min using the mixer. The tubes were stored at −20 °C for 15 min, taken out and then vortexed vigorously for 1 min. 1 g sodium chloride (NaCl) and 4 g anhydrous magnesium sulfate ($MgSO_4$) were added. The tubes was capped and immediately vortexed vigorously for 1 min and then centrifuged for 10 min at 2077 g. Then, 1.5 mL of the upper layer (ethyl acetate) was transferred into a 2.0 mL micro-centrifuge tube waiting for cleanup.

Acetonitrile as the Extraction Solvent: Ten grams of representative portion of homogenized samples was weighed into a 50 mL centrifuge tube and then 10 mL acetonitrile was added and the cap was screwed. The following steps were same as 4.1. After centrifugation, 4 mL of the upper layer (acetonitrile) was transferred to a 50 mL flask and evaporated to near dryness by rotary vacuum (evaporation at 35 °C). The residue was redissolved in 4 mL ethyl acetate, and 1.5 mL extract was transferred into a 2.0 mL micro-centrifuge tube waiting for cleanup.

Cleanup. In each case, a 1.5 mL aliquot was transferred into the dispersive-SPE tubes containing 150 mg anhydrous MgSO4, and (1) +0 mg, or 25 mg, or 50 mg, or 75 mg, or 100 mg primary secondary amine (PSA); (2) +0 mg, or 10 mg, or 20 mg, or 30 mg, or 40 mg graphitized carbon black (GCB) for test. Then the tubes were well capped and vortexed for 1 min. The tubes were then centrifuged for 5 min at RCF 2077 g. The resulting supernatants were filtered through 0.22 μm Nylon syringe filters for GC-MS/MS analysis.

Method Performance. After test, the experiments were done with ethyl acetate for extraction and cleaned up with 50 mg PSA + 150 mg anhydrous $MgSO_4$ for dispersive-SPE procedure. Matrix effect assessment, precision (when repeated independent analysis were performed), accuracy (when recovery assays were performed), sensitivity and limits of quantitation were calculated for developed analytical methodology. Recovery experiments were carried out with 5 replicates at three spiked levels (0.005, 0.05, and 0.5 mg/kg) by adding known volumes of tetraconazole standards to different matrixes (apple, tomato, cucumber, melon, strawberry and watermelon). Blank samples were performed in order to check interference from the matrix.

Field Application. The optimized procedure was applied in 2010 to determine tetraconazole in cucumber dissipation rate experiment from Shandong, Henan and Zhejiang provinces of China. The fields were divided into 15 m^2-sized blocks. Each field experiment treatment was designed with

Table 1. Recoveries of tetraconazole added to cucumber at 0.05 mg/kg using different sorbents (n = 5)

PSA (mg)	Mean recovery (%)	RSD_r (%)	GCB (mg)	Mean recovery (%)	RSD_r (%)
0	107.48	8.3	0	107.48	8.3
25	104.64	3.4	10	86.08	3.2
50	98.99	1.5	20	84.79	4.1
75	93.21	2.4	30	76.17	3.0
100	92.50	3.9	40	74.65	3.6

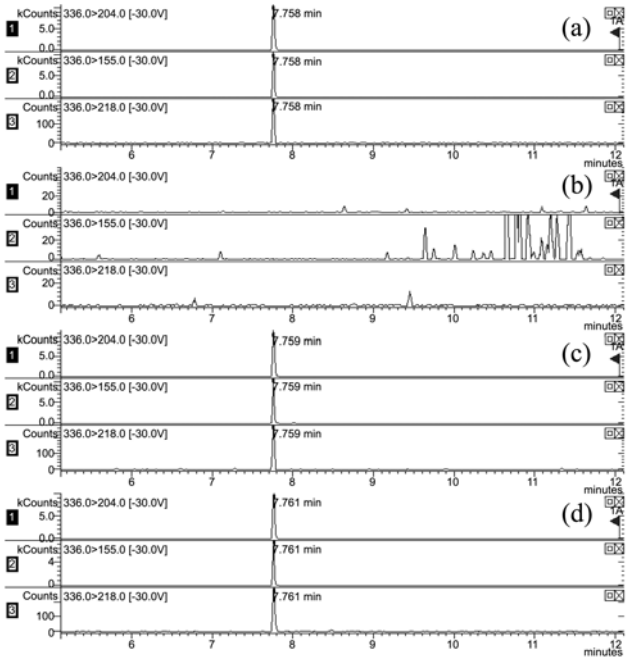

Figure 2. Chromatograms of (a) tetraconazole standard, (b) blank cucumber sample, (c) cucumber sample spiked at 0.005 mg/kg cleanup with 50 mg PSA, (d) cucumber sample spiked at 0.005 mg/kg cleanup with 20 mg GCB.

three replicate plots for the control and the dissipation rate study. The application rate in dissipation experiments was 90 g (a.i.)/ha of 4% tetraconazole aqueous emulsion with one time spray. Representative melon samples were collected in 2 h, 1, 2, 3, 5, 7, 10 and 14 days after spraying. Samples were put into polyethylene bags and deep-frozen (−20 °C) until analysis. For each batch of samples analyzed, a calibration curve at concentration between 0.005 and 2 mg/L was injected before and after the sample extracts. All samples and standards were injected in triplicate.

Results and Discussion

Optimization of the Method.

GC-MS/MS Parameters: For the optimization of the MS method, tetraconazole was monitorized in scanning mode in the range *m/z* 50-600. Then, the precursor ions were selected with the aim of achieving a compromise between both selectivity (the highest *m/z* ion is preferred) and sensitivity (the highest abundance ion). The MS/MS transition that gave the highest response for tetraconazole was selected. The precursor ion selected was *m/z* 336, which was fragmented by application of different voltages to product ions in the range *m/z* 50-400. The suitable collision energy was set at 30 V. The product quantitative ion was selected as *m/z* 204 and qualitative ions were *m/z* 155 and *m/z* 218. This enabled the MRM transitions to be selected.

Table 2. Calibration data, LOD and LOQ for tetraconazole in different matrixes

Matrix	Calibration equation	Relative coefficient	LOD (μg/kg)	LOQ (μg/kg)
cucumber	y=2E+07x + 95324	0.9999	0.002	0.007
tomato	y=3E+07x – 81969	1	0.003	0.011
apple	y=2E+07x + 194794	0.9986	0.002	0.006
strawberry	y=1E+07x + 377825	0.9973	0.002	0.008
melon	y=1E+07x + 190976	0.9984	0.004	0.012
watermelon	y=1E+07x – 135650	0.9986	0.002	0.006

Selection of Extraction Solvents: In this method, ethyl acetate and acetonitrile are selected as extraction solvents. The extract of ethyl acetate can be used for GC-MS/MS directly. Acetonitrile has a larger solvent expansion volume during GC vaporization and low volatility. In this study, acetonitrile as the solvent for the sample, evaporation and resolvation, and ethyl acetate as extraction solvent were performed and analyzed in five parallels at one spiked level (0.05 mg/kg) in cucumber. The results showed that the average extraction efficiencies of ethyl acetate (92%) were higher than that of acetonitrile (85%). This may be that there are more steps in acetonitrile extraction which lead to the comparatively lower recovery efficiency in acetonitrile extraction. Ethyl acetate is especially suitable for the extraction of high-sugar commodities like melon, apple and other fruits, since sugar has limited solubility in ethyl acetate. Therefore, ethyl acetate is more suitable for this method, which makes it simpler, quicker and more effective.

Cleanup: In the dispersive SPE, anhydrous $MgSO_4$ was used to absorb microwater in the solvent. PSA and GCB were tested as sorbents separately in this study. There are much pigment and sugar in vegetables and fruits. PSA can absorb fat acids, pigment, sugar *et al.* GCB can absorb pigment effectively. Results showed that, without the cleanup using PSA or GCB, the cucumber extract colors were much

Table 3. Average recovery and relative standard derivation of tetraconazole at various matrixes and levels (n = 5)

Matrix	Spiking 0.005 mg/kg		Spiking 0.05 mg/kg			Spiking 0.5 mg/kg	
	Mean (%)	RSD_r (%)	Mean (%)	RSD_r (%)	RSD_R (%)	Mean (%)	RSD_r (%)
cucumber	97.92	5.1	92.46	4.9	5.6	101.43	3.1
tomato	110.66	17.5	90.95	4.3	2.7	93.78	4.4
apple	93.22	7.6	94.94	4.8	1.8	93.01	2.8
strawberry	95.71	6.9	98.80	4.6	2.5	90.40	2.1
melon	90.82	8.7	105.23	1.3	4.2	85.53	2.0
watermelon	106.58	11.3	96.13	8.4	5.3	89.19	4.5

deeper than that with the sorbent. Among the different kinds and amounts of PSA and GCB, 50 mg PSA was better than the others according to purification and recovery efficiency (Table 1). Moreover GCB is more expensive than PSA. Therefore, 50 mg PSA was selected as sorbent in this method. Figure 2 shows the chromatograms of the tetraconazole standard, blank, and spiked samples cleanup with PSA or GCB.

Matrix Effect: The response of the detector system to certain agrichemicals may be affected by the presence of co-extractives from the sample.[11,12] Compared with those produced by solvent solutions of the target analyte, the matrix effects may affect response in positive or negative way, depending on the level of ion suppression. This can greatly affect the method producibility and accuracy.[13,14] The matrix effect on MS detector using the method developed here was investigated in fruits and vegetables by comparing standards in solvent with matrix-matched standards. The results showed that the matrix significantly enhanced the response of the instrument.

Validation of the Method.

Linearity, LODs and LOQs: The calibration curves obtained for tetraconazole (from 0.005 mg/L to 2 mg/L) in different matrixes were shown in Table 2. In this study, calibration was performed with external matrix-matched standards to eliminate matrix effect and to obtain more realistic results. The Limits of detection and quantification (LOD and LOQ), defined as the lowest concentration that the analytical process can reliably differentiate from background levels, were estimated for spiked samples (0.005 mg/kg) based on an S/N of 3:1 and 10:1. The LODs of tetraconazole ranged from 0.002 μg/kg to 0.004 μg/kg, and LOQs ranged from 0.006 μg/kg to 0.012 μg/kg in fruits and vegetables (Table 2), which were lower than that of the published methods.[8,9,15] It was found that the LOQs of this method were lower than the MRLs (0.05-2 mg/kg) settled by the authorities. Moreover, there were no MRLs for tetraconazole in fruits and vegetables made in China. This method may be helpful for China to settle MRLs for tetraconazole and monitor it in routine food control.

Recovery, Repeatability and Reproducibility: The recoveries of tetraconazole obtained with this method at fortification levels of 0.005, 0.05 and 0.5 mg/kg (based on five replicates) in cucumber, tomato, apple, strawberry, melon and watermelon samples were shown in the Table 3. The mean recoveries ranged from 85.53% to 110.66%, and the coefficient of variation (RSD_r) value of repeatability of the method ranged from 1.3% to 17.5%. The recoveries and relative standard deviations of this method were acceptable for pesticide residue analysis.

The repeatability of the instrument was determined by analyzing the same cucumber sample spiked at 0.05 mg/kg. When the sample was injected 10 times at one-hour-intervals, the RSD values obtained for peak areas and retention times by GC-MS/MS were 3.7% and 0.3%, respectively. The precision of the method was determined by the repeatability and reproducibility studies of method and expressed by the relative standard deviation (RSD). The repeatability RSD_r was measured by comparing standard deviation of the recovery percentage spiked samples run on the same day. The reproducibility RSD_R was determined by analyzing spiked samples (0.05 mg/kg) for 5 different days by five operators. The reproducibility RSD_R ranged from 1.8 to 5.6%, as summarized in Table 3.

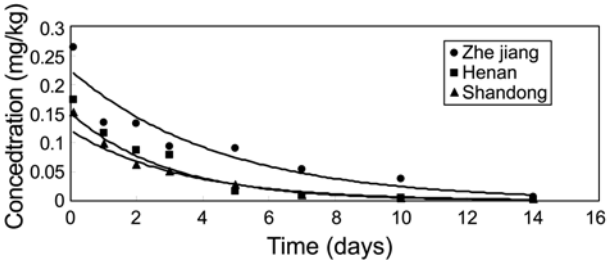

Figure 3. Dissipation of residues of tetraconazole in cucumber under field conditions in three different geographic zones.

Table 4. Half-life and other parameters for tetraconazole dissipated in cucumber

Locality	Regression equation	Determination coefficient (R^2)	Half-life (day)
Zhejiang	$y=0.224e^{-0.2211x}$	0.9360	3.1
Henan	$y=0.1509e^{-0.337x}$	0.9529	2.1
Shandong	$y=0.1209e^{-0.2928x}$	0.9652	2.4

Application to Field-treated Samples. In method development and validation, the proposed method was proven to show sensitive determination of tetraconazole for spiked cucumber, melon, tomato, apple, strawberry and watermelon samples. Validation parameters of the method were presented in terms of specificity, linearity, LODs, LOQs, recovery, precision, repeatability and reproducibility.

The application of the proposed method was assessed for analyzing tetraconazole in cucumber dissipation rate experiment from Zhejiang, Henan and Shandong provinces of China. The dissipation curves of tetraconazole in cucumber under field conditions were shown in Figure 3. The degradation process of tetraconazole appeared to follow the first-order kinetic reaction. The degradation rate constant and half-life were calculated using the first-order rate equation: $C_t = C_0e^{-kt}$. The half-lives and other parameters of tetraconazole residue dissipation were calculated from the experimental data and summarized in Table 4. The initial deposit of tetraconazole in melon were 0.264 mg/kg, 0.174 mg/kg, and 0.162 mg/kg in Zhejiang, Henan and Shandong, which declined to 0.007 mg/kg, 0.002 mg/kg and 0.003 mg/kg after14 days respectively. The dissipation rates were more than 97% by the 14th day in three geographic zones after treatment. The half-lives were observed to be 3.1, 2.1 and 2.4 days with a rate constant (k) 0.936, 0.9529 and 0.9652.

Conclusion

A GC-MS/MS method for the trace analysis of the new

generation fungicide tetraconazole in fruits and vegetables was developed and validated. The devised GC-MS/MS method combined with ethyl acetate extraction followed by the dispersive-SPE purification provided sufficient selectivity and sensitivity for the detection of tetraconazole. The method developed showed satisfactory validation parameters in terms of linearity, lower limits, accuracy and precision, which is also accurate, fast, cheap and sufficiently easy to perform that it could be used for regular monitoring of tetraconazole residue in fruits and vegetables. This study offered an effective residue analysis method for tetraconazole registration and MRL establishment in China.

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