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Optimization of Toxicological and Forensic Tools in the Investigation of Wildlife Poisoning

Optimización de Herramientas Toxicológicas y Forenses en la Lucha contra el Veneno en el Medio Natural

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Memoria presentada por
Irene Valverde Domínguez

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VºBº de los Directores
Dr. Antonio J. García Fernández
Dra. Silvia Espín Luján

A mis padres Pilar y Juanjo

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Abbreviations

AA: Ammonium acetate

AAS: Atomic absorption spectroscopy

ABERG: Animal Behaviour and Ecotoxicology Research Group

AChE: Acetylcholinesterase

ACN: Acetonitrile

AFBI: Agri-Food and Biosciences Institute

AJS-ESI: Agilent Jet Stream Electrospray Ionization Source

AMA: Ammonium acetate

AR: Anticoagulant rodenticides

ASE: Accelerated solvent extraction

AT: Acetone

B.W.: Body weight

B: Biocide

BM0: Body mass on day 0

BMn: Body mass during necropsy

C18: Octadecylsilyl

C8: Octylsilane

CAD: Centro de Análisis y Diagnóstico (Wildlife Analysis and Diagnosis Centre) in Andalusia

CEEA: Comité Ético de Experimentación Animal

CF: Chloroform

CHEX: Cyclohexane

CRM: Certified Reference Materials

CRNMFV: Centro di Referenza Nazionale per la Medicina Forense Veterinaria Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana "M. Aleandri"

CV: Coefficient of variation

CWIH: Centre for Wildlife Investigation and Health, Faculty of Veterinary Medicine, Agricultural University of Tirana

DAD: diode array detector

DD: Days of decomposition

DDAVT: Department of Drug Analysis and Veterinary Toxicology, Scientific Veterinary Institute Novi Sad, Novi Sad

DE: Diatomaceous earth

DIE: Diethyl ether

DM: Dichloromethane

dMRM: Dynamic multiple reaction monitoring mode

DPP: Dihydrogen potassium phosphate

DSC: N, N′-Disuccinimidyl carbonate

dSPE: dispersive solid-phase extraction

EA: Ethyl acetate

EPA: Environmental Protection Agency

EQC: External quality control

ESI: Electrospray ionization source

ESI: Electrospray ionization source

ET: Ethanol

ET: Ethanol

EU: European Union

EULS: Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences

F: Female

FA: Formic Acid

FERA: Fera Science Ltd

FGAR: anticoagulant rodenticides

FL: Fluorescent detector

Fluo: Fluorescence

Fr: Freezing

FVMS: Faculty of Veterinary Medicine Skopje

GC: Gas chromatography

GPC: Gel permeation chromatography

HEX: Hexane

HLB: hydrophilic–lipophilic balanced copolymer cartridge

HPLC: high-performance liquid chromatography

ICP: Inductively Coupled Plasma

IP: Isopropanol

IQC: internal quality control

IREC-CSIC-UCLM: Institute of Game and Wildlife Research from University of Castilla-La Mancha

IZSVe: Istituto Zooprofilattico Sperimentale delle Venezie

LB: Liver bromadiolone concentration

LC: Liquid chromatography

LD50: Lethal dose 50

LHAP: Laboratório de Histologia e Anatomia Patológica da Universidade de Trás-os-Montes e Alto Douro

LLE: Liquid-liquid extraction

LMUM: Ludwig-Maximilians-University of Munich, Faculty of Veterinary Medicine, Institute of Pharmacology, Toxicology and Pharmacy

LOD: limit of detection

LOQ: limit of quantification

LP: Laboratory of Pathology, Croatian Veterinary Institute, Poultry Centre

LW: Liver weight

LWC: Liver water content

M: Male

MET: Methanol

$MgSO_4$: Magnesium sulphate

MS/MS: Tandem mass spectrometry

MS: Mass spectrometry

N: Total number of samples/individuals/compounds

NA: Not available

Na_2SO_4: Anhydrous sodium sulphate

NaCl: Sodium chloride

NaOAc: Sodium acetate

nd: Not detected

Nf: Not found

N_{LOQ}: Number of compounds with LOQ ≤ 5 (in ng/ml for blood and µg/kg for liver) multiplied by a coefficient of 0.9 and divided by the total number of compounds analysed

NP: Not provided

NR: Not reported

N_R: Number of compounds with recoveries ranging from 70 to 120% multiplied by a coefficient of 1.2 and divided by the total number of compounds analysed

NSAIDs: Non-steroidal anti-inflammatory drugs

OC: Organochlorines

OP: Organophosphates

OT: Oven temperature

Pb: Lead

PCA: Principal component analysis

PCs: Principal components

PHY: Potassium hydroxide

PMR: *Post-mortem* drug redistribution

PP: Phosphate

PPP: Plant protection products

PSA: Primary secondary amine

PT: Proficiency Testing

Q-TOF Quadrupole-time-of-flight

QuEChERS: quick, easy, cheap, effective, rugged, and safe method

RCF: Relative centrifugal force

RSD: Relative standard deviation

SASA: Science & Advice for Scottish Agriculture

SCDS: Sodium citrate dibasic sesquihydrate

SCTD: sodium citrate tribasic dihydrate

SD: Standard deviation

SERTOX-ULPGC: Toxicology Unit from University of Las Palmas de Gran Canaria

SGARs: second generation anticoagulant rodenticides

SPE: solid-phase extraction

STSM: Short-Term Scientific Mission

STVF-UM: Service of Toxicology and Forensic Veterinary from University of Murcia

Ta: Ambient temperature

Ti: Internal temperature

TI: Traumatic injury

TL: Toxicology lab, Department of toxicology, residues and environmental contaminants, Ministry of Development and Food

TOXLAB: Toxicology Laboratory, Vetagro Sup, Veterinary Campus

TRIET: Triethylamine

UHPLC: Ultra high performance liquid chromatography

UK: United Kingdom

UNEX: Unit of Toxicology from University of Extremadura

US: United States

UV: Ultraviolet detector

UW: Ultrapure water

VKOR: Vitamin K epoxide reductase

W.W.: Wet weight

W: Water

WRC: Wildlife Recovery Centre

σ: Target standard deviation for proficiency assessment

Introduction

1. The use of poison to kill wildlife

Wildlife is exposed to many threats worldwide which may cause important population declines and even the extinction of species. Most of these threats are driven by human activities and/or because human-wildlife conflicts (e.g., deforestation, use of pesticides in the fields, illegal hunting, illegal trade and illegal poisoning) (Woodroffe et al., 2005; Mateo-Tomás et al., 2012; Ntemiri et al., 2018; Aguirre et al., 2020; Roe et al., 2020).

The use of poison to kill animals is a traditional hunting activity linked to the history of humanity worldwide. First poisons were extracted from plants and animals. For example, poisoned weapons have been used in Asia, America, and Africa by some communities to hunt or fish (Cole, 1998; Ogada, 2014). More recently, synthetic pesticides have replaced the use of traditional poisons, perhaps because they are easier and quicker to produce and may cause massive effects (Cole, 1998; Ogada, 2014). Although the selectivity of poisoning depends on the type of poison and the way that poison is used relative to animal capture, poisoning is generally considered a non-selective method, affecting the target species but also any other domestic animal and wildlife, including endangered species (Berny, 2007; Ogada, 2014; Cano et al., 2016). Poisoning via indiscriminate methods or substances is reported as the major cause of wildlife mortality in some countries of Europe, Africa and Asia, and predators are typically reported as the species more frequently affected (Guitart et al., 2010; Tenan et al., 2012; Richards et al., 2018; Hong et al., 2019). However, little information is available in other countries from South America or Asia, where the extent of the poisoning is under reported in the literature, partly due to lack of resources for analysis and writing up such incidents (Wobeser et al., 2004; Gwaltney-Brant, 2018; Plaza et al., 2019). In the field, wildlife poisoning cases are often not reported and/or not detected (e.g., because some poisoned animals die away from poisoning source, there are no mechanisms in place to report wildlife deceases, carcasses may be too decomposed for meaningful analysis), which makes current real impact on the

status of wildlife unknown (Ogada, 2014; Ntemiri et al., 2018; Gil-Sánchez et al., 2021).

Deliberate abuse of pesticides/poisons occurs when toxic products are used to kill animals considered harmful to certain activities, such as i) to protect livestock-farming and hunting games from predation (Villafuerte et al., 1998; Ntemiri et al., 2018); ii) to eliminate so-called 'nuisance' animals like dogs and cats in the neighbourhoods (Navas et al., 1998; Mateo & Guitart, 2000; Berny, 2007; Mateo-Tomás et al., 2012; De Roma et al., 2017, 2018), or vultures in Africa where poachers poison these scavengers because they fly over the carcasses and give away the position of the illicit hunted animal (Ogada, 2014); iii) as a retaliatory way to solve feuds between private individuals (e.g., hunters use baits to poison sheepdogs, because they might attack hunting dogs, or the deliberate poisoning of neighbouring livestock because issues on land property and grazing rights) (Ntemiri et al., 2018).

However, poisoning may also occur as a result of a misuse of a pesticide substance (i.e., bad placing of AR baits or for excessive or wrong concentrations of pesticides in legal application) (Tariq et al., 2007; Gallocchio et al., 2014). One example is the management of the outbreak of Common voles (*Microtus arvalis*) in 2006-2007 by the regional government of *Junta de Castilla y León* (JCYL) in Spain, which financed three extensive campaigns of vole poisoning using anticoagulant rodenticides (AR) in the field (including five Natura 2000 Bird Special Protection Areas) and, consequently, caused a measurable decrease of biodiversity because of poisoning of non-target animals (Olea et al., 2009; Ntemiri et al., 2018). In addition to primary poisoning that occurs when the animal is poisoned directly by the bait or toxic product, secondary poisoning may occur when an animal predates a poisoned victim. Secondary poisoning is frequently reported in raptors and scavengers, e.g., Berny et al. (1997) reported foxes (*Vulpes vulpes*) and buzzards (*Buteo buteo*) poisoned by bromadiolone after rodent predation. Other secondary poisoning case of a golden eagle (*Aquila chrysaetos*) was reported by Wobeser et al. (2004) after consuming a black-billed magpie (*Pica hudsonia*) that died in association with

the use of organophosphates (OP) to treat cattle for ectoparasites. Carbofuran secondary poisoning in a white-tailed sea eagle (*Haliaeetus albicilla*) was reported after predation on a poisoned raven (Krone et al., 2017). Carcasses with strychnine residues have also been reported as a source of secondary poisoning for diurnal birds of prey, vultures, sheepdogs, cats, badgers (*Meles meles*), weasels (*Mustela nivalis*), corvids, and gulls, among others (Sánchez-Barbudo et al., 2012; Ntemiri et al., 2018). Also, entomofauna found in the carcasses may be affected by poisoning (Verón-Fernández et al., 2021). A tertiary poisoning has been suggested, e.g., reptiles may form part of a tertiary exposure pathway for predators (López-Perea & Mateo, 2018).

The most affected species in south-west Europe by poisoning are raptors and scavengers, such as Fox (*Vulpes vulpes*), Griffon Vulture (*Gyps fulvus*), Red Kite (*Milvus milvus*) and Black Kite (*Milvus migrans*), followed by domestic mammals (mostly dogs and cats) (Bodega Zugasti, 2014; Cano et al., 2016). Some species affected by poisoning are endangered wildlife species. Using domestic and feral animals as sentinel species should be highlighted in the study of wildlife poisonings (Cenerini et al., 2012; Cano et al., 2016). A recent study suggests that, due to different reasons, there are species overrepresented (vultures) and/or underrepresented, like corvids or small mammals. The overrepresentation may happen mainly due to three reasons: the bigger animal size and therefore more likely to be found, because birds are more frequently poisoned than mammals due to secondary poisoning, and because there are conspicuous species normally target of conservation programmes which give more attention to these species (Gil-Sánchez et al., 2021).

Bait ingestion is the main way of exposure of wildlife to the different toxic products used to kill animals worldwide (Mateo-Tomás et al., 2012; RSPB, 2009). The presentation of the baits is extremely diverse because they are handmade with the intention of making them attractive to each target species (García-Fernández et al., 2006; Ntemiri & Saravia, 2016). The most common type is a piece of meat mixed with one or more pesticides for large carnivores as target species (García-Fernández et al., 1997; María-Mojica et al., 1998, 2001), but also

other types like seeds coated with the toxic or *in-vivo* baits (alive animals with the poisoning substance attached) have been reported (Friend et al., 1999; María-Mojica et al., 2006; Giorgi & Mengozzi, 2011; Ogada, 2014; Ntemiri et al., 2018).

Figure 1 shows some examples of baits found in the field and received at the Service of Toxicology and Forensic Veterinary from University of Murcia in Spain (STVF-UM). Baits may also become a public health issue since children can accidentally encounter them and be intoxicated (De Roma et al., 2018), or because they can be ingested by game species such as wild boars which may later be captured for human consumption (Gil-Sánchez et al., 2021).

Other type of baits (i.e., 'nontoxic') are instead composed of materials intended to cause irreversible physical damage, leading to painful and slow death (e.g., screws, needles, glass (Giorgi & Mengozzi, 2011; De Roma et al., 2018) or hooks) (Figure 1).

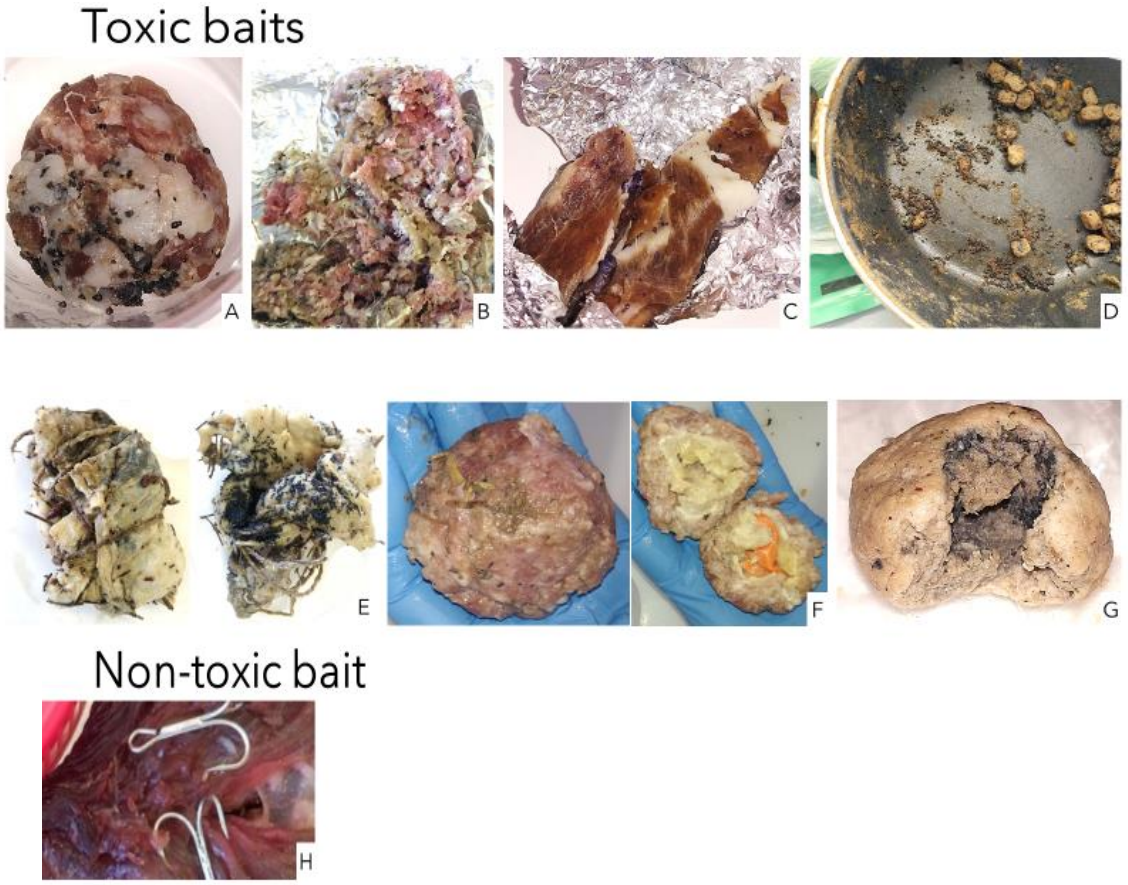


Figure 1. Examples of meat baits, handmade baits and non-toxic bait received at the STVF-UM. (*Images A-G: Irene Valverde; Image H: Isabel Navas. Property of the STVF-UM*). A. Minced meat with aldicarb; B. Minced meat with aldicarb; C. Ham with carbofuran; D. Dog feed with aldicarb

prepared in a pan; E. Chicken skin tied with a cord containing aldicarb. F. Minced meat containing a balloon filled with imidacloprid; G. Minced animal feed containing aldicarb; H. Two treble hooks hidden into a piece of fresh meat

2. Substances involved in wildlife poisoning cases

A wide variety of substances are involved in wildlife poisoning cases worldwide, however, some are more frequently encountered or detected. In this sense, the group of acetylcholinesterase inhibitors (AChE) (carbamates and OP) and AR are more commonly involved in wildlife poisoning (Caloni et al., 2012; Plaza et al., 2019; Bertero et al., 2020).

When focusing on European wildlife poisoning, different case reviews of animal poisoning have shown that the main compounds used are similar in the European countries (Berny et al., 2010; Guitart et al., 2010 a; Guitart et al., 2010 b). Table 1 shows a selection of post-2000 publications that report the toxic compounds involved in poisoning cases, as well as the species affected and country. In those publications, carbamates became the substances most frequently used in Europe, especially aldicarb and carbofuran, followed by AR, OP, strychnine and organochlorines (OC) (Motas-Guzmán et al., 2003; Berny, 2007; Wang et al., 2007; Hernández & Margalida, 2008, 2009; Berny et al., 2015; Ruiz-Suárez et al., 2015; Bille et al., 2016).

Table 1. Review of the main compounds detected in animal poisoning published since year 2000 in south-west Europe.

Toxic group	1st Toxic compound[1]	2nd Toxic compound[2]	Species	Country (Area)	Year	Toxic groups studied [a]	Reference
Carbamates, OP, AR	Aldicarb	Coumarin derivatives (AR)	Dogs, wildlife, cats, sheep, goats, bees, baits	Spain (Region of Murcia)	1992-2002	AR, Carbamates, Herbicides, OC, OP, Strychnine	Motas-Guzmán et al., 2003
Carbamates, OP	Aldicarb	Carbofuran	Birds, Mammals and Baits	Spain (Extremadura)	2002-2004	AR, Carbamates, OP, Strychnine	Soler-Rodríguez et al., 2006
Carbamates	Carbofuran	Difenacoum	Dog, cat, horse, cow, pig, sheep and rabbit	Austria	1999-2004	AR, Carbamates, Herbicides, Molluscicides, OP, Strychnine,	Wang et al., 2007
Carbamates	Carbofuran	Aldicarb	Birds of prey [b]	France (Pyrenees)	2005-2012	AR, Carbamates, Cd, OC, OP, Pb	Berny et al., 2015
AR	Bromadiolone	NR	Waterbirds [c] and raptors [d]	France (Loire Atlantique)	2003	Difenacoum, Bromadiolone, Coumatetralyl, Coumafen, Brodifacoum	Lambert et al. , 2007
Carbamates	Aldicarb	Carbofuran	Dog, wildlife, cat	Spain	2005-2010	AR, Carbamates, OC, OP, Molluscicides	Bodega Zugasti, 2014
Carbamates, AR	NR	NR	Wildlife, dog, cat	Italy	2005-2009	Carbamates, OP, RA, OC, Molluscicides, Strychnine, Zinc phosphide, alfa-Chloralose	Cenerini et al., 2012
Carbamates OP,	Carbofuran	Aldicarb	Wildlife, dog, cat	Spain (Aragón)			
Carbamates	Carbofuran	Bromadiolone, aldicarb	Wildlife, dog, cat	Spain (Canary Island)	2010-2013	Carbamates, OC, OP, Carbamates, OP, AR	Ruiz-Suárez et al., 2015
Carbamates	Aldicarb	Carbofuran	Wild birds and mammals	Spain	1992-2013	Carbamates, OP, AR, OC, Molluscicides, Strychnine	Cano et al., 2016

Table 1. Review of the main compounds detected in animal poisoning published since year 2000 in south-west Europe.

Toxic group	1st Toxic compound[1]	2nd Toxic compound[2]	Species	Country (Area)	Year	Toxic groups studied [a]	Reference
Carbamates	Aldicarb	Carbofuran	Egyptian vulture	Spain	1990-2007	OP and Car, OC, Pyrethroids	Hernández & Margalida, 2009
Metaldehyde, OC	Metaldehyde	NR	Baits	Italy (Campania and Calabria)	2013-2017	Carbamates, OP, OC, SR, Metaldehyde, Strychnine	De Roma et al., 2018

NR: Not reported
[1] First compound more detected in the study
[2] Second compound more detected in the study
[a] AR: Anticoagulant rodenticides; OC: Organochlorines; OP: Organophosphates
[b] Bearded Vulture (*Gypaetus barbatus*), Griffon Vulture (*Gyps fulvus*), Egyptian Vultures (*Neophron percnopterus*) and Red kite (*Milvus milvus*)
[c] Mallard (*Anas platyrhynchos),* Eurasian coot (*Fulica atra*), Common moorhen (*Gallinula chloropus*)
[d] Common kestrel (*Falco tinnunculus*), Buzard (*Buteo buteo*), Barn owl (*Tyto alba*), Tawny owl (*Strix aluco*)

2.1. Acetylcholinesterase inhibitors

Acetylcholinesterase inhibitors are a group of insecticides created to inhibit this enzyme in the synapsis nerves of the insect central nervous system (Casida & Durkin, 2013). This group of pesticides is represented by two groups: carbamates and OP.

Organophosphates (e.g., chlorpyrifos, diazinon, malathion, parathion, phorate, terbufos) were introduced in the 1940s and 1950s and they were insecticides widely used in agriculture or veterinary medicine, until many of them have been banned in the last years. Later, in the 1960s, carbamates were developed (e.g., aldicarb, carbofuran, methomyl, methiocarb, oxamyl) (Casida & Durkin, 2013; Vale & Lotti, 2015). Carbamates were developed to seek a more specific and less toxic alternative to mammals (Vale & Lotti, 2015). However, lethal dose 50 (at which 50% of the animals treated are dying, LD50) of some carbamates are lower than some OP, some examples are shown in Table 2. Despite all, OP and carbamates are pesticides less persistent in the food chain and in the environment compared to other insecticides more used in the past like OC (Smith, 1987).

Figure 2 represents the chemical structure of the most representative carbamates and OP due to their implication in wildlife poisoning.

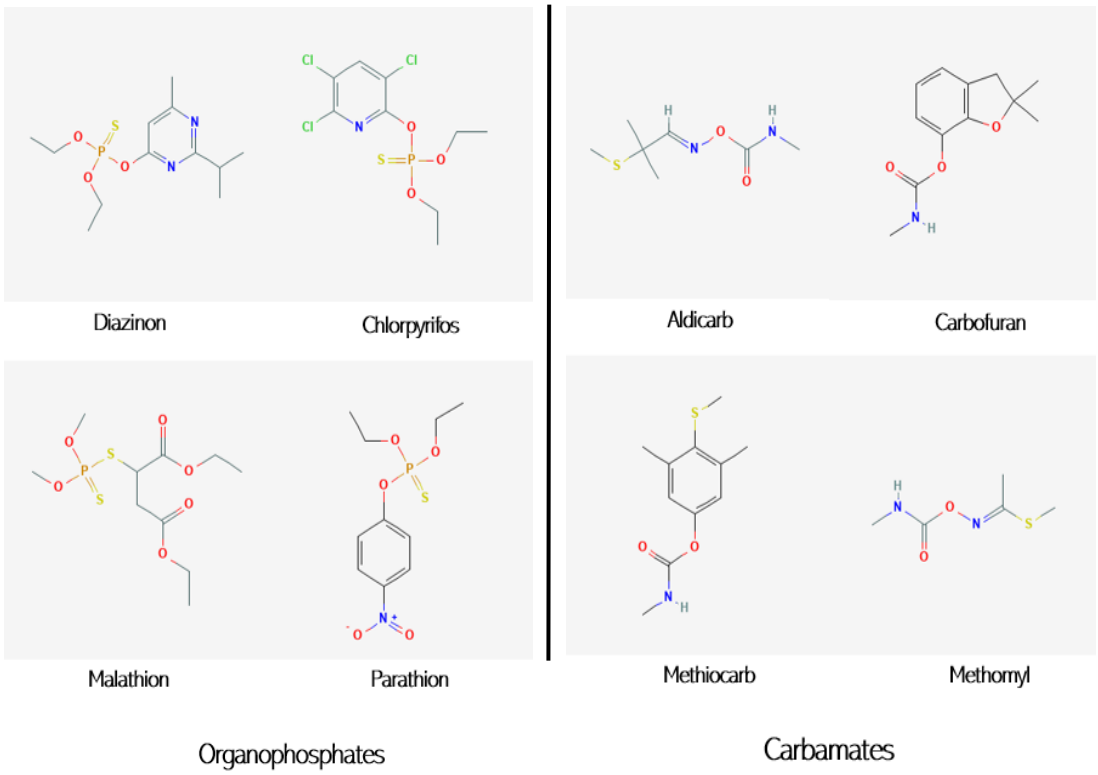


Figure 2. Chemical structure of the OP and carbamates most representative due to their implication in wildlife poisoning (Source: PubChem, 2021)

The mechanism of action of carbamates and OP consists of the inhibition of the enzyme AChE (represented in Figure 3). The acetylcholine (ACh) is a neurotransmitter present in mammals, birds, fish, reptiles and insects (Fukuto, 1990). This inhibition causes the accumulation of ACh at the synapses and stimulates the muscarinic and nicotinic receptors (Vale & Lotti, 2015). Carbamates bind less strongly than OP to AChE, and create a reversible inhibition, so they are considered less-toxic pesticides than OP, which are binding irreversibly. In addition, carbamates do not accumulate in the organs since they are rapidly metabolized and excreted (Buchweitz et al., 2013).

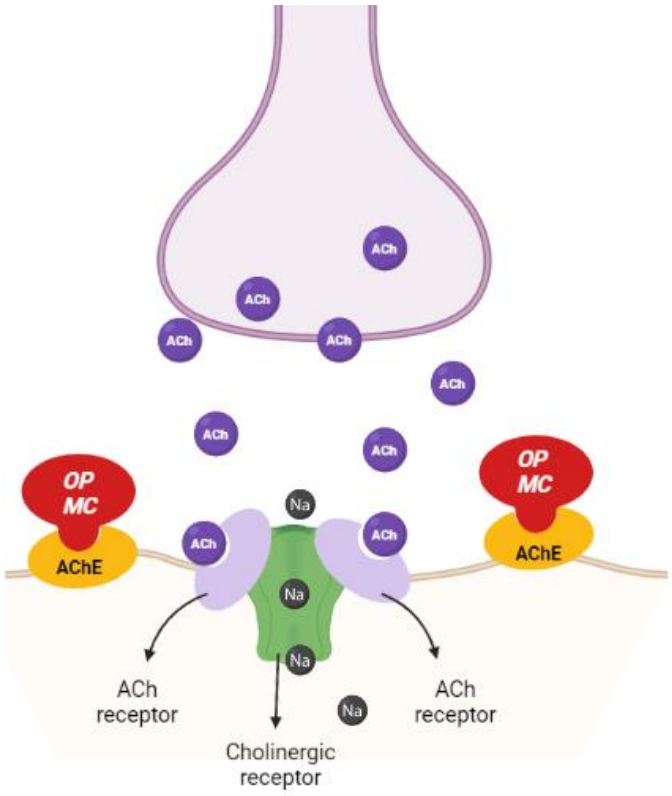


Figure 3. Inhibition of AChE activity by OP and carbamates in the nerve synapse (Created with BioRender.com by Irene Valverde). ACh: acetylcholine; AChE: acetylcholinesterase; MC: carbamate; Na: sodium; OP: organophosphate.

The first symptoms induced by AChE inhibitors are consequence of the stimulation of muscarinic receptors which causes the contraction of smooth musculature throughout the body, which symptoms are ptyalism, tears, nasal secretion, myosis, dyspnoea, vomiting, diarrhoea/involuntary defecation. After them, symptoms related to the stimulation of nicotinic receptors appear, such as fasciculations, weakness and paralysis. Finally, central nervous system disease symptoms appear, such as ataxia or paralysis, seizures, and coma. Death occurs from respiratory insufficiency or cardiac arrest (García-Fernández et al., 2006; Cenerini et al., 2012; Vale & Lotti, 2015).

Table 2. Oral LD_{50} and commercial formulations of some carbamates and OP.

	Aldicarb	Carbofuran	Methiocarb	Methomyl	Parathion	Chlorpyrifos	Diazinon	Malathion
Oral LD_{50} (mg/kg) in rat	0.9	11	15	17	3.6	97	300	1000
Commercial concentration	5, 10, 15%	50, 5 g/kg 480 g/L	500 g/L	200 g/L	15, 25% 2, 4, 6, 8% 0.5, 1, 2% 10% 10%	250, 480 g/L 50, 750 g/kg 5%	600 g/L	440 g/L
Commercial presentation	Granular	Flowable Granular	Liquid	Liquid	Wettable powders Emulsifiable dust Granular Aerosol	Liquid Granular	Liquid	Liquid

LD_{50}: Lethal dose 50

(Smith, 1987; Bradbury, 2007; FAO & PNUMA, 2017)

2.2. Anticoagulant rodenticides

In Europe, anticoagulant rodenticides are pesticides designed, registered and authorised to control rodents (Regulation (EC) No1107/2009, 2009; Regulation 528/2012, 2012). Rodents in agricultural settings pose economic repercussions when they feed on crops and compromise stored food with their excreta where they reside. In the domestic scenario, rodents are controlled to avoid diseases transmission to humans and domestic animals (Jacob & Buckle, 2018).

Before the mid-1950s, rodents were controlled worldwide by rodent proofing structures, trapping, repellents, bacterial products, or acute poisons such as arsenic or strychnine. After this period, ARs became the main method to control rodents worldwide, and warfarin was the first AR used (Shore et al., 1999; Jacob & Buckle, 2018).

Anticoagulant rodenticides are classified as first-generation ARs (FGARs), e.g., chlorophacinone, diphacinone, coumatetralyl and warfarin, and second-generation ARs (SGARs), e.g., brodifacoum, bromadiolone, difenacoum, difethialone. SGARs were developed between 1975 and 1985 following the resistance to certain FGARs observed in some rodent populations (Shore et al., 1999; Jacob & Buckle, 2018). However, the development of a third generation based on the stereochemistry of the old SGARs (e.g., Trans and Cis-Isomers of SGARs) is being suggested (Damin-Pernik et al., 2017). Figure 4 shows the chemical structure of the FGARs and SGARs more representative due to their implication in wildlife poisoning. Based on their chemical structure, they may be grouped into hydroxycoumarins or indandiones.

The mechanism of action of anticoagulant rodenticides is based on the inactivation of the vitamin K epoxide reductase (VKOR) in the liver and other tissues (Figure 5).

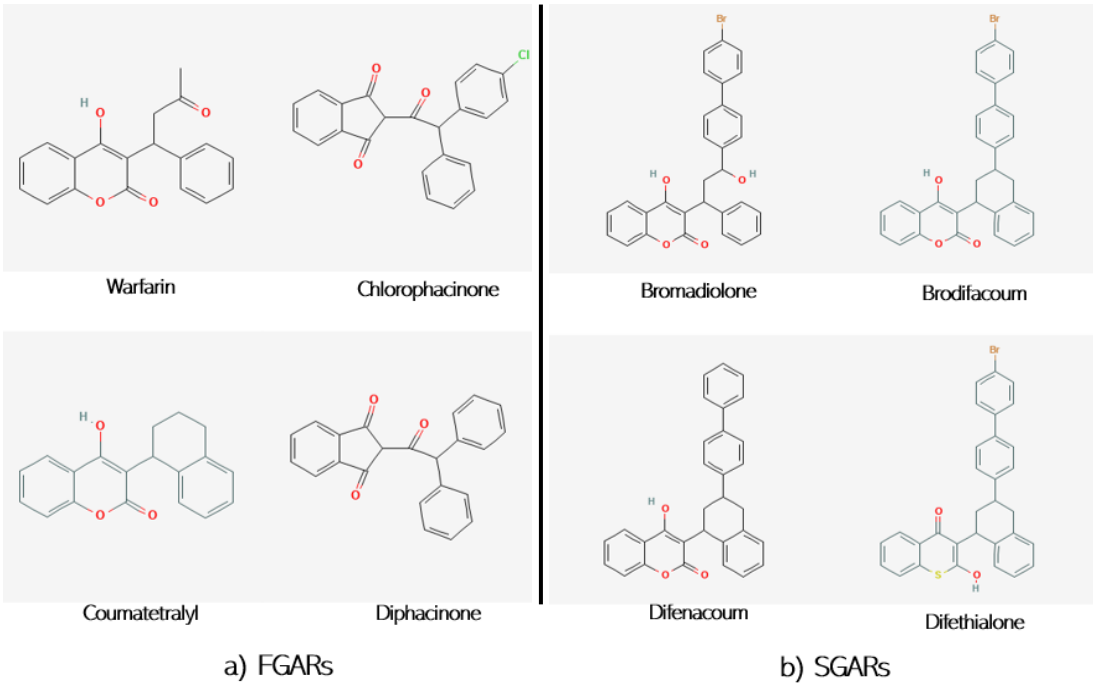


Figure 4. Chemical structure of FGARs (a) and SGARs (b) more representative due to their implication in wildlife poisoning (Source: PubChem, 2021)

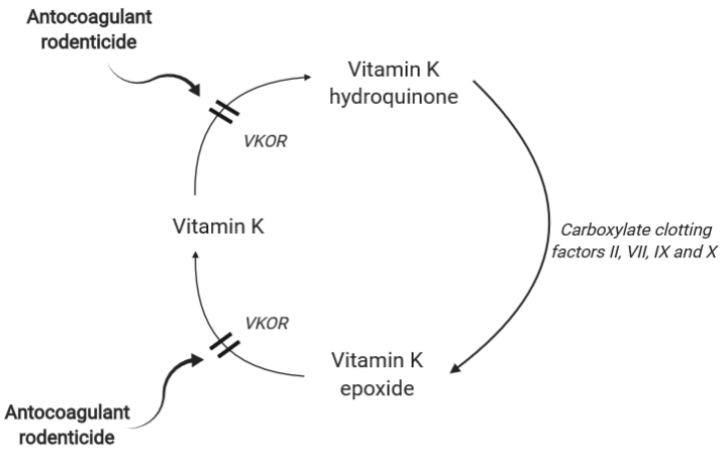


Figure 5. Mechanism of action of anticoagulant rodenticides. VKOR: vitamin K epoxide reductase (Valverde et al., 2021)

Consequently, vitamin K hydroquinone formation decreases, and causes a reduction in the carboxylation (involving the creation of a carboxylic acid group) of clotting factors II, VII, IX, and X. As a result, blood clotting is affected. Coagulation factors II, VII, IX, X are activated by the reduction of vitamin K (hydroquinone), which leads to the γ-carboxylation of the coagulation factors. When these factors are activated, vitamin K is reduced to vitamin K epoxide. Vitamin K reductase catalyses inactive epoxide to hydroquinone again (Furie et al., 1999; van den Brink et al., 2018). As a result,

the main symptom of AR intoxication is the presence of internal and/or external haemorrhage (Figure 6) (Erickson & Urban, 2004; Muscarella et al., 2016).

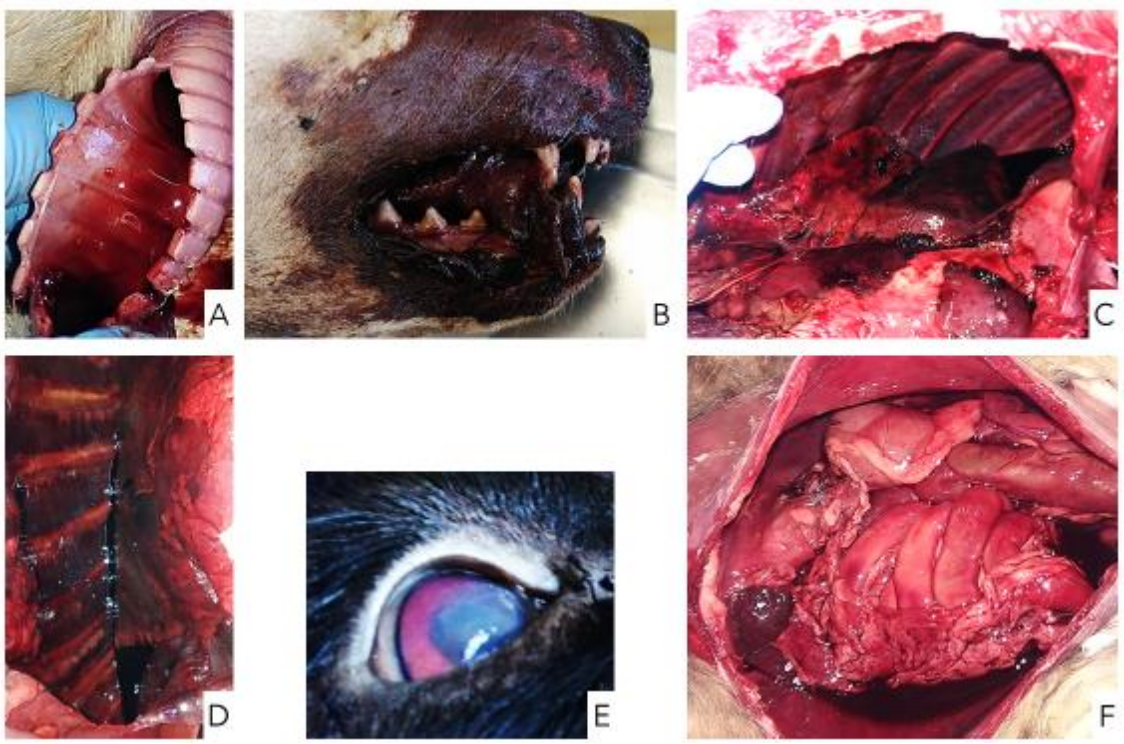
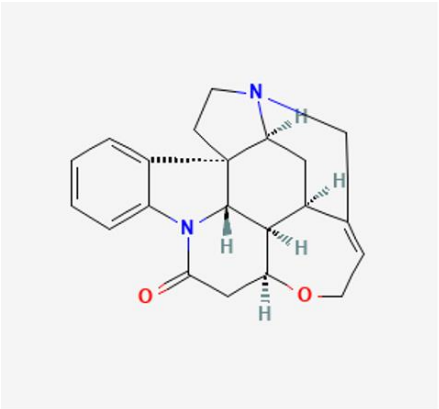


Figure 6. Examples of dog carcasses with anticoagulant rodenticides haemorrhages received at the STVF-UM (*Images: Irene Valverde. Property of the STVF-UM*).

A. Haemorrhage in trachea; B. Bleeding from respiratory tract through the nose and mouth; C. Haemorrhages in lungs and muscles; D. Blood in thoracic cavity; E. Blood in the eyeball; F. Blood in abdominal cavity and haemorrhaging organs.

2.3. Other compounds of concern in wildlife poisoning

Strychnine



Strychnine in an alkaloid extracted from the plant *Strychnos nux-vomica*. Figure 7 depicts the chemical structure of strychnine. This compound was widely used in Europe as rodenticide until its prohibition in 2004 (Commission decision 2004/129/EC, 2004).

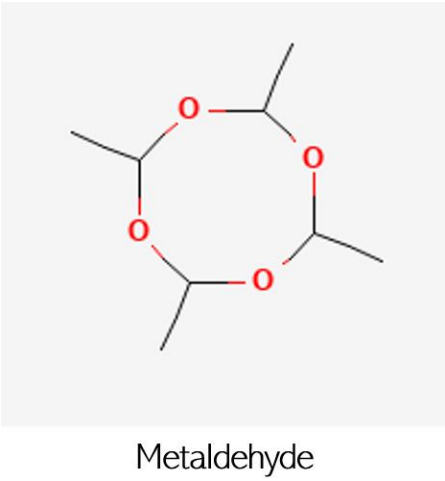
Figure 7. Strychnine chemical structure (Source: PubChem, 2021)

Strychnine is absorbed in the gastrointestinal tract of mammals, birds, reptiles and amphibians, and acts upon the central nervous system. The mechanism of neuroexcitation occurs through competitive antagonism of the inhibitory neurotransmitter glycine. This causes the inactivation of the motoneurons in the relevant segment, and it also affects the agonist and antagonist muscles producing prolonged and generalized muscle contractions with rigidity of the limbs, arching of the head, neck and back in extreme hyperextension (Chaiarch & Leitch, 1971; Rosano et al., 2000; Barroso et al., 2005) (Figure 8).

Figure 8. Rigidity of the limbs and extreme hyperextension in a dog poisoned with a bait containing strychnine (Image: Antonio J. García-Fernández, property of the STVF-UM).

Death is caused by the cessation of respiration arising from the tonic and tetanic contractions of diaphragm and thoracic abdominal muscles (Duverneuil et al., 2004; Martínez-López et al., 2006; García-Fernández et al., 2004, 2006; Cenerini et al., 2012).

Metaldehyde



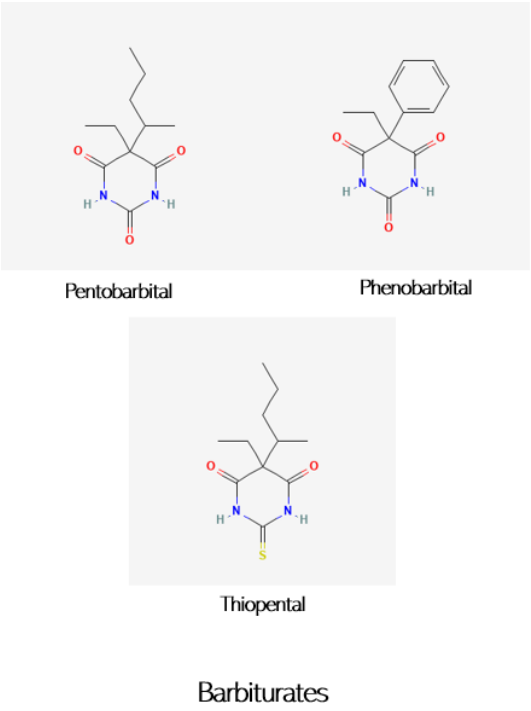
Metaldehyde is a pure tetramer of acetaldehyde used to control slugs and snails. Figure 9 represents the chemical structure of metaldehyde.

Figure 9. Metaldehyde chemical structure (Source: PubChem, 2021)

The mechanism of action of metaldehyde is based on the inhibition of gamma aminobutyric acid (GABA) norepinephrine, 5-hydroxytryptamine, 5-hydroxyindolacetic acid and an increase of monoamine oxidase activity in the

brain. Dogs fed metaldehyde exhibited vomiting, depression, incoordination, hyperpnea, tachycardia, prostration and cyanosis (Von Burg & Stout, 1991; Jones & Charlton, 1999; De Roma et al., 2017).

Barbiturates



Barbiturates are a group of drugs widely used in veterinary medicine as euthanizing agents and in human medicine are increasingly used to control epilepsy, among other uses. The primary active component in euthanasia solutions is sodium pentobarbital (Tanaka et al., 1997; Thomas, 1999; Yarema & Becker, 2005; PubChem, 2021). Figure 10 shows the chemical structure of some representative barbiturates.

Figure 10. Chemical structure of some representative barbiturates (Source: PubChem, 2021)

Barbiturates cause a swift depression of the central nervous system, starting in the cerebral cortex. The barbiturate overdose causes first deep anaesthesia and then apnoea, due to the depression of the respiratory centre, which finally causes a cardiac arrest (American Veterinary Medical Association, 2013).

3. Diagnosis of Poisoning: a challenge for laboratories

A suitable *post-mortem* examination and well-focused toxicological analyses are essential in the investigation of wildlife poisoning cases (Brown et al., 2005), but also the information obtained in the crime scene before the removal of the carcass or bait (García-Fernández et al., 2006) can help to guide the toxicological analyses and to give a diagnosis. Therefore, these cases pose a

challenge in different stages of the investigation, which in turn complicates conclusively identifying or confirming that a poisoning event has in fact occurred.

During the investigation many difficulties may appear, including (as per Wobeser et al., 2004; García-Fernández et al., 2006; Berny, 2007; Luzardo et al., 2015): i) finding poisoned animals and/or baits in the field; ii) standard tissue matrices not always being available due to the degradation during cadaveric decomposition processes; iii) the volume of sample obtained often being insufficient for toxicological analysis; iv) the possibility of a wide range of different substances being involved; v) the frequent lack of information regarding the poisoning event; vi) the lack of tissue reference concentrations or values associated with acute poisoning in wildlife species, being even less available in decomposing tissues; and vii) the difficulty to make a trial and to charge the person responsible of the illegal act.

3.1. Optimal samples for acute intoxication diagnosis

When collecting samples in a suspected poisoning event, it is important to understand the way of exposure of the possible toxic and select the best sample according to their kinetic and dynamic. However, in wildlife poisoning, the most suitable sample to investigate a case is not always available. When possible, the main samples to collect are:

Gastric content. It is the target sample when use of rapid-acting pesticides such as AChE inhibitors (carbamates and OP) or strychnine, may be suspected. Chemical substances may remain in the stomach content with negligible to no alteration which can facilitate the analytical procedures in many cases (e.g., sometimes it is possible to visually identify the commercial formulation from intact product remains). For the same reason, vomit and gastric wash (an intervention carried out in living animals that have ingested pesticide within approximately an hour) should be also analysed when they are available. If SGARs use is suspected then their delayed toxic effects will negate the viability of these samples (the compound will not be found in the gastric content),

instead liver or blood will be the more appropriate samples (Berny, 2007; Mateo et al., 2013; Vale & Lotti, 2015; Bille et al., 2016).

Liver. It is the main metabolising organ in the body. Additionally, it is considered the optimal organ in which to confirm the absorption of the chemical substance when oral intoxication is suspected. However, liver is not optimal for all potentially suspected cases of oral intoxication, it will depend on the substance and its quick action (Mateo et al., 2013; Vudathala et al., 2014). In addition, some substances may be degraded to other metabolites very quickly by liver detoxification activity, for example in ethylene glycol intoxication (Hess et al., 2004; Mateo et al., 2013). Moreover, liver may be also an easy sample to collect because it normally has a sufficient amount of sample available and remains longer during carcass decomposition (Soria-Sánchez & Valverde-Villarreal, 2015; Valverde et al., 2020). Uneven distribution of certain substances across liver tissue may exist, so homogenization before chemical analysis may be necessary for accuracy and to avoid false negatives.

Blood. It may be gathered from living or recently deceased animals. Blood reflects the pharmacological status of the animal at the time of death. However, this must be taken carefully since *post-mortem* changes may affect the initial concentration (Mateo et al., 2013; Soria-Sánchez & Valverde-Villarreal, 2015).

Brain. In fresh carcasses, brain may be used to detect a decrease of AChE activity as a biomarker of effect induced by exposure to carbamates or OP. Depending on the region of the brain, AChE values can differ significantly (Hart & Westlake, 1986). Moreover, carbamates bind to AChE and create a reversible inhibition, while OP binding is irreversible. It can also be used to detect lipophilic pesticides (e.g., OC) or drugs (e.g., morphine and cocaine) (Berny, 2007; Mateo et al., 2013; Soria-Sánchez & Valverde-Villarreal, 2015).

Plasma. Like blood, plasma may be gathered from alive or recently deceased animals. It is a useful sample for detecting a decrease of AChE activity like in brain, which could be extrapolated to mean. Other toxins (e.g., botulinum toxin) may be also detected in plasma (Mateo et al., 2013).

When the carcass is in a very advanced state of decomposition other samples may be used such as **pellets, palate, tongue, talons** or the portion of **earth beneath the carcass** (Mateo et al., 2013; Richards et al., 2014, 2015, 2017). **Cadaveric fauna** on and around the carcass can be also indicative and useful for poison detection (Gagliano-Candela & Aventaggiato, 2001; Jales et al., 2020).

Other tissues such as kidney or urine are also considered, however, they are not frequently analysed in wildlife poisoning for several reasons: they are difficult to collect, particularly in small animals such as rodents or passerines, especially when they are too decomposed, and there are no reference concentrations available in the literature (although they would be useful for qualitative analyses) (Berny, 2007; Soria-Sánchez & Valverde-Villarreal, 2015).

In spite of all samples mentioned above, in the Spanish Ministerial Order (OrdenJUS/1291/2010, 2010), the Article 11 "Types of samples for *post-mortem* toxicological studies" refers to different samples that must be collected to study toxicological cases, including blood, gastric content and liver. However, in the same Ministerial Order, the Article 25 "Sampling in cases of wildlife poisoning" only refers to gastric content as matrix in cases of dead mammals and birds, and water in cases of dead fish, which is confusing since the same samples must be collected to study human or wildlife poisoning cases.

3.2. Carcass decomposition and *post-mortem* redistribution of residues

The wildlife carcasses found in the field can be in a wide range of different **stages of decomposition**. In association, many samples that would be optimal if retrieved from a fresh carcass are very often also encountered in advanced stage of decomposition. As a result, they are not submitted to laboratories due to the uncertainty of their usefulness for toxicology analysis. Besides, only few articles mention the state of the samples when reporting poisoning cases (Berny et al., 1997, 2007; Martínez-López et al., 2006), or the related effects on the detection of the toxic compounds and their usefulness for toxicological analysis (Oates, 1984; Brooks, 2016; Jarmusz & Bajerlein, 2019).

The stage of decomposition of the carcass may affect and difficult the detection of substances involved in a poisoning case, since the availability of sample and

the compounds may be altered (Brown et al., 2005; Luzardo et al., 2014). For this reason, alternative samples have been suggested in the investigation of wildlife poisoning (e.g., talons, beaks, palate, tongue) (Richards et al., 2015, 2017).

Moreover, it is important to take into account that the bodies after death are not static. One example of these changes is *post-mortem* redistribution (PMR), which may produce changes in the drug concentration between the organs after death by two different mechanisms: diffusion through blood vessels and transparietal diffusion to the neighbour organs. PMR means that concentrations detected in the organs after death may not reflect the real concentration at the moment of death (Pélissier-Alicot et al., 2003; Yarema & Becker, 2005). In spite of the importance of this process, it is barely studied in humans and animals (McIntyre, 2014).

All these issues, favour that little information is available for the laboratories when receiving the samples.

3.3. Compound degradation and transformation

A correct interpretation of the toxicology results in living beings and in dead bodies is achieved through understanding the pathways of degradation and transformation of toxic compounds in the environment. During degradation processes, compounds disappear, and new compounds are generated. Knowing these processes will provide useful information to establish the chronology of the poisoning, possible means of exposure and to identify the most appropriate analytical methods, among others.

Toxic substances used to kill animals undergo degradation/transformation when/if exposed to environmental conditions (rain, sun, moisture, etc.); nevertheless, their persistence depends on a combination of their chemical properties and on the environmental conditions they are exposed to in a specific moment. Two main routes of degradation exist: i) biotic transformation, prompted by microorganisms; and ii) abiotic transformation, produced due to chemical and photochemical reactions (Fenner et al., 2013; Singh et al., 2014). Biotic degradation is the main route of pesticide degradation; however,

depending the chemical properties of a pesticide, will further influence the main route through which degradation occurs. For example, the carbamates main degradation routes in the environment are microbial, base catalysed transformation (hydrolysis of ester bond) or phototransformation; while OP main degradation route is microbial transformation (oxidation and hydrolysis) (Fenner et al., 2013). SGARs are considered stable in the environment and in soil because of their low solubility in water (Sage et al., 2007); barbiturates are pharmaceuticals with high persistence in the environment and in the carcasses (Harms et al., 2014; Payne et al., 2015), they may persist during years (Giusiani et al., 2012).

Moreover, the type of substratum may determine the route of decomposition of the chemical. In soil, the pesticide degradation depends on factors such as temperature, moisture, bacterial composition and bacterial activity. In aquatic environments, it depends on the presence of inorganic and organic nutrients, temperature, oxygen concentration, redox potential, bioavailability and microbial adaptation (Kurek et al., 2016).

In addition to abiotic degradation (affected by weather conditions and other factors) and biotic degradation (affected by microorganism activity), the toxic substances involved in poisoning cases may be degraded in the carcass by other factors such as tissue autolysis and cadaveric fauna involved in the decomposition process. However, little literature and information are available about the degradation process of the toxic substances in dead body of pesticide-poisoned animals (Oates et al., 1984; Martínez-López et al., 2006; Berny, 2007; Brooks, 2016; Viero et al., 2019). There is an experimental study which demonstrated the decomposition of methomyl in blood by internal bacteria such *as B. cereus, P. aeruginosa,* and *Bacillus sp.* (Kawakami et al., 2017).

An increase in temperature may accelerate the degradation of carbamates and OP (Smith, 1987), whith carbofuran and fenamiphos being degraded more rapidly in/on moist surfaces and high temperatures (Cáceres et al., 2010; Otieno et al., 2010). Post-mortem chemical changes are still imprecise, unreliable, and

impractical for use in the field (Brooks, 2016). However, some studies have demonstrated that anaerobic bacteria can degrade some drugs such as benzodiazepines, heroin or cocaine (Robertson & Drummer, 1995; Drummer, 2004). Moreover, cadaveric fauna strongly modifies the tissues after death when feeding on the carcasses, which make them less available for further forensic and toxicologic studies (Viero et al., 2019). Cadaveric fauna may also be affected from the poison and found dead around the carcass. The presence of pesticide residues from poisoning may alter insect life cycle stage development and growth which can alter rate of degradation and estimation of time of death using collected insect specimens that have themselves been exposed while interacting with a carcass (Martins, 2012; Fajardo et al., 2015, 2016; Fernández-Verón et al., 2021). The fauna might also be used for the analysis (Gagliano-Candela & Aventaggiato, 2001; Jales et al., 2020).

3.4. Chemical-toxicological analyses

Regarding the analytical methodology, there is a wide range of extraction and clean-up techniques and instrumental methods for the identification and quantification of toxic substances in biological samples. Nevertheless, most of them have been designed for the analysis of compounds within the same chemical group (e.g., different chemical groups may be destroyed or modified by the analytical techniques used for analysis of others), so it might be needed to use several different methods to optimize chances of detection if use of poisons spanning a range of chemical groups may be suspected (Tarbah et al., 2004; Barroso et al., 2005; Inoue et al., 2007; de Siqueira et al., 2015; Imran et al., 2015).

In addition to different techniques to analyse the same or different chemical groups, there are different methods using different samples, precision parameters, recoveries, detection and quantification limits (Vudathala et al., 2010; Bidny et al., 2015). As an example of this wide variety, AR may be extracted from liver, kidney, blood and bile, among others, by liquid-liquid extraction (LLE), solid-phase extraction (SPE) or dispersive solid-phase extraction (dSPE) (Valverde et al., 2021); carbofuran can be extracted from liver,

vitreous humour, blood, and serum by dSPE or SPE and detected by liquid chromatography (LC) (Molina-Ruiz & Cieslik, 2015; Sell et al., 2017); or OP can be extracted using dSPE or SPE and analysed with both LC and gas chromatography (GC) from blood, liver or stomach content (Ko et al., 2014; Lacassie et al., 2001; Sell et al., 2017; Valente et al., 2015). These variety of techniques can give results that are not comparable in certain situations, which leads to the necessity of reviewing available techniques in the literature, comparing them, and developing new standardized methods. The evaluation of methodologies and performance is important to avoid e.g., false negatives, perception certain classes of compounds are not used when in fact they would not be detected via analysis favoured by certain labs, or non-attribution of cause of death in some carcasses. This will ensure all laboratories are delivering reliable results.

Therefore, it is essential to develop sensitive and specific multiresidue methodologies, to cover a wide spectrum of unknown toxic substances in samples from different nature and state of decomposition in order to minimize costs and time, and to increase the possibilities to identify the toxics involved in poisoning cases (Luzardo et al., 2014).

4. Wildlife poisoning laboratories

In order to improve the fight against wildlife poisoning, it is important to join and coordinate efforts within and between countries to share information and maintain contact with other colleagues working in the same field (Mateo, 2010; Motas-Guzmán et al., 2003), as the use of poison evolves relatively quickly and can vary considerably depending on the region (Bodega Zugasti, 2014). Therefore, the creation of a European network, where countries can share data about toxicovigilance, poisoning cases and substances currently used in each area, has been proposed by many authors, institutions and projects (COST CA16224; Elliott et al., 2008; Mateo, 2010; EU Action Plan, 2015; Silva et al., 2018). In addition, the creation of new or harmonized/consolidated techniques and protocols of standardisation to make the results comparable within the international network are recommended.

Beyond the importance of an international network, it is also crucial to create national networks when more than one laboratory exists in the same country. For example, in Spain there are five reference laboratories with large experience in forensic veterinary toxicology, which work as a Spanish network for years. These laboratories are: the Service of Toxicology and Forensic Veterinary from University of Murcia (STVF-UM), the Unit of Toxicology from University of Extremadura (UNEX), the Institute for Game and Wildlife Research from University of Castilla-La Mancha (IREC-CSIC-UCLM), the Toxicology Unit from University of Las Palmas de Gran Canaria (SERTOX-ULPGC), and the Wildlife Analysis and Diagnosis Centre in Andalusia (CAD).

To ensure the reliability of the analytical results, laboratories have developed quality control systems and standards. The existence of these networks may allow the comparison of techniques to compare and contrast analytical performance and parameters (e.g., better recoveries, sensibility, reproducibility). There are internal quality control (IQC) and external quality control (EQC) activities. IQC is the responsibility of each team of analysts, and technicians and is defined "as the set of procedures undertaken by laboratory staff for the continuous monitoring of an operation and the measurements to decide whether results are reliable enough to be released" (Garrido Frenich et al., 2006). On the other hand, EQC provides both evidence of the quality of the laboratory's performance as a whole, and of individual analyst proficiency. EQC is typically proofed by an interlaboratory comparison (Garrido Frenich et al., 2006). Interlaboratory comparison is defined as "organisation, performance and evaluation of tests on the same sample by two or more laboratories in accordance with predetermined conditions to determine testing performance. According to the purpose the study can be classified as collaborative study or proficiency study" (Commission decision 2002/657/EC, 2002).

Collaborative studies or collaborative assessment experiments are recommended, in which the performance of each laboratory is assessed analysing the same sample (same standard measurement method on identical material) by the same method to determine the performance characteristics of

the method. The study covers random measurement error and laboratory bias (Commission decision 2002/657/EC, 2002; ISO5725-1:1994, 1994). Collaborative studies according to ISO5725-1:1994 are necessary to verify reproducibility (Commission decision 2002/657/EC, 2002).

Proficiency study is based on "the analysis of the same sample allowing laboratories to choose their own methods, provided these methods are used under routine conditions. The study has to be performed according to ISO Guide 43-1, 1997 and ISO Guide 43-2, 1997 and can be used to assess the reproducibility of methods" (Commission decision 2002/657/EC, 2002).

EQC activities and IQC measures are complementary, not exclusives (Garrido Frenich et al., 2006).

Table 3 compiles the main parameters evaluated during method validation, including precision (repeatability, reproducibility), accuracy, linearity, recovery, limit of detection (LOD) and limit of quantification (LOQ) (Commission decision 2002/657/EC, 2002; Rao, 2018).

Table 3. Main parameters to be verified during method validation

	Definition	Measurement	Formula
Precision	The closeness of agreement between independent test results obtained under stipulated conditions when the procedure is applied repeatedly to multiple samplings.	By injecting a series of standards or analysing series of samples from multiple samplings from a homogeneous lot.	RSD (%) or CV (%) = SD/Mean ×100
Repeatability (Precision)	Precision where independent test results are obtained with the same method on identical test items, in the same laboratory, by the same operator, using the same equipment.		RSD (%) or CV (%) = SD/Mean ×100
Reproducibility (Precision)	Precision where test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipment.		RSD (%) or CV (%) = SD/Mean ×100
Selectivity/Specificity	The ability to measure accurately an analyte in the presence of interferences that may be expected to be present in the sample matrix.	By examining chromatographic blanks (from a sample that is known to contain no analyte) in the expected time window of the analyte peak.	
Accuracy	The closeness of agreement between a test result and the accepted reference value.	By spiking the sample matrix of interest with a known concentration of analyte standard and analysing the sample using the method being validated.	
Trueness	The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. It is usually expressed as bias.		Trueness (%) = mean recovery-corrected concentration detected × 100/certified value.
Bias	The estimate of the systematic error.		Bias (%) $= X_{laboratory} - X_{reference} / X_{reference} \times 100$
Linearity	Capability to elicit check consequences which might be at once, or with the aid of well described mathematical adjustments, proportional to the concentration of analytes in	By injecting a series of standards of stock solution/diluted using the solvent/mobile phase, at a minimum of five different concentrations in the range of 50–150% of the expected working range.	

Table 3. Main parameters to be verified during method validation

	Definition	Measurement	Formula
	within a given range. It is determined by trueness and precision.		
Limit of Detection	The lowest concentration at which the instrument can detect but not quantify and the noise to signal ratio should be 1:3.		LOD (mg/L) = 3 × Noise/Signal × Lowest concentration of the linearity samples
Limit of Quantitation	The lowest concentration at which the instrument can detect and quantify. The noise to signal ratio should be 1:10.		LOQ (mg/L) = 10 × Noise/Signal × Lowest concentration of the linearity samples
Recovery	The percentage of the true concentration of a substance recovered during the analytical procedure. It is determined during validation if no certified reference material is available.		Recovery (%) = 100 × measured content/fortification level

CV: coefficient of variance; RSD: relative standard deviation; SD: standard deviation

(2002/657/EC, 2002; Rao, 2018)

5. Regulation in the European Union

The use of baits is an historical human practice to control predators and was even promoted in the past by law in some countries (BOE, 1953; Bodega Zugasti, 2014; Ntemiri et al., 2018).

In the last years, the use of baits has been forbidden in many countries around the world (Ogada, 2014). In Europe, the use of baits is prohibited by the Habitats and Birds directives (Directive 2009/147/EC of the European Parliment and of the Council of 30 November 2009 on the conservation of wild birds, 2010; Council Directive 92/43 EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora, 1992) as well as by the Bern Convention on the Conservation of European Wildlife and Natural Habitats. Furthermore, each country usually has its own legislation about this issue (Muscarella et al., 2016; Ntemiri et al., 2018).

Although many toxic products legally used in the past to control pests are nowadays illegal, their use is still very extended, as in the cases of aldicarb and carbofuran (Guitart et al., 2010; Grilo et al., 2021), which means that a restricted product can be currently implicated in poisoning cases (Martínez-Haro et al., 2008; Ruiz-Suárez et al., 2015). This may suggest that there is a stock or black-market available for these substances (Bodega Zugasti, 2014).

Table 4 shows information about legal status and regulation in European Union (EU) of the main compounds involved in poisoning.

In the EU, the legal use of pesticides is regulated under two main groups: as plant protection products (PPP), to protect crops, and as biocides, products against pests but not strictly related to agriculture. Thus, the same product can be regulated under both groups depending on its use (EFSA; No1107/2009, 2009; 528/2012, 2012).

Organochlorines were widely used from the 1940s to 1970-80s when they were banned in many developed countries. Their prohibition was motivated due to their high environmental persistence and negative health consequences to human health and wildlife like thyroid function disruption. However,

dichlorodiphenyltrichloroethane (DDT) continues to be used in some developing countries for malaria control (Regulation(EC)No850/2004, 2004; Meeker & Boas, 2011) because it remains one of the most effective strategies for combatting it.

Aldicarb and carbofuran were banned in Europe in 2003 and 2007, respectively (Council decision 2003/199/EC, 2003; Commision decision 2007/416/EC, 2007) since they were demonstrated to be hazardous to non-target organisms, especially to small birds, mammals and earthworms, due to their high toxicity, low handling safety and ecotoxicological effects. For the same reasons, other carbamates have been banned recently, such as methomyl (authorisation until August 2019) (Commission directive 2009/115/EC, 2009) and methiocarb (authorisation until July 2020) (Regulation (EU) 2019/1606, 2019).

Regarding OP, there are also a wide range of compounds banned due to their harmful effect(s) on human or animal health and on the environment. Many OP have been banned in the last decade such as phorate (authorisation until July 2003) and terbufos (authorisation until July 2003 and June 2007 in Germany) (Commission Regulation (EC)No2076/2002, 2002), diazinon (authorisation until December 2007) (Commission decision 2007/393/EC, 2007), or chlorpyrifos (authorisation until February 2020) (Regulation (EU) 2020/1085, 2018).

Table 4. Regulation and chemical information of the main compounds involved in animal poisoning in the EU.

GROUP	COMPOUND [a]	CAS number [b]	MW (g/mol) [b]	State in EU	LAW/REGULATION
Carbamates	Aldicarb	116-06-3	190.27	PPP: Not approved	(Council decision 2003/199/EC, 2003)
	Carbofuran	1563-66-2	221.25	PPP: Not approved	(Commision decision 2007/416/EC, 2007)
	Methomyl	16752-77-5/19928-35-9	162.21	PPP: Not approved	(Commission directive 2009/115/EC, 2009)
	Methiocarb	2032-65-7	225.31	PPP: Not approved	(Regulation (EU) 2019/1606, 2019)
Organophosphates	Diazinon	333-41-5	304.35	PPP: Not approved	(Commission decision 2007/393/EC, 2007)
	Chlorpyrifos	2921-88-2	350.6	PPP: Not approved	(Regulation (EU) 2020/1085, 2018)
	Malathion	121-75-5	330.4	PPP: Approved	(Regulation (EU) 2018/1495, 2018)
	Parathion	56-38-2	291.26	PPP: Not approved	(Commission decision 2001/520/EC, 2001)
Anticoagulant rodenticides	Bromadiolone	28772-56-7	527.4	PPP: Not approved B: Approved	(Regulation (EU)No540/2011, 2011) (Directive 2011/48/EU, 2011) (Regulation(EU)2017/1380, 2017)
	Brodifacoum	56073-10-0	523.4	PPP: Not approved B: Approved	(Regulation (EC)No1107/2009, 2009) (Regulation(EU)2017/1381, 2017)
	Difenacoum	56073-07-5	444.5	PPP: Not approved B: Approved	(Regulation (EC)No1107/2009, 2009) (Regulation (EU)2017/1379, 2017)
	Warfarin	81-81-2 5543-58-8	308.3	PPP: Not approved B: Approved	(Regulation (EU)2015/408, 2015) (Regulation (EU)2017/1376, 2017)
	Chlorophacinone	3691-35-8	374.8	PPP: Not approved B: Approved	(Regulation (EC)No1107/2009, 2009) (Regulation(EU)2017/1377, 2017)
Alkaloids (rodenticide)	Strychnine	57-24-9	334.4	Not approved	(Commission decision 2004/129/EC, 2004)

B: Biocide; CAS: Chemical Abstracts Service; EU: European Union; MW: Molar weight; PPP: Plant protection product
[a] Main compounds involved in animal poisoning are shown
[b] PubChem, 2021

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Aims and objectives

General aim

The main aim of this thesis is to provide additional toxicological and forensic tools to improve the fight against wildlife poisoning in Europe. This requires increasing the knowledge on, among other issues, standardisation and protocolisation of methods for classifying animal carcass decomposition, assessing the degradation of compounds in carcasses, collecting available information on analytical techniques and comparing the results obtained between them. Moreover, the creation of a network of pan-European laboratories to improve the exchange of information within countries will improve the fight against poison in the nature.

Specific objectives

Objective 1. (Chapter I) To search for complementary forensic data, mainly related to the date of death and the carcass decomposition, to protocolize and standardize the classification of cadaveric decomposition using a small raptor species as model.

Objective 2. (Chapter II) To evaluate for the first time the degradation of toxic compounds in poisoned carcasses using the SGARs bromadiolone and a small raptor species as models. Bromadiolone was selected as the target compound because it is used in the EU as biocide and as PPP and it is the most frequent AR detected worldwide.

Objective 3. (Chapter III) To compile and compare the analytical procedures applied for AR determination in the literature, as a first approach for future similar studies.

Objective 4. (Chapter IV) To compare the analytical procedures applied in four of the forensic veterinary laboratories of reference in Spain involved in the *Veneno-No* Life+ Project (www.venenono.org) (STVF-UM, UNEX, IREC-CSIC-UCLM and SERTOX-ULPGC), as part of an external quality assessment of analytical techniques. This study might allow the harmonisation of the results so that they can be comparable.

Objective 5. (Chapter V) To start a European network of laboratories working in forensic veterinary toxicology. Different European laboratories and institutions were contacted and asked to fill a questionnaire with basic information on their activities and capacities.

Chapter I. Protocol to classify the stages of carcass decomposition and estimate the time of death in small-size raptors

Image: Irene Valverde Domínguez

Abstract

One of the most common wildlife crimes involving birds worldwide is malicious poisoning. *Post-mortem* examination and toxicological analysis are essential for a proper diagnosis of the cause of the poisoning. However, investigators often require an estimate of the time of death, which is best determined by identifying the stage of carcass decomposition. The aim of this chapter is to propose a scoring method to classify the stages of carcass decomposition and thus provide an estimate of the time of death in small-size raptors. This protocol can be used by forensic veterinarians, researchers, authorities and personnel collecting carcasses in order to standardise methods and minimize subjectivity. For this purpose, 12 carcasses of Common kestrel (*Falco tinnunculus*) were exposed to external weather conditions (in the period 4-19 July 2019) in Murcia, Southeastern Spain. The ambient temperature and relative humidity, body core temperatures and carcass weights were measured at intervals over the study period. Necropsies were performed (2 birds at each interval) at 1-2 hours, 24 hours, 72 hours, 96 hours, 7 days and 15 days after death. The necropsy of a previously frozen bird was performed to act as a comparison with non-frozen fresh individuals. Six stages of the *post-mortem* autolytic process were selected: fresh carcass, moderate decomposition, advanced decomposition, very advanced decomposition, initial skeletal reduction and complete skeletal reduction. To classify the carcasses according to these categories, a scoring method is proposed considering 5 parameters: state of the eyeballs, tongue/oral cavity, pectoral muscle, internal organs and other features. Several parameters affecting the process of the decomposition are discussed.

Keywords: autolysis, carcass, decomposition, *Falco tinnunculus,* necropsy, time of death, forensic.

Introduction

One of the most common wildlife crimes worldwide is poisoning, being a challenge not only for wildlife managers, enforcement authorities and veterinarians but also for toxicology and forensic science laboratories. In Spain alone, between 1992-2013, 18503 animals were identified as having been poisoned, including kites, vultures, eagles, wolves and bears (Cano et al., 2016). This implies a significant threat to European wildlife biodiversity. As an example, more than 90% of European populations of Cinereous vulture (*Aegypius monachus*), Common vulture (*Gyps fulvus*) and Egyptian vulture (*Neophron percnopterus*) are breeding in this country. For that reason, current efforts within the research community are also focused on supporting the fight against wildlife crime.

Post-mortem examination and toxicological analysis are essential for the accurate diagnosis of wildlife poisoning cases (Brown et al., 2005). Equally, determining the time of death is important to help identify the circumstances surrounding the event within a time frame and to support investigations into the identity of those responsible. This is a very important issue in judicial processes, and particularly in criminal offences involving wildlife. Frequently, forensic toxicology laboratories receive a wide variety of biological matrices or full carcasses from wild mammals and birds in different decomposition stages. However, few information is available on the process of decomposition and the possibility to assess the time of death in avian species (Brooks, 2016; Jarmusz and Bajerlein, 2019; Oates et al., 1984). There is also a lack of appropriate and standardised protocols for the correct classification of the carcass decomposition in wild birds. Therefore, appropriate and easy-to-follow protocols are needed to classify the stages of carcass decomposition and estimate the time of death in wild birds, which are validated and may be used to support the investigation of wildlife poisoning crime scenes, the prosecution of those responsible and better case resolution.

The aim of this chapter is to propose a scoring method for carcass classification according to the degree of decomposition and estimation of time of death in

small-sized raptors. For this purpose, a decomposition experiment was carried out in Common kestrels. This species was selected since there was a sufficient number of wild bird carcasses (of similar morphology) available in the Wildlife Recovery Centre. This protocol can be used by forensic veterinarians or researchers in order to standardise methodologies and estimate the time of death in small-sized raptors under similar weather conditions. The protocol is also intended to provide a resource for official authorities or personnel in charge of carcass collection in the environment. We give details so that non-specialists can also follow the protocol, classify the carcass decomposition stage trying to minimize subjectivity, and estimate the time of death. This may help forensic toxicology laboratories to improve the wildlife poisoning diagnosis as the data will have been gathered from the crime scene without being lost due to delays.

Materials and Methods

The experiment was conducted using 13 carcasses of Common kestrel (test birds). The individuals selected came from the "Santa Faz" Wildlife Recovery Centre (WRC), Alicante, South-eastern Spain. Birds used in the decomposition experiment were euthanised because of an unfavourable prognosis to be released to the wild due to flight impairment caused by traumatic wing injuries. The individuals were kept for at least one month under the same management conditions at the WRC to ensure the homogeneity of the population. Detailed information of the individuals is described in Table S1 (Supplementary Material). All procedures performed were in accordance with the ethical standards of the *Comité Ético de Experimentación Animal* (CEEA) – University of Murcia (CEEA 549/2019), and all applicable institutional, local, and national guidelines and laws were followed. Euthanasia was performed by intra-venous administration of a lethal dose of sodium pentobarbital. The investigation took place at the outdoor facilities of the Toxicology and Forensic Veterinary Service at the University of Murcia, southeast of Spain. Immediately after euthanasia the carcasses were placed in sternal recumbency on a dry gravel substrate. They were left exposed continuously to outside weather conditions (see Table S2 for

additional details) and protected from predation with a wire mesh cage (Figure 1). Twelve carcasses were used to carry out the decomposition experiment, and, additionally, one carcass was frozen at -20 °C 6 hours after euthanasia to assess the effect of freezing, since the freezing process is known to cause histological changes and gross appearance of carcasses (Cooper, 2013).

Figure 1. Carcasses of Common kestrel in sternal recumbency with temperature/humidity probes inside the protective cage

The autolytic process study was carried out during the period from 4 July (8:30 p.m.) to 19 July (11:00 a.m.) 2019. The ambient relative humidity, ambient air temperature and internal temperature of the carcasses were continuously measured using probes. The mean ± SD (min-max) ambient air temperature (ºC), humidity (%), day duration (hours) and wind speed (km/h) recorded were 30 ± 2 (24-33) ºC, 54 ± 8 (45-70) %, 14:33:45 ± 0:05:05 (14:25:00-14:41:00) hours and 9.16 ± 1.17 (6.90-11.30) km/h, respectively (Table 1, Figure 2, Table S2). All carcasses (except for the 2 individuals necropsied on day 0 and the frozen carcass) were weighed daily at the same time every day (Figure 3).

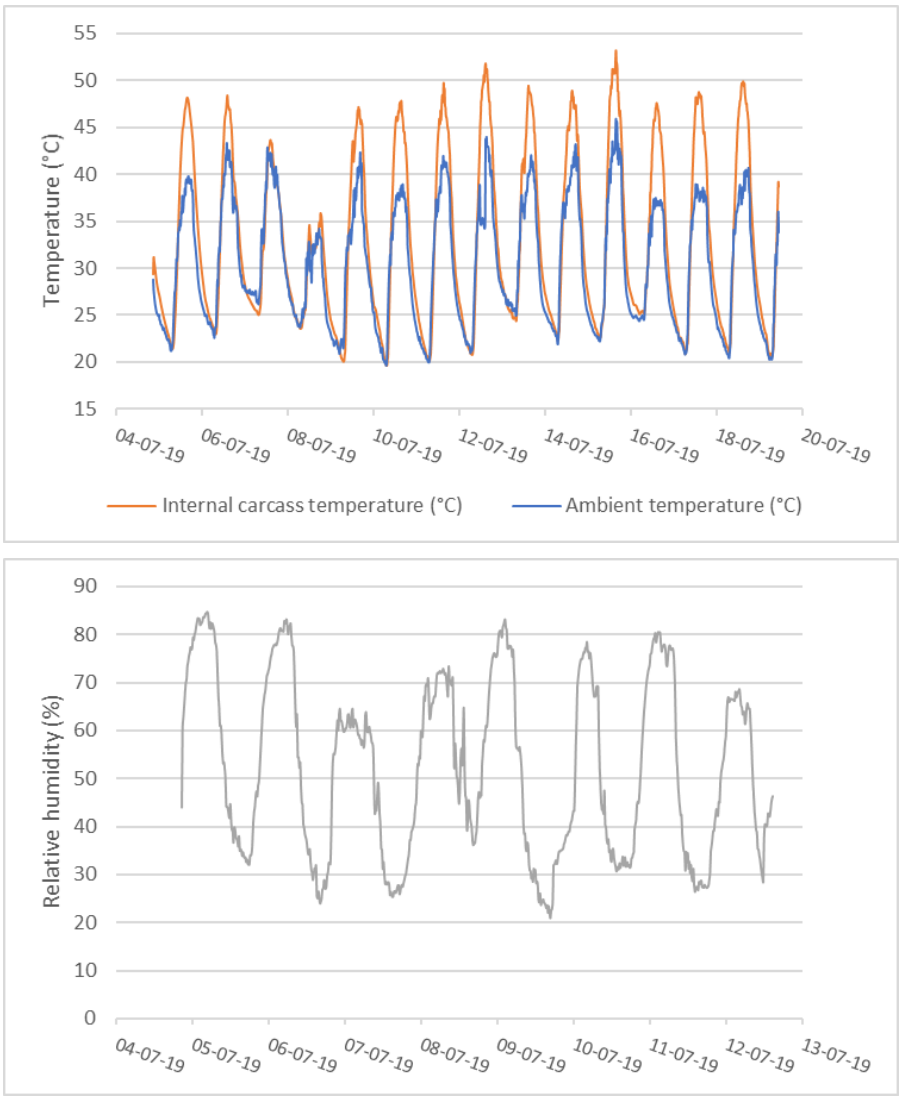


Figure 2. Values for ambient/internal temperature and relative humidity during the test period (4-19 July 2019, Murcia, Spain). *Note: due to problems with the probe the relative humidity has only been measured until 12 July.*

Necropsies were performed at the following times after euthanasia: Day 0 (1-2 hours), Day 2 (24 hours), Day 3 (72 hours), Day 4 (96 hours), Day 7 and Day 15 (2 individuals per stage). The necropsy of the (defrosted to room temperature) frozen individual was performed after 1 week of freezing for comparison with the non-frozen fresh individuals (Day 0).

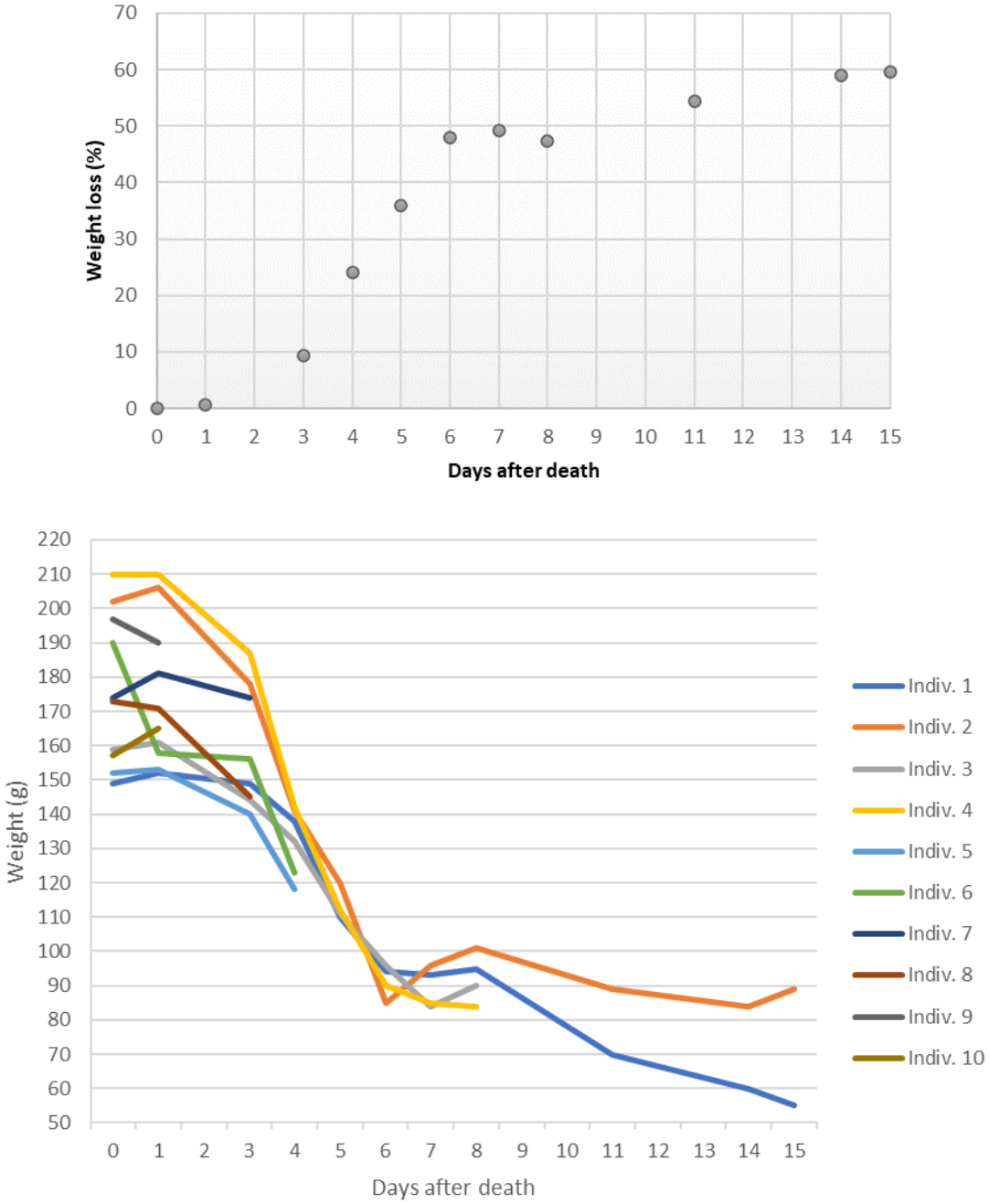


Figure 3. Values for the weight (g) of 10 carcasses of Common kestrel and average weight loss (%) in relation to the fresh carcasses (Day 0) during the test period (4-19 July 2019, Murcia, Spain).

During the necropsy, photographs were taken and detailed descriptions of the degree of external and internal decomposition of the carcasses and the presence of insect fauna were recorded. *Rigor mortis* of the carcass was also estimated. This information was collated and examined in order to select the carcass parameters, which showed identifiable signs of visible degradation that could be easily and clearly scored. Once identified, each parameter was scored

with an increasing numerical value according to the decomposition degree, together with a detailed description and picture for each numerical score. The scores obtained in the different parameters were summed and a classification system in different degradation categories developed, where each stage or category is assigned a range of scores.

Results and discussion

Carcass decomposition was categorised according to the scoring system in six categories (Table 1). After a thorough examination of the photographs taken and the detailed descriptions registered on the degree of external and internal decomposition of the carcasses during the experiment, 5 different parameters were selected to be scored since they showed clear visible degradation during the decomposition process: 1) eyeballs, 2) tongue/oral cavity, 3) pectoral (breast) muscle, 4) internal organs (mainly the liver as a reference organ) and 5) other features (blood colour and feathers status). A score system, ranging from 0 to 3, is assigned to each of these parameters according to the description and photographs presented in Table 2. After that, the scores obtained for the 5 parameters evaluated are summed in order to classify the stage of carcass decomposition into the following six categories, which were considered easily distinguishable: fresh carcass (0-2 points), moderate decomposition (3-6 points), advanced decomposition (7-11 points), very advanced decomposition (12-15 points), initial skeletal reduction (16 points) (Table 1). Complete skeletal reduction is described but it was not scored, because the study arrives until day 15.

In cases of incomplete carcasses (e. g. due to predation) the main information should be reported from the liver and other internal organs. If one of the parameters is missing, an estimation could be done considering the most frequent score for other parameters. However, this protocol cannot be applied to carcasses with more than two parameters missed.

Table 1. Time of death estimation and carcass decomposition categories. Days after death, weather conditions, carcass decomposition stages, *rigor mortis* and cadaveric fauna found in Common kestrel during the experiment (4-19 July 2019).

Days (hours) after death*	Internal temperature (°C)/ambient (°C)/Relative humidity (%) [a]	Carcass decomposition category [b]	Scoring [c]	*Rigor mortis* [d]	Cadaveric fauna[e]
Day 0 (1-2)	29.91 / 26.75 / 65.35**	Fresh	0-2	30 min: in neck and legs 60 min: more intense (almost complete) in neck and legs (less appreciable in digits and beak) 90 min: complete in neck and legs and intense in wing and beak 150 min: almost complete 390 min: complete	Absent
Day 1 (24)	32.87 / 30.17 / 58.31	Moderate decomposition	3-6	After 24 hours: slightly decreased in neck and digits (still present in beak and legs) 48 hours: more noticeable decrease	Eggs and larvae (3 mm) in oral cavity, adult flies
Day 3-4 (72-96)	32.74 / 33.17 / 44.63 28.67 / 27.97 / 60.33	Advanced decomposition	7-11	72 hours: disappeared completely	Larvae (up to 6 mm) within the carcass, coleoptera and grey fly adults
Day 7	32.81 / 30.10 / 50.65	Very advanced decomposition	12-15	-	Coleoptera and smaller number of larvae
Day 15	33.90 / 30.09 / NM	Initial skeletal reduction	16	-	Coleoptera and ants
>15 days Not studied		Complete skeletal reduction	-	-	Not studied

**It s* It should be considered that the evolution of the decomposition, cadaveric fauna and weight loss vary considerably depending on the environmental conditions (see Figure 2), the animal characteristics and death circumstances, so the time required to reach the different carcass decomposition categories must be adjusted according to each situation.*

*** Data from 8.30 p.m.*

[a] *The mean value for all individuals per day is presented. NM: Not measured.*

[b] *Based on the criteria presented on Table 2*

[c] *Total scoring = Eyeballs score (0-3) + Tongue/Oral cavity score (0-3) + Pectoral (breast) muscle score (0-3) + Internal organs score (liver, 0-3) + Others score (feathers/blood, 0-3). See the description and pictures to score the different parameters in Table 2. Initial skeletal reduction stage is easily recognizable, it receives the total scoring of 16.*

[d] *The time to reach the rigor mortis can vary depending on the species, size of the individual and the circumstances surrounding death*

[e] *See Figure 5*

Different categories have been used to classify the phases of decomposition. However, they are generally referred to human bodies and/or mammal carcasses (Brooks, 2016).

Carcass decomposition categories

The days elapsed after death in this experiment, the environmental conditions, the carcass decomposition stages and corresponding scoring, and the cadaveric fauna observed were compiled in Table 1. All the information that may be useful when estimating the carcass decomposition stage and time of death in future studies is also shown (Table 2).

The ***Fresh carcass*** (1-2 hours after death) has eyeballs with convex shape in lateral view which appear bright, the non-pigmented oral mucous membrane and the tongue remain pink and turgid, the pectoral muscle masses are red in colour and have a turgid consistency and, it is easy to separate the skin from the muscle during the necropsy, the internal organs (especially the liver as a reference organ, since not all organs decay at the same rate) maintain their turgid structure, consistency and natural colour, and the blood is red. The smell of the body is like fresh blood or has no smell, (this characteristic should be noted however it is subjective). The feathers are in good condition and do not easily detach from the body. The *rigor mortis* process begins.

The carcass with ***Moderate decomposition*** (1 day after death) has eyeballs which become opaque and lose their turgor with a collapsed appearance, the oral cavity and tongue are pale and have a dry appearance, the pectoral muscle also becomes red pale but is still easy to detach from the skin. The internal organs have a slightly dehydrated and dull structure and consistency (surface a little "wrinkled"), and the colour is darker compared with the natural colour, being generally reddish and homogeneous between the organs. The smell of decomposition begins to manifest itself and the blood becomes red dark. The *rigor mortis* still present in beak and legs.

The carcass with ***Advanced decomposition*** (2-3 days after death) has completely dehydrated eyeballs, the oral cavity and tongue are dehydrated, wrinkled and obscured. The pectoral muscle is dark brown and has medium dehydration,

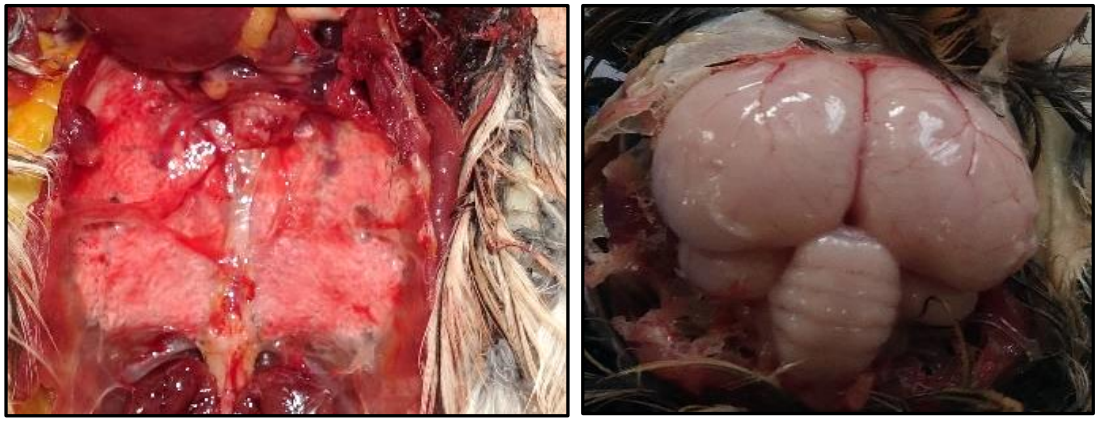
making it difficult to separate the skin from the muscle during necropsy. The internal organs lose their structure, but they remain easily identifiable, their consistency is soft and friable, and their colour is dark brown with a mixture of colours within each organ. The blood becomes brownish-black or is absent. The *rigor mortis* process is finished.

The carcass with ***Very advanced decomposition*** (7 days after death), has eyeballs which have lost their structure completely, the tongue is stiff, showing a parchment-like appearance and there is a dark colour to the oral cavity. Sometimes, detachment of the horny layer of the beak can be observed. The pectoral muscles are completely dehydrated, and the keel is clearly visualized, it is impossible to separate the skin from the muscle. The internal organs lose their structure, so it is difficult to identify them, however, some of them are guessed and others disappear, their consistency is dry or very friable, the colour is brownish, dark and uniformly homogeneous. The feathers easily detach from the skin.

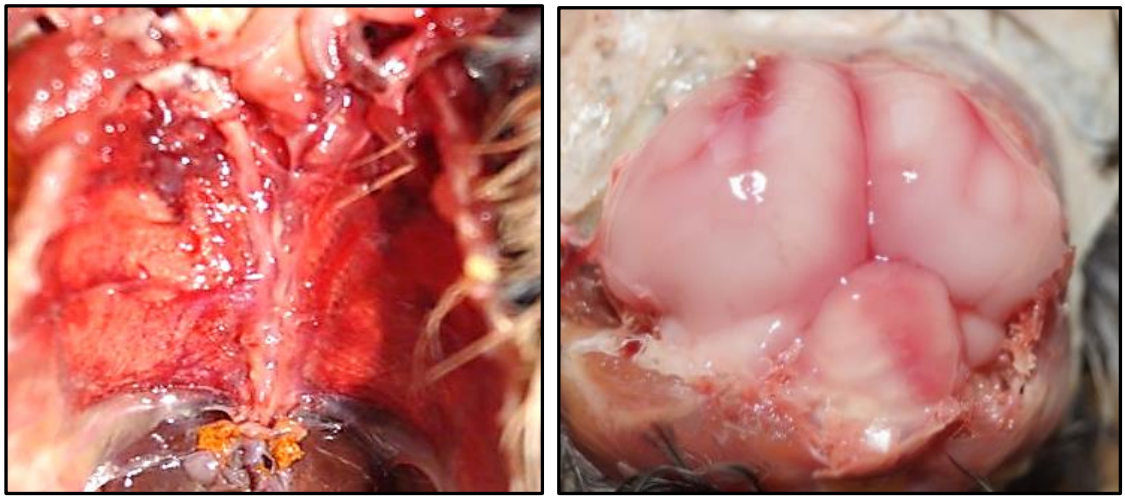
The carcass with ***Initial skeletal reduction*** (15 days after death) is characterized by a complete dehydration of the entire body, acquiring a dark and homogeneous colouration of dry, stiff and parchment-like appearance.

The carcass with ***Complete skeletal reduction*** consists of bones and feathers, the soft tissues having disappeared. This last phase may take months to complete, depending on the environmental conditions, and in this study was not evaluated.

Within the category of the ***"fresh"*** carcass decomposition, the differences between non-frozen individuals and the frozen individual were evaluated. The main differences were observed in the lungs and brain. In the non-frozen individual (Figure 4a), the lungs have their natural pink colour, and the brain has a well-defined vascularization and pale pink parenchyma. Nevertheless, the frozen individual (Figure 4b) shows congested, red lungs and the brain has a blurred vascularization and a more intense pink coloration of the parenchyma.



a



b

Figure 4. Lungs and brain of fresh carcasses of Common kestrel: a) Non-frozen carcass. Lungs show their natural pink color and brain has well-defined vascularization and pale pink parenchyma; b) Frozen carcass. Lungs are congestive and show red color and brain has blurred vascularization and deep pink parenchyma.

Determining the *degree of carcass decomposition* and the time of death is of special interest to be able to frame a crime/accident at a given time. When studying the autolysis in a corpse, which allows to *estimate the time of death*, several parameters affecting the evolution of the decomposition process should be considered, i.e.: the environmental conditions (temperature, humidity, rainfall), the species, the weight or size of the animal, its state of health or presence of wounds, the position and location of the corpse, the presence or absence of food in the gastrointestinal tract, the internal temperature of the corpse, and the circumstances of death. For example, the autolysis process is expected to be faster in the presence of open wounds, or at high temperatures and humid environments (Brooks, 2016; Cooper, 2013; Oates et al., 1984).

Therefore, it is essential to gather basic information regarding the body and the conditions that surround it. In addition, it is recommended to photograph the carcass in detail, including holes where cadaveric fauna can be observed, as well as the scene surrounding the carcass, in order to be able to consult the photographs if necessary. Document S1 (Supplementary Material) includes printable field and lab documentation, i.e., a form compiling some basic information of interest to estimate the stage of carcass decomposition and the time of death, and the scoring table with pictures.

Table 2. Parameters and scoring to classify the stages of carcass decomposition in small-size raptors.

Parameter	Description	Score
Eyeballs	They keep bright and with convex shape in lateral view.	0
	Opacity, they lose their whole structure.	1
	Completely dehydrated.	2
	Absent.	3
Tongue/Oral cavity	Pink, turgid tongue.	0
	Pale and dry.	1
	Dehydrated, dark and wrinkled.	2
	Parchment-like appearance of the tongue and loss of natural color, it turns dark. Detachment of the horny layer of the beak.	3
Pectoral (breast) muscle	Red color and turgid consistence. Easy to separate from de skin.	0
	Red Pale. Easy to separate from de skin.	1
	Dark brownish color, medium dehydrated. Difficult to separate from the skin.	2
	Completely dehydrated, keel visualized. Impossible to separate from the skin.	3
Internal organs	(Liver as reference organ)	
Structure	Turgid.	
Consistence	Bright.	
Color	Natural from each organ.	0
Green spot	No spot or only dyes tissue in direct contact.	
Internal smell	Fresh blood/No smell.	
Structure	Slightly dehydrated (Surface a little "wrinkled").	
Consistence	Slightly dehydrated and dull (Surface a little "wrinkled").	
Color	Dark compared to the natural, homogeneous between the organs (reddish).	1
Green spot	Just dyes the organs in direct contact.	
Internal smell	Decomposition smell starts.	
Structure	They lose it, but organs are well identified.	
Consistence	Softer or friable.	2
Color	Dark-brownish and mix of colors inside the same organ.	
Green spot	Dyes all intestinal handles, dark green/blackish.	
Structure	Difficult to identify the organs, some have disappeared.	
Consistence	Dry or very friable.	3
Color	Dark and homogeneous in all the organ (brownish).	
Green spot	Absent.	
Others	Feathers in good condition, they do not detach/red blood.	0
	Red Dark blood.	1
	Brownish-dark blood or blood missing.	2
	Feathers detach.	3

Eyeballs

0 points

1 points

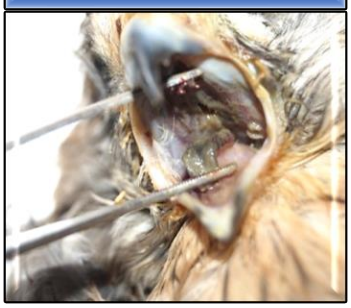
2 points

3 points

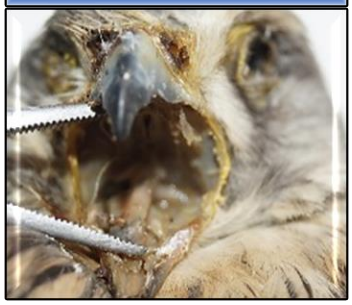
Tongue / Oral cavity

0 points

1 points

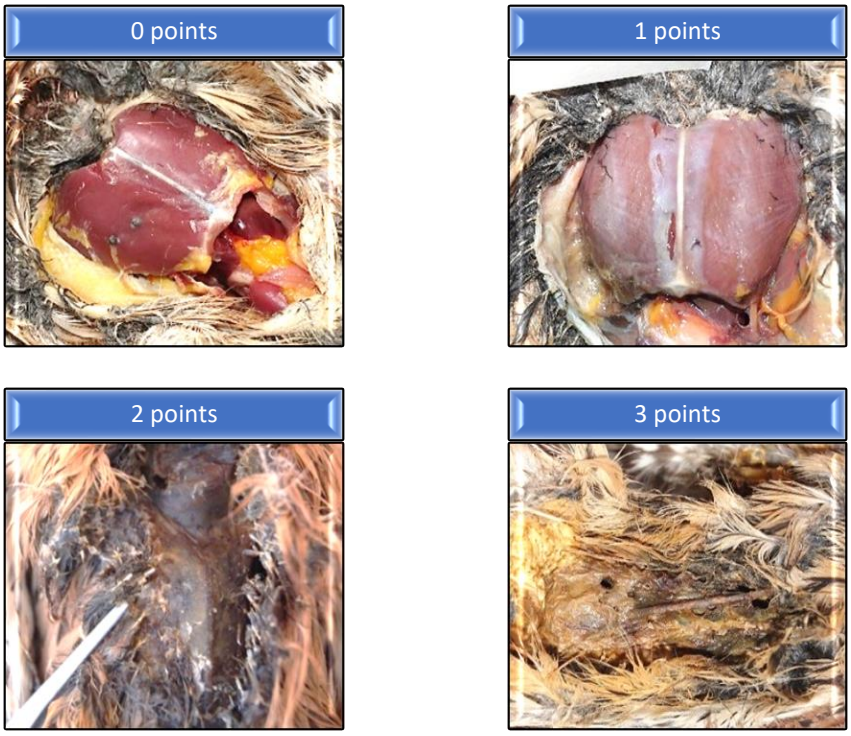


2 points

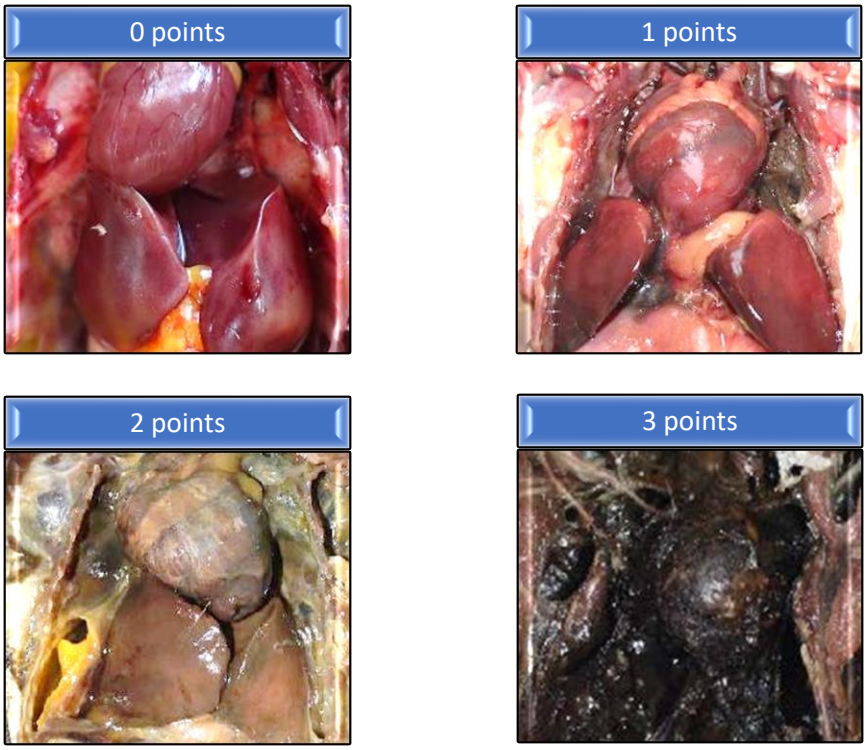


3 points

Pectoral (breast) muscle

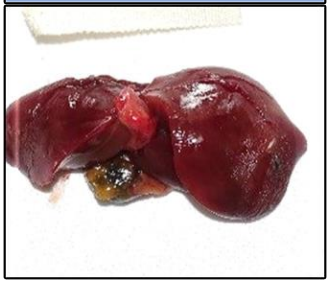


Internal organs (1st part: general view)

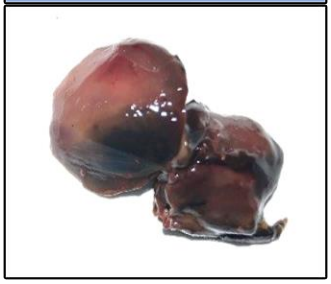


Internal organs (2nd part: liver as reference organ)

0 points



1 points



2 points

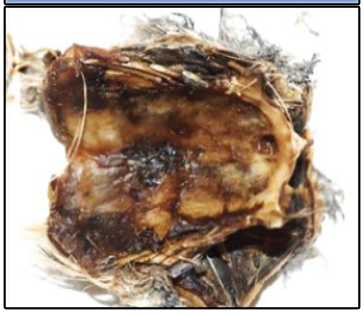
3 points

Others

0 points

1 points

2 points



3 points

Furthermore, as explained before, certain external and internal characteristics of the carcass observable during the necropsy will help to classify the stage of decomposition, such as the coloration, structure and consistency of the organs and tissues, or the degree of dehydration. Due to the reaction of the hydrogen cyanide with the haemoglobin transformed into biliverdin by bacterial activity, a greenish colour stain appears in the surrounding tissues and skin, called "green spot". This spot is found in the abdominal area. The presence and extension of a green spot can also help to classify the degree of decomposition. The period in which it appears is called the chromatic period/stage, and it manifests and evolves in the early stages of decomposition. However, this spot is not always easy to identify, depending on the size of the animal (Gisbert Calabuig et al., 2004; Brooks, 2016).

Time course of the rigor mortis

In addition to these criteria, the *rigor mortis* of the carcass will also help estimate the time of death in the early stages. According to a study in ducks, the peak of *rigor mortis* in muscles of the jaw, neck and legs is usually reached 1 hour after death, while stiffness in the pectoral muscles appears after 1.5 hours (Morrow and Glover, 1970). In other study in mallards, total stiffness was described 1-2 hours after death (Oates et al., 1984). In this study, *rigor mortis* gradually appeared after death as follows: *rigor* in neck and legs after 30 min, more intense *rigor* (almost complete) in neck and legs (less appreciable in digits and beak) after 60 min, complete *rigor mortis* in neck and legs and intense *rigor* in wing and beak after 90 min, almost complete *rigor mortis* after 150 min and complete *rigor mortis* after 390 min. The *rigor mortis* slightly decreased in neck and digits (still present in beak and legs) after 24 hours, with a more noticeable decrease after 48 hours and finally, *rigor mortis* disappeared completely after 72 hours. It should be taken into account that the time to reach the *rigor mortis* can vary depending on the species, size of the individual and the circumstances surrounding death (vigorous muscle exertion may accelerate the onset and degree of *rigor mortis*). In the protocol, *rigor mortis* is useful to distinguish between *Moderate decomposition* or *Advanced decomposition* stages when

the difference is not clear. The *rigor mortis* will appear in the last stage of *Moderate decomposition* and in the first stage of *Advanced decomposition*. Therefore, after scoring the carcasses according to the criteria shown in Tables 1 and 2, the presence or absence of *rigor mortis* will help to differentiate between both stages if they are not clear.

Forensic entomology

The *estimation of the time of death* is easier within the first 72 hours, however, the carcass provides less information after that, and *forensic entomology* can be very useful for providing estimates after days, weeks and even months after death (Barnes, 2013). An expert in entomology is needed for a proper identification and interpretation of cadaveric fauna found in a carcass, and the insects may be highly variable depending on the geographical area, season and circumstances. For this reason, this parameter is not considered in the scoring method proposed in this article, although some basic data gathered during necropsies is provided.

The identification and analysis of the insects found in the body does not indicate the exact date of death but allows us to estimate the minimum *post-mortem* interval. There are several studies investigating the temporal pattern of insects during the decomposition sequence in different animals (see review by Barnes, 2013). There are mainly three methods to carry out this estimate: by ageing blowfly larvae in the corpse, through the succession of insects or by seasonality of their activity (Barnes, 2013). The methodology used will depend on the cadaveric fauna available. Barnes (2013) provides a detailed description of these methods. Nonetheless, it should be considered that the size, smell, condition and position of the corpse affects the activity of the insects, as well as the geographical area, season and environmental factors. As for the first method, in general, the flies are the initial colonizers in a carcass, being quickly attracted to the body and depositing eggs in dark and damp areas such as eyeballs, nostrils, oral cavity, anus/cloaca, genital region, wounds, etc. The eggs hatch at larvae at a rate that will depend on the environmental conditions and the species of insect involved. The larvae, after three phases of growth, pass to

pupa, which will harden and darken to form an adult individual (Barnes, 2013). Therefore, the minimum *post-mortem* interval can be estimated by determining the time elapsed since the egg laying, calculated using local meteorological data and the identification of the species and their developmental phase (Barnes, 2013). Regarding the insect succession methodology, different species will be attracted to the body depending on the degree of cadaveric decomposition and the odour emitted. In general, fly species dominate the initial stages, while coleoptera (beetles) dominate later stages (Barnes, 2013). Finally, the activity of insects and their development depends on the environmental conditions and, therefore, on the season, so that knowledge of different insect species and their activity pattern can help frame a crime in a certain time of the year (Barnes, 2013). The presence of cadaveric fauna can complicate the identification of the organs, because of their necrophagous activity (Viero et al., 2019).

The different types of cadaveric fauna observed in our experiment were: ants, arthropods, coleoptera and dipterans, in eggs, larvae and adult stages. In individuals necropsied at 24 hours after death, eggs and 3 mm-larvae were observed only in the oral cavity, as well as flies around the corpse, eggs being found even within a few hours after death; at 72 hours after death, adult individuals of grey fly *(Sarcophagidae)*, larvae and some coleoptera were found; at 96 hours after death a large number of larvae of different sizes (maximum 6 mm) were observed throughout the interior of the body (including the brain and behind the pleura), as well as some beetles and adult flies. After 7 days, the quantity of larvae is lower and more beetles were found; and after 15 days, only ants and beetles were observed (Figure 5).

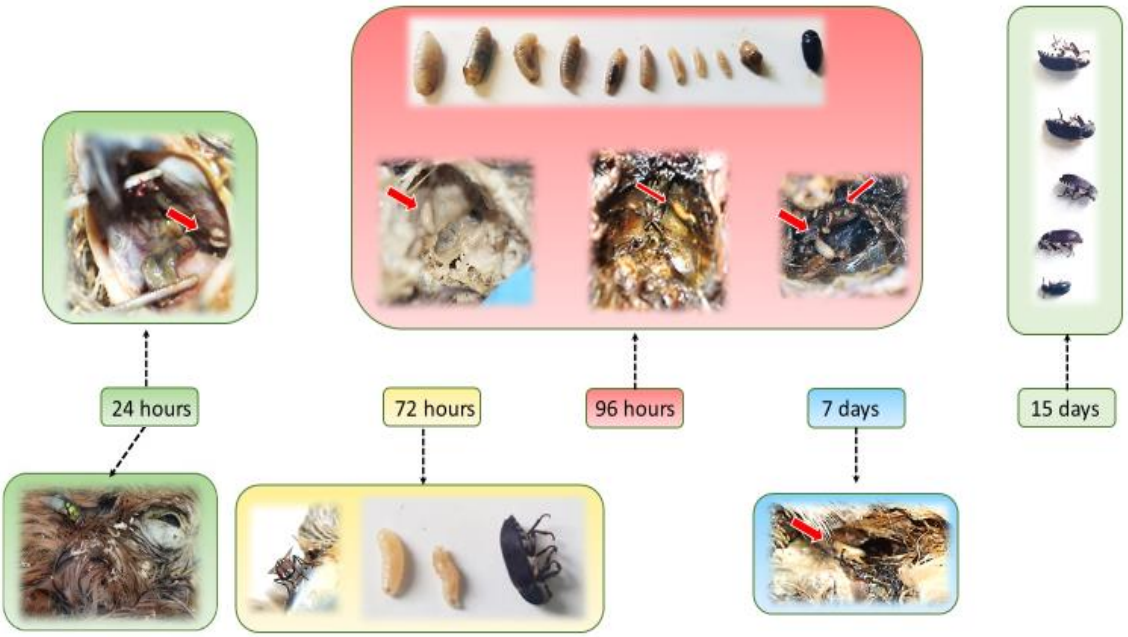


Figure 5. Cadaveric fauna found during the experiment in Common kestrel.

A few hours after death: fly eggs can be found in the oral cavity.

At 24 hours after death, eggs and 3 mm-larvae are observed only in the oral cavity, as well as mobile adult flies.

At 72 hours adult individuals of grey fly (Sarcophagidae), larvae and some beetles were found.

At 96 hours a large number of larvae of different sizes (up to 6 mm) was found throughout the interior of the body (including the brain and behind the pleura), some beetles and adult flies were found.

At 7 days plus, the quantity of larvae is smaller and more beetles were found.

At 15 days, only beetles and ants were found.

It is observed that the first phases of decomposition are rapidly reached, while the last ones (very advanced decomposition, initial and complete skeletal reduction) require more time. The loss of body weight (represented as a percentage respect to the fresh body, see Figure 3 and Table S3 in Supplementary Material) and the cadaveric fauna found are also presented. Some weak points of this study that should be considered are: (i) the limited number of individuals available for the study to make strong generalizations and interpretations; (ii) the daily handling of the carcasses to record body weights in this experiment may also affect the rate of decomposition and it may alter oxygenation and colonization by bacteria and insects; however, it was necessary to gather the body weight and assess changes in weight over time; (iii) as previously mentioned, the process of these parameters varies considerably

depending on various factors (environmental conditions, characteristics of the animal, circumstances of death, etc.), so the estimation of the time of death must be adjusted according to the conditions of each case, and iv) the barbiturate used for euthanasia could have an effect on the decomposition and/or cadaveric fauna activity, however, no studies evaluating this potential effect have been found in the literature and further studies would be needed to better understand this issue. Despite of this, the present study provides valuable information, considering that these types of studies in wild birds are scarce, that will help to standardise methodologies and minimize subjectivity: a new scoring method for carcass classification according to the degree of decomposition and estimation of time of death in small-sized raptors that can be used by forensic veterinarians, researchers, official authorities or personnel in charge of carcass collection in the environment.

Conclusions

When determining the degree of carcass autolysis and estimating the time of death, the most relevant parameters (i.e., environmental conditions, characteristics of the carcass and circumstances of the death) must be considered. This protocol proposes a scoring method that will aid the classification of the stage of carcass decomposition and estimation of the time of death in birds. Our investigation was conducted under a limited number of environmental conditions and using small-sized raptors, which had been euthanised as a reference. The principal decomposition changes were observed during the first 7 days. In 15 days, the initial skeletal reduction was reached which was progressing beyond the investigation period and it was obvious that more time was needed to reach the stage of complete skeletal reduction. Furthermore, changes in colour and vascularization definition in the frozen carcass compared with fresh carcasses were observed, confirming that potential histological changes affecting the appearance of some organs should be considered when the necropsy of a frozen carcass is carried out. It is recognised that this investigation has some inevitable limitations such as the small sample size studied, the daily handling of the carcasses (for weighing and

examination), and the specific weather conditions prevailing and the special fauna (bacteria, insects, etc.) present in the geographical area selected. It is also noted that, although the importance of using additional descriptors such as smell and colour descriptions is obvious, it may not be possible to include them in an objective scoring protocol such as the one proposed here. The investigation reported here is intended to be a starting point from which data may be collected and validated. Further studies with other avian species and different weather conditions would help to better classify carcass decomposition and estimate time of death.

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Supplementary Material

Protocol to classify the stages of carcass decomposition and estimate the time of death in small-size raptors

Table S1. Individual information of Common kestrel used in the decomposition experiment

Age	Sex[a]	Weight (g)	Health status[b]
Adult	F	183	TI
Adult	M	190	TI
Adult	F	200	TI
Adult	F	211	TI
Adult	M	161	TI
Adult	M	197	TI
Adult	M	168	TI
Adult	M	173	TI
Adult	M	166	TI
Adult	M	164	TI
Adult	M	158	TI
Adult	H	210	TI
Adult[c]	M	164	TI

[a] F=female; M=male
[b] TI=Traumatic injury
[c] Frozen individual

Table S2. Weather conditions during the decomposition experiment

Days after death*	Sunrise time	Sunset time	Day duration (hours)	Carcass internal temperature (°C) / ambient temperature (°C) / Relative humidity (%) [a]	Wind (day mean km/h)
04-jul (Day 0)	06:48	21:29	14:41	29.91 / 26.75 / 65.35**	9
05-jul (Day 1)	06:48	21:29	14:40	32.87 / 30.17 / 58.31	8.4
06-jul	06:49	21:28	14:39	33.60 / 31.62 / 54.99	8.2
07-jul (Day 3)	06:49	21:28	14:38	32.74 / 33.17 / 44.63	9.6
08-jul (Day 4)	06:50	21:28	14:38	28.67 / 27.97 / 60.33	10.5
09-jul	06:50	21:28	14:37	32.20 / 29.92 / 44.84	9.1
10-jul	06:51	21:27	14:36	32.88 / 29.31 / 51.58	9.4
11-jul (Day 7)	06:52	21:27	14:35	32.81 / 30.10 / 50.65	7.7
12-jul	06:52	21:27	14:34	34.24 / 29.02 / 53.71	8.6
13-jul	06:53	21:26	14:33	34.31 / 31.72 / NM	9.9
14-jul	06:54	21:26	14:31	34.21 / 31.66 / NM	11.1
15-jul	06:54	21:25	14:30	34.77 / 31.5 / NM	9.5
16-jul	06:55	21:25	14:29	33.35 / 29.83 / NM	11.3
17-jul	06:56	21:24	14:28	33.36 / 29.83 / NM	8.5
18-jul	06:57	21:23	14:26	33.90 / 30.09/ NM	6.9
19-jul (Day 15)	06:57	21:23	14:25	33.90 / 30.09 / NM	8.9

**It should be considered that the evolution of the decomposition, cadaveric fauna and weight loss vary considerably depending on the environmental conditions, the animal characteristics and death circumstances, so the time required to reach the different carcass decomposition categories must be adjusted according to each situation.*

***From 8:30 p.m.*

[a]The mean value for all individuals per day is presented. NM: Not measured. See Figure 2

Table S3. Weight loss during the decomposition days and the categories associated.

Days (hours) after death*	Carcass decomposition category [a]	Mean weight loss (%) [b]
Day 0 (1-2)	Fresh	0
Day 1 (24)	Moderate decomposition	1
Day 3-4 (72-96)	Advanced decomposition	9
Day 7	Very advanced decomposition	49
Day 15	Initial skeletal reduction	60
>15 days Not studied	Complete skeletal reduction	Not studied

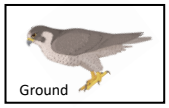
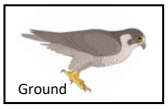
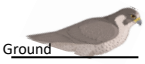
**It should be considered that the evolution of the decomposition, cadaveric fauna and weight loss vary considerably depending on the environmental conditions (see Figure 2), the animal characteristics and death circumstances, so the time required to reach the different carcass decomposition categories must be adjusted according to each situation*

[a] *Based on the criteria presented on Table 2*

[b] *See Figure 3*

Document S1. Printable field and lab documentation

Document S1 A. Form compiling basic information to estimate the stage of carcass decomposition and the time of death in birds

Name				Contact	Phone:			
					Email:			
Date		Time			Signature:			
Place	Town (country):							
	Geographic coordinates:							
Individual identification marks (number/ring (band)/tattoo/microchip/others):								
Specie	Common name:			*Scientific name:*				
Weight (g)		Complete carcass? (lost body parts)						
Sex	Male				Female		Unknown	
Age	Young				Adult		Unknown	
Carcass position	 *Right lateral decubitus*		 *Left lateral decubitus*		 *Supine decubitus*		 *Prone decubitus*	
Cadaveric fauna:								
Eggs		Larvae		Insects		Other observations:		
External signs of possible diseases/pathologies (suspected cause/s of death):								
Carcass internal temperature (°C)								
Environmental conditions								
Ambient temperature (°C)					Relative humidity (%)			
Rigor mortis	Yes				No			
Other observations								

Note: Take several photographs of the scene (from a general view to details in and around carcass) to be checked later if needed.

Document S1 B. Parameters and scoring to classify the stages of carcass decomposition in birds

Parameter	Description	Points	Score
Eyeballs	They keep bright and with convex shape in lateral view.	0	
	Opacity, they lose their whole structure.	1	
	Completely dehydrated.	2	
	Absent.	3	
Tongue/Oral cavity	Pink, turgid tongue.	0	
	Pale and dry.	1	
	Dehydrated, dark and wrinkled.	2	
	Parchment-like appearance of the tongue and loss of natural color, it turns dark. Detachment of the horny layer of the beak.	3	
Pectoral (breast) muscle	Red color and turgid consistence. Easy to separate from de skin.	0	
	Red Pale. Easy to separate from de skin.	1	
	Dark brownish color, medium dehydrated. Difficult to separate from the skin.	2	
	Completely dehydrated, keel visualized. Impossible to separate from the skin.	3	
Internal organs	**(Liver as reference organ)**		
Structure	Turgid.		
Consistence	Bright.		
Color	Natural from each organ.	0	
Green spot	No spot or only dyes tissue in direct contact.		
Internal smell	Fresh blood/No smell.		
Structure	Slightly dehydrated (Surface a little "wrinkled").		
Consistence	Slightly dehydrated and dull (Surface a little "wrinkled").		
Color	Dark compared to the natural, homogeneous between the organs (reddish).	1	
Green spot	Just dyes the organs in direct contact.		
Internal smell	Decomposition smell starts.		
Structure	They lose it, but organs are well identified.		
Consistence	Softer or friable.	2	
Color	Dark-brownish and mix of colors inside the same organ.		
Green spot	Dyes all intestinal handles, dark green/blackish.		
Structure	Difficult to identify the organs, some have disappeared.		
Consistence	Dry or very friable.	3	
Color	Dark and homogeneous in all the organ (brownish).		
Green spot	Absent.		
Others	Feathers in good condition, they do not detach/red blood.	0	
	Red Dark blood.	1	
	Brownish-dark blood or blood missing.	2	
	Feathers detach	3	
		TOTAL SCORE	

Results total scoring

Scoring*	Carcass decomposition category
0-2	Fresh
3-6	Moderate decomposition
7-11	Advanced decomposition
12-15	Very advanced decomposition
16	Initial skeletal reduction

**Total scoring = Eyeballs score (0-3) + Tongue/Oral cavity score (0-3) + Pectoral (breast) muscle score (0-3) + Internal organs score (liver, 0-3) + Other score (feathers/blood, 0-3). See the description and pictures to score the different parameters in Table 2. Initial and complete skeletal reduction stages are easily recognizable, they receive the total scoring of 16 and 17, respectively.*

Chapter II. Temporal persistence of bromadiolone in decomposing bodies of Common kestrel (*Falco tinnunculus*)

Image: Pixabay

Abstract

Bromadiolone is a SGARs used to control pest rodents worldwide. SGARs are frequently involved in secondary poisoning in rodent predators due to their persistence and toxicity. This study aims to evaluate the persistence of bromadiolone in liver at different stages of carcass decomposition in experimentally-dosed Common kestrels to understand the possibility of detecting bromadiolone in cases of wildlife poisoning and the potential risk of tertiary poisoning. Twelve individuals were divided into the bromadiolone-dose group (dosed with 55 mg/kg b.w.) and the control group. Hepatic bromadiolone concentrations found in each stage of decomposition were: 3000, 2891, 4804, 4245, 8848, and 756 ng/g dry weight at 1–2 h (fresh carcass), 24 h (moderate decomposition), 72 h, 96 h (advanced decomposition), seven days (very advanced decomposition), and 15 days (initial skeletal reduction) after death, respectively. Liver bromadiolone concentrations in carcasses remained relatively stable over the first four days and raised on day 7 of decomposition under the specific conditions of this experiment, presenting a risk of causing tertiary poisoning. However, at the initial skeletal reduction stage, liver bromadiolone concentration declined, which should be considered to interpret toxicological analyses and for proper diagnosis. This experimental study provides for the first time some light to better understand the degradation of SGARs in carcasses in the wild.

Keywords: anticoagulant rodenticides, carcass decomposition, bromadiolone degradation, wildlife poisoning, biomonitoring.

Introduction

Anticoagulant rodenticides are widely used to control pest rodents around the world (Berny et al., 1995; Hosea 2000; Winters et al., 2010). They are classified into two categories according to the period in which they were developed: FGARs and SGARs, the latter being more persistent and toxic after one dose (Ruiz-Suárez et al., 2014; van den Brink et al., 2018). The mechanism of action of AR is based on the inactivation of the membrane protein VKOR in the liver, kidney, and pancreas. This inactivation leads to a reduction in vitamin K hydroquinone, which is needed for the carboxylation of clotting factors II, VII, IX, and X (Furie et al., 1999; Crowell et al., 2013; Rattner & Mastrota 2018). The reduction of blood clotting causes death by internal and external bleeding (van den Brink et al., 2018).

Anticoagulant rodenticides are also illegally used to kill non-target species considered harmful to agriculture, livestock-farming, and/or hunting, or as revenge between private individuals. These non-selective practices are a threat to wildlife and domestic animals (Berny 2007; Mateo-Tomás et al., 2012; Ruiz-Suárez et al., 2015). This use of AR in poisoned baits is considered an illegal action in the EU (EEC 1992, 2010) as well as in other countries such as the United States (US) (Gabriel et al., 2012), Canada (Proulx & Rodtka 2015), and in 83% of African countries (Ogada, 2014).

The persistence and toxicity of SGARs have led to the problem of the secondary poisoning of rodent predators including mammals, scavengers, and raptors (e.g., barn owl (*Tyto alba*) and red kite are frequent victims) (Albert et al., 2010; Guitart et al., 2010; Sánchez-Barbudo et al., 2012; López-Perea et al., 2015; Mcfarland et al., 2017; van den Brink et al., 2018). These species can be exposed to low doses of AR over multiple days, and the proportion of individuals poisoned or containing residues in their organisms has grown in the last years (Poché 1988; van den Brink et al., 2018). The animals exposed to sub-lethal levels of AR over time could be weaker and more prone to infections, accidents, or predation (Fournier-Chambrillon et al., 2004; Berny 2007).

Bromadiolone is the AR with the most biocidal products registered in the EU (EU 2012; Jacob, J. & Buckle 2018) and, together with difenacoum, the only AR used for PPP (EC 2009; Jacob, J. & Buckle 2018). Its use is also authorized in other countries around the world (Winters et al., 2010; Lohr & Davis 2018; Slankard et al., 2019). Accordingly, bromadiolone is also the most frequently detected AR in raptors worldwide (Lambert et al., 2007; Berny & Gaillet 2008; Walker et al., 2008; Albert et al., 2010; Murray & Avian 2011; Christensen et al., 2012; Sánchez-Barbudo et al., 2012; Hughes et al., 2013; Langford et al., 2013; Ruiz-Suárez et al., 2014; Stansley et al., 2014; Hong et al., 2019). This may be due to the fact that SGARs in general, and bromadiolone in particular, are more persistent than FGARs, having a longer half-life in the liver of prey, and thus, increased risk of secondary poisoning in predators (AJ 2014). In this regard, the bromadiolone half-life in living rats' livers ranges from 170 to 318 days (Agency 2007), while the US Environmental Protection Agency (EPA) (Agency 2007) suggested that bromadiolone could persist in the liver of live rats for more than one year. However, to the best of our knowledge, data on bromadiolone persistence in decaying carcasses are lacking in the literature.

Suitable *post-mortem* examination and toxicological analysis are essential to deal with cases of wildlife poisoning (Brown et al., 2005). These poisoning cases are difficult for toxicology laboratories, mainly due to the numerous products that can be involved and the variety and complexity of biological matrices with different states of decomposition. Degradation of toxic substances involved in poisoning cases in the carcass can be affected by weather conditions (e.g., sunlight, temperature, and humidity), microorganisms and cadaveric fauna leaching from the carcass to the soil, and tissue autolysis. All these factors occur during carcass decomposition and can alter the concentrations of the toxic compounds in internal tissues, which in turn will affect the correct diagnosis of wildlife poisoning. Nevertheless, few articles mention the decay status of the matrices (Berny et al., 1997; Martínez-López et al., 2006; Berny, 2007) and, to the best of our knowledge, no studies have evaluated the persistence of AR in carcasses of poisoned animals over time. Thus, it is crucial to evaluate the effect of carcass decomposition on the stability of different toxic compounds in

tissues, so that an accurate interpretation of the toxicological analysis can be assured. In this sense, liver is the main metabolizing and accumulating organ for AR, and the tissue recommended for analysis (Valverde, et al., 2020).

The main aim of this study was to provide a first approach to evaluate the persistence of bromadiolone over time in the liver of decomposing carcasses of experimentally-dosed Common kestrels. This will improve interpretation of the presence of bromadiolone in exposed (or intoxicated) wild birds at different stages of carcass decomposition and the detection of bromadiolone in cases of wildlife poisoning as well as the risk of tertiary poisoning for scavengers.

Materials and Methods

Experimental Set-Up

Twelve Common kestrels admitted in the "Santa Faz" Wildlife Recovery Center (WRC, Alicante, southeastern Spain) were used for the experiment. These kestrels were non-releasable and destined to be euthanized due to traumatic wing injuries preventing their release and survival in the natural environment. All individuals were physiologically healthy, with normal diet and body mass. To ensure the homogeneity of the study population, individuals were kept for at least one month under the same management conditions in proper installations at the WRC. In total, eight males and four females with body weight (b.w.) ranging from 158 to 211 g were used. Common kestrels were divided into two groups: bromadiolone-dose group (n = 6 individuals, four males and two females, see details below) and control group (n = 6 individuals, four males and two females). According to the ethics in animal experimentation, the number of animals used must be minimized, and 12 individuals were considered a sufficient number to obtain reliable data. All procedures performed complied with the ethical standards of the Comité Ético de Experimentación Animal (CEEA)–University of Murcia (identification code: 549/2019; date: 24 June 2019) as well as applicable institutional, local, and national guidelines and laws.

Each individual within the bromadiolone-dose group was orally dosed by providing a small piece of chicken containing the mg of the compound (bromadiolone ≥90% purchased from Sigma-Aldrich, New Haven, CT, USA).

Individuals in the control group were also provided with a small piece of bromadiolone-free chicken. The LD50 for bromadiolone in the study species is not available, and inter and intraspecific differences in sensitivity to AR have been reported (Thomas et al., 2018). The exact dose of bromadiolone given to each individual was 55 mg/kg b.w., half the LD50 reported for multiple bird species (Coeurdassier et al., 2012; Ruiz-Suárez et al. 2014). This dose was chosen to produce hepatic residues found in real cases, since raptors can be exposed to repeated sublethal doses (through rodent predation) that can be even higher than the LD50 reported for some predators (Berny et al., 1997; Stone et al. 1999; Giraudoux et al. 2006; Coeurdassier et al. 2012).

The 12 kestrels were euthanized three days after receiving bromadiolone due to the delayed toxic action of this compound (Suárez and Cueto 2018; van den Brink et al., 2018) by administering an intravenous lethal dose of sodium pentobarbital. The carcasses were immediately moved to the outdoor facilities of the Toxicology and Forensic Veterinary Service at the University of Murcia, southeast of Spain (Figure 1). The individuals were placed in a prone position, on a gravel floor, simulating a case of poisoning. They were exposed to the weather (see Table 1) 24 h a day, but were put inside a cage to avoid scavenging by large animals (Figure 1).

Figure 1. Carcasses of Common kestrel in the prone position with temperature/humidity probes.

The decomposition experiment was performed from July 4th to July 19th 2019. The relative humidity, ambient temperature, and internal temperature of the carcasses were measured continuously using intraesophageal probes Onset TMC6-HC, and the information was recorded via Onset HOBO® U12-013 dataloggers (see graph with measurements in Valverde et al., (2020). Individuals

were weighed daily to evaluate the body weight loss over time. Necropsies were staggered over time (two individuals per stage, one from the bromadiolone-dose group and another one from the control group). The stages selected were: 1–2 h (day 0), 24 h (day 1), 72 h (day 3), 96 h (day 4), 7 days, and 15 days after death. During the necropsy, detailed data were recorded including date and time of necropsy, body mass measurements, sex and age of the individuals, cadaveric fauna found (e.g., eggs, larvae, insects, etc.), rigor mortis and the state of decomposition (structure, consistence, colour and other observations of eyes, tongue and oral cavity, pectoral muscle, and internal organs). Additional details can be found in a carcass decomposition protocol published elsewhere (Valverde et al., 2020). Several photographs were taken during each necropsy. Both ante-mortem and *post-mortem* signs related with AR intoxication were evaluated (Murray, 2018). In this line, several parameters were carefully gathered after bromadiolone administration and during the necropsies including decreased mentation, weakness, pallor of mucous membranes, evidence of external (e.g., oral cavity, nares, cloaca), and/or internal haemorrhages and haematomas (Murray, 2018). Samples of each organ were collected for further studies.

Sample Acquisition

Blood samples were collected in two stages during the experiment: (i) before bromadiolone administration to ensure that individuals did not have bromadiolone residues (in both the control and bromadiolone-dose group), and (ii) before the euthanasia (three days after bromadiolone administration) in the bromadiolone-dose group. Blood samples (ca. 2 ml) were obtained by puncturing the brachial vein with a needle (25G) and syringe and conserved in heparinized Eppendorf tubes at −20 °C until analysis.

Liver samples were taken during the necropsies and collected in polypropylene flasks and stored frozen at −20 °C until analysis. The percentage of humidity of the liver samples was calculated in an Infrared Moisture Analyzer MA35 (Sartorius) in order to indicate the results in dry weight (d.w.) and correct for different water content.

Chemicals and Reagents

Bromadiolone analytical standard (≥90%) was purchased from Sigma-Aldrich (New Haven, CT, USA). All solvents and reagents were of High Performance Liquid Chromatography (HPLC) quality (>99.9% purity). Acetonitrile was obtained from PanReac® (Darmstadt, Germany), methanol was obtained from Lab-Scan® (Gliwice, Poland), and formic acid from Probus® (Badalona, Barcelona, Spain). Magnesium sulphate, sodium chloride, sodium citrate dibasic sesquihydrate, sodium citrate tribasic dihydrate, polymerically bonded, ethylenediamine-Npropyl phase that contains both primary and secondary amines (Supelclean Primary secondary amine (PSA) bonded silica), and C18 (Discovery DSC-18: octadecylsilane 18% C) were purchased from Supelco® (Bellefonte, PA, USA).

Sample Preparation and Chemical Analysis

Bromadiolone was extracted from blood and liver samples using the dispersive solid phase extraction (dSPE) technique described by Gómez-Ramírez et al., (2012). Briefly, 2 g of blood or the whole homogenized liver was mixed with 2 ml of acetonitrile as the extractant. The tubes were vortexed vigorously for about a minute and a mixture of salts (1.33 g magnesium sulphate, 0.33 g sodium chloride, 0.17 g sodium citrate dibasic sesquihydrate and 0.33 g sodium citrate tribasic dehydrate) was added. The tubes were again vigorously shaken with vortex for one minute approximately. The tubes were centrifuged at 998 relative centrifugal force (RCF) for 5 min, and frozen at −20 °C for 1 h. After that, the tubes were again centrifuged in the same conditions, and the supernatant was then transferred to another tube and mixed with a new mix of salts (50 mg PSA, 50 mg DSC-18, and 300 mg magnesium sulphate). The tube was shaken and centrifuged again at 998 RCF for 5 min. The supernatant was evaporated until dry with a nitrogen stream, redissolved in 1 ml of methanol, and acidified by adding 10 µl of 5% formic acid in acetonitrile for HPLC/MS analysis.

Instruments and Conditions

Bromadiolone was detected and quantified using an Agilent 1290 Infinity II Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an

Automated Multisampler module and a High Speed Binary Pump, and connected to an Agilent 6550 Q-TOF Mass Spectrometer (Agilent Technologies, Santa Clara, CA, USA) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) (Agilent Technologies, Santa Clara, CA, USA) interface. Experimental parameters for HPLC and Q-TOF were set in MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.08.00).

Standards and samples (injection volume of 20 µl) were injected into a Zorbax Eclipse XDB C8, 5 µm, 150 x 4.6 mm HPLC column, at a flow rate of 0.7 ml/min. The column was thermostated at 25°C. Solvents A (MilliQ water with 20 mM ammonium acetate) and B (methanol with 20 mM ammonium acetate) were used for the compound separation. Initial conditions were 50% solvent A and 50% solvent B. After the injection, compounds were eluted using a linear gradient 50–95% B for 22 min. Then, a linear gradient from 95–50% B was applied in 3 min and finally the system was equilibrated at starting conditions (50% B) for 10 min before a new injection.

The mass spectrometer was operated in the negative mode. The nebulizer gas pressure was set to 40 psi, whereas the drying gas flow was set to 13 l/min at a temperature of 250 °C, and the sheath gas flow was set to 12 l/min at a temperature of 300 °C. The capillary spray, nozzle, fragmentor, and octopole RF Vpp voltages were 3500 V, 1000 V, 350 V, and 750 V, respectively. Profile data in the 100–1100 m/z range were acquired for MS scans in 2 GHz extended dynamic range mode. Reference masses at 525.0707 and 586.0997 were used. The data were analysed with MassHunter Qualitative Analysis Navigator software (version B.06.00, Service Pack 1, Agilent Technologies, Inc. US, 2012). Extracted ion chromatograms, obtained from bromadiolone molecular formula, were analysed.

A calibration curve was prepared using two replicates of spiked chicken liver at three levels (20, 40, and 80 ng/g) and injected in HPLC/MS-TOF following the same analytical conditions as the samples. A blank containing the mobile phases A and B was injected at the beginning and at the end of the batch of

samples to monitor for contamination. The same curve was used to calculate validation parameters, obtaining a correlation coefficient of r = 0.999 for linearity, 54.87% of recovery, and a repeatability variation coefficient of 9.59%.

Statistics

Statistical analyses and graphs were carried out using Microsoft Excel 2016 and SPSS v. 25. Data are presented as mean ± SD and range. Correlations between variables were tested with the Pearson correlation coefficient. Multivariate analyses of biological variables (i.e., sex coded as 1=male and 2=female, and body mass on day 0), weather variables (i.e., internal temperature and ambient temperature), decomposition variables (i.e., days of decomposition, body mass during necropsy, liver weight and liver water content), and bromadiolone concentration (ng/g, d.w.) were tested using principal component analysis (PCA). Tests were considered significant when $p < 0.05$.

Table 1. Weather conditions during the experiment in Common kestrel. Days after death, internal carcass temperature, ambient temperature and humidity during the experiment are provided (4–19 July 2019, sunrise 06:48–06:57 a.m. and sunset 09:31–09:26 p.m., raining 0 mm, mean wind speed 9.16 km/h, Murcia, Spain).

Days after Death	Internal Temperature (°C) [1]	Ambient Temperature (°C) [1]	Relative Humidity (%) [1]
Day 0	NM	NM	NM
Day 1	32.87	30.17	58.31
Day 3	32.74	33.17	44.63
Day 4	28.67	27.97	60.33
Day 7	32.81	30.10	50.65
Day 15	33.90	30.09	NM

Results and Discussion

The weather conditions during the experiment are presented in Table 1. Global mean of the period ± SD (min-max day mean) ambient air temperature, humidity, day duration, and wind speed recorded during the experiment period were 30 ± 2 (24–33) °C, 54 ± 8 (45–70)%, 14:33:45 ± 0:05:05 (14:25:00–14:41:00) hours:minutes:seconds and 9.16 ± 1.17 (6.90–11.30) km/h, respectively (detailed in Table 1).

Bromadiolone concentrations in blood and liver in both the bromadiolone-dose and the control groups are detailed in Table 2. Bromadiolone was only detected in one blood sample collected before bromadiolone administration and at low concentrations (4 ng/g wet weight (w.w.)). This suggests that Common kestrels were rarely exposed to bromadiolone before the experiment. In the bromadiolone-dose group, the compound was detected in all blood samples collected three days after bromadiolone administration and before euthanasia (range: 45–135 ng/g, w.w., n = 6; Table 2), reflecting bromadiolone exposure and absorption due to the experimental dosing.

Since carcasses were exposed to weather conditions, liver water content sharply decreased with time, and a negative correlation was found between tissue water content and days of decomposition ($r = -0.95$, $p < 0.01$; Figure 2). Therefore, bromadiolone concentrations are reported in both w.w. and d.w. to correct for the different water content between days (Table 2, Figure 3).

Blood is not the preferred tissue for testing AR, which raises the possibility that AR could have been present in the liver of birds before bromadiolone administration. Bromadiolone was detected at low concentrations in four control liver samples (range: 16–204 ng/g, d.w., n = 4; Table 2) and it was not detected in the other two control samples, which shows, in accordance with blood results, that individuals had low bromadiolone residues in the liver before the experimental dosing. Therefore, the presence of bromadiolone in the livers of the dose group before the administration cannot be discarded. However, the pre-experimental concentrations can be considered negligible compared to those in the dose group after the experimental dosing, where bromadiolone was detected in all livers (range: 756–8848 ng/g, d.w., n = 6). Although this was out of the scope of this article, it is important to note that free-ranging Common kestrels, as rodent predators, are exposed to sublethal doses with potential health effects, particularly reproductive effects (López-Perea et al., 2018).

Table 2. Bromadiolone concentration in blood (before dosing and euthanasia) and liver (according to the days of decomposition) of Common kestrels in the bromadiolone-dose and control groups.

ID	Sex	Group	Blood		Liver			
			Concentration before Dosing (ng/g, w.w.)	Concentration before Euthanasia (ng/g, w.w.)	Decomposition Day	Liver Weight (g)	Concentration (ng/g, w.w.)	Concentration (ng/g, d.w.)
#1	F	Bromadiolone-dose group	nd	47	0	5.0	960	3000
#3	M		nd	57	1	4.1	896	2891
#5	F		nd	45	3	2.2	2062	4804
#7	M		4	135	4	2.2	1419	4245
#9	M		nd	76	7	1.0	3794	8848
#11	M		nd	60	15	1.0	603	756
#2	M	Control group	nd	NA	0	6.0	65	204
#4	M		nd	NA	1	5.2	12	38
#6	M		nd	NA	3	2.8	36	84
#8	M		nd	NA	4	0.5	nd	nd
#10	F		nd	NA	7	0.5	nd	nd
#12	F		nd	NA	15	0.7	13	16

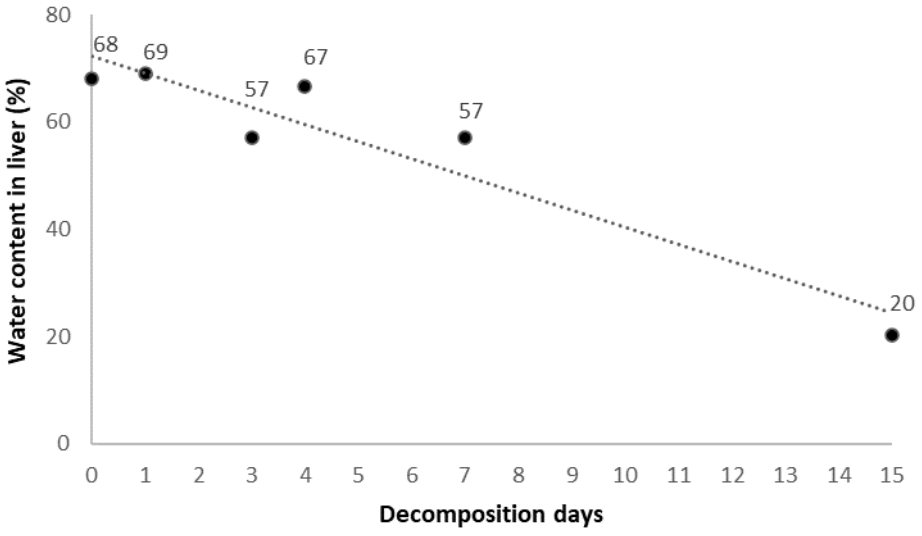


Figure 2. Water content in tissue (%) over time (decompositions days) in decaying carcasses of Common kestrel ($r = -0.95$, $p < 0.01$). Numbers above circles indicate the mean water content (%) for the control and dosed individual at each time point.

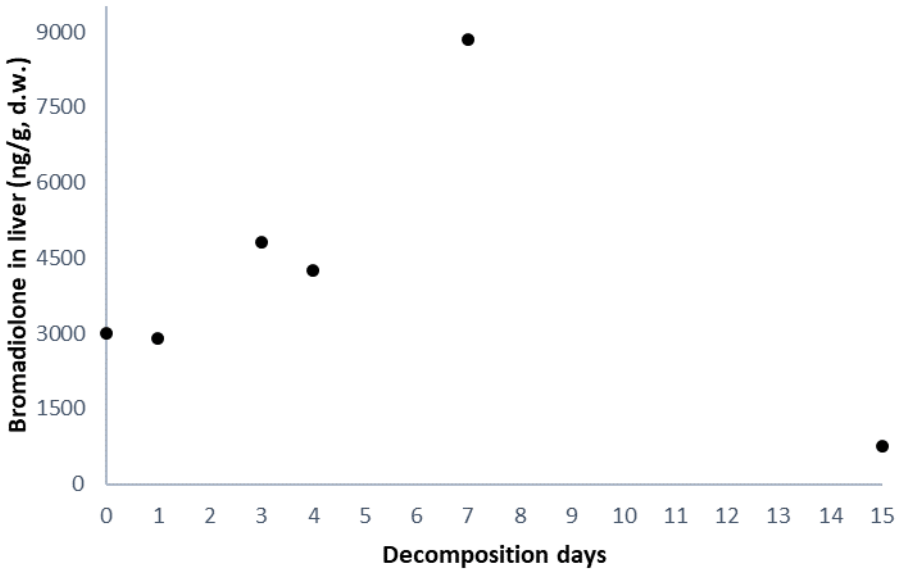


Figure 3. Bromadiolone concentration in liver (ng/g, d.w.) relative to the carcass decomposition time (days) in dosed Common kestrel (r = −0.20, p = 0.699, n = 6).

The extracted principal components (PCs) are shown in Figure 4. Two PCs were extracted (eigenvalues: PC1 3.8 and PC2 1.9), explaining 63% of the total variation (PC1 and PC2 accounted for 42% and 21% of the variance, respectively). PC1 gave a similar weight to the variables days of decomposition (−0.95) and with opposite sign to body mass during necropsy (0.84), liver weight (0.80) and liver water content (0.91), which might be described as "decomposition" variables. PC1 also gave similar loadings to internal temperature (−0.67) and ambient temperature (−0.46), described as "weather" variables. The second component (PC2) gave more emphasis to sex (0.77) and body mass on day 0 (0.93), considered as "biological" variables, and to bromadiolone concentration (−0.53), with the opposite sign (Figure 4). In addition, the "biological" variables were significantly correlated, with females showing higher body mass, as well as the "weather" variables, with increased ambient temperatures being related to higher internal temperatures in the carcasses (Figure 5). The "decomposition" variables were also correlated. In this sense, longer decomposition time (i.e., higher days of decomposition) was related to lower body mass during necropsy, lower liver weight, and liver water content; while higher body mass during necropsy was related to higher liver

weight and liver water content (Figure 5). In addition, increased internal temperatures in the carcass were related to lower liver water content (Figure 5).

PCA showed that individuals scoring highly on PC1 showed higher body mass during necropsy, liver weight and liver water content, and consequently lower days of decomposition (i.e., kestrels necropsied on days 0–1; Figure 4). Accordingly, individuals that scored highly on PC2 will have higher body mass on day 0 and lower bromadiolone concentrations in liver (Figure 4). In Figure 4, individuals in the bromadiolone-dose and control groups are indicated by different colours. In general, individuals in the bromadiolone-dose group lay below the origin and hence closer to the liver bromadiolone vector due to the higher liver bromadiolone concentrations. However, the kestrel from the bromadiolone-dose group necropsied on day 0 was positioned at the top right corner of the figure due to its highest body mass on day 0 and body mass during necropsy.

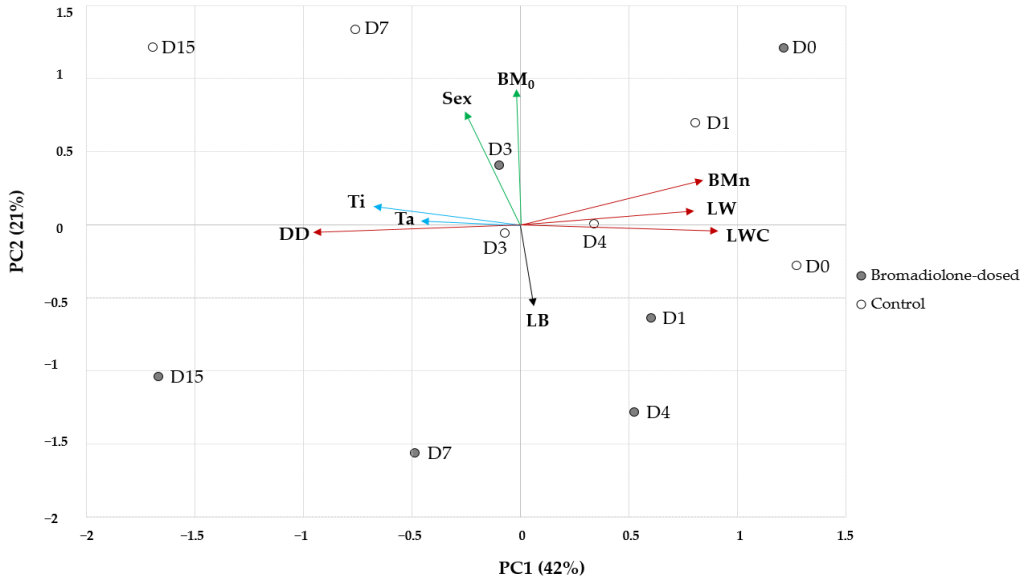


Figure 4. Principal component analysis (PCA) biplot of the Common kestrel experiment. Vectors represent: biological variables (sex and body mass on day 0, BM_0), weather variables (i.e., internal temperature, Ti, and ambient temperature, Ta), decomposition variables (i.e., days of decomposition, DD, body mass during necropsy, BMn, liver weight, LW, and liver water content, LWC), and liver bromadiolone concentration (LB). The points represent individual birds from the bromadiolone-dose (grey) or control group (white), and D0-D15 indicates the days of decomposition.

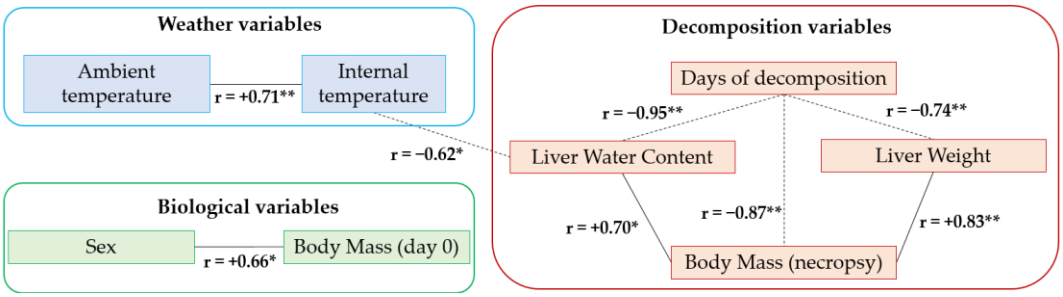


Figure 5. Significant relationships between weather variables (ambient and internal temperatures), biological variables (sex and body mass on day 0), and decomposition variables (days of decomposition, liver weight, liver water content and body mass during necropsy) in Common kestrels. Pearson correlation coefficients (r) are presented. The directions of the relationships are shown with positive and solid lines, or negative and dashed lines. * $p < 0.05$, ** $p \leq 0.01$.

The water content in a decay sample can vary greatly depending on the decomposition stage of the carcass, which may also affect the compound concentrations (discussed below). Therefore, it is important to highlight the difficulty of comparing results between studies, since these parameters are scarcely reported. For that reason, only individuals from decomposition day 0 (fresh carcass) and day 1 (carcass at moderate decomposition stage according to the protocol developed in Valverde et al., (2020) were selected to compare concentrations with other studies in the liver.

Bromadiolone concentrations in the liver of dosed Common kestrels were 960 and 896 ng/g w.w., at decomposition day 0 and 1, respectively (Table 2). No signs of AR poisoning were observed neither in the live animals after dosing nor in the necropsies (i.e., decreased mentation, weakness, pale mucous membranes, evidence of external and/or internal bleeding, hematoma (Murray 2018)). This can be explained because the dose selected (half of the LD50 reported for a variety of bird species) was aimed to provide environmentally relevant doses according to concentrations reported in free-ranging wild birds. However, this lack of direct evidence should not automatically be considered as a lack of toxicity in these animals (Ruiz-Suárez et al., 2014). As a matter of fact, some lethal cases have been related to low AR concentrations in liver, but clinical signs compatible with AR intoxication were found, and this is considered sufficient evidence of AR-related lethal poisoning by survey networks in France

and the United Kingdom (Wildlife Incident Investigation Scheme 2007; Coeurdassier et al., 2012). Liver concentrations of SGARs ranging from 100 to 200 ng/g w.w. have been suggested as levels of concern in raptors, while 200 ng/g w.w. are considered critical (Thomas et al., 2011; Christensen et al., 2012). However, data from secondary exposure studies show that evaluating dose-response relationships and estimating effect thresholds and tissue reference values are important challenges that need further research (Rattner & Mastrota 2018). In biomonitoring studies in Common kestrels, some liver concentrations of total ARs were above 100 ng/g w.w., with mean bromadiolone levels of 79.8 ± 34.4 ng/g w.w. in Canada (Christensen et al., 2012), while in livers of Common kestrels from Denmark, the median total AR (brodifacoum, bromadiolone, coumatetralyl, difenacoum, and flocoumafen) concentrations were 46 ng/g w.w., with a maximum of 679 ng/g w.w. of bromadiolone (median 0 ng/g) (Christensen et al., 2012). Bromadiolone concentrations found in liver samples in this experiment were higher than those reported in some biomonitoring studies in Common kestrels (Thomas et al., 2011; Christensen et al., 2012). The absence of AR intoxication signs in this study could be related to bromadiolone producing less pronounced signs of toxicity in raptors than other SGARs (Rattner & Mastrota 2018). In addition, effect thresholds for Common kestrels have not been reported, and both inter- and intraspecific variability in sensitivity to AR have been described. In this sense, remarkable differences in AR tolerance have been described among some bird species (Rattner & Mastrota 2018).

Since bromadiolone was detected in the liver of all dosed birds, the effect of carcass degradation in bromadiolone concentrations was evaluated. Liver bromadiolone levels were not correlated with days of decomposition ($r = -0.20$, $p = 0.699$), showing that there was no progressive reduction in liver concentrations with time in this study (Figure 3). Bromadiolone concentrations found in the liver of Common kestrel carcasses showed a slight (not significant) rise over the first days of decomposition, particularly evident on day 7 (Table 2, Figure 3), under the specific characteristics and weather conditions of this experiment (Table 1). On day 15, bromadiolone concentrations showed a non-

significant decrease of 84% compared to the mean value observed at decomposition days 0–7 (Table 2, Figure 3). Considering that, due to ethical reasons and the availability of non-releasable individuals, the bromadiolone-dose group only had one kestrel for each decomposition stage, the individual effect was strong. Therefore, due to the limited number of samples, this trend could be a random variation in bromadiolone concentrations. However, several combined factors could partially explain these results, although further studies are needed for a proper interpretation. These factors include: (i) individual-specific condition, (ii) *post-mortem* drug redistribution, (iii) *post-mortem* tissue alteration, and (iv) bromadiolone degradation.

The *post-mortem* drug redistribution (PMR), in other words, movement of drugs between organs, tissues, and fluids into the body after death (Yarema and Becker 2005), is another factor that may influence bromadiolone concentrations in liver. The PMR occurs by different mechanisms (e.g., diffusion through blood vessels, transparietal diffusion toward the surrounding organs, bacterial activity, cell death, which produces the leakage of the substances into extracellular space, pH changes, etc.). Therefore, accurate interpretation of compound concentrations in organs can be done when a carcass is fresh, while the PMR may complicate the understanding of the results in forensic toxicology and concentrations of toxic substances in internal tissues must be carefully interpreted (Pélissier-Alicot et al., 2003; Yarema & Becker 2005; Kennedy, 2015). Moreover, there is no specific marker to evaluate how long a substance is under the effects of PMR (Pélissier-Alicot et al., 2003; Yarema & Becker 2005; Kennedy 2015). Although PMR has been studied mainly in human medicine rather than in animals, as far as we are concerned, this forensic phenomenon also takes place in animals (Pélissier-Alicot et al., 2003; Yarema & Becker 2005; Kennedy 2015).

The *post-mortem* tissue alterations may also affect bromadiolone concentrations. There is a series of changes in the carcass (e.g., tissue autolysis and putrefaction) determined by different factors such as the cause of death, the size and position of the carcass, presence of cadaveric fauna or the

environmental conditions (e.g., some weather conditions may favour bacterial and cadaveric fauna and the decomposition process) (Oates, 1984; Brooks, 2016; Viero et al., 2019), leading to the loss of tissue integrity and mass. Liver weights of the individuals from decomposition day 0 to day 4 (including fresh, moderate, and advanced decomposition stages) ranged from 2.2 to 5.0 g in the bromadiolone-dose group. However, the liver weights of the individuals necropsied on days 7 and 15 (very advanced decomposition stage and initial skeletal reduction, respectively) were similar between them and decreased by 2.2–5.3 times the liver weights on days 0–4 (0.98 and 0.95 g, respectively). This suggests that liver tissue decreases in mass independently of the dehydration (as water content was 57 and 20%, respectively, Table 2).

Finally, on day 15, there was a non-significant drop in bromadiolone concentration that could be partially related with a degradation of the compound with time. For example, during the putrefactive processes, bacteria may produce and metabolize different compounds (Pélissier-Alicot et al., 2003; Kennedy, 2015), which could alter bromadiolone levels. The carcass on day 15 would be less attractive for scavengers, which would mean, together with the potential bromadiolone degradation, a lower risk of tertiary poisoning for scavengers. However, this study should be considered as a first approach for future studies, but it presents some limitations regarding the number of individuals available and cannot provide clear evidence. There is a lack of literature regarding the behaviour of AR in carcasses in the field and their potential degradation over time. Further studies including more individuals necropsied at each time point and after additional days of decomposition within this time frame (particularly from day 7 to 15) would help to properly draw the degradation curve for bromadiolone in carcasses.

Conclusions

This experimental study was limited regarding the number of individuals used at each decomposition stage due to ethical reasons and the availability of non-releasable Common kestrels. Therefore, the non-significant tendency found in bromadiolone concentrations could be a random variation. However, this is the

first study providing some light to better understand the degradation of SGARs in carcasses in the field. Our results could suggest that bromadiolone may persist in fresh, moderately, and advanced decomposed carcasses, although concentrations can be affected by individual-specific condition, PMR, and tissue degradation. Thus, carcasses in the field may be a source of secondary or tertiary poisoning for scavengers, at least during the first week after death when weather conditions are similar to those found in this study. However, when the carcass was at initial skeletal reduction (ca. 15 days after death), bromadiolone concentration in liver declined by 84% compared to the mean value observed at earlier decomposition stages. This result should be interpreted with caution since it represents data from a single individual. Therefore, additional research is encouraged to better interpret the degradation of the product with time. This information is essential to evaluate the risk of secondary and tertiary poisoning and for an accurate interpretation of the toxicological analysis and proper diagnosis. Considering our results, wildlife sampled 7–15 days *post-mortem* with low AR concentrations, but showing haemorrhaging signs, should not be immediately ruled as non-AR death due to the potential decreased trends suggested in this study.

Despite the lack of any AR intoxication sign in the experimentally-dosed Common kestrels, bromadiolone levels found in liver were higher than those reported as SGARs concentrations of concern in raptors (100–200 ng/g w.w., (Christensen et al., 2012)) and higher than those found in some biomonitoring studies. The absence of signs of toxicity could be due to bromadiolone producing less pronounced intoxication signs than other SGARs, and the potential inter- and intraspecific variability in sensitivity to AR.

New experiments including more individuals necropsied after additional days of decomposition would help to properly draw the degradation curve for bromadiolone in carcasses. It is essential to undertake complementary studies on a broader variety of weather conditions and species of different sizes for a proper assessment of the persistence of bromadiolone and other ARs on

wildlife carcasses. In addition, the dose-response relationships, effect thresholds, and tissue reference values should be further evaluated.

Finally, we encourage future studies to provide information on the water content and state of decomposition of samples to better evaluate concentrations and facilitate results comparison between studies. We recommend the use of protocols such as the one provided by Valverde et al., (2020), which is based on a scoring method to classify stages of carcass decomposition.

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Chapter III. Wildlife poisoning: a novel scoring system and review of analytical methods for anticoagulant rodenticide determination

Image: Irene Valverde Domínguez

Abstract

Anticoagulant rodenticides are commonly used to control rodent populations and frequently involved in wildlife and domestic animal poisoning. These poisoning cases (especially for AR) are a challenge for forensic toxicologists, and adequate *post-mortem* examination and toxicological analyses become essential for a proper diagnosis. Publications describing different analytical methods for AR analysis in biological samples are growing, and a clear compilation of the overall picture is needed to standardize methodologies in future research. This review aims to compile and compare the analytical procedures applied for AR determination in the literature. Using this information, a scoring system was developed for those techniques using liver and blood as matrices, and the techniques were ranked considering different criteria (i.e., sample amount required, recoveries, LOQ, number of AR analysed, points of the calibration curve and multi-class methods). This review shows an overview of the main methods used for AR analysis in forensic toxicology and will help to elucidate future directions to improve multi-residue techniques to detect the AR involved in wildlife lethal poisoning.

Keywords: anticoagulant rodenticides; analytical methods; wildlife; poisoning; forensic; scoring system.

Introduction

Anticoagulant rodenticides are compounds frequently used to control rodent populations worldwide. AR are regulated in the EU as PPP or as biocides depending on their use. A total of 14 rodenticidal compounds are authorized in Europe, 8 of them are AR (i.e., brodifacoum, bromadiolone, chlorophacinone, coumatetralyl, difenacoum, difethialone, flocumafen, warfarin) (Regulation (EC) No 1107/2009; Regulation (EU) No 528/2012). Alpha-chloralose, zinc phosphide, aluminium phosphide, calcium phosphide, magnesium phosphide and CO_2 are the non-anticoagulant rodenticides authorized in the EU (van den Brink et al., 2018).

Anticoagulant rodenticides can be classified as first-generation AR (FGARs) and second-generation AR (SGARs), the latter developed due to the resistance observed in some rodent populations to some FGARs (van den Brink et al., 2018). However, a third generation is suggested to be developed, based on the stereochemistry of the old SGARs (Damin-Pernik et al., 2017). Coagulation factors II, VII, IX, X are activated by the reduction of vitamin K (hydroquinone), which leads to the γ-carboxylation of the coagulation factors. When these factors are activated, vitamin K is reduced to vitamin K epoxide. Vitamin K reductase catalyses inactive epoxide to hydroquinone again. AR mechanism of action (Figure 1) is based on the inactivation of the VKOR in the liver and other tissues. Consequently, vitamin K hydroquinone formation decreases, and carboxylation of clotting factors II, VII, IX and X is reduced. As a result, blood clotting is affected and associated clinical signs will appear (Furie et al., 1999; Brown 2009; van den Brink et al., 2018).

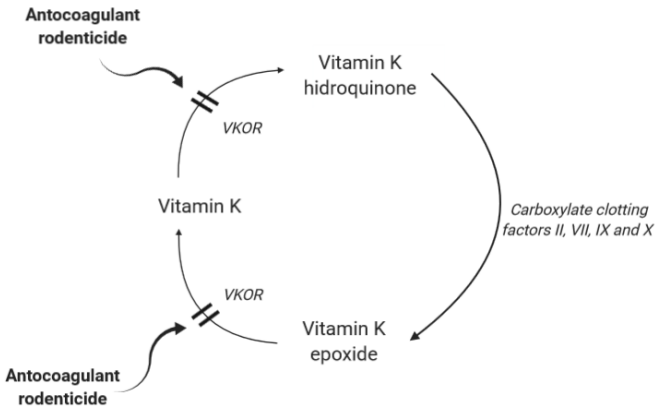

Figure 1. Mechanism of action of anticoagulant rodenticides. VKOR: vitamin K epoxide reductase.

Rodenticides have been successfully used against the adverse impacts caused by rodents since the mid-20th century. Rodents are host of bacteria, parasites and viruses posing a risk to human health, they cause crop damage but also cable or material destruction, they spread very fast and can affect local fauna, etc. (Martínez-Padilla et al., 2017; van den Brink et al., 2018). However, when these products are misused, they become a non-selective method, affecting the target species but also any other domestic or wild animal, including endangered species (Navas et al., 1998; Mateo et al., 2000; Wobeser et al., 2004; Nakayama et al., 2019). Moreover, it can be a public health hazard, as these substances can be ingested by game species (e.g., wild pigs, rabbits, etc.) and later consumed by humans (López-Perea et al., 2018). AR are sometimes used intentionally to kill non-target animals considered harmful to certain activities (agriculture, livestock-farming or hunting) or as a revenge way of solving feuds between private individuals, these practices are an important threat to wild and domestic animals (Berny 2007; Ruiz-Suárez et al., 2015). The use of the AR in poisoned baits is considered an illegal action and a criminal offence for the protection of the environment through criminal law in Europe (Council Directive 92/43 EEC; Directive 2008/99/EC; Directive 2009/147/EC).

Different publications have reviewed cases of animal poisoning involving AR in Europe, as well as their implication in secondary poisoning (Berny et al., 2010; Guitart et al., 2010; Vandenbroucke et al., 2010; McFarland et al., 2017; Nakayama et al., 2019). Some of the species most affected by AR primary or secondary poisoning are raptors with scavenging habits such as Buzzard (*Buteo buteo*), but also wild and domestic mammals (mostly dogs and cats) (Berny

2007). Using domestic and feral animals as sentinel species should be highlighted in the study of wildlife poisonings (Navas et al., 1998; Mateo and Guitart, 2000; Navas et al., 2016).

Bromadiolone and difenacoum are the AR with more products registered for biocidal use, and they are also the only two AR authorized as PPP in the EU. Bromadiolone is authorized in eight countries of EU member states, while difenacoum is permitted only in two. Van den Brink et al. (2018) also mention that, since the ban of chlorophacinone in 2009, bromadiolone is the AR most widely used in PPP. PPP may cause more environmental risks as they become more accessible for wildlife due to their widespread use in the environment (van den Brink et al., 2018). Moreover, brodifacoum, flocoumafen and bromadiolone have the longest half-life in liver of rat and mouse (Table S1). Bromadiolone is extensively used in some countries against field rodents (e.g., voles) and the amount of active ingredient used is higher than for other AR. In contrast, difenacoum is only used to control rats around fields, with very limited use (Jacob and Buckle, 2018). All these points favour the frequent involvement of bromadiolone in poisoning.

Wildlife and domestic animal poisoning are a challenge for forensic toxicologists. Adequate *post-mortem* examination and toxicological analyses are essential to determine cases of poisoning (Brown, et al., 2005; Valverde et al., 2020a; Valverde et al., 2020b). Publications describing different analytical methods for the identification and quantification of AR in biological samples are growing in the literature (Vudathala et al. 2010; Bidny et al. 2015; Imran et al. 2015). However, these techniques vary in the matrix analysed, the number of compounds measured, the extraction method and instrument used, and in characteristics and accuracy. Therefore, a proper compilation and comparison between techniques available in the literature is needed to better understand the current state of the science and to further improve the analytical methods in wildlife forensic toxicology.

The main aim of this review is to compile and compare the analytical procedures applied for AR determination in the literature. For this purpose, we have

reviewed the main publications available and prepared a database compiling the laboratory techniques used for the analysis of AR in both fauna and humans, providing mainly the type of compound analysed, the matrix used, the weight or volume of sample analysed, the extraction technique, the extractant solvents used, recoveries, LOQ) and the instrumental method applied. Using this information, a scoring system was developed for those techniques using liver and blood, and the main techniques were ranked according to the sample amount, recoveries, LOQ and number of AR analysed. This will facilitate comparison between techniques and the choice of a way forward for futures studies.

Furthermore, this review will show the main methods used for AR analysis in forensic toxicology and will help to elucidate future directions to improve multi-residue techniques suitable to detect the AR that are causing wildlife lethal poisoning nowadays.

Methods

Different databases were used to search the literature available, including PubMed, Web of Science and ResearchGate. The list of references of the different articles reviewed was also scanned to identify additional publications. Different keywords and combinations of terms were used, such as 'anticoagulant rodenticide', 'poisoning', 'forensic', 'animal', 'wildlife', 'analysis'.

A global descriptive statistical study was carried out using the data from all the publications reviewed using Microsoft Excel 2016 spreadsheet. Regarding the methodologies used for AR determination, information is provided as follows: matrices used, sample weight or volume, analytical technique, AR analysed, extraction and clean-up procedure, recovery, LOQ and chromatographic conditions reported.

Moreover, a scoring system was developed for those techniques using liver and blood as matrices (Table 1 and 2). The parameters selected for scoring the different techniques were: recoveries, LOQ (and/or LOD), sample amount and number of compounds analysed.

Table 1. Scoring for techniques using blood samples for AR analysis.

	(Bidny et al. 2015)	(Martínez-Padilla et al., 2017)	(Seljetun et al., 2018)	(Yan et al., 2012)	(Jin et al., 2007)	(Jin and Chen 2006)	(Adamowicz P. 2009)	(Adamowicz P. 2009)	(Vudathala et al. 2010)	(Gómez-Ramírez et al., 2012)	(Hao et al., 2014)	(Meister 2005)	(Qiao et al. 2018)	(Rial-Berriel et al. 2020)
Extraction technique[1]	LLE	LLE	LLE	LLE	LLE	LLE	LLE	LLE	dSPE	dSPE	ASE	SPE	LLE	dSPE
Instrument	UPLC-MS/MS	LC-MS	UHPLC-MS/MS	LC-MS/MS	LC-MS/MS	HPLC-MS/MS	HPLC-MS	LC-MS/MS	HPLC-UV and FL	LC-MS/MS	HPLC-DAD	HPLC-FL	UPLC-MS/MS	LC-MS/MS
Sample volume (ml)	0.2	0.4	0.1	1	0.2	0.2	1	1	1	2	1	5	1	0.25
Recoveries (%)														
Brodifacoum	87.5 [2]		69.5 [2]	59.03 [a]		85.75 [2]	81	81	87.34 [2]	72.59		45.07 [2]	52.93 [2]	76.6 [4]
Bromadiolone	82.5 [2]	97	48 [2]	73.3 [a]	83.65 [2]	93.55 [2]	79	79	88 [2]	128.79	97.5 [2]	51.77 [2]	57.46 [2]	76.6 [4]
Chlorophacinone	87 [2]								85.67 [2]	86.26			70.8 [2]	76.6 [4]
Coumachlor													70.76 [2]	76.6 [4]
Coumafuryl									97.67 [2]				61.43 [2]	
Coumatetralyl	90 [2]		12,5 [2]				74	74		134,54	91 [2]	87.4 [2]	75.73 [2]	76.6 [4]
Difenacoum	80 [2]		65 [2]				76	76	92 [2]	93.45		53.5 [2]	55 [2]	76.6 [4]
Difethialone	85 [2]		76.5 [2]				76	76	68 [2]			57.27 [2]		76.6 [4]
Diphacinone	75 [2]								84.34 [2]	74.15			71.66 [2]	76.6 [4]
Flocoumafen	89 [2]		85 [2]			87.05 [2]						46.05 [2]	57.7 [2]	76.6 [4]
Warfarin	95 [2]						65	65	94.67 [2]	104.06	101 [2]	57 [2]	77.4 [2]	76.6 [4]
LOQ (ng/ml)														
Brodifacoum	2		2.6	0.5		0.5	15	60	0.033 [2, 3]	5		0.3	0.5	0.8
Bromadiolone	2	0.1	2.6	0.5	0.5	0.05	50	200	0.165 [2, 3]	5	150	0.6	0.5	0.4
Chlorophacinone	2								0.165 [2, 3]	5			0.5	0.8
Coumachlor													0.5	0.2
Coumafuryl									0.33 [2, 3]				0.5	

Table 1. Scoring for techniques using blood samples for AR analysis.

	(Bidny et al. 2015)	(Martínez-Padilla et al., 2017)	(Seljetun et al., 2018)	(Yan et al., 2012)	(Jin et al., 2007)	(Jin and Chen 2006)	(Adamowicz P. 2009)	(Adamowicz P. 2009)	(Vudathala et al. 2010)	(Gómez-Ramírez et al., 2012)	(Hao et al., 2014)	(Meister 2005)	(Qiao et al. 2018)	(Rial-Berriel et al. 2020)
Coumatetralyl	2		1.5				10	10		5	50	0.4	0.5	0.4
Difenacoum	2		2.2				30	60	0.033 [2, 3]	1		0.3	0.5	0.4
Difethialone	2		2.7				200	280	0.165 [2, 3]			12.9		0.8
Diphacinone	2								0.165 [2, 3]	5			0.5	1.2
Flocoumafen	2		2.7			0.05						0.6	0.5	0.2
Warfarin	2						15	150	0.33 [2, 3]	5	60	1	0.5	0.1
Number of compounds (N)	9	1	6	2	1	3	6	6	8	7	3	7	10	10
Calibration points	7	NR	3*	3	NR	8	9	9	NR	3	7	5	6	12
N with recovery 70-120%	9	1	2	1	1	3	5	5	7	5	3	0	5	10
N with recovery <70% or >120%	0	0	4	1	0	0	1	1	1	2	0	6	5	0
N with LOQ ≤ 5 ng/ml	9	1	6	2	1	3	0	0	0	7	0	6	10	10
N with LOQ >5 ng/ml	0	0	0	0	0	0	6	6	8	0	3	1	0	0
Relative score [5]	**8.0**	**2.4**	**5.4**	**1.7**	**2.4**	**2.6**	**4.8**	**4.8**	**6.8**	**6.2**	**1.4**	**5.3**	**7.7**	**8.7**

[1] ASE: Accelerated solvent extraction, dSPE: Dispersive Solid-phase extraction, LLE: Liquid-liquid extraction. SPE: Solid-phase extraction

[2] Results obtained calculating the media.

[3] LOQ were calculated multiplying LOD by 3.3.

[4] The lowest recovery was selected, recoveries ranged from76 to 119.5.

[5] Only studies providing sample amount, recoveries and LOQ were selected to be ranked according to the equation:

Total score = $[((NR \times 1.2) / N) + ((NLOQ \times 0.9) / N) + (N \times 0.6)^{a}] + 0.3^{b} + 0.2^{c} + 0.1^{d}$; where:

N_R: Number of compounds with recoveries ranging from 70 to 120% multiplied by a coefficient of 1.2 and divided by the total number of compounds analysed.

N_{LOQ}: Number of compounds with LOQ ≤ 5 multiplied by a coefficient of 0.9 and divided by the total number of compounds analysed.

N: Total number of compounds measured in the technique.

[a] Total number of compounds measured in the technique multiplied by a coefficient of 0.6 when N ≥ 4.

[b] Sample amount: + 0.3 when the sample amount used was < 1 ml.

[c] Calibration curve points: + 0.2 when there was ≥ 3 concentration points in the curve.

[d] Multi-class methods: + 0.1 when the method analyses additional chemical groups.

* Number of calibration points were not reported but a range of concentrations was provided, assuming there were at least 3.

NR: not reported.

For this purpose, we used an equation where the different parameters had a different weight according to their importance to validate an analytical technique. In this sense, recoveries represent a 40% of the equation, LOQ account for a 30%, and the number of AR analysed (multi-residue method), and the sample amount used for analysis represent 20% and 10%, respectively. Recoveries and LOQ are provided with a higher load in the equation because they are the main parameters to assess the method validation SANTE/12682/2019. Only studies providing sample amount, recoveries and LOQ were selected to be ranked. Anticoagulant substances such as dicoumarol, phenprocumon, pindone and valone were excluded from the scoring system, because they are rarely used as AR.

The equation used in the scoring system was:

$$Total\ score = [((N_R \times 1.2) / N) + ((N_{LOQ} \times 0.9) / N) + (N \times 0.6)^a] + 0.3^b + 0.2^c + 0.1^d$$

where:

N_R: Number of compounds with recoveries ranging from 70 to 120% multiplied by a coefficient of 1.2 and divided by the total number of compounds analysed.

N_{LOQ}: Number of compounds with LOQ ≤ 5 (in ng/ml for blood and µg/kg for liver) multiplied by a coefficient of 0.9 and divided by the total number of compounds analysed.

N: Total number of compounds measured in the technique.

[a] Total number of compounds measured in the technique multiplied by a coefficient of 0.6 when N ≥ 4.

[b] Sample amount: + 0.3 when the sample amount used was < 1 ml (for blood) or ≤ 0.5 g (for liver).

[c] Calibration curve points: + 0.2 when there was ≥ 3 concentration points in the curve.

[d] Multi-class methods: + 0.1 when the method analyses additional chemical groups.

The best recovery ranges (70-120%) were selected according to SANTE/12682/2019 recommendations. The LOQ limit (≤ 5 ng/ml or µg/kg) was selected by reviewing the LOQ achieved for these compounds in the available literature. When LOQ values were not provided, the LOD values were multiplied per 3.3 (Wenzl et al., 2016). When more than one recovery or LOQ/LOD were provided, the mean value was calculated and used in the equation. When LOQ value was reported as ≥ or ≤, that value was selected to score the techniques. When analysing samples to evaluate a potential case of animal poisoning, different AR and other toxic compounds may be involved. Thus, multi-residue and multi-class techniques capable to detect a wide range of compounds are positively scored. In addition, in wildlife forensic toxicology is often difficult to gather a large amount of sample, and in some cases the sample must be fractionated to perform different analyses. Therefore, techniques using low sample amounts are required and positively evaluated.

Results and Discussion

Anticoagulant rodenticides analysed

A total of 49 articles describing 56 analytical methods for AR analysis were reviewed. Techniques working with both animal and human samples were studied together, since this does not affect the quality of the method (Table S2). The description of these methodologies has been published in the last 26 years (period 1995-2021; Table S2). Other previous articles were reviewed by World Health Organization Geneva (1995). Most of the methods described in the literature are set to detect bromadiolone, brodifacoum and difenacoum (Figure 2, Table S2). This is probably due to their common presence in cases evaluating wildlife poisoning (Fournier-Chambrillon et al., 2004; Berny and Gaillet, 2008a; Langford et al., 2013; Ruiz-Suárez et al., 2015) together with their widespread use to control rodent pests (Berny et al., 1997; Berny and Gaillet, 2008; Hernández et al., 2013).

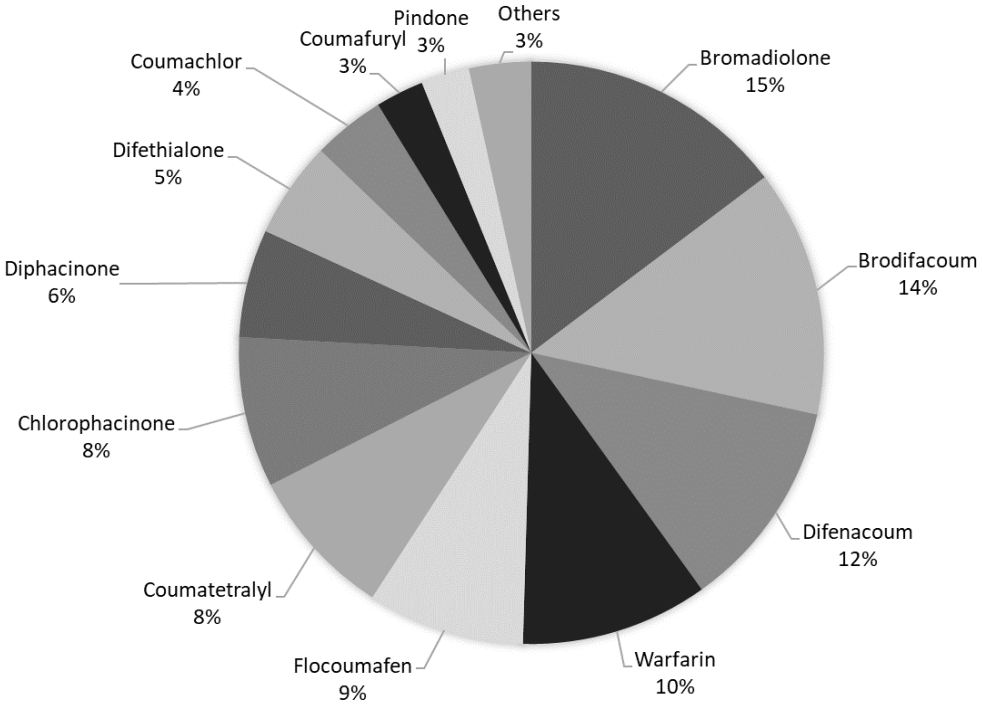

Figure 2. Frequency of methodologies analysing each AR.

Moreover, some of these techniques are able to simultaneously detect a variety of compounds in addition to AR, including non-AR pesticides, such as carbamates, OP, and human and veterinary drugs (Luzardo et al., 2014; Sell et al., 2017; Taylor et al., 2019). In wildlife poisoning cases, the list of compounds to which an animal may be exposed is extensive, and clinical signs are rarely observed as the animals are often found dead. Therefore, methods that can simultaneously detect a wide variety of compounds offer an enormous advantage to the toxicologist (Table S2).

Matrix and sample amount

In AR diagnosis, different matrices can be used. The review of methodologies showed that liver (48%) and blood (34%) were the matrices more frequently used for AR analysis (Table S2). This can be explained because, once absorbed, the AR passes into blood and is distributed to target tissues. In general, liver is an important matrix in the analysis of AR since this organ metabolizes and accumulates these substances. Nevertheless, liver is a less accessible organ for biopsies in biomonitoring studies or in sample collection form alive individuals,

in these cases, blood is mainly used (Berny et al., 1995; Vandenbroucke et al., 2008).

The amount of sample for analysis is one of the main limiting factors when working with wildlife, particularly in small species. In addition, in forensic research, the sample may be fractionated to apply different toxicological analysis. The sample amount used in the techniques reviewed in Table S2, ranges between 0.02-5 g or ml of liver and blood, respectively. On average, the most frequent mass/volume used are 1-2 g of liver and 1 ml of blood (Table S2).

Extraction and clean-up techniques

Different extraction techniques are reported according to the compounds analysed and matrices used (Table S2). Liquid-liquid extraction (LLE; 32%) and the solid-phase extraction (SPE; 32%) stand out, but other techniques such as dispersive-solid phase extraction (dSPE; 14%) are also reported. Within each extraction technique, several modifications have been proposed, even combinations of several techniques (LeDoux 2011; Imran et al. 2015).

The main extractant solvents used to analyse AR in the publications reviewed in this study are acetonitrile (38%), followed by acetone (30%) and ethyl acetate and methanol (21%) (Table S2), according to González-Curbelo et al. (2015). The solubility of ARs in different solvents is reported in Table S3. Ethyl acetate has the advantage of a partial miscibility with water, which makes the addition of non-polar solvents superfluous to separate it from water. However, the highly polar pesticides do not separate on ethyl acetate (Wilkowska and Biziuk 2011; Lucci et al., 2012). Acetone has intermediate polarity, being easily miscible with water, but the separation of water from this solvent requires a non-polar solvent (Wilkowska and Biziuk 2011). Nevertheless, acetonitrile is a polar solvent, miscible with water, but with sufficient dispersive properties to extract polar and non-polar pesticide residues from non-fatty samples (Lambropoulou and Albanis 2007).

Liquid-liquid extraction

Liquid-liquid extraction is based on the separation of the analytes according to the different solubility of the substances, that is, the different distribution of a product in coexisting liquid phases. In general, the extractant solvents most used for this technique are acetonitrile and ethyl acetate. Combinations of extractants are often used (LeDoux 2011). During LLE, the addition of salts to the solution to allow the separation of the organic phase from the aqueous phase is relatively common (Martins et al., 2013). Liquid-liquid extraction is a versatile technique with good reproducibility (Psillakis and Kalogerakis 2003).

On the other hand, some disadvantages are formation of emulsions, incomplete separation of the layers (Imran et al. 2015), as well as time consuming and laborious techniques with expensive steps regarding material and solvents. Moreover, evaporation of large amounts of solvent is required, which can lead to the loss of the analyte (LeDoux 2011).

According to the reviewed publications, LLE has been widely extended for the extraction of AR (Table S2; Imran et al. 2015). In the methodologies reported for these compounds (Table S2), the most commonly extractant solvent used in LLE was ethyl acetate (43%) (e.g., Denooz et al., 2009; Luzardo et al., 2014; Bidny et al., 2015). However, Imran et al. (2015) states that minimum recovery ranges are obtained for liver and plasma samples using ethyl acetate (from 54.3 to 96%), while the best recoveries are obtained with acetonitrile-ethyl ether (9:1) (92–109%).

Solid-phase extraction

Solid-phase extraction is based on the absorption of analytes in solid sorbents. This technique is also used for enrichment of liquid or gaseous matrices. It is carried out in four steps (Figure 3): 1) conditioning (the sorbent functional groups are solvated), 2) retention (the analytes bind to the surface of the sorbent), 3) selective washing (the unwanted particles are removed) and 4) elution (the analytes are "desorbed" and collected) (Martins et al., 2013). There are a variety of sorbents, but the most common material is silica. Silica is sufficiently reactive to allow its surface to be modified by chemical reaction and

is stable enough to be used in a wide range of solutions. The choice of the sorbent material depends on the matrix, the analytes of interest and their interferants (Martins et al., 2013).

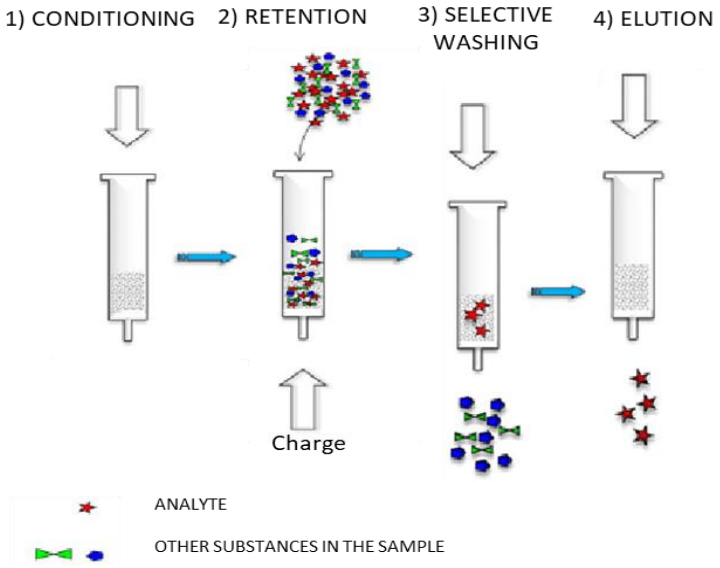

Figure 3. Scheme showing the solid phase extraction steps.

The advantage of this technique is that the drawbacks mentioned in the LLE can be avoided. In addition, a small volume of sample can be extracted, and it is possible to automatize it. The main disadvantages are the need of conditioning, washing, elution and drying of columns, and to choose an appropriate adsorbent and elution solvent (Martins et al., 2013; Imran et al. 2015).

The literature review shows that the extractant solvent most frequently used in SPE was acetone (53%) (e.g., Sage et al., 2010; Gallocchio et al., 2014; De Roma et al., 2018), and alumina is the solid sorbent most common for AR (43%, Table S2).

Dispersive solid-phase extraction

Dispersive solid-phase extraction was designed for the detection of pesticides in vegetal matrices. Acetonitrile was the extractant solvent par excellence. Subsequently, different salts were added to separate the analytes between the aqueous phase and the solvent (Anastassiades et al., 2003; Martins et al., 2013).

Different modifications of the technique have been carried out to adapt it to different biological matrices (Gómez-Ramírez et al., 2012; González-Curbelo et al., 2015).

The dSPE is, according to several authors, a simple, fast and economical method, regarding solvents consumption and what this implies. It also allows the simultaneous extraction of polar and non-polar compounds (Anastassiades et al., 2003; Martins et al., 2013; Imran et al., 2015). Extraction of a greater number of compounds groups is achieved compared with traditional techniques (Wilkowska and Biziuk 2011). This technique is a good alternative method to LLE (Imran et al. 2015). The main disadvantage is the low concentration of target compounds in the final extract compared to other traditional techniques, such as LLE and SPE (Martins et al., 2013).

Regarding the results of the present review (Table S2), the dSPE was used in the analysis of AR, but to a lesser extent than the LLE and SPE techniques. The extractant solvent used was acetonitrile (Table S2), considered the best extractant since it can be separated quite easily from water (through the addition of salts) (Wilkowska and Biziuk 2011). However, when acetonitrile is used in fatty samples, some lipids from the sample may coextract, and a clean-up is needed (Lehotay et al. 2005). In the clean-up step, PSA was more frequently used (Table S2).

Quantification methods

Different types of chromatography and detectors are combined depending on the compound of interest (Tables S2 and S4). A wide range of detectors can be coupled to chromatography techniques (Cai et al., 2009; Vudathala et al., 2010; Luzardo et al., 2014). Ultraviolet (UV) detector, fluorescent (FL) detector and diode array detector (DAD) coupled to HPLC, among others, have been used to detect AR (Tables 2 and 4). However, mass spectrometry (MS) detector has proved higher sensitivity and specificity and may identify pesticide metabolites and degradation products in the same acquisition series (LeDoux 2011).

In the present review on methodologies for AR analysis, liquid chromatography (LC in its different forms) was used in all the cases due to the non-volatile

characteristic of AR at gas chromatography (GC) temperatures (Imran et al., 2015). Only one article was found in which AR were analysed with GC-MS, due to the use of an in-injector pyrolysis of bromadiolone at 390ºC (Doubková et al. 2017). Mass spectrometry and tandem mass spectrometry (MS/MS) were the detectors most frequently coupled (24 and 61%, respectively; Tables S2 and S4). In general, LC-MS/MS (39%) was the main instrument used.

The range of recoveries observed in the different techniques reviewed is very wide (10 to 134%). This is due to the multiple factors that may affect recoveries, such as matrix and sample amount, extraction and clean-up procedure, analytical technique and number of compounds analysed (Table S2).

Regarding chromatographic conditions, the main characteristics are reported in Table S4. Most of the studies use a C18 column as stationary phase (77%). Using C18 silica column (octadecylsilyl) as stationary phase, allows the retention of hydrophilic (polar) molecules, thus a non-polar mobile phase is needed. On the contrary, a C18 column in reversed-phase retains hydrophobic (non-polar) molecules and a polar mobile phase is needed. Preparation of stationary phases with C18 does not require the exclusion of absolute water, which eases the procedure (Engelhardt et al., 2007). In some cases, reversed-phase column was used (19%). Most of the techniques report two mobile phases (A and B) with a gradient, and a third phase is less frequently used. Methanol and ultra-pure water are mostly used in mobile phase, ammonium acetate is the buffer most used.

Method validation, specificity and carry over effect

Method validation data provided in the literature is available in Table S2 (recoveries) and Table 3 (calibration function information and precision). Most of the methods reviewed accomplishes the validation criteria specified in SANTE/12682/2019.

Proper recoveries should range from 70 to 120%, however this parameter was not always achieved. Linearity of the calibration curve was tested using more than 3 concentration points. Moreover, repeatability and reproducibility (RSD/CV) were ≤ 20% in all methods (Meiser 2005; Sánchez-Barbudo et al.

2012; Seljetun et al., 2018). It should be noted that analytical methods for AR based only on internal validation may be subject to unrecognized inconsistencies in performance. This prompted the development of a consistent, robust, easily transferrable method for analysis of AR in liver, and its validation in a blinded multi-laboratory collaborative study, producing consistent analytical results (Smith et al. 2017). Measurement uncertainty needs to be established according to ISO/IEC 17025. The precision should be calculated from experiments different than those used to estimate the bias. The latter should be based on an external source such as Certified Reference Materials (CRM) and Proficiency Testing (PT) reference values (SANTE/12682/2019).

Regarding specificity of the detectors used, UV and FL provide good specificity (Meiser, 2005; Vudathala et al., 2010), although Berny et al. (1995) have reported interferences and decreased specificity using UV when liver extracts are in bad condition. MS is well known for its high specificity and sensitivity in determining chemicals and their metabolites in biological fluids compared with the other detectors. However, MS requires a more expensive experimental setup (Armentano et al., 2012).

In regard to the carryover effect, it may be a serious problem when analysing AR at high concentrations. For example, this effect has been observed for flocoumafen and brodifacoum when injected at high concentration (Carelli et al. 2020; Rial-Berriel et al. 2020). However, this parameter is not always reported in method development papers. The carryover effect can be tested by the injection of a blank after a fortified sample, and the injection of blanks after each real sample (Martínez-Padilla et al., 2017; Carelli et al., 2020; Rial-Berriel et al., 2020). To avoid this effect, the column can be rinsed several times with mobile phase after each injection (Smith et al. 2017).

Ranking of techniques analysing liver and blood

In the present review, techniques working with liver and blood as the main matrices were chosen to stablish a ranking according to a scoring system described in the Methods section. This will give some light in the comparison

of the techniques reviewed. It is important to highlight that this ranking has been created considering the specific parameters established in this review (recoveries, LOQ, sample amount, number of compounds analysed, points of the calibration curve and multi-class methods). Nevertheless, a technique may have a high analytical quality e.g., for a single compound and, however, it may obtain a low score in this review (since e.g. the sample amount or the number of compounds analysed are not positively scored). Moreover, many studies reviewed have been excluded since recoveries and LOQ were not provided. Therefore, we do not intend to rank the "better" or "more appropriate" techniques, but to positively score those methods combining good recoveries, low LOQ, low sample amounts and high number of compounds analysed.

Regarding the ranking for those techniques using blood as working matrix (Table 1), the technique of Rial-Berriel et al. (2020) stands out with 8.7 points, followed by Bidny et al. (2015) and Qiao et al. (2018) with 8.0 and 7.7 points, respectively. This is because Rial-Berriel et al. (2020) and Bidny et al. (2015) analysed 10 and 9 compounds, respectively; all of them had recoveries ranging 70-120% and LOQ below 5 ng/ml. In addition, this quality was achieved using only 0.2 ml of blood. Although Qiao et al. (2018) analysed a high number of compounds (N=10), and used 1 ml of sample, some compounds had recoveries below 70%. Vudathala et al. (2010) also analysed a good number of compounds (N=8), their LOQ were very low (0.033-0.33ng/ml), and used a small sample amount (1 ml); however, it does not provide the number of points or the range of concentrations used in the calibration curve. Gómez-Ramírez et al. (2012) analysed 7 rodenticides, but their recoveries were out of the range of 70-120% in some cases. Other studies (Jin and Chen, 2006, Martínez-Padilla et al., 2017, Jin et al., 2007) also had good recoveries and LOQ and used a low sample volume, but these techniques obtained a lower score because they analysed 1-3 compounds.

Rial-Berriel et al. (2020) used a dSPE extraction technique (acetonitrile as extractant) and LC-MS/MS. The methods described by Bidny et al. (2015) and Qiao et al. (2018) used LL extraction (ethyl acetate as extractant) and UPLC-

MS/MS determination. Other techniques using dSPE/SPE extraction (using acetonitrile) also got a high score and allow the analysis of several AR by LC-MS/MS and HPLC-UV or FL (e.g., Vudathala et al., 2010; Gómez-Ramírez et al., 2012; Seljetun et al., 2018), but a higher sample volume would be needed (1-2 ml; Table 1). Moreover, it should be highlighted that Rial-Berriel et al. (2020) was the only multi-class method in the blood scoring.

Regarding the ranking for techniques using liver (Table 2), Maršálek et al. (2015) and Taylor et al. (2019) stand out with scores of 8.0 and 7.8, respectively. Both techniques analysed 9 compounds with recoveries and LOQ within the established ranges, but they slightly differ due to the sample amount, Maršálek et al. (2015) using 0.5 g and Taylor et al. (2019) using 1 g of liver, however, Taylor et al., (2019) is a multi-class method. Although Sell et al. (2017) also analysed 9 compounds, it obtained a lower score (7.0 points) due to the LOQ (ranged 5-50 µg/kg) and the use of 2 g of liver. Maršálek et al. (2015) used SPE (with methanol) and LC/LC-MS/MS determination, while Taylor et al. (2019) used dSPE (with acetonitrile) and ultra high performance liquid chromatography (UHPLC) coupled to MS/MS and LC coupeled to MS/MS determination, respectively. All these techniques are good alternatives for analysing a high number of AR obtaining good recoveries and LOQ values.

Table 2. Scoring for techniques using liver samples for AR analysis

	(López-García et al. 2017)	(Thomas et al. 2011)	(Langford et al. 2013)	(Sánchez-Barbudo et al. 2012)	(Fourel, et al., 2017)	(Sell et al., 2017)	(Vudathala et al. 2010)	(Berny et al., 1995)	(Meiser 2005)	(Doubková et al. 2017)	(Maršálek et al., 2015)	(Taylor et al., 2019)	(Hauck et al. 2016)	(Luzardo et al., 2014)	(Armentano et al., 2012)	(Smith et al. 2017)	(Taylor et al. 2018)
Extraction technique[1]	"dilute and shoot"	SPE	NP	SPE	SLE	dSPE	dSPE	NP	SPE	in-injector pyrolysis of bromadiolone	onlineSPE	dSPE	LLE	SLE	NP	dSPE	GPC
Instrument	UHPLC-MS	LC-MS/MS	LC-MS/MS	LC-MS	LC-MS/MS	LC-MS/MS	HPLC-UV or FL	HPTLC-UV	HPLC-FL	GC-MS/MS	LC/LC-MS/ MS	UHPLC-MS/MS	LC-MS/MS	LC-MS/MS	HPLC-FL	UPLC-MS/MS	UHPLC–MS/MS
Sample amount (g)	2.5	0.5	0.5	1	0.5	2	1	1	5	0.5	0.5	1	0.1	2	4	1	>4
Recoveries (%)																	
Brodifacoum	41.8 [2]	70	53.5	70		92.2	86.0 [2]	88.5	49.6 [2]		95.5 [2]	93	93	97.4	109.1	105.66 [2]	80.66 [2]
Bromadiolone	82.3 [2]	70	74	70		93.8	77.0 [2]	90.2	43.6 [2]	96 [2]	95.5 [2]	85		94.3	81.1	106.66 [2]	83 [2]
Chlorophacinone	76.5 [2]				94.2	107.3	86.3 [2]	85.7			95.5 [2]	89		87.9		105 [2]	
Coumachlor	87.5 [2]			50		99.6		92.1			95.5 [2]				82.4	100 [2]	
Coumafen																	
Coumafuryl					87.45		66.3 [2]				95.5 [2]	92			90.6		
Coumatetralyl						109.3		94.6	74.5 [2]		95.5 [2]			89.2	98.3		105.6 [2]
Difenacoum	79.0 [2]		64.6	70		97.7	103.7 [2]	93.2	49.9 [2]		95.5 [2]	94		91.3	91.3		85 [2]
Difethialone		70	54	70			82.0 [2]	90.8	44.6 [2]			98		86.9		112.66 [2]	85.33 [2]
Diphacinone	78.3 [2]			70		106.5	66.7 [2]					91				101.33 [2]	
Flocoumafen			59	70		94.8			48.2 [2]		95.5 [2]	94			70.2		82.6 [2]

Table 2. Scoring for techniques using liver samples for AR analysis

	(López-García et al. 2017)	(Thomas et al. 2011)	(Langford et al. 2013)	(Sánchez-Barbudo et al. 2012)	(Fourel, et al., 2017)	(Sell et al., 2017)	(Vudathala et al. 2010)	(Berny et al., 1995)	(Meiser 2005)	(Doubková et al. 2017)	(Maršálek et al., 2015)	(Taylor et al., 2019)	(Hauck et al. 2016)	(Luzardo et al., 2014)	(Armentano et al., 2012)	(Smith et al. 2017)	(Taylor et al. 2018)
Warfarin	89.0 [2]			70	102.15	95.4	70.3 [2]	85.7	52.9 [2]		95.5 [2]	107		92.7	77.1	93.66 [2]	103.6 [2]
LOQ (µg/kg)																	
Brodifacoum	100	20 [3]	16.5 [3]	3.3 [3]		50	33 [3]	500	0.3		0.1	3	1	10	42.1	50	3
Bromadiolone	0.1	20 [3]	16.5 [3]	3.3 [3]		10	165 [3]	500	0.6	2	0.1	3		10	47.6	50	3
Chlorophacinone	0.5				2	25	165 [3]	500			0.1	3		30		50	
Coumachlor	0.1					5		500			0.1				43.9	50	
Coumafen																	
Coumafuryl					2		330 [3]				0.1	3			301.5		
Coumatetralyl				9.9 [3]		50		500	0.4		0.1			30	15.7		3
Difenacoum	0.2		6.6 [3]	3.3 [3]		10	33 [3]	500	0.3		0.1	3		10	44.5		3
Difethialone		50 [3]	16.5 [3]	9.9 [3]			165 [3]	500	12.9			3		30		50	3
Diphacinone	0.5			9.9 [3]		25	165 [3]					3				50	
Flocoumafen			6.6 [3]	3.3 [3]		25			0.6		0.1	3			21.5		3
Warfarin	0.1			3.3 [3]	1	10	330 [3]	500	1		0.1	3		20	95.2	50	3
Number of compounds (N)	7	3	5	8	3	9	8	8	7	1	9	9	1	7	8	7	7
Calibration points	3*	5	NR	3*	6	8	NR	5	5	6	7	3*	6	10	6	7	5
N with recovery 70-120%	7	3	1	7	3	9	6	8	0	1	9	9	1	7	8	7	7
N with recovery <70% or >120	0	0	4	1	0	0	2	0	6	0	0	0	0	0	0	0	0

Table 2. Scoring for techniques using liver samples for AR analysis

	(López-García et al. 2017)	(Thomas et al. 2011)	(Langford et al. 2013)	(Sánchez-Barbudo et al. 2012)	(Fourel, et al., 2017)	(Sell et al., 2017)	(Vudathala et al. 2010)	(Berny et al., 1995)	(Meiser 2005)	(Doubková et al. 2017)	(Maršálek et al., 2015)	(Taylor et al., 2019)	(Hauck et al. 2016)	(Luzardo et al., 2014)	(Armentano et al., 2012)	(Smith et al. 2017)	(Taylor et al. 2018)
N with LOQ ≤ 5 µg/kg	6	0	0	5	3	1	0	0	6	1	9	9	1	0	0	0	7
N with LOQ >5 µg/kg	1	3	5	3	0	8	8	8	1	0	0	0	0	7	8	7	0
Relative score[4]	6.4	1.7	3.5	6.6	2.7	7.0	5.7	6.2	5.3	2.6	8.0	7.8	2.6	5.7	6.2	5.6	6.5

[1] dSPE: Dispersive Solid-phase extraction, GPC: Gel Permeation Chromatography, LLE: Liquid-liquid extraction, NP: not provided, SLE: Solid-liquid extraction, SPE: Solid-phase extraction
[2] Results obtained calculating the media.
[3] LOQ were calculated multiplying LOD by 3.3.
[4] Only studies providing sample amount, recoveries and LOQ were selected to be ranked according to the equation:
Total score = $[((N_R \times 1.2) / N) + ((N_{LOQ} \times 0.9) / N) + (N \times 0.6)^a] + 0.3^b + 0.2^c + 0.1^d$; where:
N_R: Number of compounds with recoveries ranging from 70 to 120% multiplied by a coefficient of 1.2 and divided by the total number of compounds analysed.
N_{LOQ}: Number of compounds with LOQ ≤ 5 multiplied by a coefficient of 0.9 and divided by the total number of compounds analysed.
N: Total number of compounds measured in the technique.
[a] Total number of compounds measured in the technique multiplied by a coefficient of 0.6 when N ≥ 4.
[b] Sample amount: + 0.3 when the sample amount used was ≤ 0.5 g.
[c] Calibration curve points: + 0.2 when there was ≥ 3 concentration points in the curve.
[d] Multi-class methods: + 0.1 when the method analyses additional chemical groups
* Number of calibration points were not reported but a range of concentrations was provided, assuming there were at least 3.
NR: not reported.

Table 3. Validation method data

Matrix	r^2	Calibration function	Concentration rages in curve ng/mL**	Repeatability (RSD%)	Reproducibility (RSD%)	References
Blood	>0.98	linear	2-200	<8	<19.4	(Bidny et al. 2015)
Blood	>0.99	2nd order	1.5-1356	<9.1	17	(Seljetun et al., 2018)
Blood	0.99	linear	1-100	<14.2	<14.9	(Yan et al., 2012)
Blood	>0.99	linear	0.05-100	<7.8	<8.2	(Jin and Chen 2006)
Blood	0.99	linear	0.5-100	<7.5	<11.9	(Jin et al., 2007
Blood	>0.97	linear	10-5000	<15	<15	(Adamowicz and Kala, 2009)
Blood	0.97-0.99		0.3-206.4	<10.9	<12.5	(Meiser 2005)
Blood	≥0.90*	linear	20-80	<15% (diphacinone 32%)	<15 (diphacinone 38%)	(Gómez-Ramírez et al., 2012)
Blood Urine	0.99*	linear	20-150000	<1.1	<1.6	(Hao et al., 2014)
Blood, Liver	>0.99	linear	250-20000	NR	<14.3%***	(Armentano et al., 2012)
Liver	>0.99	linear	0.1-300	≤15	≤15	(López-García et al. 2017)
Liver	>0.99		0.0025-0.008	NR	NR	(Thomas et al., 2011)
Liver			40-2500	NR	NR	(Sánchez-Barbudo et al. 2012)
Liver	>0.99	polynomial	1-1000	<12.67***	<14.5***	(Fourel et al., 2017)
Liver	>0.99	linear	5-500	<11.3***	<21.9***	(Sell et al., 2017)
Liver Serum	>0.99	linear	100-2000	<5***	<5***	(Berny et al., 1995)
Liver, Blood	0.99-0.99		0.3- 206.4	<4.4*** <10.9***	<7.3*** <12.2***	(Meiser 2005)

Table 3. Validation method data

Matrix	r^2	Calibration function	Concentration rages in curve ng/mL**	Repeatability (RSD%)	Reproducibility (RSD%)	References
Liver	>0.99	linear	2-1000	<8.9	<7.9	(Doubková et al. 2017)
Blood Kidney	>0.99	linear	0.1-750	NR	2.8-17.5	(Maršálek et al., 2015)
Liver, Kidney, Muscle	>0.99	linear	0.5-50	NR	NR	(Taylo et al., 2019)
Tissue, Liver	>0.98	linear	0.5-500	<18	<18	(Luzardo et al., 2014)
Blood Liver	0.99	linear	1-50	<8.3***	<6.5***	(Hauck et al. 2016)
Liver	>0.99	quadratic	25- 2500	2-16		(Smith et al. 2017)
Liver	>0.99	linear	0.1-100	NR	NR	(Geduhn et al. 2014)
Blood	>0.99			<14***		(Carelli et al. 2020)
Hair				<14%***		(Carelli et al. 2020)
Blood	>0.99	linear	0.5-50	<15	<15	(Qiao et al. 2018)
Liver	≥0.96	linear	25-100	NR	NR	(Taylor et al. 2018)
Blood		linear	0.1-20	0.1-19.6	0.08-19.2	(Rial-Berriel et al. 2020)

Note: recoveries are provided in Table 1, 2 and S2.
NR: not reported
* *r* instead of r^2 is provided
**Units were transformed to ng/ml when needed
***Coefficient of variation instead of RSD was provided

In general, most of the analytical methods with the highest scores used dSPE, despite this extraction technique being less frequently used in the available literature. This is due to the combination of good recoveries, a high number of compounds detected, and good LOQ. Moreover, as described before, most techniques with the highest scores used acetonitrile as extractant and LC-MS/MS as instrument for AR determination.

Conclusions and recommendations for future research

Animal poisoning is an issue of special concern worldwide. AR are frequently involved in wildlife and domestic animal poisoning, and adequate analysis for proper diagnosis represents a challenge for forensic toxicologists.

Many different analytical methods have been described in the literature for the AR analysis in biological samples. Bromadiolone, together with difenacoum, are the AR with more products registered in Europe (mainly as biocides, but also as PPP), which explains that bromadiolone is the compound most frequently analysed in the techniques reported in the literature. Blood (distribution matrix) and liver (main metabolizing and accumulating organ) are the main matrices used for AR analysis, being the preferred tissues in live and dead animals, respectively.

Regarding the analytical techniques applied, LLE and SPE are the extraction techniques most frequently used, despite the disadvantages presented by these traditional techniques. The extractant solvents most commonly used are ethyl acetate, acetone and acetonitrile in LLE, SPE and dSPE, respectively. When the solubility of the solvent is considered, acetone would offer a better solubility for most of the AR analysed, although no data has been found regarding the solubility of AR in acetonitrile. Liquid chromatography coupled to a MS/MS detector is the instrument most frequently used to detect not only the AR but also different metabolites and degradation products.

Overall, a wide diversity of methodologies with different sensitivity to detect different compounds has been reported. Using a scoring system, we can better distinguish multi-residue techniques combining good recoveries and LOQ by

using low sample amounts and analysing a high number of AR. Some techniques can analyse up to 10 AR in 0.2 ml of blood (Rial-Berriel et al. 2020) and 0.5-1 g of liver (Maršálek et al. 2015; Taylor et al. 2019) obtaining recoveries ranging 70-120% and LOQ below 5 ng/ml or µg/kg. In general, analytical methods using dSPE (with acetonitrile as extractant) and LC-MS/MS for AR determination show a high number of compounds detected and good recoveries and LOQ.

This review provides detailed data on the AR methods reported in the literature that will help analysts to make comparisons and further develop and improve multi-residue techniques suitable to detect AR involved in wildlife poisoning. Most techniques are generally set up using fresh matrices, while the samples received in toxicology and forensic veterinary laboratories are frequently in different states of decomposition. Therefore, it is essential to develop sensitive and specific multi-residue methodologies to be able to identify different AR in samples from different nature and state of decomposition, which would help to minimize costs and time, standardize methods and maximize the possibility of identifying the AR involved in each case. In this sense, the use of protocols to classify the stages of carcass decomposition are recommended (e.g., Valverde et al., 2020a) because the degree of decomposition could affect the persistence of AR over time in carcasses (Valverde et al., 2020b). The development of protocols to harmonize and standardize analytical methods in different toxicology labs is also needed, as it is pursued by different networks such as the COST Action "European Raptor Biomonitoring Facility" (ERBFacility, CA16224). External validation (CRM and PT) and inter-laboratory comparisons are critical to harmonizing methods to ensure adequate performance and comparable results across laboratories.

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Supplementary Material

Wildlife poisoning: a novel scoring system and review of analytical methods for anticoagulant rodenticide determination

Table S1. Persistence in liver (days) of different AR [a]

	Rat	Mouse	*Falco sparverius* *Megascops asio*
Brodifacoum	80 [b]–350	307.4	
Bromadiolone	170 [b]–318	28.1	
Chlorofacinone	NA	35.4	
Coumatetralyl	55–62	15.8	
Difethialone	74–126	28.5	
Difenacoum	128	61.8	
Diphacinone	3 [b]	NA	0.325 [b] (initial) 2.49 [b] (terminal) 0.88 [b] (initial) 29.2 [b] (terminal)
Flocoumafen	220	93.8	
Pindone	2.1	NA	
Warfarin	26/66.8	66.8	

[a] Data obtained from (Epa, 2007; van den Brink et al., 2018).
[b] Half-life
NA: Not available

Table S2. Reported methodologies for determining AR in biological samples.

Weight/Volume	Analytical Technique [a]	Anticoagulant rodenticide	Extraction and clean-up procedure [b]	Recovery [c] (%)	Reference
Blood					
0.2 ml	UPLC–MS/MS	Coumatetralyl, Warfarin, Brodifacoum, Bromadiolone, Difenacoum, Flocoumafen, Difethialone, Diphacinone, Chlorophacinone	LLE [PP, EA]	70–105	(Bidny et al, 2015) [d]
1 ml	LC–DAD	Acenocoumarol, Warfarin, Phenprocoumon	LLE [DE–EA (50/50) HCl 0.2M]		(Denooz et al., 2009) [d]
0.4 ml	LC–ESI–MS	Bromadiolone	SPE [DM:AT (70:30), Na2SO4]	97	(Martínez-Padilla et al., 2017)
0.1 ml	UHPLC–MS/MS	Brodifacoum, Bromadiolone, Coumatetralyl, Difenacoum, Difethialone, Flocoumafen	LLE [ACN, EA/heptane (4:1)]	10–90	(Seljetun et al., 2018)
1 ml	LC–ESI–MS/MS	Bromadiolone, Brodifacoum	LLE [EA]	54.3–76.6	(Yan et al., 2012) [d]
1 ml	LC–MS/MS	Brodifacoum	LLE [EA]		(Yan et al., 2016) [d]
0.2 ml	HPLC–MS/MS	Bromadiolone	LLE [EA]	82.1–85.2	(Jin et al., 2007) [d]
	HPLC–ESI–MS	Bromadiolone, Flocoumafen, Brodifacoum	LLE [EA]	82.0–96.1	(Jin and Chen, 2006) [d]
	LC–MS and LC–MS/MS	Bromadiolone, Brodifacoum, Difethialone, Difenacoum, Warfarin, Coumatetralyl	LLE [CF:AT (1:1)]	65–81	(Adamowicz and Kala, 2009) [d]
0.1 ml	LC–MS/MS	Warfarin	LLE Protein-precipitation [CAN]		(Di Rago et al., 2014) [d]
2 g	LC–MS/MS	Warfarin, Coumatetralyl, Brodifacoum, Bromadiolone, Difenacoum, Chlorophacinone, Diphacinone	dSPE [ACN, SCDS, SCTD, NaCl, MgSO4, PSA, C18]	72–134	(Gómez-Ramírez et al., 2012) [d]
1 ml	HPLC–UV or FL/MS	Brodifacoum, Bromadiolone, Chlorophacinone, Coumafuryl, Difenacoum, Diphacinone Pindone, Warfarin	dSPE [ACN, PSA, Florisil, MgSO4, basic alumina]	>77[c]	(Vudathala et al., 2010) [d]

Table S2. Reported methodologies for determining AR in biological samples.

Weight/Volume	Analytical Technique [a]	Anticoagulant rodenticide	Extraction and clean–up procedure [b]	Recovery [c] (%)	Reference
2 ml	HPLC–UV/FL	Brodifacoum, Bromodiolone, Chlorophacinone, Coumafuryl, dicoumarol, Difenacoum, Difethialone, Diphacinone, Pindone, Valone, Warfarin	SPE [ACN, Alumina]		(Waddell et al., 2013)
1 ml	HPLC–DAD	Warfarin, Coumatetralyl, Bromadiolone	ASE [MET]	87–108	(Hao et al., 2014) [d]
5 g/ml	HPLC–FL	Coumatetralyl, phenprocoumon, Warfarin, Bromadiolone, Difenacoum, Flocoumafen, Brodifacoum	SPE [ACN/AT 1:1, HEX]	55.4–102.9	(Meiser, 2005) [d]
0.5 g/ml	GC–MS/MS	Bromadiolone	MET	94–98	(Doubková et al., 2017) [d]
0.5 ml	LC–MS/MS	Difenacoum, Flocoumafen	DIE		(Carelli *et al.*, 2020)
1 ml	UPLC-MS/MS	Brodifacoum, Bromadiolone, Chlorophacinone, Coumachlor, Coumafuryl, Coumatetralyl, Dicumarol, Difenacoum, Diphacinone, Flocoumafen, Pindone, Valone, Warfarin	LLE [EA]	50.1-84.7	(Qiao *et al.*, 2018) [d]
0.25 ml	LC-MS/MS	Brodifacoum, Bromadiolone, Chlorophacinone, Coumachlor, Coumatetralyl, Difenacoum, Difethialone, Diphacinone, Flocoumafen, Warfarin	dSPE [ACN, MgSO4, sodium acetate]	70-120	(Rial-Berriel et al., 2020) [d, e]
Bile					
4 g	HPLC–FL	Bromadiolone, Brodifacoum, Coumachlor, Coumafuryl, Coumatetralyl, Difenacoum, Flocoumafen, Warfarin	SPE [DM:AT (70:30), MET:glacial AA (95:5)alumina–based]	70–109	(Armentano et al., 2012) [d]
2 g	HPLC–DAD/FL	Brodifacoum, Bromadiolone, chloropahacinone, Coumachlor, Coumafuryl,	SPE [AT, Na2SO4, Florisil]		(Gallocchio et al., 2014) [d]

Table S2. Reported methodologies for determining AR in biological samples.

Weight/Volume	Analytical Technique [a]	Anticoagulant rodenticide	Extraction and clean–up procedure [b]	Recovery [c] (%)	Reference
		Coumatetralyl, Difenacoum, Diphacinone, Flocoumafen, Pindone, Warfarin			
Brain					
0.1 g	LC–MS/MS	Brodifacoum	LLE [ACN, DM]	>93	(Hauck et al., 2016) [d, e]
Faeces					
0.1 g	UHPLC–MS/MS	Brodifacoum, Bromadiolone, Coumatetralyl, Difenacoum, Difethialone, Flocoumafen	LLE [ACN, DM]	18–69	(Seljetun et al., 2018)
0.5 g	LC–ESI–MS	Bromadiolone	SPE [AT, silica]		(Sage et al., 2010) [d]
Hair					
0.02 g	LC–MS/MS	Bromadiolone, Brodifacoum	LLE [MET, EA]		(Yan et al., 2012) [d]
0.2 g	UHPLC–MS/MS	Coumatetralyl, Brodifacoum, Bromadiolone, Difenacoum, Flocoumafen, Coumachlor, Acenocoumarol, Coumafuryl, Dicoumarol	LLE [MET]		(Leporati et al., 2016)
0.025 g	LC–MS/MS	Difenacoum, Flocoumafen	MET		(Carelli *et al.*, 2020)
Liver					
	LC–MS/MS	Bromadiolone, Brodifacoum, Difenacoum Difethialone, Flocoumafen	ACN, heptane		(Langford et al., 2013)
1 g	LC–ESI–MS	Bromadiolone, Brodifacoum, Coumatetralyl, Chlorofacinone, Difenacoum, Difethialone, Diphacinone, Flocoumafen, Warfarin	SPE [Na2SO4 DM:AT, Alumina]	>60	(Sánchez-Barbudo et al., 2012)
1 g	LC–ESI–MS	Bromadiolone, Brodifacoum, Difenacoum, Difethialone, Flocoumafen, Warfarin,	SPE [Na2SO4 DM:AT, Alumina]	>70	(López-Perea et al., 2015)

Table S2. Reported methodologies for determining AR in biological samples.

Weight/Volume	Analytical Technique [a]	Anticoagulant rodenticide	Extraction and clean–up procedure [b]	Recovery [c] (%)	Reference
	HPLC	Bromadiolone, Difenacoum, Flocoumafen, Brodifacoum	SPE [AT:DM, Alumina]	53.9–83.7	(Shore et al., 2003)
	reversed phase HPLC–FL	Brodifacoum, Bromadiolone, coumafen, Coumatetralyl, Difenacoum	SPE [AT/DIE (90/10), AT/ CF (50/50)]	>80	(Lambert et al., 2007)
0.5 g	LC–MS/MS	Brodifacoum, Bromadiolone, Chlorophacinone, Diphacinone, Diphetialone, Pindone, Warfarin	SPE [ACN, Na2SO4, C18]	>70	(Albert et al., 2010)
1 g	LC–ESI/MS	Brodifacoum, Bromadiolone, Chlorophacinone, Coumatetralyl, Difencoum, Difethialone, Diphacinone, Flocoumafen, Warfarin	SPE [DM:AT (70:30), Na2SO4, Alumina]	70–50	(Sánchez-Barbudo et al., 2012)
2 g	LC–MS/MS	Brodifacoum, Bromadiolone, Chlorophacinone, Coumachlor, Coumatetralyl, Diphacinone, Difenacoum, Flocoumafen, Warfarin	dSPE [ACN, MgSO4, PSA, C18]	90–110	(Sell et al., 2017) [d, e]
1 g	HPLC–UV or FL/MS	Brodifacoum, Bromadiolone, Chlorophacinone, Coumafuryl, Difenacoum, Diphacinone Pindone, Warfarin	dSPE [ACN, PSA, Florisil, MgSO4, basic alumina, C18]	53–116	(Vudathala et al., 2010) [d]
1 g	UHPLC–Orbitrap–MS	Bromadiolone, Brodifacoum, Difenacoum, Chlorophacinone, Diphacinone, Coumachlor, Warfarin (Warfarin alcohol)	dSPE [ACN, NaCl, MgSO4]	67–104	(López-García et al., 2017) [d]
2.5 g	UHPLC–Orbitrap–MS	Bromadiolone, Brodifacoum, Difenacoum, Chlorophacinone, Diphacinone, Coumachlor, Warfarin (Warfarin alcohol)	dSPE [ACN+FA]		(López-García et al., 2017) [d]
0.5 g	LC–MS/MS	Diastereomers of Bromadiolone, Difenacoum, Brodifacoum, Flocoumafen, Difethialone	AT, ACN, HEX	72.9-109.0	(Fourel et al.,2017) [d]

Table S2. Reported methodologies for determining AR in biological samples.

Weight/Volume	Analytical Technique [a]	Anticoagulant rodenticide	Extraction and clean–up procedure [b]	Recovery [c] (%)	Reference
0.2–2 g	LC–MS/MS	Brodifacoum, Bromadiolone, Chlorophacinone, Coumatetralyl, Difenacoum, Difethialone, Flocoumafen, Warfarin			(Geduhn et al., 2015)
<4 g	LC–MS/MS	Brodifacoum, Bromadiolone, Chlorophacinone, Coumatetralyl, Difenacoum, Diphacinone, Flocoumafen, Warfarin	SLE [CF/AT (1:1, 0.075% ascorbic acid), ascorbic acid, Na2SO4, CHEX/EA (1:1), GPC]		(Ruiz-Suárez et al., 2016)
5 g/ml	HPLC–FL	Coumatetralyl, Phenprocoumon, Warfarin, Bromadiolone, Difenacoum, Flocoumafen, Brodifacoum	SPE [ACN/AT 1:1, HEX]	55.4–102.9	(Meiser, 2005) [d]
0.5 g/ml	GC–MS/MS	Bromadiolone	MET	94–98	(Doubková et al., 2017) [d]
0.1 g	LC–MS/MS	Brodifacoum	LLE [ACN, DM]	>93	(Hauck et al., 2016) [d]
0.5 g	LC/LC–ESI–MS/MS	Brodifacoum, Bromadiolone, Chlorophacinone, Coumachlor, Coumafuryl, Coumatetralyl, Difenacoum, Flocoumafen, Pindone, Warfarin	onlineSPE [MET, C18]	91–100	(Maršálek et al., 2015) [d]
0.5 g/0.1 ml	LC–HESI–MS/MS	Brodifacoum, Bromadiolone, Chlorophacinone, Coumatetralyl, Difenacoum, Difethialone, Flocoumafen, Warfarin	LLE [AT, DIE]		(Vandenbroucke et al., 2008)
2 g	LC–MS/MS	Coumatetralyl, Warfarin, Chlorophacinone, Difenacoum, Bromadiolone, Brodifacoum, Difethialone	SLE [DE, DM/EA/AT (50:30:20), Fr, CHEX]	86.9–97.4	(Luzardo et al., 2014) [d, e]
1 g	UHPLC–MS/MS	Brodifacoum, Bromadiolone, Chlorophacinone, Coumatetralyl, Difencoum, Difethialone, Diphacinone, Flocoumafen, Warfarin	dSPE [AT, ACN, HEX]	60-120	(Taylor et al., 2019) [d, e]

Table S2. Reported methodologies for determining AR in biological samples.

Weight/Volume	Analytical Technique [a]	Anticoagulant rodenticide	Extraction and clean–up procedure [b]	Recovery [c] (%)	Reference
4 g	HPLC–FL	Bromadiolone, Brodifacoum, Coumachlor, Coumafuryl, Coumatetralyl, Difenacoum, Flocoumafen, Warfarin	SPE [DM:AT (70:30), MET:glacial AA (95:5)alumina–based]	70–109	(Armentano et al., 2012) [d]
2 g	HPLC–DAD/FL	Brodifacoum, Bromadiolone, chloropahacinone, Coumachlor, Coumafuryl, Coumatetralyl, Difenacoum, Diphacinone, Flocoumafen, Pindone, Warfarin	SPE [AT, Na2SO4, Florisil]		(Gallocchio et al., 2014) [d]
1 g	UPLC–MS/MS	Brodifacoum, Bromadiolone, Chlorophacinone, Coumachlor, Dicoumarol, Difethialone, Diphacinone, Warfarin	dSPE [10% MET:ACN, MgSO4, florisil, Alumina, PSA]	51-72	(Smith *et al.*, 2017) [d]
0.2-2 g	HPLC–MS/MS	Brodifacoum, Bromadiolone, Chlorophacinone, Coumatetralyl, Difenacoum, Flocoumafen, Warfarin	SPE [MET, W, NaCl, DE]	41-118	(Geduhn *et al.*, 2014)
2 g	LC-MS/MS	Brodifacoum, Bromadiolone, Difenacoum, Difethialone, Flocoumafen	DE, DM/EA/AT (50/30/20)		(Rial-Berriel *et al.*, 2021)
1 g		Brodifacoum, Bromadiolone, Difenacoum, Difethialone, Flocoumafen	dSPE [ACN (0,5% formic acid), MgSO4, sodium acetate]	80-120	(Rial-Berriel *et al.*, 2021)
≤4 g	UHPLC–MS/MS	Brodifacoum, Bromadiolone, Chlorophacinone, Coumatetralyl, Difenacoum, Diphacinone, Difethialone Flocoumafen, Warfarin	GPC [CF:AT, AA, Na2SO4]	76-107	(Taylor et al., 2018) [d]
Kidney					
0.5 g	LC/LC–ESI–MS/MS	Brodifacoum, Bromadiolone, Chlorophacinone, Coumachlor, Coumafuryl, Coumatetralyl, Difenacoum, Flocoumafen, Pindone, Warfarin	onlineSPE [MET, C18]	89–97	(Maršálek et al., 2015) [d]

Table S2. Reported methodologies for determining AR in biological samples.

Weight/Volume	Analytical Technique [a]	Anticoagulant rodenticide	Extraction and clean-up procedure [b]	Recovery [c] (%)	Reference
1 g	UHPLC–MS/MS	Brodifacoum, Bromadiolone, Chlorophacinone, Coumatetralyl, Difencoum, Difethialone, Diphacinone, Flocoumafen, Warfarin	dSPE [AT, ACN, HEX]	22.9–77.1	(Taylor et al., 2019) [d, e]
Plasma/Serum					
0.2 ml	HPLC–MS	Chlorophacinone	SPE [10% MET in ACN, DM/CAN]	81.6–87.4	(OuYang et al., 2009) [d]
2 ml	HPLC–UV	Bromadiolone, Chlorophacinone, Warfarin	LL [CF:MET]		(Berny et al., 2006)
0.5 ml	separation IC–MS/MS detection LC–MS	Valone	SPE [MET/ACN (10:90) 1° LLE Protein-precipitation MET/W (80:20)]	81–90.1	(Cai et al., 2009) [d]
2 ml	HPLC–UV/FL	Brodifacoum, Bromodiolone, Chlorophacinone, Coumafuryl, Dicoumarol, Difenacoum, Difethialone, Diphacinone, Pindone, Valone, Warfarin	SPE [ACN, Alumina]		(Waddell et al., 2013)
Stomach content					
1.5 g	HPLC–UV	Warfarin, Chlorophacinone, Bromadiolone	LL [CF:ET]		(Berny et al., 2006)
2 g	HPLC–DAD/FL	Brodifacoum, Bromadiolone, chloropahacinone, Coumachlor, Coumafuryl, Coumatetralyl, Difenacoum, Diphacinone, Flocoumafen, Pindone, Warfarin	SPE [AT, Na2SO4, Florisil]		(Gallocchio et al., 2014) [d]
Tissues (not specified)					
4 g	HPLC–FL	Bromadiolone, Brodifacoum, Coumachlor, Coumafuryl, Coumatetralyl, Difenacoum, Flocoumafen, Warfarin	SPE [DM:AT]		(Muscarella et al., 2016)

Table S2. Reported methodologies for determining AR in biological samples.

Weight/Volume	Analytical Technique [a]	Anticoagulant rodenticide	Extraction and clean–up procedure [b]	Recovery [c] (%)	Reference
2 g	LC–MS/MS	Coumatetralyl, Warfarin, Chlorophacinone, Difenacoum, Bromadiolone, Brodifacoum, Difethialone	SLE [DE, DM/EA/AT (50:30:20), Fr, CHEX]	86.9–97.4	(Luzardo et al., 2014) [d, e]
Urine					
	UPLC–MS/MS	Brodifacoum, Bromadiolone, Warfarin, Coumachlor, Coumatetralyl, Difenacoum, Pindone, Diphacinone, Chlorophacinone	LLME [EA]	64.6–124.2	(Yan et al., 2018) [d]
1 ml	HPLC–DAD	Warfarin, Coumatetralyl, Bromadiolone	ASE [MET]	87–108	(Hao et al., 2014) [d]

[a] DAD: array diode detector, ESI: electrospray ionization, FL: fluorescence detector, GC: gas chromatography, HPLC: high performance liquid chromatography, LC: liquid chromatography, MS: mass spectrometry, UHPLC: ultra-high performance liquid chromatography, UV: ultraviolet detector.
[b] ASE: Accelerated solvent extraction, dSPE: Dispersive Solid-phase extraction, GPC: Gel Permeation Chromatography, LLE: Liquid-liquid extraction. LLME: Liquid-liquid microextraction, SLE: Solid-liquid extraction, SPE: Solid-phase extraction [AA: Acetic acid, ACN: Acetonitrile, AT: Acetone, CF: Chloroform, CHEX: cyclohexane, DE: Diatomaceous earth, DIE: Diethyl ether, DM: dichloromethane, EA: ethyl acetate, ET: Ethanol, FA: Formic Acid, Fr: Freezing, HEX: Hexane, HLB: hydrophilic–lipophilic balanced copolymer cartridge, MET: Methanol, PP: Phosphate, PSA: primary secondary amine, SCDS: sodium citrate dibasic sesquihydrate, SCTD: sodium citrate tribasic dihydrate, W: Water]
[c] The recovery of most of the rodenticides was ≥77% in blood, except for difethialone, which had a recovery of 53%.
[d] Method development papers
[e] Multi-class technique

Table S3. Solubility of AR in different solvents (20–25°C) g/L[a]

	Water (pH 9) [b]	Ethyl acetate	Acetone	Acetonitrile	Maximum solubility
Bromadiolone	0.180	25	22.3	Nf	Dimethylformamide 730
Brodifacoum		Moderately soluble	23	Nf	Dichloromethane 50
Difenacoum	0.0037–0.084	2–3.7	>50	Nf	Chloroform >50
					Dichlorometane 19.6
Warfarin	17		65	Nf	Dioxane 100
Chlorophacinone	459	Readily soluble	Readily soluble	Nf	
Flocoumafen	>10		>600	Nf	
	>600				

[a] Data obtain from Pubchem

[b] Water has been used as a reference

Nf: not found

Table S4. Reported chromatographic conditions for determining AR in biological samples.

Analytical Technique [a]	Stationary phase (column)	Size	Mobil phase A[b]	Mobil phase B [b]	Mobil phase C [b]	Flow (ml/min)	Gradient	Reference
LC–MS/MS	Accucore C18	150 × 3 mm, 2.6 µm	UW	MET		800	0–1 min: 50% A; 1–1.5 min: 50% A→5%; 1.5–3.5 min: 5% A; 3.5–3.7 min: 5% A→50% A; 3.7–5 min: 50% A	(Luzardo et al., 2014)
	C18 Phenomenex	150 × 4.6 mm, 4.0 µm	7.5 mM AF in UW	MET	2% FA	1	During the entire run, solvent C was set at 2.5%. A and B: 0–12 min: 87.5% A→7.5% A; 12–16 min: 7.5% A; 16.0–16.2 min: 7.5% A→87.5% A; 16.2– 25.0 min: 87.5% A	
LC–MS/MS	X-TerraÒ MS C18	2.1 × 100 mm, 3.5 µm	AmA 10 mM, pH 6.8	MET		0.250	0 min: 75% A, 25 % B; 10 min: 5 % A, 95 % B; 20 min: 5 % A, 95 % B; 24 min: 75 % A, 25 % B; 28 min: 75 % A, 25 % B.	(Albert et al., 2010)
UPLC–MS/MS	BEH–C18	100 × 2.1 mm, 1.7 µm	10 mmol/l aqueos AMA (pH 7.5)	MET		0.4	85% A for 1 min, followed by a gradient to 2% eluent A in 6 min.	(Bidny et al., 2015)
HPLC–DAD	Kromasil 100–5 C18	250 × 4.6 mm, 5 µm	70:30 7mmol/l AMA, MET			1.0		(Hao et al., 2014)
HPLC–FL	Reversed–phase Eurospher C18	4.6 × 250 mm, 5µm	AmA (0.39%)/AA (0.2%)/TRIET (0.2%)/W (pH 6.4)	MET		1.0	50–70% solution B 0–7 min: 70–82% B; 7–14: 82–92% B; 14–17 min: 92–95% B; 17–22 min: 95–100% B; 22–23 min: held at 100% B; 23–25 min; and linear change back to initial conditions 25–28 min.	(Meiser, 2005)
HPLC–FL	Reversed–phase Eurospher C18	4.6 × 250 mm, 5 µm	DPP (0.67%)	ACN		from 0.5 to 1.0	25–45% B; 0–12 min: 45–57% B; 12–20 min: linear increase from 57–75% B; 20–28 min: 75–85% B; 28–32 min: linear change back to initial conditions 32–37 min.	
LC–HESI–MS/MS	C18 Gravity	125 × 2.0 mm, 3 µm	5mM AF in W	5mM AF in MET		0.2	0–4 min: 50% A; 4–6 min: 10% A; 6–14 min: 10% A; 14–14.5 min: 50% A; 14.5–20 min: 50% A.	(Vandenbroucke et al., 2008)
LC–MS/MS	Luna C8 connected to a C8 pre–column	4 × 2mm, 3 µm / 75 × 2.1 mm, 3 µm	5% IP in ET	0.5% IP in 0.1% AA in W		0.3	(A:B, v/v) was 2:98 from 0 to 1 min then 98:2 at 5.0 min, and held 98:2 for 10 min. Subsequently, mobile phase composition was 2:98 at 20 and held for 6 min for re–equilibration.	(Sell et al., 2017)
LC–MS/MS	Waters Sunfire C8	150 × 4.6 mm, 5 µm	W with AmA 20 mM	MET with AmA 20 mM		0.8	gradient where at 0 min: 50% B and at 22 min: 95% B.	(Gómez-Ramírez et al., 2012)

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Analytical Technique [a]	Stationary phase (column)	Size	Mobil phase A[b]	Mobil phase B [b]	Mobil phase C [b]	Flow (ml/min)	Gradient	Reference
HPLC–UV or FL/MS	Betasil reversed–phase C18	150 × 4.6 mm, 5 µm	0.03M TBA	MET		0.6	30% B for 10 min, followed by gradient to 80% B in 4.5 min, increasing to 90% B in the next 0.5 min.	(Vudathala et al., 2010)
HPLC–UV or FL/MS							30% B for 7–10 min, a gradient to 75% B; 10–21 min: 75–80% B; 21–26 min: holding it followed by equilibration to starting conditions for the next 4 min.	
LC–MS/MS	ZORBAX SB–C18 fitted with a Phenomenex guard column	150 × 4.6 mm, 5 µm/12.5 × 2.1 mm, 5 µm	AmA in W (containing 5% MET)	MET		500	Initial composition (50% B, v/v) was maintained for 2 min, then B was increased from 50% to 85% for 2 min, held at 85% for 3.5 min, decreased to 50% for 1 min, and re–equilibrated at 50% for 3.5 min, yielding a total run time of 12 min.	(Zhu et al., 2013)
LC–LC/ESI/MS/MS	reversed–phase, Optimize Technologies EXP trap C18 the analytical column was a Thermo Scientific Hypersil C18	2.1 ×5 mm, 3 µm/2.1×100 mm, 1.9 µm	A1: 5 mM AF in water A2: 0.1 % formic acid in water	B1: MET. B2: MET	C2: ACN	Pump 1: 0.3 Pump 2: 0.6	Pump 1: 0 min: 55% A; 45% B; 0.60 min: 55% A; 45% B; 2.80 min: 20% A; 80% B; 6.30 min: 0% A; 100% B; 6.70 min: 55% A; 45% B; 8.00 min: 55% A; 45% B. Pump 2: 0 min: 70% A; 30% B; 0% C; 0.17 min: 70% A; 30% B; 0% C; 1.00 min: 50% A; 30% B; 20% C; 4.80 min: 50% A; 30% B; 20% C; 5.80 min: 0% A; 0% B; 100% C; 13 min: 0% A; 0% B; 100% C; 14 min: 70% A; 30% B; 0% C; 1 min: 70% A; 30% B; 0% C;	(Maršálek et al., 2015)
UHPLC–Orbitrap/MS		100 × 2.1 mm, 1.9 µm	MET containing 0.1% FA and AF 4 mM	W with 0.1% FA and AF 4 mM		0.25	95% of aqueous phase was set during 1 min, decreasing linearly to 0% in 7 min. After 4 min keeping 0% of aqueous phase, this percentage was increased again up to 95% in 0.5 min. Finally, the initial conditions were kept constant during 1.5 min, obtaining a total analysis time of 14 min.	(López-García et al., 2017)
LC–DAD/UV	Symmetry®C8 packed with 5µm diameter particles (Waters)	250 × 4.6 mm, 5 µm / 20 × 4.6mm	ACN	phosphate buffer		1	0–1 min: 13% A; 1–9 min: 13–35% A; 9–28 min: 35–80% A; 28–30 min, decrease from 80 to 13% A; 30–35 min, column equilibration with 13% A.	(Denooz et al., 2009)

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Analytical Technique [a]	Stationary phase (column)	Size	Mobil phase A[b]	Mobil phase B [b]	Mobil phase C [b]	Flow (ml/min)	Gradient	Reference
GC–MS/MS	VF–5 ms 5% phenyl–methyl	30 m × 0.25 mm	Helium					(Doubková et al., 2017)
LC–MS/MS	Kinetex XB–C18	4.6 × 150 mm, 5 µm	25 mM aqueous AMA	ACN		1	Equilibration time (–5.00 to 0.00 min), 20% B; 0–9 min: 95% B; 9–15 min: 95% B.	(Di Rago et al., 2014)
HPLC–DAD/FL	Supelco Ascentis 1 Express C18	150× 4.6 mm, 2.7 µm	0.01M AmA	MET		0.3	1 min: 40% A, 60% B; 20 min: 0% A, 100% B; 25 min: 0% A, 100% B; 26 min: 50% A, 50 %B; 30 min: 50% A, 50% B.	(Gallocchio et al., 2014)
LC–ESI/MS	NR	NR	W with FA (0.1%)	ACN with FA (0.1%)		1.2	25%, 75% B; 0% A, 100% B at 8 min and returning to the initial conditions by 9 min.	(Martínez-Padilla et al., 2017)
HPLC–FL	silica–based	250 × 4.6 mm, 5 µm	W with 0.25% glacial AA	MET with 0.25% glacial AA		0.5	75% A constant for 5 min, followed by an elution gradient, as reported.0 min: 75% A, 25 % B; 5 min: 5 % A, 95 % B; 20 min: 0 % A, 100 % B; 35 min: 0 % A, 100 % B; 38 min: 75 % A, 25 % B; 45 min: 75 % A, 25 % B.	(Armentano e tal., 2012)
HPLC–UV	Chromcart Nucleosil C18 pre–column	250 × 4mm column / 10 nm pores, 5 µm	MET	25mM phosphate buffer			Isocratic	(Berny et al., 2006)
IC–MS/MS LC–MS	Dionex Ionpac AS11 separation using a Dionex Ionpac AG11 guard column	250 × 4mm / 50 × 4mm	MET/30.0mmol/l KOH (10:90)			1.0	Isocratic	(Cai et al., 2009)
LC–ESI/MS	Zorbax Eclipse XDB–C18 and a Zorbax Eclipse XDB–C8 guard column from	2.1 × 100 mm, 3.5 µm/2.1 × 12.5 mm, 5 µm	10 mM AmA	MET		0.250	0 min: 30% A, 70% B; 0–5 min: 80% B; 5–6 min: 90% B, holding at 90% B during 4 min and back at 70% B at 11 min.	(Sage et al., 2010)
HPLC–MS	Dionex Ionpac® AG11 guard column and a Dionex Ionpac® AS11 separation column	50 × 4 mm/250 × 4 mm	MET–40.0 mmol/l KOH (10:90)			1.0	0–2 min: 10.0 mmol/l KOH; 2–7 min: 10–30 mmol/l KOH (linear gradient); 7–10 min: 30 mmol/l KOH.	(OuYang et al., 2009)

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Analytical Technique [a]	Stationary phase (column)	Size	Mobil phase A[b]	Mobil phase B [b]	Mobil phase C [b]	Flow (ml/min)	Gradient	Reference
UHPLC–MS/MS	Acquity UPLC® BEH C18–column	2.1 × 50 mm, 1.7 µm	5 mM AF buffer pH 10.2	MET		0.5	0–1.5 min: 10% B; 1.5–1.8 min: 30% B; 1.8–1.81 min: 58% B; 1.81–3.5 min: 60% B; 3.5–3.52 min: 60% B; 3.52–4 min: 100% B; 4–4.5 mon: 100% B; 4.5–4.51 min: 10% B.	(Seljetun et al., 2018)
HPLC–UV/FL	NR	NR		NR			NR	(Waddell et al., 2013)
LC–ESI/MS/MS	XBridge C18	50 × 2.1 mm, 5 µm	10 mM AmA	MET		0.2	0–1 min: 60% A, 40% B; 1–2 min: 85% B; 2–4.5 min: 85% B; and returned to 40% B at 5 min for a total run time of 7 min.	(Yan et al., 2012)
LC–MS/MS	Waters XBridge C18 fitted with an end–capped C18 guard column	50 × 2.1 mm, 5 µm/12.5 × 2.1 mm, 5 µm	11 mM AmA	MET		0.2	0–1 min: 60% A, 40% B; 1–2 min: 85% B; 2–4.5 min: 85% B; and returned to 40% B at 5 min for a total run time of 7 min..	(Yan et al., 2016)
LC–MS/MS	Agilent Poroshell 120 EC–C18 column	2.1× 150 mm, 2.4 µm	ACN–W, AT			0.55	6 min: linear gradient from 50–100%.	(Hauck et al., 2016)
HPLC–MS/MS	ZORBAX Eclipse XDB C18	150 × 2.1 mm, 5 µm	AA–AmA (5 mmol/l, pH 4.5)/MET (20:80)			0.50	isocratic	(Jin et al., 2007)
UHPLC–MS/MS	Kinetex C18 column protected by a C18 guard column	50 × 2.1 mm, 1.7 µm	W/AF 2 mM	MET		0.5	initial 80:20 ratio for 1 min, then linear gradient to 0:100 in 4 min; final isocratic condition at 100% B for 0.5 min.	(Leporati et al., 2016)
HPLC–ESI/MS	ZORBAX Eclipse XDB C18	150 × 2.1 mm, 5 µm	0.2% AA/MET (12:88)			0.50		(Jin and Chen, 2006)
UPLC–MS/MS	reverse–phase ACQUITY UPLC BEH C18 column, Waters	2.1 × 100 mm, 1.7 µm	5mM AF and 0.1% FA in W	5mM AF and 0.1% FA in MET		0.3	7 min: 0–1 min: 10% B; 1.01–2 min 10–90% B; 2.01–5 min: 90% B; 5.01–6 min: 10% B; 6.01–7 min: 10% B.	(Yan et al., 2018)
HPLC–FL	LiChroSpher 100–RP 18E prepacked reversed–phase	125 × 4.6 mm, 5 µm	AmA, MET, TRIET buffer pH 5.2			1.0	0–4 min: linear gradient from 62–82% MET; from 4–12 min: 82% MET; 12–17 min: a linear change back to the initial conditions.	(Lambert et al., 2007)
LC–MS LC–MS/MS	LiChroCART column filled with Purospher RP–18e (Merck)	125 × 3 mm	0.1% FA in ACN, W			0.8	(shown in relation to content of ACN): 0 min: 10% ACN; 10 min: 100% ACN; 15 min: 100% ACN; 16 min: 10% ACN: 20 min: 10% ACN. Total analysis time was 20 min.	(Adamowicz and Kala, 2009)

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Analytical Technique [a]	Stationary phase (column)	Size	Mobil phase A[b]	Mobil phase B [b]	Mobil phase C [b]	Flow (ml/min)	Gradient	Reference
LC–MS/MS	Acquity BEH C18	50 × 2mm, 1.7 µm	W (10 mM AmA)	MET (10 mM AmA)		0,6	0–2min: 50% to 1% water (10 mM ammonium acetate)	(Langford et al., 2013)
HPLC	Hypersil ODS C18	250 × 4.6 mm, 5 µm	76:24 MET:W, supplemented with 0.25% AA	AMA 40 mM		1,1	Isocratic	(Shore et al., 2003)
HPLC–FL	silica-based	250 × 4.6, 5 µm	W with 0.25% glacial AA	MET with 0.25% glacial AA		0.5	75% A, followed by an elution gradient. 5 min: 5% A, 95% B; 20 min: 0% A, 100% B; 35 min: 0% A, 100% B; 38 min: 75% A, 25% B; 45 min: 75% A, 25% B.	(Muscarella et al., 2016)
LC–ESI/MS	phenyl–hexyl	150 × 2.1 mm, 3 µm	MET	AmA 10 mM, pH 6.8		0.2	20% A, 80% B, reaching 75% A, 25% B at min 8.75. This was maintained until min 30.62, returning to the initial conditions by min 31.5. Then, column was stabilized with conditions until min 43.75 before the next sample injection.	(Sánchez-Barbudo et al., 2012)
LC–ESI/MS	Eclipse XDB–C18	4.6 × 12.5 mm, 5 µm	AmA 10 mM, pH: 6.03	MET		1.2	35% A, 65% B, reaching 15% A and 85% B at min 5. This was maintained until min 10, returning to the initial conditions by min 12. Then, column was stabilized with the initial conditions until min 15 before the next sample injection.	(López-Perea et al., 2015)
LC–MS/MS	Poroshell 120 StableBond C18	2.1 × 100 mm, 2.7 µm	AmA 10 mM	ACN		0.25	0 min: 20% B; 0.1 min: 30% B; 0.5 min: 40% B; 5–11 min: 50% B; 11.5–12.5 min: 90% B; 13.5–25 min: 20% B.	(Fourel et al., 2017)
LC–MS/MS	Waters Acquity UPLC BEH C18	50 × 2.1 mm, 1.7 µm	W/MET 95/5, 5mM AmA	MET, 5mM AmA		0.48	7 min: 0 min: 70% A; 0.52 min: 70% A; 0.66 min: 40% A; 1.05 min: 40% A; 3.31 min: 15% A; 4.90 min: 15% A; 5 min: 0% A; 6 min: 0% A; 6.05 min: 70% A; 7 min: 70% A.	(Ruiz-Suárez et al., 2016)
UHPLC–MS/MS	Kinetex C18	50 × 4.6 mm; 2.6 µm	MET/W 5/95 (5 mM AmA)	MET (5 mM AmA)		0.4	0.1 min: 25% B; 0.7 min: 60% B; 12 min: 98% B; 13 min: 98% B; 14 min: 25% B; 17 min: Stop.	(Taylor et al., 2019)
HPLC–FLD	silica–based	250 × 4.6 mm; 5 µm	W with 0.25% glacial AA	MET with 0.25% glacial AA		0.5	75% A, followed by an elution gradient. 5 min: 5% A, 95% B; 20 min: 0% A, 100% B; 35 min: 0% A, 100% B; 38 min: 75% A, 25% B; 45 min: 75% A, 25% B.	(Muscarella et al., 2016)

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UPLC-MS/MS	Accucore C18 LC	2.1 × 100 mm; 2.6 µm	AmA 10 mM, pH 9	MET		0.3	1 min: 60% A/40% B; 8 min: B increased linearly to 57%; 6 min: B increased further to 77%. 15–18 min postinjection, B increased to 81% and finally to 90% in 1 min. Mobile phase B was held at 90% for 5 min to rinse the column.	(Smith *et al.*, 2017)
HPLC–MS/MS	Luna PFP (2) / Kinetex PFP	50 × 2mm; 3 µm / 50× 2.1mm; 5 µm	MET 0.5% AA AmA 5 mM	W 0.5% AA AmA 5 mM		0.8	10% A, reaching 90% A at 3 min, continued for 1 min and then switched to 10% A for column equilibration for about 2 min.	(Geduhn *et al.*, 2014)
UPLC-MS/MS	Eclipse Plus C18 C18 guard	2.1 × 100 mm; 3.5 µm / 2.5 × 2.1 mm; 5 µm	AmA 10 mmol/l MET 5%	MET		0.2	0–1.5 min: 80% A; 1.5 to 3 min: a decrease to 40% A; 3–5 min 40% A; 5– 5.5 min: a decrease to 15% A; 5.5–7.5 min: 15% A; 10 min back to 80% A.	(Qiao *et al.*, 2018)
LC–MS/MS	Kinetex C18	100 × 2.1 mm; 2.6 µm				0.3	1 min: 85% A; 1.5 min: 85% to 2% A; up to 12 min: 2% A.	(Carelli *et al.*, 2020)
LC–MS/MS	InfinityLab Poroshell 120 InfinityLab Poroshell 120UHPLC	2.1 × 100 mm, 2.7 µm / 2.1 × 5mm, 2.7 µm	AmA 2 mM 0.1% FA in W	MET, AmA 2 mM		0.4	0.5 min: 95% A; 1 min: 80% A; 2.5 min 60% A; 8 min: 15% A; 10–14 min: 0% A; 14.01 min: 95% A	(Rial-Berriel, et al., 2020)
UHPLC–MS/MS	Kinetex C18	50 × 4.6 mm, 2.6 µm	W/MET, 5 mM AmA	MET, 5 mM AmA		0.4	0 min: 10% B; 0.3 min: 40% B; 3.1 min: 98% B; 4.1 min: 98% B; 4.2–6.00 min, equilibration time.	(Taylor et al., 2018)

NR: Not reported

[a] DAD: array diode detector, ESI: electrospray ionization, FL: fluorescence detector, GC: gas chromatography, HPLC: high performance liquid chromatography, LC: liquid chromatography, MS: mass spectrometry, UHPLC: ultra high performance liquid chromatography, UV: ultraviolet detector.

[b] AA: Acetic acid, ACN: Acetonitrile, AF: Ammonium formate, AMA: Ammonium acetate, AT: Acetone, DPP: Dihydrogen potassium phosphate, ET: Ethanol, IP: Isopropanol, MET: Methanol, PHY: Potassium hydroxide, TRIET: Triethylamine, UW: Ultrapure water, W: Water.

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Chapter IV. Interlaboratory performance comparison to determine toxic compounds involved in wildlife poisoning

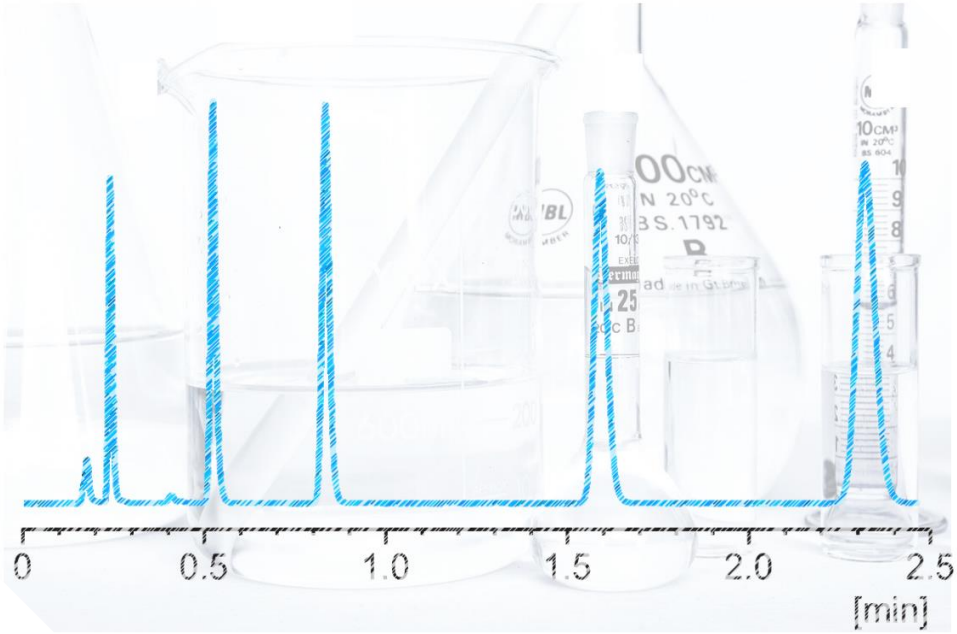

Image: Irene Valverde Domínguez composition from Pixabay

Abstract

The wide variety of compounds involved in animal poisoning poses some difficulties during the laboratory analysis. Several heterogenic techniques using diverse matrices, extractants and analytical instruments at different conditions have been described. Therefore, it is essential to monitor the performance of different laboratories applying a variety of methodologies in the determination of toxic compounds involved in wildlife poisoning worldwide, to ensure comparable results. The aim of this comparative study was to make a first approach in assessing the individual performance characteristics of the analytical procedures applied to detect toxic compounds involved in wildlife poisoning between different laboratories. For this purpose, four laboratories of reference in veterinary forensic toxicology in Spain have participated: the Service of Toxicology and Forensic Veterinary from University of Murcia (STVF-UM), the Unit of Toxicology from University of Extremadura (UNEX), and the Institute for Game and Wildlife Research from University of Castilla-La Mancha (IREC-CSIC-UCLM). the Toxicology Unit from University of Las Palmas de Gran Canaria (SERTOX-ULPGC). This study was carried out with chicken liver samples spiked with 11 strategically selected substances at STVF-UM, including anticoagulant rodenticides (AR) (bromadiolone, brodifacoum, difenacoum, warfarin and chlorophacinone), carbamates (carbofuran, aldicarb and methiocarb) and organophosphates (diazinon, chlorpyrifos and parathion). Each laboratory carried out the analyses with their routine techniques. Collaborators were required to report the mean concentrations in the spiked liver samples for each substance evaluated, repeatability, recoveries, and limits of detection/quantification of their techniques. In this interlaboratory comparison, the laboratory performance was expressed in terms of z-score in accordance with ISO13528:2015. Despite their different extraction procedures, instrumentation, and chromatographic conditions, in general all the participant laboratories have accurate and comparable results for all the compounds evaluated. However, some techniques stand out because they are more economic and environmentally respectful.

Keywords: interlaboratory comparison, forensic, toxicology, performance.

Introduction

Poisoning is an illegal activity reported as one of the main causes of death in wildlife, considered an established, predominant threat to numerous species of birds and mammals, and consequently, to the whole ecosystem (Guitart et al., 2010; Tenan et al., 2012). Primary poisoning occurs when an animal directly ingests a poisonous substance (i.e., a prepared bait), while secondary poisoning occurs when a poisoned animal is preyed upon (Berny & Gaillet, 2008; Ogada, 2014; Ntemiri et al., 2018). Even a tertiary poisoning may occur, mainly when AR are involved (Eason & Murphy, 1999; Dutto et al., 2018; Valverde et al., 2020a). Raptors and mammals, some of them scavenger species, are specially affected by poisoning, which may lead to the extintion of some species in certain areas (Berny & Gaillet, 2008; Mateo-Tomás et al., 2012; Márquez et al., 2013; Ogada, 2014; Ntemiri et al., 2018).

A wide variety of compounds involved in animal poisoning cases have been reported, which poses some difficulties during the laboratory analysis due to the wide variety of analytical techniques available (Luzardo et al., 2014). However, the main groups of compounds sought and detected in wildlife poisoning in toxicology laboratories are cholinesterase inhibitors (carbamates and OP) and AR, mainly SGARs (Berny, 2007; Hernández and Margalida, 2008; Sánchez-Barbudo et al., 2012a). Other products such as strychnine (Martínez-López et al., 2006; Hernández & Margalida, 2008; Proulx & Rodtka, 2015), metaldehyde (De Roma et al., 2018), alpha-chloralose (Guitart et al., 2010), ethylene glycol (Berny et al., 2010) or barbiturates (Wells et al., 2020; Herrero-Villar et al., 2021) have also been reported. Most of the compounds used to poison fauna are banned products in the EU, such as the carbamates aldicarb or carbofuran (Ruiz-Suárez et al., 2015); however, they are still frequently used to poison animals several years after its prohibition (both forbidden since 2003 and 2007 in the EU, respectively) (Council decision 2003/199/EC, 2003; Commision decision 2007/416/EC, 2007; Berny et al., 2015; Ruiz-Suárez et al., 2015). Among the wide variety of substances used in poisoning cases, both FGARs and SGARs are of interest since many products are still authorised as

biocide products to control rodents in the EU (e.g., the FGARs warfarin, coumatetralyl and chlorophacinone, and the SGARs difethialone, difenacoum, bromadiolone, flocoumafen and brodifacoum) (Directive 2011/48/EU, 2011; Regulation(EU) 2017/1377, 2017; Regulation(EU) 2017/1381, 2017; Regulation (EU) 2017/1376, 2017; Regulation (EU) 2017/1379, 2017). AR may cause ecological issues in predators due to their accumulation across the trophic chain when they are misused or abused, which may result in secondary and/or tertiary intoxication (Eason & Murphy, 1999; Sánchez-Barbudo et al., 2012a; Espín et al., 2016).

One of the main objectives in veterinary forensic toxicology is the proper diagnosis of animal poisoning. This requires gathering as much information as possible on the incident, including factors related to the animal, the substance involved and the scenario (García-Fernández et al., 2006). However, this information is sometimes difficult to compile in wildlife poisoning. Moreover, other factors, including the sample amount, the stage of carcass decomposition, and the *post-mortem* degradation of the poisons, pose additional difficulties to achieve a correct diagnosis, because they can significantly alter the concentrations of the compound in different tissues and/or fluids (Brown et al., 2005; Luzardo et al., 2014; Viero et al., 2019; Valverde et al., 2020a; 2020b; Valverde et al., 2021).

The analysis (detection and quantification) of toxic compounds in wildlife poisoning is a challenging area because of the wide variety of complex matrices, the large number of target analytes potentially involved, which may require multiple analyses, and these analyses are difficult in many cases because the scarce volume of sample. Thus, reliable and sensitive analytical techniques are needed. Diverse analytical methods have been reported to detect different compounds involved in poisoning cases; however, these techniques often detect a small number of compounds and/or substances within the same chemical group (Vudathala et al., 2010; Papoutsis et al., 2012; Ko et al., 2014; Gonçalves et al., 2017). Besides, several heterogenic techniques using diverse matrices, extractants and analytical instruments at different conditions have

been described (Musshoff et al., 2002; Gallardo et al., 2006; Rallis et al., 2012; Richards et al., 2015; Valverde et al., 2021). Therefore, it is essential to monitor the performance of different laboratories applying a variety of methodologies in the determination of toxic compounds involved in wildlife poisoning worldwide, because it will allow to identify gaps and standardizing or streamlining will ultimately improve chances of detection and reduce false negative determinations. The evaluation of methodologies and performance is important to avoid e.g., false negatives, perception certain classes of compounds are not used when in fact they would not be detected via analysis favoured by certain labs, or non-attribution of cause of death in some carcasses. However, to our knowledge, no studies have been published so far for external quality control in such laboratories.

The aim of this study was to make a first approach in the comparison of the performance characteristics of the analytical procedures used in four Spanish reference laboratories for wildlife toxicology to detect the toxic substances most frequently used in wildlife poisoning according to the literature and cases reports (AR, carbamates and OP) (Guitart et al., 2010; Cenerini et al., 2012). The participant laboratories were four of the reference laboratories involved in the *Veneno-No* Life+ Project (www.venenono.org): the Toxicology Unit from STVF-UM, UNEX, IREC-CSIC-UCLM and SERTOX-ULPGC, all within the network of Spain. This interlaboratory study also aimed to confirm the quality of procedures and results in order to harmonize methodologies and maximise reliability and comparability of data. Moreover, this study is intended to help establish improvements in the current analytical techniques if needed.

Material and methods

Spiked sample preparation

Chicken liver obtained from a local butcher was selected as test material. The liver was ground, homogenized and then analysed to confirm the absence of any target analytes by analysing a blank liver. Liver was selected because it is the main matrix used in forensic analysis as the main metabolizer and accumulating organ of toxic substances (Hill and Fleming, 1982; Vudathala et

al., 2014), as well as the main matrix used to analyse AR because the mode of action targets this organ (Valverde et al., 2021).

The compounds selected to prepare the final standard solution were chosen according to the substances detected in poisoning cases in Europe (Soler-Rodríguez et al., 2006; Guitart et al., 2010; Vandenbroucke et al., 2010; Bodega, 2014; Ntemiri and Saravia, 2016). A total of 11 substances were selected, including 3 SGARs (bromadiolone, brodifacoum, and difenacoum), 2 FGARs (warfarin and chlorophacinone), 3 carbamate pesticides (carbofuran, aldicarb and methiocarb) and 3 OP pesticides (diazinon, chlorpyrifos and parathion).

All analytical standards were ≥98% purity. Rodenticide chlorophacinone (96%) was obtained from Dr. Ehrenstorfer GmbH (Germany), while warfarin (98%), difenacoum (98.7%), brodifacoum (99.8%) and bromadiolone (98.8%) (New Haven, CT, USA) were purchased from Sigma-Aldrich (Saint Louis, USA). OP (Chlorpyrifos 99%, parathion 99.6%, diazinon 98.2%) and carbamates (aldicarb 100%, carbofuran 99.9%, and methiocarb 99.7%) were purchased from Sigma-Aldrich (Saint Louis, USA). All solvents and reagents were of HPLC quality (>99.9% purity). Diethyl ether, chloroform and methanol were obtained from Lab-Scan® (Gliwice, Poland). Dichloromethane was obtained from Macron Fine Chemicals™ (Gliwice, Poland) and acetone from J.T.Baker analysed Pesticide Reagent™ (Gliwice, Poland). Formic acid was obtained from Probus® (Badalona, Spain).

Rodenticides were prepared at 1.0 mg/ml in dichloromethane. Carbamates and OP were prepared at 1.2 mg/ml in acetone. After that, a final standard solution containing all the compounds at 75 µg/ml in acetone was prepared.

The homogenate of chicken liver was separated in 12 glass tubes of 30 ml (3 replicates for each laboratory containing 3.2 g of liver each). Each tube was spiked with 214 µl of the final standard solution to achieve a concentration of 5 µg/g, and the homogenates were shaken in vortex vigorously (Figure 1).

The spiked-liver tubes and the vials with the final standard solution were sealed and stored at −20 °C until their shipment to the participant reference laboratories. A chromatography vial containing 1 ml of the final standard

solution at 75 µg/ml and 3 replicates of the spiked liver samples at a final concentration of 5 µg/g for each substance were sent to each laboratory, together with an instruction sheet asking to analyse the replicates with their routine techniques using the same glass tube containing the spiked livers, if possible. Moreover, an excel sheet prepared entry template was also submitted in which to report the analytical results, including the concentrations calculated per replicate, LOD/LOQ, recoveries, repeatability, extraction and detection techniques applied. The packages were delivered by a courier company under freezing conditions and arrived at their destination before 24 hours (Figure 1).

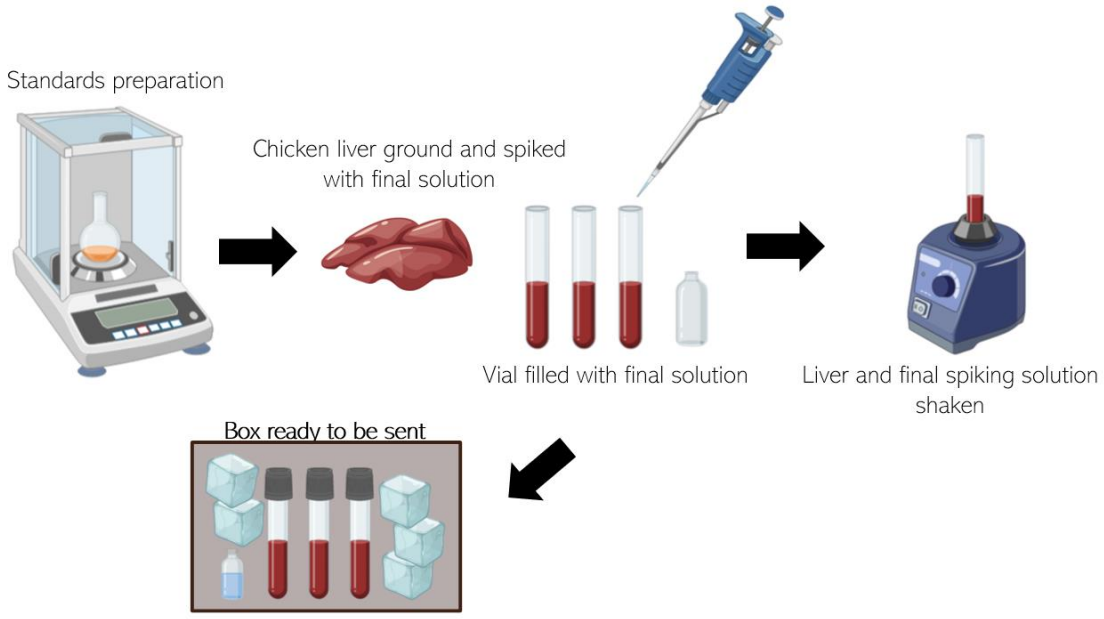

Figure 1. Diagram of the spiked liver sample preparation *(Created with BioRender.com by Irene Valverde)*.

Sample extraction

Each laboratory carried out the extraction procedures according to their routine technique. The main steps are compiled in Table 1.

In the IREC-CSIC-UCLM laboratory different techniques are used according to the compound as described by Sánchez-Barbudo et al. (2012b) and López-Perea et al. (2015). The extraction of carbamates and OP was carried out using 1 g of liver homogenised with 9 g of anhydrous sodium sulphate (Na_2SO_4) in a mortar. The homogenate was placed in a glass tube with Teflon caps. Then, 15

ml of dichloromethane were added, and the samples were mixed for 10 min in a horizontal shaker (SH30L; Finepcr, Seoul, Korea), and sonicated 5 min (Ultrasons-H Selecta; Abrera, Spain). The extracts were filtered, and the remaining sample homogenates were extracted twice again with 5 ml of dichloromethane. The extract was evaporated in a rotary evaporator (Büchi; Flawil, Switzerland) at 400 mbar and 40 °C and resuspended in 2 ml of ethyl acetate:cyclohexane (1:1 v/v). The purification of the extract was done by gel GPC at atmospheric pressure in a glass column with an internal diameter of 17.25 mm and filled with 43.5 cm of Bio-Beads S-X3 (Bio-Rad Laboratories; Madrid, Spain). Mobile phase was ethyl acetate:cyclohexane (1:1 v/ v). The fractions corresponding to 55–60 ml and 60–90 ml (both selected for pesticide analysis) were collected and evaporated with a rotary evaporator. Both fractions were resuspended in 0.5 ml ethyl acetate and placed in 2 ml vials for analysis by chromatography.

The AR extraction in the IREC-CSIC-UCLM was carried out using 1 g of liver grounded in a mortar with 9 g of Na_2SO_4 (Prolabo, Leuven, Belgium). The homogenate was transferred to a Teflon-capped 30 ml-glass tube and 20 ml of a mixture of dichloromethane:acetone (70:30) (HiperSolv Cromanorm Gradient grade, Prolabo, Leuven, Belgium) was added. The mixture was horizontally shaken for 10 min and sonicated for 5 min. The extract was filtered through a Whatman paper filter and collected in a conical tube for solvent evaporation in a rotary evaporator. The extraction was repeated with 5 ml of the solvent mixture, and the supernatant obtained was added to the previous one. After solvent evaporation, the dry extract was dissolved in 2 ml of dichloromethane:acetone (70:30). Then, this extract was cleaned-up in a SPE column of neutral alumina (SPE ALN 500 mg/3 ml, Upti-clean Interchrom, Montluçon, France). The SPE column was conditioned with 5 ml of dichloromethane and 10 ml of dichloromethane:acetone (70:30). The sample was added to the column and washed with 3 ml of dichloromethane:acetone (25:75). Finally, the AR were eluted with 3 ml of methanol:acetic acid (95:5) (Prolabo, Leuven, Belgium). The solvent was evaporated under nitrogen flow and the dried extract was reconstituted in 0.5 ml of methanol and filtered

through a 13 mm-filter with a 0.2 µm nylon membrane (Acrodisk, Pall, NY, USA) for analysis by chromatography.

In SERTOX-ULPGC, the extraction was carried out using the dSPE following a modified QuEChERS technique (Rial-Berriel et al., 2020). For this purpose, 1 g of liver was weighed into a tube suitable for homogenization with a Precellys Evolution homogenizer (Bertin Technologies, Rockville, Washington D.C., USA), operated at 6500 rpm, 2 x 30 sec. Then, the homogenate was diluted with 4 ml ultrapure water, and 1 ml of the diluted homogenate was placed in a 5 ml Eppendorf tube to be processed. First, 2 ml of acetonitrile, 0.48 g of magnesium sulphate ($MgSO_4$) and 0.12 g sodium acetate (NaOAc) were added, followed by 30 s of vortexing and 1 min of vertical-manual shaking. Finally, the Eppendorf tubes were centrifuged for 5 min, at 4500 rpm and 2°C. The supernatant was then filtered through a 0.2 µm Chromafil PET-20/15 syringe filter (polyester, certified for HPLC, Macherey-Nagel, Düren, Germany) into an amber vial directly, for analysis by chromatography.

In the STVF-UM, the extraction was carried out using a modification of the dSPE technique described by Gómez-Ramírez et al. (2012) which is based in a modified QuEChERS technique. Briefly, 3 g of liver were mixed with 3 ml of acetonitrile (PanReac®, Darmstadt, Germany) as extractant solvent. The tubes were vortexed vigorously for about a minute and a salt mixture of 1.33 g $MgSO_4$, 0.33 g sodium chloride (NaCl), 0.17 g sodium citrate dibasic sesquihydrate (SCDS) and 0.33 g sodium citrate tribasic dehydrate (SCTD) (Supelco®, Bellefonte, PA, USA) was added. The tubes were again strongly shaken with vortex for one minute approximately. The tubes were centrifuged at 998 RCF for 5 min, and frozen at −20 °C for 1 h. After that, the tubes were again centrifuged in the same conditions, and the supernatant was then transferred to another tube and mixed with a new mix of salts with 0.05 g of primary secondary amine (PSA) (Superclean PSA bonded silica), 0.05 mg Discovery DSC-18: octadecylsilane 18% C (DSC-18), and 3 g $MgSO_4$ (Supelco®, Bellefonte, PA, USA). The tube was shaken and centrifuged again at 998 RCF for 5 min. Finally, 1 ml supernatant was transferred to vials for analysis by

chromatography. For AR analysis, the vial was acidified by adding 10 µl of 5% formic acid in acetonitrile.

Finally, in the UNEX, the extraction was carried out using 2 g of homogenized liver sample and 10 ml of acetonitrile in 50 ml polypropylene Falcon tubes. The mix was homogenized in an Ultra-Turrax® (IKA-T18 Basic with S18N-19G disperser) for 30 seconds. A mixture of salts with 1 g $MgSO_4$ and 0.5 g of NaOAc (Sigma-Aldrich®, Steinheim, Germany) was added. The mixture was vortexed vigorously and then sonicated for 15 min (Ultrasounds, J.P.Selecta®, Barcelona, Spain). After that, the tubes were frozen at -20 ºC for 1 h and then centrifuged at 5500 RCF for 10 min (Digicen21R, Ortoalresa®, Madrid, Spain). Finally, the supernatant (extract) was transferred to a clean 4 ml-glass vial for chromatographic analysis. No purification was carried out on this extract. An aliquot of 200 µl of the extract was 1/10 diluted in acetonitrile to analyse AR and carbamates by LC and 200 µl of the extract was evaporated under nitrogen flow and the dried extract was resuspended in 2 ml of cyclohexene to analyse OP by GC. The diluted extracts were injected directly in the chromatographs.

Table 1. Summary of the extraction techniques used in the interlaboratory comparison.

	Sample amount (g)	Extraction technique	Extraction solvent	Solvent:Sample Ratio	Extraction Reagents	Clean-up (purification)
UNEX	2	dSPE (all compounds)	Acetonitrile 10 ml	5:1	$MgSO_4$ 1 g NaOAc 0.5 g	No purification Freezing
SERTOX-ULPGC	1	dSPE (all compounds)	Acetonitrile 2 ml	2:1	$MgSO_4$ 0.48 g NaOAc 0.12 g	No purification
STVF-UM	3*	dSPE (all compounds)	Acetonitrile 3 ml	1:1	$MgSO_4$ 1.33 g NaCl 0.33 g SCDS 0.17 g SCTD 0.33 g	PSA 0.05 g C-18 0.05 g Magnesium sulphate 3 g Freezing
IREC-CSIC- UCLM	1		Dichloromethane:Acetone 25 ml	25:1	Na_2SO_4 9 g	SPE column with neutral alumina Methanol:acetic acid (elution) (for AR)
	1		Dichloromethane 25 ml	25:1	Na_2SO_4 9 g	GPC (Bio-Beads S-X3) Ethyl acetate:cyclohexane (Mobile phase) (for carbamates and OP)

UNEX: Unit of Toxicology from University of Extremadura; SERTOX-ULPGC: Toxicology Unit from University of Las Palmas de Gran Canaria; STVF-UM: Service of Toxicology and Forensic Veterinary from University of Murcia IREC-CSIC-UCLM: Institute for Game and Wildlife Research from University of Castilla-La Mancha.
AR: Anticoagulant rodenticides; DSC: N, N′-Disuccinimidyl carbonate; dSPE: dispersive Solid-Phase extraction; GPC: gel permeation chromatography; $MgSO_4$: Magnesium sulphate; Na_2SO_4: anhydrous sodium sulphate; OP: organophosphates; PSA: primary secondary amine; SPE: Solid-phase extraction; SCDS: Sodium citrate dibasic sesquihydrate; SCTD: Sodium citrate tribasic dehydrate; NaCl: Sodium chloride; NaOAc: sodium acetate.
*STVF-UM used all the available sample volume (3g) for this interlaboratory comparison, but they obtained similar results using 1 g of sample for AR analysis.

Analytical techniques

The analytical procedures used to detect the different compounds are LC and GC coupled to MS. However, each laboratory used different chromatographic conditions. The main analytical parameters are compiled in Table 2.

In the IREC-CSIC-UCLM, the LC-MS analytical system was formed by an Agilent 1100 series chromatograph and Agilent 6110 Quadrupole with a multimode source (MM) (López-Perea et al., 2015). The nitrogen for ionization source was supplied with a high purity nitrogen generator (Whisper 2-50, Ingeniería Analítica, Sant Cugat, Spain). For the chromatography an Eclipse column XDB-C18 (4.6 × 12.5 mm, 5 µm) was used. The injection volume was 30 µl. A gradient elution of two solvents was set up (A: ammonium acetate 10 mM, pH: 6.03; B: methanol). The initial conditions were 35% A - 65% B, reaching 15% A - 85% B at min 5. This was maintained until min 10, returning to the initial conditions at min 12. Then, column was stabilized with the initial conditions until min 15 before the next sample injection. The flow rate was 1.2 ml/min. AR were detected using negative ion monitoring with the following MM-ESI source settings. Nebulizer pressure was set at 60 psi, drying gas flow was 4.8 l/min, drying gas temperature was 250 °C, vaporizer temperature was 150 °C, capillary voltage was 2000 V, charging voltage was 1000 V, and fragmentation voltage varied among compounds. Four ions previously selected for each compound by means of analysis of complete scanning and flow injection analysis of sequences (FIAS) of AR standards were monitored in SIM mode.

The GC–MS system used in the IREC-CSIC-UCLM was an Agilent-Technologies 6890N with mass selective detector 5973 Network (Sánchez-Barbudo et al., 2012b). The chromatographic conditions were controlled using the MSD ChemStation software version D.01.00 (Agilent-Technologies; Waldbronn, Germany). The GC column was a BPX5 (30 m x 0.32 mm, 0.25 µm, Agilent Technologies; Santa Clara, CA). The flow rate of helium was 34.9 ml/min. The injection volume was 1 µl in splitless mode. Injector conditions were 280 ºC and 31.5 kPa. The column oven had an initial temperature of 50 ºC and followed by a temperature ramp of 5 ºC/min to 310 ºC. Total run time was 60 min.

Identification was performed by comparison with mass spectra available in NIST MS search 2.0 library.

In the SERTOX-ULPGC, LC was performed using an Agilent 1290 Infinity II UHPLC (Agilent Technologies, Palo Alto, USA). The column was an InfinityLab Poroshell 120 (2.1 mm × 100 mm, 2.7 µm), coupled to an inline filter and an UHPLC guard column with the same characteristics as the analytical column, to protect the column. A gradient elution of two solvents was set up (A: 0.1% FA and 2 mM ammonium acetate in ultrapure water; B: 2 mM ammonium acetate in methanol). The gradient of mobile phase A was: 95% - 0.5 min; 80% - 1 min; 60% - 2.5 min; 15% - 8 min; 0% - 10 to 14 min; 95% - 14.01 min. 8 µl was injected at a flow rate set at 0.4 ml/min and an oven column temperature of 50°C. For identification and quantification, we employed an Agilent 6460 mass spectrometer (Agilent Technologies, Palo Alto, USA), operated in the dynamic multiple reaction monitoring mode (dMRM), in both positive and negative polarities, with a cycle time 800 ms, a dwell time of 8 to 60 ms, and a total run time of 18 min. The Agilent Jet Stream Electrospray Ionization Source (AJS-ESI) was operated under the following conditions: gas temperature 190°C; nebulizer gas flow and pressure were 11 l/min and 26 psi, respectively; the temperature of the sheath gas and the flow were 330°C and 12 l/min, respectively; and the positive and negative capillary voltages were 3900 V and 2600 V. The drying and desolvation gas was nitrogen provided by the Zefiro 40 nitrogen generator (F-DGSi, Evry, France). Nitrogen 6.0 (99.9999% purity, Linde, Dublin, Ireland) was used as the collision gas.

Regarding the GC used in the SERTOX-ULPGC, an Agilent 7890B gas chromatograph (Agilent Technologies, Palo Alto, USA) was employed. Two Agilent columns J&W HP-5MS (5% cross-linked phenyl-methylpolysiloxane, Agilent Technologies) ultra-inert fused silica capillary 30 m (15 + 15) length, internal diameter of 0.25 mm, and a film thickness of 0.25 µm, were employed for the separations. The columns were joined by means of a purged joint to allow the application of the back-flushing technique that reduces the background noise and extends the column lifetime. An ultra-inert glass wool

inlet liner at 250°C was used at the injection port, and the injection of 1.5 µl was performed in splitless pulsed mode. Helium 5.0 (99.999% purity) at a constant flow 1.5 ml/min, and the collision gas being nitrogen 6.0 (99.9999% purity). The initial oven temperature of 80°C was maintained for 1.8 min, then increased at a rate of 40°C/min to 170°C, then increased at a rate of 10°C/min to 310°C, and finally maintained for 3 min at 310°C. The post-run backflush to clean the column was set at 315°C for 5 min at -5.8 ml/min for the first column, and the final run time at 21.05 min. For the identification and quantification of the compounds, an Agilent 7010 mass spectrometer (Agilent Technologies, Palo Alto, USA) was used. This equipment was operated in the multiple reaction monitoring mode (MRM), with 24-time segments, cycle time between 300 and 600 ms and a dwell time between 15 and 40 ms. The electron impact (EI) and transfer line ionization source temperatures were set at 280°C, with a solvent delay of 3.7 min.

The LC-MS analytical system in the STVF-UM was formed by an Agilent 1290 Infinity II Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an Automated Multisampler module and a High Speed Binary Pump, and connected to an Agilent 6550 Q-TOF-MS (Agilent Technologies, Santa Clara, CA, USA) using an Agilent Jet Stream Dual electrospray (AJS-Dual ESI) (Agilent Technologies, Santa Clara, CA, USA) interface. Experimental parameters for HPLC and Q-TOF were set in MassHunterWorkstation Data Acquisition software (Agilent Technologies, Rev. B.08.00). Standards and samples (injection volume of 20 µl) were injected into a Zorbax Eclipse XDB C8, 5 µm, 150 x 4.6 mm HPLC column, at a flow rate of 0.7 ml/min. The column was thermostated at 25 °C. A gradient elution of two solvents was set up (A: MilliQ water with 20 mM ammonium acetate; B: methanol with 20 mM ammonium acetate). The gradient conditions were 50% solvent A - 50% solvent B. After the injection, compounds were eluted using a linear gradient 50–95% B for 22 min. Then, a linear gradient from 95–50% B was applied in 3 min and finally the system was equilibrated at starting conditions (50% B) for 10 min before a new injection. Total run time was 35 min. The mass spectrometer was operated in the negative mode. The nebulizer gas pressure was set to 40 psi, whereas the drying gas flow was set to

13 l/min at a temperature of 250 °C, and the sheath gas flow was set to 12 l/min at a temperature of 300 °C. The capillary spray, nozzle, fragmentor, and octopole RF Vpp voltages were 3500 V, 1000 V, 350 V, and 750 V, respectively. Profile data in the 100–1100 m/z range were acquired for MS scans in 2 GHz extended dynamic range mode. The data were analyzed with MassHunter Qualitative Analysis Navigator software (version B.06.00, Service Pack 1, Agilent Technologies, Inc. US, 2012).

The GC used in the STVF-UM was a Shimadzu gas chromatograph (Shimadzu GCMS-QP-2010-Plus). The analyses were carried out using a low bleed capillary GC column SLB-5ms (5% cross-linked silphenylene polymer virtually equivalent in polarity to poly (5% diphenyl/95% dimethyl siloxane) phase, Sigma-Aldrich) with 30 m length x 0.25 mm internal diameter, and 0.25 µm film thickness. The gas used was helium 5.0 (99.999% purity). Regarding injection and temperature gradient in the oven two techniques were carried out. For aldicarb, an ultra-inert glass wool inlet liner at low temperature (60°C) was used at the injection port, and the injection of 1 µl was performed in splitless pulsed mode. Helium was set at a constant flow of 2.3 ml/min, and the collision gas was nitrogen 6.0 (99.9999% purity). The initial oven temperature was 50°C and it was maintained for 12 min, then increased at a rate of 5°C/min to 65°C, then increased at a rate of 35°C/min to 250°C. Total run time was 20.30 min. For the rest of pesticides, an ultra-inert glass wool inlet liner at 200°C was used at the injection port, and the injection of 1 µl was performed in splitless pulsed mode. Helium was set at a constant flow of 1 ml/min, and the collision gas was nitrogen 6.0 (99.9999% purity). The initial oven temperature of 150°C was maintained for 2 min, then increased at a rate of 40°C/min to 200°C and maintained for 1 min, then increased at a rate of 20°C/min to 290°C and maintained for 12.5 min. Total run time was 21.25 min. For the identification and quantification of the pesticides, a MS (Shimadzu MSQP) was used. The electron impact (EI) and transfer line ionization source temperatures were set at 230°C, with a solvent delay of 4.0 min.

The LC-MS analytical system used in the UNEX was formed by Agilent 1260 series Infinity II HPLC chromatograph and Agilent 6470 Triple Quadrupole LC/MS with an Electrospray ionization source (ESI). The nitrogen for ionization source was supplied with a high purity nitrogen. For the chromatography we used an Eclipse column Poroshell-C18 (2.1 × 100 mm, 2,7 µm). The injection volume was 4 µl. A gradient elution of two solvents was set up (A: ammonium acetate 10 mM, pH: 5,5; B: methanol). The initial conditions were 95% A - 5% B, 80% A - 20% B at min 2 and reaching 5%A - 95% B at min 12. This was maintained until min 14, returning to the initial conditions at min 15. Then, column was stabilized with the initial conditions until min 5 before the next sample injection. The flow rate was 0.4 ml/min. AR were detected using negative ion monitoring with the following ESI source settings. Nebulizer pressure was set at 40 psi, drying gas flow was 10 l/min, drying gas temperature was 270 °C, capillary voltage was 2000 V, charging voltage was 3000 V, and fragmentation voltage varied among compounds. Two ions transition were previously selected for each compound.

Finally, the GC–MS system used in the UNEX was a 45-GC Bruker with Scion Triple Quadrupole detector (Bruker). The chromatographic conditions were controlled using the MS Work Station software version 6.8 (Bruker, Germany). The GC column was a DB-5MS (30 m x 0.25 mm, 0.25 µm, Agilent-Technologies; Santa Clara, CA). The flow rate of helium was 1 ml/min. The injection volume was 1 µl in splitless mode. Injector and Transfer line temperature were 280 °C. The column oven had an initial temperature of 60 °C and followed by a ramp temperature of 20 °C/min to 240°C and then a temperature gradient of 5°C/min to 300°C. Total run time was 30 min.

Table 2. Analytical techniques used in the interlaboratory comparison.

	Analytical Technique	Detector	Injection volume (µl)	Stationary phase		Mobil phase		Flow (ml/min)	Gradient	Total run time (min)
				Column	Size	A	B			
UNEX	HPLC	MS/MS	4	Eclipse Poroshell-C18	C18 2.1 × 100 mm, 2.7 µm	AA 10 mM	Methanol	0.4	95% A and 5% B, 80% A and 20% B at min 2 and reaching 5%A and 95% B at min 12	15
	GC	MS/MS	1	DB-5MS	(5%-phenyl)-methyl-polysiloxane 30 m × 0.25 mm, 0.25 µm	Helium		1	OT: 60 °C followed by 20 °C/min to 240°C and then 5°C/min to 300°C	30
SERTOX-ULPGC	UHPLC	MS/MS	8	InfinityLab Poroshell 120	C18 2.1 × 100 mm, 2.7 µm	AA 2 mM	2 mM AA in Methanol	0.4	A: 95% - 0.5 min; 80% - 1 min; 60% - 2.5 min; 15% - 8 min; 0% - 10 to 14 min; 95% - 14.01 min	18
	GC	MS/MS	1.5	J&W HP-5MS	(5%-phenyl)-methyl-polysiloxane 30 × 0.25 mm, 0.25 µm	Helium		1.5	OT: 80°C for 1.8 min, increase 40°C/min to 170°C, increase 10°C/min to 310°C, finally 310°C for 3 min	21.05
STVF-UM	HPLC	MS/MS	20	Zorbax Eclipse XDB	C8 4.6 × 150 mm, 5 µm	AA 20 mM	20 mM AA in Methanol	0.7	50% A and 50% solvent B. Linear gradient 50–95% B for 22 min. Then, linear gradient from 95–50% B in 3 min	35
	GC	MS/MS	1	SLB-5ms	(5%-phenyl) methyl-polysiloxane 30 × 0.25 mm, 0.25 µm	Helium		2.3	OT for Aldicarb: 50°C for 12 min, increased 5°C/min to 65°C, increased at a rate of 35°C/min to 250°C	20.30
								1	OT for Carbofuran, Methiocarb, Chlorpyrifos, Parathion, Diazinon: 150°C for 2 min, increased at 40°C/min to 200°C for 1 min, increased at 20°C/min to 290°C for 12.5 min	21.25
IREC-CSIC-UCLM	HPLC	MS	30	eXtra-Dense Bonding of organo-silane ligands-C18	C18 4.6 × 12.5 mm, 5 µm	AA 10 mM	Methanol	1.2	35% A and 65% B, reaching 15% A and 85% B at min 5, maintained until min 10, returning to the initial conditions by min 12	15

GC	MS	1	BPX5	(5%-phenyl)-methyl-polysiloxane	30 m x 0.32 mm, 0.25 µm	Helium	1.2	50 ºC and followed a ramp temperature of 5 ºC/min to 310 ºC	60

UNEX: Unit of Toxicology from University of Extremadura; SERTOX-ULPGC: Toxicology Unit from University of Las Palmas de Gran Canaria; STVF-UM: Service of Toxicology and Forensic Veterinary from University of Murcia; IREC-CSIC-UCLM: Institute for Game and Wildlife Research from University of Castilla-La Mancha.
AA: Ammonium acetate; GC: Gas chromatography; HPLC: High performance liquid chromatography; MS: Mass spectrometry; MS/MS: Tandem mass spectrometry; OT: Oven temperature; UHPLC: Ultra high performance liquid chromatography

Parameters evaluated

Collaborators were required to report the mean concentrations in the spiked liver samples (µg/g) received for each substance evaluated, coefficient of variation (CV, %) for repeatability, recoveries (%), LOD and/or LOQ, and the description of their techniques. The CV represents the relative dispersion of the data around the mean, and values ≤20% are considered appropriate according to SANTE/12682/2019 (2019). Recoveries (%) are the proportion of analyte remaining at the point of the final determination, following its addition (usually to a blank sample) immediately prior to extraction. It is determined during validation, if no certified reference material is available (Commission decision 2002/657/EC, 2002; SANTE/12682/2019, 2019).

The LOD is the validated lowest residue concentration which can be quantified and reported by routine monitoring with validated control methods (SANTE/12682/2019, 2019). On the other hand, LOQ is the lowest concentration or mass of the analyte that has been validated with acceptable accuracy by applying the complete analytical method and identification criteria. LOQ is preferable to LOD because it avoids possible confusion with "limit of detection" (SANTE/12682/2019, 2019).

Z-score is a standardized measure of performance, calculated using the participant results, the reference value and the standard deviation for proficiency assessment (ISO13528:2015). In this proficiency test comparison, the laboratory performance was expressed in terms of z-score in accordance with ISO13528:2015 (2015).

$$Z = (X_{lab} - X_{ref}) / \sigma$$

Where:

X_{lab} is the result reported by each collaborator

X_{ref} is the reference value (5 µg/g for all the substances evaluated)

σ is the target standard deviation. It is the maximum acceptable standard uncertainty that was set to 25% of the reference value (Dehouck et al., 2015)

The interpretation of the z-score is done according to ISO/IEC17043:2010 (2010): |score| ≤ 2 indicates a satisfactory result, 2 < |score| < 3 means questionable result, and |score| ≥ 3 indicates an unsatisfactory result. The z-score compares the deviation of each laboratory from the reference value with the target standard deviation used as common quality criterion (Dehouck et al., 2015).

Results and discussion

Table 3 describes the mean concentration of spiked liver samples (µg/g), z-score, LOD (µg/g), LOQ (µg/g), recoveries (%) and CV (%) for repeatability obtained in the four laboratories for each compound.

Spiked liver samples were prepared at 5 µg/g, which was set as the reference value. Mean concentrations of spiked liver samples (µg/g) vary in each laboratory depending on the technique and compound (Table 3). UNEX concentrations ranged 4.8-5.5 µg/g for all the substances, STVF-UM concentrations ranged 2.2-5.3 µg/g), SERTOX-ULPGC concentrations were within the range 5.0-6.8 µg/g, while concentrations in IREC-CSIC-UCLM ranged 3.1-5.2 µg/g (except for chlorophacinone that was not detected).

According to SANTE/12682/2019, good recoveries are stablished in the range of 70–120%. The techniques from UNEX and STVF-UM had recoveries within this range for all carbamates and OP, the technique from IREC-CSIC-UCLM also showed recoveries within this range except for aldicarb (62.5%) and the technique from SERTOX-ULPGC had recoveries outside that range for some pesticides (99-135%). Regarding the techniques used for AR, all recoveries were within 70-120% except for some compounds in the technique used by SERTOX-ULPGC (106-127%), STVF-UM (44-67%) and chlorophacinone in IREC-CSIC-UCLM (not detected). The CVs were ≤ 20% (SANTE/12682/2019) in all methods and for all compounds.

The LOD and LOQ established in these methods are in all cases low enough to detect and quantify the compounds evaluated in this study in cases of poisoning (Table 3). For example, red kites affected by acute poisoning showed liver bromadiolone and chlorophacinone concentrations ranging from 200-5600

ng/g and 900-5200 ng/g (w.w.), respectively (Berny & Gaillet, 2008). These concentrations are much higher than the LOQ reported for AR in this study (0.4-8 ng/ml; Table 3). On the other hand, an experimental study of aldicarb and carbofuran degradation in rats reported the lowest *post-mortem* concentrations detected in liver after the first day of death as 120 ng/g of aldicarb and 40 ng/g of carbofuran (de Siqueira et al., 2016), concentrations higher than the LOQ described in Table 3 for these carbamates (0.4-5 ng/ml). Regarding OP pesticides, Nielsen et al. (1991) carried out a kinetic study of parathion in neonatal and young pigs after intravenous administration with a dose without toxic effects (0.5 mg/kg body weight). The highest concentrations of parathion after 3 hours of dosage were detected in liver (840 ± 426 ng/g), kidneys (272 ± 122 ng/g) and plasma (83 ± 47 ng/ml). These concentrations (causing no observable effects) of parathion are also higher than the LOQ presented in this study (10-40 ng/ml; Table 3).

According to z-score, all the techniques showed satisfactory results for all compounds (z-score < 2), except for difenacoum and chlorophacinone in the method of STVF-UM with a z-score slightly higher (z-score=2.2) that can be considered 'questionable' (Dehouck et al., 2015). The main extraction method used by UNEX, SERTOX-ULPGC and STVF-UM is dSPE based on a modified QuEChERS method (Gómez-Ramírez et al., 2012; Rial-Berriel et al., 2020). QuEChERS (quick, easy, cheap, effective, rugged, and safe method) was first designed to extract pesticides from vegetable matrices. Basically, QuEChERS method is carried out in two: 1) extraction and 2) clean-up or purification. In the extraction step, acetonitrile is used as extractant solvent and then a mix of salts are added to separate the phases. Then, in the clean-up, other mix of purification compounds are added to remove undesirable substances that could interfere in the chromatographic results (Anastassiades et al., 2003). This method has more recently been adapted to extract pesticides and other types of compounds from complex matrices such as blood, liver, milk, meat or eggs (Gómez-Ramírez et al., 2012; Jeong et al., 2012; Molina-Ruiz & Cieslik, 2015; Rial-Berriel et al., 2020; Wilkowska & Biziuk, 2011).

One of the main goals of toxicology laboratories is to achieve a multiresidue technique capable of analyse the maximum number of compounds, but other parameters should be also considered. Ideally, the technique should require as little volume of sample as possible, because samples are often sparsely available in wildlife forensic toxicology or may need to be divided to carry out different toxicology analysis. In addition, whenever possible, the minimum number of extraction steps are preferred to reduce the risk of analyte loss and save time. Minimizing the use of solvents and reagents required is also ideal since these are usually expensive and, in many cases, also are themselves toxic. Moreover, the toxicity of the reagents should be taken into account to achieve more environmentally friendly techniques. Finally, those methods using a single run by GC or LC are preferred to save both time and reagents/material. Considering all these issues will help to economise both time and reagents, thus more economic and environmentally friendly techniques will be obtained.

The modified QuEChERS method used by UNEX, SERTOX-ULPGC and STVF-UM is able to extract all the target compounds (AR, carbamates and OP) using the same methodology (Table 1). The sample amount used in the techniques based on QuEChERS method in this study is 3, 2 and 1 g of liver in STVF-UM, UNEX and SERTOX-ULPGC, respectively. Although STVF-UM used all the liver sample available for this study (3 g), its technique for AR is adapted to 1 g of sample. All these techniques use acetonitrile as the extractant solvent with a solvent:sample ratio of 1:1 in STVF-UM, 2:1 in SERTOX-ULPGC and 5:1 in UNEX. In this sense, SERTOX-ULPGC and STVF-UM are the techniques using the lowest volume of solvent. UNEX and SERTOX-ULPGC use the same two extractant salts ($MgSO_4$ and NaOAc), although SERTOX-ULPGC uses a lesser amount, and STVF-UM uses four different extractant salts ($MgSO_4$, NaCl, SCDS and SCTD). In the extraction technique used by IREC-CSIC-UCLM, 25 ml of solvents to extract compounds from 1 g of liver are needed (solvent:sample ratio 25:1), the salt used is Na_2SO_4.

Regarding the clean-up of the techniques, the freezing step - used only by UNEX and STVF-UM, may be considered as a pre-purification step since it helps

to remove waxes, fat and water (Schenck et al., 2002; Hong et al., 2004; Anastassiades, 2005). Only two laboratories, STVF-UM and IREC-CSIC-UCLM, use a clean-up step, while SERTOX-ULPGC does not use any purification. In the case of STVF-UM a mix of $MgSO_4$, PSA to eliminate fatty and organic acids, and C18 to eliminate proteins, peptides and lipids is used; while IREC-CSIC-UCLM use a neutral alumina column as SPE clean-up step (López-Perea et al., 2015) for AR and a GPC clean-up with Bio-Beads S-X3 (Sánchez-Barbudo et al., 2012b) for pesticides. As purification compounds, PSA, C18, Bio-Beads S-X3 and neutral alumina are used to remove lipids (fatty acids and sterols) but also proteins and peptides (Björklund et al., 2001; Anastassiades et al., 2003; Fidalgo-Used et al., 2007; Rial-Berriel et al., 2020). Magnesium sulphate, NaCl and Na_2SO_4 are used to remove water and proteins from the sample (Aguilera-Luiz et al., 2008), since water may affect the separation and recoveries of pesticides (Schenck et al., 2002). However, the use of $MgSO_4$ was shown to remove a higher percentage of water in samples compared with Na_2SO_4 using acetonitrile and acetone (Schenck et al., 2002). Good recoveries have also been reported using Na_2SO_4 and ethyl acetate as extractant solvent (Andersson & Pålsheden, 1991). Sodium salts (NaCl, Na_2SO_4, SCDS and SCTD) are also used to control the polarity of the extraction solvent. Moreover, citrate salts in the extraction step are used to buffer and adjust the pH to around 5 (Kaczyński et al., 2017).

Regarding analytical techniques (Table 2), AR are always analysed by LC because they are non-volatile substances (Imran et al., 2015), while carbamates and OP can be analysed using both LC and GC. UNEX and SERTOX-ULPGC analyse all studied compounds by LC except for OP pesticides (GC). However, STVF-UM and IREC-CSIC-UCLM analyse all studied compounds by GC except for AR (LC).

Liquid chromatography is performed by HPLC in UNEX, STVF-UM and IREC-CSIC-UCLM and by UHPLC in SERTOX-ULPGC. Regarding the columns, UNEX, IREC-CSIC-UCLM and SERTOX-ULPGC use a C18 column, while STVF-UM uses a C8 column. C8 (octylsilane) and C18 (octadecylsilyl) columns are straight alkyl

chain, most preferred to HPLC columns as they can be used in a wide pH range as stationary phase. Both can be used interchangeably to remove fats. Preparation of stationary phases with C18 does not require the exclusion of absolute water, which eases the procedure (Engelhardt et al., 1982; Anastassiades, 2005; Martínez-Vidal & Garrido-Frenich, 2006; Kumar et al., 2012). Regarding the mobile phases, all laboratories use two phases with a gradient of injection. Total run times were 15 min for UNEX and IREC-CSIC-UCLM, and 18 and 35 min for SERTOX-ULPGC and STVF-UM, respectively.

Gas chromatography techniques use the same column size (30 m x 0.25 mm) except IREC-CSIC-UCLM that uses a higher internal diameter of the column (0.32 mm). In all participant laboratories the stationary phase used is (5%-phenyl)-methylpolysiloxane with a thickness of 0.25 µm. Columns with (5%-phenyl)-methylpolysiloxane are used in GC for general purposes, these columns are non-polar, to analyse semivolatiles compounds, halogenated compounds, pesticides, drugs of abuse or amines (Agilent). Total run times were 21.05, 30 and 60 min for SERTOX-ULPGC, UNEX and IREC-CSIC-UCLM, respectively; and 20.30 and 21.25 min in the STVF-UM for aldicarb and other pesticides, respectively, where two runs are needed to detect all carbamates and OP evaluated.

Considering the sample amount, the extraction steps and volume of solvent and other reagents needed during the extraction, and the total run time during chromatography, SERTOX-ULPGC can be considered the fastest and most economic and environmentally friendly technique. However, considering that the techniques compared in this study use different extraction techniques and chromatographic conditions, in general they all obtained satisfactory results for the 11 substances evaluated and they can report comparable results in wildlife poisoning cases. The lower recoveries obtained in the STVF-UM technique for AR may be due to the different column (C8 instead of C18) and gradient used in the LC system.

Table 3. Results obtained in the interlaboratory comparison.

		Aldicarb	Carbofuran	Methiocarb	Diazinon	Chlorpyrifos	Parathion	Bromadiolone	Brodifacoum	Difenacoum	Warfarin	Chlorophacinone
UNEX	Mean concentration of spiked liver samples (µg/g)	5.08 [a]	4.8 [a]	5.1 [a]	4.9 [b]	4.8 [b]	4.83 [b]	5.5 [a]	5.0 [a]	5.2 [a]	4.8 [a]	5.4 [a]
	z-score	0.1	0.2	0.1	0.1	0.2	0.1	0.4	0.0	0.2	0.2	0.3
	LOD (ng/ml)	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	LOQ (ng/ml)	5.0	5.0	5.0	10.0	10.0	10.0	2.0	2.0	2.0	2.0	2.0
	% Recovery	101.6	96.4	101.5	97.1	96.5	96.6	109.9	100.9	103.7	95.1	107.7
	CV (%)	0.9	3.9	2.1	1.3	0.1	0.1	0.4	1.2	1.3	4.5	0.4
SERTOX-ULPGC	Mean concentration of spiked liver samples (µg/g)	6.2 [a]	6.6 [a]	6.8 [a]	5.4 [b]	6.4 [b]	5.0 [b]	6.1 [a]	6.2 [a]	6.4 [a]	5.7 [a]	5.3 [a]
	z-score	1.0	1.3	1.4	0.3	1.1	0.0	0.9	1.0	1.1	0.6	0.2
	LOD (ng/ml)	0.8	0.4	0.4	1.2	1.6	40.0	0.4	0.4	0.8	0.8	8.0
	LOQ (ng/ml)	0.8	0.4	0.4	1.2	1.6	40.0	0.4	0.4	0.8	0.8	8.0
	% Recovery	124.0	132.7	135.2	108.5	127.6	99	121.0	124.3	127.3	113.3	106.5
	CV (%)	1.1	0.5	1.7	13.2	13.6	14.7	3.1	2.6	2.4	2.8	2.2
STVF-UM	Mean concentration of spiked liver samples (µg/g)	5.1 [b]	4.5 [b]	4.2 [b]	4.8 [b]	4.5 [b]	5.3 [b]	2.9 [a]	3.1 [a]	2.2 [a]	3.3 [a]	2.2 [a]
	z-score	0.1	0.4	0.6	0.1	0.4	0.1	1.7	1.5	2.2	1.4	2.2
	LOD (ng/ml)	0.2	3.0	5.0	2.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
	LOQ (ng/ml)	0.2	5.0	10.0	3.0	10.0	10.0	5.0	5.0	5.0	5.0	5.0
	% Recovery	102.3	89.1	84.4	96.8	89.2	106.6	58.3	62.3	44.7	66.9	44.2
	CV (%)	7.0	16.4	8.6	10.9	3.3	5.3	14.1	13.7	12.2	12.5	13.6
IREC-CSIC-UCLM	Mean concentration of spiked liver samples (µg/g)	3.1 [b]	4.6 [b]	4.8 [b]	4.3 [b]	5.2 [b]	4.6 [b]	4.8 [a]	4.2 [a]	4.8 [a]	4.7 [a]	ND
	z-score	1.5	0.3	0.2	0.6	0.2	0.3	0.2	0.6	0.2	0.2	ND
	LOD (ng/ml)	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	ND
	LOQ (ng/ml)	NR	NR	NR	NR	NR	NR	1-6	1-6	1-6	1-6	1-6

Table 3. Results obtained in the interlaboratory comparison.

	Aldicarb	Carbofuran	Methiocarb	Diazinon	Chlorpyrifos	Parathion	Bromadiolone	Brodifacoum	Difenacoum	Warfarin	Chlorophacinone
% Recovery	62.5	91.3	96.3	85.5	103.1	91.6	96.1	83.8	95.2	93.5	ND
CV (%)	0.6	0.8	0.9	0.6	0.7	0.9	0.3	1.0	0.7	0.5	ND

UNEX: Unit of Toxicology from University of Extremadura; SERTOX-ULPGC: Toxicology Unit from University of Las Palmas de Gran Canaria; STVF-UM: Service of Toxicology and Forensic Veterinary from University of Murcia; IREC-CSIC-UCLM: Institute for Game and Wildlife Research from University of Castilla-La Mancha.

CV: Coefficient of variation; LOD: Limit of detection; LOQ: Limit of quantification; ND: non detected; NR: non reported

[a] LC-MS/MS, [b] GC-MS/MS

Conclusions

This study presents, to the best of our knowledge, the first interlaboratory comparison of different analytical techniques used in the determination of toxic compounds involved in wildlife poisoning.

Overall, the participant laboratories offer techniques with satisfactory and comparable results for AR (bromadiolone, brodifacoum, difenacoum, warfarin and chlorophacinone), carbamates (aldicarb, carbofuran, methiocarb) and OP pesticides (diazinon, chlorpyrifos, and parathion).

Considering the amount of sample, the extraction steps and volume of solvent and other reagents needed, and the total run time during chromatography, the SERTOX-ULPGC technique stands out as the fastest and most economic and environmentally friendly one.

Further studies will be needed to evaluate the techniques used for other pesticides reported in wildlife poisoning including additional carbamates, OP, and other mammalicides, as well as using real samples from wildlife poisoning cases.

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Chapter V. Developing a European network of analytical laboratories and government institutions to fight against raptor poisoning

Image: Irene Valverde Domínguez composition from Pixabay

Abstract

Several cases of wildlife poisoning in Europe have been reported causing population declines, especially in raptors. Toxicovigilance and risk assessment studies are essential to reinforce the knowledge of the number of illegal poisoning cases and the substances involved in these crimes. Many researchers and projects in different institutions have suggested the creation of a network to improve communication and share information between European countries. This article presents the results of the Short-Term Scientific Mission titled "Developing a Network of Analytical Labs and Government Institutions" supported by the COST Action European Raptor Biomonitoring Facility (CA16224), which aims to start a network, focused on veterinary forensic toxicology laboratories, in order to improve communication between laboratories in the fight against wildlife poisoning, specially focused on raptors.

For this purpose, a questionnaire was designed and sent by email to 118 laboratories. It had 39 questions on different topics (e.g., laboratory activities, analytical information). A total of 28 replies were received. Most participant laboratories work on veterinary forensic toxicology research and external cases at the same time, which can give a wide overview of the real situation in the field. The different analytical techniques, and data collection should be harmonized, and a sufficient communication between laboratories is needed to create an effective network. The present study establishes a first contact between European laboratories as an initial step to create a European network and compile basic data from a questionnaire to detect strengths and pitfalls that will help to harmonize methodologies and increase pan-European capacities.

Keywords: forensics, wildlife toxicology, ecotoxicology, poison, birds of prey.

Introduction

In Europe, the use of poison to kill wildlife and domestic animals is strictly prohibited by different regulations and directives (Directive 92/43 1992; Directive 2009/147/EC 2010). According to the Directive 2008/99/EC 2008 on the protection of the environment through criminal law, "killing, destruction, possession or taking of specimens of protected wild fauna or flora species" constitutes a criminal offence. Nevertheless, several cases of illegal animal poisoning in Europe have been reported (Hernández and Margalida 2008; Guitart et al. 2010; Parvanov et al. 2018; Ntemiri et al. 2018).

Animal poisoning may occur due to different causes: (i) misuse of a registered chemical product or pesticide, (ii) abuse or illegal poisoning, when a chemical product, authorized or not, is used intentionally to kill animals using baits, (iii) as a result of a secondary poisoning (i.e., an animal predates other animal already poisoned), or (iv) as an incidental case with a substance with an approved use (Hunter et al. 2005; Berny 2007; Lambert et al. 2007; Krone et al. 2017). It has also been shown that illegal poisoning of raptors may result in population decline. The example of Red kites (*Milvus milvus*) in Spain is a good example of such a case (Mateo-Tomás et al. 2020).

The existence of conflicts between humans and wildlife is the main reason to use poison to kill animals (Berny 2007; Mateo-Tomás et al. 2012; Bodega 2014), hence this should be the first step to deal with illegal wildlife poisoning. Moreover, more restrictive and effective laws are urged by many authors as crucial measures to control the use of illegal poison, due to the high incidence of animal poisoning with current laws (Hernández and Margalida 2008; Mateo 2010; Bille et al. 2016; Parvanov et al. 2018). But also, these laws must be enforced correctly, and an appropriate training on environmental laws for public prosecutors, judges, lawyers, and land users is necessary. Additionally, the material and human resources for the prosecution of this crime must be allocated by the authorities (Ruiz-Suárez et al., 2015; Ntemiri et al. 2018; Silva et al., 2018).

Despite the laws, it has been demonstrated that prohibition of a product does not prevent it to be used to poison animals. However, the origin of these products is unclear, although old stocks, chemists or veterinarians could be the suppliers (Martínez-Haro et al., 2008). In addition, legally available products such as AR cause most of the acute poisonings in fauna, probably because they are easily available at the supermarkets and widely used (Berny et al., 2010; Mateo 2010). This scenario leads researchers to focus on product regulation, distribution and professional use, and also on the control of banned chemical stocks (Martínez-Haro et al. 2008; Ruiz-Suárez et al., 2015). The products most frequently used in illegal baits are those with a low lethal dose. Therefore, some measures suggested are to reduce the concentration of the legal pesticides and to sell products with high lethal doses (Martínez-Haro et al., 2008). The implementation of educational programs and canine teams to look for baits and dissuade poisoners are other measures recommended (Ruiz-Suárez et al., 2015; Ntemiri and Saravia 2016; Silva et al. 2018). In this sense, the EU Action Plan, 2015 to prevent illegal poisoning of wildlife made a complete list of suggestions to improve the control over legal substances used as poison and make them less available. This included actions in marketing, national legislation, setting up a system of obligatory prescription at the point of sale, and gathering detailed information in the distribution point about the amount purchased and final use of the substance, and other specific information. Regarding banned products, the EU Action Plan, 2015 also established strategic lines including a removal program of these substances, and an inspection, surveillance and control plan after the removal deadline is over.

Coming back to the example of AR, these products are frequently involved in incidental cases due to a misuse or secondary poisoning, mostly because of their widespread use to control rodent population (Lambert et al. 2007; Sánchez-Barbudo et al. 2012; Ruiz-Suárez et al. 2014), but also because of their high persistence in organs and tissues of poisoned rodents (Gray et al., 1994). These AR can also persist in carcasses, presenting a risk of causing tertiary poisoning (Valverde et al., 2020a). Controlling populations of plagues (voles and rodents) by combining mechanical traps (Thomas et al. 2011; Coeurdassier

et al. 2014) and biological and chemical tools, could help to reduce chemical control (Thomas et al., 2011). Moreover, the prohibition of chemical control in areas where biodiversity conservation is a priority over other issues should be considered (Coeurdassier et al., 2014). The addition of some repellents or the incorporation of an emetic substance in the commercial product are other measures carried out to avoid primary poisoning in non-target species (Martínez-Haro et al., 2008).

In order to support current and future regulations, it is important to carry out different toxicovigilance and risk assessment studies, to reinforce the knowledge of the number of illegal poisoning cases and the substances involved in these crimes (Elliott et al., 2008; Mateo, 2010; EU Action Plan, 2015; Bille et al., 2016; Silva et al., 2018). For this purpose, many researchers and institutions/projects have suggested the creation of a network to communicate and share information between European countries about toxicovigilance, to identify each case of poisoning, and to enhance the knowledge about wildlife poisoning cases (Motas-Guzmán et al., 2003; Elliott et al. 2008; Guitart et al. 2010; Mateo 2010; EU Action Plan 2015; Silva et al., 2018; COST CA16224).

The COST Action European Raptor Biomonitoring Facility (ERBFacility; CA16224) aims to create a European network for contaminant biomonitoring in raptors (birds of prey). In this context, a Short-Term Scientific Mission (STSM) titled "Developing a Network of Analytical Labs and Government Institutions" and held by Irene Valverde was carried out in the National Veterinary School of Lyon (VetAgro Sup) hosted by Prof. Philippe Berny (15 September 2019-15 December 2019), and co-supervised by Prof. Antonio J. García-Fernández and Dr. Silvia Espín (University of Murcia). This article presents the results of the STSM aiming to create a network, focused on veterinary forensic toxicology laboratories, and to start a communication between the laboratories in the fight against wildlife poisoning, specially focused on raptors.

Material and methods

To start with the creation of the European Network, a questionnaire was designed (Figure S1) and sent to different laboratories and institutions in Europe, and the data gathered is presented and discussed.

An email account (toxlabnetwork@hotmail.com) was created in the web www.outlook.com to start the communication with the laboratories. The questionnaire was developed using the website SurveyMonkey® (https://www.surveymonkey.com/).

Contact emails from potential laboratory candidates were obtained from different sources, including: i) internet searching using combinations of keywords (i.e., laboratory, forensic, toxicology, wildlife, veterinary, Europe); ii) contacting toxicology laboratories/departments in European veterinary faculties; iii) asking for known laboratories in different European countries to the members of the ERBFacility COST Action; and iv) personal contacts. In this sense, during the Working Group 2 Workshop on risk assessment of AR in European raptors, held in Madrid in April 2019, and the Working Groups 1 and 2 meeting on poisoning of raptors in Europe held in Bucharest in November 2019, participants provided additional contacts to the list.

On 17th October 2019, an email providing the link to the questionnaire was sent to 118 laboratories, but 6 could not reach the recipient due to some error in the email address. The period given to the candidates to respond the questionnaire was 3 weeks and reminders were sent on a regular basis.

The questionnaire had a total of 39 questions grouped by different topics (i.e., laboratory information, species, wildlife species, raptors, necropsy and necropsy protocol information, analytical information, laboratory activities, legal cases, funding and other information). All questions had closed answers with either one option or multiple choice. A gap called "Others (Please specify)" was also provided in some questions. Moreover, according to the answers, the candidates were redirected to a different block of questions. The questions were mainly focused on wildlife and domestic animal poisoning. However, two questions were exclusively focused on raptors samples. The first question was:

Does your laboratory work with veterinary forensic toxicology? If a respondent answered "No", the questionnaire was finished and the email address was saved, and if the answer was "Yes", the questionnaire continued. The diagram of the questionnaire is presented in Figure S1.

When the established deadline arrived, the results were compiled and studied. The analysis of the data was carried out using Microsoft Excel (2016).

Results and discussion

A total of 28 replies (25% of the questionnaires sent) were received (Figure 1). However, total numbers may vary along the article because some laboratories did not reply to all the questions. From the total replies, 9 (32%) laboratories answered "No" to the first question indicating that they do not work on veterinary forensic toxicology, while 13 (46%) laboratories completed the whole questionnaire and 6 (21%) sent uncompleted questionnaires. There were no responses to the questionnaire from some countries (i.e., Bulgaria, Finland, Hungary, Ireland, Latvia, Poland, Russia, Slovenia, Belgium, Luxembourg, Denmark, Bosnia and Herzegovina, and Sweden; in yellow in the map of Figure 1) and no contacts were found for the countries in grey in Figure 1 (Austria, Belarus, Czech Republic, Lithuania, Moldova, Montenegro, Slovakia, Ukraine), thus we are unaware of any laboratory focused on veterinary forensic toxicology in those countries. Therefore, there is a gap of information on veterinary forensic toxicology for part of Europe (mainly northern and eastern Europe) due to uncomplete questionnaires (some questions were not answered) and the lack of contacts/responses from certain countries.

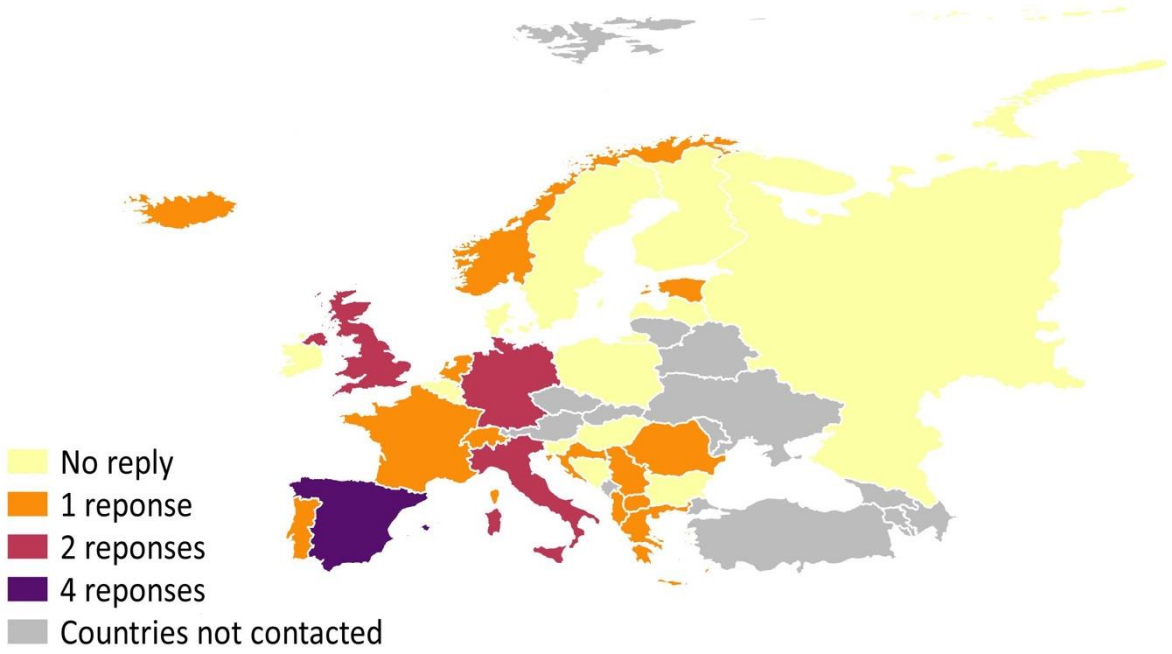

Figure 1. Number of replies per country from contacted laboratories in Europe.

A total of 19 (68%) laboratories indicated that they work on veterinary forensic toxicology in Europe and agreed to participate in the European network, are located in the following 13 countries (number of laboratories per country in brackets): Albania (1), Croatia (1), Estonia (1), France (1), Germany (1), Greece (1), Italy (2), North Macedonia (1), Portugal (1), Romania (1), Serbia (1), Spain (4), United Kingdom (UK) (3) (Table 1; Figure 2). Throughout the text, we refer to the different laboratories by their country, except for those countries with more than one laboratory. In that case, we will mention the laboratory name.

Table 1. List of laboratories in Europe working on veterinary forensic toxicology who participated in the study (n=19)

Country	Lab name
Albania	Centre for Wildlife Investigation and Health, Faculty of Veterinary Medicine, Agricultural University of Tirana (CWIH)*
Croatia	Laboratory of Pathology, Croatian Veterinary Institute, Poultry Centre (LP)*
Estonia	Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences (EULS)*
France	Toxicology Laboratory (ToxLab), Vetagro Sup, Veterinary Campus
Germany	Ludwig-Maximilians-University of Munich, Faculty of Veterinary Medicine, Institute of Pharmacology, Toxicology and Pharmacy (LMUM)*
Greece	Toxicology lab, Department of toxicology, residues and environmental contaminants, Ministry of Development and Food (TL)*
Italy	Centro di Referenza Nazionale per la Medicina Forense Veterinaria Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana "M. Aleandri" (CRNMFV)*
Italy	Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe)
Macedonia	Faculty of Veterinary Medicine Skopje (FVMS)*
Portugal	Laboratório de Histologia e Anatomia Patológica da Universidade de Trás-os-Montes e Alto Douro (LHAP)*
Romania	Animal Behaviour and Ecotoxicology research group (ABERG)*
Serbia	Department of Drug Analysis and Veterinary Toxicology, Scientific Veterinary Institute Novi Sad, Novi Sad (DDAVT)*
Spain	Instituto de Investigación en Recursos Cinegéticos (IREC-CSIC-UCLM REC)
Spain	Service of Toxicology and Forensic Veterinary, University of Murcia (STVF-UM)
Spain	Servicio de Toxicología Clínica y Analítica (SERTOX-ULPGC), University of Las Palmas de Gran Canaria
Spain	Veterinary Analytical Toxicology Laboratory, University of Extremadura (UNEX)*
UK	Agri-food and Biosciences Institute (AFBI)
UK	Fera Science Ltd (Fera)
UK	Science & Advice for Scottish Agriculture (SASA)

*Acronyms have been created when they were not available

Among the laboratories working with veterinary forensic toxicology, 14 (74%) laboratories work on both research and external cases (2 from Italy, 4 from Spain, 2 from UK - Fera and SASA (for full laboratory names see Table 1) and 1 from Portugal, France, Serbia, Albania, Estonia and Croatia). Three (16%) laboratories work only with external cases (Germany, Greece and 1 from UK – AFBI), while 2 (11%) laboratories carry out only research work (Romania and Macedonia). Fourteen (74%) laboratories work with domestic animals and wildlife samples, whereas 3 (16%) laboratories work only with wildlife samples (Romania, Albania and Estonia).

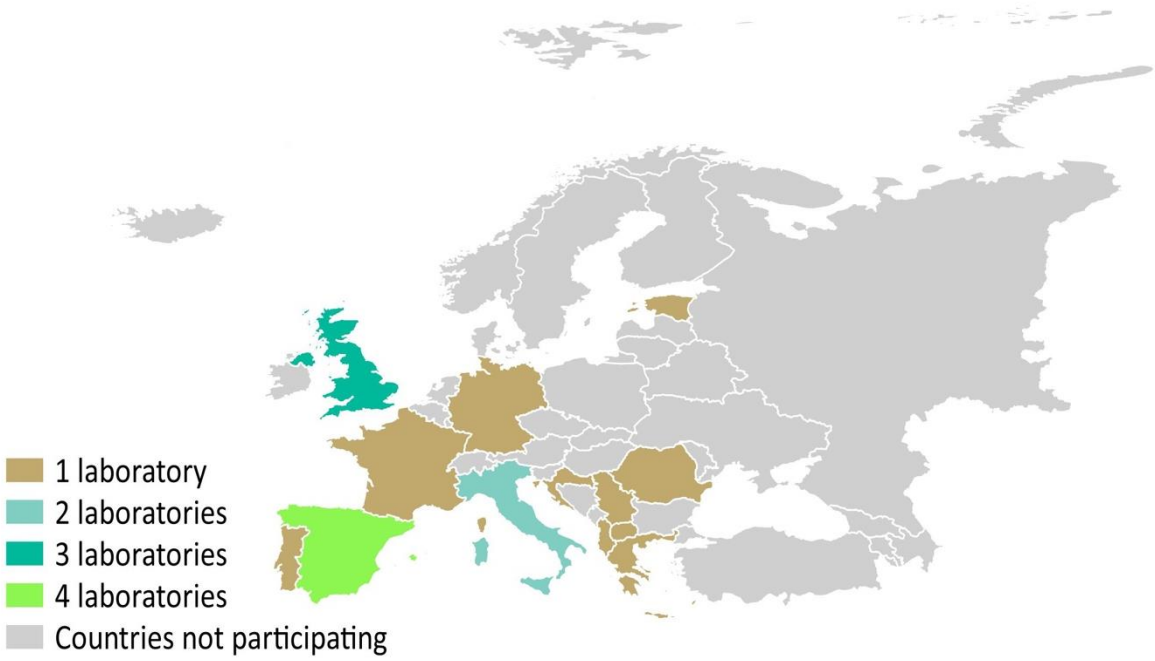

Figure 2. Number of laboratories per country interested in participating in the European network (n=19).

Domestic and wildlife groups and raptor species.

Regarding wildlife, 18 (95%) laboratories receive samples from raptors and other animal groups such as other birds, reptiles, fish, bees and mammals (Figure 3). Many of them also receive baits for analysis. The laboratory from Romania is the only one that does not work with raptor samples, but it receives samples from other birds, fish and aquatic invertebrates.

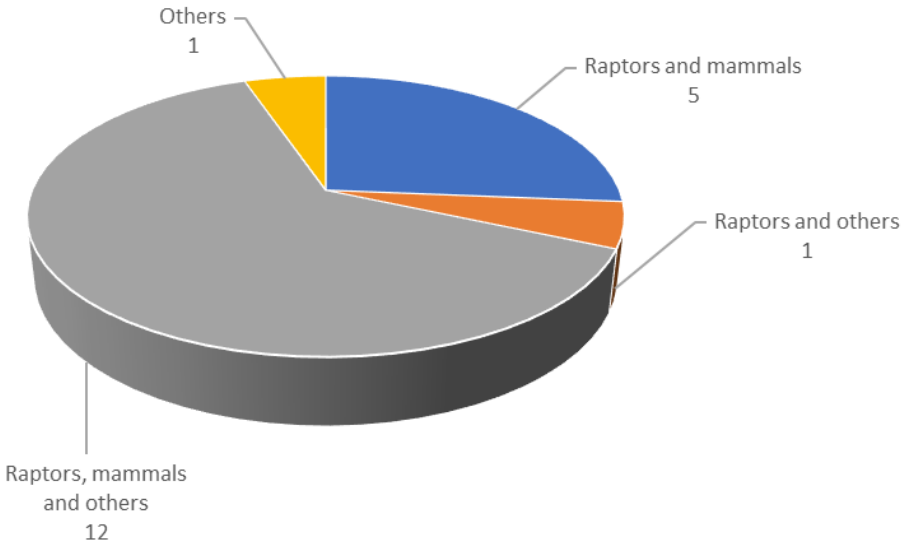

Figure 3. Wildlife species analysed in participant laboratories.

Fourteen of the most common raptor species in Europe were listed in the questionnaire to estimate the number of raptor specimens received per species and year in each laboratory (Table 2). The Common buzzard (*Buteo buteo*) (n=15, 94% laboratories), the Eurasian sparrowhawk (*Accipiter nisus*) (n=12, 75%), the Red kite (*Milvus milvus*) (n=11, 69%), the Golden eagle (*Aquila chrysaetos*) (n=11, 69%) and the Northern goshawk (*Accipiter gentilis*) (n=11, 69%) are the raptor species most frequently received per year in the participant European laboratories responding this question (n=16). The Little owl (*Athene noctua*) is the raptor species less commonly received (n=7, 44%).

Table 2. Number of laboratories in Europe receiving raptor species and number of individuals received per year (n=16)

	Not received	Received	Individuals/year			
Species			<5	5-20	20-35	>35
Buteo buteo	1	15	3	6	4	2
Accipiter nisus	3	12	9	3	0	0
Accipiter gentilis	5	11	9	2	0	0
Aquila chrysaetos	5	11	7	2	0	0
Milvus milvus	2	11	4	5	2	0
Falco peregrinus	5	10	7	3	0	0
Falco tinnunculus	4	10	7	2	1	0
Tyto alba	5	10	6	4	0	0
Bubo bubo	6	9	8	0	0	1
Gyps fulvus	5	9	2	4	1	2
Strix aluco	4	9	6	3	0	0
Circus pygargus	6	8	8	0	0	0
Milvus migrans	6	8	5	2	1	0
Athene noctua	8	7	6	1	0	0

Note: in general, 16 responses were received, but in some cases the sum of not received and received samples is lower than 16 because some laboratories did not respond for some species.

The raptor species most frequently received in the laboratories is the Common buzzard (all laboratories except for North Macedonia) probably due to their widespread distribution in the western palearctic. Moreover, this species is an active hunter and a facultative scavenger, which makes them susceptible not only to contaminants accumulated in trophic chain, but also to primary and secondary poisoning, for example, AR or lead from ammunition sources. In fact, due to both its distribution and diet, Common buzzard has been suggested as be a good key species in pan-European biomonitoring studies (Schindler et al. 2012; Badry et al. 2020).

Compounds, matrices and methods

The groups of most analysed compounds by the participant laboratories are AR (n=15, 83%), carbamates (n=15, 83%) and OC (n=15, 83%). Figure 4 represents which group of compounds is analysed per country. Regarding detection in raptor poisoning cases in the European laboratories, carbamates, AR and OP were the group of compounds most frequently detected. This is consistent with

the literature on poisoning cases (Motas-Guzmán et al., 2003; Berny et al. 2010; Guitart et al. 2010; Chiari et al. 2017; Parvanov et al. 2018; Uros and Andevski 2018; Grilo et al. 2021).

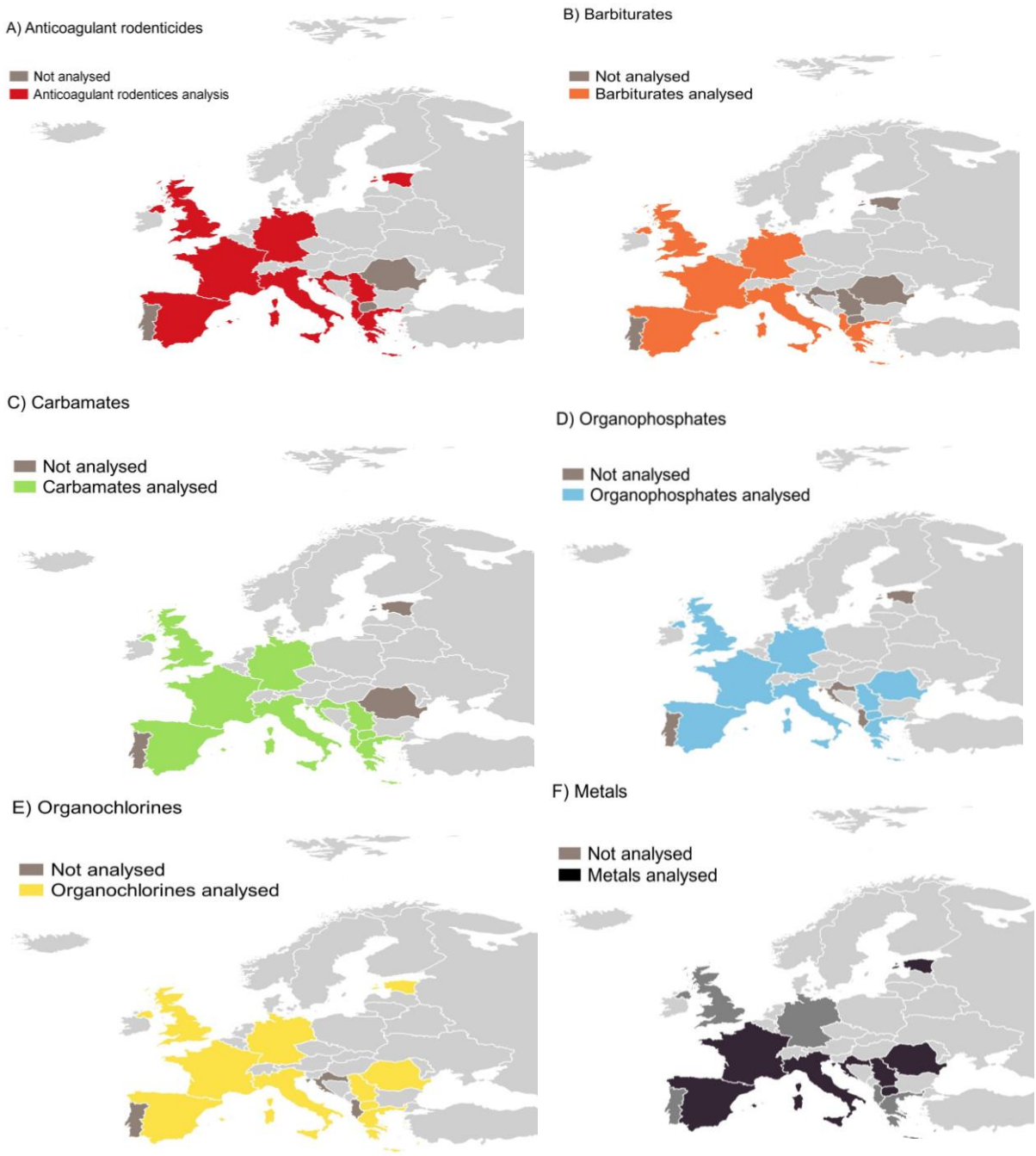

Figure 4. Group of compounds analysed in each country.

Barbiturates are pharmaceuticals widely used in veterinary medicine and they are involved in secondary poisoning (Wells et al. 2020; Herrero-Villar et al. 2021). Nevertheless, our results showed that few laboratories analysed them in

Europe (n=8, 44%), being the group of compounds less frequently analysed (Figure 4). Other compounds analysed but in a smaller number of laboratories (n=8, 44%) are: neonicotinoids (imidacloprid), pyrethroids, deltamethrin, veterinary pharmaceuticals (antibiotics, Non-steroidal anti-inflammatory drugs (NSAIDs), hormones, paracetamol, benzodiazepines, levamisole, etc.), pyrogallol, colchicine, phosphine, cyanides, brucine and ethylene glycol.

Some compounds such as glyphosate and ethylene glycol require further attention since they are barely mentioned in the literature (Modrä and Svobodová, 2009; Berny et al., 2010; Uros and Andevski, 2018). Only two (11%) laboratories analyse glyphosate (IREC-CSIC-UCLM from Spain and Fera from UK).

According to poisoning reports in the field, most laboratories have developed techniques to detect the compounds most frequently used to poison animals. Table 3 shows the matrix and the analytical methods used to analyse each compound group in the European laboratories participating in the present study. Number of laboratories not analysing each compound group is also shown.

Table 3. Number of laboratories analyzing different compound groups by matrix and analytical methods used to analyze each compound group (n=number of laboratories providing response).

		AR [a]	Barbiturates	Carbamates	Pharmaceuticals	Metals	Metaldehyde	OC [a]	OP [a]	Strychnine	α-Chloralose	Others
	Not analysed (n=18)	3	9	3	9	6	6	3	4	7	7	
Matrix (n=14)	Blood	7	5	6	5	10	3	5	5	4	4	5
	Plasma	4	3	4	6	4	3	4	4	3	3	3
	Gastric content	6	9	14	9	7	12	10	13	10	9	8
	Kidney	5	3	5	5	10	3	11	9	7	6	8
	Liver	12	6	11	7	12	3	11	9	7	6	8
	Baits	11	7	13	8	8	11	11	12	10	10	7
Methods (n=14)	HPLC [b]-UV[b]/DAD[b]/Fluo [b]	5	0	1	NR	NA	0	0	0	1	0	NR
	LC-MS-MS[b]	7	1	9		NA	3	1	4	6	4	
	GC [b]	0	0	0		NA	0	2	0	0	1	
	GC-MS	1	8	5		NA	6	11	11	6	5	
	AAS[b]	NA	NA	NA		6	NA	NA	0	NA	NA	
	ICP [b]/ICP-MS	NA	NA	NA		7	NA	NA	0	NA	NA	
	Others	2	0	1		2	1	0	0	0	0	

NA: not applicable; NR: This information was not required in the questionnaire

[a] AR: Anticoagulant rodenticides; OC: Organochlorines; OP: Organophosphates

[b]AAS: Atomic absorption spectroscopy; DAD: Diode-Array Detector; Fluo: Fluorescence; GC: Gas chromatography; HPLC: High Performance Liquid Chromatography; ICP: Inductively Coupled Plasma; MS: Mass spectrometry Detector; UV: Ultraviolet Detector.

Figure S2 represents the specific compounds analysed within each compound group in the different laboratories. Both Table 3 and Figure S2 show results from 18 laboratories.

Baits, gastric content, and liver are the matrices most frequently used to analyse poisoning substances among the respondents. The three matrices are the preferred tissues for the detection of common substances (Berny 2007), since they are linked with oral exposure, which is the most common route of exposure for animals (Mineau and Tucker, 2002; Giorgi and Mengozzi, 2011). After ingestion, the substances are absorbed and distributed through the body via

the blood. Concentrations in the blood represent a short-term exposure and they usually have short half-lives. Thus, blood is a useful sample in live animals, while it is not such a good matrix in dead animals (Mateo et al., 2013; Espín et al., 2016). In addition, the liver is the main metabolizing and in many cases accumulating organ (Watt et al. 2005), which will allow us to confirm that the substance has been absorbed from the ingesta (Thomas, 1999). Concentrations in tissues like the liver, determine medium or long-term exposure (Espín et al., 2016). Although the choice of target matrix should be determined by the toxicokinetic and toxicodynamic of the substances (García-Fernández 2014), tissues which accumulate the highest contaminant concentrations are sometimes analysed in reference to the target organ (Espín et al., 2016).

In poisoning cases, the substances most commonly involved are carbamates and OP which are quickly metabolized in the body, so the use of gastric content and liver as target sample is useful (Mateo et al. 2013). In addition, baits will help to identify the compound during the analysis because it is likely to be found at high concentrations (Motas-Guzmán et al. 2003; Mateo et al. 2013). Visual inspection of the gastric content can help to detect the compounds before the analysis (e.g., by the presence of granulated material or coloured content) and to link a bait with a poisoning event (Cenerini et al. 2012).

On the contrary, plasma and kidney are not very often analysed (Figure 5). Plasma, like blood, cannot be obtained from dead animals most of the time so it is only used for diagnostic purposes in live animals. Nevertheless, plasma concentrations of some compounds like AR can be very good predictors of clinical poisoning of raptors (Murray, 2020). Although we have collected information about the main samples used to diagnose poisoning, sometimes less suitable samples are available because of the state of decomposition of carcasses. Martínez-López et al. 2006 found strychnine in fragments of the remaining tissue adhering to the vertebral column and ribs, from the area corresponding to the anatomical location of the liver and stomach.

To sum up, matrices and analytical techniques used to analyse the same compounds are very diverse. This could be a methodological issue to harmonize among laboratories during the creation of the network.

Coinciding with bibliography, and due to their chemical and pharmaceutical properties (Espín et al. 2016; Valverde et al. 2021), AR were found to be mostly analysed in liver and baits by LC-MS-MS, except in the laboratory from Greece, where they are analysed with GC-MS technique (Table 3). Among them, bromadiolone (93%) and brodifacoum (87%) were the most frequently analysed (Figure S2), probably because they are usually found in wildlife poisoning and widely used to control rodent pests (Berny and Gaillet, 2008; Langford et al., 2013; Valverde et al., 2021).

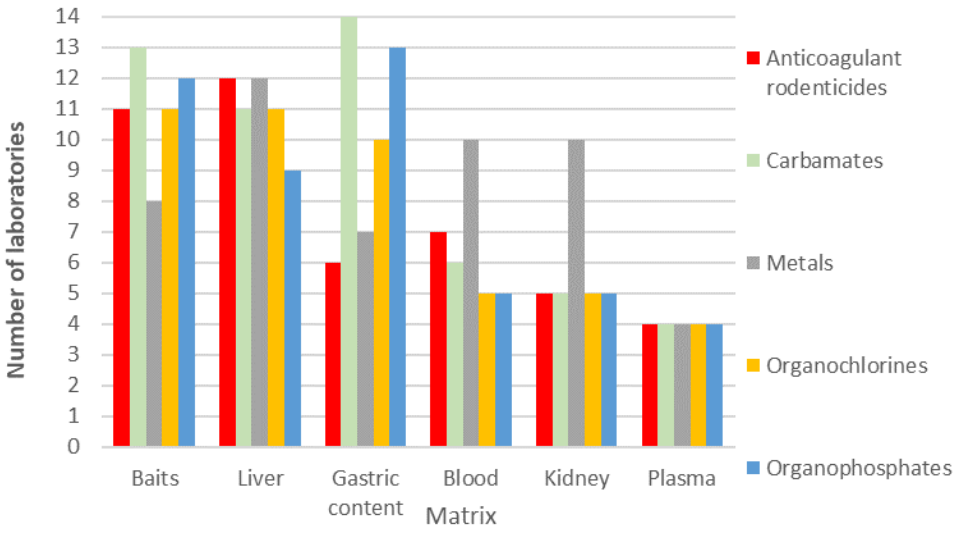

Figure 5. Matrices used in veterinary toxicology to analyse the main groups of compounds in poisoning cases (n=18 laboratories).

According to the bibliography (Espín et al., 2016) and to this questionnaire, the most common matrices to analyse carbamates, OP, metaldehyde, strychnine and α-chloralose are gastric content and baits, followed by liver. Carbamates are mostly analysed by LC-MS, while OP and metaldehyde are mainly analysed by GC-MS, and both instruments are similarly used for strychnine and α-chloralose (Table 3). Carbofuran (100%) is the carbamate most frequently analysed, and chlorpyrifos (100%) and diazinon (93%) are the OP most analysed. (Figure S2). Carbofuran, together with aldicarb, are by far the

carbamates most frequently involved in poisoning cases (Modrä and Svobodová, 2009; Guitart et al., 2010; Ruiz-Suárez et al., 2015; Ntemiri and Saravia, 2016), despite the fact that both were definitively banned in 2008 and 2007, respectively (Decision 2003/199/EC 2003; Decision 2007/416/EC 2007). Chlorpyrifos has been recently banned (Regulation (EU) 2020/1085 2018) and diazinon was banned in 2007 (Decision 2007/393/EC 2007), both are also involved in poisoning cases (Ruiz-Suárez et al., 2015; Ntemiri and Saravia, 2016). However, diazinon is rarely found.

Organochlorines are more usually analysed in liver and baits by GC-MS, except in the laboratory from Serbia, where they are analysed by LC-MS (Table 3). Lindane (87%) and endosulfan (80%) are the OC most frequently analysed (Figure S2) and also the OCs most detected in cases of poisoning (Martínez-Haro et al. 2008; Hernández and Margalida 2009; Bertero et al. 2020). Organochlorines are usually analysed in liver, fat and brain, but also in stomach content and plasma (Berny, 2007; Espín et al., 2016). In the case of metals, the most frequently used matrices are liver, kidney and blood. In general, they are analysed by ICP/ICP-MS or AAS (Table 3). Lead (Pb) (100%) is the most analysed metal (Figure S2). According to the review by Espín et al., 2016, liver and kidney are the most used tissues to analyse metals, and blood is mainly used to detect high levels of Pb. Although normally animals are non-intentionally poisoned by lead, it is a metal of concern in hunting activities, since birds, mainly scavengers and waterfowl, are highly exposed to the ingestion of lead ammunition (Mateo et al. 1997; Garcia-Fernandez et al. 2005; Guitart et al. 2010; Espín et al. 2014; Berny et al. 2015).

The matrices used to analyze pharmaceuticals are very diverse (Espín et al., 2016), since this group includes many different substances of different classes (e.g., antibiotics, NSAIDs, hormones, benzodiazepines, antiparasitics). Barbiturates are always analysed in gastric content and baits, followed by liver and blood. They are analysed with GC-MS, except in the laboratory SERTOX-ULPGC from Spain, where they are analysed with LC-MS (Table 3). Pentobarbital is the most commonly analysed compound within this group (100%) (Figure S2).

This is the most used pharmaceutical to euthanize domestic animals that may be eaten by scavengers and become a secondary-poisoning source (Wells et al. 2020; Herrero-Villar et al. 2021). Pentobarbital is well detected in gastric content and liver (Friend et al., 1999).

Necropsy

Necropsies are an important step in the study of poisoning cases since they provide much information before the laboratory analysis (Valverde et al. 2020a; 2020b). Table S1 compiles information about necropsy questions. In those laboratories that perform necropsies (n=11, 61%), the main points they focus on when they perform a necropsy are: the anamnesis, the presence of haemorrhages and the nature of gastric content. In 5 laboratories (Portugal, Serbia, Croatia, IZSVe (Italy) and STVF-UM from Spain) more than 100 necropsies per year are performed. Four laboratories provide specific necropsy veterinary forensic training to their staff (Portugal, Estonia and STVF-UM from Spain) (Table S1) and 9 (82%) laboratories have a necropsy protocol. Three (27%) of them never carry out X-Ray (Serbia, Albania and North Macedonia), three (27%) laboratories always do X-Ray because it is part of their protocol (Portugal, Estonia and IREC-CSIC-UCLM from Spain), and three (27%) laboratories do X-Ray when a trauma is suspected (STVF-UM from Spain and 2 laboratories from Italy) (Table S1). Eight (73%) laboratories estimate the date of death, and most of them use the overall status and forensic entomology, but the laboratory in Estonia uses all relevant findings in combination of weather and species biology (Table S1).

The information obtained during necropsies is essential to better study poisoning cases before performing analytical procedures (Brown et al. 2005; Mateo et al. 2013; Valverde et al. 2020b). Proper protocols for collecting information and contextual data in the field and during necropsy, as well as proper sample collection and estimation of carcass decomposition and time of death are essential for a successful resolution of poisoning cases (Mateo et al. 2013; Valverde et al. 2020b; Espín et al. 2021). These are important issues that should be considered to harmonize practices in the future.

Funding and costs

The average cost of toxicological analysis is 50-250 €, and the funding is provided mostly from the governments (Table S2). In the majority of laboratories (78%) funding comes from the government, also combined with NGO and private sources. A notable exception is the UK: the two labs participating in the survey only receive funding from the government. In the laboratory from Romania, funding comes from research projects, and the laboratory from Estonia also receives funding on a "project basis" or "through universities internal resources".

Table S2 details the costs of analysis and the number of compounds analysed, the use of reference material and the accredited labs. In general, laboratories with lower price per analysis (<50€) do not analyse more than 18 compounds, while laboratories with higher prices analyse a larger number of compounds. This may be explained because the development of new analytical techniques implies higher laboratory costs. Moreover, laboratories with prices of 50-250 or >250 € always provide interpretation of the results and legal reports, which also implies more workload, time and experienced personnel.

The origin of the funding may also determine the prices offered by the laboratories. The funding of the laboratories whose prices range between 50 and 250 € in most cases comes from the government, nevertheless, laboratories with <50 € prices are those with private or NGO financial support.

Other information

Different questions about other laboratory routines were also asked in the questionnaire (Tables 7 and 8). Nine laboratories (53%) provide toxicology training to their staff. Most of them (13 laboratories, 76%) are able to process samples from outside of the institution and/or cooperate with other countries. Laboratories from Romania, UK, Italy and Croatia publish data online (see Table S4).

In general, laboratories give results in 15-30 days. Regarding the use of reference materials, most of the laboratories (82%) use at least some analytical

reference material, while three (18%) of them do not have them available. Regarding the laboratories with accreditation (2 from UK and Italy, Serbia and North Macedonia), all of them have quality assurance ISO 17025 (Table S3).

Most laboratories (14 labs, 82%) provide interpretation of the analytical results (Table S4), which may be a helpful tool considering many clients are not specialists in toxicology.

With respect to legal cases (Table S5), 14 laboratories (82%) prepare legal reports. The legislation on animal poisoning in European countries is extensive. There is international legislation such as conventions and treaties (Directive 92/43 1992; Directive 2008/99/EC 2008; Directive 2009/147/EC 2010), and there are laws in each country regulating specifically wildlife poisoning (Bille et al., 2016; Ntemiri and Saravia, 2016). North Macedonia does not have a specific law for intentional animal poisoning. Germany and Italy have the same law for domestic animals and wildlife, and in UK, France, Serbia, Croatia and Spain there is a specific wildlife law. In Estonia there is no specific law, but it is regulated through multiple legislation indirectly, however, some are straighter forward (Figure 6).

Albania, Romania and Greece did not answer this part of the questionnaire. However, the questionnaire did not provide enough information to go in depth into the legislation and its efficacy in each country.

The creation of a coordinated European network may help European countries to keep updated regarding the current products used to poison animals, spatiotemporal differences in their use (e.g., metaldehyde baits are more often reported in southern Italy (De Roma et al. 2018), whereas insecticides and rodenticides are more frequent in northern Italy (Giorgi and Mengozzi, 2011; Chiari et al., 2017).

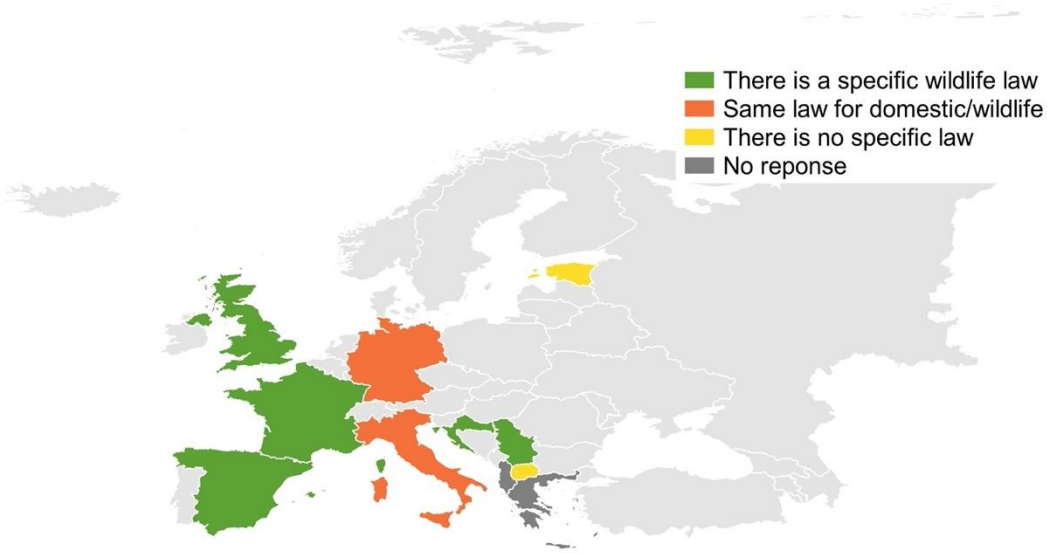

Figure 6. Wildlife legislation.

Finally, in order to avoid a lengthy and tedious questionnaire, technical questions related to sample amount, extraction technique, limits of quantification and additional questions about necropsy findings were not asked. For this reason, a new questionnaire is recommended to obtain detailed information on these issues.

Conclusions and recommendations

Illegal wildlife poisoning is a frequent issue in Europe. To evaluate and fight against these acts, a fluent communication and coordination between laboratories in Europe is required. Therefore, the present study represents a first contact between European laboratories as an initial step to create a European network and compile basic data from a questionnaire to detect strengths and pitfalls that will help to harmonize methodologies and increase pan-European capacities.

Most laboratories participating in the present study, work on veterinary forensic toxicology research and external cases at the same time, which can give a wide overview of the real situation in the field.

The different analytical techniques, samples used, and data collection should be harmonized, and a sufficient communication between laboratories is

needed to create an effective network. All respondents reacted positively to this suggestion.

To continue with the network development, some suggestions are given:

(i) An online platform should be created, with free access to detailed information on each laboratory (e.g., contact, address, analytical techniques available, prices, etc.). Data on poisoning cases should be uploaded/updated regularly by each laboratory. This data should contain, at least, information regarding the place of origin, species, samples and analytical techniques used, detected compound/s, and basic necropsy information (if it is accessible). A simple online necropsy protocol could be elaborated for this purpose to identify the main necropsy findings (see some suggestions at Mateo et al. 2013; Valverde et al. 2020b). Furthermore, a forum could be available to share opinions and seek for assistance in complex cases or for technical purposes to other colleagues in the network.

(ii) Laboratories should analyse, at least, carbamates, OP and AR in suspected poisoning cases using liver, baits and/or gastric content as key samples. If this is not possible, the laboratories could contact other laboratories from the network to send the samples and perform the analyses.

(iii) The compilation of clear protocols explaining how to collect, pack and send samples to other laboratories should be also elaborated.

(iv) A new questionnaire to gather additional information about sample amount, extraction and analytical techniques is needed to improve and harmonize methodologies in Europe.

v) Common analytical work to validate wildlife forensic toxicology analytical procedures as well as development of new / non-invasive samples such as feathers and hair are needed.

All the information gathered in the present study as well as the recommendations provided are a first step to develop a pan-European network of analytical laboratories and Government institutions to fight against wildlife poisoning.

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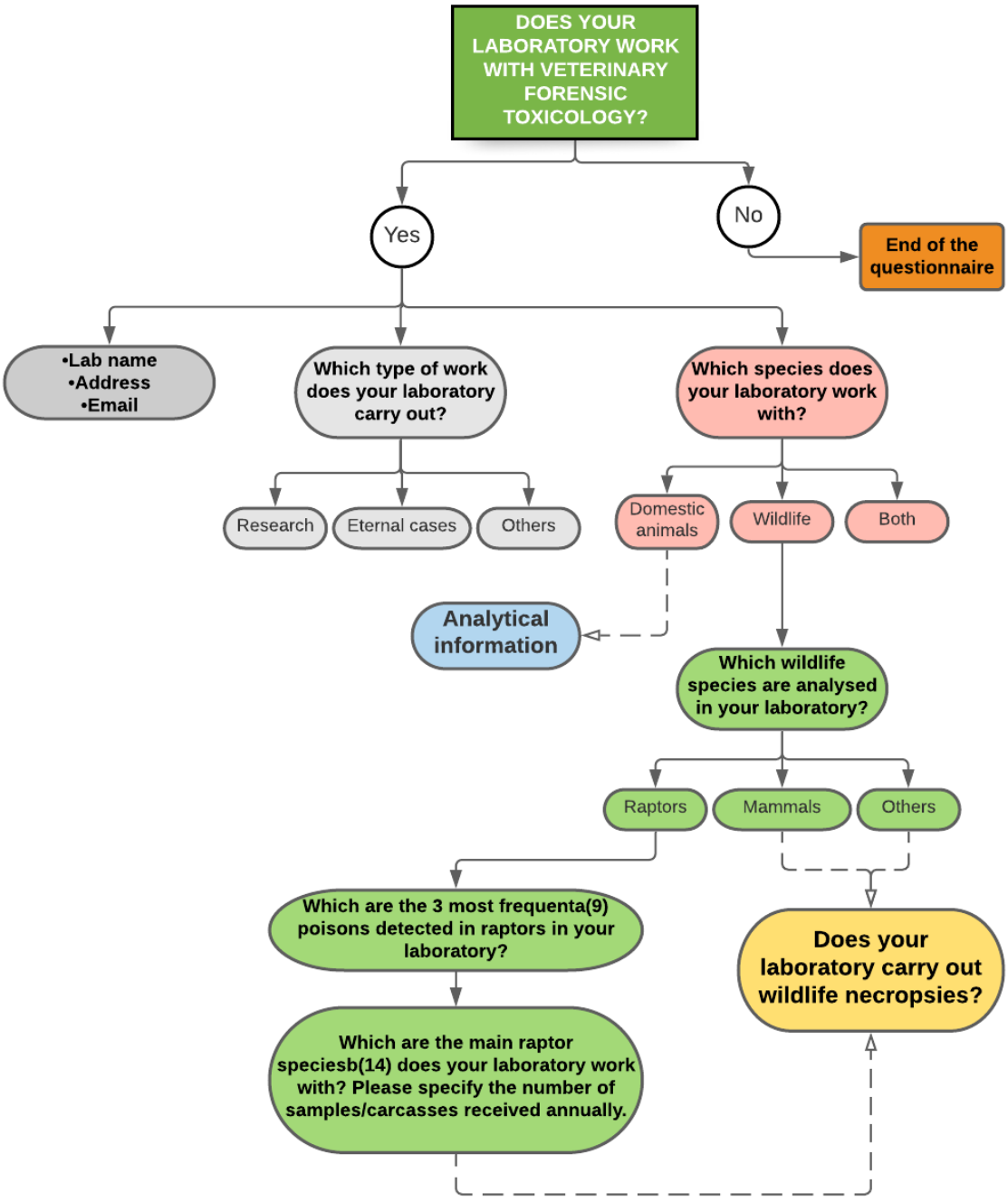
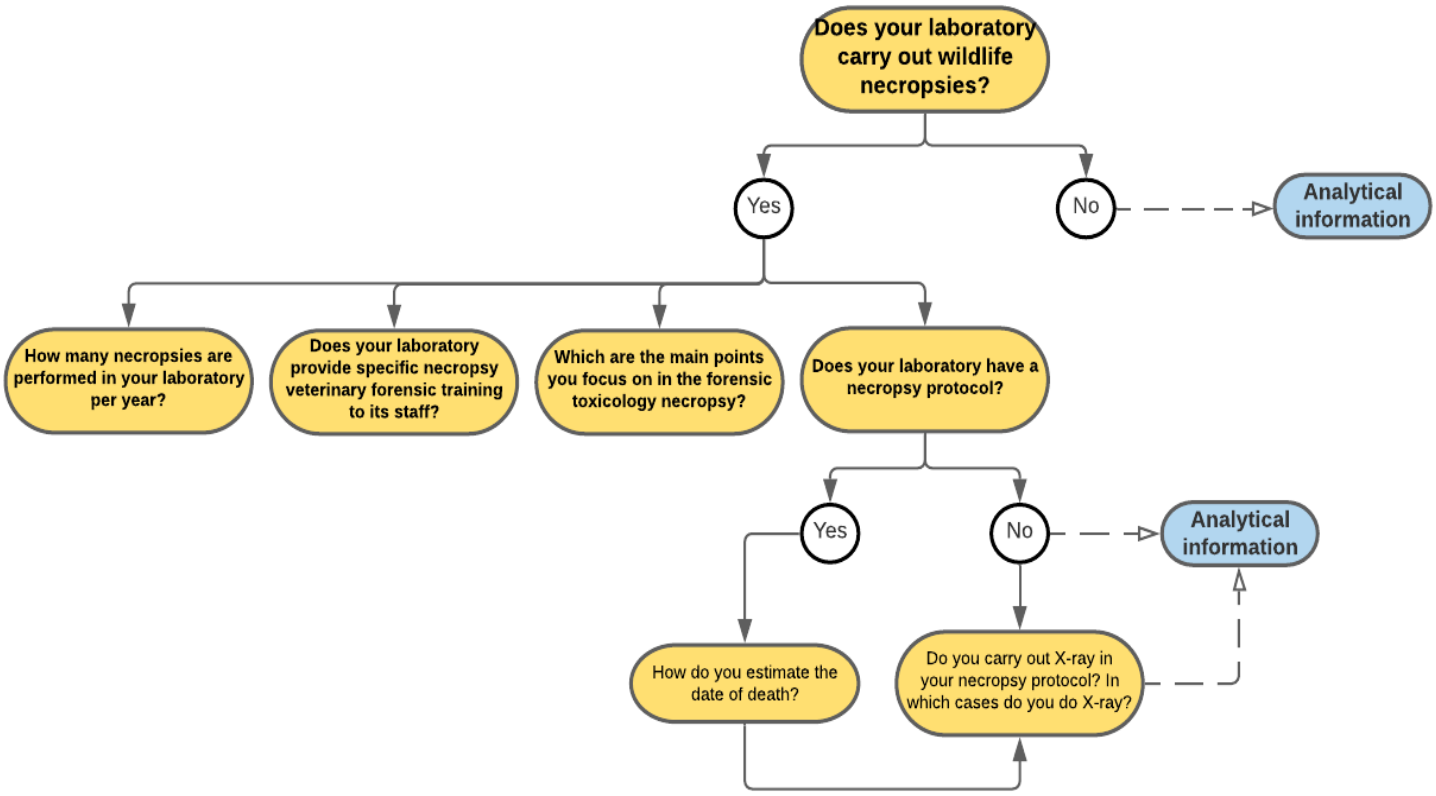
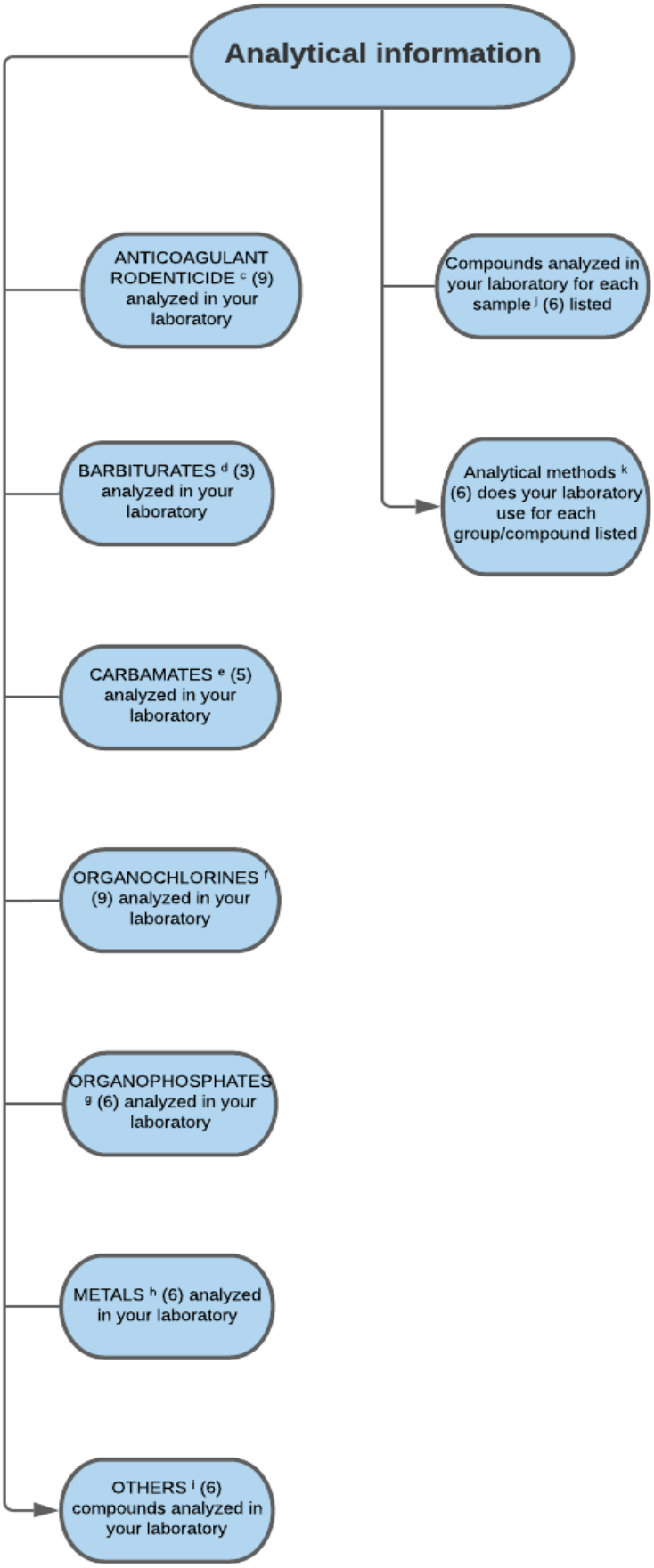
Supplementary Material

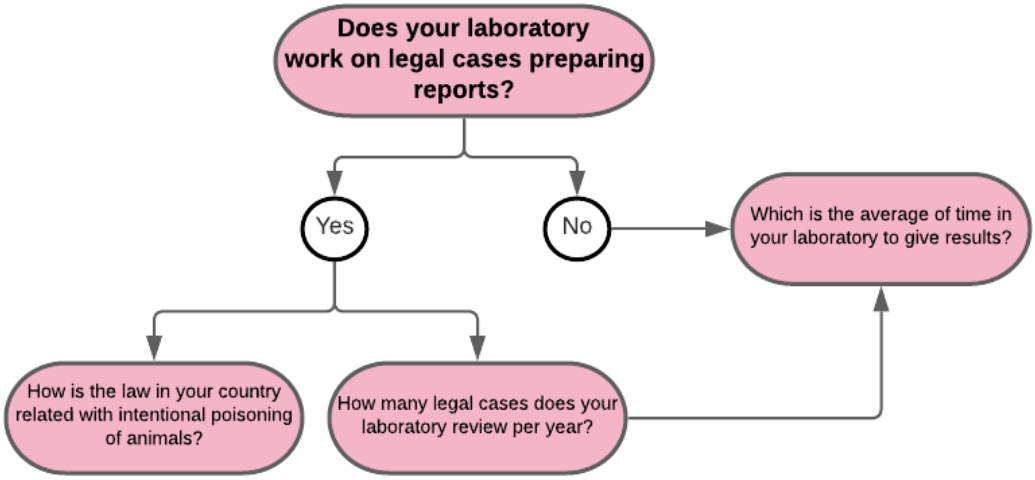
Developing a European network of analytical laboratories and government institutions to fight against raptor poisoning

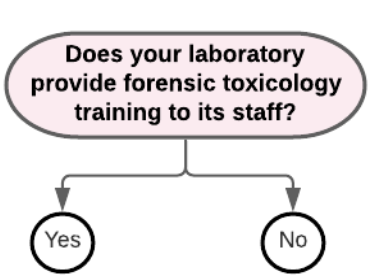
Figure S1. Diagram of the questionnaire.

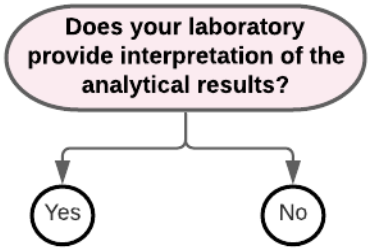


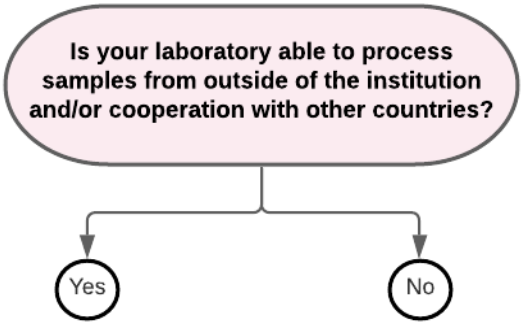

Does your laboratory carry out wildlife necropsies?
Yes
No
Analytical information
How many necropsies are performed in your laboratory per year?
Does your laboratory provide specific necropsy veterinary forensic training to its staff?
Which are the main points you focus on in the forensic toxicology necropsy?
Does your laboratory have a necropsy protocol?
Yes
No
Analytical information
How do you estimate the date of death?
Do you carry out X-ray in your necropsy protocol? In which cases do you do X-ray?

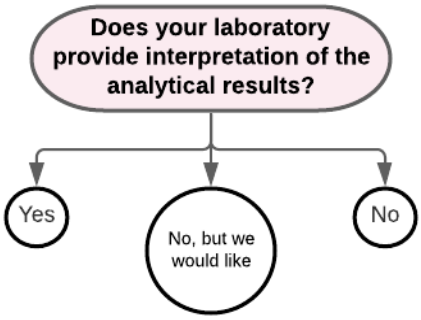

Analytical information
ANTICOAGULANT RODENTICIDE [c] (9) analyzed in your laboratory
BARBITURATES [d] (3) analyzed in your laboratory
CARBAMATES [e] (5) analyzed in your laboratory
ORGANOCHLORINES [f] (9) analyzed in your laboratory
ORGANOPHOSPHATES [g] (6) analyzed in your laboratory
METALS [h] (6) analyzed in your laboratory
OTHERS [i] (6) compounds analyzed in your laboratory
Compounds analyzed in your laboratory for each sample [j] (6) listed
Analytical methods [k] (6) does your laboratory use for each group/compound listed

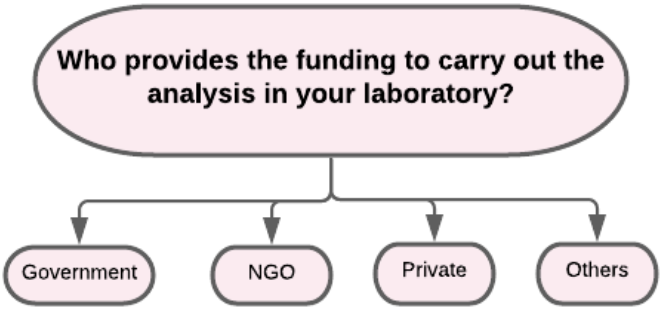

Does your laboratory work on legal cases preparing reports?
Yes
No
Which is the average of time in your laboratory to give results?
How is the law in your country related with intentional poisoning of animals?
How many legal cases does your laboratory review per year?

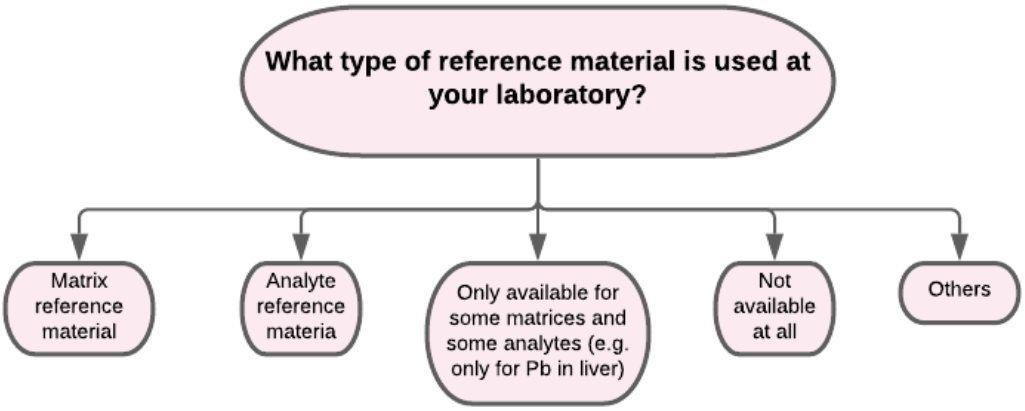

Does your laboratory provide forensic toxicology training to its staff?
Yes
No

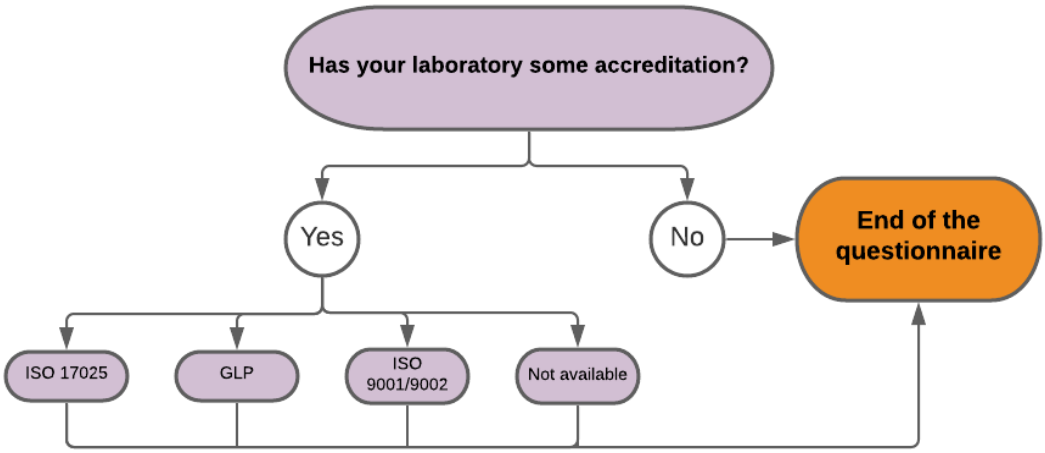

Does your laboratory provide interpretation of the analytical results?
Yes
No


Is your laboratory able to process samples from outside of the institution and/or cooperation with other countries?
Yes
No


Does your laboratory provide interpretation of the analytical results?
Yes
No, but we would like
No


Who provides the funding to carry out the analysis in your laboratory?
Government
NGO
Private
Others


What type of reference material is used at your laboratory?
Matrix reference material
Analyte reference materia
Only available for some matrices and some analytes (e.g. only for Pb in liver)
Not available at all
Others


Has your laboratory some accreditation?
Yes
No
End of the questionnaire
ISO 17025
GLP
ISO 9001/9002
Not available

[a] Anticoagulant rodenticides
Barbiturates
Carbamates
Drugs
Metals
Metaldehyde
Organochlorines
Organophosphates
Strychnine
α-Chloralose
Others

[b] *Accipiter gentilis*
Accipiter nisus
Aquila chrysaetos
Athene noctua
Bubo bubo
Buteo buteo
Circus pygargus
Falco peregrinus
Falco tinnunculus
Gyps fulvus
Milvus migrans
Milvus milvus
Strix aluco
Tyto alba

[c] Brodifacoum
Bromadiolone
Coumafuryl
Coumatetralyl
Chlorophacinone
Diphacinone
Difenacoum
Flocoumafen
Warfarin
Difethialone
Other
[c] Brodifacoum
Bromadiolone
Coumafuryl

[d] Pentobarbital
Phenobarbital
Thiopental
Other

[e] Aldicarb
Carbofuran
Oxamyl
Methiocarb
Methomyl
Carbaryl
Carbosulfan
Bendiocarb
Other

[f] Aldrin
Chlordane
DDE
DDT
Dieldrin
Endosulphan
Heptachlor
Imazalil
Lindane
Other

[g] Chlorpyrifos
Diazinon
Dimethoate
Fenthion
Malathion
Parathion
Other

[h] As
Cd
Cu
Hg
Pb
Zn
Other

[i] Glyphosate
Metaldehyde
Paraquat
Strychnine
α-Chloralose
None
Other

[j] Blood
Plasma
Gastric content
Kidney
Liver
Baits

[k] HPLC-UV/DAD/Fluo
LC-MS
GC
GC-MS
AAS
ICP/ICP-MS
Others

Figure S2. Number of laboratories analysing chemicals within different compound group

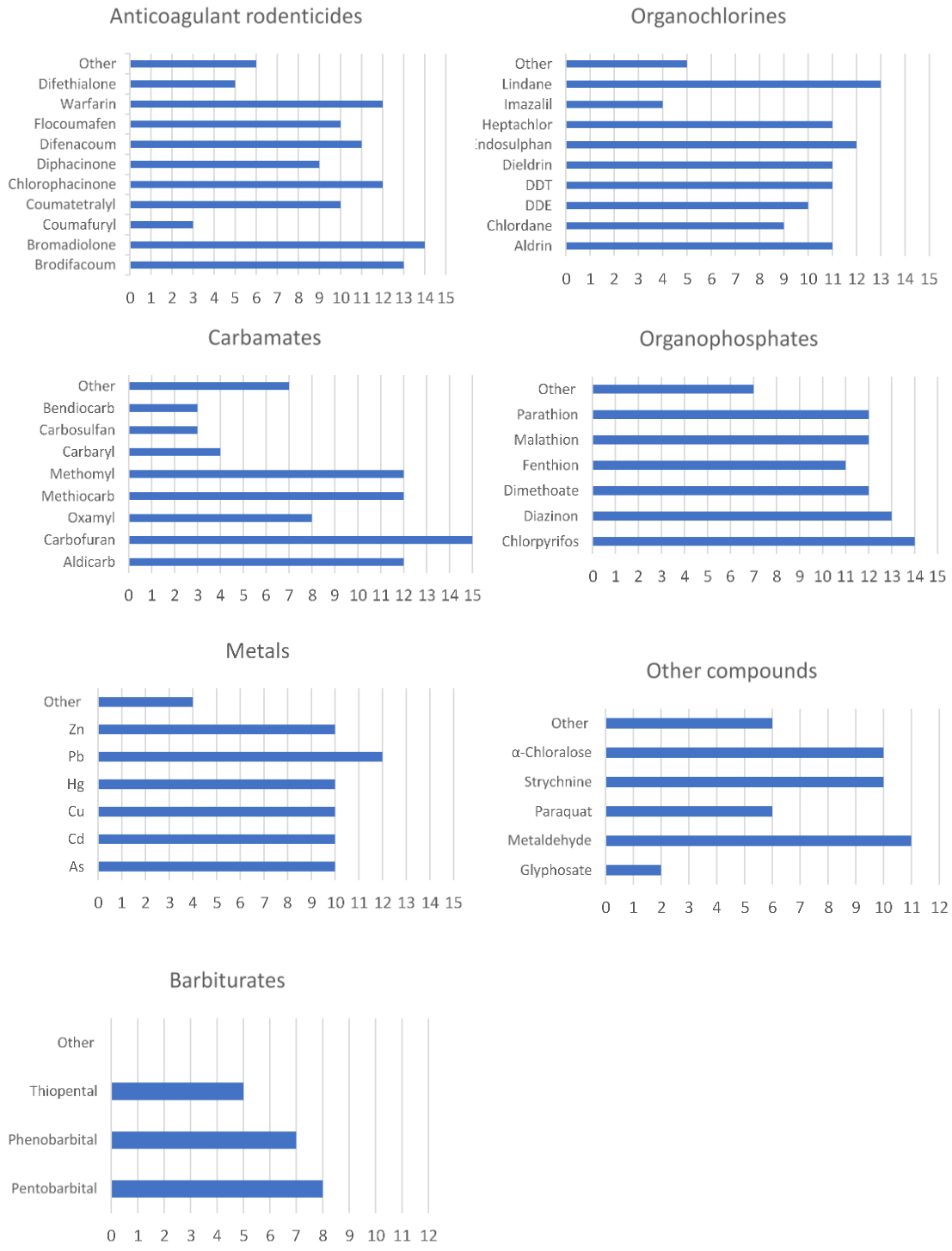


Table S1. Necropsy (n=11) and necropsy protocol (n=9) questions.

How many necropsies are performed in your laboratory per year?		
<30	30-100	>100
3	3	5

Does your laboratory provide specific necropsy veterinary forensic training to its staff?	
No	Yes
7	4

Which are the main points you focus on in the forensic toxicology necropsy?					
Haemorrhages	Trauma	Nature of gastric content	Limbs stiffness	Anamnesis	Others
10	7	10	5	8	6

Do you carry out X-ray in your necropsy protocol?		
Never	Always, it is a part of our protocol	Only when a trauma is suspected like a gunshot
3	3	3

Do you estimate the date of death in your necropsy protocol?	
No	Yes
1	8

How do you estimate the date of death?		
Forensic entomology	Overall status	Others
5	7	1

Table S2. Price and funding. (n=16)

Average price for the analysis		Funding for the analysis	
<50 €	3	Government	14
50-250 €	11	NGO	5
>250 €	3	Private	7
		Others	2

Table S3. Time to give results, reference material used and quality assurance standard questions (n=17)

Which is the average of time in your laboratory to give results?				
15-30 days	1-2 months	>3 months		
12	4	1		
What type of reference material is used at your laboratory?				
Matrix reference material	Analyte reference material	Only available for some matrices and some analytes (e.g., only for Pb in liver)	Not available at all	Others
6	10	4	3	0
What type of quality assurance standard does your laboratory work with?				
ISO 17025	GLP	ISO 9001/9002	Not available	Others
4	1	6	0	0

Table S4. Other information about the laboratory routine questions (n=17 responses)

	No	Yes	No, but we would like
Forensic toxicology training to its staff	8	9[a]	
To process samples from outside of the institution and/or cooperation with other countries	4	13	
Publish data online	7	6[b]	4
Interpretation of the analytical results	3	14	
Accreditation	11	6	

[a] Attendance to several specialized courses, analytical training for poisons, estimation of cause and time of death, use of analytical methodology and analytical techniques, lectures, meetings, external training, master course, teaching forensic science for veterinary students.

Table S5. Legal cases questions

Legal cases preparing reports (n=17)		
Yes	No	
14	3	
How is the law in your country related with intentional poisoning of animals? (n=14)		
There is no specific law	It is the same for domestic and wildlife	There is a specific wildlife law
2	4	9
How many legal cases does your laboratory review per year? (n=14)		
<30	30-100	>100
7	3	5

General discussion

Numerous challenges are inherent to veterinary forensic toxicology in the study of wildlife poisoning, some of which have been addressed in this thesis, focusing on AR. Figure 1 presents a diagram showing some of the main factors that should be considered in a poisoning case, as well as the main difficulties to approach them and the chapters developed under this thesis to solve part of these problems.

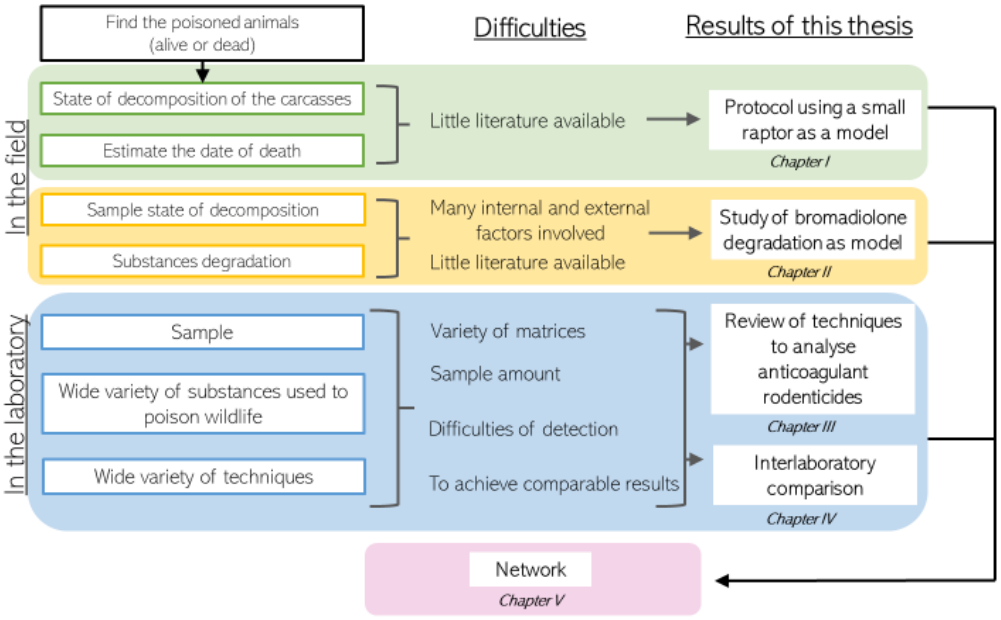


Figure 1. Diagram of the main factors that should be considered in a poisoning case, the main difficulties to approach them and the chapters developed in this thesis to solve part of these problems.

When poisoned animals are found dead in the field, as is frequently the case, this prevents the study of symptoms that could help in the diagnosis of poisoning (Valverde et al., 2021). Moreover, carcasses of poisoned animals are not found fresh but normally in different stages of decomposition (Brown et al., 2005; Cooper, 2013). Despite this, some signs, such as the position of the carcass, the ground around the limbs and especially under the oral cavity if there is bait in the mouth, the presence of vomit, blood, or dead insects around natural orifices of the body, may help to guide the diagnosis of cause of death. In other words, an exhaustive study and a thorough collection of information in the crime scene are essential (García-Fernández et al., 2006), and some manuals have been develped for environmental agents (Fajardo et al., 2015, 2016). However, regardless of the aetiology, the scientific literature generally

refers to the diagnosis of cause of death of animals which recently died, which implies the study on fresh carcasses and a toxicological analysis on fresh samples. Starting at the moment of death, changes in the natural colours, shapes or tissue sizes begin to take place. Some studies have been carried out in mammals about carcass decomposition (Brooks, 2016; Jarmusz & Bajerlein, 2019). However, few information is available on bird carcasses decomposition (Oates et al., 1984). This scarce information urges to enlarge the knowledge about decomposition processes in carcasses of birds, especially in raptor species, which are the main species reportedly affected by poisoning in the field (Guitart et al., 2010; Sánchez-Barbudo et al., 2012; Cano et al., 2016). Moreover, publications rarely report this information and there is a lack of proper protocols to harmonize the classification of the stage of decomposition.

In response, Chapter I in this thesis experimentally evaluated the decomposition of carcasses of Common kestrels under specific environmental conditions, to carefully report all the data that may be needed for future studies. Chapter I also offered printable field and laboratory documentation to help harmonising the collection of data. The field form compiles some basic information of interest to estimate the stage of carcass decomposition, the time of death and some other circumstances related to death of the animal. In many cases, these signs or evidences can help during the police investigation of the crime or accident. Although many parameters are well studied to estimate the time of death in humans such as temperature, entomology, or *rigor mortis*, they have to be extrapolated to other mammals with caution (Oates et al., 1984; Brooks, 2016), and little information is available about the time and factors that affect carcass decomposition in animals, especially in wildlife (Oates et al., 1984). In the estimation of the time of death, the environmental conditions (temperature, humidity, rainfall), the species, the weight or size of the animal, its state of health or presence of wounds, the position and location of the corpse, the presence or absence of food in the gastrointestinal tract, the internal temperature of the corpse and the circumstances of death are parameters that should be considered (Oates et al., 1984; Cooper, 2013; Brooks, 2016). All these factors, together with the *rigor mortis* and forensic entomology, are presented and discussed in the first chapter. In addition, the laboratory document proposes a scoring system, supported by photos, to classify the stages of carcass decomposition in six different categories. This protocol will help standardise methodologies for carcass classification according to the degree of decomposition in small-sized raptors, as well as minimize subjectivity. In addition, it was created and

presented in a user-friendly way for broader audience, forensic veterinarians, researchers, or personnel in charge of carcass collection (Valverde et al., 2020a).

Once the carcass has been collected, and the degree of decomposition and the time of death have been estimated, we should also consider that these processes can modify some chemical structures and alter the concentrations of the toxic compounds in the different tissues of the carcass. Samples arrive at the toxicology laboratories in different stages of decomposition, which may pose difficulties in the detection of the chemical substances and in the correct interpretation of the analytical results. In this sense, during the analysis of the sample, it is possible to detect the primary toxic compound origin of the poisoning, its metabolites and/or its *post-mortem* degradation products (Brown et al., 2005; Berny, 2007; Luzardo et al., 2014). Nevertheless, when reviewing the bibliography about poisoning reports or analytical techniques to detect substances involved in poisoning cases, information about the state of the sample is rarely mentioned (Brown et al., 2005; Valverde et al., 2020a). This may be due to the lack of easy and standardised classification methods and adapted techniques to these sample types. Chapter I can be considered a first approach and could be applied to other species in the future for the standardisation of the classification of the degree of decomposition.

In some poisoning cases, the only proof of the toxic compound without quantifying justified by standardized analyses, together with other signs or evidence, could be enough to give a definitive poisoning diagnosis. However, in other cases, it is necessary to carry out quantitative analyses of the substance and its metabolites and breakdown products in the different tissues in order to confirm a death caused by poisoning and to establish the date of death. For example, legal pesticides can be found in the tissues of dead animals as residual concentrations without presenting a cause-effect relationship (OrdenJUS/1291/2010, 2010). As mentioned above, the decomposition processes may alter concentrations of the chemicals, sometimes showing increased results (for example as consequence of the dehydration process) and others showing decreased concentrations by chemical degradation or *post-mortem* redistribution (PMR) (Valverde et al., 2020b). However, it would be necessary to provide a concentration, which must be appropriately interpreted taking into account not only the nature of the sample but also its stage of decomposition. There are scarce articles regarding these phenomena, and they are absent for raptors. For this purpose, an experimental study was designed in Chapter II using a SGARs, bromadiolone, as a

model in raptor carcasses, in order to evaluate the degradation of this commonly used biocide (Valverde et al., 2020b). This study provides, for the first time, some light about compound degradation in carcasses of raptors in the field. Bromadiolone was selected as the target compound because it is used in the EU as biocide and as PPP and it is the most frequent AR detected worldwide (Christensen et al., 2012; Sánchez-Barbudo et al., 2012; Langford et al., 2013; Ruiz-Suárez et al., 2014). In addition, it has been reported as one of the chemicals frequently involved in secondary poisoning cases (Berny & Gaillet, 2008; Walker et al., 2008; Sánchez-Barbudo et al., 2012; Montaz et al., 2014). A case report by Martínez-López et al. (2006) demonstrated that strychnine could be detected in a Bonelli's eagle in a skeletal reduction state. The results of the bromadiolone degradation study showed that bromadiolone can be detected in liver of very advanced decomposed carcasses of Common kestrels dosed at low levels even fifteen days after death, under the specific environmental conditions of the experiment (mean ambient temperature 30ºC ± 2, min. 24ºC and max. 33ºC; Murcia, SE Spain). Furthermore, bromadiolone concentrations found in liver samples in this experiment were higher than those reported in some biomonitoring studies in Common kestrels (Thomas et al., 2011; Christensen et al., 2012), which demonstrate that these carcasses in the field would represent a risk for secondary or tertiary poisoning, since the bromadiolone concentration in the carcasses (simulating levels in a secondary poisoning) could poison other scavengers predating on them.

Liver was chosen as target sample since it is an organ that remains longer in the carcasses and is a highly metabolically active organ in the body (Vudathala et al., 2014; Espín et al., 2016). Moreover, liver is the target organ of some poisons such as AR affecting the clotting factors (Valverde et al., 2020b). Although more studies should be done in other species and with other compounds under different conditions, these results show that, when a poisoning case is suspected, analytical studies can be done even in carcasses at very advanced decomposition state.

The carcass decomposition (Chapter I) and bromadiolone degradation experiments (Chapter II) presented in this thesis show some weak points that should be considered in future studies. The first factor is mainly related with ethical reasons that do not allow to use a high number of individuals. Therefore, the low number of individuals available cannot provide strong evidence. Moreover, individual-specific conditions and the barbiturate used for euthanasia could influence the decomposition and/or cadaveric fauna activity. However, no studies evaluating this potential effect have been found in

the literature and further studies are desirable to better understand this issue. In addition, there is a lack of literature regarding the behaviour of AR in carcasses in the field and their potential degradation over time, as well as PMR of drugs and *post-mortem* tissue alteration. Finally, the estimation of the time of death must be adjusted according to the conditions of each case. Thus, studies with the same and other species, including more individuals, additional days of decomposition and under different weather conditions should be carried out. Furthermore, other substances frequently used to poison animals may be selected to experimentally evaluate their degradation in the carcasses.

When a sample is received in the laboratory to apply an analytical method, the carcass state of decomposition may be critical not only because of the degradation of the chemical substances, as explained before, but also due to the sample amount available to perform the analysis. During the decomposition, a substantial loss of tissue may happen due to the autolytic process (Brown et al., 2005; Cooper, 2013), or the sample amount may be also affected by predation, since carcasses may remain in the field for a long time until they are found. Moreover, the sample in the laboratory is usually divided into analytical sub-samples to carry out different types of analysis. In addition, a variety of substances may be involved in different wildlife poisoning events (Wang et al., 2007; Cano et al., 2016; De Roma et al., 2018). Therefore, a toxicological analytical technique should be used to analyse a wide number of substances in a small sample amount.

Diverse and heterogenous analytical procedures have been described in the literature to analyse different compounds using different matrices. Hence, a proper compilation and comparison of techniques available is needed to understand and further improve the analytical procedures in wildlife forensic toxicology. As a first step, Chapter III aimed to review the literature available to report the analytical techniques applied for AR detection. Current techniques reviewed to analyse AR mainly use ≤ 3 g or ml of liver or blood, respectively, which can be considered a quiet small sample amount. A detailed database is provided in Chapter III compiling different parameters, including the compound analysed, matrix used, weight or volume of sample, the extraction techniques, recoveries, LOQ and the instrumental methods. This information was also used to score those techniques using the main matrices reported (liver and blood), which were ranked considering the number of AR analysed, sample amount, calibration curve points, recoveries, LOQ and multi-class method. To analyse AR, LLE,

SPE or dSPE using as extractant solvents ethyl acetate, acetone and acetonitrile, respectively, have been reported in liver and blood. Detection of AR has been always carried out by LC, because they are non-volatile compounds at GC temperatures (Imran et al., 2015); however, one article used GC to analyse AR due to the use of an in-injector pyrolysis of bromadiolone at 390°C (Doubková et al., 2017). Liquid chromatography coupled to a tandem mass spectrometry detector is the instrument most frequently used to detect not only the AR but also different metabolites and degradation products. Moreover, some techniques are able to analyse up to 10 different AR simultaneously. Laboratories usually work with baits, gastric content and liver to diagnose poisoning cases. This is because the three matrices are the preferred tissues for the detection of frequently used substances linked with oral exposure, which is the most common route of exposure for animals (Mineau & Tucker, 2002; Berny, 2007; Giorgi & Mengozzi, 2011). However, although blood is a difficult matrix to collect in carcasses (Mateo et al., 2013), it is frequently used for AR analysis in living or recently deceased individuals (Adamowicz & Kala, 2009; Mateo et al., 2013; Bidny et al., 2015).

Analytical methods based only on internal validation may be subject to inconsistencies in performance. Therefore, interlaboratory comparisons are desirable to confirm if different techniques currently applied by laboratories may provide consistent and robust results. For this purpose, Chapter IV aimed to perform an interlaboratory study to compare the characteristics of the analytical procedures applied to detect toxic compounds involved in wildlife poisoning by different laboratories. For this purpose, four of the reference laboratories involved in the *Veneno-No* Life+ Project (www.venenono.org) have participated: STVF-UM, UNEX, IREC-CSIC-UCLM and SERTOX-ULPGC. Laboratories were sent prepared samples (chicken livers) spiked with 11 selected compounds: five AR (SGARs: bromadiolone, brodifacoum, difenacoum, and FGARs: warfarin and chlorophacinone), three highly toxic carbamate pesticides (aldicarb, carbofuran and methiocarb) and three OP pesticides with alkyl-phosphorothioate structure (diazinon, chlorpyrifos and parathion). These aforementioned pesticides were selected to represent the three main compound groups based on their frequency of use in wildlife poisoning cases. The interlaboratory comparison ensures the quality of procedures and results, and it allows to harmonise methodologies and

maximise reliability and comparability of data. According to the laboratory comparison carried out as part of this thesis, AR, carbamates, and OP were mainly extracted with a modified QuEChERS methods. The sample amount used in the techniques in this study were 3 and 2 g in STVF-UM and UNEX, respectively, and 1 g in SERTOX-ULPGC and IREC-CSIC-UCLM. Although STVF-UM used all the sample available for this study (3 g), its technique for AR is adapted to 1 g of sample. Taking into account the solvent:sample ratios SERTOX-ULPGC and STVF-UM were the techniques using lower volume of solvent. AR were always analysed with LC (using a C18 or C8 column), and carbamates were analysed with both LC and GC, depending on the laboratory, while OP were analysed with GC (with a (5%-phenyl)-methylpolysiloxane column). Considering the sample amount, the extraction steps and volume of solvent and other reagents needed during the extraction, and the total run time during chromatography, SERTOX-ULPGC can be considered the fastest and most economic and environmentally friendly technique.

According to the parameters assessed, CVs for repeatability were ≤ 20% in all methods and for all compounds and, in general, good recoveries within the range of 70–120% (SANTE/12682/2019) were obtained. The techniques from UNEX and STVF-UM had recoveries within this range for all carbamates and OP, the technique from IREC-CSIC-UCLM also showed recoveries within this range except for aldicarb (62.5%) and the technique from SERTOX-ULPGC had recoveries outside that range for some pesticides (99-135%). Regarding the techniques used for AR, all recoveries were within 70-120% except for some compounds in the techniques used by SERTOX-ULPGC (106-127%) and STVF-UM (44-67%). The LOD and LOQ stablished in these methods are in all cases low enough to detect and quantify the compounds evaluated in this study in cases of poisoning. In this chapter, the laboratory performance was expressed in terms of z-score in accordance with ISO13528:2015. The interpretation of the z-score is done according to ISO 17043:2010: |score| ≤ 2 indicates a satisfactory result, 2 < |score| < 3 means questionable result, and |score| ≥ 3 indicates an unsatisfactory result. Most of the z-scores in this interlaboratory study were below 2, indicative of satisfactory results. Considering that the techniques

compared in this study use different extraction methods and chromatographic conditions, in general they all obtained satisfactory results based on z-score classification for the 11 substances evaluated and they can report comparable results in wildlife poisoning cases (except for difenacoum and chlorophacinone in the method of STVF-UM with a z-score slightly higher (z-score=2.2) that can be considered questionable).

Currently, there is a wide range of analytical techniques, a variety of chemical substances and matrices, and, in some cases, only small amounts of sample matrix are available relative to analytical requirements. These issues urge the necessity of homogeneous and standardised multiresidue analytical techniques able to analyse the maximum number of substances, using the minimum amount of sample, with comparable results between laboratories. However, as shown in the interlaboratory comparison carried out in this thesis (Chapter IV), the use of the same technique is not always needed to achieve comparable results.

To accomplish a proper diagnosis of poisoning, several steps in the chain of action may be standardised, from the collection of evidence (signs) and samples in the field, from the suspected crime scene, to the appropriate interpretation of the analytical results.

Data have to be shared within regions and countries in order to enlarge the knowledge involving poisoning investigations, including the carcass/sample state of decomposition, substances currently used/detected and more appropriate analytical technique to analyse each case (Chapters I-IV). To achieve this, networks of laboratories (among other institutions) should be stablished at national and international levels. To start with this idea, this thesis compiled information on different topics by sending a questionnaire to different European laboratories and institutions focused on veterinary forensic toxicology (Chapter V). Nineteen laboratories from 13 European countries (Albania, Croatia, Estonia, France, Germany, Greece, Italy, North Macedonia, Portugal, Romania, Serbia, Spain, and United Kingdom) were willing to participate as a network in the fight against illegal wildlife poisoning. However,

there is a gap of information from part of Europe (mainly northern and eastern Europe) due to uncomplete questionnaires and the lack of contacts/responses from certain countries. All the laboratories (except Romania) receive raptor species, which are the main species reportedly involved in illegal wildlife poisoning, and with the Common buzzard as the raptor species from which samples are most frequently received at the laboratories. Baits, gastric content, and liver are the matrices most favoured to analyse poisoning substances in these participating laboratories due to the reasons explained before. The groups of compounds most frequently analysed by the participant laboratories are AR, carbamates, OC, and OP. In addition, carbamates, AR, and OP were the group of compounds most frequently detected in raptor poisoning cases. Matrices and analytical techniques used to analyse the same compounds are very diverse. This could be a methodological issue that requires further studies and potential harmonisation among laboratories during the creation of the network. Necropsies are an important step in the study of poisoning cases because they provide valuable information before the laboratory analysis (Valverde et al., 2020a). However, it was observed that not all the participating laboratories perform necropsies (n=11, 61%).

Finally, to facilitate the operation of this network, further data should be gathered from these laboratories and more communication should be stablished to further ensure and increase the high analytical quality to achieve the goals of the STSM "Developing a Network of Analytical Labs and Government Institutions". For example, after the establishment of the European network, comparison and standardisation of analytical techniques should be tested using a wider network of laboratories and other techniques following a similar model as the one presented in Chapter IV, to enable the comparability and harmonization of results. Moreover, new contacts will be needed to enlarge the European network.

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General conclusions

Along with the specific conclusions of this thesis presented in each of the chapters, the general conclusions of this doctoral thesis and some recommendations for future studies are presented below.

1. A novel protocol focused on small-sized raptors exposed to elements from a Mediterranean climate has been created, which will harmonise the classification of the stage of carcass decomposition and will facilitate the estimation of the time of death in future research.

2. It was found that the SGARs bromadiolone persists in the liver of carcasses of small-sized raptors several days after death. Therefore, these carcasses with these concentrations in the field may be a source of secondary or tertiary poisoning for scavengers, at least during the first week after death when weather conditions are similar to those reported in this thesis. Further studies to assess poison degradation in carcasses under different scenarios (more individuals necropsied after additional days of decomposition, broader variety of weather conditions and species of different sizes) are needed.

3. Information gathered in a review of techniques to analyse anticoagulant rodenticides shows that different matrices are used to detect these compounds, as well as a variety of analytical methods. Although the use of the same methodology is not mandatory to achieve comparable results across laboratories, external validation and interlaboratory comparisons promote augmented and standardized performance.

4. The first comparative study between four laboratories specialized in veterinary forensic toxicology shows that the different techniques applied to analyse carbamates, organophosphates and anticoagulant rodenticides are appropriate and can obtain comparable results. These laboratories are able to provide reliable results using small sample amounts while also often adhering to more economic and environmentally friendly practices.

5. An initial step to create a European network of analytical laboratories and Government institutions to fight against wildlife poisoning was carried out in this thesis. Additional information about the different extraction and analytical techniques used, sample amounts required, and data collection is needed and should be shared to improve methodologies in the network. The network will help to harmonise methodologies and increase pan-European capacities. More laboratories/institutions are needed to cover a wider European area, and deeper analysis of how to encourage as many additional laboratories as possible to join the network is needed.

6. Further studies are highly recommended for a better fight against wildlife poisoning. For this purpose, some inconsistencies and gaps reported in this thesis should be considered. Future studies are encouraged to provide information on the moisture content and state of decomposition of samples to better evaluate detected concentrations and facilitate comparison of results between studies. Other important issues for a correct and rapid diagnosis of poisoning, include finding the bait used in a poisoning case and collecting enough sample amount from the carcass during the necropsy. The selection of samples will depend on each case but, at least, the liver (as main metabolizing and accumulating organ) and gastric content (if available) should be taken. Regarding the target compounds, carbamates, organophosphates, and anticoagulant rodenticides should be analysed together with other substances that may be involved in suspected wildlife poisoning cases. The analytical techniques used in the diagnosis of wildlife poisoning must be subject to all internal quality controls, and interlaboratory comparisons are also highly recommended.

7. An online platform with detailed data on poisoning cases should be created. This data should be uploaded/updated regularly by each laboratory to report, at least, information regarding the place of the poisoning case, species, samples used, basic necropsy findings (if available), analytical techniques used, compound/s and concentrations detected. Furthermore, a forum could be available to share opinions and

seek for assistance in complex cases or for technical purposes to other colleagues in the network. This platform should receive some support – either funding for creation of a data coordinator position or support for an external party to help collate and share this data to facilitate the participation of the laboratories.

Extended abstract

Title: *Optimization of toxicological and forensic tools in the investigation of wildlife poisoning*

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Introduction

The use of poison to kill animals is a traditional hunting activity linked to the history of humanity worldwide. Poisoning is a non-selective method, affecting the target species but also any other domestic animal and wildlife, causing important population declines and even the extinction of species (Berny, 2007; Cano et al., 2016; Ogada, 2014).

Deliberate abuse of pesticides/poison occurs when toxic products are used to kill animals considered harmful to certain activities, such as i) in the agriculture to eliminate pests in the crops; ii) to protect livestock-farming and hunting games from predation (Ntemiri et al., 2018; Villafuerte et al., 1998); iii) to eliminate animals considered annoying (Berny, 2007; De Roma et al., 2017, 2018; Mateo-Tomás et al., 2012; Mateo & Guitart, 2000; Navas et al., 1998); and iv) as a revenge way to solve feuds between private individuals (Ntemiri et al., 2018). The most affected species in south-west Europe by poison are raptors and scavengers, followed by domestic mammals (mostly dogs and cats) (Bodega Zugasti, 2014; Cano et al., 2016). Bait ingestion is the main way of exposure of wildlife to the different toxic products used to kill animals (Mateo-Tomás et al., 2012; RSPB, 2009). In addition, secondary poisoning may occur when an animal predates a poisoned victim (Berny et al., 1997; K. Ntemiri et al., 2018; Sánchez-Barbudo et al., 2012; Wobeser et al., 2004). Even tertiary poisonings have been described in some species (López-Perea et al., 2018).

In wildlife poisoning cases, there are a wide variety of substances involved, although some of them are more frequently detected. In this sense, the group of acetylcholinesterase inhibitors (carbamates and organophosphates (OP)) and anticoagulant rodenticides (AR) are more commonly involved in wildlife poisoning (Caloni et al., 2012).

During the investigation many difficulties may exist: i) finding the poisoned animals and/or baits in the field; ii) standard tissue matrices are not always available due to the degradation during cadaveric decomposition processes; iii) the sample volume is often insufficient for toxicological analysis; iv) a wide range of different substances can be involved; v) the frequent lack of information regarding the case; vi) the lack of tissue reference concentrations or values associated with acute poisoning in wildlife species, being even less available in decomposing tissues; and vii) the difficulty to make a trial and to charge the person responsible of the illegal act (Berny, 2007; García-Fernández et al., 2006; Luzardo et al., 2015; Wobeser et al., 2004).

The wildlife carcasses found in the field can be in a wide range of different stages of decomposition. The stage of decomposition of the carcass may affect and difficult the detection of substances involved in a poisoning case, since the availability of sample and the concentration of compounds may be altered (Brown et al., 2005; Luzardo et al., 2014). Toxic substances in the carcass suffer degradation when they are exposed to environmental conditions (rain, sun, moisture, etc.); and by other factors such as tissue autolysis and cadaveric fauna involved in the decomposition process. Nevertheless, their persistence depends on their chemical properties and on the environmental conditions they are exposed to in a specific moment (Fenner et al., 2013; Singh et al., 2014). However, little literature and information are available about the degradation process of the toxic substances in the body after dead in animal poisoning cases (Berny, 2007; Brooks, 2016; Martínez-López et al., 2006; Oates et al., 1984; Viero et al., 2019).

Regarding the analytical methodology, there is a wide range of extraction and clean-up techniques and instrumental methods for the identification and

quantification of toxic substances in biological samples (Barroso et al., 2005; de Siqueira et al., 2015; Imran et al., 2015; Inoue et al., 2007; Tarbah et al., 2004). These variety of techniques can give results that are not comparable in certain situations, which leads to the necessity of reviewing available techniques in the literature, comparing them, and developing new standardized methods. For this purpose, laboratories carry out external quality control by interlaboratory comparison studies (Garrido Frenich et al., 2006).

In order to improve the fight against wildlife poisoning, it is important to join and coordinate efforts between and within countries to share information and keep in touch with other colleagues working in the same field (Mateo, 2010; Motas-Guzmán et al., 2003), as the use of poison evolves relatively quickly and can vary considerably depending on the region (Bodega Zugasti, 2014). Therefore, the creation of a European network, where countries can share data about toxicovigilance, poisoning cases and substances currently used in each area, has been proposed by many authors, institutions and projects (COST CA16224; Elliott et al., 2007; Mateo, 2010; EU Action Plan, 2015; Silva et al., 2018). These networks may allow the comparison of techniques to know those obtaining the best analytical parameters (e.g., better recoveries, sensibility, reproducibility).

In Europe, the use of baits is prohibited by the Habitats and Birds directives (Directive 2009/147/EC of the European Parliment and of the Council of 30 November 2009 on the conservation of wild birds, 2010; Council Directive 92/43 EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora, 1992) as well as by the Bern Convention on the Conservation of European Wildlife and Natural Habitats. Furthermore, each country usually has its own legislation about this issue (Muscarella et al., 2016; Ntemiri et al., 2018).

In the European Union, the legal use of pesticides is regulated under two main groups: as plant protection products, to protect crops, and as biocides, products against pests but not strictly related to agriculture. Thus, the same product can be regulated under both groups depending on its use (EFSA; No1107/2009, 2009; 528/2012, 2012).

Objectives

The main aim of this thesis is to provide additional toxicological and forensic tools to improve the fight against wildlife poisoning in Europe. This requires increasing the knowledge on, among other issues, standardisation and protocolisation of methods for classifying animal carcass decomposition, assessing the degradation of compounds in carcasses, collecting available information on analytical techniques and comparing the results obtained between them. Moreover, the creation of a network of pan-European laboratories to improve the exchange of information within countries will improve the fight against poison in the nature.

Objective 1. (Chapter I) To search for complementary forensic data, mainly related to the date of death and the carcass decomposition, to protocolize and standardize the classification of cadaveric decomposition using a small raptor species as model.

Objective 2. (Chapter II) To evaluate for the first time the degradation of toxic compounds in poisoned carcasses using the SGARs bromadiolone and a small raptor species as models. Bromadiolone was selected as the target compound because it is used in the EU as biocide and as PPP and it is the most frequent AR detected worldwide.

Objective 3. (Chapter III) To compile and compare the analytical procedures applied for AR determination in the literature, as a first approach for future similar studies.

Objective 4. (Chapter IV) To compare the analytical procedures applied in four of the forensic veterinary laboratories of reference in Spain involved in the *Veneno-No* Life+ Project (www.venenono.org) (STVF-UM, UNEX, IREC-CSIC-UCLM and SERTOX-ULPGC), as part of an external quality assessment of analytical techniques. This study might allow the harmonisation of the results so that they can be comparable.

Objective 5. (Chapter V) To start a European network of laboratories working in forensic veterinary toxicology. Different European laboratories and institutions

were contacted and asked to fill a questionnaire with basic information on their activities and capacities.

Chapter I. Protocol to classify the stages of carcass decomposition and estimate the time of death in small-size raptors

The aim of this chapter is to propose a scoring method for carcass classification according to the degree of decomposition and estimation of time of death in small-sized raptors.

Materials and Methods

For this purpose, a decomposition experiment was carried out using 13 carcasses of Common kestrel (*Falco tinnunculus*). They were left exposed continuously to outside weather conditions and one carcass was frozen to assess the effect of freezing.

The autolytic process study was carried out during the period from 4 July (8:30 p.m.) to 19 July (11:00 a.m.) 2019. The mean ± SD (min-max) ambient air temperature and internal temperature of the carcasses (°C), humidity (%), day duration (hours) and wind speed (km/h) were recorded. Necropsies were performed at 1-2 h, 24 h, 72 h, 96 h, 7 and 15 days after death.

Results, discussion, and main conclusion

Six stages of the *post-mortem* autolytic process were selected: fresh carcass (1-2 hours after death), moderate decomposition (1 day after death), advanced decomposition (2-3 days after death), very advanced decomposition (7 days after death), initial skeletal reduction (15 days after death) and complete skeletal reduction. This last phase may take months to complete, depending on the environmental conditions, and in this study was not evaluated. The scoring method considers five different parameters selected during the decomposition process: 1) eyeballs, 2) tongue/oral cavity, 3) pectoral (breast) muscle, 4) internal organs (mainly the liver as a reference organ) and 5) other features (blood colour and feathers status). In cases of incomplete carcasses (e. g. due

to predation) this protocol cannot be applied to carcasses with more than two parameters missed.

The principal decomposition changes were observed during the first 7 days. Potential histological changes affecting the appearance of some organs should be considered when the necropsy of a frozen carcass is carried out. This protocol will harmonise the classification of the stage of carcass decomposition and will facilitate the estimation of the time of death in future research. The investigation reported here is intended to be a starting point from which data may be collected and validated. Further studies with other avian species and different weather conditions would help to better classify carcass decomposition and estimate time of death.

Chapter II. Temporal persistence of bromadiolone in decomposing bodies of Common kestrel (*Falco tinnunculus*)

The main aim of this study is to provide a first approach to evaluate the persistence of bromadiolone over time in the liver of decomposing carcasses of experimentally-dosed Common kestrels. This will improve interpretation of the presence of bromadiolone in exposed (or intoxicated) wild birds at different stages of carcass decomposition and the detection of bromadiolone in cases of wildlife poisoning as well as the risk of tertiary poisoning for scavengers.

Materials and Methods

Twelve Common kestrels were divided into two groups: bromadiolone-dose group (n = 6) and control group (n = 6). Bromadiolone-dose group was orally dosed with 55 mg/kg body weight. The 12 kestrels were euthanized three days after receiving bromadiolone. The carcasses were exposed to weather conditions. The decomposition stages selected were: 1–2 h (day 0), 24 h (day 1), 72 h (day 3), 96 h (day 4), 7 days, and 15 days after death.

Blood samples were collected before bromadiolone administration to ensure that individuals did not have bromadiolone residues (in both the control and bromadiolone-dose group), and before the euthanasia (three days after

bromadiolone administration) in the bromadiolone-dose group. Liver samples were taken during the necropsies. The percentage of humidity of the liver samples was calculated in order to correct for different water content.

Results, discussion, and main conclusions

Bromadiolone was detected in all blood samples collected three days after bromadiolone administration and before euthanasia (range: 45–135 ng/g, wet weight), reflecting its exposure and absorption due to the experimental dosing.

Hepatic bromadiolone concentrations in each stage of decomposition were: 3000, 2891, 4804, 4245, 8848, and 756 ng/g dry weight at 1–2 h, 24 h, 72 h, 96 h, 7 and 15 days after death, respectively.

Bromadiolone persists in the liver of carcasses of Common kestrels several days after death. Therefore, carcasses in the field may be a source of secondary or tertiary poisoning for scavengers, at least during the first week after death (under similar weather conditions of the study). Further studies to assess poison degradation in carcasses under different scenarios (more individuals necropsied after additional days of decomposition, broader variety of weather conditions and species of different sizes) are needed.

Chapter III. Wildlife poisoning: a novel scoring system and review of analytical methods for anticoagulant rodenticide determination

The main aim of this review is to compile and compare the analytical procedures applied for AR determination in the literature. For this purpose, we have reviewed the main publications available and prepared a database compiling the laboratory techniques used for the analysis of AR in both fauna and humans. Mainly the type of compound analysed, the matrix used, the weight or volume of sample analysed, the extraction technique, the extractant solvents used, recoveries, limits of quantification (LOQ) and the instrumental method applied were provided. Using this information, a scoring system was developed for those techniques using liver and blood, and the main techniques were ranked according to the sample amount, recoveries, LOQ and number of AR analysed.

This will facilitate comparison between techniques and the choice of a way forward for futures studies. Furthermore, this review will help to elucidate future directions to improve multi-residue techniques suitable to detect the AR that are causing wildlife lethal poisoning nowadays.

Materials and Methods

Different databases were used to search the literature available. Regarding the methodologies used for AR determination, information is provided as follows: matrices used, sample weight or volume, analytical technique, AR analysed, extraction and clean-up procedure, recovery, LOQ and chromatographic conditions reported.

For the development of the scoring system for those techniques using liver and blood as matrices, we used an equation where the different parameters had a different weight according to their importance to validate an analytical technique. Only studies providing sample amount, recoveries and LOQ (and/or limits of detection (LOD)), and number of compounds analysed were selected to be ranked.

Results, discussion, and main conclusion

A total of 49 articles describing 56 analytical methods for AR analysis were reviewed. Most of the methods described in the literature are set to detect bromadiolone, brodifacoum and difenacoum. Some of these techniques are able to simultaneously detect a variety of compounds in addition to AR, including non-AR pesticides, such as carbamates, OP, and human and veterinary drugs (Imran et al., 2015; Sell et al., 2017; Taylor et al., 2019).

Liver (48%) and blood (34%) were the matrices more frequently used for AR analysis. On average, the most frequent mass/volume used are 1-2 g of liver and 1 ml of blood.

Different extraction techniques are reported according to the compounds analysed and matrices used. Liquid-liquid extraction (32%) and the solid-phase extraction (32%) stand out, but other techniques such as dispersive-solid phase extraction (dSPE; 14%) are also reported. Within each extraction technique,

several modifications have been proposed, even combinations of several techniques (Imran et al., 2015; LeDoux, 2011). The main extractant solvents used to analyse AR in the publications reviewed are acetonitrile (38%), acetone (30%), ethyl acetate and methanol (21%).

A ranking of techniques has been created considering the specific parameters established in this review (recoveries, LOQ, sample amount, number of compounds analysed, points of the calibration curve and multi-class methods). Moreover, many studies reviewed have been excluded since recoveries and LOQ were not provided. Therefore, we do not intend to rank the "better" or "more appropriate" techniques, but to positively score those methods combining good recoveries, low LOQ, low sample amounts and high number of compounds analysed.

In general, most of the analytical methods with the highest scores used dSPE and acetonitrile as extractant, despite this extraction technique being less frequently used in the available literature. For the AR determination, liquid chromatography (LC) coupled to a tandem mass spectrometer was mainly used.

Information gathered shows that techniques to analyse AR use different matrices to detect these compounds, as well as a variety of analytical methods. Although the use of the same methodology is not mandatory to achieve comparable results across laboratories, external validation and interlaboratory comparisons are critical to ensure an adequate performance.

Chapter IV. Interlaboratory performance comparison to determine toxic compounds involved in wildlife poisoning

The aim of this study is to make a first approach in the comparison of the performance characteristics of the analytical procedures used in four Spanish reference laboratories for wildlife toxicology to detect the toxic substances most frequently used in wildlife poisoning (AR, carbamates and OP). The participant laboratories were STVF-UM, UNEX, IREC-CSIC-UCLM y SERTOX-ULPGC. This interlaboratory study also aims to confirm the quality of procedures and results

in order to harmonise methodologies and maximise reliability and comparability of data. Moreover, this study will help to establish improvements in the current analytical techniques if needed.

Material and methods

Spiked chicken liver was selected as test material. The compounds selected to prepare the final standard solution were chosen according to the substances detected in poisoning cases in Europe (Soler-Rodríguez et al., 2006; Guitart et al., 2010; Vandenbroucke et al., 2010; Bodega, 2014; Ntemiri and Saravia, 2016). A total of 11 substances were selected, including bromadiolone, brodifacoum, difenacoum, warfarin, chlorophacinone, carbofuran, aldicarb, methiocarb, diazinon, chlorpyrifos and parathion. Each laboratory carried out the extraction procedures with their routine technique.

Collaborators were required to report the mean concentrations in the spiked liver samples for each substance evaluated, repeatability, recoveries, and LOD/LOQ of their techniques. In this interlaboratory comparison, the laboratory performance was expressed in terms of z-score (standardized measure of performance, calculated using the participant results, the reference value and the standard deviation for proficiency assessment) in accordance with ISO13528:2015.

Results, discussion, and main conclusion

According to z-score, all the techniques showed satisfactory results for all compounds (z-score < 2), except for difenacoum and chlorophacinone in the method of STVF-UM with a z-score slightly higher (z-score=2.2) that can be considered questionable (Dehouck et al., 2015). The main extraction method used by UNEX, SERTOX-ULPGC and STVF-UM is dSPE based on a modified QuEChERS method (Gómez-Ramírez et al., 2012; Rial-Berriel et al., 2020).

Regarding analytical techniques, AR are always analysed by LC because they are non-volatile substances (Imran et al., 2015), while carbamates and OP can be analysed using both LC and gas chromatography (GC). UNEX and SERTOX-

ULPGC analyse all studied compounds by LC except for OP pesticides (GC). However, STVF-UM and IREC-CSIC-UCLM analyse all studied compounds by GC except for AR (LC).

The first comparative study between four laboratories specialised in veterinary forensic toxicology shows that the different techniques applied to analyse carbamates, OP and AR are appropriate and can obtain comparable results. These laboratories are able to provide reliable results using small sample amounts and more economic and environmentally friendly techniques.

Considering the amount of sample, the extraction steps and volume of solvent and other reagents needed, and the total run time during chromatography, SERTOX-ULPGC stands out as the fastest and most economic and environmentally friendly technique.

Further studies will be needed to evaluate the techniques used for other pesticides reported in wildlife poisoning including additional carbamates, OP, and other mammalicides, as well as using real samples from wildlife poisoning cases.

Chapter V. Developing a European network of analytical laboratories and government institutions to fight against raptor poisoning

This chapter presents the results of the COST Action European Raptor Biomonitoring Facility (CA16224) Short-Term Scientific Mission titled "Developing a Network of Analytical Labs and Government Institutions" aiming to create a network, focused on veterinary forensic toxicology laboratories, and to start a communication between the laboratories in the fight against wildlife poisoning, specially focused on raptors in Europe.

Material and methods

To start with the creation of the European Network, a questionnaire was designed and sent to different laboratories and institutions in Europe, and the data gathered is presented and discussed.

Results, discussion and main conclusion

From that questionnaire, it was achieved a list of 19 laboratories from 13 different European countries (Albania, Croatia, Estonia, France, Germany, Greece, Italy, North Macedonia, Portugal, Romania, Serbia, Spain and United Kingdom) willing to participate as a network in the fight against wildlife poisoning. However, there is a gap of information from part of Europe (mainly northern and eastern Europe) due to uncomplete questionnaires and the lack of contacts/responses from certain countries.

All the laboratories (except Romania) receive raptor species, which are the main species involved in wildlife poisoning, and Common buzzard (*Buteo buteo*) is the raptor species more frequently received in the laboratories.

Baits, gastric content, and liver are the matrices most frequently used to analyse poisoning substances in these laboratories. This is because the three matrices are the preferred tissues for the detection of common substances linked with oral exposure, which is the most common route of exposure for animals (Mineau & Tucker, 2002; Berny, 2007; Giorgi & Mengozzi, 2011).

The groups of compounds most frequently analysed by the participant laboratories are AR, carbamates, organochlorines, and OP. In addition, carbamates, AR, and OP were the group of compounds most frequently detected in raptor poisoning cases.

Necropsies are an important step in the study of poisoning cases because they provide valuable information before the laboratory analysis (Valverde et al., 2020). However, it was detected that not all the participant laboratories perform necropsies (n=11, 61%).

Finally, to make this network feasible, further data should be gathered from these laboratories and more communication should be stablished to achieve the goals of the project. For example, after the establishment of the European network, comparison and standardisation of analytical techniques should be tested following a similar model as the one presented in Chapter IV, to ensure the comparability and harmonisation of results. The network will help to

harmonise methodologies and increase pan-European capacities. More laboratories/institutions are needed to cover a wider European area.

Further studies are highly recommended for a better fight against wildlife poisoning. For this purpose, some inconsistencies and gaps reported in this thesis should be considered. Future studies are encouraged to provide information on the water content and state of decomposition of samples to better evaluate concentrations and facilitate results comparison between studies. The selection of samples will depend on each case but, at least, the liver (as main metabolizing and accumulating organ) and gastric content (if available) should be taken. Regarding the target compounds, carbamates, OP, and AR should be analysed together with other substances that may be involved in suspected wildlife poisoning cases. The analytical techniques used in the diagnosis of wildlife poisoning must be subject to all internal quality controls, and interlaboratory comparisons are also highly recommended.

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Resumen

Título: *Optimización de herramientas toxicológicas y forenses en la lucha contra el veneno en el medio natural*

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Introducción

El uso de veneno para matar animales es una actividad de caza tradicional que ha estado vinculada a la historia de la humanidad en todo el mundo. El envenenamiento es un método no selectivo, que afecta a las especies objetivo, pero también a cualquier otro animal doméstico y silvestre, causando importantes declives de las poblaciones e incluso la extinción de especies (Berny, 2007; Cano et al., 2016; Ogada, 2014).

El uso deliberado de venenos para matar animales considerados nocivos se da en determinadas actividades, como i) la eliminación de plagas en los cultivos agrícolas; ii) la protección de la ganadería y la caza de los depredadores (Ntemiri et al., 2018; Villafuerte et al., 1998); iii) la eliminación de animales considerados molestos (Berny, 2007; De Roma et al., 2017, 2018; Mateo-Tomás et al., 2012; Mateo y Guitart, 2000; Navas et al., 1998); y iv) como una forma de venganza para resolver disputas entre particulares (Ntemiri et al., 2018).

Las especies más afectadas en el suroeste de Europa por el veneno son las rapaces y las especies carroñeras, seguidas por los mamíferos domésticos (principalmente perros y gatos) (Bodega Zugasti, 2014; Cano et al., 2016). La ingestión de cebos es la principal forma de exposición de la fauna silvestre a los diferentes productos tóxicos utilizados para matar animales (Mateo-Tomás et al., 2012; RSPB, 2009). Además, el envenenamiento secundario puede ocurrir cuando un animal depreda sobre un animal envenenado previamente tras consumir directamente un veneno (Berny et al., 1997; Ntemiri et al., 2018;

Sánchez-Barbudo et al., 2012; Wobeser et al., 2004). Incluso se han descrito envenenamientos terciarios en algunas especies (López-Perea et al., 2018).

En los casos de envenenamiento de la fauna silvestre, hay una amplia variedad de sustancias involucradas, aunque algunas de ellas se detectan con mayor frecuencia. En este sentido, los inhibidores de la acetilcolinesterasa (carbamatos y organofosforados (OP)) y los rodenticidas anticoagulantes (RA) son los compuestos más comúnmente involucrados en la intoxicación de fauna silvestre (Caloni et al., 2012).

Durante la investigación de un caso de envenenamiento existen diversos factores que dificultan el éxito de la misma: i) dificultad para encontrar los animales envenenados y/o cebos en el campo; ii) las muestras y tejidos óptimos para el análisis no siempre están disponibles debido a la degradación durante los procesos de descomposición cadavérica; iii) el volumen de la muestra suele ser insuficiente para el análisis toxicológico; iv) existe una amplia gama de sustancias diferentes potencialmente sospechosas de causar la intoxicación; v) la frecuente falta de información sobre el caso; vi) la carencia de concentraciones de referencia tisular o de valores asociados con el envenenamiento agudo en especies de fauna silvestre, estando aún menos disponible para tejidos en descomposición; y vii) la dificultad de descubrir e inculpar a la persona responsable del delito (Berny, 2007; García-Fernández et al., 2006; Luzardo et al., 2015; Wobeser et al., 2004).

Los cadáveres de fauna silvestre pueden encontrarse en el campo en diferentes grados de descomposición. El estado de descomposición de los cadáveres puede afectar y dificultar la detección de sustancias implicadas en un caso de envenenamiento, ya que la disponibilidad de la muestra y la concentración de los compuestos pueden verse alteradas (Brown et al., 2005; Luzardo et al., 2014). Las sustancias tóxicas presentes en los cadáveres se degradan cuando están expuestas a condiciones ambientales (lluvia, sol, humedad, etc.), y por otros factores como la autolisis de los tejidos y la fauna cadavérica que interviene en el proceso de descomposición. Además, su persistencia depende de sus propiedades químicas y de las condiciones ambientales a las que se

exponen en un momento específico (Fenner et al., 2013; Singh et al., 2014). Sin embargo, hay poca literatura e información disponible sobre el proceso de degradación de las sustancias tóxicas en el cuerpo tras la muerte en casos de envenenamiento de animales (Berny, 2007; Brooks, 2016; Martínez-López et al., 2006; Oates et al., 1984; Viero et al., 2019).

En cuanto a la metodología analítica, existe una amplia gama de técnicas de extracción y purificación, así como métodos instrumentales para la identificación y cuantificación de sustancias tóxicas en muestras biológicas (Barroso et al., 2005; de Siqueira et al., 2015; Imran et al., 2015; Inoue et al., 2007; Tarbah et al., 2004). Esta variedad de técnicas puede dar resultados que no son comparables entre sí en ciertas situaciones, lo que lleva a la necesidad de revisar las técnicas disponibles en la literatura, compararlas y desarrollar nuevos métodos estandarizados. Para ello, los laboratorios llevan a cabo un control de calidad externo mediante el desarrollo de estudios de comparación interlaboratorio (Garrido Frenich et al., 2006).

Para mejorar la lucha contra el envenenamiento de la fauna silvestre, es importante unir y coordinar esfuerzos entre los países y dentro de cada país para compartir información y mantenerse en contacto con otros compañeros que trabajen en el mismo campo (Mateo, 2010; Motas-Guzmán et al., 2003), ya que las formas de uso del veneno evolucionan relativamente rápido y puede variar considerablemente dependiendo de la región (Bodega Zugasti, 2014). Por lo tanto, diversos autores, instituciones y proyectos han propuesto la creación de una red europea en la que los países puedan compartir datos sobre toxicovigilancia, casos de intoxicación y sustancias que se utilizan actualmente en cada área (COST CA16224; Elliott et al., 2007; Mateo, 2010; Plan de Acción de la UE, 2015; Silva et al., 2018). Estas redes pueden permitir la comparación de técnicas para conocer aquellas con los mejores parámetros analíticos (por ejemplo, mejores recuperaciones, sensibilidad, reproducibilidad).

En Europa, el uso de cebos envenenados está prohibido por las Directivas de Hábitats y Aves (Directiva 2009/147/CE del Parlamento Europeo y del Consejo, de 30 de noviembre de 2009, relativa a la conservación de las aves silvestres,

2010; por la Directiva 92/43 CEE del Consejo, de 21 de mayo de 1992, relativa a la conservación de los hábitats naturales y de la fauna y flora silvestres, 1992), así como por el Convenio de Berna sobre la conservación de la vida silvestre y los hábitats naturales europeos. Además, cada país suele tener su propia legislación para la gestión del envenenamiento de fauna silvestre (Muscarella et al., 2016; Ntemiri et al., 2018). En la Unión Europea, el uso legal de plaguicidas está dividido principalmente en dos grupos: como productos fitosanitarios, para proteger cultivos, y como biocidas, productos contra plagas pero no estrictamente relacionados con la agricultura. Así pues, el mismo producto puede regularse en ambos grupos en función de su uso (EFSA; no1107/2009; 528/2012).

Objetivos

El objetivo principal de esta tesis es proporcionar herramientas toxicológicas y forenses complementarias para mejorar la lucha contra el envenenamiento de la fauna silvestre en Europa. Esto requiere un mayor conocimiento, entre otras cuestiones, de la normalización y protocolización de métodos para clasificar la descomposición cadavérica, evaluar la degradación de los compuestos en los cadáveres, recopilar la información disponible sobre técnicas analíticas, y comparar los resultados obtenidos entre ellos. Además, la creación de una red de laboratorios para el intercambio de información mejorará la lucha contra el veneno en la naturaleza.

Objetivo 1. (Capítulo I) Buscar datos forenses complementarios, principalmente relacionados con la data de la muerte y la clasificación de la descomposición de los cadáveres, y protocolizar y estandarizar la clasificación de la descomposición cadavérica utilizando como modelo una especie de rapaz de pequeño tamaño.

Objetivo 2. (Capítulo II) Evaluar por primera vez la degradación de compuestos tóxicos en cadáveres envenenados utilizando como modelo el SGARs bromadiolona y una especie de rapaz de pequeño tamaño. La bromadiolona fue seleccionada como el compuesto diana porque se utiliza en

la UE como biocida y como PPP y es la AR más frecuente detectada en todo el mundo.

Objetivo 3. (Capítulo III) Recopilar y comparar los procedimientos analíticos aplicados para la determinación de los RA en la literatura, como primer enfoque para futuros estudios similares.

Objetivo 4. (Capítulo IV) Comparar los procedimientos analíticos aplicados en cuatro de los laboratorios veterinarios forenses de referencia en España implicados en el Proyecto Veneno-No Life+ (www.venenono.org) (STVF-UM, UNEX, IREC-CSIC-UCLM y SERTOX-ULPGC), como parte de una evaluación externa de la calidad de las técnicas analíticas. Este estudio podría permitir la armonización de los resultados para que puedan ser comparables.

Objetivo 5. (Capítulo V) Crear una red europea de laboratorios de toxicología veterinaria forense. Se contactará con diferentes laboratorios e instituciones europeas y se les pedirá que rellenen un cuestionario con información básica sobre su actividad.

Capítulo I. Protocolo para clasificar el estado de descomposición cadavérica y estimar la data de la muerte en aves rapaces de pequeño tamaño

El objetivo de este capítulo es proponer un método de puntuación para la clasificación de cadáveres de acuerdo con el grado de descomposición y estimación de la data de muerte en rapaces de pequeño tamaño.

Material y Métodos

Se realizó un experimento de descomposición utilizando 13 cadáveres de cernícalo común (*Falco tinnunculus*). Se les dejó expuestos ininterrumpidamente a las condiciones climáticas externas y se congeló un cadáver a las 6 horas tras la muerte para evaluar el efecto de la congelación.

El estudio del proceso autolítico se llevó a cabo durante el período comprendido entre el 4 de julio (8:30 p.m.) y el 19 de julio (11:00 a.m.) de 2019. Se registró la temperatura media ambiental y la temperatura interna de los

cadáveres (ºC), la humedad (%), la duración del día (horas) y la velocidad del viento (km/h). Se realizaron necropsias a 1-2 h, 24 h, 72 h, 96 h, 7 y 15 días después de la muerte.

Resultados, discusión y conclusiones

Se seleccionaron seis etapas del proceso autolítico *post mortem*: cadáver fresco (1-2 horas tras la muerte), descomposición moderada (1 día tras la muerte), descomposición avanzada (2-3 días tras la muerte), descomposición muy avanzada (7 días tras la muerte), reducción esquelética inicial (15 días tras la muerte) y reducción esquelética completa. Esta última fase puede tardar meses en completarse, dependiendo de las condiciones ambientales, y no se evaluó en este estudio. Para el método de puntuación, se seleccionaron cinco parámetros diferentes para ser evaluados durante el proceso de descomposición: 1) globos oculares, 2) lengua/cavidad oral, 3) músculo pectoral (pechuga), 4) órganos internos (principalmente el hígado como órgano de referencia) y 5) otras características (color de la sangre y estado de las plumas). En caso de cadáveres incompletos (por ejemplo, por depredación), este protocolo no podrá aplicarse a los cadáveres en los que no se puedan evaluar más de dos parámetros.

Los principales cambios en la descomposición se observaron durante los primeros 7 días. Los posibles cambios histológicos que afecten a la apariencia de algunos órganos deben considerarse cuando se realiza la necropsia de un cadáver congelado. Este protocolo armonizará la clasificación de los grados de descomposición de los cadáveres y facilitará la estimación de la data de muerte en futuras investigaciones. Esta investigación pretende ser un punto de partida a partir del cual puedan recogerse y validarse los datos. Se recomiendan nuevos estudios con otras especies de aves y diferentes condiciones climáticas que ayuden a clasificar la descomposición de los cadáveres y estimar el momento de la muerte.

Capítulo II. Degradación de la bromadiolona en cadáveres en descomposición de cernícalo común (*Falco tinnunculus*)

El objetivo principal de este estudio es proporcionar un primer acercamiento para evaluar la degradación de la bromadiolona en el hígado de cadáveres de cernícalo común en descomposición tratados experimentalmente. Este estudio mejorará la interpretación de la degradación de las concentraciones de bromadiolona en aves silvestres expuestas (o envenenadas) en diferentes grados de descomposición de los cadáveres, y la detección de bromadiolona en casos de envenenamiento de la fauna silvestre, así como el riesgo de envenenamiento terciario para las especies carroñeras.

Material y Métodos

Doce individuos de cernícalo común se dividieron en dos grupos: grupo de administración de bromadiolona (n = 6) y grupo control (n = 6). El grupo de administración de bromadiolona recibió 55 mg/kg de peso corporal de bromadiolona vía oral. Los 12 cernícalos fueron sacrificados tres días después de recibir la bromadiolona. Los cadáveres estuvieron expuestos a las condiciones climáticas. Las etapas de descomposición seleccionadas fueron: 1-2 h (día 0), 24 h (día 1), 72 h (día 3), 96 h (día 4), 7 días y 15 días después de la muerte.

Se tomaron muestras de sangre antes de la administración de bromadiolona para asegurar la ausencia de residuos de bromadiolona en los individuos (tanto en el grupo de control como en el grupo de administración de bromadiolona) y momentos antes de la eutanasia en el grupo de administración de la bromadiolona. Durante las necropsias se tomaron muestras de hígado. Se calculó el porcentaje de humedad de las muestras de hígado para corregir el contenido de agua.

Resultados, discusión y conclusiones

En todas las muestras de sangre recogidas tres días después de la administración de bromadiolona y antes de la eutanasia se detectó

bromadiolona (rango: 45-135 ng/g, peso húmedo), reflejando la exposición y absorción de esta debido a la dosificación experimental.

Las concentraciones hepáticas de bromadiolona en cada etapa de descomposición fueron: 3000, 2891, 4804, 4245, 8848 y 756 ng/g peso seco a 1-2 h, 24 h, 72 h, 96 h, 7 y 15 días después de la muerte, respectivamente.

La bromadiolona persiste en el hígado de los cadáveres de cernícalo común varios días después de la muerte. Por lo tanto, los cadáveres en el campo pueden ser una fuente de envenenamiento secundario o terciario para los carroñeros, al menos durante la primera semana después de la muerte (en condiciones climáticas similares a las del estudio). Se necesitan más estudios para evaluar la degradación de los venenos en los cadáveres bajo diferentes situaciones (más individuos necropsiados después de días adicionales de descomposición, mayor variedad de condiciones climáticas y especies de diferentes tamaños).

Capítulo III. Envenenamiento de la fauna silvestre: un nuevo sistema de puntuación y revisión de métodos analíticos para la determinación de rodenticidas anticoagulantes

El objetivo principal de esta revisión es recopilar y comparar los procedimientos analíticos aplicados para la determinación de RA en la literatura. Para ello, se han revisado las principales publicaciones disponibles y preparado una base de datos que recopila las técnicas de laboratorio utilizadas para el análisis de RA tanto en animales como en humanos. Se recopiló principalmente el tipo de compuesto analizado, la matriz utilizada, el peso o volumen de la muestra analizada, la técnica de extracción, los disolventes de extracción utilizados, las recuperaciones, los límites de cuantificación (LOQ) y la instrumentación analítica aplicada. Utilizando esta información, se desarrolló un sistema de puntuación para aquellas técnicas que utilizan hígado y sangre, y las técnicas se clasificaron según la cantidad de muestra, recuperaciones, LOQ y número de RA analizados. Esto facilitará la comparación entre técnicas y la elección del camino a seguir para los futuros estudios. Además, esta revisión ayudará a

dilucidar las direcciones futuras para mejorar las técnicas multiresiduo para la detección de RA que están causando el envenenamiento letal de la fauna silvestre hoy en día.

Material y Métodos

Se utilizaron diferentes bases de datos para buscar la literatura disponible. Respecto a los métodos utilizados para la determinación de los RA, se proporciona la siguiente información: matrices utilizadas, peso o volumen de la muestra, técnica analítica, los RA analizados, procedimiento de extracción y purificación, recuperación, LOQ y condiciones cromatográficas.

Para el desarrollo del sistema de puntuación de aquellas técnicas que utilizan hígado y sangre como matrices, se utilizó una ecuación donde los diferentes parámetros tenían un peso diferente según su importancia para validar una técnica analítica. Solo se seleccionaron para ser clasificados los estudios que proporcionaran la cantidad de la muestra, las recuperaciones, los LOQ (y/o los límites de detección (LOD)) y el número de compuestos analizados.

Resultados, discusión y conclusiones

Se revisaron un total de 49 artículos que describen 56 métodos analíticos para el análisis de RA. La mayoría de los métodos descritos en la literatura se establecen para detectar bromadiolona, brodifacoum y difenacoum. Algunas de estas técnicas son capaces de detectar simultáneamente otros compuestos además de RA, como carbamatos, OP, y fármacos humanos y veterinarios (Luzardo et al., 2014; Sell et al., 2017; Taylor et al., 2019).

El hígado (48%) y la sangre (34%) fueron las matrices más utilizadas para el análisis de RA. En general, la masa/volumen más frecuentemente utilizados son 1-2 g de hígado y 1 ml de sangre.

Se han reportado diferentes técnicas de extracción según los compuestos analizados y las matrices utilizadas. Destacan la extracción líquido-líquido (32%) y en fase sólida (32%), pero también se describen otras técnicas como la extracción en fase sólida dispersiva (dSPE; 14%). Además, se han propuesto varias modificaciones de cada técnica de extracción, incluso combinaciones de

varias técnicas (LeDoux 2011; Imran et al. 2015). Los principales solventes de extracción utilizados para analizar los RA en las publicaciones examinadas son el acetonitrilo (38%), la acetona (30%), el acetato de etilo y el metanol (21%).

El ranking de técnicas se ha creado teniendo en cuenta parámetros específicos establecidos en esta revisión (recuperaciones, LOQ, cantidad de muestra, número de compuestos analizados, puntos de la curva de calibración y métodos multiclase). Además, se han excluido muchos estudios revisados, ya que no se facilitaron recuperaciones ni LOQ. Por lo tanto, no pretendemos clasificar las mejores técnicas o las más apropiadas, sino calificar positivamente los métodos que combinan buenas recuperaciones, bajos LOQ, bajas cantidades de muestra y mayor número de compuestos analizados.

En general, la mayoría de los métodos analíticos con las puntuaciones más altas utilizaron dSPE y acetonitrilo como extractante, a pesar de que esta técnica de extracción se utiliza con menos frecuencia en la literatura disponible. Para la determinación de RA, se utilizó principalmente cromatografía líquida (LC) acoplada a un espectrómetro de masas en tándem.

La información recopilada muestra que las técnicas de análisis de RA utilizan diferentes matrices para detectar estos compuestos, así como una amplia variedad de métodos analíticos. Aunque el uso de la misma metodología no es obligatorio para lograr resultados comparables entre laboratorios, la validación externa y las comparaciones interlaboratorio son fundamentales para garantizar un rendimiento adecuado.

Capítulo IV. Comparación interlaboratorio para la determinación de compuestos tóxicos implicados en el envenenamiento de la fauna silvestre

El objetivo de este estudio es hacer un primer acercamiento a la comparación de las características de los procedimientos analíticos utilizados en cuatro laboratorios españoles de referencia en toxicología de la fauna silvestre para detectar las sustancias tóxicas más frecuentemente utilizadas en el envenenamiento de la fauna silvestre (RA, carbamatos y OP). Los laboratorios

participantes fueron STVF-UM, UNEX, IREC-CSIC-UCLM y SERTOX-ULPGC. Este estudio interlaboratorio también pretende confirmar la calidad de los procedimientos y resultados con el fin de armonizar las metodologías y maximizar la fiabilidad y comparabilidad de los datos. Además, este estudio ayudará a establecer mejoras en las técnicas analíticas actuales si es necesario.

Material y métodos

Se utilizó hígado de pollo enriquecido como material de análisis. Los compuestos seleccionados para preparar la solución estándar final fueron elegidos según las sustancias detectadas en casos de envenenamiento en Europa (Soler-Rodríguez et al., 2006; Guitart et al., 2010; Vandenbroucke et al., 2010; Bodega, 2014; Ntemiri y Saravia, 2016). Se seleccionó un total de 11 sustancias, entre ellas bromadiolona, brodifacum, difenacum, warfarina, clorofancinona, carbofurano, aldicarb, metiocarb, diazinón, clorpirifós y paratión. Cada laboratorio llevó a cabo los procedimientos de extracción con su técnica de rutina.

Se solicitó a los colaboradores que proporcionaran las concentraciones medias en las muestras de hígado enriquecido para cada sustancia evaluada, repetibilidad, recuperaciones y LOD/LOQ de sus técnicas. En esta comparación interlaboratorio, el rendimiento del laboratorio se expresó en términos de *z-score* (medida normalizada del rendimiento, calculada utilizando los resultados de los participantes, el valor de referencia y la desviación típica para la prueba de la competencia entre laboratorios) de acuerdo con la ISO13528:2015.

Resultados, discusión y conclusiones

De acuerdo con el *z-score*, todas las técnicas mostraron resultados satisfactorios para todos los compuestos (z-*score*=2), a excepción del difenacum y la clorofancinona en el método de STVF-UM con un z-*score* ligeramente superior (z-*score*=2.2) que puede considerarse cuestionable (Dehouck et al., 2015). El principal método de extracción utilizado por UNEX, SERTOX-ULPGC y STVF-UM es la dSPE basado en un método QuEChERS modificado (Gómez-Ramírez et al., 2012; Rial-Berriel et al., 2020). En cuanto a las técnicas analíticas, los RA

se analizan siempre por LC porque son sustancias no volátiles (Imran et al., 2015), mientras que los carbamatos y la OP se pueden analizar utilizando LC y cromatografía de gases (GC). La UNEX y SERTOX-ULPGC analizan todos los compuestos estudiados por LC excepto los plaguicidas OP (GC). Sin embargo, STVF-UM y IREC-CSIC-UCLM analizan todos los compuestos estudiados por CG excepto AR (LC).

Este primer estudio comparativo entre cuatro laboratorios especializados en toxicología forense veterinaria muestra que las diferentes técnicas aplicadas para analizar carbamatos, OP y RA son adecuadas y pueden obtener resultados comparables. Estos laboratorios son capaces de proporcionar resultados fiables utilizando pequeñas cantidades de muestra y técnicas más económicas y respetuosas con el medio ambiente. Considerando la cantidad de muestra, los pasos de extracción y el volumen de disolvente y otros reactivos necesarios, y el tiempo total de cromatografía, SERTOX-ULPGC destaca como la técnica más rápida y más económica y respetuosa con el medio ambiente.

Se necesitarán más estudios para evaluar las técnicas utilizadas para otros pesticidas detectados en casos de envenenamiento de fauna silvestre, incluyendo más carbamatos, OP y otros mamalicidas, así como el uso de muestras reales de casos de envenenamiento de fauna silvestre.

Capítulo V. Desarrollo de una red europea de laboratorios analíticos e instituciones gubernamentales para la lucha contra el envenenamiento de aves rapaces

Este capítulo presenta los resultados de la misión científica de corta duración de la COST Action European Raptor Biomonitoring Facility (CA16224) titulada "Desarrollo de una red de laboratorios analíticos e instituciones gubernamentales" con el objetivo de crear una red de laboratorios de toxicología veterinaria forense, y de iniciar una comunicación entre los laboratorios para la lucha contra el envenenamiento de la fauna silvestre, especialmente centrada en las aves rapaces en Europa.

Material y métodos

Se diseñó un cuestionario que se envió a diferentes laboratorios e instituciones de Europa, y en este capítulo se presentan y se debaten los datos recopilados.

Resultados, discusión y conclusiones

De ese cuestionario se obtuvo una lista de 19 laboratorios de 13 países europeos diferentes (Albania, Croacia, Estonia, Francia, Alemania, Grecia, Italia, Macedonia del Norte, Portugal, Rumania, Serbia, España y Reino Unido) dispuestos a participar como una red en la lucha contra el envenenamiento de la fauna silvestre. Sin embargo, se carece de información de parte de Europa (principalmente de Europa septentrional y oriental) debido a cuestionarios incompletos y a la falta de contactos/respuestas de algunos países.

Todos los laboratorios (excepto Rumania) reciben especies de rapaces, que son las principales especies involucradas en los casos de envenenamiento de fauna silvestre, siendo el busardo ratonero (*Buteo buteo*) la especie de ave rapaz más frecuentemente recibida en los laboratorios.

Los cebos, el contenido gástrico y el hígado son las matrices más utilizadas para analizar las sustancias tóxicas en estos laboratorios. Esto se debe a que las tres matrices son las muestras de preferencia para la detección de sustancias relacionadas con la exposición oral, que es la vía de exposición a los venenos más común para los animales (Mineau y Tucker, 2002; Berny, 2007; Giorgi y Mengozzi, 2011).

Los grupos de compuestos analizados con mayor frecuencia por los laboratorios participantes son RA, carbamatos, organoclorados y OP. Además, los carbamatos, RA y OP fueron el grupo de compuestos más frecuentemente detectados en casos de envenenamiento de aves rapaces.

Las necropsias son una parte importante en el estudio de los casos de envenenamiento porque proporcionan información valiosa antes del análisis de laboratorio (Valverde et al., 2020). Sin embargo, se detectó que no todos los laboratorios participantes realizan necropsias (n=11, 61%).

Por último, para que esta red sea factible, se deben recopilar más datos de estos laboratorios y establecer más comunicación para lograr los objetivos del proyecto. Por ejemplo, tras la creación de la red europea, la comparación y la normalización de las técnicas analíticas deben comprobarse, siguiendo un modelo similar al presentado en el capítulo IV, a fin de garantizar la comparabilidad y la armonización de los resultados. Además, serán necesarios nuevos contactos para ampliar la red europea. La red ayudará a armonizar las metodologías y a aumentar las capacidades paneuropeas. Se necesitan más laboratorios/instituciones para abarcar un espacio europeo más amplio.

Para mejorar la lucha contra el envenenamiento de la fauna silvestre se recomienda llevar a cabo más estudios. Para este propósito, se deben considerar algunas inconsistencias y lagunas descritas en esta tesis. Se anima a que en futuros estudios se proporcione información sobre el contenido de agua y el estado de descomposición de las muestras para evaluar mejor las concentraciones y facilitar la comparación de los resultados entre los diferentes estudios. La elección de las muestras dependerá de cada caso, pero al menos, el hígado (como principal órgano metabolizador y acumulador) y el contenido gástrico (si está disponible) deben ser tomados. En cuanto a los compuestos diana, los carbamatos, los OP y los RA deben analizarse junto con otras sustancias que pueden estar implicadas en casos de intoxicación de fauna silvestre. Las técnicas analíticas utilizadas en el diagnóstico del envenenamiento de la fauna silvestre deben estar sujetas a todos los controles de calidad internos, además también se recomiendan los estudios de comparación interlaboratorio.

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