

Dissipation of Propamidine Fungicide Residues in Greenhouse Tomato

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Abstract

A method of reverse phase high performance liquid chromatography (RP-HPLC) was established to analyze the dissipation of propamidine residue in tomato. Residue of propamidine was extracted from tomato using methanol buffered and determined by RP-HPLC with UV detection at 262 nm. The results showed that the average recoveries of the samples fortified with propamidine at the concentration range of 25 to 300 mg kg⁻¹ ranged from 87.972 to 106.341% with a relative standard deviation ranged between 0.169 to 3.503%. Initial deposit ranged from 2.45 to 5.70 mg kg⁻¹. The dissipation of propamidine in tomato followed the first order kinetic equation. The dissipation rate constants in tomato treated with recommended and double recommended dose applied at 4 times and 2 times ranged from 0.110 to 0.151 days, and the corresponding half-lives from 4.589 to 6.300 days. At the day 14 after the last application the residue concentrations of propamidine in tomato ranged from 0.42 to 0.54 mg kg⁻¹ from the two blocks for all treatments. These propamidine residues dissipated below the limit of detection of 0.07 mg kg⁻¹ 28 days after the last treatment. The results presented in this work and the low toxicity of propamidine for environment proved that propamidine will not pose any residual toxicity problem after 14 days of application and tomato fruits could be used safely for human consumption.

Keywords: propamidine fungicide, RP-HPLC, residue, dissipation, tomato

1. Introduction

The use of pesticides in agriculture is necessary to combat a variety of pests that could destroy crops and to improve the quality of the food produced. Agricultural use of pesticides plays a beneficial role in providing a plentiful, low cost supply of high quality fruits and vegetables. On the other hand, as a consequence of this use, the presence of residues in food that was critical elements of overall population health is unavoidable and pesticide residues in food is of great importance in the evaluation of food quality (Goto et al., 2003). The cultivation of tomato especially in greenhouse conditions demands frequent application of a large number of pesticides to control a variety of insects and diseases, but over time both insects and diseases have developed resistance to such pesticides. Grey mould is one of the most serious vegetable diseases in greenhouses in China (Ji et al., 1998). With the intensive use of pesticides in greenhouse crops, residues may be accumulated at levels higher than those permitted by the China pesticide management legislation or international Maximum Residue Levels (MRLs).

Propamidine is a novel, systemic plant fungicide which is mainly used to control various diseases caused by botrytis fungi on fruits and vegetables under field and greenhouse conditions, spraying of propamidine fungicide at 90-180 g a.i. ha⁻¹ had higher control effectiveness to disease than fungicides such as procymidone, pyrimethanil and dimethachlon at 450 g a.i. ha⁻¹ (Chen et al., 2005). It was reported that propamidine is of low toxicity, safe to human being and environment. However no published data are available concerning the residues of propamidine in plant extracts. Therefore to guarantee the use of propamidine in field according to good agricultural practices and to protect consumer health residual analysis study of propamidine in tomato become indispensable to know the residual level, the rate of dissipation and the half lives ($t_{1/2}$) of propamidine in tomato. Hence, an ultrasonication-assisted solvent extraction method using reverse phase high performance liquid chromatography was determined and validated in this study to perform propamidine fungicide residues determination in tomato for understanding the behaviour of propamidine fungicide residue in tomato fruit grown under greenhouse conditions.

2. Materials and Methods

2.1 Solvents, Reagent and Pesticide

The organic solvent, methanol used was HPLC grade and was purchased from Laiyang Shuangshuang Chemical Co., Ltd (China), sodium dodecyl sulfate (SDS), phosphoric acid, de-ionized water and the propamidine, TC > 95% were provided by Research and Development Center of Biorational Pesticide (RDCBP).

De-ionized water and methanol were degassed by ultrasonic cleaner bath. All samples and solvents were filtered through Millipore membrane filters (0.45 μm pore size) before injection on the column. The analytical stock solutions of the pesticide were prepared in methanol and stored in a volumetric flask maintained at 4°C.

2.2 Field Trial

Tomato variety Jinpeng No.1 was grown during the summer 2012 in a commercial Chinese greenhouse at the Xi Xiao-zai village (Shaanxi, China) located at fifteen kilometer in the west of Northwest A & F University. A randomized complete block design with 2 blocks, each block containing 5 plots; keeping a distance of 70 cm between rows and 30 cm within rows was performed for experiment. Propamidine was not applied to the test plots during this experiment. Irrigation and all cultural practices were carried out as local practices (OCDE, 2009). After formation of fruits the plots were treated with the commercial formulation of propamidine (TC > 95%) with a hand sprayer at the recommended rate 90 g a.i. ha^{-1} , double recommended rate 180 g a.i. ha^{-1} , and zero dose 0 g a.i. ha^{-1} sprayed with water as a control treatment (Tao et al., 2010; Zhou et al., 2004).

2.3 Sampling and Storage

About 1kg of tomato fruits was randomly collected into plastic polyethylene bags from each plot as representative samples. The fruits also were randomly sampled from the plants approximately 0 days (2 hours), 1, 3, 7, 14 and 28 days after last application of propamidine. The Samples were immediately transported to the laboratory and homogenized. Two representative subsamples of 4 g were taken for each plot. One subsample was prepared for chromatographic analysis and the other was placed into glass containers and frozen at -40°C, temperature at which enzymatic degradation of pesticide residues is usually extremely slow (CAC/GL 40, 1993; FAO, 1986) prior to use; the frozen samples were thawed at 4°C overnight.

2.4 Instrumental

The Experiments were conducted on Shimadzu Liquid Chromatograph- system equipped with a LC-6AD pump, a SCL-10A vp controller, a detector SPD-10A uv, and 10 μm Hypersil BDS C_{18} Column (4.6 mm \times 250 mm). Quantitative and qualitative analysis were conducted using the optimized chromatographic condition performed based on the report of Yuan et al. (2007) but the mobile phase was modified by adding the Phosphoric acid buffer.

2.5 Preparations of Standard Solutions

Pesticide stock standard solution (1000 $\mu\text{g ml}^{-1}$) of propamidine fungicide ($\text{C}_{17}\text{H}_{20}\text{N}_4\text{O}_2 \cdot 2\text{HCl}$) was prepared by dissolving 0.0250 g of the pesticide in 25 mL of methanol and stored in volumetric flask 25 mL in freezer at 4°C. Working standard solutions (50 - 600 $\mu\text{g mL}^{-1}$) were prepared by appropriate dilutions of the stock standard solution immediately before used. Matrix- extract standard solutions are the standards prepared in blank matrix. After extraction procedures, the extract was evaporated and reconstituted with standard solution (Kumar, 2010) to give standards of the required concentration.

2.6 Preparation of Fortified Sample

Tomato fruits free of propamidine were first washed and triturated using mortar and pestle. Sample fortification was made by adding 2 mL of appropriate concentration of the propamidine working standard solution to a 4 g sample, letting it stands for a few minutes before extraction to allow the spiked solution to penetrate into the matrix.

2.7 Sample Extraction

Sample extraction was based on ultrasonication-assisted solvent extraction method of Devanand et al. (2005); Shen et al. (2005) and Vagi et al. (2007). A volume of 10 mL of extraction solvent was added to a 4 g tomato sample and sonicated at room temperature in an ultrasonic cleaner bath for 15 min. This step was repeated two times for a total of 20 mL of extraction solvent. The extract was then separated by centrifugation at 8000 rpm for 15 minutes at 20°C using a high speed refrigerated centrifuge and the supernatant obtained was collected into an Erlenmeyer flask. The obtained organic subtract was subject to another extraction-centrifugation with 10 mL of extraction solvent. The extract from the first and the second extraction were collected in the same flask and the

combined extract was passed through a 0.45 µm Teflon filter for chromatographic analysis. The same procedure was used for propamidine extraction in tomato fortified and real sample.

2.8 Validation Method

The validation procedure was based on the investigation of the following parameters: Selectivity, analytical curve and linearity, recovery and accuracy, precision (repeatability), matrix effect, stability, detection limit and quantification limit.

The selectivity was carried out by comparing representative chromatograms of standard working solution, blank extract and fortified sample. Calibration curves were carried out with calibration solutions at 5 concentration levels (50, 100, 200, 400, and 600 µg mL⁻¹) prepared in methanol and in methanol containing matrix extract. The solutions were injected in RP-HPLC system in triplicate and chromatograms were recorded. Calibration curves were obtained by plotting average peak area versus concentrations.

The linearity based on the construction of the analytical curves was obtained using both analytical standard solutions. Accuracy of a method is defined as the closeness of a measured value to the true value. The accuracy of the method was tested (% recovery and % RSD of individual measurements) by analyzing tomato samples without the interest pesticide residue. Tomato samples were fortified at five concentration levels: 25, 50, 100, 200 and 300 mg kg⁻¹. Each concentration level was extracted and analyzed three times. Repeatability of the instrument was evaluated by calculating the relative standard deviation for six injections (Putheti & Leburu, 2008; Rahman et al., 2010) of propamidine working standard solution (200 µg mL⁻¹) in the chromatographic system.

Matrix effect was performed by comparison between calibration curves prepared in pure methanol and methanol containing tomato extract prepared as described above. Calculation was made using the following equation used by Cardoso et al. (2011).

$$\text{Matrix effect (\%)} = \frac{\text{Slope}(x_1) - \text{Slope}(x_2)}{\text{Slope}(x_2)} \times 100$$

Where x_1 = slope of the curve obtained by injection of the analytical solutions prepared in the extract and x_2 = slope of the curve obtained by injection of the analytical solution prepared in methanol.

The stability of propamidine in solvent and solvent containing extract of tomato was carried out by evaluation of the percentage deviation in chromatogram peak response. Propamidine working standard solution (200 µg mL⁻¹) and tomato fortified sample solution concentration level 100 mg kg⁻¹ freshly prepared were injected in RP-HPLC system to check the freshly chromatogram peak area. Both solutions were further stored in a freezer at 4°C, room temperature at 25 ± 2°C and oven at 54°C. Finally, After 3 days and 14 days of storage the standard working solution and sample solution were reanalyzed and the percentage deviation in peak response was evaluated.

Limit of detection (LOD) and limit of quantification (LOQ) of the method were determined using signal-to-noise ratio method (U.S. FDA, 1996; Walfish, 2006; Assis et al., 2011); in which the lowest concentration detected or measured should be the one where the peak height is 3 and 10 times respectively, the peak height of the equipment noise at the retention time of the peak of propamidine.

2.9 Statistical Analysis

The dissipation kinetic of the propamidine in tomato was determined by plotting residue concentration against time and the maximum squares of correlation coefficients found were used to determine the equations of best fit curves. For all the samples studied, exponential relationships were found to apply, corresponding to first order rate equation. Confirmation of the first order kinetics was further made graphically from the linearity of the plots of Log Concentration against time. The rate equation was calculated from the first order rate equation:

$$C_t = C_0 e^{-kt}$$

Where, C_t represents the concentration of the pesticide residue at time t , C_0 represents the initial concentration and k is the rate constant in days⁻¹.

The half-life ($t_{1/2}$) was determined from the k value for each experiment, being as calculate by Wang et al. (2007) and Liang et al. (2011).

$$t_{1/2} = \frac{\ln(2)}{k}$$

3. Results and Discussion

3.1 Chromatographic Conditions

Several tests were carried out based on the report of Yuan et al. (2007) to determine the instrumental condition. The best response analytical signals of propamidine fungicide was achieved with a mobile phase consisting of 0.1% phosphoric acid, methanol/de-ionized water (V/V, 80:20) solution containing 3.0 mmol/L SDS. For the HPLC system the best results of the analysis were obtained with a 10 μm Hypersil BDS C_{18} column (4.6 mm \times 250 mm) at the flow rate of 1.0 mL min^{-1} and the column temperature of 25°C; injection volume was 20 μL and the UV detector wavelength was set at 262 nm using an isocratic elution system.

3.2 Validation Method

The selectivity was evaluated by comparing representative chromatograms of standard working solution (100 mg L^{-1}), blank extract and fortified sample (100 mg kg^{-1}). The results are shown in figure 1.

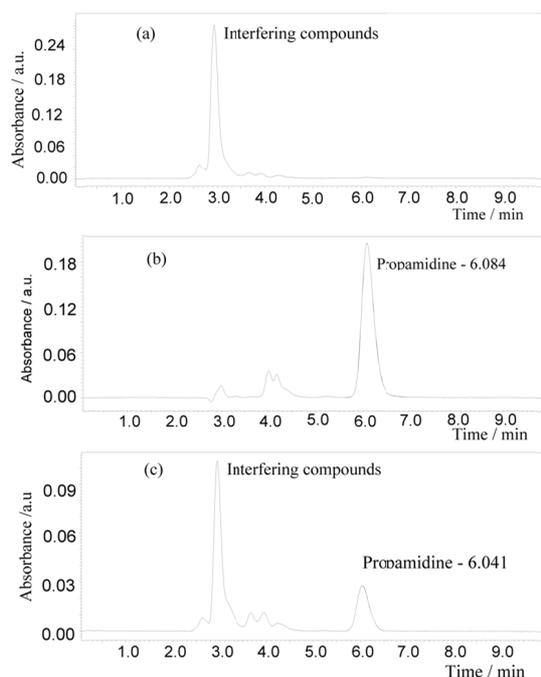


Figure 1. Chromatogram showing the retention times of propamidine in selectivity study. Chromatogram Figure 1 a, b, c presenting respectively the chromatogram of tomato blank sample, propamidine from solvent standard solution (100 mg L^{-1}), and propamidine from tomato fortified (100 mg kg^{-1}) sample solution

The absence of signal at the retention time of propamidine indicates that no interfering compounds were present. The external calibration curve (Figure 2) was obtained by plotting average peak area versus concentrations of interest pesticide.

The values obtained for the analytical curves with the solutions prepared in solvent (methanol) and in solvent containing matrix extract demonstrated satisfactory linearity with linear regression equation $y = 51987x + 311379$, $y = 50737x + 530132$ and correlation coefficients 0.9999, 0.9997 for the pesticide in solvent and solvent with matrix extract respectively. The comparison test of the intercept performed with zero showed that the intercept is not significantly different from zero at $\alpha = 5\%$ probability in the two cases with T_{cal} for equation in standard solution (SC) and standard containing matrix extract (SMC) respectively, $\text{SC } T_{\text{cal}} = 3.0647$ and $\text{SMC } T_{\text{cal}} = 2.92321 < T_{\text{tab}} = 3.1824$.

For quantification of propamidine, since the intercept was not significantly different from zero at $\alpha = 5\%$ probability and the calibration function $y = a + bx$ didn't give satisfactory recovery the slope of regression line was recalculated by forcing the calibration curve through the origin. The linear regression obtained $y = 52721x$ with $R^2 \geq 0.999$ was used for quantification of propamidine in fortified and real sample.

The accuracy of the method was tested by analyzing tomato samples free of propamidine fortified at five concentration levels ranged from 25 to 300 mg kg⁻¹. Each concentration level was extracted and analyzed three times and the results are shown in table 1.

Table 1. Accuracy data of the method (n=3)

Accuracy of the test method (Recovery studies)					
Fortification level (mg/kg)	Amount		% Recovery	Average recovery	RSD (%)
	Added (mg)	Found (mg)			
25	0.100	0.105	105.368	106.030	1.503
	0.100	0.105	104.874		
	0.100	0.108	107.848		
50	0.200	0.204	101.997	101.947	1.666
	0.200	0.200	100.224		
	0.200	0.207	103.619		
100	0.400	0.432	108.026	106.341	2.972
	0.400	0.433	108.301		
	0.400	0.411	102.694		
200	0.800	0.741	92.599	94.566	3.503
	0.800	0.787	98.391		
	0.800	0.742	92.708		
300	1.200	1.057	88.095	87.972	0.169
	1.200	1.056	88.008		
	1.200	1.054	87.813		

Recovery values of propamidine from tomatoes fortified sample were 87.972 to 106.341% with RSD (0.169 - 3.503%) less than 10% (European Commission, Directorate General Health and Consumer Protection [EC DGHC], 2010) indicates that the method was accurate.

Precision was studied by performing repeatability studies expressed as RSD. For retention time and peak area the values of RSD were respectively 0.094 and 4.982%. The RSD of repeatability lower than 10% (EC DGHC, 2010) indicates that the method was precise. Matrix effect was performed by comparison between calibration curves prepared in pure methanol and in tomato matrix extract. The signal suppression/enhancement (C %) found in this experiment was -2.43%, located in the range -20% < C % < 20% (Cardoso, 2011) indicating that the matrix effect was not significant. For stability, the values of percentage deviation in the peak response from initial data were located in the acceptance criteria ranged from -20% to 10% (VICH, 2011) so propamidine was found to be stable in methanol and in tomato extract solution in freezer, room temperature and oven condition for the period of 14 days (period of assay). Instrumental LOD based on S/N of 3:1 and LOQ based on S/N of 10:1 were 0.07 mg kg⁻¹ and 0.2 mg kg⁻¹ respectively.

3.3 Dissipation of Propamidine in Tomato

3.3.1 Propamidine Residue Levels in Fresh Harvested Tomato

Field tomatoes were treated with propamidine commercial formulation 4 times and 2 times at the recommended 90 g a.i. ha⁻¹ and double recommended rate 180 g a.i. ha⁻¹ at 7 day intervals. Tomatoes samples were collected at 0 day (2 h), 1, 3, 7, 14 and 28 days post spraying. Table 2 shows the results of analysis using RP-HPLC system.

The initial residue concentration of propamidine in tomato sample collected from block 1 and block 2 were respectively: for the plot treated at normal rate at 4 times (3.17; 2.73 mg kg⁻¹); double dose at 4 times (5.70, 5.47 mg kg⁻¹); plot treated at normal rate at 2 times (2.92, 2.45 mg kg⁻¹) and plot treated at double rate at 2 times (3.06, 3.37 mg kg⁻¹). We have not found residues of propamidine on sample from control plot.

Residues concentration decreased rapidly on the first days. At the day 14 after the last application the residue concentrations of propamidine in tomato ranged from 0.42 to 0.54 mg kg⁻¹ from the two blocks for all treatments. There was no significant difference in dissipation pattern between the two rates of applications (P > 0.05) suggesting that the dissipation of propamidine was independent of the dose and number of application. Similar results were reported by Saimandir and Gopal (2012) with indoxacarb on eggplant fruits. The residues were non detectable at day 28.

Table 2. Residues values ($\text{mg kg}^{-1} \pm \text{SD}$) of propamidine found in greenhouse tomatoes at various interval times (days) after application at recommended dose $90 \text{ g a.i. ha}^{-1}$ and double recommended dose $180 \text{ g a.i. ha}^{-1}$ ($n=2$)

Block	Dose g a.i. ha^{-1}	Application number	Residues ($\text{mg kg}^{-1} \pm \text{SD}$) at days after last application					
			0	1	3	7	14	28
Block 1 (Treatments)	90	4	3.17 ± 0.76	2.04 ± 0.04	1.78 ± 0.11	0.85 ± 0.07	0.50 ± 0.05	nd
	180	4	5.70 ± 0.37	2.33 ± 0.07	2.00 ± 0.06	1.37 ± 0.09	0.54 ± 0.02	nd
	90	2	2.92 ± 0.33	2.03 ± 0.04	1.84 ± 0.11	0.57 ± 0.18	0.47 ± 0.001	nd
	180	2	3.06 ± 0.14	1.71 ± 0.05	1.59 ± 0.08	0.52 ± 0.01	0.42 ± 0.05	nd
Block 2 (Treatments)	90	4	2.73 ± 0.09	1.86 ± 0.01	1.83 ± 0.13	0.81 ± 0.13	0.47 ± 0.03	nd
	180	4	5.47 ± 0.31	2.72 ± 0.02	2.37 ± 0.03	1.38 ± 0.05	0.51 ± 0.05	nd
	90	2	2.45 ± 0.03	1.81 ± 0.07	1.54 ± 0.01	0.91 ± 0.07	0.50 ± 0.06	nd
	180	2	3.37 ± 0.01	1.84 ± 0.02	1.18 ± 0.08	0.87 ± 0.15	0.48 ± 0.04	nd

nd: non detectable.

3.3.2 Dissipation of propamidine in tomato

Figure 3 shows the dissipation curve of propamidine in tomato from different plots sprayed at recommended rate ($90 \text{ g a.i. ha}^{-1}$) and double recommended rate ($180 \text{ g a.i. ha}^{-1}$).

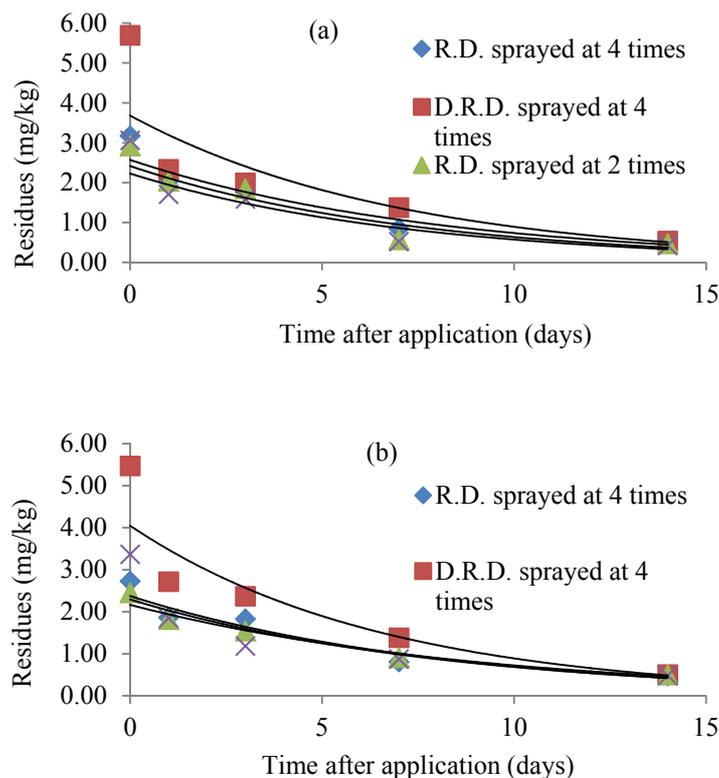


Figure 3. Dissipation curve of propamidine in tomato after repeated applications at different doses; the curves in Figure 3 a & b presents the dynamic curves of propamidine on block 1 and block 2 for recommended rate $90 \text{ g a.i. ha}^{-1}$ and double recommended dose $180 \text{ g a.i. ha}^{-1}$.

R.D., recommended dose; D.R.D., double recommended dose

The results presented in Table 2 and Figure 3 had shown a rapid decrease of residues concentration after the first time of sampling. Decrease in levels of residues of propamidine was found as an exponential decrease in the residue concentrations over the period of time and followed first-order rate of dissipation. The dynamics could be described by the following equation $C_t = C_0 e^{-kt}$. Table 3 shows the first-order kinetics equation, half-life and other parameters for propamidine dissipation in tomato.

Table 3. Half-life and other statistical parameters for propamidine dissipation in the tomato in green houses conditions after 4 and 2 times' application at the dose 90 -180 g a.i. ha⁻¹; (n=2)

block	Dose g a.i.ha ⁻¹	Application number	Kinetics Equation	Determination Coefficient (R ²)	Constant k (days ⁻¹)	Half-life (days)
Block 1 (Treatments)	90	4	$y = 2.570e^{-0.125t}$	0.942	0.125	5.544
	180	4	$y = 3.684e^{-0.142t}$	0.888	0.142	4.880
	90	2	$y = 2.415e^{-0.134t}$	0.862	0.134	5.172
	180	2	$y = 2.228e^{-0.136t}$	0.846	0.136	5.096
Block 2 (Treatments)	90	4	$y = 2.374e^{-0.123t}$	0.950	0.123	5.634
	180	4	$y = 4.048e^{-0.151t}$	0.949	0.151	4.589
	90	2	$y = 2.164e^{-0.110t}$	0.979	0.110	6.300
	180	2	$y = 2.292e^{-0.121t}$	0.872	0.121	5.727

The first-order kinetic equations determination coefficients (R²) were ranged from 0.846 to 0.979. The study revealed that propamidine dissipation rate in tomato was independent of initial deposit. The theoretical half-life of propamidine at the recommended and double recommended dose showed less variations on the trial for both cases (4 times and 2 times applications). The half life values ranged from 4.589 to 6.300 days, Hu et al. (2005) have reported similar half-life time (T_{1/2}: 6.37 days) with 2- allyphenol a new fungicide used on tomato against grey mould caused by *botrytis cinerea* in tomato.

The decline of the residue may be attributed to growth dilution between application and sampling, volatilization that occurs during the first days following application, transfer of propamidine from plant to soil due to the systemic propriety of propamidine, sunlight UV radiation, or other complex conditions. Further studies are required to assess the breakdown products, exposure risk, and the environmental fate of propamidine.

4. Conclusion

The present study revealed that the method is suitable for the determination of propamidine fungicide residues in tomato. The system was linear in the concentration range of 0.05 to 0.6 mg ml⁻¹; interfering peaks at elution times and significant matrix effects were not observed for the interest pesticide. Intermediary precision, recovery and accuracy have proved that the method is precise and accurate, also the limit of detection and quantification were satisfactory. Concerning residues dissipation study, initial deposit of double dose sprayed at 4 times were 5.70 mg kg⁻¹; 5.47 mg kg⁻¹ respectively in block 1 and 2. After 14 days of application the initial deposit dissipates respectively to 0.54 mg kg⁻¹ (90.55%) and 0.51 mg kg⁻¹ (90.74%). The longest half -life was 6.300 days. Hence the MRL of propamidine TC > 95% has not set up by China pesticide management legislation and the FAO/WHO.

According to the results presented in this work the method could be useful for the establishment of MRL of propamidine and routine residues analysis to ensure food safety routine residue analytical methods.

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