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Correlation between endemic chlordecone concentrations in three bovine tissues determined by isotopic dilution liquid chromatography–tandem mass spectrometry

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Highlights

- Occurrence and concentrations of chlordecone in fat, muscle and liver in 200 bovines.
- Chlordecone concentrations are correlated in fat, muscle and liver.
- Correlation factors: 0.54 muscle/fat, 3.75 liver/fat, and 0.14 muscle/liver.

Abstract

Chlordecone (CLD) is an organochlorine pesticide widely used from the 1970s to the 1990s in the French West Indies that induced long-term pollution of the ecosystem. Due to involuntary soil ingestion, some species bred in open-air areas can be contaminated. As CLD is distributed in various tissues depending on the breeding species, this study focuses on the distribution of CLD in bovines. For this purpose, three tissues, i.e. fat, muscle, and liver, from 200 bovines originating from Martinique and Guadeloupe were sampled in 2016 to determine their endemic contamination levels. Analyses were performed with the official method for veterinary controls, isotopic dilution liquid chromatography–tandem mass spectrometry, which has been fully validated and which reaches a limit of quantification of 3 $\mu\text{g.kg}^{-1}$ fresh weight (fw). Irrespective of the matrices, CLD was detected in 68% of samples (404 samples above the LOD) and quantified in 59% of samples (332 samples above the LOQ). Regarding contamination levels, the liver had a broader range of concentrations (LOQ up to 420.6 $\mu\text{g.kg}^{-1}$ fw) than fat (LOQ up to 124.6 $\mu\text{g.kg}^{-1}$ fw) and muscle (LOQ up to 67.6 $\mu\text{g.kg}^{-1}$ fw). This confirms the atypical behaviour of CLD compared to other persistent organochlorine pollutants. Statistical processing demonstrated a correlation between CLD concentrations among the three studied tissues. The CLD concentration ratios were 0.54 for muscle/fat, 3.75 for liver/fat, and 0.14 for muscle/liver.

39 **Keywords:** Chlordecone, ID-LC-MS/MS, bovine tissues, correlation factors

1. Introduction

Chlordecone (CLD) is an organochlorine pesticide that was widely used in the French West Indies (Martinique and Guadeloupe) to protect banana plantations from black weevil (*cosmopolites sordidus*) between 1972 and 1993. CLD is a bi-cyclic chlorinated molecule. As its octanol/water coefficient exceeds 3, CLD is classified as a lipophilic pollutant. CLD also exhibits a ketone function (C=O) and the presence of a non-saturated carbon-carbon bond that can lead to keto-enol equilibrium.

CLD was classified in 2009 by the Stockholm Convention as a persistent pollutant. It is known to be carcinogenic, mutagenic and reprotoxic, and is suspected of being an endocrine disrupter (Multigner et al., 2010). In a cohort of a study carried out in Guadeloupe (Timoun cohort), an effect of CLD exposure was found on the duration of pregnancy (Hervé et al., 2016), and on the thyroid hormone system (Cordier et al., 2015), as well as impairment of fine motor function in boys (Boucher et al., 2013; Dallaire et al., 2012), and visual contrast sensitivity (Saint-Amour et al., 2020). For male adults, an increase in the risk of prostate cancer has also been documented (Multigner et al., 2010).

Due to the former use of this pesticide, certain Martinican and Guadeloupean environmental compartments are widely contaminated with CLD. Soil contamination, linked to former use of CLD, depends on soil composition (Cabidoche et al., 2009; Woignier et al., 2013). Studies on the fate of CLD have demonstrated low and poor degradation in soils with decontamination projected to take decades to centuries (Cabidoche et al., 2009). More recent studies have also indicated that transformation of CLD occurs in soils through highly complex pathways (Benoit et al., 2017; Chevallier et al., 2019; Clostre et al., 2015). Food crops (including vegetables) through root uptake (Clostre et al., 2015; Florence et al., 2015; Létondor et al., 2015), and ruminants, through involuntary soil ingestion, can also be contaminated by CLD (Clostre et al., 2014; Collas et al., 2019; Fournier et al., 2017).

Regarding food safety, in 2007, the French Food Safety Agency re-examined toxicological reference values based on previous studies in rats dating back to 2003. No changes were made: the chronic health-based guidance value was established at 0.5 µg/kg body weight/day, and the acute value at 10 µg/kg body weight/day. The impact of food consumption, including local food supply habits, was also examined based on consumption data (AFSSA, 2007): it was determined that setting maximum concentration limits in foodstuffs would be of limited effectiveness considering subsistence production (Dubuisson et al., 2007). Moreover, it was concluded that the maximum residue levels (MRLs) established by the European Regulation on MRLs of pesticides in and on food and feed of plant and animal origin (Commission Regulation (EU) No 212/2013, 212AD ; Commission Regulation (EC) No 839/2008) , provided sufficient protection of the population regarding CLD exposure via food of animal origin (ANSES, 2017). Concerning livestock, the target tissue for official controls is perirenal fat, since it is the tissue in which non-polar organochlorine pesticides are supposed to be mostly distributed. However, some studies have shown that CLD has atypical behaviour regarding its tissue distribution, and found that CLD is not only distributed in fat but also in muscle in certain species, such as poultry (Jondreville et al., 2014a), ducks (Jondreville et al., 2014b), growing male Alpine kids (Lastel et al., 2018), ewes Saint-Hilaire et al., 2018) and pigs (Fourcot et al., 2020). This raises the question of distribution in other species such as bovines, which are largely consumed and are one of the most targeted species for official food controls.

CLD concentrations can be determined either by gas or liquid chromatography; methods are mostly adapted from Bordet et al. (Bordet et al., 2007). For gas chromatography, detection can be performed with an electron capture detector (GC/ECD) or mass spectrometry, with low or high resolution (Clostre et al., 2014; Florence et al., 2015; Fournier et al., 2017; Lastel et al., 2018; Méndez-Fernandez et al., 2018). This second type of detection is recommended in the case of positive results (i.e. > the limit of quantification - LOQ) for confirmation purposes.

Although gas chromatography is still used, most of the current methods, including the official method for controls in France, are based on isotopic dilution followed by reversed-phase liquid chromatography analysis and tandem mass spectrometry detection with negative electrospray ionization (Clostre et al., 2015; Lastel et al., 2018; Saint-Hilaire et al., 2018). Isotopic dilution is recommended to ensure accurate identification and quantification. Recent publications also report the use of high-resolution mass spectrometry with LC separation for the identification of CLD transformation products in the environment through biodegradation or food processing (Martin et al., 2020; Ollivier et al., 2020).

The present study aims at determining CLD levels in fat, muscle and liver tissues of 200 endemically contaminated bovines originating primarily from the most contaminated areas of Martinique and Guadeloupe. The analytical method used was the official method for monitoring controls in France, and is based on isotopic dilution liquid chromatography hyphenated with mass spectrometry (ID-LC-MS/MS). These data were then used to assess the correlations between CLD concentrations in fat, muscle and liver.

2. Material and methods

2.1. Chemicals and reagents

All solutions were prepared with analytical reagent grade chemicals and ultrapure water (18.2 MΩ.cm) obtained by purifying distilled water with a Milli-Q system (Merck Millipore, Saint-Quentin-en-Yvelines, France). All solvents (acetone, acetonitrile, dichloromethane, hexane, and methanol) were HPLC grade and were purchased from Fisher Scientific (Illkirch, France). Sodium hydroxide and formic acid (98%) were also purchased from Fisher Scientific (Illkirch, France). CLD (98%) and CLD ¹³C₁₀ (98%) standards were purchased from Cluzeau Info Labo (Sainte-Foy-La-Grande, France). Working solutions were prepared by dilution of the commercial standards in methanol before use, to prepare calibration standards and fortify the samples for quality controls and method validation. All solutions were stored at 5 ± 3°C.

116

117 2.2. Equipment

118 LC-MS/MS measurements were performed using a 1200 series HPLC binary pump system
119 (Agilent Technologies, Courtaboeuf, France) coupled with an API 5500 QTRAP hybrid tandem
120 mass spectrometer (AB Sciex, Les Ulis, France). An Aqua C18 column (150 mm x 2.0 mm i.d.,
121 3 µm particle size) equipped with an Aqua C18 guard column (4.0 mm x 3.0 mm i.d., 3 µm
122 particle size) was used for chromatographic separation. An Eppendorf centrifuge 5810 (Sigma
123 Aldrich, Saint-Quentin-Fallavier, France), a refrigerated Hettich 32 R centrifuge (Sigma
124 Aldrich, Saint-Quentin-Fallavier, France), and a Genie 2 vortex (Scientific Industries, Bohemia,
125 NY, United States) were used for the extraction. A Grindomix GM 200 (Retsch Industries,
126 Haan, Germany) and a Polytron PT 3100 (Fisher Scientific, Illkirch, France) were used for
127 sample preparation. An IKA Ultraturrax T25 digital Disperser (Retsch Industries) was used for
128 both sample preparation and extraction. An XS 204 balance (Mettler-Toledo, Viroflay, France)
129 was used to weigh the samples. A water bath heater from Fisher (Fisher Scientific, Illkirch,
130 France) was used for fat melting. A Reacti-Therm Pierce heating module (Thermofisher, Les
131 Ulis France) was used for solvent evaporation.

132

133 2.3. Sampling

134 From January to October 2016, an oriented exploratory survey plan was implemented by the
135 French authorities in charge of food control (Directorate for food, agriculture and forestry of
136 Guadeloupe and Martinique) toward the most probable contaminated bovines in the French
137 West Indies. In all, 200 bovines were selected, mostly in Martinique (120 bovines). In
138 Guadeloupe, open-air farms located in the most contaminated areas were chosen, whereas in
139 Martinique, animals were selected as a priority when the livestock was found to have positive
140 results in previous official control plans. Livestock number, age, and sex of the animals were

reported, as well as slaughterhouse locations (18 locations for Martinique and a single location for Guadeloupe). For each bovine, the three tissues – perirenal fat, skirt for muscle, and liver – were sampled and clearly identified for traceability.

For quality controls and method validation, liver and muscle samples were purchased from a local supermarket (Choisy-le-Roi, France) and perirenal fat was collected from a local butcher (Saint-Maur-des-Fossés, France). These matrices were determined to be CLD-free.

2.4. Sample preparation and extraction

The analytical procedure employed in this study was adapted from (Bordet et al., 2007), with minor changes regarding sample preparation and major changes regarding separation and detection: GC-ECD was switched to LC-MS/MS². This is the official method to be implemented in France for monitoring and control plans for food of animal origin by approved laboratories.

2.4.1. Preparation and extraction procedure for fat

Frozen fat samples were cut into pieces, blended with a Grindomix apparatus, heated to a maximum of 60 °C and filtered through a strainer to clean samples of residues. A total of 0.5 g was precisely weighed in a test tube. Then, 75 µL of internal standard CLD-¹³C₁₀ at a concentration of 0.5 ng.µL⁻¹ was added to melted fat. After 1 min vortex-stirring, the internal standard was kept in contact for 1 h with the matrix. CLD was extracted from the melted fat sample 3 times with 3 mL of a mix of acetonitrile/dichloromethane 75/25 (v/v). For each extraction, the test tube was briefly shaken with a vortex homogenizer and then centrifuged for 20 min at 1,200 g at a temperature of -20 °C. The supernatants were collected into another test tube and solvent was evaporated to dryness at a temperature not exceeding 40 °C under a gentle stream of nitrogen. The dry extract was dissolved in 15 mL of a mixture of hexane/acetone 85/15 (v/v) and then transferred to a 50 mL centrifuge tube. 5 mL of 0.5 M aqueous sodium

hydroxide solution was added, and the tube was vortexed for 15 s. The lower aqueous phase was collected in another centrifuge tube. This extraction step was repeated twice, and the three aqueous phases were combined in the same tube, and then washed with 5 mL of hexane by gentle manual shaking. The hexane phase was discarded after a 3 min centrifugation step at 750 g. Then, 5 mL of aqueous sulfuric acid solution at 60% were added to the aqueous extract, and the tube was shaken for 15 s with a vortex homogenizer and gently left to cool at room temperature. CLD was extracted 3 times with 5 mL of a mixture of hexane/acetone 85/15 (v/v) by shaking for 15 s with a vortex homogenizer, then centrifuged for 3 min at 750 g, and the upper organic phases were collected in a 15 mL centrifuge tube and washed with 2 mL of ultrapure water. After final centrifugation for 3 min at 750 g, the organic phase was evaporated to dryness at a temperature not exceeding 40 °C under a gentle stream of nitrogen. The dry extract was dissolved in 1 mL of methanol and transferred to a vial for chromatographic analysis.

2.4.2. Preparation and extraction procedure for muscle and liver

Due to the lower fat content of liver and muscle compared to perirenal fat, a different sample preparation method was used. Samples were cut into pieces and homogenized first with a Grindomix apparatus, then a Polytron and a T25 Ultra-Turrax apparatus. Then, 2 g of homogenized sample were precisely weighed in a 50 mL centrifuge tube before the addition of 50 µL of internal standard CLD-¹³C₁₀ at a concentration of 0.6 ng.µL⁻¹. After 1 min vortex-stirring, the internal standard was kept in contact for 1 h with the matrix. CLD was extracted by 10 mL of a mixture of hexane/acetone 85/15 (v/v) by using a T25 Ultra-Turrax for 1 min at 10,000 rpm. The organic phase was collected in another 50 mL centrifuge tube. The extracts then followed the same basic and acidic transformations as for the fat extracts.

2.5. LC-MS/MS analysis

A total amount of 5 μL of the individual final extract was injected into the chromatographic system at room temperature (Phenomenex, Le Pecq, France). Ultrapure water containing 0.1% (v/v) formic acid (mobile phase A) and methanol containing 0.1% (v/v) formic acid (mobile phase B) were used for the gradient elution at a constant flow rate ($200 \mu\text{L min}^{-1}$). The gradient started with 40% of phase B and increased to 100% within 6 min. Phase B was kept constant for the next 8 min and was then set back to 60% in 2 min before a 5 min equilibrium time, before the next injection.

Electrospray ionization was run in negative mode. The spray voltage was set at -4,500 V. The curtain gas was set at 25 psi of nitrogen. Spray gas and auxiliary gas were set at 40 and 60 psi of air, respectively. The collision gas was set at “Medium” value. The source temperature was set at 600°C . The instrument was operated in multiple reaction-monitoring mode (MRM). CLD and its isotopic standard were detected under the deprotonated hydrated form (Bichon et al., 2015). The quantifying and qualifying transitions for CLD were $506.7 > 426.7$ and $506.7 > 428.7$, respectively. For these two transitions, the decluttering potential was -160 V, entrance potential -10 V, collision cell exit potential -15 V, and collision energy -25 eV. The transition for CLD- ^{13}C was $516.7 > 435.7$ with the same parameters, except the collision energy of -28 eV. The transition for CLD- ^{13}C was $516.7 > 435.7$ with the same parameters, except the collision cell exit potential -10 V, and the collision energy -30 eV. The dwell time for each transition was set at 100 ms. Analyst 1.51 Software (AB Sciex) was used for system control and data acquisition and processing.

2.6. Method validation and quality assurance/quality control

The method was validated as per standard NF V03-110: 2010 (AFNOR, 2010), based on tolerance intervals and overall determination of the performance of the method through the accuracy profile (Mermet and Granier, 2012).

216 The accuracy profile provides a graphical representation of the risk of error for each
217 concentration level. The validated range is defined as the domain where β -expectation limits lie
218 within the acceptance limits (λ). This method also evaluates the performance of the calibration
219 model, the accuracy, and the uncertainty. The acceptance limits (λ) were set to 70-120% in
220 accordance with European Union guidelines (SANTE/11945/ 2015, 2015). Probability β was
221 set to 80%, meaning that the risk of results lying outside the limits is below 20% on average.
222 Initially, the method was validated and implemented to cover two commodity groups mentioned
223 in the SANTE document: “Fat from food of animal origin” and “Meat (muscle) and seafood”.
224 For the second commodity group, as fishes were targeted for official controls, it was decided to
225 select barracuda, a fish consumed in the French West Indies. Since (i) sample preparation is
226 identical for fish and meat samples, and (ii) the analysis is based on isotopic dilution, the method
227 was considered to be validated for the whole commodity group. This was confirmed by the
228 conformity of routine quality control, implemented for the present study, including recoveries
229 on meat as detailed below. Later, the method was extended to liver tissues. Therefore, three
230 separate profiles were built: one for the “meat and seafood” commodity, one for liver, and one
231 covering fat regarding its specific preparation. Five series spiked at 4 levels (3 levels for liver)
232 from 3 to 500 $\mu\text{g.kg}^{-1}$ fw were analyzed, each on different days in duplicate. The performances
233 of the method are presented in [Table 1](#).

234 An overall limit of quantification (LOQ) (3 $\mu\text{g.kg}^{-1}$ fw) was experimentally tested and the range
235 of application was tested up to 500 $\mu\text{g.kg}^{-1}$ fw, with success. Compliance of the test
236 concentrations with performance requirements laid down in the SANTE document
237 ([SANTE/11945/ 2015, 2015](#)) was checked, and the accuracy profiles were validated. The LOQ
238 is consistent with previous studies (Lastel et al., 2018; Saint-Hilaire et al., 2018) and the
239 European regulation on MRLs of pesticides ([Commission Regulation \(EU\) No 212/2013](#),

212AD ; Commission Regulation (EC) No 839/2008), which sets the MRL at 100 $\mu\text{g.kg}^{-1}$ fw for fat, muscle and liver.

The limit of detection (LOD) was set according to the calibration model used for quantification with standard solutions and to the enrichment factor between the matrix and the extracts: it was set at 2 $\mu\text{g.kg}^{-1}$ fw for fat, and 0.5 for $\mu\text{g.kg}^{-1}$ fw for muscle. For liver, the LOD was set at one third of the LOQ, i.e. 1 $\mu\text{g.kg}^{-1}$ fw.

The repeatability coefficients of variation for all matrices were within the range 1.2–8.3%, all concentration levels considered. The intermediate precision coefficients of variation ranged from 2.5 to 9.9%. Both criteria met the requirements of the SANTE Guidelines (SANTE/11945/2015, 2015), which recommend a maximum value of 20%.

The expanded uncertainty of the method was estimated taking into account the standard deviation of the tolerance interval and an estimation of the bias. It was set at 20 %, corresponding to the maximum estimated uncertainty for each matrix and tested concentrations. For routine analysis, quality controls were included in each sample batch, with criteria recommended by SANTE guidelines (SANTE/11945/ 2015, 2015). Reagent blanks were injected after each sample to check the absence of cross-contamination, with a tolerance corresponding to 30% of the LOQ. Bracketing calibration (6 levels) was performed, and linearity was controlled by residual deviation below 20% from the calibration curve. In every sample batch, recovery at a level corresponding to 1.5 x LOQ was checked to be in the 70–120% range. Retention times (± 0.1 min between CLD and CLD ^{13}C retention times) and ion ratio deviations ($\pm 30\%$) were systematically examined to ensure correct analyte identification in case of a result above the LOQ. Results were validated only if all criteria were within the acceptable limits.

Some of the analyses (73 out of 200 whole triplets) were subcontracted to the laboratory Inovalys, Le Mans, France. This laboratory, belonging to the network approved for French

official controls, applied the same official method described above after in-house validation and demonstration of equivalent performance. In both laboratories, analyses were performed under the international standard ISO/IEC 17025:2005 (ISO/IEC 17025, 2005) by qualified operators.

2.7. Statistical analysis

The correlation study of the CLD concentrations in the three tissues was performed in multiple steps. In order not to skew the determination of the correlation factors, results below the LOQ were not included in the statistical analysis. For dataset description, in the lower bound scenario, zero and the LOD values replaced results below LOD and LOQ, respectively whereas in the upper bound scenario, these results were replaced by LOD and LOQ, respectively. A Shapiro–Wilk test was used to determine the normality of the concentration distributions for each of the three studied tissues. Pearson tests (SPSS software, v 23.0 (IBM, New York, NY, United States)) were used to assess the correlations of CLD concentrations in the triplets (fat, muscle and liver). The statistical significance was set to $p < 0.05$. Linear regressions, prediction models, and graphical representations of the correlations were performed with Studio software, v 1.2 (R Development Core Team, 2016).

3. Results and discussion

3.1. Occurrence of CLD content in bovine perirenal fat, muscle, and liver

The main statistical descriptors are presented in [Table 2](#), and [Figure 1](#) illustrates the distribution of the determined concentrations. The whole dataset is available in the Supplementary Information. The most contaminated triplets exhibit atypically high values compared to the whole dataset: CLD concentrations in liver and muscle ($812 \mu\text{g.kg}^{-1}$ fw and $152 \mu\text{g.kg}^{-1}$ fw) are almost two fold higher than for the penultimate triplet ($420.6 \mu\text{g.kg}^{-1}$ fw and

67.6 $\mu\text{g.kg}^{-1}$ fw). This triplet was excluded from the dataset for description and statistical analysis. Irrespective of the type of matrix, CLD was detected in 68% of samples (404 samples above LOD) and quantified in 59% of samples (332 samples above LOQ). Considering the three tissues, CLD was mostly quantified in liver (65%, 130 out of 199 samples), followed by fat (53%, 106 out of 199 samples), and muscle (48%, 96 out of 199 samples). Regarding contamination levels, liver covers a broader range of concentrations (LOQ up to 420.6 $\mu\text{g.kg}^{-1}$ fw) than fat (LOQ up to 124.6 $\mu\text{g.kg}^{-1}$ fw), and muscle (LOQ up to 67.6 $\mu\text{g.kg}^{-1}$ fw), as mentioned in Table 2.

For muscle and liver, a previous study regarding food exposure of Martinicans and Guadeloupeans reported that CLD was detected in 19% of beef meat samples (n=38) ([Dubuisson et al., 2007](#)). The samples were collected during a sampling plan that was not only focussed on contaminated areas (without precision) and was organised between 2002 and 2004. The type of tissue collected was not detailed. The observed concentration range started at 0.83 $\mu\text{g.kg}^{-1}$ fw for the average lower-bound residue level, and at 1.16 $\mu\text{g.kg}^{-1}$ fw for the average upper-bound, and reached up to 2.88 $\mu\text{g.kg}^{-1}$ fw for the highest values. The higher detection occurrence (59% for muscle and 85% for liver) and contamination ranges in the present study are difficult to compare since they might be linked to a bias in sample collection, since it is oriented toward most-probable contaminated bovines in the present study.

Considering muscle tissue, the most consumed part, the results can be compared with those reported in the Kannari study ([ANSES, 2017](#)), where meat samples were collected between 2009 and 2015 from different supply chains, including slaughterhouses, local markets, subsistence production, and medium and large wholesalers. The Kannari study differentiates contaminated and non-contaminated areas of Martinique and Guadeloupe. In slaughterhouses, 64% (n=293) of the samples were above the LOQ of 1–10 $\mu\text{g.kg}^{-1}$ fw in contaminated areas.

315 These figures are consistent with those found in the present study oriented toward contaminated
316 areas: 48%, 96 samples out of 199 above the LOQ for muscle, and 65%, 130 samples out of
317 199 for liver. The median level calculated in the Kannari study ($11 \mu\text{g.kg}^{-1}$ fw in contaminated
318 areas) was higher than the median level of $4.1 \mu\text{g.kg}^{-1}$ fw found in the present study. Moreover,
319 considering the lower and upper bound hypotheses, the same trend is observed (mean
320 concentrations of 5.8 and $6.2 \mu\text{g.kg}^{-1}$ fw versus 0.6 and $4 \mu\text{g.kg}^{-1}$ fw in the Kannari study). The
321 differences regarding these figures may be due to the type of animals sampled (not detailed in
322 the Kannari study) and to the sampling strategy that included imported commodities for the
323 purpose of the Kannari plan, whereas only local production was considered in the present work.
324 Regarding other species, our results in endemically contaminated bovines are similar to those
325 reported in endemically contaminated pigs originating from Guadeloupe ([ANSES 2019](#)). In pig
326 skirt sample ($n=77$), CLD concentrations ranged from 10 to $56 \mu\text{g.kg}^{-1}$ fw with a median at 23
327 $\mu\text{g.kg}^{-1}$ fw. In three other muscles (longissimus dorsal muscle, semi-membranous muscle and
328 flank) collected on another set of 15 pigs, the concentration range was also similar (11 – 34
329 $\mu\text{g.kg}^{-1}$ fw and median within 18 – $27 \mu\text{g.kg}^{-1}$ fw). These figures document the potential endemic
330 background contamination range of non-artificially fed bovines and pigs bred in Martinique
331 and Guadeloupe. Another study measured CLD concentrations in fat, legs and liver of free-
332 range ducks bred in endemically contaminated areas of Martinique ([Jondreville et al., 2014b](#)).
333 Again, concentration ranges are consistent with those reported in the present study: 47 – $1,215$
334 $\mu\text{g.kg}^{-1}$ fw for liver, 9 – $278 \mu\text{g.kg}^{-1}$ fw for fat, and 6 – $145 \mu\text{g.kg}^{-1}$ fw for legs (without skin). In
335 duck tissues, livers were also more highly contaminated than fat and muscle.

336 The distribution of CLD in fat, muscle and liver has also been reported to document the
337 contamination and/or decontamination kinetics for male Alpine kids ([Lastel et al., 2018](#)),
338 Muscovy ducks ([Jondreville et al., 2014b](#)) and pigs ([ANSES 2019](#)). It was also documented for
339 poultry, where liver and fat were investigated ([Jondreville et al., 2014a](#)). The same structure of

contamination was observed: liver followed by fat and muscle. Since the above-mentioned studies concerned artificially contaminated animals, the comparison of both CLD levels and occurrence is not relevant.

The contamination of all studied tissues, both occurrence and concentration levels, documents the atypical behaviour of CLD in different species, including bovines. It is distributed in different tissues and does not accumulate mainly in fat. This raises the question of a potential correlation factor between CLD concentrations in tissues.

3.2. Statistical correlation between tissues

For the purposes of the correlation study, only quantified results in the three tissues were taken into account, thus limiting the dataset to 96 animals. As mentioned above, the most contaminated triplet (concentrations in fat/muscle/liver: 172/152/812 $\mu\text{g.kg}^{-1}$ fw) was excluded from data processing.

As mentioned in [Table 3](#), Pearson correlation coefficients were above 0.884, indicating a strong correlation. Moreover, a significance test was performed: values were below 0.01, which supports the strong correlation.

A linear relationship of the observed CLD concentrations was found for muscle/fat, muscle/liver, and liver/fat. The intercept was forced to zero to avoid negative values at low concentrations. The slope of the curves represents the CLD correlation factors between the tissues ([Figure 2](#)). The CLD concentration ratios were estimated: 0.54 for muscle/fat, 3.75 for liver/fat, and 0.14 for muscle/liver. The coefficients of correlation (R^2) of the model were 0.973, 0.901, and 0.906, respectively. To go further, prediction intervals were also determined, based on the dispersion of the measurements ([ANSES 2018.](#)). Depending on a two standard deviations confidence level, it was possible to determine a prediction range of CLD concentration in fat (tissue considered for controls) from a target concentration in skirt or liver (as consumed parts).

The value targeted in skirt was set at $20 \mu\text{g.kg}^{-1}$ fw on the basis of national authorities' recommendations. For the two standard deviation interval, the CLD concentration in fat ranged from 27 to $47 \mu\text{g.kg}^{-1}$ fw: a CLD concentration of $27 \mu\text{g.kg}^{-1}$ fw in fat was associated at 97.5% to a CLD concentration below the target value of $20 \mu\text{g.kg}^{-1}$ fw in skirt. For the three standard deviation interval, the range was 32 to $42 \mu\text{g.kg}^{-1}$ fw: a CLD concentration of $32 \mu\text{g.kg}^{-1}$ fw in fat was associated at 84% to a concentration of CLD below the target value of $20 \mu\text{g.kg}^{-1}$ fw in skirt. For liver, it was not possible to determine a range of concentrations of CLD in fat corresponding to $20 \mu\text{g.kg}^{-1}$ fw in liver for any of the two confidence levels (95 nor 68%). It was only possible to conclude that a concentration of $20 \mu\text{g.kg}^{-1}$ fw in liver, corresponded to a value of $5 \mu\text{g.kg}^{-1}$ fw in fat in average. These prediction interval were used to define a CLD mitigation concentration of $27 \mu\text{g.kg}^{-1}$ fw in fat for official control plans ([Ministerial decree of Mai the 23rd, 2019](#)), corresponding to a MRL of $20 \mu\text{g.kg}^{-1}$ fw in muscle and liver.

As no data on CLD distribution in fat/muscle/liver are reported in the literature for endemically contaminated bovines, these results were compared to those obtained for other species, i.e. ducks and pigs. The observed ratios of CLD concentrations in liver/fat and muscle/liver for bovines match those reported by Jondreville et al. ([Jondreville et al., 2014b](#)) on fresh matter for Muscovy ducks: 3.9 for liver/abdominal fat, 0.6 for muscle with skin/fat, and 0.5 for muscle without skin/fat. The muscle/fat ratio was also modelled with an intercept not forced to zero for endemically contaminated pigs from Guadeloupe (n=77 animals), where skirt muscle was investigated, and another set of pigs (n=15 animals) where skirt muscle but also three other types of muscles were considered (longissimus dorsi muscle, semi-membranous muscle, and flank) ([ANSES 2019](#)). A muscle/fat ratio of 0.64 was determined, considering the two groups of animals, and thus including muscle type variability. This ratio is similar to that modelled in the present study, even though animals and muscle types are different.

Fourcot et al ([Fourcot et al., 2020](#)) modelled the CLD distribution in two breeds of growing pigs (Large White and Creole pigs) contaminated with intravenous administration of CLD (n=7 for each breed). To allow comparison with our data, we calculated a muscle/fat ratio from the published figures in Fourcot *et al.*: total amount of CLD estimated in muscle or fat (Muscle: 8.9 mg for Creole pigs and 12.5 mg for Large White pigs- Fat: 13.2 mg for Creole pigs and 10.0 mg for Large White pigs) is divided by muscle or fat percentage of the animals respectively (Muscle: 43.3 % for Creole pigs and 54.8% for Large White pigs. Fat: 34.7% for Creole pigs and 22.4% for Large White pigs). Muscle/fat ratios are similar between bovine (0.54), Creole pigs (0.54) and Large White pigs (0.51).

The CLD tissue distribution in artificially fed growing kids was also assessed ([Lastel et al., 2018](#)). The observed ratios (liver/fat: 31 during the contamination period, 28 during the decontamination period – muscle (skirt) /fat: 0.5 during the contamination period and 0.6 during the decontamination period) are very different from those estimated in the present bovine study: the liver/fat ratio is 55 times higher and the skirt muscle/fat ratio is 7 times lower in goats. These differences could be linked to the general tissue composition of the different studied species, including the differences in lipid classes, the overall fat content of the whole animal, as mentioned by Lastel *et.al.*, and the metabolic pathways of CLD, as mentioned above.

4. Conclusion

An in-house validated ID-LC-MS/MS method was used to analyze CLD in 200 samples of bovine fat, liver and muscle (skirt) originating from contaminated areas of Martinique and Guadeloupe. CLD was mostly quantified in liver (65%), followed by fat (53%), and muscle (48%). These results confirmed that CLD can be distributed in the studied bovine tissues, as previously reported for goats, poultry and ducks. Strong CLD concentration correlations were found between these three tissues, with concentration ratios estimated at 0.54 for muscle/fat,

3.75 for liver/fat, and 0.14 for muscle/liver. These correlations were used to define a CLD mitigation concentration of 27 $\mu\text{g.kg}^{-1}$ fw in fat for official control plans (Ministerial decree of Mai the 23rd, 2019), corresponding to an MRL of 20 $\mu\text{g.kg}^{-1}$ fw in muscle and offal. Moreover, those data might be the support for MRL revision at European level.

It would be interesting to include other types of muscle tissue in a similar study, to take into account their different composition, including fat content. This could reinforce the correlation factors to further support consumer protection through dietary exposure.

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Boxes represent the concentrations between percentiles 25 and 75. The line dividing the box represents the median concentration.

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Figure 2: Linear model and confidence intervals on means and observances for muscle/fat and liver/fat correlations of CLD concentrations. Concentrations are expressed on fresh weight.

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Conflicts of interest

The authors declare no conflict of interest.

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Table 1: Analytical performances of the method

Matrix		Accuracy			
		Mean recoveries	Trueness	Precision	
				Repeatability	Intermediate precision
	CLD concentration levels (µg.kg ⁻¹ fw)	(%)	(relative bias %)	(%)	(%)
Perirenal fat	3	109	8.5	2.1	2.5
	10	108	7.5	1.2	2.8
	100	104	4.1	2.6	5.8
	500	99	-1.4	2.5	8.1
Meat and seafood	Liver	3	99	-1.1	4.6
		20	102	2.5	4.3
		500	106	5.9	3.5
	Other commodities	3	100	0.4	4.3
		10	101	1.1	1.7
		20	99	-0.7	3.3
		500	92	-7.9	3.6
					7.7

Table 2: Main descriptors of the dataset (n=199 animals)

	Number of detections ^a	Number of quantifications ^b	Concentration ($\mu\text{g.kg}^{-1}$ fw)				
			Maximum	Lower bound hypothesis		Upper bound hypothesis	
				Mean	Median	Mean	Median
Fat	117	106	124.6	11.4	4.2	12.2	4.2
Muscle	117	96	67.6	5.8	0.5	6.2	3.0
Liver	170	130	420.6	40.0	19.0	40.5	19.0

a:>LOD

b: >LOQ

Table 3: Pearson correlation coefficients

	Fat	Liver
Fat	-	0.884
Muscle	0.974	0.871

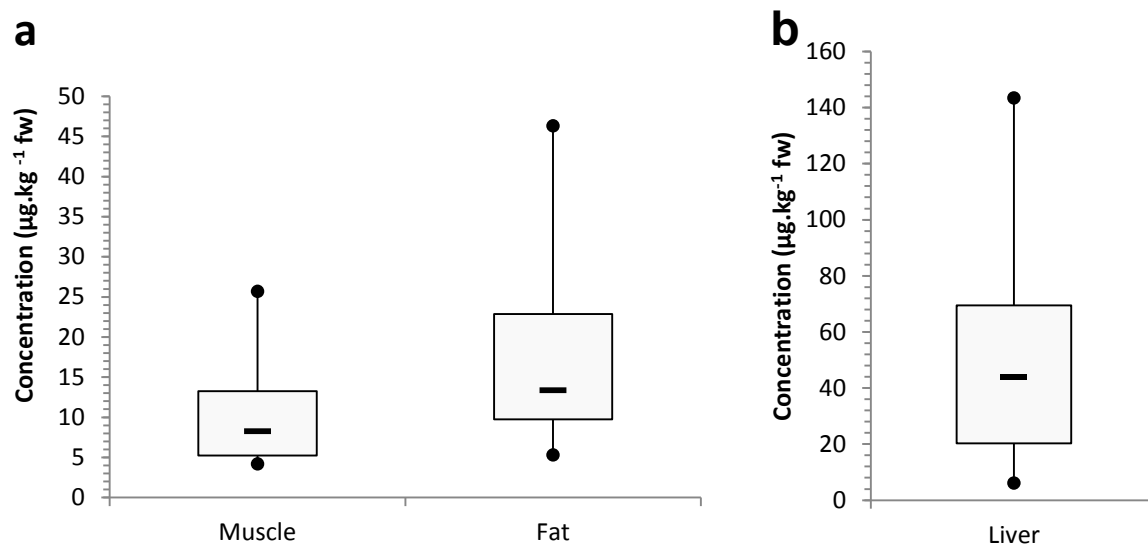


Figure 1: CLD concentrations ($\mu\text{g.kg}^{-1}\text{ fw}$) of positive results ($\geq \text{LOQ}$) in (a) muscle ($n=96$) and fat ($n=106$), and (b) in liver ($n=130$) for the studied bovines. Boxes represent the concentrations between percentiles 25 and 75. The line dividing the box represents the median concentration. The whiskers below and above the box represent percentiles 10 and 90.

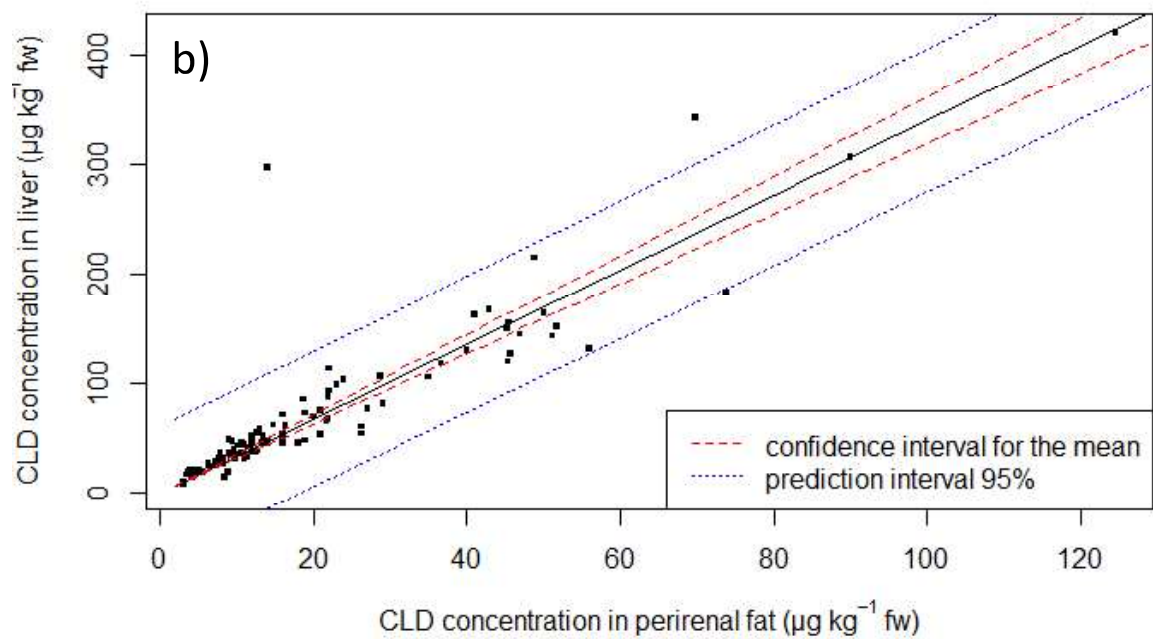
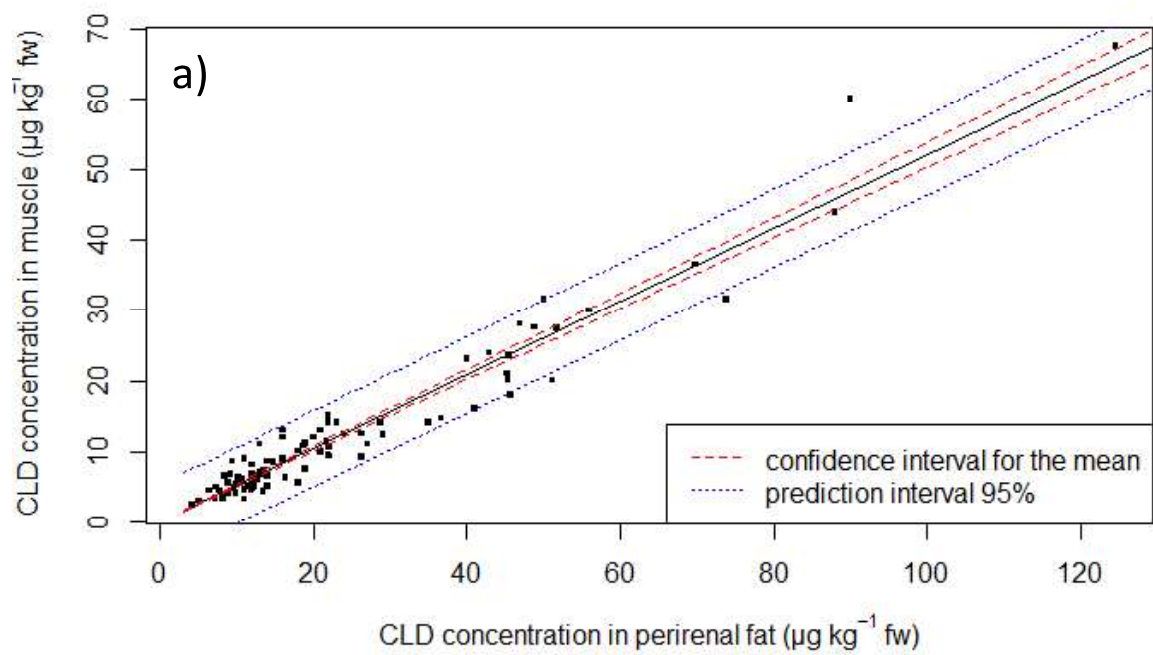
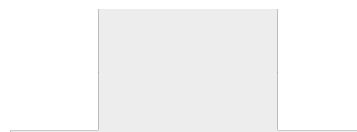
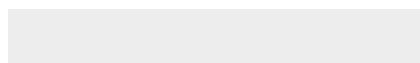
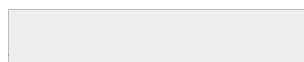


Figure 2: Linear model and confidence intervals on means and observances for (a) muscle/fat and (b) liver/fat correlations of CLD concentrations. Concentrations are expressed on fresh weight (fw).



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All authors have read and agreed to the published version of the manuscript.

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