Residue behavior and risk assessment of pyraclostrobin and tebuconazole in peppers under different growing conditions

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Abstract
This study evaluates the residue behavior and risks of pyraclostrobin and tebuconazole in peppers. An analytical method for the simultaneous determination of the concentration of these fungicides in peppers was developed using ultra-high performance liquid chromatography–triple quadrupole mass spectrometry. Pepper samples were extracted with acetonitrile and cleaned with primary secondary amine and graphitized carbon black. The average recoveries of pyraclostrobin and tebuconazole under three fortification levels were 86.7–101.4% and 81.7–104.4%, with relative standard deviations of 4.0–7.2% and 3.8–10.9%, respectively. The limit of quantification of both fungicides in peppers was 0.01 mg/kg. The terminal residue trial of 30% pyraclostrobin and tebuconazole suspension concentrate was investigated for samples cultivated in open fields and greenhouses. The results showed that the terminal residues of pyraclostrobin and tebuconazole in peppers were lower than the maximum residue limits established by GB 2763–2021 (0.5 mg/kg for pyraclostrobin and 2 mg/kg for tebuconazole). The results of a statistical t-test indicated that there was no significant difference between samples grown in open fields and greenhouses. According to the international estimate of short-term intake (IESTI) calculation model, provided by the Joint FAO/WHO Meeting on Pesticide Residues, the acute dietary exposure risk of both fungicides in peppers was acceptable for the general population, with an IESTI of 0–3% and 0–5% of the acute reference dose for pyraclostrobin and tebuconazole, respectively.

Keywords Pyraclostrobin · Tebuconazole · Residue · Peppers · Risk assessment

Introduction
Chemical pesticides are widely used to prevent and control diseases, pests, and weed damage on crops. However, their extensive use has led to residue pollution and environmental problems, including damage to water, soil, and air, which have led to significant public concern (Deng et al. 2019). Pesticide residues can represent a risk to human health considering their concentration in the food chain, passive and active exposure, and chronic or acute toxicity for users and consumers (Voltz et al. 2022). In the evaluation of pesticide residue levels, maximum residue limits (MRLs) have been established to supervise the quality of agricultural products, which substantially affects international trade.

Vegetables represent 30% of the human diet intake, and they are the main pathway of pesticide residue dietary risk to humans (Claeys et al. 2011). Pepper (Capsicum annuum) is a common type of vegetable, and it has different pharmacological functions, including gastric protection, anti-inflammation, and anti-obesity properties (Wu et al. 2020). China is their largest producer and consumer (Wang et al. 2009). Peppers have a higher output value and economic benefits than commonly consumed vegetables, such as cabbages, in China (Dai and Liu 2005). However, during their growth, peppers are susceptible to different diseases, including anthrax. Many chemicals, such as the fungicides pyraclostrobin and tebuconazole, have been marketed to prevent diseases in pepper crops.

Pyraclostrobin is a fungicide from the strobilurin group, and it was developed by the chemical company...
BASF. Pyraclostrobin acts through the inhibition of mitochondrial respiration by blocking electron transfer within the respiratory chain. This causes important cellular biochemical processes to be severely disrupted, which stops fungal growth (JMPR 2003). In China, pyraclostrobin has been registered for use on 57 categories of crops, including peppers. The GB 2763–2021 standard establishes the MRL of pyraclostrobin (0.5 mg/kg) on peppers. Pyraclostrobin is used to prevent different diseases, including mildew and powdery mildew (MacBean 2015). Pyraclostrobin has been applied with difenoconazole to prevent the infestation of four Colletotrichum species on peppers in greenhouse trials (Shi et al. 2021). It has also been used to prevent the development of Fusarium asiaticum and Fusarium graminearum on wheat without cross-resistance (Zhao et al. 2021). In another study, it was combined with dimethomorph to prevent bacterial and fungal diseases in grapes (Wang et al. 2018). However, pyraclostrobin can also negatively affect bees, which can potentially lead to significant deleterious effects in stingless bees at a colonial level (Da Costa Domingues et al., 2020). Furthermore, pyraclostrobin residues on fruits and vegetables could cause food safety problems (Yang et al. 2018). Therefore, it is necessary to investigate its residue concentrations in fruits and vegetables. The analysis methods for the identification of pyraclostrobin in fruits and vegetables include gas chromatography–tandem mass spectrometry (GC–MS/MS) (Park et al. 2021), high-performance liquid chromatography (HPLC) (Melo et al. 2006), and liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Li et al. 2020).

Tebuconazole is a broad-spectrum triazole fungicide developed by Bayer. As a seed dressing, tebuconazole is effective against various smut and bunt diseases of cereals. As a foliar spray, it controls numerous pathogens such as rust species, powdery mildew, and scale in various crops (JMPR 1994). Tebuconazole has been registered for application on peppers in China, and GB 2763–2021 establishes its MRL (2 mg/kg) for peppers. Although the pre-harvest and post-harvest applications of tebuconazole have significant effects on fungal diseases of fruits, vegetables, beans, and food crops (Shuang et al. 2021), this substance can cause negative effects on the reproduction of farmland birds upon ingestion of seeds (Lopez-Antia et al. 2021). There are many methods to determine the concentration of tebuconazole owing to its high sensitivity, including LC-UV (Miyauchi et al. 2005) and LC–MS/MS (Pallavi et al. 2021).

To the best of our knowledge, no published study has investigated the residue concentrations of the mix of pyraclostrobin and tebuconazole on peppers, their associated acute dietary exposure, and their differences considering different planting conditions (greenhouse and open field). Therefore, this study investigates these aspects so as to provide data to improve the food safety of these pesticides applied to peppers.

The purposes of this study were as follows: (1) to develop a method to simultaneously determine pyraclostrobin and tebuconazole concentrations in peppers using ultra-high performance liquid chromatography–triple quadrupole mass spectrometry (UPLC–MS/MS) combined with an optimized quick, easy, cheap, effective, rugged, and safe (QuEChERS) sample preparation; (2) investigate the residue behavior of pyraclostrobin and tebuconazole on peppers; (3) evaluate the differences caused by different planting conditions, including greenhouse and open field, by t-tests; and (4) evaluate the associated acute dietary exposure risks.

**Material and methods**

**Chemicals and reagents**

The pyraclostrobin (100 mg/L) and tebuconazole (100 mg/L) standards were obtained from the Agro-environmental Protection Institute Ministry of Agriculture (AEPI, Beijing, China). The 30% suspension concentrate (SC) containing 10% pyraclostrobin and 20% tebuconazole was obtained from Bissell, Henan Agricultural Science and Technology Co. Ltd. (Henan, China). The HPLC grade methanol and acetonitrile were purchased from Dikma Technologies Inc. The analytical grade sodium chloride (NaCl) and anhydrous magnesium sulfate (MgSO₄) were purchased from Beijing Chemical Reagent Company (Beijing, China). The formic acid (88%) was purchased from Thermo Fisher Scientific. The primary secondary amine (PSA) and graphitized carbon black (GCB) were purchased from Agela Technologies (Tianjin, China). Ultrapure water was obtained from a Master-S30UV pure water system (Hitech Instruments Co. Ltd.; Shanghai, China).

Mixed standard stock solutions (10 mg/L) of pyraclostrobin and tebuconazole were prepared in pure acetonitrile. All solutions were stored in the refrigerator at 4 °C.

**Apparatus**

The UPLC–MS/MS system consisted of a Waters Acquity UPLC connected to a Xevo TQD triple quadrupole mass spectrometer, and it was used for the instrumental analysis. Other instruments used in these experiments included the following: a JY 2002 electronic balance (Shanghai Shunyuhengping Scientific Instrument Co. Ltd.), ME155DU electronic balance (Mettler Toledo Co. Ltd.), UMV-2 multi-tube vortex mixer (Beijing Yousheng...
Field experiments

Following adequate planting scale, cultivation mode, and climatic conditions for the growth of peppers, supervised field experiments were performed in 12 different sites, including Beijing (BJ), Inner Mongolia (IM), Shanxi (SX), Henan (HeN), Hunan (HuN), Sichuan (SC), Gansu (GS), Shandong (SD), Shanghai (SH), Guangdong (GD), Jiangxi (JX), and Guangxi (GX). The experiments were conducted in 2020 and followed the NY/T 788 2018 guidelines for testing pesticide residue in crops. Under the premise of sufficient sample quantity, the field experiment plots consisted of a check experiment plot and a treatment experiment plot, which were no less than 50 m² each.

To evaluate the terminal residues of pyraclostrobin and tebuconazole in the pepper samples, 30% fungicide SC was sprayed on the surface of the peppers at the initial stage of anthracnose. The SC formulation was dissolved in water and sprayed on the treatment plot with an electric sprayer at 315 g a.i/ha, the highest recommended dosage. Fresh water was used for the corresponding check plot area, which was isolated at 3 m from the treatment plot area. The pesticides were applied three times, with a 7-day application interval, and the pre-harvest interval (PHI) was 5 days. More than 2 kg of representative pepper fruits (not less than 24 pepper fruits) were randomly collected from 12 pre-selected sampling points at days 5 and 7 after spraying, and two relatively independent samples were collected in each field trial plot.

For the laboratory pretreatment, all peppers of each independent field were cut into segments of 1 cm, mixed, and then parted using a quartation method. Finally, the peppers were separated into two samples (laboratory samples A and B), weighing more than 200 g each. All samples were stored in a deep freezer at −20 °C.

Analytical methods

The field pepper samples were appropriately homogenized with dry ice by a homogenizer before sample preparation.
The qualitative and quantitative analyses of the fungicides were conducted in a triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI+) source. Multiple reaction monitoring (MRM) was performed in the positive ionization mode, with a capillary voltage of 3.0 kV, source temperature of 150 °C, and desolvation temperature of 450 °C. Nitrogen gas (99.95%) was used as the source gas at a 50 L/h flow rate for the cone and 600 L/h for the desolvation. In addition, 99.99% argon was used as the collision gas, at a pressure of 2–3 mbar. The MS parameters of the fungicides, including cone voltage and collision energy, were individually optimized to obtain perfect sensitivity and resolution (Table 1). A Masslynx v.4.1 (Waters, USA) software with TargetLynx was used to acquire and analyze the data.
Differences between greenhouse and open field samples

The *t*-test is a common statistic method used to determine if there is a significant difference between two groups of data. In this study, the residue concentrations of pepper samples grown under different conditions was fit to the two-tailed *t*-test. A significant difference between two datasets exists if the calculated probability of the *t*-test is lower than 0.05, and an extremely significant difference exists for a probability lower than 0.01.

Acute dietary exposure risk assessment

To calculate the international estimate of short-term intake (IESTI), we used the highest expected residue (highest residue in the edible portion of a commodity...
or highest residue in a processed commodity [HR-P]) and the highest large portion (LP) data for the general population (all ages) and children (6 years and under), as per the guidelines of the Joint FAO/WHO Meeting on Pesticide Residues (JMPR). According to the national dietary survey data by WHO (World Health Organization) and FAO (Food and Agriculture Organization of United Nations), the pepper LP is 295.71 g/person and the unit weight edible portion ($U_e$) is 43.2 g for general population in China. The LP is no consumption and the $U_e$ is 9.32 g for 2–6-year children in Netherlands. The IESTI calculation for peppers fit case 1 for children (Eq. 1); the residue concentration in a composite sample (raw or processed) reflects the concentration in a LP of the commodity. The calculations also fit case 2a for the general population (Eq. 2), in which the unit weight of the edible portion ($U_e$) is lower than that of the large portion (JMPR 2019).

$$\text{IESTI} = \frac{\text{LP} \times \text{HR}}{\text{bw}} \quad (1)$$

$$\text{IESTI} = \frac{[U_e \times \text{HR} \times v + (\text{LP} - U_e) \times \text{HR}]}{\text{bw}} \quad (2)$$

In these equations, the following definitions apply.

- **LP** largest portion provided (97.5th percentile of eaters), kg/day;
- **HR** highest residue concentration found in composite sample of edible portions according to data from supervised trials, mg/kg;
- **bw** average body weight for a given population age group, kg;
- **$U_e$** edible portion of a unit weight, kg;
- **$v$** variability factor, which represents the ratio of the 97.5th percentile residue to the mean residue in single units.

### Results and discussion

#### Method validation

Serial standard calibration curves were used to evaluate the sensitivity of the method. To improve the accuracy of the method, matrix standard calibration curves were applied to the quantitative calculation instead of solvent standard
calibration curves. The calibration curves were constructed based on standard concentrations against responses of quantitative ion chromatographic peaks. The results showed that the linealities were excellent, and the correlation coefficient \((r)\) of the fungicides were higher than 0.999.

To evaluate the accuracy and precision of the developed method, recovery experiments for pyraclostrobin and tebuconazole were performed in the pepper samples. For fortified levels of 0.01, 0.5, and 2 mg/kg, the average fortified recoveries of pyraclostrobin ranged from 86.7 to 101.4%, with a relative standard deviation (RSD) of 4.0–7.2%, whereas the recoveries of tebuconazole ranged within 81.7–104.4%, with an RSD of 3.4–10.9% (Table 2). The limit of quantification (LOQ) was 0.01 mg/kg. These results indicate that the accuracy and precision of the adopted method met the guideline NY/T 788–2018. Typical chromatograms are shown in Fig. 1.

### Terminal residues

The terminal residues of pyraclostrobin and tebuconazole on pepper samples from 12 different field experiment sites

**Table 4** The terminal residues of tebuconazole in peppers

| Compound     | Planting conditions | Location | PHI (days) | Residue (mg/kg) | 1     | 2     | Average |
|--------------|---------------------|----------|------------|----------------|-------|-------|---------|
| Tebuconazole | Greenhouse          | IM       | 5          | 0.048          | 0.088 | 0.068 |
|              |                     | 7         | 0.025      | 0.048          | 0.037 |
|              | SX                  | 5         | 0.18       | 0.14           | 0.16  |
|              |                     | 7         | 0.096      | 0.15           | 0.12  |
|              | GS                  | 5         | 0.23       | 0.13           | 0.18  |
|              |                     | 7         | 0.22       | 0.17           | 0.19  |
|              | BJ                  | 5         | 0.42       | 0.34           | 0.38  |
|              |                     | 7         | 0.32       | 0.48           | 0.4   |
|              | SD                  | 5         | 0.34       | 0.38           | 0.36  |
|              |                     | 7         | 0.47       | 0.49           | 0.48  |
|              | SH                  | 5         | 1.2        | 1.1            | 1.1   |
|              |                     | 7         | 0.82       | 1.0            | 0.91  |
|              | Open field          | HeN      | 5          | 0.10           | 0.14  | 0.12  |
|              |                     | 7         | 0.10       | 0.08           | 0.09  |
|              | JX                  | 5         | 0.12       | 0.13           | 0.13  |
|              |                     | 7         | 0.084      | 0.090          | 0.087 |
|              | HuN                 | 5         | 0.63       | 0.43           | 0.53  |
|              |                     | 7         | 0.6        | 0.41           | 0.5   |
|              | GX                  | 5         | 0.22       | 0.15           | 0.19  |
|              |                     | 7         | 0.25       | 0.23           | 0.24  |
|              | SC                  | 5         | 0.085      | 0.099          | 0.092 |
|              |                     | 7         | 0.097      | 0.081          | 0.089 |
|              | GD                  | 5         | 0.55       | 0.60           | 0.58  |
|              |                     | 7         | 0.51       | 0.63           | 0.57  |

**Table 5** t-test results of pyraclostrobin and tebuconazole residue in peppers

| Compound     | Application interval | Planting conditions | Average residue | t-test result \((P)\) |
|--------------|----------------------|---------------------|-----------------|----------------------|
| pyraclostrobin | 5 days               | Greenhouse          | 0.045           | 0.097 0.088 0.28 0.21 0.48 0.532 |
|              |                      | Open field          | 0.16 0.12 0.22 0.17 0.056 0.2 |
|              | 7 days               | Greenhouse          | 0.025           | 0.09 0.12 0.22 0.19 0.4 0.622 |
|              |                      | Open field          | 0.14 0.086 0.21 0.21 0.058 0.16 |
| tebuconazole | 5 days               | Greenhouse          | 0.068           | 0.16 0.18 0.38 0.36 1.1 0.582 |
|              |                      | Open field          | 0.12 0.13 0.53 0.19 0.092 0.58 |
|              | 7 days               | Greenhouse          | 0.037           | 0.12 0.19 0.4 0.48 0.91 0.568 |
|              |                      | Open field          | 0.09 0.087 0.5 0.24 0.089 0.57 |
are shown in Table 3 and Table 4. The residues of pyraclostrobin varied from 0.032 to 0.48 mg/kg for an interval of 5 days, and 0.018 to 0.47 mg/kg for an interval of 7 days. The tebuconazole residues varied from 0.048 to 1.2 mg/kg for an interval of 5 days, and 0.025 to 1.0 mg/kg for an interval of 7 days. The highest residue values of pyraclostrobin (0.48 mg/kg) and tebuconazole (1.2 mg/kg) in the pepper samples were lower than the respective MRLs established in GB 2763–2021 (0.5 and 2 mg/kg, respectively).

The 12 different field experiment sites consisted of 6 greenhouses and 6 open fields, and the residue values from the different sites were diverse. The probability of the t-test between the two different growing conditions was calculated using a t-test function. The t-test results for the residues of the two fungicides in peppers grown under two different conditions are shown in Table 5. Based on the two-tailed test and bi-sample quadratic mean deviation assumption, the calculated t-test probabilities of the two fungicides in peppers for 5- and 7-day intervals between greenhouse and open field growth varied from 0.532 to 0.622. This indicated that there was no significant difference between the residues of peppers obtained from greenhouses and open fields.

Dietary exposure risk assessment

Considering the individual residue values close to MRLs and data variability, it was necessary to evaluate the international estimate of short-term intake (IESTI) for a dietary exposure risk assessment.

Using Eqs. 1 and 2, the IESTI of pyraclostrobin and tebuconazole in peppers was calculated, as shown in Table 6. The results show that the acute reference dose (ARfD) of pyraclostrobin was 0.7 mg/kg bw (JMPR 2003), and the calculated IESTI was 3.45 µg/kg for the general population (>1 years) and not consumption (NC) for children (2–6 years). The IESTI was 0.5% of the ARfD for the general population and 0% for children. For tebuconazole, the ARfD was 0.3 mg/kg bw (JMPR 1994), and the calculated IESTI was 8.61 µg/kg for the general population (>1 years) and NC for children (2–6 years). The respective IESTI was 3% of the ARfD for the general population and 0% for children. Therefore, the acute dietary exposure to residues of pyraclostrobin and tebuconazole in peppers is unlikely to present a public health concern.

Conclusions

Terminal residue field trials of 30% pyraclostrobin and tebuconazole SC on peppers were conducted under different planting conditions in 12 experimental field sites. A method for the simultaneous determination of pyraclostrobin and tebuconazole in peppers by UPLC–MS/MS
was developed, and the recoveries of pyraclostrobin and tebuconazole in peppers were 86.7–101.4% with RSDs of 4.0–7.2% and 81.7–104.4% with RSDs of 3.4–10.9%, respectively. The LODs and LOQs of both fungicides were 0.001 mg/kg and 0.01 mg/kg, respectively. The accuracy and precision of the developed method met the NY/T 788–2018 guidelines. The results of the terminal residue trials indicated that the residue levels of pyraclostrobin and tebuconazole in peppers were lower than their respective MRLs (0.5 and 2 mg/kg, respectively) determined in GB 2763–2021. Based on the terminal residue results, a rational application of 30% pyraclostrobin and tebuconazole SC to prevent pepper anthracnose was determined as follows: maximum of three applications at the dosage of 315 g a.i./ha during the initial stage of the disease. The application interval was 7 days, and the PHI was 5 days.

The t-test results of terminal residues of both fungicides showed that there were no significant differences between samples grown in greenhouses and open fields. The dietary risk assessment indicated that the acute dietary exposure to residues of pyraclostrobin and tebuconazole in peppers is unlikely to present a public health concern.

**Author contribution** All authors contributed to the study conception and design. The study design was performed by Ercheng Zhao. The optimization of the analysis method was performed by Dong Wang and Min He. The pretreatment of all field samples was performed by Xiaoying Du and Bingjie Liu. The preparation of all samples was performed by Anqi Xie. The UPLC–MS/MS analysis was performed by Li Chen and Pingzhong Yu. Data collection and analysis were performed by Junjie Jing. The first draft of the manuscript was written by Junjie Jing, and all authors commented on previous versions of the manuscript. All authors have read and approved the final manuscript.

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**Data availability** Not applicable.

**Declarations**

**Ethics approval and consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Competing interests** The authors declare no competing interests.

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