Enantioselective separation and zebrafish embryo toxicity of insecticide beta-cypermethrin

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Abstract

Enantioselectivity of chiral pollutants is receiving growing concern due to the difference in toxicology and environment fate between enantiomers. In this study, enantiomers of insecticide beta-cypermethrin (beta-CP) were separated on selected chiral column by HPLC, and the toxicity of enantiomers was evaluated using the zebrafish embryo-larval assays. The enantiomers of beta-CP were baseline separated on Chiralcel OD and Chiralpak AD columns and detected by circular dichroism (CD) at 236 nm. Better separation could be achieved at lower temperature (e.g., 20°C) and with lower levels of polar modifiers. Pure enantiomers were obtained on Chiralcel OD. The CD spectra of enantiomers were recorded. By comparing the elution order with a previous similar study, the absolute configuration of beta-CP enantiomers was determined. The individual enantiomers were used in zebrafish embryo test, and the results showed that beta-CP enantioselectively induced yolk sac edema, pericardial edema and crooked body. The 1*R-cis-*α*S* and 1*R-trans-*α*S* enantiomers showed strong developmental toxicities at concentration of 0.1 mg/L, while the 1*S-cis-*α*R* and 1*S-trans-*α*R* induced no malformations at higher concentration (e.g., 0.3 mg/L). The results suggest that the enantioselective toxicological effects of beta-CP should be considered when evaluating its ecotoxicological effects.

Key words: beta-cypermethrin; enantioselectivity; zebrafish embryo; chiral separation

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Introduction

A great number of pollutants are chiral, and the enantioselectivity in their fate and effects in the environment have become one of most attractive challenging subjects (Liu et al., 2005a; Garrison, 2006). For many currently used chiral pesticides, typically they are sold as racemates. It has been reported that chiral selectivity of these pesticides may occur not only in the target pests control processes but also in their biological activity to non-target organisms and environmental behaviors (Richardson and Ternes, 2005; Liu et al., 2005b). However, current information is in great paucity on the ecotoxicological effects and environmental fate of individual enantiomers of chiral pesticides.

Synthetic pyrethroid pesticides (SPs) are widely used pesticides, contain up to eight optical isomers (Chamberlain et al., 1998). The enantioselective toxicities of SPs to non-target organisms have been reported (Chen and Liu, 2008; Lin et al., 2008; Liu et al., 2008, 2009). Recent studies have showed that this kind of pesticides from both agriculture and residential run-offs induced toxicological effects on aquatic organisms (Amweg et al., 2005; Weston et al., 2005; Jin et al., 2008). SPs can exert

estrogenic effects by mimicking or inhibiting the actions of endogenous estrogens (Wang et al., 2007; Zhao et al., 2008; Jin et al., 2009), and even their metabolites can show estrogenic potentials (Tyler et al., 2000; McCarthy et al., 2006). Beta-cypermethrin (beta-CP) is an active SP containing two pairs of enantiomers, which intensively controls a wide range of pests both in agriculture and resident. Beta-CP is highly toxic to aquatic invertebrates and aquatic vertebrates. Moore and Waring (2001) reported that low level of cypermethrin in the aquatic environment can have a significant long-term effect on Atlantic salmon population through disruption of reproductive functions. Sub-lethal exposure of cypermethrin altered the biochemical, haematological parameters and enzymes of organs tissue and exerted stress on *Labeo rohita* (Das and Mukherjee, 2003). David et al. (2004) reported that, the administration of sub-lethal concentration of cypermethrin to cyprinus carpion, decreased the soluble proteins, while increased amino acids and activities of protease, aspartate and alanine aminotrasferase. However, no data are present in the open literature on early life stages such as embryo and larvae of *Danio reio*, and its enantioselectivity toxicity has not been well documented.

In this study, we evaluated the enantiomer resolution of beta-CP on HPLC columns. Circular dichroism (CD)

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detection was used to determine the chiral signals of enantiomers. Their absolute configurations were obtained by comparing the elution orders with a previous study. The enantioselectivity in toxicity of the separated enantiomers against the zebrafish embryos was evaluated.

1 Material and methods

1.1 Chemicals and reagents

Beta-cypermethrin (Fig. 1, beta-CP, 96%, enriched in 1*R*-*cis*-α*S* + 1*S*-*cis*-α*R* and 1*R*-*trans*-α*S* + 1*S*-*trans*-α*R*) was purchased from Xinhuo Technology Institute (Baoding, China). Solvents including *n*-hexane, ethanol (EtOH), isopropanol (IPA) and 1,2-dichroloethane were of HPLC grade and purchased from Tedia (Fairfield, USA). The pesticide stock solution was prepared by dissolving the beta-CP in *n*-hexane at 2000 mg/L and stored at 4°C in darkness. Working solutions were prepared daily by diluting the stock solution to 200 mg/L.
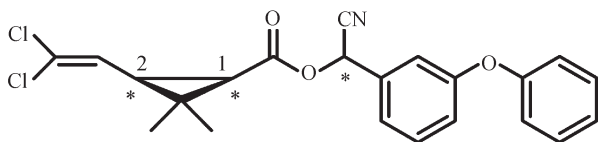
Fig. 1 Chemical structure of beta-cypermethrin. * indicates the chiral center.

1.2 Chromatographic separation and analysis

Enantiomer separation was performed on a Jasco LC-2000 series HPLC system (Jasco, Japan) equipped with a PU-2089 quaternary gradient pump, a mobile phase vacuum degasser, an AS-1559 autosampler, a CO-2060 column temperature control compartment, a UV-2075 plus UV/Vis detector, a variable-wavelength CD-2095 circular dichroism detector (CD), an OR-2090 PLUS optical rotatory dispersion detector (ORD), and a LC-Net II/ADC data collector. Chromatographic data were acquired and processed with the ChromPass software (Jasco, Japan). Chiralcel OD and Chiralpak AD columns were purchased from Daicel Chemical Industries (Japan). The signals of UV and CD detectors were recorded at 236 nm. A volume of 10 μL (200 mg/L) was injected for chiral separation in the normal-phase mode with *n*-hexane as the primary carrier, EtOH or IPA (1%–5%) as the polar modifier.

On the basis of the separation results, Chialcel OD was used to prepare the individual enantiomers that were used for subsequent absolute configuration determination and toxicity assays. The resolved enantiomers were collected manually into separate glass vials at the HPLC outlet. The fractions were evaporated to dryness under a stream of nitrogen and redissolved in *n*-hexane (for absolute configuration determination) or in acetone (for toxicity assays). The purity and concentration of the recovered enantiomers were determined by chiral HPLC and GC analysis, respectively. For GC analysis, an Agilent 6890N GC equipped with an electron capture detector (ECD)

and a HP-5 capillary column were used for concentration quantification. The flow rate of carrier gas (nitrogen) was 1.0 mL/min. The column temperature was held at 190°C. The temperature in the inlet was 280°C, and individual enantiomers were introduced through 1.0 μL injection in the splitless mode. The purity was found to be > 99.9% for each of the enantiomers in this study, and no racemization was observed during experiment.

1.3 Chiroptical detection and absolute configuration determination

The resolution by HPLC along with detection by CD was used for distinguishing the enantiomers. By using stop-flow method, the CD spectra were recorded at the top of the chromatographic peak with the wavelength ranging from 220 to 420 nm. The spectroscopic conditions were set as a resolution of 0.2 nm and a 10× accumulation. The mobile phase background CD spectra were recorded before the scanning of separated enantiomers and were subtracted from the spectrum of each peak automatically.

Enantiomers from the same compound elute in a similar order under the same chromatographic condition. Thus, obtained enantiomers (Chiralcel OD column) were injected individually on two chained Chirex 00G-3019-OD columns (Phenomenex, USA) under chromatographic conditions as described before (Liu et al., 2005a). Absolute configurations of enantiomers were obtained by comparing their elution order with previous study (Liu et al., 2005a).

1.4 Fish embryo exposure and toxicity assay

The embryo collection and preparation procedure followed that of Westerfield (1993). The embryo toxicity test was similar to the OECD test guidelines (Braunbeck and Lammer, 2006). Briefly, the eggs were placed in various exposure chambers containing the test compound with various concentrations for 1 hr. Then, fertilized eggs (i.e., embryos) were separated from the nonfertilized ones and placed in 24-well plates using a pipet. Twenty fertilized eggs were placed individually in 2 mL of the respective test solutions containing of 0.5% acetone in reconstituted water according to Westerfield (1993). The reconstituted water contained (mmol/L): 137 NaCl, 5.4 KCl, 0.25 Na_2HPO_4, 0.44 KH_2PO_4, 1.3 $CaCl_2$, 1.0 $MgSO_4$, and 4.2 $NaHCO_3$ in ultrapure water (18.3 MΩ-cm resistivity) produced by Milli-Q system (Millipore, USA). The remaining four wells of each plate used as internal control that contained only artificial water with 0.5% acetone. Three plates were used as replicates for each concentration treatment. The plates were covered with lids to minimize water evaporation. Incubation was carried out at (27 ± 1)°C and with 24-hr light daily. The development of embryos from blastula to early larval stages was monitored at specified time points (t = 12, 24, 36, 48, 60 or 72 hr). End points used for assessing the effects of beta-CP enantiomers included egg and embryo mortality, yolk sac edema, pericardial edema and crooked body. No observed effect concentrations (NOECs) were defined as the highest concentration above negative control at which a significant effect was not observed. The NOECs were estimated on the basis of the

developmental and teratogenic responses of the embryos at different concentrations.

1.5 Statistical analysis

The toxicity data of abnormality for individual enantiomers were submitted to one-way ANOVA to determine significant difference. The software package used for the statistical analysis was Origin 8.0 (OriginLab, USA). The differences were considered statistically significant at $p < 0.05$.

2 Result and discussion

2.1 Chromatographic separation

The enantioselective resolution of beta-CP was evaluated on two different columns. Optimization of the separation condition was performed by investigating the effect of the type and concentration of mobile-phase modifiers (i.e., EtOH and IPA). Figures 2 and 3 show the chromatograms of beta-CP on the Chiralpak AD and Chiralcel OD columns tested, respectively. The two columns showed different separation abilities for the insecticide. IPA was found to be a good modifier on both of the two columns. Optimum separation on the Chiralpak AD was achieved using n-hexane/IPA of 97/3 (V/V) as the mobile phase at a flow rate of 0.40 mL/min. Complete separation on the Chiralcel OD column was obtained using n-hexane/IPA as mobile phase at 0.40 mL/min and

25°C. With all the modifiers (2%–5%), the Chiralcel OD column showed a well baseline separation of beta-CP. Higher levels of alcohol modifiers resulted in reduction of retention time and suppression of the peak tailing.

A decrease in the level of a polar modifier in the mobile phase often results in an increase in chiral separation. We studied the effects of the level of modifiers in n-hexane on the separation abilities of two columns. The chromatographic parameters, including separation factor (α), resolution factor (R_s) and separation factor (k) for the resolved enantiomers of beta-CP, were calculated according to the chromatograms obtained (Table 1). The hydrogen bonding between CSPs and enantiomers weakened, as the polarity of the mobile phase increasing with the amount of modifiers. As a result, the k value for all the columns tested was decreased. By comparison, the α values varied little over the range of the IPA levels in n-hexane on Chiralpak AD column. For Chiralcel OD column, the values of α_{12} and α_{34} decreased with EtOH level increased, however, the values of α_{24} increased as mobile levels increasing. The maximum resolution may be obtained at a certain level of a polar modifier in the mobile phase (Ellington et al., 2001).

Increasing temperature can reduce the viscosity of mobile phase on the same column, thus the enantioselective abilities reduced at higher temperature. Figure 3 shows the chiral separation of temperature dependence on Chiralcel OD column. Complete separations were obtained at all the temperature tested. As temperature increased, the enantioselective separation ability decreased, however, the retention time and peak tailing of all peaks were reduced at the same time.
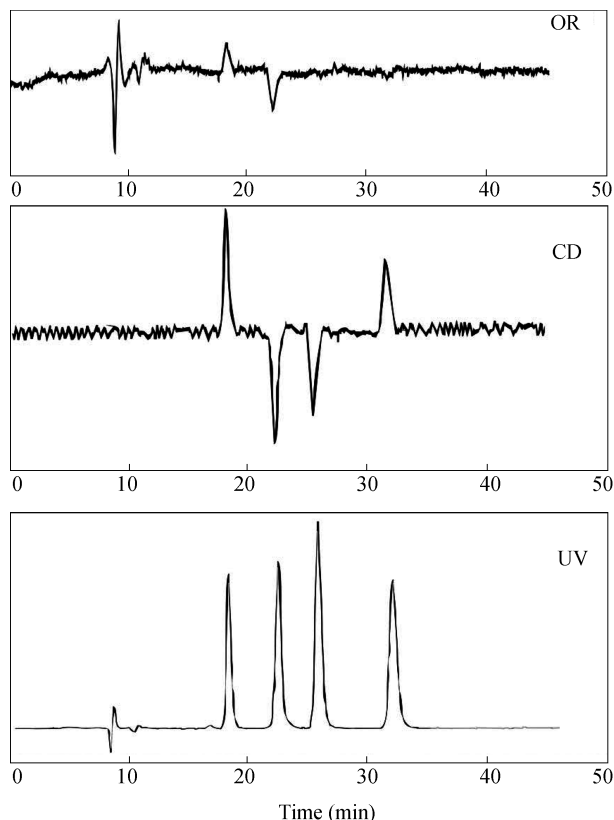
Fig. 2 Chiral separation chromatograms of beta-cypermethrin. OR: rotatory dispersion; CD: circular dichroism; UV: ultraviolet. Chromatographic condition: Chiralpak AD; mobile phase n-hexane/isopropanol (97/3); temperature 25°C; flow rate 0.40 mL/min.
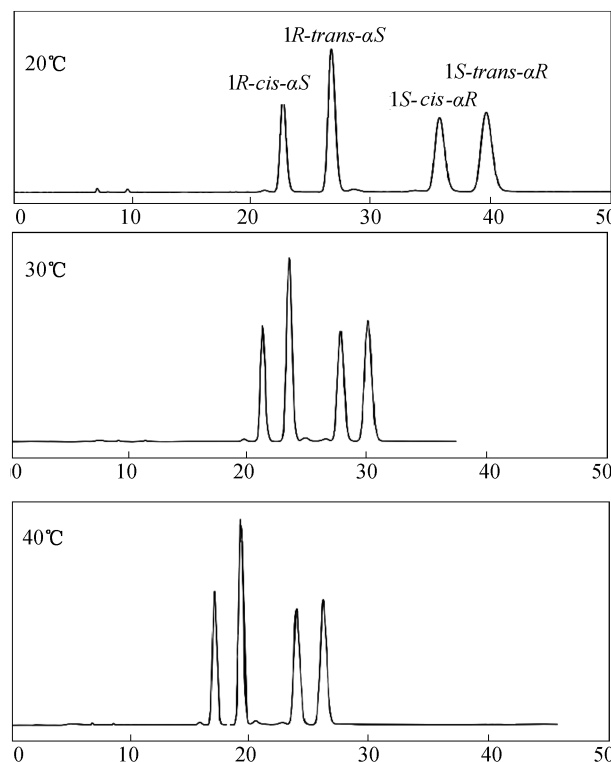
Fig. 3 Effect of temperature on enantioseparation of beta-cypermethrin on Chiralcel OD column. Mobile phase was n-hexane/isopropanol (97/3) and flow rate was 0.40 mL/min.

**Table 1**　Effect of modifiers in mobile phase on the enantiomeric separation of beta-cypermethrin

Column	Modifier (%)	k_1'	k_2'	k_3'	k_4'	α_{12}	α_{23}	α_{34}	R_{s12}	R_{s23}	R_{s34}
Chiralpak AD[a]	1	1.08	1.35	1.62	1.91	1.25	1.20	1.18	2.92	2.56	2.62
	1.5	1.05	1.29	1.56	1.84	1.23	1.21	1.18	2.59	2.66	2.48
	2	1.02	1.25	1.53	1.78	1.23	1.22	1.17	2.50	2.75	2.34
	2.5	0.86	1.06	1.32	1.54	1.24	1.24	1.17	2.40	2.78	2.19
	3	0.84	1.03	1.30	1.50	1.23	1.25	1.16	2.33	2.85	2.11
Chiralcel OD[b]	1	–	–	–	–	–	–	–	–	–	–
	2	0.78	1.25	1.36	2.23	1.61	1.09	1.63	8.23	1.72	9.46
	3	0.74	1.13	1.31	1.98	1.53	1.16	1.51	5.82	2.39	8.20
	4	0.68	1.00	1.21	1.74	1.47	1.21	1.44	5.67	3.24	7.07
	5	0.64	0.90	1.12	1.56	1.42	1.24	1.39	4.98	3.63	6.26

[a] *n*-Hexane/isopropanol, 25°C, 0.40 mL/min on Chiralpak AD; [b] *n*-hexane/ethanol, 25°C, 0.40 mL/min on Chiralcel OD.
k: capacity factor; α: separation factor; R_s: resolution factor.

2.2 Circular dichroism spectra and identification of enantiomers

Several kinds of detection techniques are available to distinguish between a pair of individual enantiomers. Recently, HPLC coupled with CD detector has been proved a powerful tool for determining the optical property of separated enantiomers (Berthod et al., 2004). Figure 2 shows that the separated enantiomers could be differentiated in terms of their CD signs detected at the wavelength of 236 nm. The respective CD spectra of separated enantiomers of beta-CP between wavelengths of 220 and 420 nm shown in Fig. 4 are consistent with the theoretical prediction, that taking the CD signal of zero as a mirror, the CD spectra of one enantiomer and its antipode are the mirror images of each other. The CD spectrum of peak 1 and peak 4 enantiomers showed intense positive cotton effect between 220 and 260 nm and weak positive cotton effect between
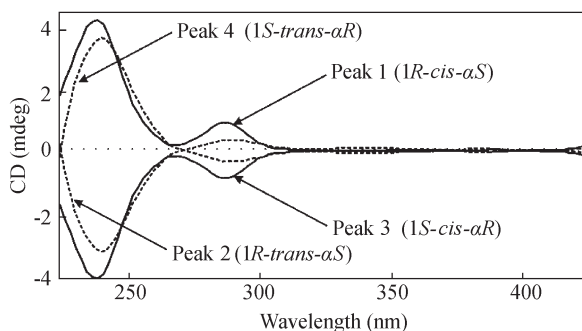
Fig. 4　Circular dichroism (CD) spectra of separated enantiomers of beta-cypermethrin. Chromatographic conditions: chiralcel OD; mobile phase: *n*-hexane/isopropanol (97/3); flow rate: 0.40 mL/min.

260 and 310 nm. In contrast, the CD spectrum of the peaks 3 and 2 had optically opposite to that of their antipodes. These results indicate that the CD detection can distinguish the enantiomer sensitively.

Obtained enantiomers was individually injected in two chained Chirex 00G-3019-OD columns under chromatographic conditions (*n*-hexane/1,2-dichroloethane/EtOH 500/30/0.15, at flow rate of 0.4 mL/min) as described before (Liu et al., 2005a). According to previous study, the retention time of enantiomers of beta-CP were 1*R*-*trans*-αS > 1*S*-*trans*-αR > 1*S*-*cis*-αR > 1*S*-*cis*-αR. And here in our study, the retention time of obtained enantiomers were in order of peak 2 > peak 4 > peak 3 > peak 1 on two chained Chirex 00G-3019-OD columns (data not shown). Therefore, the peak 1, peak 2, peak 3 and peak 4 obtained on Chiralcel OD column corresponded to enantiomers of 1*R*-*cis*-αS, 1*R*-*trans*-αS, 1*S*-*cis*-αR and 1*S*-*trans*-αR (Fig. 3). These results are in good agreement with CD spectra (Fig. 4), that peak 1 and peak 3 are pair of enantiomers, and peak 2 and peak 4 are another pair of enantiomers. This good separation and identification allowed the isolation of individual enantiomers that were used in the following toxicity assays to evaluate enantioselectivity in aquatic toxicity.
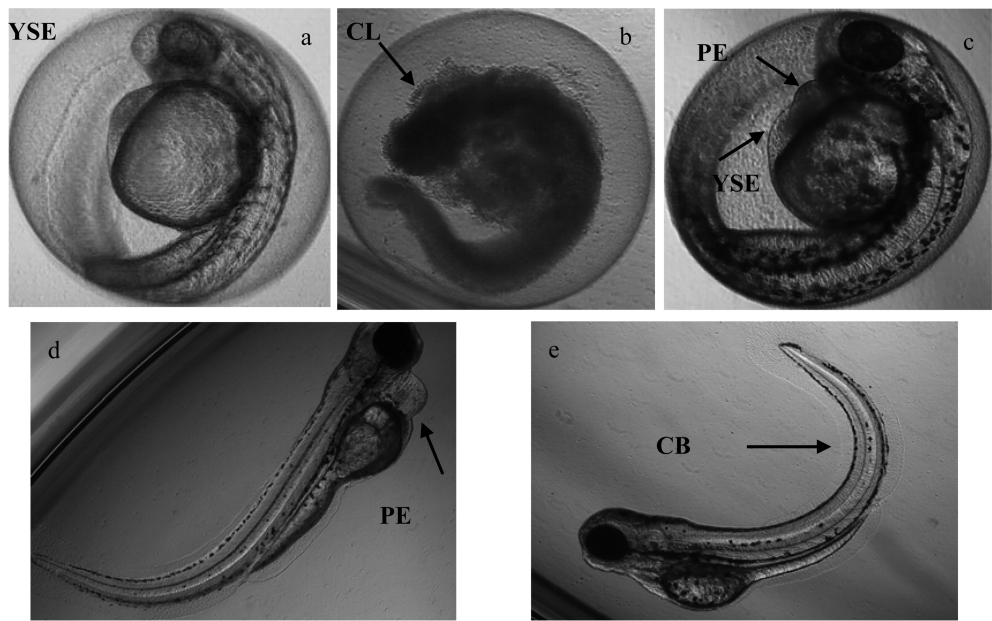
2.3 Enantioselective embryo toxicity

Exposure of zebrafish embryos-larva to enantiomers of beta-cypermethrin led to lethal as well as various nonlethal malformations (Fig. 5 and Table 2). For instance, the embryos subjected to the treatment of enantiomer of 1*R*-*cis*-αS enantiomer showed yolk sac edema and conjugation (Fig. 5a, b). Higher concentration (0.2 mg/L) of 1*R*-*cis*-αS

Table 2　Summary of response endpoints for enantioselective embryo toxicity of beta-cypermethrin

Developmental defect	48-hr exposure				72-hr exposure			
	1*R*-*cis*-αS	1*S*-*cis*-αR	1*R*-*trans*-αS	1*S*-*trans*-αR	1*R*-*cis*-αS	1*S*-*cis*-αR	1*R*-*trans*-αS	1*S*-*trans*-αR
Mortality (LC_{50}, mg/L)	0.29	–[a]	–[a]	–[a]	0.27	–[a]	–[a]	–[a]
Malformation (EC_{50}, mg/L)								
Crooked body	0.10	–[b]	0.23	–[b]	0.03	–[b]	0.08	–[b]
Yolk sac edema	0.65	–[b]	1.07	–[b]	0.55	–[b]	–[b]	–[b]
Pericardial edema	–[b]	–[b]	–[b]	–[b]	0.43	–[b]	1.25	–[b]
NOECs (mg/L)								
Crooked body	< 0.10	–[b]	< 0.10	–[b] <	0.05	–[b]	< 0.10	–[b]
Yolk sac edema	< 0.05	–[b]	< 0.20	–[b] <	0.10	–[b]	–[b]	–[b]
Pericardial edema	–[b]	–[b]	–[b]	–[b]	< 0.05	–[b]	< 0.10	–[b]

[a] No mortality observed at the highest concentration exposed; [b] no this kind of malformation observed at all concentrations.
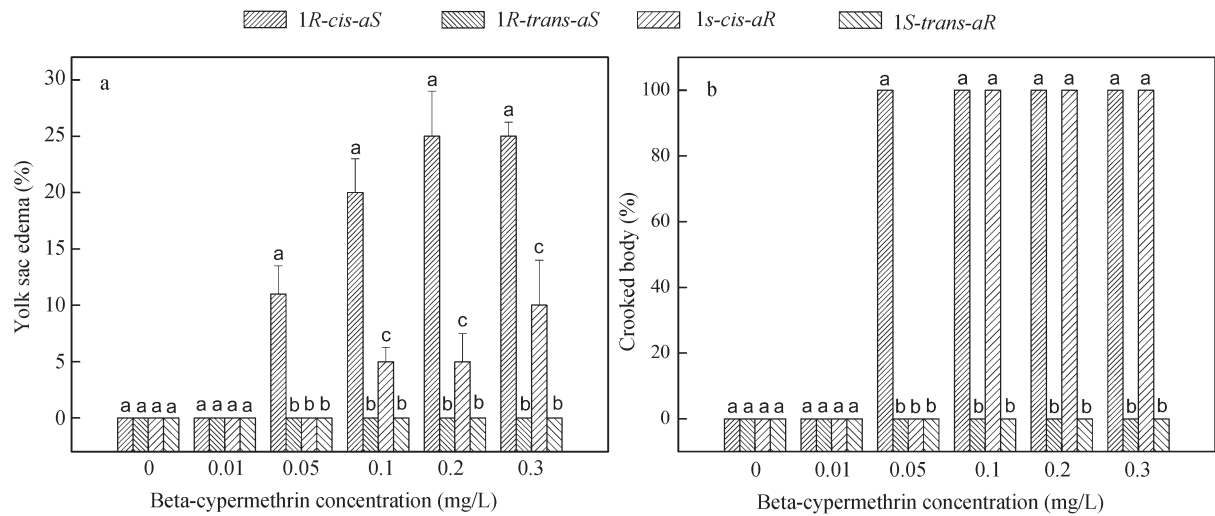
al., 2006). Thus, the embryo test offers more end points as criteria for identifying enantioselectivity in ecotoxicity.

3 Conclusions

Enantiomers of beta-CP were separated on two different polysaccharide-based CSP columns. By using the CD spectra, the two pairs of enantiomers of beta-CP can be distinguished. Compared with a previous study in beta-CP, we were able to deduce the absolute configurations of resolved enantiomers to be 1R-cis-αS, 1R-trans-αS, 1S-cis-αR, 1S-trans-αR. Significant enantioselectivity in embryo toxicity was observed between enantiomers. The 1R-cis-αS, 1R-trans-αS enantiomers were found to be more active in inducing malformation. These results imply that the ecotoxicological effects of chiral pesticides must be individually considered. Compared with conventional shot-term bioassays used for detecting enantioselective toxicity of chiral pesticides, the use of zebrafish embryos may be a rapid and inexpensive approach for evaluating the enantioselectivity of chiral compounds in the aquatic toxicity assay.

Acknowledgments

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