Research Article

Compound-specific stable carbon isotope analysis of galaxolide enantiomers in sediment using gas chromatography/isotope ratio monitoring mass spectrometry

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RATIONALE: Both chiral analysis and compound-specific stable carbon isotope analysis have limitations when applied to environmental research. However, the combination of these two techniques might overcome their respective limitation and give more insight into the enantioselective fate and source apportionment of chiral organic contaminants.

METHODS: After Soxhlet extraction and clean-up, sediment extracts were further pre-concentrated using normal-phase preparative high-performance liquid chromatography to isolate sufficient quantities of highly purified galaxolide (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(g)-2-benzopyran; HHCB). The enantiomeric fractions and stable carbon isotopes of the HHCB were determined using gas chromatography/mass spectrometry (GC/MS) and GC/isotope ratio mass spectrometry (IRMS).

RESULTS: The method was validated by analysis of the enantiomeric fractions and the stable carbon isotope ratios of the HHCB standard at each step of the pre-concentration procedure, and no significant enantiomeric and isotopic fractionation was found. The sediment sample was further used to test the developed method, and it was shown that the HHCB enantiomers in the sediment sample exhibited significantly different $\delta^{13}C$ values (–33.03 to –24.57‰) and a slight enantiomeric fractionation (0.507 and 0.490) from a HHCB standard reference compound (–26.50 to –26.21‰ for $\delta^{13}C$ values, and 0.519 and 0.497 for enantiomeric fractions).

CONCLUSIONS: This work offers a novel approach to elucidating the sources and the abiotic or biological transformation processes of HHCB in the environment and will offer a perspective for assessing the environment fate of any chiral organic compound. Copyright © 2013 John Wiley & Sons, Ltd.

Source apportionment and environmental transformation of organic contaminants are two basic topics in environmental chemistry. Because microbial and biochemical processes might result in enantiomer-specific transformation and isotope fractionation of organic contaminants, enantiomeric analysis of chiral contaminants and compound-specific stable isotope analysis (CSIA) are powerful tools for the discrimination of abiotic and biotic processes and tracing the sources of organic contaminants.[1–6] The two techniques have been widely used in the study of sources, reaction dynamics, and *in situ* biodegradation of compounds such as chlorinated ethenes (CEs),[7–10] hexachlorocyclohexanes (HCHs),[11,12] dichlorodiphenyltrichloroethane (DDT),[13,14] and polychlorinated biphenyls (PCBs).[15–17]

However, both techniques have limitations when applied to environmental research. For CSIA, distinct isotopic compositions of pollution sources are required, and the differences between sources must be greater than the measurement error. Moreover, interpretation becomes complex when there are many possible sources.[5,6,18] In enantiomeric analysis, as reported in previous studies,[12,19] substantial biodegradation may occur without significant deviations of the enantiomeric fractions (EFs). Thus, EF values are not always a reliable tool for assessing the biodegradation of certain contaminants. In recent years, the combination of enantiospecific analysis and CSIA has become a promising new approach that can provide insight into enantioselective fates and source apportionment of environmental organic contaminants.[12,20] It can overcome to some extent the limitation of using only a single technique. In 2011, Badea *et al.*[12] first developed an enantiospecific stable carbon isotope analysis (ESIA) method for α-HCH enantiomers, and three dense, non-aqueous-phase liquids obtained from an HCH-contaminated field site were analyzed to test the applicability of the method. They found that the isotopic compositions of the α-HCH enantiomers showed a range of enantiomeric and isotope patterns,

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suggesting that enantiomeric and isotope fractionation can serve as an indicator for biodegradation and source characterization of α-HCH in the environment.

Two main factors hinder the wide application of this enantiomer-specific CSIA method: amounts of analyte required and matrix effects. In environmental matrices, most organic contaminants and their metabolites are present only in trace concentrations (in the ng/mL or ng/g range). However, a more than 1000-fold higher concentration (i.e. 10–100 μg/mL) is required for CSIA or ESIA.[21] Concentration alone can provide larger amounts of analyte but cannot resolve matrix interferences. Most enantioselective gas chromatography (GC) columns have relatively low maximum operating temperatures (for example, <230°C) compared with common GC columns, and many matrix interfering substances are, therefore, not eluted from the enantioselective columns. These matrix effects and column bleed might reduce sensitivity and also add to the number of combustion products when conducting gas chromatography/isotope ratio mass spectrometry (GC/IRMS) analysis. Thus, there is a need for an efficient preparative procedure that will both provide the larger amounts of analyte required and reduce the matrix effects.[22–25]

Galaxolide (HHCB), a synthetic polycyclic musk having two pairs of enantiomers, is widely used in personal care products and has increasingly raised public concern due to its uncertain environmental fate and potentially adverse effects on human health and the environment.[26,27] In Europe, HHCB has been classified as a 'high volume chemical' and in 1995 it had an average use per capita of about 15.5 mg/day.[3] There have been some studies of HHCB, focusing on its enantioselective transformation and accumulation in water, sediment, and biota.[28–31] In the present study, normal-phase preparative high-performance liquid chromatography (HPLC) was used to isolate sufficient quantities of highly purified HHCB from a real sediment sample to determine the stable carbon isotope ratios of individual HHCB enantiomers. Before the HHCB enantiomers were analyzed by GC/IRMS, it was verified that each step involved in the preparative process of HHCB was free from obvious enantiomeric fractionation and isotope fractionation. It is critical to evaluate if this whole sample pre-concentration procedure could be used for the determination of the carbon isotope ratios of individual enantiomers. If successful, the final developed method will help to improve the characterization of the source and fate of HHCB in the environment and will offer a perspective for assessing the environmental fate of any chiral organic compound.

EXPERIMENTAL

Chemicals and other materials

1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(g)-2-benzopyran (Galaxolide, HHCB, 75%) was purchased from LGC Promochem (Weseel, Germany). Hexamethylbenzene (HMB) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). HPLC-grade n-hexane and dichloromethane were obtained from J&K Scientific (Beijing, China) and CNW Technologies GmbH (Düsseldorf, Germany), respectively. Analytical grade silica gel (80–100 mesh) and alumina

(100–200 mesh) were Soxhlet extracted for 72 h with dichloromethane, then activated for 12 h at 180 and 250°C, deactivated with 3% redistilled water, and kept in n-hexane until use. Anhydrous sodium sulfate was dried at 450°C for 5 h.

Sample preparation and cleanup

The sediment sample used in this study was collected from Dagu Drainage River in Tianjin city, China, which receives most of the wastewater from a municipal waste-treatment plant, industrial discharge, and domestic sewage. Before extraction, the sample was freeze-dried with an ALPHA 1-4 instrument (Marin Christ, Osterode, Germany) and screened through a 30-mesh sieve. The sample extraction and clean-up procedures were similar to those previously described.[27] Briefly, the sample (10 g) was Soxhlet extracted for 72 h with dichloromethane. Activated Cu was added to remove sulfur. The extract was concentrated and exchanged to hexane and then fractionated on a combined silica/alumina column. Three fractions were subsequently eluted with three different solvents: F1 (n-hexane), F2 (n-hexane/dichloromethane 3:1), and F3 (dichloromethane). The F3 dichloromethane fraction was concentrated to 0.5 mL and exchanged to n-hexane solvent under a gentle stream of nitrogen gas. Finally, a known amount of internal standard (hexamethylbenzene, HMB) was added before analysis. Before the GC/IRMS analysis, a preparative step was first performed with a normal-phase HPLC system to isolate sufficient quantities of the purified HHCB fraction. The GC/MS chromatograms of sample D4 before and after preparation are presented in Fig. 1.
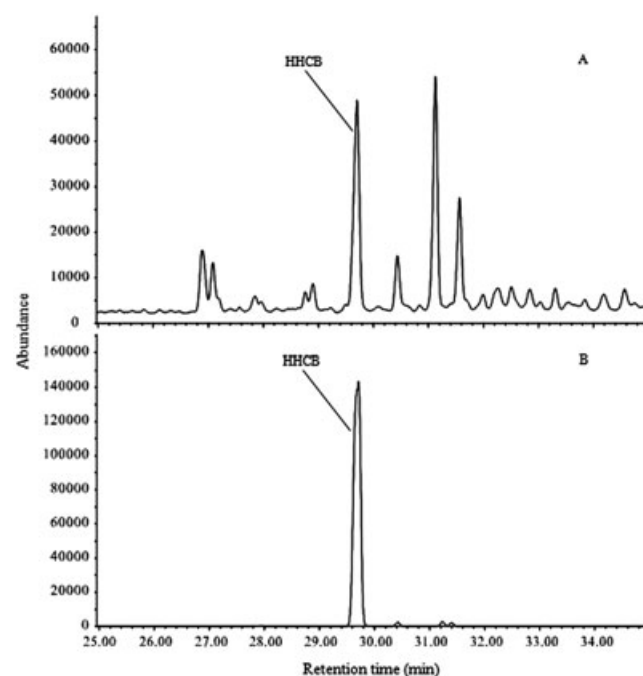

Figure 1. GC/MS chromatograms (full scan, EI) of HHCB in sample D4 (A) before and (B) after preparation.

Preparative liquid chromatography

Isolation of the target compounds was performed using a normal-phase preparative liquid chromatography system, which consisted of an HP/Agilent 1100 Series high-pressure liquid chromatography system and an Agilent 1200 Series analytical and preparative scale fraction collector (Agilent Technologies, Palo Alto, CA, USA). The target compound (HHCB) was separated on a Zorbax RX-Sil (4.6 × 250 mm, 5 μm, Agilent) reversed-phase C18 column, with *n*-hexane/dichloromethane (90:10 v/v) as mobile phase at a flow rate of 0.6 mL/min and detected with an ultraviolet detector at 245 and 280 nm.

Instrumental analysis

GC/MS analysis

GC/MS analysis of HHCB was performed on an Agilent 7890A gas chromatograph coupled to an Agilent 5975C quadrupole mass spectrometer in electron ionization mode (70 eV). The GC column was a DB-XLB capillary column (30 m × 0.25 mm, 0.25 μm; J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The temperature program was as follows: hold at 100°C for 2 min, ramp to 150°C at 8°C/min, ramp to 210°C at 5°C/min, and finally ramp to 300°C at 15°C/min and hold for 20 min. The temperatures of the injection port, transfer line, and ion source were 280, 150, and 230°C, respectively. The injection was in splitless mode and the mass spectrometer was operated in full scan mode with a range of *m/z* 35–600.

The separation of HHCB enantiomers was performed on a model 2010 gas chromatograph coupled to a QP2010 quadrupole mass spectrometer (Shimadzu, Columbia, MD, USA) in electron ionization mode (70 eV) using a Hydrodex-β-6TBDM chiral column (25 m × 0.25 mm, 0.25 μm; Macherey-Nagel, Düren, Germany). Helium was used as the carrier gas at a relatively low flow rate of 0.7 mL/min. The temperature program was as follows: hold at 110°C for 1 min, ramp to 132°C at 5°C/min and hold for 140 min, ramp to 194°C at 1.5°C/min, and finally ramp at 5°C/min to 230°C and hold for 10 min. The injection was in splitless mode, and the mass spectrometer was operated in full scan and selected ion monitoring (SIM) mode. The temperatures of the injection port, transfer line, and ion source were 220, 210, and 180°C, respectively. The enantiomeric compositions were expressed as enantiomer fractions (EFs), calculated with the peak areas (A) of the enantiomer pairs using the formula:[32]

$$EF = \frac{A1}{A1 + A2}$$

GC/IRMS analysis

For the GC/IRMS analysis of HHCB, an Agilent 6890N gas chromatograph was coupled to a Micromass Isoprime II isotope ratio mass spectrometer (Manchester, UK) through an oxidation furnace containing CuO for complete combustion of the HHCB. The oxidation furnace was operated at 850°C, and samples were introduced in continuous flow mode. The same DB-XLB capillary column (30 m × 0.25 mm, 0.25 μm) as used for the GC/MS analysis

was connected between the injection port and the oxidation furnace. Helium was used as the carrier gas, and the injection was operated in splitless mode. The temperature program was identical to that used for the GC/MS analysis (see above). For assessment of the reproducibility, at least three replicates were measured per sample. The systematic isotope effect related to the concentration of the analyte was monitored by injecting four solutions with the following concentrations of HHCB: 10, 20, 40, and 60 μg/mL.

The GC/IRMS analysis of HHCB enantiomers was performed using the same system as used for HHCB racemic mixture analysis. The same hydrodex-β-6TBDM chiral column (25 m × 0.25 mm, 0.25 μm) as used for the GC/MS analysis was used to separate the HHCB enantiomers prior to isotope analysis. Helium was used as the carrier gas at a flow rate of 0.7 mL/min. The temperature program was the same as that used for the separation of HHCB enantiomers in the GC/MS analysis (see above). The isotopic signals for HHCB were identified by injection of the reference compounds and comparison of their retention times with those from the GC/MS analysis. In order to check the reproducibility, at least three replicates were measured per sample. All stable carbon isotopic data are reported as delta (δ) values in the per mil (‰) notation, and all values reported below are relative to the international standard, Vienna Pee Dee Belemnite (V-PDB), according to the equation:

$$\delta^{13}C[‰] = \left(\frac{R_{sample} - R_{standard}}{R_{standard}} \right) \times 1000$$

where R_{sample} is the carbon isotope ratio ($^{13}C/^{12}C$) of the sample and $R_{standard}$ is the carbon isotope ratio ($^{13}C/^{12}C$) of the V-PDB standard.

RESULTS AND DISCUSSION

Carbon isotope ratio values of HHCB during the pre-concentration procedure

In order to ensure the precision of CSIA, there must be more than 10 ng of the target compound per injection, and there must be no interfering compounds co-eluting with the target compound. Therefore, the highly purified HHCB from the sediment was further pre-concentrated by normal-phase preparative liquid chromatography. Before the method was applied to sediment samples, the stable carbon isotope ratios in the HHCB standard were determined at each step of the pre-concentration procedure to check for isotopic fractionation. Four main steps were involved: (1) sample cleanup on the combined silica/alumina column, (2) concentration with a rotary evaporator and a gentle stream of nitrogen gas, (3) preparation with the normal-phase HPLC system, and (4) a second concentration with a rotary evaporator and a gentle stream of nitrogen gas.

Table 1 summarizes the carbon isotope ratios of the HHCB standards obtained at each step. As shown in Table 1, the averages ranged from –26.70 ± 0.12‰ to –26.80 ± 0.05‰. Compared with the value for the initial HHCB standard (–26.58 ± 0.06‰), the reductions in the δ¹³C values were all less than –0.30‰, on average, lower

Table 1. Carbon isotope ratios ($\delta^{13}C$ values, ‰) and enantiomeric fractions (EFs) of HHCB obtained during the pre-concentration procedure (mean $\pm$ SD, n = 6). EFs (*trans*-HHCB) = H1/(H1 + H4);   EFs   (*cis*-HHCB) = H2/(H2 + H3) (see Fig. 2)

	$\delta^{13}C$ (‰)	EFs *trans*-HHCB	EFs *cis*-HHCB
Step 1[a]	−26.80±0.05	n.a.	n.a.
Step 2/Step 4[b]	−26.70±0.12	n.a.	n.a.
Step 3[c]	−26.74±0.05	n.a.	n.a.
Whole procedure[d]	−26.76±0.17	0.516±0.001	0.500±0.003
Standard[e]	−26.58±0.06	0.519±0.004	0.497±0.002

[a]HHCB standard solution is cleaned on the combined silica/alumina column and then concentrated by rotary evaporation and a gentle stream of nitrogen gas.
[b]HHCB standard solution is concentrated by rotary evaporation and a gentle stream of nitrogen gas.
[c]HHCB standard solution is prepared by the normal-phase HPLC system and then concentrated by rotary evaporation and a gentle stream of nitrogen gas.
[d]HHCB standard solution is treated from step 1 to step 4 in turn.
[e]The initial HHCB standard solution.
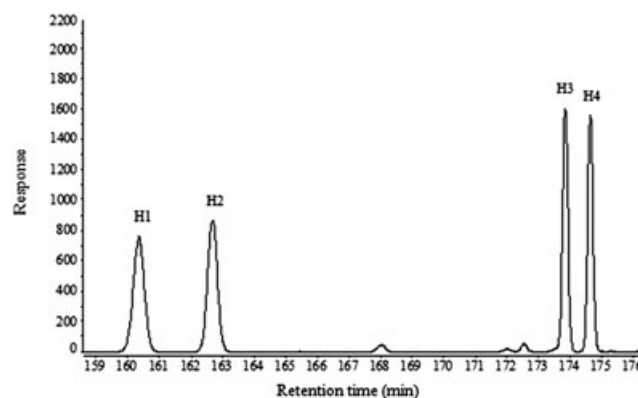n.a.- not analyzed

Figure 2. GC/MS chromatogram of HHCB enantiomers obtained during the pre-concentration procedure: H1: (–)-*trans*-HHCB; H2: (–)-*cis*-HHCB; H3: (+)-*cis*-HHCB; H4: (+)-*trans*-HHCB.

than the analytical uncertainty of 0.5‰ for the instrumental measurements. This suggests that there was no obvious isotope fractionation throughout the whole preparative procedure or at any of the individual steps. Therefore, the procedure is acceptable for the pre-concentration of HHCB from sediments.

Further analyses of standard solutions of HHCB, diluted in *n*-hexane to different final concentrations (10–60 μg/mL), were performed to evaluate the possibility of systematic isotope effects related to the concentration of HHCB. The results are shown in Table 2. The average carbon isotope values of the HHCB standard compound ranged from −26.58 ± 0.06 to −26.77 ± 0.19‰. These results are the same within instrumental measurement error (0.50‰), indicating that there were no detectable isotope effects related to concentration.

EFs of HHCB during the pre-concentration procedure

Before the GC/IRMS analysis, the enantiomers of HHCB standards were studied by GC/MS using a chiral GC column. As shown in Fig. 2, the enantiomers were successfully separated, and the elution order of the HHCB enantiomers,

according to the report by Franke *et al.*,[30] was: H1: (–)-*trans*-HHCB (4S7S); H2: (–)-*cis*-HHCB (4S7R); H3: (+)-*cis*-HHCB (4R7S); H4: (+)-*trans*-HHCB (4R7R). The EFs of the HHCB enantiomers, defined as H1/(H1+H4) for *trans*-HHCB and H2/(H2+H3) for *cis*-HHCB, were calculated and compared with those of the initial HHCB standards using independent-sample *t* tests. As can be seen in Table 1, the EFs of the *trans* and *cis* enantiomers for the initial HHCB standard were 0.519 and 0.497, respectively, whereas the mean EFs after preparation with the normal-phase HPLC system were 0.516 and 0.500. Statistical analysis showed that there were no significant differences ($p > 0.05$).

GC/IRMS separation of HHCB enantiomers and their carbon isotope ratios

The elution orders of HHCB enantiomers on the GC/IRMS system were similar to those on the GC/MS system. As can be seen in Fig. 2, complete baseline separation was achieved for each of the HHCB enantiomers by GC/MS. The difference between the retention times for the (-)-*trans*-HHCB and (-)-*cis*-HHCB enantiomers was more than 2 min, and the difference for (+)-*trans*-HHCB and (+)-*cis*-HHCB enantiomers was about 1 min. On the GC/IRMS system (Fig. 3), nearly complete baseline separation was still observed, although the differences between the isotopic signatures of the HHCB enantiomers were only 1 and 0.7 min. Table 3 lists the carbon isotope ratios of the HHCB racemic mixtures and the enantiomers for the HHCB standard obtained through the whole pre-concentration procedure. The average isotope ratios of the four HHCB enantiomers ranged from −26.21‰ to −26.50‰, very close to those of the HHCB racemic mixtures (−26.58‰), demonstrating the accuracy of the method.

Table 2. Systematic isotope effects related to the concentration of HHCB standard solutions ($\delta^{13}C$ values, ‰)

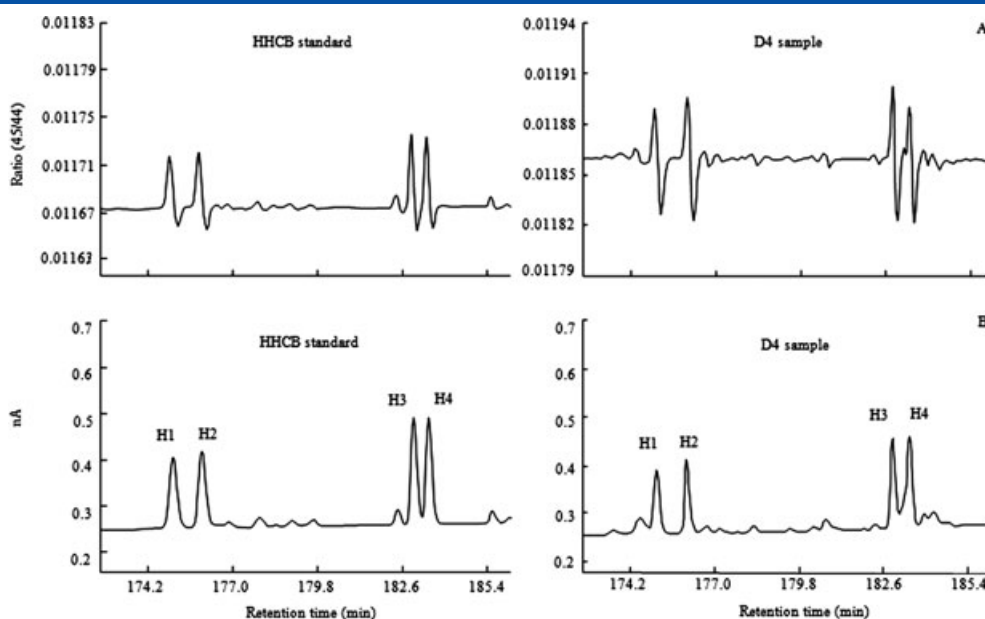| Concentration (μg/mL)	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	Average	SD
10	−26.52	−26.72	−26.66	−26.77	−26.76	−26.81	−26.71	0.10
20	−26.58	−26.55	−26.62	−26.67	−26.49	−26.59	−26.58	0.06
40	−26.63	−26.86	−26.55	−26.63	−26.94	−26.76	−26.73	0.15
60	−26.46	−26.79	−26.73	−26.76	−27.06	−26.82	−26.77	0.19

Figure 3. GC/IRMS (A) and GC/MS (B) chromatograms of HHCB enantiomers for the HHCB standard and D4 sample: H1: (–)-*trans*-HHCB; H2: (–)-*cis*-HHCB; H3: (+)-*cis*-HHCB; H4: (+)-*trans*-HHCB.

Table 3. Carbon isotope ratios ($\delta^{13}C$ values, ‰) and enantiomeric fractions (EFs) of HHCB enantiomers and racemic mixtures in standard compound and environmental sample

Samples	H1	H2	H3	H4	Racemic mixtures	EFs *trans*-HHCB	EFs *cis*-HHCB
Standard	−26.21	−26.29	−26.50	−26.42	−26.58	0.519	0.497
D4	−33.03	−27.23	−24.57	−32.69	−27.32	0.507	0.490

Note: EFs = $A1_{Peak\ area}/(A1_{Peak\ area} + A2_{Peak\ area})$; EFs (*trans*-HHCB) = H1/(H1 + H4); EFs (*cis*-HHCB) = H2/(H2 + H3) (see Fig. 2)

Tables 1–3 and Figs. 2 and 3 show that this method, which isolates sufficient quantities of highly purified target components using a normal-phase preparative liquid chromatography system, can be used to measure the stable carbon isotopic composition of HHCB enantiomers.

Application to the measurement of HHCB in sediment sample

To validate the method, a sediment sample (D4) collected from a river receiving sewage was used for the determination of the carbon isotope ratios of HHCB enantiomers. Before CSIA, the sample was subjected to the entire pre-concentration procedure developed in this study. Figure 1 presents the GC/MS chromatograms of sample D4 before and after pre-concentration, and it can be seen that the target compound HHCB was well isolated, and there were no obvious interference peaks near the HHCB peak after pre-concentration. For the HHCB standard, the mean EFs for the two pairs of enantiomers were 0.519 and 0.497 for *trans*-HHCB and *cis*-HHCB, respectively, whereas the mean EFs of *trans*-HHCB and *cis*-HHCB for sediment sample D4 were 0.507 and 0.490, respectively (Table 3). These results could

not be explained if enantioselective transformation of HHCB occurred in the sediment. There have been a few studies of the enantiomer fraction of HHCB in environmental samples. Franke *et al.*[30] first described an enantioselective and species-dependent transformation of HHCB to HHCB-lactone in the aquatic environment and attributed this pronounced deviation in enantiomeric composition from racemic HHCB to enzymatic oxidation at the benzylic position of HHCB during biotransfomation reactions. Gatermann *et al.*[31] also reported a very strong enantio-selective metabolization for *trans*- and *cis*-HHCB in crucian carp from the pond of a municipal sewage treatment plant and observed a preferential metabolization of the 4S enantiomers (4S,7S-HHCB and 4S,7R-HHCB).

Table 3 lists the carbon isotope ratios of the HHCB enantiomers for the pure HHCB standard and the D4 sample. The carbon isotope ratios of HHCB were –26.58‰ and –27.32‰ for the HHCB standard and sediment sample D4, respectively. The $\delta^{13}C$ values of the four enantiomers of the HHCB standard were –26.21‰, –26.29‰, –26.50‰ and –26.42‰ for H1, H2, H3 and H4, respectively. However, sample D4 exhibited a significantly different isotopic signature: for the *trans*-enantiomers (H1 and H4), the $\delta^{13}C$

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values were –33.03‰ and –32.69‰, respectively, whereas, for the *cis*-enantiomers (H2 and H3), the $\delta^{13}C$ values were –27.23‰ and –24.57‰, respectively. Obviously, a significant enrichment of the heavier carbon isotope (^{13}C) occurred. Although some abiotic environmental processes, such as equilibrium chemical reactions or phase-transfer processes including air-water partitioning and sorption processes, could also induce the isotope fractionation, this fractionation is generally much less than those induced by biotransformation processes and within the instrumental precision (0.5‰).[7,33,34] Therefore, this result suggests that the *cis*-HHCB enantiomers might have undergone an enantioselective biotransformation and implies that a combination of isotopic patterns and chiral signatures could be used to trace the biotransformation of chiral compounds in the environment.

CONCLUSIONS

A method combining chiral analysis with compound-specific stable isotope analysis was developed for HHCB enantiomers in the environment. The chiral GC/MS analysis of the HHCB standard showed that no enantiomeric fractionation occurred at any step of the pre-concentration procedure. Moreover, no isotopic fractionation of HHCB racemic mixtures or its enantiomers was observed in the entire procedure. These results demonstrate that the pre-concentration procedure can isolate sufficient quantities of highly purified HHCB for carbon isotope analysis of HHCB enantiomers.

The method was also applied to determine the carbon isotope ratios of the four HHCB enantiomers in a sediment sample. Although the EF values of the two pairs of HHCB enantiomers in the sediment were similar to those of the HHCB standards, the carbon isotope ratios exhibited significant isotopic fractionation, suggesting that *cis*-HHCB enantiomers had undergone enantioselective biotransformation. The isotope analysis of enantiomers might give more powerful information to enable the environmental fate of chiral organic compounds to be elucidated.

Acknowledgements

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