Supplementary material

Exposure to Hexabromocyclododecanes via Dust Ingestion, but not Diet, Correlates with Concentrations in Human Serum

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Analytical methods.

^{13}C- labeled α-HBCD, β-HBCD and γ-HBCD were used as internal standards for the diastereomer- and enantiomer-selective analysis of HBCDs.

Food.

Extraction and clean-up for GC-MS. All samples were screened for the presence of HBCD when they were analyzed for PBDEs. The method used for the analysis of food samples is based closely on that described previously (Voorspoels et al., 2003; 2007). An accurately weighed aliquot (3-8 g) of the freeze-dried sample was transferred to an extraction thimble and Soxhlet extracted with 100 mL n-hexane/acetone mixture (3:1, v/v) for 2 h. An aliquot (~1/8th) of the extract was used for gravimetrical lipid determination. Clean-up was achieved by column chromatography on 8 g silica impregnated with concentrated sulfuric acid (44%, w/w). After elution with 15 mL of hexane and 10 mL dichloromethane (DCM), the cleaned extract was concentrated with a rotary-evaporator and further to dryness by a gentle stream of nitrogen. The dried extract was solubilized in 100 µL and transferred in a vial for injection into the gas chromatographic (GC) system.

Extraction and clean-up for LC-MS. Samples where a peak corresponding to the retention time of HBCD was seen in the GC-ECNI/MS chromatogram, were once more extracted, clean-up and analyzed by LC-MS/MS based on the method described previously (Abdallah et al., 2008). An accurately weighed aliquot (0.5-1 g) of the freeze-dried sample was extracted using pressurized liquid extraction (Dionex Europe, UK, ASE 300). Samples were loaded into pre-cleaned 66 mL cells containing 1.5 g Florisil and Hydromatrix (Varian Inc., UK) to fill the void volume of the cells, spiked with 25 ng of each of ^{13}C-labelled α-, β- and γ-HBCD as internal (surrogate) standards (i.e. standards used for determination of analyte concentrations) and extracted with hexane:dichloromethane (1:9, *v/v*) at 90 ˚C and 1500 psi. The heating time was 5 minutes, static time 4 min, purge time 90 s, flush volume 50 %, with three static cycles.
Clean up: The crude extracts were concentrated to 0.5 mL using a Zymark Turbovap® II (Caliper Life Sciences, Hopkinton, MA, USA) then cleaned up by loading onto SPE cartridges filled with 8 g of pre-cleaned acidified silica (44% concentrated sulfuric acid, *w/w*). The analytes were eluted with 25 mL of hexane:dichloromethane (1:1, *v/v*). The eluate was evaporated to dryness under a gentle stream of N_2 then reconstituted in 200 µL of d_{18}-γ-HBCD (25 pg μL^{-1} in methanol) used as recovery determination (or syringe) standard, used to determine the recoveries of internal standards for QA/QC purposes.

Dust. All dust samples were analyzed by LC-MS/MS. The method used for the analysis of dust samples is based closely on that described previously (Abdallah et al., 2008). An accurately weighed aliquot of each sample (typically 200 mg) was extracted using pressurized liquid extraction (Dionex Europe, UK, ASE 300). Samples were loaded into pre-cleaned 66 mL cells containing 1.5 g Florisil and Hydromatrix (Varian Inc., UK) to fill the void volume of the cells, spiked with 25 ng of each of ^{13}C-labelled α-, β- and γ-HBCD as internal (surrogate) standards (i.e. standards used for determination of analyte concentrations) and extracted with hexane:dichloromethane (1:9, *v/v*) at 90 ˚C and 1500 psi. The heating time was 5 minutes, static time 4 min, purge time 90 s, flush volume 50 %, with three static cycles.
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Serum. Preparation, extraction and clean-up of serum samples were as described by Covaci and Voorspoels (2005). All samples were first thawed and then homogenized by shaking for 1 min. Internal standards (7.5 ng for each ^{13}C-HBCD isomer) were added to the serum samples (typically 3.5 mL), together with 1 mL formic acid for protein denaturation and 3 mL Mili-Q water (for dilution, mixed and sonicated for 20 min. Prior to sample application, the SPE cartridges (OASIS HLB cartridges, 6 mL/500 mg) were washed with 5ml DCM and activated with 5ml methanol and 5ml Milli-Q water. After sample loading, the SPE cartridges were rinsed with 3 ml Milli-Q water. The sorbent bed was dried thoroughly under vacuum for 30 min. The SPE cartridges were eluted with 3×3mL DCM in a separate tube and the eluate was concentrated to ~ 1 mL under nitrogen. An empty cartridge (6 ml) filled (from bottom to top) with 2 g of acid silica and 0.5 g Na_2SO_4 was pre-washed with 5 mL DCM. The concentrated eluate obtained from the SPE cartridge was then loaded on the clean-up cartridge and the analytes were eluted with 8 ml DCM. The final eluate was concentrated under a gentle nitrogen stream until dryness, dissolved in 100 µL iso-octane and transferred to a vial for LC analysis. As procedural blank, 5ml of distilled water in place of serum sample was subjected to the same procedure.

Chemical Analysis

LC-MS/MS. All purified extracts were subjected to analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Separation of α-, β- and γ-HBCDs was achieved using a dual pump Shimadzu LC-20AB Prominence liquid chromatograph equipped with SIL-20A autosampler, a DGU-20A3 vacuum degasser and a Varian Pursuit XRS3 C_{18} reversed phase analytical column (150 mm × 2 mm i.d., 3 µm particle size). A mobile phase program based upon (a) 1:1 methanol/water and (b) methanol at a flow rate of 150 µL/min was applied for elution of the target compounds; starting at 50% (b) then increased linearly to 100% (b) over 4 min, held for 7 min followed by a linear decrease to 60% (b) over 4 min, held for 1 min and finishing with 100% (a) for 10 min. The three HBCD diastereomers were baseline separated with retention times of 10.6, 11.2 and 11.7 min for α-, β- and γ-HBCD, respectively (See Supplemental Material, Figure 1).

Separation of HBCD enantiomers was performed on a chiral permethylated *β*-cyclodextrin LC column (200 mm x 4 mm I.D., 5 µm particle size) (NUCLEODEX beta-PM, Macherey-Nagel; GmbH & Co, Düren, Germany). A mobile phase of (a) 1:1 methanol/water with 2 mM ammonium acetate and (b) 3:7 methanol/acetonitrile at a flow rate of 500 µL/min was applied for elution of the target compounds; starting at 50 % (b) then increased linearly to 100 % (b) over 4.5 min, held for 5.5 min, followed by a linear decrease to 65 % (b) over 4 min and held for 2 min (Harrad et al., 2009).

Mass spectrometric analysis was performed using a Sciex API 2000 triple quadrupole mass spectrometer operated in electrospray negative ionization (ESNI) mode. MS/MS detection operated in the MRM mode was used for quantitative determination based on *m/z* 640.6→79, *m/z* 652.4→79 and *m/z* 657.7→79 for the native, ^{13}C-labelled and d_{18}-labelled HBCD diastereomers, respectively. Specific instrumental calibration parameters are given in Table 1.

GC-ECNI/MS. For screening of food extracts, an Agilent Technologies 6890 GC (Palo Alto, CA, USA) connected with an Agilent 5973 MS system was used with a 15 m x 0.25 mm x 0.10 µm DB-5 capillary column (J&W Scientific, Folsom, CA, USA). The carrier gas was helium at a ramped flow rate of 1.0 mL/min (for 20 min) and then of 2.0 mL/min. The temperature program was starting from 90 °C, kept for 1.25 min, then with 10 °C/min to 200 °C, then with 20 °C/min to 300 °C, held 8 min. One µL of the cleaned extract was injected into a programmable temperature vaporizer operated in solvent vent mode (injector temperature: 90 °C, held for 0.05 min, then with 700 °C/min to 295 °C, and kept for 25 min;

vent flow was set at 75 mL/min and the purge vent opened at 1.25 min). For HBCD, ions *m/z* = 79 and 81 were monitored throughout the chromatographic run.

Quality control

Multi-level calibration curves ($r^2 > 0.999$) in the linear response interval of the detector were created for quantification. The identification of HBCDs was based on their relative retention times (RRTs) to the internal standard, ion chromatograms and intensity ratios of the monitored ions for quantification. The quality control was performed by regular analysis of procedural blanks (1 in each batch of 8 samples), blind duplicate samples (1 in each batch of 8 samples with RSD < 15%).

Method recoveries were 73 ± 9 % at a spiking level of 500 pg/mL. None of the target compounds were detected in method blanks. Therefore, the limit of quantification (LOQ) was calculated based on S/N ratio of 10:1. LOQs in food ranged between 5-20 pg/g ww, LOQ in dust was 500 pg/g dw. LOQs in serum were 0.5 ng/g lw. Samples with concentrations below LOQ were replaced by *f**LOQ with *f* being the fraction of samples above LOQ.

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Supplemental Material, Table 1: Optimized MS/MS parameters* for the analysis of HBCDs.

Parameter	Value (units)
Curtain gas	35 (a.u.)
Turbo gas temperature	100 (°C)
Ion spray voltage	- 4500 (V)
Declustering potential	-5 (V)
Focusing potential	-365 (V)
Collision gas	5 (a.u.)
Collision energy	40 (eV)
Cell entrance potential	-6 (V)
Collision cell exit potential	-10(V)

a.u. – arbitrary units

* These parameters were obtained using direct infusion experiments of the target compounds (α-, β- and γ- HBCDs, native d_{18}- and ^{13}C- labelled isomers, 2 ng μL^{-1} each in MeOH) into the MS/MS system via a built-in Harvard syringe pump at a flow rate of 10 μL min^{-1}.

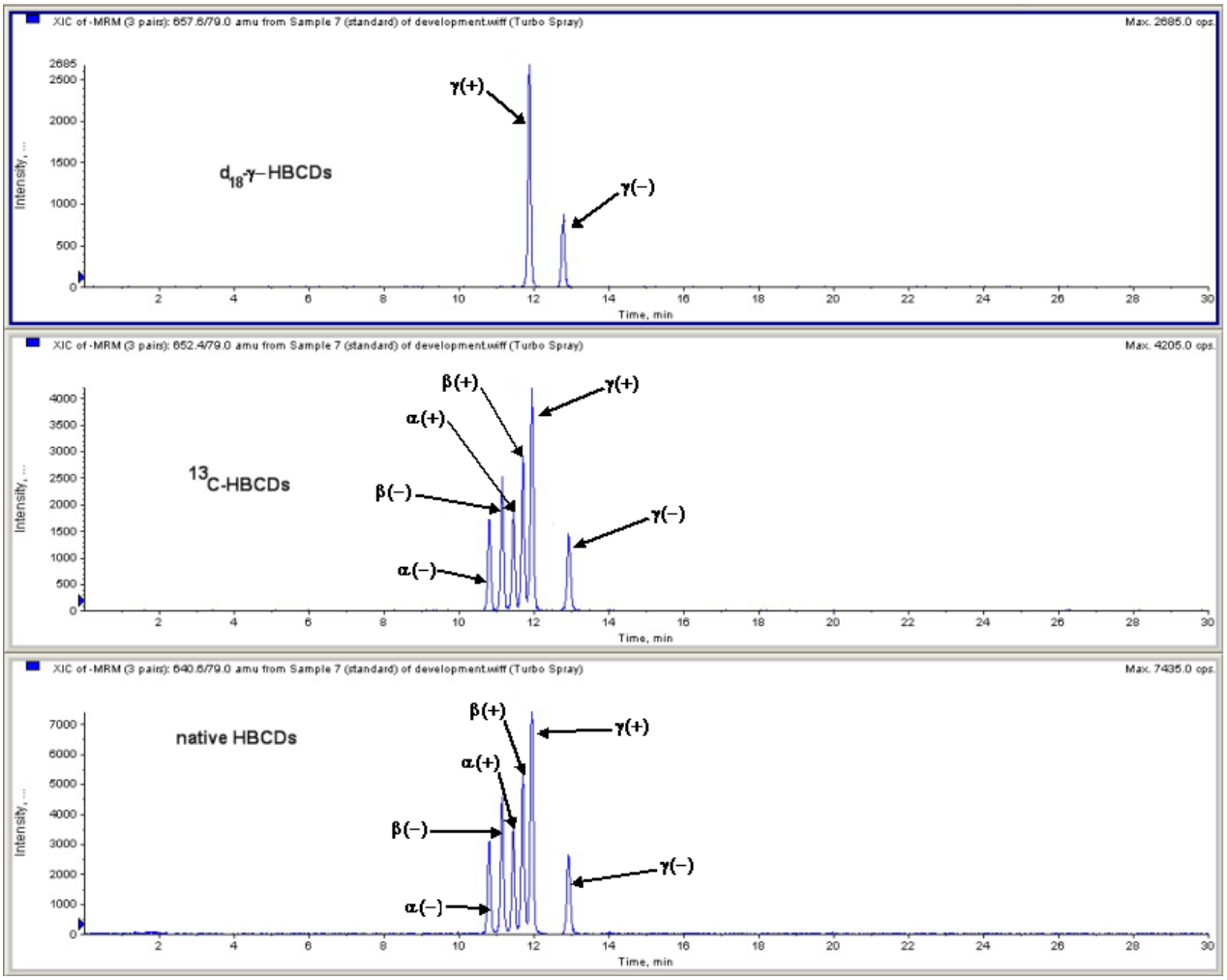
Supplemental Material, Table 2: Concentrations (ng/g dry weight) of HBCD diastereomers Detected in **SRM 2585** (House Dust) Compared to the Indicative Values as given in reference (Keller et al., 2007).

	Average concentration (ng/g dry weight) ± standard deviation (n=10)	
	Measured	**Indicative**
α-HBCD	20 ± 3.1	19 ± 3.7
β-HBCD	4.3 ± 0.5	4.3 ± 1.1
γ-HBCD	121 ± 19	120 ± 22

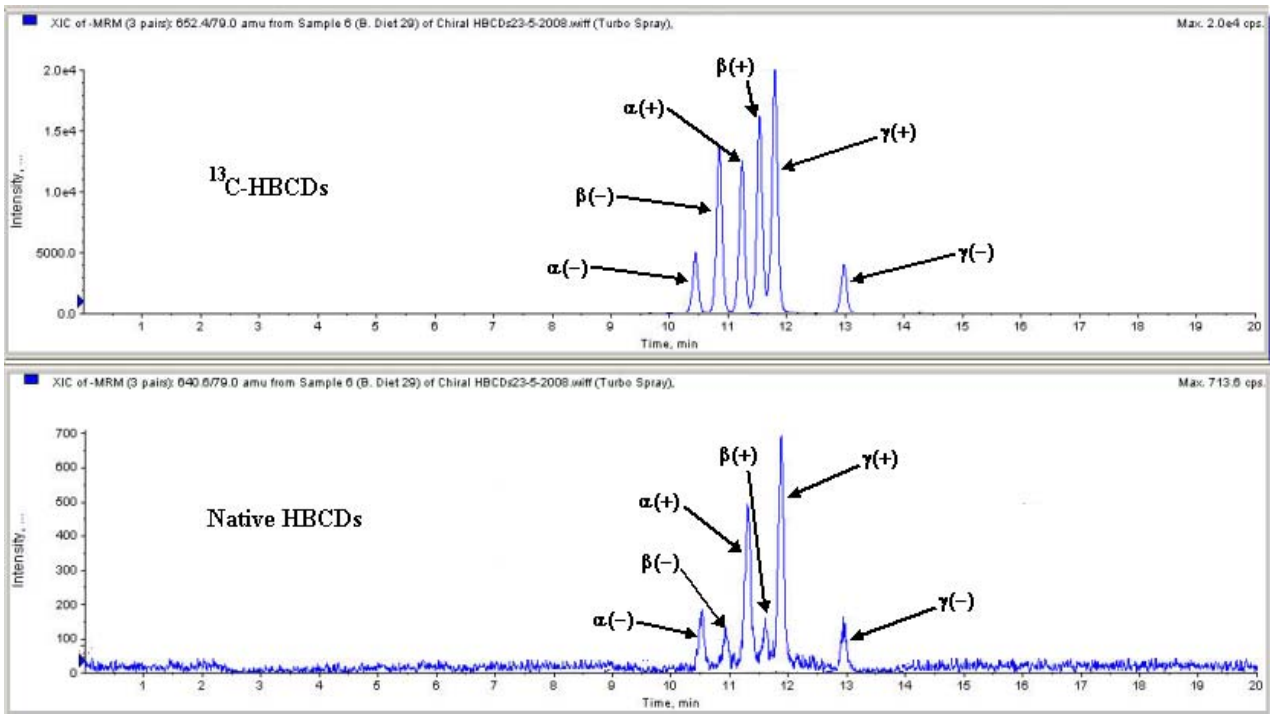
Supplemental Material, Table 3: Concentrations (pg/g ww or pg/µl) of HBCD measured during the interlaboratory test organized by the Norwegian Institute of Public Health (Haug et al., 2008).

	UA	**Assigned**
	Value	**Mean ± SD**
Fish oil (pg/g ww)	7150	7040 ± 1480
Herring (pg/g ww)	580	400 ± 140
Std solution (pg/µl)	485	500 ± 25

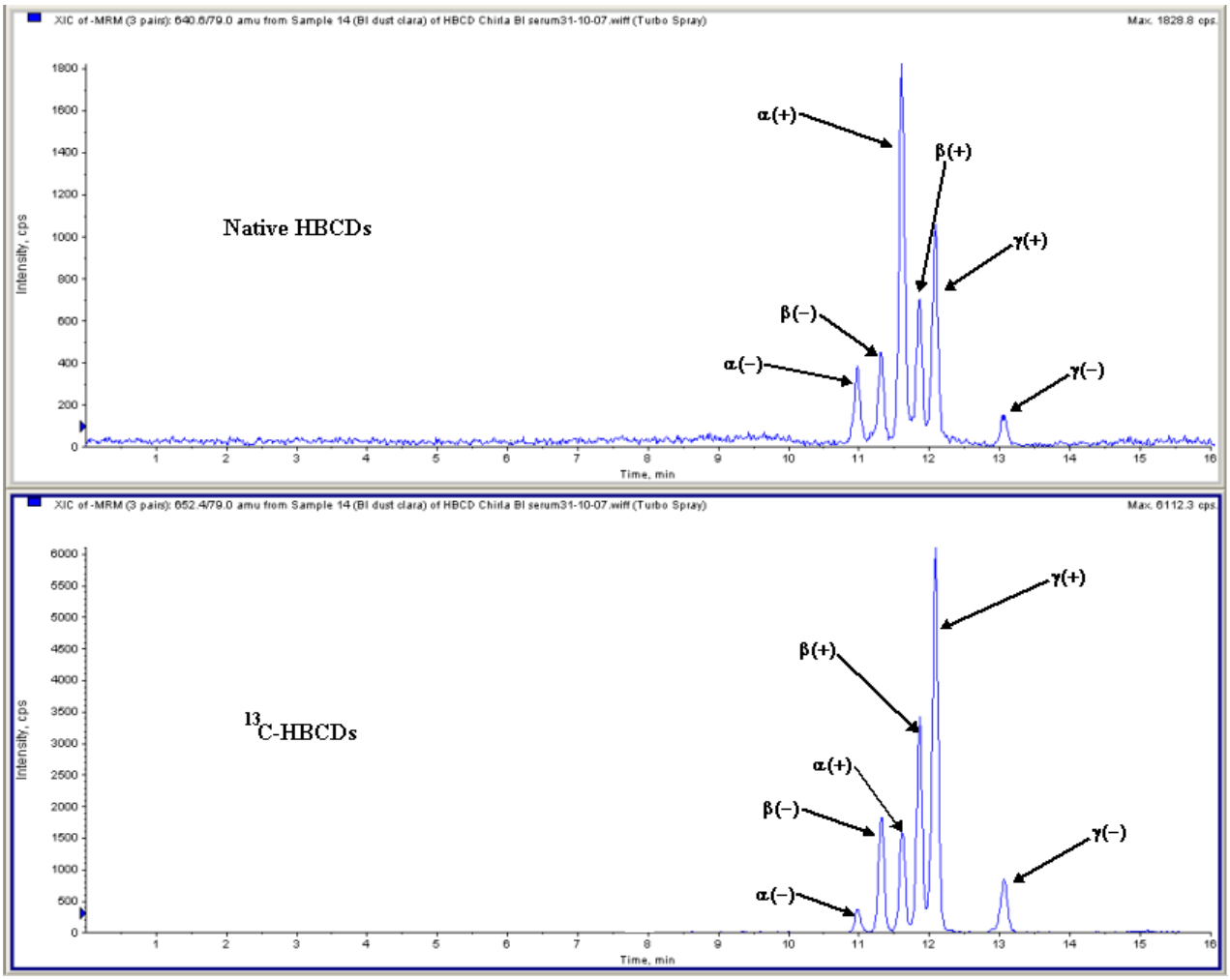
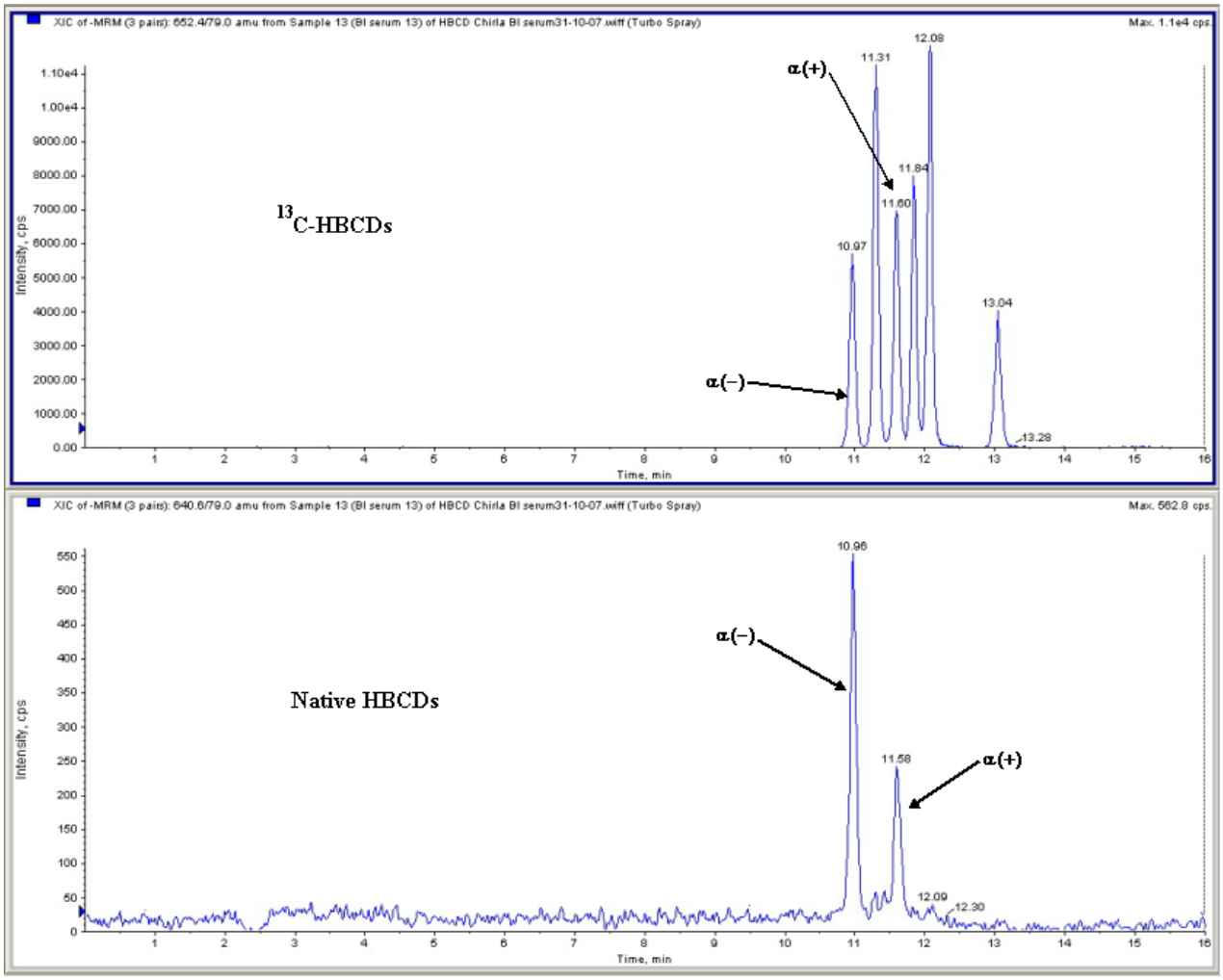
Supplemental Material, Figure 1. Chiral LC/MS/MS chromatogram showing separation of enantiomers in d_{18}-, ^{13}C- and native HBCDs standard solutions (500 pg/µl in methanol).



Supplemental Material, Figure 2: Chiral LC/MS/MS chromatogram of a diet sample.



Supplemental Material, Figure 3: Chiral LC/MS/MS chromatogram of a dust sample.



Supplemental Material, Figure 4: Chiral LC/MS/MS chromatogram of a serum sample.