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Agnieszka Chłopaś-Konowałek[1], Marcin Zawadzki[2], Łukasz Kurach[3], Olga Wachełko[1], Rafał Ciaputa[4], Kaja Tusiewicz[2], Paweł Szpot[2]

Simultaneous poisoning of 48 birds of prey – bendiocarb determination with the use of UHPLC-ESI-MS/MS method in fatal case from Eastern Europe

Celowe zatrucie 48 ptaków drapieżnych – oznaczenie bendiokarbu metodą UHPLC-ESI-MS/MS – śmiertelny przypadek w Europie Wschodniej

1 Institute of Toxicology Research, Borowa, Poland
2 Wroclaw Medical University, Department of Forensic Medicine, Wroclaw, Poland
3 Medical University of Lublin, Independent Laboratory of Behavioral Studies, Lublin, Poland
4 Wroclaw University of Environmental and Life Sciences Department of Pathology, Division of Pathomorphology and Veterinary Forensics, Faculty of Veterinary Medicine, Wroclaw, Poland

Abstract

Aim: Bendiocarb is used against a wide range of insects but has already been withdrawn from the market in some countries. It poses a high risk to birds as they can accidentally ingest it while searching for food, followed by toxic effects. This paper presents the results of toxicological and histopathological studies of 48 cases of intentional birds of prey poisoning with bendiocarb in Eastern Europe, specifically Poland.

Materials and methods: A novel ultra-high-performance liquid chromatography-triple quadrupole-tandem mass spectrometry (UHPLC-ESI-MS/MS) method for bendiocarb determination in animal liver samples was developed and fully validated. The sample preparation technique was based on one-step precipitation of proteins with cold acetonitrile. The internal standard used was carbaryl-d_7. Full time of analysis was less than 10 minutes. The application of the UHPLC-ESI-MS/MS method allowed us to achieve the lowest LOQ (1 ng/g) of bendiocarb in biological samples to date.

Results: Necropsies and histopathological examinations of common ravens (*Corvus corax*), western marsh harriers (*Circus aeruginosus*), red kites (*Milvus milvus*), and a white-tailed eagle (*Haliaeetus albicilla*) revealed multi-organ toxicity manifested as congestion, oedema, or stagnation of blood. An analytical investigation confirmed the presence of bendiocarb in liver in the 1808–7721 ng/g range. Furthermore, the presence of this compound was qualitatively confirmed in the stomach and beak contents and also in the bait located near the deceased animals.

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Conclusions: A comprehensive forensic examination is crucial to monitor wildlife fatalities, especially applying a combined analytical and histopathological approach to identify and eliminate highly toxic substances which pose a threat to the ecosystem.

Key words: carbamate pesticides, bendiocarb, raptor poisoning, wildlife crime

Streszczenie

Cel: Bendiokarb jest insektycydem o szerokim spektrum działania, aczkolwiek w niektórych krajach został wycofany z użytku. Stanowi on duże zagrożenie dla ptaków jako że najczęściej ulegają one zatruciu na skutek zjedzenia padliny lub przynęty zawierającej bendiokarb. W pracy przedstawiono wyniki badań toksykologicznych i histopatologicznych obejmujące 48 przypadków celowego zatrucia ptaków drapieżnych bendiokarbem w Europie Wschodniej, a konkretnie w Polsce.

Materiały i metody: Nowa metoda do oznaczania bendiokarbu w próbkach wątrób ptaków została opracowana i zwalidowana z wykorzystaniem ultra-wysokosprawnej chromatografii cieczowej sprzężonej ze spektrometrią mas typu potrójny kwadrupol (UHPLC-ESI-MS/MS).

Próbki zostały poddane jednoetapowej precypitacji białek zmrożonym acetonitrylem. Karbaryl-d_7 został użyty jako wzorzec wewnętrzny. Zastosowanie metody UHPLC-QqQ-MS/MS umożliwiło uzyskanie najniższego jak dotąd parametru LOQ – na poziomie 1 ng/g bendiokarbu w próbkach biologicznych.

Wyniki: Sekcja zwłok oraz badania histopatologiczne kruków pospolitych (*Corvus corax*), błotniaków stawowych (*Circus aeruginosus*), kani rudej (*Milvus milvus*) oraz orła bielika (*Haliaeetus albicilla*) ujawniły toksyczność wielonarządową charakteryzującą się przekrwieniami, obrzękami lub zastojem krwi. Badania analityczne potwierdziły obecność bendiokarbu w wątrobie w zakresie stężeń 1808–7721 ng/g. Ponadto obecność tego związku potwierdzono jakościowo w treści żołądka i dziobie, a także w przynęcie znajdującej się w pobliżu padłych zwierząt.

Wnioski: Kompleksowe badania kryminalistyczne mają kluczowe znaczenie w monitorowaniu przypadków śmiertelnych zatruć wśród dzikich zwierząt. Zastosowanie połączonego podejścia analitycznego i histopatologicznego może pomóc w identyfikacji i eliminacji wysoce toksycznych substancji mogących stanowić zagrożenie dla ekosystemu.

Słowa kluczowe: pestycydy karbaminianowe, bendiokarb, zatrucie ptaków, przestępczość wobec dzikich zwierząt

Introduction

Intentional or accidental poisoning of wildlife causes significant mortality of protected vertebrate species worldwide, especially birds. Bird poisoning occurs accidentally through pesticides used in agriculture [1] but also intentionally by poisoned bait, which is what happens most often [2].

Carbamate (CB) poisoning is the most frequently reported in studies across Europe concerning cholinesterase inhibitor pesticides [1,3,4]. These insecticides have a broad spectrum of activity, are moderately toxic, and cause serious environmental effects [5]. Highly water-soluble carbamates, like methomyl, oxamyl, and aldicarb, are more prone to run-off and leaching into groundwater and water bodies. Aromatic-based carbamate pesticides can be adsorbed onto soil particles and enter water bodies. Apart from the presence in water and soil, carbamates are also detected in the air due to spray drift or volatilization [6]. Literature reported that mortality may occur as a result of substance accumulation through multiple consumptions of a small amount of plant protection products at different intervals [7,8]. Acute poisoning is a consequence of eating a bait in the form of meat pieces or a dead animal soaked or immersed in poison. Due to this fact, the massive mortality of birds is presumed to be intentional poisoning [2,9]. The consequences of these actions pose a great risk for the ecosystem because secondary poisoning may occur, which is described as the consumption of dead birds containing intoxicants by other animals in the food chain [10]. Birds of prey, such as the white-tailed eagle *(Haliaeetus*

albicilla) or common buzzard *(Buteo buteo)*, are the most frequent victims of this harmful practice. Epidemiological studies revealed that 52.2% of bird and wild mammal deaths are related to intentional poisoning with pesticides [11].

Carbamates exhibit low toxicity towards mammals, but high toxicity for birds, bees and fish [12]. Carbamate pesticides showing high toxicity (LD50 <0.1 μg/bee) include bendiocarb, dicrotophos, diazinon and chlorpyrifos. Mammals are particularly sensitive to broad-spectrum carbamate insecticides. Especially toxic (LD50 <10 mg/kg body weight) are aldicarb (0.9 mg/kg), oxamyl (2.8 mg/kg), thiophanox (8.5 mg/kg) and carbofuran (8.9 mg/kg). Birds are very vulnerable to the neurotoxic effects of carbamates [13]. Some of the most toxic insecticides to birds (LD50 <10 mg/kg body weight) are carbofuran, aldicarb, oxamyl, methiocarb and triazamate [13].

Cases of intentional or accidental poisoning with bendiocarb are rarely reported, while frequent poisonings with another widely used and highly toxic carbamate pesticide like carbofuran are known in the literature [14,15,16], which is a broad-spectrum, high-efficiency, low-residue, and highly toxic carbamate pesticide like bendiocarb [15].

Within the group of carbamate pesticides, aldicarb and carbofuran were the most commonly used in the past [3,4]. However, the European Union banned these pesticides in 2003 [17] and 2007 [18], respectively. For this reason, there is a high risk that bendiocarb will be the next pesticide from this group that will be used to poison wildlife.

Bendiocarb (2,2-dimethyl-1,3-benzodioxol-4-yl-*N*-methyl carbamate) is a broad-spectrum carbamate insecticide, classified as a reversible inhibitor of acetylcholinesterase (AChE) [19]. It is sold under various trade names: Ficam, Dycarb, Garvox, Turcam, Niomil, Turcam, Multamat, Seedox, Tattoo [20]. On the market, it is available as a powder (1%), in granules (2.5, 5, and 10%), oil suspension ULV (25%), and as a wettable powder (76%). Bendiocarb is a highly lipophilic compound, very well absorbed from the gastrointestinal tract, through mucous membranes, skin, and the respiratory system [21]. Bendiocarb is rapidly metabolized and excreted mainly in the urine at 86-99% within 24 hours, with fecal excretion of about 3-8% [22]. Bendiocarb transforms into metabolites: 7-hydroxybendiocarb, *N*-hydroxymethylbendiocarb, 2,2-dimethyl-1,3-benzodioxol-4-ol and methyl carbamate [21]. Taking into consideration oral exposure, bendiocarb belongs to the acute toxicity category I, the highest of four categories regarding this effect. In addition, bendiocarb falls into the acute toxicity category II for dermal and inhalation routes of exposure, the acute toxicity category III for primary dermal irritation and the acute toxicity category IV for primary eye irritation [23,24].

According to Directive 1012/3/UE, bendiocarb is approved for use against mosquitoes, flies, wasps, ants, fleas, cockroaches, ticks, and snails in the European Union and recommended by the World Health Organization (WHO) against malaria-transmitting insects. Bendiocarb's toxicity manifests rapidly, usually within 1 hour after dosing in animals and within a few minutes in humans [5]. The first intoxication case was reported by Humphreys and Stodulski in 1981 and involved sparrows, blackbirds, and dogs. The animals developed convulsions and died within 30 minutes to one hour after ingestion of bendiocarb-soaked pieces of bread [25]. Acute bendiocarb intoxication causes tachycardia, pupil dilation, muscle contraction, excessive salivation, nausea, vomiting, as well as ataxia, seizures and coma [26,27,28]. The symptoms after intoxication include paralysis of muscles of the respiratory system with intense constriction of the pulmonary alveolus [20].

Numerous cases of poisoning with carbamate compounds have been reported in the literature to date, but there are no data on poisoning birds of prey with bendiocarb, so the purpose of this study was to determine the concentrations of bendiocarb in biological materials from birds suspected of being poisoned with this xenobiotic.

Case report

At the end of May 2020, shelter employees were informed about the discovery of several dozen dead birds of prey in central-western Poland, in the Greater Poland Voivodeship. According to police findings, the bodies of the birds were several meters apart and found intact. Pieces of meat, probably chicken, were scattered around the dead birds. This may suggest that wild birds were poisoned on purpose.

Aim of the study

This is the first report describing deliberate and mass poisoning of different species of birds of prey with bendiocarb. The present study aims to present the analytical (UHPLC-ESI-MS/MS) and histopathological findings of serial cases of bendiocarb poisoning concerning 39 common ravens (*Corvus corax)*, 6 western marsh harriers (*Circus aeruginosus*), 2 red kites (*Milvus milvus*), and 1 white-tailed eagle (*Haliaeetus albicilla*) of samples collected from deceased animals (**Fig. 1**) and to highlight the important contribution of such an analysis to criminal investigations.

Materials and methods

Chemicals

Water (Chromasolv® LC–MS), acetonitrile (Chromasolv® LC–MS), and formic acid were purchased from Sigma-Aldrich (Steinheim, Germany); ammonium formate was purchased from Sigma-Aldrich (Bangalore, India); bendiocarb was purchased from Sigma Aldrich (Steinheim, Germany) as analytical standard (PESTANAL®); carbaryl-d_7, used as the internal standard (IS), was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Standard solutions of bendiocarb and internal standard (IS) carbaryl-d_7, at concentration of 10 mg/mL, were prepared in acetone.

Fig. 1. Poisoned birds of prey during the autopsy: common ravens (*Corvus corax*) (A and C), western marsh harriers (*Circus aeruginosus*) (A), red kites (*Milvus milvus*), and a white-tailed eagle (*Haliaeetus albicilla*) (B)

Biological material

Drug-free blank liver samples used for the development and validation of the method were obtained from a butcher store. Blank samples were screened before spiking to ensure that they were free from drugs and pesticides.

The birds and remains of meat that were sent to our laboratory for analysis were secured and transported to the Institute of Pathomorphology and Forensic Veterinary Medicine in Wrocław. Dead remains of game and meat were preserved and stored at -20°C until pathological and veterinary examination. The forensic livers of 48 birds were sent to our laboratory for routine toxicological analysis. In accordance with the prosecutor's order, an untargeted analysis was performed, which indicated the presence of only bendiocarb in the collected biological material.

Working solutions, calibration points, and quality control samples

The working solutions of different concentrations were prepared by dilution of the standard solution with acetone. The stock solution and standard solutions were stored at -20°C. Calibration points were prepared by spiking the appropriate working solution into drug-free liver homogenate samples. The final concentrations of the calibrators were 1 (lower limit of quantification [LLOQ]), 10, 50, 100, 250, 500, 1000, 2500, and 5000 (upper limit of quantification [ULOQ]) ng/g for bendiocarb in liver homogenate. Quality control samples were prepared by spiking the blank liver with bendiocarb standard to a final concentration of 1, 250, and 2500 ng/g.

Sample preparation

Liver tissues samples were homogenized using an Q55 sonicator (QSonica, Newtown, USA). In order to homogenize the tissue samples, 0.5 g of the solid specimen was transferred to a plastic tube (12 mL) and mixed with 0.5 mL of water (Chromasolv® LC–MS). The tube was placed in a glass beaker containing ice cubes. Tissues were disrupted by the use of ultrasonic probe (5 kHz frequency). The total time of homogenization was 10 min. Liver homogenate (100 µL) was transferred into 2-mL Eppendorf tubes. Next, 10 µL of internal standard solution (carbaryl-d_7, 500 ng/mL) was added. The sample was mixed with 0.5 mL of cold acetonitrile (kept on ice) and vortexed to precipitate the proteins and perform simultaneous liquid-liquid extraction (LLE). The precipitated proteins were removed by centrifugation at 13 500 rpm at 4°C for 10 min and 100 µL of clear supernatant was transferred into glass inserts of autosampler vials and analysed by UHPLC-ESI-MS/MS. The injection volume was 1.0 µL. Since the concentrations of bendiocarb in most biological specimens were markedly above ULOQ (5000 ng/g), the assay was repeated. Liver homogenates were diluted 100-fold with blank liver homogenate.

Chromatographic and spectrometric conditions

Analyses were performed using an ultra-high-performance liquid chromatograph Nexera LC-40 Shimadzu (Kyoto, Japan). The separation was done using a Kinetex Biphenyl 2.6 µm 2.1 × 50 mm column (Phenomenex, Torrance, CA, USA) with the thermostat set at 40°C. The mobile phase consisted of a mixture of 10 mM ammonium formate and 0.1% formic acid in water (A) and a mixture of 10 mM ammonium formate and 0.1% formic acid in methanol (B). The gradient elution was carried out at a constant flow of 0.4 mL/min. The gradient applied was as follows: 0 min, 5% B; 6 min, 95% B. Return to the initial gradient compositions (95% A and 5% B) was performed for 2 min.

Detection of the bendiocarb was achieved using a triple-quadrupole mass spectrometer (LCMS-8060, QqQ, Shimadzu, Kyoto, Japan). The spectrometer was equipped with an electrospray ionization (ESI) source; determination of the bendiocarb was carried out in the multiple reaction monitoring (MRM) mode. The following MS parameters were fixed: nebulizing gas flow, 3 L/min; heating gas flow, 10 L/min; interface temperature, 300°C; desolvation line temperature, 250°C; heat block temperature, 400°C; and drying gas flow, 10 L/min. A summary of precursor and product ions, collision energies, dwell time, Q1–Q3 pre-bias voltages, and retention time for each compound is presented in **Table I**.

Table I. MRM conditions used in the UHPLC-ESI-MS/MS method for quantification of bendiocarb in liver homogenate

Compound	Retention time [min]	Precursor Ion [m/z]	Product Ion [m/z]	Dwell Time (msec)	Q1 Pre-Bias [V]	Collision Energy [V]	Q3 Pre-Bias [V]
Carbaryl- d_7	4.67	209.0	152.4* 133.3 124.5	30.0	-20.0 -21.0 -22.0	-12.0 -24.0 -24.0	-16.0 -22.0 -14.0
Bendiocarb	4.50	224.0	167.4 109.3* 81.3	30.0	-10.0 -10.0 -15.0	-9.0 -18.0 -33.0	-17.0 -19.0 -14.0

* ions selected for quantitative analysis

Validation

Selectivity

Five different lots of blank liver homogenate from different origins were tested for possible endogenous interference peaks at the retention time of the bendiocarb or internal standard.

Linearity

Linearity was evaluated by analysis of bendiocarb working solutions with liver homogenate in the final concentration range of 1–5000 ng/g. The linear calibration model was applied. The coefficient of determination (R^2) was determined. According to the acceptance criteria used, the coefficient of determination should meet the condition: $R^2 \geq 0.995$.

Precision and accuracy

The intra-day and inter-day precision and accuracy were estimated by replicating analysis ($n = 5$) of QC samples at three concentration levels: 1, 250, and 2500 ng/g. The precision was defined as relative standard deviation (RSD%). The accuracy was expressed as a mean relative error (RE%).

The LOQ and the LOD

The lower limit of quantification (LLOQ) was defined as the concentration at which the relative standard deviation (RSD%) and relative error (RE%) do not exceed 20% and 15%, respectively [29]. LOD was considered to be the lowest concentration of the sample for which the signal-to-noise ratio met the condition at least: S/N ≥ 3.

Carryover

To investigate the carryover, three samples without analytes were analysed after a calibration sample at the bendiocarb concentration of 5000 ng/g (highest calibration level). Unacceptable carryover was when peak area ratio in a zero sample after analysis of a sample containing a high concentration of bendiocarb exceeded 20% of the area ratio observed for the LOQ samples.

Recovery and matrix effect

The recovery and matrix effect values were determined at each of the three concentration levels: 1 (low QC), 250 (medium QC), and 2500 (high QC) ng/g. The recovery (in percent, n = 5) was determined by comparing the response of extracted analyte in spiked blank matrix with the response of analyte spiked after the extraction of a blank matrix. The matrix effect (in percent, n = 5) was determined by comparing the response of analyte spiked after the extraction of a blank matrix with the response of analyte in a neat solution. Matrix effect was calculated using the equation described by Chambers et al. [30].

Histopathology examination

Kidney, liver, lung, and small intestine specimens were fixed in 7% buffered formalin for 24 hours then embedded in paraffin blocks and cut into 4 μm sections. The sections were stained with haematoxylin and eosin (H-E) by standard histological methodology and underwent histopathological assessment. Microscopic photographs underwent computer-a-

ided image analysis using a computer coupled with an Olympus BX53 optical microscope (Olympus, Japan) and equipped with a digital Olympus ColorView IIIu camera (Olympus, Japan). The measurements were taken using cell^A software (Olympus Soft Imaging Solution GmbH, Germany).

Results and discussion

Illegal poisoning of wildlife is a worldwide issue. Although the sale and distribution of some carbamate pesticides have become more restrictive in Europe and other countries, they still cause accidental and deliberate poisoning of animals [31]. The investigation of intentional animal poisoning cases is as serious as that in a human but also is a challenging process. In this context, the continuous and accurate collection of epidemiological data on animal poisoning is crucial to obtain useful information on toxicant trends and rise of new substances to carry out and enhance preventive measures for appropriate risk management, to help veterinarians to manage cases of suspected poisoning, and finally to draw attention towards an issue that has also environmental and human health implications [32].

For the first time, the present study reports toxicological and histopathological findings of massive bendiocarb-intoxication of several species of birds of prey in the Eastern part of Europe.

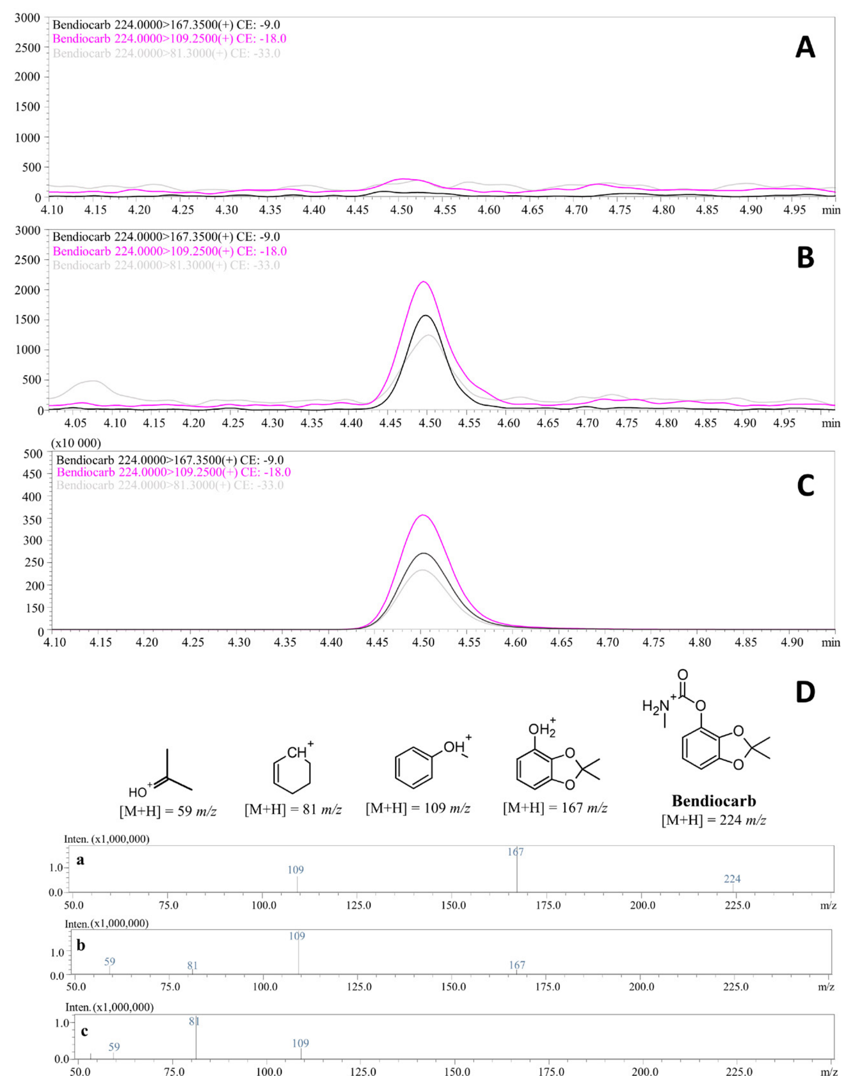

Fig. 2. Multiple reaction monitoring (MRM) chromatograms of (A) blank liver sample; (B) bendiocarb at the concentration of LLOQ: 1.0 ng/g; (C) real analysis of white-tailed eagle (*Haliaeetus albicilla*) liver sample; and (D) product ion spectra of bendiocarb with proposed fragmentation pathway of this substance; collision energy: a–10, b–20, and c–35 V

Validation process

Under the chromatographic conditions, the MRM transitions (*m/z*) of 224.0→167.4, 224.0→109.3, 224.0→81.3, and 209.0→152.4, 209.0→133.3, 209.0→124.5 were selected for optimal monitoring of bendiocarb and carbaryl-d_7, respectively. No interfering ion current signals were observed at the retention times of bendiocarb or IS. Chromatograms of the blank sample, bendiocarb at a concentration of LOQ, real analysis of white-tailed eagle (*Haliaeetus albicilla*) liver sample, and product ion spectra with proposed fragmentation pathway of bendiocarb are presented in **Fig. 2**. Summary of the calibration curve parameters and validation results are presented in **Table II**. Intra-day and inter-day precisions (RSD%) and accuracies (RE%) examined at bendiocarb concentrations of 1, 250, and 2500 ng/g did not exceed 10%. Recovery and matrix effect values were in the ranges of 91.5%–118.1% and -8.5%–18.1%, respectively. The studied dilution effect for the samples expressed as RE was equal -0.34%, thus it did not exceed ±15%. In addition, it did not differ significantly from the RE values of undiluted solutions. Thus, it can be concluded that the 100-fold dilution of the test sample does not significantly affect the result of the analysis of the tested compounds. Furthermore, there were no substances carried over between samples.

Table II. Parameters of the method for quantification of bendiocarb in liver tissue and validation results

Calibration curve						**Validation parameters**						
Biological matrix	**The linear concentration range [ng/g]**	**Calibration regression equation**	**The coefficient of determination (R^2)**	**LLOQ [ng/g]**	**LOD [ng/g]**	**QC concentration level [ng/g]**	**Intraday (n=5)**		**Interday (n=5)**		**Recovery (%) (n=5)**	**Matrix effect (%) (n=5)**
							Precision RSD (%)	**Accuracy RE (%)**	**Precision RSD (%)**	**Accuracy RE (%)**		
Liver homogenate	1 – 5000	y=1.78220x + 0	0.999	1.0	0.5	1	4.1	-1.4	3.3	-1.9	91.5	-8.5
						250	9.8	3.5	9.6	5.4	118.1	18.1
						2500	3.9	-0.9	2.6	0.1	98.0	-2.0

Comparison of methods applied in bendiocarb determination in biological samples

The most widespread methods used for the bendiocarb determination are chromatographic techniques (liquid and gas chromatography) coupled with UV detection at 220 nm [33] and 280 nm [34] as well as mass spectrometry (MS) [35] and tandem mass spectrometry (MS/MS) in multiple reaction monitoring mode (MRM) [36, 37]. These methods, as opposed to spectrophotometry [25], are characterized by high selectivity due to the chromatographic separation of substances contained in samples. In previously described methods, methyl benzoate [34], bromophos-methyl [35], and triphenylphosphate [37] were applied as internal standards. However, in each case, the use of isotopic-unlabelled analytical standards as IS can lead to unreliable analysis results due to the possibility of the presence of such compounds in tested samples. Reported to date, limits of quantification were 50 ng/mL [35,36] and 13 ng/mL [37], while the detection limits were 4 ng/mL [37] and 25 ng/mL [35]. The preparation procedure (based on dilution of the sample) applied by Menezes et al. [33] in their HPLC-UV method is not suitable for analysis of such complex matrix as biological specimens (especially tissues homogenates). In turn, other sample preparation techniques applied for biological samples (whole blood and hen eggs) were time-consuming due to the use of multistage preanalytical techniques, such as liquid-liquid extraction [36] and QuEChERS combined with dispersive solid-phase extraction (d-SPE) procedure [37]. The comparison of the methods for the determination of bendiocarb in biological samples is presented in **Table III**. The sample preparation procedure presented in this paper is fast and simple (one-stage precipitation of proteins with cold acetonitrile). In addition, by the development of a novel

Table III. Comparison of the methods for the determination of bendiocarb in biological samples.

Biological sample (volume / weight)	Sample preparation	Method	Column	Recovery [%] / Internal Standard	Linearity range [ng/mL or ng/g]	LOQ [ng/mL or ng/g]	LOD [ng/mL or ng/g]	References
Wool [a]	Extraction with methanol	HPLC-UV 280 nm	Zorbax ODS	96 – 103 Methyl benzoate	–[a]	–	–[a]	[34]
Intestine content (–)	LLE with hexane	UV-Spectrophotometry 276 nm	Information not applicable to the method	Information not applicable to the method	–	–	–	[25]
Raw milk (50 μL)	Dilution with phosphate buffer (pH 6) with ACN	HPLC-UV 220 nm	BSA-Si-C_8 Bovine serum albumin-dimethyl-octyl silica gel	99.6 –	–	–	–	[33]
Whole blood (1000 μL)	LLE with DCM / ethyl acetate / acetone	GC-MS EI/– (SIM)	HP 5-MS Dimethyl-phenyl-silicone	87 – 94 Bromo-phos-methyl	50 – 4000	50	25	[35]
Whole blood (2000 μL)	LLE (pH 7) with DCM / ethyl acetate / acetone	GC-MS/MS EI/triple quadrupole (MRM)	BPX5 SGE Phenyl-polysilphenylene-siloxane	93 – [b]	0.5 – 500 [c]	50 [c]	–	[36]
Hen eggs (5 g)	QuEChERS ($MgSO_4$: CH_3COONa) with d-SPE	HPLC-MS/MS ESI/QTRAP (MRM)	Zorbax Eclipse Plus C_{18}	75.7 – 81.5 Triphenyl-phosphate	5 – 100 [d]	13 [d]	4 [d]	[37]
Liver homogenate (100 μL)	Precipitation with ACN	UHPLC-MS/MS ESI/triple quadrupole (MRM)	Kinetex Biphenyl	91.5 – 118.1 Carbaryl-d_7	1 – 5000	1	0.5	Presented method

— Information not provided; [a] The authors did not weight the wool swatch (7.6 x 10.2 cm), therefore linearity (0.001-0.02% by weight) and limit of detection (4 ppm) could not be converted to an unified unit; [b] In described method 90 pesticides were determined by GC-MS/MS with the use of five different internal standards, however there is no specify information which IS was used for quantification of bendiocarb; [c] linearity was defined in the unit μg/kg while limit of quantification value in ng/mL; [d] expressed in unit ng/g

Abbreviations: LOQ – Limit of quantification; LOD – limit of detection; HPLC – high performance liquid chromatography; UHPLC – ultra-high performance liquid chromatography ; UV – ultra-visible detection; GC – gas chromatography; MS – mass spectrometry; MS/MS – tandem mass spectrometry; QTRAP – triple quadrupole linear ion trap; EI – electron ionization; ESI – electrospray ionization; SIM – single ion monitoring; MRM – multiple reaction monitoring; d-SPE – dispersive solid-phase extraction; LLE – liquid-liquid extraction; ACN – acetonitrile; DCM – dichloromethane

UHPLC-ESI-MS/MS method, the lowest limits of quantification (1 ng/g) and detection (0.5 ng/g) were achieved. The utilization of carbaryl-d_7 as an IS resulted in very good recovery values in the range of 91.5%–118.1%.

Analytical findings

The mean concentration of bendiocarb in livers of the common raven was found to be 7721 ng/g. In the case of the western marsh harrier, the mean concentration was 2266 ng/g, while the mean concentration in red kites and the white-tailed eagle were 4963 ng/g and 1808 ng/g, respectively (**Table IV**).

Table IV. Quantitative findings of bendiocarb (presented case) and carbofuran intoxication in livers of various bird species

Bird species	Organ	Concentration [ng/g]	Reference
common raven	liver	7721	Presented method
western marsh harrier	liver	2266	Presented method
red kite	liver	4963	Presented method
white-tailed eagle	liver	1808	Presented method
common buzzard	liver	14–1890	[14]
white-tailed eagle	liver	11–699	[14]
black kite	liver	270	[15]
duck	liver	370–480	[16]

The concentrations are in a similar range, but the time since ingestion and what effective concentration was delivered are not known. Furthermore, the bendiocarb LD50 value is unknown for the bird species we have analysed. The baits and food content extracted from the beaks and stomachs of the birds were characterized only by a qualitative analysis due to the very high concentrations of bendiocarb found in these materials.

Acute oral toxicity (LD50) was investigated in different adult mammals: rat 34–156 mg/kg, guinea pig 35 mg/kg, rabbit 35–40 mg/kg; and also in non-mammalian species like birds: mallard duck 3.1 mg/kg, bobwhite quail 16 mg/kg, hen 137 mg/kg; fish 0.7–1.8 mg/l, and bee 0.1 μg per bee [38].

Birds are more sensitive to acute exposure to carbamate pesticides than mammals due to a reduced level of anticholinesterase detoxifying enzymes [39] and high activity of AChE in the brain [40], resulting in the rapid rate of binding to carbamate pesticides. AChE inhibition leads to the accumulation of acetylcholine in the gap junction, causing hyperstimulation of cholinergic receptors and leading to a so-called cholinergic crisis manifested as miosis, hypersecretion of exocrine glands, bradycardia, and convulsions [41]. Although bendiocarb intoxication is rare among birds of prey, the most common poisoning occurs with carbofuran, which also belongs to carbamate pesticides and that is the reason why authors decided to compare their results with carbofuran data [8,10, 14,15,16]. Based on studies of acetylcholinesterase activity in brain tissue in 1967–2002 in Canada, massive carbofuran poisoning of many eagles species, as well as coyotes, foxes, skunks, and black-billed magpies as a result of secondary intoxication was identified [10]. Although the use of carbofuran is banned in the European Union from 2008, reports of intentional poisoning continue to appear; for example, white-tailed sea eagles dead on an island by the Baltic Sea were reported [42]. In a study conducted in northeastern Poland between 2008 and 2019, carbofuran was detected in 62% of samples during the first 3 years after the withdrawal of carbofuran-containing products from the market, while in subsequent years the percentage dropped to 44% and then 33% [14]. In this study, the presence of carbofuran in the livers of protected birds of prey was confirmed in 18 of the 33 common buzzard (*Buteo buteo*) liver samples and in 5 of the 15 analyzed liver samples from the white-tailed eagles (*Haliaeetus albicilla*), with concentrations ranging from 14-1890 ng/g liver for common buzzards and 11-699 ng/g liver for white-tailed eagles [14]. A case of possibly intentional carbofuran poisoning of poultry on a farm was also confirmed in China [16]. Toxicological testing of livers seized from two ducks confirmed the presence of carbofuran at 370 ng/g in the first and 480 mg/g in the second duck, respectively.

In general, it is difficult to extrapolate the toxicity of carbamate compounds within different species of wild birds. This is due to a number of aspects, which include the size of the bird's body, feeding habits and the physiology of the different animal species. These differences can affect metabolic activity, which also affects the concentration of the pesticide in the analyzed biological material.

The problem of wildlife intoxication with the use of pesticides is still present and an increasing num-

ber of bendiocarb-related cases may be expected, especially as a result of banning other widely used carbamate pesticides in the past–carbaryl and aldicarb.

Pathomorphological findings

In all birds, external examination showed that the continuity of the tissues was preserved, and there was no damage to the skeletal system. In some of the birds, in the beak and the throat, food remains were found, which corresponded to the bait secured in the field, i.e., minced meat.

In the oesophagus and the stomach, insignificant amounts of food were found. Trachea and lungs were intensely congested. The heart muscle was very hyperaemic. The gastric mucosa was hyperaemic, as was the intestinal mucosa. The liver and spleen were strongly congested and brittle. Kidneys were swollen and congested.

Histopathological findings

Histopathological examination of the kidneys revealed severe hyperaemia and blood stasis, as well as small foci of extravasation. Severe congestion and blood stasis were observed in the lungs. Within the intestines and stomach, there was evidence of hyperaemia and autolysis of the mucosa, as well as exfoliation of the cellular detritus into their lumen. Strong congestion and blood stasis were observed in the liver, numerous grains of hemosiderin, and bilirubin in hepatocytes. Moreover, an initial stage of autolysis was found. Blood stagnation and hyperaemia were found in the spleen. Within the heart muscle fibres, blood congestion and stagnation were found.

The histopathological characterization presented by us (**Fig. 3**) is consistent with what was described in the literature for bendiocarb and carbamates in general for other species.

According to a review conducted by Novotný concerning 89 cases of carbofuran poisoning between 2004 and 2009 in the Czech Republic, necropsies showed dried saliva around the oral cavity, congestion of the organs, and haemorrhagic necrosis of the small gut. While the histopathological examination revealed congestion of the kidney, liver, and lungs, granular dystrophy of the liver, and haemorrhagic necrosis of the small intestine [43]. Most of the histopathological analyses describing bendiocarb effects have been performed in rabbits under laboratory-controlled conditions. After the 30th day of bendiocarb *per os* administration, examination of the liver revealed dilated bile capillaries with reduced microvilli, irregularly shaped nuclei, and no visible alterations in the intercellular contacts [44]. Another long-term treatment with bendiocarb increased cell death, perisinusoidal fibrosis, and steatosis in liver [45]. The effects on kidney tissue have also been studied in rabbits. Bendiocarb caused extensive cytoplasmic vacuolization and degenerative changes,

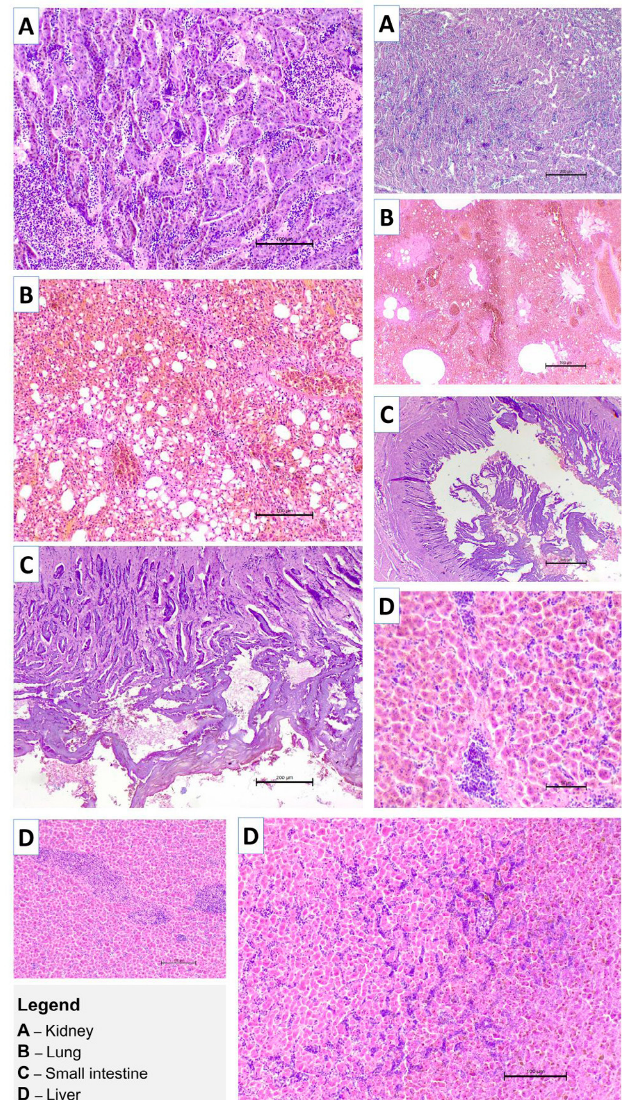

Fig. 3. Histopathology of tissues after bendiocarb ingestion. (A) kidney, (B) lung, (C) small intestine, and (D) liver of common raven (*Corvus corax*)

such as mitochondrial swelling and shortening of basal infoldings within proximal and distal tubules. Moreover, within Henle's loop, vacuolized cytoplasm was noticed and tubular sections revealed cellular detachment between the adjacent epithelial cells and rare necrotizing epithelial cells [26]. Kidney function in bendiocarb-treated rats was also compromised and was characterized as a disruption of antioxidative barrier (CAT, SOD, GPx) together with an increased level of malondialdehyde, creatine, urea, and uric acid. The histopathological analysis showed tubular degeneration and glomerular atrophies. Swelling of mitochondria and vacuolization in basal cytoplasm of proximal tubule cells of the kidney were also noticed [46].

Conclusions

In this study, we presented 48 cases of birds of prey poisoning with bendiocarb, characterized by toxicological and histopathological studies. High concentrations of bendiocarb were found in the livers of birds, which may indicate incidental and deliberate poisoning. Each organ subjected to histopathological examination showed damage, oedema, or congestion, suggesting a systemic mechanism of bendiocarb. This observation could be an indication of carbamate pesticide intoxication for clinicians, especially if it is associated with suggestive clinical signs or sudden death in the animals. Intentional poisoning of animals is a cruel crime, and its investigation should be based on reliable evidence provided by veterinarians and toxicologists to know the full scale of this phenomenon and be able to take appropriate measures to eliminate and prevent it. A novel UHPLC-ESI-MS/MS method was developed, fully validated, and applied for analysis of authentic samples from a case of bird intoxications after the ingestion of poisoned bait laced with bendiocarb. The presented sample preparation technique (one-step precipitation of proteins) is fast and simple. The time of analysis was shortened to less than 10 minutes and the lowest limit of quantification was achieved (1 ng/g) to date. For this reason, the developed UHPLC-ESI-MS/MS method possesses a great potential to be applied in routine environmental analysis.

References

1. Berny P. Pesticides and the intoxication of wild animals. J Vet Pharmacol Ther 2007; 30(2):93–100.
2. Chiari M, Cortinovis C, Vitale N, Zanoni M, Faggionato E, Biancardi A, Caloni F. Pesticide incidence in poisoned baits: A 10-year report. Sci Total Environ 2017; 601–602:285–292.
3. Guitart R, Croubels S, Caloni F, Sachana M, Davanzo F, Vandenbroucke V, Berny P. Animal poisoning in Europe. Part 1: Farm livestock and poultry. Vet J 2010; 183(3):249–254.
4. Guitart R, Sachana M, Caloni F, Croubels S, Vandenbroucke V, Berny P. Animal poisoning in Europe. Part 3: Wildlife. Vet J 2010; 183(3):260–265.
5. Vale A, Lotti M. Organophosphorus and carbamate insecticide poisoning. In: Handbook of Clinical Neurology. Vol. 131. Elsevier B.V. 2015; 149–168.
6. Malhotra H, Kaur S, Phale PS. Conserved Metabolic and Evolutionary Themes in Microbial Degradation of Carbamate Pesticides. Front Microbiol 2021; Jul 7;12:648868.
7. Lehel J, Laczay P, Déri J, Darin EG, Budai P. Model study on the clinical signs and residue concentrations of sublethal carbofuran poisoning in birds. J Wildl Dis 2010; 46(4):1274–1278.
8. Krone O, Auls S, Neurath H. Case report: secondary poisoning in a white-tailed sea eagle caused by carbofuran. Eur J Wildl Res 2017; 63(6):1–4.
9. Caloni F, Cortinovis C, Rivolta M, Davanzo F. Suspected poisoning of domestic animals by pesticides. Sci Total Environ 2016; 539:331–336.
10. Wobeser G, Bollinger T, Leighton FA, Blakley B, Mineau P. Secondary poisoning of eagles following intentional poisoning of coyotes with anticholinesterase pesticides in westen Canada. J Wildl Dis 2004; 40(2):163–172.
11. Ruiz-Suárez N, Boada LD, Henríquez-Hernández LA, González-Moreo F, Suárez-Pérez A, Camacho M, Zumbado M, Almeida--González M, Del Mar Travieso-Aja M, Luzardo OP. Continued implication of the banned pesticides carbofuran and aldicarb in the poisoning of domestic and wild animals of the Canary Islands (Spain). Sci Total Environ 2015; 505:1093–1099.
12. Sánchez-Bayo F. Insecticides Mode of Action in Relation to Their Toxicity to Non-Target Organisms. J Environment Analytic Toxicol 201; S:4.
13. Walker CH. Neurotoxic pesticides and behavioural effects upon birds. Ecotoxicology 2003; 12:307-316.

14. Kitowski I, Łopucki R, Stachniuk A, Fornal E. A pesticide banned in the European Union over a decade ago is still present in raptors in Poland. Environ Conserv 2020; 47(4), 310-314.
15. Hong SY, Lin HS, Walther BA, Shie JE, Sun YH. Recent avian poisonings suggest a secondary poisoning crisis of black kites during the 1980s in Taiwan. J Raptor Res 2018; 52(3):326–337.
16. Chen S, Yu Y, Ma J, Wen C, Wang X, Zhou Q. Simultaneous determination of carbofuran and 3-hydroxycarbofuran in duck liver by an UPLC-MS/MS. Acta Chromatogr 2020; 33(4), 354-360.
17. Council Decision of 18 March 2003 concerning the non-inclusion of aldicarb in Annex I to Council Directive 91/414/EEC and the withdrawal of authorisations for plant protection products containing this active substance. Off J Eur Union 22/03/2003; L 076:0021–4. [accessed 2021 May 23].
18. Commission Decision of 13 June 2007 concerning the non-inclusion of carbofuran in Annex I to Council Directive 91/414/EEC and the withdrawal of authorisations for plant protection products containing that substance. Off J Eur Union 16/06/2007;L156:0030–1. [accessed 2021 May 23].
19. Jensen BH, Petersen A, Christensen T. Probabilistic assessment of the cumulative dietary acute exposure of the population of Denmark to organophosphorus and carbamate pesticides. Food Addit Contam – Part A Chem Anal Control Expo Risk Assess 2009.; 26(7):1038–1048.
20. Flesarova S, Lukac N, Danko J, Massanyi P. Bendiocarbamate induced structural alterations in rabbit thymus after experimental peroral administration. J Environ Sci Health B . 2007; 42:329–334.
21. Whyatt RM, Barr DB, Camann DE, Kinney PL, Barr JR, Andrews HF, Hoepner LA, Garfinkel R, Hazi Y, Reyes A, Ramirez J, Cosme Y, Perera FP. Contemporary-use pesticides in personal air samples during pregnancy and blood samples at delivery among urban minority mothers and newborns. Environ Health Perspect 2003; 111:749– 756.
22. Aly HAA, Domènech O. Cytotoxicity and mitochondrial dysfunction of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in isolated rat hepatocytes. Toxicol Lett 2009; 191(1):79-87.
23. World Health Organization (WHO). The WHO recommended classification of pesticides by hazard and guidelines to classification. WHO Press, World Health Organization, Geneva, Switzerland, 2004. [accessed 2022 October 27].
24. EPA. R.E.D. FACTS: Bendiocarb. U.S. EPA: Washington, D.C, 1999. https://www3.epa.gov/pesticides/chem_search/reg_actions/reregistration/fs_PC-105201_1-Sep-99.pdf [accessed 2022 October 27].
25. Humphreys DJ, Stodulski JBJ. Bendiocarb poisoning in birds and a dog. J Appl Toxicol 1981; 1(6):332–333.
26. Almášiová V, Holovská K, Cigánková V. Structural and ultrastructural study of the rabbit kidney exposed to carbamate insecticide. Acta Vet Brno 2014 83(4):295–298.
27. Smulders CJ, Bueters TJ, Van Kleef RG, Vijverberg HP. Selective effects of carbamate pesticides on rat neuronal nicotinic acetylcholine receptors and rat brain acetylcholinesterase. Toxicol Appl Pharmacol 2003; 193:139–146.
28. Jokanovic M. Medical treatment of acute poisoning with organophosphorus and carbamate pesticides. Toxicol Lett 2009; 190:107–115.
29. Peters FT, Drummer OH, Musshoff F. Validation of new methods. Forensic Sci Int 2007; 165:216–224.
30. Chambers E, Wagrowski-Diehl DM, Lu Z, Mazzeo JR. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. J Chromatogr B 2007; 852:22–34.
31. Bertero A, Chiari M, Vitale N, Zanoni M, Faggionato E, Biancardi A, Caloni F. Types of pesticides involved in domestic and wild animal poisoning in Italy. Sci Total Environ 2020; 707:136129.
32. Newbery S, Munro R. Forensic veterinary medicine 1. Investigation involving live animals. In Pract 2011; 33(5):220–227.
33. Menezes ML, Fèlix G.On Line Extraction and Separation of Bendiocarb, Methomyl, Methylparathion, and Pentachlorophenol Pesticides from Raw Milk. J Liq Chromatogr Relat Technol 1998; 21:2863–2871.
34. Zehner JM, Simonaitis RA, Bry RE. High Performance Liquid Chromatographic Determination of Bendiocarb on Wool. JAOAC 1980; 63:47–48.
35. Frenzel T, Sochor H, Speer K, Uihlein M. Rapid Multimethod for Verification and Determination of Toxic Pesticides in Whole Blood by Means of Capillary GC-MS. J Anal Toxicol 2000; 24:365–371.
36. Luzardo OP, Almeida-González M, Ruiz-Suárez N, Zumbado M, Henríquez-Hernández LA, Meilán MJ, Camacho M, Boada LD. Validated analytical methodology for the simultaneous determination of a wide range of pesticides in human blood using GC-MS/MS and LC-ESI/MS/MS and its application in two poisoning cases. Sci Justice 2015; 55:307–315.
37. Song NE, Lee JY, Mansur AR, Jang HW, Lim MCh, Lee Y, Yoo M, Nam TG. Determination of 60 pesticides in hen eggs using the QuEChERS procedure followed by LC-MS/MS and GC-MS/MS. Food Chem 2019;125050.
38. Petrovova E, Sedmera D, Luptacova L, Mazensky D, Denko J. Chick development and high dose of bendiocarb. J Environ Sci Health A Tox Hazard Subst Environ Eng 2012; 47(9):1312-8.
39. Parker ML, Goldstein MI. Differential toxicities of organophospate and carbamate insecticides in the nestling European starling (Sturnus vulgaris). Arch Environ Contam Toxicol . 2000; 39(2):233–242.
40. Westlake GE, Martin AD, Stanley PI, Walker CH. Control enzyme levels in the plasma, brain and liver from wild birds and mammals in Britain. Comp Biochem Physiol Part C, Comp 1983; 76(1):15–24.
41. O'Malley M. 1997. Clinical evaluation of pesticide exposure and poisonings. Lancet 1983; 349(9059):1161–1166.
42. Krone O, Auls S, Neurath H. Case report: secondary poisoning in a white-tailed sea eagle caused by carbofuran. Eur J Wildl Res 2017; 63(6):63–66.

43. Novotný L, Misík J, Honzlová A, Ondráček P, Kuča K, Vávra O, Rachač V, Chloupek P. Incidental poisoning of animals by carbamates in the Czech Republic. J Appl Biomed 2011; 9(3):157–161.
44. Holovska K, Almasiova V, Cigankova V. Ultrastructural changes in the rabbit liver induced by carbamate insecticide bendiocarb. J Environ Sci Heal – Part B Pestic Food Contam Agric Wastes 2014; 49(8):616–623.
45. Petrovova E, Purzyc H, Mazensky D, Luptakova L, Torma N, Sopoliga I. Morphometric Alterations, Steatosis, Fibrosis and Active Caspase-3 Detection in Carbamate Bendiocarb Treated Rabbit Liver. Env Toxicol 2015; 30:212–222
46. Adiguzel C, Kalender Y. Bendiocarb-induced nephrotoxicity in rats and the protective role of vitamins C and E. Environ Sci Pollut Res 2020; 27(6):6449–6458.

ORCID

Agnieszka Chłopaś-Konowałek – 0000-0003-2814-8114
Marcin Zawadzki – 0000-0003-2146-9724
Łukasz Kurach – 0000-0002-1005-8068
Olga Wachełko – 0000-0003-4068-2475
Rafał Ciaputa – 0000-0002-8816-7723
Kaja Tusiewicz – 0000-0002-9968-2042
Paweł Szpot – 0000-0002-5352-3492

CORRESPONDING AUTHOR

Kaja Tusiewicz
Wroclaw Medical University, Department of Forensic Medicine,
4 J. Mikulicza-Radeckiego Str., Wroclaw 50345, POLAND
e-mail: kajatusiewicz@gmail.com