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Analysis of Insecticide Clothianidin and Its Metabolites in Rice by Liquid Chromatography with a UV Detector

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ABSTRACT

An HPLC method for simultaneous determination of insecticide clothianidin and its metabolites (TZNG, TMG, TZMU, and MNG) in rice was developed and is described in this paper. We extracted the parent clothianidin compound and its metabolites from samples with methanol and an aliquot of the extract was evaporated in a rotary evaporator to remove methanol. The residue was dissolved in 1 mL of 0.005 M 1-sodium octansulfonate (pH 8.3) and transferred to the ENVI-CARB cartridge coupled with an HLB cartridge for solid phase extraction (SPE). Clothianidin and its 4 metabolites were eluted from the cartridges with 3.5 mL of 70% acetonitrile in 0.005 M 1-sodium octansulfonate (pH 7). Determination and separation of clothianidin and its four metabolites were performed in an Agilent Aq column using gradient mobile phase and different UV wavelengths. The average recoveries of clothianidin, TZNG, TMG, TZMU, and MNG were 110%, 87%, 113%, 108%, and 61%, respectively, spiked at 2 ppm into rice. The relative standard deviation (RSD) was determined to be less than 10% in a triple-repeated confirmation test. The limits of quantification (LOQ) were 0.05 ppm for clothianidin, TZNG, TZMU and MNG, and 0.1 ppm for TMG in rice. The method was applied for the determination of clothianidin and its metabolites in 8 polished and 4 unpolished rice grain samples, each of which was collected from a different market in Taiwan. No residue of clothianidin or any of its metabolites was found at levels or at greater than the LOQ in these rice samples.

Key words: clothianidin, metabolites, residue, analysis, rice

INTRODUCTION

Clothianidin, (*E*)-1-(2-chloro-1,3- thiazol-5-yl-methyl) -3-methyl-2-nitroguanidine, is a neonicotinoid used in systemic insecticide. This compound is highly effective in controlling hemipterous insects as well as coleopterous, thysanopterous, and certain lepidopterous pests[(1)]. As with other neonicotinoid insecticides, clothianidin acts agonistically on insect nicotinic acetylchloine receptors[(2)]. Clothianidin is considered an alternative to imidacloprid in the control of aphids and viral diseases in sugar beet[(3)] and has been used to control rootworms (*Diabrotica* spp.) in corn[(4)]. In Taiwan, a water-soluble granule (SG) of 16% clothianidin is commonly applied to commercial rice fields in a concentration of 64 g active ingredient (A.I.)/ha to control Brown planthopper, leafhopper, and stinkbug populations.

When orally administered to rats in a test, clothianidin was almost completely excreted through urine and faeces within 2 days of consumption[(5)]. The major compound detected in excreta was the original clothianidin. The metabolites were *N*-(2-chlorothiazol-5-yl-methyl)-*N*'-nitroguanidin (TZNG) and *N*-methyl-*N*'-nitroguanidine (MNG)[(5)]. The metabolic pathways of clothianidin in maize and sugar beets after seed dressing were similar to that described for rats[(6)]. The unaltered parent compound is also the major residue component found in maize and sugar beet at harvest[(6)]. The major metabolic pathway involves *N*-demethylation of clothianidin to produce TZNG[(6)], hydrolysis of the nitroimino moiety to form *N*-(2-chlorothiazol-5-yl-methyl)-*N*'-methylurea (TZMU), denitrification to form *N*-(2-chlorothiazol-5-yl-methyl)-*N*'-methylguanidine (TMG), and cleavage of C-N bond within TMG to form methylguanidine (MG). MG is a naturally occurring compound and, as such, is not a residue of concern with regard to crops[(6)]. Figure 1 shows the chemical structures of clothianidin and its four metabolites. Because thiamethoxam, another neonicotinoid insecticide, metabolizes quickly into clothianidin[(7)], it may also be necessary to determine the residue profile on plants for clothianidin and its metabolites following thiamethoxam application.

Kim *et al*. (2004) developed an enzyme-linked immunosorbent assay (ELISA) method for assaying the insecticide imidacloprid using monoclonal antibodies (MAb). They found similar affinities between one MAb for acetamiprid and clothianidin, but noted they were 50-times weaker than one MAb of imidacloprid[(8)].

The purpose of our study is to develop a simple and rapid method for simultaneous determination of clothianidin and its four metabolites in grains of rice. This is the first report on analytical methods that can be used to detect clothianidin and its metabolites in crops.

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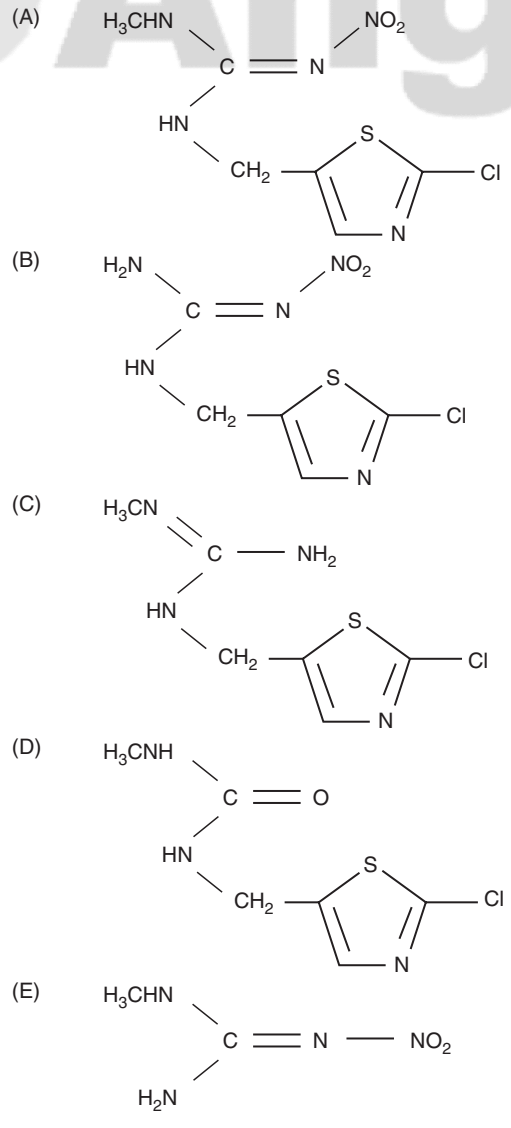

Figure 1. The chemical structures of clothianidin (A) and major associated metabolites (B, TZNG; C, TMG; D, TZMU; E, MNG).

MATERIALS AND METHODS

I. *Materials and Reagents*

The certified reference standards of clothianidin and its four metabolites, TZNG, TZMU, MNG, and TMG, were provided by Sumitomo Chemical Takeda Agro Company, Ltd. (Tokyo, Japan) and Taiwan Lih-Nung Agricultural Chemical Industries, Ltd. A stock standard solution was prepared with methanol for TMG. The other four compounds were prepared with acetonitrile to a concentration of 1000 μg/mL. Working solutions were prepared by diluting the stock solution with either methanol or acetonitrile. Methanol, acetonitrile (HPLC grade) and glacial acetic acid were purchased from Merck (Darmsadt, Germany). Water was purified through a Millipore Milli-Q water purifier. 1-Octanesulfonic acid (sodium salt) was purchased from J. T. Baker (NJ, USA). 1-sodium octansulfonate solution (0.005 M) was prepared by dissolving 1.08 g 1-octanesulfonic acid (sodium salt) in 1 L of water and adjusting the pH to either 7 or 8.3 with the addition of 0.1 N hydrochloric acid or sodium hydroxide solution. ENVI-CARB (250 mg, 3 mL) and Oasis HLB (60 mg, 3 mL) cartridges were purchased from Supelco (PA, USA) and Waters (Mass, USA), respectively.

Table 1. Gradient mobile HPLC separation phases for clothianidin and its four metabolites

Time	(%)				Flow rate
(min)	A[a]	B[b]	C[c]	D[d]	(mL/min)
0-7	100	0	0	0	0.10
7.1-27	0	20	80	0	0.37
27.1-33	10	30	0	60	0.40
33.1-40	100	0	0	0	0.35
40.1-43	100	0	0	0	0.10

[a]Water.
[b]Acetonitrile.
[c]0.005 M 1-sodium octansulfonate (pH 7).
[d]Methanol.

II. *High Performance Liquid Chromatography*

HPLC analysis was conducted on an Agilent 1050 system (Agilent Technologies, Taipei, Taiwan) equipped with a variable wavelength detector (VWD) and an Agilent Aq (15 cm × 2.1 mm I.D.; particle size, 5 μm) column. All runs were acquired and processed using the Agilent ChemStation software. Detector wavelength was set at 269 nm for MNG and at 254 nm for clothianidin and the other three metabolites. HPLC conditions are shown in Table 1. Clothianidin and its metabolites were eluted from the LC column by mobile phase consisting of 20% acetonitrile and 80% 0.005 M 1-sodium octansulfonate (pH 7). For regenerating the LC column and maintaining the repeatability of retention times, a mobile phase consisting of 60% methanol, 30% acetonitrile, and 10% water was employed to wash the column (Table 1). Injection volume was 50 μL. Standards were injected between sample injections. Identification and quantification of clothianidin and its four metabolites in test samples were accomplished by comparing the retention time and peak area of samples to those of the standards.

III. *Extraction and Cleanup*

A 5 g sample of unpolished rice was transferred into a glass jar and homogenized for 1 min with 50 mL of methanol. The homogenate was then filtered under reduced pressure through a funnel using Advantec No. 2 filter paper (Toyo Roshi Kaisha, Japan). The volume of filtrate was raised to 200 mL by the addition of methanol. A 10 mL portion of sample was evaporated to remove methanol under reduced pressure. The residue was dissolved in 1 mL of 0.005 M 1-sodium octansulfonate (pH 8.3) and applied to an ENVI-CARB cartridge coupled with a HLB cartridge for solid phase extraction (SPE). The combined cartridges were previously conditioned with 5 mL of methanol, 3 mL

of water, and 3 mL of 0.005 M 1-sodium octansulfonate (pH 8.3), followed by sample loading with 6 mL of 0.005 M 1-sodium octansulfonate (pH 8.3) twice, and washed with 3 mL of 1% acetonitrile in 0.005 M 1-sodium octansulfonate (pH 8.3). Clothianidin and its four metabolites were eluted from cartridges using 3.5 mL of 70% acetonitrile in 0.005 M 1-sodium octansulfonate (pH 7). The eluent was evaporated to ~2.5 mL under nitrogen and reconstituted to 5 mL with 0.1% acetic acid for HPLC analysis. A recovery study was carried out in triplicate by spiking 10 μg standards into 5 g testing samples to achieve a 2 ppm concentration level. Blank tests without spiking standards were also performed to determine the contribution of background factors.

IV. *Survey Study and Storage Test*

Four unpolished and 8 polished rice grain samples were collected from 12 markets in eight Taiwan counties. Each rice grain sample was ground with a mill into powder (500 g). At least 100 g of each milled sample (laboratory sample) was stored at -20°C for further analysis. The stability of clothianidin in rice during freeze storage was evaluated using a blank sample of 5 g. The prepared blank rice samples in triplicate were fortified using 2, 10, and 20 ppm of clothianidin, respectively. The analytical procedures used to measure and analyze the clothianidin and metabolites in our rice samples have been previously described.

RESULTS AND DISCUSSION

I. *Liquid Chromatography*

Figure 2 shows HPLC chromatograms of clothianidin and metabolite standards, rice samples, and rice samples spiked with clothianidin and metabolites. There was generally good resolution, with the exception of MNG on the HPLC analysis graph. The retention times of MNG, TZMU, TZNG, clothianidin, and TMG were 8.8, 13.7, 14.7, 15.7, and 16.7 min, respectively. We constructed an eight-point standard curve plotting peak area response versus concentration in μg/mL using data obtained from our injection of standards of known concentrations. Concentrations of the standard solutions ranged from 0.0025 to 0.5 μg/mL (0.005 to 0.5 μg/mL for TMG). Linearity of HPLC/UV detection was evaluated using the square correlation coefficients (R^2) of the standard curves generated by clothianidin and its 4 metabolite standard mixtures. Table 2 shows calibration data. We evaluated repeatability of peak areas by analyzing the relative standard deviation (RSD) of five injections carried out on the same day. We tested reproducibility by conducting five injections at random over a 10-day period. RSD ranged from 1.7% to 6.5% (n = 5) for intra-day and inter-day analyses. The limit of detection (LOD) and limit of quantification (LOQ) were defined as a signal to noise (S/N) ratio of 3 and 10-20, respectively. The LOD and LOQ of clothianidin and its metabolites ranged from 0.0025 to 0.005 and 0.05 to 0.1 ppm, respectively. The SPE procedures and pH of 1-sodium octansulfonate solution can be expected to affect the clean-up efficiency of clothianidin and its metabolites. If the solution was directly eluted with 3.5 mL of 70% acetonitrile in 0.005 M 1-sodium octansulfonate (pH 7) in SPE procedures without washing with 3 mL of 1% acetonitrile in 0.005 M 1-sodium octansulfonate (pH 8.3), there would be significant background noise (Figure 2D) that could unnecessarily complicate efforts to identify clothianidin and its metabolites in the HPLC chromatogram. Washing and the elution of clothianidin and its metabolites with pH 7 of 0.005 M 1-sodium octansulfonate

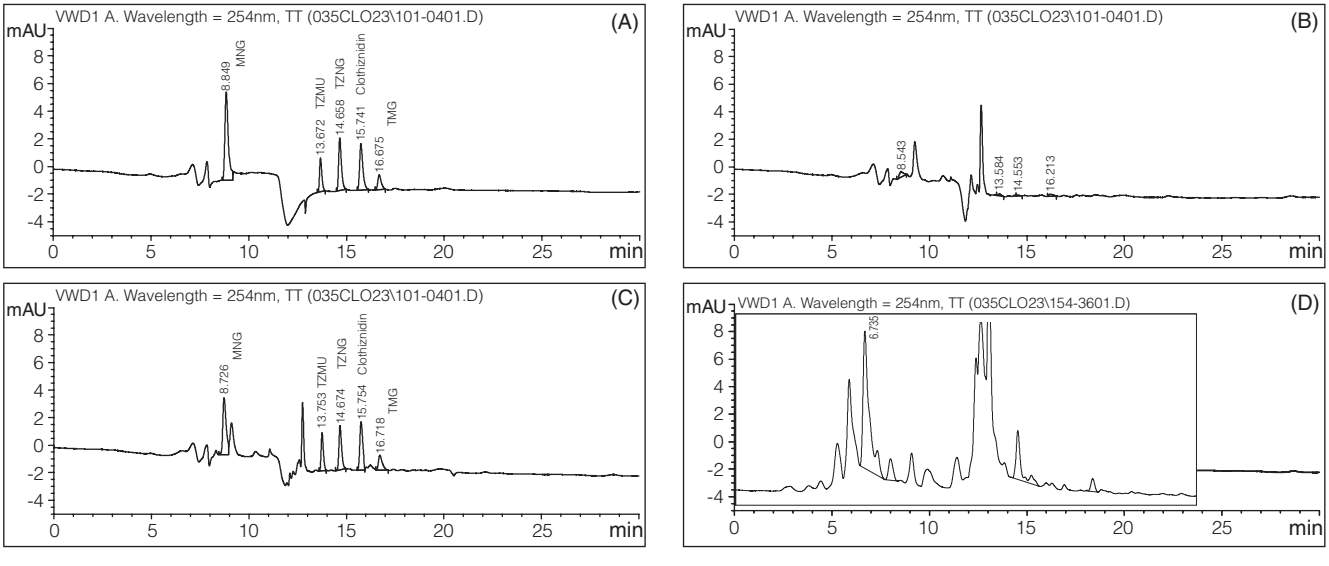

Figure 2. HPLC chromatograms of clothianidin and metabolite standards (A, 0.1 μg/mL), the control rice sample (B), the spiked sample (C), and the unwashed control sample during the SPE procedure (D).

(Figure 2B and C) could reduce rice matrix interference. We reconstituted the eluent with 0.1% acetic acid following SPE procedures to improve resolution of clothianidin and its metabolites under HPLC. The sensitivity and resolution of these five peaks can expected to be poor if the residue is dissolved in either 70% acetonitrile in 0.005 M 1-sodium octansulfonate (pH 7, Figure 3D) or methanol (Figure 3E). The retention time of TMG will be longer if the residue is dissolved either in water (Figure 3B) or in 0.005 M 1-sodium octansulfonate (pH 8.3, Figure 3C).

II. *Recovery Study*

Average recoveries of clothianidin, TZNG, TMG, TAMU, and MNG were 110%, 87%, 113%, 108%, and 61%, respectively. The RSD, when spiked at 2 ppm into rice, was determined to be < 10% (see Table 3). The low recovery of MNG in rice may be due to loss during washing procedures related conducted during SPE clean-up caused by MNG's higher polarity (MNG will not dissolve in most organic solvents). Further studies will be conducted in order to improve MNG recovery. According to a study by Klein[6], the metabolite MNG is present in minute quantities in plants (0.7-5.7% of the total radioactive residue) following clothianidin application and, therefore, should not be considered a relevant factor in the residue definition[6]. While additional and individual clean-up procedures reduced some matrix interference, they were time-consuming.

Based on our studies, we propose the following methodology to quickly measure levels of clothianidin and its four metabolites in rice simultaneously. The use of the ENVI-CARB cartridge in combination with a strong anion-exchange and amine cartridge for SPE of pesticides in apples, oranges, carrots, and wheat was found to reduce the matrix enhancement effect more than that of other SPE approaches. However, even with these three cartridges, the effect was not completely eliminated[9]. Our results

Table 2. Calibration data for clothianidin and its metabolites under HPLC analysis

Compounds	Range of concentration (μg/mL)	Equation	R^2
Clothianidin	0.0025-0.5	y = 691.295 x - 0.093	0.999
TZNG	0.0025-0.5	y = 678.122 x - 0.316	0.999
TMG	0.0050-0.5	y = 88.536 x + 0.306	0.989
TZMU	0.0025-0.5	y = 371.481 x + 1.072	0.998
MNG	0.0025-0.5	y = 1356.659 x + 5.032	0.999

Table 3. Recovery data for clothianidin and its major metabolites spiked in rice (spike level, 2 ppm, n = 3)

Compound	Recovery (%)	RSD (%)
Clothianidin	109.7	8.7
TZNG	86.8	9.8
TMG	113.0	4.3
TZMU	107.8	4.9
MNG	61.0	4.0

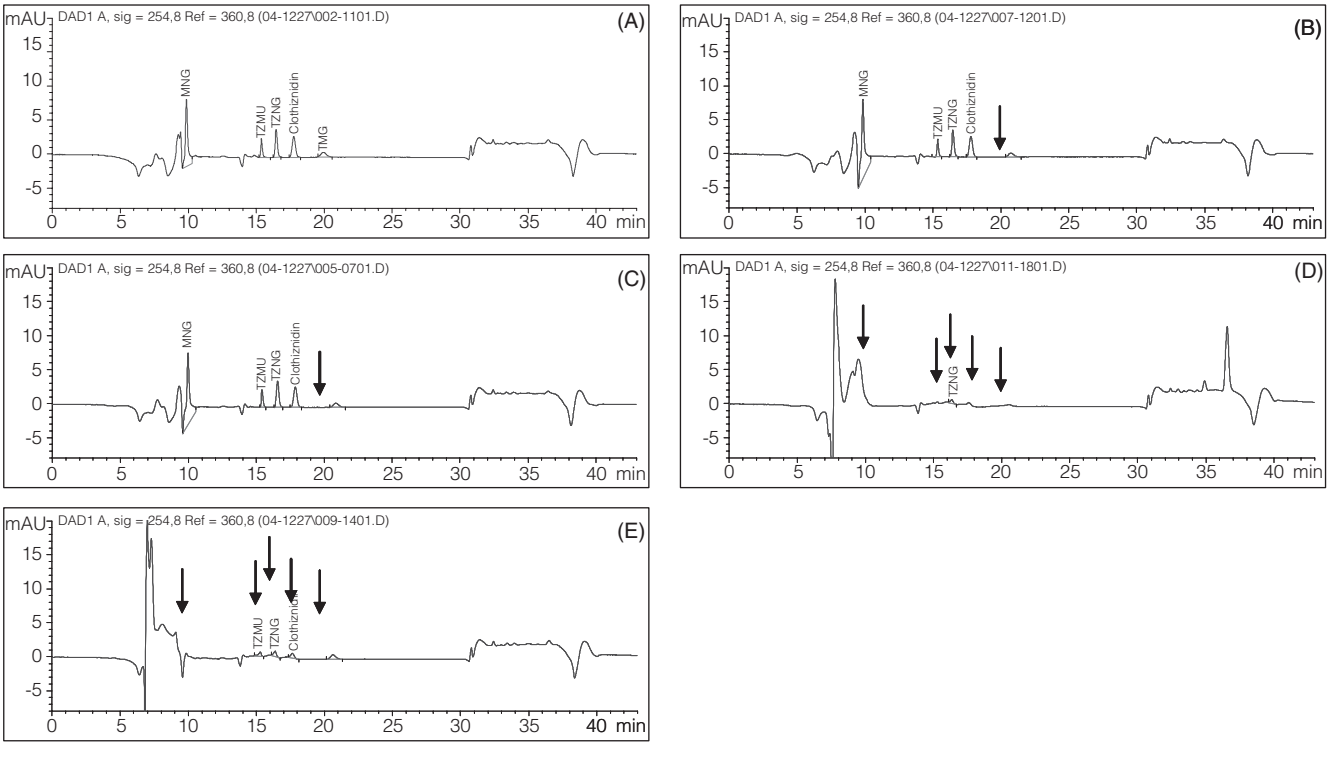

Figure 3. HPLC chromatograms of clothianidin and its metabolite standards (0.1 μg/mL) in 0.1% acetic acid (A); pure water (B); 0.005 M 1-sodium octansulfonate (pH 8.3)(C); 70% acetonitrile in 0.005 M 1-sodium octansulfonate (pH 7)(D); and methanol (E). The arrows indicate the peaks of standards.

Table 4. Summary of data on the stability of clothianidin in rice grains kept in frozen storage

Days frozen	Fortification date	Analysis date	Recovery % (RSD, n = 3)			
			Control	Spike level (ppm)		
				2	10	20
40	11 Aug 04	20 Sep 04	NPF[a]	82.1 (3.2)	94.2 (2.8)	98.6 (1.3)

[a]Denotes no peak found.

indicate that simple SPE procedures using an ENVI-CARB cartridge, when coupled with an HLB cartridge (a macroporous copolymer made from a balanced ratio of two monomers–lipophilic divinylbenzene and hydrophilic *N*-vinylpyrrolidone) and a gradient mobile phase HPLC conditioned with an Agilent Aq column, comprise a quick and accurate method by which clothianidin and its metabolites can be identified and measured in rice.

III. *Residues in Rice Samples*

No residues (< LOQ) of clothianidin or its metabolites were identified in rice samples collected for this study. All study samples were kept in frozen storage for no longer than 40 days between time of sampling and time of extraction. Fortified and unfortified rice samples were analyzed to determine the storage stability of clothianidin in rice extracts during storage at -20 ± 5°C for up to 40 days. Results indicate that clothianidin in rice will remain stable under the conditions stated for up to 40 days (Table 4).

CONCLUSIONS

We developed through this study a simple and accurate HPLC method by which clothianidin and its metabolites, TZNG, TMG, TZMU, and MNG, could be identified and measured in rice. Results suggest that the method proposed is less time-consuming. Clothianidin and associated metabolite residues in rice samples collected from 12 different markets fell below levels of 0.05-0.1 ppm.

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