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Food Chemistry 374 (2022) 131504

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Identification by volatolomics of hydrocarbon, oxygenated, sulfur and aromatic markers of livestock exposure to α-hexabromocyclododecane

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ARTICLE INFO

Keywords:
Volatolomics
α-HBCDD
Headspace-SPME
GC–MS
Livestock
Food safety

ABSTRACT

Volatile organic compounds (VOC)-based metabolomics, or volatolomics, was investigated for revealing livestock exposure to chemical contamination. Three farm animals, namely laying hens, broilers, and pigs, were experimentally exposed to 5 or 50 ng α-HBCDD g^{-1} feed. Liver and egg yolk for hens were analysed by headspace-SPME-GC–MS to reveal candidate markers of the livestock exposure to α-HBCDD. For hens, 2-butanol was found as marker in egg. In liver, twelve VOCs were highlighted as markers, with three aromatic VOCs – styrene, *o*-xylene, α-methylstyrene – highlighted for the two α-HBCDD doses. For broilers, six markers were revealed, with interestingly, styrene and phenol which were also found as markers in hen liver. For pigs, ten markers were revealed and the seven tentatively identified markers were oxygenated and sulfur VOCs. The candidate markers tentatively identified were discussed in light of previous volatolomics data, in particular from a γ-HBCDD exposure of laying hens.

1. Introduction

Hexabromocyclododecane (HBCDD) is a brominated flame retardant that has been a targeted substance in the Stockholm Convention since May 2013. HBCDD is classified as a substance of very high concern (SVHC) on the REACH candidate list due to its persistent bioaccumulative and toxic (PBT) properties. For non-occupationally exposed persons, dietary exposure is a major route of total HBCDD intake (Covaci et al., 2006). Food contaminations by HBCDD are especially problematic because of sometimes high levels, so that the ingestion of a single contaminated animal-derived food can significantly increase consumer exposure to it (Ratel et al., 2017). Based on direct HBCDD detection in food, the current analytical methods for the surveillance of HBCDD contamination are efficient and sensitive but are hindered by cumbersome implementation related in particular to the ubiquitous occurrence of HBCDD. These routine monitoring techniques thus do not allow rapid cost-effective large-scale methods, which seem essential for strengthening food safety with respect to this contaminant (Meurillon et al., 2018).

To overcome these limitations, alternative approaches based on omics have emerged to detect contaminations. They are inspired by research showing the usefulness of the rapid cost-effective analysis of expired volatile organic compound (VOC) markers in clinical diagnosis (Hakim et al., 2012). Berge et al. (2011) proved the concept that the signature of the liver metabolome in small compounds, the volatolome, was modified for chickens in response to exposure in their diet to different xenobiotics including brominated flame retardants such as polybrominated diphenyl ethers. Liver is a key target for the determination of markers of exposure to contaminants because of its major function in protecting the organism from potentially toxic chemical insults. Bouhlel et al. took this concept and identified some VOCs that were impacted in the liver volatolomes of chickens exposed to three types of

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https://doi.org/10.1016/j.foodchem.2021.131504
Received 2 March 2021; Received in revised form 20 October 2021; Accepted 27 October 2021
Available online 30 October 2021

micropollutants – a pesticide, an environmental contaminant and a coccidiostatic agent (Bouhlel et al., 2018). As HBCDD impacts the metabolism of exposed organisms with clear indications of toxicological effects in the liver (Cantón et al., 2008; Germer et al., 2006), liver volatolomics could be useful for identifying markers of HBCDD exposure and back-tracing HBCDD food contamination.

Ratel et al. used volatolomics to reveal exposure of laying hens to γ-HBCDD (Ratel et al., 2017) and reported a list of VOCs in livers impacted by γ-HBCDD exposure. The study of Ratel et al. (2017) showed that at least four additional issues needed to be addressed: (i) this first study performed on HBCDD focused on the γ-isomer, whereas the α-HBCDD isomer is probably more relevant because it predominates in the environment, in animal tissues and in animal-derived food (Marvin et al., 2011; Rivière et al., 2014; Koch et al., 2015; Dominguez-Romero et al., 2016), (ii) this first list of VOC candidate markers needed filtering to screen for the most robust one, (iii) the proof of concept had to be confirmed on more realistic HBCDD levels of feed contamination, and (iv) the scope had to be extended to other farm animals.

Based on control/test experiments, the present study aimed first to confirm the utility demonstrated by Ratel et al. (2017) of liver volatolomics in demonstrating the exposure of laying hens to HBCDD, by considering the α-HBCDD isomer. In addition, realistic exposure levels of laying hens were implemented and the potential of egg volatolomics was explored. Egg is widely consumed and the literature has reported high HBCDD levels in chicken eggs, sometimes above the mg kg^{-1} LW level (Hiebl and Vetter, 2007; Dominguez-Romero et al., 2016). According to its lipid rate, egg yolk may contain many volatile compounds and it is therefore very relevant for considering the implementation of non-invasive volatolomics methods for food safety surveillance. Liver volatolomes were also investigated for two other major monogastric farm animals, namely broilers and pigs. The potential markers discovered are discussed especially in the light of the candidates found in the two main studies carried out from liver volatolomics of livestock exposed or not to micropollutants: the study of Ratel et al. (2017), based on the exposure of laying hens to γ-HBCDD, and that of Bouhlel et al. (2018), based on the exposure of broilers to different micropollutants.

2. Materials and methods

2.1. Animal feeding

Compositions of experimental feeds for the three farm animals were detailed in Table S1. Contaminated diets were obtained by replacing 5 g of clean soy oil in the control diet by 5 g of soy oil spiked at appropriate levels with α-HBCDD. The synthesis of α-HBCDD has been described by Dominguez-Romero et al. (2016). Briefly, technical HBCDD, containing 1, 5, and 93% of α-, β-, and γ-HBCDD, respectively, was enriched in α-isomer by thermal rearrangement (172 ± 0.4 °C, 6 h), according to a method adapted from that described by Szabo et al. (2011). Purification was performed on neutral silica gel and magnesium silicate manually packed solid phase extraction (SPE) columns and also by preferential precipitation at −20 °C, using dichloromethane and *n*-hexane. The purity of resulting crystals has been estimated at 99.3% α-HBCDD, η-HBCDD being the only identified impurity. Crystals of α-HBCDD were dissolved in acetone used to spike soy oil at the targeted concentration. All spiked feeds were prepared at the same time in the INRAE PEAT feed mixing unit (Nouzilly, France) by using dilutions of a single 50 μg g^{-1} contaminated oil prepared by LABERCA (Nantes, France). However, for pigs, the volume of contaminated feeds needed for all the experiment was too high to be produced in the INRA PEAT facilities. To overcome this issue, the contaminated feeds for pigs have been concentrated 10 times more than the expected level, then contaminated feed was brought each day of the experiment in the pig trough in a 1:10 ratio (w/w) mixed with uncontaminated feed.

For the three farm animals, a contaminated diet with the target concentration of 50 ng α-HBCDD g^{-1} was prepared. This concentration was expected to enable the animal-derived products to reach several hundreds of ng of HBCDD g^{-1} LW, as previously reported in heavily contaminated samples shown by French monitoring plans (Jondreville et al., 2017). For laying hens and pigs, a second lower dose of 5 ng g^{-1} feed was tested to obtain animal-derived products with more realistic α-HBCDD contamination levels. For broilers, the high occupancy of the INRAE facilities did not allow testing two exposure doses with individual animal cages.

2.2. Animal testing

Animal experiments have been designed to study the exposure to α-HBCDD in feed of laying hens, slow-growing broiler chickens and growing pigs. These experiments were conducted in compliance with Directive 2010/63/EU in France and approved by the relevant ethics committee. Animal experiments were conducted in appropriate facilities with cages allowing feed ingestion of individual animals to be monitored. Laying hens, broilers and pigs were fed with non-contaminated feeds (control groups) or with α-HBCDD contaminated feeds (exposed groups) for 18, 12 and 16 weeks, respectively. The main features of the three experiments conducted in laying hens, broilers and pigs are summarized in Table 1, with the detailed conditions of each animal experiment which are given in Table S2. Information about body weight and feed ingested of animals during animal testing is given in Table S3.

2.3. Slaughtering and sampling

At the end of the exposure period, all animals were sacrificed and the weight of each warm carcass was recorded at slaughter (Table S3). Poultry and pigs were killed after a 12-hour and a 18-hour fast, respectively, by electronarcosis followed by exsanguination (electric stunner for poultry, Ducatillon, Cysoing, France; Morphee M4 electric stunner for pigs, Lelong & Cie, Savigny, France). "Control samples" and "exposed samples" correspond to the samples collected from control and exposed animal carcasses, respectively. For eggs, the eggs laid before slaughter and on the day of slaughter were collected. The egg laid before slaughter was dedicated to HBCDD analysis. It was weighed and removed from its shell before separating and storing at −20 °C the white and yolk. The egg laid on the day of slaughter was dedicated to volatolomics analysis. It was collected, immersed in liquid nitrogen and stored at − 80 °C. For fat and muscle samples dedicated to HBCDD quantification, abdominal fat and muscles from one thigh (without skin and bone) for poultry and samples of back fat and semi-membranosus muscle for pigs were collected, weighed and stored at −20 °C. For liver samples dedicated to HBCDD quantification and volatolomics, the whole liver for poultry and a sample prepared from the left lateral and right medial lobes for pigs were collected, weighed, immersed in liquid nitrogen, wrapped in aluminum foil, vacuum-packed, and stored at −80 °C. No visible sign of pathology was detected during the autopsy carried out during the cutting of the post-slaughter animals. Data about weight and lipid content of collected samples are given in Table S3.

2.4. HBCDD quantification

HBCDD isomers (α, β and γ) were analysed in feed and animal

Table 1
Summary of the experiments conducted in laying hens, broilers and pigs.

Livestock animals	Laying hens			Broilers		Pigs		
Strain	Novo Brown			JA657		(LW × Ld) × Piétrain		
Initial age (day)	210			1		70		
Duration of exposure (week)	18			12		16		
Target dose of α-HBCDD (ng g^{-1} feed)	50	5	0	50	0	50	5	0
Number of animals	3	3	2	5	4	3	3	3

samples according to a method covered by the scope of the ISO/IEC 17025:2005 accreditation of the LABERCA laboratory, and described by Dominguez-Romero et al. (2016). Briefly, all glassware and Na2SO4 were baked prior to use at 400 °C for 4 h or at 650 °C for 6 h, respectively. Feed was dried in an oven at 80 °C. Other matrices, animal tissues and egg yolk, were lyophilized. The sample size ranges used for HBCDD quantification was given for each matrix in Table S4. Lipids were extracted by pressurized liquid extraction (PLE) over three successive static cycles (100 bar, 120 °C) (SpeedExtractor, Büchi, Switzerland) with a toluene/acetone mixture (70:30, v/v), evaporated to dryness, and weighed. Purification steps were conducted on a SPE column manually packed with Na2SO4, neutral, and acidic (H2SO4) silica gel using hexane and dichloromethane, followed by partitioning between *n*-hexane and 1 N NaOH. Final extracts were reconstituted into a mixture of methanol/water 80:20 (v/v). HBCDD isomers were analyzed by LC-ESI (−)-MS/MS (6410, Agilent Technologies). Separation was achieved on a Hypersil Gold column (100 mm × 2.1 mm, 1.9 μm) (Thermo Scientific, San Jose, CA, USA) fitted to a 1260 series HPLC pump. The mobile phase was constituted of 20 mM ammonium acetate (A) and a mixture of acetonitrile/methanol 1:1 (v/v) (B) in isocratic conditions (A/B 30:70, v/v). The transitions monitored through the triple-quadrupole mass filter corresponded to $[M - H]^- \rightarrow [Br]^-$. Each analytical series comprised a procedural blank and a quality control sample. Quantification was achieved according to the isotopic dilution principle (13C-labeled isomers as internal standards and 2H18-β-HCBDD as external standard). Limits of quantification (LOQ) were determined individually for each sample and isomer, based on a signal-to-noise ratio of 3. A limit of reporting (LoR) higher than each LOQ was established by matrix, species and isomer (considering procedural contamination as well), as presented in Table 2.

2.5. Volatolome analysis

All of each egg yolk and liver were ground in liquid nitrogen for 1 and 3 min, respectively, into a fine homogeneous powder using a homemade stainless steel ball mill. A 1.2 g aliquot of powder was placed in a 20 mL glass vial (Supelco, Sigma-Aldrich, St. Louis, MO), sealed under a nitrogen flow with magnetic caps with PTFE/silicone septa (Supelco, Sigma-Aldrich), and stored at −80 °C. The volatolome of powdered liver and egg yolk samples was analyzed by headspace-solid-phase microextraction (HS-SPME) coupled to gas chromatography - mass spectrometry (GC–MS) according to Ratel et al. (2017). Briefly, the following steps were carried out with an automated sampler (AOC-5000 Shimadzu, Japan): (i) preheating of the sample for 10 min in the agitator (500 rpm), (ii) SPME trapping (75 μm carboxen/polydimethylsiloxane, 23-gauge needle, Supelco) of the volatile organic compounds (VOC) for 30 min. For the liver samples, the extraction temperature was set at 40 °C, as recommended by Bouhlel et al. (2017) for better extraction of liver VOCs with a narrower analytical variability and improved sensitivity. For egg yolk samples, this temperature had to be increased to 60 °C to boost extraction rates. After extraction, thermal desorption was performed at 250 °C for 2 min in the GC inlet. Further VOC analysis was performed by GC/MS-full scan (GC2010; QP2010+, Shimadzu). VOCs were injected in splitless mode into a DB-5MS capillary column (60 m × 0.32 mm × 1 μm; Agilent J&W) with helium as carrier gas at a flow rate of 1 mL min^{-1}. Oven temperature was held at 40 °C for 5 min, ramped to 230 °C at 3 °C min^{-1}, and held at 230 °C for 10 min. The temperature of the transfer line between GC and MS was set at 230 °C. Temperature was

Table 2
HBCDD concentrations determined in feeds and animal tissues. Values are means ± standard error. Levels of α- and γ-HBCDD measured in liver samples of control laying hens were not zero because of an accidental contamination during liver sample treatment (see paragraph 3.2. in Results section).

Samples	Laying hens			Broilers		Pigs		
	dose 50[a]	dose 5[a]	control[a]	dose 50[a]	control[a]	dose 50[a]	dose 5[a]	control[a]
Feed (ng g^{-1} fw)								
LoR [b]		0.04		0.03			0.04	
α-HBCDD	40	3.62	< LoR	38.0	< LoR	31.8	3.42	< LoR
β-HBCDD	< LoR	< LoR	< LoR	< LoR	< LoR	< LoR	< LoR	< LoR
γ-HBCDD	0.18	0.14	< LoR	< LoR	< LoR	< LoR	< LoR	< LoR
Liver (ng g^{-1} lw)								
LoR		0.4		0.2			0.08	
number of samples	3	3	2	5	4	3	3	3
α-HBCDD	142 ± 22*	11.0 ± 2.2*	2.2 ± 1.8	100 ± 16	0.42 ± 0.29	15.6 ± 3.2	1.71 ± 0.17	< LoR
β-HBCDD	< LoR	< LoR	< LoR	< LoR	< LoR	< LoR	< LoR	< LoR
γ-HBCDD	5.6 ± 4.1	2.6 ± 1.7	5.0 ± 3.3	< LoR	< LoR	< LoR	< LoR	< LoR
Egg (ng g^{-1} lw)								
LoR		0.1						
number of samples	3	3	2	5	4	3	3	3
α-HBCDD	242 ± 8.5	23.3 ± 1.5	< LoR	N/A	N/A	N/A	N/A	N/A
β-HBCDD	< LoR	< LoR	< LoR	N/A	N/A	N/A	N/A	N/A
γ-HBCDD	< LoR	< LoR	< LoR	N/A	N/A	N/A	N/A	N/A
Fat[c] (ng g^{-1} lw)								
LoR		0.1		0.2			0.1	
number of samples	3	3	2	5	4	3	3	3
α-HBCDD	302 ± 6.6	33.1 ± 6.3	0.26 ± 0.10	384 ± 82	0.46 ± 0.18	179 ± 21	14.1 ± 0.3	0.32 ± 0.19
β-HBCDD	< LoR	< LoR	< LoR	< LoR	< LoR	< LoR	< LoR	< LoR
γ-HBCDD	0.89 ± 0.42	0.46 ± 0.29	0.45 ± 0.29	< LoR	< LoR	< LoR	< LoR	< LoR
Muscle[d] (ng g^{-1} lw)								
LoR		0.2		0.3			0.1	
number of samples	3	3	2	5	4	3	3	3
α-HBCDD	378 ± 41	N/A	N/A	260 ± 63	0.88 ± 0.64	142 ± 15	13.0 ± 2.8	< LoR
β-HBCDD	< LoR	N/A	N/A	< LoR	< LoR	0.7 ± 0.5	< LoR	< LoR
γ-HBCDD	< LoR	N/A	N/A	< LoR	< LoR	< LoR	< LoR	< LoR

* Each α-HBCDD concentration in liver of exposed hens was corrected in removing the part of α-HBCDD brought by the polystyrene box used during liver grinding, according to Dominguez-Romero et al. (2016) and Jondreville et al. (2017).

[a] Diet contaminated with the target concentration of 5 or 50 ng α-HBCDD g^{-1}.

[b] Limit of Reporting.

[c] Abdominal fat for hens and broilers, back fat for pigs.

[d] Thigh muscle for hens and broilers, semi-membranosus muscle for pigs.

fixed at 180 °C in the MS source and 150 °C in the MS quadrupole. Electron impact energy was set at 70 eV, and data was collected in the range *m/z* 33–250 at 10 scans per second.

2.6. Data treatment

All calculations and statistical analyses applied to animal testing and HBCDD quantification data have been previously detailed (Dominguez-Romero et al., 2016; Jondreville et al., 2017). Peak areas of the VOCs were integrated from the SPME-GC–MS signals using a mass fragment selected for both being specific to the sought-after molecule and free of any co-elution with a home-made automatic algorithm developed in Bouhlel et al. (2017) under Matlab R2017 (The MathWorks, Natick, USA). VOCs were tentatively identified on the basis of both mass spectra, by comparison against the NIST 17 mass spectral library (version 2.3, build May 4, 2017), and retention indices (RI), by comparison with published RI values and those of our internal database. For calculation of the experimental RI, an alkane standard solution (Supelco, Sigma-Aldrich) was analyzed by SPME-GC–MS at the end of the analytical campaign. Data were processed using Statistica (version 12, StatSoft) and R (version 2.1.4., http://www.R-project.org) software. Student's *t*-test and one-way ANOVA were performed on the abundances of the VOCs monitored in volatolomes for the comparisons of control *vs*. one or two groups of α-HBCDD contaminated animals.

3. Results

3.1. HBCDD in feed

HBCDD concentrations determined in feed and animal tissues are summarized in Table 2. Control feeds used for the three animals tested were HBCDD-free according to limits of reporting. For contaminated feed intended to contain 50 ng α-HBCDD g^{-1} spiked feed, compliant concentrations of 40, 38 and 31.8 ng α-HBCDD g^{-1} feed were measured for laying hens, broilers and pigs, respectively. The concentration of α-HBCDD in contaminated feeds was 20–36% lower than expected. For the second lot of contaminated feed intended to contain 5 ng α-HBCDD g^{-1} spiked feed for laying hens and pigs, concentrations of 3.62 ng and 3.42 ng α-HBCDD g^{-1} feed were measured, respectively. The intended 1:10 ratios between the two α-HBCDD levels (5 and 50 ng α-HBCDD g^{-1}) in spiked feed were moderately well kept following the preparation of contaminated feed, with a 1:9.0 ratio for laying hens and 1:9.3 for pigs. The difference observed in feed between the expected and measured concentrations is probably related to the uncertainty in the weight of the produced crystals of α-HBCDD used for spiking oils. But no degradation product of α-HBCDD has been identified in feed and only α-HBCDD measured values were considered for further calculations. No β-HBCDD was detected in any spiked feed. Some γ-HBCDD was quantified at levels of 0.18 and 0.14 ng g^{-1} fw in the high- and low-level feeds, respectively, representing 0.45 and 3.9% of the total HBCDD.

3.2. HBCDD in animal samples

The results of HBCDD quantification in animal samples obtained at the end of the exposure period are presented in Table 2. The results of tissue distribution and transfer to eggs of ingested α-HBCDD have been detailed in laying hens and in broilers by Dominguez-Romero et al. (2016) and Jondreville et al. (2017), respectively. The exposed animals, at any level, performed as well as the control animals in terms of body weight, growth rate, feed efficiency, and laying rate, and the weight and lipid content of their tissues were not significantly different (Dominguez-Romero et al., 2016, Jondreville et al., 2017). While the HBCDD levels in ground liver samples from control laying hens should have been almost zero, traces of α- and γ-HBCDD isomers have been detected, with 2.2 ± 1.8 ng and 5.0 ± 3.3 ng g^{-1} lw for α- and γ-isomer, respectively (Table 2). The γ-HBCDD content was very similar to that from exposed hens (2.6 ± 1.7 ng and 5.6 ± 4.1 ng γ-HBCDD g^{-1} lw for the exposure "dose 5" and "dose 50", respectively; Table 2), which suggested a systematic contamination in all ground liver samples. The profile in HBCDD isomers determined in ground control liver samples was fairly constant. Dominated by the γ-isomer (29%, 5%, 66% of α-, β- and γ-HBCDD, respectively), it matched that of a technical mixture. HBCDD is known as a ubiquitous environmental contaminant commonly found in industrial and domestic polymeric materials. The analysis of the polystyrene box used during the liver grinding (Laberca analysis ID 14.1842.3) has revealed a similar profile in HBCDD isomers (26%, 14%, 60% of α-, β- and γ-HBCDD, respectively). HBCDD traces detected in ground control liver samples could thus be ascribed to the polystyrene box. The analyses of HBCDD quantification pointed out that the feeds given to control animals were α/β/γ-HBCDD-free and that the liver grinding step was the source of the contamination revealed in control ground liver samples. Accordingly, the volatolome of the liver from control hens can then be used to determine α-HBCDD-exposure markers by comparison with the volatolome of liver from exposed animals deliberately fed with α-HBCDD-contaminated feeds. In the experiments conducted on pigs and laying hens, the α-HBCDD concentration measured in tissues was proportional to the level of diet exposure of the pigs and laying hens.

3.3. Change in volatolomes in response to α-HBCDD exposure

To confirm the promising interest of liver volatolomics (Ratel et al., 2017) and to explore the potential of egg volatolomics for revealing livestock exposure to HBCDD, changes in volatolomes were investigated in SPME-GC–MS signals obtained from livers and eggs of control and α-HBCDD exposed animals. Analyses of liver volatolomes found 98, 105, and 134 VOCs for laying hens, broilers and pigs, respectively, and 51 VOCs were detected in egg yolk volatolomes (Table S5).

3.3.1. Laying hens

The results of liver volatolomics presented in Table 3 show that 12 VOCs were impacted in the livers of exposed laying hens. Fig. S1 presents the first map of PCA plotted on these candidate markers. The hepatic disturbance was thus visible in the volatolome even for realistic low doses of α-HBCDD. Levels of HBCDD in feed in our study (5 and 50 ng α-HBCDD g^{-1} feed) were reduced by a factor of 20 and 200 compared to the levels of HBCDD used in Ratel et al. (2017) (0.1 and 10 μg γ-HBCDD g^{-1} feed). Table 3 includes mainly hydrocarbon, oxygenated (alcohols, ketones, acids), aromatic and sulfur compounds. These results are consistent with the list of candidate markers published by Ratel et al. (2017) in response to γ-HBCDD exposure, the authors highlighting in the livers of laying hens mainly hydrocarbon compounds (alkanes and branched alkanes), oxygenated compounds (alcohols, aldehydes, ketones) and aromatic compounds. **Hydrocarbons.** Heptane (increased in exposed animals) and 2,2,4-trimethylpentane (decreased in exposed animals) were found as significant markers of α-HBCDD exposure. Several hydrocarbons were previously found as markers in the livers of laying hens after a γ-HBCDD exposure, with levels in these compounds also increased or decreased in exposed animals according to the compound considered (Ratel et al., 2017). The changes in the hydrocarbon content of livers in response to α-HBCDD exposure may result from an imbalance between their production, mainly due to unsaturated fatty acid peroxidation by reactive oxygen species (ROS), and their hydroxylation, by detoxifying enzymes resulting in the production of alcohols (Hakim et al., 2012). **Alcohols.** Table 3 includes 2-butanol. In response to the exposure to toxic xenobiotics, the level of alcohols in livers could result from an equilibrium between anabolism (e.g. hydroxylation of hydrocarbons) and catabolism (e.g. activation of CYP-450) reactions. Several alcohols were included in the list of Ratel et al. (2017), and primary and secondary alcohols have been proposed as candidate markers for VOC-based clinical diagnoses (Hakim et al., 2012). 2-Butanol can be thus considered as particularly useful for revealing exposure of laying hens to α-HBCDD. **Ketones.** Like for alcohols, these

Table 3
Laying hens - Volatile compounds in liver volatolome impacted by the exposure to a-HBCDD. Values are the mean of abundances ($\times 10^4$) of each candidate marker with its standard deviation (in brackets). Values in bold correspond to levels found higher in liver from "control" animals compared to "exposed animals".

Candidate markers	Tentatively identification[a]	m/z[b]	Exp. RI[c]	Ref. RI[d]	Liver			Egg yolk		
					control	*dose 50[e]*	*dose 5[e]*	*control*	*dose 50[e]*	*dose 5[e]*
Hydrocarbons										
2,2,4-trimethylpentane	MS+RI	57	688	687 [1]	5.7 (9.1%)		**1.4**** (24.7%)			
heptane	MS+RI	100	700	700	13.7 (26.9%)		21.8* (5.3%)			
Alcohols										
2-butanol	MS+RI	59	602	603 [1]	13.4 (32.8%)	23.7* (3.9%)		42.5 (22.0%)		82.8* (1.1%)
Ketones	MS+RI									
2,5-octanedione	MS+RI	99	985	985 [2]	2.0 (47.1%)		6.6* (26.1%)			
Acids										
acetic acid	MS+RI	60	575	602 [1]	512.8 (29.7%)	775.5* (2.6%)				
Aromatic compounds										
styrene	MS+RI	104	898	897 [1]	4.0 (11.2%)	**2.7*** (15.7%)	**2.6*** (16.4%)			
o-xylene	MS+RI	91	898	898 [3]	5.8 (1.6%)	**3.7*** (15.9%)	**3.5*** (26.6%)			
α-methylstyrene	MS+RI	117	994	994 [4]	1.1 (0.3%)	2.1* (19.6%)	4.1** (13.7%)			
Sulfur compounds										
sulfur dioxide	MS	64	<500		32.7 (7.2%)	**20.9*** (18.6%)				
dimethylsulfone	MS+RI	79	906	915 [1]	9.7 (6.1%)	38.3* (23.3%)				
Unknown										
unknown		54	1028		0.4 (31.3%)	1.7** (11.7%)	3.5* (26.2%)			
unknown		100	1141		0.3 (39.4%)		1.5** (4.7%)			

[a]MS + RI, mass spectrum and RI agree with literature data; MS, mass spectrum agrees with literature spectrum.
[b]ass fragment used for area determination.
[c,d]Retention indices on a DB5 capillary column from experimental run (c) or bibliographic data (d).
[1] Engel and Ratel, 2007; [2] Xie et al., 2008; [3] Vasta et al., 2007; [4] Čajka et al., 2007.
[e]Diet contaminated with the target concentration of 5 or 50 ng α-HBCDD g^{-1}
*$p<0.05$.
**$p<0.01$.

compounds are at the crossroads of cell metabolism elicited in response to exposure to toxic xenobiotics. The levels of 2,5-octanedione could be modified because of an impact of the toxic exposure on the lipid metabolism with a high oxidation rate of fatty acids or on the protein metabolism with amino acid metabolism-induced ketone formation (Hakim et al., 2012). **Sulfur compounds**. Two compounds (sulfur dioxide, dimethylsulfone) were identified as markers at the highest HBCDD dose. Among the candidate markers not classified according to their chemical structure ("others" class) by Ratel et al. (2017), we can note two sulfur-containing compounds (thiazole and thiadiazole). Bouhlel et al. (2018) also highlighted dimethylsulfone and carbon disulfide among the most important liver VOC contributors to the separation of control chickens and exposed chickens to micropollutants such as pesticides or polychlorobiphenyls. In the review of Schubert et al. (2004) on the medical diagnostic potential of endogenous VOCs, the authors report a possible relation between impairment of liver function, which could be initiated by exposure to α-HBCCD given its toxicological effects reported in the liver (Cantón et al., 2008; Germer et al., 2006), and level of sulfur-containing compounds. The generation of these compounds may be connected to an incomplete metabolism of methionine in the transamination pathway. **Aromatic compounds.** Table 3 shows 3 aromatic compounds derived from alkylbenzenes among candidate markers: styrene, o-xylene and α-methylstyrene. Alkylbenzenes were already highlighted by Ratel et al. (2017) when animals were exposed to γ-HBCDD. In their study, the levels of all alkylbenzene candidate markers were higher in exposed laying hens, like in our study. *o*-Xylene and α-methylstyrene were also among the VOCs identified by Bouhlel et al. (2017) as the major contributors to the separation of control and exposed chickens based on liver volatolomics. Although it is largely agreed that alkylbenzenes have an exogenous origin (Hakim et al., 2012), their level in the liver could be impacted by cellular and enzymatic defense mechanisms elicited to eliminate hazardous compounds such as α-HBCDD. It is of note that we found styrene, o-xylene, α-methylstyrene and one unknown VOC (RI 1028) as candidate markers in the livers of laying hens for both α-HBCDD doses. However, the abundance of these VOCs and the α-HBCDD concentration in liver were not significantly correlated according to the R-Pearson test (r Pearson indices between −0.13 and −0.46; p values between 0.26 and 0.75).

Regarding the egg volatolome, only 2-butanol was affected by the α-HBCDD exposure (Table 3). This VOC was also identified as a marker in the livers of laying hens exposed to the higher dose of α-HBCDD. 2-Butanol is a secondary alcohol in one of the VOC families reported as likely to be impacted by a chemical risk exposure (Ratel et al., 2017). Given the high utility of eggs in any non-invasive volatolomics-based control, it would be interesting to deepen our knowledge of the potential impact of liver disorders on egg composition. Saraswati et al. (2013) showed that liver functions modified yolk precursor synthesis and depositions in the developing follicles. It would also be of interest to improve egg volatolome analysis by implementing recent advances in sample preparation and volatile fraction collection (Majchrzak et al., 2018).

3.3.2. *Broilers*

The exposure to α-HBCDD generates a detectable metabolic disturbance in the liver volatolome of broilers. The first map of PCA plotted on the candidate markers allows the separation of case/control groups to be visualized (Fig. S1). With levels higher in control animals, the candidate markers listed in Table 4 are hydrocarbon (2-methylbutane) or aromatic (phenol, 2-phenoxyethanol, methoxybenzene and styrene) compounds. These chemical families were already highlighted in the liver volatolome of laying hens exposed to α-HBCDD (Table 3) and to γ-HBCDD (Ratel et al., 2017). Concerning aromatic compounds, styrene was already identified as affected in liver of laying hens for the two exposure

Table 4
Broilers - Volatile compounds in liver volatolome impacted by the exposure to a-HBCDD. Values are the mean of abundances ($\times 10^4$) of each candidate marker with its standard deviation (in brackets). Values in bold correspond to levels found higher in liver from "control" animals compared to "exposed animals".

	Tentatively identification[a]	m/z[b]	Exp. RI[c]	Ref. RI[d]	*control*	*dose 50*[e]
2-methylbutane	MS	72	508		63.9 (31.2%)	**28.2*** (45.1%)
styrene	MS+RI	104	898	897 [1]	13.7 (19.2%)	**8.9*** (32.2%)
methoxybenzene	MS+RI	108	922	918 [2]	9.3 (29.5%)	**3.2**** (21.0%)
phenol	MS+RI	94	976	983 [3]	92.4 (23.2%)	**38.3**** (54.3%)
2-phenoxyethanol	MS+RI	94	1207	1220 [4]	270.7 (26.2%)	**38.9**** (100.9%)
unknown		77	1132		10 (16.2%)	**3.4**** (35.2%)

[a]MS + RI, mass spectrum and RI agree with literature data; MS, mass spectrum agrees with literature spectrum
[b]Mass fragment used for area determination
[c,d]Retention indices on a DB5 capillary column from experimental run (c) or bibliographic data (d) [1] Engel and Ratel, 2007; [2] Leffingwell and Alford, 2011; [3] Vasta et al., 2007; [4] de Simón et al., 2009.
[e]Diet contaminated with the target concentration of 50 ng α-HBCDD g^{-1}.
*p <0.05.
**p <0.01.

doses of α-HBCDD (Table 3). Styrene levels were detected significantly higher in control hens than in exposed hens. These results make it promising candidate marker. Phenol was already highlighted in the livers of laying hens as a candidate marker of γ-HBCDD exposure by Ratel et al. (2017), with levels also higher in the livers of controls. To explain changes in liver phenol content in response to γ-HBCDD exposure, the authors hypothesize an imbalance between production and degradation of phenol related to (i) the double involvement of CYP-450 enzymes in the detoxication process of toxic xenobiotics and in microsomal hydroxylation of phenol in liver, and (ii) the hepatic degradation of tyrosine and tryptophan amino acids responsible for phenol generation, which could be increased in the case of liver function impairment. Our result thus confirms that phenol is a promising candidate marker to reveal differences in liver metabolism after a diet exposure to HBCDD.

3.3.3. Pigs

Table 5 shows that 1 and 9 VOCs were significantly impacted when animals were exposed to 5 and 50 ng α-HBCDD g^{-1} feed, respectively. This result suggests a dose effect and possibly a higher metabolic response threshold in the case of this animal. Fig. S1 presents the first map of PCA plotted on the candidate markers revealed for the exposure at 50 ng α-HBCDD g^{-1} feed. The candidate markers, levels of which were all higher in exposed animals, were mainly oxygenated compounds (alcohols, ketones, lactones) and one sulfur-containing compound (carbon disulfide). Among alcohols, we found the primary alcohol 3-methyl-1-butanol. This compound was reported as a hepatic candidate marker for exposure of laying hens to γ-HBCDD (Ratel et al., 2017), with levels also higher in exposed animals. Carbon disulfide was reported in the liver volatolome of chickens by Bouhlel et al. (2017) as a major contributor to the discrimination between control chickens and chickens exposed to pesticide. Further work is needed to identify the unknown VOC (RI 1132), which is a candidate marker for pigs and broilers for the higher α-HBCDD level.

4. Conclusion

The present paper confirms that liver volatolome is relevant to highlight metabolic disturbances induced by the exposure of animals to a chemical contamination with α-HBCDD. Based on realistic α-HBCDD exposures, our work supports the study of Ratel et al. (2017) by detecting, by liver volatolomics, the α-HBCDD exposure of laying hens. It shows the effectiveness of this approach in the case of two other farm animals, namely broilers and pigs. But given the numbers of animals involved in this proof-of-concept study, further work is needed to assess the robustness of the markers identified before considering to use them for food safety surveillance. The chemical families of most candidate markers are consistent with the two main cellular reactions put forward as affecting the anabolism and catabolism of VOCs studied as marker of

Table 5
Pigs - Volatile compounds in liver volatolome impacted by the exposure to a-HBCDD. Values are the mean of abundances ($\times 10^4$) of each candidate marker with its standard deviation (in brackets). Values in bold correspond to levels found higher in liver from "control" animals compared to "exposed animals".

Candidate markers	Tentatively identification[a]	m/z[b]	Exp. RI[c]	Ref. RI[d]	*control*	*dose 50*[e]	*dose 5*[e]
Alcohols							
3-methyl-1-butanol	MS+RI	55	733	734 [1]	5.0 (28.5%)	8.7* (20.5%)	
2,6-dimethylcyclohexanol	MS+RI	95	1119	1114 [2]	832.2 (33.0%)	2116.5* (32.7%)	
Ketones							
2,4,4-trimethylcyclopentanone	MS	83	1010		15.1 (19.3%)	62.2* (44.6%)	
2,2,6-trimethylcyclohexan-1-one	MS+RI	82	1042	1047 [3]	104.2 (32.6%)	229.8** (13.9%)	
isophorone	MS+RI	82	1062	1080 [4]	51.5 (36.7%)	151.0** (18.2%)	
Lactones							
dihydro-5-methyl-2(3H)-furanone	MS+RI	56	953	954 [1]	31.1 (6.3%)	50.5* (22.3%)	
Sulfur compounds							
carbone disulfide	MS+RI	76	570	568 [5]	526.8 (46.3%)	1102.0* (9.5%)	
Unknown							
unknown		69	1087		1.7 (41.3%)		3.4* (12.8%)
unknown		77	1132		1.6 (18.6%)	3.1* (28.1%)	
unknown		55	1162		4.7 (18.5%)	15.1** (12.8%)	

[a]MS + RI, mass spectrum and RI agree with literature data; MS, mass spectrum agrees with literature spectrum.
[b]Mass fragment used for area determination.
[c,d]Retention indices on a DB5 capillary column from experimental run (c) or bibliographic data (d) [1] Engel and Ratel, 2007; [2] Radulovic et al., 2010; [3] Čajka et al., 2007; [4] Aaslyng et al., 1998; [5] Beaulieu and Grimm, 2001.
[e]Diet contaminated with the target concentration of 5 or 50 ng α-HBCDD g^{-1}.
*$p < 0.05$.
**$p < 0.01$.

pathologies as cancers (Hakim et al., 2012). First, oxidative stress could be induced, with synthesis of reactive oxygen species that leak from the mitochondria or from peroxidated polyunsaturated fatty acids in the cell membranes. Second, detoxifying enzymes like cytochrome P-450 enzymes could be induced with catalysis of the oxidation of organic substances. Additional case/control experiments need to be investigated to go further in assessing the robustness of the candidate markers, understanding of the cellular reaction mechanisms which affect the VOC production and ascertaining the relationship between level of risky exposure and volatolomic response. In this purpose, repeating *in vivo* experiments with higher numbers of animals, especially for volatolomic studies on pig livers and poultry eggs, or implementing *in vitro* experiments with hepatocyte culture cells are two very useful opportunities. The recent advances in sensors for non-invasive and early detection of VOCs (Li et al., 2020) should be followed since they may lead rapidly to the design of routine sensors for easy, rapid detection of volatolomic markers useful for food chemical safety surveillance.

CRediT authorship contribution statement

Jérémy Ratel: ethodology, Writing – original draft. **Frédéric Mercier:** Methodology. **Magaly Angénieux:** Methodology. **Nathalie Kondjoyan:** Methodology. **Saïd Abouelkaram:** Methodology. **Patrick Blinet:** Methodology. **Angélique Travel:** Funding acquisition, Project administration. **Eric Royer:** Methodology. **Elisabeth Baéza-Campone:** Methodology. **Ronan Cariou:** Methodology. **Catherine Jondreville:** Funding acquisition, Project administration. **Erwan Engel:** Writing – review & editing, Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors thank the French Ministry for Food, Agriculture and Fisheries for financial support from the Compte d'Affectation Spéciale Développement Agricole et Rural (CASDAR project 1256). They are also grateful to the technical staff of PEAT and BOA (INRAE Nouzilly) and of GIE VGS (IFIP Villefranche-de-Rouergue) for diet preparation, animal rearing and sample collection.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.131504.

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