

*Sensors* **2007**, *7*, 1271-1286

**sensors**

ISSN 1424-8220

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*Full Research Paper*

## **Hazards of Secondary Bromadiolone Intoxications Evaluated using High-performance Liquid Chromatography with Electrochemical Detection**

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*Received: 28 June 2007 / Accepted: 18 July 2007 / Published: 20 July 2007*

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**Abstract:** This study reported on the possibility of intoxications of non-target wild animals associated with use of bromadiolone as the active component of rodenticides with anticoagulation effects. A laboratory test was done with earthworms were exposed to bromadiolone-containing granules under the conditions specified in the modified OECD 207 guideline. No mortality of earthworms was observed during the fourteen days long exposure. When the earthworms from the above test became a part of the diet of common voles in the following experiment, no mortality of consumers was observed too. However, electrochemical analysis revealed higher levels of bromadiolone in tissues from earthworms as well as common voles compared to control animals. There were determined comparable levels of bromadiolone in the liver tissue of common voles after primary ( $2.34 \pm 0.10 \mu\text{g/g}$ ) and secondary ( $2.20 \pm 0.53 \mu\text{g/g}$ ) intoxication. Therefore, the risk of secondary intoxication of small mammalian species feeding on bromadiolone-containing

earthworms is the same as of primary intoxication through baited granules. Bromadiolone bio-accumulation in the food chain was monitored using the newly developed analytical procedure based on the use of a liquid chromatography coupled with electrochemical detector (HPLC-ED). The HPLC-ED method allowed to determine the levels of bromadiolone in biological samples and is therefore suitable for examining the environmental hazards of this substance.

**Keywords:** anticoagulant rodenticide; electrochemistry, cyclic voltammetry; animal tissues; non-target organisms; *Eisenia fetida*; *Microtus arvalis*; *Lepus europaeus*

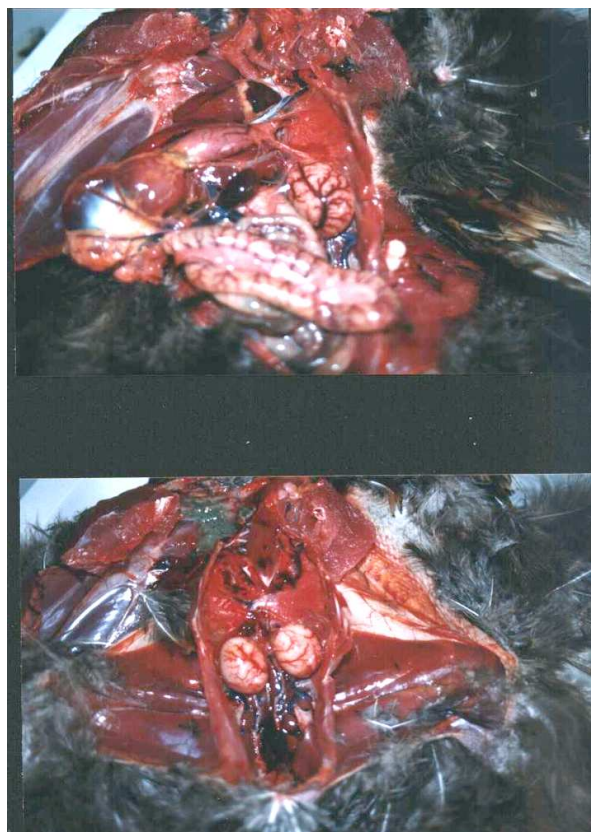
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## 1. Introduction

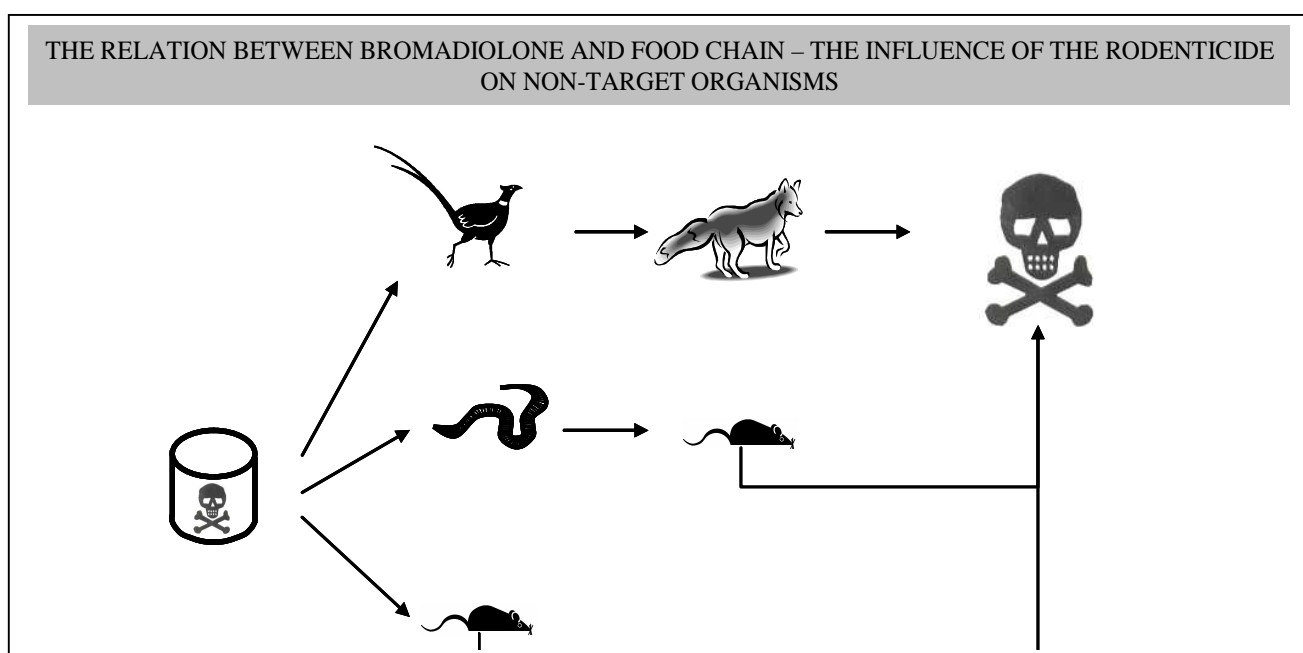
Industrial pollutants come from various anthropogenic sources into the environment [1,2]. Agricultural pesticides including rodenticides belong to such group of pollutants [3-6]. Rodenticides are used to control pest rodents that may be harbouring zoonotic infectious agents [7-11]. However, rodenticides may have also direct or indirect effects on non-target organisms [12,13]. Detrimental effects as well as mortality in both domestic [14] and wild non-target animals [4,15] have been shown after use of anticoagulant rodenticides. One of the so-called second-generation anticoagulant rodenticide, bromadiolone, can be found in livers of predators of small rodents such as the fox, marten, and birds of prey at sublethal concentrations [16-18]. Bromadiolone acts as the antagonist of vitamin K1 and inhibits thus blood clotting (Fig. 1). It may be characterized as having a very low LD50 value and high stability [19]. Therefore bromadiolone may persist in the environment (particular in the soil), bio-accumulate in an organism and cause acute or chronic intoxications [20]. In addition the interaction of bromadiolone and blood plasma proteins is not clear yet. Recently, a study reporting on the interaction of bromadiolone and albumin was published [21].

Several products containing bromadiolone have been approved for use as baited granules in the Czech Republic. The granules disintegrate quickly in the fields of application, thus there is a risk of both primary and secondary intoxications of non-target organisms, because the carrying substances of granules may attract earthworms. The insectivorous and other small mammalian species may possibly become intoxicated by this way (Fig. 2).

Great attention has been paid to the surveillance of residues of pesticides both in the environment and foods [22-27]. Several analytical approaches such as gas chromatography mass spectroscopy, thin layer chromatography or immunochemistry [28,29] may be used to determine bromadiolone. However, high performance liquid chromatography (HPLC) coupled with various detectors (fluorescence or mass detectors) is the most commonly used instrument to detect bromadiolone [30-34]. These instruments, however, suffer from various reason, most of all, high cost. Electrochemical sensors and biosensors have superior properties over the other existing measurement systems because they can provide quick, simple and low-cost on-field determination of many biologically active species and a number of dangerous pollutants [35-57].



**Figure 1.** Autopsy of pheasants killed at the end of the experiment revealed no marked pathological changes except for occasional haemorrhages within the breast muscles. The figure shows a typical finding in animals poisoned with rodenticides, so it indicates that the pathological changes were found in opposite to the relatively good health state of pheasants [58].



**Figure 2.** Scheme of the model experiment simulating the bromadiolone distribution within the food chain.

Considering the above facts, the aim of this work was to propose a new method of bromadiolone detection. We optimized high performance liquid chromatography with electrochemical detection (HPLC-ED) for this purpose and employed this approach to determine the concentration of the target molecule in tissues of animals from two trophic levels.

## 2. Experimental Section

### 2.1 Chemicals

Acetic acid, sodium acetate, acetonitrile (HPLC grade) and bromadiolone (Pestanal - analytical standard) were purchased from Sigma Aldrich (Steinheim, Germany). Methanol was purchased from Fluka (Steinheim, Germany). Water for the preparation of buffers underwent demineralization by reverse osmosis using the instruments Aqua Osmotic 02 (Aqua Osmotic, Tisnov, Czech Republic). Then, it was purified using Millipore RG (Millipore Corp., USA, 18 M $\Omega$ ). Working solutions of the bromadiolone standard were prepared daily by diluting the stock solution of 1 mg/ml concentration with methanol.

### 2.2 Rodenticide-containing product

The experiment was carried out with a commercially available rodenticide (with 0.005% of bromadiolone; thereafter referred as PRODUCT), which is used commonly for the control of common voles in gardens, orchards, vineyards, and non-agricultural land. The PRODUCT is not appropriate for large-scale use but for the application in the voles' burrows. The recommended dose is 5-10 granules per open burrow.

### 2.3 Biotests

#### 2.3.1 Toxicity for earthworms – test in the artificial soil substrate

The test was carried out according to the modified OECD 207 guideline [59]. Glass bottles (1,000 ml) were filled with 500 g of the artificial soil test substrate consisting of peat (10 %), kaolin clay (20 %), and silica sand (70 %). The substrate was mixed with 10 granules of the PRODUCT. Demineralised water was added at a level corresponding to 35% of dry weight of the substrate. Four bottles with the PRODUCT and four control bottles were used in the experiment. Ten laboratory reared individuals of *Eisenia fetida* were weighed prior to the experiment and placed on the surface of the substrate in each bottle. Only adult individuals with the clitellum were used; the minimum average weight was 400 mg. The test was carried out under conditions as follows: temperature of 20 $\pm$ 2°C and continual lightning (450-600 lx). The evaluation of the test was performed after 14 days. One half of control earthworms and one half of the earthworms exposed to the PRODUCT were frozen immediately after collecting. The remaining earthworms were used in the following experiment with common voles.

### 2.3.2 Primary and secondary intoxication of common voles

Nine common voles (*Microtus arvalis*) from the laboratory rearing were used to experimentally study the possibility of primary and secondary intoxication by bromadiolone. Voles were kept individually in 9 l glass aquaria with wood shavings as bedding. Three groups of voles were employed in the experiment lasted 5 days. The first group consisted of control animals were fed with common commercially available granules for laboratory mice. The second group of voles was fed with the PRODUCT granules of which they received 0.086 g daily. The third group was fed with earthworms, which were previously exposed to PRODUCT granules for 14 days. Drinking water was available *ad libitum*. To ensure adaptation of voles to earthworms as a feed, the voles used in the experiment were fed with earthworms from the laboratory rearing for a period of 7 days prior to the experiment. At the start of the experiment the average weight of voles from the first, second and third group was 16.6 g, 15.5 g and 15.6 g, respectively. At the end of the 5-days long experiment, the weight of animals from the fore mentioned groups was 17.3, 15.9 and 15.2 g, respectively. Voles were weighed, euthanized and then subjected to autopsy. No gross pathological lesions were found. The liver was collected from each animal. The average weight of the liver of animals from the fore mentioned groups was 0.95, 0.93 and 0.94 g, respectively.

### 2.3.3 Hares

Three hare cadavers from hunting grounds on agricultural premises in districts to the east of the city of Brno (South Moravia, Czech Republic), where bromadiolone-containing granular baits was used, were collected in November 2005 and used in our experiments.

## 2.4 Preparation of biological samples

The samples of 0.5 g tissue (earthworm, liver from the treated voles or liver and stomach content of hares) were homogenized with 0.5 ml of methanol using a homogenizer (Ultraturax) for 15 min. The homogenates was sonicated at 150 W at 4 °C for 15 min. using K5 Sonicator (Slovakia) and subsequently vortexed for 15 min. using Vortex-2 Genie (Scientific Industries, New York, USA). The mixture was centrifuged for 20 minutes at 14,000 g at 4 °C using Universal 32 R centrifuge (Hettich-Zentrifugen GmbH, Tuttlingen, Germany). Subsequently, the supernatant liquor was collected and stored at -20 °C until the next analysis. Prior to the analysis, the samples were diluted 2 × with methanol.

## 2.5 Electrochemical measurements in the stationary system

Electrochemical cyclic voltammetric measurement was carried out using the AUTOLAB Analyser (EcoChemie, The Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland). The three-electrode system was used; it consisted of the carbon paste electrode (paste composition: 70% carbon powder and 30% mineral oil, w/w; the teflon body in which the diameter of the active electrode surface was 2.5 mm; the surface of the electrode was polished prior to each measurement using a fine filtration paper according to [60-62]); reference electrode (Ag/AgCl/3 mol l<sup>-1</sup> KCl), and the auxiliary electrode from glassy carbon. GPES software (EcoChemie, The Netherlands) was employed to process raw data

using Level 4 Savitzky and Golay filter. Experiments were carried out at room temperature. The measurement was performed in the potential range from 0.6 to 1 V using the following parameters: the potential step of 2.4 mV, the scan rate of 0.3 V.s<sup>-1</sup>. Acetate buffer (0.2 M, pH 4.0) was used as a supporting electrolyte.

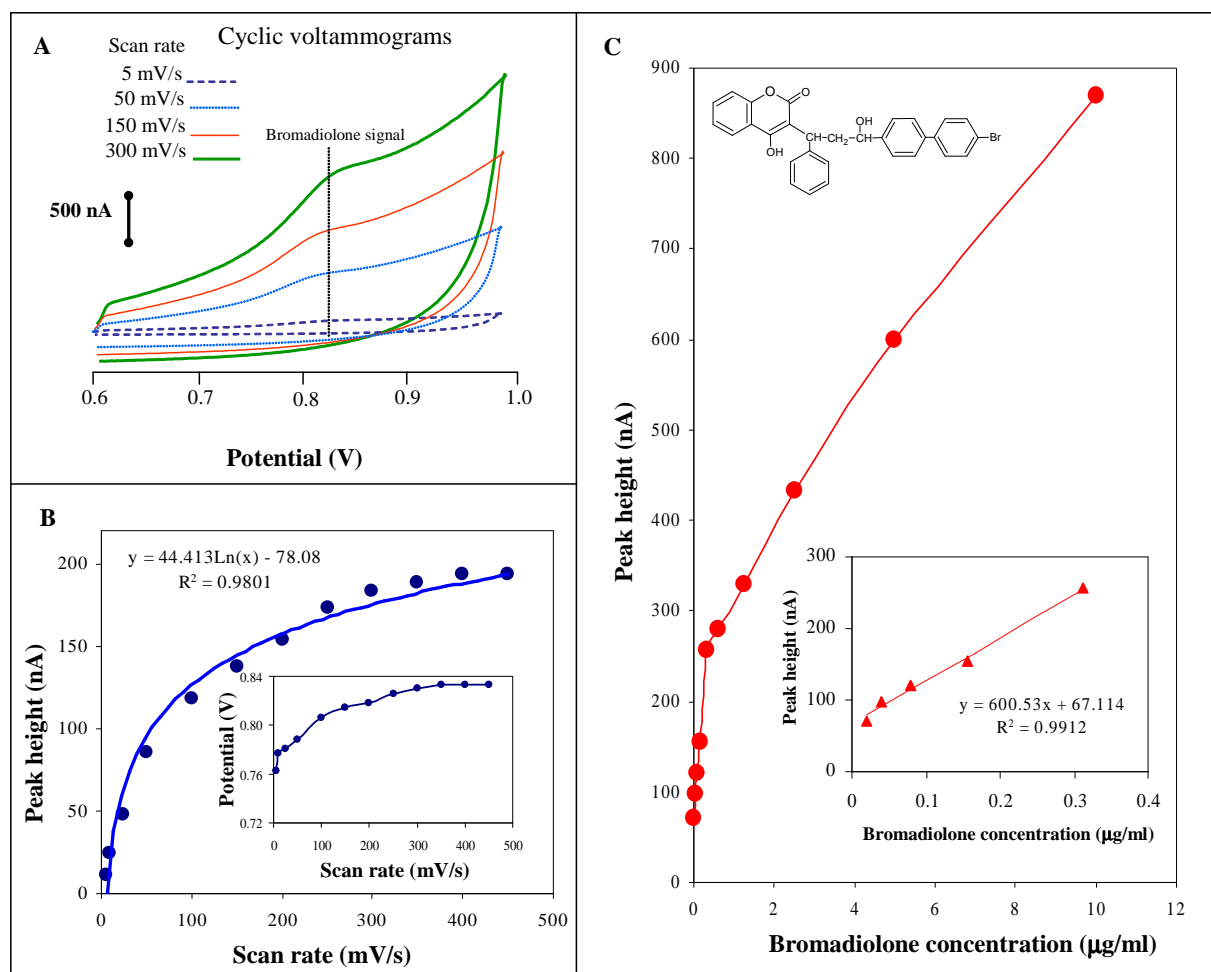
### 2.6 Electrochemical measurements in the flow system

The flow system consisted of the chromatographic pump (Model 583 ESA Inc., Chelmsford, MA, USA), Guard cell (Model 5020 ESA, USA), reaction loop (1m) for Flow Injection Analysis (FIA), and the chromatographic column Polaris (C18-A, 150 × 4.6 mm, with particle size of 3 μm; Varian, Inc.) and the electrochemical detector. The detector consisted of parts as follows: flow amperometric analytical cell (Model 5040, ESA, USA) containing the reference (hydrogen-palladium), auxiliary carbon and the working electrode made of glassy carbon, the Coulochem III control module. The sample (5 μl) was injected manually using a 5 μl injection loop (Supelco, Bellefonte, PA, USA). The data from the detector were processed using the CSW 32 software. The isocratic mobile phase consisted of 0.2 M acetate buffer (pH 4.0/acetonitrile, 40:60, v/v). The detection potential was optimized; see in “Results” section.

## 3. Results

### 3.1 Detection of bromadiolone using cyclic voltammetry

To employ HPLC with electrochemical detection for measurement of bromadiolone we aimed on study of the basic electrochemical behaviour of the target molecule using cyclic voltammetry at the carbon paste electrode. The voltammograms with a distinct oxidation signal at potential of 836 mV due to bromadiolone are shown in Fig. 3A. Dependence of the peak height on the scan rate was logarithmic ( $y = 44.413 \ln(x) - 78.08$ ). In addition, the potential of the peak shifted gradually to positive potentials (Fig. 3B). The dependence of the peak height on the concentration of bromadiolone was also determined. The signal enhanced sharply with the concentration in the given concentration range up to a concentration of 1 μg/ml, then more gradually (Fig. 3C). In spite of the fact that the peak height of bromadiolone enhanced with its increasing concentration, but the dependence measured is not strictly linear within the interval. For analytical purposes this dependence can be split into two strictly linear lines at a concentration of bromadiolone ranging from 0.625 to 10 μg/ml ( $y = 600.53x + 67.114$ ,  $R^2 = 0.9912$ ); and at a concentration of bromadiolone ranging from 20 to 300 ng/ml ( $y = 62.576x + 259.78$ ,  $R^2 = 0.9927$ , inset in Fig. 3C). The limit of detection for bromadiolone measured on the carbon paste electrode by cyclic voltammetry was evaluated as 5 ng/ml. It can be concluded that CV is a method suitable for fundamental studying of electrochemical behaviour of a target molecule; however, it can not be used for analysis of real sample due to relative low selectivity. Therefore the experimental results obtained were used in an optimization procedure for the detection of bromadiolone using flow analysis.

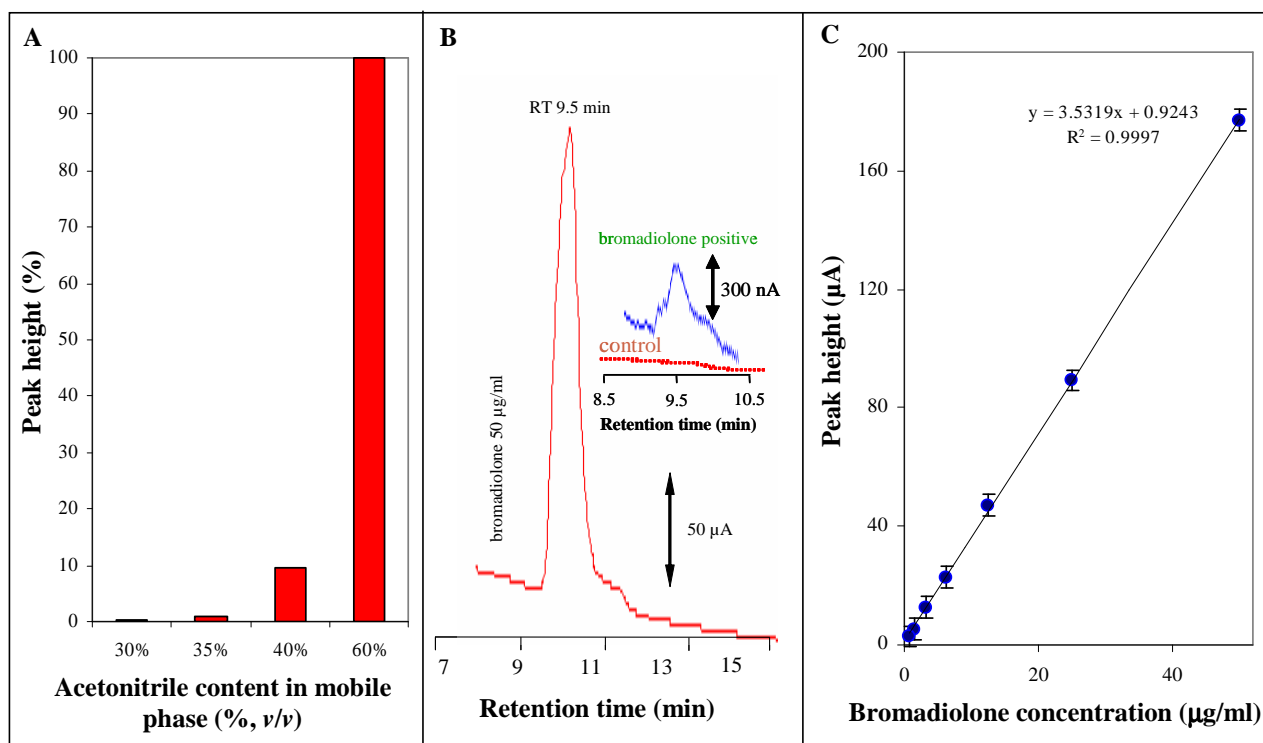


**Figure 3.** The electrochemical analysis of bromadiolone using cyclic voltammetry. **(A)** Cyclic voltammograms of bromadiolone (1  $\mu\text{g/ml}$ ) at scan rate of 5, 50, 150, and 300 mV/s. **(B)** Dependence of the height and position of potential of the bromadiolone signal on the scan rate. **(C)** Variation in the height of the bromadiolone signal on the concentration at a scan rate of 300 mV/s. Parameters of measurement: 0.2 M acetate buffer (pH 4.0), in the potential range from 0.6 to 1 V, potential step: 2.4 mV, experiments were carried out at room temperature.

### 3.2 Detection of bromadiolone using flow injection analysis with glassy carbon electrode

To make the analysis of bromadiolone-containing samples rapid and suitable for routine, a new approach of the detection of bromadiolone using Flow Injection Analysis (FIA) was proposed. The electrochemical response of bromadiolone was studied at a constant flow rate of the mobile phase (0.5 ml/min) and a varying working electrode potential. The detection potential of 840 mV was selected based on the hydrodynamic voltammogram (not shown). To obtain the maximum resolution of the bromadiolone peak and the maximum intensity of the signal, the effect of an organic component of the mobile phase (acetonitrile) was studied (Fig. 4A). The increased portion of the organic phase was found beneficial for the detection of bromadiolone. This fact is likely to be associated with the physicochemical properties of bromadiolone as a non-polar compound (more soluble in organic solvents) although our previous studies indicated a significant decrease in the electrochemical signal in the presence of the increased portion of the organic component in the mobile phase [58,63-66]. The flow rate of the mobile phase also affects the electrochemical signal. Variations in the bromadiolone

signal with the flow rate ranging from 0.1 ml/min to 2.0 ml/min were determined. The bromadiolone signal increased at a flow rate up to 0.8 ml/min. Then it did not change (not shown).



**Figure 4.** The analysis of bromadiolone using high performance liquid chromatography with electrochemical detection. (A) The effect of the organic component in the mobile phase (acetonitrile) on the bromadiolone peak height. (B) Chromatogram of bromadiolone at a concentration of 50 µg/ml, retention time of the peak of 9.5min. (C) Dependence of the height of the signal on the bromadiolone concentration. Experimental conditions were as follows: chromatographic column Polaris C18-A, 150 × 4.6 mm, diameter: 3 µm (Varian, Inc.). The sample was injected manually. Isocratic mobile phase consisted of 0.2 M acetate buffer (pH 4.0)/acetonitrile (40:60, v/v) and the detection potential of 840 mV were used. The flow rate of the mobile phase was 0.8 ml/min.

### 3.3 High performance liquid chromatographic analysis of bromadiolone

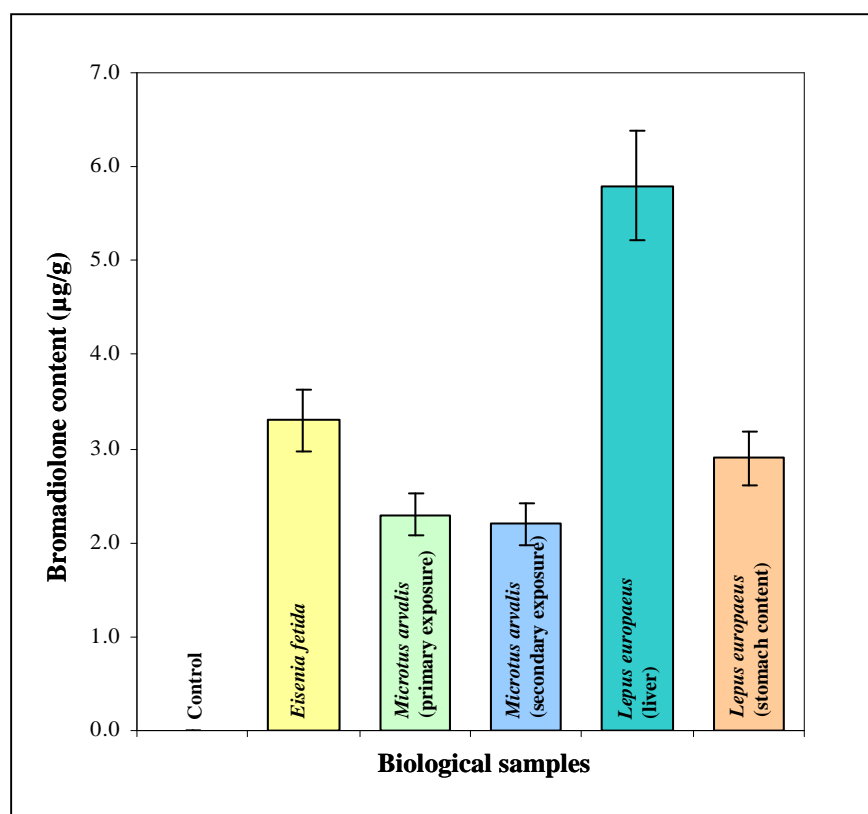
Under the experimental conditions optimized using FIA (working electrode potential 840 mV, mobile phase 0.2 M acetate buffer (pH 4.0)/acetonitrile (40:60, v/v), the flow rate of the mobile phase 0.8 ml/min) high performance liquid chromatography coupled with electrochemical detector was used to determine the content of bromadiolone in real samples. The signal of bromadiolone was observed with the retention time of 9.5 min (Fig. 4B). In addition, the calibration dependence for bromadiolone at a concentration range from 0.3 to 50 µg/ml was determined. The strictly linear calibration curve was obtained:  $y = 3.5x + 0.9$ ;  $R^2 = 0.9997$ , with a relative standard deviation of about 4 % (Fig. 4C). When we proposed the technique for determination of bromadiolone, the toxic effects of the target molecule on various experimental models were investigated.



### 3.4 Bromadiolone exposure

#### 3.4.1 Earthworms

During the 14-days long experiment, all earthworms exposed to bromadiolone survived. At the beginning of the experiment their average weight was 430 mg. However, the weight of control earthworms was 293 mg while earthworms exposed to the PRODUCT granules weighed 344 mg at the end of the experiment. The decrease in the weight of control earthworms (more than 30 %) was caused by starvation since the substrate contained no suitable nutrients. Earthworms exposed to the PRODUCT granules lost only 20 % of their original weight. They were assumed to have received granules containing bromadiolone. Using the proposed approach of homogenization and extraction, the samples of earthworms (*Eisenia fetida*) were prepared and subsequently analyzed by HPLC-ED. The average level of bromadiolone was  $1.7 \pm 0.2 \mu\text{g}$  per earthworm, which corresponds to  $3.3 \pm 0.9 \mu\text{g}$  per g of earthworm weight (Fig. 5). No signal due to bromadiolone was determined in samples from control earthworms.



**Figure 5.** The content of bromadiolone in the tissues from experimental animals. No bromadiolone signal was determined in samples from the controls (voles, earthworms). The levels of bromadiolone in earthworms were recalculated relative to the weight of earthworms. The levels of bromadiolone in voles were recalculated to the weight of the liver. For other details see in Fig. 3 and “Experimental section”.

### 3.4.2 Common voles

The level of bromadiolone in liver tissue from voles fed by the PRODUCT granules determined by HPLC-ED was  $2.3 \pm 0.1 \mu\text{g/g}$ . The average level of bromadiolone in liver tissue of voles fed with earthworms exposed to bromadiolone was  $2.2 \pm 0.5 \mu\text{g/g}$  (Fig. 5).

### 3.4.3 Hares

If we clinically examined the hares, two of them had no symptoms of intoxication by a rodenticide. The last hare (adult female) showed clinical symptoms of the intoxication. It was depressed, weak and the visible mucous membranes were pale. Blood was present in the nostrils. Autopsy revealed the hemothorax, hemoperitoneum, heart chambers and aorta without blood clots. The spleen was not enlarged. Samples of the liver and stomach content from all hares were collected for toxicological analysis. Moreover using HPLC-ED the samples of liver tissues and stomach content of three hares suspected from rodenticide poisoning were analyzed. We found out that the content of bromadiolone were  $5.8 \pm 0.2 \mu\text{g/g}$  in the liver and  $2.9 \pm 0.3 \mu\text{g/g}$  in the stomach content of the last hare (Fig. 5). The determined bromadiolone content in analyzed samples was higher than 0.2 mg/kg, which is considered as rodenticide poisoning. The samples of liver tissues and stomach content from other two hares were bromadiolone negative.

## 4. Discussion

Earthworms (*Eisenia fetida*) are standard species used in terrestrial ecotoxicology [59,67]. It is an epigeal species living in the humus layer of soil rich in organic components. The artificial soil substrate used in this test is rather deficient in the organic components, as documented by the decreasing weight of earthworms at the end of the test. However, the significantly lower decrease in the weight of earthworms kept in the substrate containing the PRODUCT, as compared with the control substrate, confirms the intake of the PRODUCT granules. Thanks to their composition (99.995% of a bonding agent and attractants such as lucerne, maize meal, etc.), the PRODUCT granules can be particularly a suitable feed for earthworms in low-productive lands [67]. Due to this the occurrence of bromadiolone can be very expected in tissues of many predators although the respective concentration may not always result in toxic effects [16]. Close attention is being devoted to the occurrence of residues in the environment, with pesticide-derived compounds representing an important group of residues [68]. Moreover earthworms are useful bioindicators of agroecosystem polluting [69]. It has been observed that some copper-containing fungicides decrease soil microbial biomass, lowering the population of earthworms. Contrary to this, it clearly follows from the obtained results that rodenticide-based bromadiolone-containing products have no lethal effects on earthworms and do not reduce the population of these significant soil decomposers.

The common vole (*Microtus arvalis*) is an herbivorous animal predominantly. Earthworms are therefore not a typical component of its diet. The reason why we used common voles in the experiment was that the common vole is not a protected and, thus endangered species. Moreover we can use voles from our laboratory rearing both for the evaluation of primary and secondary exposures. It is also representative of other small mammalian species that may be endangered by the use of rodenticides for

plant protection. There are only a few papers reported on the evaluation of secondary poisonings in insectivorous mammals such as shrews fed earthworms exposed to pesticides [70]. The levels of bromadiolone in all samples of the liver collected from common voles exceeded 0.2 mg/kg. Such levels are considered to be beyond the limit of bromadiolone intoxication [16,71]. No pathological changes were observed in experimental voles probably due to the short time of exposure after which they were killed and submitted for laboratory toxicological analysis. In the case of the bromadiolone intake through earthworms (secondary intoxication), the ratio between the values found and the maximum amount of the received bromadiolone is 0.50 while in the case of the PRODUCT granules the analogous ratio is 0.14. The difference between these two ratios values may indicate that bromadiolone bound in the organic matter is accumulated better in the subsequent consumer as compared with the PRODUCT baited granules. Since the experimental common vole as a herbivorous mammal does not prefer animal-based components in the diet, a greater risk of bromadiolone accumulation mediated by earthworms may be expected in higher trophic levels of the food chain, i.e., many species such as amphibians, reptiles, birds, mammals, and fish for which earthworms are a source of feed.

The routine analysis of pesticide-derived products is based on chromatographic methods with UV detection or less frequently used fluorimetric and mass detection [72-75]. However, electrochemical techniques for the study of these substances have not been used so far. The use of HPLC-ED permitted determining the levels of bromadiolone in biological samples. Thus, this method may be suitable for the evaluation of environmental hazards posed by this substance. At the same time, this method can also be used to study pharmacokinetic properties of bromadiolone. All current HPLC methods proposed for the determination of this substance require expensive devices such mass spectrometer. An analysis using the proposed method is rapid, relatively low cost and also applicable for the determination of trace levels of bromadiolone.

## Acknowledgements

This work was supported by grants of the Ministry of Education of the Czech Republic (project MSMT 6215712402).

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