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Analysis of hexabromocyclododecane diastereomers and enantiomers by liquid chromatography/tandem mass spectrometry: Chromatographic selectivity and ionization matrix effects

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Abstract

Hexabromocyclododecane (HBCD) is a flame retardant that is undergoing environmental risk assessment. The liquid chromatographic retention and electrospray ionization matrix effects were investigated for HBCD methods of analysis for environmental matrices. Column selectivity towards HBCD diastereomers was evaluated for C_{30} and C_{18} stationary phases under different mobile phase conditions and column temperatures. The HBCD elution order was dependent on the shape selectivity of the stationary phase and the mobile phase composition. Greater resolution, on columns with reduced shape selectivity, of β-HBCD and γ-HBCD was achieved with the use of an acetonitrile/water (compared with a methanol/water) mobile phase composition. A liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) method for the analysis of HBCD in biological tissues was evaluated for potential matrix effects. The influence of extracted matrix components on HBCD diastereomer and enantiomer analysis was investigated using a postextraction addition approach. Although the analysis of HBCD diastereomers was relatively unaffected by the sample matrix, the responses of the HBCD enantiomers in tissue samples were significantly influenced by matrix effects and other changes to the ionization conditions. The use of racemic ^{13}C-labeled HBCD diastereomers as internal standards for enantiomer fraction measurements corrected for the changes in the mass spectrometer response.
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Keywords: Hexabromocyclododecane; Flame retardant; Shape selectivity; Matrix effects; Racemic internal standards

1. Introduction

The flame retardant hexabromocyclododecane (HBCD) is currently undergoing environmental risk assessment within the European Union and preliminary risk assessments are taking place in other countries [1,2]. HBCD shares some characteristics with persistent organic pollutants such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), and has been shown to undergo atmospheric transport [3] and to bioaccumulate [4].

Commercial HBCD mixtures consist primarily of three diastereomers, termed α-HBCD, β-HBCD, and γ-HBCD [1,5]. Each of these diastereomers has two enantiomers. Quantification of the individual diastereomers is necessary to properly determine the environmental behavior and risk of HBCD. The most abundant diastereomer (>70%) in commercial mixtures is γ-HBCD [5]. Sediment has been found to contain primarily γ-HBCD [6], but α-HBCD dominates in aquatic biota [5,7]. The diastereomer distribution in the atmosphere is currently unclear [3]. Enantiomer specific accumulation has been observed in fish [8], indicating that like the pesticide hexachlorocyclohexane [1,9], the enantiomers may be selectively degraded in biota. Further research is necessary to more completely understand the enantioselective behavior of HBCD.

Due to separation difficulty and thermal instability associated with HBCD diastereomer analysis by gas chromatography/mass spectrometry (GC/MS), the diastereomers are often analyzed by liquid chromatography/single quadrupole mass spectrometry (LC/MS) [6,7] or LC/MS/MS [8,10]. The elution order with most C_{18} columns is α-, β-, γ-HBCD. The diastereomers have also been separated using a C_{30} column, with an elution order of α-, γ, β-HBCD [11]. The change in elution order observed

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Table 1
Columns used in selectivity measurements

Column	Supplier	Chain length	Bonding chemistry	Code
Carotenoid S-5	Waters/YMC (Milford, MA, USA)	C_{30}	Polymeric	C
Pinnacle II PAH	Restek (Bellefonte, PA, USA)	C_{18}	Polymeric	P
Suplex PKB-100	Supelco (Bellefonte, PA, USA)	–	Monomeric	S
Eclipse XDB-C18	Agilent (Palo Alto, CA, USA)	C_{18}	Monomeric	E
Zorbax ODS (Classic)	Agilent (Palo Alto, CA, USA)	C_{18}	Monomeric	Z

for the C_{30} and C_{18} columns may be useful for the development of independent separation methods. For example, if co-elution is suspected on a C_{18} column, the sample could be run on a C_{30} column to verify the results of a quantitative analysis. Columns with different retention properties can also be used to confirm the identities of analytes [12]. In this study, the mechanisms that cause the HBCD elution order change were investigated on various stationary phases, at different column temperatures, and with different mobile phase solvents.

Electrospray ionization is subject to sample matrix effects that can cause enhancement or suppression of the target analytes' signal and can adversely affect their quantification [13–16]. Matrix effects are caused by coeluting compounds that interfere with the ionization of the analyte. Often, matrix effects can be avoided through the use of the isotope dilution approach to quantification; however, methods for the analysis of the three HBCD diastereomers have typically used a single labeled HBCD diastereomer or a different compound as the internal standard. Although $^{13}C_{12}$-α-HBCD, $^{13}C_{12}$-β-HBCD, and $^{13}C_{12}$-γ-HBCD standards are commercially available, simultaneous use of the labeled diastereomers as internal standards may not be considered cost effective. Tomy et al. [17] recommended the use of three labeled internal standards for HBCD diastereomer analysis based on an analysis of matrix effects. Internal standards have not been previously used for the determination of enantiomer fractions (EFs) of HBCD, even though it has been suggested that matrix effects influence the LC/MS/MS measurement of EFs in biota [8]. In this study, matrix effects associated with the analysis of HBCD diastereomers and the determination of enantiomer fractions in tissue samples were investigated to assess the need for and utility of matched stable isotope labeled HBCD internal standards.

2. Experimental

Standards of unlabeled and $^{13}C_{12}$-labeled α-HBCD, β-HBCD and γ-HBCD were purchased from Wellington Laboratories (Guelf, Ontario, Canada). All solvents were HPLC grade.

2.1. Diastereomer selectivity factor measurements

HBCD retention behavior was studied under a variety of stationary phase and mobile phase conditions. The measurements were made on an Agilent 1100 series HPLC (Palo Alto, CA, USA) coupled to an Applied Biosystems/Sciex API 4000 (Foster City, CA, USA) triple quadrupole mass spectrometer. Three parameters were studied using standards of the three HBCD diastereomers: stationary phase type, column temperature, and mobile phase composition. The flow rate was 0.5 mL/min, and 10 ng of each HBCD isomer was injected onto the column. All runs were isocratic. At room temperature, two mobile phase systems were studied: methanol/water and acetonitrile/water, both 90:10 volume fractions. At 0 °C, the methanol/water and acetonitrile/water systems were both 95:5 volume fractions. The five columns that were evaluated are listed in Table 1. All columns were 250 mm × 4.6 mm I.D. and had a 5 μm particle size. The Suplex PKB-100 stationary phase consisted of amide polar-embedded alkyl chains. The one letter code for each column given in Table 1 is used in Fig. 1. The tandem mass spectrometer parameters were similar to those used for the matrix effect measurements; see below.

The shape recognition characteristics of the LC columns were measured using National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 869a [18]. This SRM is a mixture of three polycyclic aromatic hydrocarbons (PAHs) in acetonitrile: benzo[*a*]pyrene (BaP), 1,2:3,4:5,6:7,8-tetrabenzonaphthalene (TBN), and phenanthro[3,4-*c*]phenanthrene (PhPh). The void volume was measured in the same run by spiking a void volume marker, uracil, into SRM 869a. This mixture was run on each LC column under each of the conditions. The absorbance at 254 nm was monitored. Columns with reduced shape selectivity give a PhPh, BaP, TBN elution order. Columns with enhanced shape selectivity give a PhPh, TBN, BaP elution order [18].

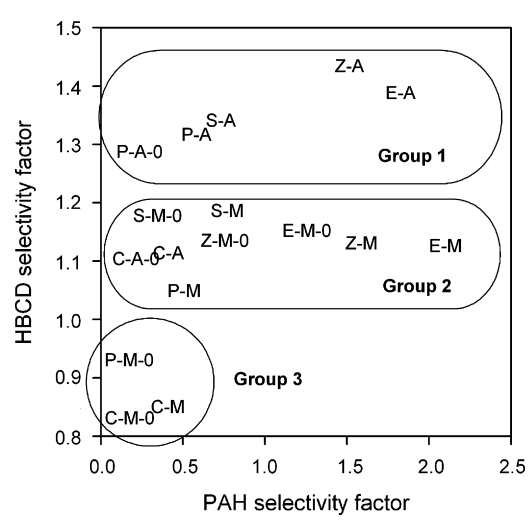

Fig. 1. Selectivity factor plot. For each data point, the first letter refers to the LC column (see Table 1), and the second letter refers to either methanol (M) or acetonitrile (A) based mobile phase. Data points with a zero refer to measurements taken with a column temperature of 0 °C, instead of at room temperature.

Thus, in terms of elution order, SRM 869a is analogous to a mixture of the HBCD diastereomers. Each is a mixture of three compounds, two of which switch elution order depending on the separation conditions. Example chromatograms of SRM 869a and the three HBCD diastereomers under enhanced shape selectivity and reduced shape selectivity conditions are given as Supplementary material.

The chromatographic selectivity factor is a measure of the relative retention of two analytes. The void volume, t_0, was used to calculate the retention factor k', $k' = (t_R - t_0)/t_0$, for each of the HBCD isomers and the PAHs. The HBCD and PAH selectivity factors were calculated using the following equations.

$$\text{HBCD selectivity factor} = \frac{k'_{\gamma-\text{HBCD}}}{k'_{\beta-\text{HBCD}}} \quad (1)$$

$$\text{PAH selectivity factor} = \frac{k'_{\text{TBN}}}{k'_{\text{BaP}}} \quad (2)$$

2.2. Matrix effect measurements

The effect of the extracted sample matrix on the ionization of the HBCD diastereomers and enantiomers was investigated using three tissue samples: Organics in Whale Blubber (NIST SRM 1945), white-sided dolphin blubber (from the National Marine Mammal Tissue Bank, maintained by NIST), and Lake Superior Fish Tissue (NIST SRM 1946). The matrix effects were studied using a postextraction addition approach in which HBCD is extracted from a tissue sample and the extract is either analyzed as is, or is spiked with a solution of HBCD standards before analysis. If matrix effects are insignificant, then the response (R), or peak area of the analyte, of the spiked matrix solution will equal the sum of the unspiked matrix solution response plus the pure standard solution response (Eq. (3)). Matrix effects are evident if the response of the spiked matrix does not equal the sum of the unspiked matrix solution response plus the pure standard solution response:

$$R_{\text{spiked matrix}} = R_{\text{unspiked matrix}} + R_{\text{standard}} \quad (3)$$

Preparation of the extract was as follows. Each tissue sample (1.5 g SRM 1945, 0.63 g white-sided dolphin blubber, and 3.0 g SRM 1946) was mixed with ~30 g Na_2SO_4 and loaded into pressurized fluid extraction cells. An internal standard solution containing the three labeled HBCD diastereomers was added to each sample cell. Extraction was carried out by pressurized fluid extraction using five extraction cycles of dichloromethane at 100 °C and 2000 psi. Lipids and other interferences were then removed from each sample by processing twice through a 600 mm × 25 mm I.D. gel permeation chromatography column (PLgel, Polymer Labs, Amherst, MA, USA) [19]. Samples were solvent exchanged into isooctane and processed by solid phase extraction using ~1.8 g of 5% deactivated alumina to further remove potential ionization interferents. HBCD was eluted with 9 mL of 35% dichloromethane in hexane using an automated solid phase extraction apparatus. The extracted matrix solutions were solvent exchanged into acetonitrile, and a portion of each solution was removed and spiked with the three unlabeled HBCD diastereomers (these spiked extracted matrix solutions are represented by $R_{\text{spiked matrix}}$ in Eq. (3)). The standard solution used to evaluate the matrix effects (represented by R_{standard} in Eq. (3)) contained the same mass of HBCD that was used to spike the extract.

Measurements were made on an Agilent 1100 series LC coupled to an Applied Biosystems/Sciex API 4000 triple quadrupole mass spectrometer. For both the diastereomer and enantiomer separations, the column temperature was 27 °C, the autosampler temperature was 20 °C, and the injection volume was 10 μL. For the standard solution used to evaluate the matrix effects, the mass of α-HBCD, β-HBCD and γ-HBCD injected on column was 429 ng, 474 ng and 474 ng, respectively. The three diastereomers were separated using a 250 mm × 5 mm I.D., 5 μm particle size, Agilent Zorbax Eclipse XDB C_{18} column. The mobile phase consisted of methanol, acetonitrile, and water, at a constant flow rate of 300 μL/min. The initial solvent composition was 60% water/30% methanol/10% acetonitrile. The mixture was changed linearly over 4 min to a final composition of 50% methanol/50% acetonitrile. This was achieved using two solvent mixtures of 50% methanol/50% acetonitrile (A) and 75% water/25% methanol (B), and an initial flow composition of 20% A/80% B ramped linearly over 4 min to 100% A. The final composition was held for 10 min, and then a linear ramp over 4 min returned the mobile phase to the initial composition. The column was equilibrated for 7 min between analyses.

The HBCD enantiomers were separated on a 200 mm × 4 mm Nucleosil β-PM cyclodextrin column (Phenomonex, Torrance, CA, USA) using a mobile phase of water, methanol, and acetonitrile. The flow rate was 500 μL/min. The initial solvent composition was 49% water/30% methanol/21% acetonitrile. The mobile phase composition was changed linearly over 4 min to a final composition of 30% methanol/70% acetonitrile. This was achieved using two solvent mixtures of 30% methanol/70% acetonitrile (A) and 70% water/30% methanol (B), and an initial flow composition of 30% A/70% B ramped linearly over 4 min to 100% A. The final composition was held for 6 min before a linear ramp over 7 min returned the mobile phase to the initial solvent composition. The column equilibrated for 8 min between analyses. The diastereomer and enantiomer separations were based on previously published methods [8]. Example chromatograms are given as Supplementary material.

The mass spectrometer was operated in negative mode electrospray ionization (−ESI) with multiple reaction monitoring (MRM). The $(M-H)^- \rightarrow Br^-$ transitions at m/z 640.6 → 78.9 and 640.6 → 80.9 were monitored for unlabeled HBCD. The labeled HBCD was monitored at the 652.6 → 78.9 and 652.6 → 80.9 transitions. The optimized ionization and collision induced dissociation parameters used to investigate the matrix effects and measure the enantiomer fractions are given as Supplementary material. The matrix effects associated with the diastereomer and enantiomer LC/MS/MS methods were evaluated with three replicate injections of each of the six (three spiked and three unspiked) extracted matrix solutions. Both −ESI and negative mode atmospheric pressure chemical ionization (−APCI) were evaluated for sensitivity. It was found that −ESI was roughly 30 times more sensitive than −APCI.

2.3. Calculation of enantiomer fractions

The EF describes the difference in the amount, or mass, of two enantiomers in a sample; see Eq. (4). The mass of the analyte ($mass_a$) can be determined by the response (R) of the analyte and its response factor (RF), in which case Eq. (4) becomes Eq. (6). If the response factors of the two enantiomers are equal ($RF_1 = RF_2$), then Eq. (6) simplifies to Eq. (7). Eq. (7) is the general approach for calculating EFs [20]. The (+) and (−) forms of the enantiomers were not identified under these chromatographic conditions; therefore, R_1 is defined as the first enantiomer to elute. Note that this is a different definition than used by Janák et al. [8], in which the (+) and (−) forms were identified and R_1 was defined as the (+) form. For α-HBCD and β-HBCD the (−) form eluted first; for γ-HBCD the (+) form eluted first.

$$\mathrm{EF} = \frac{\mathrm{mass}_1}{\mathrm{mass}_1 + \mathrm{mass}_2} \tag{4}$$

$$\mathrm{mass}_a = R_a(\mathrm{RF}_a) \tag{5}$$

$$\mathrm{EF} = \frac{R_1(\mathrm{RF}_1)}{R_1(\mathrm{RF}_1) + R_2(\mathrm{RF}_2)} \tag{6}$$

$$\mathrm{EF} = \frac{R_1}{R_1 + R_2} \tag{7}$$

To test for matrix effects, the combined EF of the unspiked extracted matrix and the standard solution were calculated using Eq. (8), where $R_{a,matrix}$ refers to the response of enantiomers in the unspiked extracted matrix. If there are no matrix effects, the EF of the spiked extracted matrix (calculated using Eq. (7)) will equal the combined EF of the unspiked extracted matrix and the standard solution (calculated using Eq. (8)):

$$\mathrm{EF}_{\mathrm{combined}} = \frac{R_{1,\mathrm{matrix}} + R_{1,\mathrm{std}}}{(R_{1,\mathrm{matrix}} + R_{1,\mathrm{std}}) + (R_{2,\mathrm{matrix}} + R_{2,\mathrm{std}})} \tag{8}$$

When using LC/MS and LC/MS/MS, the response factors for two enantiomers may be different due to matrix effects or other changes in the ionization conditions. This ionization variability was corrected for by using ^{13}C-labeled HBCD internal standards for each pair of enantiomers. Solving the relative response factor (RRF), Eq. (9), for $mass_a$, and substitution into Eq. (4) yields Eq. (10). Two assumptions simplify Eq. (10) to Eq. (11). First, the ^{13}C-labeled HBCD standards are racemic ($mass_{IS,1} = mass_{IS,2}$). Second, the enantiomer response factors are equal, in the absence of any differences in their ionization conditions; therefore, the relative response factors are equal ($RRF_1 = RRF_2$). Eq. (11) was used to calculate the EFs using racemic internal standards (ISs).

$$\mathrm{RRF}_a = \frac{(\mathrm{mass}_a/\mathrm{mass}_{\mathrm{IS,\,a}})}{(R_a/R_{\mathrm{IS,a}})} \tag{9}$$

$$\mathrm{EF} = \frac{(R_1/R_{\mathrm{IS},1})(\mathrm{mass}_{\mathrm{IS},1})(\mathrm{RRF}_1)}{(R_1/R_{\mathrm{IS},1})(\mathrm{mass}_{\mathrm{IS},1})(\mathrm{RRF}_1) + (R_2/R_{\mathrm{IS},2})(\mathrm{mass}_{\mathrm{IS},2})(\mathrm{RRF}_2)} \tag{10}$$

$$\mathrm{EF} = \frac{R_1/R_{\mathrm{IS},1}}{(R_1/R_{\mathrm{IS},1} + R_2/R_{\mathrm{IS},2})} \tag{11}$$

3. Results and discussion

3.1. Diastereomer retention behavior

Solute retention in reversed phase LC is largely correlated with the polarity of the solute. In addition, the shape selective properties of the stationary phase can influence the separation of compounds with constrained molecular structure [21]. Shape selectivity has been shown to increase with increasing alkyl chain length [22]. C_{30} phases have deeper slots between the alkyl chains and, compared to C_{18} phases, will more strongly retain molecules whose shape allows them to fit into these slots. Alkyl chain order is increased with increased packing density, and this configuration is thought to result in more well defined stationary phase slots and enhance the shape selectivity [21,23]. Monomeric stationary phases have a relatively low surface density; polymeric stationary phases have a higher surface density and enhanced shape selectivity. The column temperature will also affect the shape selectivity [24]. At lower temperatures, the alkyl chains become more ordered and more shape selective. Other factors, such as the mobile phase composition [21], can also affect the shape selectivity.

The hypothesis for this HBCD selectivity experiment was that the three HBCD isomers have different molecular shapes, and differences in stationary phase shape selectivity cause the different elution orders that are observed when using C_{18} and C_{30} columns. In this experiment, the shape selectivity of the column was measured using SRM 869a and was represented by the PAH selectivity factor (Eq. (2)). Column selectivity towards HBCD was represented by the HBCD selectivity factor (Eq. (1)). The results from the selectivity measurements are presented in Fig. 1. This plot shows how the HBCD selectivity factor varies with the PAH selectivity factor. Lower values of the PAH selectivity factor represent greater shape selective properties.

As an aid to discussing the information in Fig. 1, the data is divided into three groups. Most of the data points are in Groups 1 and 2. Group 1 contains samples run with the 90% acetonitrile mobile phase. Group 2 contains samples run with the 90% methanol mobile phase, except for the room temperature and 0 °C Carotenoid C_{30} samples run with 90% acetonitrile. Samples in Groups 1 and 2 both have the α-, β-, γ-HBCD elution order. Group 1 has a greater HBCD selectivity factor than Group 2 and a greater resolution of β-HBCD and γ-HBCD. Groups 1 and 2 have different HBCD selectivity factors, but within the groups the HBCD selectivity factor is similar. The shape selectivity of the stationary phase, represented by the PAH selectivity factor, did not correlate with the HBCD selectivity factor as well as the mobile phase solvent. Group 3 has a α-, γ-, β-HBCD elution order. Although Group 3 has a low PAH selectivity factor (high shape selectivity), samples in Groups 1 and 2 also have PAH selectivity factors less than 0.5. Therefore, shape selectivity is not the only mechanism involved in producing the α-, γ-, β-HBCD elution order. The results show, however, that enhanced shape selectivity is necessary to produce the α-, γ-, β-HBCD elution order. When the Pinnacle C_{18} column was held at room temperature (data point P-M), the sample had an α-, β-, γ-HBCD elution order and was classified in Group 2. When the

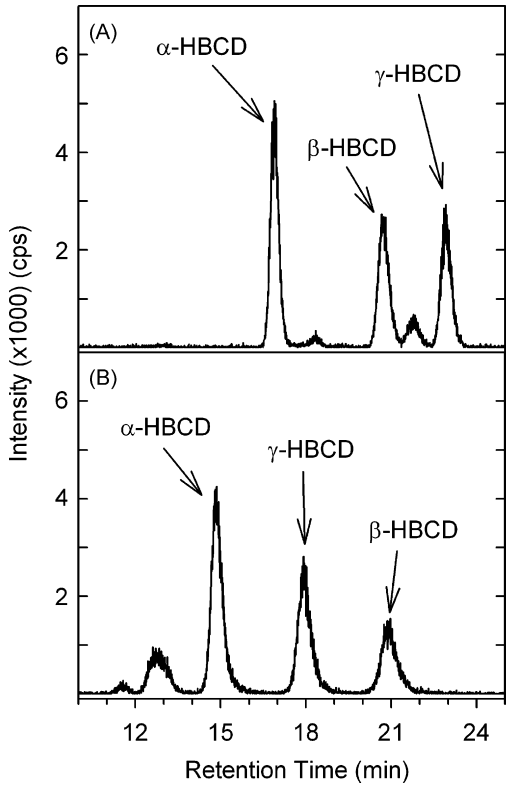

Fig. 2. Atmospheric particle-phase MRM chromatograms. The same sample was analyzed using an Eclipse C_{18} column (A) and a Carotenoid C_{30} column (B), both 250 mm × 4.6 mm. In both cases, the mobile phase was 90% methanol, 10% water.

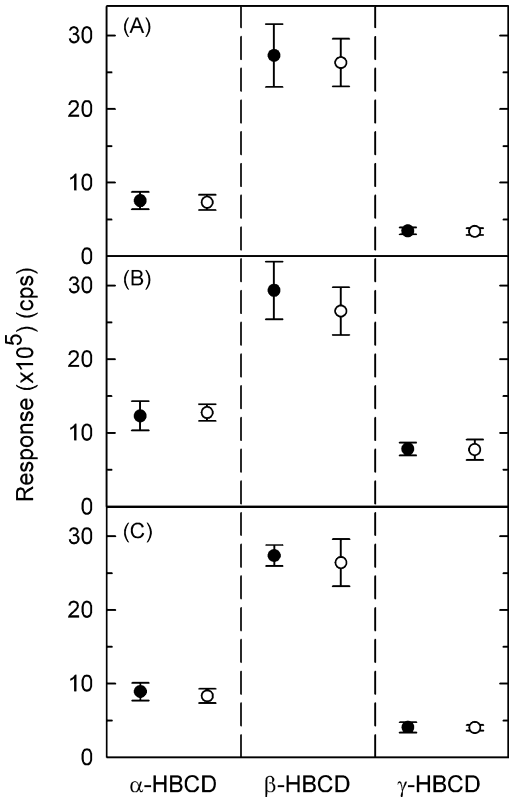

Fig. 3. Matrix effects on the HBCD diastereomer response in whale blubber (A), dolphin blubber (B), and fish tissue (C). Closed circles represent the response of the spiked extracted matrix. Open circles represent the response of the unspiked extracted matrix plus the response of the standard solution. Error bars are the 95% confidence intervals of three replicate measurements.

temperature was lowered to 0 °C (data point P-M-0), the sample had an α-, γ-, β-HBCD elution order and was classified in Group 3. As discussed above, lowering the column's temperature will increase its shape selective properties. This is evidence that shape recognition influences the elution order.

The change in HBCD elution order (i.e., α-, γ-, β) only resulted when methanol was used as the mobile phase modifier (see Fig. 1, Group 3). Separations carried out with the Pinnacle C_{18} and Carotenoid C_{30} columns with acetonitrile did not produce this elution order.

Fig. 2 shows chromatograms of an atmospheric particle-phase sample taken near Rohwer, AR, USA, and run on two columns that give different HBCD elution orders. Details on the sampling and preparation method for this sample have been published by Hoh and Hites [3]. Both chromatograms contain two peaks not attributed to α-, β-, and γ-HBCD. The identities of these components are unknown; however, commercial HBCD mixtures are known to contain small amounts of two other HBCD isomers [25]. Of seven atmospheric samples analyzed [3], one contained these two unknown peaks.

3.2. Matrix effects on diastereomer analysis

Diastereomer levels for tissue extracts are shown in Fig. 3. Diastereomer response was not significantly influenced by the presence of extracted matrix components. β-HBCD gives the largest response in Fig. 3 due to its relatively high response factor. These tissues did not contain detectable levels of β-HBCD or γ-HBCD; their response is due to the spiked compound.

These results indicate that extracted matrix components will not interfere with the accuracy of HBCD diastereomer measurements in certain biological samples, even when only one labeled HBCD diastereomer is used. This conclusion is dependent on the removal of a majority of potential interferences from the sample extracts, similar to the procedure used in this study, and use of the same LC separation. Alternate procedures should be independently evaluated. Tomy et al. [17], using a different extraction method, LC separation, and technique for measuring matrix effects, found that matrix effects influenced the LC/MS/MS measurement of HBCD diastereomers in fish and sediment samples.

3.3. Matrix effects on enantiomer analysis

Extracted matrix components more strongly influenced the measurement of HBCD enantiomers than HBCD diastereomers (Fig. 4). In this figure, the EF of the spiked extracted matrix solutions, calculated using Eq. (7), are compared to the $EF_{combined}$ of the unspiked extracted matrix solutions and the standard solution, calculated using Eq. (8). Internal standards were not used to correct for matrix effects. Extracted matrix components significantly influenced the measurement of the EFs for all three diastereomers. These matrix effects were dependent on the tis-

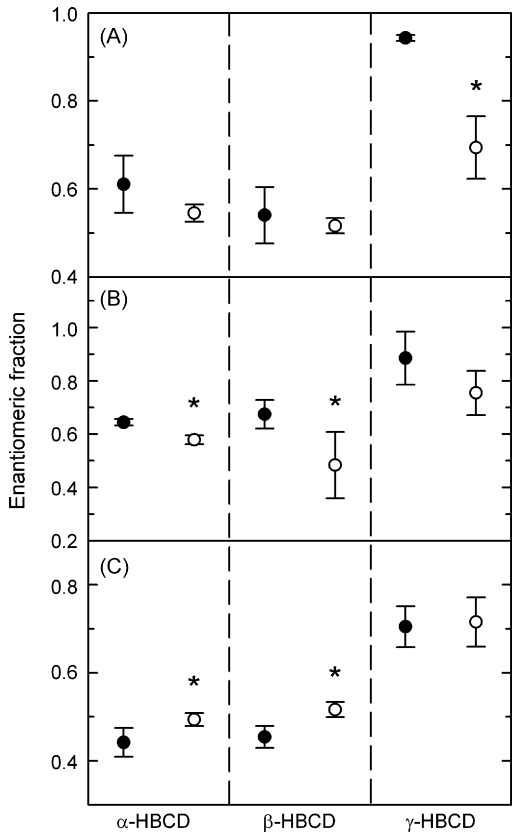

Fig. 4. Matrix effects on EF measurements in whale blubber (A), dolphin blubber (B), and fish tissue (C). Closed circles represent the EF of the spiked extracted matrix solution, calculated using Eq. (7). Open circles represent the $EF_{combined}$, of the unspiked extracted matrix and standard solution, calculated using Eq. (8). Error bars are the 95% confidence intervals of three replicate measurements. An asterisk indicates a statistically significant difference between responses.

sue type. α-HBCD and β-HBCD in whale blubber, and γ-HBCD in dolphin blubber and fish tissue, did not show a difference at the 95% confidence level using a t-test assuming unequal variances.

Since the unspiked samples did not contain detectable levels of β-HBCD or γ-HBCD, the $EF_{combined}$ value for those enantiomers is entirely due to the pure standard solution injected without the presence of a matrix. If the standard solution is (as assumed) racemic, then the expected $EF_{combined}$ value is 0.5.

Table 2
Calculated EFs in standard solutions

	Mean	Std. error	% Std. error	% Deviation from 0.500
α-HBCD				
Without IS	0.438	0.072	17	12
With IS	0.495	0.004	0.89	1.0
β-HBCD				
Without IS	0.326	0.045	13	35
With IS	0.507	0.002	0.48	1.4
γ-HBCD				
Without IS	0.859	0.028	3.2	72
With IS	0.481	0.007	1.5	3.8

The EFs were calculated by Eq. (7) (without the internal standards) and Eq. (11) (with the internal standards). The errors are the standard error of the mean of four replicate injections.

However, the $EF_{combined}$ value for β-HBCD, and particularly γ-HBCD, was observed to deviate from 0.5. This deviation is likely due to changes in the response of the mass spectrometer between the elution of the two enantiomers. Stable isotope labeled internal standards can be used to correct for this variation in response. Table 2 shows the EFs of a standard solution calculated using Eq. (7) (without the internal standards) and Eq. (11) (with the internal standards). The EFs calculated using the internal standards are much closer to the expected racemic value of 0.5. There is also less variability between measurements when using the internal standards. Variation in the response of the mass spectrometer had the largest effect on the accuracy of the γ-HBCD enantiomer fraction measurement. Janák et al. [8] also observed non-racemic EF values for standard solutions that were calculated without internal standards.

To illustrate the utility of using internal standards to measure EFs in tissue samples, Table 3 shows the repeatability of EF measurements on five Atlantic white-sided dolphin blubber samples, obtained from the National Marine Mammal Tissue Bank. The samples were analyzed using the method described above. The α-HBCD enantiomer fraction in each sample was measured six times: three times on different dates without the internal standards and three times on different dates with the internal standards. The samples did not contain detectable levels of β-HBCD or γ-HBCD. Use of the internal standards improved

Table 3
Reproducibility of α-HBCD EFs in white-sided dolphin blubber, measured without (Eq. (7)) and with (Eq. (11)) the internal standards

Dolphin sample	Without IS August 2005	Without IS September 2005	Without IS October 2005	RSD (%)	With IS August 2005	With IS September 2005	With IS October 2005	RSD (%)
1	0.290	0.247	0.321	13	0.561	0.564	0.573	1.1
2	0.616	0.519	0.191	50	0.539	0.565	0.537	2.8
3	0.348	0.366	0.325	5.9	0.572	0.565	0.558	1.3
4	0.351	0.290	0.493	28	0.548	0.569	0.582	3.1
5	0.390	0.411	0.507	14	0.575	0.569	0.545	2.9
Average	0.399	0.367	0.367	22	0.559	0.566	0.559	2.2
Std. error	0.057	0.048	0.059		0.007	0.001	0.008	

RSD is the relative standard deviation. The calculation of the EFs was dependent on the elution order of the enantiomers, not the identity of the (+) and (−) forms (see text).

the reproducibility of the EF measurement. There was also a source of bias, generally towards lower EF values, due to matrix effects when the internal standard was not used.

4. Conclusions

LC columns with enhanced shape selectivity and a methanol/water mobile phase produce a different HBCD diastereomer elution order compared to columns with reduced shape selectivity. The different properties of the two column types can be used to check for coeluting compounds that will affect the quantitative accuracy of diastereomer analyses, and to assist in identifying unknowns. Using the analytical method described in this study, potential matrix interferences did not significantly influence the LC/MS/MS analyses of the diastereomers in the biological tissues. The use of racemic ^{13}C-labeled α-HBCD, β-HBCD, and γ-HBCD internal standards can substantially improve the accuracy and precision of LC/MS/MS enantiomer fraction measurements in biological tissues. This method for the measurement of enantiomer fractions is applicable to other compounds for which racemic stable isotope labeled internal standards are available.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2006.09.024.

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