Effects of glycosylation on the accumulation and transport of fipronil in earthworm (Eisenia feotida)

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Abstract

In this study, the differences in the accumulation of fipronil (F) and the glycosylated product glucose-fipronil (GTF) in earthworm within 48 hours were investigated, and the reason for these differences was explored. The accumulation concentrations of F and GTF for 48 hours in earthworm were determined and studied by HPLC after simple, rapid pretreatment; the mean recoveries were 84.79~95.83%, and the relative standard deviations were 3.39~9.21%, indicating that the method could accurately detect the accumulation concentrations of F and GTF in earthworm. Results showed that the accumulation of F and GTF in earthworm increased with the treatment time, the accumulation concentrations of F in earthworm were significantly higher than those of GTF, and the accumulation concentrations of F were 3.1~6.2 times those of GTF. In addition, the half-lives of GTF in soil (16.90~18.24 days) were significantly lower than those of F (24.75~26.65 days). After adding a hexose transport inhibitor phlorizin, the accumulation change of F in earthworm were not significant, but the accumulation of GTF in earthworm were significantly inhibited, and the accumulation concentrations of GTF in earthworm after adding phlorizin were 32.71~59.07% of those without phlorizin. Overall, our results indicated that the uptake and transport of F and GTF in earthworm were significantly different, the uptake and transport of GTF was relate to monosaccharide transporters, and glycosylation could reduce the toxicity of fipronil to earthworm and the environment, providing an important reference for the application of glucose-fipronil.

1. Introduction

Pesticides have an indispensable position in agricultural production and can improve the productivity and profitability of agricultural products (Yao et al., 2021). As a highly effective phenylpyrazole insecticide, fipronil (CAS Number 120068-37-3) was invented by May and Baker in 1987 and entered the Chinese market in 1993 (Li et al., 2014; Liu et al., 2008), and can be used to control a wide range of pests, such as leaf-cutting ant, fire ant, and *Spodoptera litura* (Mota et al., 2021; Hano et al., 2019; Jameel et al., 2019). Fipronil is known to target the gamma amino butyric acid (GABA) receptor, thereby blocking the chloride channels in the neurons of the central nervous system (CNS), which leads to excessive neuronal stimulation and, eventually, the death of the pests (Kumar et al., 2012; Li et al., 2007; Ikeda et al., 2004).

Due to the broad spectrum and high efficiency, fipronil has increased in popularity during the past few decades (Regan et al., 2017; Mandal et al., 2013). However, the environmental impact of fipronil has always received great attention because of its strong toxicity to some non-target organisms (Kasai et al., 2016; Morrissey et al., 2015; Pisa et al., 2015). Previous studies have reported that fipronil can significantly change the physiological and biochemical characteristics of fishes (Clasen et al., 2012; Hayasaka et al., 2012). Although the European Union has banned the use of fipronil in crops, it is still often used in crops such as sugarcane and citrus in some countries (Farder-Gomes et al., 2021). A recent study has shown that more than 480 million bees died in three months, possibly due to fipronil exposure in Brazil (Castilhos et al., 2019). It is expensive and difficult to develop a novel insecticide. Therefore, an alternative efficient strategy is to research and find ways to reduce the toxicity of fipronil to non-target organisms.

Properly modifying the structure of pesticides can change the toxicity and systemic properties of the pesticides, and the glycosylation of pesticide plays an irreplaceable role in looking for targeting carriers and reducing toxicity and side effects (Duhan et al., 2015). Early on, our group has synthesized a novel conjugate of the insecticide fipronil which containing a glucose moiety (Fig. 1). It is proved that the permeability and water solubility of glucose-fipronil is better than fipronil, and glucose-fipronil has better mobility in the sieve tubes of *Ricinus communis* L. seedlings (Yang et al., 2011). Subsequently, Wu et al. found that the transport mechanism of glucose-fipronil in the phloem of *R. communis*, their study showed that the transport of glucose-fipronil in the phloem was relate to monosaccharide transporters (Wu et al., 2021). In a recent paper, Wen et al. indicated that glucose-fipronil also has good insecticidal activity against *Plutella xylostella*, a target pest in cruciferous plants (Wen et al., 2018). Furthermore, the current focus of our research is to study whether fipronil could reduce its toxicity to non-target organisms after glycosylation, as this would provide important references for the application of glucose-fipronil.

Large scale use and higher doses of pesticides lead to adverse effects on the non-target organisms, pesticide residues in food, toxic effects on human beings and environmental pollution (Kumar et al, 2012). It is well recognized that there are risks attached to the consumption of pesticide-treated crops because of the presence of residues on them. Therefore, rational recommendation of a pesticide requires that it must not only provide an effective control of pests, but at the same time its residues on the other beneficial organisms and humans must be toxicologically acceptable (Kumar et al, 2013). Earthworms (*Eisenia foetida*) are widely distributed and frequently dominant in freshwater benthic communities (Palacios et al., 2010). As a representative organism used in the bioassay of the soil system, *E. foetida* plays an important role in ecological assessment, especially in the bioaccumulation of chemicals and heavy metals (Ciutat et al., 2005; Pasteris et al., 2003). In China, fipronil is mainly used as a seed-coating preparation for agricultural purposes to control soil pests (Qin et al., 2015). Like other agrochemicals, fipronil can enter into soil and aquatic system via direct spraying, rain wash and surface runoff (Chandler et al, 2010). When taking benthic organisms into consideration which use sediment and organic matter as a food resource and habitat, the increase of fipronil in sediment may bring greater risk (Chandler et al, 2010). Meanwhile benthic organisms may accumulate these sediment-associated chemicals and then pose a risk to higher trophic level organisms via food chain and help these compounds input into environment again through bioturbation (Liu et al, 2012).

In this research, methods for extraction, cleaning, and detection of fipronil and glucose-fipronil in earthworm tissue and soil were developed. In order to determine the toxic change of fipronil to worms after glycosylation, two types of uptake kinetics were examined and compared resulting from fipronil and glucose-fipronil treatment in worms and soil, respectively. And the effects of adding phlorizin on the accumulation of fipronil and glucose-fipronil in worms and soil were studied to determine whether the transport of glucose-fipronil was related to monosaccharide transporters.

2. Materials And Methods

2.1. Chemicals, soil, and test organism

Glucose-fipronil was prepared based on our previously described (Yang et al., 2011). Phloridzin (CAS Number 60-81-1) were purchased from and Sigma-Aldrich. Acetonitrile and acetone were purchased from Guangzhou chemical reagent factory.

The experimental soil was collected from the Insecticidal Botanical Garden of South China Agricultural University, Guangzhou, China, and had not been treated with fipronil in the past five years. After removing the superficial layer of 1–2 cm, the soil was collected to a depth of 10 cm, then sieved through 500 m mesh, air dried at room temperature and avoid light, and used within a few days. Physicochemical properties of the soil were as follows: the organic carbon content was 4.04 ± 1.33%; the pH was 4.7 ± 0.4; the soil type was red loam; and the soil cation exchange capacity was 6.2 cmol/kg.

The earthworm (*E. foetida*), medium size, about 10 cm long, weighing 300–500 mg, was purchased from Earthworm Breeding Factory in Jiangmen, Guangdong, and reared in large pots of soil at 20 ± 2 ℃ with frequent water spraying to keep the soil humidity at 80 ± 5%. Before the bioassay test, these worms were allowed to live in these breeding conditions for at least one week to adapt. Adult *E. foetida* (aged 5–6 weeks) was used for the experiments below.

2.2. Extraction of fipronil (F) and glucose-fipronil (GTF) from earthworm and soil

According to our preliminary tests, acetonitrile was used for the extraction of F and GTF. Briefly, the earthworm samples were crushed and accurately weighed 1 g into a 10-mL tube, then added 5 mL of acetonitrile. After mixing well, the tubifex sample was extracted by ultrasonic extraction for 30 min. Finally, the extract was centrifuged at 3000 r/min for 5 min, and an amount of 1 mL of the supernatant was taken and filtered through a 0.22-μm filter membrane. The filtrate was stored in a chromatographic analysis vial at − 20°C and used for HPLC analysis of F and GTF.

The soil samples were accurately weighed 10 g into a 50-mL tube, then added 25 mL acetonitrile and 2 g anhydrous sodium sulfate. After mixing well, the soil sample was extracted by ultrasonic extraction for 30 min and centrifuged at 3500 r/min for 5 min. An amount of 5 g of anhydrous sodium sulfate was added for dehydration. The extract was transferred to a round-bottomed flask and rotary-evaporated at 35 ℃ to dryness under a vacuum. Finally, the flask was washed with 1 mL acetonitrile, and the washing solution was passed through a 0.22-μm filter membrane. The filtrate was used for subsequent further HPLC detection.

2.3. HPLC analysis of fipronil (F) and glucose-fipronil (GTF) in earthworm and soil

The standard curve was established using standard working solutions ranging from 0.01 to 10 mg/L. And recovery tests were used to evaluate the accuracy and precision. As previously described, preparation of earthworm and soil samples fortified with F and GTF at concentrations of 0.1, 1 and 1 mg/L were

performed respectively and replicated five times for each fortification level. Subsequently, the spike recoveries and relative standard deviation (RSD) at various levels were measured.

F was determined using a Shimadzu LC-20A HPLC (equipped with UV detector, Shimadzu Company of Japan). HPLC was performed with an Agilent SB-C_{18} column (150 mm × 4.6 μm × 5 μm) using a mobile phase of acetonitrile-water (60:40), with a flow rate of 1 mL/min, column temperature of 30 ℃, detection wavelength of 210 nm, and injection volume of 10 μL.

GTF was also determined using a Shimadzu LC-20A HPLC (equipped with UV detector). HPLC was performed with an Agilent SB-C_{18} column (150 mm × 4.6 μm × 5 μm) using a mobile phase of acetonitrile-water (50:50), with a flow rate of 1 mL/min, column temperature of 30 ℃, detection wavelength of 210 nm, and injection volume of 10 μL.

2.4. Effects of fipronil (F) glycosylation on the uptake and accumulation in earthworm

To determine the effects of glycosylation of F on the total accumulation in earthworms, the uptake kinetics of F and GTF were examined, respectively. The effects of glycosylation on the transfer of F from soil to earthworm were also determined. Two groups of experiments were conducted, one of which was treated with F and the other was treated with GTF. The experiments were repeated three times. In order to disperse the test substances homogeneously in the soil, the following dilution procedure was carried out in steps. First, put 200 g of dry soil into the two 1000-mL beakers, respectively, and added 30 mL of F (1000 mg/L) and GTF (1000 mg/L) acetone solution to the two 1000-mL beakers, then stirred well. Adjusted the moisture content of the soil sample to 80% before adding the worms, and renewed the water every 8 hours to maintain a certain level of humidity in the soil. In each treatment, 20 worms were placed, sealed with aluminum foil, and reserved 5–6 small holes in the bottle mouth to ensure the circulation of air place. The soil and earthworm samples were collected after 2, 4, 8, 12, 24, and 48 hours, respectively. And the collected worms were washed with deionized water, then the peripheral water of the samples were dried using absorbent paper. Finally, all the samples were stored at -20 ℃ until further sample processing.

2.5. Degradation of fipronil (F) and glucose-fipronil (GTF) in soil

In order to determine the degradation of F and GTF in the soil, two groups of experiments without adding earthworm were established. The experiments were repeated three times. During 14 days of pesticide exposure, 10 g soil samples were collected at 1, 3, 5, 7, 10 and 14 days, and then transferred to 50-mL plastic centrifuge tube for extraction and analysis. The degradation of F and GTF in soil over time was evaluated using a first-order kinetic equation, and the degradation dynamic equation and half-life were calculated according to Eq. (1) and Eq. (2), respectively (Wang et al. 2019; Liu et al. 2014), where C_0 is the initial concentration (mg/kg), k is the degradation coefficient, and $t_{1/2}$ is the required time of pesticide residue level decreased to half of the initial residue concentration.

$C_t = C_0 e^{-kt}$ (1)

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

2.6. Effects of phlorizin on the transport of F and GTF in earthworm

The differences in the uptake kinetics of F and GTF with and without the addition of phlorizin were compared to investigate whether the uptake and transport of F and GTF in earthworm was relate to monosaccharide transporters. There were two groups of experiments in this part, the soils were treated with F and GTF respectively according to method 2.4. Before adding worms, 30 mL of phlorizin aqueous solution (1000 mg/L) was added and mixed well respectively. And then adjusted the moisture content of the soil sample to 80%. Finally, 20 worms were placed in each treatment and the earthworm and soil samples were collected after 2, 4, 8, 12, 24 and 48 hours, respectively. The experiments were repeated three times. All the samples were stored at -20 ℃ for further sample processing.

2.7. Data analysis

Collected data from the before-mentioned experiments were subjected to one-way analysis of variance (ANOVA) using SPSS Statistics, Version 17.0, 2009 (International Business Machines Corporation, Armonk, NY, USA). If significant differences occurred between the treatments, means were separated by Tukey's honestly significant difference (HSD) test at $p < 0.05$ level. Data presented corresponds to means with standard error of three independent experiments.

3. Results And Discussion

3.1. Method validation

Accurate and efficient detection and quantification of F and GTF in earthworm and soil were achieved by developing a HPLC analysis method. The calibration curve had good linearity in the linear range (0.01~10 mg/L), and the correlation coefficient was 0.9999. The average recovery of five replicates of each matrix was determined at spiked levels of 0.01, 0.1 and 1 mg/L to verify and evaluate the accuracy of the method. As shown in **Table 1**, the average recoveries of F in earthworm and soil were 89.20~95.78% and 84.79~91.84%, respectively, and the relative standard deviations were 3.39~6.24% and 5.73~9.21%, respectively. And the average recoveries of GTF in earthworm and soil were 89.18~95.83% and 85.09~91.53%, respectively, and the relative standard deviations were 4.34~6.51% and 5.14~9.07%, respectively. The recovery test results showed that the analytical method had good linearity and accuracy, and this method could accurately detect and analyze the contents of F and GTF in earthworm and soil samples.

3.2. Accumulation of fipronil (F) and glucose-fipronil (GTF) in earthworm

To determine the effects of F glycosylation on accumulation in earthworm, the accumulation curves of F and GTF in earthworm tissue were detected and shown in **Figure 2A**. It can be seen that both the accumulation of F and GTF in earthworm increased with the treatment time within 48 hours. The accumulation concentrations of F and GTF in earthworm tissue were 2.05 mg/kg and 0.46 mg/kg respectively after 2 h, and the accumulation concentrations gradually increased with time and reached 8.91 mg/kg and 2.85 mg/kg respectively after 48 hours of processing. In addition, there was a significant difference between the two accumulation concentrations of F and GTF in earthworm tissue at the same time point, the concentrations of F were always significantly higher than those of GTF. The results showed that compared with F, GTF was more difficult to accumulate in earthworm, indicating that glycosylation could reduce the accumulation of F in earthworm.

To more precisely express the differences in accumulation of F and GTF in earthworm, meanwhile the q values were calculated, and the data were shown in **Figure 2B**. The q values were defined as: $q = C_F / C_{GTF}$; where C_F and C_{GTF} were the accumulation concentrations of F and GTF in earthworm, respectively. It can be seen that the accumulation concentrations of F in earthworm were 3.1~6.2 times those of GTF, indicating that the accumulation of F in earthworm was reduced 3.1~6.2 times after glycosylation.

3.3. Transport of fipronil (F) and glucose-fipronil (GTF) from soil to earthworm

In this work, we used AF (accumulation factor) to express the accumulation of F and GTF in earthworm tissue. AF is a function of the relative absorptive capacity of the soil versus the earthworm, and for this treatment it was defined as: $AF = C_{earthworm} / C_{soil}$; where $C_{earthworm}$ and C_{soil} were the accumulation concentrations of F and GTF in earthworm and soil, respectively (Liu et al. 2012). The AF value at each sampling time point was plotted against time. As shown in **Figure 3**, the AFs of F and GTF increased over time within 48 hours, the AFs of F and GTF were 0.026 and 0.006 respectively after 2 h, and reached 0.185 and 0.052 after 48 h. The results also showed that AF values of F were always significantly higher than those of GTF, indicating that F was more easily transferred from soil to earthworm tissue than GTF. The overall results showed that F was more difficult to transfer from soil to earthworm after glycosylation, which indicated glycosylation could prevent the transfer of F from the soil to earthworm, and F and GTF may had different mechanisms of transmission in earthworm.

3.4. Degradation of fipronil (F) and glucose-fipronil (GTF) in soil

Degradation rate of pesticides after application is an important factor and useful tool for evaluating their residue behavior (Yang et al. 2020). **Tables 2** detailed the degradation regression equation, half-life, and other relevant data of F and GTF in soil. It can be seen that the half-lives of F were 24.75~26.65 days, and the half-lives of GTF were 16.90~18.24 days, the half-lives of GTF in soil were significantly shorter than those of F. The results indicated that compared with F, GTF was safer and more easily accepted by the environment.

3.5. Effects of phlorizin on the uptake of fipronil (F) and glucose-fipronil (GTF) in earthworm

To explore the difference of absorption patterns of F and GTF in earthworm, the accumulation changes of F and GTF in earthworm within 48 hours with and without phlorizin supplementation were compared. Phlorizin is a hexose transport inhibitor commonly used to study active cellular uptake of glucose (Wu et al. 2021). As shown in **Figure 4**, after adding phlorizin, the accumulation of F in earthworm changed but not significant **(Fig. 4A)**, while the accumulation of GTF in earthworm decreased significantly **(Fig. 4B)**. It can be seen that the addition of phlorizin had no effect on the absorption of F, but affected the absorption of GTF by earthworm. The results showed that the addition of phlorizin made the GTF harder to enter the earthworm and the absorb of GTF may be related to transporters. It may be that F is a passive transport when it enters the earthworm, while GTF is an active transport when it enters the earthworm, which needs to consume glycoprotein.

To more precisely express the differences in accumulation concentration of GTF in earthworm after adding phlorizin, meanwhile the t values were calculated, and the data were shown in **Table 3**. The t values were defined as: $t = C_1 / C_2$; where C_1 was the accumulation concentration of GTF with phlorizin supplementation, and C_2 was the accumulation concentration of GTF in the absence of phlorizin. It can be seen that with the addition of phlorizin, the accumulation concentrations of GTF in earthworm were 32.71%~59.07% of those without phlorizin, which indicated that the transport of GTF in earthworm became more difficult after adding phlorizin and the transport of GTF was related to monosaccharide transporters.

4. Conclusions

In conclusion, the accumulation differences of fipronil (F) and the glycosylated product glucose-fipronil (GTF) in earthworm tissue were investigated to determine the effects of F glycosylation on reducing the toxicity of non-target organism, and the reason for these differences was also explored. The methods for pretreatment and detection of F and GTF in soil and earthworm were established and verified. Results showed that GTF was more difficult to accumulate in earthworm than F, indicating that glycosylation could reduce the toxicity of F to earthworm. After adding a hexose transport inhibitor phlorizin, the uptake and accumulation of F in earthworm tissue changed but not significant, while the uptake and accumulation of GTF decreased significantly in earthworm tissue, indicating that the addition of phlorizin inhibited the accumulation of GTF in earthworm tissue and the transport of GTF was related to monosaccharide transporters. Moreover, GTF was more easily degraded in soil than F and more easily accepted by the environment. Overall, our results demonstrated that glycosylation could reduce the toxicity of fipronil to non-target organisms and the environment, providing an important reference for the application of glucose-fipronil.

Declarations

Ethics approval and consent to participate

No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with an unregulated species.

Consent for publication

Not applicable for this section.

Availability of data and materials

All data generated or analysed during this study are included in this published article (and its supplementary information files).

Competing interests

The authors declare no conflict of interest.

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Author contributions

Conceptualization, D. C; methodology, R. H.; investigation, P. Z.; resources, Z. Z.; data curation, L. Z.; writing—original draft preparation, S. L.; writing—review and editing, M. K. and S. F.; supervision, D. C. and Z. Z.; project administration, Z. Z.; funding acquisition, Z. Z.

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Tables

Table 1

Accuracy and precision of analytical methods for the determination of fipronil (F) and glucose-fipronil (GTF) in earthworm and soil (n = 5).

Composition	Matrix	Spiked level (mg/L)	Mean recovery (%)	RSD (%)
F	Earthworm	0.01	89.20	3.39
		0.10	92.89	6.24
		1.00	95.78	5.81
	Soil	0.01	86.69	8.65
		0.10	84.79	5.73
		1.00	91.84	9.21
GTF		0.01	91.68	4.34
	Earthworm	0.10	89.18	4.57
		1.00	95.83	6.51
		0.01	91.53	9.07
	Soil	0.10	87.15	7.79
		1.00	85.09	5.14

Table 2

Regression equation, correlation coefficient (R^2) and half-life of fipronil (F) and glucose-fipronil (GTF) in soil, "*" indicated significant difference between the two half-lives at $p < 0.05$ level based on Tukey's HSD test (n = 3).

Pesticide	Repetition	Regression equation	Correlation Coefficient (R^2)	Half-life (days)
F	1	$y = 81.508\ e^{-0.028x}$	0.9920	24.75
	2	$y = 82.235\ e^{-0.026x}$	0.9979	26.65
	3	$y = 84.055\ e^{-0.027x}$	0.9983	25.67
	Average	$y = 82.597\ e^{-0.027x}$	0.9977	25.67*
GTF	1	$y = 82.901\ e^{-0.041x}$	0.9933	16.90
	2	$y = 84.054\ e^{-0.039x}$	0.9892	17.77
	3	$y = 86.629\ e^{-0.038x}$	0.9906	18.24
	Average	$y = 84.526\ e^{-0.039x}$	0.9918	17.77*

Table 3

The t value of GTF in earthworm, the t value was defined as: $t = C_1 / C_2$; where C_1 was the accumulation concentration of GTF with phlorizin supplementation, and C_2 was the accumulation concentration of GTF in the absence of phlorizin.

	Exposure time (h)					
	2	4	8	12	24	48
t value (%)	32.71±1.81	41.20±2.24	49.72±2.67	45.85±3.89	52.84±3.19	59.07±3.12

Figures

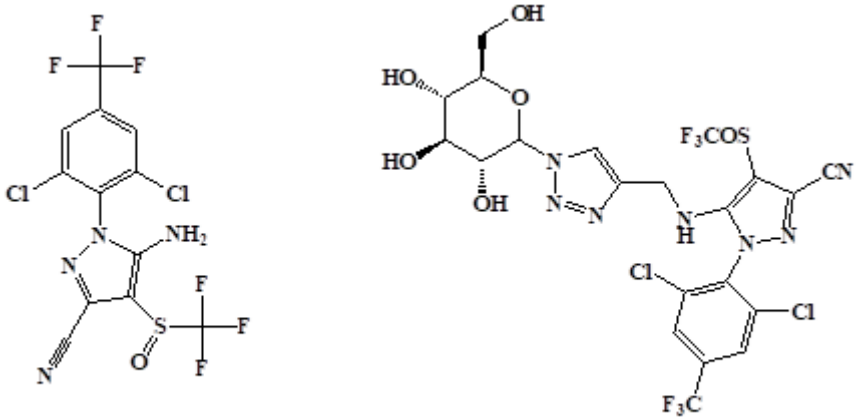

Figure 1

Structure of fipronil (F) and glucose-fipronil (GTF).

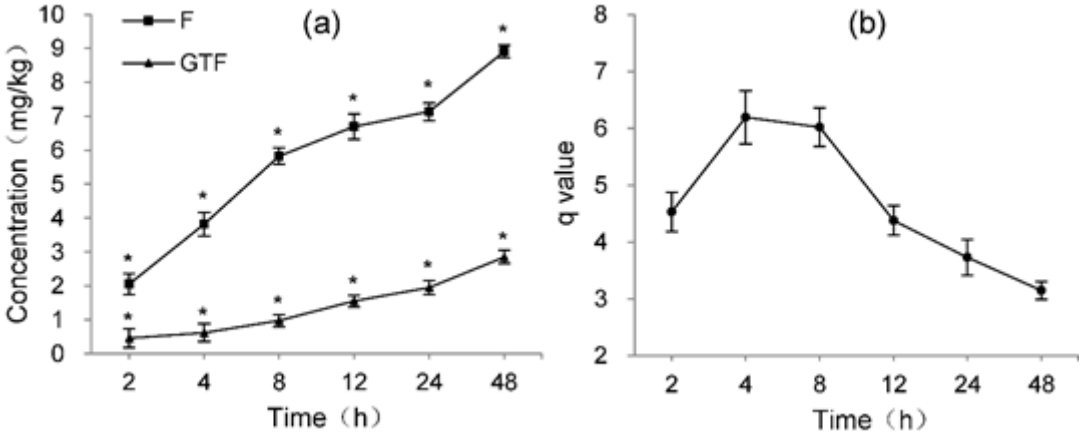

Figure 2

Accumulation of fipronil (F) and glucose-fipronil (GTF) in earthworm (a) and q value (b) at different time point, the q value was defined as: $q = C_F / C_{GTF}$, where C_F and C_{GTF} were the accumulation concentrations of F and GTF in earthworm, respectively, the bars are standard error, '*' indicated significant difference between the two concentrations at the same time point ($p < 0.05$, Tukey's HSD test).

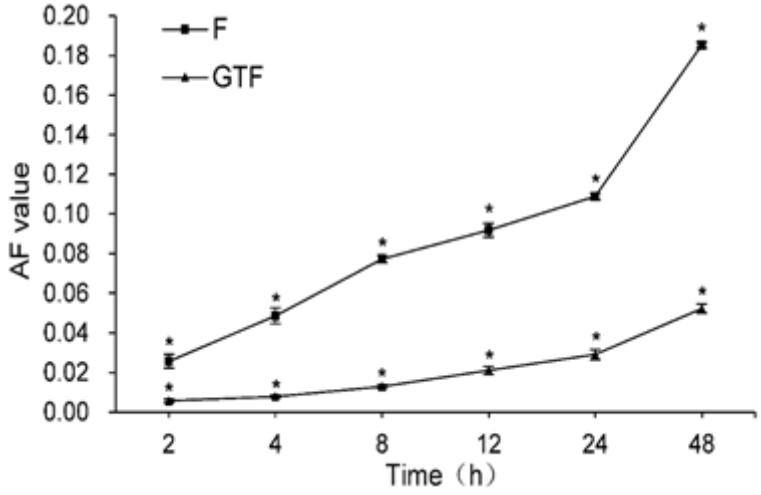

Figure 3

AF value at different time point, the AF value was defined as: $AF = C_{earthworm} / C_{soil}$; where $C_{earthworm}$ and C_{soil} are the accumulation concentrations of F and GTF in earthworm and soil, respectively, the bars are standard error, '*' indicated significant difference between the two concentrations at the same time point ($p < 0.05$, Tukey's HSD test).

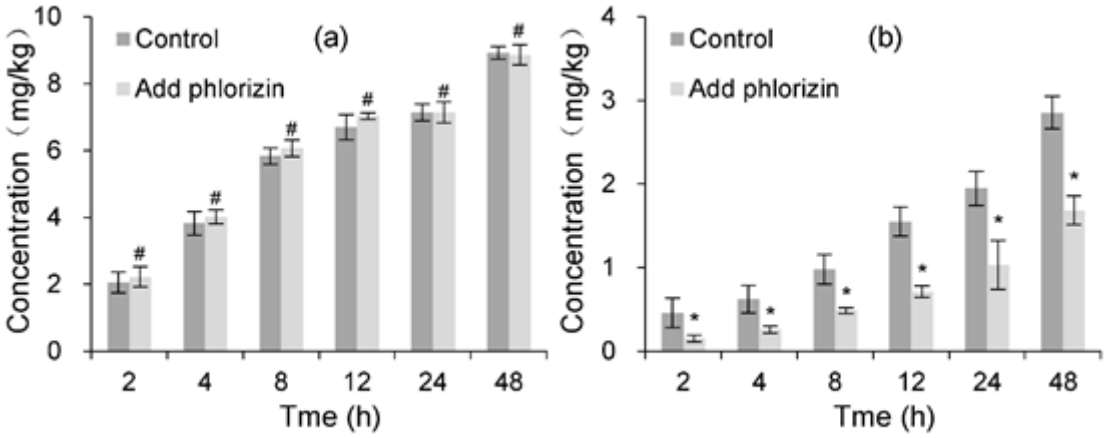

Figure 4

Accumulation concentration of fipronil (a) and glucose-fipronil (b) in earthworm at different point with phlorizin supplementation, the bars are standard error, ‘Control’ indicated the accumulation concentration of fipronil and glucose-fipronil in earthworm at different point without phloridzin, ‘#’ indicated that there was no significant difference between the two concentrations at the same time point, ‘*’ indicated significant difference between the two concentrations at the same time point ($p < 0.05$, Tukey’s HSD test).