Bull Environ Contam Toxicol (2012) 89:868–872
DOI 10.1007/s00128-012-0765-x

Comparison of Greenhouse and Field Degradation Behaviour of Isoprocarb, Hexaflumuron and Difenoconazole in *Perilla frutescens*

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Received: 11 April 2012 / Accepted: 26 July 2012 / Published online: 8 August 2012

Abstract Isoprocarb, hexaflumuron and difenoconazole were used in *Perilla frutescens* at 600, 60 and 75 g a.i./ha respectively. High performance liquid chromatography-tandem mass spectrometry was used for residue determination because of high selectivity and simple treatment. The results showed that the half-lives of isoprocarb, hexaflumuron and difenoconazole at greenhouse condition were 0.71, 1.63 and 1.21 days respectively, and at field condition, the values were 1.13, 1.07 and 0.92 days respectively.

Keywords Isoprocarb · Hexaflumuron · Difenoconazole · *Perilla frutescens*

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Perilla frutescens is not only a traditional Chinese medicine but also an edible vegetable. It has been planted in China for more than 2,000 years and is also in the list of "*Articles that Belong to Both Foods and Medicines*" issued by Minstry of Health of the People's Republic of China in 2008. Its leaves have a very pleasant sweet taste and are used as a spice, cooked as potherbs or fried, and combined with fish, rice, vegetables and soups. *P. frutescens* leaves have become one of the favourite vegetables in China today and are exported to Japan and Korea. In international agricultural trade, it is now widely acknowledged that technical measures such as food quality and sanitary and phytosanitary (SPS) requirements can impede trade, particularly in the case of developing countries (Henson and Loader 2001). In recent years, *P. frutescens* exported from China to Japan was often detected because of pesticides exceeding the standard, especially isoprocarb, hexaflumuron and difenoconazole. In Japanese "*Positive List System for Agricultural Chemical Residues in Foods*", there is no maximum residue limits (MRLs) of isoprocarb on *P. frutescens*, and then "the uniform limit", that is 0.01 mg/kg, is suitable. The MRLs of hexaflumuron and difenoconazole were 0.02 and 0.2 mg/kg. However, in China, to our knowledge, there is no regulation on the MRL in *P. frutescens*. Many researchers studied the degradation of difenoconazole on main foods, such as tomato, apple and Chinese cabbage (Kong et al. 2012; Rueegg and Siegfried 1996; Wang et al. 2008). However, as *P. frutescens* is a minor breeding crop and its leaves are mainly eaten as a vegetable in Asian countries, few studies focusing on pesticides degradation have been carried out on *P. frutescens*. In this study, degradation studies of isoprocarb, hexaflumuron and difenoconazole were conducted in *P. frutescens*, under the greenhouse and field planting conditions, the residues were determined by high

performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS).

The objective of this present study was to determine the residues and degradation dynamics of the isoprocarb, hexaflumuron and difenoconazole, to compare the difference between greenhouse and field planting conditions.

Materials and Methods

The analytical standard of isoprocarb (99 %, purity), hexaflumuron (99 %, purity) and difenoconazole (99 %, purity) were obtained from Environmental Protection Monitoring Institute, Tianjin, China. 20 % EC isoprocarb was purchased from Lvyin agro-chemical Co, Ltd (Guangzhou, China). 5 % EC hexaflumuron was purchased from Pinghu pesticide Company (Zhejiang, China). 10 % AS difenoconazole was purchased from Syngenta (Syngenta, Sweden). *P. frutescens* seed was supplied by Fengyangshan Green foods Co., Ltd (Longquan city, Zhejiang, China). Guarantee reagent grade acetonitrile and toluene were purchased from Merck (Darmstadt, Germany). HPLC-grade water was supplied by Wahaha (Hangzhou, China). All other chemicals were of analytical reagent grade and obtained from commercial sources.

The field experiment was conducted during May to August, 2011, in the experimental field of Zhejiang Academy of Agriculture Science (Hangzhou, China). Each experimental plot was 30 m^2, with 1 m distance interval. Blank experimental plots were set at the same time. The application dose of experimental plot was arranged orderly. Greenhouse and field planting conditions were applied at the same time, on considering of the vegetable production practice in China. Isoprocarb, hexaflumuron and difenoconazole were applied at the recommended doses of 600, 60 and 75 g a.i./ha, respectively. The testing seed was planted on 5 May 2011, and the pesticides were applied during July to August in 2011, which was the middle growing stage of *P. frutescens*. The leaves were collected randomly from each treated plot as well as from the blank plot on 0 (1 h after application), 1, 3, 6, 7, and 14 days after pesticide application. About 200 g *P. frutescens* leaves were collected at different time intervals, and they were homogenized by a blender (Philips, China) and then stored at −20°C until they were analyzed.

In the open field, the maximum and minimum air temperatures during this experiment were close to 20 and 36°C, respectively. The mean monthly rainfalls were 109 and 285.5 mm in July and August 2011, during the pesticides application period. The relative humidity ranged from 60 % to 94 %. The wind speed ranged from 2.4 to 6.1 km/h. In the greenhouse, the air temperature ranged from 25 to 46°C and the relative humidity was from 70 % to 95 %.

HPLC–MS/MS detection was used for residue analysis by TSQ Quantum Discovery mass spectrometer system (Thermo Fisher Scientific, USA) equipped with an electrospray interface. Thermo Fisher Xcalibur 2.0.7 software was used to control the instrument and collect and analyze data. Separation was carried out on a chiral column Lux Cellulose-1 [cellulose tris (3,5-dimethylphenylcarbamate)] supplied by Phenomenex (Torrance, USA). The mobile phase consisted of 90 % (*v*/*v*) acetonitrile and 10 % (*v*/*v*) 0.1 % formic acid solution. The flow rate was set at 0.2 mL/min, column oven temperature was set at 25°C, and autosampler temperature was set at 4°C. The injection volume was 5 μL, and the total run-time was 15 min. Electrospray in positive mode was used and the spray voltage was set at 4.0 kV. The capillary temperature was set to 350°C. Aux auxiliary gas and sheath gas were normal nitrogen. Collision gas was high pure argon with pressure at 0.2 Pa in collision cell. The first mass transition was used for quantification, while the second mass transition was used for confirmation of the residues. Two transitions were used for quantification and confirmation, with m/z $194 > 95$ and $194 > 137$ for isoprocarb, m/z $461 > 141$ and $461 > 158$ for hexaflumuron, m/z $406 > 251$ and $406 > 188$ for difenoconazole. The retention times (t_R) of isoprocarb, hexaflumuron and difenoconazole were about 2.39, 2.95 and 3.17 min.

The homogenized *P. frutescens* leaves (25.0 g/sample) were weighted into a 250 mL conical flask with stopper, followed by addition of 30 mL water and stand for 30 min, and then 80 mL acetonitrile were added and shaken for 30 min. After 30 mL supernatant was taken into plastic centrifuge tube, 3 g magnesium sulfate, 2 g sodium chloride and 10 mL hexane were added, and shaken for 5 min. The extract was centrifuged for 5 min at 3,000 r/min and 10 mL middle layer was transferred, and waiting for purifying by solid phase extraction (SPE).

For the purifying, the SPE column was rinsed with 10 mL (acetonitrile + toluene) (3 + 1). 10 mL of the middle layer was transferred into the SPE column and the fraction was collected. 30 mL (acetonitrile + toluene) (3 + 1) were used for elutriation and the elution liquid was collected. The elution liquid was immersed in a constant temperature bath with 40°C and concentrated to dryness. Finally 5 mL methanol was added to the volume for solution, the liquid was filtered by 0.22 μm membrane, and then determined by HPLC–MS-MS.

A series of standard solutions of isoprocarb, hexaflumuron and difenoconazole were constructed with mixed working standard solutions at concentrations of 0.01, 0.1, 1.0 and 10 mg/kg. According to procedure described in samples extraction and purification, blank matrix was prepared and a series of matrix matched calibration standards with the same concentrations were also prepared. Isoprocarb, hexaflumuron and difenoconazole were added

to untreated control samples at 4 concentration levels (0.01, 0.1, 1.0 and 10 mg/kg for each pesticide based on five replicates). The samples were left for 1 h to equilibrate the analytes in the sample. The fortified samples were analyzed based on the described procedure and the recoveries were calculated. The precision of the method was determined by the repeatability and expressed by relative standard deviation (RSD %).

Results and Discussion

HPLC–MS/MS has been used extensively in drug metabolism for high selectivity, high sentivity and simple preliminary treatment (Zhang et al. 2011; Qian et al. 2011; Liu et al. 2005; Fernandez et al. 2000). Under the HPLC–MS-MS condition, the 3 pesticides were separated completely (shown in Fig. 1). The recoveries of isoprocarb were varied from 82.5 % to 100.6 % with RSD from 3.0 % to 12.6 % at 4 spiking levels (0.01, 0.1, 1.0 and 10 mg/kg); that of hexaflumuron were varied from 81.7 % to 96.8 % with RSD from 3.8 % to 12.4 %; that of difenoconazole were varied from 84.2 % to 94.3 % with RSD from 6.3 % to 11.4 %. The mean recoveries were all exceeded of 80 %, confirmed that the method is sufficiently reliable for pesticide analysis in this study. The limit of detection (LOD) and the limit of quantification (LOQ) were defined as the concentration giving a signal to noise ratio of 3 and 10. In this article, the LOD and LOQ for these 3 pesticides were estimated to be 0.0006 and 0.002 mg/kg, respectively.

The residues of isoprocarb, hexaflumuron and difenoconazole in *P. frutescens* leaves were processed and fitted by first-order kinetics, $C = C_0\, e^{-kt}$, employing a non-liner least-squares regression analysis of residue concentration against time, where C is the concentration at time t, C_0 is the initial concentration and k is the rate constant. Half-life of pesticides in plant was an important indicator of pesticide efficacy and pollution, and the half-life ($t_{1/2}$, day) was estimated from equation, $t_{1/2} = ln\ 2/k = 0.693/k$.

The pesticides residues of isoprocarb, hexaflumuron and difenoconazole in *P. frutescens* from 0 to 14 days are shown in Table 1, and the regression functions are shown in Table 2.

In the control, isoprocarb, hexaflumuron, and difenoconazole residues were below 0.01 mg/kg. After treatment, the concentration of the 3 pesticides decreased gradually with time. The results in Tables 1 and 2 showed that with application dose of 600 g a.i./ha, the initial deposit of

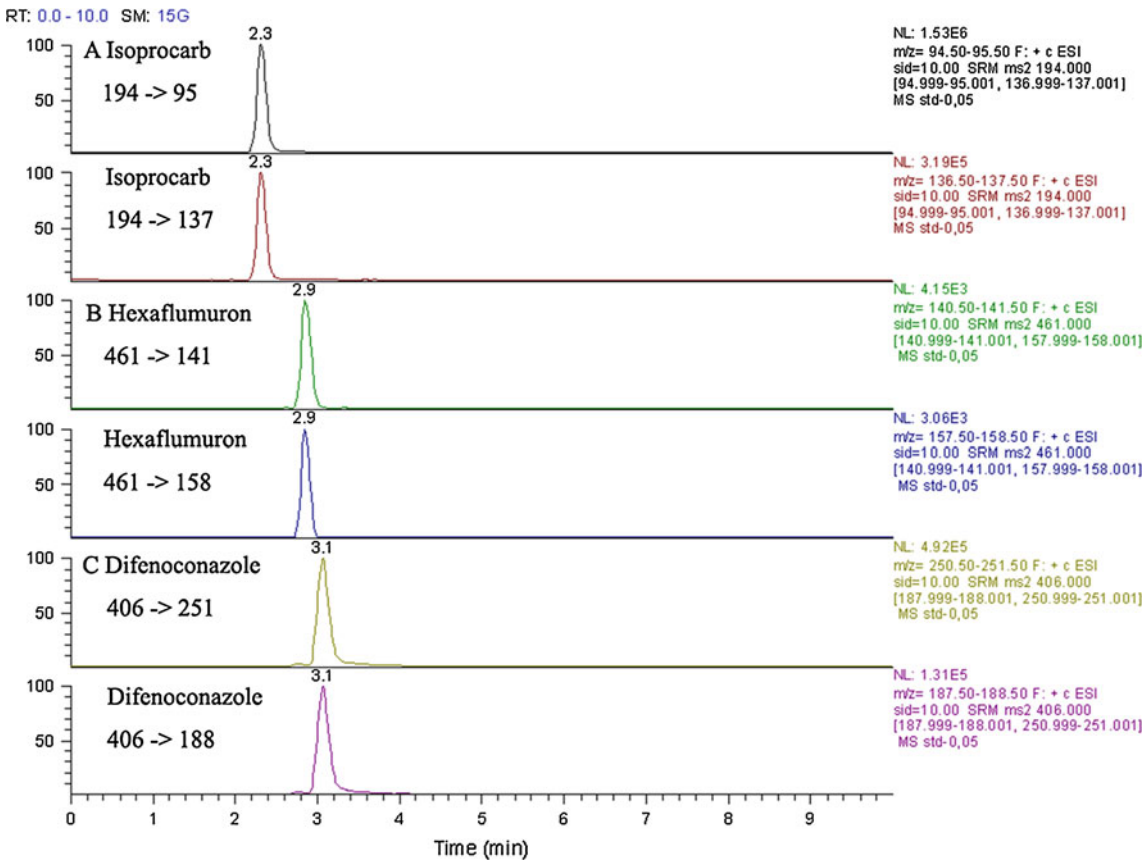

Fig. 1 Typical MRM chromatograms of isoprocarb, hexaflumuron and difenoconazole in standard solutions (*A* quantification and *B* confirmation)

Table 1 Pesticides residues (mg/kg) in *P. frutescens* at different times[a]

Days	Isoprocarb		Hexaflumuron		Difenoconazole	
	Greenhouse	Field	Greenhouse	Field	Greenhouse	Field
0	11.7 ± 0.20	4.96 ± 0.36	9.63 ± 0.09	8.83 ± 0.11	14.4 ± 0.26	12.3 ± 0.20
1	6.41 ± 0.19	2.36 ± 0.28	7.88 ± 0.14	6.75 ± 0.08	13.9 ± 0.30	8.93 ± 0.23
3	3.74 ± 0.18	1.40 ± 0.14	4.91 ± 0.04	3.60 ± 0.05	6.83 ± 0.03	3.68 ± 0.08
6	1.80 ± 0.13	1.06 ± 0.05	3.19 ± 0.08	2.17 ± 0.04	3.55 ± 0.05	2.04 ± 0.11
7	1.02 ± 0.04	0.51 ± 0.04	2.04 ± 0.15	0.93 ± 0.02	2.22 ± 0.04	1.06 ± 0.03
14	0.26 ± 0.03	0.18 ± 0.02	1.09 ± 0.04	0.39 ± 0.03	0.90 ± 0.04	0.25 ± 0.02

[a] Values represent the means ± SDs (n = 3)

isoprocarb were 11.7 and 4.96 mg/kg at greenhouse and field conditions respectively, which were found to be 0.26 and 0.18 mg/kg on 14th day after application, the percentages of dissipation recorded on 14th day were 97.7 % and 96.3 %. The initial deposit of isoprocarb at greenhouse and field had great difference and it also degraded greatly on the 1st day of application, which was 6.41 and 2.36 mg/kg respectively, with the dissipation percentages of 45.2 % and 52.4 %.The results indicated that isoprocarb was easy to be adhered on *P. frutescens* at greenhouse condition. With application dose of 60 g a.i./ha, the initial deposit of hexaflumuron were 9.63 and 8.83 mg/kg at greenhouse and field conditions respectively, which were found to be 1.09 and 0.39 mg/kg on 14th day after application, the percentages of dissipation recorded on 14th day were 88.7 and 95.5 %. With application dose of 75 g a.i./ha, the initial deposit of difenoconazole were 14.4 and 12.3 mg/kg at greenhouse and field conditions respectively, which were found to be 0.90 and 0.25 mg/kg on 14th day after application, the percentages of dissipation recorded on 14th day were 93.7 % and 97.9 %. The dissipation of isoprocarb, hexaflumuron and difenoconazole in *P. frutescens* leaves followed first-order kinetics under both greenhouse and field conditions. At greenhouse condition, the half-lives of isoprocarb, hexaflumuron and difenoconazole were 0.71, 1.56 and 1.21 days respectively, and at field condition, the values were 1.13, 1.10 and 0.92 days respectively. The results showed that when isoprocarb was applied on *P. frutescens* leaves, the initial deposit in greenhouse was much higher than that in open field with the same dose, while the degradation rate of greenhouse condition was faster than that of field condition. Therefore the dissipation rates of isoprocarb were affected by many factors, and that of lower concentration dissipated more slowly than that of higher concentration. When hexaflumuron and difenoconazole were applied on *P. frutescens* leaves, the degradation rate of field condition was faster than that of greenhouse condition. In this study, the temperature variety range in greenhouse and field was different. The weather was windy and there was plenty of rain during the field experiment process. It was shown that the hexaflumuron and difenoconazole deposited on *P. frutescens* leaves was easier to be removed in field condition.

Residues of these 3 pesticides with 14 days Pre-harvest interval (PHI) are shown in Table 3.

The results in Table 3 showed that at greenhouse condition and at the recommended doses, the MRLs of isoprocarb,

Table 2 Regression functions of the 3 pesticides in *P. frutescens* leaves[a]

Site	Regression equation	R^2	Half-life ($t_{1/2}$) (days)
Isoprocarb			
Greenhouse	$y = 28.334e^{-0.722t}$	0.9706	0.71 ± 0.06
Field	$y = 9.2378e^{-0.613t}$	0.9705	1.13 ± 0.12
Hexaflumuron			
Greenhouse	$y = 17.308e^{-0.439t}$	0.9832	1.56 ± 0.23
Field	$y = 21.328e^{-0.63t}$	0.9731	1.10 ± 0.12
Difenoconazole			
Greenhouse	$y = 34.191e^{-0.572t}$	0.9639	1.21 ± 0.08
Field	$y = 34.608e^{-0.756t}$	0.9632	0.92 ± 0.06

[a] The regressive functions and half-lives were obtained based on the mean value of three replicates

Table 3 Residues with 14 days pre-harvest interval (PHI) and Japanese MRLs[a]

Pesticides	Site	Application dose (g a.i./ha)	Residues with 21 days PHI (mg/kg)	Japanese MRLs (mg/kg)
Isoprocarb	Greenhouse	600	0.26	0.01
	Field		0.18	
Hexaflumuron	Greenhouse	60	1.09	0.02
	Field		0.39	
Difenoconazole	Greenhouse	75	0.90	0.2
	Field		0.25	

[a] The values were the means of three replicates; the MRLs were searched in Japanese "Positive List System for Agricultural Chemical Residues in Foods"

hexaflumuron and difenoconazole were 0.26, 1.09 and 0.90 mg/kg respectively with 14 days PHI. At open field condition, the values were 0.18, 0.39 and 0.25 mg/kg respectively. According to Japanese "*Positive List system*", the standards of isoprocarb, hexaflumuron and difenoconazole were 0.01, 0.02 and 0.2 mg/kg respectively. The MRLs with 14 days PHI all exceeded the standards. As *P. frutescens* grows fast and the PHI of 14 days was considered long enough, the results showed that in international *P. frutescens* trade, isoprocarb, hexaflumuron and difenoconazole are not recommended to be used on *P. frutescens* with the recommended dose. In addition, the results showed that controlled dose setting for the use of isoprocarb, hexaflumuron and difenoconazole, controlled open field and greenhouse treatments, and the PHI have a crucial role in the reduction of pesticide residue. Unfortunately, as far as we know, there is little information about the pesticide behavior in *P. frutescens* in open field and greenhouse. Therefore, a deeper investigation is necessary and further studies should also be performed to better assess the pesticide behavior in *P. frutescens*, which are commonly used as a fresh food.

Acknowledgments This work was supported by The Key SQT Innovation Team of Zhejiang Province for Agro-Products Standards and Testing Technology (Grant No. 2010R50028).

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