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Detection of Hexabromocyclododecane and Its Metabolite Pentabromocyclododecene in Chicken Egg and Fish from the Official Food Control

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During routine gas chromatography with electron capture detection (GC/ECD) analysis of chicken eggs, we observed that the most prominent peak in some samples did not match the retention time of any of the food contaminants screened. Subsequent GC coupled with mass spectrometry (GC/MS) studies clarified that the mass spectrum of the peak was very similar to hexabromocyclododecane (HBCD), which was also identified by GC/MS in the egg. The unknown compound was positively identified as pentabromocyclododecene (PBCDE), a metabolite of HBCD detected for the first time in foodstuffs. Studies of the analytical method used for the analysis of pesticides and contaminants showed that this cleanup method was suitable for the determination of HBCD and PBCDE, but storage of sample extracts resulted in the loss of HBCD when the sample extracts were not sufficiently purified. The concentrations of HBCD and PBCDE in the high polluted sample were 2.0 and 3.6 mg/kg egg fat. HBCD and PBCDE were also detected in two additional eggs at lower levels (<0.15 mg/kg), whereas 75 eggs did not contain these compounds (<0.02 mg/kg). We also detected HBCD and PBCDE in two samples of whitefish (*Coregonus* sp.), while an eel sample (*Anguilla anguilla*) positively tested for HBCD did not contain PBCDE. Surprisingly, the potential metabolite of HBCD, PBCDE, has not been detected before in any food or environmental sample. The present results indicate that more attention should be paid to the detection of HBCD and its metabolite PBCDE in chicken eggs.

KEYWORDS: Polybrominated flame retardants; hexabromocyclododecane; pentabromocyclododecene; chicken eggs; fish fillet

INTRODUCTION

In Germany alone, the annual economic costs due to fire accidents amount to ∼6 billion Euros (*1*). Hence, fire prevention is an important task in human society. A key compound class used in this field are brominated flame retardants (BFRs). BFR is a summarizing term for ∼75 different compounds or mixtures, which represent ∼20% of all compounds used in fire prevention (*2*). The worldwide and European demand for BFRs is about 200000 and 30000 t/a, respectively (*3*). Eight (worldwide) or 30% (in Europe) of these application rates arise from technical hexabromocyclododecane (HBCD; **Figure 1**) (*3*). Restrictions for polybrominated diphenyl ethers (PBDEs), particularly in Europe, appear to be compensated by the increased use of technical HBCD. The production of technical HBCD is based on the addition of bromine to 1-*cis*,5-*trans*,9-*trans*-cyclododecatriene, which yields a mixture of α- (∼6%), *β*- (∼8%), and *γ*-HBCD (∼80%) (*4*). The isomers are labeled in the order of their liquid chromatography (LC) elution (*5*). HBCD is used in diverse applications, which include admixtures in polystyrene, upholstery textiles, and to a lesser degree in electronic equipment (*6*).

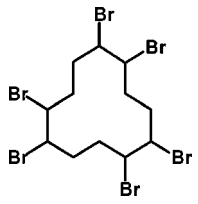

Figure 1. General structure of HBCD. Diastereomers result from *exo*- and *endo*-orientation of the bromine substitutents, which are generally in *anti*-orientation.

The white crystalline powder with an elemental composition of $C_{12}H_{18}Br_6$ has a melting point of 195 °C, a degree of bromination of 74.7%, and a molecular weight of 641.7 g/mol. Different from other organobromine compounds (e.g., PBDEs and polybrominated biphenyls), the solubility of technical

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HBCD is low in both water and pentane (0.01 wt % at 25 °C; *7*) but relatively high in other slightly polar organic solvents. HBCD has been detected in sewage sludge and river sediments but also in humans and food (*6*). In a recent report, composite egg samples from 17 countries were analyzed and HBCD concentrations ranged from <3 to 91 μg/kg egg fat (*8*).

LC/tandem mass spectrometry (MS/MS) is the method of choice for the individual quantitation of HBCD diastereomers that cannot be obtained by gas chromatography/mass spectrometry (GC/MS) (*6*). The fact that α-, β-, and γ-HBCD elute from GC columns as one peak is—at least in part—due to the isomerization of HBCD at elevated temperatures (>160 °C) (*9*, *10*). Furthermore, HBCD isomers are prone to decomposition in hot, dirty GC split/splitless injectors (*11*). Therefore, GC analysis will not provide information on the enantiomeric distribution of HBCDs, which can be achieved as well by using LC/MS/MS (*10*, *12*).

Despite these disadvantages, GC has its merits as well in the field of HBCD determination. Different to technical HBCD, PBDEs and many other BFRs cannot be analyzed by LC/MS since these BFRs are not sufficiently ionized. Thus, a thorough, isomer-specific analysis of BFRs requires both GC and LC, which is costly and time-consuming. On the other hand, the highly selective and sensitive LC/MS/MS methods in use for HBCD isomers do not permit the identification of other contaminants in the sample, even if they are more abundant than HBCD. Such nontarget screening for BFRs by GC in combination with electron capture negative ion mass spectrometry or GC/electron capture detection (ECD) can be used to identify samples with relevant residues of technical HBCD and related contaminants. In this study, we report on such a case. During routine analysis in food control, an abundant peak was detected in the GC/ECD chromatograms of purified chicken egg extracts. GC/MS verification brought our attention to HBCD and a potential metabolite.

MATERIALS AND METHODS

Chemicals and Samples. Standards of technical HBCD and isodrin were from Dr. Ehrenstorfer (Augsburg, Germany). A solution of α-HBCD was kindly obtained from M. Schlabach (Norwegian Institute of Air Research, Kjeller/Norway). Pentabromocyclododecene (PBCDE) described in this study was not commercially available. The identity of PBCDE was derived from GC/MS analysis (see below). Solvents were of "residue analysis grade" (LGC Promochem, Wesel, Germany). Chicken eggs and fish samples were taken as part of the official food control in Bavaria (Germany) from local retailers and wholesalers.

Sample Cleanup. Twenty grams of homogenized chicken egg (mixed pool of 12 individual eggs) or fish fillets was ground with sodium sulfate and sea sand (*13*). The dry and homogeneous material was subjected to column extraction of lipids and lipophilic substances using 300 mL of *n*-hexane/acetone (2:1, v/v) according to Ernst et al. (*14*). A 0.5 g amount of the resulting fat and 50 ng of isodrin (internal standard, IS) were dissolved in 5 mL of ethyl acetate/cyclohexane (1:1, v:v). Sample cleanup was performed according to the official German procedure, developed for the determination of pesticides and PCBs in fatty animal food (*15*). In brief, after gel-permeation chromatography (GPC) with bio-beads S-X3 and elution with ethyl acetate/cyclohexane (1:1, v:v), the solvent was concentrated to 4 mL (extract A). For further purification, ethyl acetate/cyclohexane was exchanged by isooctane, concentrated to 1 mL, and fractionated on 1 g of silica, deactivated with 1.5% water (w:v). Elution with 8 mL of *n*-hexane provided a solution with mainly aromatic organohalogen compounds, such as HCB, DDE, and PCBs. Subsequent elution with 8 mL of *n*-hexane/toluene (63:35, v:v) quantitatively yielded chloropesticides as well as technical HBCD and PBCDE. For determination of HBCD and PBCDE, the solvent of the latter fraction was changed to ethyl acetate/cyclohexane (1:1,v:v). The final volume was adjusted to 4 mL (extract B). Either extract A or extract B was analyzed by GC/ECD.

Gas Chromatographic Parameters. Quantitative analyses were performed with a Hewlett-Packard 6890 GC/ECD system using parameters recently described in detail (*13*). In brief, samples (1 μL) were splitless injected at 285 °C. He (constant pressure of 1.5 bar) and 40 mL/min Ar/CH_4 (90/10) were used as carrier and makeup gases, respectively. Capillary columns (30 m length, 0.25 mm internal diameter) coated with DB-5 or DB-1 (Agilent J&W Scientific) were used in parallel mode (*13*). The limit of detection (LOD) in samples was about 0.02 mg/kg egg fat for technical HBCD and PBCDE (based on the ECD response of technical HBCD). The LOD can be easily improved by reducing the final volume of the sample extract (4 mL, see above) or by a higher injection volume. GC/EI-MS analyses were carried out with a Hewlett-Packard 6890/5973 instrument using a HP5-MS capillary column and parameters reported previously except for a slightly higher column head pressure of 1.4 bar (*16*). In the full scan mode, *m*/*z* 40 to *m*/*z* 650 was recorded.

Quality Control. To demonstrate that PBCDE was not primarily a degradation product formed due to inappropriate sample cleanup or GC parameters, we performed the following experiments. First, technical HBCD (which is dominated by γ-HBCD) was spiked into an aliquot of an egg previously found to be free of HBCD. The spiked sample was purified as shown above, and the recovery of the spiked amount of HBCD was >90%. In a second experiment, technical HBCD was added to a HBCD-free egg extract (extract A and extract B). Because biological samples mainly contain α-HBCD, we repeated the later spiking experiment (extract A and extract B) with this isomer.

RESULTS AND DISCUSSION

Mass Spectrometry of HBCD and PBCDE. During routine examination under the Bavarian official food control, an abundant signal subsequently labeled PBCDE was determined in a chicken egg sample (chicken egg 1) whose retention time did not agree with any standard analyzed in routine (**Figure 2a**). When further purification steps (group separation of organohalogen compounds and separation of sterols and other nonsaponifiable lipid components, see Materials and Methods) were carried out, PBCDE was determined in the fraction of organobromine and nonaromatic organochlorine compounds (**Figure 2b**). PBCDE had previously not been detected in other samples at comparable relevance so that a deeper investigation was justified. The follow-up GC/EI-MS determination indicated five bromine substituents on PBCDE (**Figure 3**). The characteristic fragment ions of PBCDE were very similar with the GC/EI-MS data of technical HBCD as shown in the NIST library as implemented in our GC/MS software. Remberger et al. (*17*) also presented an EI-MS spectrum of technical HBCD slightly different to the NIST spectrum, which indicated variations from instrument to instrument and probably also from condition to condition. For instance, in their paper, *m*/*z* 159 was more abundant than *m*/*z* 157, which is different to the spectrum in the NIST library. Furthermore, the isotope pattern of the fragment ion at *m*/*z* 237 is also different in both literature sources. Because the mass spectrum of PBCDE was more similar to the NIST data of technical HBCD, we based the following comparison on this spectrum. However, the retention time of PBCDE was similar to PCB 170 and slightly shorter than that of 2,7-dibromo-4a-bromomethyl-1,1-dimethyl-2,3,4,-4a,9,9a-hexahydro-1H-xanthene (TriBHD) on both columns (DB-5 and DB-1), whereas HBCD was eluting much later in the retention time range of 2,5,7-tribromo-4a-bromomethyl-1,1-dimethyl-2,3,4,4a,9,9a-hexahydro-1H-xanthene (TetraBHD) (*13*, *18*). Thus, PBCDE was not identical with HBCD, which was proven by injection of an authentic technical HBCD standard (**Figure 2c**). These GC/MS measurements confirmed that HBCD

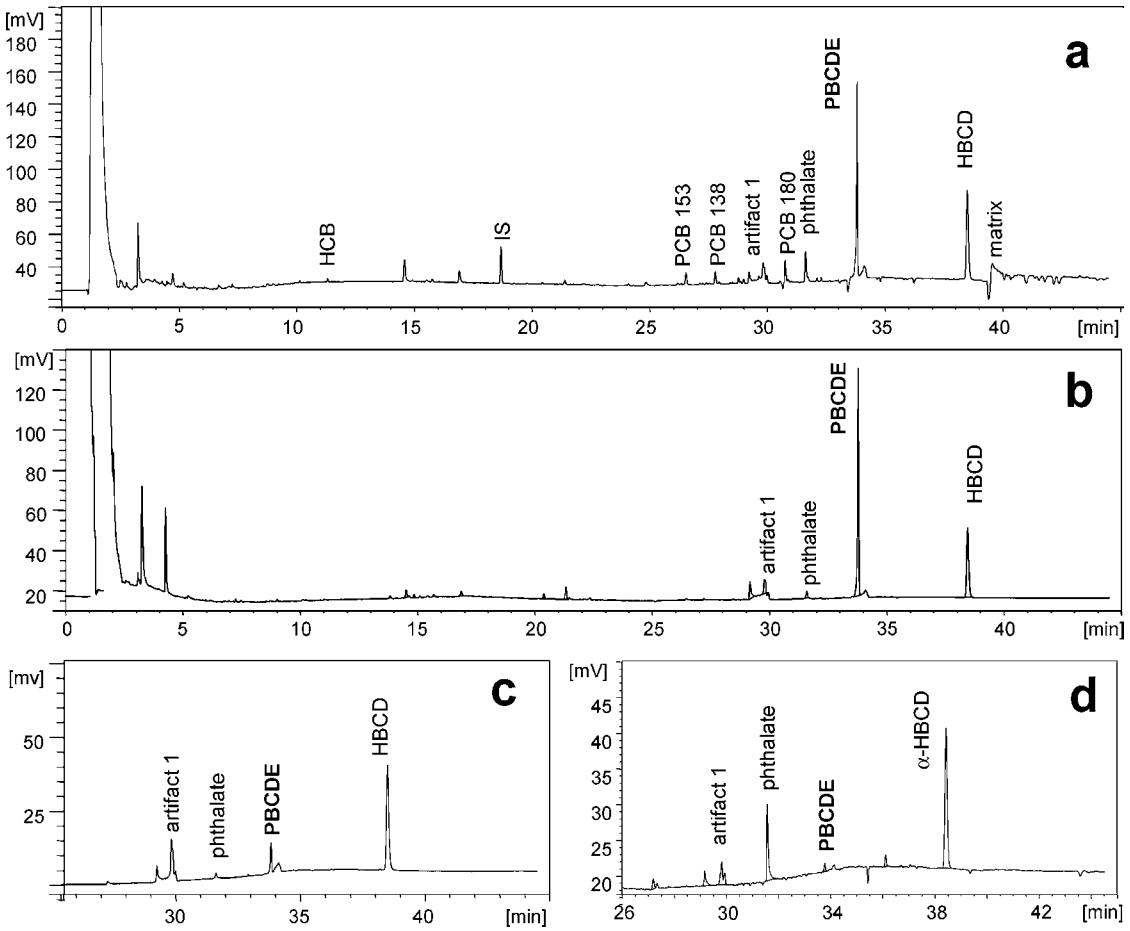

Figure 2. GC/ECD chromatograms of samples related to the detection of HBCD and PBCDE in chicken egg as determined on the DB1 column using splitless injection at 285 °C. (**a**) Full egg extract (extract A), (**b**) fraction obtained after additional cleanup (extract B), (**c**) technical HBCD standard (without matrix), and (**d**) α-HBCD isomer spiked into a chicken egg free of HBCD contamination. Artifact 1 is a thermal decomposition product of HBCD, and phthalate (DEHP) is an artifact from sample cleanup. HCB, PCB congeners, and IS (extract A) are separated during an additional cleanup step that leads to extract B.

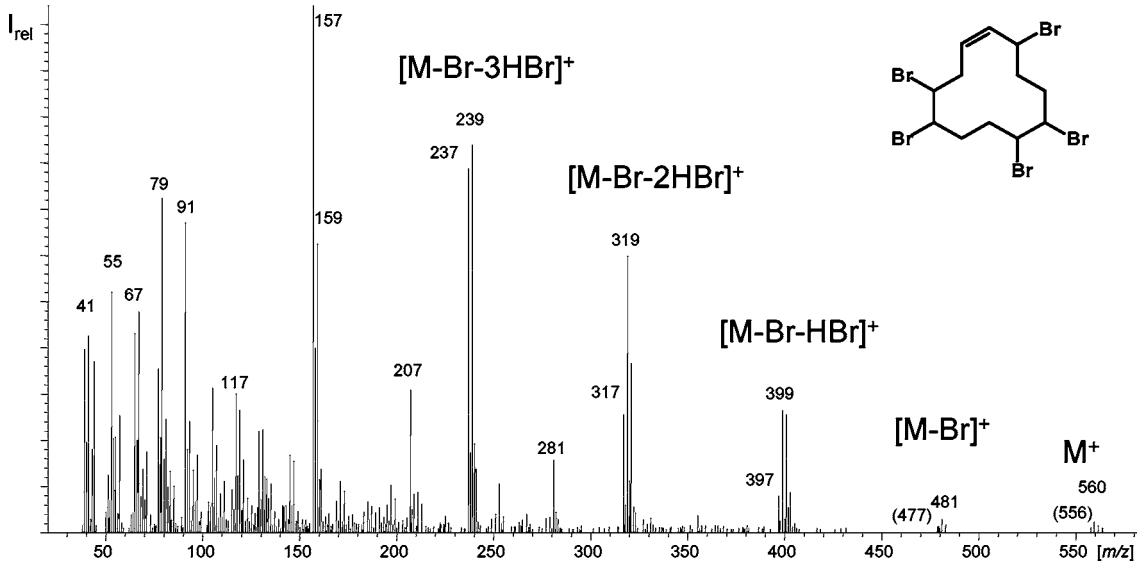

Figure 3. GC/EI-MS of PBCDE with one possible general structure inserted. Masses in parentheses are the assigned monoisotopic peak, which was not detected because of low isotope abundance.

was also present in chicken egg 1 (**Figure 2**). Still, the similarity of the spectra of technical HBCD and PBCDE was remarkable. For instance, the base peak at *m*/*z* 157 was also highly abundant in technical HBCD (NIST library). The low-mass fragment ions at *m*/*z* 41, 53, 65/67, 79, 91, 105, and 117 deepened the assumption of an aliphatic backbone of PBCDE. All fragment ions mentioned are also present in the GC/EI-MS of technical HBCD albeit at different relative abundances. At higher mass, the fragment ions at *m*/*z* 317 (two Br) and *m*/*z* 397 (three Br) are also in agreement with technical HBCD. At low intensity,

we also detected the molecular ion at *m*/*z* 556 (dominated by *m*/*z* 560) and the $[M - Br]^+$ fragment ion at *m*/*z* 477 (dominated by *m*/*z* 481). Note that the $[M - Br]^+$ fragment of technical HBCD is at *m*/*z* 557 (dominated with *m*/*z* 561 and 563) and hence shifted by 1 u to higher mass as compared to M^+ of PBCDE (data not shown). Apart from the molecular ion and the M^+ of PBCDE and $[M - Br]^+$ of HBCD, both spectra were virtually identical within the frame of variations from instrument to instrument. This feature and the additional identification of HBCD in the sample (**Figure 2**) fully support the interpretation of the compound under investigation as PBCDE (**Figure 3**). GC/MS does not allow the clarification if the detected peak represents one or a mixture of more than one PBCDE isomer.

Surprisingly, PBCDE(s) have only been reported on two occasions in the scientific literature. Barontini et al. investigated the thermal stability of technical HBCD and found 174 decomposition products (*19*). A PBCDE isomer was found to be a key intermediate of this process (*19*). The PBCDE was formed by the elimination of HBr, and the resulting double bond was adjacent to the initial Br−Br carbons (**Figure 3**, inserted structure) (*19*). This fact and the observation that, albeit to a minor degree, PBCDE was also detected during the GC analysis of the technical HBCD standard (**Figure 2c**) strongly indicates that PBCDE is identical with the key transformation product described by Barontini et al. (*19*). Likewise, Gard et al. produced PBCDE from technical HBCD by treatment with potassium *tert*-butoxide, but the positions of the bromine substituents were not determined in their study (*20*). However, both papers support the positive identification of PBCDE in the chicken egg sample.

GC Determination of HBCD and PBCDE. As mentioned before, GC analysis of technical HBCD is not trivial, and this was also suspected to be valid for PBCDE. Therefore, we had to check both the sample cleanup and the GC determination of technical HBCD. Tomy et al. reported that technical HBCD standard was not stable in acetonitrile (*5*). Our own experiments clarified that storage of standards in *n*-hexane and isooctane resulted in loss of compound most likely by glass absorbance due to the low solubility of technical HBCD in these solvents. Switching to ethyl acetate/cyclohexane (i.e., the solvent mixture used during the sample clean up by GPC), however, provided the desired long-term stability of the technical HBCD standard (see Materials and Methods). Furthermore, a thorough investigation of the sample cleanup method clarified that HBCD was not lost at the end of the whole procedure used in this study.

GC/ECD analysis of technical HBCD resulted in surprisingly good results (**Figure 2c**) although a split/splitless injector was used at high temperature (see Materials and Methods). Under these conditions, some decomposition of technical HBCD was observed. The GC/ECD chromatograms of technical HBCD also contained PBCDE and another earlier eluting decomposition product (hereafter named artifact 1) of technical HBCD (**Figure 2c**). Artifact 1 was composed of a defuse peak pattern typically for GC breakdown products. In contrast, PBCDE eluted as a very sharp peak. The relative intensity of technical HBCD was ∼66% and thus represented the most abundant peak. Artifact 1 amounted to ∼20% and PBCDE amounted to ∼14% of the area of the technical HBCD peak. These ratios obtained for the technical HBCD standard were virtually constant for several concentrations of technical HBCD including that found in chicken egg 1. Lowering the injector temperature did not reduce the decomposition of technical HBCD. Thus, this standard method was maintained for all subsequent measurements. Because HBCD residues in bird eggs mostly originate from α-HBCD (typically >80%) (*6*), we performed additional experiments with this HBCD isomer. Analysis of an α-HBCD standard alone as well its addition to an egg extract free of HBCD and PBCDE led only to 3% PBCDE of the area of α-HBCD (**Figure 2d**). This also confirms that α-HBCD is the most stable HBCD isomer (*21*).

Table 1. Concentrations (mg/kg Lipids) of HBCD and PBCDE in Chicken Eggs and Fish from Bavaria (Southern Germany)

species (farming type or origin)	lipid content (%)	HBCD[a]	PBCDE[a]
chicken egg 1 (free-range chicken eggs)	10.1	2.0	3.6
chicken egg 2 (free-range chicken eggs)	9.0	0.03	0.07
chicken egg 3 (deep litter eggs)	13.1	0.12	0.02
eel, *Anguilla anguilla* (river)	27	0.04	<0.02[b]
whitefish, *Coregonus* sp. 1 (lake)	5.3	0.07	0.03
whitefish, *Coregonus* sp. 1 (lake)	5.1	0.06	0.03

[a] Determined with the GC/ECD response of a technical HBCD standard. [b] LOD.

The performance on DB-5 and DB-1 columns was similar, but the more nonpolar DB-1 column provided sharper signals for technical HBCD and performed a better separation from egg−matrix peaks (e.g., sterols). Both technical HBCD and TetraBHD (see above) gave almost identical response factors in the ECD, and this was also assumed to be valid for PBCDE. Note, however, that the GC/ECD response of PCB 180 was ∼seven-fold higher than the response of technical HBCD (and the proposed for PBCDE). In the investigated egg sample, the response of technical HBCD was lower than PBCDE whereas the ratio of artifact 1 to HBCD was identical in technical HBCD standard and sample (**Figure 2a**,**c**). Comparison of peak abundances in technical HBCD standard and egg sample enabled us to estimate that <15% of PBCDE originates from the decomposition of HBCD and ∼85% originates from PBCDE as present as contaminant in the egg sample. Noteworthy as well, the GC stability of PBCDE was much better than that of technical HBCD. This also underlines that the abundant peak of PBCDE in the chicken egg sample was not (exclusively) produced in the GC system but an original contaminant accumulated or formed in the egg.

Concentrations of HBCD and PBCDE in Foodstuff. On the basis of identical GC/ECD responses for technical HBCD (standard available) and PBCDE (no standard available), chicken egg 1 was contaminated with 2.0 mg HBCD and 3.6 mg PBCDE per kg egg fat (**Table 1**). HBCD has previously been detected in chicken eggs as well as in eggs of wild birds, but none of these papers reported on the co-occurrence of PBCDE in bird eggs (*8*, *22*−*28*). Remarkably as well, the HBCD concentration in our sample was 25-fold higher than the highest reported to date in chicken eggs (*8*). According to European law, the origin of eggs must be labeled on the shell. Hence, tracing back chicken egg 1 to a small producer of eggs from free-range chicken was possible. The chicken had (nonintended) contact with different materials, and picking on these was most likely the reason for the uptake and presence of HBCD and PBCDE in the eggs.

Surprisingly, reanalysis of egg extracts (extract A) stored at both 4 °C or room temperature over several weeks resulted in a very low abundant peak of HBCD as compared to the initial result, whereas PBCDE remained unchanged. This effect was not found for the technical HBCD standard solution. However, subsequent repurification of aliquots of frozen chicken egg 1 resulted in the same concentrations of PBCDE and HBCD as

reported above. This indicated that HBCD was most selectively degraded or otherwise lost from the solution during the storage time. Interestingly, this effect was only found in extract A but not in extract B. The difference in both samples is the low but significant amount of sample matrix residues. Because this problem also occurs under LC/MS analysis, sample extracts to be inspected for the presence of HBCD should be analyzed without delay or a particularly good sample cleanup is required.

It must be stressed that only three out of 78 samples of pooled chicken egg examined in 2006 contained HBCD and PBCDE. The concentrations of HBCD and PBCDE in the additional positive chicken eggs 2 and 3 were much lower (<0.15 mg/kg egg fat, **Table 1**). However, the concentration of HBCD in these chicken eggs was still higher than the highest concentration determined in a screening of chicken eggs collected close to dumpsites from 17 countries (*8*). Under the Bavarian official food control, hundreds of samples of various meat and cow milk have been analyzed for pesticides and PCBs with the described method, but HBCD and PBCDE have not been detected by GC/ECD except for three samples of local fish (**Table 1**). Note that PBCDE was only detected in whitefish but not in eel (**Table 1**).

Evaluation of the HBCD and PBCDE Concentrations in the Chicken Eggs. The daily intake of HBCD via food in Sweden was recently estimated to be <3 ng (kg body weight)$^{-1}$ days^{-1} (*29*). For a 75 kg test person, the food-based intake of HBCD would thus be <225 ng/day (*29*). An average chicken egg weighs ~60 g and has a fat content of ~11% (*30*). Thus, an average chicken egg contains ~6.6 g of fat. Hence, the high-polluted chicken egg 1 accumulated ~13.2 μg of HBCD or ~37 μg of sum(HBCD + PBCDE). This is ~60-fold of the average daily HBCD intake calculated by Lind et al. (*29*). Although such heavy HBCD contamination of eggs appears to be scarce, our data indicate that single events may have a great impact on the uptake of HBCD via foodstuffs. Thus, a more thorough control of the HBCD contamination of chicken eggs and egg products in routine analysis is recommended in favor of consumers protection.

Along with this result, the determination of PBCDE in chicken egg represents the first data on this compound in food or environmental samples. This may be explained with the high-selective LC/MSMS and GC/MS methods currently used for the determination of HBCD, which give no response for PBCDE. Thus, we assume that in our particular case, the nontarget analysis including screening by GC/ECD, which detects all compounds with affinity to electrons, was suitable to detect PBCDE. It should however be noted that it remained unclear if PBCDE was formed from HBCD by the hen or in the egg or if it was already present in the habitat and only taken up by the hen as can be predicted for HBCD. Because HBCD and PBCDE were only detected in <4% of the chicken eggs, it is unlikely that uptake of HBCD (and PBCDE) occurred from commercial chicken feed.

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