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Preservative Effects of Strobilurin Fungicides on Citrus Storage Diseases and Residue Safety Assessment

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Abstract

Ethofenprox, pyraclostrobine, trifloxystrobin and enestroburin are strobilurin fungicides that can effectively control fungal diseases caused by ascomycetes, zygomycetes, imperfect fungi, etc. With the purpose of guaranteeing the safe use of strobilurin fungicides in the prevention and treatment of citrus diseases, toxicity and control effects of the fungicides on citrus storage diseases and GC-MS (gas chromatography-mass spectrometry) were applied in this study to determine its residual safety. The results indicated that ethofenprox, trifloxystrobin, pyraclostrobine, and enestroburin had excellent inhibitory effects on citrus storage diseases at concentration of 200-400 μg/mL. Degradation dynamics of 4 fungicides during the storage period of citrus could be expressed as the first-order kinetics equation. The fungicides could penetrate into flesh through peels slowly. Therefore, the residue content of the fungicide on peels was higher than that in the flesh of the same citrus. After citrus fruits were treated for 90 d, the residues were lower than the maximum residue limits in all the countries, so the citrus were safe. This investigation provided the theoretical guidance and technical support for the quality evaluation of citrus products.

Keywords: strobilurin fungicide, preservative effects, citrus storage disease, residue, safety assessment

1. Introduction

Citrus is the most widely planted fruit in the world and takes the second place in China (Fan et al., 2010; Liu & Wu, 2014). The citrus fruit may be infected with different kinds of fungi during storage, which leads to the serious fruit decay and causes economic loss of 20%-30% per year, or even 50% sometimes (Liu & Wu, 2014). Particularly, it can be infected by more than 10 kinds of microbes, such as *Penicillium italicum* Sacc and *P. digitatum* Wehmer which are largely responsible for 70%-80% fruit decay (Liu & Wu, 2014). Serious losses occur after harvest during marketing and storage of citrus fruit mainly due to green mold caused by *P. digitatum*, followed by blue mold and sourrot respectively caused by *P. italicum* and *Geotrichum candidum* (Sholberg et al., 2005; Ma et al., 2015). Inhibiting the infection and proliferation of *P. italicum* and *P. digitatum* is of great importance, through which we can reduce the unnecessary loss of harvested citrus. The biological control has been reported to be effective. Nevertheless, the control of plant diseases is mainly dependent on chemical fungicides (Russell, 2005). Toxic chemical fungicides are massively used to control postharvest storage losses. Postharvest green and blue molds of citrus are controlled by sodium bicarbonate, imazalil, thiabendazole, pyrimethanil and fludioxonil (Adaskaveg et al., 2004; Kanetis et al., 2007; Liu et al., 2007, 2014; Ma et al., 2015). Chemical control of these pathogens on a commercial scale is mainly based on postharvest treatments with strobilurin fungicides, which are the solely registered fungicides applied legally to citrus fruits in the European Union (Kanetis et al., 2007, 2008).

Strobilurin fungicide performance against *P. digitatum* has been widely explored (Balba, 2007). Specifically, strobilurin fungicide has a broad spectrum of activity and can be used against fungal pathogens effectively which is a synthetic analogue of fungal metabolites of strobilurins and oudemansins (Bartlett et al., 2002; Balba, 2007; BASF Corporation, 2008; Lu et al., 2015; Xie et al., 2015). Strobilurin fungicides such as ethofenprox, pyraclostrobine, trifloxystrobin and enestroburin are bactericides, but they can also control fungal diseases caused by ascomycetes, zygomycetes and imperfect fungi with protection, treatment, eradication, penetration and absorption activities effectively (Kanetis et al., 2004, 2007, 2008). Compared with commonly used fungicides, strobilurin fungicides including ethofenprox, trifloxystrobin, pyraclostrobine and enestroburin have wider bactericidal spectrum, higher bactericidal activity and the mechanism of action is unique. Strobilurin fungicides can be quickly degraded in plants, soils and water . Moreover, they are environmentally friendly and can effectively protect a variety of crops from diseases. So, strobilurin fungicides have been intensively adopted by many countries such as America and Brazil to control the diseases of citrus or other diseases (OChoa-Acuna et al., 2009; Mahoney & Gillard, 2014; Benelli et al., 2016; Debona et al., 2016a, 2016b; Woodward et al., 2016). The residue levels and degradation pattern of strobilurin fungicide on various horticultural crops were widely investigated (Marin et al., 2003;Wang et al., 2014; Xue et al., 2014; You et al., 2015; Campillo et al., 2015; Raina-Fulton, 2015; Wu et al., 2016; Li et al., 2016; Guo et al., 2017; Kolosova et al., 2017; Zhao et al., 2017). However, the levels, dose and effectiveness of strobilurin fungicide used in postharvest treatments for citrus fruit were seldom explored in terms of controlling decay. In this paper, we explored the effects of ethofenprox, pyraclostrobine, trifloxystrobin and enestroburin on the prevention and control of the fungus *P. italicum* and *P. digitatum* of citrus as well as the inhibition effects along with residue safety assessment during citrus storage. The study provided the theoretical basis and technical support for the quality and safety inspection of Chinese citrus and other agricultural products.

2. Materials and Methods

2.1 Reagents and Materials

The fungus *P. italicum* and *P. digitatum* were obtained from China General Microbiological Culture Collection Center.

Citrus were harvested on April 21 2017 in an experimental orchard under standard horticultural practices in Zhaoqing City, Guangdong Province, China. Citrus were randomly harvested from 10 trees. The fruits were picked from the outer edge of the canopy of each tree, placed in plastic boxes, delivered to the laboratory immediately after harvest, graded, sized, returned to the boxes (40 fruits individually numbered per box), and grouped into eight treatments (each treatment containing nine fruit boxes).

2.2 Inhibition Effects of the Fungicides on P. digitatum and P. italicum of Citrus

The antifungal activities of different fungicides on citrus against *P.digitatum* and *P.italicum* were determined by mycelial growth assays (Liu et al., 2007). The antifungal activities were determined in vitro by transferring plugs (5 mm in diameter) of mycelium from the leading edge of an actively growing colony to a series of PDA (potato dextrose agar) plates containing 0.5, 1.0, 2.0, 4.0 and 8.0 μg/mL fungicides. Added with distilled water, PDA plates were used as the control. For each concentration, three replicates were arranged. The diameters (minus the diameter of the inoculation plug) of the colonies were measured in 7 days after incubation at 28 °C in darkness. The median effective concentration of the fungicides (EC_{50}) for the isolates were calculated based on linear regression of colony diameter on log-transformed fungicide concentration. The experiment was performed in three replicates. The growth inhibition rates of fungicides against *P. digitatum* and *P. italicum* are calculated as:

$$\text{Inhibition rate (\%)} = \frac{\text{Colony diameter of control} - \text{Colony diameter of treatment}}{\text{Colony diameter of control} - 0.5} \times 100\% \quad (1)$$

2.3 Control Effects of the Fungicides on Citrus Storage Diseases

Dip treatments were performed according to the method described by Schirra et al. (2002). The experiments were carried out with freshly harvested citrus. Fruits were submerged in the commercial fungicide solution (5% of ethofenprox, trifloxystrobin, pyraclostrobine and enestroburin) for 5 min and then washed by tap water and naturally dried. Fruits were punctured to generate the wounds (approximately 5 mm in depth) with a nail at four sites around the equator. Fungal strains used in these experiments were *P. digitatum* and *P. italicum*. Inoculums contained conidia (10^4 conidia/mL) and antifungal compounds (strobilurin fungicide, at desired final concentrations). 10 μL of the inoculum were applied onto each wound. For each treatment, three replicates (five fruits per replicate and four wounds per fruit) were prepared in each experiment. Then the treated fruits were stored at 20 °C under the relative humidity of 90%. Symptoms were recorded at different days post-inoculation (dpi) as the number of infected wounds in each replicate. The percentage of infected wounds and mean values for each treatment were calculated as:

$$\text{Rotten fruit rate (\%)} = \frac{\text{Rotten fruit number}}{\text{Total fruit number}} \times 100\% \quad (2)$$

$$\text{Control efficiency (\%)} = \frac{\text{Rotten fruit rate of control} - \text{Rotten fruit rate of treatment}}{\text{Rotten fruit rate of control}} \times 100\% \quad (3)$$

2.4 Extraction, Clean-Up Procedures and Detection of the Fungicides From Citrus Samples

2.4.1 Preparation of Standard Solutions

Standard solutions were prepared as follows. The stock solutions of the fungicides were diluted by adding hexane to obtain the following final concentrations: 0.05, 0.1, 0.2, 0.4 and 0.8 μg/mL. All standard solutions were stored at 4 °C before using. The concentrations of the fungicide samples were determined by GC-MS. About 1.0 μL of aliquots were injected directly into the GC-MS system. Quantifications of strobilurin fungicide (ethofenprox, trifloxystrobin, pyraclostrobine and enestroburin) were based on the external standard method.

2.5 Extraction, Clean-Up Procedures of the Fungicide Residues From Citrus Samples

To investigate the efficiency of extraction and clean-up procedures, recovery experiments were carried out at different levels to establish the reliability and validity of the analytical method. Fruit samples were weighed and peeled. The peel was weighed and its percentage with respect to the whole fruit weight was calculated. The peel was then minced with a mincing knife and was homogenized. The samples were stored in a freezer at -20 °C until analysis. Citrus samples (20 g) were homogenized in 30 mL of acetonitrile with a high-speed homogenizer for 3 min. In recovery experiments, ethofenprox, trifloxystrobin, pyraclostrobine and enestroburin were added to the samples respectively. The three final concentration of ethofenprox was 0.005, 0.5 and 1.0 μg/g, so was that of trifloxystrobin. The three final concentration of pyraclostrobine was 0.01, 0.5 and 1.0 μg/g, so was that of enestroburin. A blank control group was also arranged and all the experiments were repeated three times. Ethofenprox, trifloxystrobin, pyraclostrobine and enestroburin were extracted from citrus samples according to QuEChERS method after different cleaning treatments (Anastassiades et al., 2003; Schenck & Hobb, 2004). PSA (Primary secondary amine) (150 mg) and anhydrous magnesium sulfate (6 g) were added to the samples before centrifuging at 4000 rpm for 5 min. About 20 mL of the supernatant was collected and added into the centrifuge tubes containing Na_2SO_4. The supernatant was evaporated to near dryness with a vacuum rotary evaporator at 45 °C and to dry under a gentle nitrogen stream. The residue was redissolved in 1 mL of acetonitrile for clean-up.

TPH SPE sorbent column was activated with 10 mL of acetonitrile-toluene (V/V = 3/1) solutions and the sample extracts were loaded onto the SPE tubes. Then the SPE column was washed by 10 mL of acetonitrile-toluene (V/V = 3/1) solutions. Finally, the elution was collected in the conical vial and the obtained elution was evaporated to near dryness with a vacuum rotary evaporator at 45 °C and to dryness under a gentle nitrogen stream. The residue was redissolved in 1 mL of hexane prior to the GC-MS analysis.

2.6 Detection Method of the Fungicides From the Citrus Samples

The optimal operating parameters of GC-MS analysis were as follows. A Agilent Technologies Model GC 7890 series gas chromatograph coupled with an Agilent 5975 series mass-selective detector quadrupole mass spectrometer (Agilent Technologies, CA, USA) was employed for all analyses. Samples were separated on HP-5 MS capillary column. The oven temperature was initially maintained at 160 °C for 1 min and the temperature was raised to 280 °C at a rate of 15/min followed by isothermal period of 4 min. Then the temperature was raised to 300 °C at a rate of 10 °C /min followed by isothermal period of 4 min. The injector was heated to 280 °C and operated in the splitless mode and the injection volume was 1.0 μL. Ionization methods was EI^+ and the quadrupole temperature was 150 °C. Ion source temperature was 230 °C and the detection mode was the selective ion mode. The carrier gas was helium at a flow rate of 1.0 mL/min.

2.7 Degradation Dynamics of the Fungicides in the Citrus

After the fruits were treated with the fungicides, 5 citrus were collected from randomly selected sampling points at different time. Each citrus was divided into two parts: peel and flesh. Then the samples were mixed according to the quadrant sampling method. Finally, the samples were extracted, purified and analyzed according to the method described above.

2.8 Statistical Analysis

All statistical analyses were performed by the GraphPad InStat software and Statistica 8.0 software. The 95% significance level was adopted for all comparisons. The EC_{50} and 95% confidence limits were estimated by Probit analysis.

3. Results and Discussion

3.1 Antifungal Activity Assays of the Fungicides Against P. digitatum and P. italicum of Citrus

The EC_{50} values of the *P. digitatum* exposed to ethofenprox, trifloxystrobin, pyraclostrobine and enestroburin were respectively 1.74, 4.54, 6.95 and 5.26 µg/mL at 24 h (Table 1). The EC_{50} values of the *P. italicum* exposed to ethofenprox, trifloxystrobin, pyraclostrobine and enestroburin were 3.43, 2.01, 3.54 and 4.76 µg/mL at 24 h respectively. The EC_{50} value of the *P.italicum* exposed to prochloraz at 24 h was 5.28 µg/mL, which was below that of strobilurin fungicide (Table 2).

Table 1. Inhibition effects of strobilurin fungicides against citrus *P. digitatum*

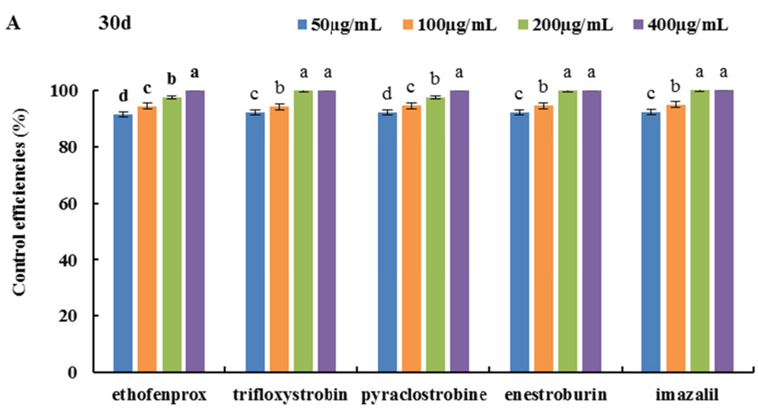
Fungicides	Toxicity regression equations	EC_{50} values (µg/mL)	Correlation coefficients	Confidence intervals (95%)
Ethofenprox	y=4.8279+0.7125x	1.74	0.9601	0.54-5.61
Trifloxystrobin	y=4.3311+1.0185x	4.54	0.9921	2.57-8.02
Pyraclostrobine	y=3.9488+1.2484x	6.95	0.9759	4.72-10.24
Enestroburin	y=4.3906+0.8455x	5.26	0.9718	2.85-9.09
Prochloraz	y=4.6401+0.5788x	4.20	0.9865	1.61-10.97
Imazalil	y=4.3631+1.0866x	3.86	0.9813	2.11-7.09

Table 2. Inhibition effects of the fungicides against citrus *P. italicum*

Fungicides	Toxicity regression equations	EC_{50} values (µg/mL)	Correlation coefficients	Confidence intervals (95%)
Ethofenprox	y=4.0194+1.8132x	3.43	0.9973	2.16-5.44
Trifloxystrobin	y=4.7052+0.9743x	2.01	0.9520	0.84-4.82
Pyraclostrobine	y=4.5192+0.8758x	3.54	0.9852	1.69-7.39
Enestroburin	y=4.3793+0.9163x	4.76	0.9936	2.59-8.75
Prochloraz	y=4.2942+0.9769x	5.28	0.9875	3.06-9.11
Imazalil	y=4.7329+1.0685x	1.78	0.9908	0.71-4.44

3.2 Control Effects of the Fungicides Against Storage Diseases of Citrus

The rates of rotten fruits between 30-d storage and 90-d storage showed no significant differences after the citrus were treated with ethofenprox, trifloxystrobin, pyraclostrobine and enestroburin at concentrations 200 µg/mL and 400 µg/mL for 90 d (Figure 1). The results indicated that ethofenprox, trifloxystrobin, pyraclostrobine and enestroburin within the concentration range of 200-400 µg/mL had the good control effect on citrus storage diseases.



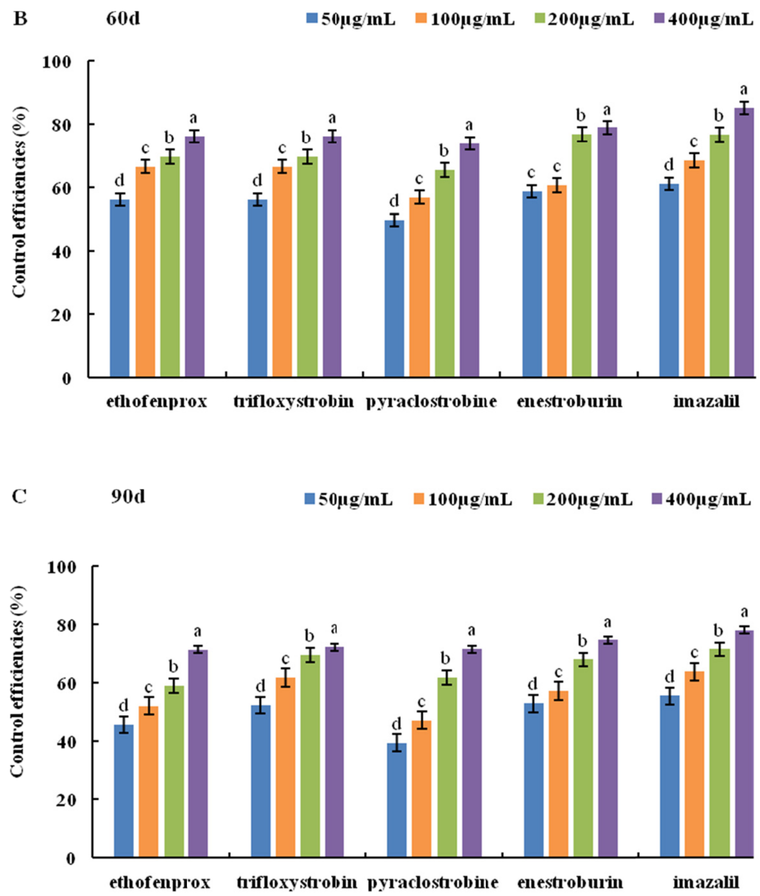


Figure 1. Control effects of the fungicides on citrus storage diseases

3.3 Fortified Recoveries and Precision of the Fungicides From the Citrus Samples

Under the selected experimental conditions, the total ion chromatogram of ethofenprox, trifloxystrobin, pyraclostrobine and enestroburin were obtained. The observed mass transitions and the collision energy used for quantitation of ethofenprox, trifloxystrobin, pyraclostrobine and enestroburin were shown in Table 3. Parent compound of the four Strobilurin fungicides were subjected to collision-induced dissociation in the MRM positive mode. Chromatographic separation of strobilurin fungicides were conducted using temperature-programmed route, with retention times of 7.739 min for ethofenprox, 8.150 min for trifloxystrobin, 10.097 min for pyraclostrobine, and 13.991 min for enestroburin (Figure 2).

Table 3. Quantitative and qualitative ions of the fungicides

Fungicides	Retention time	Quantitative ions	Qualitative ions I	Qualitative ions II	Qualitative ions III
Ethofenprox	7.739	206	131	116	313
Trifloxystrobin	8.150	131	116	172	377
Pyraclostrobine	10.097	325	132	133	
Enestroburin	13.991	205	189	145	146

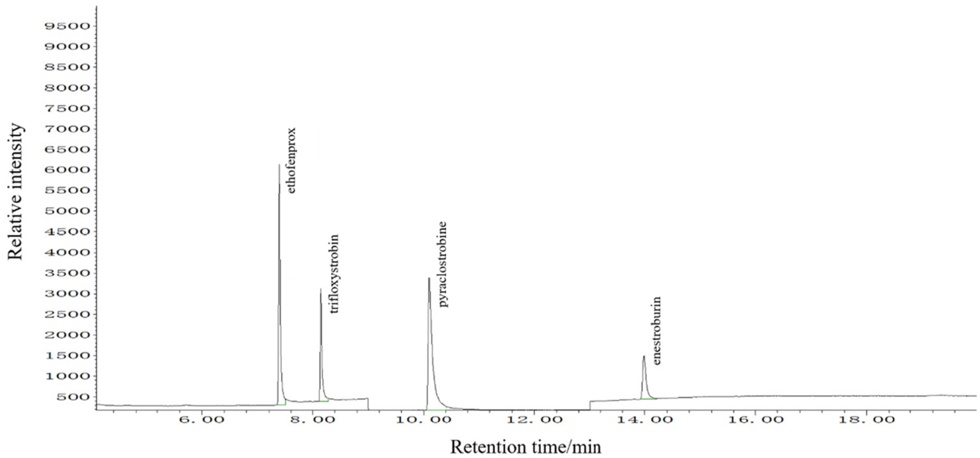


Figure 2. GC-MS chromatogram of ethofenprox, trifloxystrobin, pyraclostrobine, and enestroburin standards

The average recovery was above 80% and the coefficients of variation were respectively 4.20%-7.09%, 6.15%-6.78%, 2.77%-5.90% and 2.49%-4.81%, which met the requirements of pesticide residue analysis (Table 4). The average recoveries and coefficients of variance were within the range required for residue analysis (Commission, 2007). Based on the noise signal of GC-MS, the lower limits of detection of ethofenprox, trifloxystrobin, pyraclostrobine and enestroburin were 0.005, 0.005, 0.01 and 0.01 mg/kg, respectively (Table 5). GC-MS chromatograms of different samples were shown in Figures 3-4.

Table 4. Fortified recoveries and precisions of the fungicides in citrus samples (n = 10)

Fungicides	Fortification levels (mg/kg)	Average recovery rates (%)	Standard deviations	Coefficients of variation (%)
Ethofenprox	0.005	87.68	0.0002	4.33
	0.5	90.04	0.0189	4.20
	1.0	88.10	0.0625	7.09
Trifloxystrobin	0.005	92.55	0.0003	6.45
	0.5	95.32	0.0293	6.15
	1.0	92.72	0.0629	6.78
Enestroburin	0.01	86.64	0.0003	3.24
	0.5	92.74	0.0275	5.90
	1.0	89.35	0.0248	2.77
Pyraclostrobine	0.01	87.72	0.0003	3.65
	0.5	92.16	0.0222	4.81
	1.0	87.97	0.0200	2.49

Table 5. Lower limits of detection of the fungicides in citrus by GC-MS

Fungicides	Linear range (mg/kg)	Correlation coefficients	Lower limits of detection (mg/kg)
ethofenprox	0.05-0.8	0.9997	0.005
trifloxystrobin	0.05-0.8	0.9999	0.005
pyraclostrobine	0.05-0.8	0.9989	0.01
enestroburin	0.05-0.8	0.9980	0.01

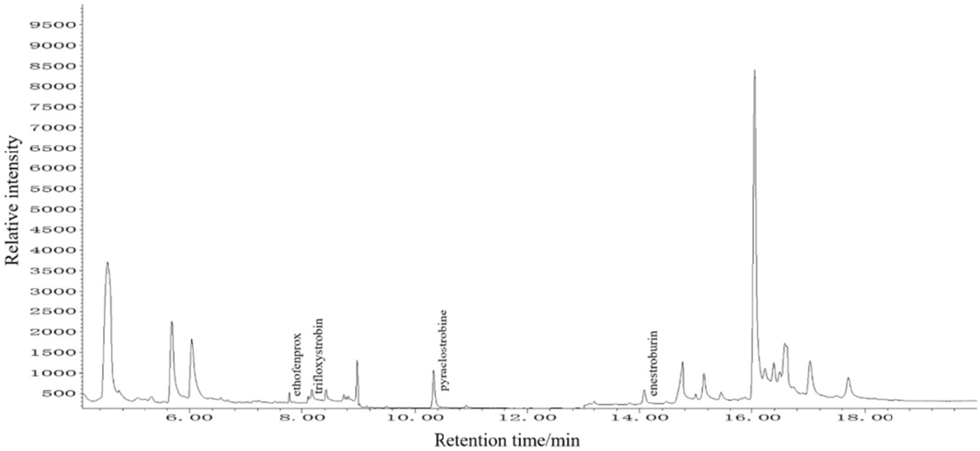


Figure 3. GC-MS chromatogram of blank citrus sample

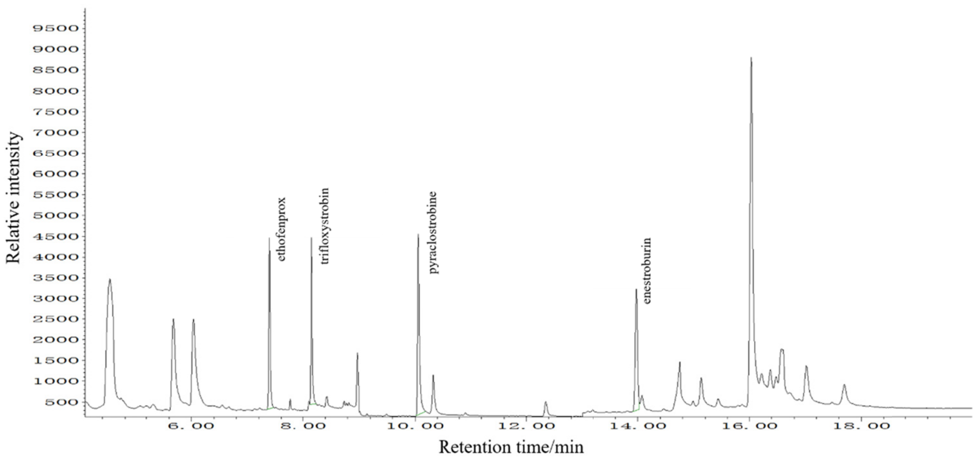


Figure 4. GC-MS chromatogram of citrus sample

3.4 Residues of the Fungicides in the Citrus After Different Storage Time

3.4.1 Residual Dynamics of the Fungicides in Citrus

The results indicated that the degradation dynamic equations of ethofenprox, trifloxystrobin, pyraclostrobine and enestroburin in the citrus could be expressed as the first-order reaction dynamic equations. The half-lives of the four Strobilurin fungicides in the citrus peels were respectively 14.56, 10.85, 22.87 and 16.50 d. The half-lives of them in the citrus flesh were 12.74, 9.39, 20.09 and 13.00 d, respectively (Table 6). The half-lives of four fungicides in the citrus were all less than 30 d, indicating that the four fungicides were degradable low-residue fungicides.

Table 6. Degradation kinetic parameters of the fungicides during citrus storage

Fungicides	Citrus parts	Degradation kinetics equations	Velocity constants (K)	Correlation coefficients (r)	Half-lives (d)
ethofenprox	peel	C_t=1.7025e$^{-0.0476t}$	0.0476	0.9870	14.56
	flesh	C_t=0.1584e$^{-0.0544t}$	0.0544	0.9433	12.74
trifloxystrobin	peel	C_t=1.4345e$^{-0.0639t}$	0.0639	0.9978	10.85
	flesh	C_t=0.1223e$^{-0.0738t}$	0.0738	0.9522	9.39
pyraclostrobine	peel	C_t=1.4739e$^{-0.0303t}$	0.0303	0.9979	22.87
	flesh	C_t=0.1141e$^{-0.0345t}$	0.0345	0.9252	20.09
enestroburin	peel	C_t=1.1955e$^{-0.0420t}$	0.0420	0.9938	16.50
	flesh	C_t=0.0880e$^{-0.0533t}$	0.0533	0.9793	13.00

3.5 Residues of the Fungicides in Citrus Peels and Fruit Flesh After Different Storage Times

The terminal residue of trifloxystrobin in the citrus peels was less than 0.3 mg/kg after the citrus were treated with trifloxystrobin WDG (50%) at concentrations of 400 μg/mL for 30 d. The terminal residue of pyraclostrobine in the citrus peels was 0.13 mg/kg after the citrus were treated by pyraclostrobin EC (250 g/L) at the concentration of 400 μg/mL for 90 d (Table 7). The terminal residues of four kinds of fungicides in the citrus fruit flesh were lower than those in the peels after the citrus fruits were treated with four fungicides at the concentration of 400 μg/mL for 30, 60 and 90 d (Table 8).

Table 7. Residues of the fungicides in citrus fruit peels after different storage times

Fungicides	Residues of fungicides in citrus fruit peels after different storage time (mg/kg)		
	30 d	60 d	90 d
Ethofenprox	0.44	0.20	0.11
Trifloxystrobin	0.23	0.07	0.03
Pyraclostrobine	0.58	0.22	0.13
Enestroburin	0.41	0.09	0.04

Table 8. Residues of the fungicides in citrus fruit flesh after different storage times

Fungicides	Residues of fungicides in citrus fruit flesh after different storage time (mg/kg)		
	30 d	60 d	90 d
Ethofenprox	0.041	0.014	0.01
Trifloxystrobin	0.027	0.011	0.006
Pyraclostrobine	0.041	0.027	0.016
Enestroburin	0.023	0.011	ND

Strobilurin fungicides such as ethofenprox, trifloxystrobin, pyraclostrobine and enestroburin had different inhibition effects on the *P. italicum* and *P. digitatum.* In recent years, fungicides still play an important role in pest management and the substitutes are still not found. The baseline sensitivities of *P. digitatum* populations to novel fungicides from different chemical classes such as azoxystrobin, fludioxonil and pyrimethanil were established by Kanetis et al. (2004). Investigations on strobilurin fungicide in postinoculation treatments on pear (*Pyrus communis* L.) showed that the new "low-risk" fungicides such as fenhexamid, fludioxonil and strobilurin fungicide had high activity against the gray mold caused by *Botrytis cinerea*, while blue mold caused by *Pyrus expansum* can beeffectively controlled only by fludioxonil and strobilurin fungicide (Adaskaveg & Forster, 2004). In preinoculation treatments, fenhexamid effectively controlled gray mold, nevertheless, strobilurin fungicide effectively controlled blue mold. Sholberg et al. (2005) indicated that pre- or post-harvest applications of strobilurin fungicide could control gray and blue mold on stored apples effectively. Especially at 50 °C, postharvest treatments with strobilurin fungicide can effectively control green and blue mold in citrus. The maximum residue limits of ethofenprox, trifloxystrobin and pyraclostrobine show differences in different countries. In the European Union, the maximum residue of trifloxystrobin and pyraclostrobine on fruits like citrus were 0.3 and 1 mg/kg (CAC, 2016; European Commission, 2016; GB2763-2016, 2017). In Japan, the maximum residue of ethofenprox, trifloxystrobin and pyraclostrobine are 1, 0.3, and 10 mg/kg, respectively. Therefore, it is essential to regulate the applied doses of the fungicides to remove the barrier of international trade and make the pulp processing safe. In this study, after the leaching treatment with 400 μg/mL ethofenprox, trifloxystrobin, pyraclostrobine and enestroburin, the residue of fungicides in the whole storage process was lower than 0.3 mg/kg, which was below the maximum residue limit. Degradation dynamics of 4 fungicides during the citrus storage period was proposed on the basis of the first-order kinetics equation. As a result, strobilurin fungicides could penetrate into flesh through the peels and the residues in the peels were higher than that in the flesh in the same citrus. After being treated for 3 d, the residues in the citrus were lower than the maximum residue limits required in European Union and Japan. The residues in the citrus were lower than the maximum residues in other countries and the treated citrus were safe fruits after the citrus were treated for 90 d.

4. Conclusions

This study revealed that ethofenprox, trifloxystrobin, pyraclostrobine and enestroburin could efficiently control the storage diseases of citrus at the concentration of 200-400 μg/mL. The fungicides could penetrate into flesh through the peels when the citrus were treated with strobilurin fungicide and the residues of the peels were higher than that of the flesh in the same citrus. After the citrus were treated for 90 d, the residue level in the citrus was lower than the maximum residue limits specified in different countries and the citrus were safe.

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