Manuscript Number: STOTEN-D-17-01205R2

Title: Cis-bromadiolone diastereoisomer is not involved in bromadiolone
Red Kite (*Milvus milvus*) poisoning

Article Type: Research Paper

Keywords: Anticoagulant rodenticides; Stereoisomers; Tissue persistence;
Conservation; Predators; Ecotoxicology

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Order of Authors: Isabelle Fourel, Ph.D; Marlène Damin-Pernik; Etienne
Benoit; Virginie Lattard

Abstract: Anticoagulant rodenticides (ARs) are widely used pesticides to control rodent populations. Bromadiolone, a second generation anticoagulant rodenticide (SGARs), is authorized in France to control the population of water voles (*Arvicola scherman*). The persistence of SGARs in rodents is responsible for secondary exposure or poisoning of predators and scavengers, and is of ecological concern for the conservation of endangered species. Commercial formulations are a mixture of two diastereoisomers of bromadiolone: 70-90% is trans-bromadiolone and 10-30% is cis-bromadiolone. Both diastereoisomers have been shown to inhibit coagulation function with the same potency. On the other hand, cis-bromadiolone has been shown to be less tissue-persistent than trans-bromadiolone in rats. This difference led to residue levels in rats with substantially weakened proportion in cis-bromadiolone compared to the composition of baits.

In this study, a multi-residue LC-MS/MS method for the quantification of the diastereoisomers of SGARs was used to investigate their proportions in field samples of predators. In 2011, 28 red kites (*Milvus milvus*) were found dead within a few months of bromadiolone application in grassland to control water vole outbreaks. In this study, we report the concentrations of the two diastereoisomers of bromadiolone measured in the livers of thirteen red kites. Exposure to bromadiolone was apparent in all the kites with hepatic concentrations of trans-bromadiolone ranging from 390 to 870 ng/g (89 to 99% of summed SGARs). However, cis-bromadiolone was not detected in 5 of 13 red kites and was present at very low concentrations (below 2.2ng/g) in 8 of 13 kites, demonstrating that cis-bromadiolone is not involved in this red kite poisoning event. The results suggest that a change of the proportions of bromadiolone diastereoisomers in baits could reduce the risk of secondary poisoning of predators, but retain primary toxicity for control rodent outbreaks.

Response to Reviewers: May 31, 2017

Dear Daniel A. Wunderlin

Thank you for your remarks on our manuscript entitled « Cis-bromadiolone

diastereoisomer is not involved in bromadiolone Red Kite (*Milvus milvus*) poisoning» by Fourel et al. We carefully re-revised the manuscript to address the reviewers' concerns. Specific responses to each question are given below.

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Although the grammar and language are much improved on the original ms, there is still some improvement that is necessary. I have split my comments on this into two parts on the attached pdf, the first that covers small scale questions re meaning where language has been ambiguous to some extent and needs clarification, the second are simply suggestions to improve the syntax and grammar. They are included as a pdf file and I hope they are all useful.

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Table 1 legend. State what n.d. means (not determined presumably). Done

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Line 191. Was there evidence of external trauma in any of the 10 kites that had macroscopic haemorrhages, if so which. This is useful information that should be included in the text. I agree it is important to clarify this point: there was no external trauma in any of the 10 kites and it has been included in the text.

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I would rephrase as. "Bromadiolone residues were found in all the 13 red kites and the measured quantities were significant and at concentrations between 390 and 871 ng/g (mean value 642 ng/g). Done

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Line 252. Suggest rephrase to "... difenacoum in their body (Damin-Pernik et al., 2016, 2017; Fourel et al., 2017). Proportions of the minor isomers cis-bromadiolone and trans-difenacoum (minor isomers) in the livers liver of rats were very different..." This has been corrected with the suggested phrase.

There is also a need to clarify what is meant here, "different from" what? This has been clarified.

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Presumably the authors mean that predatory wildlife is not actually exposed to the minor isomers rather than that they do not absorb the isomers? Suggest change wording. Done

Line 282- 8. Rephrase for clarity as ".....it seems that the proportions of brodifacoum diastereoisomers is also modified by rodents and/or predators. Although trans-brodifacoum is less persistent than cis brodifacoum, it appears to be is obviously more persistent than cis-bromadiolone and trans-difenacoum (both minor isomers). As shown in figure 3, trans-brodifacoum was is still present in red kites liver (5.7% of trans-brodifacoum and 94.3% of cis-brodifacoum) when whereas cis-bromadiolone was almost totally absent from the kite livers (only 0.1% of total bromadiolone present and in contrast to) has nearly disappeared, although trans-bromadiolone which comprised represents 95.5% of the total ARs residues residues). Thank you for reformulating this complicated but important sentence.

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Lines 66-68. "...and in France it may be applied in fields to control 67 rodents, most and notably water voles (*Arvicola scherman*) outbreaks, that may affect the harvest of fodder harvest. Field applications of"

Line 69-70. ".....been reported as being responsible for exposure and poisoning of foxes (*Vulpes vulpes*) exposure and poisoning (Sage et al., 2010; Jacquot et al., 2013, 2014)....."

Line 72-74. "...list of mitigation measures was developed elaborated in the early 2000s and was proposed to farmers to lower the ecological consequences of bromadiolone treatments."

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I would like to submit the manuscript entitled “Cis-bromadiolone diastereoisomer is not involved in bromadiolone red kite (*Milvus milvus*) poisoning” by Fourel *et al.* for consideration of publication in *Science of Total Environment*.

Bromadiolone is used as a pesticide to control or eradicate voles in meadows, enters persistently the food chain and is responsible for secondary poisoning of predators. This manuscript reports data that describes for the first time the hepatic residues of diastereoisomers of second generation anticoagulant rodenticides in a rodent predator species, the red kite (*Milvus milvus*). The method used allows the biomonitoring of each diastereoisomer and permits to evidence the evolution of diastereoisomers ratios of bromadiolone (trans-bromadiolone and cis-bromadiolone) between the baits and the liver of red kites through the different filters of the food chain. It differentiates the biological persistence of trans-bromadiolone and cis-bromadiolone and evaluates the ecological consequences of each diastereoisomer. This study demonstrates the need to monitor the residues of each diastereoisomer for a better assessment of the risk for non-target wildlife and suggests how the use of baits weakened with trans-bromadiolone might be more ecotoxicological.

Thanks for your kind considerations.

Isabelle Fourel, PhD

Responses to Reviewers Comments

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Line 125- 127. "... A recently described multi-residue LC-MS/MS method (Fourel et al., 2017) provides will help resolve this issue. It is an appropriate tool to start investigating SGAR diastereoisomers proportions in non-target wildlife (even if residues are very low), and to evaluate their respective persistence in predators...."

Line 135-6. "To our knowledge, these are the first available data on SGAR SGARs diastereoisomers residues in non-target wildlife after using...."

Line 145- 154. ".....Sample collection was previously described in Coeurdassier et al. 2014b. Briefly, during the winter 2011-2012, 28 dead kites were discovered in areas located in Auvergne (Centre Central France) where bromadiolone was applied in grassland. First, twelve Twelve of the kites were retained for clinical investigations and residues analysis; results are presented in Coeurdassier et al. 2014b. Of Among the 16 remaining individuals, 13 were stored at -20°C by the Ligue pour la Protection des Oiseaux (LPO) Auvergne for 18 months. Then, after thawing, post-mortem examinations were conducted on these 13 kites to look for clinical signs of AR poisoning, mainly macroscopic haemorrhages in the organs of the abdominal cavity or in the brain. Microscopic haemorrhages were not investigated. The gender and class age (juvenile or adult) were also noted. The liver was sampled and stored at -20°C until analysis analysed."

Line 161-2. "... and there was little evidence of matrix effects during the validation process. ..." low matrix effect was evidenced.

Line 163. "...quantification of ARs in the livers liver of red kites has been used. The ARs analysed in this study are were three first generation anticoagulant rodenticides...."

Line 170-177. "...The chromatographic separation of the two diastereoisomers of each SGAR was is achieved with a semi-porous Poroshell 120 StableBond C18 column (2.1*100mm, 2.7µm; Poroshell 120 StableBond) and MS/MS detection was carried out by a 6410B Triple Quadrupole from (Agilent Technologies, Palo Alto, CA, USA) equipped with ElectroSpray Ionization source in negative mode. Two fragment ions were recorded in dynamic Multiple Reaction Monitoring mode for each analyte. Calibration curves were constructed, one for each first generation AR and one for each diastereoisomer of the SGARs. The limits of quantification varied between 1 - 2 ng/g wet weight ..."

Line 198. "Differences in" not "differences of"

Line 203. Delete "Amazingly"....its not a very scientific term for use here.

Line 206. "...Hepatic residues of cis-bromadiolone diastereoisomer in red kites were non-detectable null in 5 of 13 livers"...,

Line 213. Space needed before "ng/g"

Line 217. "Difethialone residues were found in 6 of 13 red kites and the maximum concentration was with maximum 24ng/g...."

Line 219. "Summed SGARs concentrations (ΣSGARs) were between 412 and 912 ng/g; ..."

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Line 246 "...already eliminated all or most part of the cis-bromadiolone diastereoisomer when they were

Line 248 "...documented but isomer isomers patterns were not described (Sage et al., 2008). At present...."

Line 257. "faster" rather than "quicker"

Line 260-5. Suggest rephrase as: Our results are consistent with the concept that the pharmacokinetic behaviour of ARs in rats (rapid metabolism of the minor isomer) also occurs in water voles but this This suggests that ARs in water voles and rats should probably have the same pharmacological behaviours, but it still remains needs to be confirmed as Indeed, metabolic capacity often varies between depends on species. and results with rats cannot could not definitively predict these similarities. We also need to investigate as

well which species is most efficient in eliminating cis-bromadiolone, : water voles or red kites. Additional experiments will be conducted in the near future to explore the pharmacokinetics of the diastereoisomers of bromadiolone in water voles and birds to address these questions."

Line 276. "... of detection was is lng/g (European...."

Lines 281 "...not possible to compare with liver residues of wild rats from our previous study (Fourel et al.,2017),

It is not clear from the grammar what the authors are comparing with what. Clarify.

Line 297 "explains" instead of "explained" and "found" instead of "evidenced"

Line 335. "...information on multiple exposure to SGARs multi-exposure with quantitative as well as qualitative data on the proportions of different respective diastereoisomers proportions

Highlights :

Commercial bromadiolone is a mixture of 70% trans-isomer and 30% cis-isomer

Cis-bromadiolone is not found in liver of red kites after bromadiolone poisoning

Cis-bromadiolone does not seem to be persistent in the food chain

Rodents and/or red kites metabolize the two diastereoisomers of bromadiolone differently

Monitoring of rodenticides should differentiate diastereoisomers in non-target species

1 **Cis-bromadiolone diastereoisomer is not involved in bromadiolone**
2 **Red Kite (*Milvus milvus*) poisoning**

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Abstract:

Anticoagulant rodenticides (ARs) are widely used pesticides to control rodent populations. Bromadiolone, a second generation anticoagulant rodenticide (SGARs), is authorized in France to control the population of water voles (*Arvicola scherman*). The persistence of SGARs in rodents is responsible for secondary exposure or poisoning of predators and scavengers, and is of ecological concern for the conservation of endangered species. Commercial formulations are a mixture of two diastereoisomers of bromadiolone: 70-90% is trans-bromadiolone and 10-30% is cis-bromadiolone. Both diastereoisomers ~~were~~ have been shown to inhibit coagulation function with the same potency. On the other hand, cis-bromadiolone ~~was~~ has been shown to be less tissue-persistent than trans-bromadiolone in rats. This difference ~~leads~~ leads to residue levels in rats with substantially weakened proportion in cis-bromadiolone compared to the composition of baits.

In this study, a multi-residue LC-MS/MS method for the quantification of the diastereoisomers of SGARs was used to investigate their proportions in field samples of predators. In 2011, 28 red kites (*Milvus milvus*) were found dead within a few months of bromadiolone application in grassland to control water vole outbreaks. In this study, we report the concentrations of the two diastereoisomers of bromadiolone measured in the livers of thirteen red kites. Exposure to bromadiolone was apparent in all the samples kites with ~~important~~ hepatic concentrations of trans-bromadiolone ranging from 390 to 870 ng/g (89 to 99% of summed SGARs), ~~that is far above the toxic threshold of 200ng/g~~. However, cis-bromadiolone was not detected in 5 of 13 red kites and was present at very low concentrations (below 2.2ng/g) in 8 of 13 kites, demonstrating that cis-bromadiolone is not involved in this red kite poisoning event. The results suggest that a change of the proportions of bromadiolone diastereoisomers in baits could reduce the risk of secondary poisoning of predators, but retain primary toxicity for control rodent outbreaks.

Key words: Anticoagulant rodenticides, Stereoisomers, Tissue persistence, Conservation, Predators, Ecotoxicology

1. Introduction

Anticoagulant Rodenticides (ARs) are used worldwide to control or eradicate rodent populations. These pesticides have often been associated with non-target secondary poisonings of predatory and scavenging birds (Sanchez-Barbudo et al., 2012; Hughes et al., 2013; Ruiz-Suarez et al., 2014; Langford et al., 2013; Jaffe et al., 2016; Christenssen et al., 2012; Hughes et al., 2013; Coeurdassier et al., 2014a) and carnivorous mammals (Geduhn et al., 2016; Sage et al., 2010; López-Perea et al., 2015; Ruiz-Suarez et al., 2016; Jacquot et al., 2014) through the ingestion of contaminated rodents. Bromadiolone is an authorized AR in Europe for plant protection purposes, and in France it may be applied in fields to control rodents, and most notably water voles (*Arvicola scherman*) outbreaks, that may affect ~~fodder~~ harvest of fodder. Field applications of bromadiolone in Franche-Comté and Auvergne regions have been reported as being responsible for exposure and poisoning of foxes (*Vulpes vulpes*) ~~exposure and poisoning~~ (Sage et al., 2010; Jacquot et al., 2013, 2014), and represents a threat to endangered species such as the red kite (*Milvus milvus*) that consume water voles during outbreaks (Coeurdassier et al., 2012, 2014a, 2014b; Decors et al., 2012). A list of mitigation measures was ~~elaborated~~ developed in the early 2000s and was proposed to farmers to lower the ecological consequences of bromadiolone treatments. In Franche-Comté region, adoption of these measures led to a substantial decrease in non-target wildlife mortality since 2006 (Jacquot et al., 2013). However, secondary exposure has not completely disappeared and new additional approaches are required to reduce adverse effects of ARs on predators and scavengers (Rattner et al., 2014; Coeurdassier et al., 2014b; Elliott et al., 2016; Smith and Shore, 2015).

As previously reported, Second Generation Anticoagulant Rodenticides (SGARs; i.e., bromadiolone, difenacoum, brodifacoum, flocoumafen and difethialone) are a mixture of two diastereoisomeric forms (1R,3R)(1S,3S)-isomers and (1R,3S)(1S,3R)-isomers, according to their chemical structure owning two stereogenic centres (Cort et al., 2012; Damin-Pernik et al., 2016, 2017; Fourel et al., 2017). Both forms are present in all commercial baits in proportions that are controlled by the authorities. For example, according to the European official values, commercialized bromadiolone in baits is composed of 70 to 90% of (1R,3S)(1S,3R)-isomers (i.e. trans-bromadiolone), the major diastereoisomeric form, and 10 to 30% of (1R,3R)(1S,3S)-isomers (i.e. cis-isomers), the minor diastereoisomeric form (EC, 2008).

The bBiological properties of both diastereoisomers of all SGARs were recently compared in rats by means of *in vitro* and *in vivo* experiments. For bromadiolone, both the cis- and trans-isomers were shown to have similar potency for ~~inhibiting the~~ Vitamin K epoxide reductase

activity, which is involved in the recycling of vitamin K and thus in the coagulation function.,
~~with the same potency.~~ Moreover, the use of only cis-bromadiolone or only trans-bromadiolone in baits was shown to lead to 100% of mortality of rats exposed to either baits. On the other hand, it was shown in laboratory rats that cis-bromadiolone and trans-bromadiolone had very different pharmacokinetic properties with a 3-fold shorter half-life for cis-bromadiolone compared to trans-bromadiolone, and consequently shorter tissue persistence of cis-bromadiolone. Similar observations were made for the other SGARs with identical inhibitory activity between diastereoisomers as demonstrated by their inhibition constant towards VKOR activity, but with different tissue persistence (Damin-Pernik et al., 2016, 2017). Consequently, for all SGARs, the diastereoisomer with greatest tissue persistence should be ~~linked to~~associated with a greater risk of secondary poisoning. To investigate this hypothesis, the livers of wild rats (*Rattus norvegicus*) that had been trapped ~~on in an the urban park fields~~ were analysed with a new multi-residue LC-MS/MS method ~~permitting the quantification of that quantified~~ the two diastereoisomeric forms of each of the five SGARs. It was demonstrated that diastereoisomers proportions of some SGARs found in liver of those rodents were effectively different from diastereoisomers proportions in baits. In all ~~the~~ cases, the minor diastereoisomer forms (cis-bromadiolone and trans-difenacoum) in hepatic residues were ~~most of the time none~~either not detected or ~~at least those were present in a lower proportion than found in~~ minor stereoisomers proportions were always substantially reduced compared to the ones of baits (Fourel et al., 2017). These field observations were consistent with pharmacokinetic and persistence behaviour of SGARs isomers observed from laboratory rats.

As wildlife secondary poisonings was correlated with AR usage and biological persistence (Rattner et al., 2014; Shore et al., 2015), ~~it was proposed by~~ Damin et al., (2016, 2017) ~~to proposed~~ lowering the proportion of the most persistent isomer (trans-bromadiolone) to ~~obtain produce~~ a new bait with the same ability to kill rodents (because both isomers have identical toxicity) but with decreased ecological risk for predators (because cis-bromadiolone is less persistent in tissue). This ~~hypothesis is proposal was~~ based on pharmacological data ~~of from~~ laboratory and wild rats (Damin et al., 2016, 2017; Fourel et al, 2017) ~~while although~~ the pharmacokinetics of SGARs diastereoisomers ~~for in~~ other species (other rodents and non-target-species) ~~is are~~ unknown. The persistence of isomers and their respective proportions in other rodents and predators with secondary exposure should be evaluated before modification of the actual baits is instituted.

A recently described multi-residue LC-MS/MS method (Fourel et al., 2017) will help resolve this issue. It is an appropriate tool to start investigating SGAR diastereoisomers proportions

in non-target wildlife (even if residues are very low), and to evaluate their respective persistence in predators. In autumn 2011, exceptional quantities of bromadiolone baits were used in Auvergne region, France, to control outbreaks of voles when hundreds of red kites were residing in this area. During the months that followed, 28 red kites (*Milvus milvus*) and 16 common buzzards (*Buteo buteo*) were found dead in the affected area, and this episode is described as the worse lethal poisoning in France since 2003 (Coeurdassier et al., 2014b, 2014c; Decors et al., 2012; LPO, 2012). The AR liver residues of thirteen of these red kites have been measured and the results are presented. In addition, the proportions of cis- and trans-diastereoisomers and necropsy findings are described. To our knowledge, these are the first available data on SGARs diastereoisomers residues in non-target wildlife after using them as a biocide or plant protection product. Consequences in term of measurement of exposure to SGARs in non-target wildlife and risk assessment are discussed.

2. Material and methods

2.1. Sample collection

Sample collection was previously described in Coeurdassier et al. 2014b. Briefly, during the winter 2011-2012, 28 dead kites were discovered in areas located in Auvergne (Centre France) where bromadiolone was applied in grassland. First, twelve kites were retained for clinical investigations and residues analysis; results are presented in Coeurdassier et al. 2014b. Among the 16 remaining individuals, 13 were stored at -20°C by the Ligue pour la Protection des Oiseaux (LPO) Auvergne for 18 months. Then, after thawing, post-mortem examinations were conducted on these 13 kites to look for clinical signs of AR poisoning, mainly macroscopic haemorrhages in organs of the abdominal cavity or brain. Microscopic haemorrhages were not investigated. The gender and class age (juvenile or adult) were also noted. The liver was sampled and stored at -20°C until analysis.

2.2. Anticoagulant rodenticide analysis

The analysis of ARs was carried out following the method recently described by Fourel et al., (2017) and complete validation has been achieved according to European Medicines Agency bio-analytical guideline (EMA, 2011). Chicken liver was used for validation purposes as it was done before in Ruiz-Suarez et al. (2016), instead of wildlife matrices, ~~as it was done before (Ruiz-Suarez, 2016)~~, and during the validation process low matrix effect was

evidenced. Briefly, a LC-MS/MS for multi-residual quantification of ARs in liver of red kites has been used. The ARs analysed in this study are three first generation anticoagulant rodenticides: warfarin, coumatetralyl, chlorophacinone and the two diastereoisomers of the five SGARs potentially used in European countries: bromadiolone, difenacoum, brodifacoum, flocoumafen and difethialone. The trans-bromadiolone is the major isomer in the bromadiolone commercial formulation and the cis-bromadiolone is the minor form. For difenacoum, brodifacoum, flocoumafen and difethialone commercial formulations, the cis-isomers are the major forms and the trans-isomers are the minor forms. The chromatographic separation of the two diastereoisomers of each SGAR is achieved with a semi-porous Poroshell 120 StableBond C18 column (2.1*100mm, 2.7µm) and MS/MS detection was carried out by a 6410B Triple Quadrupole from Agilent Technologies (Palo Alto, CA, USA) equipped with ElectroSpray Ionization source in negative mode. Two fragment ions were recorded in dynamic Multiple Reaction Monitoring mode for each analyte. Calibration curves were constructed, one for each first generation AR and one for each diastereoisomer of the SGARs. The limits of quantification varied between 1 - 2 ng/g ~~and are the same that the ones detailed for each isomer in as also reported by~~ Fourel et al.⁷ (2017). The recovery rates of spiked samples exceeded 70%.

3. Results

3.1. Post-mortem examinations

The 13 red kites were discovered in November and December 2011, except for one found in May 2012, and all were from the Puy-de-Dôme department. Ten of them were found in three municipalities close to each other (area of 300km²) where the bromadiolone treatments had been undertaken as described in Coeurdassier et al., 2014b and in table 1. The results of post-mortem examinations as well as sex and class age of the thirteen red kites are reported in table 1. Macroscopic haemorrhages ~~(but no external trauma)~~ were evident in ten of the red kites (77%, 3 females, 5 males, sex could not be determined in 2; 5 were adults and 5 were juveniles). There were no macroscopic haemorrhages in three of the red kites (3 females, 2 juveniles and 1 adult), and one of the three had signs of external trauma

3.2 Bromadiolone residues in liver of the 13 red kites

When an AR had two diastereoisomers, the total concentration (ng/g) and the proportions of the major and minor forms are reported (figures 1 and 2). There was no difference of ARs concentrations (means $\pm$ standard deviation) between the birds with macroscopic haemorrhages ($681\pm88\text{ng/g}$) and the birds without macroscopic haemorrhages ($671\pm153\text{ng/g}$). Bromadiolone residues were found in all the 13 red kites at concentrations and the measured quantities were significant and between 390 and 871 ng/g (mean value 642 ng/g). Amazingly, proportion of trans-bromadiolone diastereoisomer was between 99.5 and 100% (mean value 99.9%) of total bromadiolone residues for all the red kites, when European official values for proportions of trans-diastereoisomer in bromadiolone baits is between 70 and 90%. Hepatic residues of cis-bromadiolone diastereoisomer in red kites were null in 5 of 13 livers, or below 2.2ng/g and obviously negligible (i.e. 0.005 to 0.001 of the concentration of bromadiolone), and were negligible in the remaining kites as they were below 2.2ng/g and comprised less than 0.5% of total bromadiolone concentration.

3.3 Exposure of the 13 red kites to other ARs

Difenacoum (100% cis-difenacoum) residues in liver were quantified in 9 of 13 red kites and were below 11.8ng/g. Brodifacoum residues were found in 12 of 13 livers with a maximum ~~non-negligible~~ value of 59.9ng/g. Cis-brodifacoum was found in all of them and trans-brodifacoum in only 4 of 12 livers of red kites. Proportions of cis-brodifacoum were between 77.1 and 100% (mean value 94.3%). Flocoumafen residues were quantified in 2 of 13 red kites, with values below 3.3ng/g. Difethialone residues were found in 6 of 13 red kites, with maximum value at 24ng/g, and trans-difethialone was not detected. No first generation ARs were detected. Summed SGARs (Σ SGARs) were between 412 and 912 ng/g; 99.7% of these total residues were major diastereoisomers (i.e. trans-bromadiolone, cis-difenacoum, cis-brodifacoum, cis-difethialone) and 0.3% was minor diastereoisomers (i.e. cis-bromadiolone and trans-brodifacoum).

4. Discussion

4.1 Exposure to trans- and cis-bromadiolone

The presence of large quantities of trans-bromadiolone in liver (390-871ng/g), and macroscopic haemorrhages in 77% of the birds provides strong evidence that bromadiolone was involved in the death of the red kites. It is likely that the kites consumed poisoned voles after bromadiolone was applied to control rodent populations (Coourdassier et al., 2014b;

Decors et al., 2012). However, hepatic cis-bromadiolone (minor-isomer) residues were very low (figure 2) providing evidence that this isomer is unlikely to be responsible for the poisonings.

As illustrated in figure 3, the proportion of the bromadiolone diastereoisomers are very different in baits (European official values: 70-90% trans-bromadiolone) and in liver of red kites (99.9% trans-bromadiolone). Instability of the active substance in baits can be excluded because stability of the active ingredient and diastereoisomers proportions in bromadiolone baits have to be demonstrated by its manufacturer through mandatory aging tests. Moreover, to definitively exclude this hypothesis, diastereoisomers proportions of commercial baits containing bromadiolone have been evaluated before and after use in the field, and the results were in accordance with regulatory documents and rules (data not shown). Our findings may be interpreted in two ways: (i) red kites consumed the cis-bromadiolone diastereoisomer when feeding on voles and eliminated it before dying, and/or (ii) voles had already eliminated all or most part of cis-bromadiolone diastereoisomer when they were eaten by red kites. The pharmacokinetics of bromadiolone in voles has already been documented but isomers patterns were not described (Sage et al., 2008). At present, there are no data available on the comparative pharmacokinetics of bromadiolone diastereoisomers in voles. However, previous results reported that wild rats and exposed laboratory rats differentially metabolized the diastereoisomers of bromadiolone and of difenacoum in their body (Damin-Pernik et al., 2016, 2017; Fourel et al., 2017). Proportions of the minor isomers cis-bromadiolone and trans-difenacoum (~~minor isomers~~) in the livers of rats were very different, ~~and much lower~~ compared to those observed in commercial baits. ~~m~~More than 75% of the wild rats with bromadiolone or difenacoum residues had only the major diastereoisomer. This means that between the time they fed on baits and the moment they were trapped, the rats eliminated bromadiolone and difenacoum minor diastereoisomers quicker than the major diastereoisomers. Consequently, predatory wildlife should not absorb be exposed to little or no bromadiolone and difenacoum minor diastereoisomers (or to little quantities) when feeding on exposed rats. This suggests that ARs in water voles and rats should probably have the same pharmacological behaviours, but it still remains to be confirmed. Indeed, metabolic capacity often depends on species and results with rats could not definitively predict these similarities. We need to investigate as well which species is most efficient in eliminating cis-bromadiolone: water voles or red kites. Additional experiments will be conducted in the near future to explore pharmacokinetics of the diastereoisomers of bromadiolone in water voles and birds to address these questions.

4.2 Exposure to other SGARs diastereoisomers

As previously reported, but without information on diastereoisomers proportions, non-target wildlife species have often been found to contain multiple ARs (Christensen et al., 2012; Lopez-Perea et al., 2015; Geduhn et al., 2015; Sanchez-Barbudo et al., 2012; Hughes et al., 2013; Ruiz-Suarez et al., 2014, 2016; Langford et al., 2013; Shore et al., 2015). The 13 red kites of our study were also exposed to several SGARs. Although difenacoum residues (max. 12ng/g) in the red kites are negligible compared to trans-bromadiolone residues, it is remarkable that the trans-difenacoum (minor diastereoisomer) was not found while the limit of detection is 1ng/g (European official values for proportions of trans-diastereoisomer in difenacoum baits is between 20 and 50%). This is consistent with the above observations in rat liver. In commercial baits, cis-brodifacoum has been evaluated as being between 48% and 60% (Cort et al., 2012, Fourel et al., 2017). Proportions of trans-brodifacoum (minor isomer) are substantially less in the red kites compared to the baits (figure 3). Although it was not possible to compare with liver residues of wild rats from our previous study (Fourel et al., 2017), it seems that the proportions of brodifacoum diastereoisomers is also modified by rodents and/or predators. Although trans-brodifacoum is less persistent than cis-brodifacoum, it ~~is obviously~~ appears to be more persistent than cis-bromadiolone and trans-difenacoum (both minor isomers). As shown in figure 3, trans-brodifacoum was still present in red kites liver (5.7% of trans-brodifacoum and 94.3% of cis-brodifacoum) ~~when-whereas~~ was almost totally absent from the kite livers (only 0.1% of total bromadiolone present and in contrast to ~~has nearly disappeared, although~~ trans-bromadiolone ~~represents which comprised~~ 95.5% of the total ARs residues). ~~It was found no correlation between the proportion in trans-brodifacoum and the total amount of brodifacoum in the liver.~~ These results for brodifacoum are preliminary and need to be documented with further investigations and other sets of wildlife samples. Flocoumafen is not commercially available in France. Low levels of this persistent AR may be the result of winter migrations of red kites through Europe, as flocoumafen is used in a few European countries and has been detected in non-target wildlife species in Spain and Germany (Lopez-Perea et al., 2015; Geduhn et al., 2015). One of the red kites found dead in Auvergne during the winter 2011/2012 had been banded in Germany (Coeurdassier et al., 2014b), so possibly exposed to flocoumafen. Difethialone baits contain mainly cis-difethialone, which explained why only cis-difethialone was evidenced in red kites, as already reported in wild rat liver (Fourel et al., 2017).

4.3 Exposure to trans-bromadiolone above toxic threshold

Hepatic concentrations of ARs above 200ng/g have been associated with mortalities in raptors and small mustelids from ~~Denan~~ Denanmark (Elmeros et al., 2011; Christensen et al.,

2012), in raptors and hedgehogs from Mediterranean region of Spain (Lopez-Perea et al., 2015), and in six raptor species from Canary Islands, Spain (Ruiz-Suarez et al., 2014). The use of these limits was based on several experimental observations or probabilistic studies with collections of numerous individuals (Newton et al., 1999; Shore et al., 2014; Thomas et al., 2011; Huang et al., 2016). In England, nineteen red kites were diagnosed as AR-poisoned because they had internal haemorrhages without associated trauma and summed liver ARs concentrations of 100ng/g or above (Jaffe et al., 2016). We could then consider that a limit of 100 or 200ng/g might be extrapolated to the thirteen red kites as representing a minimum toxic threshold for them. It is obvious that the major-isomers residues (between 410 and 911ng/g), mainly trans-bromadiolone (95.5%), are far above this limit, and the minor-isomers (below 15ng/g), mainly trans-brodifacoum, are far below this limit (figure 2a and 2b). Moreover, it has been shown that cis-bromadiolone baits are as efficient as trans-bromadiolone baits in killing rodents and no choice feeding tests showed that the hepatic residues were 4-fold reduced with cis-bromadiolone baits (Damin-Pernik et al., 2017). We might then consider that the use of cis-bromadiolone would have allowed the reduction of 4-fold the amount of bromadiolone in liver of rodents and would have enabled a significant decrease of the exposure of the red kites. Moreover, it is probable that some of them would not have been exposed to SGARs above the potentially lethal limit of 0.1-0.2mg.kg⁻¹. It seems obvious that the proportions of diastereoisomers in baits should be revised as an important step towards more ecological use of ARs. This would limit the exposure of non-target wildlife and complement established mitigation measures restricting AR treatments by farmers when endangered species like the red kite are present.

5. Conclusions

The new fully validated analytical LC-MS/MS method is a powerful tool to start the characterisation of SGARs diastereoisomer proportions in non-target wildlife, and to evaluate possible ecotoxicological consequences. In this study, the Coreshell LC-MS/MS multi-residue method demonstrated that only one diastereoisomer of the bromadiolone (trans-isomers) was involved in the poisoning of the red kites. This method provides complementary information on SGARs multi-exposure with quantitative as well as qualitative data on respective diastereoisomers proportions. It evaluates the evolution of these proportions in the predators' body compared to the baits or to the rodents' body, and in this way it demonstrates the potential towards more ecological use of anticoagulant rodenticides as one of the two diastereoisomers is less persistent than the other one. The other advantages of

352 this analytical tool are the specificity of the detection allowing confident identification of the
353 compounds, and sensitivity with hepatic low limits of quantification of 1-2 ng/g for each
354 SGAR diastereoisomer.

355 When finding field carcasses, especially endangered red kites, and other non-target species,
356 SGARs hepatic residues and diastereoisomers proportions should be used with clinical signs
357 as criteria to diagnose anticoagulant rodenticides exposure. This should facilitate estimation
358 of the degree of ecological risk of each isomer and help evaluate the hazard of new baits
359 compared to existing bait formulations.

360
361 Liver residues have often been measured in wildlife but without specifying the
362 diastereoisomers proportions. The significance of such information is now apparent, and will
363 become more important if development occurs to change the diastereoisomers proportions in
364 baits. A change in the diastereoisomers proportion in favour of the less persistent isomers
365 should reduce residue concentrations and modify the diastereoisomers proportions found in
366 wildlife. It seems reasonable to monitor the effects of such modifications. Future surveys
367 should then specify the diastereoisomers proportions to give qualitative as well as
368 quantitative evaluation of diastereoisomer residues of each SGAR in predators.

371 **Aknowlegments**

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376 **Footnotes**

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Legends of figures

Figure 1: Summed SGARs concentrations (Σ SGARs - represented by the dotted line), summed ~~major SGARs~~ major diastereoisomeric forms of SGARs - (Σ major SGARs - represented by white circle) and summed minor diastereoisomeric forms of SGARs (Σ minor SGARs - represented by black triangle) concentrations in the liver of the thirteen red kites from 412 to 912ng/g and detailed percentages of bromadiolone, cis-difenacoum, brodifacoum, flocoumafen and cis-difethialone. Trans-difenacoum and trans-difethialone were not detected. Bromadiolone percentage was between 89 and 99.5% for all the birds. ~~Summed major SGARs are far above the toxic threshold of 200ng/g and summed minor SGARs are far below this toxic threshold.~~

Figure 2: Proportions and concentrations of diastereoisomers (%) of trans- and cis-bromadiolone and, cis- and trans-brodifacoum in the liver of the 13 red kites. Trans-bromadiolone was at least 99.5% of bromadiolone residues (mean 99.9%) and cis-bromadiolone proportion is 0-0.5% only (mean 0.1%). ~~Trans-bromadiolone concentration is far above the toxic threshold of 200ng/g and cis-bromadiolone concentration is far below this toxic threshold. Cis-brodifacoum is 77.1-100% of total brodifacoum residues (mean 94.3%) and trans-brodifacoum is found in only 4 red kites (mean 5.7% of total brodifacoum residues).~~

Figure 3: Proportions of major and minor diastereoisomers for bromadiolone, difenacoum, brodifacoum and difethialone in liver of the thirteen red kites, and in commercial baits dosed in Fourel et al., (2017). The proportions of minor diastereoisomers are ~~were~~ substantially lowered in liver of red kites compared to commercial baits.

Table 1: Post-mortem examination results with presence or absence of macroscopic haemorrhages, sex and class age of the thirteen red kites. Municipalities, department and date for the discovery of the carcasses of red kites.

n.r., not reported; n.d., not determined

<u>Red kite</u>	<u>sex</u>	<u>age</u>	<u>macroscopic haemorrhages</u>	<u>discovery date</u>	<u>Department</u>	<u>Municipality</u>	<u>Total bromadiolone (ng/g)</u>	<u>Total difenacoum (ng/g)</u>	<u>Total brodifacoum (ng/g)</u>	<u>Total flocoumafen (ng/g)</u>	<u>Total difethialone (ng/g)</u>
<u>1</u>	<u>F</u>	<u>juvenile</u>	<u>yes</u>	<u>21/11/2011</u>	<u>Puy-de-</u>	<u>Saint Bonnet Près Orcival</u>	<u>390</u>	<u>5,9</u>	<u>12,7</u>	<u>3,3</u>	<u>1,8</u>
<u>2</u>	<u>n.d.</u>	<u>juvenile</u>	<u>yes</u>	<u>01/12/2011</u>	<u>Dome</u>	<u>Heume l'Eglise</u>	<u>669</u>		<u>3,1</u>		
<u>3</u>	<u>F</u>	<u>adult</u>	<u>yes</u>	<u>01/12/2011</u>	<u>Puy-de-</u>	<u>Heume l'Eglise</u>	<u>565</u>	<u>4,6</u>	<u>50,5</u>		<u>13,3</u>
<u>4</u>	<u>M</u>	<u>juvenile</u>	<u>yes</u>	<u>01/05/2012</u>	<u>Dome</u>	<u>Saint Bonnet Près Orcival</u>	<u>664</u>	<u>2,1</u>	<u>59,9</u>	<u>1,6</u>	<u>7,9</u>
<u>5</u>	<u>F</u>	<u>juvenile</u>	<u>no</u>	<u>27/11/2011</u>	<u>Puy-de-</u>	<u>Saint Bonnet Près Orcival</u>	<u>636</u>	<u>1,7</u>			<u>11,2</u>
<u>6</u>	<u>M</u>	<u>adult</u>	<u>yes</u>	<u>30/11/2011</u>	<u>Dome</u>	<u>Heume l'Eglise</u>	<u>837</u>		<u>57</u>		
<u>7</u>	<u>M</u>	<u>adult</u>	<u>yes</u>	<u>30/11/2011</u>	<u>Puy-de-</u>	<u>Heume l'Eglise</u>	<u>684</u>	<u>9,1</u>	<u>7,5</u>		
<u>8</u>	<u>F</u>	<u>adult</u>	<u>no</u>	<u>30/11/2011</u>	<u>Dome</u>	<u>Heume l'Eglise</u>	<u>607</u>	<u>2,8</u>	<u>5,8</u>		
<u>9</u>	<u>n.d.</u>	<u>juvenile</u>	<u>yes</u>	<u>28/11/2011</u>	<u>Puy-de-</u>	<u>Saint Bonnet Près Orcival</u>	<u>643</u>	<u>2,9</u>	<u>1,4</u>		
<u>10</u>	<u>F</u>	<u>adult</u>	<u>yes</u>	<u>nov-11</u>	<u>Dome</u>	<u>n.r.</u>	<u>527</u>		<u>11,9</u>		<u>17,9</u>
<u>11</u>	<u>F</u>	<u>juvenile</u>	<u>no</u>	<u>nov-11</u>	<u>Puy-de-</u>	<u>n.r.</u>	<u>766</u>	<u>11,8</u>	<u>3,1</u>		
<u>12</u>	<u>M</u>	<u>juvenile</u>	<u>yes</u>	<u>nov-11</u>	<u>Dome</u>	<u>n.r.</u>	<u>489</u>		<u>56,1</u>		
<u>13</u>	<u>M</u>	<u>adult</u>	<u>yes</u>	<u>24/11/2011</u>	<u>Puy-de-</u>	<u>Saint Sulpice</u>	<u>871</u>	<u>3</u>	<u>15,2</u>	<u>-</u>	<u>24,8</u>

Red kite	Sex	Age	Macroscopic haemorrhages	Discovery date	French department	Municipality
1	F	juvenile	yes	21/11/2011	Puy-de-Dome	Saint-Bonnet-Près-Orcival
2	n.d.	juvenile	yes	01/12/2011	Puy-de-Dome	Heume-l'Eglise
3	F	adult	yes	01/12/2011	Puy-de-Dome	Heume-l'Eglise
4	M	juvenile	yes	01/05/2012	Puy-de-Dome	Saint-Bonnet-Près-Orcival
5	F	juvenile	no	27/11/2011	Puy-de-Dome	Saint-Bonnet-Près-Orcival
6	M	adult	yes	30/11/2011	Puy-de-Dome	Heume-l'Eglise
7	M	adult	yes	30/11/2011	Puy-de-Dome	Heume-l'Eglise
8	F	adult	no	30/11/2011	Puy-de-Dome	Heume-l'Eglise
9	n.d.	juvenile	yes	28/11/2011	Puy-de-Dome	Saint-Bonnet-Près-Orcival
10	F	adult	yes	nov-11	Puy-de-Dome	n.r.
11	F	juvenile	no	nov-11	Puy-de-Dome	n.r.
12	M	juvenile	yes	nov-11	Puy-de-Dome	n.r.
13	M	adult	yes	24/11/2011	Puy-de-Dome	Saint-Sulpice

Figure 1

Figure 2

A/

B/

Figure 3

1 **Cis-bromadiolone diastereoisomer is not involved in bromadiolone**
2 **Red Kite (*Milvus milvus*) poisoning**

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Abstract:

Anticoagulant rodenticides (ARs) are widely used pesticides to control rodent populations. Bromadiolone, a second generation anticoagulant rodenticide (SGARs), is authorized in France to control the population of water voles (*Arvicola scherman*). The persistence of SGARs in rodents is responsible for secondary exposure or poisoning of predators and scavengers, and is of ecological concern for the conservation of endangered species. Commercial formulations are a mixture of two diastereoisomers of bromadiolone: 70-90% is trans-bromadiolone and 10-30% is cis-bromadiolone. Both diastereoisomers have been shown to inhibit coagulation function with the same potency. On the other hand, cis-bromadiolone has been shown to be less tissue-persistent than trans-bromadiolone in rats. This difference led to residue levels in rats with substantially weakened proportion in cis-bromadiolone compared to the composition of baits. In this study, a multi-residue LC-MS/MS method for the quantification of the diastereoisomers of SGARs was used to investigate their proportions in field samples of predators. In 2011, 28 red kites (*Milvus milvus*) were found dead within a few months of bromadiolone application in grassland to control water vole outbreaks. In this study, we report the concentrations of the two diastereoisomers of bromadiolone measured in the livers of thirteen red kites. Exposure to bromadiolone was apparent in all the kites with hepatic concentrations of trans-bromadiolone ranging from 390 to 870 ng/g (89 to 99% of summed SGARs). However, cis-bromadiolone was not detected in 5 of 13 red kites and was present at very low concentrations (below 2.2ng/g) in 8 of 13 kites, demonstrating that cis-bromadiolone is not involved in this red kite poisoning event. The results suggest that a change of the proportions of bromadiolone diastereoisomers in baits could reduce the risk of secondary poisoning of predators, but retain primary toxicity for control rodent outbreaks.

Key words: Anticoagulant rodenticides, Stereoisomers, Tissue persistence, Conservation, Predators, Ecotoxicology

1. Introduction

Anticoagulant Rodenticides (ARs) are used worldwide to control or eradicate rodent populations. These pesticides have often been associated with non-target secondary poisonings of predatory and scavenging birds (Sanchez-Barbudo et al., 2012; Hughes et al., 2013; Ruiz-Suarez et al., 2014; Langford et al., 2013; Jaffe et al., 2016; Christenssen et al., 2012; Hughes et al., 2013; Coeurdassier et al., 2014a) and carnivorous mammals (Geduhn et al., 2016; Sage et al., 2010; López-Perea et al., 2015; Ruiz-Suarez et al., 2016; Jacquot et al., 2014) through the ingestion of contaminated rodents. Bromadiolone is an authorized AR in Europe for plant protection purposes, and in France it may be applied in fields to control rodents, most notably water vole (*Arvicola scherman*) outbreaks, that may affect harvest of fodder. Field applications of bromadiolone in Franche-Comté and Auvergne regions have been reported as being responsible for exposure and poisoning of foxes (*Vulpes vulpes*) (Sage et al., 2010; Jacquot et al., 2013, 2014), and represents a threat to endangered species such as the red kite (*Milvus milvus*) that consume water voles during outbreaks (Coeurdassier et al., 2012, 2014a, 2014b; Decors et al., 2012). A list of mitigation measures was developed in the early 2000s and was proposed to farmers to lower the ecological consequences of bromadiolone treatments. In Franche-Comté region, adoption of these measures led to a substantial decrease in non-target wildlife mortality since 2006 (Jacquot et al., 2013). However, secondary exposure has not completely disappeared and new additional approaches are required to reduce adverse effects of ARs on predators and scavengers (Rattner et al., 2014; Coeurdassier et al., 2014b; Elliott et al., 2016; Smith and Shore, 2015).

As previously reported, Second Generation Anticoagulant Rodenticides (SGARs; i.e., bromadiolone, difenacoum, brodifacoum, flocoumafen and difethialone) are a mixture of two diastereoisomeric forms (1R,3R)(1S,3S)-isomers and (1R,3S)(1S,3R)-isomers, their chemical structure owning two stereogenic centres (Cort et al., 2012; Damin-Pernik et al., 2016, 2017; Fourel et al., 2017). Both forms are present in all commercial baits in proportions that are controlled by the authorities. For example, according to the European official values, commercialized bromadiolone in baits is composed of 70 to 90% of (1R,3S)(1S,3R)-isomers (i.e. trans-bromadiolone), the major diastereoisomeric form, and 10 to 30% of (1R,3R)(1S,3S)-isomers (i.e. cis-isomers), the minor diastereoisomeric form (EC, 2008).

The biological properties of both diastereoisomers of all SGARs were recently compared in rats by means of *in vitro* and *in vivo* experiments. For bromadiolone, both the cis- and trans-isomers were shown to have similar potency for inhibiting Vitamin K epoxide reductase activity, which is involved in the recycling of vitamin K and thus in the coagulation

function. Moreover, the use of only cis-bromadiolone or only trans-bromadiolone in baits was shown to lead to 100% of mortality of rats exposed to either baits. On the other hand, it was shown in laboratory rats that cis-bromadiolone and trans-bromadiolone had very different pharmacokinetic properties with a 3-fold shorter half-life for cis-bromadiolone compared to trans-bromadiolone, and consequently shorter tissue persistence of cis-bromadiolone. Similar observations were made for the other SGARs with identical inhibitory activity between diastereoisomers as demonstrated by their inhibition constant towards VKOR activity, but with different tissue persistence (Damin-Pernik et al., 2016, 2017). Consequently, for all SGARs, the diastereoisomer with greatest tissue persistence should be associated with a greater risk of secondary poisoning. To investigate this hypothesis, the livers of wild rats (*Rattus norvegicus*) that had been trapped in an urban park were analysed with a new multi-residue LC-MS/MS method that quantified the two diastereoisomeric forms of each of the five SGARs. It was demonstrated that diastereoisomers proportions of some SGARs found in liver of those rodents were effectively different from diastereoisomers proportions in baits. In all cases, the minor diastereoisomer forms (cis-bromadiolone and trans-difenacoum) in hepatic residues were either not detected or were present in a lower proportion than found in baits (Fourel et al., 2017). These field observations were consistent with pharmacokinetic and persistence behaviour of SGARs isomers observed from laboratory rats.

As wildlife secondary poisonings was correlated with AR usage and biological persistence (Rattner et al., 2014; Shore et al., 2015), Damin et al. (2016, 2017) proposed lowering the proportion of the most persistent isomer (trans-bromadiolone) to produce a new bait with the same ability to kill rodents (because both isomers have identical toxicity) but with decreased ecological risk for predators (because cis-bromadiolone is less persistent in tissue). This proposal was based on pharmacological data from laboratory and wild rats (Damin et al., 2016, 2017; Fourel et al., 2017) although the pharmacokinetics of SGARs diastereoisomers in other species (other rodents and non-target-species) are unknown. The persistence of isomers and their respective proportions in other rodents and predators with secondary exposure should be evaluated before modification of the actual baits is instituted.

A recently described multi-residue LC-MS/MS method (Fourel et al., 2017) will help resolve this issue. It is an appropriate tool to start investigating SGAR diastereoisomers proportions in non-target wildlife (even if residues are very low), and to evaluate their respective persistence in predators. In autumn 2011, exceptional quantities of bromadiolone baits were used in Auvergne region, France, to control outbreaks of voles when hundreds of red kites were residing in this area. During the months that followed, 28 red kites (*Milvus milvus*) and 16 common buzzards (*Buteo buteo*) were found dead in the affected area, and this episode

is described as the worse lethal poisoning in France since 2003 (Coeurdassier et al., 2014b, 2014c; Decors et al., 2012; LPO, 2012). The AR liver residues of thirteen of these red kites have been measured and the results are presented. In addition, the proportions of cis- and trans-diastereoisomers and necropsy findings are described. To our knowledge, these are the first available data on SGARs diastereoisomers residues in non-target wildlife after using them as a biocide or plant protection product. Consequences in term of measurement of exposure to SGARs in non-target wildlife and risk assessment are discussed.

2. Material and methods

2.1. Sample collection

Sample collection was previously described in Coeurdassier et al. 2014b. Briefly, during the winter 2011-2012, 28 dead kites were discovered in areas located in Auvergne (Centre France) where bromadiolone was applied in grassland. First, twelve kites were retained for clinical investigations and residues analysis; results are presented in Coeurdassier et al. 2014b. Among the 16 remaining individuals, 13 were stored at -20°C by the Ligue pour la Protection des Oiseaux (LPO) Auvergne for 18 months. Then, after thawing, post-mortem examinations were conducted on these 13 kites to look for clinical signs of AR poisoning, mainly macroscopic haemorrhages in organs of the abdominal cavity or brain. Microscopic haemorrhages were not investigated. The gender and class age (juvenile or adult) were also noted. The liver was sampled and stored at -20°C until analysis.

2.2. Anticoagulant rodenticide analysis

The analysis of ARs was carried out following the method recently described by Fourel et al. (2017) and complete validation has been achieved according to European Medicines Agency bio-analytical guideline (EMA, 2011). Chicken liver was used for validation purposes as it was done before in Ruiz-Suarez et al. (2016), instead of wildlife matrices,, and during the validation process low matrix effect was evidenced. Briefly, a LC-MS/MS for multi-residual quantification of ARs in liver of red kites has been used. The ARs analysed in this study are three first generation anticoagulant rodenticides: warfarin, coumatetralyl, chlorophacinone and the two diastereoisomers of the five SGARs potentially used in European countries: bromadiolone, difenacoum, brodifacoum, flocoumafen and difethialone. The trans-bromadiolone is the major isomer in the bromadiolone commercial formulation and the cis-

bromadiolone is the minor form. For difenacoum, brodifacoum, flocoumafen and difethialone commercial formulations, the cis-isomers are the major forms and the trans-isomers are the minor forms. The chromatographic separation of the two diastereoisomers of each SGAR is achieved with a semi-porous Poroshell 120 StableBond C18 column (2.1*100mm, 2.7µm) and MS/MS detection was carried out by a 6410B Triple Quadrupole from Agilent Technologies (Palo Alto, CA, USA) equipped with ElectroSpray Ionization source in negative mode. Two fragment ions were recorded in dynamic Multiple Reaction Monitoring mode for each analyte. Calibration curves were constructed, one for each first generation AR and one for each diastereoisomer of the SGARs. The limits of quantification varied between 1 - 2 ng/g as also reported by Fourel et al. (2017). The recovery rates of spiked samples exceeded 70%.

3. Results

3.1. Post-mortem examinations

The 13 red kites were discovered in November and December 2011, except for one found in May 2012, and all were from the Puy-de-Dôme department. Ten of them were found in three municipalities close to each other (area of 300km²) where the bromadiolone treatments had been undertaken as described in Coeurdassier et al., 2014b and in table 1. The results of post-mortem examinations as well as sex and class age of the thirteen red kites are reported in table 1. Macroscopic haemorrhages (but no external trauma) were evident in ten of the red kites (77%, 3 females, 5 males, sex could not be determined in 2; 5 were adults and 5 were juveniles). There were no macroscopic haemorrhages in three of the red kites (3 females, 2 juveniles and 1 adult), and one of the three had signs of external trauma

3.2 Bromadiolone residues in liver of the 13 red kites

When an AR had two diastereoisomers, the total concentration (ng/g) and the proportions of the major and minor forms are reported (figures 1 and 2). There was no difference of ARs concentrations (means ± standard deviation) between the birds with macroscopic haemorrhages (681±88ng/g) and the birds without macroscopic haemorrhages (671±153ng/g). Bromadiolone residues were found in all the 13 red kites at concentrations between 390 and 871 ng/g (mean value 642 ng/g). Amazingly, proportion of trans-bromadiolone diastereoisomer was between 99.5 and 100% (mean value 99.9%) of total

bromadiolone residues for all the red kites, when European official values for proportions of trans-diastereoisomer in bromadiolone baits is between 70 and 90%. Hepatic residues of cis-bromadiolone diastereoisomer in red kites were null in 5 of 13 livers and were negligible in the remaining kites as they were below 2.2ng/g and comprised less than 0.5% of total bromadiolone concentration.

3.3 Exposure of the 13 red kites to other ARs

Difenacoum (100% cis-difenacoum) residues in liver were quantified in 9 of 13 red kites and were below 11.8ng/g. Brodifacoum residues were found in 12 of 13 livers with a maximum value of 59.9ng/g. Cis-brodifacoum was found in all of them and trans-brodifacoum in only 4 of 12 livers of red kites. Proportions of cis-brodifacoum were between 77.1 and 100% (mean value 94.3%). Flocoumafen residues were quantified in 2 of 13 red kites, with values below 3.3ng/g. Difethialone residues were found in 6 of 13 red kites, with maximum value at 24ng/g, and trans-difethialone was not detected. No first generation ARs were detected. Summed SGARs (Σ SGARs) were between 412 and 912 ng/g; 99.7% of these total residues were major diastereoisomers (i.e. trans-bromadiolone, cis-difenacoum, cis-brodifacoum, cis-difethialone) and 0.3% was minor diastereoisomers (i.e. cis-bromadiolone and trans-brodifacoum).

4. Discussion

4.1 Exposure to trans- and cis-bromadiolone

The presence of large quantities of trans-bromadiolone in liver (390-871ng/g), and macroscopic haemorrhages in 77% of the birds provides strong evidence that bromadiolone was involved in the death of the red kites. It is likely that the kites consumed poisoned voles after bromadiolone was applied to control rodent populations (Coeurdassier et al., 2014b; Decors et al., 2012). However, hepatic cis-bromadiolone (minor-isomer) residues were very low (figure 2) providing evidence that this isomer is unlikely to be responsible for the poisonings.

As illustrated in figure 3, the proportion of the bromadiolone diastereoisomers are very different in baits (European official values: 70-90% trans-bromadiolone) and in liver of red kites (99.9% trans-bromadiolone). Instability of the active substance in baits can be excluded because stability of the active ingredient and diastereoisomers proportions in bromadiolone baits have to be demonstrated by its manufacturer through mandatory aging tests. Moreover,

to definitively exclude this hypothesis, diastereoisomers proportions of commercial baits containing bromadiolone have been evaluated before and after use in the field, and the results were in accordance with regulatory documents and rules (data not shown). Our findings may be interpreted in two ways: (i) red kites consumed the cis-bromadiolone diastereoisomer when feeding on voles and eliminated it before dying, and/or (ii) voles had already eliminated all or most part of cis-bromadiolone diastereoisomer when they were eaten by red kites. The pharmacokinetics of bromadiolone in voles has already been documented but isomers patterns were not described (Sage et al., 2008). At present, there are no data available on the comparative pharmacokinetics of bromadiolone diastereoisomers in voles. However, previous results reported that wild rats and exposed laboratory rats differentially metabolized the diastereoisomers of bromadiolone and of difenacoum in their body (Damin-Pernik et al., 2016, 2017; Fourel et al., 2017). Proportions of the minor isomers cis-bromadiolone and trans-difenacoum in the livers of rats were very different compared to those observed in commercial baits: more than 75% of the wild rats with bromadiolone or difenacoum residues had only the major diastereoisomer. This means that between the time they fed on baits and the moment they were trapped, the rats eliminated bromadiolone and difenacoum minor diastereoisomers quicker than the major diastereoisomers. Consequently, predatory wildlife should not be exposed to bromadiolone and difenacoum minor diastereoisomers (or to little quantities) when feeding on exposed rats. This suggests that ARs in water voles and rats should probably have the same pharmacological behaviours, but it still remains to be confirmed. Indeed, metabolic capacity often depends on species and results with rats could not definitively predict these similarities. We need to investigate as well which species is most efficient in eliminating cis-bromadiolone: water voles or red kites. Additional experiments will be conducted in the near future to explore pharmacokinetics of the diastereoisomers of bromadiolone in water voles and birds to address these questions.

4.2 Exposure to other SGARs diastereoisomers

As previously reported, but without information on diastereoisomers proportions, non-target wildlife species have often been found to contain multiple ARs (Christensen et al., 2012; Lopez-Perea et al., 2015; Geduhn et al., 2015; Sanchez-Barbudo et al., 2012; Hughes et al., 2013; Ruiz-Suarez et al., 2014, 2016; Langford et al., 2013; Shore et al., 2015). The 13 red kites of our study were also exposed to several SGARs. Although difenacoum residues (max. 12ng/g) in the red kites are negligible compared to trans-bromadiolone residues, it is remarkable that the trans-difenacoum (minor diastereoisomer) was not found while the limit of detection is 1ng/g (European official values for proportions of trans-diastereoisomer in

difenacoum baits is between 20 and 50%). This is consistent with the above observations in rat liver. In commercial baits, cis-brodifacoum has been evaluated as being between 48% and 60% (Cort et al., 2012, Fourel et al., 2017). Proportions of trans-brodifacoum (minor isomer) are substantially less in the red kites compared to the baits (figure 3). Although it was not possible to compare with liver residues of wild rats from our previous study (Fourel et al., 2017), it seems that the proportions of brodifacoum diastereoisomers is also modified by rodents and/or predators. Although trans-brodifacoum is less persistent than cis-brodifacoum, it appears to be more persistent than cis-bromadiolone and trans-difenacoum (both minor isomers). As shown in figure 3, trans-brodifacoum was still present in red kites liver (5.7% of trans-brodifacoum and 94.3% of cis-brodifacoum) whereas cis-bromadiolone was almost totally absent from the kite livers (only 0.1% of total bromadiolone present and in contrast to trans-bromadiolone which comprised 95.5% of the total ARs residues). These results for brodifacoum are preliminary and need to be documented with further investigations and other sets of wildlife samples. Flocoumafen is not commercially available in France. Low levels of this persistent AR may be the result of winter migrations of red kites through Europe, as flocoumafen is used in a few European countries and has been detected in non-target wildlife species in Spain and Germany (Lopez-Perea et al., 2015; Geduhn et al., 2015). One of the red kites found dead in Auvergne during the winter 2011/2012 had been banded in Germany (Coeurdassier et al., 2014b), so possibly exposed to flocoumafen. Difethialone baits contain mainly cis-difethialone, which explained why only cis-difethialone was evidenced in red kites, as already reported in wild rat liver (Fourel et al., 2017).

4.3 Exposure to trans-bromadiolone above toxic threshold

Hepatic concentrations of ARs above 200ng/g have been associated with mortalities in raptors and small mustelids from Denmark (Elmeros et al., 2011; Christensen et al., 2012), in raptors and hedgehogs from Mediterranean region of Spain (Lopez-Perea et al., 2015), and in six raptor species from Canary Islands, Spain (Ruiz-Suarez et al., 2014). The use of these limits was based on several experimental observations or probabilistic studies with collections of numerous individuals (Newton et al., 1999; Shore et al., 2014; Thomas et al., 2011; Huang et al., 2016). In England, nineteen red kites were diagnosed as AR-poisoned because they had internal haemorrhages without associated trauma and summed liver ARs concentrations of 100ng/g or above (Jaffe et al., 2016). We could then consider that a limit of 100 or 200ng/g might be extrapolated to the thirteen red kites as representing a minimum toxic threshold for them. It is obvious that the major-isomers residues (between 410 and 911ng/g), mainly trans-bromadiolone (95.5%), are far above this limit, and the minor-isomers (below 15ng/g), mainly trans-brodifacoum, are far below this limit (figure 2a and 2b).

Moreover, it has been shown that cis-bromadiolone baits are as efficient as trans-bromadiolone baits in killing rodents and no choice feeding tests showed that the hepatic residues were 4-fold reduced with cis-bromadiolone baits (Damin-Pernik et al., 2017). We might then consider that the use of cis-bromadiolone would have allowed the reduction of 4-fold the amount of bromadiolone in liver of rodents and would have enabled a significant decrease of the exposure of the red kites. Moreover, it is probable that some of them would not have been exposed to SGARs above the potentially lethal limit of $0.1\text{-}0.2\text{mg.kg}^{-1}$. It seems obvious that the proportions of diastereoisomers in baits should be revised as an important step towards more ecological use of ARs. This would limit the exposure of non-target wildlife and complement established mitigation measures restricting AR treatments by farmers when endangered species like the red kite are present.

5. Conclusions

The new fully validated analytical LC-MS/MS method is a powerful tool to start the characterisation of SGARs diastereoisomer proportions in non-target wildlife, and to evaluate possible ecotoxicological consequences. In this study, the Coreshell LC-MS/MS multi-residue method demonstrated that only one diastereoisomer of the bromadiolone (trans-isomers) was involved in the poisoning of the red kites. This method provides complementary information on SGARs multi-exposure with quantitative as well as qualitative data on respective diastereoisomers proportions. It evaluates the evolution of these proportions in the predators' body compared to the baits or to the rodents' body, and in this way it demonstrates the potential towards more ecological use of anticoagulant rodenticides as one of the two diastereoisomers is less persistent than the other one. The other advantages of this analytical tool are the specificity of the detection allowing confident identification of the compounds, and sensitivity with hepatic low limits of quantification of $1\text{-}2\text{ ng/g}$ for each SGAR diastereoisomer.

When finding field carcasses, especially endangered red kites, and other non-target species, SGARs hepatic residues and diastereoisomers proportions should be used with clinical signs as criteria to diagnose anticoagulant rodenticides exposure. This should facilitate estimation of the degree of ecological risk of each isomer and help evaluate the hazard of new baits compared to existing bait formulations.

Liver residues have often been measured in wildlife but without specifying the diastereoisomers proportions. The significance of such information is now apparent, and will

become more important if development occurs to change the diastereoisomers proportions in baits. A change in the diastereoisomers proportion in favour of the less persistent isomers should reduce residue concentrations and modify the diastereoisomers proportions found in wildlife. It seems reasonable to monitor the effects of such modifications. Future surveys should then specify the diastereoisomers proportions to give qualitative as well as quantitative evaluation of diastereoisomer residues of each SGAR in predators.

Aknowlegments

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Footnotes

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Legends of figures

Figure 1: Summed SGARs concentrations (Σ SGARs - represented by the dotted line), summed major diastereoisomeric forms of SGARs (Σ major SGARs - represented by white circle) and summed minor diastereoisomeric forms of SGARs (Σ minor SGARs - represented by black triangle) concentrations in the liver of the thirteen red kites from 412 to 912ng/g and detailed percentages of bromadiolone, cis-difenacoum, brodifacoum, flocoumafen and cis-difethialone. Trans-difenacoum and trans-difethialone were not detected. Bromadiolone percentage was between 89 and 99.5% for all the birds.

Figure 2: Proportions and concentrations of diastereoisomers (%) of trans- and cis-bromadiolone and, cis- and trans-brodifacoum in the liver of the 13 red kites. Trans-bromadiolone was at least 99.5% of bromadiolone residues (mean 99.9%) and cis-bromadiolone proportion is 0-0.5% only (mean 0.1%).

Figure 3: Proportions of major and minor diastereoisomers for bromadiolone, difenacoum, brodifacoum and difethialone in liver of the thirteen red kites, and in commercial baits dosed in Fourel et al. (2017). The proportions of minor diastereoisomers were substantially lowered in liver of red kites compared to commercial baits.

Table 1: Post-mortem examination results with presence or absence of macroscopic haemorrhages, sex and class age of the thirteen red kites. Municipalities, department and date for the discovery of the carcasses of red kites.

n.r., not reported; n.d., not determined

Red kite	sex	age	macroscopic haemorrhages	discovery date	Department	Municipality	Total bromadiolone (ng/g)	Total difenacoum (ng/g)	Total brodifacoum (ng/g)	Total flocoumafen (ng/g)	Total difethialone (ng/g)
1	F	juvenile	yes	21/11/2011	Puy-de-Dôme	Saint_Bonnet_Près_Orcival	390	5,9	12,7	3,3	1,8
2	n.d.	juvenile	yes	01/12/2011	Puy-de-Dôme	Heume_l'Eglise	669		3,1		
3	F	adult	yes	01/12/2011	Puy-de-Dôme	Heume_l'Eglise	565	4,6	50,5		13,3
4	M	juvenile	yes	01/05/2012	Puy-de-Dôme	Saint_Bonnet_Près_Orcival	664	2,1	59,9	1,6	7,9
5	F	juvenile	no	27/11/2011	Puy-de-Dôme	Saint_Bonnet_Près_Orcival	636	1,7			11,2
6	M	adult	yes	30/11/2011	Puy-de-Dôme	Heume_l'Eglise	837		57		
7	M	adult	yes	30/11/2011	Puy-de-Dôme	Heume_l'Eglise	684	9,1	7,5		
8	F	adult	no	30/11/2011	Puy-de-Dôme	Heume_l'Eglise	607	2,8	5,8		
9	n.d.	juvenile	yes	28/11/2011	Puy-de-Dôme	Saint_Bonnet_Près_Orcival	643	2,9	1,4		
10	F	adult	yes	nov-11	Puy-de-Dôme	n.r.	527		11,9		17,9
11	F	juvenile	no	nov-11	Puy-de-Dôme	n.r.	766	11,8	3,1		
12	M	juvenile	yes	nov-11	Puy-de-Dôme	n.r.	489		56,1		
13	M	adult	yes	24/11/2011	Puy-de-Dôme	Saint_Sulpice	871	3	15,2		24,8

Figure 1

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Figure 1

Figure 2

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Figure 2

Figure 3
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