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Article

High-Performance Liquid Chromatographic Determination of Tricyclazole Residues in Rice Grain, Rice Straw, and Soil

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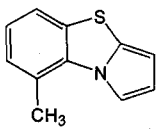
An analytical method was developed to determine tricyclazole residues in rice grain, straw, and soil using high-performance liquid chromatography (HPLC) with ultraviolet absorption detection. Tricyclazole was extracted with methanol from moist rice grain, straw, and soil samples. *n*-Hexane washing was employed to remove nonpolar co-extractives during liquid-liquid partition. Tricyclazole was then extracted with dichloromethane from alkaline aqueous phase, while acidic interferences remained in the phase. Dichloromethane extract was further purified by silica gel column chromatography prior to HPLC determination. Reverse-phase HPLC using an octadecylsilyl column was successfully applied to separate and quantitate the tricyclazole residue in sample extracts monitored at λ_{max} 225 nm. Recoveries from fortified samples averaged 95.5± 3.0% (n=6), 87.5±2.0% (n=6), and 84.3±2.8% (n=12) for rice grain, straw, and soil, respectively. Detection limit of the method was 0.02 mg/kg for rice grain and soil samples while 0.05 mg/kg for rice straw samples. The proposed method was reproducible and sensitive enough to evaluate the safety of tricyclazole residues in rice grain, straw, and soil.

Key words : *Tricyclazole, residue analysis, HPLC determination, rice, soil.*

Tricyclazole (5-methyl-1,2,4-triazolo[3,4-*b*][1,3]benzothiazole) is a systemic fungicide widely used for control of rice blast, *Pyricularia oryzae*. Its long-term efficacy makes it possible that one or two applications give a season-long control of the disease.[1,2] Conversely, this may also give rise to residue problems, such as high level of terminal residues in the harvest or long persistence in the soil.[3] Development of the highly reliable methods is, therefore, required to precisely track the residues in the paddy environment. A few had been studied to develop the analytical method for tricyclazole residues in rice and soil using GLC with flame photometric detection.[4,5] However, relatively low recovery (60~80%) and poor reproducibility (more than 10% RSD) were reported because of highly polar nature of the compound. The present paper describes a new analytical method for tricyclazole residues in rice grain, straw, and soil samples using high-performance liquid chromatography. The method was developed not only to achieve reliability higher than current methods but to fulfill required sensitivity and readiness for analytical operation.



Tricyclazole

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Abbreviations: GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography; RSD, relative standard deviation.

Materials and Methods

Chemicals. Analytical standard of tricyclazole (98.5% pure) was kindly supplied by Kyungnong Co., Korea. Stock standard solution of 1000 mg/l was prepared in acetonitrile. The stock solution was stable at 4°C for at least 6 months. Acetonitrile and deionized water were HPLC grade. All other solvents were pesticide residue grade or reagent grade freshly redistilled in glass. Silica gel (70~230 mesh, CC grade) was purchased from Merck, Germany and activated at 130°C for more than 5 h prior to use. All other reagents were reagent grade unless specified.

Rice and soil samples. At maturity, rice plants (Dongjin variety) were harvested from a paddy field located in Chilgok, Kyungbuk Province, where no tricyclazole had been applied during the whole cultivation period. Grain and straw parts were separated from the plants and air-dried. Rough rice grains were husked and finely ground to pass 40-mesh sieve using Wiley mill. Straw samples

were chopped, air-dried, and also finely ground to pass 40-mesh sieve. Bulk soil samples were collected from two paddy fields, Kyungsan and Youngchun in Kyungbuk Province, to the soil depth of 10 cm during the cultivating season, air-dried, and passed through 10-mesh sieve before use. Physicochemical characteristics of soils are shown in Table 1.

Extraction and partition. Twenty-five grams of soil or rice grain samples were moistened with 25 ml distilled water. After standing for 10 min, 100 ml methanol was added and extracted for 1 h on gyrotatory shaker at 200 rpm. To a 10 g rice straw sample weighed in homogenizer cup, was added 150 ml of methanol/water mixture (2/1, v/v) and extracted for 2 min at 10,000 rpm. The extract was suction-filtered through the filter paper (Toyo No. 6, Japan) on porcelain Büchner funnel. The flask and filter cake were rinsed with fresh 50 ml methanol, and the rinsate was combined with the previous filtrate. The filtrate was quantitatively transferred into a separatory funnel, and sequential addition of 300 ml distilled water, 50 ml saturated NaCl and 100 ml n-hexane was followed. After vigorous shaking for 1 min and standing until two layers clearly separated, the upper hexane phase was discarded. In the aqueous phase was added 5 ml of 1 N aqueous sodium hydroxide solution, swirled briefly, and then vigorously extracted with two 50 ml portions of dichloromethane. The lower dichloromethane phase was dried over 20 g anhydrous sodium sulfate layer, collected in 250 ml distilling flask, and evaporated just to dryness *in vacuo* at 40°C. The residue was dissolved in 10 ml dichloromethane and subjected to silica gel column chromatography.

Silica gel column chromatogaphy. Chromatographic column was plugged with glass wool, dry packed with 5 g activated silica gel and topped with *ca.* 2 cm layer of anhydrous sodium sulfate. The column was pre-washed by passing 25 ml dichloromethane through it until the solvent level reached the top of sodium sulfate layer. The dichloromethane extract from the partition step was poured into the column, and the column wall was rinsed twice with 2 ml portions of dichloromethane. When the liquid drained to sodium sulfate layer, the column was eluted with 50 ml ethyl acetate/dichloromethane mixture (15/85, v/v), and the fraction was discarded. The column was then eluted with 50 ml of ethyl acetate/dichloromethane mixture (80/20, v/v), and the fraction was collected. The eluate was concentrated just to dryness, and the residue was reconstituted with 10 ml acetonitrile/water (20/80, v/v) for HPLC determination.

High-performance liquid chromatography. HPLC was performed using a Waters (USA) HPLC system equipped with two 510 pumps, 680 gradient controller, 486 tunable UV/VIS absorbance detector, Rheodyne (USA) 7125 injector and Hewlett Packard (USA) 3396 Series II integrator. Nova-Pak C18 (3.9 mm i.d.×150 mm, 4 μm spherical, Waters Associates, USA) was used as the analytical column. Operating parameters used for the determination of tricyclazole residues are as follows; mobile phase, acetonitrile/water (20/80, v/v), isocratic; flow rate 1.0 ml/min; detection, UV absorption at 225 nm, 0.032 AUFS; sample size, 50 μl; chart speed 0.5 cm/min. Under these conditions, retention time of tricyclazole was 7.6 min.

Validation of the analytical method. Recovery experiments were run on control rice grain, straw, and soil samples to validate the analytical method proposed for tricyclazole residues. Prior to extraction, series of control samples were fortified with tricyclazole standard solution in acetonitrile at specified concentrations. After standing for 2 h, analytical procedures mentioned above were carried out to produce quality assurance data.

Persistence of tricyclazole in soils. Each 500 g (oven-dry basis) of Kyungsan and Youngchun soil samples was evenly spreaded on stainless-steel dissecting pan. Tricyclazole standard solution, 50 mg/l, dissolved in acetonitrile was carefully treated dropwise on the soil surface at the rate of 0.75 mg/kg soil. After evaporating the organic solvent, treated soils were thoroughly mixed for 30 min, weighed into the wide-mouth test tube (27 mm i.d.×17 cm H) by 25 g portions per tube. Distilled water was added to make it submerged to 2 cm depth for paddy conditions. Contents in the tube were then mixed, loosely covered with aluminum foil, and incubated at 27±1°C until sampled. Water loss by evaporation was replenished every one week during the trial. Two tubes were taken at specified intervals, sealed tight, and stored at -20°C until analyzed.

Results and Discussion

A preliminary study was conducted to find out whether GLC can be applied to determine the tricyclazole residue as earlier studies did. Using nitrogen-phosphorus detector, the chromatographic behavior of tricyclazole on SPB-5 capillary column (0.53 mm i.d. ×30 m, 0.5 μm film thickness) was investigated. Under the condition of column temperature 190°C, isothermal, and helium 15 ml/min as

Table 1. Physicochemical characteristics of soils.

Soil designation	Soil texture	Soil separate (%)			pH	OM (%)	CEC (cmol/kg)
		Sand	Silt	Clay			
Kyungsan	SiCL	62.3	15.5	22.2	5.5	1.9	13.7
Youngchun	SL	78.1	11.2	10.7	5.8	4.1	13.7

carrier gas, tricyclazole showed a skew peak with severe backward tailing (peak asymmetry factor=1.9)[6] presumably due to its polar nature. During six consecutive injections, moreover, large variation in peak area was observed with 9.8% RSD. Therefore, GLC was inadequate as a routine tool for the determination of the compound.

When reverse-phase HPLC using an octadecylsilyl column was employed, tricyclazole showed a sharp symmetrical peak under the mobile phase of acetonitrile/water mixture. In the range of 20% to 50% of acetonitrile contents in water, its capacity factor increased almost double as acetonitrile contents decreased by 10%. This indicates that tricyclazole exists as neutral form in the mobile phase of acetonitrile/water mixture, thus, there was no need for ion-suppression.[7] As tricyclazole is not readily oxidized nor reduced and has no fluorophore, ultraviolet absorption detector was the only choice among common HPLC detectors. Although extinction coefficient was high (2.36×10^4 $cm^{-1}M^{-1}$ at λ_{max} 225 nm) enough for residue analysis, its chromophore, unfortunately, absorbed only short ultraviolet region. This led to lower selectivity in the determination step, thus rigorous purification of sample extracts might be required. The study was, therefore, mainly focused on the development of efficient but simple cleanup methods.

Considering the polar nature of tricyclazole, an attempt was done at partition step to remove nonpolar co-extractives. From the methanol extract diluted with saline water, tricyclazole was not partitioned into hexane phase but all extracted into dichloromethane phase. Employing a washing step with hexane, 34 and 20% of co-extractives from rice grain and straw samples were removed, respectively. Adsorption chromatography was applied to further purify the extracts. Elution profiles of tricyclazole on silica gel and Florisil columns, are shown in Fig. 1. Silica gel exhibited more favorable elution pattern of tricyclazole than Florisil. More polar solvents were required to elute tricyclazole from the Florisil column and, even in methanol/acetonitrile (10/90, v/v), all the tricyclazole were not recovered. Moreover, the elution pattern appeared less resolved to fractionate the analyte from interferences than that of silica gel. Though ethyl acetate and dichloromethane were known to have similar degree of solvent strength,[7] elution of tricyclazole on the silica gel column was largely affected by the ratio of two solvents. Therefore, solvent selectivity was expected to better separate the analyte from interferences.

Coupling with the proposed partition and adsorption chromatography, while rice grain and soil extracts showed clean HPLC chromatograms free of interference, the rice straw sample did not. Overlapping of tricyclazole with co-extractives made the method unacceptable for determination, thus, additional cleanup method was needed. A coagulation technique[8] used for the analysis of polar pesticides was tried

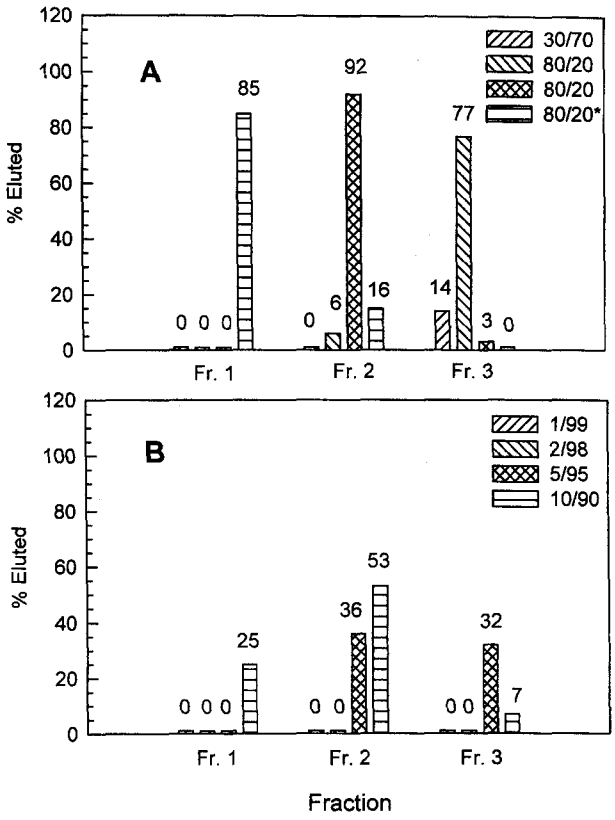

Fig. 1. Elution profile of tricyclazole on silica gel and Florisil columns. A, Silica gel eluted with ethyl acetate/dichloromethane mixtures (v/v); B, Florisil eluted with methanol/acetonitrile mixtures (v/v). The fraction was collected in 25 ml portions. * Pre-eluted with 50 ml of ethyl acetate/dichloromethane mixture (15/85, v/v).

but failed to reduce the interference even when all the tricyclazole was recovered. As coagulation technique is based on precipitation of compounds with low water solubility under acidic condition, the interferences might be regarded as highly polar compounds, mostly having ionizable functional groups. When sodium hydroxide was added to the aqueous phase during partition step, interference level was remarkably decreased and the quantitation could be made as shown in Fig. 2. Under the basic condition of 0.01~1.0 N sodium hydroxide, tricyclazole was stable at least 6 h, showing less than 2% of degradation, which is sufficient time to complete the partition step. By adjusting the aqueous phase to be basic as well, 33 and 44% of co-extractives were additionally removed from rice grain and straw extracts, respectively.

Typical HPLC chromatgrams of rice grain, straw and soil extracts are shown in Figs. 3-5. The proposed method produced very clean HPLC chromatograms for rice grain and soil samples. Chromatograms of rice straw were rather complicated but quite acceptable for quantitation. Detection limit of the proposed method was 0.02 mg/kg for rice grain and soil samples while 0.05 mg/kg for rice straw samples based on 3% full scale deflection (S/N >10). These

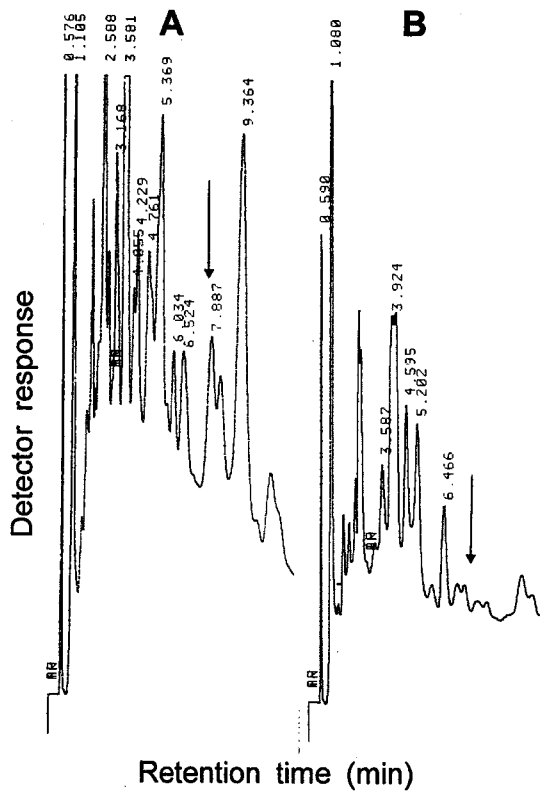

Fig. 2. Effect of alkalization of the aqueous phase on the removal of co-extractives from rice straw extract during partition. A, before NaOH addition; B, after NaOH addition

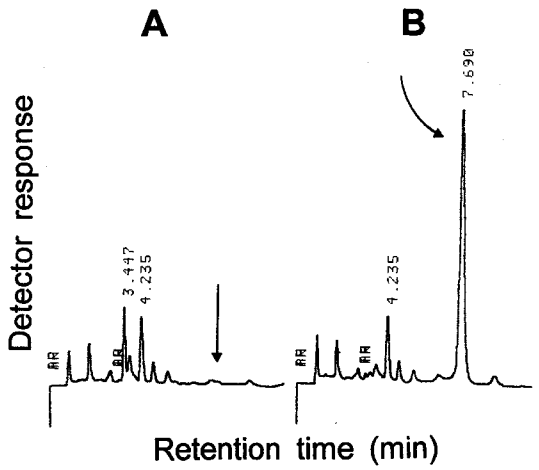

Fig. 3. Typical chromatograms of extracts from the rice grain. A, control; B, fortified with tricyclazole at 0.2 mg/kg

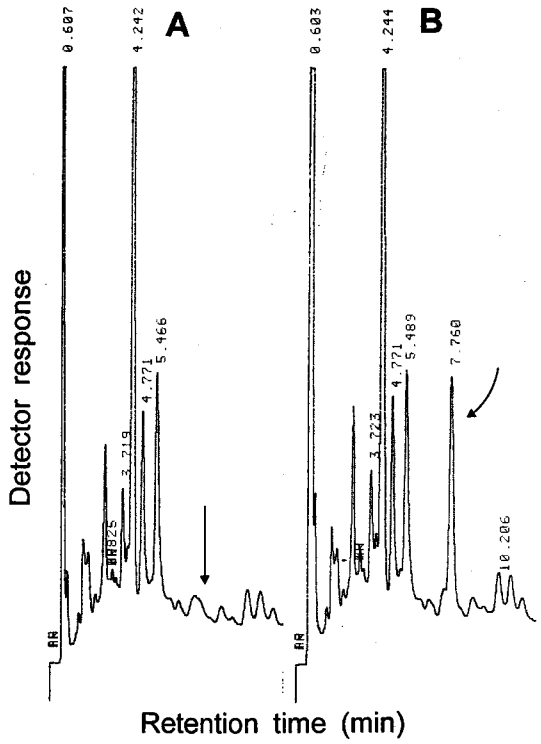

Fig. 4. Typical chromatograms of extracts from the rice straw. A, control; B, fortified with tricyclazole at 0.5 mg/kg

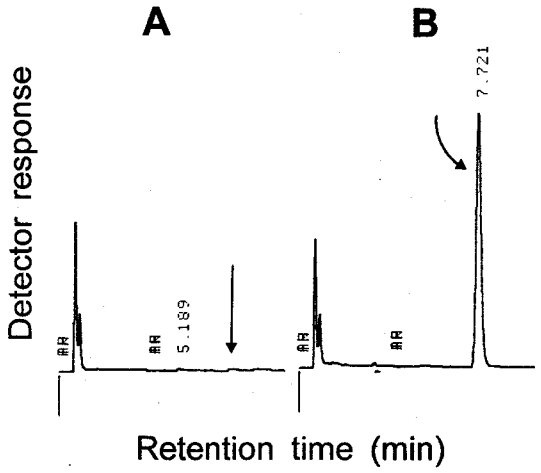

Fig. 5. Typical chromatograms of extracts from the soil. A, control; B, fortified with tricyclazole at 0.2 mg/kg.

sensitivities were sufficiently high to track the behavior of tricyclazole in the environment as well as to evaluate its terminal residues.

Percent recoveries generated during the validation of analytical methods are presented in Table 2. Recoveries averaged 95.5±3.0% (n=6), 87.5±2.0% (n=6), and 84.3 ±2.8% (n=12) for rice grain, straw, and soil samples, respectively. The analytical method was successfully validated as measured by mean recoveries of more than 70% by 6 to 12 replicates per sample type. Coefficients of variation (CV) over all types of samples were less than 10%, indicating that the method could be reproducibly applied to analyze tricyclazole residues in rice and soil samples.

A residue trial was performed to verify the extraction efficiency of the proposed method and to investigate the persistence of tricyclazole residues in soils. As shown in Fig. 6, tricyclazole was rapidly dissipated during initial period of 15 days after treatment. After that, however, the rate reduced greatly showing typical multiple first-order kinetics. This means that at least two factors affecting fast and slow rates, are associated to the dissipation of

Table 2. Recovery and detection limit of tricyclazole residues.

Sample	Fortification (mg/kg)	Recovery±SD (%)*	Detection limit (mg/kg)
Rice grain	0.2	94.5±0.9	0.02
	1.0	96.5±4.3	
Rice straw	0.5	87.8±2.5	0.05
	2.5	87.2±1.9	
Kyungsan soil	0.2	85.9±0.7	0.02
	1.0	87.7±0.8	
Youngchun soil	0.2	81.1±0.7	0.02
	1.0	82.5±1.0	

*Mean values for triplicate samples with standard deviations

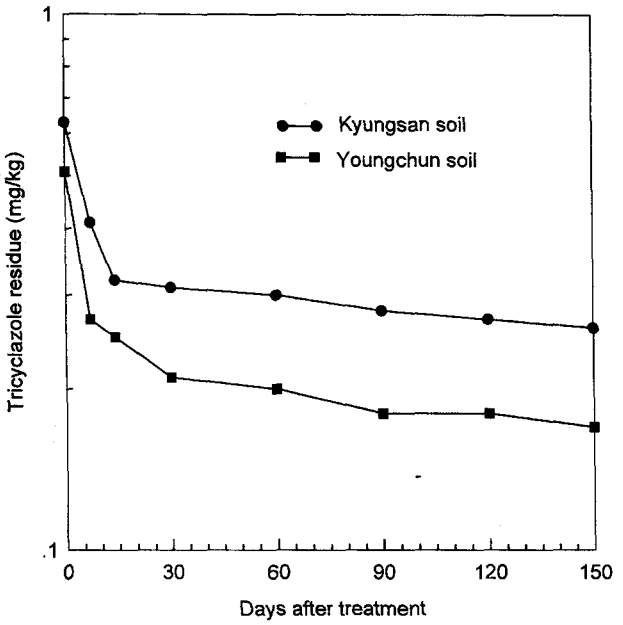

Fig. 6. Persistence of tricyclazole in soils under laboratory conditions.

tricyclazole in soils. Consequently, tricyclazole has much possibility to remain in soils for considerable period after application, therefore a reliable method is needed to track its residues. Meanwhile, comparing the residue level in soil samples collected 30 and 150 days after treatment, the residue level of 150-day samples still reaches 81~84% of 30-day samples. Assuming no degradation occurred during the period, the proposed method was verified to extract more than 80% of tricyclazole residues in 120-day soil samples.

The proposed method satisfies criteria of the analytical method for pesticide residues, which are more than 70% of recovery, less than 10% of CV, and more sensitive than 0.05 mg/kg of detection limit, on Test Guidelines for Pesticide Persistence notified by Rural Development Administration.[9] Analytical procedures do not require any special apparatus or instruments but consist of currently available techniques familiar to the residue analyst. The method is so quick to operate that one experienced person can analyze 12 samples per day. Therefore, authors suggest that the proposed method could be sufficiently applied to the routine analysis of tricyclazole residues in rice and soil samples.

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References

1. Tomlin, C. (1997) *The Pesticide Manual* (11th ed.) pp. 1239-1241, British Crop Protection Council, Surrey, UK.
2. Agricultural Chemicals Industrial Association (ACIA) (1998). *The Pesticide Use Guidelines,* pp. 206-208, ACIA, Korea.
3. Lee, Y.D. (1996) Persistence of tricyclazole in paddy soils, Research Report of the Institute of Science and Technology, pp. 1-55, Taegu University, Korea.
4. Day, E.W. Jr., Koons, J.R., and O.D. Decker (1980) Tricyclazole. In *Analytical methods for pesticides and plant growth regulators,* vol. XI, pp. 263-273, Academic Press, New York, USA.
5. Koons, J.R., D.P. Rainey, and W.L. Sullivan (1985) Determination of tricyclazole and alcohol metabolite in rice grain and straw. *J. Agric. Food Chem.* **33**, 552-555.
6. Willard, H.H., L.L. Merritt, Jr., J.A. Dean, and F.A. Settle, Jr. (1988). Chromatography: general principle. In *Instrumental method of analysis* (7th ed.) pp. 520-521, Wadworth, Belmont, CA, USA.
7. Snyder, L.R. and J.J. Kirkland (1979) *Introduction to modern liquid chromatography* (2nd ed.) pp. 281-288 and 365-374, John Wiley, New York, USA.
8. AOAC (1995) Pesticide and industrial chemical residues. In *Official method of analysis* (16th ed.) pp. 32-33, AOAC International, Arlington, VA, USA.
9. Rural Development Administration (1997) Test guidelines for pesticide persistence. In *Criteria and guidelines for pesticide registration,* Annex 7, Korea.