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Analysis of anticoagulant rodenticide residues in *Microtus arvalis* tissues by liquid chromatography with diode array, fluorescence and mass spectrometry detection

Alma M. Hernández[a], José Bernal[a,*], José L. Bernal[a], María T. Martín[a], Constantino Caminero[b], María J. Nozal[a]

[a] *I.U. CINQUIMA, Analytical Chemistry Group, University of Valladolid, 47011 Valladolid, Spain*
[b] *Instituto Tecnológico Agrario de Castilla y León (ITACyL), Ctra. Burgos km. 119, 47071 Valladolid, Spain*

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ABSTRACT

We describe here a fast and selective analytical method to determine the levels of four anticoagulant rodenticides (chlorophacinone, bromadiolone, brodifacoum and difenacoum) in animal tissues by liquid chromatography (LC) using different detection methods: fluorescence (FLD), diode array (DAD) and electrospray ionization-mass spectrometry (ESI-MS). Rodenticides were extracted from freeze-dried and homogenized tissue samples (liver, intestine and muscle) that had been obtained from the common vole (*Microtus arvalis*). These samples were diluted in 5 mL of methanol, the solution was shaken and centrifuged, and the supernatant was removed and evaporated to dryness. The residue was reconstituted in 1 mL of methanol (liver samples) or 1 mL of the mobile phase (muscle and intestine samples), and injected onto an LC–DAD–FLD–MS system coupled to electrospray ionization (ESI) in negative mode. After conducting an LC optimization study, we selected a Gemini 5 μm C_{18} column, a mobile phase composed of a mixture of 30 mM ammonium formate in water and methanol (26:74, v/v), and we used an isocratic elution mode. The method was fully validated and shown to be selective, precise, accurate, and linear in the range from ~5 μg/kg (ESI-MS) or ~50 μg/kg (DAD–FLD) to 10,000 μg/kg, depending on the compound analyzed. Recoveries ranged from 82% to 103%, while the limits of detection and quantification ranged from 9–89 μg/kg (FLD–DAD) and 0.6–4.6 μg/kg (ESI-MS). This method was successfully used to simultaneously measure the aforementioned compounds in *M. arvalis* tissues.

1. Introduction

Anticoagulant rodenticides are used in Spain to control rodent infestation and to reduce the associated crop damage, particularly that caused by the common vole (*Microtus arvalis*). In recent years these campaigns have been expanded in response to pressure from farmers on the authorities to reduce the impact of several recent plagues. Cyclic plagues of common voles in the region of "Castilla y León" have prompted the implementation of control strategies based on the use of anticoagulant rodenticides. The last plague was so intense that an extensive treatment with chlorophacinone (CP) followed by bromadiolone (BD) was applied as a last resort. Moreover, when combating a vole plague in 2007, several groups of voles were found to resist up to 25 times the lethal dose of rodenticide. This apparent resistance suggests that new control strategies are required using other authorized compounds, such as difenacoum (DF) and brodifacoum (BF). However, when using alternative strategies the risk of secondary toxicity to non-target species must be evaluated (*e.g.*, scavengers or carnivorous animals). One means of assessing this risk is to quantify the levels of residues of anticoagulant rodenticides in dead voles to determine whether they pose a risk to other animal species.

Anticoagulant rodenticides like CP, DF, BF and BD are normally used to control mouse and rat populations [1,2]. There active constituents are 4-hydroxycoumarins (BD, BF and DF) and indandione (CP) derivatives [3] (see structures in Fig. 1), and these compounds block the vitamin K epoxide reductase required to reduce vitamin K epoxide, an essential factor in the biosynthesis of clotting factors [2]. The liver is the main

Abbreviations: CP, chlorophacinone; BD, bromadiolone; BF, brodifacoum; DF, difenacoum; FC, flocoumafen; IS, internal standard; QC, quality control; SIM, selected ion monitoring; FIA, flow injection analysis; SANCO, Santé et Consommateurs; S/N, signal-to-noise ratio; IUPAC, international union of pure and applied chemistry; FDA, food and drug administration; ITACyL, Instituto Tecnológico Agrario de Castilla y León.

* Corresponding author. Tel.: +34 983 186347; fax: +34 983 186347.
E-mail addresses: jose.bernal@qa.uva.es, pepinho@qa.uva.es (J. Bernal).

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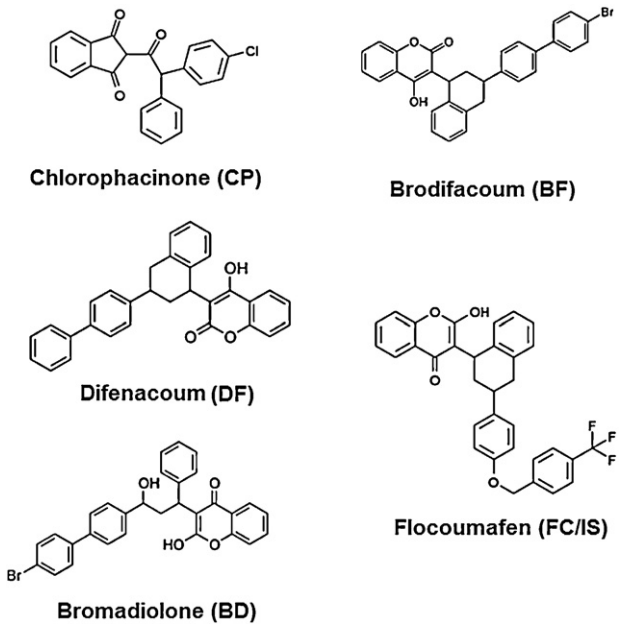

Fig. 1. Chemical structures of the studied anticoagulant rodenticides.

organ in which anticoagulant rodenticides accumulate and it is thus the most informative tissue in which to evaluate their presence [3].

Several analytical methods have been used to determine levels of anticoagulant rodenticides, involving flurorimetric, spectrophotometric and thin layer or ion chromatography [1,4–6]. However, due to their poor specificity and low sensitivity, these techniques are not suitable to quantifying the low levels of residues present in animal tissues [1]. Gas chromatography has been also employed in some cases [7,8] but its use is limited due to lengthy procedure times and poor recoveries. The most commonly used method to identify these compounds in animal tissues is liquid chromatography (LC) in conjunction with UV [9,10], FLD [11,12] or both [13–17]. While indandione-based rodenticides do not produce fluorescence and they can therefore not be accurately detected using these methods, their detection using LC coupled to mass spectrometry (MS) has been recently reported [3,18–21].

Very few studies have determined the levels of rodenticides in tissues from the common vole (*M. arvalis*). Indeed, there is only one report describing an extraction procedure to determine CP levels in *Microtus* spp. carcasses [22] using a time consuming solvent extraction procedure followed by solid phase extraction (SPE). In other studies were these compounds have been determined in animal matrices [1–3,6,10,12–16,18,20], the procedures usually involve time consuming treatments that increase the overall analytic time, including SPE [1,2,6,12–16,18,20], or they use large amounts of solvents [1,12,15,16].

The main goal of the present work was to develop an analytical method to evaluate anticoagulant rodenticide residues over a wide range of concentrations in tissues (liver, intestine and muscle) from *M. arvalis*, based on a single LC procedure coupled to multiple detection methods (DAD, FLD, MS). In achieving this goal, we devised a new way to treat the samples that significantly reduces the inherent time and cost of previously described approaches, without affecting the selectivity or extraction efficiency. The new LC–DAD–FLD–ESI-MS method was fully validated and the results obtained were compared with earlier data. Finally, the proposed method was used to analyze rodenticide residues in tissues from dead voles (*M. arvalis*) collected in the "Castilla y León" region of Spain.

2. Materials and methods

2.1. Materials and chemicals

The rodenticide standards for chlorophacinone (CP, Det. Purity 96%), bromadiolone (BD, Det. Purity 98%), brodifacoum (BF, Det. Purity 96%), difenacoum (DF, Det. Purity 97%) and flocoumafen (FC, Det. Purity 96.5%, used as an internal standard (IS) for LC–MS analyses) were provided by Ehrenstofer Gmbh (Augsburg, Germany), and they were used within their established reanalysis dates. Ammonium formate was supplied by SIGMA Aldrich Chemie GmbH (Steinheim, Germany), and methanol (LC grade) was purchased from Labscan (Dublin, Ireland). All other chemicals were obtained at the highest quality grade from commercial sources. LC-grade water was obtained by purifying demineralized water using a Milli-Q system (Millipore, Bedford, MA, USA). A vibromatic mechanical shaker and an ultrasonic bath (Ultrasons) were supplied by J.P. Selecta S. A. (Barcelona, Spain). A 5810R centrifuge (Eppendorf–Netheler–Hinz GmbH, Hamburg, Germany) and an R-210/215 rotary evaporator (Buchi, Flawil, Switzerland) were used for all extractions. Nylon syringe filters (17 mm, 0.45 μm) were purchased from Nalgene (Rochester, NY, USA).

2.2. Standard solutions

Standard stock solutions were prepared by dissolving approximately 10 mg of each compound in 10 mL of LC-grade methanol to a final concentration of approximately 1000 mg/L. To determine how accurately the initial stock solutions were prepared, the concentrations of three different solutions were compared. Standard deviation (%RSD) values of <0.5% were obtained in all cases, indicating a high degree of accuracy when preparing the solutions. The concentration of all stock solutions was corrected for purity, based on the purity levels summarized in Section 2.1. These individual standard solutions were combined and further diluted with LC-grade methanol to prepare the working solutions, which contained all the analytes (100,000 μg/L, 10,000 μg/L, 5000 μg/L, 4500 μg/L, 2000 μg/L, 1000 μg/L, 500 μg/L, 250 μg/L, 100 μg/L, 25 μg/L, 10 μg/L, 5 μg/L, 3 μg/L and 1.5 μg/L).

Calibration standards were prepared distinctly depending on the type of calibration curve (standard or matrix-matched). To prepare non-matrix matched standard calibration solutions, aliquots were taken from each of the working solutions, which contained all analytes, and they were further diluted with LC–methanol to obtain the desired final concentration. For DAD–FLD we used a concentration range of ~25 μg/L (limit of quantification, LOQ)–5000 μg/L, and made 6 more dilutions within this range (100 μg/L, 250 μg/L, 500 μg/L, 1000 μg/L, 2000 μg/L and 4500 μg/L). For ESI-MS, linearity was studied between 1.5 μg/L (LOQ) and 5000 μg/L, and 6 standards within this range were used to construct the calibration curve (10 μg/L, 25 μg/L, 100 μg/L, 250 μg/L, 500 μg/L, 1000 μg/L and 2000 μg/L). The IS was added to all calibration standards at 75 μg/L. Blank tissue samples (50 mg) from *M. arvalis* were spiked with 100 μL of standard solutions in methanol at different concentrations prior to the proposed sample treatment in order to prepare the corresponding calibration solutions for DAD–FLD (from the LOQ (~50 μg/kg) up to 10,000 μg/kg: 200 μg/kg, 500 μg/kg, 1000 μg/kg, 2000 μg/kg, 4000 μg/kg and 9000 μg/kg) and for ESI-MS (from the LOQ (~5 μg/kg) up to 10,000 μg/kg: 20 μg/kg, 50 μg/kg, 200 μg/kg, 500 μg/kg, 1000 μg/kg and 4000 μg/kg). An additional point corresponding to a blank sample (the matrix processed without an internal standard) was included in the calibration curves for all detection modes, and a zero sample (matrix sample

processed with IS) was also prepared for ESI-MS. The blank and zero samples were analyzed to confirm the absence of interference but they were not used to construct the calibration function. For the ESI-MS analysis, the IS was added to the 100 μL methanol solution with the other standards at a concentration of 150 μg/kg.

Each quality control (QC) sample was prepared using 50 mg of blank tissue (intestine, liver or muscle) that was spiked prior to the proposed treatment with 100 μL of the individual rodenticide standards (or with the IS only in the case of ESI-MS analyses), made up at different concentrations in methanol. In this way, we obtained QC samples that were prepared in the same way as the matrix-matched standards. The concentrations of the QC samples were: low QC, 100 and 200 μg/kg (DAD–FLD) and 6 and 10 μg/kg (ESI-MS); medium QC, 1000 μg/kg of each compound for both detection methods; high QC, 9000 μg/kg of each compound for both detection methods. When preparing the matrix-matched standards for the calibration curves and QC samples, we used pooled tissue samples taken from 6 different animals that had been previously analyzed by LC–ESI-MS to confirm the absence of rodenticide residues.

All standards (stock, working and calibration) and tissue sample solutions (matrix-matched, calibration and QC) were stored in glass amber containers at −20 °C in the dark to avoid photodegradation, where they were stable for over one month. All calibration (non-matrix and matrix) and QC samples were prepared in bulk in advance and were stored frozen, where they were stable for over one month.

2.3. Chromatography systems

We used an Agilent Technologies (Palo Alto, CA, USA) 1050 series LC–FLD–DAD and an Agilent Technologies 1100 LC–MS, controlled using ChemStation software. Both systems consisted of a vacuum degasser, a quaternary solvent pump and an autosampler with a column oven. The 1050 LC system was equipped with fluorescence (FLD) and diode array (DAD) detectors, and the 1100 series LC with a single quadrupole MS analyzer with an electrospray (ESI) interface. A Gemini 5-μm C_{18} 110 Å (150 mm × 4.6 mm i.d.) analytical column (Phenomenex, Torrance, CA, USA) was used for the LC analyses, and was protected with a Gemini C_{18} security guard cartridge (4 mm × 3.0 mm i.d.; Phenomenex). Based on the findings of the optimization study, the mobile phase selected was a mixture of 30 mM ammonium formate (pH 6.5) in water and methanol (26/74, v/v), which was applied at a flow rate of 1 mL/min in isocratic mode. The injection volume was set at 20 μL (draw speed 50 μL/min) and the temperature at 25 °C. The FLD detection wavelengths were 263 nm (excitation) and 390 (emission) for quantification of BD, DF and BF, while CP was measured at 285 nm by DAD. CP could not be directly detected by FLD as it produces no fluorescence, while BD, DF and BF were detectable by DAD but with a signal intensity that was much lower than those obtained by FLD. Flow injection analysis (FIA) tests of the MS parameters were carried out to define the operating conditions of the ESI interface in negative mode that provided the greatest sensitivity for anticoagulant rodenticides The conditions adopted were: drying gas (N_2) temperature, 350 °C; drying gas flow (N_2), 12 L/min; nebulizer gas (N_2) pressure, 60 psi; capillary voltage, 3500 V; fragmentor voltage, 180 V (CP, BD, DF) and 220 V (BF, FC). Full-scan LC–MS spectra were obtained by scanning from m/z 100 to 700. The most abundant ion for each compound was quantified in selected ion monitoring (SIM) mode, and two more ions were use to confirm the presence of each analyte (Table 1). Chromatograms for all detection modes were analyzed using the Data Analysis program in ChemStation (Rev.A.10.02 (1757); Agilent Technologies), and the analytes were quantified using peak areas (DAD–FLD) or ratios (ESI-MS).

Table 1
Quantification and confirmation ions selected for each anticoagulant rodenticide to perform the ESI-MS detection in negative SIM mode.

Compound	Quantification ion	Confirmation ions
Chlorophacinone (CP)	373	374, 375
Bromadiolone (BD)	527	525, 526
Difenacoum (DF)	443	444, 445
Brodifacoum (BF)	523	521, 524
Flocoumafen (FC, IS)	541	382, 289

2.4. Sample procurement and treatment

2.4.1. Sample acquisition

Liver, intestine and muscle tissue samples from 30 voles (*M. arvalis*) were supplied by the "Instituto Tecnológico Agrario de Castilla y León" (ITACyL), whose technicians collected the dead animals in the provinces of Valladolid and Palencia (Castilla y León).

2.4.2. Tissue homogenization

Whole organs were removed from each animal by ITACyL technicians, and they were blended and homogenized with a IKA® ULTRA-TURRAX® T 18 basic (IKA-Werke GmbH & Co. KG, Staufen, Germany). The samples were subsequently freeze-dried and then ground with a mortar and pestle for maximum sample homogeneity. All samples were then stored in the dark at −20 °C.

2.4.3. Blank tissue preparation

Tissues samples from dead voles collected in areas in which no rodenticide treatment has been applied were first analyzed by LC–ESI-MS to confirm the absence of rodenticides. Subsequently, these samples were pooled and subsamples of the corresponding tissues pools were used as blanks for the validation studies, and to prepare the QC and matrix-matched samples.

2.4.4. Sample extraction

Similar extraction procedures were used for the three different tissue matrices to analyze all samples, including the matrix-based calibrators and QC samples, with the exception of the B tissue samples. Briefly, subsamples of 50 mg of each tissue were weighed and 100 μL of a rodenticide standard mixture was added at different concentrations (also containing IS (150 μg/kg) in the case of ESI-MS analyses). A drying time of 5 min was used to allow the rodenticide standards to be absorbed into the sample. It should be noted that 100 μL of methanol was added to each unknown tissue sample for FLD–DAD analysis, while 100 μL of methanol containing 150 μg/kg IS was added for ESI-MS analyses.

Next, 5 mL of methanol was added to each sample and after mechanical shaking (5 min), the mixture was centrifuged for 10 min at 9000 rpm and 10 °C. The supernatant was collected and transferred to a glass flask, and then evaporated to dryness in a rotary evaporator. The residue was reconstituted depending on the tissue matrices: 1 mL of methanol was used for the liver tissue samples, while 1 mL of mobile phase (30 mM ammonium formate:methanol, 26:74, v/v) was used to reconstitute the muscle and intestine samples. The resulting solution was passed through a syringe filter, after which a 20 μL aliquot was injected onto the corresponding LC system.

2.5. Method validation

The method was validated following some of the recommendations of the technical report of the international union of pure

and applied chemistry (IUPAC) [23] (2002), the Guidance for Industry (Validation of Bioanalytical Methods) of the US Food and Drug Administration (FDA) [24], and the SANCO/825/00 document [25]. Accordingly, we determined the recovery, selectivity, limits of quantification and detection, carry-over effect, reinjection reproducibility, linearity, precision, accuracy and stability. Basic but efficient chemometric statistical tools from Excel (Microsoft Office 2010, Microsoft Corporation, Redmond, WA, USA) and ChemStation (Rev.A.10.02 (1757); Agilent Technologies) software were used to analyze the data obtained during the validation process.

The recovery and efficiency of the sample treatment for the rodenticides studied was determined in six replicates at three different concentrations (low, medium and high QC), comparing the peak areas of the compounds in: (i) extracted blank vole tissue samples spiked with the same amounts of the compounds and treated as described in Section 2.4 (A tissue samples) and (ii) extracted blank vole tissue samples treated as described in Section 2.4, and then spiked with the same amounts of rodenticide and IS when reconstituting the dry residues (B tissue samples). The internal standard (FC) was added in equal amounts (150 μg/kg) to the rodenticide standard solutions, and subsequently to the samples for ESI-MS analysis. To investigate possible matrix effects, which is particularly important for ESI-MS, the peak areas of the rodenticides from standard solutions were compared with those from the B tissue samples.

To determine the selectivity of the method, we assayed extracts from the blank and spiked tissue samples. The limits of detection (LOD) and LOQ were determined by injecting a number of extracts from blank tissue samples ($n = 6$) and measuring the magnitude of the background response. We experimentally estimated the LOD and LOQ as three and 10 times the signal-to-noise ratio (S/N), respectively.

Standard calibration curves were used to quantify rodenticides in vole liver and intestine samples using FLD or DAD, as neither the matrix nor the sample treatment significantly affected the analyte signal. The standard solutions used to construct the calibration curve were prepared as described in Section 2.2. By using on-line coupling and Chemstation software, it was possible to simultaneously obtain data for both detectors. Matrix-matched standard calibration curves were required to quantify the analytes in vole tissues analyzed by ESI-MS (muscle, liver and intestine), due to a strong matrix effect that influenced the ionization of the compounds, and in muscle samples, due to the loss of up to 18% of the analytes during the extraction procedure. Blank tissue samples were treated by the procedure proposed and prepared as described in Section 2.2. The samples were injected onto the LC–FLD–DAD and LC–MS systems, and the analyte signal (area FLD–DAD, ratios ESI-MS) obtained for each concentration and detector was used to construct the standard or matrix-matched calibration curves. In the case of the ESI-MS analyses, because an IS was used, the analyte signals were divided by the IS signal to obtain the corresponding ratio of areas, which was then used to construct the corresponding calibration curves. All samples were injected three times, from low to high concentrations, and between each entire calibration curve, QC samples were injected in triplicate (also from low to high concentrations). In addition, calibration curves for all the assays were fitted by the peak area against the analyte concentrations (or using the ratio for ESI-MS) with a $1/x$ weighted linear least-squares regression model. Linearity was evaluated by visual analysis of the plots, calculating the correlation coefficients (R^2), back calculation of the concentrations of the individual calibration standards, and analysis of response factors.

The carry-over effect was assessed for each analyte in each matrix by injecting blank tissue samples with a high concentration sample (high QC) or calibration standard (5000 μg/L or 10,000 μg/kg), and evaluating the responses at the retention times of the analytes and IS. Reinjection reproducibility was evaluated by re-injecting previously acceptable standards (25 μg/L and 4000 μg/L) three times per day and QCs (low and high) that had been stored for 1 week at −20 °C.

Intra-day precision and accuracy was determined concurrently by repeated sample analysis of QC samples on the same day. In each run a calibration curve was established and six replicates of low, medium and high QC samples were analyzed. Inter-day precision and accuracy were assessed by analyzing six sample replicates at the three concentrations of the rodenticides against a calibration curve on three consecutive days. Precision was expressed as the percentage of the relative standard deviation (%RSD) at a given concentration for each QC sample. Accuracy was determined based on the relative error (%RE).

We evaluated the stability of the analytes during sample preparation, after long-term (frozen at the intended storage temperature) and short-term (room temperature) storage, and after undergoing freeze and thaw cycles, as well as evaluating the autosampler stability. To perform these experiments, a set of samples prepared from a freshly made stock solution of the analytes was used in the appropriate analyte-free tissue matrix.

The stability of the anticoagulant rodenticides was determined after three freeze/thaw cycles. Three aliquots at each of the low and high QC concentrations were tested in standard and matrix solutions and no unstable analytes were observed, as none of the peak parameters were affected in any case. To determine the room temperature matrix stability, three aliquots of each of the low and high concentrations (low and high QCs) were thawed at room temperature, maintained at this temperature for 4–24 h, and subsequently analyzed. We observed no significant variation in the stability of the analytes. Long term stability was studied by storing at least three aliquots of each of the low and high concentrations in the same conditions as the study samples (−20 °C). These solutions were analyzed twice weekly over five weeks and they were found to be stable, as the results were comparable with the back-calculated values for the standards at the appropriate concentrations from the first day of the long-term stability analysis. Autosampler stability was evaluated to demonstrate the stability of the processed sample in the auto sampler compared with that of fresh processed samples. To determine the autosampler stability of the analytes in the three tissue matrices, three aliquots (low, medium and high QCs) were stored in the autosampler for 6 h, subsequently analyzed and their concentrations compared with the actual values.

3. Results and discussion

3.1. Sample preparation

To the best of our knowledge, only one previous study has been published in which the levels of an anticoagulant rodenticide (CP) were determined in a *Microtus* spp. matrix (carcass) [21]. That study employed a solvent extraction procedure followed by SPE. Due to the longer preparation times previously reported when analyzing these compounds in other matrices using SPE or multiple solvent extraction procedures [1–3,12–18,20], neither SPE nor multiple extraction were considered as options for sample extraction in the present study.

We first selected the amount of tissue to be used in the experiments, and found that 50 mg of tissue was sufficient to obtain good results with a high degree of sensitivity. We also optimized the volume (50–500 μL) of the rodenticide standard solution used to spike the A tissue samples, and the drying time (2–20 min) required to allow the complete adsorption of the rodenticides. After several tests, 100 μL and 5 min were chosen as the optimal values, as higher volumes increased the drying time, and lower volumes did

not allow for homogenous and complete spiking of the sample. A drying time of 5 min was sufficient to allow complete adsorption of the analytes by the sample.

We next tested the volume (2–15 mL) of the extractant (methanol) required to provide the best results in terms of recovery. Volumes less than 5 mL were insufficient to obtain recoveries greater than 80% in the three matrices assayed, while higher volumes did not produce significant improvements in the percentage recovery. Accordingly, 5 mL of methanol was selected as the optimal extraction volume. Analysis of the agitation (5–30 min) time in the Vibromatic mechanical shaker revealed similar and adequate recovery percentages (>80%) for all tissue matrices at all the shaking times tested. Thus, a 5 min shaking period was selected to reduce the length of the extraction procedure. The resulting mixture was centrifuged and the supernatant placed in a 25 mL conical flask, and it as gently evaporated to dryness at 40 °C in a rotary evaporator.

The nature and amount of the solvent used to reconstitute the dry extract were also optimized. As the standards were prepared in methanol, we first selected this compound to dissolve the dry extract. While this caused no problems with the liver tissue samples, a single interference peak that affected the detection of BF fluorescence, was observed for the muscle and intestine samples. This interference was eliminated by using the mobile phase as a solvent. In optimizing the volume required to reconstitute the extract (0.5–3.0 mL), we found that 1 mL provided the best results, taking both recovery and sensitivity into account.

To assess the efficiency of the proposed sample treatment, we compared the results obtained for liver, muscle and intestine samples spiked with three different rodenticide concentrations, either before or after sample treatment. The resulting recovery ranged from 82% to 103%, indicating that the sample treatment procedure selected was adequate (Tables 2 and 3). Lower recovery of all the compounds were obtained from muscle samples. No significant differences in the recovery percentages of the compounds analyzed were observed between detection modes.

3.2. Chromatography

The separation of some anticoagulant rodenticides in similar matrices has been described previously in reverse phase mode using C_{18}-based stationary phases columns with water/methanol, water/acetonitrile or combinations of aqueous buffers with methanol or acetonitrile [1–3,12–18,20]; although some other approaches using normal phase, ion pair and ion-exchange LC techniques have also been reported [1,10,15–17,20]. We selected a Gemini C_{18} column as it exhibited good stability over a wide pH range (1–12). In selecting the mobile phase, we initially chose methanol instead of acetonitrile as the rodenticides were dissolved in this organic solvent and because previous experiments revealed poorer analyte peak shapes with acetonitrile, particularly for BF. Ammonium formate was selected as the buffer for experiments using MS detection.

We first studied the influence of ionic strength on separation by varying the buffer concentrations (10–50 mM). We observed a decrease in peak widths and a slightly shorter analysis time when the ionic strength was increased to 30 mM. As there was little change at higher values, 30 mM was selected as the optimal buffer concentration. The influence of the ammonium formate pH (2.5, 4.5, 6.5 and 9.0) was also investigated and at acid pH values (2.5 and 4.5) the analyte signals were poorly defined, with longer retention times for all the compounds. At neutral and basic pH values these issues were not observed, although at pH 9 the CP peak exhibited a tail that overlapped with a matrix peak at higher concentrations. As this did not occur pH 6.5, we selected 6.5 as the working pH, which was obtained directly by preparing the ammonium formate 30 mM solution in water. The reproducibility of the analyte retention times at pH 6.5 was adequate, with a variation in retention time of less than 0.5% in all cases for day-to-day or batch-to-batch tests. The percentage of each of the components of the mobile phase was also varied to decrease chromatographic run time as much as possible. After performing several tests, we selected a mobile phase composed of 30 mM ammonium formate (pH 6.5) and methanol (26:74, v/v) in isocratic elution mode at 1 mL/min.

We analyzed the influence of column temperature (from 25 to 50 °C in 5 °C intervals) and as expected, we detected a slight decrease in retention times as the temperature increased, although this improvement did not compensate the loss of symmetry observed, particularly for CP, BD and DF. Thus, we selected 25 °C as the optimal value. We investigated the possibility of enhancing the LOD and LOQ by injecting larger sample volumes The injection of control blank tissue samples spiked with 150 μg/kg of rodenticides in volumes ranging from 10 μL to 50 μL resulted in an increase in the S/N ratio for all compounds when up to 20 μL was injected, above which the S/N ratio did not improve significantly. Joint FLD and DAD was required due to the fact that three rodenticides (BD, DF, BF) presented an intense fluorescence signal that provided good sensitivity values, while CP presented a weak fluorescence signal, and was better detected by DAD. After performing FLD and DAD scanning to obtain the best S/N values for each detector and compound, excitation and emission wavelengths of 263 nm and 390 nm, respectively, were selected for FLD detection of BD, DF and BF, and we chose 285 nm as the optimal wavelength for CP detection.

The chromatographic conditions proposed generated narrow and reproducible chromatographic peaks and allowed the compounds of interest to be separated in less than 15 min, as observed in the FLD–DAD chromatograms of non-spiked and rodenticide-spiked vole tissue samples (summarized in Fig. 2). Because the FLD–DAD chromatograms were quite similar for the different tissue matrices assayed, the FLD–DAD chromatogram for muscle tissue is presented as an example of the chromatographic separation. In the FLD chromatogram, BD, DF and BF were resolved at baseline and exhibited good symmetry, while a BD isomer peak was observed close to BD at longer retention times. The CP peak in the DAD chromatogram had good symmetry, although its retention time at baseline was slightly higher due to the previous elution of some matrix components. However, this did not affect the quantification of CP, which was resolved perfectly from the matrix interference peaks.

To the best of our knowledge, this is the first study in which these compounds have been analyzed in this matrix. However, comparing our proposed method with those used previously to analyze multiple rodenticides in other animal matrices demonstrates that most of the limitations of the procedures used previously are addressed by our method. These limitations include: (i) the use of mobile phases that are incompatible with MS detection [1,2]; (ii) lengthy analysis times [3,10,13,14,17,18]; (iii) the use of different methods for UV and FLD detection [13]; (iv) high flow-rates (>1.2 mL/min) [1,12]; (v) separation of a limited number of compounds in the same run [1,6,13] and (vi) partial separation of the analyte s (full separation was not required in these studies as quantification was performed by MS/MS [3,17,18,21]).

3.3. Mass spectrometry optimization

The first LC–MS experiments to select the optimal ESI-MS parameters and the appropriate ions were carried out by flow injection analysis (FIA) of the individual rodenticide solutions to monitor MS intensity. These compounds are usually analyzed in ESI negative ion mode [3,20] and although atmospheric pressure chemical ionization (APCI) has also been used [17], we found that using ESI in negative ion mode was more sensitive. A major

Table 2
Recovery data obtained for blank tissue samples treated accordingly to the procedure described in Section 2.4 and Section 3.1, which were analyzed by LC–DAD–FLD ($n = 6$).

Compound	Concentration (μg/kg)	Evaluation of the sample treatment			Evaluation of the matrix effect		
		Mean (%) ± RSD (%)			Mean (%) ± RSD (%)		
		Liver	Muscle	Intestine	Liver	Muscle	Intestine
CP[A]	200	95 ± 4	85 ± 4	92 ± 5	93 ± 5	96 ± 6	95 ± 5
	1000	95 ± 5	86 ± 4	93 ± 5	95 ± 5	94 ± 5	97 ± 5
	9000	99 ± 4	84 ± 4	93 ± 5	98 ± 6	99 ± 5	100 ± 6
BD[B]	200	99 ± 4	82 ± 5	99 ± 5	99 ± 5	101 ± 4	97 ± 4
	1000	98 ± 5	83 ± 4	97 ± 5	102 ± 5	97 ± 5	99 ± 5
	9000	99 ± 4	85 ± 4	97 ± 4	97 ± 4	103 ± 5	102 ± 5
DF[B]	100	91 ± 5	82 ± 5	101 ± 6	95 ± 6	92 ± 5	93 ± 5
	1000	96 ± 5	85 ± 5	99 ± 5	98 ± 5	95 ± 5	96 ± 5
	9000	94 ± 5	86 ± 6	98 ± 5	93 ± 5	91 ± 6	91 ± 5
BF[B]	100	92 ± 6	84 ± 4	98 ± 5	99 ± 5	94 ± 5	103 ± 5
	1000	92 ± 5	82 ± 5	96 ± 4	95 ± 6	97 ± 4	99 ± 4
	9000	92 ± 5	82 ± 5	98 ± 4	101 ± 5	98 ± 5	100 ± 5

A, DAD; B, FLD.

ion for each compound was evident in the mass spectra of the compounds, corresponding to the deprotonated molecular ions $[M–H]^-$. These ions were used to quantify the rodenticides when using SIM mode in order to obtain the maximum sensitivity for quantitative analysis (the ions selected to quantify and confirm each of the studied rodenticides are summarized in Table 1). In the LC–ESI-MS chromatograms of muscle tissue samples analyzed under the selected conditions (see Fig. 3), the peaks are baseline-resolved and exhibit good shape and symmetry. As also observed for LC–FLD–DAD, we found no significant differences between the chromatograms obtained for the different matrices. An internal standard (FC) was required due to the poor reproducibility of ESI-MS analyses (%RSD > 15). This compound, which is also an anticoagulant rodenticide, was chosen as the IS because it possesses similar physico-chemical properties to the analytes of interest and its retention time did not affect the chromatographic separation (Figs. 3 and 4).

To evaluate the effects of the matrix on ESI ionization, the peak areas for rodenticides in standard methanol solutions were compared with those obtained for the B tissue samples. Responses less than 75% were obtained for all compounds at the three concentrations assayed, and no significant differences were observed (Table 3). We therefore concluded that the matrix (vole tissue) affected the electrospray ionization of the selected rodenticides, provoking ion suppression in this case. As seen for the LC–FLD–DAD chromatograms, no significant differences were observed between the chromatograms obtained for the different matrices.

3.4. Method validation

To assess the selectivity of the method, extracts from blank tissue samples (intestine, liver and muscle) were assayed along with blank tissue samples spiked with the rodenticide standards. No matrix interference was evident in the DAD, FLD or ESI-MS chromatograms obtained for liver and muscle tissue samples (Figs. 2 and 3). The LOD and LOQ values were calculated (see Tables 4 and 5) and the LOQ values were determined quantitatively with adequate precision and accuracy. In all cases, better LOD and LOQ values were obtained when ESI-MS was used, and in most of the cases these values were better (LOD values ranging from 0.5 μg/kg to 40 μg/kg) than when anticoagulant rodenticides in similar matrices (animal tissues or fluids) were analyzed using LC with UV/DAD [1,2,13,14], FLD [2,6,12,14,15] or MS detectors [3,18]. While the LOD and LOQ values for DAD–FLD were slightly worse than those obtained with ESI-MS or those previously reported [6,12,14,15], DAD–FLD remains an economic alternative for experiments in which a high degree of sensitivity is not required.

The extraction efficacy for the rodenticides analyzed in the different tissue matrices ranged from 82% to 103%. We

Table 3
Recovery data obtained for blank tissue samples treated accordingly to the procedure described in Section 2.4 and Section 3.1, which were analyzed by LC–ESI-MS ($n = 6$).

Compound	Concentration (μg/kg)	Evaluation of the sample treatment			Evaluation of the matrix effect		
		Mean (%) ± RSD (%)			Mean (%) ± RSD (%)		
		Liver	Muscle	Intestine	Liver	Muscle	Intestine
CP	10	95 ± 4	83 ± 4	91 ± 5	63 ± 6	67 ± 5	70 ± 5
	1000	97 ± 5	87 ± 4	94 ± 6	65 ± 5	64 ± 6	66 ± 6
	9000	98 ± 4	82 ± 4	95 ± 5	61 ± 5	62 ± 5	69 ± 5
BD	10	101 ± 4	83 ± 5	103 ± 4	64 ± 5	68 ± 5	66 ± 5
	1000	100 ± 5	85 ± 4	99 ± 5	60 ± 4	68 ± 6	68 ± 4
	9000	97 ± 4	83 ± 4	98 ± 4	60 ± 4	62 ± 5	63 ± 4
DF	6	93 ± 5	83 ± 6	98 ± 5	71 ± 6	70 ± 5	73 ± 6
	1000	95 ± 6	84 ± 5	96 ± 5	67 ± 5	73 ± 6	68 ± 6
	9000	93 ± 6	85 ± 5	100 ± 5	70 ± 6	69 ± 5	71 ± 4
BF	6	89 ± 5	82 ± 4	95 ± 5	66 ± 4	70 ± 5	72 ± 5
	1000	91 ± 6	86 ± 4	93 ± 4	68 ± 5	67 ± 5	74 ± 4
	9000	92 ± 5	82 ± 5	98 ± 4	70 ± 5	65 ± 4	70 ± 4
FC (IS)	150	95 ± 4	86 ± 5	99 ± 4	66 ± 6	67 ± 5	69 ± 6

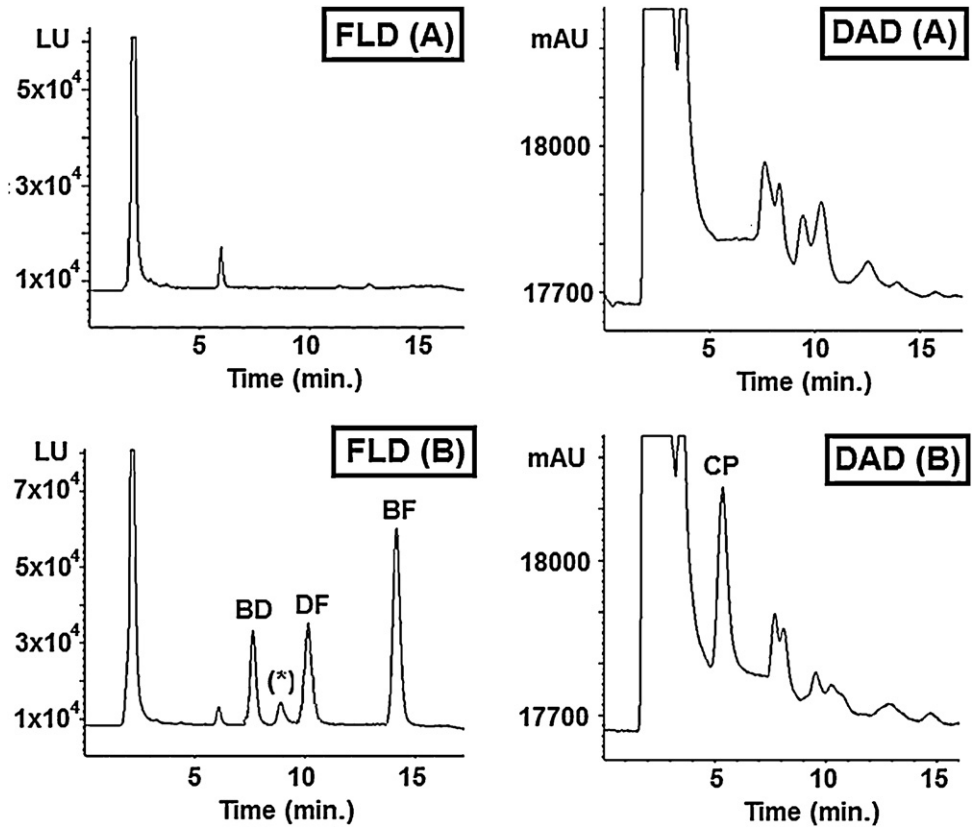

Fig. 2. LC–FLD (λ_{ex} 263 nm and λ_{em} 390 nm) and LC–DAD (λ 285 nm) chromatograms of (a) a blank muscle tissue sample and (b) a blank muscle tissue sample spiked with the studied rodenticides (chlorophacinone (CP), bromadiolone (BD), bromadiolone isomer (*), brodifacoum (BF) and difenacoum (DF)) at 250 μg/kg. The LC–FLD–DAD conditions are described in detail in Section 2.3.

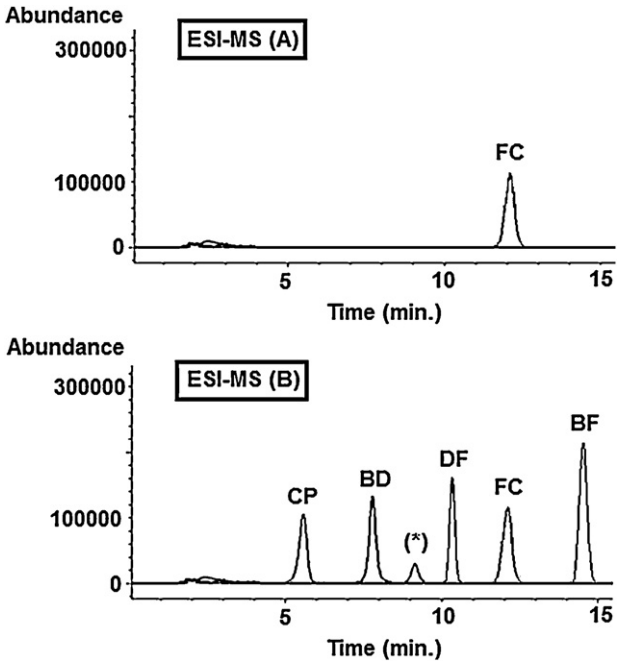

Fig. 3. LC–ESI-MS (SIM) chromatograms in negative mode of (a) blank liver tissue sample with the internal standard (flocoumafen (FC) at 150 μg/kg and (b) blank liver tissue sample spiked at 150 μg/kg with the studied rodenticides (chlorophacinone (CP), bromadiolone (BD), bromadiolone isomer (*), brodifacoum (BF) and difenacoum (DF). The LC–ESI-MS conditions are described in Section 2.3 and Table 1.

observed no significant differences in recovery using different concentrations or detectors, although slightly lower recoveries were obtained for muscle samples (Tables 2 and 3). Considering the simple extraction procedure used to process the samples, these results were very good, particularly when compared with the often tedious and time consuming procedures commonly used for these compounds, such as SPE. In fact, our recoveries were similar [1,2,6,12,15,16], or better [10,13,14,17,18] than those previously reported using SPE and other more laborious sample treatments.

As mentioned above, different calibration curves were used to quantify the compounds of interest depending to the detection mode used. Non-matrix standard calibration curves were used to quantify the analytes in liver and intestine by DAD and FLD. The resulting plots gave straight lines ($p < 0.05$) and they were linear

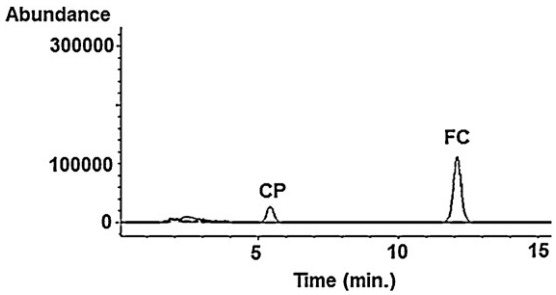

Fig. 4. LC–ESI-MS (SIM) chromatogram in negative mode of a liver tissue sample with the internal standard (flocoumafen (FC) at 150 μg/kg, where residues of chlorophacinone (CP, 30 μg/kg) were detected. The LC–ESI-MS conditions are described in Section 2.3 and Table 1.

Table 4
Method validation parameters and mean calibration curves for anticoagulant rodenticides LC–DAD–FLD determination in *Microtus arvalis* tissue samples (I = intestine; L = liver; M = muscle).

Validation parameter		CPA			BDB			DFB			BFB		
		I	L	M	I	L	M	I	L	M	I	L	M
Intraday precision	Low	5.1	4.2	4.1B	4.1	3.0	4.2	3.1	3.0	4.1	5.2	5.1	4.0
(%RSD)	Medium	4.3	4.7	5.2	2.4	3.3	3.4	4.2	2.2	3.2	2.2	4.4	3.8
	High	3.2	2.3	5.9	4.2	3.9	2.2	3.8	3.3	2.8	3.4	4.0	6.4
Interday precision	Low	7.2	5.1	6.2	5.8	5.1	6.8	6.2	5.0	5.1	6.0	5.3	5.1
(%RSD)	Medium	4.3	2.8	4.3	5.1	3.4	2.2	4.4	3.2	3.2	4.2	2.1	4.2
	High	4.8	4.2	5.0	3.9	4.8	3.0	4.1	4.1	4.0	5.9	4.2	4.1
Intraday accuracy	Low	5.1	5.4	4.3	4.3	3.0	4.3	−6.0	−4.1	−3.3	4.2	3.1	6.2
(%RE)	Medium	−2.3	−2.3	−2.2	3.2	4.2	−2.1	−2.2	−2.3	−3.2	−4.0	−4.3	−4.2
	High	−5.2	−7.1	−6.1	−4.2	−5.0	−6.2	−2.3	−5.1	−5.0	3.2	7.2	4.8
Interday accuracy	Low	4.9	6.3	5.7	2.8	5.3	5.7	6.2	4.8	7.1	5.8	4.3	4.2
(%RE)	Medium	−4.2	−4.0	−5.0	−3.1	−4.1	−4.2	−3.1	−3.1	−4.0	−6.1	−3.1	−5.3
	High	6.2	5.8	5.2	−5.2	−5.3	−5.0	−4.0	−5.2	−6.1	5.2	4.8	4.1
LOD (μg/kg)		25	22	28	16	17	19	14	14	16	9A	9	11
LOQ (μg/kg)		80	70	89	50	56	63	49	49	54	33	33	36
Linear range (μg/kg)		80–10,000	70–10,000	89–10,000	50–10,000	56–10,000	63–10,000	49–10,000	49–10,000	54–10,000	33–10,000	33–10,000	36–10,000
Correlation coefficient (R^2)		0.999	0.998	0.996	0.999	0.997	0.998	0.995	0.999	0.999	0.998	0.998	0.997

A, DAD; B, FLD.

Table 5
Method validation parameters and mean calibration curves for anticoagulant rodenticides determination by LC–ESI-MS in *Microtus arvalis* tissue samples (I = intestine; L = liver; M = muscle).

Validation parameter		CP			BD			DF			BF		
		I	L	M	I	L	M	I	L	M	I	L	M
Intraday precision	Low	6.2	5.2	5.0	5.7	4.8	4.8	4.4	4.8	5.3	6.1	5.9	4.8
(%RSD)	Medium	5.3	6.1	7.1	4.4	4.3	3.9	4.8	3.7	4.2	4.2	5.4	6.2
	High	4.1	4.3	6.9	4.8	4.9	3.3	5.1	4.1	5.0	5.3	5.2	7.0
Interday precision	Low	7.8	6.8	7.7	7.9	6.2	7.6	6.9	5.7	7.3	7.8	6.7	7.3
(%RSD)	Medium	6.0	5.4	5.2	6.3	4.4	3.8	5.4	4.0	5.4	5.3	4.2	6.0
	High	6.3	6.0	6.3	5.2	6.6	5.0	6.1	5.2	6.2	6.9	6.3	5.8
Intraday accuracy	Low	5.8	6.2	6.2	5.0	5.3	5.3	−6.8	6.1	5.3	5.8	5.1	7.6
(%RE)	Medium	−3.3	−4.0	−3.1	4.3	5.1	−3.4	−3.3	−4.2	−4.1	−5.0	−5.6	−5.3
	High	−6.8	−7.8	−7.3	−6.2	−5.8	−7.2	−4.3	−6.0	−6.9	5.2	7.8	6.2
Interday accuracy	Low	6.7	7.2	7.6	4.4	6.3	7.7	7.2	7.2	7.9	7.4	5.9	5.4
(%RE)	Medium	−5.0	−6.1	−5.8	−5.2	−5.2	−6.3	−5.1	−4.0	−6.2	−7.1	−6.0	−6.3
	High	7.9	6.7	7.2	−6.0	−7.4	−7.0	−5.8	−7.3	−6.9	6.6	7.2	5.9
LOD (μg/kg)		1.5	1.5	1.5	1.2	1.2	1.2	0.8	0.8	0.8	0.6	0.6	0.6
LOQ (μg/kg)		4.6	4.6	4.6	3.8	3.8	3.8	3.0	3.0	3.0	2.0	2.0	2.0
Linear range (μg/kg)		4.6–10,000	4.6–10,000	4.6–10,000	3.8–10,000	3.8–10,000	3.8–10,000	3.0–10,000	3.0–10,000	3.0–10,000	2.0–10,000	2.0–10,000	2.0–10,000
Correlation coefficient (R^2)		0.997	0.996	0.998	0.997	0.996	0.995	0.996	0.998	0.998	0.997	0.999	0.998

across the ranges studied (Table 4). Moreover, the slopes of the standard calibration curves and the matrix-matched calibration curves for each tissue type fell within the same confidence interval. These findings indicate that the standard calibration curve for each analyte was suitable to quantify all the vole tissue matrices analyzed here (intestine, liver and muscle). As already mentioned, we observed a strong matrix effect that affected analyte ionization in ESI-MS analyses. In agreement with this finding, the confidence intervals for the slopes of the standard and matrix-matched curves did not overlap. Hence, when quantifying anticoagulant rodenticides in vole tissues using LC–ESI-MS, matrix-matched calibration curves should be used. Muscle tissue samples should be also quantified using matrix-based calibration curves due to the loss of analytes during the extraction procedure, an effect that could not be compensated for without using matrix-matched standards. Linearity was evaluated by visual inspection of the calibration curves and by calculating the regression equation and R^2, the latter determined as >0.99 (see Tables 4 and 5). The deviation of the back-calculated standard concentrations was equal to or less than 10% in all cases from the nominal values. The assessment of the linearity was supported by analyzing the response factor plots, and the linearity of the calibration curves was corroborated by the near-zero slopes of the curves and RSD values (<3%) across all concentrations, and for all detection methods and matrices analyzed. The absence of bias was confirmed by a *t*-test and by studying the distribution of residuals.

No peaks were observed at the retention times of the analytes or the IS when blank tissue samples were examined after running samples spiked with high concentrations of the rodenticides of interest and the IS. This observation is corroborated by the blank tissue chromatograms (see Figs. 2 and 3), and indicates a negligible carry-over effect when using the proposed method to analyze these anticoagulant rodenticides in the tissues of interest. Evaluation of the reinjection reproducibility revealed %RSD values <1.5% in all cases, demonstrating that the samples could be safely re-analyzed within one week in the case of instrument failure.

The intra-day precision (%RSD) for the rodenticides studied ranged from 2% to 7% for all the detection methods and tissues analyzed, while the %RSD values for the inter-day assays ranged from 2% to 8% (Tables 4 and 5). The accuracy (%RE) for each compound ranged from 2% to 8% (absolute values) for the intra-day readings, and 3–8% (absolute values) for the inter-day assays. Accuracy and precision were slightly better for DAD–FLD than ESI-MS, although both were sufficiently high. These results indicate that the method proposed is both precise and accurate. Moreover, while an IS was recommended in most validation guidelines and was used here to correct for the lack of reproducibility in the ESI-MS analyses, it was not necessary when using DAD and FLD due to the high degree of precision and accuracy of these detection methods.

No instability was observed for any of the rodenticides in freeze/thaw stability studies, as the parameters of the analyte peaks and the concentrations were unchanged by these test conditions. Similar, no variations were evident in other stability tests: room temperature matrix stability, long term stability and autosampler stability. All the analytes of interest were stable in the different matrices assayed under our experimental conditions and we found no significant differences when comparing values obtained from our samples with those of fresh samples. In all cases the absolute %RSD values were less than 10%.

3.5. Method application

When we analyzed liver, muscle and intestine samples from *M. arvalis* (30) using the proposed method, no DF or BF residues were detected at concentrations above the LODs in any of the samples analyzed. This was as expected given that these compounds have not yet been used to control vole plagues, although their use had been authorized in Spain at the time that the samples were collected. Residues of BD and CP were detected at levels much lower than the existing LD_{50} values (see Section 3.4) in three (BD: 10 μg/kg, 15 μg/kg and 25 μg/kg) and five (CP: 10 μg/kg, 15 μg/kg, 20 μg/kg, 30 μg/kg and 45 μg/kg) liver samples, but not in muscle or intestine samples, and never in the same sample (an LC–ESI-MS chromatogram of a liver tissue sample in which 30 μg/kg CP was detected is shown in Fig. 4). These residues were detected with LC–ESI-MS, due to the insufficient sensitivity of LC–DAD–FLD. The detection of BD and CP residues in liver tissues is consistent with previous studies that proposed the liver to be a likely site of accumulation of anticoagulants [3,22]. In summary, the anticoagulant rodenticide residues detected in some of the samples analyzed appear to be too low to pose a serious risk to other animal species. However, this tentative conclusion should be confirmed by further analyses on larger sample sizes, and using samples collected after rodenticide administration.

4. Conclusions

In conclusion, we present here a fast, simple and sensitive LC–DAD–FLD–MS method to measure anticoagulant rodenticides (chlorophacinone, bromadiolone, brodifacoum and difenacoum) in *M. arvalis* tissues (liver, muscle and intestine), which offers many advantages over previous approaches in terms of analysis time and sensitivity (ESI-MS). This is the first method reported in which the same chromatographic and extraction conditions were used successfully with three different detection methods. Our findings demonstrate that this method is consistent and reliable, with low %RSD values, little bias and good recovery. As DAD–FLD determination was not influenced by the matrix, quantification of the rodenticides could be performed using standard calibration curves. However, matrix-matched calibration curves were required when using ESI-MS due to the observed matrix effect. We employed this method to analyze rodenticide residues in 30 tissue samples from *M. arvalis*. Only very low concentrations of CP and BD were detected in 8 liver samples, and not in intestine or muscle, suggesting that anticoagulant rodenticide residues do not pose an imminent risk to other animal species.

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References

[1] K. Hunter, J. Chromatogr. 321 (1985) 255.
[2] M.G. Palazoglu, E.R. Tor, D.M. Holstege, F.D. Galey, J. Agric. Food Chem. 46 (1998) 4260.
[3] V. Vandenbroucke, N. Desmet, P. De Backer, S. Croubels, J. Chromatogr. B 869 (2008) 101.
[4] Y. Kawano, W. Chang, J. Assoc. Off. Anal. Chem. 62 (1980) 996.
[5] P.J. Berny, T. Buronfosse, G. Lorgue, J. Anal. Toxicol. 19 (1995) 576.
[6] M.-C. Jin, X.-H. Chen, Y. Zhu, J. Chromatogr. A 1155 (2007) 57.
[7] R.W. Bullard, G. Holguin, J.E. Peterson, J. Agric. Food Chem. 23 (1975) 72.
[8] R.W. Bullard, R.D. Thompson, G. Holguin, J. Agric. Food Chem. 24 (1976) 261.
[9] A. Medvedovici, F. David, P. Sandra, Talanta 44 (1997) 1633.
[10] F. Guan, A. Ishii, H. Seno, K. Watanabe, T. Kumazawa, O. Suzuki, J. Pharm. Biomed. Anal. 21 (1999) 179.
[11] M.J. Kelly, J. Chambers, A.D. MacNicoll, J. Chromatogr. 620 (1993) 105.
[12] K. Hunter, J. Chromatogr. 270 (1983) 267.
[13] V. Fauconnet, H. Pouliquen, L. Pinault, J. Anal. Toxicol. 21 (1997) 548.
[14] W. Langseth, U. Nymoen, Fresen. J. Anal. Chem. 339 (1991) 249.
[15] K. Hunter, E.A. Sharp, A. Newton, J. Chromatogr. 435 (1988) 83.
[16] K. Hunter, E.A. Sharp, J. Chromatogr. 437 (1988) 301.
[17] F. Guan, A. Ishii, H. Seno, K. Watanabe-Suzuki, T. Kumazawa, O. Suzuki, J. Chromatogr. B 731 (1999) 155.
[18] L.J. Marek, W.C. Koskinen, J. Agric. Food Chem. 55 (2007) 571.

[19] M.Z. Mesmer, R.A. Flurer, J. Chromatogr. A 891 (2000) 249.
[20] M.-C. Jin, G.-Z. Xu, Y.-P. Ren, X.-H. Chen, X.-M. Xu, J. Appl. Toxicol. 28 (2008) 621.
[21] R.A. Middleberg, J. Homan, Methods Mol. Biol. 902 (2012) 139.
[22] T.M. Primus, J.D. Eisemann, G.H. Matschke, C. Ramey, J.J. Johnston, in: J.J. Johnston (Ed.), Pesticides and Wildlife, American Chemical Society, Washington, 2001, p. 164.
[23] M. Tompson, S.L. Ellison, R. Wood, Pure Appl. Chem. 74 (2002) 835.
[24] Guidance for Industry, Bioanalytical method validation. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf, 2001 (accessed 19.02.13).
[25] Document No. SANCO/825/00 rev.8.1, European Commission, Directorate general health and consumer protection, Guidance document on residue analytical methods. http://ec.europa.eu/food/plant/protection/resources/guide_doc_825-00_rev7_en.pdf, 2010 (accessed 19.02.13).