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연구보문

Residual Analysis of Insecticides (Lambda-cyhalothrin, Lufenuron, Thiamethoxam and Clothianidin) in Pomegranate Using GC-μECD or HPLC-UVD

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In this study, the residual levels of four insecticidal compounds (lambda-cyhalothrin, lufenuron, thiamethoxam, and clothianidin) were monitored in the pomegranate, in order to assess the risk to consumers posed by the presence of such residues. The insecticides were applied at the recommended dose rates onto pomegranate trees. The samples were then collected at harvesting time after several treatments (two, three, and four treatments). After sample preparation progressed through the clean-up procedure, lufenuron, thiamethoxam, and clothianidin residues were analyzed via a HPCL-UVD, and the lambda-cyhalothrin residue was analyzed via a GC-μECD. The versatility of this method was evidenced by its excellent linearity (>0.9998 to 1) at broad concentration ranges. The mean recoveries evaluated from the untreated sample spiked with two different fortification levels ranged from 72.45 to 113.90%, and the repeatability (as a relative standard deviation) resulted from triplicate recovery tests was in a range from 0.80 to 11.75%. The residues of all insecticides determined from treated pomegranate samples and their LOD levels (lunfenuron, 0.01; lambda-cyhalothrin, 0.005; thiamethoxam, 0.01; clothianidin, 0.02 mg/kg) were much lower than their MRLs (0.5 mg/kg).

Key Words: GC-μECD, HPLC-UVD, Insecticides, Pomegranate

INTRODUCTION

Pomegranate aril juice provides approximately 16% of an adult's daily vitamin C requirement per 100 mL serving, and is also a good source of vitamin B5, potassium, and antioxidant polyphenols; the marked antioxidant activities of the pomegranate have been well established, and many clinical studies have demonstrated that pomegranate consumption contributes to the prevention of several diseases, such as coronary heart disease and certain types of cancer (Palou *et al*., 2007). Consumer demand for this fruit has recently been trending upward (Suhaibani and Ali, 2004). Pesticides have been employed broadly for pest control and weed control purposes, and also to promote crop growth; this has increased crop productivity in general.

Lambda-cyhalothrin, (*R*,*S*)-α-cyano-3-phenoxybenzyl (1*S*)-*cis*-3-[(*Z*)-2-chloro-3,3,3-trifluoropropenyl]-2,2-dim ethylcyclopropanecarboxylate, is a pyrethroid insecticide with a unique chemical configuration consisting principally of a dimethyl cyclopropane carboxylate moiety (Bonafos *et al*, 2007; Seenivasan and Muraleedharan, 2004; Çavaş *et al*., 2003). It exhibits marked activity against a broad range of chewing and sucking pests--in particular, Lepidoptera, Coleoptera, and mites infesting fruits, cereals, maize, cotton, wheat, pulses, and oilseeds; from a public health standpoint, lambda-

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cyhalothrin also functions effectively as a vector control agent.

Lufenuron, (*RS*)-1-[2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)phenyl]-3-(2,6-difluorobenzoyl) urea, is a benzoylphenylurean (BPU)-class insecticide, which functions as a chitin synthesis inhibitor (CSI). Doses of lufenuron higher than that recommended for anti-flea treatment have also proven quite effective in the treatment of dermatomycosis in dogs and cats (Ahrie *et al*., 2008; Khay *et al*., 2008). The compound appears to be minimally toxic to mammals, since its activity is highly specific to immature insects at the molting stage.

Thiamethoxam, 3-[(2-chloro-5-thiazoly)methyl] tetrahydro-5-methyl-N-nitro-4H-1,3,5-oxadiazin-4-imine, has been newly introduced to the American market in the past few years and has been approved for use as a soil, foliar, and seed treatment agent for the control of aphids, whiteflies, and certain beetles, among others(Campbell *et al*., 2005; Pandey *et al*., 2009; Rancan *et al*., 2006). It is applied during the growing season in different vegetable crops in India to control insects and fungal diseases (Singh *et al*., 2004).

Intensive pesticide use has resulted in contaminations of agricultural products, as well as soil and water. Pesticide residue analysis is essential to address rising consumer concerns regarding possible contamination issues. Cyhalothrin has been investigated in this regard primarily via HPLC methods, but a few studies using a HPLC-MS have also been performed recently (Seccia *et al*., 2008). Some authors' studies have dealt with the determination of thiamethoxam in various real samples using different methods; the LC-MS technique was employed by Obana *et al*. (Rancan *et al*., 2006; Pandey *et al*., 2009; Campbell *et al*., 2005; González *et al*., 2008). Five benzoylureas, including lufenuron in ground water samples and Chinese Cabbage, were previously evaluated using HPLC with different detectors—fluorescence (Garía *et al*., 2006) and ultraviolet detection as previously described by Gamon *et al*. (1998) (Khay *et al*., 2008). Lambda-cyhalothrin is mainly detected and analyzed via Gas Chromatography, but there have also been some research determinations of lambda-cyhalothrin in various samples using GC-ECD (Seenivasan and Muraleedharan, 2009; Bouldin *et al*., 2006).

The principal objective of this study was to develop and carry out a routinizable monitoring of the residue levels of lambda-cyhalothrin, lufenuron, thiamethoxam, and clothianidin in the pomegranate.

MATERIALS AND METHODS

Chemicals

Pesticides (lambda-cyhalothrin, lufenuron, thiamethoxam and clothianidin), all at above 98% purity, were provided by the Society of Pesticide Industries, Republic of Korea.

All reagents and solvents employed herein were of analytical-grade or HPLC-grade. Organic solvents were purchased from Baker NJ (USA), and sodium sulfate (anhydrous) and sodium chloride were supplied by Junsei Chemical Co., Ltd (Japan). The silica gel used for column chromatography cleanup was purchased from Sigma-Aldrich (USA). Solid phase extraction (SPE; florisil) cartridge was purchased from phenomenex (USA).

Sample preparation

Lambda-cyhalothrin, lufenuron, and thiamethoxam, including its metabolite clothianidin were extracted individually from pomegranate samples. Exactly 20 g of samples were homogenized with 100 mL of methanol-water (50:50, v/v), methanol, or acetone, respectively, at 1200 rpm for 5 min (WiselMix™ HG-150; Daehan Scientific, Korea). The homogenates were then filtered through Whatman filter paper (No. 6) (Whatman International Ltd, England) topped with Celite 545 (Daejun Chemicals and Materials Co., Ltd. Korea) in a porcelain Büchner funnel, and subsequently washed with the same extraction solvent. The filtrates for lambda-cyhalothrin and lufenuron were partitioned with 100 mL dichloromethane and n-hexane, respectively; whereas the filtrates for thiamethoxam and its metabolite clothianidin were filled up to a volume of 200 mL and a 50 mL sample was subjected to portioning with 100 mL of *n*-hexane. Partitioning was enhanced by salting-out with 50 mL saturated NaCl. The partitioned organic layers were then dehydrated through sodium sulfate (anhydrous) and evaporated to dryness in a rotary vacuum evaporator (Büchi Rotavapor R0114, Switzerland) at 40℃. The residues for lambda-cyhalothrin and lufenuron were dissolved in 4 mL *n*-hexane and that for thiamethoxam and

clothianidin in 5 mL dichloromethane for cleanup.

Clean-up for lambda-cyhalothrin and lufenuron was conducted using open preparative chromatographic columns packed with 5 g silica gel and 2 g sodium sulfate anhydrous (Na_2SO_4) placed atop the columns. The columns were then activated with 30 mL *n*-hexane followed by sample extract loading, after which the analytes were eluted with the appropriate solvents. Lambda-cyhalothrin was eluted with 50 mL acetone-n-hexane (10:90, v/v), while lufenuron was eluted with 50 mL acetone-n-hexane (15:85, v/v) after continuous washing with 50 mL *n*-hexane-acetone (95:5, v/v) and 50 mL *n*-hexane-acetone (90:10, v/v). The eluates were evaporated *in vacuo* at 40℃ and then lambda-cyhalothrin was re-dissolved in 2 mL *n*-hexane and lufenuron in n-hexane-propanol-methanol (90:5:5, v/v), lambda-cyhalothrin and lufenuron were analyzed via GC-μECD and HPLC-UVD, respectively.

Thiamethoxam and clothianidin were purified using SPE cartridges (florisil 1000 g/6 mL), conditioned with 5 mL dichloromethane. Sample extracts were loaded onto the cartridges. The samples were washed with 10 mL dichloromethane-acetone (96:4, v/v) and eluted with 25 mL dichloromethane-acetone (55:45, v/v). The eluates were evaporated *in vacuo* at 30℃ and re-dissolved in 2 mL methanol-water (50:50, v/v), and then analyzed via HPLC/UVD.

Method validation

Stock solutions at 100 μg/mL of lambda-cyhalothrin, lufenuron, thiamethoxam, and clothianidin were prepared in acetone, respectively. Working solutions of the analytes were prepared with an adequate solvent at different six concentrations as follows; 0.025, 0.05, 0.1, 0.25, 0.5, and 1 μg/mL in n-hexane for thiamethoxam; 0.1, 0.5, 1, 2, 4, and 5 μg/mL in *n*-hexane-propanol-methanol (90:5:5, v/v/v) for lufenuron; 0.025, 0.05, 0.1, 0.5, 1, and 2 μg/mL for thiamethoxam and 0.05, 0.1, 0.2, 1, 2, and 4 μg/mL for clothianidin in methanol-water (50:50, v/v), respectively. All stock and working solutions were stored at -24℃ during study. Calibration curves were created by the peak area versus the concentration of the analytes' working solutions.

To validate the analytical method, recovery tests were examined by spiking working solutions into blank samples at different two concentrations in triplicate. The spiked levels were 0.05 and 0.2 mg/kg for lambda-cyhalothrin, 0.2 and 0.4 mg/kg for lufenuron, 0.1 and 0.5 mg/kg for thiamethoxam, and 0.2 and 1 mg/kg for clothianidin.

Limits of detection (LODs) of the analytes were assessed with a signal-to-noise ratio (S/N ratio). Appropriate concentrations prepared in blank extracts were detected by their own analytical instruments, individually, and every peak height of the analytes was compared with the blank signals.

CONDITIONS OF ANALYTICAL INSTRUMENTS

GC-μECD

Lambda-cyhalothrin analysis was conducted using an Agilent Technologies 7890 A GC System (USA) consisting of a model 7683B autoinjector and an μ-electron capture detector. Chromatographic separation was conducted using an HP-5 (50 m × 0.25 mm, 0.25 μm film thickness) column. The oven temperature was held at 120℃ for 5 min and then increased to 270℃ at a rate of 5℃/min for 5 min. The injection port and detector temperatures were maintained at 250℃ and 280℃, respectively. The injection volume was 2 μL, and the column was flowed with nitrogen gas at 1 mL/min.

HPLC-UVD

Lufenuron analysis was conducted using a Kontron HPLC system (Italy) consisting of a 355 UV-detector and a 322 pump. Chromatographic separation was conducted using a Waters Spherisorb® 5 μL NH_2, 4.6 × 250 mm. The mobile phase was a mixture of n-hexane-propanol-methanol (90:5:5, v/v) and 20 μL of sample was injected into an HPLC column. Lufenuron was detected at a wavelength of 250 nm, and the detected time of lufenuron was 12.92 min.

Analysis of thiamethoxam and clothianidin was conducted using the Shimadzu liquid chromatography system equipped with a SCL-10AVP system controller, LC-6AD pumps, and a SPD-10AVP UV-vis detector (Shimadzu, Kyoto, Japan). An Aqua C18 200Å (4.6×250 mm, 5.0 μm, Phenomenex, USA) was employed as an analytical column for the target compound. The mobile phase was a mixture of methanol-water (30:70, v/v), and the flow rate was 0.6 mL/min. 20 μL of sample was injected onto the HPLC column. Thiamethoxam

and clothianidin were detected at a wavelength of 230 nm; the detection time of thiamethoxam was 10.60 min, and 16.57 min for clothianidin.

RESULTS AND DISCUSSION

Extraction

In this study, pesticides were extracted from pomegranate fruits via liquid-liquid extraction. The whole procedures were simply divided into the following steps: extraction with a polar solvent, liquid-liquid partition with a polar solvent, and cleanup with an open preparative chromatographic column packed with a polar sorbent or an SPE cartridge. A few methods have been previously developed for the determination of lufenuron residues in various matrices, including fruit, vegetables, blood, and groundwater. These studies have generally employed liquid chromatography with mass spectrometric, fluorescence, or diode-array detection, and the procedures have generally been derived from solvent partitioning and solid phase extraction protocols (Brito *et al*., 2002; Khay *et al*., 2008). The n-hexane partition cleanup procedure appears to effectively remove interfering co-extractives for HPLC analysis (Singh *et al*., 2004). The n-hexane partition procedure for the analysis of the insecticide lambda-cyhalothrin was previously developed and described by Seenivasan *et al*. (2009) (Seenivasan and Muraleedharan, 2009), in a study of the residues of lambda-cyhalothrin in tea, and Singh *et al*. (2004) (Singh *et al*., 2004) used a similar method to analyze thiamethoxam residues in fresh and cooked vegetable samples. The sample preparation method employed in this study also resulted in reliable results, which were demonstrated in the validation results.

Linearity

Lambda-cyhalothrin, lufenuron, thiamethoxam and clothianidin evidenced good linearity at the different concentrations employed. The linear equations were as follows: Y=21565.02x + 650.47; Y=61.009x + 3.6892; Y=104382.92x - 628.87; and Y=62164.83x - 374.25, respectively. The correlation coefficients (r^2) ranged from 0.9998 to 1.

Recovery

The analytical methods were validated for the black pomegranate prior to actual analysis. In an effort to validate the analytical method, the recovery percentage was established via the fortification of standard solutions of lambda-cyhalothrin, lufenuron, thiamethoxam, and clothianidin. The achieved recoveries ranged from 73.61% to 108.89% with a standard deviation of ±11.45%, which indicated that the mean recovery should be within a range of 70-110% in order to sufficiently validate the quantitative methods. These results were also fitted in accordance with the relevant international guidelines (SANCO, 2004).

Limit of detection (LOD)

The LODs were calculated considering a value 3 times the background noise obtained for blank samples, which also included the instrument noise. The calculated LODs were 0.005 (lambda-cyhalothrin), 0.01 (lufenuron), 0.01 (thiamethoxam), and 0.02 mg/kg (clothianidin) (Table 1). All limits were substantially lower than the MRLs of 0.5 mg/kg established by the Korea Food and Drug Administration (KFDA, 2005).

Stability of the analytes

The residue analyses of the field samples were conducted subsequent to the completion of the experimental procedures. As the determination of lambda-cyhalothrin, lufenuron, thiamethoxam, and clothianidin residues could be delayed as the result of unforeseen systematic errors and/or poor preliminary experimental results, the actual residues could be altered by chemical or metabolic reactions with sample matrices during storage. Therefore, it is necessary that the stability of the analytes be evaluated in samples stored

Table 1. Calibration curve and linearity of the analytes

Pesticides	Concentration range (mg/kg)	Equation	r^2 value
Lambda-cyhalothrin	0.025 - 6	Y=21565.02x + 650.47	1
Lufenuron	0.1 - 5	Y=61.009x + 3.6892	0.9998
Thiamethoxam	0.025 - 2	Y=104382.92x - 628.87	1
Clothianidin	0.05 - 4	Y=62164.83x - 374.25	1

Table 2. Validation of the analytical method of compounds in pomegranate

Insecticides	Concentration (mg/kg)	Recovery (%)			Average (%)	SD (%)	LOD (mg/kg)
		I	II	III			
Lambda-cyhalotherin	0.05	85.41	91.04	90.00	82.70	5.75	0.005
	0.2	95.46	81.59	85.97	87.67	7.09	
Lufenuron	0.2	95.75	101.59	88.52	95.15	6.35	0.01
	0.4	113.9	105.14	91.19	103.41	11.45	
Thiamethoxam	0.1	86.64	90.49	84.34	87.15	3.11	0.01
	0.5	72.45	74.50	73.89	73.61	1.05	
Clothianidin	0.2	87.37	90.89	110.40	108.89	3.53	0.02
	1	87.37	90.89	88.72	88.99	1.77	

Table 3. Storage stability of the analytes

Pesticides	Spiked concentration	Recovery (%)			Average (%)
		I	II	III	
Lambda-cyhalothrin	5	93.97	98.42	80.62	91.03 ± 9.27
Lufenuron	0.4	105.05	85.61	106.27	99.18 ± 11.75
Thiamethoxam	0.5	71.06	69.45	70.93	70.48 ± 0.89
Clothianidin	1	85.04	90.16	84.00	86.40 ± 3.30

Table 4. Residue levels of the analytes in field-incurred pomegranates

Spraying times	Lambda-cyhalothrin (mg/kg)	Lufenuron (mg/kg)	Thiamethoxama (mg/kg)
Control	<0.01	<0.01	<0.01
2	0.011	0.063	0.100
2	0.004	0.049	0.110
3	0.011	0.118	0.130
3	0.014	0.08	0.100
4	0.016	0.12	0.160
4	0.010	0.097	0.080
MRLs		0.5 mg/kg	

[a]Thiamethoxam residues were calculated in conjunction with clothianidin residues.

for experimental periods of time. Blank samples were fortified with lambda-cyhalothrin, lufenuron, thiamethoxam, and clothianidin at levels of 5, 0.4, 0.5, and 1 mg/kg for each matrix in triplicate in order to test storage stability of the analytes. The storage stability was evaluated by recovery test, and their rates were 91.03 ± 9.27, 99.18 ± 11.75, 70.48 ± 0.89 and 86.40 ± 3.30%, respectively. This indicates that all of these compounds are relatively stable in samples under storage conditions during the experimental time period of this study.

Analysis of field-incurred samples

The analytical method was applied to analyses of the treated samples. Very small quantities of lambda-cyhalothrin, lufenuron, thiamethoxam, and clothianidin were detected in pomegranates treated for different application times. Although the insecticides were detected in the pomegranate, the detected levels were lower than the MRLs for each compound established by the Korea Food and Drug Administration (2005). The residues obtained can be attributed principally to growth dilution occurring between application and sampling, as well as to the volatilization associated with application, removal by weathering, heat decomposition, sunlight UV radiation, or other complex conditions.

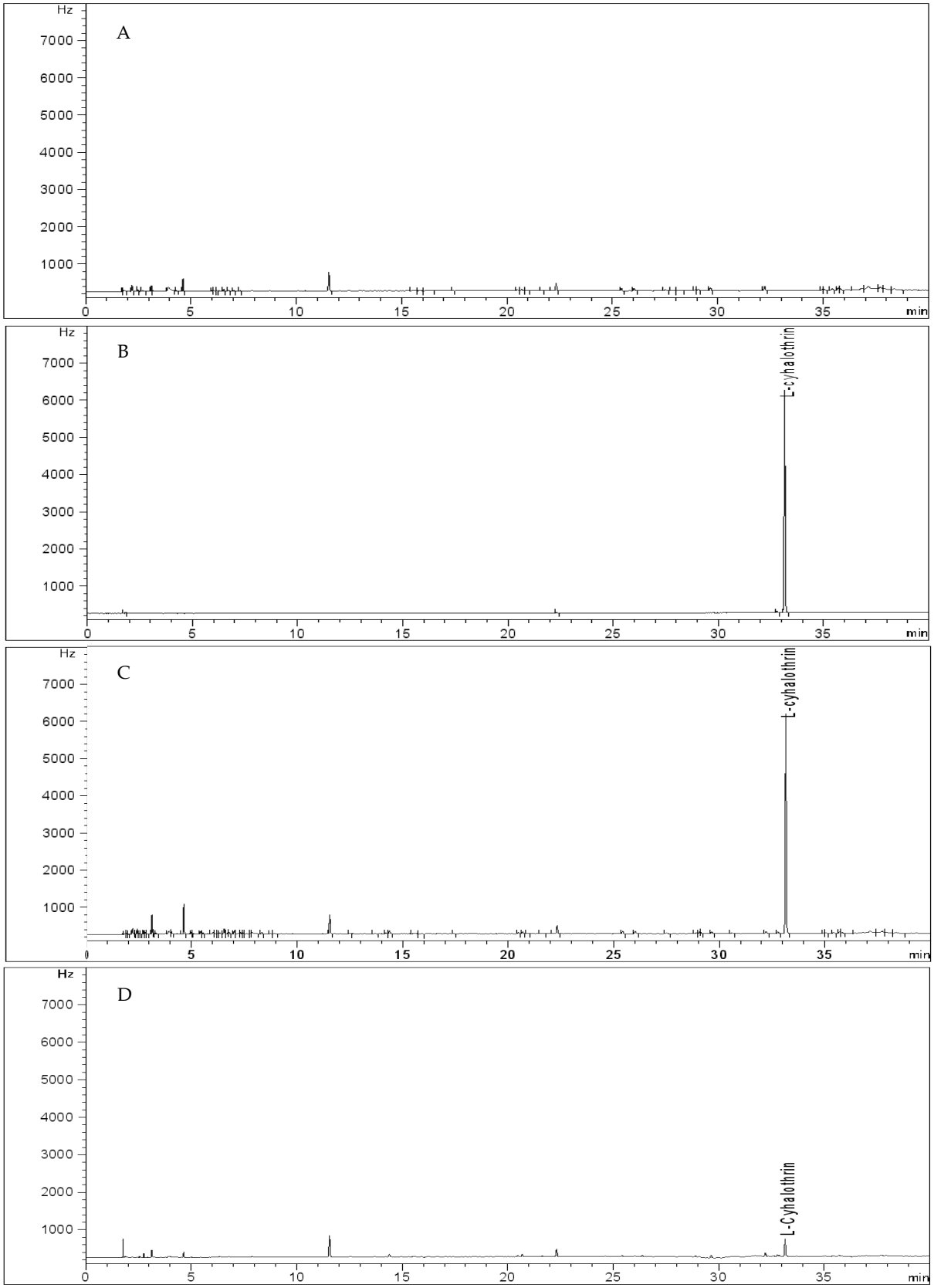

Fig. 1. Typical GC-μECD chromatograms of lambda-cyhalothrin; control pomegranate (A), standard at 1 μg/ mL (B), recovery sample at 0.2 mg/ kg (C), and treated sample 4 times (D).

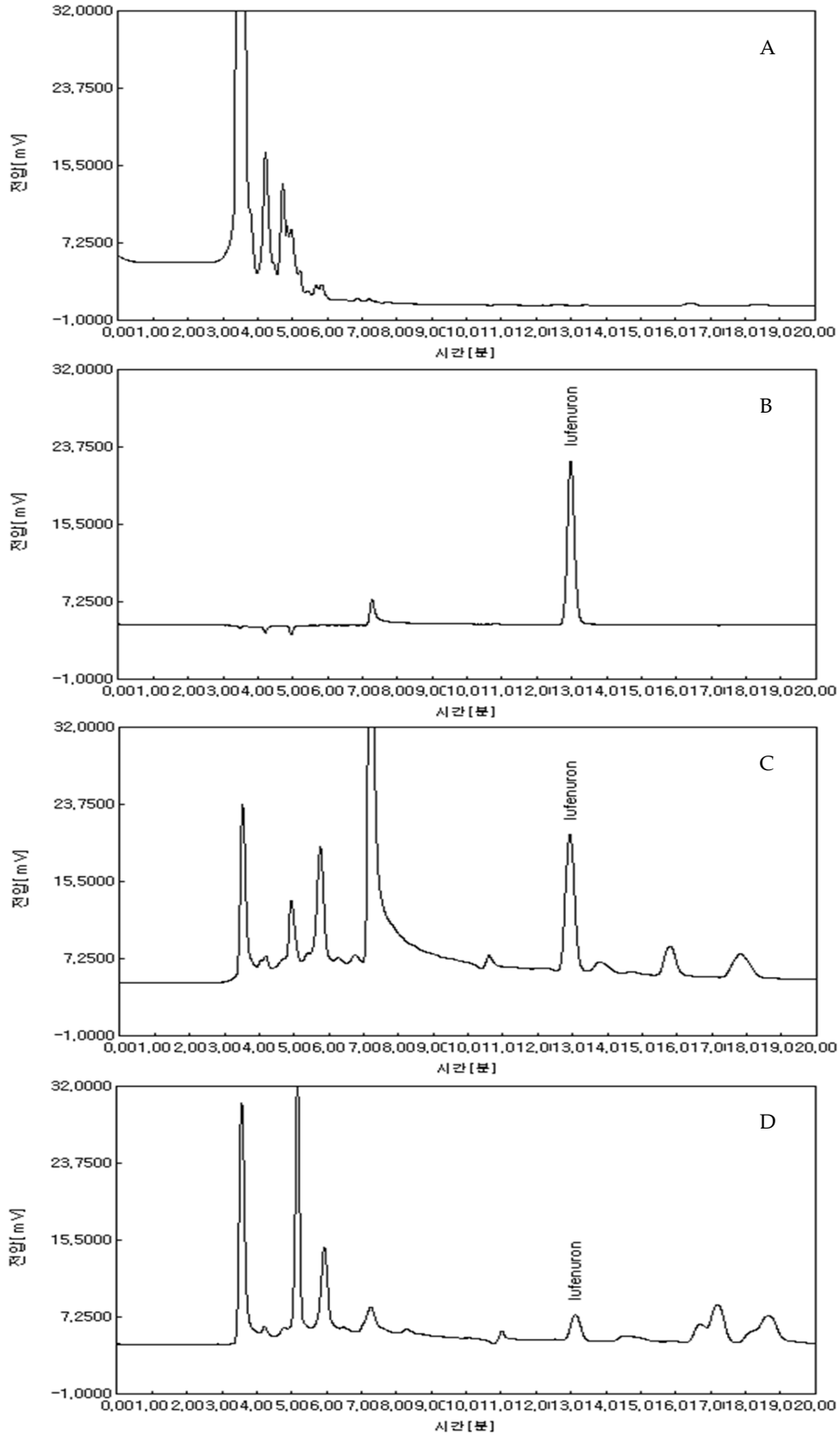

Fig. 2. Typical HPLC chromatograms of lufenuron; control pomegranate (A), standard at 4 μg/ mL (B), recovery sample at 0.4 mg/ kg (C), and treated sample 2 times (D).

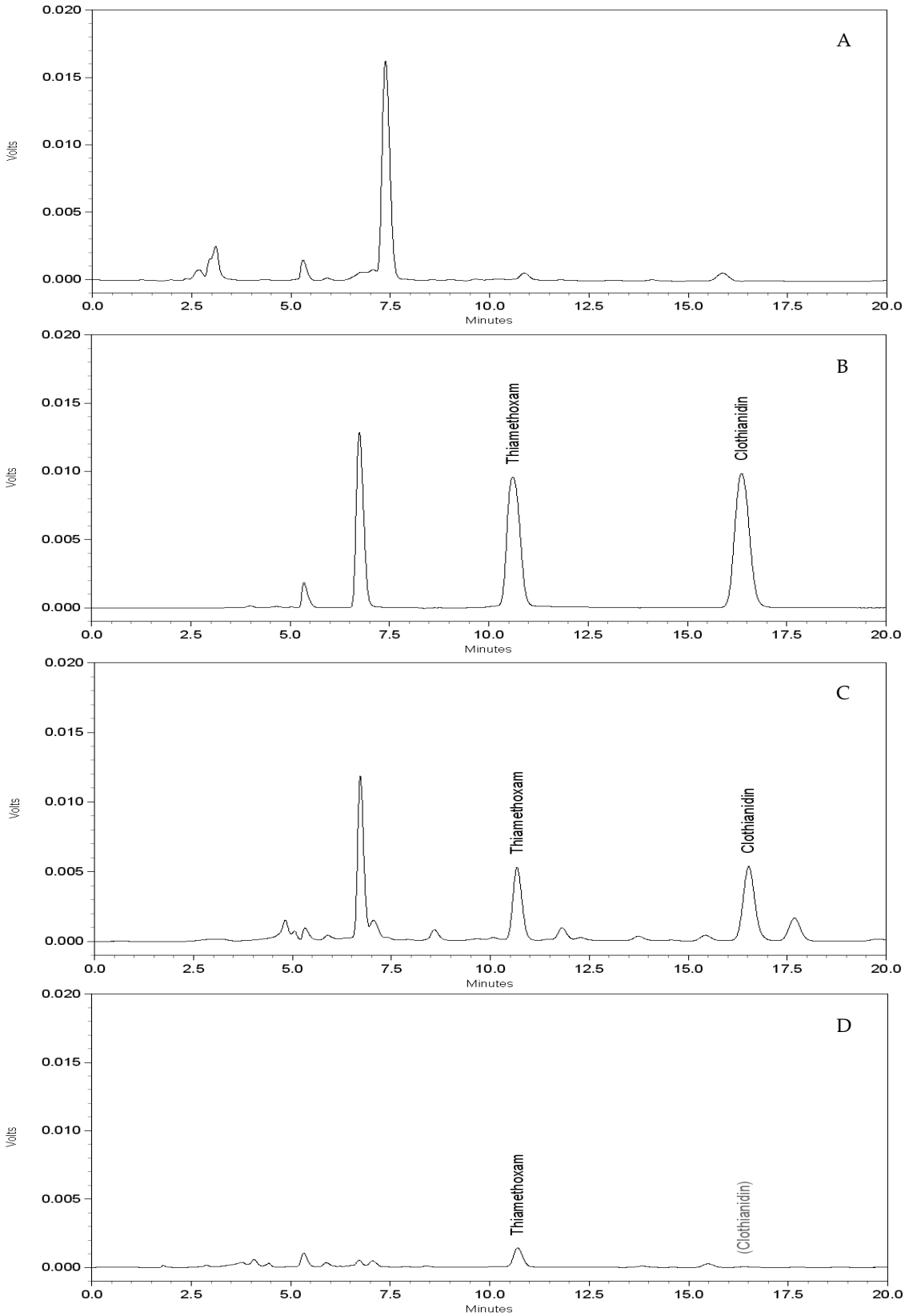

Fig. 3. Typical HPLC chromatograms of thiamethoxam and clothianidin; control pomegranate (A), standard thiamethoxam at 1 μg/ mL and clothianidin at 2 μg/ mL (B), recovery sample thiamethoxam at 0.5 mg/ kg and clothianidin at 1 mg/ kg (C), and treated sample 2 times (D).

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