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Monitoring of Carbamate Insecticide Residues in Cucumbers Produced from Conventional and Greenhouse Farming Systems

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Abstract

The study was designed to investigate carbamate insecticide residues in cucumbers and soil from two different farming systems located in Phitsanulok, Thailand.Ten carbamate insecticides were analyzed using the quick easy cheap effective rugged and safe (QuEChERS) multi-residue extraction, followed by high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (HPLC-QTOF-MS). The results showed the level of insecticide residues detected in cucumbers and soil from greenhouse farming, were at a lower level than those of conventional farming. Conventionally-farmed cucumbers contain methiocarb, methomyl, aldicarb sulfoxide, carbofuran and carbofuran-3-hydroxy ranging from 0.437 to 1.721 mg/kg, which exceeds Maximum Residue Levels (MRLs), whereas greenhouse-farmed cucumbers contain less insecticide residue which ranges from 0.132 to 0.439 mg/kg. The difference in the distribution patterns of insecticides in cucumbers and soil was detected from the study samples collected at a conventional farm. The level of methiocarb contained in cucumbers and the level of methomyl contained in soil from conventional farming were significantly higher than that of insecticide levels in greenhouse farming by approximately 13-fold ($p < 0.05$) and 3-fold ($p < 0.05$), receptively. Our results suggested that residue of CIs contained in cucumbers and soil is affected by the two different farming systems. The reduction of insecticide use needs to be enhanced within agriculture, and the routine monitoring of them is necessary to increase public awareness of food safety and the reduction of health risks associated with contaminated foods and the environment.

Keywords: Carbamate; Insecticide; Cucumber; Greenhouse farming; Conventional farming

1. Introduction

Carbamate Insecticides (CIs) are one of the most accessible insecticides in worldwide, particularly in the agricultural sector due to their broad spectrum of biocide activity (Dhouib *et al*., 2016; Wei *et al*., 2018). The use of CIs in Thailand have been extensively reported throughout the past decade, due to the increasing incidences of insecticide poisoning. It has been documented that from 2012-2016, Thailand imported a great amount of active insecticide ingredients that averaged 4216.67 tons/year, with a market value of 89.56 million USD/year (Sapbamrer, 2018). While the amount of imported insecticides and costs are rapidly rising, there has also been an increase in the reported cases of insecticide poisoning among farmers and the population (Panuwet *et al*., 2012).The uses of insecticides are not only a threat to public health, but also a global ecological threat. A previous report showed that the use of insecticide may affect pollinator abundance and the richness of diversity, which will subsequently affect agriculture (Evans *et al*., 2018). Decreasing the number and variety of beneficial insects such as bees, stingless bees and wild pollinators, may directly cause a reduction in agricultural production creating economic concerns at both local and international levels.

The CIs are N-methyl carbamates derived from a carbamic acid which is also known as a cholinesterase inhibitor; a cholinesterase is one of the most important enzymes which is involved in the hydrolysis of the neurotransmitter acetylcholine that maintains a proper function of the nervous system (Colovic *et al*., 2013). The inhibition process exerted through carbamylation of the serine hydroxyl group in the active site of cholinesterase, results in the degradation of an inactive and/or blocked neurotransmission in the central and peripheral nervous systems (Jardim *et al*., 2018). The rapid onset of CIs toxicity can manifest in both insects and humans.

Normally, humans can be exposed to CIs through the contamination of food, soil, particles of CIs in the air and surface water (Dhouib *et al*., 2016; Wanwimolruk *et al*., 2016; Kanthawongwan *et al*., 2019). There is growing evidence that mammals exposed to CIs, especially through oral exposure, can suffer various pathophysiological aberrations via the generation of oxidative stress. A recent study of rats showed that chronic exposure to CIs affected their cellular enzymes and non-enzyme-mediated antioxidant defense systems, which promoted immunotoxicity (Dhouib *et al*., 2014). Several studies clearly demonstrated effects of CIs-induced oxidative stress in a dose-dependent manner (Jaiswal *et al*., 2014; Rai and Sharma, 2007) which may contribute to tumorigenesis (Dhouib *et al*., 2016). Previously studies in humans showed that cumulative exposures to CIs varied among populations but is not considered of health concern (EPA, 2007; Jardim *et al*., 2018) however; there are other factors that should be taken into consideration, such as other insecticides that share a similarity in their ability to inhibit the cholinesterase inhibitor, and the different exposure scenarios such as time of initial exposure, duration of exposure, dose, and the susceptibility among the population.

Cucumbers (*Cucumis sativus L*.) are one of the most cultivated plants in Thailand. It has been reported that cucumbers are one of the economically important vegetables that are very popular among consumers. They can be grown in various farming systems, mainly in open fields and greenhouses. The important aim of growing cucumbers in a greenhouse is to reduce the use of insecticides, because of reduced pest infestations. In contrast, in conventional farming, most cucumbers are grown in open fields, which face the risk of pest infestation. It has been reported that during cucumber cultivation, insect infestation seriously affects the yield and quality of production, therefore, the application of insecticides are commonly applied in ordinary farming systems. Due to the broad application of insecticides internationally in agricultural farms, contamination of their residues are commonly reported as well as the reduced cucumber production (Mansour *et al*., 2009). There is little known information on the incidence of CIs contamination of cucumbers from various farming systems. Therefore, this study aimed to determine the carbamate residue contents in cucumbers grown in conventional and greenhouse farming using QuEChERS multi-residue extraction, followed by high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (HPLC-QTOF-MS). The provided data on CIs in cucumbers and the soil designated according to the systems of their production, could be used as surrogate data for food safety, as well as assist in the understanding of the environmental burden of insecticide exposure in Thailand.

2. Materials and Methods

2.1 Study area and sample collection

This experiment was conducted in two cucumber farms from different locations in Phitsanulok province, Thailand. The study sites were located in the lower Northern area of Thailand, which is one of the most important and largest producer of agricultural crops. The average annual temperature was 27.8 °C, and the average annual rainfall was 1,317.0 millimeter (Department of Meteorology of Thailand, 2017). The inside temperature of the greenhouse farming system was about 34°C during the day and about 25°C at night, with a 79% relative humidity. The experimental design was split-plot with three replicates for conventional and greenhouse farming systems. When planting the cucumbers, we used commercial mixed-soil which consisted of manure, rice husk and coconut husk. After planting, we sprayed both farming systems with CIs, to protect the seedling from ants and other ground-dwelling pests. Seven days after planting, carbosulfan and cypermethrin were applied in both farming systems. Afterwards, in the conventional farming system, cypermethrin and imidacloprid were applied to control the insects by 3 weekly applications, until they were 28 days old. After flowering, insecticide applications ceased 7 days before the cucumbers were harvested for analysis at 35 days old, while in the greenhouse farming system, insecticide applications were discontinued 7 days after planting. During the flower blooming period, two hives of stingless bees (silver hair stingless bee; *Tetragonula pagdeni*) were employed as pollinators, therefore, insecticide applications were limited in the greenhouse farming system. For the analysis of the insecticide residue, the cucumbers were collected randomly from each growing system by harvesting them at their market ready stage. The soil samples were collected from a depth of 25-50 cm without topsoil, according to a previous study (Joko *et al*., 2017), then the cucumbers and samples from each system were collected in triplicate, transported to the laboratory and stored at 4°C until analysis.

2.2 Analysis of insecticide residues

2.2.1 Materials

The triphenyl phosphate (TPP) solution which is used as the internal standard, and the standard mixture of CIs (M-531M) are alicarb sulfoxide, aldicarb sulfone, oxamyl, methomyl, carbofuran-3-hydroxy, aldicarb, propoxur, carbofuran, carbaryl and methiocarb were all purchased from AccuStandard® (New Haven, CT, USA), and the standard stock solution was stored at 0 to 5°C.The calibration standards and working standards were prepared by dilution with water: acetronitile (10:90) on the day of analysis.

Acetronitrile, methanol and water were of LC-MS grades and obtained from RCI labscan LTD, Thailand, while the analytical grade of formic acid was obtained from Merck, Germany. The QuEChERS extraction and dispersive kits were purchased from Agilent Technologies (Santa Clara, CA, USA).

2.2.2 Extraction and clean-up

The extraction and cleanup of insecticides were conducted according to QuEChERS method as previously described (Arienzo *et al*., 2013; Leili *et al*., 2016). Ten gram of homogenized cucumber was mixed with 10 mL of acetonitrile in a 50 mL centrifuge tube, and 5.0 g of the soil sample (sieved by 2 mm mesh) was mixed with 5 mL of water and 10 mL of acetonitrile in a 50 mL centrifuge tube. Then the internal standard solution (TPP at 100 μg/mL, 10 μL) was added into all samples, followed by 4 g of anhydrous magnesium sulfate, 1 g of sodium chloride, 1 g of sodium citrate and 0.5 g of disodium citrate sesquihydrate being added, then the mixture was shaken and centrifuged for phase separation. The organic phase and the proteins were cleaned-up using dispersive solid phase extraction (D-SPE) through displacement of the supernatant into another tube, comprising of 150 mg of primary secondary amine (PSA), and 900 mg of magnesium sulfate. After shaking and centrifugation, the extracted samples were transferred into other tubes and evaporated until dry. The samples were reconstituted by 1 mL of 10% acetonitrile, then filtered through a 0.45 μm syringe filter prior to the insecticide residues being determined via HPLC-QTOF-MS.

2.2.3 Apparatus and insecticide residues analysis

The determination of the insecticide residues was performed using an Agilent series 1260 Infinity HPLC instrument (Agilent, Waldbronn, Germany) coupled to an Agilent 6540 QTOF mass spectrometer (Agilent Technologies, Singapore) equipped with an electrospray ionization (ESI) interface. Details of the HPLC-QTOF-MS determination and apparatus were modified according to previously described methods (Ferrer *et al.*, 2005; Ngamdokmai, *et al.*, 2017). Briefly, 10 μL of extracted samples were subjected to analysis with HPLC-QTOF-MS, using Luna C18 (2) column (4.6 mm x 150 mm, 100Å 5 μm, Phenomenex Torrance, CA, USA). The mobile phase comprised of 0.1% formic acid in water and methanol, the gradient elution was: 0 min, 95:5, (A: B v/v); 5 min, 10:90, 15 min, 10:90. The MS operating parameters were as follows: gas temperature 350°C, drying gas 10 L/min, nebulizer 30 psig, capillary 3500 V, fragmentor 100 V, skimmer 65 V and OCT 1RF Vpp 750 V. All acquisitions and analysis data were controlled by MassHunter software (Agilent Technologies, USA). The samples were analyzed in positive ESI mode. The calibration curve was prepared from the stock solution of CIs standard mixture at 5 different concentration levels from 0.05-2 mg/mL, were prepared by diluting them with acetonitrile/water (10/90) which presented correlation coefficients of ≥ 0.93.

2.2.4 Quality assurance and quality control

For quality control, recovery studies for method validation were performed by adding appropriate volumes of working solutions of CIs standard mixture to blank cucumber and soil samples at concentrations of 0.10 mg/L. The obtained recoveries of the 10 insecticides are given in Table 1, and the limits of detection were ≤ 0.0075 mg/kg for them. The Maximum Residue Levels (MRLs) for each insecticide in cucumbers were cited from the recommended MRLs that were established by the Thailand National Bureau of Agriculture Commodity and the EU food standards (Codex International Food Standards, 2015; Thailand National Bureau of Agriculture Commodity and Food, 2014).

Table 1. Recovery and standard deviation of the recovery of the 10 CIs in cucumbers and soil

Compounds	Spiked level (mg/kg)	Recovery (%) (n = 3)		%CV (n = 3)	
		Cucumbers	Soil	Cucumbers	Soil
aldicarb sulfoxide	0.10	114.86	105.00	8.44	0.91
aldicarb sulfone	0.10	100.78	98.35	1.56	1.03
oxamyl	0.10	93.22	104.20	8.30	7.84
methomyl	0.10	112.87	91.81	7.93	0.89
carbofuran-3-hydroxy	0.10	105.54	88.18	0.47	2.21
aldicarb	0.10	83.86	116.44	5.69	4.51
propoxur	0.10	85.15	111.68	2.29	3.92
carbofuran	0.10	86.88	89.19	4.33	3.73
carbaryl	0.10	86.23	88.10	1.74	3.58
methiocarb	0.10	112.38	91.81	2.74	3.51

n: represents the number of replications in the experiment

2.3 Statistical analysis

Data analysis was carried out using statistical software. The differences of insecticide residues in cucumbers and soil samples from different farming systems, were determined by using an independent sample t-test, and a p-value less than 0.05 was considered a statistical significance.

3. Results and Discussion

Ten insecticides were determined from cucumbers grown in our greenhouse and conventional farms and the soil. These insecticides were designated among those widely used in the cucumber growing process in Thailand, including in Phitsanulok province. We found that both cucumber and soil samples from our conventional farm, contained a number of insecticide residues higher than those found in our greenhouse. Of the 10 types of insecticides analyzed, only 6 were found in both of our farms. In the conventional farm, there were 5 types of insecticide residue detected in the cucumbers (methiocarb, methomyl, aldicarb sulfoxide, carbofuran and carbofuran-3-hydroxy), and 4 (methiocarb, methomyl, propoxur and carbofuran-3-hydroxy) in the soil samples. The samples from our greenhouse contained less insecticide residues in both cucumbers and soil (methiocarb, methomyl and carbofuran-3-hydroxy). The only residue difference between both farms were propoxur, aldicarb sulfoxide and carbofuran. The comparison between both cucumber samples, showed that methiocarb was significantly higher ($p < 0.05$) in the conventional farm, while significantly higher ($p < 0.05$) of methomyl were detected in the soil samples from our conventional farm, compared to the greenhouse samples. In conventional farming, growers apply several agricultural chemicals, which is a common practice of Thai farmers, in order to promote yields and protect them from pests. CIs are well-known insecticides which are claimed to have a short half-life in the environment, while the soil conditions and the growing systems, may have an effect on the degradation rate of them. Accumulated evidence has shown that different environmental conditions have increased the longevity of CIs; in aerobic soil carbaryl quickly degraded (half-life in 4 days) whereas in anaerobic soil its degradation was slower (half-life in 72 days) (Tiwari *et al*., 2019).

Our results demonstrated that the most commonly detected insecticides of the carbamate group in cucumbers and soil samples were, methomyl, methiocarb and carbofuran-3-hydroxy, which were detected in both conventional and greenhouse farming, however, the levels detected in both in the greenhouse, tended to be at a lower level (Table 2.).

Table 2. Detected insecticide residues (mg/kg: fresh weight) in cucumbers from our greenhouse and conventional farms.

Insecticides	Conventional farming (mg/kg) (n = 3)		Greenhouse farming (mg/kg) (n = 3)	
	Cucumbers	Soil	Cucumbers	Soil
methiocarb	$1.721 \pm 0.004^{*}$	1.746 ± 0.002	0.132 ± 0.080	1.540 ± 0.030
methomyl	0.437 ± 0.059^{a}	$1.499 \pm 0.074^{*}$	0.377 ± 0.009	0.480 ± 0.170
propoxur	nd	0.123 ± 0.007	nd	nd
aldicarb sulfoxide	0.354 ± 0.107	nd	nd	nd
carbofuran	0.118 ± 0.057	nd	nd	nd
carbofuran-3-hydroxy	0.533 ± 0.001	1.071 ± 0.070	0.439 ± 0.004	1.046 ± 0.190

[a]The values are expressed as mean ± SD.
nd: non-detectable
* represents significant difference from greenhouse farming, $p < 0.05$
n: represents number of samples in each group

Our results discovered that some of the CIs detected in cucumbers grown in conventional and greenhouse farms, exceeded the MRLs (Table 3). Conventionally-farmed cucumbers contained methiocarb, methomyl, aldicarb sulfoxide, carbofuran and carbofuran-3-hydroxy ranging from 0.118 to 1.721 mg/kg which are higher than the MRLs value from both the Thai Agricultural standards and the EU regulations, except for the carbofuran level (0.118 ± 0.057 mg/kg) which was lower than the recommended Thai Agricultural standards of 0.5 mg/kg (Codex International Food Standards, 2015; Thailand National Bureau of Agriculture Commodity and Food, 2014). In the field study, some of the CIs could potentially transfer to other metabolites due to the oxidation and hydrolysis processes; carbosulfan is first metabolized to carbofuran, then to carbofuran-3-hydroxy and subsequently to 3-ketocarbofuran (Tomlin, 1995).This is in line with the previous report from China on residue behavior of carbosulfan in cucumbers. The report claimed that even though the insecticide was applied at the recommended dosage, the residue of carbofuran varied from 0.008 to 0.123 mg/kg which exceeded the MRLs (Song *et al*., 2018). It is worth noting that greenhouse-farmed cucumbers contained lesser amounts of insecticide residue which ranged from 0.132 to 0.439 mg/kg, and none of them exceeded the MRLs of Thai Agricultural standard. However, methomyl and carbofuran contained in greenhouse-farmed cucumbers exceeded the MRLs of EU standard. Variations of insecticide residue in cucumbers grown in different farming systems may depend on many factors, such as the prevalence of pests and diseases, the time of harvest, and the time and quantity of insecticide applications (Leili *et al*., 2016: Mansour *et al*., 2009).

The level of methiocarb contained in cucumbers from conventional farming, was significantly higher than that found in the greenhouse ones, by approximately 13-fold ($p < 0.05$). Methiocarb is a commonly preferred insecticide for plant cultivation instead of organochlorines, due to their lesser extent in the environment. In this study, the level of methomyl contained in both the cucumbers and the soil on our conventional farm was significantly higher than that in the greenhouse, by approximately 3 fold

Table 3. The comparison of insecticide residues and MRLs (mg/kg) in cucumbers, from our greenhouse and conventional farms

Insecticides	MRL value (mg/kg)		Present study (mg/kg)	
	Thailand	EU	Conventional farming (n = 3)	Greenhouse farming (n = 3)
methiocarb[1]	-	0.200	1.721 ± 0.004	0.132 ± 0.080
methomyl	0.100	0.010	0.437 ± 0.059	0.377 ± 0.009
aldicarb[2]	-	0.200	0.354 ± 0.107 (aldicarb sulfoxide)	nd
carbofuran[3]	0.500	0.002	0.118 ± 0.057 (carbofuran)	nd (carbofuran)
			0.533 ± 0.001 (Carbofuran-3-hydroxy)	0.439 ± 0.004 (Carbofuran-3-hydroxy)

Note. MRL values for each insecticide in cucumbers (*Cucumis sativus L.*) were cited from recommended MRLs established by the European commission, 2015 and the Thailand National Bureau of Agriculture Commodity and Food, 2014.

[1] represents the sum of methiocarb and methiocarb sulfoxide and sulfone, expressed as methiocarb in the EU regulations for MRLs.

[2] represents the sum of aldicarb sulfoxide and sulfone, which are expressed as aldicarb for the EU regulations for MRLs.

[3]represents the sum of carbofuran (including any carbofuran generated from carbosulfan, benfuracarb or furathiocarb) and 3-OH carbofuran, are expressed as carbofuran in the EU regulations for MRLs.

($p < 0.05$). Moreover, in our conventional farm more CIs were detected, such as aldicarb sulfoxide in the cucumbers and propoxur in the soil. In the environment, aldicarb gradually oxidized into aldicarb sulfoxide which is a more stable metabolite. (Paliwal, 1994).Propoxur toxicity has been linked to the induction of oxidative stress in mammals (Tsitsimpikou *et al*., 2013). Propoxur is one of the CIs, which has a long residue effect, and a previous study showed that it was highly persistent, with a half-life of almost 3 months in slit foam soil. The biodegradation of propoxur is dependent on many environmental factors, such as aerobic conditions and soil properties (National Center for Biotechnology Information, 2020). Therefore, as a result of the persistence of this chemical, it increases its ability to transfer from spraying the fruits to contaminating the soil, which is classified as off-target contamination. In this study, the difference in the distribution pattern of propoxur insecticide in the soil was detected from the samples collected in our conventional farm. Our results may vary according to soil and climate conditions, growing seasons and related agricultural practices, whereas in the greenhouse, the soil conditions remained stable. Carbofuran was detected in cucumbers from the conventional farm but not in greenhouse farm, whereas carbofuran-3-hydroxy was detected in both farms, which may be due to the properties of carbosulfan in the environment that easily transform into carbofuran and 3-hydroxy carbofuran as previously described (Soler *et al*., 2006; Tomlin, 1995). A previous study on carbosulfan and its metabolites detected in cucumbers in China, showed that the level of carbofuran, which had a half-life of approximately 45 days, varied after being applied (Song *et al*., 2018). Trevisan *et al* (2004) stated that repeat spraying of carbosulfan consequently converted to carbofuran, which remained in the bagasse of oranges for at least 28 days after application. Therefore, the application of insecticides should be stopped 7 days before harvesting (Song *et al*., 2018). One of the toxic compounds of carbofuran is an endocrine disruptor, which has the potential to change the expression of genes that leads to hormonal distribution in an organism (Ibrahim and Harabawy, 2014). The contamination of soil by these insecticides may affect not only cucumber absorption of them, and also affect the levels cycling in the environment which can cause bioaccumulation in the food chain that in turn affects beneficial species.

In the soil samples from the greenhouse, even though no insecticides had been applied during the study period, the levels of methiocarb and carbofuran-3-hydroxy residues were very high at 1.540 mg/kg and 1.046 mg/kg, respectively, compared to the residue found in the cucumbers. When we tested the soil samples from the conventional farm, they were also found to be similar to those of the greenhouse ones. The result from the greenhouse farm was ambiguous, because we had discontinued the applications of insecticides 7 days after planting. However, the high levels of insecticides found, may be due to the commercial planting materials we purchased which contained reused-soil, which might have been previously contaminated with insecticides. Methiocarb has a short half-life, which is one of the favorite insecticides widely used in Thailand, therefore, its residues may commonly persist in agricultural farms, planting materials and soil. The residue of carbofuran-3-hydroxy found, indicated that these planting materials might have been contaminated by carbofuran from previous applications, therefore may not be safe from contamination. Our results proposed that residues of CIs contained in cucumbers and the soil had a direct effect on farming and the environment. Greenhouse farming may be an option for farmers to reduce the application of insecticides, which will decrease the residues in both the products and the environment. Insecticide usage needs to be reduced within the agricultural system, and routine monitoring is necessary to increase public awareness of food safety and health risks associated with contaminated foods in the environment.

4. Conclusion

Both of our farming systems were affected by the residue of CIs found in the cucumbers and soil. Ten CIs were determined, but only 6 were detected in the cucumber and the soil samples. Growing cucumbers in the conventional farm resulted in a higher number and level of insecticide residues than those found in the greenhouse. Therefore, our results revealed a higher level of residues from CIs in the soil samples than the cucumbers, both in conventional and greenhouse farming, which may be due to the spraying methods adopted. The majority of the residues detected in our study, exceeded the MRLs of both the Thai Agricultural standard and the EU regulations. Therefore, we should be concerned that the application of insecticides on cucumbers may result in residue contamination of the crops, soil, and the environment.

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