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Field Efficacy of Acaricides on Citrus Red Mite and Dissipation Study for Cyflumetofen and Pyridaben in Lemon Fruit Using QuEChERS Method and LC-ESI-MS/MS

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Abstract

The present study was conducted to evaluate field efficacy of the recommended doses of five acaricides (cyflumetofen, 20% SC; pyridaben, 15%EC; spiromesifen, 24% SC; spirodiclofen, 24% SC and fenpyroximate, 5% EC) followed by dissipation study for cyflumetofen and pyridaben, against *Panonychuscitri (*McGregor*)* on lemon trees. The mean number of motile stages was significantly (p < 0.05) reduced in all treatments after spraying the acaricides compared with the control. The cyflumetofen and spirodiclofen showed tangible efficacy decreasing *P. citri* movable stages numbers. During two consecutive seasons, the highest efficacy was recorded seven days after application, while the lower one recorded after three days of application. Results showed that the population of *P. citri* examined in the treated plot was these pesticides had been used, was significantly reduced. Based on these results, the cyfumetofen and spirodiclofen are to be recommended for citrus red mite control programs. The analytical procedures were done using QuEChERS method and LC-MS/MS for determination. The recoveries ranged between 92 and 117% with ≤ 4.2 % RSD and ± 6% combined uncertainty. The half-lives were calculated using the first-order kinetic model for both pesticides, cyflumetofen (5.33 days) and pyridaben (7.29 days).

***Keywords*:** Acaricides residues; LC-MS/MS; Lemon; *Panonychus citri*; QuEChERS and Toxicity.

1. Introduction

The lemon plays a significant role in human health as a raw material or after extraction process; due to its high content of active substance defined as secondary metabolites like vitamin C complex (ascorbic acid and citrus bioflavonoids). Several mite species commonly attack citrus around the world. It has been reported that there are many phytophagous species (104) that severely affect lemon fruits, leaves and buds [1]. Lemon like any other agricultural products can be susceptible to pesticides used in field or store. mites were found to be a harmful species on citrus in many parts of the world including Egypt [2].

Panonychuscitri McGregor (citrus red mite) is an important citrus pest causing yield loss worldwide. Citrus red mite prefers sweet orange as a host followed by lemon trees [3]. The control of this mite depends on acaricides, and it tends to develop resistance to that acaricides widely used in its control. A lot of pesticides were developed for red mites control, such as organophosphates, bifenazate, pyrethroids [4] and avermectins [5]. Also, etoxazole as chitin synthesis inhibitor [6, 7], clofentezine and hexythiazox were industrialized for the control of red mites.

Cyflumetofen has been proved to be effective against several mite species including two-spotted spider mite (*Tetranychus urticae* Koch), citrus red mite (*P. citri*) and the European red mite *(Panonychus ulmi* Koch). On the other hand, it is safe for non-targeted beneficial organisms [8]; so, it has been documented as an appropriate acaricide for integrated pest management (IPM) as it is used to controlspider mite species while using predatory mites [9, 10]. On the contrary, abamectin, fenpropathrin, hexythiazoxand pyridaben acaricides are still commonly used even though mites have acquired some resistance to them.

The nature of these toxic pesticides plays a role in developing residues analysis techniques to assess the human intake to facilitate picking up safe, healthy, chemical-free food. Also, the use of citrus products in

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nutrition and as dietary supplements enhanced the efforts to control the quality and safety by residues methodology.

The scope of this study is to evaluate field efficacy of five acaricides (cyflumetofen, 20% SC; pyridaben, 15%EC; spiromesifen, 24% SC; spirodiclofen, 24% SC and fenpyroximate, 5% EC) against the red citrus mite. Also, a dissipation studies and dynamic distribution including half-lives times of cyflumetofen and pyridaben residues in lemon were executed using QuEChERS analytical procedures and liquid chromatography tandem mass spectrometry. Furthermore, this study is aiming to shed the light on mite's resistance to acaricides to provide basic information that enhances the management programs of mites in citrus orchards.

2. Experimental

2.1. Field efficacyof acaricides

Evaluation of the effectiveness of five acaricides, namely Danisaraba 20% SC (Cyflumetofen), Acaronein 15%EC (Pyridaben), Oberon® 24% SC (Spiromesifen), Envidor 24% SC (Spirodiclofen) and Ortus Super 5% EC (Fenpyroximate) against the red citrus mite on citrus lemon trees (*Citrus limon* L.) was conducted in Tantaat Al GharbiaGovernorate.

The trial was carried out in lemon orchard (about 5000 m^2) commonlyinfested by *P. citri* populations and the lemon trees received all normalagricultural processes without any pesticide's application. The trial included five treatments acaricides and water spray as blank control. All the treatments with four replicated plots (each including 8–10 trees) were arranged in randomized block design and sprayed. The application rate of Danisaraba 20% SC (40 ml/100 L), Acaronein 15%EC (150 ml/100L), Oberon® 24% SC (60 ml/100 L), Envidor 24% SC (25 ml/100 L) and Ortus Super 5% EC (50 ml/100 L) was the recommended dose on citrus red mite. Two rows at edges of each orchard and one row between every two plots were not sprayed as buffer areas.The spray was applied in the first week of Novemberduring 2019 and 2020 seasons by a compressor spryer (600 litters capacity).

Initial counts of *P. citri* motile stages were made before the acaricides spray and after 3, 7 and 14 days of spraying. Twenty leaves were randomly taken as samples from the trees located in the middle of eachreplicated plot(80 leaves / treatment). Leaf samples were kept into perforated polyethylene bags,closed with rubber bands, and kept in an ice box then transferred to the laboratory for examination using a stereomicroscope. Motile stagesof *P. citri* were counted and recorded.

2.2. Chemicals and reagents

Acetonitrile and Methanol were produced by Merck as HPLC grade. Ammonia solution (33%) and Formic acid (98-100%) made by Riedel–de Haen. LC-MS quality de-ionized water was generated by Millipore instrument. Magnesium sulfate, sodium chloride, sodium citrate and citric acid disodium salt (QuEChERS extraction reagents) ready collected and weighted in one package from Agilent technologies. Syringe filter 0.45 µm made by Millipore. Cyflumetofen and Pyridaben pesticide reference standards purity were more than 95% (Dr. Ehrensdorfer).

2.3. LC-MS/MS analysis

Exion HPLC series connected to Qtrap API 6500+ triple mass was used in separation with Poroshell 120 EC column (C18) 2.7 µm particle sizes and 3 x 50 mm dimension from Agilent. LC-MS/MS was established with an ESI (electro spray ionization) interface. Liquid solid separation was performed by 0.3 ml/min flow rate; 60, 60, 10, 10, 60 and 60 % from A bottle during time; 0, 1, 5, 8, 9 and 11 min respectively, while A was prepared to be 10mM ammonium formate solution pH 4 in water – Methanol (9:1) and B was only Methanol. MRM (Multiple Reaction Monitoring) separation and detection mechanism was used to support quantitation by confirmation ion using positive ionization. Nitrogen gas was used in nebulizing and as curtain gas which was optimized with other parameters to get the best sensitivity in accordance with manufacturer recommendation to be as follows: 5500 volts and 400 ^{0}C in ionization point, declutter and collision potential were optimized individually for each compound using syringe pump built in instrument.

2.4. Pesticide standards preparation

Both pesticides were prepared individually as stock standard solutions (1000 µg/ml) in Toluene. Aliquots from tow pesticide stock solution were collected and mixed to be intermediate solution at 10 µg/ml concentration in acetonitrile. Five serial dilutions (0.005, 0.01, 0.05, 0.1 and 0.5 µg/ml) were prepared in acetonitrile to be used as calibration mixtures. Stock solutions were stored at -18 ± 2 ºC while other prepared solutions were at specific degree.

2.5. LC Mobile Phase: 1.73 ml formic acid was added into 900 ml di-ionized water and adjusted to pH4 by ammonia solution before completing the volume to 1 liter by Methanol to be 50 ml Molar as stock buffered solution. The working buffered solution mobile phase was prepared by the dilution of stock solution 5 times in methanol/ di-ionized water 1/9.

2.6. Lemon Sample extraction: 10g sample was added into 50 ml tube Polyethylene (PFTE) and shacked by Geno shaker for homogenizing (700 rpm for 5 min) after adding 10 ml Acetonitrile and one more time after adding buffer-salt mixture. Centrifugation was 5 min at 4000 rpm. An aliquot from upper layer was filtrated by 0.45 µm syringe filter before injecting 2 µl into LC system. Final extract should be diluted if the found concentration exited the calibration limits. Horizontal shaker (Sample Prep 2010-230 Geno/ Grinder) made by SPEX Sample pre (UK) coupled with 15 and 50 ml tube holder.

Data analysis

Statistical studies of the data were done using one-way analysis of variance (ANOVA) tracked by a comparison test (Tukey-Kramer HSD) to determine any significant differences in between the treatments on each sampling date ($P < 0.05$; using the software SPSS 16.0. The Reduction percentages of the *P. citri* were calculated according to Henderson and Tilton [11].

$$\text{Reduction } \% \equiv \left(1 - \frac{\text{Treatment after} \times \text{Control before}}{\text{Treatment before} \times \text{Control after}}\right) \times 100$$

3. Result and discussion

3.1. Field Efficacy of Acaricides

The efficacy of five acaricides; Cyfumetofen, Pyridaben, Spiromesifen, Spirodiclofen and Fenpyroximate applied at the recommended rates against motile stages of *P. citri* was estimated after 3, 7 and 14 days from the application. The mean number of motile stages was significantly ($p < 0.05$) reduced in all treatments after applying acaricides compared with the control.

The highest reduction of citrus red mite motile stages recorded with cyfumetofen and spirodiclofen treatments during the two seasons, while pyridaben recorded lowest reduction in motile stages compared to other treatments (Table 1).

During 2019 and 2020 seasons, the acaricides evaluation for 7 days after treatment revealed that Cyfumetofen and Spirodiclofen showed good efficacy decreasing citrus mite movable stages from 9.59 to 0.81 individual /leaf and from 10.96 to 1.03 individual /leaf respectively in the first season, and from 10.75 to 1.06 individual /leaf and from 11.66 to 1.09 individual /leaf respectively in the second season. Spiromesifen and Fenpyroximate gave moderate efficacy in decreasing mite population from 9.56 to 1.44 and from 11.53 to 1.91 respectively in the first season, while it was found from 12.24 to 1.69 individual /leaf and from 11.98 to 1.63 individual /leaf respectively in the second season. Pyridaben was the least efficient one decreasing the population of *P. citri* from 10.41 to 1.90 and from 11.08 to 2.31 individual /leaf in 2019 and 2020 seasons respectively.

The relative efficacy of the acaricides; Cyfumetofen, Pyridaben, Spiromesifen, Spirodiclofen and Fenpyroximate applied at the recommended rates against motile stages of *P. citri* was (93.71& 92.46%), (86.47& 83.15%), (88.85& 86.61%), (90.43& 92.48%) and (89.77& 89.05%) on the 7 day during 2019 and 2020 seasons respectively but decreased on the 14th day from 3- 5% in all treatments.

No previous data were found to evaluate field efficacy of cyflumetofen on *P. citri* in Egypt and to compare it with four acaricides that are commonly used as highly effective against economically important mite species developing resistance to acaricides on many fruit crops.

Our results demonstrated that the cyflumetofen and spirodiclofen showed significant decrease of *P. citri* movable stages. The highest efficacy was recorded after 7 days from spraying with all acaricides tested but the lower efficacy recorded after three days during the two seasons.

There are several studies indicating the acaricides efficacy of tetranychid mites. Cyflumetofen efficacy against *P. citri*. was better than that for *T. urticae* [9]. That may be due to the AB-1 inhibition of mitochondrial complex II in *P. citri* at lower concentrations is greater than that in *T. urticae* and the metabolic rate to AB-1 from cyflumetofen in *P. citri* is higher than that in *T. urticae*. In addition, cyflumetofen showed considerable negative effects on *T. urticae*life-tables but it had low effects on *Phytoseiulus persimilis*Athias-Henriot [10].

Also, cyflumetofen and spirodiclofen had significant effects on *P. citri* biological parameters including developmental time, survival rate, and fecundity, which considerably reduced net reproductive rate (R_0), finite rate of increase (λ) and intrinsic rate of increase (r) [12].

Also, the spirodiclofen gave moderate reduction efficacy in the population of *P. citri.* movable stages. These outcomes are consistent with a report in 2007 which stated thatspirodiclofen showed good reduction efficacy of *P. ulmi*, but a lower efficacy was only recorded in the evaluation three days after treatment in the first year of experiment [13]. This initial effect of spirodiclofen comes as a result of its slower initial activity against spider mite females: after direct treatment, most female's take several days to die, but fertility and fecundity are significantly decreased [14, 15, 16].

Table 1. The efficacy of five acaricides on *Panonychuscitri* movable stages on lemon trees during 2019 and 2020 seasons

Treatments	Mean numbers of *P. citri* movable stages / lemon leaf during 2019 season				Mean after spraying
	Before spraying	After 3 days	After 7 days	After 14 days	
Check	9.51^{b}	10.25^{a}	12.83^{a}	15.19^{a}	12.75^{a}
Cyfumetofen	9.59^{b}	2.65^{b}	0.81^{c}	1.30^{c}	1.59^{c}
Pyridaben	10.41^{ab}	3.28^{b}	1.90^{b}	2.53^{b}	2.57^{b}
Spiromesifen	9.56^{b}	2.50^{b}	1.44^{bc}	2.23b^{c}	2.05^{bc}
Spirodiclofen	10.75^{ab}	2.29^{b}	1.06^{c}	1.44^{c}	1.60^{c}
Fenpyroximate	12.24^{a}	2.80^{b}	1.69^{bc}	2.41^{bc}	2.30b^{c}
F	3.35	162.74	534.88	406.49	512.48
P	0.03	0.000	0.000	0.000	0.000
	Mean numbers of *P. citri* movable stages / lemon leaf during 2020 season				
Check	11.39^{a}	12.61^{a}	14.11^{a}	15.96^{a}	14.23^{a}
Cyfumetofen	10.96^{a}	2.44^{b}	1.03^{c}	1.93^{c}	1.80^{c}
Pyridaben	11.08^{a}	3.29^{b}	2.31^{b}	3.16^{b}	2.92^{b}
Spiromesifen	11.53^{a}	3.31^{b}	1.91b^{c}	2.79b^{c}	2.67^{b}
Spirodiclofen	11.66^{a}	2.38^{b}	1.09^{c}	1.75^{c}	1.74^{c}
Fenpyroximate	11.98^{a}	3.16^{b}	1.63b^{c}	2.66^{bc}	2.48^{bc}
F	0.78	151.61	610.87	495.90	669.83
P	0.141	0.000	0.000	0.000	0.000

The means in each column with the same letters are not significantly different using Tukey's HSD, ($P \leq 0.05$).

Table 2. The reduction percent of *P. citri* movable stages after pesticides application on lemon trees during 2019 and 2020 seasons

Acaricides	Reduction % of *P. citri* during 2019 season			
	After 3 days	After 7 days	After 14 days	Mean
Cyfumetofen	74.35	93.71	88.89	85.65
Pyridaben	70.81	86.47	84.81	80.70
Spiromesifen	75.74	88.85	85.43	83.34
Spirodiclofen	80.25	90.43	88.57	86.41
Fenpyroximate	78.77	89.77	87.65	85.40
	Reduction % of *P. citri* during 2020 season			
Cyfumetofen	79.92	92.46	87.47	86.62
Pyridaben	73.20	83.15	79.63	78.66
Spiromesifen	74.05	86.61	82.75	81.14
Spirodiclofen	81.61	92.48	89.30	87.79
Fenpyroximate	76.16	89.05	84.14	83.11

In this study, Spiromesifen and Fenpyroximate gave moderate efficacy decrease in the population of *P. citri* movable stages during 2019 and 2020 seasons. Similarly, Marčić in 2007 stated that the fenpyroximate was achieved 43% efficacy 45 days after treatment [13]. Spiromesifen is considered suitable for IPM systems in apple orchards as a supporting and correcting agent for mite population management [17].

Based on the data obtained from evaluating field efficacies of the five acaricides on *P. citri*, aiming to provide basic information for improving the management programs of mite in citrus groves, we found that the cyfumetofen and spirodiclofen are more effective in reducing population of *P. citri*, therefore, we recommend that they should be used in the control programs of citrus red mite.

3.2. LC-ESI-MS/MS

An ESI source adjusted in positive mode was used connectedly with direct continuous infusion to optimize all tested pesticides in full scan mode before and after fragmentation to choose the best precursor and produce ions than optimized DP (decluster potential) and CE (collision energy) to get the highest sensitive parameter. Two MRM were chosen for each compound for quantitation and conformation and collected with their optimized potential to build an acquisition method performed with HPLC separation conditioning to study pesticides recovery.

All reported MRM's were in accordance with those obtained by EURL Datapool [18] MRM's. The injection of 2 µl from extract solution into LC-MSMS prevents sensitivity from matrix effect.

3.3. Matrix effect

Analyte signal deviation caused by the presence of some sample components in final extract determined as matrix effect. However, these effects can be found as signal enhancement or suppression; so, we need to correct this deviation when data obtained.

Pesticides standard solutions matching with sample matrix can describe the amount lost by suppression; so, we can correct results. During this recovery study all spiked samples were injected with compound standards in solvent (for calibration) plus another one in matrix (Fig. 1) to remove suppression effect.

3.5. Method validation

Method validation protocols in Eurachem guideline were used for in-house method validation [19]. Also, the acceptance criteria were in use from the Document Guidance of Analytical Quality Control and Method Validation for Pesticide Residues Analysis in Food and Feed Procedures [20].

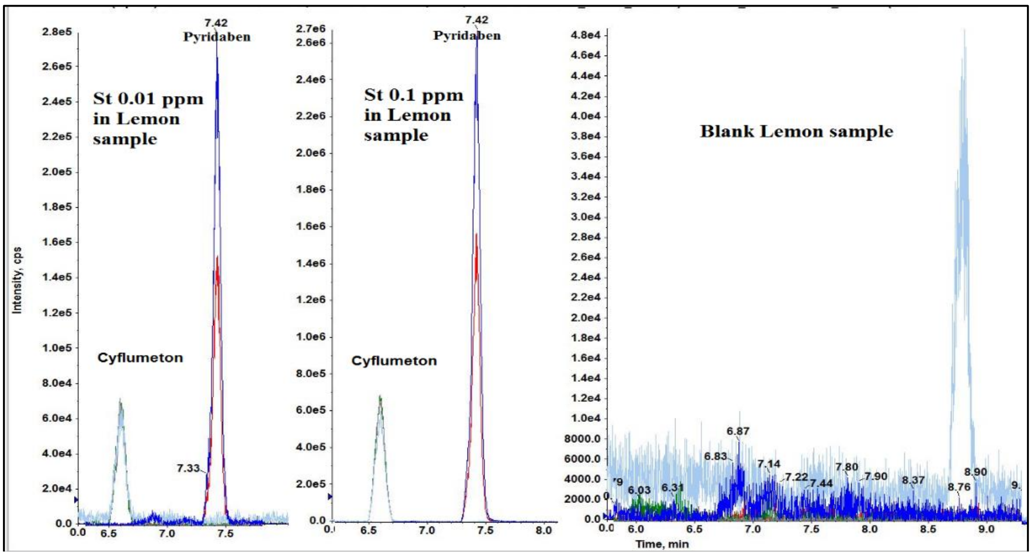

Fig 1. Tested pesticides chromatogram includes blank sample and standard matched sample

3.4. Limit of quantitation

The minimum concentration of analyte (LOQ) was that can be determined with repeatability (acceptable precision) and recovery below the quantified conditions of the test. LOQ was valued using repeated spiked samples at the estimated lowest quantitation level of 0.01 mg/kg. Relative standard deviation (RSD %) and recoveries at LOQ are presented in Table 3.

3.6. Method linearity

Six levels of calibration curve; 0.001, 0.002, 0.005, 0.01, 0.05 and 0.1 µg/ml were used for quantitative analysis which covered all determined concentrations. The correlation coefficient was found to be 0.9982, 0.9992 for standards curve in between 0.001 to 0.1 ppm for pyridaben and cyflumetofen respectively.

3.7. Recovery test

The analytical method procedures were established using six spiked replicates at three different concentration levels (0.01, 0.05 and 0.1 mg/kg) for 2 pesticides using LC-MS/MS.

Overall good recoveries were showed in Table 3 showed an in between 92% and 117% for Pyridaben and Cyflumetofen pesticides with ≤ 4.2% RSD. Good recoveries were found due to method computability for analysis with both analytes due to physicochemical properties of tested pesticides. These properties like log octanol water (cyflumetofen: 4.3 at 25 °C and pyridaben: 6.37 23 °C) support extraction efficiency as moderately nonpolar analytes, and PKa (-4.19, -2.69 for cyflumetofen and pyridaben respectively) led to excellent ionization which supports sensitivity using mobile phase pH 4.

Uncertainty measurements were done for tested pesticides, it was found to be as follow: Uprec, Relative standard uncertainty due to precision investigates (4.2%) calculated using RSD of repeated spiked samples results. Ubias, the bias of the analytical procedure (1.9%) was studied for the lowest mean recovery (88%) (SD = 2.3% and n = 6) for pyridaben while t tab and t calc were found to be 2.57 and 7 respectively for 5 degrees of freedom.

The possible uncertainties attributable to reference standard preparation (analyte standard purity, balance, pipettes, micropipettes, solvents and volumetric flasks) were 0.7%.

Finally, the combined uncertainty (UC) and expanded uncertainty were found to be 2.9% and 6% respectively when using 95% confidence level and a coverage factor (k = 2).

Table 3. Tested pesticides determination method and recovery evaluation

	mean% ± RSD% (0.01, 0.05 and 0.1 mg/kg)			Q_{type}	RSD_{pooled}
Cyflumeton	111 ± 4	101 ± 2	92 ± 2	101.3%	2%
Pyridaben	111 ± 4.2	99 ± 1.4	88 ± 2.3	99.5%	0.03%
			Multiple reaction monitoring		
	RT (min)	DP	Precursor	Fragments	CE
Cyflumeton				249	19
	6.67	71	465	173	49
Pyridaben				309	19
	7.63	64	365	147	31

RT (retention time), DP (declaster potential) and CE (collision energy).

Table 4. Cyflumetofen and pyridaben dissipation rate

Lemon sample residues analysis				
	Cyflumetofen		Pyridaben	
Time/Day	Residues mean (mg/kg)	Dissipation %	Residues mean (mg/kg)	dissipation
Initial	1.360	0.00	1.704	0.00
1	0.984	27.65	0.860	36.76
3	0.884	35.00	0.652	52.06
5	0.740	45.59	0.648	52.35
7	0.692	49.12	0.520	61.76
10	0.584	57.06	0.452	66.76
15	0.136	90.00	0.293	78.46

Table 5. Cyflumetofen and Pyridaben regression equation, correlation coefficient, half-life ($t_{1/2}$)

	Regression equation	Correlation coefficient (R^2)	$t_{1/2}$ (days)
Cyflumetofen	$C_t=1.3906e^{-0.13t}$	0.871	5.33
Pyridaben	$C_t=1.1121e^{-0.095t}$	0.838	7.29

The first-order kinetic model (Ct =C0e_kt) was subjecting to investigate data while Ct is the concentration at time t, C0 is the initial concentration, and k is the dissipation rate constant.

Result showed in table 4 and 5 showed a dramatical dissipation in both pesticides. The reduction in residue concentration was 90% in cyflumetofen after 15 days (45.3% from EU MRL) while pyridaben was still 78.46 % (97.6 % from EU MRL). On the other hand, the first pesticide was found in initial determination 1.3 time more than the second, when the spray concentrations were 20% SC (40 ml/100 L) for cyflumetofen and 15%EC (150 ml/100L) for pyridaben. But after 3 days pyridaben residue was dissipated with 52% while cyflumetofen residue was 65% from initial concentration that can explain the efficacy power of the second acaricide on tested mites. Besides, pyridaben was more stable for 15 days (78.4% dissipation rate) posing a threat to the environment in comparing with cyflumetofen, this stability can be cleared due to their physicochemical properties. A high vapors pressure was found in cyflumetofen (< 5.9x10-6 Pa at 25 °C (98.4%) [21] while pyridaben (0.25 mPa at 25 °C) (pesticides manual) which explained the fast losing of pyridaben in first 3 days. In addition, the water solubility of cyflumetofen was double that found in pyridaben (28 and 12 ug/L) [22, 23] which led to more distribution concentration in the inside area of lemon fruits (acidic water content). That could explain why pyridaben showed more stability in total area of fruits due to its ability to be stored in outside area of lemon more than cyflumetofen (fatty part).

Moreover, the dilution in pesticide concentration caused by the growth of citrus may also play a significant role in analytes concentration loss by time, which led to the similar dissipation rate in both pesticides, but pyridaben was still more persistence. The half-lives of cyflumetofen (5.33 d) when compared with that in Pyridaben (7.29 d).

These half-lives of pyridaben were also different from that reported in Zhejiang (12.8 d) and in Guizhou (13.8 d) but similar to others in 2011 at both two sites (3.32 and 8.11 d) informed in 2012 [24].

Also, these data were in agreement with PHI recommended by BASF Corporation approved by EPA for their cyflumetofen product (at least 7 days) and 14 days for pyridaben in lemon by European Food Safety Authority (EFSA) [25, 26, 27].

4. Conclusion

Cyfumetofen and spirodiclofen were found to be more effective in *P. citri* population reduction which supports their use in the control programs of citrus red mite. Moreover, cyflumetofen give less dissipation time with good efficacy which led to good control and less risk.

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