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*Research Article*

# Investigation and Validation of Detection of Storage Stability of Difenoconazole Residue in Mango

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To investigate the stability of the pesticide residue in storage samples is a part of detection, which is also an improvement to the accuracy of analytical results. In this work, the UPLC-MS/MS method with perfect accuracy and stability was established for determining residues of difenoconazole in mango. The stability of the residue under different temperatures (4°C and −20°C) and media (fruit samples and pretreated sample solution) was investigated. At 0.1 mg/kg, the residue degraded in 6 months by 12% when at −20°C, while in a week by only 12.2% at 4°C. However, when pretreated and preserved in the solution, the residue remained more than 90% for 6–8 weeks. The results indicated that the main causes of degradation are biochemical factors, and the factors are affected by temperature. The findings also provided appropriate conditions for sample storage. This investigation promotes the accuracy in detection and hence guarantees food safety and quality.

## 1. Introduction

Difenoconazole is a synthetic fungicide which acts by inhibiting demethylation of fungus during the synthesis of ergosterol [1, 2] (the molecular structure is shown in Figure 1). It is widely used for crop protection against fungal pathogens, such as ascomycetes, basidiomycetes, and deuteromycetes [3–5]. Although it degrades rapidly, low level of residue in agrofood can threaten human life especially in raw foods, such as vegetables and fruits [6–8]. Thus, determining the pesticide residue is an effective way to guarantee agrofood safety. Mango is one of the most popular fruits, and its farming is threatened by fungal diseases. Consequently, difenoconazole is often applied to overcome this. Codex Alimentarius Commission (CAC) regulates the MRL of difenoconazole in mango to be as low as 0.07 mg/kg [9].

Gas/liquid chromatography (GC/LC) and gas/liquid chromatography-mass spectrometry (GC/LC-MS) methods are mainly used for determination of difenoconazole [10–13]. Besides being accurate, these methods are time-consuming due to complex processes involved, and thus, a large number of samples need to be stored for analysis. During storage, although samples are stored in relatively low temperature, their components can exhibit drastic changes due to their volatility, hydrolysis, photolysis degradations, and enzymatic reactions. Nevertheless, it is not clear what happens to the pesticide residues under different storage conditions. Therefore, it cannot be concluded that the original level of the residue remains constant in storage, or errors do not occur during detection. However, there are few studies on stability of the pesticide in stored samples and insufficient data to explain this, hence making it a blind spot to detection.

Evidently, storage is a part of the detection course, and to analyze the stability of pesticide in samples accuracy test method is critical [14, 15]. This is also necessary to guarantee

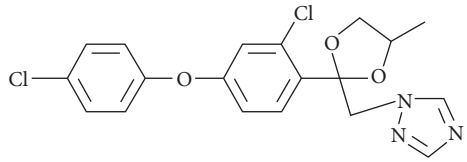

Figure 1: The molecular structure of difenoconazole.

the safety of agrofood. The accurate information on residual characteristics is important for the application of pesticides. To the best of our knowledge, the storage stability of difenoconazole in mango has not been reported.

In this work, a method to determine stability of difenoconazole residues in mangoes was established, and then the residue of difenoconazole in mango stored under different conditions was dynamically studied. This work investigated the storage stability of the difenoconazole in mango and the main effects on its stability. Reasonable storage conditions for samples were proposed to improve the accuracy of detection. This guarantees the data validity of pesticide residue test and eliminates potential errors. Storage stability data also perfected the evaluation system of the pesticide characteristics. This would be effective guide to reasonable application of pesticides and measures to guarantee quality and safety of agrofood.

## 2. Materials and Methods

*2.1. Reagents and Instruments.* Standard difenoconazole was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). HPLC grade acetonitrile (ACN), methyl alcohol (MeOH), ethyl acetate, and acetone were obtained from Fisher (Thermo Fisher, USA). Primary secondary amine (PSA) was supplied by Agilent Technologies (Santa Clara, CA, USA). Stock standard solution (1000 mg/L) of difenoconazole was prepared in MeOH. Series of working standard solution were prepared in stock solution and then diluted with MeOH. NaCl and $MgSO_4$ were obtained from Guoyao Chemicals Co., Ltd. (Shanghai, China), and purified water was prepared using Milli-Q apparatus (Ultima Duo 200, COMBI).

The ultra-high-performance liquid chromatography (Acquity; Waters Corporation, Milford, MA) system coupled with tandem mass spectrometry (API 5000, AB SCIEX Crop., USA), analytical weighing balance (AUY 220, Shimadzu, Tokyo, Japan), homogenizer (IKA-T18), and high-speed freezing centrifuge (Himac CR 22N, Hitachi) were the main instruments used in the experiment.

*2.2. Development of Detection Method for Difenoconazole*

*2.2.1. Sample Pretreatment.* 666 $m^2$ field of mango trees in Danzhou city of Hainan province were employed in this study. In a whole growing season, no difenoconazole was used on these trees. Mango fruits were uniformly collected from 12 sampling points in the field and assigned blank samples. After homogenization, the mango sarcocarps were uniformly spiked with the standard solution at three different concentrations (0.01, 0.05, and 0.1 $\mu$g/g) and kept open for 30 min for the evaporation of the solvent. Half an hour later, the spiked samples were mixed. 10.0 g of the spiked samples and 20 mL of the different solvents (acetonitrile, ethyl acetate, and acetone) were homogenized for 2 min and then centrifuged at 8000 rpm/min for 5 min. The supernatants were transferred into a centrifuge tube with 5.0 g sodium chloride and homogenized for 1 min. The mixture was centrifuged at 8000 rpm for 2 min. 10 mL of the resultant supernatants was added into a mixture of 0.8 mg $MgSO_4$ and different concentrations of PSA and homogenized for 1 min [16]. Then, the mixture was centrifuged at 8000 rpm for 5 min, and 5 mL of the supernatants was filtered using 0.22 $\mu$m organic phase filter membrane for assays.

*2.2.2. Analytical Conditions.* Analysis was performed using UPLC-MS/MS equipment. Analytes were separated on an ACQUITY UPLC® BEH $C_{18}$ (2.1 mm × 100 mm, 1.7 $\mu$m) column at 35°C. The mobile phase consisted of acetonitrile (A) and water (B). The gradient elution program was as follows: 0–3 min, 10% A; 3–5 min, 90% A; 5–5.2 min, 10% A; and 5.2–7 min, 10% A. Positive electrospray ionization mode ($ESI^+$) was run using multiple reaction monitoring (MRM) with two mass transitions where the target analytes yielded $[M + H]^+$ precursor ions. Two of the strongest daughter ions were used for quantification and confirmation in the assay. The basic MS source conditions were as follows: ion spray voltage (IS), 5500 V; source temperature, 650°C; atomizing air (GS1), 55 psi; auxiliary heater (GS2), 55 psi; curtain gas (CUR), 20 psi; collision gas (CAD), 6 psi; entrance voltage (EP), 10 V. The analyst software platform enabled instrument control and data processing using version 1.6.2 of AB SCIEX mass spectrometry system.

*2.2.3. Validation of the Method.* The precision, stability, and sensitivity of the method were evaluated. Blank samples were used for method validation [17]. A linear calibration curve was constructed by plotting targeted pesticide peak areas ratio against the concentration values. Linearity, representing the usability, was assessed as a coefficient ($R^2$) calculated from five points (0.005, 0.01, 0.020, 0.030, and 0.050 $\mu$g/mL) of the calibration curves. The recovery for the precision of the method was evaluated by carrying out five consecutive extractions ($n = 5$) of the spiked samples at three concentration levels (0.01, 0.05, and 0.10 mg/kg). The RSD of these results reflects the stability of the methods. The LOD and LOQ were determined according to the signal-to-noise ratios 3 and 10, which indicated the sensitivity of the method.

*2.2.4. Effect of Matrix on the Method.* The matrix in the sample would affect the detection of the target compounds. Thus, matrix effect (ME) of the sample was also investigated to evaluate the method. Blank samples were treated as above, and standard stock solution was added into the extraction solution at three different concentrations (0.01, 0.05, and

0.1 $\mu$g/mL). Standard solution in purified reagent (MeOH) was labelled A, and spiked solution was labelled B. A and B solutions of same concentration were analyzed by the UPLC-MS/MS method. The peak areas of the quantitative ion were designated as area A and area B. The ME was calculated according to the following formula:

$$\mathrm{ME} = \frac{\mathrm{area\,(B)}}{\mathrm{area\,(A)}} \times 100\%. \quad (1)$$

The analysis was conducted 5 times.

*2.2.5. Analysis of the Uncertainty of the Method.* According to the characteristic results in this work, evaluation of uncertainty of type A showed this method was suitable for detection. Bessel formula (2) was used to evaluate the method. 0.05 $\mu$g/mL of the spiked samples was evaluated using the developed method. The analysis was conducted 5 times. The uncertainty ($u$) was calculated by using the following formulas:

$$s = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \overline{x})^2}{n-1}}, \qquad u = \frac{s}{\sqrt{n}}. \quad (2)$$

*2.3. Storage Conditions for the Samples*

*2.3.1. Storage Temperatures.* Stored samples were spiked with the pesticide as described above and then stored at different temperatures (−20°C and 4°C). For samples at −20°C, detection of residues was done at the intervals of 0, 1, 3, 6, and 12 months and 1, 2, 3, and 4 weeks for 4°C. Quality control samples, prepared by adding standard into the blank samples at the same concentrations, were analyzed simultaneously. Detections were all repeated three times.

*2.3.2. Storage Modes.* Typically, mango sarcocarp and analyte solutions are preserved at low temperature in actual detection operation. In this work, these modes of storage were also investigated. The samples were pretreated as before and the extract preserved at 4°C for 2, 4, 6, and 8 weeks hermetically. The solution would be topped up if the solvent levels went down. The quality control and blank samples were also prepared in the same way.

*2.4. Statistical Data Analysis.* Data were expressed as mean ± standard error (SE). Variance analysis was conducted by SPSS Statistics (version 19.0, IBM). Results of inhibition of seedling growth were analyzed by one-way ANOVA (analysis of variance) and Fisher's LSD (least significant difference) test at probability levels of 0.05 and 0.01.

## 3. Results and Discussion

*3.1. Selection of the Extraction Solvent.* To choose an appropriate type of extraction solvent, three types of solvents were compared: ethyl acetate, acetone, and acetonitrile. Low recoveries, from 55% to 65%, were obtained when ethyl acetate was used (Table 1). The color of acetone extract was darker than that of acetonitrile, meaning that more impurities were obtained. High levels of impurities will affect the purification and the detection system. Thus, acetone was not an appropriate extraction solvent although having 90–105% recoveries (Table 1). At the same time, less impurity was extracted from the matrix by acetonitrile. Excellent recoveries of the residue extracted using acetonitrile had low RSDs, 4.0–7.0%. Therefore, acetonitrile was used.

Detection of pesticide residue mainly involves sample preparation (extraction and purification) and instrumental analysis. Outstanding sample preparations should be rapid, simple, inexpensive, and environmentally friendly. For detection, extraction is the first and most crucial step, needing less impurities and highly targeting. Perfect matched polarity between solvent and analyte is important to improve the extraction efficiency and minimize the interference from the matrix. In this work, ethyl acetate was immiscible with water phase, and this inhibited the permeation between solvent and target compounds. Consequently, lower extraction efficiency was obtained. Accordingly, acetone and acetonitrile are miscible with water, and this improves the extraction efficiency. At the same time, acetonitrile extracted less impurities than acetone. Thereby, acetonitrile was used as a solvent for accuracy of the column and instrument.

*3.2. Sample Purification Process.* The effects of different amounts of PSA (50, 75, 100, 125, and 150 mg) on the detection method were investigated. Figure 2 shows that excellent recoveries were obtained when 75–125 mg PSA was employed and that excess or insufficient of its amounts would lead to poor recoveries. Insufficient PSA could not easily absorb the impurities in the samples to produce detectable matrix effects. Excess PSA lowered the recoveries. Generally, the optimal amount of PSA in the test was 100 mg. In the detection of contamination, solid phase extraction (SPE) column purification method involves complex operations. This is a key process which determines the efficiency as it involves series of physical reactions such as adsorption and desorption [18]. Compared with the SPE method, the dispersive-solid phase method for cleanup was chosen for this work [19–21]. It is flexible and involves simple processes, and it also would serve as a modification template depending on the analyte properties, sample composition, equipment, and analytical techniques [22].

*3.3. Optimization of Instrument Condition.* Standard solutions of difenoconazole were used to optimize the conditions to develop excellent analytical method. Prior to the analysis of the samples, target fragmental ions of difenoconazole were monitored in 100–500 m/z scan range so as to obtain the best spectrum of the positively charged precursor ions.

Table 1: Results of recoveries from different solvents ($n = 3$).

| | 0.01 $\mu$g/g | | 0.05 $\mu$g/g | | 0.1 $\mu$g/g | |
|---|---|---|---|---|---|---|
| | Recovery (%) | RSD (%) | Recovery (%) | RSD (%) | Recovery (%) | RSD (%) |
| Ethyl acetate | 56.29 | 5.00 | 61.63 | 5.62 | 62.98 | 4.70 |
| Acetone | 101.34 | 6.25 | 96.52 | 8.53 | 93.43 | 5.80 |
| Acetonitrile | 106.92 | 3.86 | 96.38 | 6.45 | 99.14 | 4.86 |

The results were obtained at 125 mg of PSA.

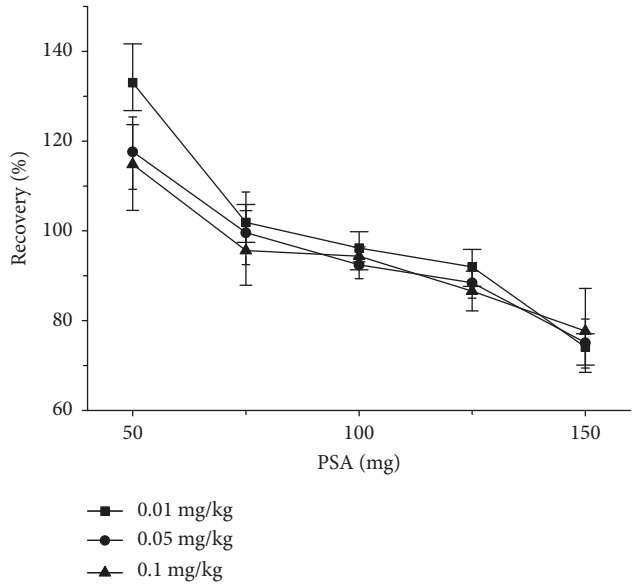


Figure 2: The optimization for amount of PSA ($n = 3$).

The results showed that 251.00 and 337.00 m/z ($[M + H]^+$) were the two strongest daughter ions of difenoconazole. Reversed-phase HPLC is appropriate for difenoconazole due to its polarity. Proper retention time, perfect shaped peak, high resolution, and strong intensity molecular ion peak were obtained after optimization.

To obtain high intensity, declustering potential (DP) voltage and collision energy (CE) for precursor ions were optimized (Figure 3). CE affects ions in a more intense way than DP on optimization. Initially, intensities of ions quantitatively (251.00) and qualitatively (337.00) were highest when DP was fixed at levels of 50–150 V and CEs at 32.98 V and 35.70 V. Then, CEs were fixed and DP was adjusted to 125.9 V to obtain the highest ion intensities. Quantitative ion chromatogram of difenoconazole in quality control samples, blank samples, storage samples, and standard solution is shown in Figure 4.

### 3.4. Method Validation

*3.4.1. Accuracy and Stability of the Method.* Linearity shown in this work is from 0.005 to 0.050 $\mu$g/mL with a good correlation coefficient of 0.9967. Recovery analyses were carried out for three concentrations (0.01, 0.05, and 0.1 mg/kg). Precision and accuracy of the method were expressed by determining the recoveries and their relative standard deviations (RSD, %), as shown in Table 2. The results exhibited recovery rates of 70% to 105% with less than 9% RSDs consistent with determined residues in the samples [9, 23]. These results ascertain that residues in the extract can be accurately determined at concentrations between 0.005 and 0.05 $\mu$g/mL.

*3.4.2. Sensitivity of the Method.* Difenoconazole standard solution was added into blank mango samples to determine the LOD and LOQ of the method. The residue concentrations were considered to be the LOD and LOQ of the equipment when the quantitative ion peak heights were 3 and 10 times to the noise heights. According to the results, LOD and LOQ of the method were 0.002 and 0.01 mg/kg, respectively. LOQ was quite lower than the MRL (0.07 mg/kg) which is regulated by CAC, and hence this is the most competent method to determine the concentrations of the residue.

*3.4.3. Matrix Effect on the Method.* Matrix effects, at different concentrations, are shown in Table 3. All MEs were over 100%, manifesting that the components in the matrix promoted the ionization of the difenoconazole. The trend shows that the effects increased with the target compound decrease. ME affected the detection accuracy but this could be eliminated by developing a calibration curve using the blank sample extract solution. The data were obtained based on this method.

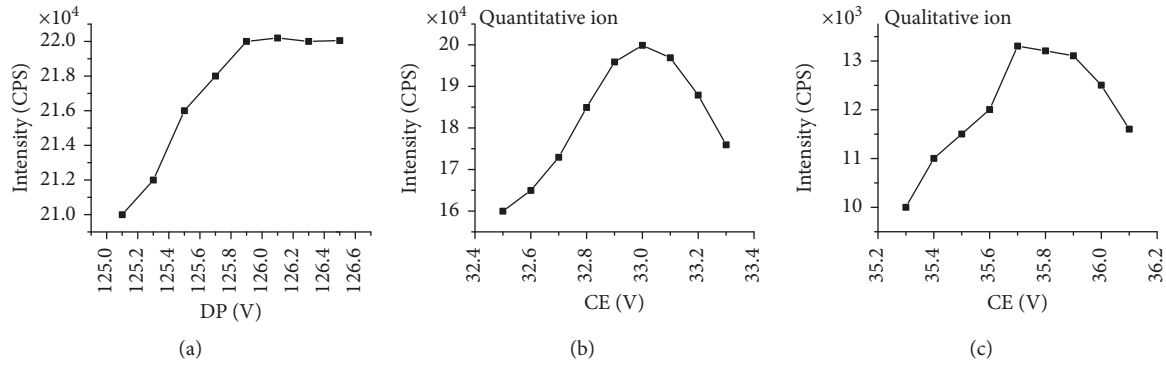


Figure 3: The optimization of the DP voltage and CE.

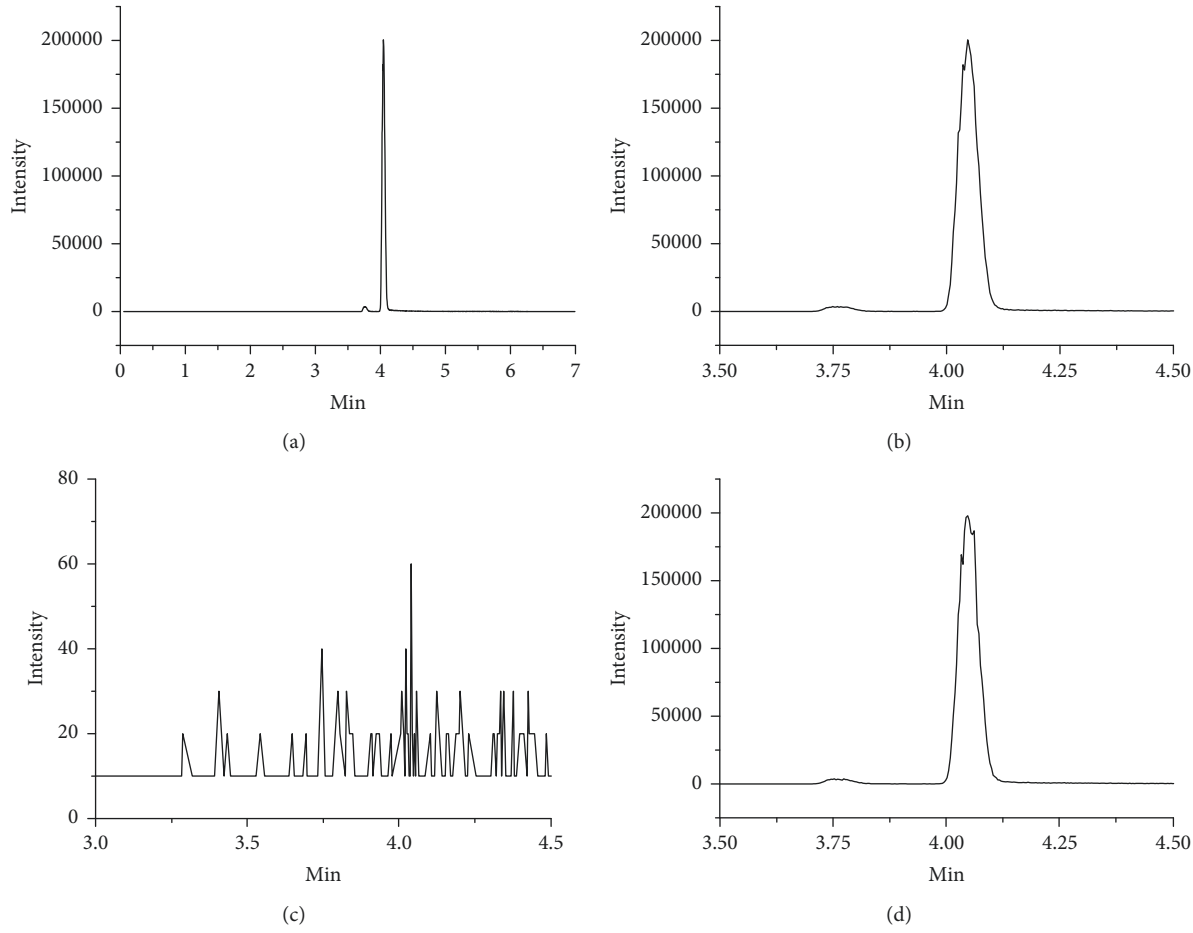


Figure 4: The ion flow chromatogram of difenoconazole. (a) Standard total ion. (b) Standard quantitative ion. (c) Blank sample quantitative ion. (d) Control sample quantitative ion.

*3.4.4. Measurement of Uncertainty of the Method.* The recovery results in Section 3.4.1 (0.05 $\mu$g/mL) were used for calculating the uncertainty according to the formula in Section 2.2.5. The uncertainty of the method was calculated as 0.001, and its relative standard uncertainty was 0.025. This value is quite low and reflects the high accuracy and stability of this method. According to the mathematical model, the uncertainty was due to several aspects, such as the repeatability of detection, the accuracy of the standard, the accuracy of the constant volume, and the measurement of the initial samples. The uncertainty is a comprehensive embodiment of all the processes and operations.

*3.4.5. Storage Stability at Different Temperatures.* The results of storage stability of residue in mango sarcocarp at low temperature are shown in Figure 5. There were low

Table 2: Recoveries and RSD of difenoconazole spiked pulp ($n = 5$).

| Sample | Addition concentration (mg/kg) | Recovery (%) | | | | | | RSD (%) |
|---|---|---|---|---|---|---|---|---|
| | | I | II | III | IV | V | Average | |
| Mango | 0.01 | 82 | 72 | 86 | 82 | 71 | 79 | 8.6 |
| | 0.05 | 79 | 85 | 74 | 83 | 83 | 81 | 5.4 |
| | 0.1 | 88 | 88 | 94 | 98 | 105 | 95 | 7.3 |

Table 3: Matrix effect and RSD ($n = 5$).

| Concentration ($\mu$g/mL) | Average of ME (%, $n = 5$) | RSD (%) |
|---|---|---|
| 0.005 | 114.69 | 3.42 |
| 0.025 | 111.54 | 1.73 |
| 0.05 | 104.13 | 2.59 |

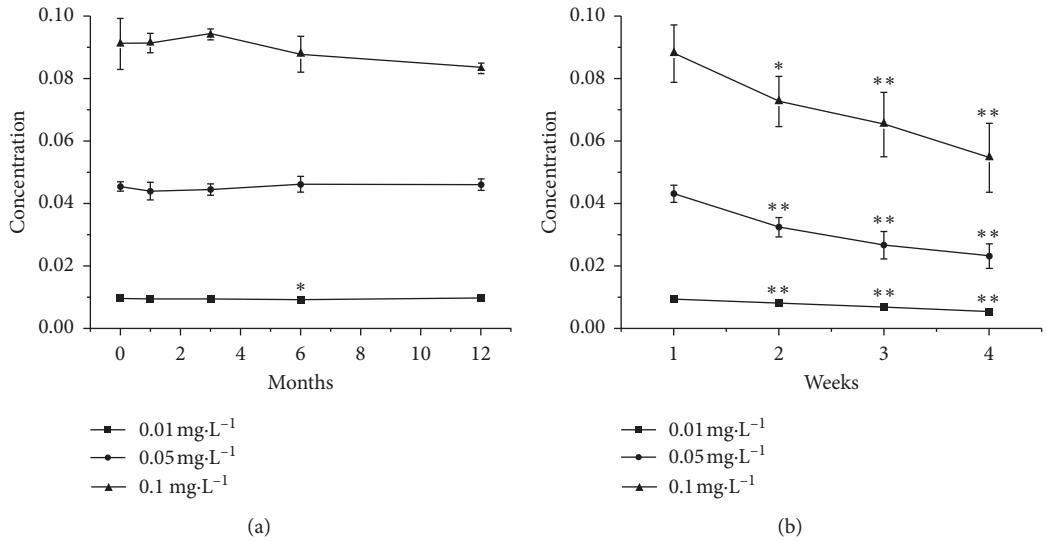


Figure 5: Storage stability of difenoconazole in mango stored at −20°C and 4°C over time. Concentrations are presented as the mean percentage compared to initial values. Values significantly lower than the controls are indicated with a single asterisk (LSD test, $^{*}p < 0.05$) or double asterisk ($^{**}p < 0.01$). Error bars are standard errors of the means ($n = 3$).

variations of residue concentrations when the samples were stored at −20°C. Twelve months later, only about 8.63% and 0.52% of the residue degradation of 0.1 mg/kg and 0.010 mg/kg concentration occurred. At 4°C, the concentration of residue remained more than 86% for one week, after which it exhibited a downward trend. In three weeks, only 66%, 43%, and 68% of the residue at the three concentrations, respectively, remained in the samples. This indicates that storage temperature is a critical factor affecting the stability of the residue. The results show that low storage temperature greatly inhibited the degradation of difenoconazole, and the process is promoted by relatively high temperature (4°C). Thus, samples can be kept at −20°C for a long time before analysis, and 4°C is only appropriate for temporary storage.

Residue degradation in preserved samples was mainly affected by chemical and biochemical factors. Sample matrix has abundant enzymes and microbes, probably causing degradation of the residue but this slows down when the samples are stored at low temperature. In contrast, relative high temperature would enhance their activities, which would promote the degradation of pesticide. Chemical degradation, such as hydrolysis, oxidation, and photolysis, was also influenced by the temperature. The results were consistent with the reports [24, 25]. Therefore, relative high temperature promotes the residue degradation. However, the main factor that affects the stability of this residue remains unclear, and this calls for further investigations.

*3.4.6. Storage Stability in Different Storage Modes.* To further clarify the cause of degradation of the residue, more investigations were conducted. Samples were thoroughly pretreated, and the concentrated residue was stored in the solvent. This is also a frequently used storage mode of the sample in actual analysis operation. Results indicated that there were little changes (less than 10%) of the residue concentration for 6–8 weeks at 4°C although much of the solvent had evaporated (Figure 6). In the solvent, enzyme and microbe would lose their activity, excluding biochemical degradation, and chemical degradation would be the only

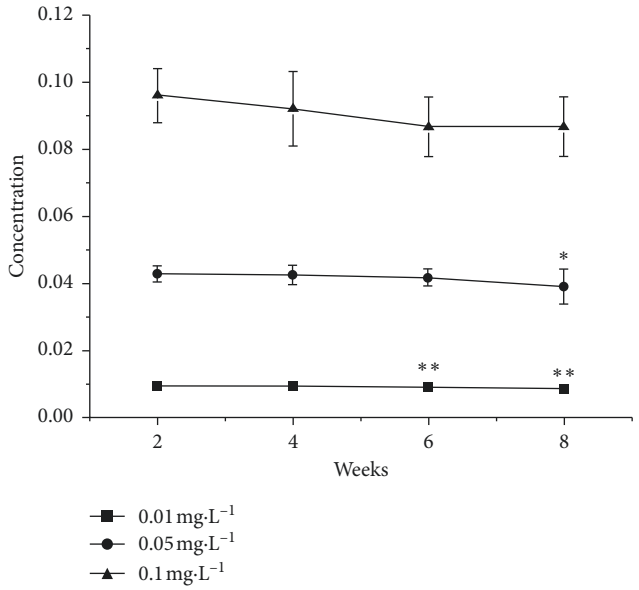


Figure 6: Storage stability of the difenoconazole concentration in mango extract solution at 4°C over time (weeks). Concentrations are presented as the mean percentage compared to the initial values. Values significantly lower than the initial controls are indicated with a single asterisk (LSD test, $^{*}p < 0.05$) or double asterisk ($^{**}p < 0.01$). Error bars are standard errors of the means ($n = 3$).

way. The results showed that little chemical degradation occurred to the residue, although not fully exhibited. Thus, biochemical factors would be determined as the main reason for the degradation of the pesticide residue.

## 4. Conclusion

Results from this work show that biochemical factors mainly cause the degradation of difenoconazole, and the factors are sensitive to temperature. It is a stable mode for the residue preservation to store the extracted samples in the solution. These findings not only provide basic data on the storage of the difenoconazole in the samples but also confirm the validity of the analysis. The investigation eliminates the potential errors in detection and also promotes a reasonable guidance for application of pesticides. All these measures are important in guaranteeing food safety and quality.

## Data Availability

The data used to support the findings of this study are included within the article.

## Disclosure

Fangfang Zhao and Jingkun Liu are co-first authors.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Acknowledgments

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