Doctoral Dissertation

Dissipation pattern of some pesticides in/on pepper, pomegranate and perilla leave: Contribution to safety guideline

Department of Agricultural Chemistry
Graduate School Chonnam National University

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Dissipation Patterns of Some Pesticides in/on Specific Crops: Contribution to Safety Guideline

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Supervised by Professor Jae-Han Shim

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Dissipation Patterns of Some Pesticides in/on Specific Crops: Contribution to Safety Guideline

(SYNOPSIS)

The aim of this study was to establish the residue limits and to predict the final residual levels of the tested analytes such as (bitertanol, chlorfluazuron, fludioxonil, flufenoxuron, pyridalyl, lambda-cyhalothrin, lufenuron, and pyridalyl). Persistence and degradation behavior of the pesticides (abamectin and acequincocyl) were also studied in order to estimate the pre-harvest residue limits for food safety.

Pesticides were applied in/on target crops (pepper fruit, perilla leaves and pomegranate fruit) following the KFDA guidelines for safety usage at the recommended doses in addition to double the recommended doses. The analysis methods for pesticide residues in pepper fruits and perilla leaves and pomegranate fruits have been studied and the method performance was evaluated. Most methods were modified including liquid-liquid extraction with additional column clean-up procedure. The final extracts were analyzed using gas chromatograph coupled with micro-electron capture detector (μ-ECD) and nitrogen phosphorus detector (NPD), and high performance liquid chromatograph equipped with either ultraviolet or fluorescence detector (HPLC-UVD/FLD). The methods were found to be precise and accurate with good correlation coefficients (r^2>0.95) that fitted with the acceptance value in the Codex guidelines. All recoveries ranged from 73.6 to 126.8% with relative standard deviations less than 12. Furthermore, the limits of quantification

(LOQs) were much lower than the maximum residue limits (MRLs) in the tested samples. All trials were proved to be simple, reliable, accurate, precise, and applicable to field incurred samples.

The dissipation pattern and the residual determination of 11 pesticides were analyzed from 3 kinds of samples. The residue levels of both chlorfluazuron and pyridalyl were below the MRLs 2 hour after application, while those of the other 3 pesticides (bitertanol, fludioxonil, and flufenoxuron) were below the MRLs after 4 and 6 days of application. The half-life was ranged from 1.9 days (flufenoxuron) to 8.7 days (pyridalyl).

It was shown that application of two, three, four and five times for Lambda-cyhalothrin, lufenuron, thiamethoxam, and clothianidin in/on pomegranate fruits, gave a safe residue levels for consumers. 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 levels were lower than the MRLs for each compound established by the Korea Food and Drug Administration (KFDA).

The residual levels of abamectin substantially decreased to 100%, 7 days post-application to perilla leaves, whereas acequinocyl decreased to half concentration at 7 days post-application.

Chapter I. General Introduction

Agriculture produces foods, which are fundamental for human health. It is therefore seems obvious that agriculture, food, and health are related (Lang, 2006).Modern agricultural production in major countries depends heavily on the use of pesticides, mainly to protect fruits and vegetable products. The use of pesticides is also considered as one of the most important pre- and post-harvest treatment methods to avoid damage of plants and keep away from product reduction (Garía *et al*., 2010). Pesticides clearly have assisted in controlling pests and maintaining the availability of low cost and high quality food. For example, Own and Jayasuriya (1989) reports that crop and livestock production in the US would drop by 25-30% and prices of agricultural products would increase by 50-75%, if pesticides were completely withdrawn from uses. Public health risks were, however, associated with the use of pesticide. These risks include on-farm ingestion by workers, discharge of toxic chemicals into the air and water, and consumption of foods that contain pesticide residues by consumers (Wilson and Otsuk, 2004). The active substances contained in pesticides and biocides are very large and diverse array of chemicals and include many biological agents. Throughout the globe, there may be up to 1,500 compounds, including fungicides, herbicides, and insecticides in descending order of importance. Pesticides are transported mainly by rain and wind from their points of application to neighboring crops of (Fenik *et al*., 2011). The uses of pesticides have been linked to a wide spectrum of human health hazards, ranging from short-term impacts such as headaches and nausea to chronic impacts including cancer, reproductive harm, and endocrine disruption (Blasco *et al*., 2006).

In the present, there are two opposite trends regarding the uses of pesticides; each one

related to a geographic region. The developed countries, including European Union, USA, and Canada, approved new laws restraining the use of agrochemicals. This legislation aims at protecting consumers through a more thorough toxicological testing of compounds and enforcement of lower concentration limits for the residues tolerated in food and water (Harris, 2002; Chun and Kang, 2003; Carvalho, 2009). On the contrary, the developing countries go in a different direction in these matters. They need to increase the agriculture production and the use of crop protection chemicals seems a simple way for obtaining better crop yields.

In the international market, the maximum residue limit (MRL) regulations are stringent in most countries. Thus, the recommendations regarding the dose-specific pre-harvest interval (PHI) are essential to ensure dissipation of any applied chemical below the prescribed MRL at harvest to provide safety to the consumers **(**Benerjee *et al*., 2008**)**. It is the responsibility of the governmental authorities to register and set MRLs to regulate the residues of pesticides in fruits and vegetables. In developing countries, residue problem gained much importance, due to the lack of governmental inspections and awareness of the producers as well as the consumers about issue. As a consequence, food consumers are face-to-face with food products which have high residue levels (Cengiz *et al*., 2006)**.**

The acceptable daily intake (ADI) of the pesticide residue, which is established "on the basis of a complete review of the available information, including data on the biochemical, metabolic, pharmacological, and toxicological properties of the pesticide, is derived from studies of experimental animals and observations in human" (Renwick,2002). The total dietary intake of a particular pesticide residue in a food product is calculated by summing the consumption of all the food containing the residue, weighted by the MRL of a particular pesticide in each food. The MRL is an index that represents the maximum concentration of a pesticide residue (expressed as mg/kg) legally permitted in food commodities and animal feeds.

MRLs on food imports are set by each country and are imposed as regulatory standards at the border (Nasreddine and Parent-Massin, 2002; Chun and Kang, 2003; Wilson and Otsuk *et al*., 2004). Therefore, it is significant to find out the dissipation patterns of pesticide in/on crops or fruits for producing safe food and protecting the environment. The dissipation rate of a pesticide after application is a useful tool for the assessment of the behavior of pesticide residues (Omirou *et al*., 2009).

Pesticides are being used in vegetables, grown under greenhouse conditions in many countries throughout the globe due to; unfavorable climate conditions; easy to control pest damages; and to meet the consumer off-season demand (Khay *et al.*, 2006a;b).

Pre-harvest residue limit (PHRL) is the recommended residue level at certain time and is calculated based on the dissipation curve obtained from supervised field study. If the residue at certain time is same as lower than the PHRL, it can be predicted that the residue in/on crop will be same as or lower than MRL at harvest. PHRL also can be used for the prediction of Pre-harvest interval (Chang *et al*., 2011).

The determination of pesticides at residue levels in vegetables is a hard task, due to the complexity of the matrix along with the low concentration levels at which the analytes are usually found. Therefore, a highly sensitive technique, together with an extraction and an addition sample clean-up steps required to remove the possible interferences in the analysis. An extraction process is required to separate the analyte from the matrix; typically an organic solvent or a mixture of solvent is used in the extraction process. After that, a suitable dilution and filtration (Zamora *et al*., 2004), solid phase extraction (SPE) (Štajnbaher and Zupancic-Kralj, 2003; Štajnbher and Kralj, 2008; Juan-García *et al*., 2010), gel permeation chromatography are required before injection in/onto the chromatograph.

To assess the safety of food against the target pesticides, reliable and sensitive analytical

method including sample preparation, clean-up, and instrumental analysis are required in advance.

The accuracy of an analytical method is the extent to which test results generated by the method and the true value agree. The true value for accuracy assessment can be obtained in several ways. It can also be described as the closeness of agreement between the value that is adopted, either as a conventional, true or accepted reference value, and the value found (reference). Accuracy is usually reported as percent recovery by assay, using the proposed analytical procedure, of known amount of analyte added to the sample. The range for the accuracy limit should be within the linearity range. The international conference on harmonization (ICH) recommended assessing a minimum of nine determinations over a minimum of three concentration levels covering the specified range. The typical accuracy of the recovery of the drug substance in the mixture is expected to be about 98 to 102% (Guidance for industrial 1996).

The limit of detection (LOD) is the smallest measured concentration of an analyte from which it is possible to deduce the presence of the analyte in the test sample with acceptable certainty. The LOQ is the smallest measured amount of an analyte above which the determination can be made with the specified degree of accuracy and precision. There are several scientifically valid ways to determine LOD and LOQ and any of these may be used as long as scientific justification is provided (The VICH GL 49 (MRK)-method used in residue depletion studies, 2009).

The International Conference on harmonization “ICH” introduced an easy and simple calculation (signal-to-noise ratio) as an approach for the determination of LOD and LOQ (Chan *et al*., 2004).

LOD = 3:1 signal-to-noise

LOQ = 10:1 signal-to-noise

Or

LOD = 3.3 δ/S

LOQ = 10 δ/S

Where δ is the standard deviation of the responses and S is the slope of the calibration curve. The slope S may be estimated from the calibration curve of the analyte. The estimate of δ may be carried out in a variety of ways, including:

- **Base on the standard deviation of the blank**: measurement of the magnitude of analytical background response can be performed via analyzing an appropriate number of blank samples and calculating the standard deviation of their responses.

- **Based on the calibration curve**: it is recommended that a specific calibration curve be studied using samples containing an analyte in the range of the LOQ. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines could be used as a standard deviation.

Objectives

This study was carried out on 11 pesticides in/on pepper, pomegranate, and perilla leaves. Among them, 5 pesticides; including bitertanol, chlorfluazuron, fludioxonil, flufenoxuron, and pyridalyl were tested in pepper, 4 members, including lambda-cyhalothrin, lufenuron, thiamethoxam, and clothianidin in pomegranate, and 2 members (abamectin and acequinocyl) were analyzed in perilla leaves.

The main purposes of this study were:

- To generate data regarding the persistence and pattern of decline and residue levels of pesticides and fungicide in/on pepper cultivated under experimental greenhouse conditions
- To predict the final residue levels in/on the crops
- To suggest the Pre-harvest residue level (PHRL)
- To calculate the biological half-life of the tested pesticides

Chapter II. Determination of Residual Limit of Bitertanol, Chlorfluazuron, Fludioxonil, Flufenoxuron, and Pyridalyl in/on Pepper Grown Under Greenhouse Conditions

1. ABSTRACT

In this study, four insecticides and one fungicide were applied either in single, double doses to pepper grown under greenhouse conditions. Persistence and degradation behavior of the tested analytes were determined using simple sample extraction and finally analyzed by gas chromatography or liquid chromatography with difference detectors. Calibration curves were linear over the concentration ranges of the tested analytes with $r^2 \geq 0.994$. The limits of detection and quantification were ranged from 0.004 to 0.02 mg/kg and 0.013 to 0.066 mg/kg, respectively. The method was validated in triplicate at two fortification concentrations and gave a good recovery ranged between 74.9 and 126.8% with relative standard deviations less than 11%. The rates of disappearance of tested compounds for single and double doses were described as first-order kinetics with half-lives of 1.9 to 7.6 and 1.9 to 8.7days, respectively.

2. INTRODUCTION

Peppers are native plant to America, and their fruits (pericarps) are consumed as vegetable, spices, and external medicines. They are also considered as source of vitamins A, C, and E (Belano, 2005). The pepper (*Capsicum annuum*L.) is one of the staple spices in the Korean diet and is consumed in various forms such as fresh, salted, dried or fermented. To fulfill the off-season demand for this crop, it has become a common practice to be cultivated under greenhouse conditions. Based on the economic value of the industry, capsicum or chili pepper is the second most important crop for Koreans (Dömötörová *et al.*, 2005).

Pesticides are a numerous and diverse group of chemical compounds, which are used to eliminate pests in agriculture and households. They increases the quantities and the quality of crops and food to be controlled, and help to limit many human diseases transmitted by insects or rodent vectors. However, despite their merits, some of them are toxic, environmentally stable, and move into the environment. The widespread use of pesticides not only contaminates water, soil, and air, but also accumulated in crops (e.g., fruits and vegetables).

Bitertanol (BIT) (β-(1,1'-biphenyl)-4-yloxy-α-(1,1-dimethyethy1)-1H-1,2,4-trixole-1-ethanol) is a systemic azole fungicide and is widely used for the protection of plants. In general, the determination of BIT was carried out using either GC or HPLC (Mendes, 1985; Chan *et al*., 2004; Eulogio *et al*., 2007; Llorent-Martínez *et al*., 2007; Mei *et al*., 2009).

The insect growth regulator chlorfluazuron [1-(3,5-dichloro-4-(3-chloro-5-trifluoromethyl-2-pyridyloxy) phenyl)-3-(2,6-difluorobenzyl) urea] is a potent insecticide by virtue of its ability to inhibit the synthesis of cuticle chitin, thus disrupting the normal insect growth and development (Tomlin, 2000). The specificity of benzoylureas (BUs) to species whose structural integrity depends upon chitin, their low acute toxicity to mammal along with

their high biological activity, make them suitable for inclusion in integrated pest management programs for fruits and vegetables (Shim *et al*., 2007., KovalczukT *et al*., 2008., Choi *et al*., 2011). Benzoylphenylurea insecticides are used as non-systemic insect growth regulators for control of a wide range of leaf-eating insects and their larvae in vegetables, poem fruits, and mushroom. Because of their high selectivity and rapid degradation in soil and water, benzoylphenylurea insecticides have a slight effect on the natural enemies of various harmful insect species. Thorough monitoring of pesticide residues is therefore, crucial for proper assessment of human exposure to pesticides through food. A variety of analytical methods have been developed for determination of benzoylphenylurea insecticides in various matrices. LC coupled to MS (LC/MS) has been reported as a viable technique for determination of various classes of pesticide residues, including, benzoylphenylurea insecticides, through electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) MS (Barnes *et al*., 1995; Choi *et al*., 2011). Flufenoxuron 1-[4-(2-chloro-α, α, α-trifluoro-*p*-tolyloxy)-2-fluorophenyl]-3-(2,6-difluorobenzoyl)urea is an insect larvae and nymph growth regulator. It is a growth regulator, which kills insect pests through interference with chitin formation. Although not an adulticide, a very high proportion of the eggs laid by an adult that has been exposed to this insecticide are non-viable. It has been tested against a wide range of pest species including ants, cockroaches, fleas, and mosquitoes. Because of its novel mode of action, flufenoxuron can give outstanding performance against pest strains, which have developed resistance to other insecticides. It also extremely stable and compatible with pyrethroid insecticide, alpha-cypermethrin (Boonchiangma *et al*., 2012). Reversed-phase HPLC with UV detection is predominantly employed for the determination of BUs because they are thermally unstable and, consequently, their GC properties are poor. Most of the above analytical studies were concerned with the determination of a single BU only (Tomšj and Hajslova., 1995; Martínez-Galera *et al*.,

2001).

Pyridalyl [2, 6-dichloro-4-(3,3-dichloroallyloxy)phenyl 3-[5-(trifluoromethyl)-2-pyridyloxy] propyl ether] belongs to a recent generation of chemical class of insecticides, Pyridalyl-APVMA 6, that exhibits high insecticidal activity against Lepidoptera and Thysanoptera. It was suspected that pyridalyl has a novel molecular mode of action compared to other insecticides (Powell *et al*., 2011). It also showed an excellent efficacy against the leaf miner flies. On the other hand, the toxicity of this compound on various beneficial insect is very low (Isayama *et al*., 2005).

After primary distribution, dissipation processes dominate the evolution of residues in crops until harvest. These processes depend on several factors including weather condition, doses applied, chemical formulation, application methods, and chemical phenomena (Garau *et al*., 2002). Using MRLs as a measure to ensure product safety, governmental and international organizations have regulated the use of pesticides by fixing MRLs for commercial purposes. Another value which is usually fixed in order to comply with MRLs, is the pre-harvest interval (PHI). This value is usually fixed at country level and can be described as the time period (in day) between the last pesticides application and a safe harvest of the treated crop. In the Republic of Korea, MRLs have been established at a range of 0.1 to 2 mg/kg for 13 vegetables and fruits. For instance, the MRL for bitertanol was set at 0.7 mg/kg, chlofluazuron at 0.5 mg/kg, fludioxonil 0.3 mg/kg, flufenoxuron 0.3 mg/kg, and pyridalyl was 2 mg/kg.

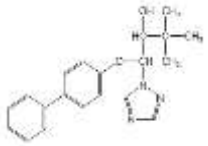
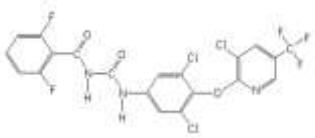
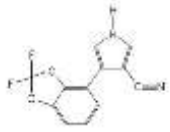
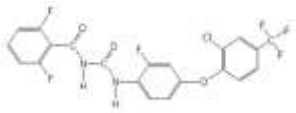
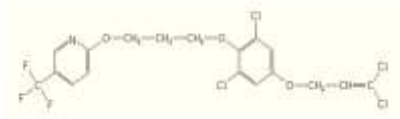
In this work we analyzed the dynamic fate of 5 pesticides in/on pepper grown under greenhouse conditions.

3. MATERIALS AND METHODS

3.1. Chemicals and reagents

Pesticides standard were kindly provided by the National Agricultural Products Quality Management Services (Gwangju, Republic of Korea). The structural formulas of these pesticides are shown in **Table 1**. Acetonitrile, acetone, ethyl acetate, *n*-hexane, dichloromethane, and sodium chloride were of HPLC grade and obtained from Merck (Darmstadt, Germany). Sodium sulfate anhydrous was from Yakuri Pure Chemicals Co. LTD (Kyoto, Japan). Solid-phase extraction (SPE) cartridges 1000 g Strata Florisil and silica were purchased from Phenomenex (USA). Mobile phases were filtered through a 0.45 µm cellulose acetate (Waters) or polytetrafluoroethylene (PTFE). Water was obtained by filtering deionized water through a 0.45 µm filter with a Waters Millipore (Milford, MA, USA) system.

Table 1. Characteristic properties of the tested pesticides

Common Name	Structure	Molecular mass	Solubility in water (mg/L)	K_{ow}
Bitertanol		337.4	2.7mg/L	4.1
Chlofuazuron		540.7	<0.01mg/L	5.8
Fludioxonil		248.2	1.8mg/L	4.12
Flufenoxuron		488.8	$7x10^{-11}$g/L	4.0
Pyridalyl		491.1	0.15µg/L	8.1

3.2. Preparation of standards and spiked samples

Standard solutions of pesticides (100 mg/L) were prepared by exactly weighing and dissolving the corresponding compounds in organic solvents. Dilutions were freshly prepared for the working solutions. All solution was prepared in brown glass vial to protect them from decomposition by light and was stored in a refrigerator at -2ºC. For recovery determinations, samples (20 g) of finely chopped samples were spiked by addition of a standard stock solution (10 mg/L) at two fortification levels per each tested pesticides. The spiked samples were allowed to stand for a 20 min before extraction to allow the spiked solution to penetrate the tested material. The spiked concentrations were as follow:

- Bitertanol: 0.1 and 0.5 mg/kg
- Chlorfluazuron: 0.2 and 1.0 mg/kg
- Fludioxonil: 0.1 and 0.5 mg/kg
- Flufenoxuron: 0.2 and 1.0 mg/kg
- Pyridalyl: 0.08 and 0.2 mg/kg

3.3. Field experiment and sampling

The experimental trial was carried out in greenhouse at Chonnam National University, Republic of Korea (**Figure 1**). Peppers were planted 1 x 3 m (width and length) apart from each other in rows, and the distance between the rows was 0.5 m. The experimental area consisted of thirty three plots for all tested pesticides. Each pesticide was applied at 2 different doses (recommended and double the recommended dose), 3 plots for each dose and 3 plots were used as control. After spraying, samples were collected at 0 days (two hours after spraying), 1, 2, 4, 6, 8, 11, and 14 days after applications. The samples were forwarded to the laboratory, where they were chopped, packed with plastic bags, labeled, and stored in individual bags at -24ºC

until extraction. All environmental variables, including temperature and humidity were estimated throughout the experimental period (**Figure 2**).

Figure 1. Plantation and spaying of pesticides under greenhouse conditions.

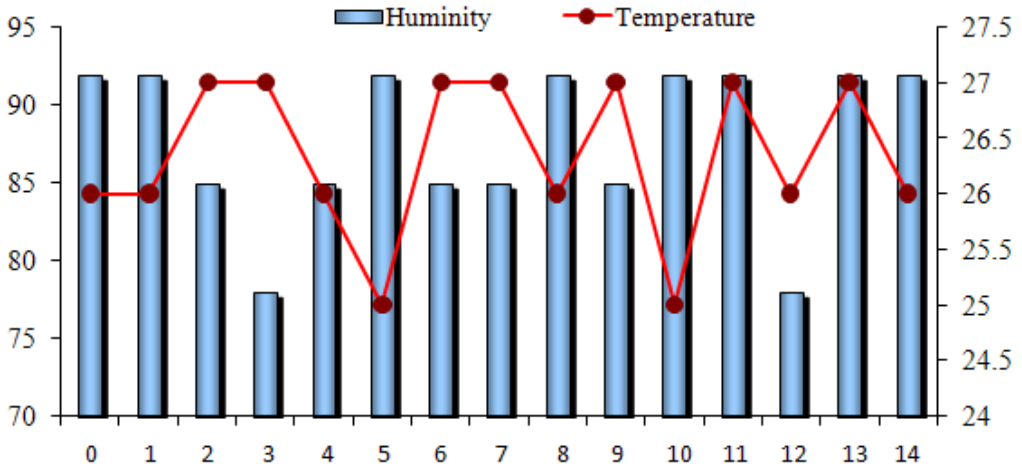


Figure 2. Temperature and humidity during cultivation period of pepper grown under greenhouse conditions.

3.4. Sample extraction

Twenty grams of each chopped pepper was extracted with 100 mL of acetonitrile in an Erlenmeyer flask. The mixture was homogenized by homogenizer (WiseMixTM HG-150) for 3 min at 2500 rpm. The samples were filtrated through Büchner funnel.

Bitertanol

Ten grams of sodium chloride (NaCl) was added to the filtrate content. The content was shacked for 5 min and allowed to stand for about an hour to get clear separated layers. A 50 mL of upper clear layer was taken and evaporated to dryness in a rotary vacuum evaporator under 40 ^{0}C. The residues were dissolved in 4 mL of *n*-hexane.

Clean-up: Florisil cartridge column (2 g/12 mL) was used to remove the interference compounds. The cartridge was conditioned with 5 mL of *n*-hexane and 4 mL of sample was loaded and washed with 10 mL of *n*-hexane. The residue was eluted with 15 mL of acetone: *n*-hexane (15:85). The eluent was evaporated at 40 ^{o}C and re-dissolved in 2 mL acetonitrile and analyzed by HPLC-UVD.

Chlorfluazuron

A mixture of 100 mL of saturated sodium chloride and 50 mL of water was added to chlorfluazuron filtrate, which is then partitioned with dichloromethane for 2 times (first 100 mL and second 50 mL). The organic layers were combined dehydrated through sodium sulfate (anhydrous) and evaporated to dryness in a rotary vacuum evaporator under 40 ^{o}C. The residue was then dissolved in 4 mL of *n*-hexane.

Clean-up: Florisil cartridge column (1g/6 mL) was conditioned with 5 mL of *n*-hexane; and then 4 mL of the residue was loaded. The residues were eluted with 20 mL of 20% acetone

in *n*-hexane. The eluent was then evaporated to dryness and re-dissolved in 2 mL of acetonitrile and analyzed by HPLC-UVD.

Fludioxonil, flufenoxuron, and pyridalyl

Ten grams of sodium chloride (NaCl) was added to the filtrate. The content was shacked for 5 min and allowed to stand for about an hour to get clear layers. A 50 mL of upper clear layer was taken and evaporated to dryness in a rotary vacuum evaporator under 40 ^{o}C. The residue was dissolved in 4 mL of *n*-hexane.

Clean-up: Florisil cartridge column (1g/6 mL) was conditioned with 5 mL of *n*-hexane followed by 4 mL of residue. Ten mL of 5% acetone in *n*-hexane was loaded and discarded. The residues were eluted with 10 mL (20 ml in case of flufenoxuron and pyridalyl) of 15% (25% for flufenoxuron and 5% in case of pyridalyl) acetone in *n*-hexane. The eluent was then evaporated to dryness and re-dissolved in 2 mL of acetone and analyzed by GC-NPD (HPLC in case of flufenoxuron and GC-µECD in case of pyridalyl).

3.5. Instrumental conditions

HPLC-FLD

Bitertanol analysis was carried out using a Shimadzu HPLC-fluorescence detector with a SCL-10AVP system controller, LC-6AD pumps. Chromatographic separation was conducted using an Atlantis C_{18} (4.6×250 mm, 5 µm, Waters) column. The mobile phase was a mixture of acetonitrile: water (60:40, v/v) and 20 µL of sample were injected in/onto an HPLC column. Bitertanol was detected at an excitation wavelength of 254 nm and emission wavelength of 322 nm, and the flow rate was 1 mL/min. Under these conditions, the detection time of bitertanol was 13.2 min.

Analysis of chlorfluazuron and flufenoxuron were carried out 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 C_{18} 200Å(4.6×250 mm, 5.0 µm, Phenomenex, USA) was employed as an analytical column for chlorfluazuron and Atlantis C_{18} (4.6×250 mm, 5 µm, Waters) column was employed for analysis of flufenoxuron. The mobile phase was a mixture of acetonitrile: methanol: water (75:5:20, v/v/v) for chlorfluazuron and acetonitrile: water (75:25, v/v) for flufenoxuron with flow rate of 1 mL/min. A 20 µL of sample was injected in/onto the HPLC column. Chlorfluazuron and flufenoxuron were detected at a wavelength of 254 nm; and the detection times were 10.62 min and 12.1 min for chlorfluazuron and flufenoxuron, respectively.

Gas chromatography

Fludioxonil and pyridalyl analysis were conducted using an Agilent Technologies 7890 A GC System (USA) consisting of a model 7683B autoinjector and an µ-electron capture detector and nitrogen phosphorus detector (Agilent 6890), respectively. Chromatographic separation of fludioxonil was conducted using an DB-5 (30 m × 0.25 mm × 0.25um, Agilent Technologies, USA) column. The oven temperature was held at 80 ºC for 2 min and increased to 200 ºC at a rate of 10 ºC /min and then increased to 220 ºC for 4 min by increasing 2 ºC/min until temperature reach to 300 ºC by increasing 10 ºC/min. The injection port and detector temperatures were maintained at 250 ºC and 320 ºC, respectively. The injection volume was 1 µL, and the column was flowed with nitrogen gas at 1 mL/min and the compound was detected at 22.5 min. On the other hand, chromatographic separation of pyridalyl was employed using an HP-5 (30 m × 0.25 mm × 0.25 µm, Agilent Technologies, USA) column. The oven

temperature was held at 250 ºC for 1 min and increased to 265 ºC at a rate of 5 ºC/min and then increased to 270 ºC for 1 min by increasing 1 ºC/min. The injection port and detector temperatures were maintained at 250 ºC and 300 ºC, respectively. The injection volume was 1 μL, and the column was flowed with nitrogen gas at 1 mL/min. Under these conditions, the detection time of the tested analyte was 11.2 min.

4. RESULTS AND DISCUSION

4.1. Extraction

In this study, pesticides were extracted from pepper via liquid-liquid extraction. Fruit and vegetable samples are matrices that do not allow direct SPE of pesticides. Pesticides must be extracted with polar solvents to have then in the aqueous extract. Acetonitrile was selected as an extraction solvent because it contains smaller amount of co-extractives compared with extracts obtained with other solvents (Anastassiades *et al*., 2003, Lambropoulou and Albanis *et al*., 2007). Acetonitrile and sodium chloride were selected for the first step extraction because sodium chloride could facilitate separation between acetonitrile and water from pepper very well than acetone.

The most common clean-up technique for crop extracts containing residues of pesticides is gel permeation chromatography or solid-phase extraction (SPE). These methods have been evaluated for their efficiencies to clean-up the crop extracts for the determination of benzoylphenylurea insecticides in fruits and vegetables (Himstra *et al*., 1999). Singh and Kulshrestha, 2005 found that partitioning with *n*-hexane could remove chlorophyll and no matrix interference could be observed during the HPLC analysis.

4.2. Detector linearity

Detector linearity was evaluated by calibration plots constructed through the different range according to the sensitivity of each pesticide.

Bitertanol, chlorfluazuron, fludioxonil, flufenoxuron, and pyridalyl gave good linearities at the tested concentrations. The linear equations were as follows: Y= 1895848x – 296608; Y= 48650x + 22.59; Y= 72142x - 3025.1; Y= 43963x – 4064; and Y= 13064x – 399.6, respectively. The correlation coefficients curves (r^2) were ranged from 0.994 to 1 (**Table 2**).

Table 2. Concentration range, correlation coefficients, and limit of detection of the tested pesticides in pepper

Pesticides	Conc. range (mg/kg)	Equation	r^2 values
Bitertanol	0.05 – 10	Y=1895848x - 296608	1
Chlofluazuron	0.1 – 6	Y=48650x + 22.59	0.999
Fludioxonil	0.05 - 5	Y=72142x - 3025.1	0.999
Flufenoxuron	0.1-6	Y=43963x - 4064	0.994
Pyridalyl	0.02-2	Y=13064x – 399.6	0.994

4.3. Limits of detection and quantification

The limits of detection and quantification were shown in Table 3. The LODs ranged from 0.004 to 0.02 mg/kg. Tomsj and Hajslova (1995) determined diflubenzuron, flufenoxuron, flucycloxuron, chlorfluazuron, and triflumuron in apples using isocratic LC and found that the LODs ranged between from 0.01 to 0.03 mg/kg. On the other hand, Himstra *et al*., (1999) found that the LODs were ranged between 0.02 to 0.05 mg/kg in mushroom, Chinese cabbage, apple, and cucumber treated with 7 benzoylureas. Additionally, Gamon *et al*., (1998) detected and quantified diflubenzuron, hexaflumuron, teflubenzuron, flufenoxuron, and lufenuron in peppers, tomatoes, eggplants, cucumbers, and oranges at a level of 0.01 and 0.04 mg/kg, respectively. Higher LODs were also reported by Brito *et al*. (2002) for lufenuron and Miliadis *et al*. (1999) for diflubenzuron, triflumuron, teflubenzuron, flufenoxuron, and lufenuron. Martnez-Galera *et al*. (2001) reported that the LODs of benzoylureas were significantly lowered by PIF detector than those obtained by UV-DAD, and they are in the same order as those obtained by LC/MS.

4.4. Recovery

Recovery studies were carried out by spiking fresh samples, which did not contain any pesticides (**Figure 3-4**), with known volume of the appropriate working mixtures of pesticides (**Figures 3-4**). Recovery rates between 73.9.3% and 126.8% were obtained (**Table 3**). Our finding were supported by the international guidelines (SANCO, 2004), which indicated that the mean recovery should be within the range of 70-120% to sufficiently validate the quantitative methods.

Table 3. Recoveries, relative standard deviations, LOD, and LOQs of the tested pesticides in pepper

Pesticides	Concentration (mg/kg)	Average recovery ± RSD (%)	LODs (mg/kg)	LOQs (mg/kg)
Bitertanol	0.1	86.0 ±4.13	0.01	0.033
	0.5	80.3 ± 5.63		
Chlorfluazuron	0.2	105.5 ± 5.7	0.02	0.066
	1	95.1 ± 3.97		
Fludioxonil	0.1	103.6 ±1.51	0.01	0.033
	0.5	126.8 ±0.84		
Flufenoxuron	0.2	83.5 ± 2.66	0.02	0.066
	1	89.9 ± 10.3		
Pyridalyl	0.04	96.1 ± 4.7	0.004	0.013
	0.2	74.9 ± 0.5		

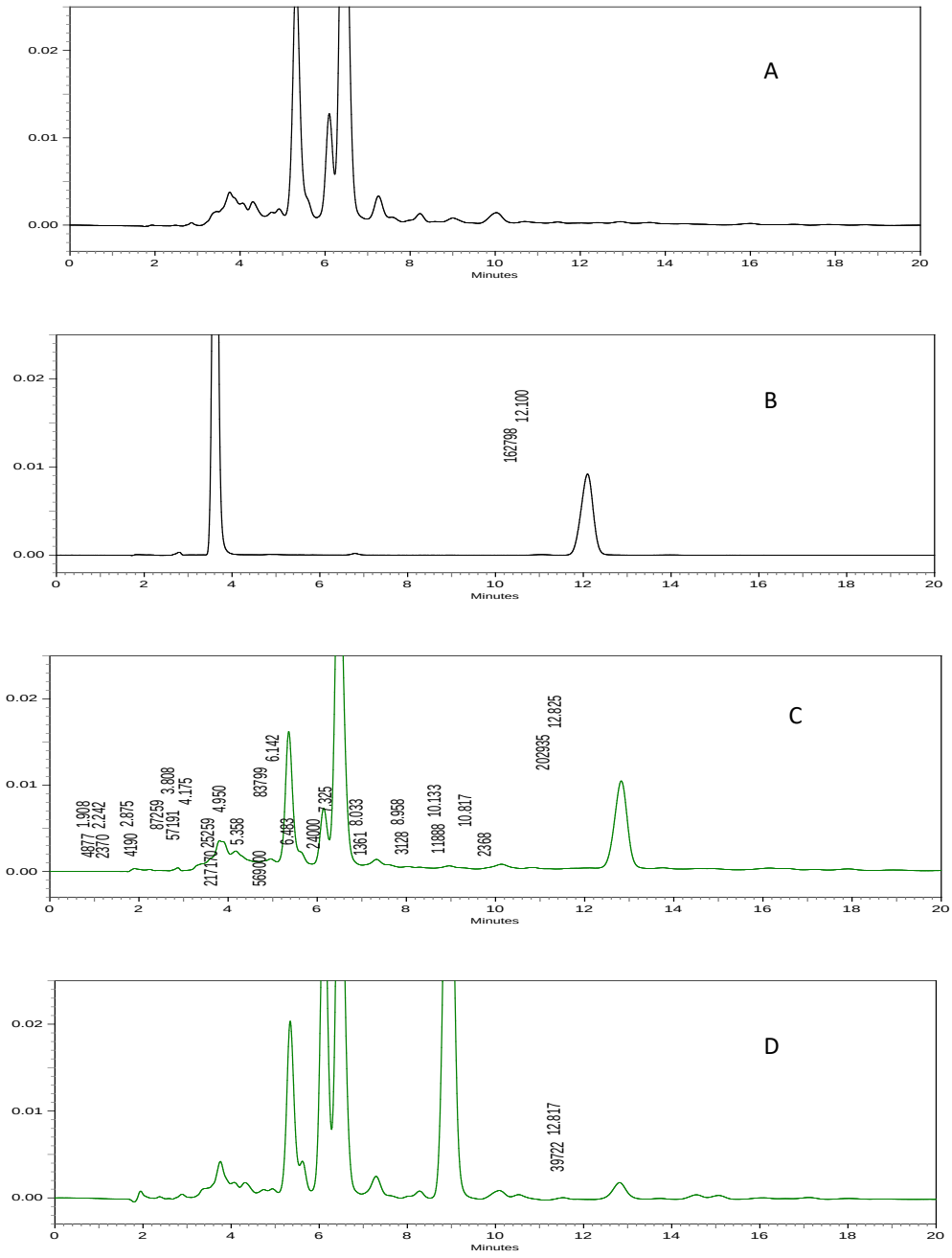


Figure 3. Chromatograms of flufenoxuron (A. control, B. standard, C. recovery, and D. real sample).

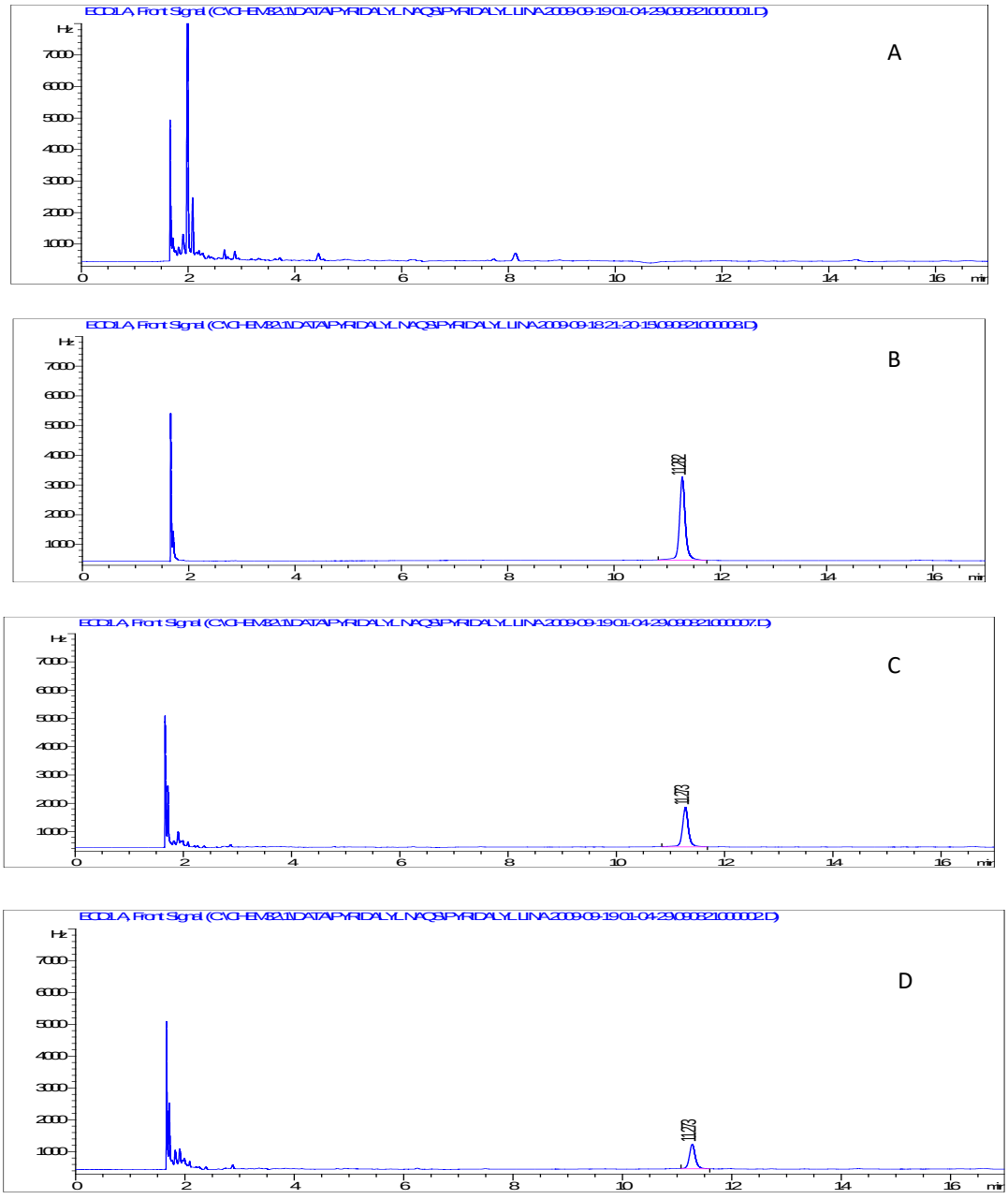


Figure 4. Chromatograms of pyridalyl (A. control, B. standard, C. recovery, and D. real sample).

4.5. Dissipation pattern of tested pesticides

Thirty samples obtained from greenhouse experiment were analyzed to assess the performance of the described method with real samples and also to determine whether the concentration of the pesticides used exceeded their MRLs. Bitertanol and fludioxonil residues were found to be lower than their MRLs 4 days post-application, whereas, chlorfluazuron and pyridalyl was safe enough in single dose application compared to their MRLs. On the contrary, flufenoxuron residues were decreased to a level below the corresponding MRL at 8 days of application (**Table 4**). Pesticides decrease on account of fruit growing, evaporation, co-distillation, thermo degradation, application method, and chemical degradation, (Garau *et al*., 2002; Marín *et al*., 2003; Moon *et al*., 2003; Sung, 2010). Because of the fruit weight was constant (±0.2 g) during the experiment, there was no dilution effect on the residual concentration.

Table 4. Residue levels of the tested pesticides in/on pepper grown under greenhouse conditions

Pesticides	Dose	0	1	2	4	6	8	11	14	MRLs	Half-life
Bitertanol	S	1.6	1.4	0.9	0.8	0.6	0.5	0.2	0.2	0.7	4.2
	D	2.2	1.7	1.6	1.0	0.7	0.9	0.2	0.2		3.9
Chlorfluazuron	S	0.3	0.2	0.2	0.2	0.2	0.1	0.1	0.06	0.5	7.6
	D	0.5	0.4	0.3	0.3	0.2	0.1	0.1	0.1		6.0
Fludioxonil	S	5.9	3.1	0.9	0.3	0.3	0.2	0.2	0.2	0.3	3.1
	D	7.0	4.6	4.0	3.7	1.0	1.0	0.8	0.4		3.4
Flufenoxuron	S	0.6	0.5	0.4	0.4	0.4	0.2	0.1	0	0.3	1.9
	D	0.6	0.6	0.4	0.4	0.3	0.2	0.1	0		1.9
Pyridalyl	S	0.5	0.4	0.4	0.4	0.3	0.2	0.2	0.1	2	6.15
	D	0.7	0.7	0.6	0.5	0.5	0.4	0.3	0.2		8.74

5. CONCLUSIONS

Methods for pesticide residue determination in peppers were successfully modified and validated. The mean recoveries ranged from 74.9 to 126.8% with relative standard deviation (RSDs) ranged from 0.5 to 10.3%. All the LOQs values were lower than the established MLRs, which indicated that the methods were sensitive and possible to detected lower concentration of pesticides in pepper. Bitertanol and fludioxonil residues were detected in concentration lower than their MRLs 4 days post-application. Whereas, chlorfluazuron and pyridalyl residues were safe enough compared to their MRLs. On the contrary, flufenoxuron residue was lower than its MRL 8 days of post-application. Pesticides should be applied correctly, according to good agricultural practice, using only the required amount. Culinary applications are necessary to decrease the intake of pesticide residues.

Chapter III. Residual Pattern of Acequinocyl and Hydroxyacequinocyl in Perilla Leaf Grown Under Greenhouse Conditions Using UPLC-PDA with Tandem Mass Confirmation

1. ABSTRACT

Persistence and degradation behavior of acequinocyl and hydroxyacequinocyl were determined in perilla leaf grown under greenhouse conditions. Acequinocyl (15%, SC) was sprayed on perilla leaf at the recommended dose rate of 37.5 g/250 L water/10 a with single and double dose applications. Leaf samples were collected randomly at 0 (2 h after application), 1, 3, 5, and 7 days post-application from the two different plots. The samples were extracted with acetonitrile, purified through a solid phase extraction procedure, and analyzed via ultra-performance liquid chromatography coupled with photo diode array detector (UPLC-PDA). Residues were confirmed via liquid chromatography tandem mass spectrometry (LC–MS/MS) in positive-ion electrospray ionization (ESI^+) mode. Calibration curves were linear over the concentration ranges for the analytes with $r^2 \geq 0.992$. The limits of detection and quantification were 0.05 and 0.165 mg/kg for both acequinocyl and hydroxylacequinocyl. The method was validated in triplicate at two fortification concentrations in the matrix. Good recoveries were observed for the target analytes and ranged between 94.95 and 113.26% with relative standard deviations less than 6%. The rate of disappearance of total acequinocyl on perilla leaf for single and double doses were described as first-order kinetics with half-life of 2.8 and 3.1days, respectively.

2. INTRODUCTION

The use of pesticides is essential to control pests in horticultural crops for the production of adequate food supply for an increasing world population and for the control of insect- borne diseases. These pesticides are used to decrease crop loss both before and after harvest (Clarke *et al*., 1997). Pesticide residues in food and crops are a direct result of the application of pesticides to crops growing in the field (Businelli *et al*., 1992). However, many pesticides are toxic substances and persistent in character. There is a growing social desire to reduce the use of pesticides in agriculture and horticulture (Freidberg, 2003; Pretty and Hine, 2005).

Acequinocyl (2-acetoxy-3-n-dodecyl-l, 4-naphthoquinone, **Figure 5**) is an acaricidal compound containing a quinone moiety in its structure that does not occur in the existing commercial miticides and possesses unique miticidal activity (Kinoshita *et al*., 1999). It also provides excellent control of many species of agricultural mite at all growth stages. It does not adversely affect beneficial mites (Kim and Yoo, 2002) and has low mammalian toxicity (LD_{50} to rat, 5000 mg/kg) (Dekeyser, 2005). The activity of this acaricide is due to its hydrolyzed derivative hydroxyacequinocyl (2-hydroxy-3-dodecyl-1,4-naphthoquinone), which inhibits complex III (bc1 complex) binding at the ubiquinol oxidation site (Q0) center, and blocks cellular respiration (Koura *et al*., 1998). Because it acts at the complex III stage, it can be used to control mite populations that are resistant to other miticides.

The levels of pesticide residues in foodstuffs are generally legislated so as to minimize the exposure of consumers to harmful or unnecessary intakes of pesticides; to ensure the proper use of pesticides in terms of granted authorization and registration (application rates and pre-harvested intervals); and to permit the free circulation of pesticide-treated products,

as long as they comply with the fixed MRLs. Pesticide residues are violative when they exceed the allowable level known established specifically for a particular pesticide on a particular food item (Osman *et al*, 2010). Acequinocyl is registered in Japan, the USA, Republic of Korea, and Taiwan for many crops, and field trials are being conducted in Europe (Caboni *et al*., 2004). In the Republic of Korea, the maximum residue limits (MRLs) have been established in the range of 0.1 to 2.0 mg/kg for 13 vegetables and fruits, however is not yet registered for perilla leaf (KFDA, 2011).

In the published literature for the determination of acequinocyl and its hydrolyzed derivative hydroxyacequinocyl in vegetable samples, only one methodology developed by Caboni *et al*. This involved high-performance liquid chromatographic (HPLC) detection with DAD detection and tandem mass spectrometric confirmation of the compounds (Caboni *et al*., 2004).

Ultra-performance liquid chromatography (UPLC) utilizes sub-2 μm particles for stationary phase which operate at elevated mobile phase linear velocities to effect a dramatic increase in resolution, sensitivity and speed of analysis (Jerkovich *et al*., 2003). Owing to its speed and sensitivity, this technique has been gaining considerable attention in recent years. In the present work, this technology has been applied to the method development and validation study for acequinocyl and hydroxyacequinocyl.

The principal objective of this study was to generate data regarding the persistence, pattern of decline and residue levels of total acequinocyl in perilla leaves cultivated under experimental greenhouse conditions via UPLC, with confirmation via tandem mass spectrometry (LC-MS/MS).

Acequinocyl Hydroxyacequinocyl

Figure 5. Chemical structure of acequinocyl and hydroxyacequinocyl.

3. MATERIALS AND METHODS

3.1. Standards and reagents

Standard acequinocyl (98.5%) and hydroxyacequinocyl (94.6%) were purchased from Agro-Kanesho Co. Ltd. (Tokyo, Japan). Ethyl acetate (EtOAc), acetonitrile (ACN), and *n*-hexane of HPLC grade were obtained from Burdick and Jackson (SK chemical, Ulsan, Republic of Korea). Acetic acid (CH_3COOH) and formic acid (HCOOH) were obtained from Junsei Chemical Co. Ltd. (Kyoto, Japan). Analytical-grade anhydrous magnesium sulfate ($MgSO_4$) was obtained from Junsei Chemicals Co., Ltd. (Tokyo, Japan). A Silica solid-phase extraction (SPE) cartridge (1 g, 6 mL) was provided by Phenomenex (CA, USA).Water was distilled and filtered through an Ultima DUO 200 (Balmann Tech, Daegu, Republic of Korea) water system prior to use.

3.2. Standard Solution

Standard stock solutions of individual acequinocyl and hydroxyacequinocyl (100 mg/L) were prepared in ACN and stored at 4°C. Fortification solution and working calibration solutions for UPLC analysis were acquired after mixing appropriate dilutions of the stock solutions with the same solvent, achieving concentrations in a range of 0.05 to 40 mg/L. Fortified and calibrated standard solutions were stored at a refrigerator (4°C).

3.3. Greenhouse experimental design

Experiments were conducted in a greenhouse in the experimental fields of Chonnam National University, Gwangju, Republic of Korea. The experimental area comprised 2 plots, in which a random block scheme was established with three replicates (plot A for single spraying;

plot B for double spraying). In addition, control samples were cultivated in a separate plot without receiving any treatment with insecticides. Acequinocyl 15% wettable powder (WP; Kyung Nong Co., Seoul, Republic of Korea) was diluted 1000 times with water and sprayed at a.i.0.0375 kg/10 a, as recommended by the manufacturer. Pesticides were sprayed first on 5 October, 2011 in plot B, and then on 12 October, 2011 in plots A and B of the greenhouses. Perilla leaves were collected at 0, 1, 3, 5, 7, 10, and 14 days after the second application. The spraying and harvesting schedule is also shown in **Table 5**. Climatic conditions were monitored using a thermo-hygrometer (model DK-012, Daekwang Inc., Seoul, Republic of Korea); temperatures ranged from 20 to 35°C, and relative humidity ranged from 15 to 55% throughout the experimental periods. The samples were collected and transferred to the laboratory where they were chopped and blended. Sub-samples weighing approximately 50 g each were stored in a freezer pending analysis.

Table 5. Time table to spray pesticide and harvest onto perilla leaves

Final spraying day	Interval prior to harvest (days)		Harvest day
	Treatment frequency=1	Treatment frequency = 2	
12 October, 2011	0	7 - 0	12 October, 2011
	1	8 – 1	13 October, 2011
	3	10 - 3	15 October, 2011
	5	12 - 5	17 October, 2011
	7	14 - 7	19 October, 2011
	10	17 - 10	22 October, 2011
	14	21 - 14	26 October, 2011
Harvest day of the untreated sample (control) - 12 October, 2011			

3.4. Sample extraction

Approximately 10 g of blended perilla leaf samples were weighed into 50 mL centrifuge tubes, and 20 mL of ACN was added to the samples, which were shaken for 1 min. Six g of anhydrous $MgSO_4$ was added and shaken again for 1 min. The extract was then centrifuged for 5 min at 5000 rpm at 4 °C. Four mL of upper layer were transferred to a round-bottomed flask and evaporated to dryness below 40 °C under vacuum.

The extract was dissolved in 4 mL *n*-hexane, and loaded into a previously conditioned 1 g SPE Silica cartridge with 5 mL of *n*-hexane. The cartridge was primarily washed with 10 mL of *n*-hexane and then co-extractives were eliminated with 10 mL of 2% of EtOAc in *n*-hexane. Finally, acequinocyl and hydroxyacequinocyl were eluted with 15 mL of 10% EtOAc in *n*-hexane. The eluate was evaporated to dryness below 40 °C under vacuum and reconstituted in 2 mL of ACN for UPLC-DAD analysis.

3.5. Instrumental conditions

Ultra-performance liquid chromatography

UPLC was performed using a Waters H-Class system equipped with a binary solvent manager, an auto sampler and PDA detector. The system was controlled by Waters Empower 3 software. The chromatographic separation was performed using a Waters Acquity BEH 100×2.1 mm, 1.7 μm, C_{18} column maintained at 35 °C. The mobile phase containing a mixture of 0.1% aqueous CH_3COOH and ACN in a ratio of 15:85(v/v) in isocratic mode was used. The detection was obtained at a wavelength of 250 nm. The flow rate was 0.8 mL/min, and the injection volume was 10 μL.

LC-ESI-MS/MS

The determined residues of acequinocyl and hydroxyacequinocyl in perilla leaves via UPLC-PDA were further confirmed using LC-tandem mass spectrometry (LC–MS/MS). The liquid chromatography was performed by a Waters Alliance 2695 Separation Module (Waters, Manchester, UK). Chromatographic separation was conducted on a Gemini C_{18} column (50 ×2.0 mmi.d., 3 μm particle size, Phenomenex, CA, USA). The binary solvent system consist of 0.1% HCOOH in ACN (A) and 0.1% HCOOH in water (B), with a linear gradient. The mobile phase gradient consisted of 0-1 min 5:95, 1-3 min ramp to 90: 10; 3-9 min hold at 90:10, 9-10 min ramped back to the initial conditions and hold until 15 min. The flow rate was 0.25 mL/min with a column temperature maintained at 30 °C. The injection volume was 5 μL. MS/MS was conducted with a triple Quadrupole LC-MS (QQQ) (Waters TQ Detector) in positive electrospray ionization (ESI^+) and multiple reactions monitoring (MRM) mode.

MassLynx V4.1 software was used for instrument control and data acquisition. The MS source conditions were as follows: capillary voltage 4000 V, source temperature 150°C, desolvation temperature 350°C, desolvation gas(N_2) with flow rate of 600 L/h and Cone gas(N_2) with flow rate of 50 L/h. Argon was used as the collision gas with a collision cell pressure of 3×10^{-3} mbar, and flow rate was 0.15 mL/min. The optimization of the precursor ion, product ions, and collision energy (eV) was carried out via direct injection of the individual pesticide standard solutions (1 μg/mL) into the mass spectrometer. Both MS1 and MS2 quadrupoles were maintained at unit resolution, and the other conditions are presented in **Table 6**.

Table 6. The optimal transition parameters of LC–ESI–MS/MS for the acequinocyl and hydroxyacequinocyl

Compound	MW	Precursor ion (*m/z*)	Product ion (*m/z*)[a]		Cone (V)	RT[b] (min)
			Quantitation	Confirmation		
Acequinocyl	384.51	343.096	188.948 (56)	114.930(26)	44	12.274
Hydroxy acequinocyl	342.47	343.138	114.990(48)	188.994(28)	44	11.540

[a] Collision energy (eV)

[b] Retention time

3.6. Method Validation

The validation of the analytical method was performed with respect to the following parameters: linearity, limits of detection and quantification, precision and accuracy, and repeatability. All the analyses were carried out using the same blank sample and solvent.

In order to construct standard calibration curves, a mixture of acequinocyl and hydroxyacequinocyl solution was serially diluted with the acetonitrile to prepare eleven different concentrations ranging from 0.05 to 40 mg/L. The calibration curves were acquired by plotting the peak area against the concentration of the corresponding calibration standards.

The LOD of the target compounds were determined using a signal-to-noise ratio of 3 with reference to the background noise obtained for the blank sample, whereas the LOQ were determined with a signal-to-noise ratio of 10.

Accuracy and precision data were obtained with recovery studies carried out by spiking samples with pesticide standards at levels of 0.5 and 2.5 mg/kg, which were ten times and fifty times the LOD, respectively. The spiked samples as well as the unspiked controls were analyzed in three replicates. The repeatability of the method was evaluated by the relative standard deviation (RSD, %) associated with the measurements of the pesticide performed during recovery analyses.

4. RESULTS AND DISCUSSION

4.1. UPLC determination and method validation

The chromatograms obtained by UPLC-PDA corresponding to standard solutions of acequinocyl and hydroxyacequinocyl prepared in the pure solvent, blank extract and spiked blank sample extracts are shown in the **Figure 6**. All peaks exhibiting RTs identical to their corresponding standards without any interference with matrix, representing the selectivity and specificity of the developed method.

The results of the analytical curves obtained with the analytical solutions prepared in pure solvent are presented in **Table 7**, with coefficient of determination (r^2) values higher than 0.990. The detector response was linearly dependent of the concentration up to 40.0 mg/L. Comparing the responses with the baseline noise, the limit of detection was found to be 0.05 mg/kg, and the limit of quantification was 0.165 mg/kg. Recoveries were measured by comparing peak areas of the spiked samples with external standards in acetonitrile. The precision (repeatability) reflects the variation in results when repetitive analyses were carried out under the same conditions (Cao *et al*. 2005). The numerical value used is the relative standard deviation for repeatability (RSD). The recovery and repeatability for acequinocyl and hydroxyacequinocyl at two different levels are summarized in **Table 7**. The precision ranged from 1.60 to 5.08%. The values are good because all measurements should be within 15% for all concentrations. The recoveries obtained for acequinocyl and hydroxyacequinocyl ranged from 94.95 to 113.26% and are considered satisfactory because all values are between 85 and 115% (Causon, 1997).

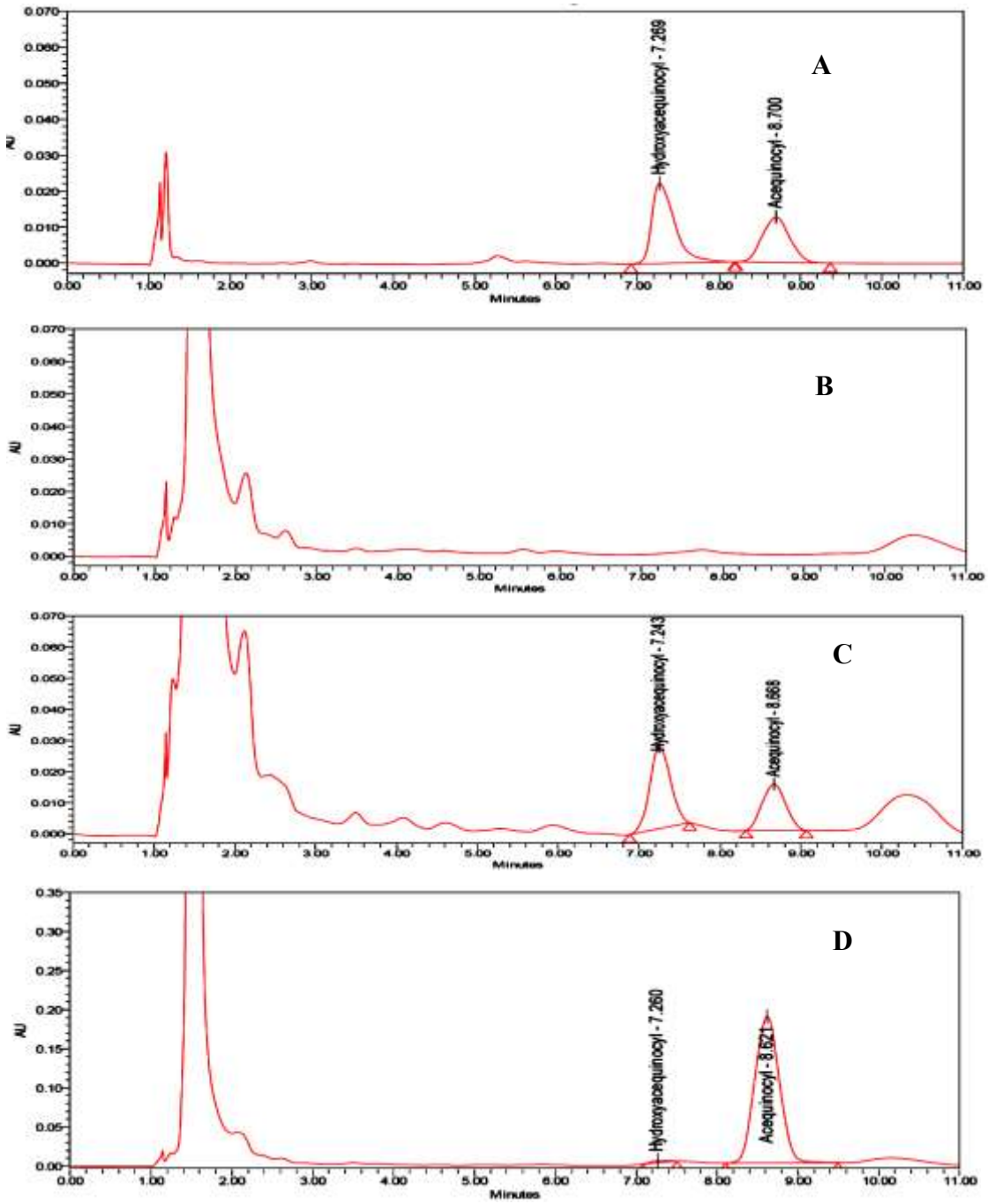


Figure 6. UPLC–PDA chromatograms of standard acequinocyl and hydroxyacequinocyl (A) an untreated perilla leaf (B), fortified perilla leaf samples (C) and field sample collected (D).

Table 7. Linearity, limit of detection, limit of quantification and recovery of acequinocyl and hydroxyacequinocyl in/on perilla leaves

Pesticides	Linearity (r^2)	[a]Recovery ±(RSD, %) (Spiked conc.)		LOD (mg/kg)	LOQ (mg/kg)
		(0.5 mg/kg)	(2.5 mg/kg)		
Acequinocyl	0.998	113.26±1.60	108.85± 1.79	0.05	0.165
Hydroxyacequinocyl	0.992	94.95± 5.08	113.24± 1.79	0.05	0.165

[a] Mean of three replicates.

4.2. Degradation dynamic

Acequinocyl degradation dynamics

The residues of acequinocyl found in perilla leaf over the testing time period are shown in **Table 8**. As expected, a gradual and continuous deterioration of the pesticide residues in and on the treated plants was observed as a function of time after application. Levels of residue can generally be interpreted by the use of a first-order model, which allows linearization of data by plotting the residues versus time. Statistical interpretation of acequinocyl dissipation was carried out by assuming that the residues degrade with first-order kinetics ($\ln C = \ln C_o - kt$), in which C is the residual concentration at time t after pesticide application, C_o is the residual concentration at time t = 0, and k is the dissipation rate constant. The residue levels of acequinocyl in mg/kg were plotted versus time, and the results are demonstrated in the **Figure 7**.

The coefficients of determination (R^2) values confirm that the degradation behavior of acequinocyl on perilla leaf can be described as a first-order reaction under the experimental conditions.

In dissipation studies, the establishment of the half-life time ($T_{1/2}$) of the residues that provides information about the persistence of pesticides in crops was calculated as 0.693/k (Putnam *et al*., 2003). For the recommended single and double doses, the average half-lives ($T_{1/2}$) for total acequinocyl in perilla leaves were 2.8 and 3.1 days, respectively. The results (DT_{50} 3 days) reported by Dekeyser (2005) evidenced the half-life of acequinocyl in perilla leaf. The dynamics could be described by the equation $C= 46.90\ e^{-0.25t}$, with square of coefficient $R^2=0.775$; and $C=46.49\ e^{-0.22\,t}$. with square of coefficient $R^2= 0.828$, for single and double doses, respectively.

Table 8. Acequinocyl and hydroxyacequinocyl residue in/on perilla leaves at various time intervals following application

Spray frequency	Day after application	Residue of Acequinocyl (Average ± SD) (mg/kg)	Residue of Hydroxyacequinocyl (Average ± SD) (mg/kg)	Total residue of Acequinocyl (mg/kg)
Control	-	ND	ND	ND
Spray 1 time (plot-A)	0	28.72 ± 1.12	0.88 ± 0.06	29.71 ± 1.14
	1	24.13 ± 0.26	0.73 ± 0.13	24.95 ± 0.42
	3	22.24 ± 0.72	0.24 ± 0.05	22.52 ± 0.74
	5	18.54 ± 0.23	0.24 ± 0.06	18.81 ± 0.16
	7	14.57 ± 0.71	0.37 ± 0.09	14.98 ± 0.62
	10	8.25 ± 0.03	0.89 ± 0.03	9.26 ± 0.07
	14	_	0.42 ± 0.01	0.48 ± 0.02
Spray 2 times (plot-B)	0	33.75 ± 0.27	0.36 ± 0.15	34.16 ± 0.44
	1	29.62 ± 0.16	0.41 ± 0.05	30.09 ± 0.17
	3	20.04 ± 0.15	0.71 ± 0.49	20.84 ± 0.60
	5	19.97 ± 1.03	0.52 ± 0.09	20.56 ± 0.92
	7	15.23 ± 2.56	0.67 ± 0.10	15.99 ± 2.66
	10	8.78 ± 0.24	1.11 ± 0.10	10.03 ± 0.34
	14	_	0.73 ± 0.04	0.96 ± 0.05

Total residue = Acequinocyl + (hydroxyacequinocyl * 1.123)

1.123 = Acequinocyl M.W. (384.51) / hydroxyacequinocyl M.W (342.47).

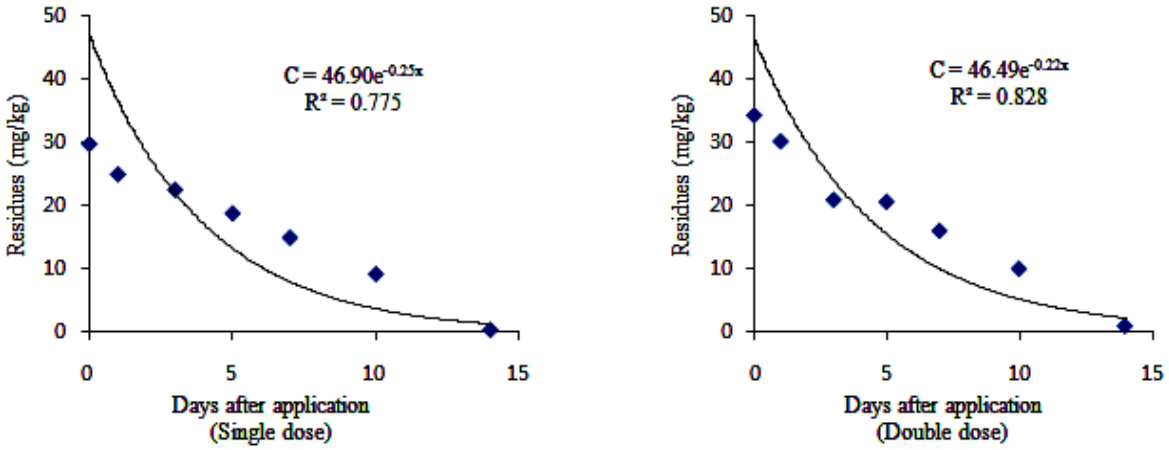


Figure 7. Degradation of total acequinocyl in/on perilla leaves for single and double doses of application.

4.3. Confirmation of field-incurred residues via LC–ESI-MS/MS analysis

For unambiguous identification of contaminants in food, mass spectrometry has proved to be a valuable technique since detection may be complicated by false-positive results using conventional LC detectors. However, acequinocyl detected by PDA in perilla leaves were confirmed through LC-MS/MS in positive electrospray ionization (ESI^+) mode using multiple reaction monitoring (MRM) with two mass transitions. The selected ion chromatograms are shown in **Figure 8**. In addition, LC-MS/MS spectra are also shown in **Figure 9**.

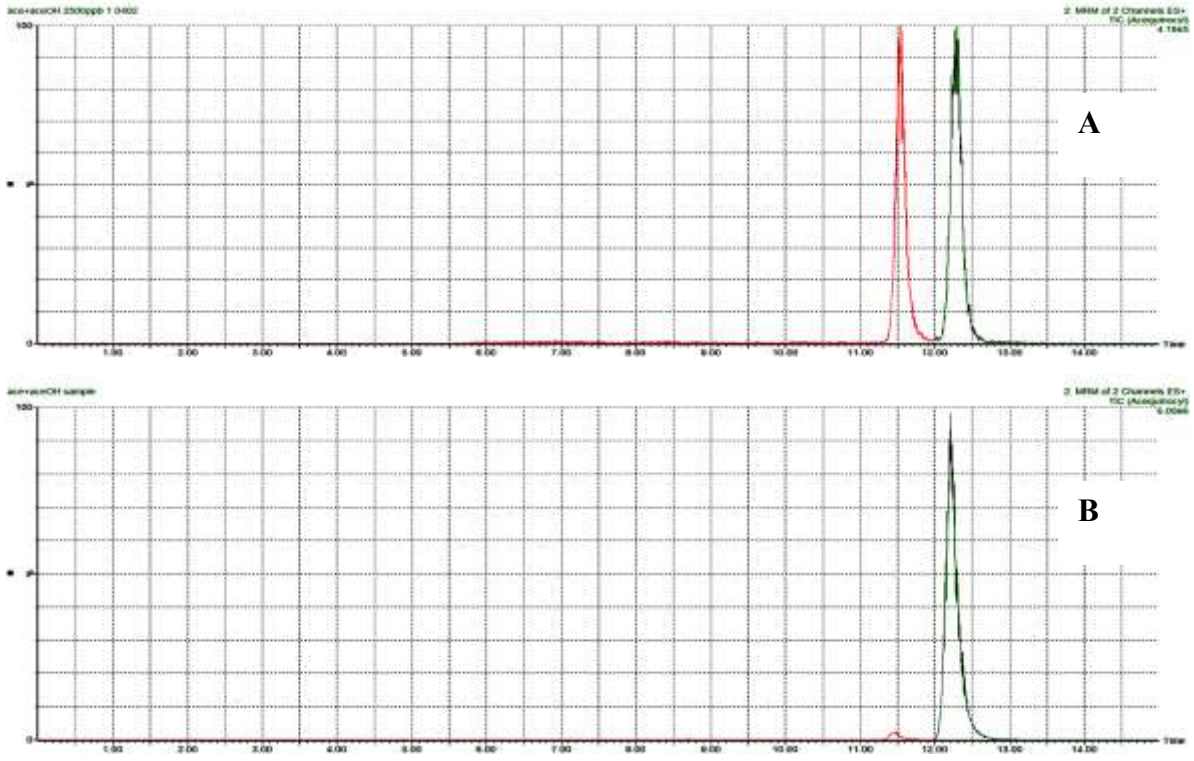


Figure 8. LC–ESI-MS/MS selected ion chromatograms of standard acequinocyl and hydroxyacequinocyl (A), and field-incurred sample (B).

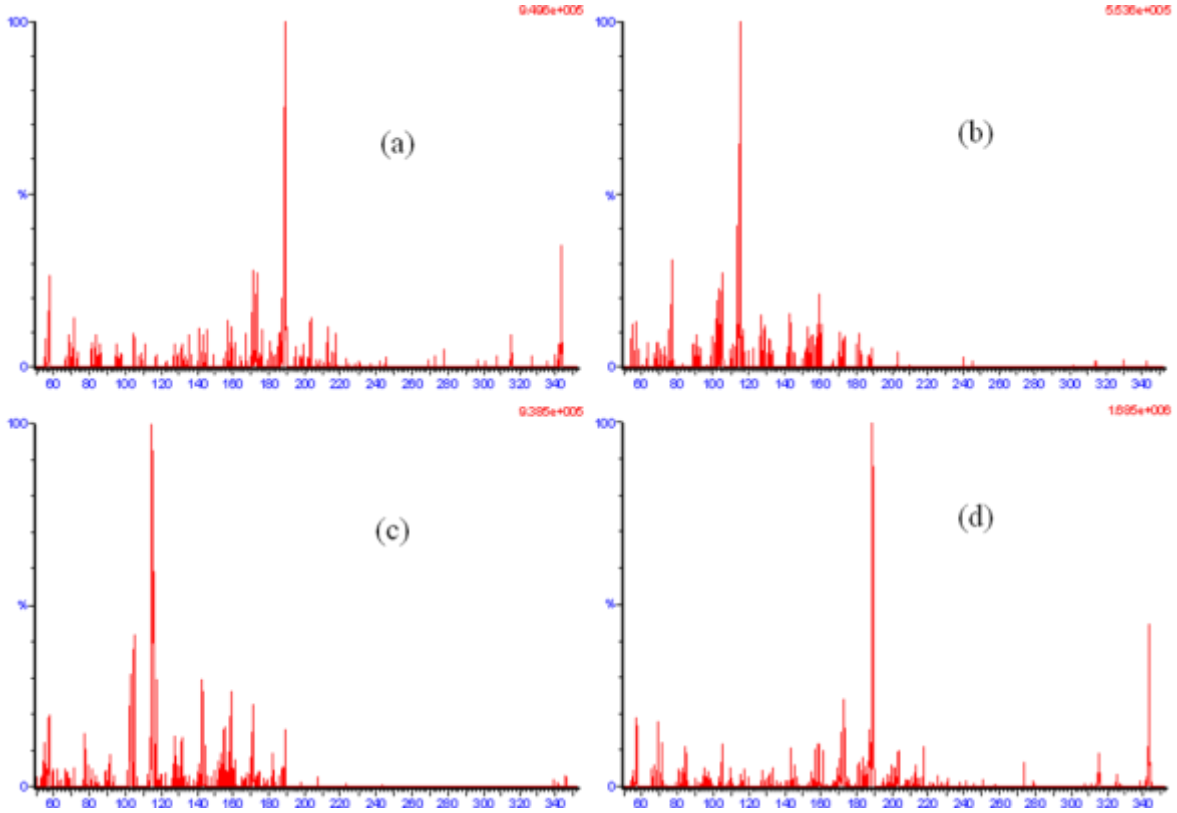


Figure 9. LC-MS/MS spectra for acequinocyl quantitation (a) and confirmation (b) and for hydroxylacequinocyl quantitation (c) and confirmation (d).

5. CONCLUSIONS

The present work was designed to investigate the residues of acequinocyl in/on perilla leaves grown under greenhouse conditions so as to determine the interval between spraying and harvest required for safe use of this crop, and to prevent any health problems for consumers. This would help to establish adequate monitoring of the residue of this insecticide/acaricide, and its judicious incorporation in pest management strategies in vegetable fields.

Chapter IV. Dissipation Pattern and Pre-Harvest Residue Limit of Abamectin in Perilla Leaves

1. ABSTRACT

The PHRL of abamectin (abamectin B1a and B1b) in *Perilla frutescents* leaves grown under green house conditions were investigated using high performance liquid chromatograph with a fluorescence detector. Samples were extracted with acetonitrile. The extract was purified through a solid phase extraction procedure (SPE). Then the purified extract was derivatized with trifluoroacetic anhydride (TFAA) and N-methylimidazole (NMIM) to form a strong stable fluorescent derivative of abamectin. Finally derivatized abamectins were conveyed to the detector via an Atlantis C_{18} column, with water and methanol as amobile phase. Calibration curves were linear over the calibration ranges with coefficients of determinants $r^2 \geq 0.999$. The limits of detection and quantification were 0.0033 and 0.01 mg/kg for abamectin B1a and B1b, respectively. Recovery was assessed in a control matrix at two different fortification concentrations, with three replicates for each concentration. Good recoveries were obtained for the target analytes, and ranged from 82.11 to 93.03%, with relative standard deviations of less than 8%. The rate of disappearance of total abamectin on perilla leaves for recommended and double the recommended doses were described as first-order kinetics with a half-life of 0.7 days. Using the PHRL curve, we could predict the residue level of total abamectin to be 0.92 mg/kg at 7 days before harvest or 0.26 mg/kg at 4 days before harvest, which would be below the provisional MRL designed by the Korea Food and Drug Administration (KFDA).

2. INTRODUCTION

During the last number of decades, the demand for increased food safety has stimulated research regarding the risk associated with the consumption of foodstuffs contaminated by pesticides. It is well known that the level of risk is closely related to the daily intake of contaminated food, and that for certain products which are frequently consumed, the risk is higher (Mestres, 1988). Perilla leaves, one of the principal minor crops in the Republic of Korea and Asia, are grown and cultivated on limited acreage. Minor crops are produced as a strain of major crops for niche markets and receive limited or no research investments in either the public or private sectors. Governments and international organizations are regulating the use of pesticides and are setting the acceptable MRLs established for crops and foods (Philp, 2003). MRLs represent the maximum quantity of residues in raw commodities of plants or animal origin, and should encourage the use of minimal quantities of active substances while still achieving adequate pest control, being applied in such a manner that the residues are as low as practicable, and ensuring that the product is toxicologically acceptable.

As of 2012, the maximum residue limit for 41 pesticides in perilla leaves had been established. Almost all of the pesticides show high residue levels in perilla leaves. Abamectin, the macrocyclic lactone class of avermectins, is comprised of at least 80% avermectin B_{1a} and less than 20% avermectin B_{1b}. It is produced by the soil bacterium *Streptomyces avermitilis* (Campbell *et al*. 1983; Sun *et al*. 2005). Abamectin is widely used to control insects because of its high toxicity. It was reported that the oral LD_{50} of abamectin for rats is about 11 mg/kg (Lanka's and Gordon 1989). In the Republic of Korea, its MRLs have been established at a range of 0.01 to 0.2 mg/kg for 17 vegetables and fruits; however, this MRL has not yet been applied to perilla leaves (KFDA 2011).

To assess such levels of target pesticides, reliable, and sensitive analytical methods are required. There are several analytical method reported for abamectin in crops or fruits, such as high-performance liquid chromatograph (HPLC) coupling with UVD of FLD (Hernandez-Borges *et al*. 2007; Xie *et al*., 2010), as well as ESI-MS/MS (Pozo *et al*., 2003). FLD is more sensitive and selective than UVD, and the initial installation cost is cheaper than MS/MS based detection.

The dissipation rate of synthetic chemicals after application depends heavily on several factors, including chemical and photochemical degradation, climatic conditions, volatilization, cultivated species, formulation class, and application mode (Papadopoulos *et al.,*1995; Sur *et al.*,2000). Thus, the dissipation curves reported in literature are valid only for a given crop under specific conditions (Hem *et al.,* 2011). Therefore, it is essential to study the dissipation kinetics and bioaccumulation of pesticides in organisms or the environment, which could help improve our understanding of pesticide safety to humans, animals and the environment

Therefore the principal objective of this study was to determine the residue levels of abamectin in perilla leaves in order to generate data regarding persistence, pattern of decline and residual levels of total abamectin.

3. MATERIALS AND MEHTODS

3.1. Chemicals and reagents

Standard abamectin containing abamectin B_{1a} 94.03% and abamectin B_{1b} 5.97% were purchased from Agro Dragon co. Ltd. (Shanghai, China). *n*-hexane, ethyl acetate (EtOAc), acetonitrile (ACN), and acetone of HPLC grade were obtained from Burdick and Jackson (SK

Chemical, Ulsan, Republic of Korea). Analytical-grade anhydrous magnesium sulfate ($MgSO_4$) was obtained from Junsei Chemicals Co., Ltd. (Tokyo, Japan). A silica solid-phase extraction (SPE) cartridge (1 g, 6 mL) was provided by Phenomenex (CA, USA). Trifluoroacetic anhydride (TFAA) and N-methylimidazole (NMIM) were of analytical grade, and were obtained from Sigma Aldrich (USA). Water was distilled and filtered through an Ultima DUO 200 water system (Balmann Tech, Daegu, Republic of Korea) prior to use.

3.2. Preparation of standard solutions

Stock solutions of abamectin B_{1a} were prepared by weighing 0.0106 mg of abamectin B_{1a} in a 100 mL volumetric flask, and diluting up to the mark with acetonitrile. Abamectin B_{1b} was prepared by diluting 10.05 mL of abamectin B_{1a} in 1.95 mL of acetonitrile to reach a concentration of 5 mg/L. Fortification levels and working calibration solutions for HPLC-FLD analysis were acquired via appropriate dilution of the stock solutions with the same solvent, achieving concentrations ranging from 0.01 to 5 mg/L. Fortified and calibrated standard solutions were stored in a refrigerator (4°C).

3.3. Greenhouse experimental design

Experiments were conducted in a greenhouse in the experimental fields of Chonnam National University, Gwangju, Republic of Korea. The experimental area comprised 2 plots, in which a random block scheme was established with three replicates (plot A for single spray; plot B for double spray). In addition, control samples were cultivated in a separate plot without receiving any treatment. Abamectin emulsifiable concentrate (EC; Kyung Nong Co., Seoul, Republic of Korea) was diluted 2000 times with water and was sprayed at a.i 0.0023 kg 10 a^{-1}, as recommended by the manufacturer. Climatic conditions were monitored using a thermo-

hygrometer (model DK-012, Daekwang Inc., Seoul, Republic of Korea), and temperatures ranged from 20 to 35°C, and relative humidity ranged from 15 to 55% throughout the experimental period, as shown in **Figure 10**.

Perilla leaves were collected from each replicated plot at 0 (after 2 h), 1, 2, 3, 4, 5, 6, and 7 days after the application in plot A, and at the same periods after a second application in plot B. The collected samples were transferred to the laboratory where they were chopped and blended. Sub-samples weighing approximately 100 g each were stored at -24°C prior to analysis. At the same time 10 g control samples in 50 mL centrifuge tubes were fortified with standard abamectin at level of LOQ×50 and were frozen as three replicates, in order to determine the stability during storage.

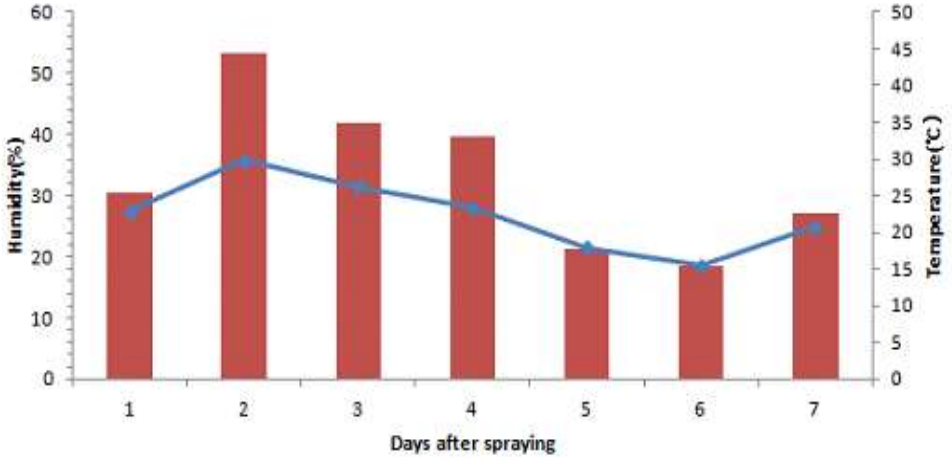


Figure 10. Temperature and humidity during cultivation period.

3.4. Analytical procedure

Sample extraction

Approximately 10 g of blended perilla leaf samples were weighed into 50 mL centrifuge tubes, and 30 mL of acetonitrile were added to the samples, which were shaken for 1 min. Six grams of anhydrous magnesium sulfate was added and shaken again for 30 s. The extract was then centrifuged for 5 min at 5000 rpm at 4°C. Fifteen mL of the upper layer were transferred to a round-bottomed flask and evaporated to dryness below 40°C under vacuum.

Sample clean-up

The extract was dissolved in 5% acetone in *n*-hexane, and was loaded into a previously conditioned 1 g SPE Silica cartridge with 6 mL of *n*-hexane. The cartridge was washed with 10 mL of 20% acetone in *n*-hexane, and abamectin was eluted with 10 mL of 30% acetone in *n*-hexane. The elutant was evaporated to dryness below 40°C under vacuum, and was then reconstituted in 3 mL of acetonitrile and 1 mL of ethyl acetate.

Derivatization

For derivatization, trifluoroaceticanhydride (TFAA) and *N*-methylimidazole(NMIM) reagent was used, which could react rapidly and form a strong stable fluorescent derivative to abamectin (Xie *et al*., 2011). The reconstituted extract was mixed with 0.3 mL of NMIM and 0.3 mL TFAA: ACN (2:1, v/v) reagent in this order. After 5 min, 0.4 mL of D.W was added to stop the derivatization reaction. Standard solutions were treated in the same manner. NMIM and TFAA-ACN reagents were kept in the dark at 4ºC pending analysis. After derivatization, samples and standards were injected quickly, due to the possibility of decreasing fluorescent signal.

3.5. Instrumental determination

HPLC was performed using a Shimadzu liquid chromatography system equipped with an SCL-10AVP system controller, LC-6AD pumps, an auto sampler (SIL 20 A) and a fluorescence detector (Shimadzu, Kyoto, Japan). The chromatographic separation was performed using an Atlantis 250×4.6 mm (Waters, Ireland), 5 μm, C_{18} column, which was maintained at 35 ºC. The mobile phase containing a mixture of water and methanol at a ratio of 6: 94 (v/v) was operated in isocratic mode. The fluorescence detector was set at an excitation wavelength of 365 nm and an emission wavelength of 470 nm. The flow rate was 1 $mL.min^{-1}$, and the injection volume was 100 μL. Same time injection analysis was performed following the order of injection as: standard, blank, recovery, and field samples in order to avoid run to run variation due to flow rate and other instrumental condition.

3.6. Method validation

Recovery studies were carried out by fortifying blank perilla leaf samples with abamectin B_{1a} and B_{1b} separately at ten times (0.1 mg/kg) and fifty times (0.5 mg/kg) concentration of LOQ with three replicates for each concentration. The fortified samples were thoroughly mixed, and were allowed to stand for 30 min prior to extraction. The samples were then extracted, purified, derivatized, and finally analyzed via the sample preparation procedures described above. Repeatability of the method was evaluated through the relative standard deviation (RSD, %) associated with the measurements of the pesticide performed during recovery analyses.

3.7. Storage stability

Storage stability was assessed via determination of recovery of the samples fortified

during freezing at -24°C. This experiment was carried out at the final stage of field sample analysis in order to determine the stability of standard in sample during the freezing period.

3.8. Statistical analysis

The dissipation kinetics of total abamectin in perilla leaf was determined by plotting the residue concentration against time. The residual concentration and half-life of abamectin were calculated by the first-order kinetics equations $C_t = C_0 e^{-kt}$ and $t_{1/2} = \ln 0.5/k$, respectively, where t is the time (days) after pesticide application, C_t is the residue concentration of the pesticide at time t, C_0 is an initial pesticide concentration after application (at t=0), k is a dissipation coefficient and $t_{1/2}$ is defined as the time required for the pesticide residue level to fall to half the initial residue level after application. The PHRL was calculated by an automated computational system using a statistical technique. This program was developed exclusively by the SPSS for the National Agricultural Products Quality Management Service (NAQS), Republic of Korea. After using a maximum residue limits and minimum dissipation constants k_{min}, as determined through SPSS, the first-order kinetics equation for pre-harvest residue limits was calculated as: PHRL = MRL × $e^{k_{min}t}$.

4. RESULTS AND DISCUSSION

4.1. Extraction assays

Extraction of perilla leaves was performed following QuEChERS sample preparation method after modification. In order to produce working samples >LOQ (0.01 mg/kg), 10 g samples were extracted with 30 mL solvent, with 15 ml supernatant removed for purification following extraction and centrifugation. Since dispersive PSA clean-up failed to provide perfect clean extract for derivatization, SPE silica cartridges were used to purify abamectin with different polarity solvents.

4.2. Method performance

Method performance was assessed by evaluating quality parameters, such as selectivity, limits of detection and quantification, linearity, recovery, and reproducibility.

The selectivity of the method was evaluated by injecting extracted blank samples following derivatization. The absence of signals above a signal-to noise ratio of 3 at the retention times of the target compounds showed that the method is free of interferences. After derivatization, different concentrations (0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1 mg/mL) of abamectin B_{1a} and B_{1b} were injected separately to the HPLC-FLD. The results showed good linearity with a correlation coefficient (r^2) of >0.999 being achieved for both analytes. The LOD was 0.003 mg/kg at a signal-to-noise ratio of 3, and the LOQ was 0.01 mg/kg at a signal-to-noise (S/N) ratio of 10. The typical HPLC-FLD chromatograms are shown in **Figure 11 and 12**. The efficiency of the method has been evaluated separately by spiking blank perilla leaf samples with known concentrations of abamectin B_{1a} and B_{1b} working solution (0.1 and 0.5 mg/kg). Three samples of each concentration were processed. The recoveries for spiked perilla leaf samples ranged

from 82.11 to 93.03%, and the relative standard deviations ranged from 3.22 to 7.67%. All values indicated good accuracy and repeatability, as these are consistent with SANCO guidelines (European Commission 2007). The recovery and relative standard deviations are shown in **Table 9**.

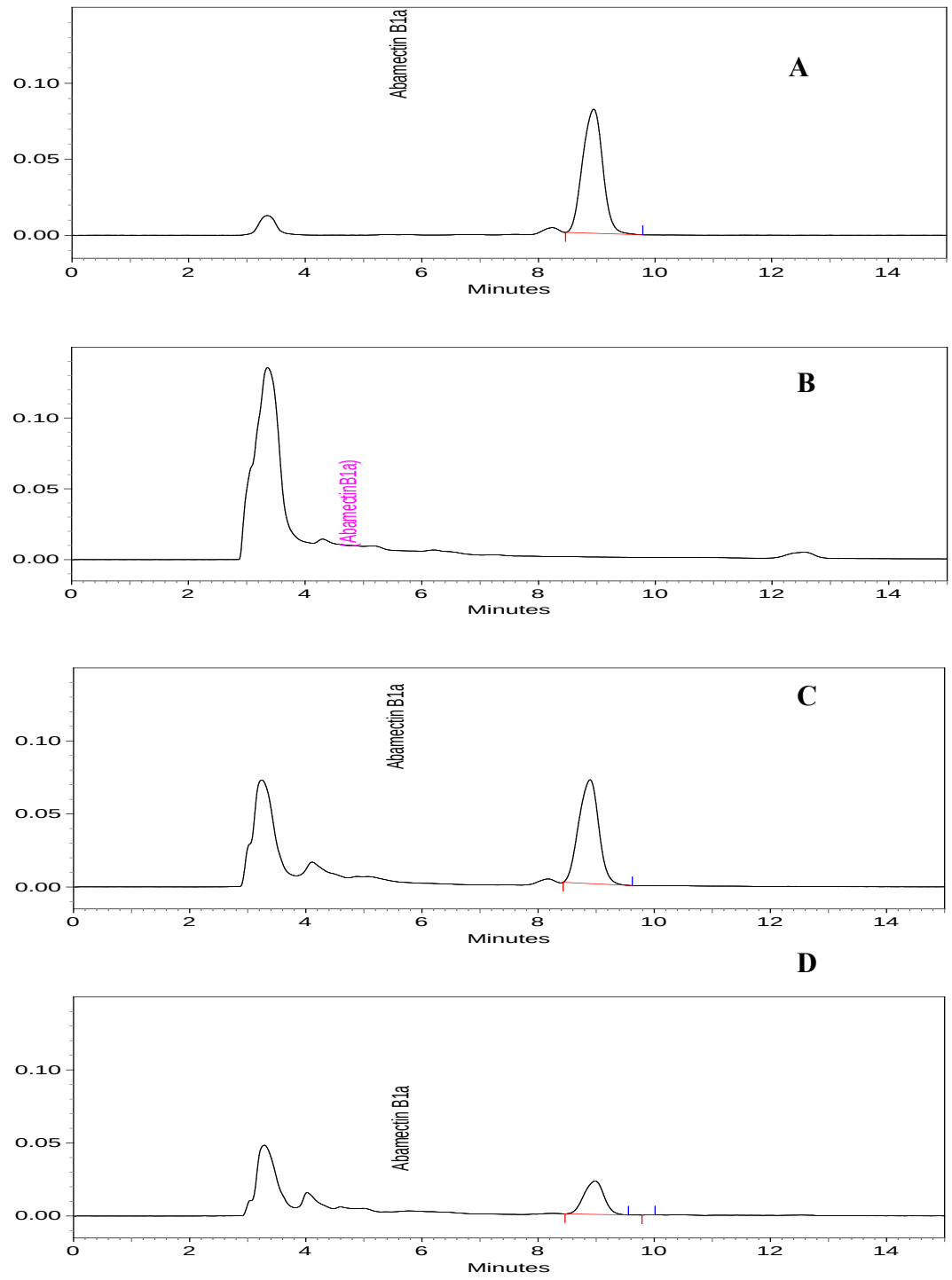


Figure 11. HPLC–FLD chromatograms of (A) standard abamectin $B_{1a,}$ (B) blank perilla leaf, (C) fortified perilla leaf samples and (D) field sample collected.

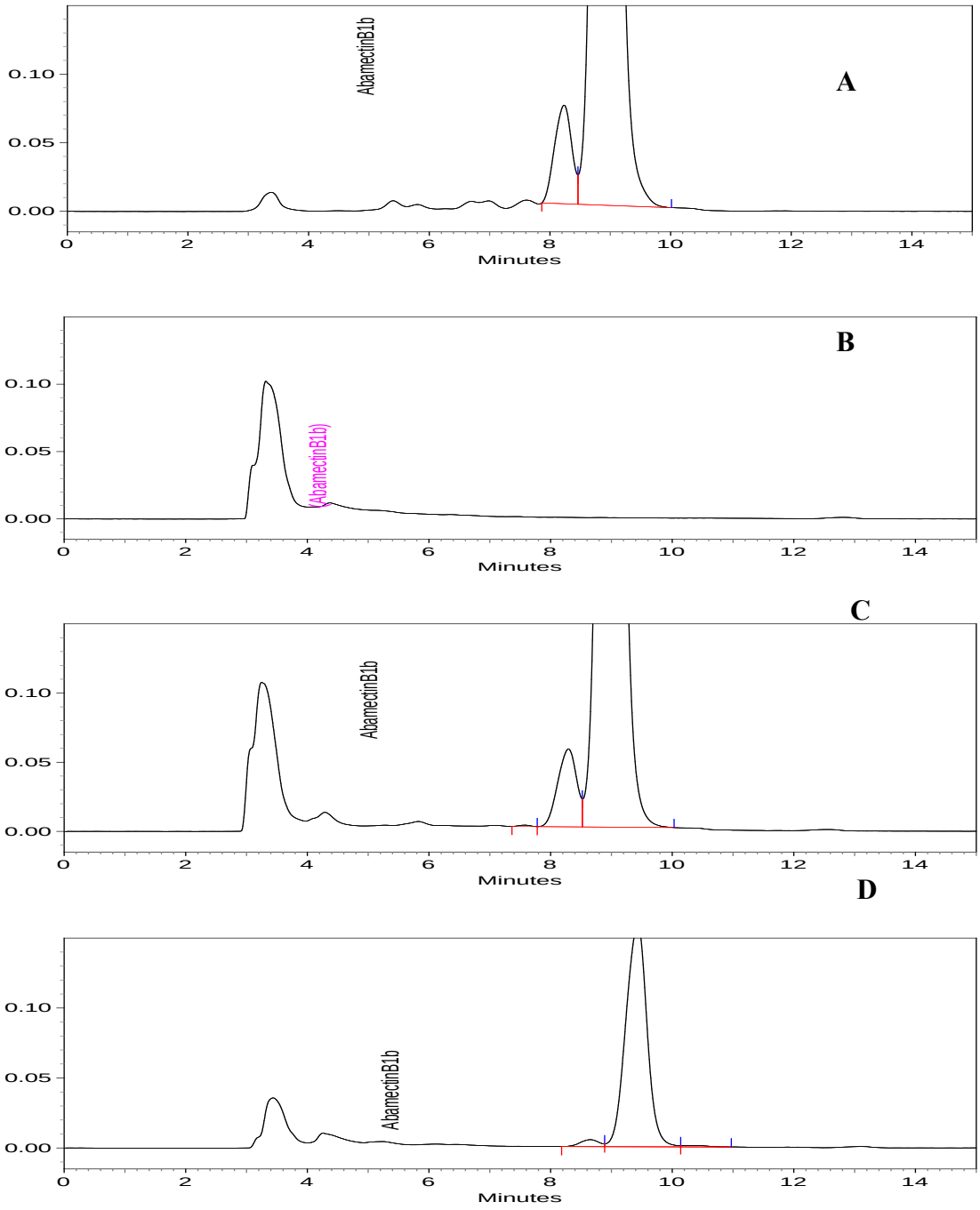


Figure 12. HPLC–FLD chromatograms of (A) standard abamectin B_{1b}, (B) blank perilla leaf, (C) fortified perilla leaf samples and (D) field sample collected.

Table 9. Linearity, limit of detection, limit of quantification and recovery for abamectin in/on perilla leaves

Compounds	Linearity (r^2)	[a]Recovery, (RSD, %) (Spiked conc.)		LOD (mg/ kg)	LOQ (mg/ kg)
		(0.1 mg/kg)	(0.5 mg/kg)		
Abamectin B1a	0.999	88.99 (3.22)	90.49 (4.28)	0.0033	0.01
Abamectin B1b	0.999	93.03 (7.67)	82.11 (3.89)	0.0033	0.01

[a] Mean of three replicates.

4.3. Dissipation of abamectin in perilla leaf

The analytical method was applied to field samples to determine the dissipation pattern of total abamectin in perilla leaves. The half-life ($t_{1/2}$) of the residues was established that provides information about the persistence of pesticides in crops and calculated as ln 0.5/k (Putnam *et al*., 2003). The initial deposit of total abamectin in perilla leaves was 0.97 and 1.01 mg/kg for single and double doses, respectively (**Table 10**). The dissipation regressive equation could be described by the following equations: $C_t = 0.4322e^{-0.688t}$ ($r^2 = 0.8937$) for single doses of application and $C_t = 0.3576e^{-0.538t}$ ($r^2 = 0.8508$) for double doses of application (**Figure 13**). For the recommended single and double dose, the average half-lives ($t_{1/2}$) for total abamectin in perilla leaves was calculated to be 0.7 days (approximately, 17 h). This quick degradation of abamectin in perilla leaves might be attributed to the effect of oxidative and/or photochemical action (Maynard *et al*., 1989).

4.4. Pre-harvest residue limit of abamectin in perilla leaf

PHRL is the recommended residue level at a given time, and is calculated based on the dissipation curve obtained from a supervised field study and half-life time ($t_{1/2}$).

The PHRL for abamectin in perilla leaves was calculated using the following equation:

$$PHRL = MRL \times e^{k_{min}t}$$

MRL denotes the maximum residue limits. In the Republic of Korea, a MRL value for abamectin in perilla leaves has not yet been established. Therefore, a provisional MRL of 0.05 mg/kg was used, following KFDA guidelines (KFDA 2011). The minimum dissipation constant k_{min} was 0.4154, subtracted from the confidence interval (CI) (automatically computed by the SPSS program). The dissipation constant k_{min}, proportionally represents the decline of residues

after pesticide application, and varies according to the type of pesticide used, the samples to which they are applied, and the elapsed time since application. *T* is time before harvest. Therefore, the equation of PHRL for abamectin was represented as $y = 0.05e^{-0.4154t}$

Using the PHRL curve, we can predict the pesticide residue level at harvest time. If the residue level of total abamectin is below 0.92 mg/kg at 7 days or 0.26 mg/kg at 4 days before harvest, then the residue of abamectin should be below MRL at harvest (**Figure 14**).

Table 10. Residues of abamectin in/on perilla leaf

Spray frequency	Day after application	Residue of abamectin B_{1a} (Average ± SD) (mg/kg)	Residue of abamectin B_{1b} (Average ± SD) (mg/kg)	Total residue of Abamectin (mg/kg)
Control	-	ND[a]	ND	ND
1 time spray (plot-A)	0	0.93 ± 0.032	0.04 ± 0.001	0.97 ± 0.031
	1	0.16 ± 0.010	ND	0.16 ± 0.010
	2	0.08 ± 0.001	ND	0.08 ± 0.001
	3	0.03 ± 0.009	ND	0.03 ± 0.009
	4	0.02 ± 0.002	ND	0.02 ± 0.002
	5	0.02 ± 0.0002	ND	0.02 ± 0.0002
	6	0.01 ± 0.001	ND	0.01 ± 0.001
	7	ND	ND	ND
2 time spray (plot-B)	0	0.97 ± 0.049	0.04 ± 0.002	1.01 ± 0.050
	1	0.15 ± 0.006	ND	0.15 ± 0006
	2	0.07 ± 0.005	ND	0.07 ± 0.005
	3	0.04 ± 0.003	ND	0.04 ± 0.003
	4	0.03 ± 0.002	ND	0.03 ± 0.002
	5	0.03 ± 0.002	ND	0.03 ± 0.002
	6	0.02 ± 0.001	ND	0.02 ± 0.001
	7	0.01 ± 0.001	ND	0.01 ± 0.001

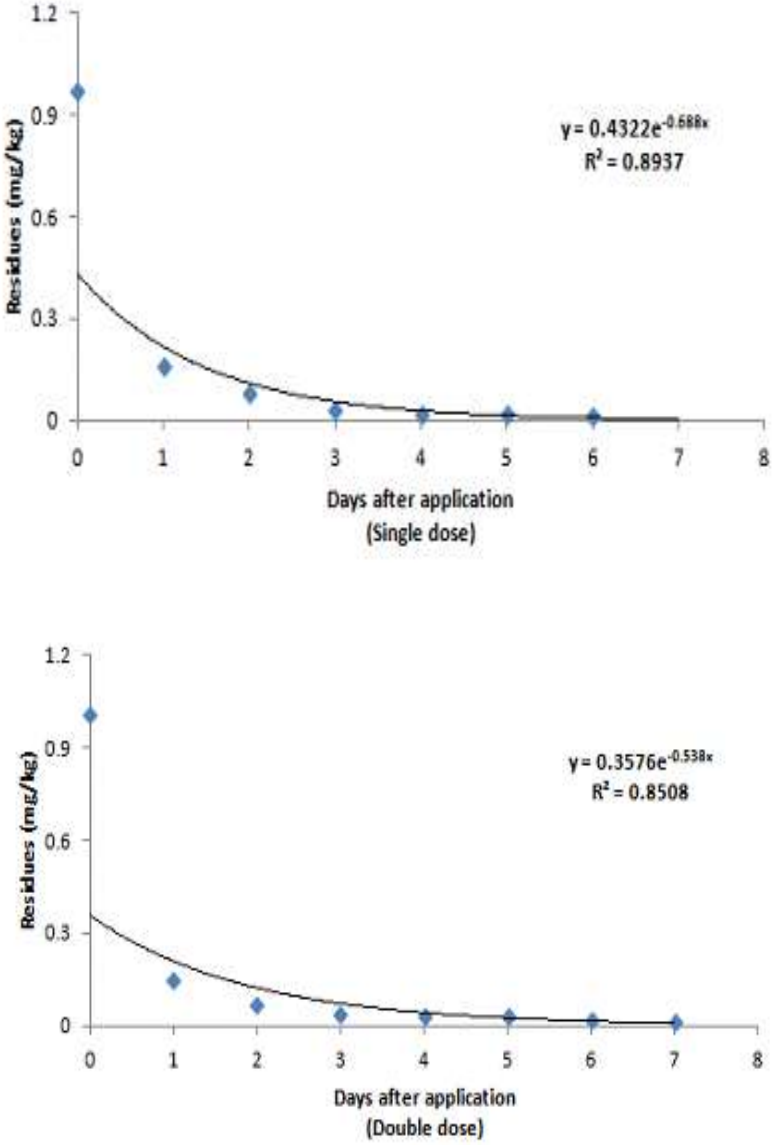


Figure 13. Degradation of total abamectin in/on perilla leaves following single and double applications.

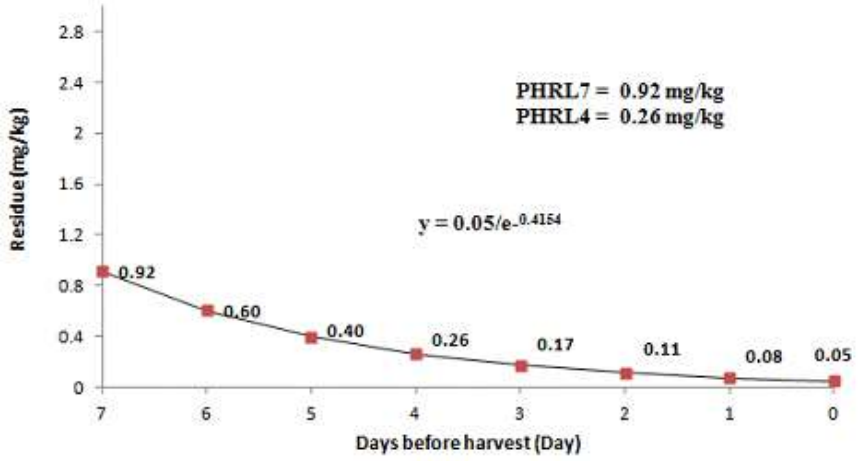


Figure 14. The proposed PHRL curve of total abamectin in/on perilla leaves during cultivation

5. CONCLUSION

The analytical method for abamectin determination in perilla leaves was sufficiently accurate in terms of consistent detection limits with MRL, recovery and repeatability, and could successfully generate data to investigate dissipation patterns of abamectin in perilla leafs under greenhouse conditions. From the dissipation pattern, the pre-harvest residue limit was calculated for proper prediction of residues during the cultivation period.

Chapter V. Residual Analysis of Insecticides, Lambda-Cyhalothrin, Lufenuron, Thiamethoxam, and Clothianidin in/on Pomegranate Using GC-μECD and HPLC-UVD

1. ABSTRACT

In this study, the residual levels of four insecticides namely; lambda-cyhalothrin, lufenuron, thiamethoxam, and clothianidin were monitored in pomegranate to assess their risk to consumers posed by the presence of such residues. The insecticides were applied at the recommended dose rates in/onto pomegranate. The samples were then collected at harvesting time after several treatments (two, three, and four treatments). Following sample preparation and clean-up procedure, lufenuron, thiamethoxam, and clothianidin residues were analyzed via HPLC-UVD, and lambda-cyhalothrin was analyzed via a GC-μECD. The versatility of this method was evidenced by its excellent linearity (>0.999 to 1) at broad concentration ranges. The mean recoveries evaluated from the untreated sample spiked with two different fortification levels were ranged from 73.61 to 108.89%, and the repeatability (relative standard deviation) was ranged from 1.05 to 11.45%. The LOQs were ranged from 0.016 to 0.066 mg/kg, and were much lower than the MRLs established by the KFDA of 0.5 mg/kg.

2. INTRODUCTION

Pomegranate juice provides approximately 16% of an adult's daily vitamin C requirement per 100 mL serving. It is also a good source of vitamin B5, potassium, and antioxidant polyphenols. The marked antioxidant activities of 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 (Al-suhaibani and Ali, 2004).

Pesticides have been employed broadly not only for pest and weed control purposes, but also to promote crop growth, which in turn increased crop productivity. Intensive pesticide use has resulted in contaminations of agricultural products, as well as soil and water. Lambda-cyhalothrin, (*R*,*S*)-α-cyano-3-phenoxybenzyl (1*S*)-*cis*-3-[(*Z*)-2-chloro-3,3,3-trifluoropropenyl]-2,2-dimethylcyclopropanecarboxylate, is a pyrethroid insecticide with a unique chemical configuration consisting principally of a dimethyl cyclopropane carboxylate moiety (Çavaş and Gözükara *et al*., 2003; Bonafos *et al*, 2007; Seenivasan and Muraleedharan, 2009;). 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-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 benzoylphenylurea (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, (Campbell *et al*., 2005; Rancan *et al*., 2006; Pandey *et al*., 2009).

Clothianidin, (*E*)-1-(2-chloro-1,3-thiazol-5-yl-methyl)-3-methyl-2-nitroguanidine, a neonicotinoid insecticide, has been used broadly for the long-term control of a wide variety of insect pests including Hemiptera, Thysanoptera, Coleoptera, Lepidoptera, and Diptera with excellent systemic action, using a variety of application methods (Uneme, 2011). The insecticidal activity of neonicotinoids is caused by their modes of action on nicotinic acetylcholine receptors (nAChRs). Neonicotinoids are active as acetylcholine agonists at the postsynaptic insect nAChRs with much higher affinity, and the toxicity of these compounds plays a major role in pest control (Tomizawa and Casida, 2005, Muccio *et al*., 2006, Rodrigues *et al*., 2010,Van Scoy *et al*., 2010). However, the neurotoxicity of these compounds also affects useful insects such as bees (EFSA, 2010); this non-targeted property may constitute a fatal flaw of the neonicotinoid insecticides.

Pesticide residue analysis is essential to address rising consumer concerns regarding possible contamination issues. Cyhalothrin has been investigated in this regard primarily via HPLC andHPLC-MS (Seccia *et al*., 2008). Thiamethoxamhas been determined using different methods in addition to LC-MS (Campbell *et al*., 2005; Rancan *et al*.,2006; González *et al*., 2008; Pandey *et al*., 2009). 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 (Gamon *et al*., 1998; Khay *et al*., 2008). Lambda-cyhalothrin is mainly detected and analyzed via gas chromatography (Bouldin *et al*., 2006; Seenivasan and Muraleedharan, 2009).

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/on the pomegranate.

3. MATERIALS AND METHODS

3.1. Chemicals reagents

Pesticides including lambda-cyhalothrin, lufenuron, thiamethoxam, and clothianidin (purity>98%) were kindly 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 clean-up was purchased from Sigma-Aldrich (USA).

3.2. Sample preparation

Lambda-cyhalothrin, lufenuron, thiamethoxam, and clothianidin were extracted individually from pomegranate samples. Exactly 20 g of samples were homogenized (WiselMix^{TM}HG-150; Daehan Scientific, Republic of Korea) with 100 mL of methanol: water (50:50, v/v), methanol, or acetone, respectively, at 1200 rpm for 5 min. 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. Republic of 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 clothianidin were filled up to a volume of 200 mL and a 50 mL sample was subjected to partitioning with 100 mL of *n*-hexane. Partitioning was enhanced by salting-out with 50 mL saturated NaCl. The organic layers were then dehydrated through sodium sulfate (anhydrous) and evaporated to dryness in a rotary vacuum evaporator (BüchiRotavapor R0114, Switzerland) at 40°C. The residues for lambda-cyhalothrin and lufenuron were dissolved in 4 mL *n*-hexane and that for thiamethoxam and clothianidin dissolved in 5 mL dichloromethane for clean-up.

Clean-up: For lambda-cyhalothrin and lufenuron, it was conducted using an open preparative chromatographic columns packed with 5 g silica gel and 2 g sodium sulfate anhydrous (Na_2SO_4) placed at the top of 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 eluants were evaporated *in vacuo* at 40 °C 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 cleaned 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 eluants were evaporated *in vacuo* at 30 °C and

re-dissolved in 2 mL methanol: water (50:50, v/v), and then analyzed via HPLC/UVD.

3.3. 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 °C for 5 min and then increased to 270 °C at a rate of 5 °C/min for 5 min. The injection port and detector temperatures were maintained at 250 °C and 280 °C, respectively. The injection volume was 2 μL, and the column was flowed with nitrogen gas at 2.25 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 C_{18} 200Å (4.6×250 mm, 5.0 μm, Phenomenex, USA) was employed as an analytical column for the target compounds. The mobile phase was a mixture of methanol: water (30:70, v/v), and the flow rate was 0.6

mL/min. A 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.

4. RESULTS AND DISCUSSIONS

4.1. Extraction

In this study, pesticides were extracted from pomegranate via liquid-liquid extraction. The whole procedures were simply divided into the following steps: extraction with polar solvent, (liquid-liquid partition), and clean-up with an open preparative chromatographic column packed with a polar sorbent or an SPE cartridge. Few methods have been previously developed for the determination of lufenuron residues in various matrices, including fruits, vegetables, blood, and groundwater. These studies have generally employed liquid chromatography with mass spectrometry, 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 clean-up 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 and Muraleedharan (2009).

4.2. Linearity

Lambda-cyhalothrin, lufenuron, thiamethoxam, and clothianidin evidenced good linearity at 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 (**Table 11**).

Table 11. Calibration curve and linearity of the tested analytes in pomegranate

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

4.3. Recovery

The analytical methods were validated for the blank 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% (**Table 12**). The findings of the current study are satisfactory to those recommended by SANCO, 2004.

4.4. Limit of detection (LOD) and limit of quantitation (LOQ)

According to the ICH Q2B (Guidance for Industry, 1996) methodology guidelines, the limits of detection (LODs) was calculated as follows:

$LOD = 3.3\delta/S$

$LOQ = 10\delta/S$

Where δ is the standard deviation of blank samples analyzed and S is the slope of the standard curve in matrix. The calculated LODs were 0.005 (lambda-cyhalothrin), 0.01 (lufenuron), 0.01 (thiamethoxam), and 0.02 mg/kg (clothianidin) (**Table 12**). All LOQs (0.016, 0.033, 0.033, and 0.066 mg/kg, respectively) were substantially lower than the MRLs of 0.5 mg/kg established by the Korea Food and Drug Administration (KFDA, 2011).

Table 12. Recoveries, relative standard deviation, limit of detection, quantification, and MRLs for validation of the analytical method for the tested compounds in pomegranate

Insecticides	Concentration (mg/kg)	RSD (%)	LOD (mg/kg)	LOQ (mg/kg)	Average recoveries (%)	MRL (mg/kg)
Lambda-cyhalothrin	0.05	5.75	0.005	0.016	82.70	0.5
	0.2	7.09			87.67	
Lufenuron	0.2	6.35	0.01	0.033	95.15	0.5
	0.4	11.45			103.41	
Thiamethoxam	0.1	3.11	0.01	0.033	87.15	0.5
	0.5	1.05			73.61	
Clothianidin	0.2	3.53	0.02	0.066	108.89	0.5
	1	1.77			88.99	

4.5. Stability of the analytes

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 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 in triplicate 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 (**Table 13**). This indicates that all of these compounds are relatively stable in samples under storage conditions during the experimental time period of the study.

Table 13. Storage stability of the tested 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

4.5. 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 (**Table 14**, **Figures 15-17**). Although the insecticides were detected in pomegranate, the detected levels were lower than the MRLs for each compound established by the Korea Food and Drug Administration (2011).

Table 14. Residue levels of the tested analytes in field-incurred pomegranates

Spraying times	Lambda-cyhalothrin (mg/kg)	Lufenuron (mg/kg)	Thiamethoxam[a] (mg/kg)	Clothianidin (mg/kg)
Control	<0.01	<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			0.5mg/kg	

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

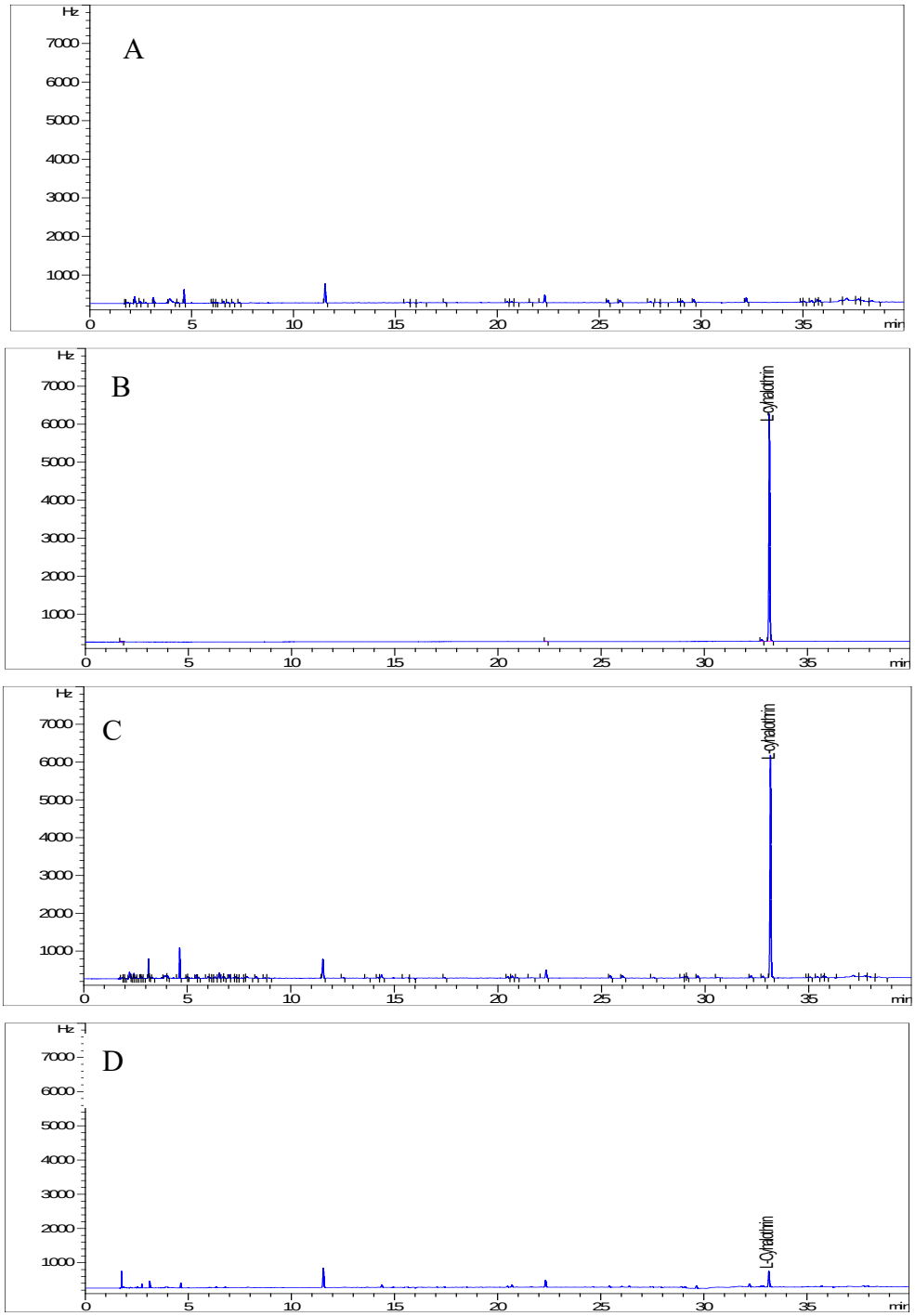


Figure 15. Typical GC-μECD chromatograms of L-cyhalothrin; control pomegranate (A), standard (B), recovery sample (C), and treated sample (D).

A

B

C

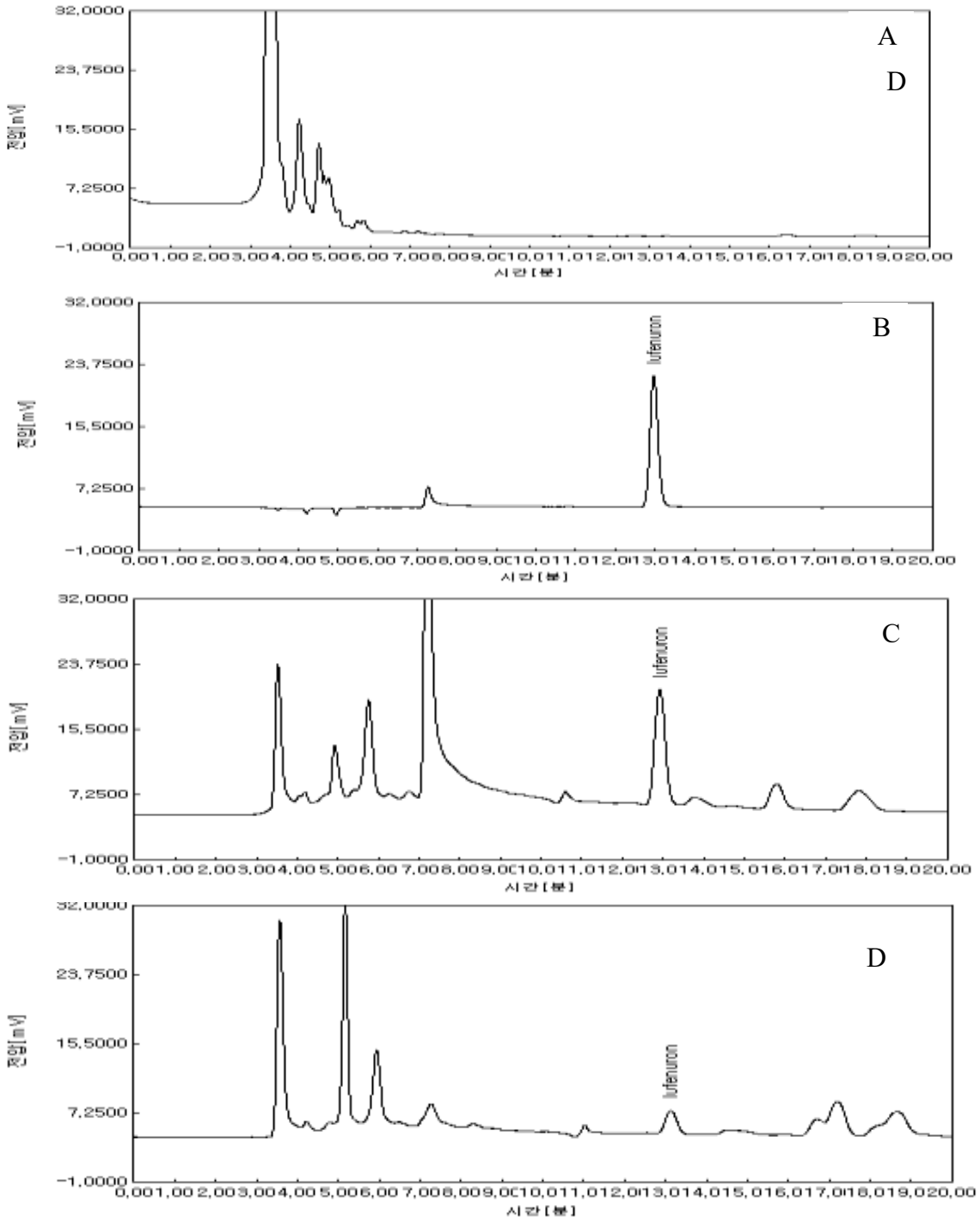


Figure 16. Typical HPLC chromatograms of lufenuron; control pomegranate (A), standard (B), recovery sample (C), and treated sample (D).

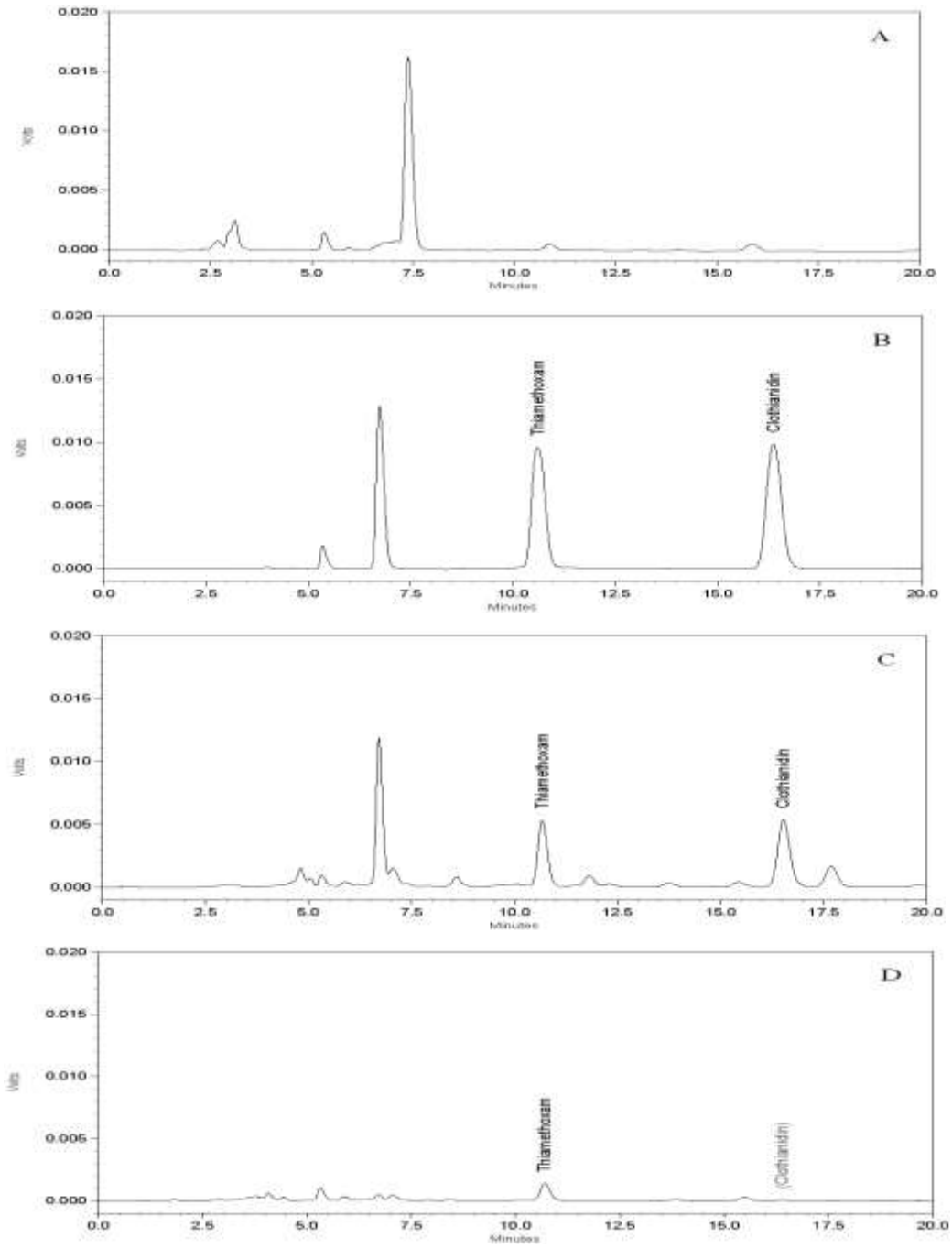


Figure 17. Typical HPLC chromatograms of thiamethoxam and clothianidin; control pomegranate (A), standard thiamethoxam and clothianidin (B), recovery sample thiamethoxam and clothianidin (C), and treated sample (D).

5. CONCLUSION

In the present study, the residual determinations of 4 insecticides were analyzed. Application of two, three, four, and five times of lambda-cyhalothrin, lufenuron, thiamethoxam, and clothianidin in/on pomegranate fruits, gave a safe residue levels for consumers. 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 KFDA.

Chapter VI. General conclusions

In the present study, the dissipation pattern and the residual determination of 11 pesticides were analyzed from 3 kinds of samples using various instruments. .

The pesticide residue levels of both chlorfluazuron and pyridalyl were below the MRLs following 0 day (2 hours) application, while the residue levels of the other 3 pesticides (bitertanol, fludioxonil, and flufenoxuron) were below the MRLs after 4 and 6 days of application. The half-life was ranged from 1.9 days (flufenoxuron) to 8.7 days (pyridalyl).

Application of two, three, four and five times of Lambda-cyhalothrin, lufenuron, thiamethoxam, and clothianidin in/on pomegranate fruits, gave a safe residue levels for consumers. 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.

The residual levels of abamectin substantially decreased to 100%, 7 days post-application, whereas acequinocyl decreased to half concentration at 7 days post-application.

This study showed the need to carry out further monitoring studies in order to improve the food safety since these pesticides represent a potential risk to human health.

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SYNOPSIS IN KOREAN

농업에 있어서 농약사용의 목적은 병해충과 잡초방제를 통하여 농산물의 생산량증대, 품질향상, 노동력 감소 및 수확기 조절 등을 위한 것으로 현대농업의 필수적인 영농자재이다. 그러나, 살충제나 살균제 같은 농약성분이 함유된 물질들에는 내분비계교란추정물질에 해당되는 성분이 포함되어 있고, 이러한 독성물질들은 주요한 신체장기에 영향을 미칠 가능성이 크다. 그러므로, 작물에 사용되는 농약들의 잔류량에 대한 모니터링이 필요하고, 작물 잔류성 평가시험을 통한 안전사용기준을 설정하는 연구가 필요하다. 본 논문의 목적은 식품안전을 위한 분석물질의 잔류한계를 설정하고 나아가 최종적으로 작물에 잔류하는 잔류수준을 파악하는 데에 있다. 또한 농약의 안전사용기준의 설정을 위해 농약의 잔류와 감소경향을 조사하였다. 이와 같은 목적에 따른 실험을 수행하기 위하여 대상으로 하는 작물에 대하여 기준량과 배량에 해당하는 농약을 처리하여 실험을 진행하였다.

농약의 잔류량을 파악하기 위해서는 각각의 작물에 대한 대상 농약의 분석방법의 확립이 선행되어야 하며, 본 논문에서는 고추, 들깻잎, 석류를 대상으로 하여 고추 중 bitertanol, chlorfluazuron, fludioxonil, flufenoxuron, pyridalyl, 들깻잎 중 acequinocyl과 abamectin, 석류 중 lambda-cyhalothrin, lufenuron, thiamethoxam, chlothianidin 등 11종의 농

약에 대한 분석법을 확립하였다. 분석법은 액-액추출법을 개선하여 추출을 위해 사용하였고, 추출된 시료는 칼럼 정제를 통해 정제한 뒤 최종적으로 GC-μECD, GC-NPD, HPLC-UVD/FLD, HPLC-PAD를 이용하여 농약의 잔류량을 분석하였다. 검증된 분석법의 직선성은 모두 r^2>0.95으로 이는 Codex 기준에 적합하였다. 또한 회수율 실험을 위하여 두 가지의 다른 농도를 처리하여 실험을 수행한 결과, 모든 실험의 회수율은 73.6%에서 126.8%으로 나타났고, 상대표준편차(RSD) 값은 12%를 넘지 않았다. 분석법의 정량한계 (LOQ) 또한 각 시료의 지정된 최대잔류허용량(MRL) 보다 아주 낮은 수준으로 설정되었으며, 위 시료에 사용된 모든 분석법은 간편하고, 정확하고, 정밀하고 신뢰성 있는 분석법 이었다.

작물에 기준량과 배량으로 처리된 농약의 감소경향은 일차반응속도론에 적용할 수 있었고, 이를 이용하여 각각의 농약에 대한 반감기를 구하였다. 고추에 처리된 농약의 반감기는 기준량 처리에서 1.9-7.6일이었고, 배량 처리에서 1.9-8.7일 이었다. 들깻잎에 처리된 acequinocyl의 경우 기준량과 배량 처리에서 각각 2.8일, 3.1일의 반감기를 보였다. 들깻잎에 처리된 abamectin의 생산단계 농산물의 잔류허용기준 (PHRL)을 확인한 결과, 수확 7일전 0.92 mg/kg, 수확 4일전 0.26 mg/kg으로 확인되었다. 석류에 처리한 4종의 잔류량을 확인한 결과 국내에 설정된 최대잔류허용기준보다 낮은 수준으로 잔류함을 확

인할 수 있었다.

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EDUCATIONAL PROFILES

1. EDUCATION

03.2009-08.2013: Ph.D study in Natural Products Chemistry Laboratory, Department of Agricultural Chemistry, College of agriculture and life science. Chonnam National University. South Korea.

Dissertation: “Dissipation pattern of some pesticides in/on specific crops: Contribution to safety guideline”, supervised by Prof. Jae-Han Shim.

03.2007-03.2009: MS.c study in Natural Products Chemistry Laboratory, Department of Agricultural Chemistry, College of agriculture and life science. Chonnam National University. South Korea.

Dissertation: “Determination of acrinathrin, denotefuran, fenhexamid, methoxyfenozide and tetradifon in pepper fruit”, supervised by Prof. Jae-Han Shim.

2. PUBLICATIONS AND POSTERS PRESENTATION

2.1. Publiscations

Sreiny Taing, **Lina Hem**, Sathya Khay and Jae-Han Shim.

"Residues analysis of deltamethrin by gas chromatography equipped with electron capture detector". Agriculture science and technology research vol. 42 (2007).

Sreiny Taing, **Lina Hem**, Sathya Khay and Jae-Han Shim.

"Study on dissipation pattern of pyridaben in pepper grown under greenhouse condition". Agriculture science and technology research vol. 42 (2007).

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"Determination of Cyhalofop-butyl and its Metabolite in Water and Soil by Liquid Chromatography". Original article/Residue and safety (2008).

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"Residual pattern of fenhexamid on pepper fruit grown under greenhouse conditions using HPLC and confirmation via tandem mass spectrometry". Journal of Food Chemistry (2011).

2.2. POSTERS

A. Abroad

1- Hem Lina, Sathya Khay, Sreiny Taing, Eun-Ho Shin, A. M. Abd El-Aty, Jin Kyoung Kim, Moo-Ki Hong and Jae-Han Shim (June 1-5, 2008): "Development of an extraction procedure for the analysis of trichlorfon in cow fresh milk using GC/μ-ECD". The 4th Pan Pacific Conference on Pesticide Science, Hawaii, USA.

2- Sathya Khay, Sreiny Taing, **Lina Hem**, Eun-Ho Shin, A. M. Abd El-Aty, Young-Seok Park, Ju Yoon Jeong and Jae-Han Shim (June 1-5, 2008): "Development and validation of an analytical method for the determination of deltamethrin in chicken muscles and eggs using GC/μ-ECD". The 4th Pan Pacific Conference on Pesticide Science, Hawaii, USA.

3- Sathya Khay, **Lina Hem**, Sreiny Taing, Jeong-Heui Choi, A. M. Abd El-Aty, Hye-Jin Park and Jae-Han Shim (June 1-5, 2008): "Method establishment and simultaneous multi-residue determination of pyrethroid insecticides in pig muscle and cow fresh milk using GC/μ-ECD". The 4th Pan Pacific Conference on Pesticide Science, Hawaii, USA.

B. Domestic

1- **Lina Hem**, Sathya Khay, Sreiny Taing, Jeong-Heui Choi, Hyeon-Ju Cho and Jae-Han Shim (July 3-4, 2008): "Dissipation pattern of fenitrothion, folpet, myclobutanil and tolyfluanid pesticide residue in pepper fruit grown under greenhouse condition". The Korean Society of Agriculture and Environment, Muju, South Korea. (Oral presentation)

2- Sreiny Taing, **Lina Hem**, Sathya Khay, Jeong-Heui Choi, Soon-Kil Cho and Jae-Han Shim (July 3-4, 2008): "Study on the dissipation pattern of pyridaben residues in pepper fruit

grown under greenhouse". The Korean Society of Agriculture and Environment, Muju, South Korea.

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5- Sathya Khay**, Lina Hem**, Sreiny Taing, Eun-Ho Shin, Jeong-Heui Choi, A. M. Abd El-Aty, Moo-Ki Hong and Jae-Han Shim (July 3-4, 2008): "Development of gas chromatographic analytical method for trichlorfon residue in cow milk using electron capture detector". The Korean Society of Agriculture and Environment, Muju, South Korea.

6- Sathya Khay**,** A. M. Abd El-Aty, **Lina Hem**, Soon-Kil Cho, Jeong-Heui Choi, and Jae-Han Shim (October 11-13, 2007): "Imidachoprid decline curves and residue levels after treatment with emulsifiable concentrate and wettable powder formulations to Chinese cabbage via analysis with liquid chromatography without a clean-up procedure". International symposium and meeting of the KSABC on plant stress and metabolism, Gyeongju, South Korea.

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