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HBCD Stereoisomer Pattern in Mirror Carps Following Dietary Exposure to Pure γ-HBCD Enantiomers

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1,2,5,6,9,10-Hexabromocyclododecane (HBCD) is a brominated flame retardant consisting of a mixture of diastereomeric pairs of enantiomers that is a known omnipresent, environmental contaminant. The present study investigated the possibility of bioisomerization of HBCD stereoisomers. Therefore, mirror carps (*Cyprinus carpio morpha noblis*) were exposed to pure (+)- and (−)-γ-HBCD, randomly sampled biweekly over a period of three and a half months and the fillets were subjected to enantiomer-specific determination of HBCD. Considering the background contamination of the fish at the beginning of the feeding period, significant enrichment of the respectively fed γ-enantiomer was already detectable after two weeks of exposure. However, no significant enrichment of the respectively expected α-enantiomer was observed within this period. Thus, no evidence for the isomerization of HBCD stereoisomers was found in mirror carp under the applied conditions.

KEYWORDS: Enantiomer-specific determination; HPLC-MS/MS; brominated flame retardant; fish feeding experiment; enantiomer fraction; bioisomerization

INTRODUCTION

1,2,5,6,9,10-Hexabromocyclododecane (HBCD) is a high production volume chemical with production globally in excess of 20000 tons and worldwide one of the most widely used brominated flame retardants (BFRs). This nonaromatic, brominated cyclic alkane is increasingly utilized primarily as an additive in polystyrene foams and in minor applications such as upholstery textiles, adhesives, and styrene-acrylonitrile resins (*1*−*3*).

Like other major BFRs, HBCD isomers show a strong tendency to bioaccumulate through food chains, persist in the environment, and have the potential for long-range environmental transport. The levels of HBCD detected in biota and other environmental matrices have been found to be increasing during recent decades. Rising HBCD concentrations were found in guillemot eggs from the Baltic Sea (*4*), in peregrine falcon eggs from South Greenland (*5*), in sediments of a Swiss lake (*6*), in northern fur seals from the Asia-Pacific region (*7*), and even in breast milk of Japanese women (*8*). Furthermore, investigations allocate the appearance and accumulation of HBCD in different fish species from various parts of the globe (*3*, *9*, *10*) showing the ubiquitous distribution of this stereochemically complex group of compounds.

The commercial HBCD mixture consists largely of a mixture of three diastereomeric pairs of enantiomers, termed (±)-α, β, and γ-HBCD with the γ-isomers as the main component (*11*−*13*). HBCD decomposes at temperatures above 220 °C (*14*, *15*), and at temperatures between 160 and 200 °C, thermal rearrangement of the HBCD isomers takes place (*13*, *16*). Recent studies have examined the isomerization of all six stereoisomers at elevated temperatures in detail (*17*, *18*). The kinetic analysis revealed that the α-enantiomers are the energetically most favorable configurations and that under thermal stress the reactions (+)-γ-HBCD → (+)-α-HBCD and (−)-γ-HBCD → (−)-α-HBCD are the most rapid ones.

Interestingly, previous investigations of biota samples have shown a dominance of the α-diastereomer comparable to the isomeric pattern of thermally treated HBCD (*16*, *19*−*21*). Recently reported enantiomer fractions (EFs) of HBCD stereoisomers in various biota samples indicate enrichment of the first eluting enantiomers (−)-α-, (−)-β-, and (+)-γ-HBCD (*22*−*24*). The interconversion of γ-HBCD to α-HBCD under thermal stress on the one hand and the diastereomer-specific enrichment of α-HBCD in biota on the other hand give rise to the question if these observations are at least partly due to isomerization of γ-HBCD to α-HBCD under physiological conditions rather than due to a combination of diastereomer-specific uptake and metabolism. Such a bioisomerization was suggested for rainbow trout after separate exposure to racemic α-, β-, and γ-HBCD (*25*). As the exposure of (±)-γ-HBCD leads to the formation of (±)-α-HBCD, the not completely avoidable background levels of (±)-α-HBCD may interfere with the unambiguous interpretation of (±)-α-HBCD levels during and after the feeding period. Because (+)-γ-HBCD isomerizes to (+)-α-HBCD and (−)-γ-HBCD isomerizes to (−)-α-HBCD (**Figure 1**) after exposure to pure γ-HBCD enantiomers, a clear increase of the respective single α-HBCD enantiomer must be observed in case of bioisomerization.

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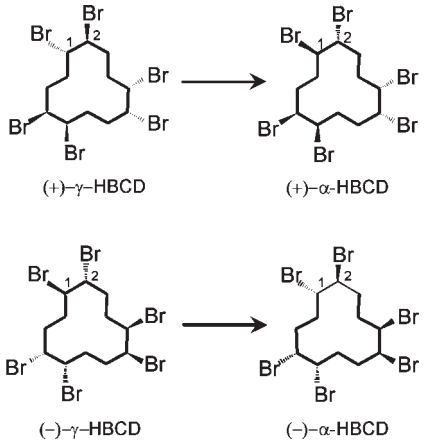

Figure 1. Interconversion pathways of γ-HBCD to α-HBCD.

The exposure to racemic γ-HBCD would lead to an increase of both α-HBCD enantiomers. This, however, would need to be separated from any increase of (±)-α-HBCD from the background content of HBCD in the untreated food.

Therefore, in this study, mirror carps were exposed to food fortified with enantiomerically pure (+)- and (−)-γ-HBCD to provide unequivocal evidence for the formation of the respective α-enantiomer and thus for or against the bioisomerization of HBCD.

Mirror carp (*Cyprinus carpio morpha noblis*) belongs to the freshwater fish family of the Cyprinidae and is widely cultivated due to its fast growth rate and high feed efficiency. This stocking fish is widespread in Europe's rivers and lakes and a popular prey for fishermen.

Mirror carps were selected for the feeding experiment with respect to their easy maintenance in closed aquatic circulation systems, their good food acceptation, as well as their growth rates.

MATERIALS AND METHODS

Chemicals. Technical HBCD was purchased from Fluka (Buchs, Switzerland). Ethanol (absolute, 99.8%) was obtained from Chemsolute (Th. Geyer GmbH & Co. KG, Renningen, Germany). Native and [$^{13}C_{12}$]-labeled α-, β-, and γ-HBCD standards as racemic solutions in toluene (chemical purity >98%) were provided by Wellington Laboratories, Inc. (Ontario, Canada). Ammonium acetate, sea sand (washed and ignited), HPLC grade acetonitrile, methanol, dichloromethane, and *n*-hexane were obtained from J. T. Baker (Deventer, Netherlands). Picograde cyclohexane and ethyl acetate were purchased from Promochem (Wesel, Germany). High-purity water was prepared with a Milli-Q system (Millipore, Bedford, MA). SPE cartridges (80 mm × 15 mm, J. T. Baker, Deventer, Netherlands) were filled with 1 g of activated Florisil (60−100 mesh, Aldrich, Steinheim, Germany).

Separation of γ-HBCD by High Performance Liquid Chromatography. The enantiomer-specific separation was performed using an Varian HPLC system (Varian Deutschland GmbH, Darmstadt, Germany) consisting of two PrepStar SD-1 pumps, a ProStar 410 HPLC autosampler, a ProStar 335 HPLC diode array detector (DAD), and a ProStar 701 fraction collector. Data were collected and processed with the Galaxy Chromatography Data System (Varian). The separation was done using a chiral NUCLEODEX β-PM preparative column (5 μm, 250 mm × 21 mm ID) from Macherey-Nagel GmbH & Co. KG (Düren, Germany) with an isocratic flow of acetonitrile: water (80:20; v/v, 5 mL min^{-1}). The column temperature was set to 30 °C. For the detection of the HBCD isomers, the DAD was set to 208 nm. The injection volume was 100 μL (about 11.3 mg HBCD). The collected fractions were concentrated under reduced pressure and freeze-dried (*26*). The purity of the separated enantiomers was determined by using an analytical HPLC-system, equipped with an NUCLEODEX β-PM chiral analytical column (5 μm, 200 mm × 4.6 mm ID, Macherey-Nagel GmbH & Co. KG). The mobile phase consisted of acetonitrile/water and was used at a constant flow of 1.0 mL min^{-1} at 30 °C. The separation of HBCD enantiomers was obtained by increasing the initial ratio (65% acetonitrile) gradually to 100% over 20 min. Identification of the separated enantiomeric pairs and their elution order were done using the commercially available racemic solutions of native α-, β-, and γ-HBCD. The purity of (+)-γ- and (−)-γ-HBCD was in both cases found to be (99.4 ± 0.1)%.

Food Preparation. Commercial fish food (DANA FEED A/S, Denmark, DAN-EX 1750, fully extruded grower feed, 3 mm pellets, 50% protein, 17% lipid, 2.6% fiber) was treated separately with a known quantity of the respective γ-HBCD enantiomer as solution in ethanol (0.996 μg g^{-1}) using a 2 L round-bottom flask. The solvent was slowly removed using a rotary evaporator, dried at 60 °C for 36 h, followed by blending with the same quantity of untreated food and homogenization. The untreated food was used as control material and its background content of (−)-α-HBCD was 0.10 ng g^{-1} and that of (+)-α-HBCD 0.15 ng g^{-1}. β- and γ-HBCD were below LOQ. The contents of the fortified γ-HBCD enantiomers were determined to be 26.0 ± 1.6 ng g^{-1} in the case of (+)-γ-HBCD and 16.8 ± 0.4 ng g^{-1} for (−)-γ-HBCD. The food was stored in the dark at −23 °C.

Maintenance of Fish and Exposure to HBCD. One year old mirror carps (*Cyprinus carpio morpha noblis*) with initial weights between 61.9 and 74.1 g obtained from a regional breeder (Spreeauen Teiche; Teichwirtschaft Eulo; Forst, Germany) were kept in three 800 L fish tanks (56 fishes each), each equipped with a separate warm water circulation system (22−24 °C) with the concentrations of ammonium, 0.41 ± 0.47 mg L^{-1}; nitrite, 1.26 ± 1.20 mg L^{-1}; and nitrate, 362 ± 168 mg L^{-1}. The dissolved oxygen was always close to the level of saturation, and the pH value was between 5.5 and 7.6. Temperature, content of oxygen, and pH value were tested daily, and verification of remaining parameters was performed weekly. Fish husbandry and feeding were conducted by the Institute of Inland Fisheries Potsdam-Sacrow (Potsdam, Germany).

Mirror carps were randomly divided over the three tanks. After an acclimatization period of seven days, control group A received untreated food while group B was fed with (+)-γ-HBCD fortified food and group C with the (−)-γ-HBCD fortified food. The daily amount of feeding was equal to 1.1% of the mean body weight and the uptake phase lasted for 107 days.

The fish feeding experiment was performed in compliance with the appropriate laws and institutional guidelines, approved by the Landesamt für Verbraucherschutz, Landwirtschaft and Flurneuordnung (Frankfurt/Oder, Germany).

Sampling of Specimens for Analysis. Between six and eight specimens were randomly sampled from each group during the uptake phase on days 0, 14, 28, 42, 56, 73, 90, and 107, animals were stunned by aimed hits on the head, killed by stabbing through the heart, weighed and transported and cooled at 0 °C to sample preparation, which took place on the same day. Carps were eviscerated, and heads, scales, and skins were detached before cutting into fillets. Liver and perivisceral body fat did not suffice to allow a complete time series. Fillets from a given feeding group were pooled, cut to small pieces, and separately cryoground down to submillimeter size through a 500 μm sieve on a centrifugal mill (ZM 1000; Retsch GmbH, Haan, Germany) using liquid nitrogen for cooling. The cryoground material were lyophilized on a Lyovac GT2 (Finn-Aqua Santasalo−Sohlberg GmbH, Hürth, Germany), homogenized, and stored at −20 °C until further processing. To avoid cross-contamination, the centrifugal mill was cleaned after each usage.

Sample Extraction and Cleanup. The fish powder was submitted to pressurized fluid extraction on a Dionex ASE-200 instrument (Dionex Corporation, Sunnyvale, CA). Stainless steel extraction cells and glass collecting vials with 11 and 60 mL volumes, respectively, were used. Sample sizes ranged from 1.0 to 1.5 g and were spiked with 50 μL of a 450 ng g^{-1} solution of $^{13}C_{12}$-labeled α-, β-, and γ-HBCD.

The cells were heated to 100 °C for 5 min and extracted with ethyl acetate at 140 bar. The flush volume was 60% over 3 static cycles. Extracts were collected in 60 mL vials and concentrated to 10 mL under a stream of nitrogen. Co-extracted lipids were removed using an automated GPC-system (GPC VARIO, LCTech, Dorfen, Germany) and equipped with an automatic injector and a fraction collector. Six mL of the fish extract was injected into an S-X3 Bio-Beads gel permeation column (500 mm × 40 mm, L × OD, 50 g, 200−400 mesh).

A mixture of cyclohexane/ethyl acetate (1:1, v/v) was used as mobile phase with a flow rate of 4 mL min^{-1}. The fraction containing HBCD was collected in a 100 mL GPC-bottle, evaporated to dryness, redissolved in *n*-hexane, and cleaned additionally on 1 g pretreated florisil (heated at 120 °C for 24 h) with the following mobile phases: *n*-hexane (5 mL) and *n*-hexane/dichloromethane (1:1, v/v, 13 mL). Extracts were concentrated to dryness using a gentle stream of nitrogen and redissolved in 300 μL of methanol for HPLC-MS/MS analysis. The lipid contents of the samples were determined gravimetrically using the noninjected portions of the concentrated extracts after GPC cleanup.

Preparation of Standard Solutions. Stock solutions (450 ng g^{-1}) of the native and mass labeled HBCD standards were prepared by gravimetric dilution in methanol and stored in light protected amber glass Certan capillary vials in a refrigerator at 4 °C. Aliquots of the stock solutions were used for the preparation of calibration solutions by subsequent gravimetric dilution and in case of the [$^{13}C_{12}$]-labeled HBCD stock solution also for spiking of the sample materials.

HPLC-ESI(−)-MS/MS Analysis. Determination of HBCD in fish samples was performed on a LC−MS/MS system with electrospray negative ionization (ESI−). In detail, an Agilent 1100 series HPLC binary pump system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, a thermostatted autosampler, and a thermostatted column compartment was coupled with an API 4000 triple-stage quadrupole mass spectrometer from Applied Biosystems/MDS SCIEX (Foster City, CA/Concord, Ontario, Canada).

The chromatographic separation of the analytes was performed using a combination of a Zorbax XDB-C_{18} (Agilent Technologies, Waldbronn, Germany) and a chiral NUCLEODEX β-PM (Macherey-Nagel GmbH & Co, Düren, Germany) analytical column (both columns: 5 μm particle size, 200 mm × 4.6 mm) maintained at 15 °C. The mobile phase for the isocratic separation of the HBCD isomers consisted of a mixture of 10 mM ammonium acetate buffer and acetonitrile: methanol (90:10, v/v) in the ratio of 90:10. The flow rate was set to 300 μL min^{-1}. The six stereoisomers were quantified by isotope dilution mass spectrometry and the transitions monitored for native HBCD were 640.6 → 79.0 and 652.6 → 79.0 for the $^{13}C_{12}$ labeled HBCD. The first and third quadrupoles were set to unit resolution. Source parameters were as follows: ion spray voltage, −4500 V; declustering potential, −30 V; desolvation temperature, 450 °C; ion source gas 1, 40 arbitrary units (a.u.); ion source gas 2, 30 au; curtain gas, 20 au; collision energy, −40 eV and collision gas, 4 au. Data were collected and processed with the Analyst 1.4.1 software package (Applied Biosystems/MDS SCIEX).

Calculation of Enantiomer Fractions. Enantiomer fractions (*27*), representing the chiral signature, were calculated as follows:

$$EF_\alpha = \frac{(-)\alpha}{(-)\alpha+(+)\alpha} \text{ and } EF_\gamma = \frac{(+)\gamma}{(-)\gamma+(+)\gamma}$$

in which (−)α, (+)α, (−)γ, (+)γ represent the peak areas. By convention the respectively first eluting enantiomers (−)-α- and (+)-γ-HBCD are placed in the numerator. Racemic mixtures show an EF of 0.5, whereas EFs of 0 or 1 indicate pure first or second eluting enantiomer, respectively. The uncertainties of the derived EF values include among others especially the contributions from sample extraction and chromatographic determination on racemic (±)-α-HBCD and (±)-γ-HBCD standard solutions.

Quality Aassurance. For quality assurance and quality control method, blank samples (n = 6) were analyzed with each batch to monitor possible HBCD contaminations. Additionally, quadruplicate analyses were done on each pool (standard deviations ≤5.2%). Furthermore, quality control for the HPLC analyses was done by repeated injections of solvent blanks (methanol). All samples were injected in triplicate (standard deviations ≤2.1%). For the quantification, an external calibration (13 points) in the range from 23.2 pg g^{-1} to 139 ng g^{-1} was performed, showing good linearity (R^2 ≥ 0.9992). The recovery of the surrogate standard was between 96.7% and 104%. The LOQ was 23 pg g^{-1} ((−)-α-HBCD), 22 pg g^{-1} ((+)-α-HBCD), 73 pg g^{-1} ((+)-γ-HBCD), and 72 pg g^{-1} ((−)-γ-HBCD), while the measured HBCD levels in the fillet were between 0.48 and 125 ng g^{-1}. The method's trueness and precision was verified by use of an interlaboratory study material (eel, Interlaboratory Comparison on Dioxins in Food 2008 (ninth Round), Norwegian Institute for Public Health, received through German Federal Environmental Agency). Quality control/quality assurance for the analysis of HBCD in biota is done by means of an in-house reference material made of chicken eggs fortified with α-, β-, and γ-HBCD.

RESULTS AND DISCUSSION

The exposure period was chosen with regard to experiences with rainbow trouts reported in the literature (*25*), and the whole experiment was designed exclusively to reveal evidence for or against bioisomerization. The level of the respectively fortified γ-HBCD enantiomers is much higher (about 15600:1) than the background amount of (±)-α-HBCD, (±)-β-HBCD, and (±)-γ-HBCD in the unspiked food, and any significant isomerization of the fed γ-HBCD enantiomer should clearly be observable. The formation of the respective α-enantiomer should lead to the decrease of the EF_α value in case of (+)-γ → (+)-α and to its increase in case of (−)-γ → (−)-α. Beyond the question of whether bioisomerization takes place or not, no further conclusions were intended from the experiment.

Effects on Fish Health. The exposure to pure (+)-γ and (−)-γ-HBCD did not seem to affect the health of carps under the applied conditions. There was no significant difference between the two exposure groups and the control group at any point in lipid or water content of the fillets. The mortality (one specimen of group C fed with (−)-γ-HBCD died on day 77) and behavior of the carps were not affected by (+)-γ and (−)-γ-HBCD exposure. The weight increase of the fish may be described with the specific growth rate (SGR), eq 1 (*28*), defined as the average relative increase in biomass e.g. fishes over a period of time.

$$SGR(\%) = \frac{\ln(W_f) - \ln(W_i)}{t} \times 100 \quad (1)$$

with ln W_f = the natural logarithm of the final weight, ln W_i = the natural logarithm of the initial weight, and t = time (days) between ln W_f and ln W_I.

The mean final wet weights of the mirror carps at day 107 were 295 g (control group A, n = 12), 311 g (group B, n = 11), and 329 g (group C, n = 10). Fishes of all three groups showed similar SGR, calculated to 1.28% day^{-1} for control group A, 1.32% day^{-1} for the group B, and 1.38% day^{-1} for the group C. The lipid content of the fillets on day 0 was 13.3% and ranged in the three groups between 13.3% and 13.9% until day 73. The mean lipid contents of days 90 and 107 were between 14.8% and 16.7%. The water content of the fish remained stable within (77.6 ± 0.6)% over groups and the whole period. The food conversion ratios ranged between 1.13 and 1.16 kg growth kg^{-1} food. All remaining water parameters ranged within common fluctuations.

Bioaccumulation and Bioisomerization. The levels of the fed γ-HBCD enantiomers increased significantly in the fillet of both exposure groups after the second sampling on day 14. None of the γ-HBCD enantiomers reached steady-state within the uptake period. **Figure 2** shows the enrichment of (−)-γ-HBCD in the corresponding feeding group after 107 days. Because of the HBCD background contamination of the commercial fish food the chromatograms show traces of the other five isomers alongside the fed γ-HBCD enantiomer. As β-HBCD displays the greatest MS/MS-response factor its content may easily be overestimated by eyesight.

This background contamination does not significantly interfere with the interpretation of the results. **Figure 3** depicts the absolute content of α- and γ-HBCD in the pool sampled on a given day from both feeding groups expressed in μmol. Contents of the control group were subtracted from those of the exposed groups. Data were also corrected for the growth using the mean specimen weight of the respective sampling day.

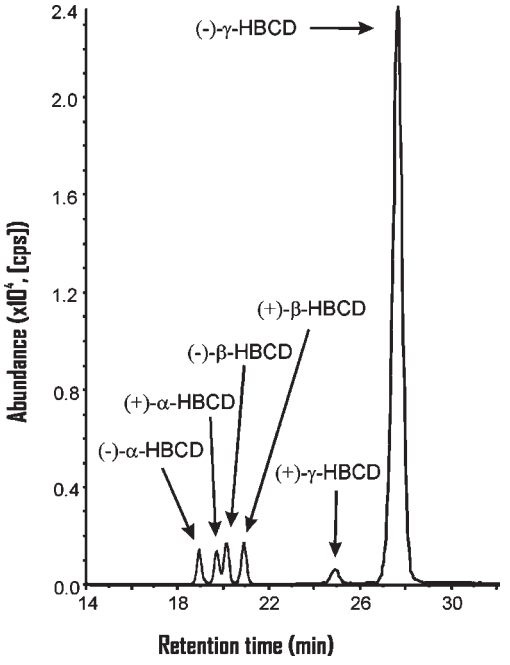

Figure 2. LC-MS/MS chromatogram of a fillet extract after feeding with (−)-γ-HBCD (group C, day 107).

The contents of all HBCD isomers in both feeding groups remain constant until day 14 followed by an increase of the content of (+)-γ- and (−)-γ-HBCD, respectively. It remains unclear why depuration initiates at day 73 in both groups while the feeding rate was kept constant. Metabolism or excretion might occur, however, bioisomerization to the respective α-isomer does not take place as is clearly seen from the stable (±)-α-HBCD ratios.

A similar depuration phenomenon was observed by Law et al. (*25*) during their exposure experiments of rainbow trout with racemic α-, β-, and γ-HBCD. After an uptake phase of 56 days, the depuration of HBCD diastereomers was investigated over 112 days, during which all fish were fed untreated food. During the depuration phase from day 63 of experiment, a decrease of the (±)-β- and (±)-γ-HBCD concentrations was observed and at the same time an increase of the (±)-α-HBCD content. This was interpreted tentatively as possible evidence for bioisomerization under the applied conditions. In the experiments reported here, any significant deviation of the EF_α values would provide unequivocal evidence for isomerization. **Figure 4** depicts the time series of the contents of (±)-α- and (±)-γ-HBCD in the different feeding groups in terms of EF values as measured in the mirror carp fillets. While a bioaccumulation of the fed γ-HBCD enantiomer is reflected by the change of the EF_γ values, the EF_α values remain grossly constant within their expanded uncertainties U_c as expected for absence of any significant isomerization.

Because U_c includes systematic contributions, contrasts between the differently fed carp groups had to be analyzed more in detail. The following relationships were investigated using the techniques indicated below: deviation of the mean EF_α of control group A from 0.5 (*t* test): the total mean EF_α of the all groups is 0.509 with a half-width of the confidence interval (99% confidence level) of 0.010 and thus does not differ from 0.5. To compare the mean EF_α of the exposed groups (B: 0.496 ± 0.022; C: 0.511 ± 0.011) with the mean EF_α of group A (0.520 ± 0.019), E_n values were calculated and tested for significance at a level of α = 0.01. All three groups are (in a statistical sense) indistinguishable from each other.

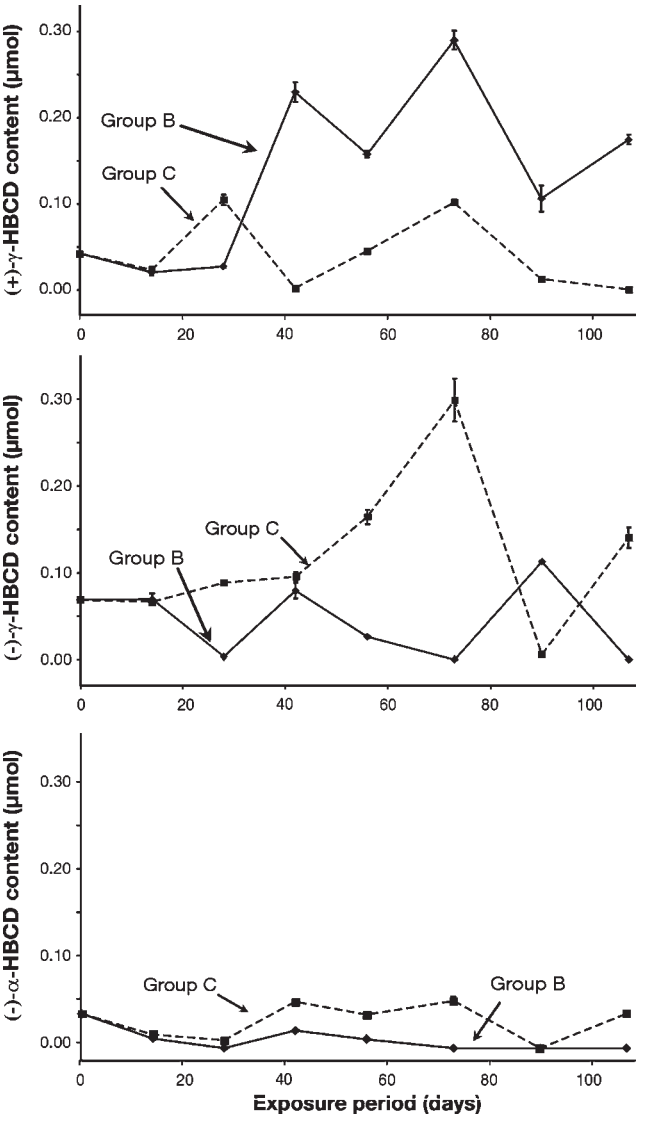

Figure 3. Development of the absolute contents of selected HBCD stereoisomers in fillets from the exposed groups during the feeding period expressed in µmol. Group B: exposure to (+)-γ-HBCD; group C: exposure to (−)-γ-HBCD; means (n = 4); bars on the values: expanded uncertainties U_c.

The existence of a trend over time in the EF_α for the three groups was tested using ordinary least-squares regression (OLS) analysis of the EF_α assuming a linear trend. Groups A and C show a significant trend with enrichment of (+)-α-HBCD toward the end of the feeding experiment, while the trend for group B is significant on the α = 0.05 level only. Excess probabilities P of the regressions are 0.028 for group B and smaller than 10^{-6} for the other two groups. The residual scatter is, however, different from group to group and largest for group B. The latter is due to the specific behavior of group B (see below), and explains the lower significance of the trend for group B.

The existence of differences in the trends of the EF_α for the two exposed groups was investigated by OLS regression analysis of the EF_α differences between the feeding groups, assuming a linear trend. No significant overall trend was observed (P = 0.995).

Instead, a certain customization with the exposed food is noted, having two features. First, an "echoing" of the food adulteration leading to oscillation of the (+)-α-HBCD fraction toward enrichment (days 0 to 40), and second an asymptotic

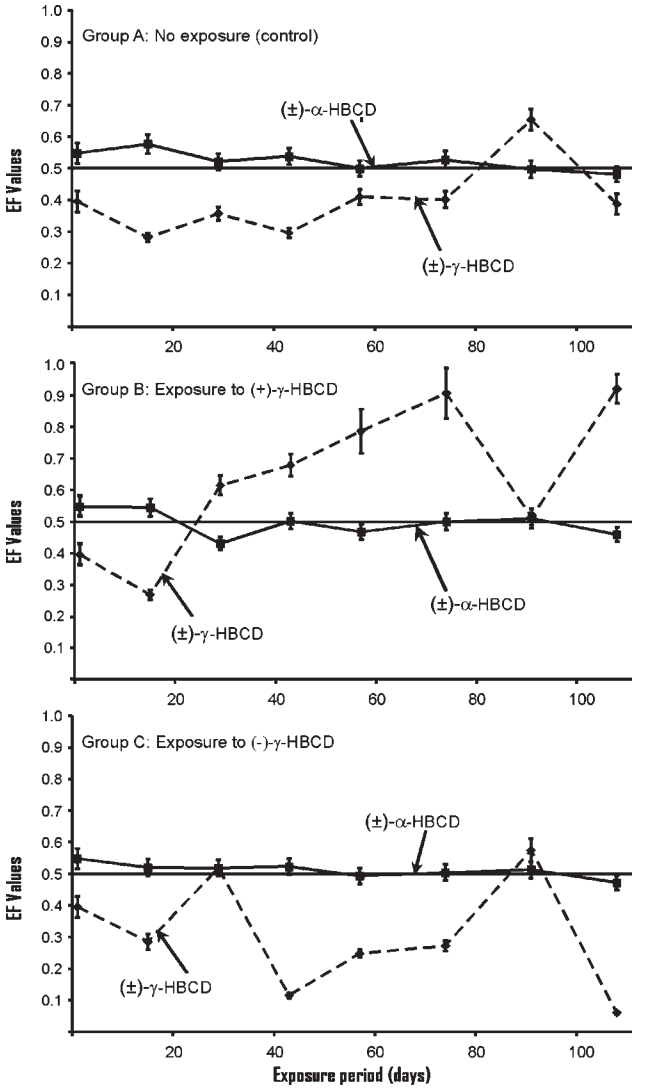

Figure 4. Development of EF_α and EF_γ values in mirror carp fillets after exposure to HBCD. Group A: control (no HBCD); group B: (+)-γ-HBCD; group C: (−)-γ-HBCD; means (n = 4); bars on the values: expanded uncertainties U_c.

decay of the difference between the two exposed groups toward zero (days 40−107, see **Figure 5**).

This observation indicates along with the depuration phenomenon regarding the fed γ-HBCD enantiomers an adaptation of the response of the biological system to the exposure with HBCD.

After 107 days, the three groups are undistinguishable with respect to their EF_α values. Therefore, no evidence for bioisomerization of HBCD stereoisomers was observed during this experiment with mirror carps. It should be taken into account that different fish species might exhibit different metabolism pathways and rates. In summary, it may be concluded that if any bioisomerization of HBCD is taking place in mirror carps at all, it must be a slow process without perceptible impact on the amount of incorporated α-HBCD.

This is supported by the estimation of the minimum bioisomerization of γ-HBCD to α-HBCD that would be significantly detectable against the uncertainty of EF_α values. The minimum measurable EF value difference is a combination of the analytical capability (expanded uncertainty, estimated as 5%) and the natural variability of EF_α. The corresponding expanded uncertainty $U(EF_\alpha)$ is assessed from the residual scatter of regression as 20%. At a value of EF_α around 0.5, it holds $(+)\alpha \approx (-)\alpha$ and

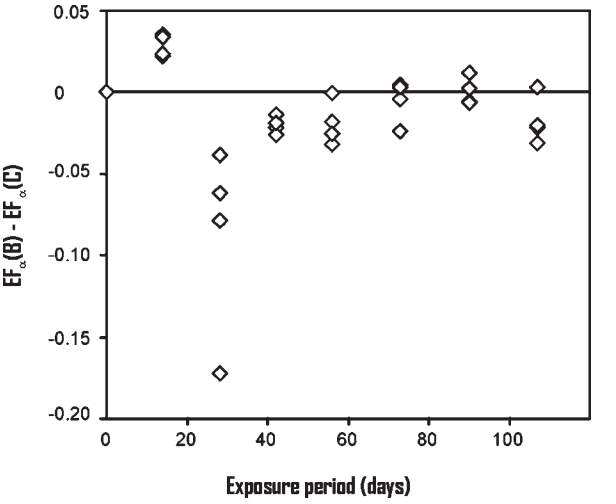

Figure 5. Differences of the EF_α values of the exposed groups B and C.

$U(\alpha)$ amounts to 16.3%. Given the observed levels of (+)-α and (−)-α, one might identify as significant any change δ, e.g. in (+)-α which increases (or decreases) the EF_α value beyond the limits of the expanded uncertainty. For $\delta > 0$, there are four possible cases (i−iv).

$$\frac{(-)\alpha + U(-\alpha)}{(+)\alpha + \delta + (-)\alpha + U(-\alpha)} \le EF_\alpha - U(EF_\alpha) \qquad \text{(i)}$$

$$\frac{(-)\alpha - U(-\alpha)}{(+)\alpha + \delta + (-)\alpha - U(-\alpha)} \le EF_\alpha - U(EF_\alpha) \qquad \text{(ii)}$$

$$\frac{(-)\alpha + U(-\alpha)}{(+)\alpha - \delta + (-)\alpha + U(-\alpha)} \le EF_\alpha + U(EF_\alpha) \qquad \text{(iii)}$$

$$\frac{(-)\alpha - U(-\alpha)}{(+)\alpha - \delta + (-)\alpha - U(-\alpha)} \le EF_\alpha + U(EF_\alpha) \qquad \text{(iv)}$$

Case i is worst in the sense of minimum EF_α value change with respect to a change in (+)α while cases ii−iv are much more sensitive with respect to a changing δ and provide better minimum limits for bioconversion. Therefore, case i is covered in detail. By rearranging i, one obtains at the limit:

$$(-)\alpha + U(-\alpha) - [EF_\alpha - U(EF_\alpha)]\cdot[(-)\alpha + U(-\alpha)] = [EF_\alpha - U(EF_\alpha)]\cdot(+)\alpha + [EF_\alpha - U(EF_\alpha)]\cdot\delta$$

and, with the assumption $(+)\alpha \approx (-)\alpha$

$$\alpha + U(\alpha) - 2\cdot[EF_\alpha - U(EF_\alpha)]\cdot\alpha - [EF_\alpha - U(EF_\alpha)]\cdot U(\alpha) = [EF_\alpha - U(EF_\alpha)]\cdot\delta$$

This leads finally to

$$\delta = \frac{2\cdot U(EF_\alpha)\cdot\alpha + [1 - EF_\alpha + U(EF_\alpha)]\cdot U(\alpha)}{EF_\alpha - U(EF_\alpha)}$$

On inserting the values ($EF_\alpha = 0.5$, $U(EF_\alpha) = 0.2 \times 0.5 = 0.1$, $U(\alpha) = 0.163 * \alpha$), one gets

$$\delta = \frac{0.2\cdot\alpha + [1 - 0.5 + 0.1]\cdot 0.163\cdot\alpha}{0.4} \text{ or}$$

$$\delta = \frac{0.2978}{0.4}\cdot\alpha = 0.744\cdot\alpha$$

This means that, with the average (±)-α-HBCD values actually observed (0.005 μmol, see **Figure 3**, or 0.97 ng g^{-1} toward the end

of the study), a conversion product at an amount-of-substance fraction as low as 0.72 ng g^{-1} would have been identified as significant, corresponding to the same amount of (+)-γ-HBCD administered. The actual dose was 1.3 orders of magnitude greater, thus any considerable bioconversion (if present) above 5% of the dose administered should have clearly been identified.

LITERATURE CITED

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