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# Interest of the Faecal and Plasma Matrix for Monitoring the Exposure of Wildlife or Domestic Animals to Anticoagulant Rodenticides

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## Abstract

Anticoagulant rodenticides (ARs), particularly second-generation compounds (SGAR), are known to be a potential threat to unintended species due to their tissue persistence. The liver is the storage tissue of ARs and is a matrix of choice in diagnosing exposure and intoxication of non-target fauna. However, it is only available on dead animals. Blood and faeces can be used on living animals. These two biological matrices were compared in terms of their relevance to exposure to ARs. In addressing this question, we compared the faecal, plasma and liver concentrations of bromadiolone, one of the SGAR frequently implicated in wildlife exposure. We studied this comparison at the individual level and at the population level, considering three influencing factors: dose, sex and time. Our findings demonstrate that faecal analyses are more valuable than plasma analyses for monitoring AR exposure of domestic and wild animals, even if faecal concentrations cannot be correlated with liver concentrations.

## Keywords

Anticoagulant rodenticides, Exposure, Biological matrix, Faeces, Plasma, Liver

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## Contents

<b>Introduction</b>	<b>1</b>
<b>1 Material and methods</b>	<b>2</b>
1.1 Chemicals	2
1.2 Animals	2
1.3 Animal study and sample collection	2
1.4 Analysis	2
Determination of bromadiolone concentration in the liver •	
Determination of bromadiolone concentration in faeces •	
Determination of anticoagulant concentration in the plasma	
1.5 Data analysis	3
<b>2 Results</b>	<b>3</b>
2.1 Comparison of faecal and plasma versus liver bromadiolone concentrations at the population level	3
2.2 Factors influencing liver/faeces concentration ratios at the population level	4
Depending on the post-exposure period • Depending on the dose of bromadiolone administered • Depending on the gender	
2.3 Comparison of faecal and hepatic concentrations at the individual scale	4

2.4 Comparison of the diastereomeric composition of faecal and hepatic residues at the individual level	4
<b>3 Discussion</b>	<b>4</b>
<b>4 Conclusion</b>	<b>8</b>
<b>References</b>	<b>8</b>

## Introduction

Anticoagulant rodenticides (hereafter ARs) have been widely used as an effective chemical solution to control rodent populations. These compounds were developed in two generations: first-generation ARs (FGARs), including warfarin, coumatralyl and chlorophacinone, for which resistance has been a limitation to their use, and second generation ARs (SGARs), such as bromadiolone, brodifacoum, difenacoum, difethialone, and flocoumafen, which were developed to improve efficacy and overcome FGAR resistance. Although developed in two generations, all of these compounds are vitamin K antagonists that cause coagulopathy in vertebrate animals due to a deficiency of vitamin K, required for activation of clotting factors II, VII, IX and X [1].

Anticoagulant rodenticides have been used for many years either as plant protection products or as biocides in and around buildings. Numerous studies mention exposure of non-target

species either through these uses allowing animals to come into direct contact with the baits (primary exposure) or through the food chain by consumption of poisoned prey or carrion (secondary exposure). Accidental exposures and poisonings to ARs have been widely documented worldwide in humans, pets, livestock, and wildlife, posing a public health concern due to this non-selectivity [2]. The involvement of the different ARs in the exposure of non-target wildlife has been reported to differ depending on the properties of the ARs and their generation, with longer tissue persistence and stronger anticoagulant potency for SGARs [3, 4]. Concurrently, the consequences of exposure to ARs have been suggested to vary between species with species more sensitive and more frequently exposed to ARs [5], but also according to different individual factors such as sex, diet, physiological and nutritional status. The main signs associated with AR exposure are hemorrhagic, that can lead to the death of the animal. Nevertheless, other adverse effects have been suggested during sublethal exposure to ARs [6–8].

The increasing detection of AR in wildlife [9–16] (and in domestic animals [17–24]) has raised awareness of the environmental risks posed by these compounds. This has prompted authorities to regulate the use practices in many countries, but also to implement exposure monitoring to prevent poisoning. To carry out this monitoring, the systematic question of the choice of the matrix to be used has often arisen. The liver has of course been the chosen matrix, since it is the target tissue and the storage tissue of ARs [25] with a proposed toxic threshold of 100 to 200 ng per gram of liver [26]. However, as this matrix is only available on dead animals, it does not allow for the assessment of the level of exposure on a population scale in order to prevent the mortality of animals due to a source of contamination present in their close environment. In this perspective, two biological matrices, blood and faeces, have been used on living animals in various scientific studies (for plasma, [24, 27–30]; for faeces, [31–33]). Nonetheless, few data are currently available concerning their relevance to reflect the exposure of an animal to ARs. Considering the aforementioned difficulties in diagnosing AR poisoning versus simple exposure, it was necessary to determine whether the residues detected in the faeces or the plasma reasonably reflect those present in the liver. In addition, it is also necessary to assess if AR residues in these matrices provide valuable information to distinguish primary from secondary exposure of non-target species.

Hence, we conducted a study in rats exposed to bromadiolone, the latter being one of the ARs most frequently implicated in wildlife poisonings [25] i/ in order to compare quantitatively and qualitatively the hepatic, plasma and faecal residues, and ii/ to evaluate the conclusions that may be reached from these determinations

## 1. Material and methods

### 1.1 Chemicals

Bromadiolone (3-[3-[4-(4-bromophenyl)phenyl]-3-hydroxy-1-phenylpropyl]-4-hydroxychromen-2-one) was provided by Liphatech (Pont-du-Casse, France). It was a mixture containing 85 % of the trans-isomers in racemic proportion (1S, 3R and 1R, 3S) and 15 % of the cis-isomers in racemic proportion (1S, 3S and 1R, 3R). Isoflurane® and vitamin K1 were purchased from Alcyon (Miribel, France).

### 1.2 Animals

Seven-week-old male (n=30) and female (n=30) OFA-Sprague Dawley rats were obtained from a local commercial breeder Charles Rivers in l'Arbresle, France. Animals were housed three per standard cage (Eurostandard, Type IV, Tecniplast, Limonest, France) for rats in the institutional animal facility and kept in a controlled environment (ambient temperature 20 °C ± 1 °C and 12-h light/dark cycle). They were given standard laboratory food (Scientific Animal Food and Engineering, reference A04) and water ad libitum. Animals were acclimated for at least one week prior to use and were maintained in accordance with the European and French legislation guidelines on animal studies.

### 1.3 Animal study and sample collection

Experimental research on the rodents was carried out according to an experimental protocol following international guidelines and approved by the ethics committee of the Veterinary School of Lyon (authorization n°201704190941578). Male and female OFA-Sprague Dawley rats received through per os administration 0,5 mg/kg and 3 mg/kg of bromadiolone dissolved in 5 % DMSO and 95 % vegetable oil. Rats were maintained alive by daily subcutaneous administration of vitamin K1 (10 mg.kg<sup>-1</sup>). At 24, 72, 168, 336 and 720 hours after bromadiolone administration, 3 rats were anesthetized with isoflurane, and blood was drawn by cardiac puncture into citrated tubes. Then, rats were euthanized with CO<sub>2</sub> and the liver of each rat was immediately excised and taken. Faeces of the day were collected directly from the cage at the same time points. Samples were weighed and stored directly at -20 °C until analysis.

### 1.4 Analysis

#### 1.4.1 Determination of bromadiolone concentration in the liver

The method of [34] was used in this study. Briefly, a solid-liquid extraction was performed to extract bromadiolone from rats' liver. The sample (1 g of liver tissue) was homogenized with 10 mL of acetone using an Ultra Turrax tissue disperser. The extract was subsequently centrifuged (2817.6 × g at 4 °C) for 10 min. The resulting supernatant was transferred in a test tube and evaporated at 60 °C under a gentle nitrogen flow. The test tube was rinsed and cleaned up with 2 mL of methanol and evaporated to dryness. Dry extract was then reconstituted in acetonitrile/hexane (50 % / 50 %). The top layer was removed, and the rest was dried at 60 °C. The final dry extract was dissolved in 1 mL methanol and the

bromadiolone concentration was analyzed by LC-MS/MS as previously described [33]. The extraction recovery of AR in liver using this protocol has been previously reported to be above 70% [35].

#### 1.4.2 Determination of bromadiolone concentration in faeces

Faecal samples were extracted according to the protocols described by [31] and [32]. Briefly, the faecal samples were first dried for 48 h in an oven at 50 °C. An aliquot of  $0.50 \pm 0.01$  g of crushed dried faeces was weighed and placed in a 50 mL polypropylene Falcon tube containing 10 mL of acetone. The whole was homogenized by shaking with a vortex. The homogenate was left for one hour without agitation. Then the samples were placed in a rotator PTR-60 during 10 min and were then centrifuged ( $2817.6 \times g$  at 4 °C) for 5 min. Six millilitres of supernatant was transferred to test tubes and evaporated to dryness under nitrogen stream at 40 °C. The residue was taken up with 1 mL of acetonitrile, shaken twice for 10 s with a vortex. Another 1 mL of hexane was added to each tube and left for a minimum of 30 minutes. The upper layer was discarded, and the remainder was evaporated to dryness. The analytes were resuspended with 200  $\mu$  L of methanol, vortexed for 20 s. and filtered through a 0.2  $\mu$  m phenex filter (Phenomenex, Torrance, CA, USA) before LC-MS/MS analysis as detailed in [35]. The extraction recovery of AR in faeces following this protocol has been previously reported to be above 70% [35]. Bromadiolone concentrations in faeces were expressed as  $\mu$ g bromadiolone per g dry matter.

#### 1.4.3 Determination of anticoagulant concentration in the plasma

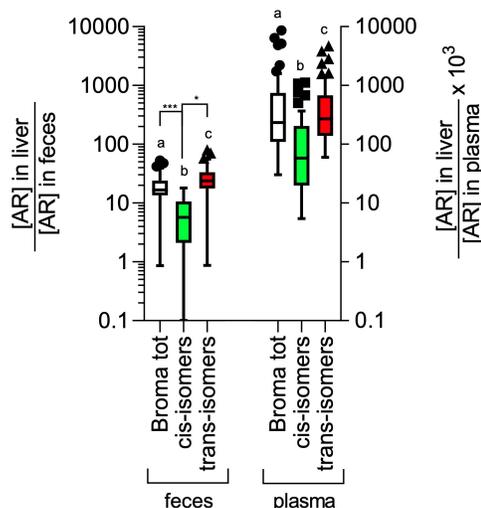
Liquid-liquid extraction was used to extract anticoagulant rodenticide from rat plasma. A volume of 200  $\mu$  L of plasma was mixed with 4 mL of acetone using a programmable rotary shaker in a sequence of cycles with orbital, reciprocal, and vortex rotation for 10 minutes. The mixture was centrifuged ( $2817.6 \times g$  at 4 °C) for 5 minutes. The liquid phase was collected and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The extraction recovery of AR in plasma following this protocol was above 70%. The dry extract was reconstituted with 200  $\mu$  L of methanol and filtered through a 0.2  $\mu$  m phenex filter (Phenomenex, Torrance, CA, USA) before LC-MS/MS analysis (Fourel et al. 2017; Moriceau et al. 2020).

### 1.5 Data analysis

Data handling and statistical analyses were conducted with GraphPad Prism 8 software (GraphPad, San Diego, CA, USA). Data are presented as the median and its 95% confidence interval as box-and-whisker plots. Statistical analysis was done by non-parametric analysis using the Tukey's multiple comparisons test or the Mann Whitney tests.  $P < 0.05$  was the accepted level of significance.

### 2.1 Comparison of faecal and plasma versus liver bromadiolone concentrations at the population level

Faecal, plasma, and liver concentrations were compared from samples taken at the same time from rats exposed to bromadiolone (containing 85% trans and 15% cis isomers) at lethal or sublethal concentrations, 24 to 720 hours prior to sampling. Bromadiolone was detected in all faecal samples ( $n=60$ ) in which bromadiolone was present in the liver ( $n=60$ ), whatever the dose and the post-administration period. In contrast, no bromadiolone was detectable in 3 plasma samples out of 58 (for 2 animals, blood collection was not successful). The median value of liver concentrations was 4.90  $\mu$ g/g, 20 times higher than that of faeces and  $350 \times 10^3$  times higher than that of plasma (Table 1). The concentration ratios between liver and faeces ranged from 0.86 to 52.74, between liver and plasma from  $30 \times 10^3$  to  $8597 \times 10^3$  (Figure 1). Bromadiolone residues found in faecal samples were all composed of cis- and trans-isomers (Table 1,  $p < 0.0001$  for cis and trans-isomers between liver and faeces), while 4 liver samples did not contain cis residues. The concentration ratios between liver and faeces ranged from 0.00 to 18.03 for cis-isomers; from 0.87 to 79.13 for trans-isomers (Figure 1). For plasma in which bromadiolone was present, 6 of 55 samples did not contain cis-isomers; 9 of 55 were negative for trans-isomers. The concentration ratios between liver and plasma (including only those containing cis and/or trans-residues) ranged from  $5.4 \times 10^3$  to  $1113 \times 10^3$  for cis-isomers; from  $60 \times 10^3$  to  $4617 \times 10^3$  for trans-isomers (Figure 1).



**Figure 1.** Ratio of AR concentrations between liver and corresponding faeces or plasma (in white, total bromadiolone; in green, cis-bromadiolone; in red, trans-bromadiolone). Statistical analysis was done using a non-parametric Tukey's multiple comparisons test.  $P < 0.005$  was the accepted level of significance. The same letter indicates a statistical difference between plasma and faeces. \*, indicates a statistical difference within the same matrix.

## 2. Results

	Bromadiolone	Cis-bromadiolone	Trans-bromadiolone
<b>Liver (<math>\mu\text{g/g}</math>)</b>			
Median [95% CI]	4.90 [3.67-7.60]a,b	0.65 [0.38-1.02]d,e	4.16 [3.07-6.02]g,h
Lower value	1.67	0.00	1.67
Upper Value	69.44	10.05	59.39
<b>Feces (<math>\mu\text{g/g}</math>)</b>			
Median [95% CI]	0.25 [0.20-0.34]a,c	0.08 [0.07-0.10]d,f	0.17 [0.13-0.27]g,i
Lower value	0.12	0.06	0.07
Upper Value	20.1	1.89	18.25
<b>Plasma (ng/mL)</b>			
Median [95% CI]	0.014 [0.006-0.039]b,c	0.007 [0.002-0.014]e,f	0.009 [0.003-0.033]h,i
Lower value	0.00	0.00	0.00
Upper Value	1.63	1.20	0.43

**Table 1.** AR concentration in liver, feces and plasma in rat population. Statistical analysis was done using a non-parametric Tukey's multiple comparisons test.  $P < 0.005$  was the accepted level of significance. The same letter indicates a statistical difference.

## 2.2 Factors influencing liver/faeces concentration ratios at the population level

### 2.2.1 Depending on the post-exposure period

Liver/faeces concentration ratios were grouped according to the time between exposure to bromadiolone and the time of sampling (from 1 day to 30 days) (Figure 2). The ratio of liver to faecal bromadiolone residue concentrations increased significantly from 24 hours postadministration to later time points. One day post administration, the mean value of the liver to faecal total bromadiolone concentrations ratio was  $6.6 \pm 5.1$ , increased to  $26.6 \pm 11.9$  at 7 days post-administration and then stabilized. (Figure 2 A). The variations observed were due to variations in liver and faecal trans-bromadiolone concentrations, whereas the ratios of cis residues did not change with post-exposure times.

### 2.2.2 Depending on the dose of bromadiolone administered

Ratios of liver and faecal concentrations were grouped according to the dose administered, i.e. sublethal or lethal. No change in ratios was observed as a function of the dose, for total bromadiolone residues and their trans-isomers. However, a significant difference was observed for cis-isomers with a ratio decreasing by a factor close to 3.5 when the dose increased from 0.5 mg/kg to 3 mg/kg.

### 2.2.3 Depending on the gender

Ratios of liver and faecal concentrations were grouped by gender (Figure 4). A significant difference between males and females was observed for total bromadiolone residues and their cis-isomers with a higher ratio in females.

## 2.3 Comparison of faecal and hepatic concentrations at the individual scale

Liver concentration of each individual was presented according to its corresponding faecal concentration (Figure 5). No correlation was observed between faecal and liver concentrations. The highest faecal concentrations did not correspond to

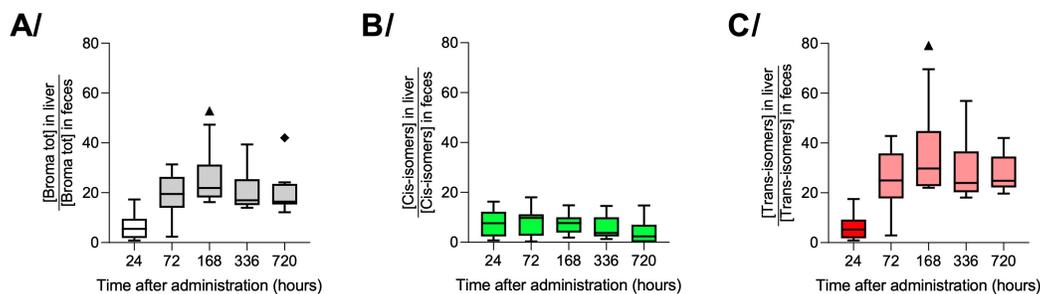
the highest liver concentrations, faecal concentrations below  $1.0 \mu\text{g/g}$  corresponded to liver concentrations ranging from  $1 \mu\text{g/g}$  to  $20 \mu\text{g/g}$  without any possible modelling (Figure 5, insert). Nevertheless, the liver concentrations could be grouped into two statistically different groups based on a threshold faecal concentration of  $0.5 \mu\text{g/g}$  (Figure 6). For cis- and trans-isomers, similar profiles were observed (data not shown) with no correlation between faecal and liver concentrations.

## 2.4 Comparison of the diastereomeric composition of faecal and hepatic residues at the individual level

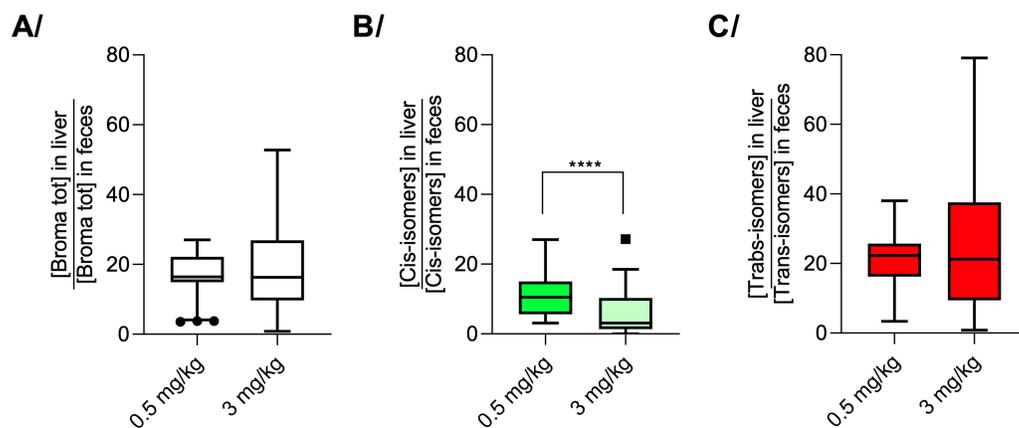
Diastereoisomeric composition of the hepatic residues of each individual was analyzed according to the diastereoisomeric composition of the residues of the corresponding faecal sample. Diastereoisomeric percentages were similar between liver and faeces for samples collected less than 48 hours after bromadiolone administration; they were statistically different ( $p < 0.0001$ ) for samples collected more than 48 hours after bromadiolone administration (Figure 7).

## 3. Discussion

Our study first demonstrates the a meaningful comparison between the potential of the faecal matrix compared to the plasma matrix regarding extrapolation to liver values. For this, it was necessary to be able to compare the concentrations of AR on the three matrices in parallel (liver, blood, faeces) in order to evaluate the reflectiveness of the concentrations of AR in faeces or plasma with regard to those measured in the liver. This comparison was carried out on laboratory rats exposed to bromadiolone, since it is difficult to obtain in the field enough individuals of the same species exposed to the same anticoagulant, and especially at a known date and in a known amount of AR, to have statistically analyzable and interpretable data. These rats were exposed to bromadiolone (until very recently, this AR was used as plant protection product in Europe and has been widely reported in non-target



**Figure 2.** Ratio of AR concentrations between liver and corresponding faeces according to the delay post-exposition. In A/ ratio for total bromadiolone residues, in B/ ratio for cis-isomers of bromadiolone, in C/ ratio for trans-isomers of bromadiolone. Statistical analysis was done using a non-parametric Tukey’s multiple comparisons test.  $P < 0.005$  was the accepted level of significance. In the same panel, different colors within the same graph are statistically different.

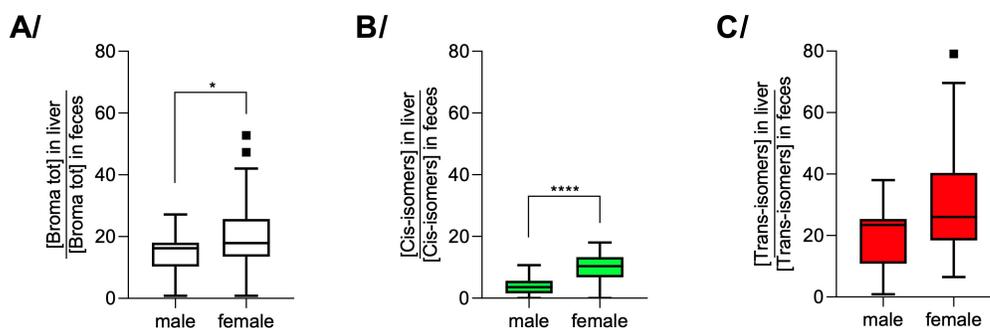


**Figure 3.** Ratio of AR concentrations between liver and corresponding faeces according to the concentration of bromadiolone administered. In A/ ratio for total bromadiolone residues, in B/ ratio for cis-isomers of bromadiolone, in C/ ratio for trans-isomers of bromadiolone. Statistical analysis was done using a non-parametric Mann-Whitney test.  $P < 0.005$  was the accepted level of significance. \*, indicates a statistical difference between doses.

wildlife) by gavage to control the administered dose. These rats were exposed to two different doses, one lethal and one sublethal, to rule out any dose effect. The population of exposed rats consisted of an equal number of females and males to avoid the influence of the sex, as the metabolism of xenobiotics being largely influenced by the sex of the animal. Finally, to consider the different situations in the field, the period between exposure and sampling was different for each animal (from 1 to 30 days with 3 animals per period), in order to have recently exposed animals as well as animals that have been exposed for a long time, as animals can survive in the field after ingestion of ARs.

While detection of ARs in plasma has been suggested as a possible indicator of toxicity [murrayContinuedAnticoagulantRodenticide2020], the use of the faecal matrix seems more relevant for exposure assessment. Although bromadiolone residue concentrations are consistently lower in faeces and plasma than in liver (while extraction rates are comparable between matrices), the concentration ratios between liver and faeces are always much lower, by a factor of nearly 50, than the concentration ratios

between liver and plasma. Residues in plasma are therefore minor compared to those in liver and even those in faeces. Consequently, in our population, a diagnosis of exposure based on the plasma matrix generates 8% of false negatives, whereas based on the faeces matrix, no false negatives are detected. Only the detection and quantification of the parent compound was investigated in this study. However, second generation anticoagulants have been reported to be not or only slightly metabolized [36]. Our study was performed using LC-MS/MS. This technology allows reaching extremely low limits of quantification of the order of a few ng/g for all ARs [37–39]. Of course, the false negative percentages on our sample would be much higher if other detection methods had been used. Indeed, different technologies are used for the detection and quantification of ARs, by liquid chromatography coupled to mass spectrometry as performed in our study, but also by liquid chromatography with UV detection [40, 41] or fluorescence detection [42, 43], leading to much higher detection limits (of the order of a few 100 ng/g, with variations depending on the AR) [25, 39]. These latter methods of detection are not very suitable for ARs



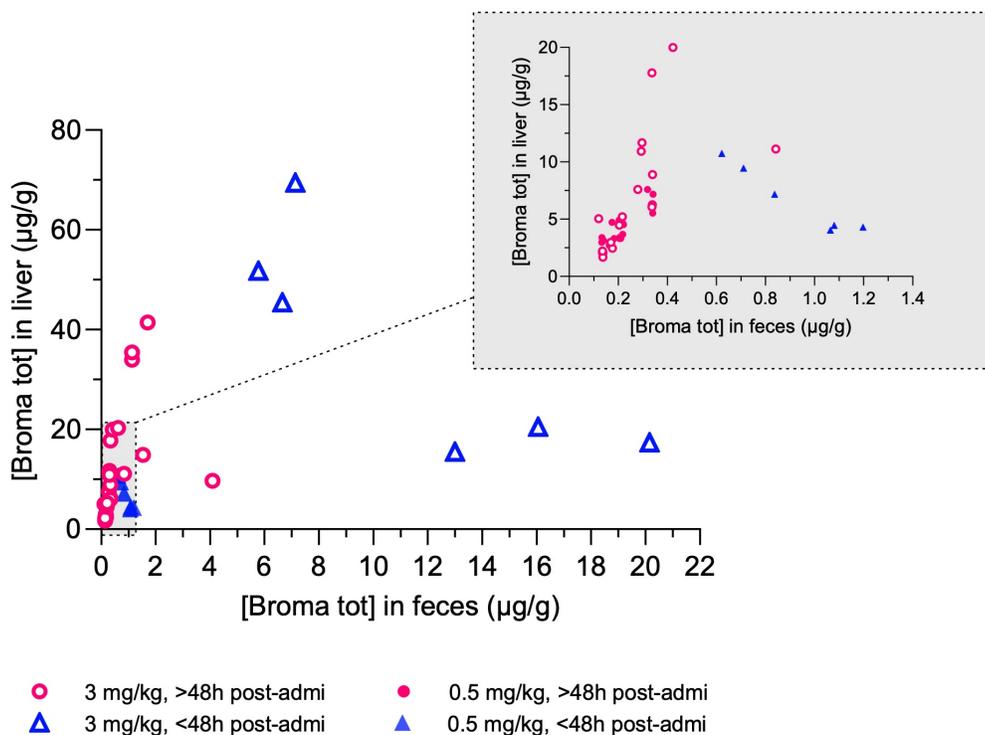
**Figure 4.** Ratio of AR concentrations between liver and corresponding faeces according to the gender of animals. In A/ ratio for total bromadiolone residues, in B/ ratio for cis-isomers of bromadiolone, in C/ ratio for trans-isomers of bromadiolone. Statistical analysis was done using a non-parametric Mann-Whitney test.  $P < 0.005$  was the accepted level of significance. \*, indicates a statistical difference between gender..

quantification on faecal or plasma matrices.

Let us now compare the results obtained on faeces and plasma at the finer scale of ARs stereoisomers. Indeed, the ARs are molecules containing one or two asymmetric carbons, at the origin of stereoisomers of configuration [44]. Thus, bromadiolone contains two asymmetric carbons and consists of a mixture of four stereoisomers, easily separable on achiral column by group of two isomers, called diastereomeric pair or cis and trans isomers [45]. Separation into four stereoisomers requires a chiral separation [46] that is more complex to perform routinely on field samples from various biological matrices. We will therefore focus on the diastereomeric scale considering that the administered bromadiolone contains 85% trans isomers and 15% cis isomers. For trans isomers, an exposure diagnosis using plasma generates, in our population, more than 23% of false negatives, while the use of the faeces matrix generates none. For cisomers, 11% of false negatives are observed among our samples using the plasma matrix versus 0 using faeces. Nevertheless, the use of faeces leads to the observation of 6% of false positives among our sampling, i.e. containing cis-isomers in faeces whereas they are undetectable in the liver. Here again, it is important to note that the use of liquid chromatography coupled to mass spectrometry leads to very few false positives because the specificity of the analyses is high due to the detection principle (based on the molar mass and fragmentation of the molecule) [47–49]. The use of liquid chromatography coupled with UV or fluorescence detection would certainly lead to observe many more false positives, especially with faeces generating a very high background noise level (mass effect) with these detection methods [38, 50, 51].

How to explain the high proportion of false negatives via the use of the plasma matrix? Bromadiolone is a second-generation AR molecule, like difenacoum, difethialone, brodifacoum or flocoumafen [1]. These molecules, unlike the first-generation molecules, are characterized by a very long persistence in the body, up to several hundred days according to some studies [3]. Their distribution is essentially intracellular [52] with a very strong hepatic extraction coefficient,

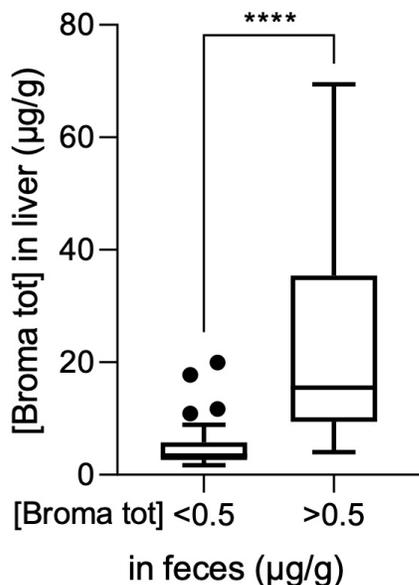
the proportion of the molecule distributed in the plasma being infinitesimal, of the order of 0.4 of the total quantity for bromadiolone [52]. This plasma circulation is low, but also very transient, whereas the presence of the molecule persists for several weeks or even months in the liver. Thus, previous studies show that after a single or repeated administration, bromadiolone becomes undetectable in plasma about 7 days after administration [38, 53]. In our study, it becomes undetectable in plasma between 14 and 30 days after initial administration. Because the distribution of bromadiolone is similar to other second-generation molecules [3, 54]), it is highly likely that using the plasma matrix to track exposure to other SGARs would lead to the same biases. As the pharmacokinetics of first generation ARs (chlorophacinone, coumatetralyl, warfarin) are different with a higher plasma distribution [52], the plasma matrix might be more relevant for monitoring exposure to these molecules. Nevertheless, since their use tends to decrease due to resistance in rodent populations [55, 56] and their tissue persistence is lower, monitoring the exposure of wildlife to these molecules is of less importance. The observation of false negatives in the field is problematic because it minimizes the prevalence of exposure. The purpose of field monitoring is to assess and prevent ecotoxicological risk to the non-target populations present. According to our results, the use of the faecal matrix avoids or, at least, minimizes the proportion of false negatives, making this matrix more relevant. Indeed, faecal excretion is the major route of elimination of most SGAR. Faecal residues consist of either direct eliminated non absorbed compound by the gastrointestinal tract or excreted unchanged unbound compounds. Biliary excretion and enterohepatic circulation also contribute to the continued elimination in faeces over a long period [36, 53, 57]. If the faecal matrix appears to be more relevant for monitoring the exposure of domestic and wild animals, is it possible to extrapolate the information from the analysis of this matrix to liver contamination? Quantitatively, faecal concentrations are much lower than liver concentrations. It is not feasible to correlate faecal concentrations with liver concentrations. This lack of correlation of these concentrations seems to be due



**Figure 5.** Hepatic bromadiolone concentration in function of its corresponding faecal concentration for each individual of the population ( $n=60$ ), according to the dose and delay between bromadiolone administration and sampling. In Insert/ Focus on faecal concentrations between  $0 \mu\text{g/g}$  and  $1.5 \mu\text{g/g}$ .

mainly to three factors. Firstly, the post-exposure period, with a more rapid decreasing of the faecal concentrations of AR than the hepatic ones. This is valid for total bromadiolone, for trans residues, but not for cis residues, whose hepatic persistence is lower [54]. The second factor contributing to this lack of correlation between faecal and hepatic concentrations relies on differences in metabolism depending on the sex of the animal. Indeed, it seems that bromadiolone, particularly the cis-isomers and eventually the trans-isomers, are more persistent in the liver of females than males. This finding is not surprising since the metabolism of xenobiotics is known to be largely sex-dependent [58–60]. This difference was recently illustrated for difethialone [61]. The third factor seems to be the ingested dose with possibly a rate of elimination depending on this concentration. However, there is not enough data to confirm this hypothesis. From a qualitative standpoint, are the faecal bromadiolone residues similar to the hepatic residues? Cis- and trans- isomers were systematically detected in liver and faeces, regardless of sex and dose (except for four individuals without cis isomers in the liver, for whom samples were taken 30 days after exposure). For recent exposure (i.e. within the first 48h post-exposure), the qualitative composition of the faecal residues is completely similar to that of the liver. However, beyond 48h post-exposure, we observe an increase in the proportion of cis-isomers in the faecal matrix while at the same time the proportion of trans-isomers increases in the liver. This is related to the greater hepatic persistence

of trans-isomers compared to cis-isomers. This evolution is responsible for the occurrence of the 4 false positives for cis-isomers observed in our population. Despite this drawback, the qualitative analysis of isomers present in faeces and liver could have several major interests: dating of exposure and possible differentiation between primary and secondary exposure. Indeed, similar diastereoisomeric proportions between liver and faeces suggest a recent exposure and the more these proportions deviate, the older the exposure. Dating an exposure is important in the field to motivate the search for the source of contamination in the area where the dead, weakened or sentinel animal is found. Identifying whether the exposure is primary or secondary is also crucial to identifying the origins of an exposure. Finding only trans-isomers of bromadiolone in liver and faeces suggests secondary exposure via ingestion of an animal that has eliminated or nearly eliminated cis-isomers, which are less persistent than trans-isomers [54]. The presence of cis-isomers of bromadiolone in faeces and even in the liver would suggest primary exposure. Larger studies will be necessary to develop these hypotheses. In parallel, it will be necessary to verify the conservation of the diastereoisomer proportions in faeces and liver, depending on their state of conservation [62], as well as the homogeneity of concentrations and proportions of diastereoisomers in faeces. Indeed, previous studies suggest that ARs are not homogeneously distributed in the latter [32]. In our study, faeces samples were mixed and homogenized prior to bromadiolone extraction, which does



**Figure 6.** Hepatic concentration of bromadiolone based on a faecal threshold concentration of 0.5 µg/g. Statistical analysis was done using a non-parametric Mann-Whitney test.  $P < 0.005$  was the accepted level of significance. \*, indicates a statistical difference.

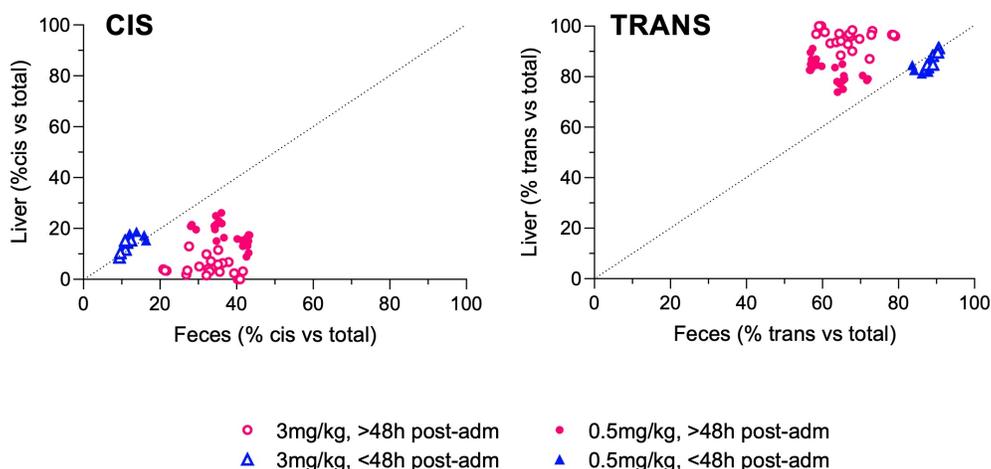
not allow us to understand this methodological aspect.

#### 4. Conclusion

This study underlines the potential of faecal samples for meaningful monitoring of the exposure of non-target animal populations to ARs. Indeed, for the possible dating of the exposure and the epidemiology of this exposure (primary or secondary), our investigational approach should nevertheless be continued. In addition, our approach is valid for bromadiolone, although it will be necessary to repeat the study for the other ARs. It is likely that the results will be similar for all SGARs, considering the similarity of their pharmacokinetic properties. However, as mentioned, the relevance of this matrix for the detection of the FGARs may be limited due to their different pharmacokinetic properties. The results obtained will need to be confirmed in the different animal species given the differences in pharmacokinetics between species. In addition, the stability of AR residues in faeces will need to be further investigated in order to explore the influence of different parameters (light, temperature, humidity, etc).

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**Figure 7.** Percentage of cis- or trans-isomers in liver bromadiolone residues as a function of its corresponding percentage of faecal residues (n=60), according to dose and time between bromadiolone administration and sampling.

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