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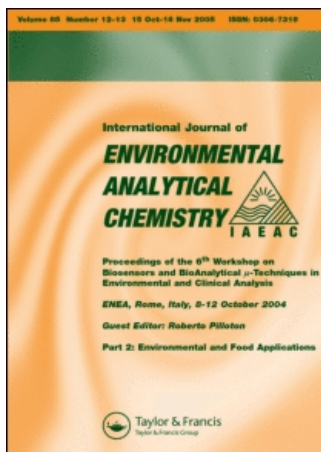
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Determination of bromadiolone in pheasants and foxes by differential pulse voltammetry

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Bromadiolone, a commercially used anticoagulant rodenticide, was determined in tissues of various animals by differential pulse voltammetry with a carbon-paste electrode. Under the most suitable experimental conditions (step potential of 25 mV s^{-1} and 0.2 mol L^{-1} acetate buffer, pH 4.2), the limit of detection was 0.5 ng mL^{-1} . The electroanalytical method was consequently used to investigate the bromadiolone transport within the food chain. Pheasants were exposed to bromadiolone and then used as feed for a fox. The average levels were 528 and 198 ng of bromadiolone per gram of fresh weight of liver of pheasant and fox, respectively. Due to the surprisingly lower content of bromadiolone in the latter, a basic biochemical analysis, particularly blood coagulation, was performed. Among the parameters studied (thrombin time, prothrombin time, activated partial thromboplastin time, and fibrinogen), there was prolongation of the prothrombin time and activated partial thromboplastin time.

Keywords: Bromadiolone; Superwarfarines; Differential pulse voltammetry; Cyclic voltammetry; Carbon-paste electrode; Electrochemistry; Animals

1. Introduction

Rodenticides are a sub-group of pesticides killing rodents. The ‘second generation’ of rodenticides called superwarfarines has the widest spectrum of use now. According to

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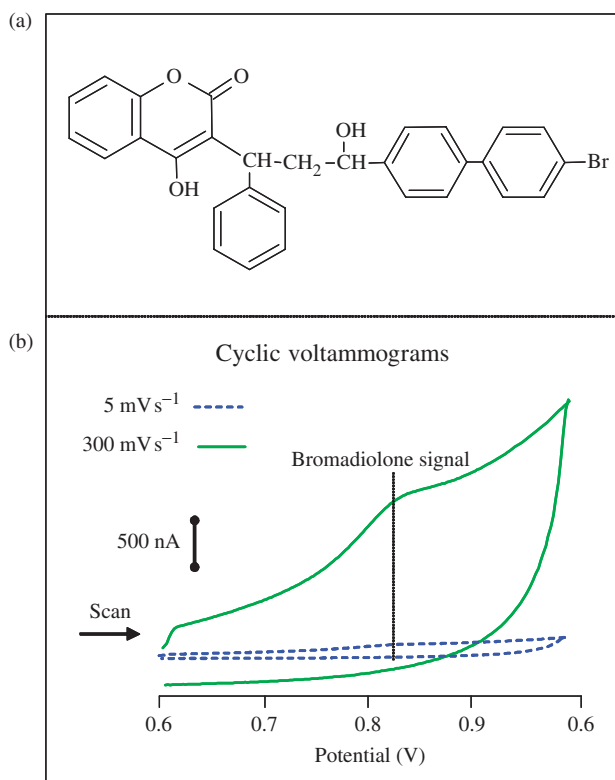


Figure 1. Chemical formula of bromadiolone (3-(3-(4'-brom-1,1'-biphenyl-4-yl)-3-hydroxy-1-phenylpropyl)-4-hydroxy-coumarine) (a). Typical cyclic voltammograms of bromadiolone ($10 \mu\text{g mL}^{-1}$) measured at two scan rates (5 and 300 mV s^{-1}). CV parameters: initial potential of 600 mV, end potential 1000 mV, step potential 2.4 mV s^{-1} , supporting electrolyte: 0.2 mol L^{-1} acetate buffer, pH 4.0 (b).

their chemical structure, they belong to hydroxycoumarines and their derivatives. They can be taken up by an organism through the digestive and respiratory system, eventually through skin. After ingestion of these substances, the liver is the main organ involved in its metabolism [1].

Bromadiolone (3-(3-(4'-brom-1,1'-biphenyl-4-yl)-3-hydroxy-1-phenylpropyl)-4-hydroxy-coumarine, figure 1a) belongs to the group of superwarfarines and is a commonly used anticoagulant rodenticide, commercially available as numerous rodenticide preparations. Its LD_{50} for a rodent is about 1 mg kg^{-1} , whereas the LD_{50} for other mammals oscillates around several tens of milligrams per kilogram. Thus, a single dose can be lethal. Nevertheless, this compound can be toxic to other non-mammalian species such as fishes, where an LC_{50} of 1.4 mg L^{-1} per 96 h has been described. Like other second-generation rodenticides, bromadiolone acts as a vitamin K_1 (fytomenadion) antagonist. This rodenticide inhibits the production of factors II, VII, IX, and X in the liver. This results in prolongation of the extrinsic, intrinsic, and common coagulation pathways leading to coagulopathy [2–4]. Besides this, the interaction of bromadiolone with blood plasma proteins is not yet clear, although a recently published article focused on the interaction of bromadiolone with albumin [5].

As a consequence of bromadiolone ingestion, the death of house and/or free living non-target animals has been observed [6]. Sub-lethal concentrations of bromadiolone have been found in livers of animals consuming rodents like foxes, buzzards and polecats, thus reflecting its transport through the food chain [6–9].

The monitoring of bromadiolone action and its distribution in the environment require rapid, low-cost, and easy-to-use analytical methods for the detection of this compound. A broad range of analytical methods have been used for these purposes, e.g. gas chromatography coupled with mass spectrometry (GC–MS) [10], thin-layer chromatography (TLC) [11], or immunochemical methods [12]. High-performance liquid chromatography (HPLC) is the most commonly used method for bromadiolone determination [8]. The use of HPLC coupled with fluorescence, mass, and UV-Vis detectors has been published [8, 13–20]. Each method has its advantages and limitations, and may serve a particular need in analysis. Besides these robust analytical techniques, electrochemical detection (ED) offers an attractive alternative method for electroactive species detection, because of its inherent advantages of simplicity, high sensitivity, relatively low cost, and potential for miniaturization with detection limits comparable with other modern analytical techniques [21–25]. The electrochemical behaviour of various 4-hydroxycoumarines was studied using different electrochemical methods [13, 26–30]. In the present work, we aimed to utilize differential pulse voltammetry with a carbon-paste electrode as a new tool for determination of bromadiolone. By substantially increasing the ratio between the faradaic and non-faradaic currents, such techniques permit convenient quantitation down to the 10^{-8} mol L⁻¹ concentration level [31]. The electroanalytical method was consequently used to investigate the bromadiolone transport within the food chain.

2. Experimental

2.1 Chemicals

HPLC-grade acetonitrile (>99.9%; v/v) and methanol (>99.9%; v/v) from Merck (Darmstadt, Germany) were used. Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise. Stock standard solutions of bromadiolone (1 mg mL⁻¹) was prepared using methanol and stored in the dark at -20°C. Working standard solutions were prepared daily by dilution of the stock solutions. The animal experiment was performed with a commercially available rodenticide Lanirat[®] Micro containing 0.005% of bromadiolone (w/w). The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled using MultiLab Pilot software. The pH electrode (SenTix H, pH 0.14/0.100°C/3 mol L⁻¹ KCl) was regularly calibrated with WTW buffers (Weilheim, Germany).

2.2 Electrochemical measurement

Cyclic and differential pulse voltammetric measurements were performed with an Autolab Analyser (EcoChemie, Netherlands) connected to a VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. The working electrode was a carbon-paste electrode. The reference electrode was an Ag/AgCl/3 M KCl electrode,

and the auxiliary electrode was a graphite electrode. For smoothing and baseline correction, the software GPES 4.4 supplied by EcoChemie was employed. The cyclic voltammetric parameters were as follows: an initial potential 600 mV, end potential 1000 mV, step potential 2.4 mV s^{-1} , and supporting electrolyte 0.2 mol L^{-1} acetate buffer, pH 4.0. The differential pulse voltammetric parameters parameters were as follows: initial potential 450 mV, end potential 1100 mV, modulation time 0.057 s, time interval 0.2 s, modulation amplitude 40 mV, and supporting electrolyte 0.2 mol L^{-1} acetate buffer. The step potential and pH of acetate buffer were optimized (see section 3). All experiments were carried out at room temperature (25–27°C).

2.2.1 Preparation of carbon-paste electrode. The carbon paste (about 0.5 g) was made from 70% graphite powder (Sigma-Aldrich) and 30% mineral oil (Sigma-Aldrich; free of DNase, RNase, and protease) according to the literature [32, 33]. This paste was housed in a Teflon body (2.5-mm diameter disk surface). Prior to measurements, an electrode surface was renewed by polishing with a soft filter paper. Then, the surface was ready for measurement of a sample volume of $5 \mu\text{L}$.

2.3 Distribution tests

2.3.1 Earthworms and voles. Ten earthworms (*Eisenia fetida*) were kept in the presence of 10 granules (approx. 1.64 g) of Lanirat[®] Micro preparation (bromadiolone content 0.005%) in a 1 L vessel with artificial soil (OECD 207) for 14 days. Then, they were used as feed for three voles (*Microtus arvalis*, 3 pieces per individual), whose livers were analysed. In addition, three voles were fed by granules of Lanirat[®] Micro, and then their livers were analysed. As a control, livers from three voles fed with the usual feed for rodents were also analysed.

2.3.2 Pheasants. Tests on the attractiveness of bait granules and toxicity of bromadiolone were based on guidelines for testing plant protection products in registration (i.e. BBA methods) published by the Federal Biological Agricultural and Forestry Institute Berlin and Braunschweig [34, 35]. For the test, 10 male adult pheasants (*Phasianus colchicus* L. 1758) were used. The animals were in good health and artificially reared. The test preceded the adaptation on test conditions for 1 week. During the adaptation time, the pheasants were fed with a mixture of common feed (commercially available standard feed mixture for a pheasant layer) and a support of granulated bait in ratio 1 : 1. This mixture was administered to pheasants in a feeder *ad libitum*. One day before the test, the pheasants were not fed, but the water was administered *ad libitum*. During the test and adaptation time, the pheasants were kept individually in an outdoor aviary commonly used for pheasants breeding. The daily feed dose per pheasant was 70 g. The ratio of tested substance and common feed was 3 : 1 (52.5 g = 75%, approx. 320 granules) and common feed (12.5 g = 25%). As a control, we used ten pheasants bred the same as the exposed animals, but fed with the same dose of rodenticide-free granules in the same ratio with common feed.

The test was divided into a 16 h starvation and 8 h exposition phase. This cycle was repeated three times. After the last exposition phase, the 14-day-long observations followed. In this period, the pheasants were fed with a common feed mixture, and water

was administered *ad libitum*. The pheasants were weighed in the following phases of the experiment: at the beginning of the adaptation time, one day before the first exposition phase, at the end of the last exposition phase, and on the 15th day of observation. One day before the first application of the tested substance and the 15th day of the experiment, blood was collected for biochemical tests. The blood serum was frozen and analysed 2 h later. At the end of the observing period, all test animals were killed.

2.3.3 Fox. A healthy 36-month old fox (*Vulpes vulpes*) was fed with meat from five pheasants for 5 days, and then the observation phase followed. On the 20th day of experiment, blood was collected for blood-clotting tests. As a control, blood tests obtained from a 6-month old healthy fox were taken. After the fox died, the dissection and blood-clotting tests were carried out.

2.3.4 Hares. In addition to the experiments under control conditions, liver samples from wild animals, namely hares (*Lepus europaeus*), were also analysed.

2.4 Preparation of samples for electrochemical measurement

Samples of animal tissues (*M. arvalis*, *L. europaeus*, *P. colchicus*, and *V. vulpes*) were provided by the University of Veterinary and Pharmaceutical Sciences in Brno, Czech Republic. Sonication of the tissues (approximately 0.5 g) was performed at 4°C for 15 min at 38 kHz and 150 W using a K5 Sonicator (Slovakia). Then, the mixture was homogenized by shaking on a Vortex-2 Genie (Scientific Industries, New York) at 4°C for 15 min. The homogenate was centrifuged (14,000 g) for 20 min at 4°C using a Universal 32 R centrifuge (Hettich-Zentrifugen GmbH, Tuttlingen, Germany). The supernatant was stored in the dark at -20°C. Prior to analysis, the samples were diluted twice with methanol. An animal-tissue sample was measured in five replicates.

2.5 Recovery

Accuracy, precision, and recovery of bromadiolone determination were evaluated using homogenates of the analysed animal tissues spiked with standard. Before the extraction was carried out, 100- μ L bromadiolone standards (concentration of 2 μ g mL⁻¹) and 100 μ L of water were added to the animal-tissue sample. Homogenates were assayed blindly, and bromadiolone concentrations were plotted from the calibration curve. Calculation of recovery was expressed according to references [21, 22, 36–38].

2.6 Determination of various biochemical parameters of pheasants

To examine the state of the internal environment of pheasants, various biochemical parameters were measured in blood collected on the 15th day of the experiment. As a control, blood samples collected from a control group of pheasants were used. The biochemical parameters determined were as follows: stress indicators (glucose), fitness indicators (whole proteins), tests of permeability and integrity of cell membranes (alanine aminotransferase, ALT; aspartate aminotransferase, AST; alkaline

Table 1. Changes in body weight of the pheasants during the experiment.^a

Weight of the pheasants during the experiment	Average weight (g)	SEM (g)	SD (g)	Min (g)	Max (g)
(1) At the beginning of the experiment (29 April)	1136	25.61	57.27	1060	1200
(2) One day before the first exposition (6 May)	1110	21.45	47.95	1040	1160
(3) At the end of last exposition phase (10 May)	1118	12.81	28.63	1080	1150
(4) Fifteen days after last administration of bromadiolone (25 May)	1108	12.41	27.74	1070	1140

^aSEM: standard error of mean; SD: standard deviation; Min: lowest pheasant weight; Max: highest pheasant weight.

phosphatase, ALP), and electrolytes (Na, K, Ca, P). The values from the biochemical profile were obtained using an automatic analyser.

The parametric *t*-test (for independent experiment design) was used to determine the differences between the control and exposed groups. A value of $p < 0.05$ was considered significantly different. Before the use of the *t*-test, the homogeneity of samples was evaluated by the *F*-test.

3. Results and discussion

3.1 Tests on the attractiveness of bait granules and toxicity of bromadiolone for pheasants

Based on the feeding experiment, it was demonstrated that the tested rodenticide bait granules are a very attractive food for pheasants. Each experimental pheasant was administered 52.5 g of bait granules, which equated to 960 granules for 3 days. Only two pheasants did not eat the entire administered portion, particularly the amounts of 3.5 g or of 1 g left. Neither symptoms of intoxication nor behavioural changes were observed 14 days after administration of the rodenticide.

The body weight of experimental animals was measured four times during the experiment: (1) at the beginning of the experiment; (2) one day before the first exposition—their average weight decreased about 26 g in comparison with the pheasants weighed at the beginning of the experiment; (3) at the end of the last exposition phase (no significant average weight change was observed); and (4) 15 days after the last administration of bromadiolone. During this period when the pheasants were fed with a standard feed mixture, no significant body weight change was observed. The changes in body weight of the experimental animals are summarized in table 1.

3.2 Biochemical parameters of blood of bromadiolone-treated pheasants and dissection

Necropsy of pheasants killed at the end of the experiment did not reveal any pathological changes except for occasional haemorrhages in the breast muscles. Fundamental biochemical blood parameters were determined at the end of the experiment. The tested group of pheasants can be characterized by significantly lower

($p < 0.05$) levels of glucose, creatinine, and sodium in comparison with both the control and pre-exposure values. On the other hand, uric acid levels were significantly higher in the experimental group. Levels of total proteins, AST, ALP, ALT, potassium, calcium, and phosphorus were not statistically different.

3.3 Optimization of an electrochemical method for bromadiolone determination

Based on previous experience in the field of electrochemical determination of various biologically active compounds [25, 39–52], cyclic voltammetry was utilized to study the electroactivity of bromadiolone, because this technique allows the study of the basic electrochemical properties and behaviour of various compounds of interest [53, 54]. A typical cyclic voltammogram of bromadiolone is shown in figure 1(b). Bromadiolone gave an oxidation signal at a potential of 840 mV. The detection limit of this method, which was estimated by dilution of a standard sample until the bromadiolone signal disappeared, was about 50 ng mL^{-1} . Due to the high detection limit of this technique the electrochemical behaviour of bromadiolone was consequently studied by differential pulse voltammetry (DPV), where the lower detection in comparison with CV could be expected [24]. The DP voltammogram of bromadiolone is shown in figure 2(a). The compound of interest gave a well-developed signal at a potential of 730 mV. In order to reach a low detection limit various experimental conditions were optimized, particularly, the step potential and pH of acetate buffer. The dependence of the peak height of bromadiolone measured in the presence of acetate buffer (0.2 mol L^{-1} , pH 4.0) on the step potential is shown in figure 2(b). The height of the peak increased markedly up to 25 mV s^{-1} and then increased more gradually. Thus, a step potential of 25 mV s^{-1} was used in the following experiments.

After that, the issue of how the different pHs of the acetate buffer can influence the signal of bromadiolone was studied. The highest signal of bromadiolone was measured in the presence of 0.2 mol L^{-1} acetate buffer, pH 4.2. Under the most suitable experimental conditions (step potential of 25 mV s^{-1} and 0.2 mol L^{-1} acetate buffer pH 4.2), the dependence of peak height of bromadiolone on its concentration within the range of $0.2\text{--}5 \text{ } \mu\text{g mL}^{-1}$ (figure 2c) was studied. The dependence was strictly linear ($y = 199.18x + 30.031$, $R^2 = 0.9978$) with a detection limit of 0.5 ng mL^{-1} . The limit was estimated by dilution of a standard sample until the bromadiolone signal disappeared.

Then, the experimental results obtained were utilized for bromadiolone determination in the biological samples by the standard addition method to evaluate the influence of other components. The content of bromadiolone in a tissue sample was 599 ng g^{-1} , as determined by the standard addition method, and 553 ng g^{-1} plotted from the calibration curve. Due to the difference (8%) between the results obtained, the bromadiolone content in the tissues of interest was evaluated by plotting from the calibration curve due to a lower experimental error. In addition, the extraction efficiency of the bromadiolone from animal tissues was determined. In particular, recovery was checked for the compounds of interest by the addition of known amounts of bromadiolone working standards to homogenates (table 2). Recovery was about 91% (table 2).

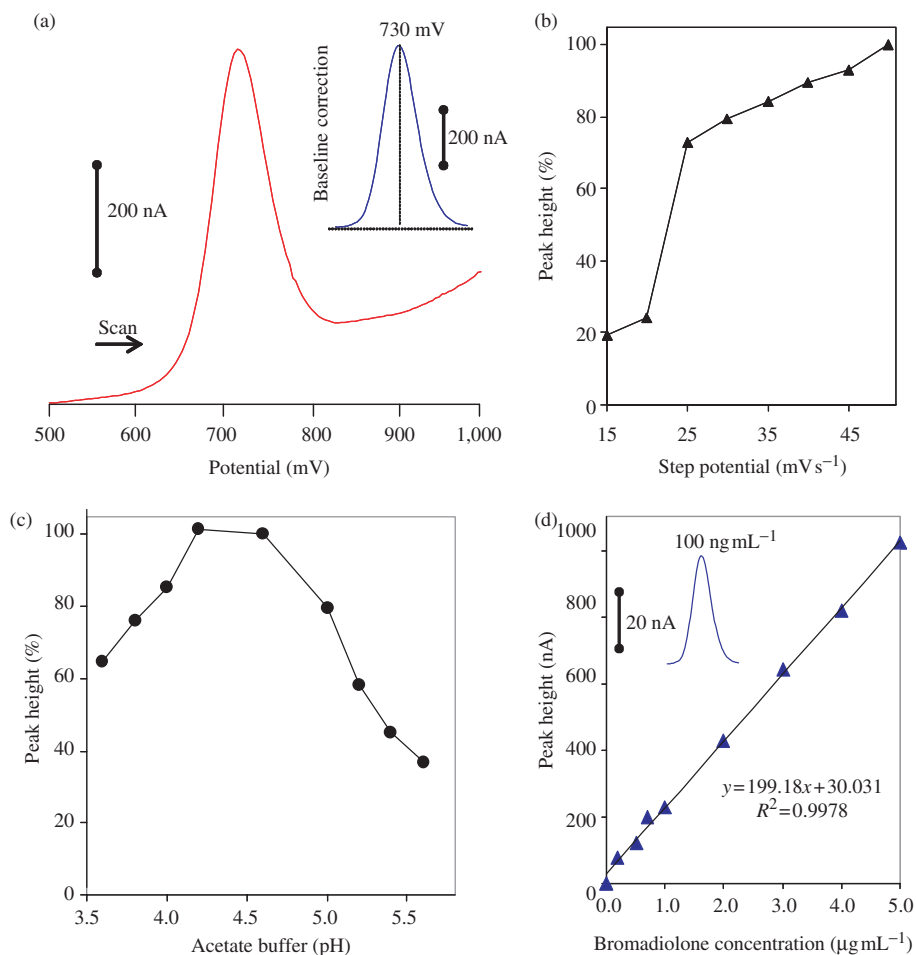


Figure 2. Typical DP voltammograms for bromadiolone (3 µg mL⁻¹) (a); insert: peak of bromadiolone after baseline correction. Dependences of peak height of bromadiolone on step potential (b), pH of acetate buffer (c) and its concentration within the range of 0.2–5 µg mL⁻¹ (d). DPV parameters: an initial potential of 450 mV, end potential 1100 mV, modulation time 0.057 s, time interval 0.2 s, modulation amplitude of 40 mV, supporting electrolyte 0.2 mol L⁻¹ acetate buffer.

Table 2. Recovery of bromadiolone for pheasant tissue analysis ($n = 5$).

Compound of interest	Homogenate (µA) ^a	Spiking (µA) ^{a,b}	Homogenate + spiking (µA) ^{a,b}	Recovery (%)
Bromadiolone	147 ± 5 (3.4)	353 ± 10 (2.8)	456 ± 19 (4.2)	91.2

^aPeak height measured by DPV; expressed as mean ± SD (CV%).

^bBromadiolone spike (2 µg mL⁻¹) was added.

3.4 Bromadiolone content in tissues of the exposed animals

The muscles and livers of pheasants exposed to bromadiolone and the liver of the fox feed with five bromadiolone-exposed pheasants were prepared according to the

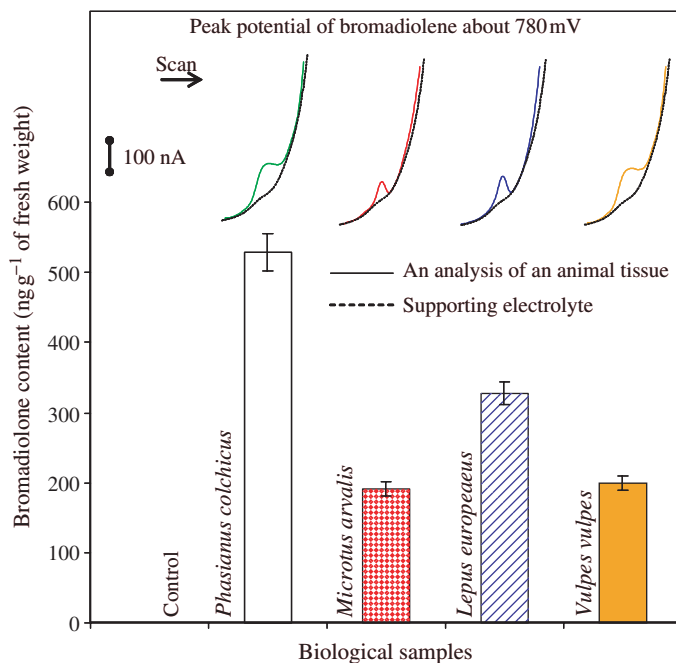


Figure 3. Bromadiolone content (nanograms of bromadiolone per fresh weight of tissue) in various animals: vole (*M. arvalis*), pheasant (*P. colchicus*), fox (*V. vulpes*) and hare (*L. europeaeus*) measured by differential pulse voltammetry. DPV parameters: step potential 25 mV s^{-1} , supporting electrolyte 0.2 mol L^{-1} acetate buffer, pH 4.2. Other details as in figure 2.

procedure mentioned in section 2 and analysed using the optimized electrochemical technique. The average level of bromadiolone was 528, 191, and 327 ng of bromadiolone per gram of fresh weight of liver of the pheasants, voles, and hares, respectively. The content of bromadiolone in the liver of fox was 198 ng of bromadiolone per gram of fresh weight of fox liver (figure 3). The potential of the bromadiolone peak for the standard and the peak determined in a real sample differed due to the complexity of the sample matrix, whereas the signal measured in real samples shifted to a more positive potential (about 5 mV). It clearly follows from the results obtained that the lowest contents of bromadiolone were determined in vole and fox. This phenomenon can be associated with the fact that the rodenticides are obviously stored and metabolized in liver and excreted from mammals via faeces. Also, their biotransformation in animals occurs very slowly [1, 55].

Due to the surprisingly lower content of bromadiolone in fox, we have done a basic biochemical analysis, particularly blood coagulation, of fox tissues. There are several clinical laboratory tests to monitor blood coagulation. Reference values for the fox (*V. vulpes*), however, are lacking. As the fox and dog are related species, it may be assumed that the physiological values of thrombin time (TT), prothrombin time (PT), activated partial thromboplastin time (APTT), as well as fibrinogen (F) are similar. Blood collected from two 6-month old healthy fox individuals provided the following coagulation parameters: 13.6–15.0 TT, 7.7–9.2 PT, 15.4–17.0 APTT, and 1.75–2.19 F; these fall within the reference range for the dog. The fox that consumed five

bromadiolone-containing pheasants died 36 days after the consumption of the first. Blood collected 20 days after consumption of the first pheasant yielded 15.3 TT, 15.5 PT, 25.4 APTT, and 1.95 F; i.e. there was a prolongation of PT and APTT. Therefore, it can be concluded that the bromadiolone-exposed fox had a worse blood sedimentation rate in comparison with non-treated animals. These signs related to bleeding in the fox included depression, weakness, pallor, and melena. On autopsy, there were petechiae and ecchymoses, and blood within body cavities.

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Note

The co-authors of the article are holders of the Certificate issued by the Central Commission for Animal Welfare (CCAW), completed the preparation, and successfully passed the qualifying examination according to § 17 of the Act No. 246/1992 Coll. of Laws on Prevention of Cruelty to Animals, as amended.

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