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Determination of brodifacoum, bromadiolone and difenacoum in commercial rodenticides by using high-performance liquid chromatography with UV detection

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Small vertebrates are carriers of infectious diseases. Recently, brodifacoum, bromadiolone, difenacoum are used in rodenticides more often. This work describes simple methods for determination of brodifacoum, bromadiolone and difenacoum in all types of rodenticide baits. For analysis of wheat-based and grain baits, about 10 g of the sample is sonicated in acetone for 3 hours. Then, the grain or flour is separated. Then the acetone is evaporated, the residue is dissolved in acetonitrile. Solid briquettes are a mixture of paraffin, poison and filler, which can be used as grain, flour and the like. Extraction in the hexane-acetonitrile system solves the problem of difficult analysis of baits containing paraffin. Paraffin dissolves in hexane, while brodifacoum, bromadiolone and difenacoum are not. If the bait contains any filler, it is filtered and the poison is extracted from it as described above. The resulting extract was examined using reversed phase HPLC with a diode array detector. The best separation of the components was achieved using a Thermo Acclaim Surfactant 5 μm (4.6 × 250 mm) column with a mobile phase consisting of acetonitrile and 0.1 M aqueous ammonium acetate solution (pH 5.4) in a gradient elution mode. Linearity for considered rodenticides varies from 0.00067 to 0.010 %. Depending on the type of bait limits of detection for bromadiolone was from 0.000102 to 0.000143 %, for brodifacoum from 0.000101 to 0.000255 % and for difenacoum from 0.000156 to 0.000313 %. The recovery of bromadiolone was 94 %, for brodifacoum – 98 %, for difenacoum – 90 %.

Keywords*: brodifacoum, bromadiolone, difenacoum, rodenticides, HPLC*

INTRODUCTION

Infectious diseases are caused by various pathogens, such as fungi, viruses, bacteria, helminths, etc. Rodents carry about 60 infectious diseases, many of which pose a serious threat to human health [1]. Such diseases include hemorrhagic fevers, Born's disease, Lassa fever, hepatitis E, plague, tularemia, salmonellosis, and others [2-5]. In addition, rodents disrupt human activities causing damage to communications and foodstuffs.

Recently, the total number of rodents and the number of rats in particular have been increasing [2,6]. Chemical rodenticides are most commonly used to control rodent numbers [7].

Rodenticide bait has several main components - poison, attractant, preservative, as well as an additive that protects the bait from environmental exposure. There are grain baits, as well as hard (paraffin briquettes) and soft briquettes (wheat-based baits). As active ingredients use poisons of acute action (zinc phosphide) or chronic action (blood anticoagulants) [8]. Anticoagulants of blood are divided into first and second generation. The first generation includes: warfarin, diphacinone, coumatetralyl, ethylphenacin, chlorophacinone etc. To achieve efficiency, the bait, including anticoagulants of the first generation, must be eaten by mouse-like rodents many times. The second generation anticoagulants include: difenacoum, brodifacoum, difethialone, flocoumafen, bromadiolone, isoindane. Anticoagulants of the second generation cause death of rodents for 3-5 days, which is faster than from anticoagulants of the first generation [9].

Recently, the majority of new rodenticides as active substances contain anticoagulants of the second generation - bromadiolone, brodifacoum or difenacoum (Fig. 1).

To extract bromadiolone from baits in the form of granules in [10] it was proposed to use a 2% solution of formic acid in methanol. A sample of the product was ground in a mortar, then 50 mg was taken, 2 ml of formic acid solution was added and placed in an ultrasonic bath for 15 minutes. Then the sample is centrifuged and chromatographed on a C18 column using a mixture of acetonitrile, methanol and water as eluents. To increase the sensitivity a fluorescent detector was used. The detection limit of bromadiolone was 0.004 mg, the recovery rate was from 86 to 99 %.

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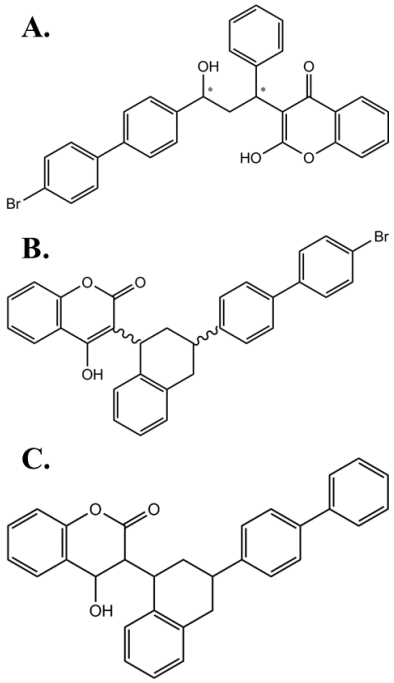

Figure 1. Bromadiolone (A), brodifacoum (B) and difenacoum (C)

Later, the same group of authors used similar conditions for the extraction of chlorophacinone and diphacinone from paraffin briquettes [11]. To prepare model samples, a solution of the active substance in ethyl acetate was added to the molten wax, then the solvent was removed at 70 °C under a nitrogen atmosphere. The detection limit of both substances was about 20 ng, which was achieved through the use of a mass spectrometric detector.

However, no universal method has been proposed for the determination of poisons in rodenticides. In this paper, a universal method is proposed for the determination of second-generation anticoagulants in grain baits and soft briquettes. A new method for extracting bromadiolone, brodifacoum and difenacoum from paraffin briquettes has also been proposed.

EXPERIMENTAL

Materials

Bromadiolone, brodifacoum and difenacoum (Pestanal®, Sigma-Aldrich), acetonitrile HPLC-grade (Merck, Germany), sodium acetate HPLC-grade (Acros Organics, USA), hexane (analytical grade, Russia), deionized water with resistance less than 18.2 MOM × cm. Other reagents used were analytical grade or higher. Commercial reagents were used without further purification.

Instrumentation and chromatographic conditions.

The HPLC system used was a Thermo ULTIMATE 3000 equipped with a DAD-3000 diode array detector. This device is also supplied with column thermostat, auto-sampler with a 20 μL loop and gradient pump with mixing on the low pressure side for 4-component gradient with a built-in degassing device. The separation was conducted using Thermo Acclaim Surfactant column, 5 μm (4.6 × 250 mm).

The mobile phase consisted of acetonitrile (A) and 0.1 M ammonium acetate solution pH 5.4 (B) in a gradient elution programmed as follows: 0–5.0 min, linear gradient from 50% to 40% B; 5.0–10.0 min, linear gradient from 40% to 5% B, maintain at 5% B until 10.0 min.

The solvent flow rate was 1.0 $ml \cdot min^{-1}$ and the temperature of the column oven was 25 °C. The analysis was carried out within the wavelength interval of 190-400 nm and the optimal wavelength value for the detection of rodenticides is 264 nm.

Identification of substances was carried out according to the retention time comparing with reference sample.

Preparation of stock solution.

A 0.500 % stock standards of brodifacoum, bromadiolone, and difenacoum in acetonitrile were prepared. Working standards for a five-point calibration curve were prepared from the stock standards by making appropriate dilutions with acetonitrile.

Preparation of model baits.

For the preparation of grain baits to the grain (about 9 g), 1 g of a 0.050 % solution of poison in ethylene glycol was added. The mixture was thoroughly mixed and dried at 60 °C. Similarly, prepared and wheat-based bait.

For the preparation of solid briquettes 1 g of a 0.050 % solution of poison in ethylene glycol was added to molten paraffin (about 9 g). Then ethylene glycol was evaporated at 60 °C and the bait was cooled.

Calculations and data processing.

Collection and processing of chromatographic data were conducted using Chromeleon 6 software (Thermo Fischer Scientific, USA). Excel 2016 (Microsoft Corporation) was used for detailed calculation.

The limit of detection LOD was set at the three times the noise level of the baseline in the chromatogram, while the limit of quantification LOQ was set at three times the LOD.

RESULTS AND DISCUSSION

Fig. 2 shows a chromatograms of standard solutions contained brodifacoum, bromadiolone and difenacoum, obtained using the ternary mobile phase

for the separation. The cis and trans forms of brodifacoum and difenacoum are well resolved.

Five sets of each form were analysed to determine methods validity. The standard of brodifacoum was assumed to consist of 56.3 % of the cis isomer and 43.7 % of the trans isomer. As the response factors for the two isomers are different, the levels for the two isomers were quantitated separately and added together for total brodifacoum in the sample. The same situation was with difenacoum. Quantification was done by using a five-point calibration curves.

Based on previous studies, we have suggested that extraction in an ultrasonic bath is the most suitable and simplest method for baits based on grain and flour. Optimization of extraction conditions was determined by the extraction coefficient. Acetonitrile, acetone and chloroform were used as solvents. To determine the recovery at each point, five baits of each type were prepared. Table 1 shows the dependence of recovery on extraction time and solvent.

Despite the different structure and properties of the matrix, it was found that extraction of acetone in an ultrasonic bath is best suited for the analysis of grain and wheat-based baits. The 3 h sonication is enough for recover more than 90 % of active substance. For better recovery from the wheat-based baits they have been previously grinded up.

Analysis of paraffin containing baits is very difficult because paraffin with incomplete removal from a solution can precipitate, which leads to contamination of the analytical column. Usually to remove the paraffin using a difficult and long-lasting filtration. We suggest a two-phase hexane-acetonitrile extraction system. In this case, the paraffin solves into hexane, while the poison remains in acetonitrile. After stirring for 12 hours, the system is placed in a separatory funnel and the acetonitrile fraction is separated. If the bait contains any filler, it is filtered and sonicated for 3 h in acetone as described above.

Percentage recoveries for each substance in each form and the associated RSD are given in Tables 2-4.

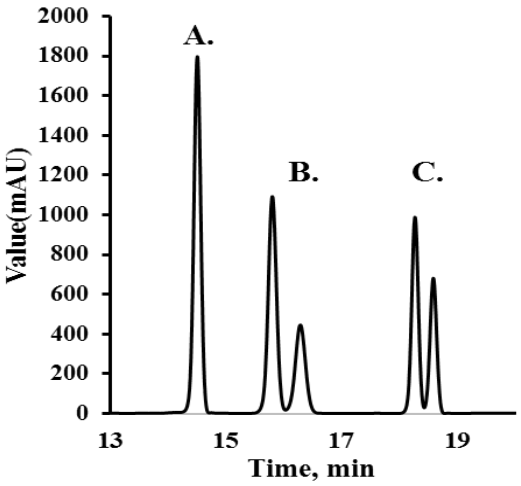

Figure 2. Chromatograms of bromadiolone (A), brodifacoum (B) and difenacoum (C) standard solutions

Table 1. The dependence of the recovery (%) from the solvent and sonicating time (calculated for five samples)

Bromadiolone		**Sonicating time, h**		
		1	**2**	**3**
Acetone	Wheat-based baits	55.5	80.0	**97.7**
	Grain baits	58.9	81.2	**95.2**
Acetonitrile	Wheat-based baits	55.4	69.1	85.2
	Grain baits	58.3	74.3	87.6
Chloroform	Wheat-based baits	60.2	71.3	84.2
	Grain baits	63.5	77.4	88.8
Brodifacoum		**Sonicating time, h**		
		1	**2**	**3**
Acetone	Wheat-based baits	64.5	84.6	**99.8**
	Grain baits	67.2	89.0	**98.0**
Acetonitrile	Wheat-based baits	62.1	72.3	84.3
	Grain baits	64.7	75.8	90.1
Chloroform	Wheat-based baits	65.8	84.3	93.6
	Grain baits	67.2	85.3	94.5
Difenacoum		**Sonicating time, h**		
		1	**2**	**3**
Acetone	Wheat-based baits	56.1	78.2	**94.2**
	Grain baits	57.2	77.6	**90.0**
Acetonitrile	Wheat-based baits	49.9	67.9	82.3
	Grain baits	50.1	68.4	84.6
Chloroform	Wheat-based baits	52.4	71.9	86.9
	Grain baits	53.8	72.3	88.6

Table 2. Recovery of brodifacoum, bromadiolone and difenacoum from the grain baits (calculated for five samples and three injections)

Object	Range, %	Recovery, %	RSD, %
Brodifacoum	0.00067 – 0.010	98.0	0.073
Bromadiolone	0.00067 – 0.010	95.2	0.073
Difenacoum	0.00067 – 0.010	90.0	0.147

Table 3. Recovery of brodifacoum, bromadiolone and difenacoum from the soft baits (calculated for five samples and three injections)

Object	Range, %	Recovery, %	RSD, %
Brodifacoum	0.00067 – 0.010	99.8	0.147
Bromadiolone	0.00067 – 0.010	97.7	0.058
Difenacoum	0.00067 – 0.010	94.2	0.214

Table 4. Recovery of brodifacoum, bromadiolone and difenacoum from the block baits (calculated for five samples and three injections)

Object	Range, %	Recovery, %	RSD, %
Brodifacoum	0.00067 – 0.010	98.5	0.058
Bromadiolone	0.00067 – 0.010	94.0	0.081
Difenacoum	0.00067 – 0.010	90.0	0.107

We have been found that recoveries from unshredded wheat-based baits are lower that for grain baits or hard briquette. This is due to the fact that the solvent does not completely penetrate the bait under the action of ultrasound. This problem disappears when the sample was grinded. It does not occur when analyzing grain and paraffin baits, since in the first case the poison is on the surface of the grain, and in the second, the bait is completely dissolved. The method showed satisfactory results in the analysis of real samples (Figures 3-5).

The results of chromatographic study showed that the peaks of the poisons and the other components of real rodenticide baits were clearly separated. This is why we conclude that this method is selective and it is appropriate for identification and quantitative analysis of brodifacoum, bromadiolone and difenacoum in wheat-based, grain and paraffin baits.

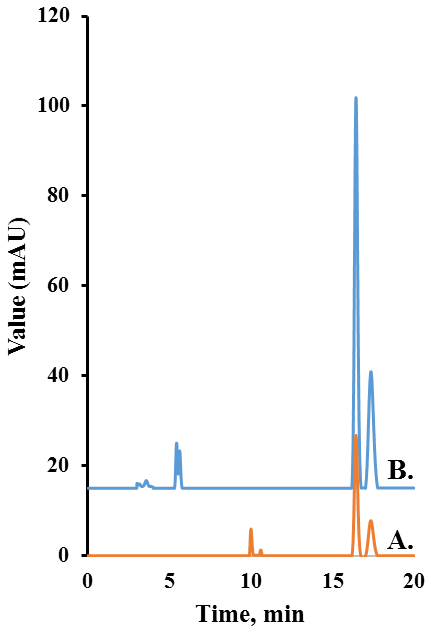

Figure 3. Chromatograms of commercial brodifacoum wheat-based (B) and paraffin (A) baits

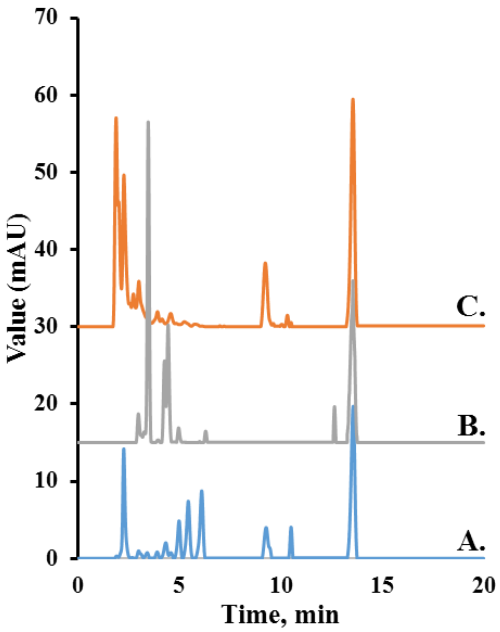

Figure 4. Chromatograms of commercial bromadiolone wheat-based (A) and grain (B) baits and solid briquettes (C)

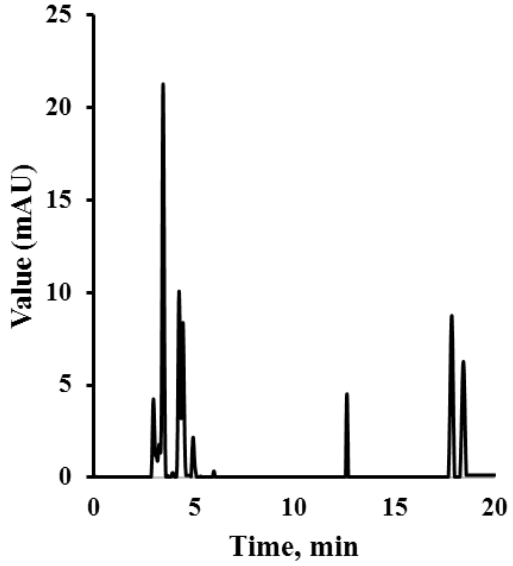

Figure 5. Chromatogram of commercial difenacoum wheat-based bait

CONCLUSIONS

The described methods provide a simple and sensitive procedure for the determination of brodifacoum, bromadiolone and difenacoum in different preparative forms of rodenticides. The methods can be recommended for routine analysis.

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