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Comparison of the Variability in the Levels of Pesticide Residue Observed in Japanese Cabbage and Grape Units

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Supporting Information

ABSTRACT: To estimate variations in pesticide residue levels in crops, the variability factors (VFs, the 97.5th percentile of the residue levels in the sample divided by the average residue levels in the lot) in residue levels of acetamiprid and cypermethrin applied to cabbage and grapes were investigated, respectively. The VFs in the residue levels of both pesticides in cabbage (2.00 and 2.39, respectively) were clearly higher than those in grapes (1.82 and 1.63, respectively). Although the residue levels of both pesticides in grapes showed a normal distribution, those values in cabbage were slightly skewed at lower residue levels. Individual residue levels in grapes had a good agreement between acetamiprid and cypermethrin. In contrast, the distribution of cypermethrin residue levels in cabbage was slightly skewed at higher residue levels as compared to that of acetamiprid. These results indicate that the difference in the relative distribution of the two pesticides between cabbage and grapes might be due to the influence of various factors such as differences in crop species, plant cultivation methods, and physicochemical properties of the pesticides.

KEYWORDS: *variability factor, pesticide residue, agricultural commodity*

INTRODUCTION

In a commodity previously treated with a pesticide, the level of pesticide residue remaining on or in a single unit varies depending on many factors such as physicochemical properties of pesticides, direction of application, application rate, preharvest interval, agricultural practices, agricultural conditions, weather conditions, sampling procedures, and growth rates. These variations should be taken into consideration when acute dietary exposure of consumers to pesticides is evaluated for risk assessment. Many reports on the variability of pesticide residue levels in agricultural commodities were aimed at predicting the acute reference dose for human health.[1−8] Those reports provided the information on individual pesticide residue variations for an index in the variability factor (VF), which is calculated as the 97.5th percentile of the residue population divided by the average residue levels in the lot.

Agriculture in Japan yields good crops even in small farms because of the favorable climate and efficient mechanized agricultural procedures. However, there is little information available on some of the local agricultural practices based on geographical conditions. There is also limited data regarding the effects of physicochemical properties of pesticides and plant crop species. Therefore, in 2007 and 2008, we attempted an estimation of individual cypermethrin residue variations in sweet peppers and apples.[9] In 2009, we further estimated the individual acetamiprid residue variations in broccolis. These investigations were conducted independently of each other. The measured VFs of the above tested commodities ranged from 1.48 for cypermethrin in sweet peppers to 1.76 for cypermethrin in apples. These studies provided a valuable piece of information to estimate the variations in pesticide residues under normal Japanese agricultural practices.[10] However, these results are not sufficient to clarify the influence of complex factors such as differences in crop species, plant cultivation methods, and physicochemical properties of the pesticides.

In 2010, a tank-mix application technique with two different pesticides, acetamiprid and cypermethrin, was applied to this study in consideration of the requirements mentioned above. Acetamiprid ((*E*)-*N*-[(6-chloro-3-pyridyl)methyl]-*N*′-cyano-*N*-methylethanimidamide) is a neonicotinoid insecticide popularly used worldwide which has a relatively low log P_{OW} of 0.80 and a relatively high water solubility of 4250 mg/L.[11] Cypermethrin (cyano(3-phenoxyphenyl)methyl 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate) is a synthetic pyrethroid insecticide popularly used worldwide which has a relatively high log P_{OW} of 6.6, and a relatively low water solubility of 0.004 mg/L.[11] The two pesticides differ significantly in their respective physicochemical properties, to facilitate an accurate determination of the variations in the individual pesticide residues. A cabbage field and a grape vineyard were selected as the test sites in this study, because the formulations of acetamiprid and cypermethrin are routinely used in cabbage fields and grape vineyards in Japan. In addition, cabbage as a leafy vegetable has not been investigated in our previous research, and the cultivation on horizontal shelves in Japanese grape vineyards is different from that of global agricultural practices.

MATERIALS AND METHODS

Field Experimentation. Field experiments were supervised by the Institute of Environmental Toxicology and were carried out by the Japan Plant Protection Association in accordance with the Japanese

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Guidelines,[12] which are implemented in accordance with OECD Guideline.[13] The pesticide formulations were applied at the maximum label rates, the maximum number of applications, and the minimum preharvest intervals for cabbage and grapes allowed by and in accordance with the Japanese Agricultural Practice.

The field experiment on cabbage (variety: *Kinkei-201*) was conducted in a field (field area, 86.4 m^2; planting rate, ca. 3300 plants/10 a) in Ibaraki prefecture from August 26 to November 5, 2010. After diluting by a factor of 1:1000, pesticides were sprayed 5 times at an approximate 7-day interval between each treatment, the final application being 7 days before harvest. The sprays were administered with a tank-mix combination of wettable powder formulations as Mospilan 20% a.i. (Nippon Soda, Japan) and Agrothrin 6% a.i. (Sumitomo Chemical, Japan), by using a back-carry type sprayer connected to a corn nozzle (Maruyama, MSB151, Japan). Application rates ranged from 213 to 292 L/10 a. The field experiment on grape (variety: *Delaware*) was conducted in an in-house orchard (field area, 41 m^2; planting rate, 25 vines/10 a) in Yamanashi prefecture from July 8 to August 5, 2010. Eleven-year grapevines were on a horizontal shelf approximately 1.5 m in height. After diluting by a factor of 1:1000, Agrothrin sprays were applied 5 times at an approximate 7-day interval between each treatment, by using a back-carry type sprayer connected to a corn nozzle (Maruyama, MSB151, Japan). After diluting by a factor of 1:2000, Mospilan sprays were applied 3 times at an approximate 7-day interval between each treatment. The intermediate sprays were applied with a tank-mix combination of the two formulations. Application rates were 300 L/10 a. The final applications of Agrothrin and Mospilan were 7 and 14 days before harvest, respectively.

Sampling and Sample Preparation. We randomly selected 130 heads of cabbage and bunches of grapes from each field, which were separately collected each in polyethylene bags to reduce cross contamination, and packed in hard boxes. Cabbage samples were brought to our institute on the sampling day. Grape samples were shipped by Yamato Transport (Japan) to our institute, at a standard temperature of 3 °C.

Each cabbage with the outer leaves or cores removed and each grape with the stems removed constituted a single commodity. The weight of each individual commodity was measured. Cabbage and grape samples were individually homogenized without sample size reduction by BLIXER-5Plus (Robot Coupe, Jackson, MS, USA) and 3901JP (Russell Hobbs Limited, U.K.) blenders, respectively. All samples were analyzed on the day following their arrival at our institute prior to storage.

Five additional heads of cabbage were collected for a separate similar analysis. The five cabbage commodities for the additional separate analysis were divided into three equal portions on weight basis (Figure 1). Portions from cabbage in the core side leaves, middle position leaves, and outer side leaves were separately acquired and homogenized for the additional analysis.

Chemicals and Reagents. Standards for acetamiprid (purity, 99.9%), and cypermethrin (purity, 94.0%) were purchased from Kanto Chemical (Japan), and Dr. Ehrenstorfer (Germany). Pesticide analysis-grade acetone, HPLC-grade tetrahydrofuran, LC−MS-grade acetonitrile, and certified-grade acetic acid and ammonium acetate were purchased from Wako Pure Chemical (Japan). Water used for the experiments was purified by a Milli-Q system (Millipore, MA, USA).

Standard stock solutions (500 mg/L) of acetamiprid and cypermethrin were separately prepared with acetonitrile. Portions of the acetamiprid stock solution were diluted with water/acetonitrile (6:4, v/v) to make standard solutions in the range of 0.025−1 μg/L to prepare a calibration curve. Portions of the cypermethrin stock solution were diluted with acetonitrile to give standard solutions in the range of 0.25−10 μg/L for the calibration curve.

Extraction. A portion (20 g) of the homogenized sample was weighed into an Erlenmeyer flask and extracted with 100 mL of acetone by shaking for 30 min using a reciprocal shaker. The mixture was then filtered by vacuum suction using a funnel (Kiriyama Glass, Japan) with a piece of filter paper (No. 704 × 60 mm; Nippon Rikagaku Kikai, Japan); the residual cake was washed with 50 mL of acetone and then filtered again. The filtrates were combined and made up to a 200 mL volume with acetone.

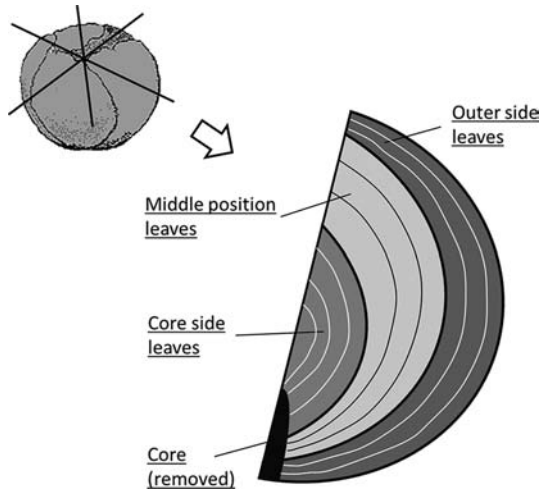

Figure 1. Diagram illustrating the removal of the cabbage portions that were used for analysis (removal of core).

Cleanup. A portion of the acetone extract (2 mL as 0.2 g of sample) was cleaned up using solid-phase extraction with an octadecyl silica cartridge (1 g/6 mL, Inert Sep C18-C; GL Science, Japan). For the cleanup step, water (8 mL) was added to the 2 mL of acetone extract, and the mixture was loaded onto the cartridge. A 10 mL mixture of acetonitrile:water (6:4, v/v) was passed through the cartridge, and the eluate was collected in a round-bottom flask (acetamiprid fraction). The cartridge was aspirated for 1 min and dried. A 10 mL mixture of acetic acid:tetrahydrofuran (1:1000, v/v) was passed through the cartridge, and the eluate was collected in another round-bottom flask (cypermethrin fraction). The eluted fraction of cypermethrin was evaporated to dryness under nitrogen stream.

The acetamiprid eluate was diluted with a suitable volume (20−2000 mL) of acetonitrile:water (6:4, v/v), and then, an aliquot (2 μL) of the diluted test solution was injected into the LC−MS/MS system. The residue containing the cypermethrin fraction was dissolved in a suitable volume (2−160 mL) of acetonitrile, and then, an aliquot (10 μL) of the diluted test solution was injected into the LC−MS/MS system. Each amount of acetamiprid and cypermethrin in the injected solution was determined by linear regression analysis of each standard calibration curve by comparing the peak area to each concentration of the respective compound in the cabbage and grape samples.

LC−MS/MS Analysis. A liquid chromatography (LC, model 1290 Infinity pumping system; Agilent, USA)−tandem mass spectrometry (MS/MS, model 6460 triple quadrupole tandem mass spectrometer; Agilent, USA) equipped with an electrospray interface (ESI) operating in positive ion modes was used. Data were processed with Agilent Mass Hunter software (version B03.01).

The LC conditions for acetamiprid were as follows: LC separation was performed on a Zorbax Eclipse Plus C18 (50 mm × 2.1 mm, 1.8 μm; Agilent) at 40 °C. The pump was set in isocratic mode at the rate of 0.3 mL/min with a mobile phase of acetonitrile and 5 mmol/L ammonium acetate aqueous solution (2:8, v/v). The retention time of acetamiprid was 2.2 min. The MS parameters for acetamiprid were as follows: capillary voltage, 4000 V (positive); nebulizer gas, 45 psi; drying gas, 5 L/min (400 °C); and fragmentor voltage 100 V. Nitrogen was used as the collision gas at 20 V. A precursor ion was selected for m/z 223.0, and a product ion for m/z 126.0 was detected in multiple-reaction monitoring mode.

The LC conditions for cypermethrin were as follows: LC separation was performed on a Unison UK-C18 (75 mm × 2.0 mm, 3 μm; Imtact, Japan) at 40 °C. Acetonitrile and 5 mmol/L ammonium acetate aqueous solution were used as the mobile phase at a flow rate of 0.3 mL/min. In gradient-elution analysis, the initial mobile phase

Table 1. Quantity of the Residues of Pesticides and the Calculated Variability Factors

crop + pesticide, no. of applications[a]	PHI,[b]	residue,[c] mg/kg (min to max)	VF[d], (97.5th percentile)	result of Shapiro–Wilk test
cabbage + acetamiprid, 5 (1000 times; 213–292 L/10 a)	7	0.129 ± 0.056 (0.044–0.288)	2.00 (0.258)	$W = 0.94229$, $p < 0.0000$
cabbage + cypermethrin, 5 (1000 times; 213–292 L/10 a)	7	0.066 ± 0.042 (<0.005–0.266)	2.39 (0.158)	$W = 0.84912$, $p < 0.0000$
grape + acetamiprid, 3 (2000 times; 300 L/10 a)	14	1.34 ± 0.578 (0.114–3.12)	1.82 (2.44)	$W = 0.98599$, $p < 0.2050$
grape + cypermethrin, 5 (1000 times; 300 L/10 a)	7	1.87 ± 0.578 (0.574–3.38)	1.63 (3.05)	$W = 0.98418$, $p < 0.1360$

[a]Dilution factors of the formulations and application volumes are expressed in the parentheses. [b]Preharvest intervals after final application. [c]Mean values ± standard deviations (minimum to maximum values). [d]Variability factor (coefficient of variation at the 97.5th percentile of the mean residue value).

was 50% acetonitrile, increased linearly to 95% in 2 min, and held at 95% for 8 min. The retention time of cypermethrin was 4.2 min as a single peak. The MS parameters for cypermethrin were as follows: capillary voltage, 4000 V (positive); nebulizer gas, 35 psi; drying gas, 5 L/min (350 °C); and fragmentor voltage 100 V. Nitrogen was used as the collision gas at 10 V. A precursor ion was selected for m/z 433.2 and a product ion for m/z 191.1 was detected in multiple-reaction monitoring mode.

Recovery Test. Specificity of the analytical method was confirmed by analysis of duplicate blank samples of cabbage and grapes, and by processing through the extraction and sample preparation procedures described above. No interference peak was observed on the chromatograms from the blank samples. Accuracy and precision of the analytical method were confirmed by the recovery test of acetamiprid and cypermethrin. The mean recoveries of triplicate spikes of cabbage and grape of acetamiprid at 0.005, 0.1, and 5 mg/kg were between 102% and 107%, and the relative standard deviations (RSD) for all were ≤4.8%. The mean recoveries of the triplicate spikes of cabbage for cypermethrin at 0.005, 0.1, and 1 mg/kg were between 79% and 95%, with their relative standard deviations being ≤3.8%. The mean recoveries of the triplicate spikes of grapes for cypermethrin at 0.005, 0.1, 2, and 4 mg/kg were between 74% and 98%, with their relative standard deviations were ≤5.1%.

Accurate and consistent instrument performance was determined by the use of additional recovery at 0.1 mg/kg and blank tests and by running a control after every 20 samples, in accordance with the Japanese Guidance.[14] A total of 28 recoveries from the additional recovery samples ranged from 74% to 102%. No interference peak was observed around the retention time of acetamiprid or cypermethrin on the chromatograms from the total 28 additional blank samples.

RESULTS AND DISCUSSION

Individual Residue Data. The results of the measured residue data and the calculated variability factors are summarized in Table 1. The individual residue values of acetamiprid and cypermethrin in cabbage ranged from 0.044–0.288 mg/kg and <0.005–0.266 mg/kg, with the means (±standard deviation) being 0.129 ± 0.056 mg/kg and 0.066 ± 0.042 mg/kg, respectively. Similarly, the values of the residues of the 2 pesticides in grapes ranged from 0.114 to 3.12 mg/kg and 0.574 to 3.38 mg/kg, with the means being 1.34 ± 0.578 mg/kg and 1.87 ± 0.578 mg/kg, respectively. All the mean residue values of acetamiprid and cypermethrin in the tested commodities were lower as compared to the maximum residue limits (MRL of acetamiprid in cabbage, 3 mg/kg; MRL of cypermethrin in cabbage, 5 mg/kg; MRL of acetamiprid in grapes, 1 mg/kg; and MRL of cypermethrin in grapes, 2 mg/kg;), as specified by the Japanese Food Sanitation Law.[15]

Distribution of Pesticide Residues. Frequency distributions of acetamiprid and cypermethrin residues in cabbage and grapes are shown in Figures 2 and 3, respectively. The statistical results from the Shapiro–Wilk test indicate that both pesticide residues in grapes have a normal distribution ($p > 0.01$). However, the distributions of acetamiprid and cypermethrin residues in cabbage were slightly skewed at the lower residue

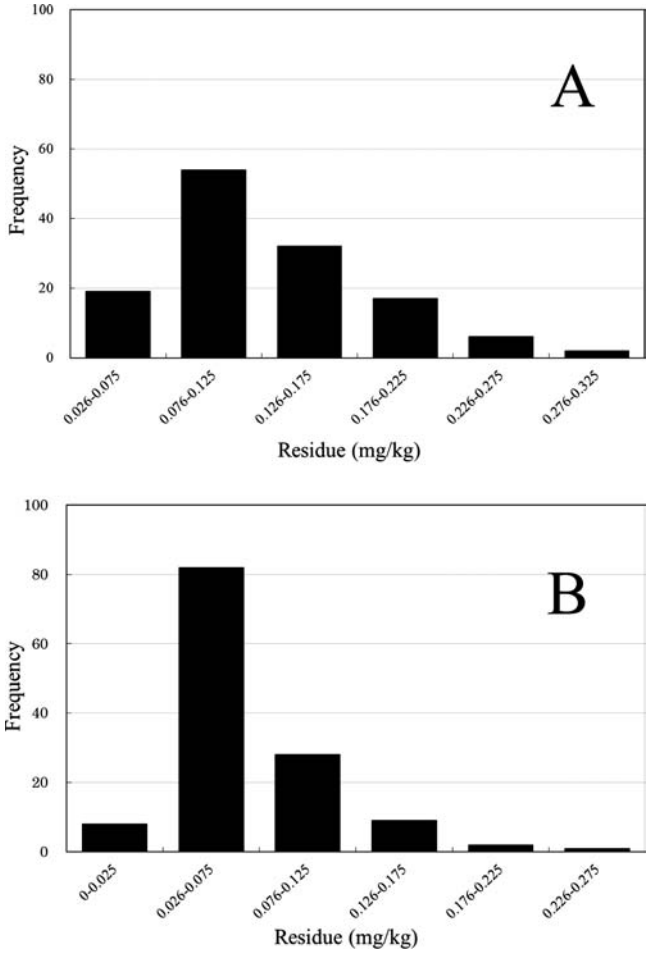

Figure 2. Frequency distribution of acetamiprid (A) and cypermethrin (B) residue levels in cabbage.

levels. The median residues in cabbage were 0.116 and 0.056 mg/kg for acetamiprid and cypermethrin, respectively, which were lower than their mean values described above. The skewed distribution of pesticide residues in cabbage was not observed for the other raw agricultural commodities, such as apples, broccolis, and sweet peppers.[9,10] The median residues in grapes were 1.33 and 1.86 mg/kg for acetamiprid and cypermethrin, respectively, which were the same as their mean values.

Sampling Size. The individually measured weights (including core) of 130 samples for each cabbage ranged from 0.841 to 2.03 kg. The mean weight of cabbage was 1.33 kg (RSD, 19.6%). The individually measured weights (including stems) of 130 samples for each grape ranged from 129 to 180 g. The mean grape weight was 155 g (RSD, 8.1%). These test sample sizes were within the acceptable range according to the

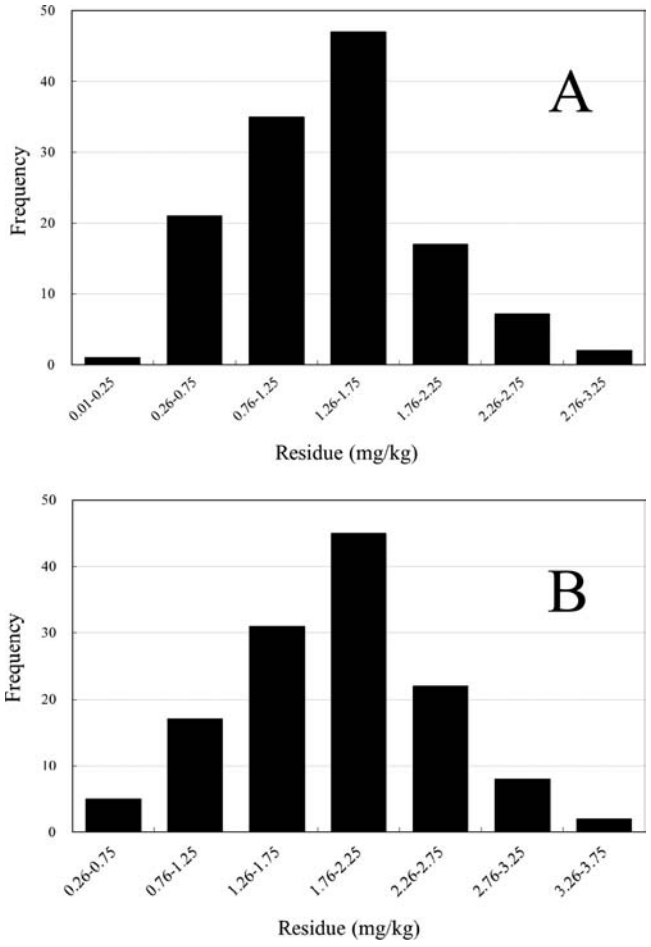

Figure 3. Frequency distribution of acetamiprid (A) and cypermethrin (B) residue levels in grapes.

Japanese local market regulation. We did not observe any correlations between the weights of individual cabbage and grapes and the residue levels of acetamiprid and cypermethrin.

Relationship between the Residue Levels of Acetamiprid and Cypermethrin. Individual residue levels of cypermethrin and acetamiprid in cabbage and grapes are summarized in Figures 4 and 5, respectively. Correlation

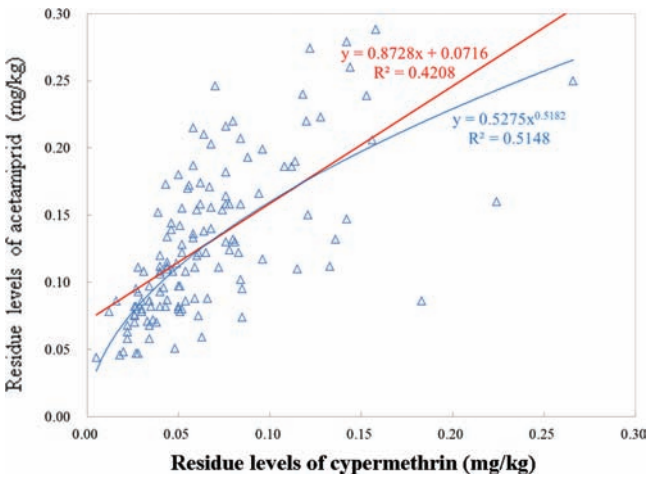

Figure 4. A scatter plot summarizing the correlation between the individual residue levels of acetamiprid and cypermethrin in cabbage.

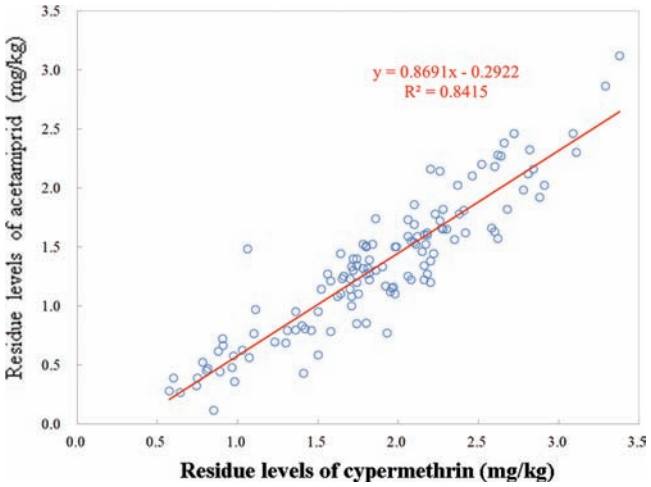

Figure 5. A scatter plot summarizing the correlation between the individual residue levels of acetamiprid and cypermethrin in grapes.

between the individual residue levels of acetamiprid and cypermethrin in grapes was found to have a good agreement (γ^2 = 0.8415). In this study, the formulations of two pesticides were applied under different conditions to the grape vineyard. The acetamiprid formulation was applied a total of three times, with the final application being 14 days before harvest. The cypermethrin formulation was applied a total of five times, with the final application being 7 days before harvest. The level of pesticide residues depends on preharvest intervals on field base,[16,17] whereas no significant difference was found between the individual cypermethrin and acetamiprid residue levels on unit base.

In contrast, there was not a clear correlation between the individual residue levels of cypermethrin and acetamiprid in cabbage, even though the two pesticide formulations were applied under identical conditions in the cabbage field. It seems that the individual residue levels of cypermethrin in cabbage diffused to a higher residue area as compared to each residue level of acetamiprid. The coefficients of correlation by log−normal power approximation (γ^2 = 0.5148) were greater than the normal base approximation (γ^2 = 0.4208) for cabbage.

Estimation of Variations in Pesticide Residue Levels. The VFs of the residues in raw agricultural commodities were calculated using Microsoft Excel, according to the reference procedure as the ratio of the 97.5th percentiles to the mean residue values.[5] A single non-detect value for cypermethrin in cabbage (<0.005 mg/kg) was replaced by half of the limit of quantification in subsequent studies.[5]

The VFs of acetamiprid and cypermethrin in cabbage were 2.00 and 2.39, respectively, while those values in grapes were 1.82 and 1.63, respectively. The VFs of both pesticides in cabbage were higher than those obtained in grapes. The higher VFs in cabbage may reflect the influence of complex factors such as different growing conditions. One of the most possible factors assumed is that grapevines were grown in the greenhouse, and cabbage in the open field: therefore, the residue levels in cabbage would be varied and influenced possibly due to pesticide dilution and degradation from sunlight and/or rain. Furthermore, while grapevines were on a horizontal shelf approximately 1.5 m in height, cabbage were grown in the field at ground level: therefore, deposition and distribution of pesticides would be different. For example, Travis et al. reported variations in the deposition and

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distribution of pesticides by a sprayer in apple trees,[18] and Tanigawa et al. also reported the variation of pesticide residues by a sprayer in eggplants.[19]

Comparison to the Reported Data. The VFs of cypermethrin in this study were 2.39 in cabbage and 1.63 in grapes, respectively, which were similar in range to the VFs obtained from cypermethrin residues in lettuce (VF: 1.73) and mangos (VFs: 2.16−2.31) reported by Ámbrus.[8] Nevertheless, it is impossible to compare the VFs of acetamiprid with the other reported data. The Joint Division of the FAO and the International Atomic Energy Agency (IAEA) reported that the mean VF obtained from 4 pesticides in 860 cabbage samples was 1.85, and the mean VF obtained from 15 pesticides in 2426 grape samples was 2.67.[5] The measured VFs in this study ranged from 1.63 for cypermethrin in grapes to 2.39 for cypermethrin in cabbage. The resultant VFs in this study were within the same range as their reported mean VFs in cabbage and grapes by FAO/IAEA, and also within the default VF of 3 that was proposed by the World Health Organization.[4]

Separate Analysis of Cabbage. To further investigate the distribution of pesticides in cabbage, additional separate analysis was performed on three equal portions on weight basis from each commodity as described in Figure 1. The mean residues of acetamiprid in the portions of the core side, middle position, and outer side leaves of cabbage were 0.054, 0.060, and 0.586 mg/kg, respectively (Table 2). The majority of acetamiprid residues (84%) existed in the portions of the outer side leaves of cabbage, while relatively minimal parts of the residues existed in the other leaves (8% or 9%). Meanwhile, the mean residues of cypermethrin in the portions of the core side, middle position, and outer side leaves of the cabbage were <0.005, <0.005, and 0.406 mg/kg, respectively. Thus, most of the cypermethrin residues existed in the portions of the outer side leaves. It is well-known that the neonicotinoid insecticides, including acetamiprid, have penetration and translocation abilities.[20] These results suggest that removal of the outer leaves in cabbage has a more significant effect on cypermethrin residues due to sampling or the sample preparation process compared to the acetamiprid residues.

Table 2. Distribution of the Residue Levels of Acetamiprid and Cypermethrin in Cabbage

	mean residue[b]	
portion to be analyzed[a]	acetamiprid	cypermethrin
core side leaves	0.054 mg/kg (8%)	<0.005 mg/kg (<1%)
middle leaves	0.060 mg/kg (9%)	<0.005 mg/kg (<1%)
outer side leaves	0.586 mg/kg (84%)	0.406 mg/kg (>98%)

[a]Portions were divided into three equal portions on weight basis; refer to Figure 1. [b]Mean residue levels in a homogenized sample from five heads of cabbage (n = 2). Percentages relative to the total residue amount are expressed in the parentheses.

Throughout this study, the effects of different growth conditions were observed on the VFs between the cabbage grown in greenhouses and the grapes grown outdoors without houses. Furthermore, the different characteristics relating to the physicochemical properties of pesticides were revealed by separate analysis of the acetamiprid and cypermethrin residues in cabbage. In conclusion, the present study demonstrated that the distribution patterns of both pesticide residues in cabbage and grapes were influenced by complex factors such as differences in crop species, plant cultivation methods, application rates, preharvest intervals, and physicochemical properties of the pesticides.

■ ASSOCIATED CONTENT

Supporting Information

Photographs of pesticide applications (Figure S1), representative chromatograms (Figure S2), and the results of the recovery tests (Table S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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