Journal of Food and Drug Analysis, Vol. 11, No. 4, 2003, Pages 296-301 藥物食品分析 第十一卷 第四期

High Performance Liquid Chromatographic Determination of Imazalil Residue in Agricultural Products

HSIU-CHIN SU* AND A-YANG LIN

Southern District Laboratory, Bureau of Food and Drug Analysis, Department of Health, Executive Yuan
180 Zihyou 2nd Rd., Zuoying District, Kaohsiung City 813, Taiwan (R.O.C.)

(Received: April 25, 2002; Accepted: November 7, 2002)

ABSTRACT

A simple, rapid and reproducible residue analytical method for imazalil in fruits and vegetables has been developed. The method involved the use of an ion-exchange cartridge for sample clean up followed by high performance liquid chromatography with ultraviolet detector. Acetic ether was used for extraction. Bond Elut PRS and Bond Elut C18 were used for purification. The chromatography column used Inertsil ODS-3 and the mobile phase was created by acetonitrile-10 mM KH_2PO_3 solution (pH 2.5), in the ratio of 35:65. The detection limit is 0.01 ppm. Rape, cabbage, honeydew melon, cumquat, mango and corn were each spiked with 0.05-3.0 ppm imazalil to be compared with the recovery study. The yield was 88.7-97.9% for rape, 72.5-87.4% for cabbage, 81.4-90.5% for honeydew melon, 81.9-87.2% for cumquat, 75.0-85.3% for mango, and 86.8-99.0% for corn.

When the method was applied in analyzing commercial fruits and vegetables, no imazalil residue was detected in 20 selected samples.

Key words: imazalil, HPLC (high performance liquid chromatography), residue analysis

INTRODUCTION

Located in the subtropical area, the weather in Taiwan is warm and humid, which leads to the growth of insects and molds. To effectively avoid mildew and increase agricultural production, it is hard to avoid the use of insecticides. Currently, there are more than 400 registered insecticides in Taiwan. Any inappropriate use of insecticides could cause residue problems. Therefore, insecticide residue is gradually becoming an important issue leading to increasing public concerns. In September 2001, the Department of Health (DOH) announced safety limits for residue in 20 categories including 308 insecticides[1]. Obviously the residue of pesticides is an important issue causing DOH concern.

After the harvesting of agricultural products, preservative processes are commonly used to prevent rotting caused by fungus during transportation. Imazalil is a broad spectrum anti-fungus, especially effective for green mold and Aspergillus. It is even more effective for benzimidazole-resistant strains. In the mean time, it can also control molds spore. After processing products with the Smoke Method, imazalil is more effective than thiabendazole[2]. Generally, 1,000 ppm water suspension or 2,000 ppm water-wax solution is utilized during the post-harvesting processes[3]. According to DOH announcements, the upper limit of residue is 0.5 ppm for leaflet vegetables and cabbages, 1.0 ppm for melon and kernels, 2.0 ppm for citruses, and 0.1 ppm for grains[1].

Imazalil 1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy) ethyl]-1H-imidazole (Figure 1) is a white crystal, with a boiling point at 347˚C and a melting point at 52.6˚C. It does not burn at 340˚C, and has a density of 1.242. Imazalil is soluble in low chain length alcohols and aromatic hydrocarbons such as methanol, ethanol, propyl alcohol, butanol, xylene, toluene, and benzene, with solubility > 50%. In ethane, the solubility is 5.21%, and in petroleum ether, 6.16%. It is less soluble in paraffin wax and slightly soluble in water (0.03%). It is stable at room temperature. The acute oral LD_{50} in rats is 227~343 mg/kg. Transdermal LD_{50} in rabbits is 200~4,880 mg/kg. Inhalation LD_{50} in rats > 16 ppm. It bears no irritation to eyes and skin. Subacute non-toxic oral dose is 14~19 mg/kg/day. Transdermal LD_{50} in rats is > 552~667 mg/kg/day. Chronic non-toxic dose is 15~17 mg/kg/day. LD_{50} for rainbow fish is 2.5 ppm, and for bluegill sunfish is > 3.2 ppm. Low toxic to fowl[4]. Imazalil is a systemic pesticide. Its mechanism is to inhibit 14-α-demethylation or 24-methylenedihydro-lanosterol of lanosterol in the fungus cell[5]. Imazalil is effective for fungus-induced mildew of fruit trees, vegetables and flowers. Also, it can be used in the spraying or soaking process of banana and citric fruits. It can be mixed with other pesticides like thiabendazole or carbendazim.

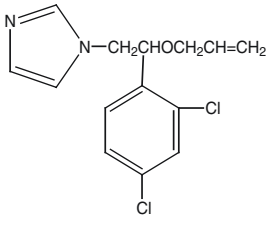

Figure 1. Chemical structure of imazalil.

* Author for correspondence. Tel: 886-7-5581884;
Fax: 886-7-5584711; E-mail: suchin1291@nlfd.gov.tw

Journal of Food and Drug Analysis, Vol. 11, No. 4, 2003

Acetic ether[6-8], acetone[8,13] and dichloromethane[9] have all been used as the extraction solvent in imazalil detection methods. After different extraction procedures, GC-ECD[8,10,11], GC-NPD[6], GC-TID[12] and HPLC-UV[7,9,13] were used as detection instruments. Although higher detection sensitivity can be achieved using GC-ECD and GC-NPD analyzers, complicated extraction procedures and large volumes of solvent can hardly be avoided to clean up the impurities. Based on supporting references[7], in this study, we applied a purification column to clean the impurities. With a simple extraction process and purification column, we expected to develop a method that showed high recovery, good reproducibility, and offered detection limits lower than the official specification limit.

MATERIALS AND METHODS

I. *Materials and Instruments*

(I) *Samples*

Analytical samples were purchased from supermarkets and grocery stores located in Kaohsiung, Taiwan.

(II) *Reagents and chemicals*

Residual grade of anhydrous sodium sulfate, LC grade of acetonitrile, distilled water, methanol, acetic ether, reagent grade of phosphoric acid, KH_2PO_4, K_2HPO_4 and 99% standard imazalil were purchased from Chem Service, Inc., U.S.A. Bond Elut PRS cartridges (500 mg, 3 mL) and Bond Elut C18 cartridges (500 mg, 3 mL) were purchased from Varian, U.S.A.

(III) *High performance liquid chromatography (HPLC)*

The HPLC system consists of two solvent transportation systems (Shimadzu LC-10AD), UV detector (Shimadzu SPD-10AV), photodiode array UV detector (Shimadzu SPD-M10A), degasser (GASTORR GT-103), thermoreactor (Shimadzu CTO-10AS), integrator (SISC Computer Integrator). The column used was Inertsil ODS-3 (4.6 × 150 mm i.d), with acetonitril-10 mM KH_2PO_4 (pH2.5, in the ratio of 35:65) as the mobile phase (in the rate of 1.0 mL/min). UV wavelength was in 225 nm and the sample volume was 20 μL.

II. *Methods*

(I) *Preparation of standard solution and standard curve*

Accurately weighed 50 mg of imazalil was dissolved in acetonitrile and volumetrically adjusted to 100 mL as the 500 μg/mL standard solution. 1 mL of 500 μg/mL standard solution was diluted with acetonitrile to 50 mL to make 10 μg/mL standard solution. Serial dilution was performed with the mobile phase solution to make solutions in the concentration range of 0.5~5.0 μg/mL. 20 μL of the solution was injected into the HPLC, in triplicate, to generate a regression curve with area under the curve against solution concentration.

(II) *Preparation of sample solution*

1. Leaflet Vegetables, Leaf Vegetables, Melons, Kernels, and Citruses

Sample was homogenized with a blender, and then 20 g of accurately weighed sample was added with 1.5 g KH_2PO_4. After high-speed mixing with 30 mL acetic ether and left for 30 min stand still, the supernatant was collected. The residue was extracted with 20 mL of acetic ether 2 times and the extractants were pooled together. After dehydration with 20 g of anhydrous sodium sulfate, the extractants were concentrated to approximately 10 mL in a 40°C water bath for the application in the clean up procedure.

2. Miscellaneous Grain Crops

The sample was homogenized with a blender, and then 50 g of accurately weighed sample was added with 1.5 g of KH_2PO_4. Dry sample should be rinsed with water and left to stand still for 30 min before blending. After high-speed mixing with 60 mL of acetic ether for 10 min and left to stand still, the supernatant was collected. The residue was extracted a second time with 40 mL of acetic ether for 2 times and the extractants were pooled together. After dehydration with 20 g of anhydrous sodium sulfate, the extractants were concentrated to approximately 10 mL in a 40°C water bath for the application in the clean up procedure.

3. Clean Up

After Bond Elut C18 extraction cartridge was activated

Table 1. Recoveries of imazalil from fruits and vegetables.

Sample	Spiked level (μg/gm)	Recovery[a] (%)[b]
Rape	0.75	95.5 (1.8)
	0.50	88.7 (4.8)
	0.25	97.9 (2.8)
Cabbage	0.75	82.5 (2.7)
	0.50	87.4 (4.2)
	0.25	72.5 (2.7)
Honeydew Melon	1.50	81.4 (2.1)
	1.00	90.5 (1.4)
	0.50	88.9 (2.3)
Tangerine	3.00	82.9 (4.9)
	2.00	81.9 (5.4)
	1.00	87.3 (3.8)
Mango	1.50	85.3 (1.8)
	1.00	75.0 (0.9)
	0.50	79.3 (3.8)
Corn	0.15	99.0 (2.5)
	0.10	87.6 (4.1)
	0.05	86.8 (4.7)

[a]Average of triplicate.
[b]Value in the parenthesis is coefficient of variation (CV, %).

sequentially with 10 mL of methanol and 10 mL of acetic ether, and Bond Elut PRS extraction cartridge was activated sequentially with 10 mL of distilled water and 10 mL of methanol, the two cartridge were connected to each other where the Bond Elut C18 cartridge was on the upper layer and Bond Elut PRS cartilage was on the lower layer. The prepared acetic ether solution, II.(1) or II.(2), was applied at the rate of 1 mL/min to elute through the connected cartridge, and then with 5 mL of acetic ether. Bond Elut C18 cartridge was discarded, and the Bond Elut PRS cartridge was washed sequentially with 10 mL of methanol and 10 mL of distilled water. Imazalil was eluted with 10 mL of mobile solution and volumetrically adjusted to make a 10 mL sample solution.

4. Identification Test and Quantitative Analysis

Accurately pippetted 20 μL of sample solution and standard solution were injected into a HPLC for analysis. The peak retention time of sample solution and standard solution was compared and the concentration of imazalil was estimated by the equation below:

$$\text{Imazalil in sample (ppm)} = \frac{C \times V}{M}$$

Wherein; C was imazalil concentration estimated from the standard curve; V was the final volume (mL) in volumetric bottle; and M was the original sample weight (g).

(III) *Recovery test*

The samples were spiked with 0.5x, 1x, and 1.5x of the allowance pesticide residues according to the DOH's announcement. The spiked samples were prepared with method (II) and analyzed, in triplicate, with an HPLC. Results were compared with the spiked amount of standard solution and the recovery rate was estimated by comparing the area under the curves of the sample and the standard. Blank analysis was performed at the same time as the control group.

(IV) *Accuracy*

Standard solution, in 3 different concentrations, was spiked into a blank extract sample before analysis. The accuracy was measured by comparing the area under the curve of spiked sample and the standard solution.

(V) *Precision*

1. Repeatability of injection: The coefficient of variance (CV) was estimated by injecting a standard solution 6 times.

2. Repeatability of analysis: Standard solution, in 3 different concentrations, was spiked into a blank extract sample, in triplicate, before analysis. The accuracy was measured by comparing the area under the curve of spiked sample and the standard solution.

(VI) *Detection limit*

Detection limit (DL) was estimated by the equation: DL = 3.3 σ/S. Wherein σ was the standard deviation, and S was the slope of the standard curve.

The slope, S, was estimated from the imazalil standard curve, and the standard deviation, σ, was estimated by measuring the background data from sufficient number of blank samples.

(VII) *Quantitative limit*

Quantitative limit (QL) was estimated by the equation: QL = 10 σ/S. Wherein σ was the standard deviation, and S was the slope of the standard curve.

The slope, S, was estimated from imazalil standard curve, and the standard deviation, σ, was estimated by measuring the background data from sufficient number of blank samples.

RESULTS AND DISCUSSION

I. *Discussion of HPLC Condition*

(I) *Selection of optimal detection wavelength*

When UV spectrophotometer was used for the detection of imazalil dissolved in the mobile phase solution, a major absorption peak appeared at wavelength 225 nm. UV225 nm is therefore selected as the wavelength used for the HPLC detector (Figure 2).

(II) *Selection of mobile phase*

The mobile phase in this study, according to Ito et al.[7], was 10 mM Sodium 1-tridecanesulfonate in acetonitrile: methanol:H_2O (4:3:3, v/v), and phosphoric acid is used to adjust the pH to 2.5. Through the preliminary finding, the addition of ion pairs caused delayed retention time of imazalil to about 15 min. This is inefficient for a

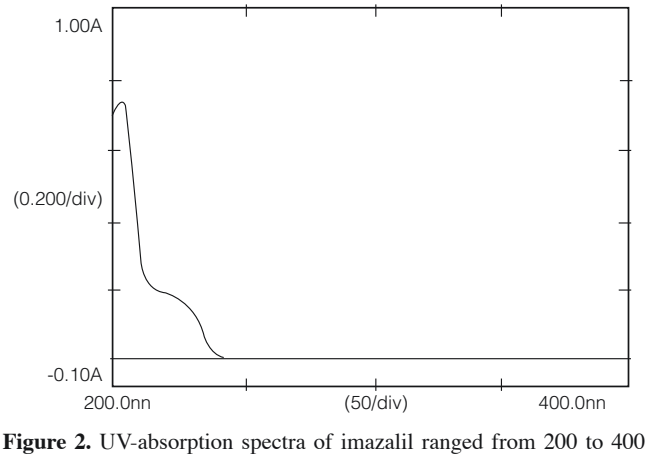

Figure 2. UV-absorption spectra of imazalil ranged from 200 to 400 nm.

Journal of Food and Drug Analysis, Vol. 11, No. 4, 2003

single test item. In the mean time, sodium 1-tridecanesulfonate is not cost-effective when used for routine analysis.

Another mobile phase, acetonitrile:0.01M K_2HPO_3 solution (6:4), was tested in this study according to Yoshiyuki et al.[13]. After a series of tests, the proportion of acetonitrile:0.01M K_2HPO_3 was changed to 35:65, and phosphoric acid was used to adjust the pH to 2.5. Imazalil can be well-separated in this condition. The retention time is 5.2 min and RSD of retention time is less than 1% (n = 7). RSD of repeatability of injection is less than 1% (n = 6). This mobile phase solution is suitable as the elution solvent for the purification process (Figure 3).

II. *Discussion of Sample Preparation*

(I) *Discussion of extraction solvent*

In most articles, acetic ether[6-8], acetone[8,13] and dichloromethane[9] are used as the extraction solvent in sample preparation of fruits and vegetables during the imazalil residue analysis. After surveying references and conducting real tests, acetic ether is selected as the extraction solvent. While using solid-phase extraction cartridge, the best extraction effect for imazalil was observed. Therefore, acetic ether is selected as the extraction solvent in this study.

(II) *Discussion of purification condition*

Samples were extracted with acetic ether and concentrated by dehydrated sodium sulfate. After direct analysis with HPLC, the spectrum was found to contain too much impurity interference. The peak of imazalil was covered by impurities and unable to be quantified (Figure 4A, B). Therefore, the sample extract has to be further purified.

In the articles, solid-phase extraction was made

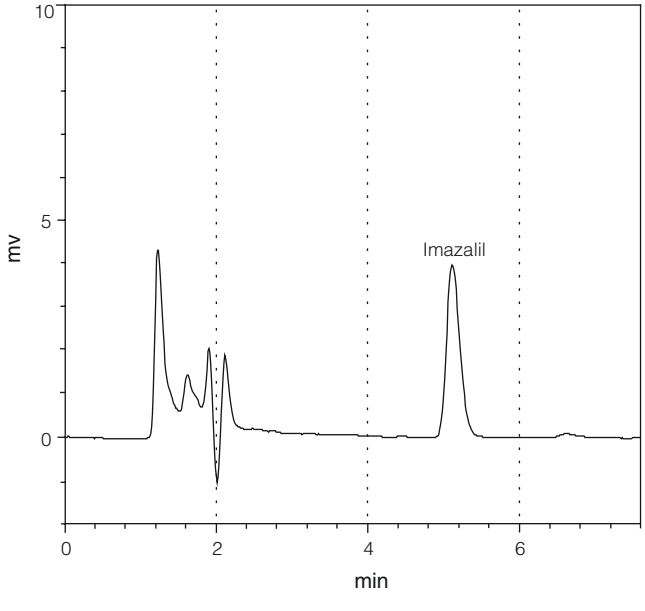

Figure 3. HPLC chromatogram of 0.5 ppm imazalil.

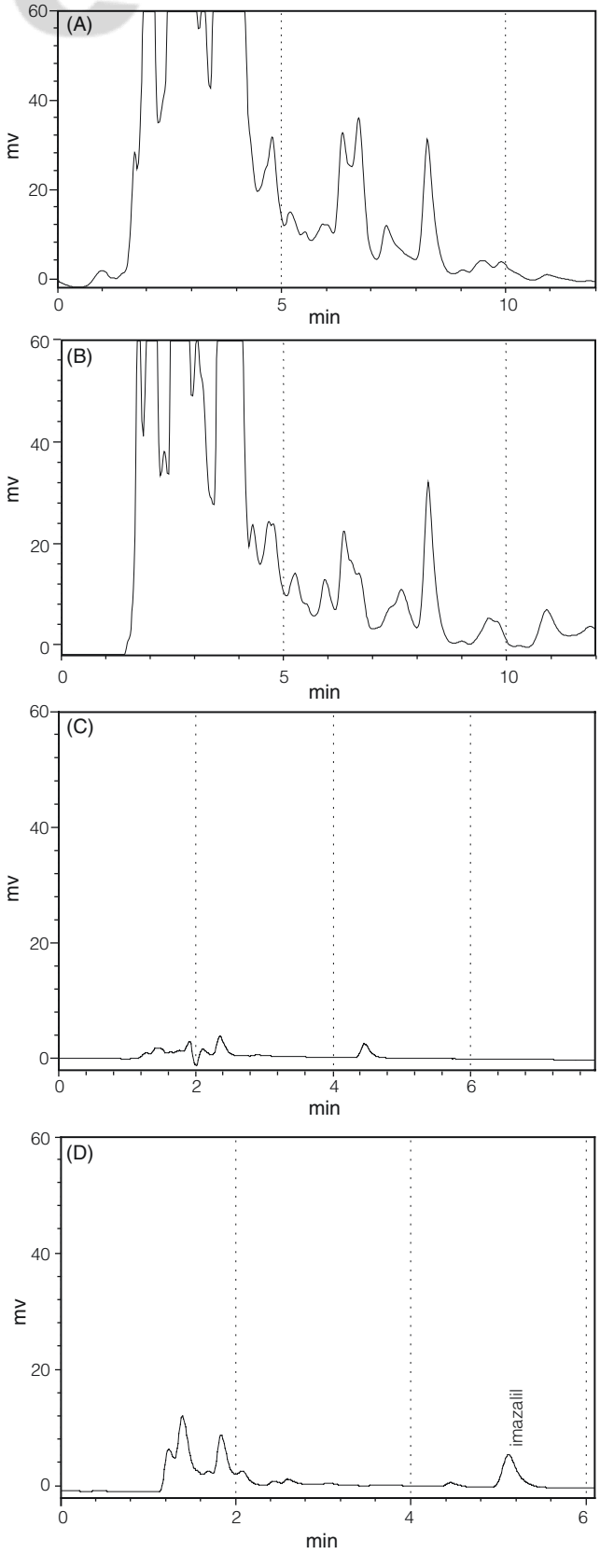

Figure 4. HPLC chromatograms of vegetable samples.
(1): Before cleanup: pakchoi(A) and rape(B).
(2): Cleanup with 500 mg of C18 cartridges and 500 mg of PRS cartridges: pakchoi(C) and rape spiked with 1.0 ppm imazalil (D).

through a Matrix solid-phase dispersion C18 column[12] or a Bond Elut PRS solid-phase extraction cartridge[7]. Alternatively, further purification can be done by changing the pH to perform liquid-liquid partition extraction[6,9,13]. As indicated by the preliminary test results, liquid-liquid partition extraction with various pH has to go through complicated procedures and could cause a loss of detection target and a decrease of the yield.

Yoshiyuki et al.[13] applied the C18 solid-phase extraction cartridge or magnesium silicate solid-phase extraction cartridge to purify vegetable/fruit extract. After eluting with methanol or acetonitrile, the HPLC analysis results showed that the impurities still could not be eliminated.

Ito et al.[7] used Bond Elut PRS as the packing material of solid-phase extraction cartridge. Bond Elute PRS is a high polarity compound with a propylsulfonic acid functional group. Because imazalil has low polarity, it is well absorbed within the ion exchange mechanism during the clean up process, and eluted with high ionic solvent. The preliminary test results indicated that if we use phosphoric acid to adjust the pH of the mobile phase (0.01M K_2HPO_3) to 2.5 to increase ionic strength, the mobile phase solvent can be used as the elution solvent to recover imazalil.

A Bond Elut C18 solid-phase extraction cartridge is set before the Bond Elut PRS solid-phase extraction cartridge. Because imazalil cannot be absorbed by C18 in acetic ether, it can flow into the Bond Elut PRS. Therefore, impurities can be removed by Bond Elut PRS solid-phase extraction cartridge and Bond Elut C18 solid-phase extraction cartridge (Figure 4 C, D). We can get good yield and found that it is suitable for the fruits and vegetables in this study.

III. *Standard Curve*

Imazalil standard curve obtains good linear relationship in the range of 0.1~10 ug/mL. The linear regression equation is: Y = 88359X + 6760.1, within 1.0~5.0 ug/mL, with coefficient correlation (r) equals to 0.9965. It indicates a good linear correlation.

IV. *Studies of Precision and Accuracy*

The studies of precision and accuracy, according to methods VI and VII, show that the precision CV from the repeatability of injection is 0.57% (n = 6), and the CV from the repeatability of analysis is 2.3% (n = 9). The recovery rate when in accuracy of 1.0 ppm, 1.5 ppm, and 2.0 ppm is 99.9%, 101.2%, and 100.9%, respectively; with CV 3.1~3.9%.

V. *Study of Recovery*

Generally, the minimum acceptable recovery rate is from 60% to 120%. The reproducible recovery rate within ±10% standard deviation is considered reliable in sample analysis. The pesticide residue and the allowance standard deviation were set at 0.1 ppm ±20%, and >1 ppm ±10%, respectively, as the references in pesticide analysis, according to the description of Gunther 1969, 1970, and 1971.[14]

When rape, cabbage, honeydew melon, tangerine, mango, and corn were spiked with imazalil in 0.25~0.75 ppm, 0.25~0.75 ppm, 0.5~1.5 ppm, 1.0~3.0 ppm, 0.5~1.5 ppm, and 0.05~0.15 ppm, respectively, with the same extraction method as the sample, and analyzed with an HPLC, the recovery rate was 88.7~97.9%, 72.5~87.4%, 81.4~90.5%, 81.9~87.2%, 75.0~85.3%, and 86.8~99.0%, respectively. The coefficient of variance was <6%. Figure 5 illustrated good separation and good recovery of mango blank sample when spiked with 1 ppm imazalil and analyzed with an HPLC.

VI. *Detection Limit (DL) and Quantitation Limit (QL)*

When analyzed with the methods VI and VII, the detection limit of imazalil was 0.01 ppm from the equation: DL = 3.3 σ/S; while the quantitation limit was 0.025 ppm from the equation: QL = 10 σ/S (Figure 6).

VII. *Imazalil Investigation from Commercial Vegetables and Fruits*

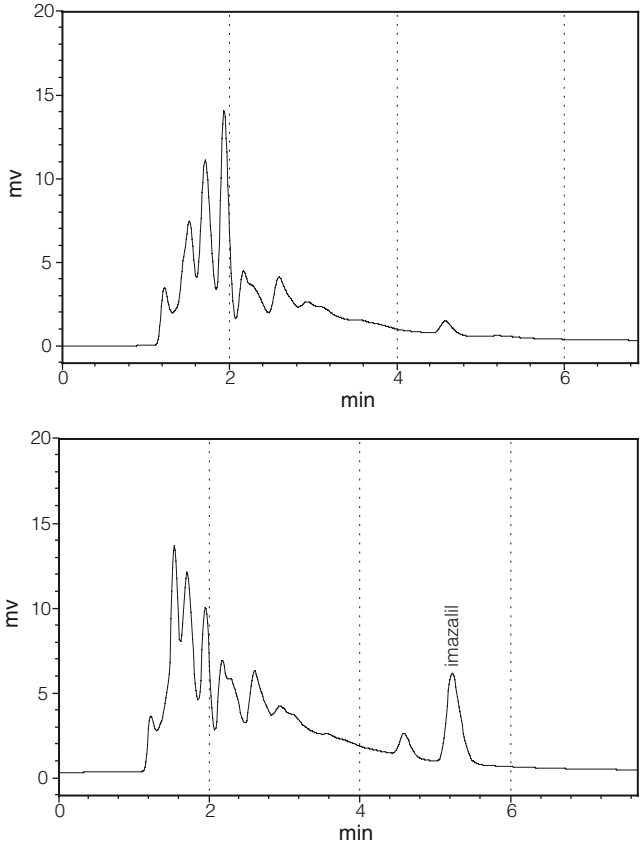

Figure 5. HPLC chromatograms for the recovery.
(A) mango blank.
(B) mango spiked with 1 ppm imazalil.

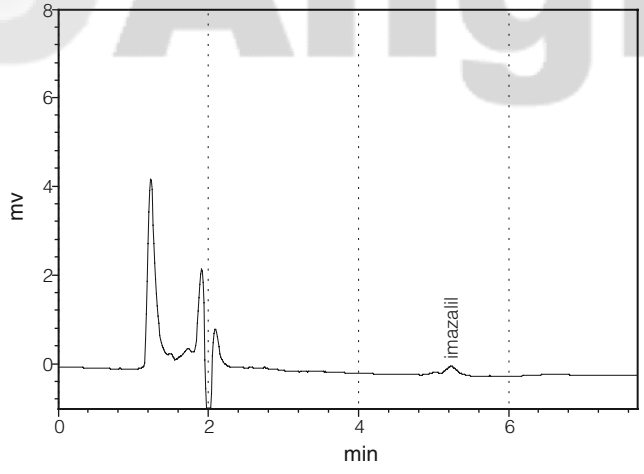

Figure 6. HPLC chromatogram of the quantitation limit for rape sample spiked with 0.025 ppm imazalil.

Based on DOH's announcement on the pesticide tolerance levels of leaflet vegetables, leaf vegetables, melons, kernels, and citruses, we investigated the imazalil residues in these 6 categories.

In summary, 20 samples from supermarkets and conventional markets in Kaohsiung were analyzed with the study method; none of them was identified with imazalil residue.

CONCLUSIONS

The method we developed was the application of an HLPC in combination with a UV detector for the detection of suspected pesticide residual samples. The method can be used as a confirmative assay in cooperation with a photodiode array for the comparison of the absorbance mappings. Not only because the pretreatment procedures are simple and easy to operate, but the impurity can be removed when a sample is passed through the Bond Elut PRS solid-phase extraction cartridge and Bond Elut C18 filtration cartridge. The study method can be applied in the routine imazalil pesticide residual analysis for agriculture products, with recovery rates >80% and detection limit <0.01 ppm.

ACKNOWLEDGMENTS

We thank Dr. Hui-Cheng Chen for his translation work.

REFERENCES

1. Department of Health, Executive Yuan. 2001. Issue of Food Sanitation Section. Announcement NO. 0900058040. (In Chinese)
2. Van, C. J., Van, G. F., Janssen, P. A. J. 1988. In vitro activity of enilconazole against *Aspergillus spp*. and its fungicidal efficacy in a smoke generator against *Aspergillus fumigatus*. Mycoses 31(3): 143-147.
3. The Horticultural Sciences Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida. Publication date: May 1993
4. Liu, L. S. 1990. Pesticides Practice. 7th ed. pp. 259-260. Hwa-chin press. Taichung, Taiwan. (In Chinese)
5. Vanden, B. H., Marichal, P. J., Coene, M. C., Will, E. G., Bellen, D., Roelslt, M. H., Jansse, P. A. J. 1989. Biochemical approaches to selective antifungal activity. Focus on azole antifungals. Mycoses 32 (SUPPL. 1): 35-52.
6. Garrido, J., Alba, M., Jimenez, I., Casado, E. and Folgueiras, M. L. 1997. Chromatographic analysis of imazalil and carbendazim in fruits. Method validation and residue monitoring program 1995. J. Chromat. A. 765: 91-97
7. Ito, Y., Ikai, Y., Oka, H., Hayakawa, J. and Kagami, T. 1998. Application of ion-exchange cartridge clean-up in food analysis I. Simultaneous determination of thiabendazole and imazalil in citrus fruit and banana using high-performance liquid chromatography with ultraviolet detection. J. Chromatogr. A 810: 81-87.
8. Matsumoto, H. 2001. Simultaneous determination of imazalil and its major metabolite in citrus fruit by solid-phase extraction and capillary gas chromatography with electron capture detection. J. AOAC Int. 84(2): 546-550.
9. Phobe, M. K. F. and Stewart, L. R. 1994. The effect of home processing on postharvest fungicide residues in citrus fruits: residues of imazalil, 2-phenylphenol and thiabendazole in "home-made" marmalade, prepared from late valencia oranges. Food Addit. Contam. 11(1): 57-70.
10. Torres, C. M., Pico, Y. and Manes J. 1997. Comparison of octadecylsilica and graphitized carbon black as materials for solid-phase extraction of fungicidal and insecticide residues from fruit and vegetables. J. Chromat. A 778: 127-137.
11. Stein, E. R., Carter W. W. and Murray, A. T. 1981. A modification of imazalil residue analysis in treated grapefruit. J. Environ. Sci. Health B 16(4): 427-437.
12. Sendro, N. K. and Maria L. R. 1999. Rapid method for the determination of thiabendazole and imazalil residues in orange by capillary column gas chromatography. J. High Resol. Chromatogr. 22(5): 303-304.
13. Yoshiyuki, C., Takeji, C., Misako, M., Tohru , A. and Masamoto, K. 1996. Systematic determination of 6 postharvest pesticides in imported citrus fruits. J. Food Hyg. Soc. Japan 137(4): 187-194.
14. Li, H. P., Wong, S. S., Li, G. C. 1996. The establishment of method for pesticide residue analysis on agricultural products. Taiwan Agricultural Chemicals and Toxic Substance Research Institute, COA. No: DOH85-FS001. (In Chinese)