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## Optimization of an HPLC-MS/MS method to analyze chlordecone in bovine serum and correlations with levels in liver, muscle and fat

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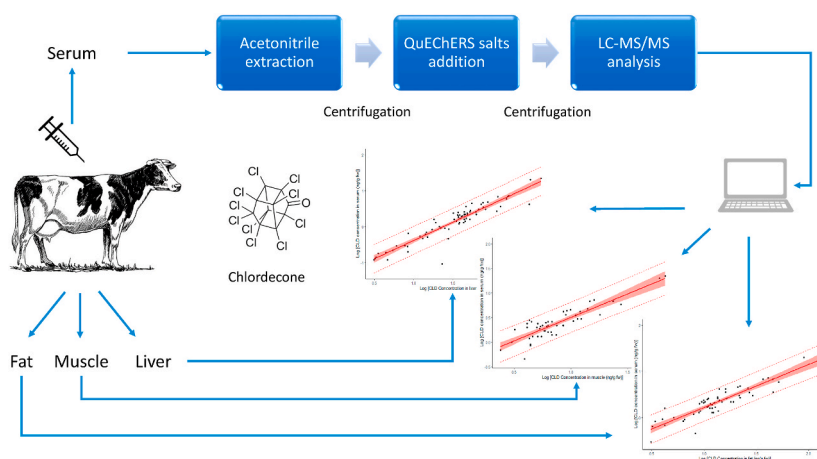
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### HIGHLIGHTS

- A QuEChERS protocol was optimized and validated for analyzing chlordecone in bovine serum.
- The method was applied to 121 bovine serum samples from the French West Indies.
- Serum chlordecone levels were correlated with levels found in fat, muscle and liver.
- The ratios allow an in-vivo monitoring and pre-control of bovines before slaughter.
- This approach is relevant to assess the contamination level of living farm animals.

### GRAPHICAL ABSTRACT



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### ABSTRACT

Chlordecone is an organochlorine pesticide used from 1972 to 1993 in the French West Indies. Its extensive use and high persistence in soils induced massive contamination of the environment and of the food chain, especially in cattle through contaminated soil ingestion. To ensure suitability for consumption of bovine meat, monitoring plans are set up based on perirenal fat concentrations after slaughtering. In the present study, we have investigated an in-vivo monitoring approach by measuring chlordecone levels in serum samples. For this purpose, a sensitive high-performance liquid-chromatography-tandem mass spectrometry (HPLC-MS/MS) method following a QuEChERS extraction method was successfully optimized and validated, reaching a limit of quantification of 0.05 ng g<sup>-1</sup> fresh weight. This method was applied to 121 serum samples collected from bovines originating from contaminated areas of Martinique and Guadeloupe. Chlordecone was detected in 88% of the samples, and quantified in 77% of the samples, with concentrations ranging from 0.05 to 22 ng g<sup>-1</sup>. Perirenal fat, liver, and

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muscle were also sampled on the same animals and the measured concentrations of chlordecone were statistically correlated to the levels determined in serum. Mean concentration ratios of 6.5 for fat/serum, 27.5 for liver/serum, and 3.3 for muscle/serum were calculated, meaning that chlordecone was not only distributed in fat (as expected), muscle and liver, but also in serum. Good correlations were found to allow prediction of chlordecone concentrations in muscle based on concentrations measured in serum. This study opens the door to possible pre-control of bovines before slaughter. In cases of probable non-compliance with maximum residue levels (MRLs), farm management could proceed to allow for depuration under controlled conditions. This would have a strong impact on both economic and food safety management measures.

## 1. Introduction

Chlordecone (also known as its commercial name Kepone) is an organochlorine insecticide responsible for widespread, long-term soil pollution in the French West Indies (Guadeloupe and Martinique), where it was used intensively from 1972 to 1993 to control banana weevil (*Cosmopolites sordidus*) in crops. The substance was banned in 1990 in the French territories by the competent authorities and was used by exemption in the French West Indies until 1993. It was classified as a persistent organic pollutant in 2009 under the Stockholm Convention (UNEP, 2019) and is considered a lipophilic compound because its logarithmic value of octanol/water partition coefficient ( $\log K_{ow}$ ) exceeds 3. The adverse effects of chlordecone are well documented, with reproductive and developmental impairments, endocrine disruption, neurotoxicity, a suspected increased risk of prostate cancer, and impairment of visual and fine motor functions in boys, in particular through results obtained based on the Timoun cohort study, which presents the results of a prospective study conducted in Guadeloupe between 2004 and 2007 on a mother-child cohort to assess the risk of congenital anomalies in relation with the exposure to chlordecone (Cordier et al., 2015; Dallaire et al., 2012; Desrochers-Couture et al., 2022; Multigner et al., 2010, 2016; Oulhote et al., 2023; Rouget et al., 2020). Its extensive use, combined with its high persistence in soils (Cabidoche et al., 2009), led to massive contamination of the environment and consequently of the food chain, including root vegetable crops, fish/seafood, and also livestock and poultry through involuntary soil intake (Cabidoche et al., 2009; Clostre et al., 2015; Collas et al., 2019; Dromard et al., 2018). For all these reasons, human exposure to chlordecone through food consumption is a paramount health concern and chlordecone has therefore been largely studied in recent decades (AFSSA, 2007; ANSES, 2017; Dereumeaux et al., 2020; Dubuisson et al., 2007). Chlordecone concentrations in the serum of Guadeloupean and Martinican human populations are well described (ANSES, 2017; Dereumeaux et al., 2020). These studies showed that chlordecone was detected in more than 90% of serum samples, with mean values of  $0.14 \mu\text{g L}^{-1}$  ( $\approx 0.14 \text{ ng g}^{-1}$ ) for Martinique and  $0.13 \mu\text{g L}^{-1}$  ( $\approx 0.13 \text{ ng g}^{-1}$ ) for Guadeloupe.

Survey and monitoring plans are set up each year in the French West Indies by the competent authorities in order to ensure food safety. Foods of animal origin are considered the main contributors to chlordecone exposure, with fish/seafoods being the most frequently quantified matrices, followed by chicken eggs and meat (excluding poultry) (ANSES, 2017). Regarding meat, bovines are the most commonly sampled species in the monitoring plans due to a high exceedance rate of the maximum residue level (MRL). Perirenal fat is sampled rather than muscle because chlordecone is expected to concentrate in fat due to its classification as a lipophilic compound. However, it has been demonstrated that chlordecone exhibits atypical behavior since it is distributed not only in fat, but also in muscle and the liver in poultry (Jondreville et al., 2014), Alpine kids (Lastel et al., 2018), ewes (Saint-Hilaire et al., 2018a), pigs (Fourcot et al., 2020) and bovines (Lavison-Bompard et al., 2021), with much higher concentrations in the liver than in fat. Studies on the distribution of chlordecone in fat, muscle, and liver tissues in bovines (ANSES, 2018; Lavison-Bompard et al., 2021) and in swine (ANSES, 2019) have led to changes in the MRL for chlordecone at the

national level, setting mitigation concentration levels in fat (as the sampled matrix in monitoring plans) in order not to exceed the MRL of  $20 \mu\text{g kg}^{-1}$  fw in muscle and offal (as edible parts) (Ministerial Decree, 2019), and further setting the MRL at  $20 \mu\text{g kg}^{-1}$  fw in the same commodities at a European level (Commission Regulation, 2021). This is the limit at which the whole carcass should be withdrawn from the market.

Bovine perirenal fat remains the targeted matrix in monitoring plans. Sampling fat is a destructive approach as it can be performed only after slaughter. A less invasive approach, based on blood sampling, is a potentially useful alternative: beyond the fact that it does not require the slaughter of the animal, it would also allow for possible depuration of the cattle considered unsuitable for consumption before slaughter. Most of the studies that focus on chlordecone content in serum (either human or animal) (Dereumeaux et al., 2020; Fourcot et al., 2020; Fournier et al., 2017; Saint-Hilaire et al., 2020) are based on the extraction protocol of Multigner et al. (2010) (further improved by Fourcot et al., 2021), which is derived from Debier et al. (2003), originally intended for polychlorobiphenyl (PCB) analysis. The protocol consists in a first step of protein denaturation, followed by solid phase extraction (SPE). After a final acidic washing step, the extract is analyzed by gas chromatography coupled with an electron capture detector (GC-ECD). Bichon et al. (2015) described a similar method with slight modifications and analysis by high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS), since LC is considered more robust than GC separation for chlordecone analysis (Bichon et al., 2015). An alternative method based on QuEChERS (Quick, Easy, Cheap, Efficient, Rugged and Safe) extraction has been described by Saint-Hilaire et al. (2021) for quantifying chlordecone and its metabolite, chlordecol, in ewe serum, and by Saint-Hilaire et al. (2023) for quantifying chlordecone in human serum. Molecularly imprinted material (MIP) can also be used for extracting and analyzing chlordecone and chlordecol in serum samples, following HPLC-MS/MS detection (Bosman et al., 2021), with enhanced selectivity and sensitivity. However, MIP is not yet commercially available.

The aim of this work was to optimize and validate a sensitive method based on a QuEChERS protocol followed by HPLC-MS/MS analysis to quantify chlordecone concentrations in serum sampled together with perirenal fat, skirt muscle, and the liver from a large collection of contaminated bovines bred in the French West Indies. We assessed concentrations in serum samples, and also established correlations between chlordecone levels in serum and in other tissues with a prospective approach to support the competent authorities in food safety monitoring, which may lead to further mitigation measures.

## 2. Material and methods

### 2.1. Reagents and solvents

All the chemicals and solvents used were of high-quality grade (purity 98%) or HPLC grade. Acetonitrile, formic acid, and propanol-2 were purchased from Fisher Scientific (Illkirch-Graffenstaden, France). Ultrapure water ( $18.2 \text{ M}\Omega \text{ cm}$ ) was obtained by purifying distilled water with a Milli-Q system (Merck Millipore, Saint-Quentin-en-Yvelines, France). QuEChERS Extraction Packets EN Method (4.0 g of magnesium sulfate ( $\text{MgSO}_4$ ), 1.0 g of sodium chloride ( $\text{NaCl}$ ), 1.0 g of sodium

citrate dehydrate and 0.5 g of sodium hydrogencitrate sesquihydrate), and QuEChERS Extraction Packets Original Method (4.0 g of  $\text{MgSO}_4$ , 1.0 g of NaCl) were purchased from Agilent Technologies (Santa Clara, CA, USA). Supel™ QuE PSA/ $\text{C}_{18}$ /ENVI-Carb (50 mg of PSA, 50 mg of  $\text{C}_{18}$ , 50 mg of ENVI-Carb and 150 mg of  $\text{MgSO}_4$ ) was obtained from Sigma-Aldrich (Steinheim, Germany).

## 2.2. Standards

Powdered chlordecone (purity >98%) was supplied by Azur Isotopes SAS (Marseille, France). A chlordecone stock solution at  $255 \mu\text{g mL}^{-1}$  was prepared in acetonitrile. Further dilutions of this stock solution were used as working solutions to spike test samples and to establish calibration curves. The isotope-labeled solution  $^{13}\text{C}_{10}$  chlordecone (purity >98%), used as internal standard (IS) at  $100 \mu\text{g mL}^{-1}$  in nonane, was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Dilutions at 2500 and 250  $\text{ng mL}^{-1}$  of  $^{13}\text{C}_{10}$  chlordecone were prepared in acetonitrile. They were used for spiking samples and for calibration solutions, respectively. Calibration solutions were prepared in acetonitrile at concentrations between 0.005 and  $20.6 \text{ ng mL}^{-1}$  for chlordecone, each containing  $12.5 \text{ ng mL}^{-1}$  of  $^{13}\text{C}_{10}$  chlordecone.

## 2.3. Equipment

An HP1200 Series HPLC (Agilent) composed of a binary pump and an autosampler coupled to a 5500 Q-Trap mass spectrometer (AB Sciex, Darmstadt, Germany) with an electrospray ionization source (ESI) was used for all experiments. An Aqua  $\text{C}_{18}$  column ( $150.0 \text{ mm} \times 2.0 \text{ mm i.d.}$ ;  $3.0 \mu\text{m}$  particle size) provided with an Aqua  $\text{C}_{18}$  guard column ( $4.0 \text{ mm} \times 2.0 \text{ mm i.d.}$ ;  $3.0 \mu\text{m}$  particle size) supplied by Phenomenex (Torrance, CA, USA) was used for the chromatographic separation. An Eppendorf centrifuge 5810 (Hamburg, Germany) and a Genie vortex homogenizer supplied by Scientific Industries (Bohemia, NY, USA) were used for the extraction. A Mettler Toledo XS 204 balance supplied by Mettler-Toledo (Columbus, OH, USA) was used to weigh the samples.

## 2.4. Sample selection and collection

The samples came from an exploratory survey plan implemented in the French West Indies (Guadeloupe and Martinique) by the French authorities in charge of food control for the analysis of chlordecone in various bovine tissues. Bovines (200 in total) were selected from January to October 2016, according to 2 criteria: animals from farms located in areas where the soil is contaminated with chlordecone, or those from farms that had positive chlordecone results during previous official controls. Three tissues were collected from these bovines to assess chlordecone distribution: perirenal fat, and liver and skirt muscle (Lavison-Bompard et al., 2021). Of these 200 sampled animals, 121 were also subjected to blood sampling (50 from Martinique and 71 from Guadeloupe).

The statistical analysis for determining potential correlation factors between the concentrations of chlordecone in serum, fat, the liver and muscle was carried out on 121 “serum – fat – liver – muscle” quadruplets.

For optimization and validation of the analytical method, and quality controls, bovine serum was purchased from Sigma-Aldrich (Saint-Louis, MO, USA). The absence of chlordecone in this matrix was verified upstream.

## 2.5. Sample preparation

A specific protocol was performed immediately after blood collection to obtain serum. The animal blood was collected in a tube without anticoagulant (called a “dry” tube) and was decanted at room temperature for at least 2 h, or at  $4^\circ\text{C}$  for 4–5 h. After centrifugation, the obtained serum was transferred to another tube (such as a cryo

polypropylene tube) before being frozen. The samples were kept frozen during shipment and until analysis.

## 2.6. Sample extraction

Samples of homogenized bovine serum ( $1.50 \text{ g} \pm 0.02 \text{ g}$ ) were weighed in 50 mL centrifuge tubes.  $^{13}\text{C}_{10}$  chlordecone at  $2500 \text{ ng mL}^{-1}$  in the amount of 25  $\mu\text{L}$  were added to each test sample tube; the tube was shaken for about 5 s with a vortex homogenizer and kept in contact for 30 min. Then, 5 mL of acetonitrile were added to the mixture and stirred for 1 min with a vortex homogenizer, before centrifugation for 5 min at room temperature (centrifugal force  $>3000\times\text{g}$ ), to achieve simultaneous extraction of chlordecone with a residual protein precipitation from serum. The supernatant fluid was transferred to another 50 mL centrifuge tube containing 4 mL of water. The QuEChERS EN method extraction salts were then added and the mixture was vigorously shaken by hand for a few seconds, then vortexed for 1 min, before centrifugation for 5 min at room temperature (centrifugal force  $>3000\times\text{g}$ ). The supernatant organic phase was transferred into a 2 mL amber vial for HPLC-MS/MS analysis. A diagram of the extraction procedure is presented in Fig. 1.

## 2.7. HPLC-MS/MS instrumental analysis conditions

The HPLC-MS/MS conditions used in this study are based on the parameters optimized by Saint-Hilaire et al. (2018a). The chlordecone molecule and its isotopic standard ( $^{13}\text{C}_{10}$  chlordecone) were eluted at a constant flow rate of  $0.2 \text{ mL min}^{-1}$  through the column maintained at  $40^\circ\text{C}$ , using a mobile phase gradient, consisting of eluent A (Milli-Q water) and B (acetonitrile), each containing 0.1% of formic acid. After a column equilibration phase with 60% B for 5 min, the elution program began at 60% B, increased to 98% over 4 min and was kept constant for 8 min, then decreased to 60% B over 5 min. The injection volume was set at 5  $\mu\text{L}$ .

Electrospray ionization was run in negative mode (ESI-) and the ion spray voltage of the 5500 Q-trap was set at  $-4.5 \text{ kV}$ . The source temperature was set at  $600^\circ\text{C}$ . The curtain gas was set at 25 psi of nitrogen; the spray gas and auxiliary gas were set at 40 and 60 psi of air, respectively. The collision gas was set at “medium”. The 5500 Q-trap was used in multiple reaction monitoring mode. The monitored transitions for chlordecone were  $506.7 > 426.7$  (quantification transition) and  $508.7 > 428.7$  (qualification transition) with declustering potential set at  $-150 \text{ V}$ , entrance potential at  $-10 \text{ V}$ , collision energy at  $-25 \text{ eV}$ , and collision cell exit potential at  $-15 \text{ V}$ . The precursor ion corresponds to

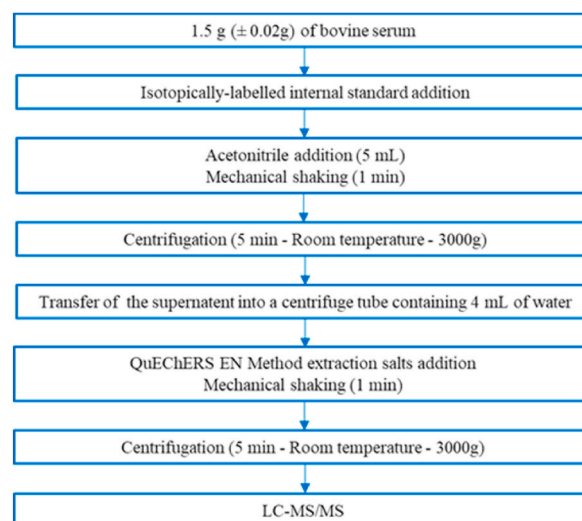


Fig. 1. Extraction procedure.

deprotonated hydrated chlordecone  $[M\cdot H_2O-H]^-$  and the product ion is the result of  $CO_2$  and  $HCl$  neutral losses  $[M\cdot H_2O-CO_2-HCl-H]^-$  (Bichon et al., 2015). The monitored transition for  $^{13}C_{10}$  chlordecone was  $516.7 > 435.7$  with declustering potential set at  $-165$  V, entrance potential at  $-10$  V, collision energy at  $-28$  eV, and collision cell exit potential at  $-10$  V. The dwell times for each transition were set at 280 ms.

Equipment control and data acquisition and processing were performed with Analyst 1.5.1 software (AB Sciex).

## 2.8. Method validation and QC routine analysis

The quantification was performed in solvent with the use of the internal isotopically labeled standard  $^{13}C_{10}$  chlordecone. The choice of the calibration function and the weighting model was statistically evaluated using a Fisher-Snedecor test, with a significance level of  $\alpha = 0.01$  and by plotting the studentized residuals (the model must have a minimum of studentized residuals between 2 and 3 in absolute value). The linearity of calibration curves was also studied by analysis of variance (ANOVA) tests, with a significance level of  $\alpha = 0.05$ .

The developed method for the analysis of chlordecone in bovine serum was validated according to the accuracy profile approach (AFNOR, 2010; Mermet and Granier, 2012) and followed the European Union guidelines specifications (SANTE/11312/2021, 2021). The accuracy profile is a statistical model for estimating the trueness and the intermediate precision of a method. It is based on the estimation of method accuracy through the evaluation of the total error (systematic and random error) and consists in building a graphical representation that makes it possible to confirm the choice of the best calibration function and to determine the validated concentration range. The results collected under intermediate precision conditions allow for the calculation of the tolerance interval based on a probability  $\beta$ , where a high proportion of future results will lie within acceptable limits ( $\pm \lambda$ ).

The validity domain can be defined between the limit of quantification (LOQ) and the upper tested concentration, as the  $\beta$ -expectation limits lie within the acceptance limits ( $\lambda$ ). The LOQ corresponds to the lowest validated level. The limit of detection (LOD) was set at one third of the LOQ.

For routine analysis, certain parameters are checked to ensure the quality of the results, and all defined criteria were set according to European recommendations (SANTE/11312/2021, 2021) and the French accreditation body recommendations (COFRAC, 2019). Quality controls were included for each sample batch, such as reagent blanks, calibration standards and two recovery check samples. In the absence of certified material, blank serum samples were spiked at levels covering the range of validity of the method (i.e.,  $0.05$  ng  $g^{-1}$  fw and  $50$  ng  $g^{-1}$  fw).

For each sample, retention times ( $\pm 0.1$  min between chlordecone and the internal isotopic standard ( $^{13}C_{10}$  chlordecone) and chlordecone ion ratio deviations between sample extracts and standards ( $\pm 30\%$ ) were systematically examined to ensure analyte identification.

Calibration (from  $0.005$  to  $21$  ng  $mL^{-1}$ ) was performed before and after sample analysis, and the response factor of bracketing calibration standards at each level should not differ by more than 20%. A reagent blank was prepared and injected under the same conditions to check the absence of any cross-contamination and of any peak interference, with a maximum signal tolerance corresponding to 30% of the LOQ. The recovery results for each level had to be between 70% and 120%. The recovery rate of the internal isotopic standard was also studied and was expected to be between 30% and 140%.

To validate the whole series, all the criteria had to be respected.

## 2.9. Statistical analysis

The descriptive statistic of chlordecone content in serum was performed using Microsoft Office Professional Plus Excel, version 2016 (Redmond, WA, United States). The statistical correlation study between chlordecone levels in the quadruplet samples of serum, fat, muscle and

liver was performed using Rstudio 1.2 software (R development Core Team, 2016). It included (i) a Shapiro-Wilk test to assess the normality of the residuals for the pairs serum-fat/muscle/liver, (ii) linear regressions to determine correlation factors between chlordecone content in serum vs. the other three matrices, (iii) prediction models, and (iv) their graphical representations. The statistical correlation study was carried out on quantified results (above the LOQ) only.

## 3. Results and discussion

### 3.1. Method optimization

The QuEChERS method is widely used for pesticide analysis (AFNOR, 2018) due to its efficiency and robustness, and because it avoids complex and time-consuming analytical steps. It is based on liquid-liquid extraction with acetonitrile in presence of salts that promote layer separation and the transfer of pesticides to the organic layer, possibly followed by purification by dispersive SPE (dSPE). However, optimizations may be required depending on the type of matrix and/or the analyte studied. This type of protocol has been successfully applied for the analysis of chlordecone in the urine and feces of ewes and in animal livers (Saint-Hilaire et al. 2018a, 2018b).

Optimization tests were thus carried out in the laboratory to optimize a suitable QuEChERS protocol for the extraction of chlordecone from bovine serum and to obtain a sufficient LOQ for our study. In order to assess the suitability and efficiency of the tested protocols, the recovery results of the different assays obtained with external calibration were compared to each other, and had to comply with the SANTE guidelines criterion of 70%–120%.

Due to the small volumes of serum samples available, the test portion was set at 1.5 mL and the volume of acetonitrile was reduced to 5 mL, instead of the usual volume of 10 mL. The usual amount of QuEChERS salts of 6.5 g (QuEChERS Extraction Packets EN Method) was then too high to obtain optimal layer separation between the aqueous and the organic layers. After centrifugation, clumping of the salts was observed without correct layer separation. Two options were considered: reducing the quantity of QuEChERS salts or adding water, miscible with the test portion. Optimizing the amount of QuEChERS salts was considered restrictive due to its unavailability in smaller commercial packaging, and its preparation in the laboratory is time-consuming. We decided to add 4 mL of water to obtain correct separation between the two layers.

The use of two different types of salts, mainly employed in pesticide analyses, was then compared: QuEChERS Extraction Packets EN Method, which are buffering salts to improve recoveries of pH-dependent analytes, and QuEChERS Extraction Packets Original Method. A total of  $3$  ng  $g^{-1}$  fw were supplemented on the matrix and the chlordecone recoveries obtained were comparable (96% for QuEChERS Extraction Packets EN Method and 95% for QuEChERS Extraction Packets Original Method). However, separation between the aqueous and organic layers was clearer with the use of the QuEChERS EN Method salts, which was retained.

Other parameters were investigated, in order to purify the extract, to decrease the matrix effects, and to obtain the lowest LOQ. The first parameter was the influence of clean-up of the extract. The purification sorbent used by Saint-Hilaire et al. (2018b) for the extraction of chlordecone in animal livers, namely Supel™ QuE PSA/C<sub>18</sub>/ENVI-Carb, was tested. Lower chlordecone recovery rates were obtained when using this clean-up phase, compared to non-purified extracts: 73% for a spiking level of  $3$  ng  $g^{-1}$  fw and 62% for a spiking level of  $0.1$  ng  $g^{-1}$  fw. In our case, this sorbent was not suitable for our analyte/matrix combination, especially as we observed pollution of the analytical chain while using the Supel™ QuE PSA/C<sub>18</sub>/ENVI-Carb phase.

As acetonitrile is very commonly used for QuEChERS extraction methods and is known to be an efficient organic solvent used in the clinical field to precipitate proteins (Polson et al., 2003), its addition at

the beginning of the protocol was tested in order to avoid a dSPE clean-up step. It was therefore added to the serum sample before the addition of water and the QuEChERS salts to allow precipitation of proteins and simultaneously extraction of chlordecone from the matrix. The chlordecone recovery rates obtained by this process were on average equal to 102% for a spiking level of  $0.05 \text{ ng g}^{-1} \text{ fw}$ . There were no differences in chlordecone recovery rates between this protocol and the usual QuEChERS protocol tested at the initial stage of method development (96% for a spiking level of  $3 \text{ ng g}^{-1}$ ), but the extract was cleaner and potentially free from matrix effects linked to serum proteins.

Finally, the concentration of the extract was tested, in order to decrease the LOQ. The extract was evaporated to dryness under nitrogen flow at a temperature below  $40 \text{ }^\circ\text{C}$  and then dissolved in acetonitrile. The recovery rates decreased to 42% for a spiking level of  $0.05 \text{ ng g}^{-1} \text{ fw}$ . These results, obtained at a very low concentration of chlordecone, were confirmed at a higher concentration of  $50 \text{ ng g}^{-1} \text{ fw}$  with a recovery rate of 38%. Moreover, yellowish deposits were formed during this step which were not dissolved by acetonitrile. A concentration step was therefore not further considered.

In summary, our method is based on the extraction of chlordecone, with simultaneous precipitation of residual proteins from the serum by acetonitrile, and addition of citrate QuEChERS salts.

### 3.2. Method validation results

The calibration function of chlordecone in acetonitrile was studied. Five calibration series, including nine concentration levels (ranging from  $0.005 \text{ ng mL}^{-1}$  to  $42.4 \text{ ng mL}^{-1}$ ) were prepared independently the same day and injected successively in a single run. Two weighting methods were tested: no weighting and  $1/x$  weighting. Regardless of the weighting method applied, the ANOVA was satisfactory with a significance level  $\alpha$  of 0.05. The calibration model chosen and applied throughout the study was a linear model with non-forced origin and  $1/x$  weighting (the model without weighting was rejected due to two outlier values ( $\geq 3$ ) among the studentized residues).

The accuracy profile was built on a range of concentration levels comprised between  $0.050 \text{ ng g}^{-1} \text{ fw}$  (the estimated LOQ) and  $51 \text{ ng g}^{-1} \text{ fw}$  of chlordecone spiked on blank bovine serum.

Three validation series were performed on three different days, and for each series, five spiked levels of concentration were tested in duplicate. The probability  $\beta$  was set to 85% for chlordecone, which means that the risk that future expected results would fall outside these limits was below 1% on average (Mermet and Granier, 2012). The acceptance limits ( $\lambda$ ) were set between 70% and 120% according to the SANTE/11312/2021 (2021) reference guideline. The obtained accuracy

profile representation and the performance criteria are presented in Fig. 2 and Table 1. All the  $\beta$ -expectation tolerance intervals were within the acceptability limits. The trueness bias ranged from 2.2% to 11.8%. The estimated repeatability (coefficient of variation  $\text{CV}_r$ ) varied from 0.7% to 4.5% and the intermediate precision (coefficient of variation  $\text{CV}_R$ ) varied from 2.2% to 6.3%, both below the limit value of 20% recommended in SANTE/11312/2021 (2021). The present method was therefore considered valid and reliable to accurately quantify chlordecone in fresh bovine serum between 0.050 (LOQ) and  $51 \text{ ng g}^{-1} \text{ fw}$ . The LOD has been set at  $0.017 \text{ ng g}^{-1} \text{ fw}$ .

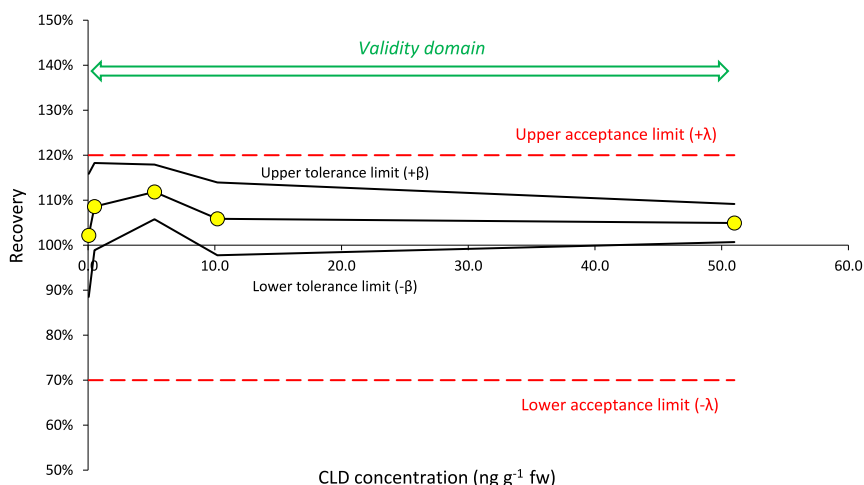
The results and performances presented in Table 1 are in accordance with the SANTE guideline (SANTE/11312/2021, 2021) (mean recoveries between 70% and 120%,  $\text{CV}_r$  and  $\text{CV}_R < 20\%$ ).

Our method with QuEChERS extraction for the analysis of chlordecone in bovine serum demonstrated enhanced sensitivity (LOQ at  $0.05 \text{ ng g}^{-1} \text{ fw}$ ) compared to most of the studies found in the literature, due to the use of HPLC-MS/MS rather than GC-ECD (and more rarely GC-MS/MS), and the use of isotopic dilution. The LOQs most often mentioned are between  $0.06 \text{ } \mu\text{g kg}^{-1} \text{ fw}$  ( $0.06 \text{ ng g}^{-1} \text{ fw}$ ) in lamb, goat, ewe and pig serum (Jurjanz et al., 2014; Lastel et al., 2016; Fournier et al., 2017; Saint-Hilaire et al., 2020; Fourcot et al., 2020), and  $0.75 \text{ ng mL}^{-1}$  ( $0.75 \text{ ng g}^{-1} \text{ fw}$  assuming that  $1 \text{ mL}$  of serum is equivalent to  $1 \text{ g}$ ) (Guldner et al., 2010) in human serum. The corresponding protocols were adapted from those for PCB analysis using a surrogate internal standard, and including numerous and longer steps for sample extraction (Debiez et al., 2003; Multigner et al., 2010).

Bichon et al. (2015) described a more sensitive method in HPLC-MS/MS using isotopic dilution. The LOQ determined at  $25 \text{ pg mL}^{-1}$  ( $0.025 \text{ ng g}^{-1} \text{ fw}$ ), is lower than our LOQ of  $0.05 \text{ ng g}^{-1} \text{ fw}$ , but this level does fit the needs of our study. The validated concentration range was determined to be between  $25 \text{ pg mL}^{-1}$  and  $5000 \text{ pg mL}^{-1}$  ( $0.025\text{--}5$

**Table 1**  
Method performance.

Concentration levels ( $\text{ng g}^{-1}$ , fw)	Mean Recovery (%)	Accuracy		
		Trueness (relative bias, %)	Repeatability ( $\text{CV}_r$ , %)	Intermediate precision ( $\text{CV}_R$ , %)
0.050	102	2.2	4.2	6.3
0.51	109	8.6	4.5	4.7
5.2	112	11.8	1.6	2.5
10	106	5.9	0.7	3.0
51	105	4.9	2.2	2.2



**Fig. 2.** Accuracy profile for chlordecone in fresh bovine serum with a probability  $\beta$  set at 85% and acceptance limits ( $\lambda$ ) set at 70% and 120%.

ng g<sup>-1</sup> fw), which is ten times less extended than our method (0.05–51 ng g<sup>-1</sup> fw). Sample extraction is also more complex and longer in the Bichon et al. (2015) protocol, with a prior hydrolysis step and a SPE.

In 2018, Saint-Hilaire et al. (2018a) published a rapid method for the quantification of chlordecone and its metabolites in a biological fluid (urine of ewes). The method developed in our laboratory is similar, based on QuEChERS extraction, but is followed by concentration of final extract and LC-MS/MS analysis. The LOQ was determined at 0.5 µg L<sup>-1</sup> (0.51 ng g<sup>-1</sup> fw, according to the density of ewe urine). The validated concentration range was from 0.5 to 200 µg L<sup>-1</sup> (0.51–203 ng g<sup>-1</sup> fw). The authors applied this method in ewe serum with slight modifications (Saint-Hilaire et al., 2021), but performance values of the method for chlordecone quantification in serum are not available. In 2023, Saint-Hilaire et al. (2023) developed an HPLC-MS/MS method with a similar QuEChERS extraction to analyze chlordecone in human serum. This method, validated between 0.06 µg L<sup>-1</sup> and 1.00 µg L<sup>-1</sup> (0.06 ng g<sup>-1</sup> and 1.00 ng g<sup>-1</sup> fw), is comparable to ours, but it includes an additional step of concentration of the extract to obtain a better sensitivity. The LOQ determined at 0.06 µg L<sup>-1</sup> (0.06 ng g<sup>-1</sup>) in human serum is similar to ours (0.05 ng g<sup>-1</sup>) in bovine serum. It corresponds to the LOQs often mentioned in the literature for the analysis of chlordecone in serum of animal origin (Jurjanz et al., 2014; Lastel et al., 2016; Fournier et al., 2017; Saint-Hilaire et al., 2020; Fourcot et al., 2020), but the QuEChERS extraction method is simpler and faster.

Bosman et al. (2021) described another type of analytical method in bovine serum, based on the use of a MIP, synthesized and designed for the chlordecone target molecule, coupled with HPLC-MS/MS analysis. The LOQ was estimated at 4.4 ng L<sup>-1</sup> (0.0044 ng g<sup>-1</sup> fw). It was determined by a signal-to-noise ratio, which reflects an instrumental LOQ, and not an experimental LOQ performed on samples that have undergone all analytical steps, as carried out in our method. The low LOQ is also explained by the use of MIP, which is an extremely selective support. In the absence of commercial MIP or appropriate laboratory synthesis equipment, our method is a suitable alternative because it is easy to apply and offers very low sensitivity, along with a broad concentration range (from 0.050 to 51 ng g<sup>-1</sup> fw).

### 3.3. Chlordecone content in bovine serum

As in the previous study (based on the same animal data set (Lavison-Bompard et al., 2021)), a quadruplet sample was removed from the statistical analysis because of its atypically high values (60/172/152/812 ng g<sup>-1</sup> fw in serum/fat/muscle/liver), far from the study area of the whole dataset (lying between “not quantified” (below the LOQ) and 22 ng g<sup>-1</sup> fw for serum). The descriptive statistics were thus performed on 120 serum results and are presented in Table 2. The data obtained for fat, muscle and liver were presented in our previous work (Lavison-Bompard et al., 2021). Data were processed according to the lower bound (LB) and upper bound (UB) hypotheses. Values below the LOD have been replaced by 0 in the LB hypothesis and by the LOD in the UB hypothesis; values between the LOD and LOQ have been replaced by the LOD in the LB hypothesis and by the LOQ in the UB hypothesis.

Of 120 analyzed samples, 88% (105 of 120) were detected (above the LOD) and 77% (92 of 120) were quantified (above the LOQ), which means that only a few results (12%) were censored. No differences were observed between the mean/medians/percentiles under the LB and UB scenarios, due to the low values of LOD and LOQ, highlighting high

method sensitivity, and robust and accurate suitability for risk assessment studies.

Whereas endemic chlordecone levels in bovine fat, liver and muscle are widely documented in the literature through many years of food monitoring (ANSES, 2017; Dubuisson et al., 2007; Lavison-Bompard et al., 2021), endemic chlordecone occurrence in bovine serum (through contaminated soil or water ingestion, as opposed to artificial contamination), have to our knowledge not been described. This is also the case in other animal species. Only chlordecone distributions in animals following artificial oral or intravenous supplementation are documented elsewhere (Fourcot et al., 2021; Fournier et al., 2017; Jondreville et al., 2014; Saint-Hilaire et al., 2021) and will be discussed in part 3.4. Other studies dealt with chlordecone measured in animal serum (Fourcot et al., 2020; Jurjanz et al., 2014; Lastel et al. 2016, 2018), still following artificial intravenous supplementation; however, these results could not be used for comparison since serum levels were either not given, or measured on freeze-dried samples.

### 3.4. Statistical correlation with fat, liver and muscle

The statistical correlation of the chlordecone content between serum on the one hand, and fat, liver or muscle on the other, was based on the concentrations measured in our previous work in fat, liver and muscle (Lavison-Bompard et al., 2021) and on the concentrations measured in serum samples in the present study. Only the quadruplets (serum – fat – liver – muscle) exhibiting chlordecone concentrations above the LOQ were considered for statistical correlation analysis. As explained in part 3.3, a quadruplet was excluded from this analysis, which was thus performed on a total of 248 quantified results, consisting in 56 serum/fat pairs, 71 serum/liver pairs and 50 serum/muscle pairs. The statistical correlation was carried out using the same methodology as in ANSES public notices (ANSES, 2018, 2019) which enabled the update of the MRL in meat, fat and offal from bovines, pigs, sheep, goats and poultry (Ministerial Decree, 2019; Commission Regulation, 2021).

Concentration ratios were calculated for each pair of tissues, and mean ratio of 6.5, 27.5 and 3.3 were obtained for fat/serum, liver/serum and muscle/serum, respectively. Because of a lack of studies on endemic chlordecone occurrences in animals, correlation comparisons could only be made on artificially contaminated animals (oral or intravenous administration, chlordecone being considered almost fully absorbed after oral administration in ruminants (Fournier et al., 2017)). Regarding the bovine species, no distribution data for chlordecone in different compartments, including serum, have been reported so far to our knowledge. Fourcot et al. (2021), who studied chlordecone toxicokinetics in ewes orally fed chlordecone, estimated linear models relating chlordecone concentrations between shaft muscle, liver and perirenal fat as  $C_{\text{tissue}2} = a C_{\text{tissue}1} + b$ . The intercept parameter “b” being statistically non-significant in this study, the slope parameter “a” can be assimilated with a concentration ratio that can be compared to ours. The correlations obtained between the tissues and serum in bovines and ewes, both ruminant species, follow the same trend, with fat/serum concentration ratios of 7.1 (ewes) and 6.5 (bovines), liver/serum concentration ratios of 26.1 (ewes) and 27.5 (bovines), and muscle/serum concentration ratios of 2.8 (ewes) and 3.3 (bovines).

Jondreville et al. (2014) reported chlordecone concentrations in serum, liver, abdominal fat, breast and leg muscles of laying hens orally exposed to chlordecone. The concentration ratios for the different

**Table 2**  
Descriptive statistics of serum chlordecone content according to the LB and UB hypotheses.

	Number of detections	Number of quantifications	Chlordecone concentration (ng g <sup>-1</sup> fw)							
			Minimum	Maximum	Mean	Standard deviation	P25	P50	P75	P95
LB hypothesis	105	92	0	22	1.5	3.0	0.051	0.23	2.1	4.8
UB hypothesis			0.017	22	1.5	3.0	0.051	0.23	2.1	4.8

LB, Lower bound; UB, Upper bound; P25, 25th percentile; P50, 50th percentile/median; P75, 75th percentile; P95, 95th percentile.

tissues versus serum varied, depending on the duration of exposure or depuration, whereas they remain constant between fat, liver and muscle. The steady-state was expected to be reached at 5 weeks of exposure. We thus calculated the concentration ratios for this time in order to compare to ours. The ratios obtained for laying hens were 3.5–6 times lower than those obtained for bovines, with values of 1.6 for fat/serum, 7.7 for liver/serum and 0.6 for muscle/serum. These differences may be attributed to different distribution pathways between poultry and bovines (as mammals), since chlordecone can be excreted through eggs (with high laying rates related to high excretion rates, Jurjanz et al. (2020), in the same way as for other similar contaminants, such as PCBs (Fournier et al., 2015).

The chlordecone concentration correlations obtained between serum and the other tissues were considered for potential application to risk management. In order to evaluate the relevance of the linear regressions, the normality of distribution of the residuals for the serum-fat/liver/muscle pairs was first assessed through a Shapiro-Wilk test. The distribution of the residuals for the serum/liver and serum/fat pairs was not considered normal with  $p$ -values of  $2.05e-12$  and  $2.03e-05$ , respectively below the reference  $p$ -value of 0.05. On the contrary, distribution was considered normal for the serum/muscle pair ( $p$ -value of 0.1141). In some cases, mathematical data transformations may help to obtain normal distributions. When concentration data were expressed as logarithms with base 10, the resulting Shapiro-Wilk test  $p$ -values ( $4.83e-08$  for serum/liver, 0.314 for serum/fat, and 0.510 for serum/muscle) showed that the distribution of the residuals was normal for the serum/fat and serum/muscle pairs. Despite a distribution of residuals that was still not normal for the serum/liver pair, logarithmic transformation of the data appeared to be more suitable than no transformation to obtain more reliable linear regressions and was thus applied to all data.

Linear modelling (not forced to zero) of logarithmic values of chlordecone concentrations in serum as a function of logarithmic values of chlordecone concentrations in fat, liver and muscle is presented in Fig. 3 and led to the following correlation equations:

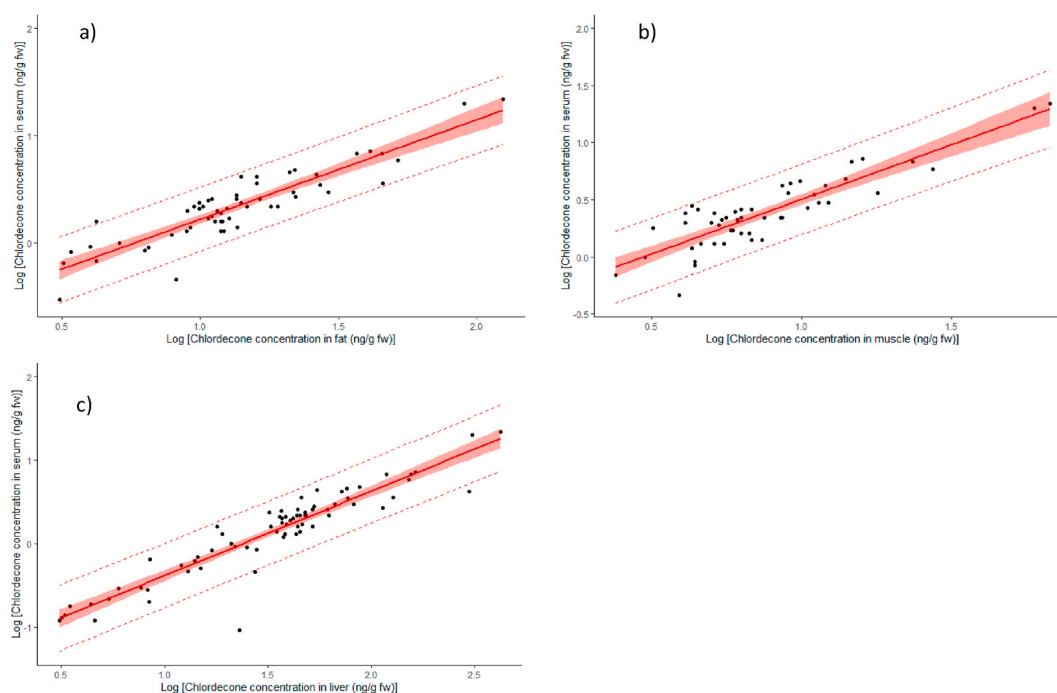
$$\text{Log } C_{\text{serum}} = 0.9541 \times \text{Log } C_{\text{muscle}} - 0.44964 \quad (R^2 = 0.7769)$$

$$\text{Log } C_{\text{serum}} = 0.92798 \times \text{Log } C_{\text{fat}} - 0.70491 \quad (R^2 = 0.8174)$$

$$\text{Log } C_{\text{serum}} = 1.01133 \times \text{Log } C_{\text{liver}} - 1.39174 \quad (R^2 = 0.8675)$$

where  $C$  is the chlordecone concentration in the different matrices.

Based on these transformed data, prediction intervals were calculated to estimate chlordecone concentrations in serum from a measured chlordecone concentration in fat, liver or muscle corresponding to the current MRL, taking into account the statistical variability of the correlation and a given confidence level. The current MRL for chlordecone in bovine muscle and liver is set at  $20 \text{ ng g}^{-1} \text{ fw}$  (Commission Regulation, 2021). Following the previous study (Lavison-Bompard et al., 2021), a management value of  $27 \text{ ng g}^{-1} \text{ fw}$  of fat was set based on a prediction interval calculated with a confidence interval of 95% (two standard deviations), corresponding to the MRL in muscle and liver (ANSES, 2018). The same confidence level (95%) was thus applied in the current study, and the target values used for calculating the prediction intervals were the MRL of  $20 \text{ ng g}^{-1} \text{ fw}$  for muscle and liver, and the management value of  $27 \text{ ng g}^{-1} \text{ fw}$  for fat. For a target value of  $20 \text{ ng g}^{-1} \text{ fw}$  in muscle, the concentration in serum lies between  $3.0$  and  $12.8 \text{ ng g}^{-1} \text{ fw}$  with a 95% confidence level, meaning that a concentration in serum of  $3.0 \text{ ng g}^{-1} \text{ fw}$  corresponds in 97.5% of cases to a concentration in muscle less or equal to  $20 \text{ ng g}^{-1} \text{ fw}$ . A target value of  $27 \text{ ng g}^{-1} \text{ fw}$  in fat corresponds to a concentration in serum lying between  $2.1$  and  $8.4 \text{ ng g}^{-1} \text{ fw}$  with a 95% confidence level. No prediction interval was calculated for the liver/serum pair because of the non-normal distribution of residuals, making the prediction not relevant. For information, prediction intervals were also calculated on non-transformed data and led to comparable results (chlordecone concentrations in serum lying within  $4.5$ – $8.4 \text{ ng g}^{-1} \text{ fw}$  for a target value of  $20 \text{ ng g}^{-1} \text{ fw}$  in muscle, and within  $2.2$ – $7.0 \text{ ng g}^{-1} \text{ fw}$  for a target value of  $27 \text{ ng g}^{-1} \text{ fw}$  in fat, with the same confidence level (still no prediction interval for the liver/serum pair was calculated). It is important to mention that the correlations established between the chlordecone concentrations in serum and muscle, and the calculated prediction intervals, apply only to the type of muscle sampled in this study, namely skirt muscle. Other muscles may have different composition, especially as regards fat content, and may lead to different correlation factors and prediction intervals. A similar



**Fig. 3.** Linear model and confidence intervals on means and observances for (a) serum/fat, (b) serum/muscle, and (c) serum/liver, based on logarithmic values of measured chlordecone concentrations (expressed in  $\text{ng g}^{-1} \text{ fw}$ ). The colored filled interval represents the confidence interval for the mean and the dashed lines represent the prediction interval, with a confidence level of 95%.



work was conducted on pigs (ANSES, 2019) and no significant impact of the type of muscle (skirt, flank, semimembranosus and longissimus dorsi) was observed in the fat/muscle correlations. This aspect should be investigated in a further work regarding the bovine species to reinforce the prediction models, limit the statistical uncertainty, and thus improve consumers' protection through dietary exposure.

#### 4. Conclusion

A sensitive HPLC-MS/MS method based on a QuEChERS protocol for quantifying chlordecone in bovine serum was optimized and validated, achieving an LOQ of 0.05 ng g<sup>-1</sup> fw. Based on the chlordecone concentrations found in 121 serum, fat, muscle, and liver samples collected from bovines bred in Guadeloupe and Martinique, prediction intervals were calculated for assessing chlordecone concentration ranges corresponding to the MRL (or mitigation level) in the different tissues, with a 95% confidence level. These prediction intervals led to a limit value of 3.0 ng g<sup>-1</sup> fw in serum in order not to exceed the MRL value of 20 ng g<sup>-1</sup> in muscle (limit value of 2.1 ng g<sup>-1</sup> fw in serum when considering the mitigation level of 27 ng g<sup>-1</sup> in fat). No relevant prediction interval could be calculated for the liver/serum pair. The robustness of the correlations and predictions may be improved by increasing the number of samples, and by extending the study to other types of muscles that have a different composition.

The validated analytical method and the prediction data may be helpful in supporting the chlordecone monitoring process in Guadeloupe and Martinique. The method could also potentially be used to assess the contamination level of a living animal, leaving the possibility open to depurate the animal when necessary prior its slaughter (e.g., by grazing in non-contaminated areas) until reaching chlordecone levels that are suitable for the consumption of meat.

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#### CRedit authorship contribution statement

**Marine Lambert:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Karelle Huby:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Julien Parinet:** Writing – review & editing, Visualization, Software, Methodology, Formal analysis, Data curation. **Thierry Guérin:** Writing – review & editing, Supervision, Data curation. **Gwenaëlle Lavison-Bompard:** Writing – review & editing, Validation, Supervision, Data curation, Conceptualization. **Chanthadary Inthavong:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization.

#### 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.

#### Data availability

Data will be made available on request.

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