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Full method validation for the determination of hexachlorobenzene and hexachlorobutadiene in fish tissue by GC–IDMS

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ABSTRACT

This paper summarizes the validation strategy and the results obtained for the simultaneous determination of hexachlorobenzene (HCB) and hexachlorobutadiene (HCBD) in fish tissue with a maximum of about 10% m/m fat content using a GC–IDMS technique. The method is applicable for the determination of HCB and HCBD at trace levels in different kinds of fish tissue samples in accordance with the requirements of the EU Directive 2008/105/EC establishing Environmental Quality Standard (EQS) levels for biota in aquatic ecosystems (10 ng/g for HCB and 55 ng/g for HCBD).

The method validation aimed to assess performance parameters such as linearity, limit of detection/limit of quantification (LOD/LOQ), trueness, selectivity, intermediate precision, repeatability, stability of the extracts and robustness. The validation experiments have been performed by using uncontaminated fish tissue. Trueness was evaluated by using a certified reference material (NIST SRM 1947) (where applicable) and by the standard addition method. Very good linear signal-concentration curves were obtained for both analytes over the whole range of calibration. The repeatability and the intermediate precision of the method, expressed as relative standard deviation (RSD) and calculated at the EQS level, were estimated to be below 3% both for HCB and HCBD. The limits of quantification were 3.7 ng/g for HCB and 15.7 ng/g for HCBD in the fish.

An uncertainty budget for the measurement of both HCB and HCBD in fish at about the EQS levels, applying the described method, has been established in the order of 10%.

The analytical method and its performance characteristics take into account the requirements of EU Directive 2009/90/EC regarding the establishment of minimum performance criteria for the methods of analysis to be used in the water monitoring activity of the Water Framework Directive. Finally, the validated method was successfully tested on contaminated *Silurus glanis* from Ebro River (Spain). The method will be used in the homogeneity, stability and interlaboratory comparison studies for the characterization of a new candidate certified reference material.

1. Introduction

Hexachlorobenzene (HCB) and hexachlorobutadiene (HCBD) are considered as harmful for the environment and especially for aquatic ecosystems. HCB was first used in agriculture as fungicide on several seeds of crops as well as a wood preserving agent. Furthermore, it was applied as a porosity-control agent in the manufacturing of graphite anodes, as a fluxing agent in the production of aluminum, as a peptizing agent in the manufacturing of nitroso and styrene rubber for tires, and as a chemical intermediate in dye manufacturing [1]. HCBD has been used in a wide range of industrial applications [2], for instance, as a hydraulic fluid, a heat transfer liquid, by-product in the synthesis of chlorinated hydrocarbons, used in the production of aluminum and graphite rods, as solvent for polymers as well as a pesticide and herbicide in agricultural activities.

The EU Water Framework Directive (WFD) [3] lays down a strategy against pollution of all EU waters (rivers, lakes, ground and coastal waters) and requires Member States to implement specific measures for pollution control and prevention. In this respect one of the "daughter" Directives [4] lays down the Environmental Quality Standards (EQS) for 33 priority substances and other 8 pollutants with the aim of achieving a good chemical status of surface water before 2015. As foreseen in Article 3 of this Directive, EU Member States that opt to apply EQS for sediment

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and biota, shall apply an EQS of 10 μg/kg for HCB and 55 μg/kg for HCBD, respectively. These EQSs are established for prey tissue (i.e. wet weight) choosing the most appropriate indicator among fish, mollusks, crustaceans and/or other biota. Although the level of these contaminants has decreased over the last decades [5,6], both HCB and HCBD are substances of major concerns for affecting not only the environment but also human and animal health. Therefore, their contamination levels should be monitored on a regular basis.

Several studies have been carried out to determine HCB in different matrices such as sediment [7–10], biota [11–19] and environmental waters [20–22]. In many cases, not only HCB but also HCBD was detected in sediment [8–10], biota [16,17] and water [20,22]. In the context of the monitoring activities of the priority substances, Article 6 of the Directive 2009/90/EC [23] prescribes that the laboratories appointed as responsible for the water monitoring shall demonstrate their competence by analysis of available reference materials.

The long-term goal of this project is to support the implementation of the Water Framework Directive through provision of a tailored certified reference material (CRM) [24]. In order to properly characterize the CRM during its development and production, a validated analytical method has to be available. This is a prerequisite for working according to ISO Guide 34 and ISO/IEC 17025 [25] which contain the general requirements for the competence of reference material producers and for the competence of testing and calibration laboratories, respectively.

This paper summarizes the method validation of a 'fit-for-purpose' analytical procedure for the determination of HCB and HCBD in a fish matrix according to Directive 2009/90/EC. The method is based on accelerated solvent extraction (ASE) followed by column clean-up and GC-IDMS (gas chromatography isotopic dilution mass spectrometry) separation and detection [26,27]. Utmost care was placed on the full method validation assessing linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability and reproducibility [20] but also recovery [28–30] and trueness (using a CRM) [31] as well as robustness and stability as also recommended by the European Commission [32]. In addition, estimations of the individual uncertainty contributions of each parameter as well as of the final expanded uncertainties have been performed.

2. Materials and methods

2.1. Reagents and certified reference material used

Acetone was provided by VWR International (Fontenay-sous-bois, France), while *n*-hexane Emsure® grade, *iso*-octane Suprasolv® grade, anhydrous Na_2SO_4 and Florisil® for column chromatography (0.150–0.250 mm) were obtained from Merck (Darmstadt, Germany). Diatomaceous earth Celite® 545 AW – reagent grade was purchased from Supelco (Bellafonte, PA, USA).

SRM 1947 – Lake Michigan Fish Tissue was provided by the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA).

2.2. Calibration standards

Isotopically labeled HCB-$^{13}C_6$ (99.5% m/m) and HCBD-$^{13}C_4$ (99% m/m), used as internal standards, both in acetone (100 ng/μL) and neat crystals of HCB (99.5% m/m) and HCBD (98.5% m/m) were supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany). Purities of the standards were taken as declared in their accompanying certificates. From the latter, stock and calibration solutions were all gravimetrically prepared in *iso*-octane.

Subsequent dilutions were performed to finally obtain stock solutions containing mass fractions of ~1 μg/g and ~5.5 μg/g for both native and labeled HCB and HCBD, respectively.

The prepared native and isotopic stock solutions were distributed in 5 mL portions to be kept at −20 °C for a maximum of 4 years. Six calibration solutions were prepared at equidistant levels in the approximate range of 28–170 ng/g for hexachlorobenzene and 166–990 ng/g for hexachlorobutadiene corresponding to the studied working range. The mass fraction of the isotopically labeled standards was adjusted for all levels to about the middle of the calibration range,~100 ng/g for HCB and ~550 ng/g for HCBD.

2.3. Sample treatment, extraction and clean-up

Fish tissue from catfish (*Anarhichas lupus*) used as blank matrix was obtained from a local fishmonger in Belgium. It was cut in 1 cm cubes, milled using an A10 Analytical mill (IKA®-Werke, GmbH & CO. KG, Staufen, Germany) and stored at −20 °C.

The analytical procedure has previously been developed and optimized in-house and is based on accelerated solvent extraction (ASE) of the fish samples with a mixture of *n*-hexane/acetone by using a Dionex ASE 200 (Amsterdam, The Netherlands), followed by column clean-up [33–35] and GC–MS detection. A precise amount of fish tissue between 2 and 3 g was weighted in an 11 mL ASE cell and mixed with 1 g of diatomaceous earth. The internal standard solution (50 μL) was carefully added drop wise to the solid powder in the cell using a gas-tight syringe (SGE, Australia). The extraction in the ASE was carried out applying the following parameters: pressure 13.34 MPa, temperature 100 °C, solvent mixture acetone/*n*-hexane (50:50% v/v), pre-heating time 5 min, static time 10 min, static cycles 3, purge time 60 s and flush volume 140%.

Afterwards, 10 mL of *iso*-octane as keeper was added to the extract in the ASE vial and was concentrated under a gentle stream of nitrogen. A polyethylene SPE clean-up column was prepared by filling with 15 g of Florisil® and 10 g of Na_2SO_4 and conditioned with 40 mL of *n*-hexane. The extract was then loaded onto the column. The column was eluted with a total of 50 mL *n*-hexane, collected in a round bottom flask, in which 5 mL of *iso*-octane was previously added as keeper. The solvent was reduced with a rotary evaporator to approximately 3–4 mL and the remaining extract was evaporated under a gentle stream of nitrogen to obtain a final volume of approximately 1.5 mL. This sample was transferred to the 1.8 mL GC vial and evaporated further under nitrogen stream until 500 μL. At this stage the sample was ready to be injected into the GC–MS system for analysis.

2.4. GC–MS analysis

Instrumental analysis was carried out by a Thermo Trace™ Finnigan 2000 GC equipped with DSQ™ mass spectrometry single quadrupole detector (Thermo Electron Corporation, Austin, TX, USA) operating in the selected ion monitoring (SIM) mode. An SGE-HT8 (SGE, Australia) fused silica column, 50 m × 0.22 mm film thickness × 0.25 μm ID was installed. A programmed temperature vaporizing (PTV) injector operating in splitless mode was used (temperature program started at 100 °C and increased to 200 °C by 14.5 °C/min) to allow rapid transfer of the analytes onto the column avoiding possible degradation in the inlet. The glass split liner (Restek, Bellefonte, PA, USA) with a dimension of 2 mm × 2.75 × 120 was cleaned and/or changed after each measuring sequence. One μL of sample was injected into the GC–MS. Helium was used as a carrier gas with a flow of 1 mL/min. The ion source and transfer line temperatures were kept at 250 °C and 280 °C, respectively. The oven temperature program started at 90 °C (hold

Table 1
Quantification and qualification ions for measurements of HCB and HCBD.

Analyte	Quantification ion (*m/z*)	Qualification ion (*m/z*)
HCB	284	282
$^{13}C_6$-HCB	292	294
HCBD	225	223
$^{13}C_4$-HCBD	231	233

time 1.2 min), then increased to 215 °C by 25 °C/min (hold time 8 min) and finally increased to 300 °C by 25 °C/min (hold time for 8 min). Two SIM windows were set with the parameters detailed in Table 1 for a total run of 25 min. The data acquisition was managed using XCalibur® Software (Thermo Electron Corporation).

2.5. Fat content determination

The fat content determination of the blank matrix fish has been carried out by applying ASE extraction with the same settings as described in section 2.3. The extract was concentrated under a gentle stream of nitrogen, placed in the oven for 3 h at 60 °C and finally dried at 105 °C until constant mass was reached. The obtained mass of the residue represented the fat content of the fish tissue calculated as % m/m.

2.6. Experimental setup of the method validation

Different experiments were set up in order to investigate the performance characteristics of the method in terms of linearity, repeatability (REP), intermediate precision (IP), limit of detection (LOD), limit of quantification (LOQ), robustness, trueness and stability of the extracts. In our approach 4 samples were analyzed in triplicate in each sequence corresponding to a maximum of 12 injections.

The measurement sequence was as follows:

1. solvent blank
2. method blank
3. calibration standards (random order)
4. solvent blank
5. samples (including standard addition sample and/or matrix CRM as control sample) – max 12 injections
6. solvent blank
7. calibration standards (random order)
8. solvent blank

The method blank underwent the same analytical procedure as the fish samples without adding fish matrix into the ASE cell. This sample was prepared in order to check contingent cross contaminations during the validation study.

The validation experiments were performed on 13 different days. On each day three independent sample preparations have been carried out. Some of the experiments were used in the estimation of different parameters. The calculation for the analyte mass fractions in the sample was based on Eq. (1) and the response factors applied were determined using the average values as obtained from the two calibration curves run in each sequence:

$$C_x = \frac{A_x \cdot n_{ISx}}{A_{ISx} \cdot RF \cdot M_{sample}} \quad (1)$$

where C_x is the mass fraction of the individual HCB or HCBD congener × in unknown sample (ng/g), A_x is the area of the native HCB or HCBD congener × in unknown sample, n_{ISx} is the amount of added internal standard (ng), A_{ISx} is the area of the labeled HCB or HCBD congener ×, *RF* is the response factor, M_{sample} is the sample mass (g).

2.6.1. Linearity/working range

The set of standards (6 calibration levels) covering the studied working range was freshly prepared from the stock solutions every new week during the validation.

2.6.2. Repeatability/intermediate precision

Blank fish was spiked at EQS level for both HCB and HCBD and analyzed in triplicate during five days for a total of 15 independent sample preparations.

2.6.3. LOD/LOQ

Blank fish was spiked at half of the EQS level for both HCB and HCBD and analyzed in triplicate on 4 days.

2.6.4. Trueness

Standard addition: Blank fish was spiked at EQS level and at half of them for HCBD and HCB and analyzed in triplicate on 9 days each.

Measurement of a CRM: a certified reference material (Lake Michigan Fish Tissue, SRM 1947, NIST) was analyzed in six replicate measurements during two different days.

2.6.5. Robustness

During the development of the method, it was found that the most critical parameters of the analytical procedure were the SPE elution volume and the volume of the final extract. Therefore, SPE elution volumes of ±10% compared to the optimized 10 mL, as well as extracts of ±60% compared to the final volume of 500 μL were tested.

2.6.6. Stability of the extracts

Three extracts of blank fish spiked at EQS level of HCB and HCBD were re-injected after one week storage at 4 °C in dark.

3. Results and discussion

3.1. Method validation

Before method validation, several steps of the analytical procedure have been optimized such as the extraction step using various ASE parameters, different types and amounts of solid phase adsorbent, to set the best fat/fat retainer ratio for the clean-up. In addition, several types and volumes of the elution solvents in the clean-up step were tested until the optimal conditions were found. The GC–MS parameters have also been studied in detail and optimized.

The in-house validation procedure was performed on the basis of the EURACHEM [36] and IUPAC [37] guidelines. In our laboratory a quality management system in accordance with EN ISO/IEC 17025 is applied since many years.

3.2. Linearity, working range and response factors

The calibration was performed using six levels. These mixtures (calibration standards) containing both native and isotopically labeled analytes were injected on different days. Fig. 1 shows the chromatogram corresponding to the lowest level of the calibration curve.

The calibration curve was established by plotting the peak area ratios for HCB/HCB-$^{13}C_6$ 284/292 vs. the mass fraction ratio of HCB/HCB-$^{13}C_6$. Similarly the peak area ratio HCBD/HCBD-$^{13}C_4$ 225/231

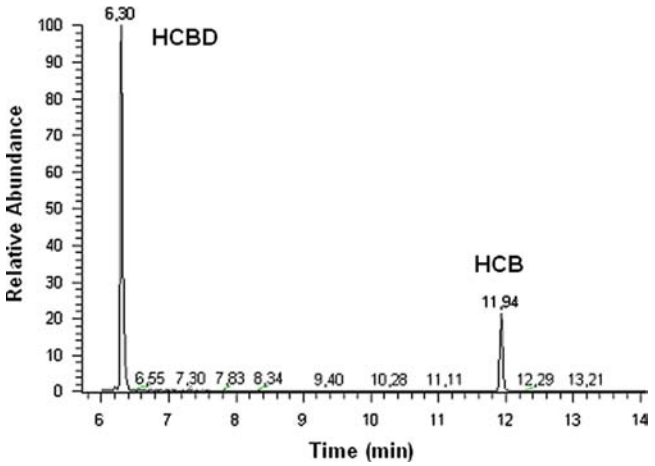

Fig. 1. Part of the chromatogram of a calibration solution at the lowest level corresponding to approximately 2.5 × EQS (~28 ng/g for HCB and ~160 ng/g for HCBD).

vs. the mass fraction ratio of HCBD/HCBD-$^{13}C_4$ was plotted. The most abundant ion of both the native and the labeled analytes were selected for quantification purposes. All data of the calibration curves injected over five days were pooled and linearity was demonstrated by correlation coefficients of about 0.998 for HCBD and of about 0.992 for HCB. The response factor values were 1.12 for HCB and 1.54 for HCBD, respectively. both calculated for all pooled data. None of the data was excluded. Linearity was also assessed through the RSD of the average response factor.

Average response factors and corresponding RSD were calculated for each day. The criterion initially set in the method validation plan for the RSD was 5%. This could not always be met for HCB due the fact that the liner of the GC injector became dirty after the injection of a few samples. As a preventive action the liner was systematically changed after each sequence resulting in a much better RSD of the RF values. On the contrary, the RSD values of the RF for HCBD showed to be less affected by the renewal of the liner.

Nevertheless, it was noticed during the course of the validation that the upper limit set for RSD of the RF value of HCB was rather ambitious. Therefore it was decided to allow a slightly higher RSD of 7%, seen that the target performance criteria of repeatability and intermediate precision were anyway satisfactorily met (see Section 3.5).

3.3. Investigation of the blank matrix and selectivity

In order to check the blank fish matrix for any contamination, fish from a local Belgian fisherman has been spiked with the isotopic standard and the resulting mass fractions of HCB and HCBD were measured. The peaks of native HCB and HCBD were negligible compared to the peaks of the internal standards (Fig. 2). Therefore, this matrix was regarded as suitable starting material in the experiments for the determination of LOD and LOQ. The fat content of the blank fish has been found to be in the range of 5–6% m/m.

Furthermore, full scan chromatograms of the samples have been checked during method validation for selectivity. Interferences by co-elution were never detected for the peaks of interest and, thus, method selectivity was ensured.

3.4. Limit of detection and limit of quantification

Twelve independent replicate analyses have been carried out on four days. All samples were spiked at half of the EQS and consecutively measured. The half EQS level corresponds roughly to mass fractions of ~30 ng/g for HCB and ~180 ng/g for HCBD calculated in the final extract and to mass fractions of ~4 ng/g for HCB and ~25 ng/g for HCBD calculated in the fish tissue.

The standard deviations (SDs) of all measurements have been calculated to estimate LOD (3 × SD) and LOQ (10 × SD) as summarized in Table 2 calculated for both analytes in the fish tissue and in the final extract.

The results are fulfilling the performance criteria set in the validation plan, i.e. LOQ lower than ~30 ng/g for HCB and lower then ~180 ng/g for HCBD, calculated in the final extract. According to the Directive 2009/90/EC, the minimum performance criteria for the LOQ of methods of analysis to be used in chemical monitoring program under the WFD, should be equal or below 30% of the EQS. Our results show that this criterion was reached for HCBD, while for HCB the determined value is sufficiently close.

3.5. Repeatability and intermediate precision

In order to evaluate the repeatability and intermediate precision of the method, five days experiments were planned in the validation. On each day, three independent replicates of blank fish tissue were spiked at EQS level for HCB and HCBD and injected in the GC–MS. The results of the experiments are shown in Table 3.

One-way ANOVA was applied to the results of these set of experiments estimating the repeatability as *within group* SD and intermediate precision as *between group* SD. The repeatability of the method, RSD_{rep}, revealed to be 2.8% for HCB and 1.6% for HCBD, respectively. The intermediate precision of the method, RSD_{ip}, was 0.9% for HCB and 2.4% for HCBD. The initial target set in the method validation plan for the RSD of the repeatability and intermediate precision was $< 4\%$ for both analytes. Therefore, the results confirmed the precision of the method.

3.6. Trueness

3.6.1. Trueness assessment for HCB measurement using SRM 1947

For the assessment of the trueness for HCB measurement, a CRM from NIST was used (SRM 1947). Trueness was assessed by measuring six replicate samples of the CRM carried out on two days by two different operators. The trueness experiments, considering the individual results by each operator or the pooled values by two operators, showed good agreement between the measured and certified values (see the results summarized in Table 4). For the trueness assessment, the comparison of the measurement result with the certified value has been performed according to ERM Application Note 1 [38].

The conclusion of the trueness assessment revealed no statistically significant difference between the measured value and the certified value, because $\Delta_m = U_\Delta$.

3.6.2. Trueness assessment using standard addition

Since there was no CRM for HCBD available on the market [24], the trueness was investigated as recovery of HCBD in spiked samples. The mean of the average HCBD concentrations found was compared to the added (theoretical) concentrations in order to estimate the recovery as slope of the regression line. Recovery values in the range between 90% and 110% were considered as satisfactory. The recovery obtained for HCBD was 94%. Similarly, the trueness for HCB was also evaluated using the data from the standard addition experiments. The obtained recovery for HCB was 97%.

3.7. Robustness

Two steps of the analytical procedure were selected to be tested for robustness. The factors considered as potentially critical and therefore investigated were the clean-up step and the volume

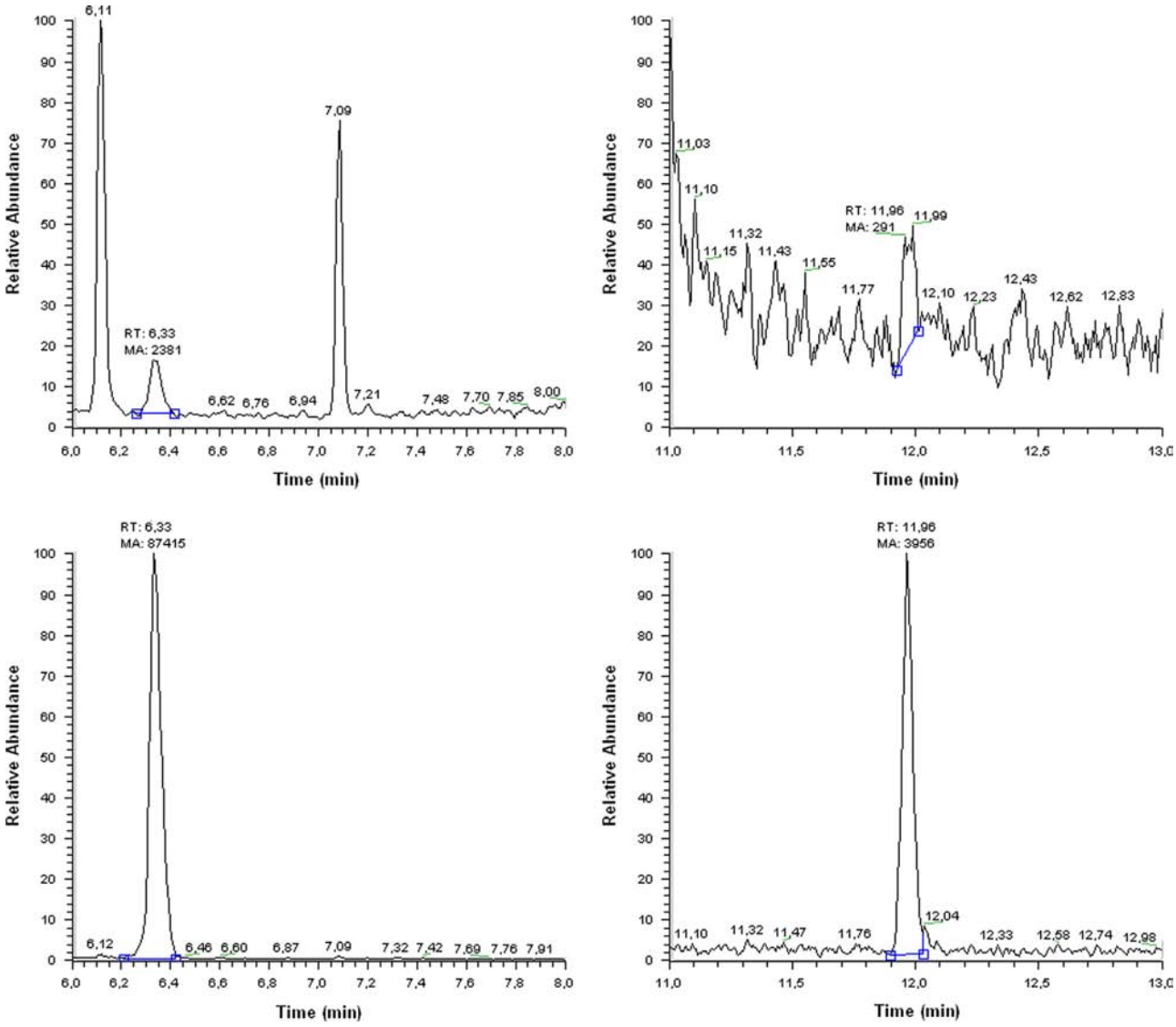

Fig. 2. Chromatograms of the blank fish matrix spiked with a mixture of internal standards (~100 ng/g for HCB and ~550 ng/g for HCBD) showing peaks of native HCBD (integrated top left) and isotopically labeled HCBD (integrated down left) as well as native HCB (integrated top right) and isotopically labeled HCB (integrated down right).

Table 2
LOD and LOQ for HCB and HCBD as 3 and 10 times the SD of 12 replicate analysis, respectively.

Analyte	LOD (ng/g)	LOQ (ng/g)	Remark
HCB	1.1	3.7	In the fish
	8.1	26.9	In the final extract
HCBD	4.7	15.7	In the fish
	34.0	113.7	In the final extract

Table 3
Mean of the mass fractions of HCB and HCBD (ng/g) in the fish tissue obtained from the repeatability and intermediate precision measurements, three replicates on each day.

Analyte	Day of validation	Mean (ng/g)	SD (ng/g)	RSD_{rep} (%)	RSD_{ip} (%)
HCB	Day 1	12.05	0.06		
	Day 2	11.95	0.16		
	Day 3	12.07	0.39	2.8	0.9
	Day 4	12.15	0.25		
	Day 5	11.54	0.36		
HCBD	Day 1	69.71	0.67		
	Day 2	68.78	0.97		
	Day 3	67.17	1.42	1.6	2.4
	Day 4	69.17	0.67		
	Day 5	64.28	1.51		

of the final extract. For the clean-up step, a 10% lower (5×9 mL) and 10% higher (5×11 mL) amount of elution solvent (*n*-hexane) was tested in triplicate. The influence of the final extract volume (set to 500 μL) was also investigated considering two different final volumes of 200 μL and 800 μL.

To test robustness, plus and minus extreme values around the optimized parameter are set for the critical identified factors as presented elsewhere [39]. Considering the elution clean-up volume and the final extract volume as critical parameters, Student's t-test has been applied to compare the means of the results (x_1 and x_2) when changing these parameters in the analytical method. The obtained results (t_{calc}, calculated according to Eq. (2)) were compared to the theoretical value (t_{crit}, $\alpha = 0.05$, 4 degrees of freedom) and showed that statistically there is no difference between the extreme conditions both for HCB and HCBD (see Table 5). The two parameters can be easily controlled between the set limits and, thus the method is demonstrated to be

Table 4
Results of trueness check using the pooled values for the calculation.

SRM 1947		HCB found		Comparison of the measured value with the certified value		
Certified value (ng/g)	u_{CRM} (ng/g)	c_m (ng/g)	u_m (ng/g)	u_Δ (ng/g)	Δ_m (ng/g)	U_Δ (ng/g)
7.48	0.33	6.55	0.33	0.46	**0.9**	**0.9**

u_m, standard uncertainty of the measurement results (calculated from the method validation study); u_{CRM}, standard uncertainty of the certified value (from the certificate of the SRM 1947); c_m, mean measured value (mean of the means of the measurement results); Δ_m, absolute difference between mean measured value and certified value; u_Δ, combined uncertainty of the difference between the measured results and certified value; U_Δ, expanded uncertainty ($k=2$).

Table 5
Outcome of robustness test varying both the elution volume during SPE clean-up (A) and the final extract volume (B) before injection into the GC–MS.

A	x_1 – 10% SPE elution volume (ng/g)	x_2+10% SPE elution volume (ng/g)	t_{calc}	t_{crit}
HCB	11.9	12.1	0.57	2.78
HCBD	66.2	65.5	0.56	2.78
B	x_1 – 60% final extract volume (ng/g)	x_2+60% final extract volume (ng/g)	t_{calc}	t_{crit}
HCB	11.8	11.4	0.62	2.78
HCBD	62.4	60.6	2.58	2.78

robust concerning these parameters:

$$t_{calc} = (x_1 - x_2)/s(1/n_1 + 1/n_2)^{1/2} \quad (2)$$

where s is the pooled estimation of the individual standard deviation, n_1, n_2 are the number of experiments.

3.8. Stability of the extracts

The means of the results for both components were compared using Student's t-tests in the same way as it was applied for robustness. Both HCB and HCBD resulted to be stable after one week of storage at+4 °C in the dark, because the condition $t_{calc} < t_{crit}$ is fulfilled for each case as highlighted in Table 6.

Table 6
Outcome of stability test at 4 °C for HCB and HCBD.

Analyte	$x_{t=0}$	$x_{t=7\ days}$	SD_1	SD_2	t_{calc}	t_{crit}
HCB	11.54	11.54	0.44	0.07	0.01	2.78
HCBD	64.28	65.89	1.51	0.79	1.63	2.78

x=sample means (based on 3 replicate measurements).

3.9. Uncertainty estimation

The relative expanded uncertainty (U) of the measurements results is estimated taking into account all the different contributions obtained during the method validation according to the following equation:

$$U = k \cdot \sqrt{u_t^2 + u_{rep}{}^2 + u_{ip}{}^2 + u_{cal}{}^2} \quad (3)$$

where U is the expanded relative uncertainty, k is the coverage factor ($k=2$), u_t is the relative standard uncertainty of trueness estimation, u_{rep} is the relative standard uncertainty of repeatability, u_{ip} is the relative standard uncertainty of intermediate precision, u_{cal} is the relative standard uncertainty related to the calibration standards, including weighing, purity and dilutions step contributions.

3.9.1. Uncertainty of trueness

When using the NIST reference material certified for the HCB content, the contribution of the relative standard uncertainty of the trueness was calculated as $u_{t,HCB}$=4.8% according to the following equation:

$$u_{t,\ HCB} = \sqrt{\frac{SD_{rep}^2}{n_{rep,t}} + \frac{SD_{ip}^2}{n_{days,t}} + u_{CRM}^2} \quad (4)$$

where $u_{t\ HCB}$ is the relative standard uncertainty of trueness for HCB, SD_{rep} is the standard deviation of repeatability measurements, SD_{ip} is the standard deviation of intermediate precision measurements, $n_{rep,t}$ is the number of replicates used for trueness check, $n_{days,t}$ is the number of days used for trueness check, u_{CRM} is the standard uncertainty of the CRM value (from certificate).

When using the standard addition approach, the contribution of the relative standard uncertainty of the trueness for HCBD, $u_{t,\ HCBD}$ was equal to 2.1% according to Eq. (5). For comparison, the relative standard uncertainty of the HCB, using the standard addition experimental setup, have also been calculated based on Eq. (5) and resulted in an $u_{t,HCB}$ of 2.2%.

$$u_t(\%) = uncertainty\ of\ the\ slope/slope \times 100 \quad (5)$$

As it can be seen, a very good agreement of the relative standard uncertainty of trueness for both analytes calculated based on the standard addition experimental setup has been achieved.

3.9.2. Considerations on trueness assessment for HCB and HCBD

Recoveries obtained for both HCB and HCBD are in good agreement with the acceptance criteria and, therefore, a recovery correction factor will not be applied to the results. When comparing the two approaches used for the estimation of the uncertainty contribution due to trueness for HCB, it becomes clear that a major contribution is given by the standard uncertainty of the CRM value (u_{CRM}). The following has finally been considered: (a) assessing the trueness through a CRM is the most reliable approach; (b) the estimation of the uncertainty contributions using the standard addition is similar for both HCBD and HCB. For these reasons, it has been suggested to replace the relative standard uncertainty of the trueness for HCBD as obtained from the standard addition experiment (2.1%) by the uncertainty of the trueness obtained for HCB using the CRM (5%). This is a more conservative approach in the estimation of the final expanded uncertainty taking into account the limitations of the standard addition approach.

3.9.3. Uncertainty of repeatability and intermediate precision

The uncertainty contributions related to repeatability and intermediate precision were calculated applying ANOVA according

to Eqs. (6) and (7) taking into account a total of 15 analyses carried out on 5 days:

$$u_{ip} = \sqrt{\frac{RSD_{ip}{}^2}{n_{days}}} \quad (6)$$

$$u_{rep} = \sqrt{\frac{RSD_{rep}{}^2}{n_{rep}}} \quad (7)$$

n_{days} = number of days
n_{rep} = number of replicates

The relative uncertainties were u_{rep} = 0.72% and u_{ip} = 0.41% for HCB and u_{rep} = 0.42% and u_{ip} = 1.07% for HCBD, respectively.

3.9.4. Uncertainty of the calibration solution preparation

The preparation of the calibration solutions has a contribution to the final measurement uncertainty through the purity of the standards and the weighing steps. The largest uncertainty contribution comes from the preparation of the lowest level calibration point, Standard 1 (2.5 EQS level), corresponding to approximate mass fractions of about 28 ng/g for HCB and 160 ng/g for HCBD. As a conservative approach, it was decided to use the highest uncertainty contribution (Standard 1) among all preparations of the calibration solutions as uncertainty contribution corresponding to the calibration preparation. Furthermore, the uncertainty of the purity of the standards has also been assessed to estimate the total uncertainty contribution of the calibration. The relative standard uncertainty of the calibration resulted in 0.9% by taking into account the uncertainty contribution of the preparation of the calibration solution of the lowest level and all additional contributions coming from the stock solution preparation.

3.9.5. Final uncertainty budget

The dominating uncertainty contribution for both analytes is originating from the trueness assessment with 4.8% for HCB measuring SRM 1947 and with an extended 5% for HCBD (Table 7). The additional uncertainty contributions from repeatability, intermediate precision and calibration were all around 1% or below.

The expanded relative uncertainty for HCB was estimated as 9.9% based on 15 replicate measurements on 5 days and as 10.5% based on 3 replicate measurements on 1 day, respectively. Following the same way of calculation, the estimation of the expanded relative uncertainty for HCBD provided 10.5% based on 15 replicate measurements on 5 days and 11.4% based on 3 replicate measurements on 1 day, respectively. As it can be seen the expanded relative uncertainty slightly decreases as the number of measurements increases. In all cases the coverage factor applied was 2 corresponding to a level of confidence of about 95% [40].

A summary of all uncertainty contributions obtained during the full method validation of HCB and HCBD measurements in fish tissue is provided in Table 7.

Table 7
Uncertainty budget for measurement of HCB and HCBD in the fish tissue.

Estimated uncertainties	HCB (%)[a]	HCBD (%)[a]
u_t	4.8	5.0
u_{rep}	0.7	0.4
u_{ip}	0.4	1.1
u_{cal}	0.9	0.9
Expanded relative uncertainty (U) (k=2)	**9.9**	**10.5**

[a] Estimations based on 15 analyses in 5 days.

Table 8
Results of the analysis of the fish specimens (based on fresh mass).

Specimen	Fish mass (kg)	Fish length (cm)	Analyzed pieces	Mass fraction of HCB (ng/g)	Mass fraction of HCBD (ng/g)
Fish 1	55	187	Tail	15.6	< LOD
			Head	10.5	< LOD
Fish 7	28	121	Tail	43.8	LOD < (7.3) < LOQ
			Head	6.6	< LOD
Fish 27	10	85	Head	< LOD	LOD < (7.7) < LOQ

3.10. Analysis of fish samples

The validated method was applied to the analysis of naturally contaminated fishes (*Silurus glanis*) from the Flix reservoir, Ebro River (Spain). Lacorte et al. [41] investigated the presence of organic pollutants in sediment and fish species originating from the same location in Spain during a study carried out in 2006. It could be concluded that the contamination level of sediment was overall low to medium and most of the fish samples contained several organic compounds such as organochlorine compounds (OCs) and polybrominated diphenyl ethers (PBDEs). The most significant levels of OCs were found in benthic type fishes in the Flix reservoir. Three specimens of different size with a fat content below 10% m/m (Fish 1, Fish 7, Fish 27) were investigated (see Table 8). The part of the analyzed specimen was the fillet and its fatty part, divided into head and tail tissues.

After evaluation of these preliminary data, two tendencies can be observed: (a) the tail is usually more contaminated compared to the head; (b) accumulation of HCB in the fish tissue is higher than of HCBD. The in-house validated method was successfully tested on real matrices and the mass fraction levels detected for both HCB and HCBD were in line with the results published by Lacorte et al. [41].

3.11. Practical observations

The method presented in this paper has proven to be suitable for the determination of HCB and HCBD in fish tissue. Some further considerations have to be taken into account when working with it.

The fat content of the fish can crucially influence the performance of the method. The whole measurement sequence has been designed to prevent the GC–MS system from becoming rapidly dirty due to the possible high fat content of the samples. The fishes used in the validation study contained about 5% m/m fat in the blank fish tissue and about 10.4 ± 0.5% m/m in the reference material SRM 1947, respectively.

It is of utmost importance to change the liner of the GC injector after each sequence injected (corresponding to a maximum of 12 injections), otherwise the intensity of the peak will decrease significantly.

The simple one column clean-up step works well when the fat content of the sample does not exceed 10% m/m. If a fish matrix with higher fat content is processed, it is advised to apply a more thorough clean-up to avoid adverse effects in the GC–MS system.

4. Conclusion

To our knowledge this paper presents the first fully validated method that allows the simultaneous quantification of HCB and HCBD in fish tissue at EQS levels. The obtained results fulfill the requirements of Article 4 of EU Directive 2009/90/EC with regard

to the uncertainty of the measurement of 50% or below estimated at the level of relevant EQS. Regarding the limit of quantification equal or below a 30% of the relevant EQS, the minimum criteria is fulfilled for HCBD, while is sufficiently close to obtain reliable data for HCB.

The developed method will be used for the CRM production at JRC-IRMM, but this simple method can also be applied by control laboratories responsible for the mandatory chemical monitoring prescribed to the EU Member States under the WFD.

The scope of the presented method could be extended for the determination of other organochlorines in similar biotic matrices. The method has been developed focusing on using only the edible part of the fish tissue without skins, bones or other parts of the specimen.

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