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Enantioseletive bioaccumulation of tebuconazole in earthworm *Eisenia fetida*

Dingyi Yu, Jianzhong Li*, Yanfeng Zhang, Huili Wang, Baoyuan Guo*, Lin Zheng

Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China. E-mail: yudingyi1990@sina.com.cn

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Abstract

Methods of extraction and determination of tebuconazole enantiomers in earthworm (*Eisenia fetida*) were developed by capillary electrophoresis (CE) and high performance liquid chromatography (HPLC). Both CE and HPLC have excellent resolution and recovery. The linearity ranges were 2.9–102.4 mg/kg and 3.0–99.6 mg/kg for (+)-R-tebuconazole and (–)-S-tebuconazole respectively in CE, and from 0.56 to 1000 mg/kg for both enantiomers in HPLC. Enantioselective bioaccumulation in earthworms from soil was investigated under laboratory condition at concentrations of 10 and 50 mg/kg dw in soil. The uptake kinetics of (+)-R-tebuconazole fitted the first-order kinetics well with r^2 0.97 and 0.94 under 10 and 50 mg/kg dw exposure condition, respectively, while (–)-S-tebuconazole with r^2 0.75 and 0.22 did not show the same. Bioaccumulation of tebuconazole in earthworm tissues was enantioselective with a preferential accumulation of (+)-R-tebuconazole. The (+)-R-tebuconazole might also have biomagnifying effect potential in earthworm food chain with biota-sediment accumulation factor (BSAF) of 1.64 kg OC/kg lip in 10 mg/kg dw exposure group and 2.61 kg OC/kg lip in 50 mg/kg dw exposure group from soil to earthworm after 36 days. Although (–)-S-tebuconazole shares the same physicochemical properties with (+)-R-tebuconazole, it did not biomagnify. BSAFs of (–)-S-tebuconazole were 0.50 kg OC/kg lip (10 mg/kg dw tebuconazole exposure) and 0.28 kg OC/kg lip (50 mg/kg dw tebuconazole exposure) after 36 days, which was possibly owing to biotransformation or metabolism in earthworm tissues.

Key words: tebuconazole; earthworm; bioaccumulation; enantioselectivity

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Introduction

It has been reported that more than 25% of pesticides are chiral compounds with at least two mirror image enantiomers (Williams, 1996). Enantiomers usually differ in their biological properties such as bioactivity, toxicity, metabolism, accumulation and degradation behaviors due to their interaction with enzymes or other naturally occurring chiral molecules (Hegeman and Laane, 2002). Triazole derivatives represent an important category of fungicides, and chirality is almost ubiquitous among them (Wu et al., 2001). Tebuconazole, (RS)-1-*p*-chlorophenyl)-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol, is a broad-spectrum triazole fungicide used to control soil-borne and foliar diseases in peanuts and other crops. Tebuconazole has one chiral carbon atom and a pair of enantiomers as shown in Fig. 1. Its absolute configuration is left-optical (–) rotation of S-enantiomer and right-optical (+) rotation of R-enantiomer (Kaulen, 1989). The (+)-R-enantiomer shows faster degradation than that of (–)-S-enantiomer in cabbage, rat liver microsomes and rabbit plasma, while (–)-S-enantiomer dissipates faster than (+)-R-form in cucumber fruit and soil (Zhu et al., 2007; Shen et al., 2012; Wang et al., 2012). It is important to further understand the biological behavior of tebuconazole enantiomers in ecosystem.

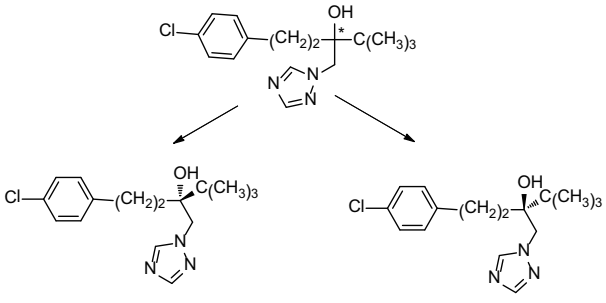

Fig. 1 Chemical structures of two enantiomers from tebuconazole.

Earthworms are exposed to a wide variety of agricultural chemicals such as insecticides, fungicides and herbicides and are recognized as effective indicators of environmental pollution. Earthworms are appropriate model organisms for bioavailability as they live in close contact with the soil, have a thin and permeable cuticle, and also consume large amounts of soil (Jager et al., 2005). Environmental Protection Agency, Organization for Economic Cooperation and Development, and Ministry of Agriculture of the People's Republic of China have already used the earthworm (*Eisenia fetida*) as an indicator organism to assess the potential biological effect of the chemicals if released into

* Corresponding author. E-mail: jzli@rcees.ac.cn (Jianzhong Li); guoby@rcees.ac.cn (Baoyuan Guo)

the environment. Equilibrium partitioning theory model has been developed to assess the bioaccumulative potential of commercial chemicals (Connell and Markwell, 1990; Armitage and Gobas, 2007). Enantioselective toxicity and bioaccumulation of pesticides in earthworms have got wide attentions in recent years. Alpha-cypermethrin (Diao et al., 2011), metalaxyl (Xu et al., 2011), and benalaxyl (Xu et al., 2009) were demonstrated to bioaccumulate enantioselectively in earthworm tissues.

Tebuconazole is often used as a seed coating agent applied in agricultural soil, therefore, earthworms may be exposed to tebuconazole of high dose directly. No research has yet been reported for bioactivity of tebuconazole enantiomers in earthworms. In this article, an effective method was developed to determine enantiomers of tebuconazole in earthworm samples by capillary electrophoresis (CE) and high performance liquid chromatography (HPLC). The enantioselective bioaccumulation of tebuconazole in earthworm (*Eisenia fetida*) from soil was investigated under laboratory conditions.

1 Materials and methods

1.1 Chemicals and reagents

Tebuconazole (purity > 98.8%) was donated by Institute for the Control of Agrochemicals, Ministry of Agriculture. Stock solutions of tebuconazole were prepared in acetone at 1000 and 5000 mg/L and kept in darkness under refrigeration at 4°C.

Water was purified by a Milli-Q system (Millipore, USA). Methanol (HPLC grade), *n*-hexane (HPLC grade) and 2-propanol (HPLC grade) were obtained from Dikma Company (USA). Acetone, petroleum ether, acetonitrile (analytical grade), sodium dodecyl sulfate (SDS), sodium hydroxide (NaOH), potassium hydroxide (KOH), phosphoric acid (H_3PO_4), potassium dihydrogen phosphate (KH_2PO_4) and urea were purchased from Beijing Chemical Reagent Co. Ltd. (China). Sulfated β-cyclodextrin (S-β-CD, typical degree of substitution, 7-11) was obtained from Fluka (Schnelldorf, Germany).

1.2 Earthworms

Mature earthworms (*Eisenia fetida*) purchased from northeastern farm, Beijing, were maintained in a wooden breeding box (60 cm × 50 cm × 40 cm) containing a mixture of soil and cattle manure. The earthworms were healthy and active before introduced into the experiment.

1.3 Apparatus

The CE experiments were carried out on a Beckman P/ACE MDQ Capillary Electrophoresis System (Beckman, USA) equipped with a UV detector. The electropherograms were recorded and integrated by an IBM PC running 32 Karat software version 4.0 (Beckman, USA). Separation was performed in a bare fused-silica capillary (Yongnian Optical Fiber, China) with a total length of 50 cm (40 cm effective length) and an inside diameter of 75 μm.

The HPLC experiments were performed using an Agilent 1100 Series HPLC (Agilent, USA) equipped with G1322A degasser, G1311A pump and G1314B VWD. AT-930 heater and cooler column attemperator (Tianjin Automatic Science Instrument Co., Ltd., China) was used to control column temperature. The signal was received and processed by Agilent chemstation software.

1.4 Capillary electrophoresis conditions

A new capillary was initially washed with methanol for 10 min, followed by ultrapure water for 5 min, 1 mol/L HCl for 10 min, ultrapure water for 5 min, 1 mol/L NaOH for 10 min, and ultrapure water for 5 min. As a daily routine procedure, the capillary was rinsed with 1 mol/L NaOH for 10 min followed by a 10-min rinse with ultrapure water, and then flushed with running buffer for 3 min before sample injection. Before each run, the capillary was rinsed with 1 mol/L NaOH for 2 min, ultrapure water for 2 min, and running buffer for 2 min. To achieve reproducible separations, all experiments were performed at (20 ± 0.1)°C and were run in triplicate.

The optimal background electrolyte (BGE) in the CE experiments was 20 mmol/L KH_2PO_4 and 10 mmol/L H_3PO_4 (pH 2.16), containing 1% S-β-CD and 2 mol/L urea. The running buffer was filtered with a 0.22 μm filter before use. The power supply was operated in the reversed-voltage mode and the analyte migrated toward the positive pole. The separation voltage was –25 kV, and sample injection was performed hydrodynamically under pressure of 3.45 kPa for 5 sec, corresponding to an injection volume of approximately 5 nL. The UV detection wavelength was set at 220 nm.

1.5 HPLC Conditions

A commercial HPLC cartridge ChiralPAK IC (cellulose tris-(3, 5-dichlorophenyl-carbamate)) was used to separate tebuconazole. The cartridge purchased from Daicel Chemical Industries (Tokyo, Japan) was 250 mm × 4.6 mm i.d. with the cellulose tris (3,5-dichlorophenyl polymer) immobilized on a 5-μm silica gel substrate. A mixture of *n*-hexane and 2-propanol (90:10, *V*/*V*) was used as mobile phase at a rate of 1.0 mL/min. The injection volume was 20 μL and the UV detection wavelength was set at 220 nm. To achieve reproducible separations, all experiments were performed at (20 ± 0.1)°C and were run in triplicate.

1.6 Sample treatment

1.6.1 Soil collection and earthworm exposure

Surface soils (0–10 cm) were collected from a farm in Changping District, Beijing, China and detected no tebuconazole residue. The soil samples were sieved (2 mm) and air-dried at room temperature and stored in a dark dry place before experiments. Physicochemical properties of the soil were as follows: organic matter (OM), 2.00%; clay, 2.73%; sand, 62.29%; silt, 32.98%; water holding capacity, 25%; and pH 7.5 ± 0.2 (determined according to GB 15618-1995).

The experiment was carried out in two levels: soil of each level was contaminated with 10 and 50 mg/kg dw

(dry weight) tebuconazole respectively. Each level had triplicate groups. We did the procedure in steps to ensure that soils were treated homogeneously. The 500 g of air-dried soil was spiked with 100 mL racemic stock solutions of 1000 and 5000 mg/L tebuconazole respectively and mixed thoroughly. After solvent evaporation, 4.5 kg of the untreated soil was added to each spiked soil and then mixed thoroughly. The soil was stationarily incubated for 2 days before the experiment. Pure water was added to restore 25% water content. Earthworms were allowed to live in the uncontaminated soil one week to acclimate before they were introduced. After their gut contents depurated on moist filter paper for 3 hr at 20°C, 120 mature earthworms were put into the contaminated soil in each group. The exposure experiment was carried out in glass boxes in man-made climate equipment. The loss of water by evaporation was compensated by addition of pure water every two days. The temperature was set at 20°C and the humidity 50%. The 5 earthworms as well as soil (10 g wet soil) were collected after exposure periods (0.25, 0.5, 1, 3, 5, 7, 10, 14, 22, 36 days). Earthworms were rinsed by pure water and placed at moist filter paper to depurate most of their gut contents for about 3 hr. After that, earthworms were dried with clear filter paper, and then both earthworms and soil were weighed and put into sample bags to freeze at –20°C.

1.6.2 Soil sample treatment

Soil samples were mixed with 5 g anhydrous sodium sulfate and 25 mL acetonitrile in a 50-mL polypropylene centrifuge tube. The tube was stirred for 3 min on a vortex mixer, exposed to ultrasonic vibration for 10 min and then centrifuged at 3000 r/min for 5 min. The extraction was repeated again following the same step. The extracts were combined and then filtered through anhydrous sodium sulfate (5 g) for dehydration and evaporated to dryness on a vacuumed rotary at 45°C. The residue was reconstituted in 1 mL of 2-propanol and filtered through a 0.22-μm filter prior to HPLC analysis.

1.6.3 Earthworm sample treatment

Before treated with extractants, earthworm samples were thawed for 10 min at room temperature. They were blended with 15 mL acetonitrile in a 50-mL polypropylene centrifuge tube and homogenized with IKA Turax T25 homogenizer at 13,600 r/min for 1 min. The mixture was vortex-mixed for 3 min, exposed to ultrasonic vibration for 10 min and then centrifuged at 3000 r/min for 5 min. The supernatants were transferred to a separatory funnel. The sample was re-extracted in the same way and the supernatants were combined. Next, 3×20 mL *n*-hexane was added for liquid-liquid partition to extract most of lipid. The upper layer of *n*-hexane was discarded, and the layer of acetonitrile was passed through a funnel with about 15 g anhydrous sodium sulfate to round bottom flask to evaporate to dryness at 40°. After that, the residue was purified by florisil-SPE cartridge (1 g, 6 mL, Dikma, USA). The cartridge was preconditioned by rinsing with 5 mL acetone followed by 5 mL petroleum ether and 5 mL acetone-petroleum (1:9, *V*/*V*). The sample of dry extract was dissolved in 3×1 mL of acetone-petroleum (1:9, *V*/*V*), and transferred into the cartridge. The eluate was discarded. Then the SPE cartridge was eluted with additional 5 mL acetone-petroleum (1:2, *V*/*V*) and the eluate was collected into a clear flask and evaporated to dryness at 40°C. Finally the residue in the flask was dissolved by 1.0 mL of 2-propanol for HPLC determination or 1.0 mL CE BGE for CE determination.

2 Results and discussion

2.1 Method development

2.1.1 Optimization of CE separation of tebuconazole

β-Cyclodextrin (β-CD) and its derivatives 2'-hydroxypropyl-β-cyclodextrin (HP-β-CD) and sulfated-β-cyclodextrin (S-β-CD) were evaluated as chiral selectors. In phosphate buffers (pH 1.87, 2.16, 3.79) containing β-CD and HP-β-CD, no enantiomeric separation was observed, which may due to the weak basicity of tebuconazole (Wu et al., 2001). The negatively charged S-β-CD could provide stronger interactions of coulombic forces, hydrogen bond and steric hindrances (Berthod, 2009) with tebuconazole than β-CD and HP-β-CD, leading to a better chiral recognition. Electroosmotic flow (EOF) was minimized in fused-silica capillary at low pH (pH < 3), which decreased the migration time toward the anode inlet. Thus, 1% S-β-CD was selected as chiral selector, 20 mmol/L KH_2PO_4 was selected as BGE, and 10 mmol/L phosphate was used to adjust pH to 2.16 initially. A reversed separation voltage of 25 kV was employed. Enantiomers were separated at this condition (Fig. 2a).

Two additives of SDS and urea were then selected to enhance the solubility of tebuconazole in buffer. The 10, 50, and 100 mmol/L SDS was added to the running buffer and no enantiomeric separation was found and only one sharp peak of the racemate was observed at about 3 min (Fig. 2c). While 1, 2, and 4 mol/L urea was added to the running

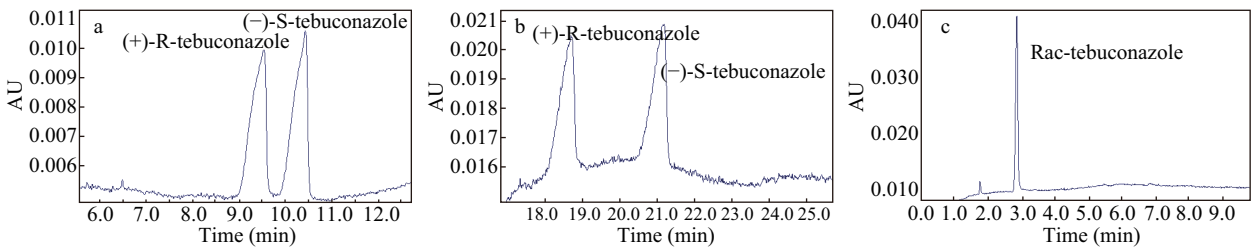

Fig. 2 Optimization of tebuconazole enantioseparation in CE. (a) buffer containing 1% S-β-CD, 20 mmol/L KH_2PO_4, 10 mmol/L phosphate; (b) addition of 2 mol/L urea; (c) addition of 50 mmol/L sodium dodecyl sulfate (SDS).

Table 1 Comparison of CE and HPLC method in the determination of tebuconazole enantiomers in earthworm samples

		RT	*a*	*b*	r^2	R_S	LOD (mg/L)	RSD (%) (n = 3)	Linearity range (mg/kg)	Recovery (%) (5 g sample)
CE	(+)-R-tebuconazole	19.62	1287.0	204.31	0.983	2.14	0.13	6.7	2.9–102.4	70.3–107.7
	(–)-S-tebuconazole	22.15	1354.1	361.58	0.984		0.17	5.7	3.0–99.6	78.2–113.3
HPLC	(–)-S-tebuconazole	20.19	23.12	–3.91	0.999	2.79	0.021	1.3	0.56–1000	82.3–105.5
	(+)-R-tebuconazole	26.21	22.89	–3.41	0.999		0.023	0.9	0.56–1000	84.1–105.1

RT is migration time for CE and retention time for HPLC.

buffer containing 1% S-β-CD, 20 mmol/L KH_2PO_4 and 10 mmol/L phosphate, current was inhibited and the migration time exhibited significant increase. Migration time as well as resolution doubled when 2 mol/L urea was added (Fig. 2b). Maximum detection concentration was enhanced from 50–100 mg/kg to over 100 mg/kg. Thus, a solution of 1% S-β-CD, 20 mmol/L KH_2PO_4, 10 mmol/L phosphate and 2 mol/L urea was employed as buffer in CE separation.

2.1.2 Optimization of SPE clear-up

The complicated matrix of earthworms might disturb CE and HPLC separations. In order to meet the requirements of both CE and HPLC analysis, further clear-up was introduced. Solid phase extraction (SPE) was evaluated to exploit the polarity difference between matrix and tebuconazole. Florisil, neutral alumina, and pesticarb/NH_2 cartridges (Agela, China) were evaluated in normal phase mode with elution of analyte achieved using solvents of higher polarity than the solvent in which the analyte was transferred into the cartridge. After dry extract was dissolved in 3×1 mL of acetone-petroleum ether (1:9, *V*/*V*) and transferred into the cartridge, 5 mL solvent was used to elute the analyte. Neutral alumina required acetone-petroleum ether (1:1, *V*/*V*) to elute all the analyte, while florisil required the less polar solvent (acetone-petroleum ether (1:2, *V*/*V*)). Pesticarb/NH_2 has the strongest retention on tebuconazole, and even with the pure acetone, there were still 10%–25% tebuconazole retained in the cartridge.

Florisil was the preferred absorbent as it provided maximum removal of interfering coextractives with the least loss among the three cartridges, so that samples were suitable for both CE and HPLC determination.

2.1.3 Comparison between CE separation and HPLC separation

The HPLC chiral separation method for tebuconazole was developed on ChiralPAK IC with *n*-hexane and 2-propanol (90:10, *V*/*V*) as mobile phase (detected at 220 nm). Several key parameters of CE and HPLC separation of tebuconazole were compared (Table 1). The elution orders of right and left-rotation enantiomers in HPLC method were measured by Chiralyser-MP optical rotation detector produced by IBZ Messtechnik Company (Germany). The optical signals were received and processed by Beijing Separation Science & Technology Development Co., Ltd. (China). The result showed that the first eluted enantiomer was laevo (–)-S-tebuconazole and the second eluted enantiomer was dextro (+)-R-tebuconazole (Fig. 3). The migration orders in CE method were then identified by HPLC orders, which was opposite to the elution orders of HPLC.

RT is the migration time for CE and retention time for HPLC. Calibration curves are expressed as regression lines ($y = ax + b$), where, y is the ratio of the peak area of enantiomeric compound, x (mg/L) is enantiomer concentration, a is slope, b is intercept, and r^2 is the correlation coefficient. The resolution for a pair of enantiomers was calculated as R_S (Eq. (1)).

$$R_S = \frac{2(RT_2 - RT_1)}{\omega_1 + \omega_2} \quad (1)$$

where, ω is the width of the peak at the baseline. LODs, recoveries, RSDs and calibration curves for both tebuconazole enantiomers are shown in Table 1.

Both CE and HPLC have excellent resolution and recovery, but HPLC has lower LOD. Moreover, low solubility of tebuconazole in BGE and the instability CE itself result in narrow linearity range and large RSD. All real earthworm samples were detected by HPLC initially and CE method was then used as a validation.

2.2 Enantioselective degradation in soil

To investigate enantioselective bioaccumulation in earthworms, enantiometric changes in soil was evaluated firstly. The enantiometric ratio (R_e) (Yi et al., 2007) was employed

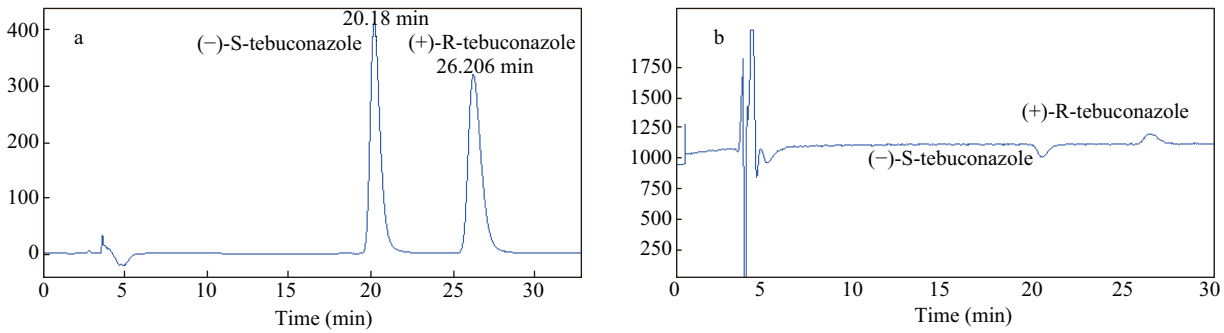

Fig. 3 UV detection and optical detection of two enantiomers of tebuconazole. (a) UV detection of HPLC method; (b) optical detection (Chiralyser optical detector). Flow rate: 1 mL/min, UV: 220 nm, *n*-hexane/2-propanol = 90/10 (*V*/*V*).

to evaluate the enantioselectivity of two enantiomers during the experiments (Eq. (2)).

$$R_e = A_1 - A_2 \quad (2)$$

where, A_1 is the peak area of (–)-S-tebuconazole, A_2 is that of (+)-R-tebuconazole and $R_e = 1$ represents the racemic mixture. R_e values were all average values of the triplicate in this study.

Concentrations and R_e values of two enantiomers in soil during the period of 36 days of incubation are shown in Table 2. Concentration of tebuconazole decreased about 27% and 17% at low and high dose soil respectively at the end of 36 days exposure. The decrease may result from degradation and biotransformation (Bending et al., 2007). No significant difference was observed between the two enantiomers in both low and high dose soil.

2.3 Enantioselective bioaccumulation in earthworms

Concentrations of tebuconazole enantiomers in exposed earthworm increased in the initial days as shown in Fig. 4. The increase rates were different from concentration to concentration, and from enantiomer to enantiomer. (+)-R-tebuconazole in low dose group experienced a steady increase and reached equilibrium at 3-day-point at 13.08 mg/kg. From 14 days to 36 days, the concentration had a slightly decline, which may due to the concentration decline in soil. In high dose group, equilibrium came later until 7 days with concentration staying at around 100 mg/kg. Concentrations of (–)-S-tebuconazole in low dose group surged rapidly to 8.78 mg/kg at 0.5 days and then slightly increased to peak at 9.90 mg/kg. From that time, concentration began to decrease and the concentration was only around 3 mg/kg after 22 days exposure. In high dose group, (–)-S-tebuconazole stopped increase at 1 day and turned to drop consistently. At 29 day point, the concentration was the minimum only 7.63 mg/kg.

Organic pollutants were assumed to be partitioned between soil and earthworms (Connell and Markwell, 1990; Krauss et al., 2000). Equilibrium partitioning theory (EPT) model was introduced to describe the uptake process of tebuconazole enantiomers (Connell and Markwell, 1990; Krauss et al., 2000). The uptake of tebuconazole enantiomers are considered to follow a first-order kinetics equation:

$$dC_{Worm}/dt = k_1 C_{Soil} - k_2 C_{Worm} \quad (3)$$

$$C_{Worm} = \frac{k_1 C_{Soil}}{k_2}[1 - \exp(-k_2 t)] = K C_{Soil}[1 - \exp(-k_2 t)] \quad (4)$$

$$K = k_1/k_2 \quad (5)$$

where, t is the exposure time, C_{Worm} (mg/kg) is the concentration of tebuconazole enantiomers bioaccumulated in earthworm tissues, C_{Soil} (mg/kg dw) is the concentration in

Table 2 Concentrations and R_e values of the two enantiomers in soil during the period of 36 days of incubation

Time (day)	10 mg/kg dw				50 mg/kg dw				R_e	
	(–)-S-tebuconazole		(+)-R-tebuconazole		(–)-S-tebuconazole		(+)-R-tebuconazole		10 mg/kg dw	50 mg/kg dw
	Avg. Con.* (mg/kg dw)	RSD (%)	Avg. Con. (mg/kg dw)	RSD (%) (n = 3)	Avg. Con. (mg/kg dw)	RSD (%) (n = 3)	Avg. Con. (mg/kg dw)	RSD (%) (n = 3)		
0	10.11	2.97	10.02	2.96	49.77	1.57	49.81	2.74	1.01	1.00
0.25	9.72	5.92	9.71	3.87	50.47	1.67	49.89	2.15	1.00	1.01
0.5	10.08	3.64	10.15	2.14	50.91	3.29	50.68	3.07	0.99	1.00
1	9.61	2.38	9.65	1.06	48.85	4.11	48.91	5.13	1.00	1.00
3	9.75	2.84	9.61	2.05	47.80	5.47	47.97	5.63	1.01	1.00
5	9.29	2.74	9.36	2.95	47.27	3.04	47.35	2.78	0.99	1.00
7	8.88	2.55	8.80	3.34	46.69	13.14	46.35	3.13	1.01	1.01
10	8.43	5.98	8.37	2.78	45.64	5.26	45.37	2.17	1.01	1.01
14	8.28	4.00	8.29	3.45	45.01	4.21	44.89	4.75	1.00	1.00
22	7.91	4.63	7.94	6.78	43.00	2.31	42.38	5.35	1.00	1.01
29	7.80	3.15	7.72	3.56	42.55	5.08	42.48	4.76	1.01	1.00
36	7.40	4.33	7.28	4.14	41.84	3.93	41.89	2.16	1.02	1.00

* Avg. Con. represents average concentration.

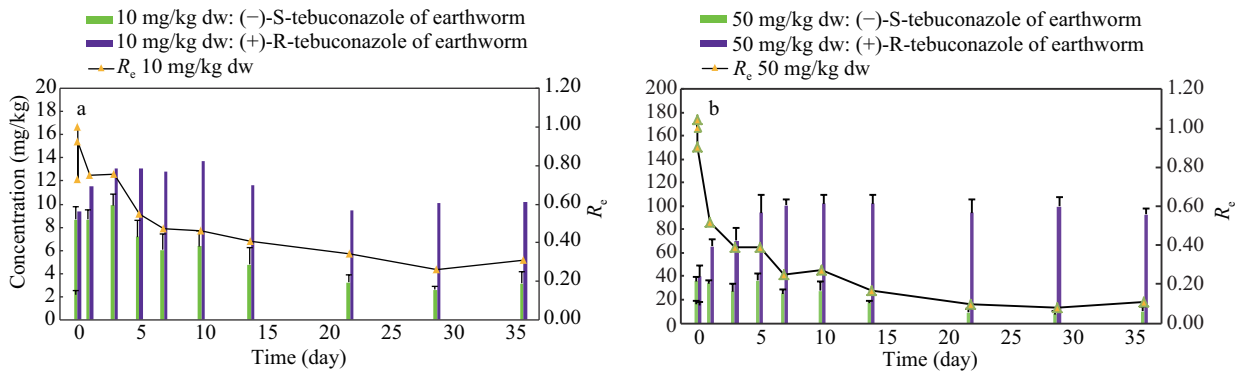

Fig. 4 Concentrations of tebuconazole enantiomers in earthworm samples and R_e values. (a) 10 mg/kg dw exposure group (b) 50 mg/kg dw exposure group. Data are expressed as mean ± SD.

Table 3 Calculated uptake rate coefficient (k_1), elimination rate coefficient (k_2), K and r^2 of tebuconazole enantiomers in low and high dose exposure

		K	k_1	k_2	r^2
10 mg/kg dw	(–)-S-tebuconazole	1.402 ± 0.082	2.445 ± 0.520	1.744 ± 0.371	0.969
	(+)-R-tebuconazole	0.831 ± 0.121	1.662 ± 0.432	1.953 ± 0.521	0.734
50 mg/kg dw	(–)-S-tebuconazole	1.916 ± 0.145	1.760 ± 0.433	0.919 ± 0.226	0.942
	(+)-R-tebuconazole	0.548 ± 0.132	1.924 ± 11.9	3.511 ± 21.7	0.222

soil, k_1 (day^{-1}) denotes the uptake rate constant from soil, and k_2 (day^{-1}) denotes the elimination rate constant, K is the constant ratio of k_1 and k_2.

By fitting the average concentrations of triplicate in earthworm and time into Eq. (4), a nonlinear dynamic fitting technique provided by SigmaPlot (SPSS Science, Chicago, IL, USA) gave the estimated value of K and k_2 (Table 4). k_1 was calculated with Eq. (5). The treads of (+)-R-tebuconazole increase fitted the first-order kinetics well under both high and low concentration groups (r^2 = 0.97, 0.94). This result indicated that (+)-R-tebuconazole in earthworms was proportional to the concentration in soil and accumulation from soil by earthworms was primarily an equilibrium partition process driven by fugacity (Liang et al., 2010). However, (–)-S-tebuconazole does not obey the first-order kinetics under both high and low concentration exposure (r^2 = 0.75, 0.22). It was suggested that the accumulation of (–)-S-tebuconazole by earthworm was more complicated than the equilibrium partition possess. Considering that the enantiomers of tebuconazole were of the same physicochemical properties, and (–)-S-tebuconazole might have processed biotransformation and metabolism beyond the equilibrium partition of (+)-R-tebuconazole.

Since the uptake kinetics of tebuconazole enantiomers was different, the uptake of tebuconazole had significant enantioseletivity as shown in Fig. 4. In low dose group (Fig. 4a), R_e dropped during the whole exposure time and stayed around 0.30 at the end of the exposure. This result suggested that the accumulation of tebuconazole in earthworm tissues was enantioselective with preferential accumulation of (+)-R-tebuconazole. In high dose group (Fig. 4b), preferential accumulation of (+)-R-tebuconazole was even stronger, and R_e decreased to only 0.11 at the end of the exposure.

In this study, biota to soil accumulation factor (BSAF) was used to express the bioaccumulation of tebuconazole enantiomers. In order to compare with cases in former literatures, the activities were normalized to lipid content of earthworm and organic carbon (OC) of soil, assuming a lipid content of 1% and a factor of 1.7 between OC and organic matter (OM) (Jager et al., 2003). The Eqs. (6) and (7) are as follows (Xu et al., 2011):

$$\mathrm{BSAF} = C_{\mathrm{EW}} / C_{\mathrm{S}} \tag{6}$$

or

$$\mathrm{BSAF} = C_{\mathrm{EW}} F_{\mathrm{OM}}(\mathrm{soil}) / 1.7 C_{\mathrm{S}} F_{\mathrm{lip}}(\mathrm{earthworm}) \tag{7}$$

where, C_{EW} and C_S are concentrations of tebuconazole enantiomers in earthworm and soil respectively. F_{OM} (soil) is fraction of OM in soil and F_{lip} (earthworm) is fraction of lipid in earthworm. A measure of BSAF in (Eq. (7)) is kg OC/kg lip. The calculated BSAFs of two enantiomers are shown in Fig. 5.

Armitage and Gobas (2007) pointed out that chemicals with a K_{OA} (octanol-air coefficient) $\geqslant 10^{5.25}$ and a K_{OW} (octanol-water coefficient) between $10^{1.75}$ and 10^{12} have a biomagnification potential in the soil-earthworm-shrew food-chain unless they are metabolized at a sufficiently rapid rate. The constant K_{OA} was estimated from the ratio of the dimensionless constants K_{OW} ($K_{OW} = 10^{3.7}$ for tebuconazole)) according to correlation:

$$K_{\mathrm{OA}} = K_{\mathrm{OW}} \mathrm{RT} / H \tag{8}$$

where, H represents Henry's law constant. K_{OA} of tebuconazole was then obtained as $10^{15.7}$. Therefore, tebuconazole is expected to have biomagnification potential in the earthworm food chain by this theory. The theory has the assumption that elimination in earthworm was considerably low. (+)-R-tebuconazole was accumulated in the earthworm body with BSAF value of 1.64 kg OC/kg lip in 10 mg/kg dw exposure group and 2.61 kg OC/kg lip in 50 mg/kg dw exposure group after 36 days. It was suggested that (+)-R-tebuconazole might have biomagnifying effect potential (BSAF > 1 kg OC/kg lip) in the earthworm food chain, which was up to expectation. Compared with (+)-R-tebuconazole, (–)-S-tebuconazole was less strongly accumulated in the earthworm tissues, of

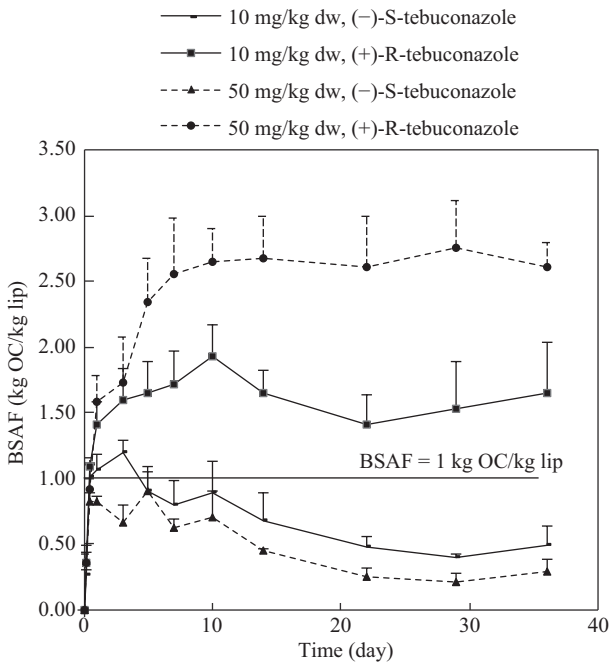

Fig. 5 Calculated BSAFs of two enantiomers in low and high dose exposure. Data are expressed as mean ± SD.

which BSAF values were 0.50 kg OC/kg lip (10 mg/kg dw tebuconazole exposure) and 0.28 kg OC/kg lip (50 mg/kg dw tebuconazole exposure) after 36 days.

3 Conclusions

Enantioselective analytical method of tebuconazole enantiomers was established and applied in the earthworm bioaccumulation experiment. The uptake kinetics of (+)-R-tebuconazole fitted the first-order kinetics well, while (–)-S-tebuconazole did not obey. Bioaccumulation of tebuconazole in earthworm tissues was enantioselective with a preferential accumulation of (+)-R-tebuconazole. In addition, (+)-R-tebuconazole might have biomagnifying effect potential (BSAF > 1 kg OC/kg lip) in this experiment, while (–)-S-tebuconazole did not have biomagnifying effect.

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