A modification of QuEChERS method to analyse anticoagulant rodenticides using small blood samples

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Abstract: The use of anticoagulant rodenticides is the most common method to control rodent plagues. Due to their physicochemical characteristics and particular mechanism of action, the application of these compounds in rural areas can pose a risk of secondary poisoning for their predators. In order to evaluate the risk of these compounds for wildlife, especially raptors that feed on rodents, biomonitoring programmes are undertaken. A fast, easy and low cost technique was needed to analyse small volumes of blood samples. Therefore, three different modifications of QuEChERS methodology have been compared, and one of them selected to detect and quantify these compounds. The process prior to analysis of the extracts involves two simple steps: the sample is extracted and partitioned using an organic solvent and salt solution. The supernatant is then cleaned using a dispersive solid phase extraction (dSPE) technique. Detection and quantification of the anticoagulant rodenticides were performed using LC-MS/MS on an Agilent 1100 VL Series ESI/LC/MSD, with an electrospray ionisation (ESI) source and ion trap analyser. The method finally chosen provides a 72-134% recoveries for the seven rodenticides (warfarin, coumatetralyl, brodifacoum, bromadiolone, difenacoum, chlorophacinone, diphacinone), higher than in other methods to analyse similar compounds. Sensitivity of our method is also higher than in other methods. In order to prove the utility of the technique, a total of 50 blood samples of free-living Eagle owls (Bubo bubo) were analysed.

Key words: Anticoagulant rodenticides, blood, quechers, biomonitoring

Resumen: Adaptación del método QuEChERS para el análisis de rodenticidas anticoagulantes en pequeños volúmenes de sangre. El uso de rodenticidas anticoagulantes es el método más frecuentemente utilizado para el control de plagas de roedores. Debido a sus características físico-químicas y particular mecanismo de acción, la utilización de estos compuestos en zonas rurales puede suponer un riesgo de intoxicación secundaria de sus depredadores. Para evaluar el riesgo a estos compuestos para la fauna silvestre, especialmente en aves rapaces que se alimentan de roedores, se llevan a cabo los programas de biomonitorización. Se ha desarrollado un método rápido, fácil y económico que permita el análisis de pequeños volúmenes de muestra de sangre. En el presente trabajo se han comparado tres diferentes modificaciones de la metodología “QuEChERS”, y posteriormente uno de ellos fue elegido para la detección y cuantificación de estos compuestos. El proceso previo al análisis de los extractos incluye dos pasos sencillos: La muestra es extraída usando un solvente orgánico y una solución salina y, posteriormente, el sobrenadante es purificado usando una técnica de extracción en fase solid dispersive. La detección y cuantificación de los rodenticidas anticoagulantes se realizó por cromatografía líquida acoplada a un detector de masas Agilent 1100 VL con trampa de iones y fuente de electrospray para ionización. La técnica finalmente elegida permite una recuperación entre 72-134% para los siete rodenticidas objeto de estudio (warfarina, cumatetralilo, difenacoum, clorofacinona, brodifacoum, bromadiolona, difacinoa), la cual es superior a la obtenida con otras técnicas que analizan compuestos similares. Además, la sensibilidad de esta técnica es mayor a la que ofrecen otras técnicas. Con el fin de comprobar la utilidad de la técnica validada, se analizaron un total de 50 muestras de sangre de búho real (Bubo bubo) capturados en su hábitat natural.

Palabras clave: Rodenticidas anticoagulantes, sangre, quechers, biomonitorización

Introduction
Rodent infestations have posed serious economic and sanitary problems for the humanity during centuries. Nowadays it still constitutes an issue of concern, especially for the agriculture, due to the economical damages to crops. Because physiology and ethology of rodents complicate their eradication, the use of chemical compounds have become necessary, being anticoagulant rodenticides (ARs), such as warfarin, coumatetralyl, difenacoum, brodifacoum, bromadiolone and difacinoa, the most commonly used [1].

The mechanism of action of these compounds is based on the inhibition of active vitamin K epoxide reductase, which results in a lack of active vitamin K and a disruption in the activation of blood clotting factors (II, VII, IX and X) [2,3]. Due to the plasma clotting factors half life, a lag time between the ingestion of anticoagulants and the onset of clinical signs exists [4]. As soon as circulating clotting factors are lost by normal attrition, fatal haemorrhages may happen [5]. On the other hand, poisoned animals can suffer somnolence, weakness, pale mucosa, decreased or lacking appetite, decreased locomotion and perception and rapid and easy exhaustion [6]. As a consequence, these individuals are more susceptible to be predated or suffer accidents [5]. In addition, sublethal levels of rodenticides may persist up to a year in the body [8], and repeated low-dose exposure can lead to accumulation and fatal haemorrhages [5]. This high persistence in the body implies a higher risk of secondary poisoning in predators, especially in birds like owls that mainly prey on rodents [5,7,9]. In fact, several cases of secondary poisoning have been documented in different raptor species [10-15]. In the case of birds inhabiting rural areas, this risk can even be higher [16]. The assessment of toxic exposure in wildlife is carried out through monitoring studies, best using non-destructive samples such as blood. In the case of birds, the small sampling volume is a limiting factor in most species, since only a 10% of the total blood volume in body is
usually collected. Hence, analytical methods that use the less sample volume as possible are needed.

An increasing number of techniques to analyse anticoagulant rodenticides in small samples of animal tissues have been developed [9,14-18]. However, they usually consist in extractions using organic solvents and purifications by solid phase extraction columns, which can sometimes be both expensive and time-consuming, due to the large volumes of solvent for activation of the columns, washing of the sample, and elution of the analytes [19]. Because an appropriate clean up of samples is essential prior chromatographic analyses, special care should be taken in order to obtain the best accuracy but the less matrix effect as possible. In this sense, methods like QuEChERS (short name for quick, easy, cheap, effective, rugged, and safe) have been successful in the analyses of several compounds, including anticoagulant rodenticides in animal tissues [19]. Regarding instrumental, ultraviolet spectrometer or fluorescence detectors coupled to HPLC have been used to detect anticoagulant rodenticides [9,11,18]. However, since mass spectrometer detectors have proved higher sensitivity, the use of HPLC-UV may have markedly underestimated the true scale of exposure of other non-target species to anticoagulant rodenticides [20].

As mentioned above, wildlife biomonitoring studies using bird samples demand for the use of very small sample amounts but sufficient to detect very low levels of contaminants. This implies the improvement of analytical techniques in order to become the most sensitive as possible. According to this, and based on existing QuEChERS method to analyse trace levels of pesticides in food [21], slight modifications were made to reduce solvent, sample amount and reagents.

**Material and methods**

**Biological samples**

Whole blood samples were obtained from healthy dogs kept in the Murcia City’s Municipal Zoonosis Control Centre. Blood was taken by puncturing the jugular vein with a 23G needle and a 10 ml syringe, using heparin (Analema, Vorquimica S.L.) as anticoagulant. In order to prove the applicability of the validated technique, a total of 50 blood samples from free-ranging Eagle owls (*Bubo bubo*) (9 adults and 41 one month-old nestlings) were analysed. These samples were obtained by puncturing brachial vein with a 23G needle and a 5 ml syringe, using heparin as anticoagulant. Samples were immediately taken to the laboratory under refrigerated conditions and frozen to -40°C until processing.

**Sample preparation**

The method used is a modification of the technique described by Anastassiades et al. [21], based on dispersive solid phase extraction (dSPE), commonly known as QuEChERS.

About 2 ml of sample (spiked dog blood or eagle owl blood) were mixed with 2 ml of solvent (according to method; table 1). The mix was shaken vigorously with a vortex for about a minute and a combination of salts (1.33 g magnesium sulphate, 0.33 g sodium chloride, 0.17 g sodium citrate dibasic sesquihydrate and 0.33 g sodium citrate tribasic dehydrate) was then added. Tubes were again vigorously shaken with vortex. This mix separates the liquid phase and stabilizes the analytes. The tubes were centrifuged at 3000 rpm for 5 minutes with a centrifuge Sanyo® MSE MISTRAL 2000 R and frozen at -4°C for 1 hour. The supernatant was then transferred to another tube and mixed with a mix of 300 mg magnesium sulfate, 50 mg PSA y 50 mg DSC-18. The tube was shaken similarly to dryness and redissolved in 1ml solvent (according to method; table 1) acidified with 10 µl of formic acid 5% in acetonitrile.

**Chemicals and standards**

Rodenticide reference standard chlorophacinone (96%) was obtained from Dr. Ehrenstorfer GmbH (Germany) while warfarin (98%), difenacoum (98.7%), coumatetralyl (99.5%), diphacinone (99%), brodifacoum (99.8%) and bromadiolone (98.8%) were purchased from Sigma-Aldrich (USA). Acetonitrile and methanol were obtained from Lab-Scan® (Poland) and formic acid from Probus® (Spain). All these solvents and reagents were of residue quality (>99.9% purity). Magnesium sulfate, sodium chloride, sodium citrate dibasic sesquihydrate, sodium citrate tribasic dihydrate, PSA bonded silica (supleclean PSA: Polymerically bonded, ethylenediamine-N-propyl phase that contains both primary and secondary amines) and C18 (Discovery DSC-18: octadecylsilane 18% C) were purchased from Supelco® (USA).

Stock solutions of 1.0 mg/ml of the standard compounds were prepared by dissolving 10.0 mg of each compound in 10 ml of methanol. A standard mix containing all the rodenticides was made at 1000 µg/ml by mixing a portion of stock solution of each compound with an appropriate amount of HPLC grade methanol. This mix was used to spike the dog blood samples.

**Instruments and conditions**

Detection and quantification of the anticoagulant rodenticides were performed on an Agilent 1100 Series ESI/LC/MSD ion Trap VL, consisting of a non-line solvent degasser, binary pump, autosampler and column temperature module, with an electrospray ionisation (ESI) source and ion trap analyser, all controlled with the HP Chemstation software (Agilent Technologies, Palo Alto, CA, USA). The separation was performed on a Waters Sunfire C8 column of 150mm x 4.6 and 5 µm particle size. Column was held at a constant temperature of 25°C. The mobile phase was water with ammonium acetate 20 mM (A) and methanol with ammonium acetate 20 mM (B) at a flow rate of 0.8 ml/min and a gradient where at t=0 min, 50% B and at t=22 min 95% B. The electrospray interface was set in negative ionization mode, selecting the precursor ions and introducing helium gas into the trap to induce collision. Throughout all the measurements the analytes were detected in multiple reaction monitoring (MRM) mode. Detection parameters are described in table 2.

Limit of detection (LOD) and qualification (LOQ) were defined as the lowest concentration detected and quantified under the established conditions for which the method was validated. LOD and LOQ were 5ppb for all the compounds except for difenacoum which was 1 ppb.
Table 2. Data acquisition parameters for seven anticoagulant rodenticides in multiple reaction monitoring (MRM) analysed by LC-MS/MS with an electrospray ionisation (ESI) source and ion trap analyser:

<table>
<thead>
<tr>
<th>Rodenticide</th>
<th>Precursor (m/z)</th>
<th>Product (m/z)</th>
<th>Reaction time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warfarin</td>
<td>357.0</td>
<td>165.7, 249.7</td>
<td>6.5-6.6</td>
</tr>
<tr>
<td>Coumatrelol</td>
<td>291.0</td>
<td>246.8, 166.7</td>
<td>7.6-7.9</td>
</tr>
<tr>
<td>Diphacinone</td>
<td>540.0</td>
<td>143.8, 167.8</td>
<td>11.3</td>
</tr>
<tr>
<td>Chlorophacinone</td>
<td>373.0</td>
<td>200.7, 144.7</td>
<td>14.2-14.3</td>
</tr>
<tr>
<td>Bromadiolone</td>
<td>526.0</td>
<td>464.9, 360.6, 308.7</td>
<td>16.3</td>
</tr>
<tr>
<td>Difenacoum</td>
<td>442.0</td>
<td>292.9, 708.0, 442.9, 180.8</td>
<td>17.2</td>
</tr>
<tr>
<td>Brodifacoum</td>
<td>522.0</td>
<td>520.9, 476.8, 188.8</td>
<td>19.2</td>
</tr>
</tbody>
</table>

Statistical analyses
Statistical analysis of the data was performed using SPSS v15.0 statistical software (SPSS Inc., 1989-1999).

Results and discussion

Extraction optimization
Three analytical methods were compared in order to improve accuracy and precision of the analyses of anticoagulant rodenticides (Table 1). These methods are based on the dispersive solid phase extraction (dSPE), commonly known as QuEChERS described by Anestessiades et al [21].

Method validation
The objective of the validation of an analytical method is to provide evidence that a method is fit for the purpose for which it is to be used, by being tested for accuracy and precision [22]. These parameters were studied for each of the methods, in order to select the best for the analyses of rodenticides.

Accuracy
Accuracy of the method was assessed by studying the recovery of rodenticides in dog blood samples spiked with a mix of the compounds of interest (warfarin, coumatrelol, brodifacoum, bromadiolone, difenacoum, chlorophacinone, diphacinone). Dog blood samples were analysed as blank in quintuplicate in order to ensure that they were free from rodenticides and to test matrix effect. Recoveries of rodenticides were tested using five replicates of spiked blood samples at three levels (20, 40 y 80 ppb). The extraction recoveries were determined by comparing peak heights obtained from extracted spiked samples with peak heights obtained in the working standard solutions. The method that provided the best recovery frequency was chosen. This frequency was calculated as follows:

\[ \text{Recovery (\%)} = \left( \frac{C_m}{C_p} \right) \times 100 \]

where \( C_m \) is the concentration of each compound in the sample and \( C_p \) is the concentration of each compound in the standard solution.

Linearity
Linearity of an analytical method is the ability to elicit test results that are directly proportional to the concentration of analytes in samples within a given range. The range and number of levels of fortification are highly related to the applicability of the method. In this case, linearity was calculated using a blank sample as 0 and five replicates of spiked blood samples with the mix of 7 rodenticides at three levels (20, 40 y 80 ppb).

Linear regression of data to a calibration curve was performed using the method of least squares. The acceptance criterion for linearity was a correlation coefficient \( r \geq 0.9 \).

Precision
Precision of a method can be defined by repeatability and reproducibility tests. Repeatability is used to prove the ability to provide similar results when the technique is repeated in the same sample, by the same operator. The acceptance criteria is based on the relative standard deviation (RSD), which is calculated analysing five replicates of spiked blood samples with the mix of 7 rodenticides at three levels (20, 40 y 80 ppb). This is calculated as follows: \( \text{RSD (\%)} = \left( \frac{SD}{Xm} \right) \times 100 \) where \( SD \) is the standard deviation of the whole series of measurements, whose mean is \( Xm \). The acceptance criterion for repeatability was RDS \( \leq 20\% \).

Reproducibility proves the ability to provide similar results when the technique is repeated in the same sample but by different operators or different laboratories. To validate reproducibility of our technique, 5 aliquots of the same sample of dog spiked blood (40 ppb) were analysed by different analysts and different days. Reproducibility acceptance criterion was RDS \( \leq 20\% \).

Method development
As mention above, accuracy or recovery is assessed using spiked samples. Mean recoveries for each of the methods evaluated are presented in Table 3. Although recoveries for method B could be considered acceptable for most of the compounds analysed (57-97%), method C was definitely chosen because it provided the maximum recovery values. Range of mean recoveries for method C was 72-134% for all the 7 anticoagulant rodenticides (Table 3). A representative chromatogram of spiked blood samples is shown in Figure 1.

Table 3. Accuracy, linearity and precision of seven anticoagulant rodenticides from spiked blood samples analysed by LC-MS/MS with an electrospray ionisation (ESI) source and ion trap analyser.

<table>
<thead>
<tr>
<th>Rodenticide</th>
<th>Method A</th>
<th>Method B</th>
<th>Method C</th>
<th>Linearity (R)</th>
<th>CV Repetitability (%)*</th>
<th>CV Repeatability (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warfarin</td>
<td>60.04</td>
<td>88.74</td>
<td>104.66</td>
<td>0.96</td>
<td>11.64</td>
<td>7.95</td>
</tr>
<tr>
<td>Coumatrelol</td>
<td>50.50</td>
<td>56.94</td>
<td>124.54</td>
<td>0.95</td>
<td>9.20</td>
<td>8.09</td>
</tr>
<tr>
<td>Diphacinone</td>
<td>28.05</td>
<td>44.88</td>
<td>74.15</td>
<td>0.99</td>
<td>12.33</td>
<td>37.55</td>
</tr>
<tr>
<td>Chlorophacinone</td>
<td>39.78</td>
<td>74.36</td>
<td>86.26</td>
<td>0.97</td>
<td>10.24</td>
<td>11.87</td>
</tr>
<tr>
<td>Bromadiolone</td>
<td>28.07</td>
<td>96.95</td>
<td>128.70</td>
<td>0.90</td>
<td>14.06</td>
<td>12.01</td>
</tr>
<tr>
<td>Difemacoum</td>
<td>43.38</td>
<td>85.80</td>
<td>91.45</td>
<td>0.97</td>
<td>5.84</td>
<td>10.51</td>
</tr>
<tr>
<td>Brodifacoum</td>
<td>29.07</td>
<td>61.92</td>
<td>72.59</td>
<td>0.93</td>
<td>7.36</td>
<td>3.07</td>
</tr>
</tbody>
</table>

* R = Correlation coefficient, RSD = Relative standard deviation, in % *Average recovery for three spiking levels, **Average of five measurements at one spiking level.

Fig.1 Chromatogram of a spiked dog blood sample analysed by LC-MS/MS with an electrospray ionisation (ESI) source and ion trap analyser using the extraction method C (2 ml acetonitrile and 1 ml methanol).
Regarding linearity, a good correlation between concentrations of rodenticides/area of chromatographic peak was found for each compound, since correlation coefficients were above 0.95 with the only exception of bromadiolone (r=0.90) and brodifacoum (r=0.93). RSD of repeatability and reproducibility indicated a good precision of the method, as they were lower than 15%, lower values than the threshold of 20% that had been set to accept the validation of the analytical method. The only exception was difacinone, whose RSD of repeatability and reproducibility were 32% and 38%, respectively. Mean recoveries for other methods described to analyse anticoagulant rodenticides in animal samples range between 52-70% [9, 15, 18, 19], which were lower than the values obtained in our technique. On the other hand, also our detection limits are in general lower than in those techniques, were the lowest was 2 ppb for the same group of compounds as we analysed [15, 17]. While this is not the first modification of a QuEChERS method to analyse anticoagulant rodenticides in blood [19], a better accuracy and sensitivity has been obtained, since their lowest LOD was 10 ppb and recoveries ranged between 68-97% [19]. In addition, the validation of our method has reduced both the use of solvents and reagents and the time required for the analyses. This implies a great advantage in order to reduce laboratory expenses and to optimize laboratory work.

Applicability of the technique

Eagle owl blood samples from free-ranging animals were analysed to assess their exposure to anticoagulant rodenticides. However, any compound could be detected in any sample. This could be due to the toxicokinetics of these compounds: after ingestion of contaminated prey items, concentrations of anticoagulants in blood usually persist only a few days and are rapidly transported and accumulated in liver, where they may persist up to a year [8].

Conclusions

The technique herein described can be considered acceptable as a method for the analyses of residues of multiple anticoagulant rodenticides in whole blood samples. The fact that a small sample amount (2 ml) can be used is especially of interest in the case of biomonitoring programs, particularly in raptors that mainly prey on rodents.

Acknowledgements

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